Lymphocyte dynamics in healthy and lymphopenic conditions

Vera van Hoeven

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Lymphocyte dynamics in healthy and lymphopenic conditions

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(met een samenvatting in het Nederlands)

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General introduction

1

Maintenance of lymphocytes

The lymphocyte pool, largely consisting of B cells and T cells, forms an essential cellular component of the immune system. By the unique ability of B cells and T cells to recognize specific antigens through antigen-specific receptors, and their capacity to generate long-lasting immunological memory, they play an important role in the defense against pathogens. The importance of lymphocytes is underscored by the fact that lymphocyte deficiencies, e.g. caused by congenic disorders, chronic viral infections, or therapy-induced lymphocyte depletion, inevitably result in increased susceptibility to infectious and malignant diseases and are associated with increased mortality. Hence, to sustain immune competence into old age, it is essential that lymphocyte populations are well maintained. By a balance of cell production and cell loss, B-cell and T-cell populations are generally maintained at a relatively stable size throughout life.

Development and maintenance of B-cell populations

B cells exert their protective effect mainly by the production of antigen-specific antibodies. *De novo* development of B cells from the hematopoietic stem cell takes place in the bone marrow, and during several developmental stages immunoglobulin (Ig) gene segments rearrange to form a B-cell receptor (BCR). Once a functional BCR is formed B cells exit the bone marrow, and upon further maturation and selection they become part of the peripheral naive B-cell pool. In addition to bone marrow output, naive B cells are maintained by antigen-independent peripheral division, which is believed to be regulated by cytokines.

Specific antigen-encounter drives rapid B-cell expansion and induces entry into either the marginal zone of the spleen (T-cell independent response) or into a germinal center (GC, T-cell dependent response). In humans, marginal zone B cells can also enter the circulation, where they are called natural effector B cells. In fact, next to naive B cells and class-switched memory B cells, they form one of the three most abundant B-cell subsets in peripheral blood. Natural effector B cells carry mutated Igs, and they form an important line of defense against blood-borne pathogens. Through a final antigen-dependent differentiation step, they can develop into antibody-producing short-lived plasma cells. The most profound B-cell proliferation takes place during the GC reaction. In the germinal center, interaction of GC B cells with T-helper cells initiates somatic hypermutation and Ig class switching, and after extensive selection, the surviving B cells will eventually differentiate into memory cells and long-lived plasma cells.

Regulation of B-cell survival is not completely understood. B-cell maintenance depends on the expression of a functional BCR, and the interaction with survival and proliferation factors BAFF (B-cell activator of the TNFα-family, also known as BLYS) and APRIL (a proliferation-inducing ligand). BAFF binds to three receptors (BAFF-R; TACI, and BCMA) that are differentially expressed on different B-cell subsets. While persistence of naive B cells and MZ B cells depends on signaling through the BAFF-R, expression of BCMA is essential for proliferation and selection of germinal center B cells, and survival of long-lived plasma cells in the bone marrow[1-3].

Development and maintenance of T-cell populations

T cells play an important role in the production of cytokines and are able to directly recognize and kill virus-infected or otherwise disordered cells in an antigen-specific manner. T-cell progenitors arise from hematopoietic stem cells in the bone marrow and migrate to the thymus, where they further develop into T cells. Successful T-cell receptor (TCR) gene rearrangement, resulting in the formation of a functional T-cell receptor, is an absolute requirement for completion of thymus-mediated T-cell development. As thymus-mediated T-cell development generates new T-cell specificities, it is essential for the establishment of a diverse repertoire in the peripheral T-cell pool[4].

Once thymocytes have completed their development and enter the periphery, they are called recent thymic emigrants (RTE). RTE are thought to be functionally immature and to require yet unknown maturation signals before being incorporated into the resident, mature naive T-cell pool[5;6]. In humans, thymus output is not the main source of new T cells, however. With age, the thymus undergoes a profound involution, and even in young adults naive T-cell production occurs almost exclusively through peripheral T-cell division[7]. It is important that such proliferation occurs non-specifically, to ensure maintenance of a diverse naive T-cell pool. Upon encounter with cognate antigen in complex with the appropriate MHC molecule, T cells expand rapidly and differentiate into effector cells. After the expansion phase, the majority of cells go into apoptosis, and the remaining cells form an antigen-specific memory T-cell population and sustain immunological memory.

Maintenance of the T-cell population is generally thought to be accomplished through peripheral competition for limiting survival factors. Naive T cells in humans are very long-lived cells, and under lymphoreplete conditions they hardly proliferate[7]. Contact of the TCR with self-peptide MHC complexes, and cytokine signaling induced by IL-7 are both critical for naive T-cell survival and the same stimuli are thought to regulate peripheral T-cell proliferation. During an immune response, expanding effector T cells produce IL-2, and pro-inflammatory cytokines temporarily regulate their survival. Maintenance of memory T cells appears to be largely independent of specific MHC molecules[8], and both survival and proliferation are controlled by binding of IL-7 and IL-15[9-11].

Immune reconstitution after lymphocyte depletion

The combination of *de novo* cell production in the bone marrow and thymus, peripheral cell division, cell survival and cell differentiation needs to be well-balanced to maintain lymphocyte populations at a steady state. Knowing how these four dynamic processes contribute to lymphocyte maintenance under normal, healthy circumstances is essential for our understanding of how, for example, advanced aging, infectious diseases, and therapeutic interventions affect these dynamics.

An extreme example of a disturbed lymphocyte compartment is the lymphopenic condition after therapeutic lymphocyte depletion, which is generally applied to prevent

rejection of allogeneic solid organ grafts, or to deplete either autoreactive or cancerous cells from the hematopoietic system, and sometimes combined with a hematopoietic stem cell transplantation. For an efficient recovery from severe lymphopenia, ideally the production rate of new lymphocytes would be increased, or the rate at which lymphocyte are lost from the periphery would be decreased. In rodents, such extreme conditions of severe lymphopenia were observed to induce homeostatic expansion of T cells[12-14] and B cells[15-19], suggesting that the immune system has the capacity to adapt lymphocyte production rates when cell numbers change. Whether (and for which lymphocyte subsets) a similar homeostatic (i.e., cell-density dependent) response is induced in lymphopenic humans is unclear, however. Following various lymphocyte-depleting therapies, reconstitution of lymphocyte subsets was observed to take months to years[20-22], even though the serum levels of the essential proliferation and survival factors BAFF and IL-7 have been found to be increased in lymphopenic individuals[23-26]. For T cells, increased expression of the proliferation marker Ki-67 has been observed following allogeneic stem cell transplantation[27] and anti-thymocyte globulin (ATG)-mediated lymphocyte depletion[28]. However, since in both studies Ki-67-expression declined when peripheral counts were still low[27;28], and in one study increased Ki-67 expression was found to coincide with episodes of immune activation[27], it remained disputable whether proliferative responses are induced by lymphopenia in humans.

Stable isotope labeling: a tool to measure lymphocyte turnover *in vivo*

To unravel the mechanism of human lymphocyte recovery, immune reconstitution ideally needs to be investigated in a lymphopenic study population with low risk of complications that may cause antigen-specific immune activation. Moreover, accurate measurement and quantification of lymphocyte production and death rates would be informative on whether lymphocyte dynamics are changed in lymphopenic conditions. Approximately 1.5 decades ago, introduction of stable isotope labeling has permitted the investigation of human lymphocyte dynamics *in vivo*[29] and this technique has in the meantime been applied to study a variety of lymphocyte subsets in a variety of conditions (see e.g. reference [30-42]). In low tracer doses, the stable isotope *deuterium* can be safely administered to humans as deuterated glucose or as deuterated water (^2H_2O). For investigation of cells with a relatively slow turnover rate, such as naive T cells, ^2H_2O is most suitable as it can easily be administered for longer periods of time[43]. Ideally, human ^2H_2O-labeling studies consist of an up-labeling phase of several weeks, and a substantial down-labeling phase thereafter, allowing for a frequent collection of blood samples and measurement of deuterium enrichment in the cell populations of interest. During the up-labeling phase, deuterium enrichment in the body water is maintained at a constant level by a small daily dose of ^2H_2O, administered orally. Cells that divide during this period, incorporate deuterium into newly synthesized DNA strands. Hence, the rate and level of deuterium incorporation in the DNA of a cell population is a reflection of how fast new cells are being produced. Under steady state conditions, this production rate is equal to the

rate at which cells are lost from a cell compartment, which occurs either via cell death or by differentiation to the next compartment[44;45].

Assessment of lymphocyte production by the thymus and the bone marrow

As deuterium is incorporated into any dividing cell, regardless of whether it was formed in a primary lymphoid organ or in the periphery, $^{2}H_{2}O$ labeling studies do not give information on how much thymus output and bone marrow output contribute to the production of new T cells and B cells respectively. For estimation of thymus function in humans, many laboratories still rely on the analysis of CD31 and PTK7 expression, two surface-molecules that have been proposed as RTE-specific markers[6;46]. These markers are, however, limited to the $CD4^{+}$ T-cell lineage, and their expression appeared to be either maintained on proliferating naive T cells[7;47] or maintained on non-dividing, long-lived naive T cells despite weeks of residence in the periphery[6;48].

Measurement of the content of T-cell receptor excision circles (TRECs) in naive T cells is an alternative tool to study thymic output. TRECs are stable extrachromosomal DNA circles that are formed during TCR gene rearrangement in the thymus, and they are not duplicated upon peripheral cell division. Although TRECs are uniquely formed in the thymus, the average TREC content in peripheral cell populations is highly influenced by the peripheral division rate and the death rate of the T-cell population. Therefore, TREC contents are not directly representative for the level of thymic output, and TREC analyses are difficult to interpret[49]. Still, TREC contents are informative about the relative contribution of thymic output and peripheral cell division. Combining deuterium labeling and TREC analyses was recently proposed as an approach to estimate absolute daily thymic output in humans[7].

During BCR gene rearrangement in the bone marrow, stable DNA circles similar to TRECs are excised from the genome, which are called kappa-deleting recombination excision circles (KRECs). KRECs are also diluted twofold upon every cell division. Recently, the use of a KREC assay was introduced to determine the replication history of B-cell subsets[50]. The KREC assay, and the combination with deuterium labeling, will form a useful tool to assess the contribution of bone marrow output in various conditions.

Scope of this thesis

In this work we investigated how lymphocyte populations are maintained in healthy conditions and how they reconstitute after lymphocyte depletion by determining their turnover dynamics. For the majority of our studies we used *in vivo* heavy water labeling to measure how fast lymphocyte subsets are being produced and how long they reside in a specific cell compartment, before they are lost either by differentiation or by cell death.

There is controversy in literature on whether recent thymic emigrants, the direct descendants of thymic output, form a subset of naive T cells that are either shorter, or longer-lived than resident, mature naive T cells. In **chapter 2** we investigate the dynamics of RTE

in mice by combining thymus transplantation experiments with *in vivo* deuterium labeling studies.

Aging is associated with a decline in immune competence, reflected by an increased susceptibility to infectious diseases and malignancies, and a decreased efficiency of vaccinations. This decrease in immune function is often attributed to the profound decline in thymic output over age. In **chapter 3** we study how *healthy* aging affects thymic output and the turnover rate of various B-cell and T-cell subsets by performing TREC analysis and *in vivo* deuterium labeling in 20-25 year old healthy young adults, and in 65-75 year old healthy aged individuals. An important aim of this study was to determine how lymphocyte numbers are maintained into old age, and whether the decline in thymic output needs to be compensated for by an increase in peripheral cell division.

High-dose chemotherapy in combination with a hematopoietic stem cell transplantation is widely applied for the treatment of hematological malignancies. Although such treatment effectively eliminates tumor cells, it adversely causes long-term immunodeficiency, which is associated with an increased risk of infectious complications and tumor relapse. **Chapter 4** describes how lymphocyte numbers recover after stem cell transplantation, and how the lymphopenic environment affects the turnover of B-cell and T-cell subsets. By performing TREC analysis we also investigate whether the thymus plays a role in T-cell reconstitution. While we found no signs of a homeostatic response for the maintenance of T cells and B cells in healthy aging (chapter 3), we report in this chapter that severe lymphopenia induces a homeostatic increase in peripheral cell division to promote immune reconstitution. Moreover, we found evidence that despite the advanced age of the stem-cell transplanted individuals, the thymus still contributed substantially to T-cell reconstitution.

To prevent rejection after allogeneic solid organ transplantation, allograft recipients are generally treated with a cocktail of immunosuppressive agents, sometimes including specific lymphocyte-depleting antibodies. In **chapter 5** we investigate the influence of immunosuppressive therapy in renal transplant recipients who were or were not lymphocyte depleted with anti-thymocyte globulin, and study whether a lymphopenia-induced response occurs in the presence of immunosuppressive agents.

Finally, **chapter 6** summarizes the preceding chapters of this thesis and discusses the results in a broader perspective.

References

1. Pieper K, Grimbacher B, Eibel H. B-cell biology and development. J.Allergy Clin.Immunol. 2013;131:959-971.
2. van Zelm MC, van der Burg M, van Dongen JJ. Homeostatic and maturation-associated proliferation in the peripheral B-cell compartment. Cell Cycle 2007;6:2890-2895.
3. Crowley JE, Treml LS, Stadanlick JE, Carpenter E, Cancro MP. Homeostatic niche specification among naive and activated B cells: a growing role for the BLyS family of receptors and ligands. Semin. Immunol. 2005;17:193-199.
4. Schluns KS, Lefrancois L. Cytokine control of memory T-cell development and survival. Nat.Rev. Immunol. 2003;3:269-279.
5. Fink PJ, Hendricks DW. Post-thymic maturation: young T cells assert their individuality. Nat.Rev. Immunol. 2011;11:544-549.
6. Haines CJ, Giffon TD, Lu LS et al. Human CD4+ T cell recent thymic emigrants are identified by protein tyrosine kinase 7 and have reduced immune function. J.Exp.Med. 2009;206:275-285.
7. den Braber I, Mugwagwa T, Vrisekoop N et al. Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. Immunity. 2012;36:288-297.
8. Tanchot C, Lemonnier FA, Perarnau B, Freitas AA, Rocha B. Differential requirements for survival and proliferation of CD8 naive or memory T cells. Science 1997;276:2057-2062.
9. Surh CD, Sprent J. Homeostasis of naive and memory T cells. Immunity. 2008;29:848-862.
10. Takada K, Jameson SC. Naive T cell homeostasis: from awareness of space to a sense of place. Nat.Rev. Immunol. 2009;9:823-832.
11. Bradley LM, Haynes L, Swain SL. IL-7: maintaining T-cell memory and achieving homeostasis. Trends Immunol. 2005;26:172-176.
12. Miller RA, Stutman O. T cell repopulation from functionally restricted splenic progenitors: 10,000-fold expansion documented by using limiting dilution analyses. J.Immunol. 1984;133:2925-2932.
13. Bell EB, Sparshott SM, Drayson MT, Ford WL. The stable and permanent expansion of functional T lymphocytes in athymic nude rats after a single injection of mature T cells. J.Immunol. 1987;139:1379-1384.
14. Freitas AA, Rocha B. Population biology of lymphocytes: the flight for survival. Annu.Rev.Immunol. 2000;18:83-111.
15. Cabatingan MS, Schmidt MR, Sen R, Woodland RT. Naive B lymphocytes undergo homeostatic proliferation in response to B cell deficit. J.Immunol. 2002;169:6795-6805.
16. Woodland RT, Schmidt MR. Homeostatic proliferation of B cells. Semin.Immunol. 2005;17:209-217.
17. Meyer-Bahlburg A, Andrews SF, Yu KO, Porcelli SA, Rawlings DJ. Characterization of a late transitional B cell population highly sensitive to BAFF-mediated homeostatic proliferation. J.Exp.Med. 2008;205:155-168.
18. Agenes F, Freitas AA. Transfer of small resting B cells into immunodeficient hosts results in the selection of a self-renewing activated B cell population. J.Exp.Med. 1999;189:319-330.
19. Sprent J, Schaefer M, Hurd M, Surh CD, Ron Y. Mature murine B and T cells transferred to SCID mice can survive indefinitely and many maintain a virgin phenotype. J.Exp.Med. 1991;174:717-728.
20. Bosch M, Khan FM, Storek J. Immune reconstitution after hematopoietic cell transplantation. Curr. Opin.Hematol. 2012;19:324-335.
21. Hill-Cawthorne GA, Button T, Tuohy O et al. Long term lymphocyte reconstitution after alemtuzumab treatment of multiple sclerosis. J.Neurol.Neurosurg.Psychiatry 2012;83:298-304.
22. Chang YJ, Zhao XY, Huang XJ. Immune reconstitution after haploidentical hematopoietic stem cell transplantation. Biol.Blood Marrow Transplant. 2014;20:440-449.

23. Thompson SA, Jones JL, Cox AL, Compston DA, Coles AJ. B-cell reconstitution and BAFF after alemtuzumab (Campath-1H) treatment of multiple sclerosis. J.Clin.Immunol. 2010;30:99-105.
24. Napolitano LA, Grant RM, Deeks SG et al. Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. Nat.Med. 2001;7:73-79.
25. Bolotin E, Annett G, Parkman R, Weinberg K. Serum levels of IL-7 in bone marrow transplant recipients: relationship to clinical characteristics and lymphocyte count. Bone Marrow Transplant. 1999;23:783-788.
26. Fry TJ, Connick E, Falloon J et al. A potential role for interleukin-7 in T-cell homeostasis. Blood 2001;97:2983-2990.
27. Hazenberg MD, Otto SA, de Pauw ES et al. T-cell receptor excision circle and T-cell dynamics after allogeneic stem cell transplantation are related to clinical events. Blood 2002;99:3449-3453.
28. Bouvy AP, Kho MM, Klepper M et al. Kinetics of homeostatic proliferation and thymopoiesis after rATG induction therapy in kidney transplant patients. Transplantation 2013;96:904-913.
29. Macallan DC, Fullerton CA, Neese RA et al. Measurement of cell proliferation by labeling of DNA with stable isotope-labeled glucose: studies in vitro, in animals, and in humans. Proc.Natl.Acad.Sci.U.S.A 1998;95:708-713.
30. Macallan DC, Asquith B, Irvine AJ et al. Measurement and modeling of human T cell kinetics. Eur.J.Immunol. 2003;33:2316-2326.
31. Macallan DC, Wallace D, Zhang Y et al. Rapid turnover of effector-memory CD4(+) T cells in healthy humans. J.Exp.Med. 2004;200:255-260.
32. Macallan DC, Wallace DL, Zhang Y et al. B-cell kinetics in humans: rapid turnover of peripheral blood memory cells. Blood 2005;105:3633-3640.
33. Mohri H, Perelson AS, Tung K et al. Increased turnover of T lymphocytes in HIV-1 infection and its reduction by antiretroviral therapy. J.Exp.Med. 2001;194:1277-1287.
34. van Gent R, Kater AP, Otto SA et al. In vivo dynamics of stable chronic lymphocytic leukemia inversely correlate with somatic hypermutation levels and suggest no major leukemic turnover in bone marrow. Cancer Res. 2008;68:10137-10144.
35. Vrisekoop N, den Braber I, de Boer AB et al. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. Proc.Natl.Acad.Sci.U.S.A 2008;105:6115-6120.
36. Vukmanovic-Stejic M, Zhang Y, Cook JE et al. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. J.Clin.Invest 2006;116:2423-2433.
37. Wallace DL, Zhang Y, Ghattas H et al. Direct measurement of T cell subset kinetics in vivo in elderly men and women. J.Immunol. 2004;173:1787-1794.
38. Messmer BT, Messmer D, Allen SL et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. J.Clin.Invest 2005;115:755-764.
39. Pillay J, den B, I, Vrisekoop N et al. In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. Blood 2010;116:625-627.
40. Zhang Y, Wallace DL, de Lara CM et al. In vivo kinetics of human natural killer cells: the effects of ageing and acute and chronic viral infection. Immunology 2007;121:258-265.
41. Zhang Y, de LC, Worth A et al. Accelerated in vivo proliferation of memory phenotype CD4+ T-cells in human HIV-1 infection irrespective of viral chemokine co-receptor tropism. PLoS.Pathog. 2013;9:e1003310.
42. Westera L, van Hoeven V, Drylewicz J et al. Lymphocyte maintenance during healthy aging requires no substantial alterations in cellular turnover. Aging Cell 2015;14:219-227.
43. Neese RA, Misell LM, Turner S et al. Measurement in vivo of proliferation rates of slow turnover cells by 2H2O labeling of the deoxyribose moiety of DNA. Proc.Natl.Acad.Sci.U.S.A 2002;99:15345-15350.
44. Busch R, Neese RA, Awada M, Hayes GM, Hellerstein MK. Measurement of cell proliferation by heavy water labeling. Nat.Protoc. 2007;2:3045-3057.

45. Westera L, Zhang Y, Tesselaar K, Borghans JA, Macallan DC. Quantitating lymphocyte homeostasis in vivo in humans using stable isotope tracers. Methods Mol.Biol. 2013;979:107-131.

46. Kimmig S, Przybylski GK, Schmidt CA et al. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. J.Exp.Med. 2002;195:789-794.

47. Kilpatrick RD, Rickabaugh T, Hultin LE et al. Homeostasis of the naive CD4+ T cell compartment during aging. J.Immunol. 2008;180:1499-1507.

48. Bains I, Yates AJ, Callard RE. Heterogeneity in thymic emigrants: implications for thymectomy and immunosenescence. PLoS.One. 2013;8:e49554.

49. Hazenberg MD, Borghans JA, de Boer RJ, Miedema F. Thymic output: a bad TREC record. Nat.Immunol. 2003;4:97-99.

50. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J.Exp.Med. 2007;204:645-655.

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Dynamics of recent thymic emigrants in young adult mice

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Abstract

The peripheral naive T-cell pool is generally thought to consist of a subpopulation of recent thymic emigrants (RTE) and a subpopulation of mature naive (MN) T cells with different dynamics. However, thymus transplantation and adoptive transfer studies in mice have provided contradicting results, with some studies suggesting that RTE are relatively short-lived cells and another suggesting that they are relatively long-lived cells in the naive T-cell pool. One possible explanation for these discrepancies is the difference in the ages of the mice whose cells were investigated. To follow the dynamics of RTE, we here performed thymus transplantations in mice of at least 12 weeks old, an age at which the size of the T-cell pool has stabilized. We found that the rate at which donor-derived RTE were lost was approximately twofold slower than in previously studied 5-6 week old thymus-engrafted mice, suggesting that age differences may indeed be a confounding factor. We performed long-term and short-term deuterium labeling experiments to estimate the lifespans of naive T cells and RTE, respectively, and used the thymus-transplantation data to estimate the survival of RTE from donor-thymus origin. Both comparisons suggested that in mice, RTE form a subpopulation of short-lived naive T cells.

Introduction

The naive T-cell pool is maintained at a relatively stable size throughout life, which is accomplished by self-renewal of naive T cells in the periphery and *de novo* production of T cells in the thymus. *De novo* produced T cells that have completed their development in the thymus and have recently entered the peripheral naive T-cell pool are often referred to as recent thymic emigrants (RTE). RTE constitute the only source of T cells with newly rearranged T-cell receptors, and are therefore crucial for the establishment of a diverse T-cell repertoire[1].

It is generally thought that RTE are relatively short-lived, and therefore, RTE have been held responsible for large and rapid changes in the naive T-cell pool, for example in HIV infection[2]. However, the existing literature on the survival of RTE and mature naive (MN) T cells in mice has provided conflicting results[3;4]. Despite the clear relevance of RTE in establishing a diverse T-cell population, their dynamics are understudied, mainly because of the lack of a reliable marker that phenotypically distinguishes RTE from MN T cells[5]. In mice, the need for a phenotypical marker to investigate RTE biology has been circumvented by the use of RAG2p-GFP transgenic mice[6], in which all new T cells that recently emigrated from the thymus express GFP for a period of approximately 3 weeks[7], and by thymus transplantation studies, in which thymic graft-derived congenic T cells can be followed in the periphery [8;9].

In the RAG2p-GFP transgenic system, the frequency of GFP-expressing RTE in the splenic T-cell pool was reported to decline from 100% in neonates to less than 20% in 6 month old adult mice [10], reflecting the establishment of a MN T-cell pool. By performing adoptive transfer experiments, two separate laboratories have studied the survival of GFP^+ RTE and final-stage single-positive thymocytes (pre-RTE) relative to other naive T cells. The reported results were conflicting; in one study GFP^+ RTE persisted shorter than co-transferred GFP^- MN T cells[4], while the other study reported a significantly better survival of pre-RTE and GFP^+ RTE compared to co-transferred lymph node-derived naive T cells[3]. Dong et al. proposed that the discrepancy between these studies could be due to the difference in the ages of the mice that were used to isolate RTE and naive T cells; whereas Dong et al.[3] isolated both RTE and naive T cells from 6-8 week old mice, Houston et al.[4] isolated RTE from 5-week old mice and MN T cells from mice that were at least 12 weeks old. Since the expected lifespan of naive T cells has been shown to increase with the age of the donor mouse [11], these age differences may indeed underlie the observed differences in dynamic behavior between RTE and other naive T cells.

Based on thymus transplantation studies, it has been suggested that RTE survive for a period of 3 weeks in the peripheral T-cell pool [8;9]. The increase in the peripheral T-cell pool size of thymus grafted mice was almost identical to the total number of T cells exported from the grafts in 3 weeks[9], and beyond 3 weeks post-transplantation, donor-derived T cells were rapidly lost from the periphery of the acceptor mice[8]. As the average lifespans of naive

$CD4^+$ and $CD8^+$ T cells estimated in deuterium labeling studies are longer than 21 days (i.e. 47 days for $CD4^+$ and 80 days for $CD8^+$ naive T cells[12], this would suggest that RTE have a shorter lifespan than the average naive T cell. However, these transplantation studies were performed in 5-6 week old mice, in which the naive T-cell pool is at its maximum size, while the deuterium labeling estimates come from 12 week old mice, an age at which the naive T-cell pool has stabilized[12;13]. Before a conclusion on the relative survival of RTE can be drawn, it is important to exclude differences in the ages of the mice as a possible confounder.

We performed thymus transplantation and short- and long-term deuterium labeling studies in mice of approximately 12 weeks of age, to study whether RTE form a subpopulation of short-lived naive T cells. We found that in young adult mice, donor-derived T cells survived approximately twofold longer than what was previously observed in 5-6 week old mice, suggesting that recipient age may indeed affect RTE lifespan. Nevertheless, the average lifespans of $CD4^+$ naive T cells from donor-thymus origin as well as naive T cells from short-term deuterium labeled mice – which are both strongly enriched for RTE – appeared to be shorter than the average lifespan of $CD4^+$ naive T cells in age-matched long-term deuterium-labeled mice. A similar trend was observed for $CD8^+$ naive T cells. These data thereby provide further support for the view that in young adult mice, RTE form a subpopulation of relatively short-lived cells in the naive T-cell pool.

Materials and Methods

Mice. Ly5.1, Ly5.2, and Ly5.1/5.2 congenic C57BL/6J mice were maintained by in-house breeding at the Central Laboratory Animal Research Facility of Utrecht University under conventional conditions. Adult mice were male and one-day old pups were of both genders. The animal studies were performed in accordance with institutional and national guidelines.

Thymic grafting. Thymic lobes were isolated from one-day old pups and engrafted under the kidney capsule of 13-15 week old Ly congenic mice according to a previously described procedure[8] with minor modifications. Briefly, acceptor mice were anesthetized by isoflurane (IsoFlo, Abbott Laboratories), incisions were made in the skin and body wall and two separate thymic lobes were implanted under the left kidney capsule. The body wall and skin were closed by interrupted sutures. Buprenorphine (Temgesic, 50 µg/kg) was administered subcutaneously twice daily for 3 days to relieve pain. Mice with unsuccessful grafts upon harvest were excluded from further analyses.

^2H_2O labeling. For finite-term labeling, 12-week old unmanipulated mice received an intraperitoneal (i.p.) injection of 15 mL/kg of deuterated water (^2H_2O) (99.8%; Cambridge Isotopes Laboratories) in phosphate-buffered saline and received 4% ^2H_2O for either 1 week

(short-term labeling) or 8 weeks (long-term labeling). To achieve prenatal $^{2}H_{2}O$ labeling in unmanipulated and thymus graft recipient mice, female mice were injected i.p. with 15 ml/kg 99.8% $^{2}H_{2}O$, housed with male mice, and fed with 4% $^{2}H_{2}O$ until conception. The offspring received 4% $^{2}H_{2}O$ until the day they were grafted with congenic thymic lobes, or until 16 weeks of age in mice that were not grafted.

Cell preparation, flow cytometry and sorting. At the indicated time points after thymus transplantation, blood, lymph nodes (2 axillary, 2 brachial, 2 inguinal, 6 superficial cervical), natural and (if present) grafted thymus, and spleen were isolated, and single cell suspensions were obtained as previously described[14]. Cell counts were determined manually, and total peripheral cell counts were estimated as the cell count in spleen plus twice that in peripheral lymph nodes[15]. Cell suspensions were stained with monoclonal antibodies (mAb) to CD3-APC (eBioscience), CD4-APC/H7 (BD), CD8-FITC (BD), CD45.2-PerCP/Cy5.5 (BD) or CD45.1-PerCP/Cy5.5 (Biolegend), CD44-eFluor450 (eBioscience), and CD62L-PE (BD) for 20 min at 4°C in the presence of blocking 2.4G2 mAb (CD16/CD32). For staining of splenocytes obtained from unmanipulated mice, a slightly different antibody mixture was used: CD62L-FITC, CD44-eFluor450 (eBioScience, SanDiego,CA),CD4-APC-H7, and CD8-PerCP (BD PharMingen, San Jose, CA). Cells were analyzed on an LSR II flow cytometer (BD) or sorted with a FACSAria II using FACSDiva software (BD). Naive T cells were defined as $CD62L^{+}$ and $CD44^{-}$.

Measurement of deuterium enrichment in DNA and plasma. Genomic DNA was isolated from sorted cell samples using the ReliaPrep Blood gDNA Miniprep system (Promega) according to manufacturer's instructions. ^{2}H incorporation in DNA was measured by gas chromatography and mass spectrometry (GC/MS) as previously described[16] with minor modifications. Briefly, after enzymatic hydrolysis of the DNA, purine deoxyribonucleotides were derivatized to perfluorotriacetyl (PFTA) and injected into the gas chromatograph (DB-17MS column; 7890A GC System, Agilent Technologies). The mass of the derivate was measured by negative chemical ionization mass spectrometry (5975C inert XL EI/CI MSD; Agilent Technologies) at m/z 435 (M0) and 436 (M1). Standards of known isotopic enrichments were used to control for varying sample concentrations, as reported previously[17]. Plasma was obtained by centrifugation of whole blood samples and deuterium enrichment was measured on the same GC/MS system (using a PoraPLOT Q 25x0.32 column, Varian) as described by Westera et al[18].

Lifespan calculation. To estimate the average lifespan of RTE from the thymus transplantation study, we fitted a linear decay model to the log-transformed declining numbers of T cells (either total CD3+, CD4+, CD8+ or naive) from donor-thymus origin in the periphery of the acceptor mice. The average lifespans were estimated as the inverse of the slopes of these regression lines. To estimate the expected lifespan of naive (RTE+MN) T cells from our *in*

vivo deuterium labeling studies, we fitted a multi-exponential model[19] to the deuterium enrichment data of naive T cells of young adult mice, which had either received deuterated water for 8 weeks or had been labeled prenatally. The resulting average turnover rates *p* of naive T cells were transformed into expected lifespans as 1/*p* (see Supplemental Materials for details). 95% confidence intervals were determined by bootstrap analysis on the residuals. As an independent measure of the lifespan of RTE we estimated the rate *d** at which labeled naive T cells disappeared[19;20] from young adult mice who had been labeled with deuterated water for 1 week, and translated to expected lifespans as 1/*d**.

Results

The natural outgrowth of murine T-cell subsets

To study the establishment of the naive and memory T-cell pools, we determined the number of thymocytes and naive and memory T cells in the spleen and lymph nodes of unmanipulated C57BL/6J mice that were bred in our facility. We observed that both $CD4^+$ and $CD8^+$ single positive (SP) thymocyte populations, which are the direct precursors of RTE (see model in Figure 1A), expand rapidly in the first few weeks after birth, reaching a peak size at the age of ~4 weeks and contracting in the 4 weeks thereafter (Figure 1B). Though less pronounced, and with a delay of a few weeks compared to the thymocyte populations, the peripheral naive T-cell pools (i.e., containing both RTE and MN T cells) follow a similar growth pattern, as indicated by the T cell numbers in both spleen and peripheral lymph nodes (Figure 1C-D). Outgrowth of the effector/memory (E/M) T-cell pools is also quite extensive immediately after birth. Similar to the naive T-cell pools, the size of the E/M pools is maintained at a relatively stable size from the age of ~12 weeks onward (Figure 1C-D).

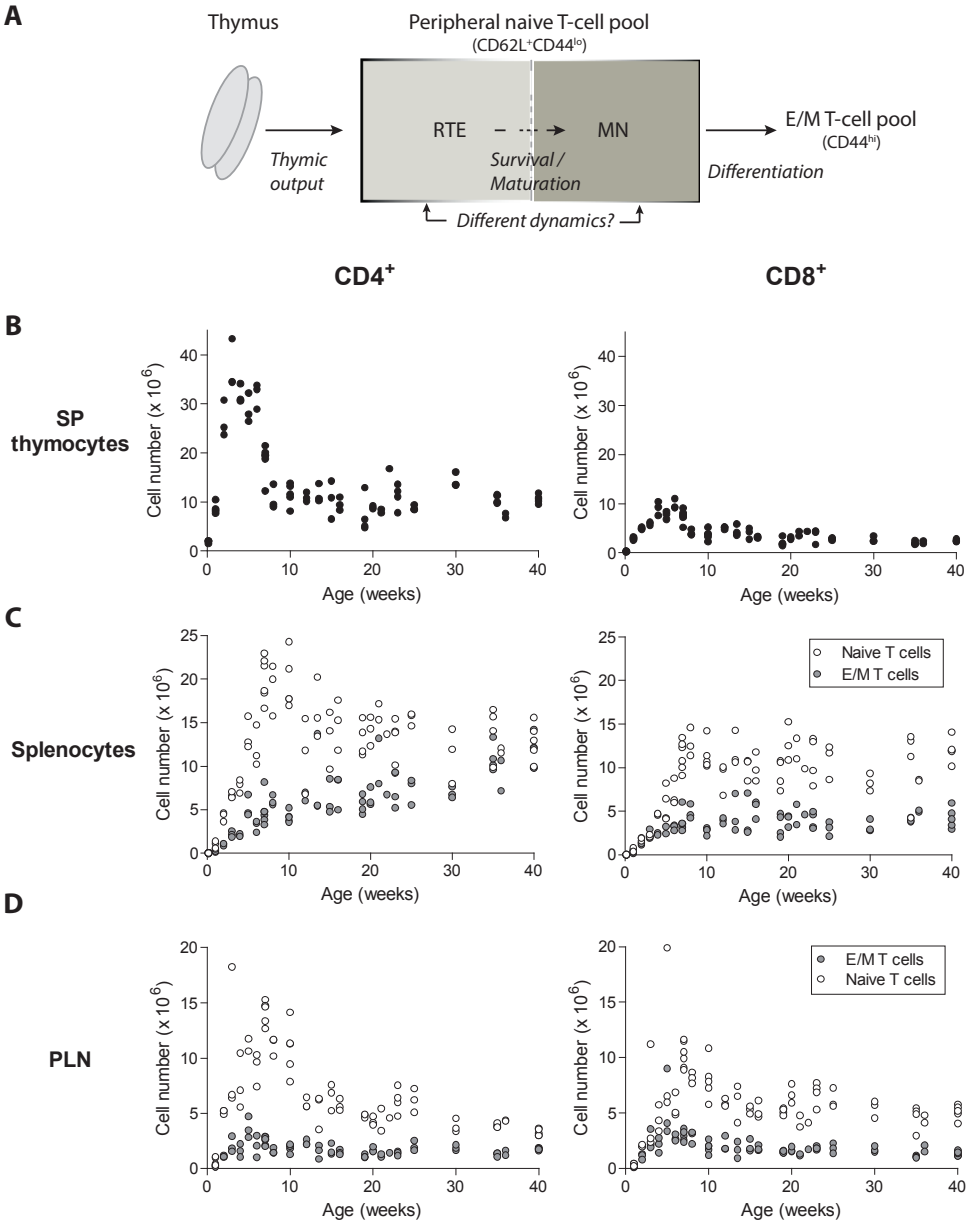

Figure 1. Natural outgrowth of T-cell subsets in C57BL/6J mice. (A) Schematic illustration of the naive T-cell pool, which is composed of a subpopulation of recent thymic emigrants (RTE) and a subpopulation of mature naive (MN) T cells. T cells produced by the thymus enter the peripheral naive T-cell pool via the RTE compartment, and become MN T cells upon survival and maturation. Part of the naive T cells will exit the compartment by differentiation into effector-memory (E/M) T cells. (B-D) Absolute number of $CD4^+$ (left panels) and $CD8^+$ (right panels) single positive thymocytes (B) and naive and effector/memory (E/M) T cells in the spleen (C) and peripheral lymph nodes (D) of unmanipulated C57BL/6J mice of varying age. Total cell numbers were determined manually and distribution of T-cell subsets was determined by flow cytometry. Naive T-cell numbers in spleen and lymph nodes were previously published by Den Braber et al[12].

Thymus transplantation studies: engraftment of functional thymic lobes

Based on these observations we decided to avoid the transiently high peripheral naive T-cell densities around week 7, and studied the dynamics of RTE in mice that were at least 12 weeks of age. We performed thymus transplantations by grafting two neonatal thymic lobes underneath the kidney capsule of congenic acceptor mice. Upon harvest, visual inspection and thymocyte counts of the grafts indicated that, in general, thymic lobes were well accepted (Figure 2A-B) and had a growth pattern similar to that of a normal neonatal thymus (Figure 2B-C). In approximately 4 weeks, the total number of thymocytes from both grafted lobes was comparable to that of the thymus in a non-grafted control mouse with an age similar to that of the acceptor mouse (Figure 2C). The presence of implanted thymic lobes did not affect the thymic cellularity of the host thymus (Figure 2C). Grafts were functional and vascularized within one week after transplantation, as donor-derived T cells were detectable in the periphery of the recipient mice by day 8 post-transplantation (supplemental Figure 1A). After 4-5 weeks, the fraction of donor-derived cells among the single positive thymocytes in the graft had declined to less than 5% (supplemental Figure 1B), indicating that most donor thymocytes had been exported to the periphery, and that the grafted thymus had been populated by host cells.

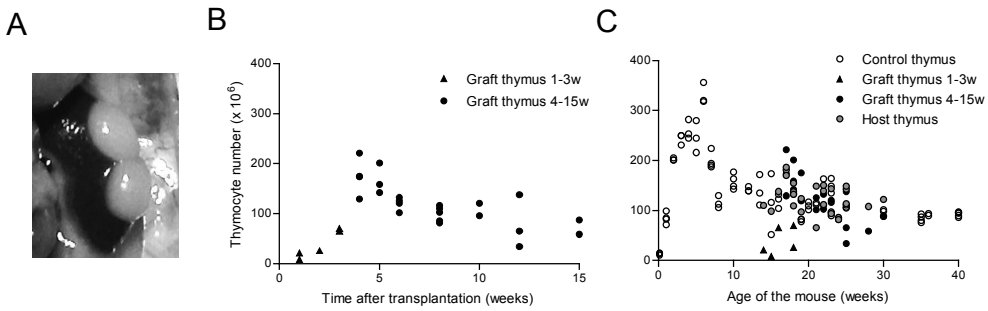

Figure 2. Successful engraftment of two thymic lobes in mice 13-15 weeks of age. Mice were sacrificed at various time points after transplantation of two neonatal thymic lobes underneath the kidney capsule. The natural thymus and thymus grafts were isolated and processed to obtain single-cell suspensions. (A) In situ picture of two grafted thymic lobes underneath the capsule of the left kidney in a mouse that was sacrificed 36 days after transplantation. (B) Total number of thymocytes in grafted thymic lobes plotted against the time post-transplantation. (C) Total number of thymocytes plotted against the age of the mouse. The thymocyte count of the natural 'host' thymus is depicted by grey filled circles (C), of grafted thymic lobes by black filled triangles (1-3wk post-transplantation, not yet full-grown) or black filled circles (full-grown graft, from 4wk post-transplantation), and of the natural thymus in control mice by open circles (B-C). From 4 weeks post-transplantation onward, thymocyte numbers were not different between full-grown thymus grafts and natural thymuses in age-matched control mice (p=0.96). The depicted value for the grafted thymus is the sum of the number of thymocytes in two grafted thymic lobes. Each data point represents one mouse.

Survival of donor-derived T cells in thymus-grafted adult mice

To investigate the dynamics of RTE, we monitored the survival of donor-derived T cells in the periphery of thymus-grafted mice from 4 weeks post-transplantation onward. To be able to make a direct comparison between our thymus transplantation results and those of Berzins et al.[8], we first analyzed our data using the same approach, i.e. by following the accumulation and survival of all $CD3^+$ donor-derived T cells in the periphery of the acceptor mice, irrespective of their $CD4^+$, $CD8^+$, naive or memory phenotype. The total number of $CD3^+$ donor-derived T cells in the periphery accumulated to a median of 8.84×10^6 cells (Figure 3A) during the first 4 weeks post-transplantation. Four weeks later (at week 8 post-transplantation), a median number of 3.27×10^6 $CD3^+$ donor-derived T cells was still present in the periphery. The estimated loss rate of 1.57×10^6 cells/week was approximately twofold slower than the decline that was observed in younger mice[8] (i.e. 3.15×10^6 per week, Figure 3A).

As we know from our previous studies that $CD4^+$ and $CD8^+$ T cells have different dynamics[12;19], we next sorted the donor-derived T cells from the thymus-transplanted mice into $CD4^+$ and $CD8^+$ and analyzed their rates of decline. Although all these cells by definition recently emigrated from the transplanted thymus, we were not sure whether these cells would still be phenotypically naive. Given the substantial population of E/M T cells observed just after birth in unmanipulated mice (Figure 1C,D) and because naive and E/M T cells have distinct kinetics[19], we decided to also phenotypically distinguish between naive and E/M $CD4^+$ and $CD8^+$ T cells in our thymus-transplanted mice. We indeed observed a substantial number of T cells with an E/M phenotype among the donor-derived T cells in both the $CD4^+$ and the $CD8^+$ T-cell compartments of the thymus-transplanted mice; at multiple time points the donor-derived E/M T-cell population was even as large as the donor-derived naive T-cell population (Figure 3B). Given these large numbers of donor-derived cells with phenotypic memory markers, it remains unclear which of the two populations – total donor-derived or donor-derived naive – provides the best estimate of the lifespan of RTE. Using the decay of donor-derived T-cell numbers from week 4 to week 8 post-transplantation (as shown in Figure 3B), we estimated that the average lifespan of donor-derived naive $CD4^+$ T cells was 23 days (95% confidence interval [CI]= 17-39), which is very similar to that of total donor-derived $CD4^+$ T cells (29 days, CI=20-51). The average lifespan of donor-derived naive $CD8^+$ T cells was 47 days (95% CI= 24-1064) and hence somewhat longer than that of total donor-derived $CD8^+$ T cells (i.e. 39 days, CI= 24-119).

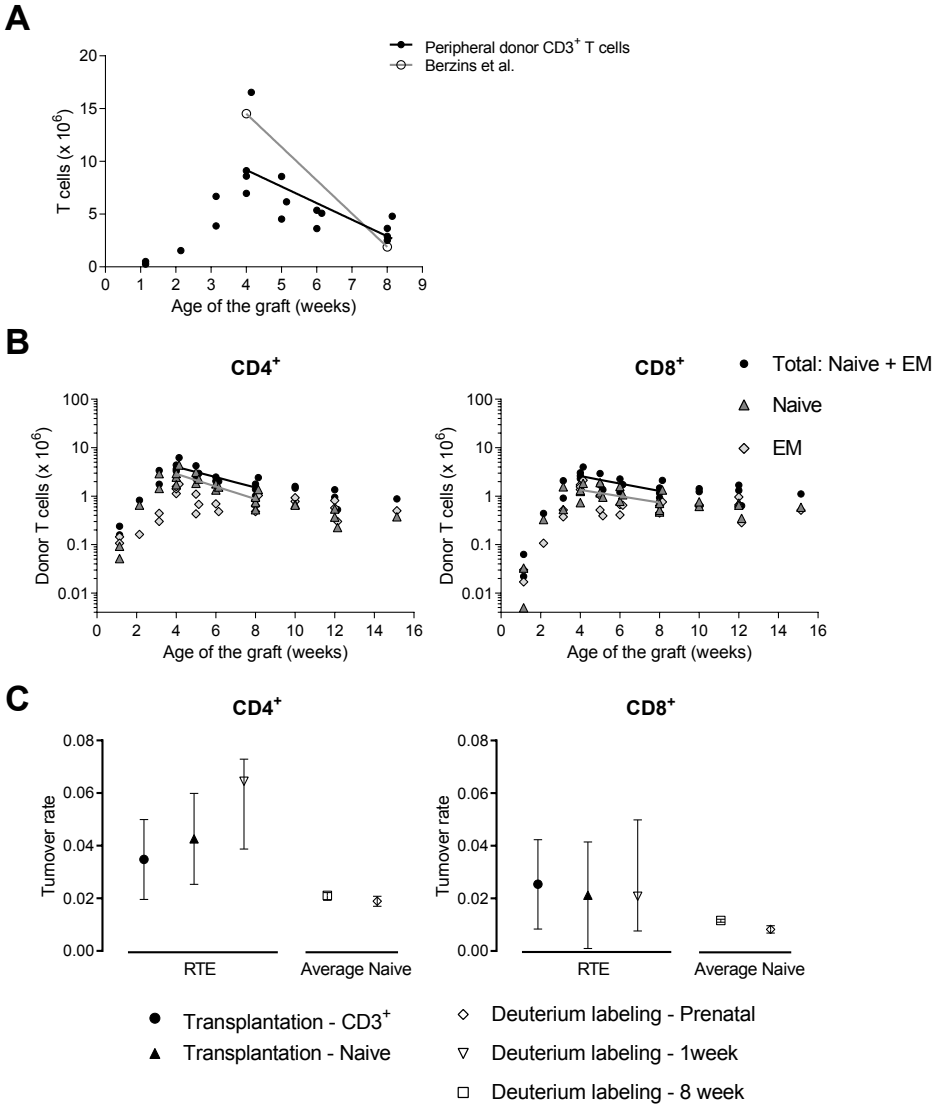

Figure 3. Dynamics of donor-derived T cells after thymus transplantation. (A) Two neonatal thymic lobes were transplanted underneath the kidney capsule of congenic 13-15 week old adult mice (our data, filled circles), or of 5-6 week old young mice (data by Berzins et al[8], open circles). Donorderived T cells were tracked over time to investigate RTE dynamics. At various time points after transplantation the fraction of donor-derived T cells in spleen and peripheral lymph nodes was determined by flow cytometry and used to calculate the absolute number of donor-derived T cells present in the periphery of the acceptor mouse. The black line (our data) and gray line (data by Berzins et al[8]) depict the best fit of an exponential decay model to the data. Filled circles each represent data of one mouse, open circles represent mean values reported for 3-10 mice[8]. (B) Number of total $CD4^+$ (left panel) and $CD8^+$ (right panel) donor-derived T cells (filled circles), naive (triangles), and effector memory (diamonds) donor-derived T cells in the $CD4^+$ (left panel) and $CD8^+$ (right panel) compartments, retrieved from the periphery of thymus-grafted mice. Exponential declines are depicted by solid lines (black for total and gray for naive subsets). (C) Turnover rates estimated by exponential decays of total donor-derived and naive T cells shown in (B) and of naive T cells from prenatal and finite term deuterium labeling data in unmanipulated mice (supplemental Figure 2).

Table 1. Lifespan of total and naive CD4+ and CD8+ T-cells estimated from thymus transplantation data and deuterium labeling.

		CD4+		CD8+	
		RTE	**Average Naive**	**RTE**	**Average Naive**
Transplantation		23 [17-39]	-	47 [24-1064]	-
Deuterium labeling	**1 wk**	15 [14-26]	-	48 [20-131]	-
	8 wk	-	47 [45-52]	-	86 [83-91]
	Prenatal	-	53 [48-59]	-	121 [104-146]

Comparing the lifespans of RTE and naive T cells in age-matched adult mice

We subsequently investigated how the lifespan of RTE compared to the average lifespan of naive T cells in control mice of similar age. The latter was based on an *in vivo* deuterium labeling study in which 12-week old mice received deuterated water (^2H_2O) for 8 weeks, during and after which the level of deuterium in the DNA of their naive T cells was measured[19]. Label was given for as long as 8 weeks to achieve a labeled cell population that would be representative for the naive T-cell pool as a whole. Alternatively, we performed a "prenatal labeling" protocol (as previously described by Westera et al.[19]), in which female mice were labeled with ^2H_2O before conception and throughout pregnancy, so that they gave birth to pups in which all cells were equally labeled. These pups received ^2H_2O until the age of 16 weeks, after which ^2H_2O was withdrawn from their drinking water. The loss of deuterium enrichment was subsequently measured to estimate the average turnover rate of their naive T cells (supplemental Figure 2 B,C). Both methods yielded comparable average turnover rates for naive $CD4^+$ and $CD8^+$ T cells of approximately 0.02 and 0.01 per day, respectively (Figure 3C), corresponding to average lifespans of 50 days for $CD4^+$ and 100 days for $CD8^+$ naive T cells (see Table 1 for respective estimates and confidence intervals).

These results suggest that the lifespans of $CD4^+$ and $CD8^+$ RTE (whether sorted for the expression of naive T-cell markers or not) are shorter than the average $CD4^+$ and $CD8^+$ naive T-cell lifespans in age-matched control mice, although for $CD8^+$ T cells the confidence intervals on the lifespans of RTE and naive T cells were overlapping.

To exclude the possibility that the difference in estimated lifespan between donor-derived T cells and unmanipulated naive T cells was caused by the presence of an additional thymus in thymus-engrafted mice, we prenatally labeled thymus acceptor mice with ^2H_2O and compared the deuterium incorporation in the DNA of recipient naive T cells to the enrichment in naive T cells of unmanipulated mice (see Materials and Methods). The level of deuterium incorporation in $CD4^+$ and $CD8^+$ naive T cells in hyperthymic mice was comparable to that in euthymic mice (Figure 4), suggesting that the additional thymus did not substantially influence the peripheral naive T-cell dynamics in thymus-grafted mice. Note that the numbers of donor-derived naive T cells in these mice were too low for measuring their deuterium enrichment.

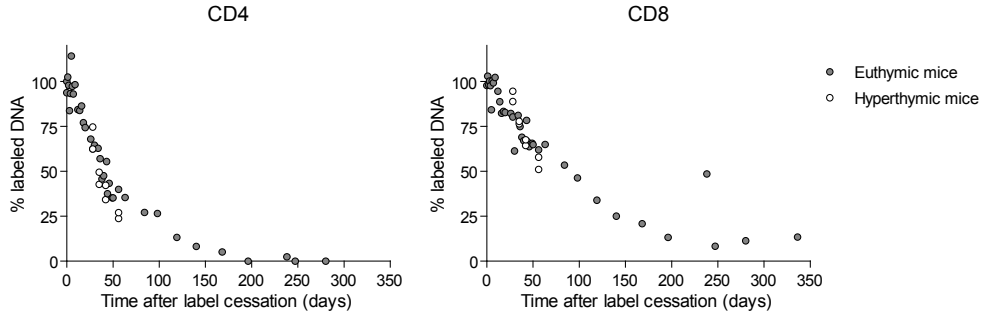

Figure 4. Prenatal deuterium labeling in thymus-grafted and unmanipulated mice. Mice were *prenatally* labeled as described in Materials and Methods. At various time points after label administration, mice were sacrificed and deuterium enrichment was determined in naive $CD4^+$ and $CD8^+$ T cells isolated from the spleen. For comparison of deuterium enrichment in naive T cells between euthymic and hyperthymic mice, donor-derived T cells were excluded from the analysis (i.e., deuterium enrichment was only measured in recipient T cells). Each data point represents one mouse.

Lifespan estimates from 1-week ^2H_2O labeling confirm RTE are relatively short-lived

As a separate confirmation that RTE form a subpopulation of short-lived naive T cells, we compared the average turnover rates of naive $CD4^+$ and the $CD8^+$ T cells in young adult mice which were either prenatally labeled, or received ^2H_2O for 8 weeks, to the loss rate of labeled cells in mice after 1 week of deuterium labeling (see Figure 3C). The rationale behind this comparison is that the labeled naive T-cell population after short-term deuterium labeling will be biased in favor of RTE, because the majority of newly produced naive T cells in mice are formed in the thymus[12]. The survival of labeled naive T cells in 1-week labeled mice turned out to be 15 days for $CD4^+$ T cells and 48 days for $CD8^+$ T cells (Table 1), which is very comparable to the RTE lifespan estimates based on the thymus transplantation experiments (Figure 3C). Taken together, these data suggest that RTE have an approximately 2-fold shorter expected lifespan than the average lifespan of naive $CD4^+$ and $CD8^+$ T cells.

Discussion

Based on a combination of thymus transplantation and *in vivo* ^2H_2O labeling in mice of similar ages, our data suggest that in young adult mice, RTE form a population of relatively short-lived cells within the naive T-cell pool. This conclusion is in line with the study by Houston et al.[4], but not with the study by Dong et al[3].

Although our thymus transplantation studies and the studies by Berzins et al[8] followed a very similar approach, the age of the acceptor mice is an important difference. While the acceptor mice in Berzins' experiments were 5-6 weeks of age when the donor thymus was transplanted, we specifically studied the survival of RTE in mice of at least 12 weeks old, as from that age onward the size of the naive T-cell pool remains relatively stable. In our

study, the loss of $CD3^+$ donor-derived T cells from 4-8 weeks after transplantation occurred at a slower rate than in the 5-6 week old mice studied by Berzins et al[8]. Since the peripheral naive T-cell pool in 5-6 week old mice is at its maximum size (Figure 1B,C), competition for survival factors due to high peripheral T-cell densities[21] may very well underlie the relatively short lifespans of donor-derived RTE in 5-6 week old mice that were observed by Berzins et al[8]. Despite the longer lifespan of RTE in 12 week old mice, RTE still had shorter expected lifespans compared to the average naive T cell in 12 week old mice.

While our data thus suggest that RTE are relatively short-lived cells, existing literature on the survival of RTE compared to mature naive T cells is conflicting. Based on a thymus-induced GFP expression system, Houston et al[4] found that after adoptive co-transfer of recently produced GFP^+ RTE and "older" GFP^- MN T cells into lymphoreplete congenic mice, the older T cells survived longer than the RTE, also suggesting that RTE are relatively short-lived naive T cells. Young RTE (GFP^+ cells in RAG2p-GFP transgenic mice) have been shown to be functionally immature in terms of proliferation and cytokine production[7;22], and it has been suggested that RTE undergo a process of post-thymic maturation[5], which may be associated with an increase in their survival capacity. Since the RTE in the study by Houston et al.[4] were isolated from 5-week old mice and the MN T cells from mice that were at least 12 weeks old, it has been suggested that the observed differences in dynamic behavior between RTE and MN T cells may in fact have been caused by the different ages of the donor mice[3]. Indeed, the expected lifespan of adoptively-transferred naive T cells has been found to increase with the age of the donor mouse, and the longer peripheral survival of "old" naive T cells was found to be associated with a lower expression of the pro-apoptotic molecule Bim[11;23]. Since the RTE in our thymus transplantation experiments came from younger donor mice than the peripheral naive T cells of the deuterium labeling studies, we cannot exclude the possibility that the age of the donor mice also played a role in the results of our thymus transplantation experiments.

Dong et al. recently followed the dynamics of final stage single positive thymocytes ("pre-RTE"), RTE and mature naive T cells, which were all isolated from donor mice of the same age[3]. In contrast to our own results and the findings by Houston et al.[4], these experiments showed that pre-RTE and RTE outcompeted co-transferred naive lymph node T cells in lymphoreplete mice over a time course of 1 week, suggesting that RTE have a survival advantage over other naive T cells[3]. Although the different observations made by Dong et al. on the one hand, and the study by Houston et al. and our own thymus transplantation study on the other hand could be related to the age of the donor mice, our own conclusion that RTE have a shorter expected lifespan than other naive T cells is not merely based on thymus transplantation studies. When we compared the survival of RTE-enriched naive T cells in a 1-week deuterium labeling study with the average turnover rate of naive T cells in prenatal or 8-week deuterium labeling studies in 12-week old mice, we again found that RTE are shorter lived than other naive T cells. Thus, even if age was excluded as a potential confounder, we still found that RTE are relatively short-lived.

Alternatively, it has been proposed[3] that the different conclusions drawn by Dong et al. and Houston et al. may be due to the different follow-up times post adoptive transfer, which was only 1 week in the study by Dong et al. and 6 weeks in the study by Houston et al. Some data suggest that the survival capacity of RTE may change within the first few weeks after thymic egress. It has been suggested that expression of early growth response gene 1 (Egr1), which is required for positive selection in the thymus, provides a survival signal to RTE ensuring their survival during the first weeks following thymic egress[24]. Thus, in the 1 week follow-up by Dong et al., pre-RTE may have persisted better than other naive T cells, because they may not yet have required survival signals, while after a longer follow-up such as in the current study and the one by Houston et al., the need for IL-7 and TCR signaling may cause RTE to be at a disadvantage in their competition with other naive T cells. Our results do not support the latter explanation, however, because the loss of donor-derived T cells from 4 weeks post thymus-transplantation onward showed no evidence of a slower decline in the first weeks. Similarly, in one of the first thymus transplantation studies[25] the initial decline of donor-thymus derived T cells was not slower than the eventual decline.

The exact signals that drive RTE maturation and survival have not yet been discovered [26]. Functional and phenotypical maturation of RTE was shown to require entry to secondary lymphoid organs and presence of dendritic cells, but was, remarkably, independent of self-peptide/MHC engagement and IL-7 signaling[27-29]. Conversely, TCR and IL7 signaling are critical for the general *survival* of naive T cells[30;31], and as the TCR repertoires of RTE and MN T cells differ[28], a certain degree of peripheral repertoire selection seems to take place at the RTE stage.

By phenotypically distinguishing between $CD62L^{+}CD44^{-}$ naive and $CD44^{+}$ effector/memory $CD4^{+}$ and $CD8^{+}$ donor-derived T cells, we observed that a substantial proportion of donor-derived T cells acquired an E/M phenotype shortly after transplantation. Why such a large fraction of RTE expresses a memory phenotype remains unclear. We observed that in neonatal unmanipulated mice, the number of E/M T cells also increased rapidly, while establishment of the naive T-cell pool was still ongoing (Figure 1B,C). Min et al. previously reported that the TCR diversity of the $CD44^{+}$ E/M T-cell population in newborns is similar to that of the naive T-cell population, suggesting that neonatal development of the E/M population is not attributed to an antigen-specific immune response[32]. As the number and diversity of memory-phenotype cells have previously been found to be similar in germ-free and conventional young adult mice, a significant proportion of the memory-phenotype population may in fact arise in the absence of foreign antigen[33]. Similarly, acquisition of a memory phenotype by donor-derived T cells after thymus transplantation may be unrelated to foreign antigen stimulation. In neonates, the lymphopenic environment was proposed to be an important trigger for expansion and differentiation of E/M T cells[32]. Our findings show that RTE also rapidly adopt an E/M phenotype in a lymphoreplete periphery, suggesting that the neonatal origin of the T cells may as well underlie their rapid phenotypic differentiation.

Indeed, neonatal RTE were recently shown to differ from adult RTE, as in neonatal mice RTE tend to leave the thymus in a more immature state[3]. Possibly, these immature RTE are more prone to express a memory phenotype, explaining the large fraction of E/M phenotype cells in both young unmanipulated mice (Figure 1) and after thymus transplantation.

In summary, our data suggest that in young adult mice with stable naive T-cell numbers, RTE form a subset of relatively short-lived naive T cells. It remains challenging to translate these insights to humans. Our deuterium labeling studies in healthy volunteers pointed out that human naive T cells are extremely long-lived, and provided little evidence for the existence of a substantial short-lived RTE compartment[14]. Moreover, we have shown that the contribution of thymic output to human naive T-cell maintenance is minor throughout adulthood[12;34]. Hence, if a short-lived RTE compartment does at all exist in humans, it must be small. Future studies on human RTE and naive T-cell dynamics in health and in disease-related or therapy-induced lymphopenia will be valuable for our understanding of the role of RTE in humans.

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References

1. Tanchot C, Rocha B. Peripheral selection of T cell repertoires: the role of continuous thymus output. J.Exp.Med. 1997;186:1099-1106.
2. Dion ML, Poulin JF, Bordi R et al. HIV infection rapidly induces and maintains a substantial suppression of thymocyte proliferation. Immunity. 2004;21:757-768.
3. Dong J, Chen Y, Xu X et al. Homeostatic properties and phenotypic maturation of murine CD4+ pre-thymic emigrants in the thymus. PLoS.One. 2013;8:e56378.
4. Houston EG, Jr., Higdon LE, Fink PJ. Recent thymic emigrants are preferentially incorporated only into the depleted T-cell pool. Proc.Natl.Acad.Sci.U.S.A 2011;108:5366-5371.
5. Fink PJ, Hendricks DW. Post-thymic maturation: young T cells assert their individuality. Nat.Rev. Immunol. 2011;11:544-549.
6. Yu W, Nagaoka H, Jankovic M et al. Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. Nature 1999;400:682-687.
7. Boursalian TE, Golob J, Soper DM, Cooper CJ, Fink PJ. Continued maturation of thymic emigrants in the periphery. Nat.Immunol. 2004;5:418-425.
8. Berzins SP, Boyd RL, Miller JF. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. J.Exp.Med. 1998;187:1839-1848.
9. Berzins SP, Godfrey DI, Miller JF, Boyd RL. A central role for thymic emigrants in peripheral T cell homeostasis. Proc.Natl.Acad.Sci.U.S.A 1999;96:9787-9791.
10. Hale JS, Boursalian TE, Turk GL, Fink PJ. Thymic output in aged mice. Proc.Natl.Acad.Sci.U.S.A 2006;103:8447-8452.
11. Tsukamoto H, Clise-Dwyer K, Huston GE et al. Age-associated increase in lifespan of naive CD4 T cells contributes to T-cell homeostasis but facilitates development of functional defects. Proc.Natl.Acad. Sci.U.S.A 2009
12. den Braber I, Mugwagwa T, Vrisekoop N et al. Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. Immunity. 2012;36:288-297.
13. Sempowski GD, Gooding ME, Liao HX, Le PT, Haynes BF. T cell receptor excision circle assessment of thymopoiesis in aging mice. Mol.Immunol. 2002;38:841-848.
14. Vrisekoop N, den Braber I, de Boer AB et al. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. Proc.Natl.Acad.Sci.U.S.A 2008;105:6115-6120.
15. Rocha B, Dautigny N, Pereira P. Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. Eur.J.Immunol. 1989;19:905-911.
16. Busch R, Neese RA, Awada M, Hayes GM, Hellerstein MK. Measurement of cell proliferation by heavy water labeling. Nat.Protoc. 2007;2:3045-3057.
17. Patterson BW, Zhao G, Klein S. Improved accuracy and precision of gas chromatography/mass spectrometry measurements for metabolic tracers. Metabolism 1998;47:706-712.
18. Westera L, Zhang Y, Tesselaar K, Borghans JA, Macallan DC. Quantitating lymphocyte homeostasis in vivo in humans using stable isotope tracers. Methods Mol.Biol. 2013;979:107-131.
19. Westera L, Drylewicz J, den Braber I et al. Closing the gap between T-cell life span estimates from stable isotope-labeling studies in mice and humans. Blood 2013;122:2205-2212.
20. Asquith B, Debacq C, Macallan DC, Willems L, Bangham CR. Lymphocyte kinetics: the interpretation of labelling data. Trends Immunol. 2002;23:596-601.
21. Freitas AA, Rocha B. Population biology of lymphocytes: the flight for survival. Annu.Rev.Immunol. 2000;18:83-111.
22. Hendricks DW, Fink PJ. Recent thymic emigrants are biased against the T-helper type 1 and toward the T-helper type 2 effector lineage. Blood 2011;117:1239-1249.

23. Tsukamoto H, Huston GE, Dibble J, Duso DK, Swain SL. Bim dictates naive CD4 T cell lifespan and the development of age-associated functional defects. J.Immunol. 2010;185:4535-4544.
24. Schnell FJ, Kersh GJ. Control of recent thymic emigrant survival by positive selection signals and early growth response gene 1. J.Immunol. 2005;175:2270-2277.
25. Wallis VJ, Leuchars E, Davies AJ. Studies of hyperthymic mice. II. The influence of thymus grafts on cell flow through the peripheral T-cell pool. Immunology 1978;35:1037-1043.
26. Fink PJ. The biology of recent thymic emigrants. Annu.Rev.Immunol. 2013;31:31-50.
27. Houston EG, Jr., Boursalian TE, Fink PJ. Homeostatic signals do not drive post-thymic T cell maturation. Cell Immunol. 2012;274:39-45.
28. Houston EG, Jr., Fink PJ. MHC drives TCR repertoire shaping, but not maturation, in recent thymic emigrants. J.Immunol. 2009;183:7244-7249.
29. Houston EG, Jr., Nechanitzky R, Fink PJ. Cutting edge: Contact with secondary lymphoid organs drives postthymic T cell maturation. J.Immunol. 2008;181:5213-5217.
30. Surh CD, Sprent J. Homeostasis of naive and memory T cells. Immunity. 2008;29:848-862.
31. Takada K, Jameson SC. Naive T cell homeostasis: from awareness of space to a sense of place. Nat. Rev.Immunol. 2009;9:823-832.
32. Min B, McHugh R, Sempowski GD et al. Neonates support lymphopenia-induced proliferation. Immunity. 2003;18:131-140.
33. Younes SA, Punkosdy G, Caucheteux S et al. Memory phenotype CD4 T cells undergoing rapid, nonburst-like, cytokine-driven proliferation can be distinguished from antigen-experienced memory cells. PLoS.Biol. 2011;9:e1001171.
34. Westera L, van Hoeven V, Drylewicz J et al. Lymphocyte maintenance during healthy aging requires no substantial alterations in cellular turnover. Aging Cell 2015;14:219-227.
35. R Development Core Team. R: A language and environment for statistical computing. 2010. Ref Type: Generic

Supplemental Materials

Mathematical modeling of prenatal ^2H_2O data

Following Westera et al.[19], the availability of deuterium in plasma at any moment in time was calculated by fitting the following equations:

(i) For finite-term labeling experiments:

$S(t) = f(1-e^{-\delta t}) + S_0 e^{-\delta t}$ during label intake ($t \le \tau$), (eq1)

$S(t) = \left[f(1-e^{-\delta\tau}) + S_0\, e^{-\delta\tau}\right] e^{-\delta(t-\tau)}$ after label intake ($t > \tau$). (eq2)

(ii) For prenatal labeling experiments: $S(t) = \beta e^{-\delta t}$. (eq3)

In these equations, *S(t)* represents the fraction of ^2H_2O in plasma at time *t* (in days), *f* is the fraction of deuterium in the drinking water, labeling was stopped at $t = \tau$ days, δ represents the turnover rate of body water per day, S_0 is the plasma enrichment level attained after the i.p. ^2H_2O boost at day 0 of finite-term labeling experiments, and β the plasma enrichment level at the start of the de-labeling period in the prenatal labeling experiments. The best fits to the plasma data are shown in Westera et al[19].

Labeling data from naive T-cells were fitted using a multi-exponential model in which each sub-population *i* contains a fraction α_i of cells with turnover p_i per day. Assuming a steady state for each kinetic subpopulation, the fraction of labeled deoxyribose residues of adenosine in the DNA of each subpopulation *i* was modeled by the following differential equation:

$$\frac{dL_i}{dt} = p_i c S(t) - p_i L_i . \quad \text{(eq4)}$$

For naive T cells, T-cell production may occur both in the thymus and in the periphery. The fraction of labeled DNA in the total T-cell population under investigation was subsequently derived from $L(t) = \sum_i \alpha_i L_i(t)$ and the average turnover rate *p* was calculated as $p = \sum_i \alpha_i p_i$.

For finite-term labeling experiments, the analytical solutions are:

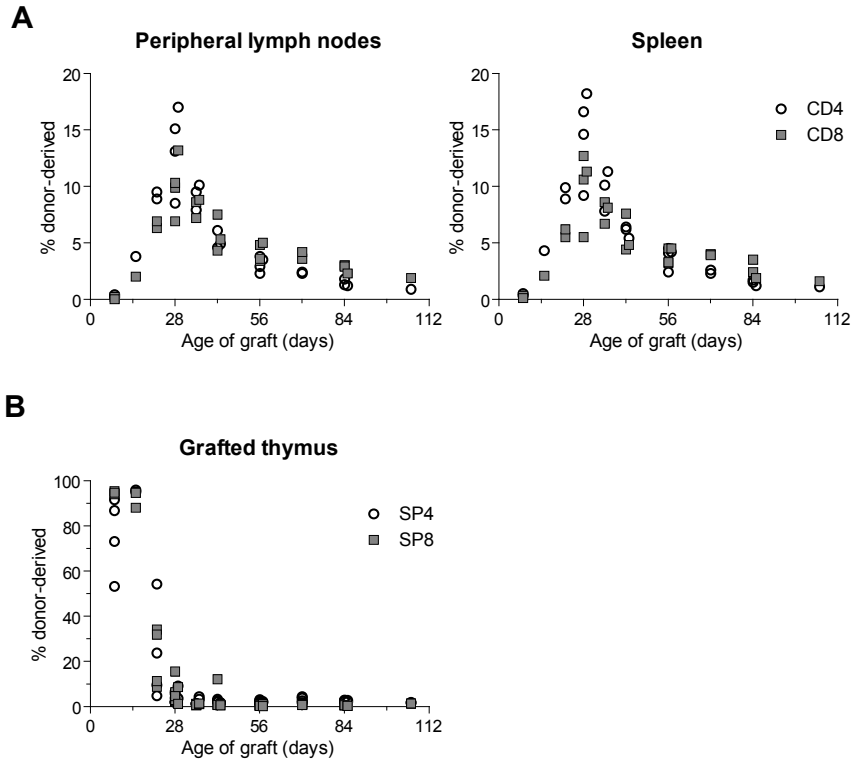
if $t \le \Delta$: $L_i(t) = 0$,

if $\Delta < t \le \tau$: $L_i(t) = \frac{c}{\delta - p_i}\left[p_i(S_0 e^{-p_i(t-\Delta)} - S(t-\Delta)) + f(1-\mathrm{e}^{-p_i(t-\Delta)})\right]$, (eq5)

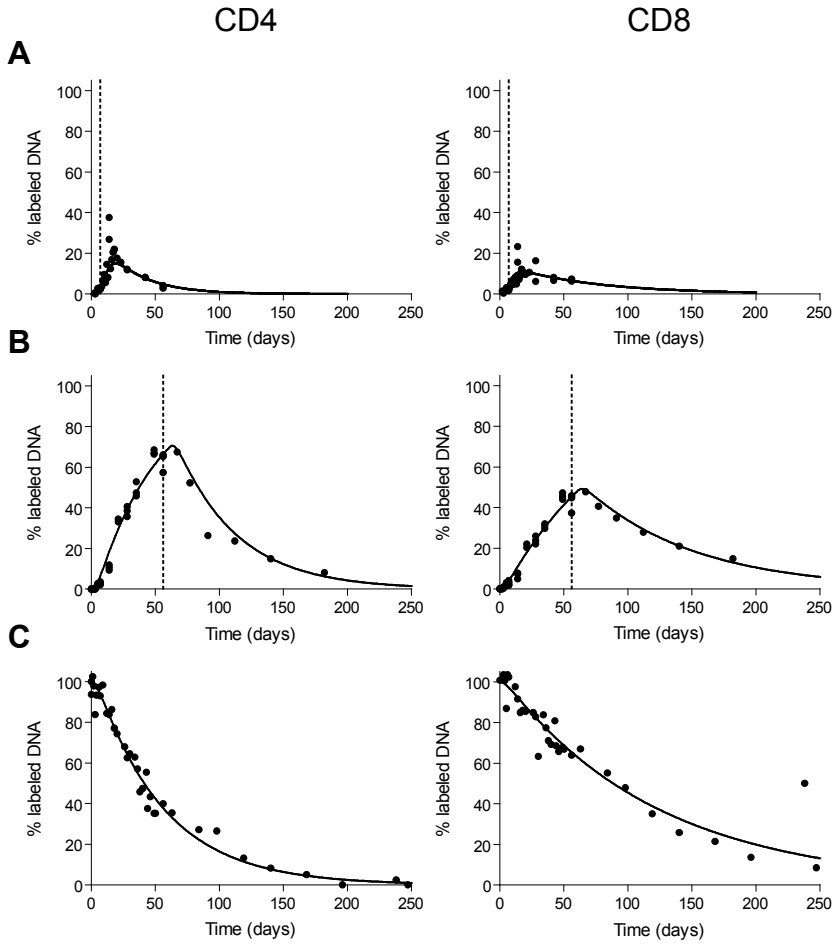
if $t > \tau + \Delta$: $L_i(t) = \frac{p_i c}{\delta - p_i}\left[S(\tau)\mathrm{e}^{-p_i(t-\Delta-\tau)} - S(t-\Delta)\right] + L_i(\tau)\, e^{-p_i(t-\Delta-\tau)}$. (eq6)

For prenatal labeling experiments, the analytical solution is: $L_i(t) = \frac{\beta c}{\delta - p_i}\left[\delta e^{-p_i t} - p_i e^{-\delta t}\right]$.

Best fits were determined by minimizing the sum of squared residuals using the R[35] function nlminb. The 95% confidence intervals were determined using a bootstrap method where the residuals to the optimal fit were resampled 500 times.



Supplemental Figure 1. Distribution of donor-derived T cells. The percentage of donor-derived T cells was determined at various time points after thymus transplantation in the $CD4^+$ and $CD8^+$ naive T-cell pool in peripheral lymphoid organs (A) and among $CD4^+$ and $CD8^+$ single positive (SP) thymocytes in grafted thymus lobes (B). In (A) each data point is derived from a single mouse and in (B) each symbol represents the percentage of donor-derived thymocytes in a single thymus lobe.



Supplemental Figure 2. Deuterium labeling of naive T cells. Percentage of deuterium-labeled DNA in $CD4^+$ (left panels) and $CD8^+$ (right panels) naive T cells. (A) Data obtained from 1 week labeling experiments in ~12 week old mice which were previously published by Westera et al[19]. (B) Data obtained from 8 week labeling experiments in ~12 week old mice which were previously published by Westera et al[19]. (C) Data obtained from prenatally labeled, unmanipulated mice. Sample processing and naive T-cell sorting is described in Materials and Methods. The solid lines represent the best fit of a single-exponential model in (A) and of a multi-exponential model to the data in (B) and (C).

2

3

Lymphocyte maintenance during healthy aging requires no substantial alterations in cellular turnover

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Abstract

In healthy humans, lymphocyte populations are maintained at a relatively constant size throughout life, reflecting a balance between lymphocyte production and loss. Given the profound immunological changes that occur during healthy aging, including a significant decline in T-cell production by the thymus, lymphocyte maintenance in the elderly is generally thought to require homeostatic alterations in lymphocyte dynamics. Surprisingly, using *in vivo* $^{2}H_{2}O$ labeling we find similar dynamics of most lymphocyte subsets between young adult and elderly healthy individuals. As the contribution of thymic output to T-cell production is only minor from young adulthood onward, compensatory increases in peripheral T-cell division rates are not required to maintain the T-cell pool, despite a tenfold decline in thymic output. These fundamental insights will aid the interpretation of further research into aging and clinical conditions related to disturbed lymphocyte dynamics.

Introduction

Advanced aging is associated with greater susceptibility to infections, reduced vaccine efficacy, and a higher incidence of cancer and autoimmune disease[1;2]. This is believed to be at least partly due to aging of the immune system. Immunological aging is a process characterized by several micro-environmental and cellular changes in the hematopoietic system which collectively affect both the production and functioning of the peripheral blood lineages. Particularly in the adaptive immune system profound age-associated changes take place at the cell population level, both in the T-cell and B-cell pools.

In the human peripheral T-cell pool, immunological aging is reflected by a numerical decline of naive T cells, loss of T-cell receptor diversity, and changes in T-cell subset distribution[3-7]. The widely held view is that these changes are caused by a combination of life-long exposure to various pathogens and the gradual involution of the thymus, an irreversible process during which functional thymic tissue becomes progressively replaced by fat[8]. Also the total number of circulating γδ T cells has been reported to decline with age, which is mainly due to a reduction in the most dominant Vδ2 subset [9;10]. The composition of the γδ T-cell population changes with age, with a skewing towards differentiated effector cells in the elderly[11]. The observation that absolute γδ T-cell numbers in young adults thymectomized during early childhood were similar to those in healthy controls[12] suggests that the decline of γδ T cells during healthy aging may be independent of thymic involution. An alternative explanation could, however, be that thymic tissue may have regrown[13]. Despite large inter-individual variation in peripheral B-cell numbers, studies have uniformly reported a numerical decline of total $CD19^+$ B cells with age[14;15]. How different B-cell subsets are affected by aging is less clear, with conflicting literature reporting e.g., decreased or unchanged naive B-cell numbers and increased or decreased memory B-cell numbers[14;15]. There is no unambiguous evidence for declining B-cell production by the bone marrow in humans with age, although an age-related reduction in B-cell progenitors has been reported by a few studies[16-18] and a pronounced loss of B-cell receptor repertoire diversity was observed in some elderly[19], which could reflect decreased bone marrow output.

The occurrence of lymphopenia-induced proliferation in rodents[20-22] has suggested that the immune system has an intrinsic capacity to maintain cell numbers at sufficiently high levels by inducing a compensatory homeostatic response when cell numbers are low. Because such responses rely on a general principle of cellular competition for limiting resources (e.g., stimulatory signals from endogenous peptide/MHC complexes and cytokines such as IL-7[23]), it is thought that similar compensatory mechanisms can also be called into action in humans, for example in response to lymphopenia in HIV infection or following stem cell transplantation (SCT), and in response to thymic involution during aging. Indeed, previous aging studies have reported increased percentages of proliferating $Ki\text{-}67^+$ naive T cells in the elderly, correlating with reduced naive T-cell pool size, and hence suggestive of a homeostatic increase in cell

production triggered by low numbers[24;25]. Aging is, however, a complex, multifactorial process with a highly variable impact on health status, which makes it difficult to determine to what extent chronological age contributes to age-associated changes in the immune system. Here, we selected only individuals with a particularly good health status, and used *in vivo* labeling with deuterated water (^2H_2O) to quantify the turnover rates of naive, memory, and natural effector B cells, naive and memory $CD4^+$ and $CD8^+$ T cells, and $\gamma\delta$ T cells in young and elderly healthy individuals. In contrast to the analysis of Ki-67 expression, providing a snapshot of the fraction of cells dividing at a single moment, labeling with ^2H_2O allowed us to record lymphocyte turnover over a longer period of time, thereby providing a very robust and reliable tool to quantify these dynamics. By combining the parameters obtained by ^2H_2O labeling and TREC analysis in a mathematical model devised previously by den Braber *et al.*[26], we also quantified to what extent thymic output declines during healthy aging, and whether and how this decline is compensated for by peripheral homeostatic mechanisms. Our data show that the turnover rates of almost all lymphocyte subsets hardly change during healthy aging. Only naive $CD8^+$ T cells had a significantly faster turnover in elderly individuals, which was related to a larger fraction of $CD95^+$ T cells in older individuals. Despite the observation that $CD4^+$ T-cell production by the thymus declines at least tenfold between the third and seventh decade of life, we find no signs of peripheral compensation for this loss of naive T-cell production.

Materials and Methods

Subjects and in vivo ^2H_2O labeling. Five young and ten elderly healthy volunteers (Table 1) were enrolled in the study after having provided written informed consent. On day 1, volunteers received an oral ramp-up dose of 7.5ml of ^2H_2O (99.8% enriched, Cambridge Isotope Laboratories, Tewksbury, MA, USA) per kg body water, in small portions throughout the day. Body water was assumed to be 60% (males) and 50% (females) of body weight [42]. Blood was drawn before the first portion and urine was collected after the last portion. As maintenance dose, volunteers drank 1.25ml/kg body water at home daily for the duration of the labeling period (9 weeks; for logistic reasons the labeling period was ~7.5 weeks and ~10 weeks for two subjects). Urine was collected an additional 15 times during the first ~100 days of the study. Blood was drawn 6 more times during labeling and 8 times during delabeling, with the last withdrawal ~1 year after stop of ^2H_2O administration. All volunteers were healthy and did not take drugs (a questionnaire was taken to confirm that subjects were healthy and did not have serious illnesses (e.g., malaria; cancer) in the past; serological testing was performed to exclude infection with HIV, HBV, and HCV). To determine CMV serostatus, CMV-specific IgG antibodies were determined in plasma by ELISA according to manufacturer's instructions (IBL International GmbH). For the purpose of analyzing the T-cell compartment,

in particular CD95 expression on naive T cells, additional blood samples were specifically collected from healthy volunteers not following the labeling protocol after having provided written informed consent. This study was approved by the medical ethical committee of the University Medical Center Utrecht and conducted in accordance with the Helsinki Declaration of 1975, revised in 2008.

Cell isolation, flow cytometry and sorting. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque (GE Healthcare, Little Chalfont, UK) density gradient centrifugation from heparinized blood. Granulocytes were obtained by erythrocyte lysis of the granulocyte/erythrocyte layer. Total PBMC were frozen as a sample with baseline enrichment on the first study-day (t=0).

Absolute cell numbers were determined using TruCOUNT tubes (BD Biosciences, San Jose, CA, USA), in which whole blood was stained using CD45-PerCP, CD3-FITC (BioLegend, San Diego, CA, USA), CD8-V500 (BD Biosciences, San Jose, CA, USA), CD4-APC-eF780, and CD19-eFluor450 (eBioscience, San Diego, CA, USA). After erythrocyte lysis with FACS Lysing Solution (BD Biosciences, San Jose, CA, USA) tubes were instantly analyzed.

CD95 expression on $CD27^+CD45RO^-$ naive T cells was measured using CD3-eFluor450, CD27-APCeFluor780 (eBioscience, San Diego, CA, USA), CD8-PerCP (BioLegend, San Diego, CA, USA), CCR7-APC (R&D systems, Minneapolis, MN, USA), CD45RO-PE-Cy7, CD95-APC, and CD28-FITC (BD Biosciences, San Jose, CA, USA). To analyze the expression of cell-cycle marker Ki-67,cells were stained with extracellular markers (CD3-eFluor450, CD4-APCeFluor780 (eBioscience, San Diego, CA, USA), CD8-PerCP, CD27-PE (BioLegend, San Diego, CA, USA), CD45RO-PE-Cy7, CD95-APC (BD Biosciences, San Jose, CA, USA)), fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences, San Jose, CA, USA), and stained intracellularly with Ki-67-FITC (DAKO, Glostrup, Denmark). Washing steps were done using Perm/Wash buffer (BD Biosciences, San Jose, CA, USA). Absolute numbers of cell subsets (e.g. $CD95^+$ naive) were calculated using the absolute number of $CD4^+$, $CD8^+$ T cells or $CD19^+$ B cells from TruCount analysis. All cells were analyzed on an LSR-II flow cytometer using FACSdiva software (BD Biosciences, San Jose, CA, USA).

For sorting of T-cell subsets, cells were incubated with CD3-FITC, CD4-Pacific Blue, CD8-PerCP-Cy5.5, CD45RO-PE (BioLegend, San Diego, CA, USA), and CD27-APC (eBioscience, San Diego, CA, USA). For sorting of B-cell subsets and $\gamma\delta$ T cells, cells were incubated with CD3-eFluor450, CD27-APC (eBioscience, San Diego, CA, USA), CD19-PerCP (BioLegend, San Diego, CA, USA), F(ab')2 IgM-FITC (Southern Biotech, Birmingham, AL, USA), and TCR-pan-$\gamma\delta$-PE (Beckman Coulter, Brea, CA, USA). Naive ($CD27^+CD45RO^-$) and memory ($CD45RO^+$) $CD4^+$ and $CD8^+$ T cells, or naive (IgM^+CD27^-), memory (IgM^-CD27^+), and natural effector (IgM^+CD27^+) $CD19^+$ B cells and pan$\gamma\delta^+$ T cells were sorted on a FACSAria II cell sorter (BD Biosciences, San Jose, CA, USA). Flow cytometric analysis and sorting were always done on freshly isolated cells. Representative density dot plots and the gating strategy for all sorted subsets are shown in supplemental Figure 1.

DNA isolation. Genomic DNA was isolated from granulocytes, total PBMC (t=0) and sorted cells using the Blood QuickPure kit (Macherey-Nagel, Dueren, Germany) or the Reliaprep Blood gDNA Miniprep System (Promega, Madison, WI, USA), and stored at -20°C before processing for gas chromatography/mass spectrometry (GC/MS).

TREC analysis. In sorted naive $CD4^+$ T-cell samples of elderly individuals, signal joint TREC numbers and DNA input were quantified with a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and calculated as described previously[43].

Measurement of deuterium enrichment in body water and DNA. Deuterium enrichment in DNA from granulocytes and sorted T-cell fractions was measured according to the method described by Busch *et al*. with minor modifications[44]. Briefly, DNA was enzymatically hydrolyzed into deoxyribonucleotides and derivatized to penta-fluoro-triacetate (PFTA) before injection (DB-17MS column, Agilent Technologies, Santa Clara, CA, USA) into the gas chromatograph (7890A GC System, Agilent Technologies, Santa Clara, CA, USA). PFTA was analyzed by negative chemical ionization mass spectrometry (5975C inert XL EI/CI MSD with Triple-Axis Detector, Agilent Technologies, Santa Clara, CA, USA) measuring ions m/z 435 and m/z 436. Deuterium enrichment in urine was analyzed on the same GC/MS system (using a PoraPLOT Q 25x0.32 column, Varian Medical Systems, Palo Alto, CA, USA) by electron impact ionization as previously described[45]. For quantification of ^2H enrichment, standard solutions with known enrichment (Tracer-to-Tracee ratios ([M+1]/[M+0]) 0, 0.0016, 0.0032, 0.0065, 0.0131, 0.0265, 0.0543, and 0.1140) were made by mixing 1-^{13}C-deoxyadenosine (Cambridge Isotopes Inc.; generates an 'M + 1' ion) with unlabeled deoxyadenosine (Sigma). To correct for abundance sensitivity of isotope ratios, we followed the approach proposed by Patterson *et al.*[46] on log10-transformed enrichment data.

Statistical analyses. Our study was designed to detect a 2-fold or greater difference between groups with a power of 80%, based on a 2-sided test with an error α of 5%. The power calculation was based on our previous deuterium labeling studies among healthy young individuals[27], which revealed a mean turnover of naive T cells of 0.00042 per day with a standard deviation of 0.00015 per day, and a mean turnover of memory T cells of 0.0056 per day with a standard deviation of 0.0028 per day, and assuming similar standard deviations at old age. Of note, with the group sizes that were used even 50% differences should be detected with a power larger than 70%.

Medians were compared between age groups using Mann-Whitney tests (GraphPad Software, Inc, La Jolla, CA, USA). Differences with a p-value<0.05 were considered significant. Correlations were analyzed using Pearson's correlation coefficient. Deuterium-enrichment data were fitted with the functions nlme and nlm in R. The 95% confidence intervals were determined using a bootstrap method where the residuals to the optimal fit were resampled 500 times.

Results

Individuals and follow-up

To quantify the dynamics of different leukocyte subsets in healthy aging, five young individuals (plus five from a previous study by Vrisekoop *et al.*[27]) and ten elderly individuals were enrolled in a heavy water (^2H_2O) labeling study (Table 1). During a 9-week labeling period, and a subsequent delabeling period of approximately 1 year, we frequently collected blood samples for measurement of deuterium enrichment in the DNA of granulocytes, B-cell subsets, total γδ T cells, and αβ T-cell subsets (for details on sort gating strategy, see supplemental Figure 1). The average turnover rate, i.e., the percentage of a cell population that is replaced by new cells per day, was estimated from the enrichment data using a multi-exponential model, which takes into account that populations can contain cells with different turnover rates[28;29]. The enrichment curves of all leukocyte subsets were normalized to the estimated maximum level of label incorporation in peripheral blood granulocytes, as this cell population is known to turn over rapidly. The dynamics of granulocytes were similar between young and elderly individuals (supplemental Figure 2).

Table 1. Subject characteristics.

Age group	Subsets	ID	Symbol	Age at start protocol	Gender
Young[27]	T cells				
		A	○	24	M
		B	□	22	M
		C	△	25	M
		D	◇	20	M
		E	▽	22	M
Young	B cells, γδ T cells				
		Y01	○	24	F
		Y02	□	23	F
		Y03	△	21	M
		Y04	◇	20	M
		Y05	▽	21	M
Aged	T cells				
		A01	●	66	M
		A02	■	72	M
		A04	▲	68	M
		A07	◈	68	M
		A09	▼	69	M
Aged	B cells, γδ T cells				
		A03	●	67	F
		A05	■	66	M
		A06	▲	75	F
		A10	◆	69	F
		A11	▼	67	F

Dynamics of naive, memory and natural effector B cells

To investigate whether aging is associated with alterations in peripheral B-cell dynamics we first determined the absolute number and distribution of the three main B-cell subsets present in the circulation, i.e., naive (IgM^+CD27^-), memory (IgM^-CD27^+), and natural effector (IgM^+CD27^+) B cells. For none of the subsets we found significant age-related differences in absolute B-cell counts, although the interindividual variation in naive B-cell numbers in the aged was relatively large (Figure 1A, Table 2). As there are indications that B-cell production by the bone marrow declines with age[16-18], B-cell numbers may stay constant because of compensatory changes in peripheral B-cell dynamics. Fitting the multi-exponential model to the enrichment data of each individual (supplemental Figures 3 and 4) revealed that the average turnover rates of all B-cell subsets in elderly individuals were not significantly different from those in young individuals (Figure 2A, Table 2). The interindividual variation in memory B-cell turnover rates between young subjects was rather large; which was not related to the relative abundance of the different B-cell subsets in these individuals. In conclusion, variation in B-cell dynamics between individuals seemed to be related to factors other than aging.

Table 2. Median (range) of average turnover rates, cell numbers, and total daily production of the different lymphocytes.

	Average turnover rate (%/day) median (range)		**Cell number (per µl blood)** median (range)		**Total production (x10^6 cells/day)** median (range)	
	Young	*Aged*	*Young*	*Aged*	*Young*	*Aged*
Naive B cells	0.23 (0.16-0.36)	0.34 (0.16-0.37)	108 (85-117)	170 (13-196)	67 (33-101)	78 (11-167)
Mem B cells	2.29 (0.42-3.89)	0.69 (0.20-1.15)	18 (9-33)	26 (14-47)	85 (17-136)	36 (19-75)
Nat Eff B cells	0.47 (0.42-0.70)	0.44 (0.26-0.61)	21 (12-39)	14 (11-63)	31 (12-41)	19 (7-54)
γδ T cells	0.52 (0.14-1.21)	0.20 (0.05-7.86)	38 (25-53)	30 (3-78)	33 (18-120)	15 (5-53)
Naive $CD4^+$ T cells	0.04 (0.03-0.07)	0.07 (0.05-0.16)	534 (307-898)	337 (192-417)	89 (20-137)	40 (31-138)
Naive $CD8^+$ T cells	0.03 (0.03-0.05)	0.09 (0.05-1.86)	254 (185-484)	31 (21-54)	24 (15-39)	7 (6-98)
Mem $CD4^+$ T cells	0.60 (0.22-1.02)	0.45 (0.44-0.80)	403 (221-448)	470 (339-546)	391 (221-911)	542 (393-936)
Mem $CD8^+$ T cells	0.53 (0.23-0.98)	0.36 (0.26-9.12)	90 (53-165)	113 (55-153)	109 (70-227)	107 (35-3497)

Dynamics of γδ T cells and naive and memory CD4⁺ and CD8⁺ T cells

We compared absolute numbers of naive ($CD27^+CD45RO^-$) and memory ($CD45RO^+$) $CD4^+$ and $CD8^+$ αβ T cells and γδ T cells in young and elderly individuals (Figure 1B and C, Table 2). Naive T-cell numbers tended to be lower in elderly subjects; this difference was significant for $CD8^+$ (p-value=0.008; Figure 1C) but not for $CD4^+$ (p-value=0.06) naive T cells. The number of memory $CD4^+$ T cells was significantly higher in elderly subjects (p-value=0.008), whereas the number of memory $CD8^+$ T cells was not different in young and aged individuals (Figure 1D). We observed considerable inter-individual variation in the number of γδ T cells, but did not find a significant difference between the age groups (Figure 1B). Within the γδ T-cell pool, the fraction of $V\delta2^+$ cells was not different in young and elderly subjects (median values were 40% for young, and 57% for elderly individuals).

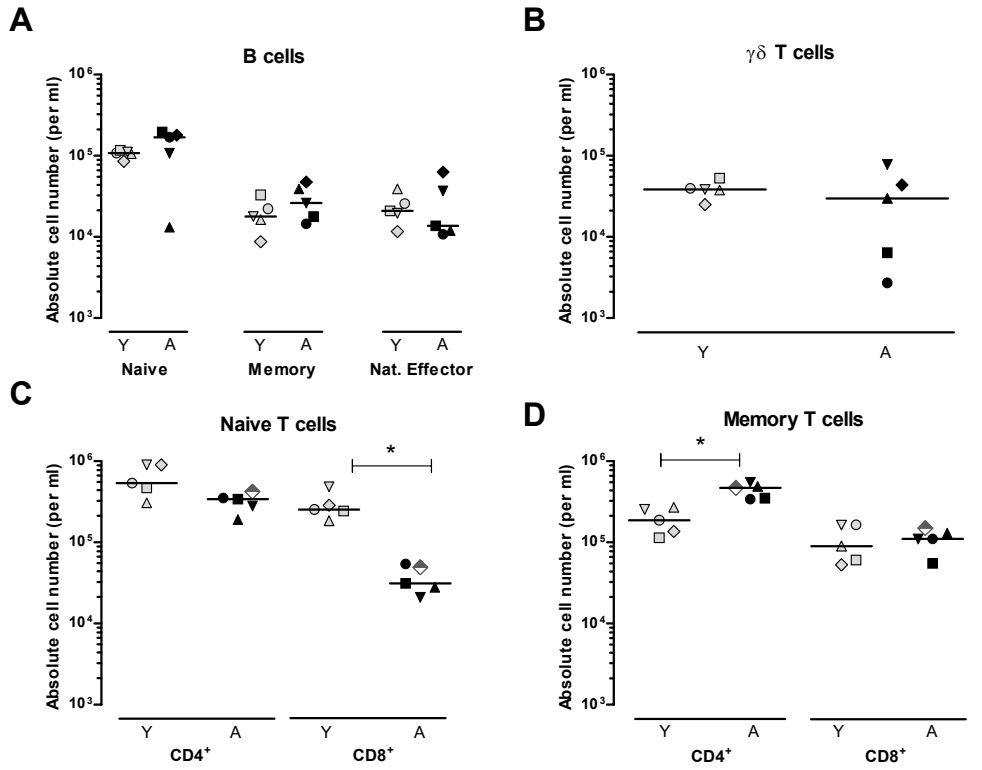

Figure 1. Absolute numbers of B cells and T cells in young and elderly individuals. Absolute numbers (per ml of blood) of (A) naive, memory, and natural effector B cells, (B) γδ T cells, (C) naive $CD4^+$ and $CD8^+$ T cells, and (D) memory $CD4^+$ and $CD8^+$ T cells, in young (gray symbols) and aged (black symbols) individuals. The elderly male tested seropositive for CMV is depicted by a semi-filled diamond (C+D). Horizontal lines represent median values. Asterisks mark significant differences (p-value<0.05) between young and aged individuals. Different symbols indicate different individuals (see Table 1) within panels A+B and within panels C+D; note that different individuals were included for analysis of B-cell subsets and γδ T cells (A+B) and T-cell subsets (C+D).

As changes in the size of the T-cell subsets might be related to changes in their dynamics, we quantified their turnover rates. For γδ T cells, fitting the multi-exponential model to the deuterium-enrichment data (supplemental Figure 3 and 4) yielded no different average turnover rates between young and aged individuals (Figure 2B, Table 2). For memory T cells, the average turnover rates estimated from the enrichment data (Westera *et al.*[29] and supplemental Figure 5) were also not different between young and elderly subjects (Figure 2D, Table 2). Hence, the age-related increase in memory $CD4^+$ T-cell numbers was not concomitant with altered turnover rates.

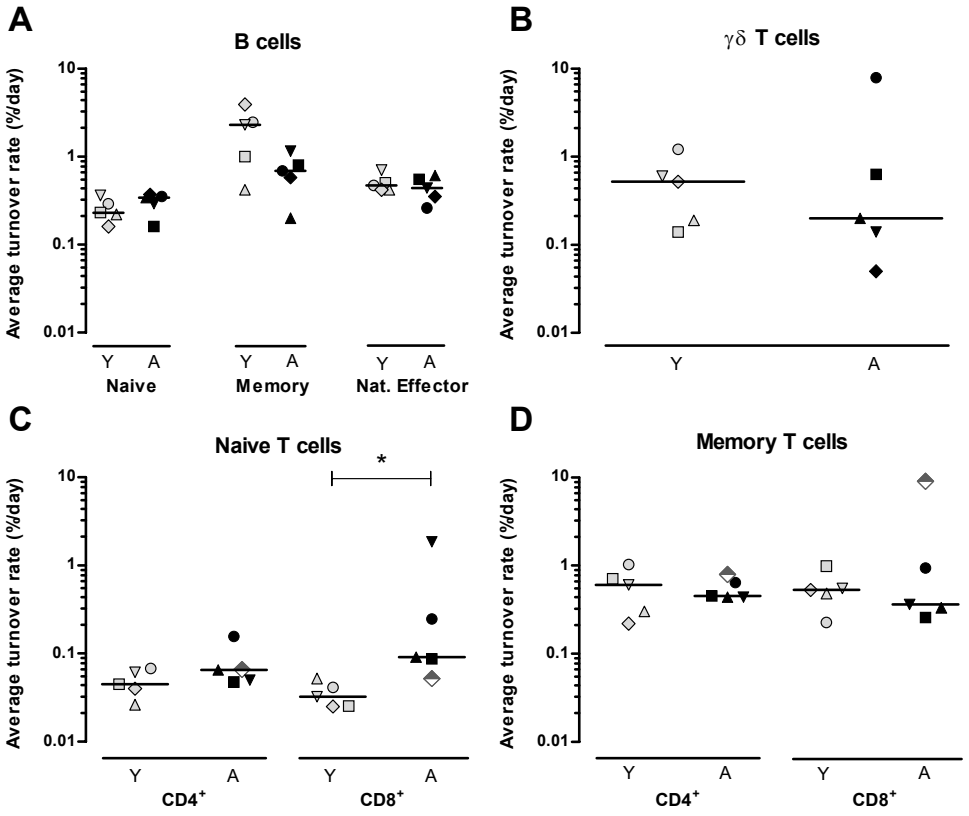

Figure 2. Summary of estimated average turnover rates in young and elderly individuals. Estimates of the average turnover rate of (A) naive, memory, and natural effector B cells, (B) γδ T cells, (C) naive $CD4^+$ and $CD8^+$ T cells, and (D) memory $CD4^+$ and $CD8^+$ T cells, in young (gray symbols) and aged (black symbols) individuals. The elderly male tested seropositive for CMV is depicted by a semi-filled diamond (C+D). All estimates were obtained by fitting the multi-exponential model to the individual data sets (see Supplemental Information). Horizontal lines represent median values. The asterisk marks a significant difference (p-value<0.05) between young and aged individuals. Individual fits are shown in supplemental Figures 3-5. Different symbols indicate different individuals within panels A+B and panels C+D.

Deuterium enrichment in naive T cells tended to be higher in elderly than in young individuals (Figure 3A), especially in the case of naive $CD8^+$ T cells, suggesting a faster turnover for this subset in elderly individuals. Fitting the multi-exponential model to the enrichment data of each individual (supplemental Figure 5) revealed that the average turnover rate of naive $CD4^+$ T cells was not significantly different between the age groups (median p_{young} = 0.04% and p_{aged} = 0.07% per day, p-value = 0.2), whereas the average turnover rate of naive $CD8^+$ T cells was significantly higher in elderly subjects (median p_{young} = 0.03% and p_{aged} = 0.09% per day, p-value = 0.02; Figure 2C, Table 2). Even though this increase should be interpreted with caution in light of the small group sizes and the large interindividual variation in the elderly group, the data show that the turnover rate of naive $CD8^+$ T cells in 4 out of the 5 elderly individuals was higher than in the young (Figure 2C).

Turnover-associated changes in the naive $CD8^+$ T-cell pool in elderly subjects

To study whether the increased turnover of naive $CD8^+$ T cells concurred with other alterations within this subset we analyzed the naive T-cell pools of the elderly subjects in more detail. Because samples of the young subjects who received ^2H_2O were no longer available, we also analyzed the composition of the naive $CD8^+$ T-cell pool in 41 additional healthy controls of different ages. In both young and elderly individuals, naive $CD4^+$ and $CD8^+$ T cells had a high expression of CCR7 and CD28 (median values for naive $CD4^+$: 98% $CCR7^+$ and 98% $CD28^+$; for naive $CD8^+$: 95% $CCR7^+$ and 96% $CD28^+$), confirming their naive phenotype. However, we found substantial age-related differences in the percentage of naive $CD8^+$ T cells expressing CD95 (Figure 3B). Whereas naive $CD4^+$ T cells had a low expression of CD95 at any age (generally around 5% $CD95^+$), the percentage of naive $CD8^+$ T cells expressing CD95 increased over age, comprising between 18% and 36% in the elderly subjects (Figure 3B), and appeared to be inversely correlated with the number of naive T cells (for $CD4^+$: r=-0.37, p-value=0.01; for $CD8^+$: r=-0.54, p-value<0.0001; Figure 3C). To investigate whether the relatively large $CD95^+$ fraction of naive $CD8^+$ T cells in elderly subjects could have contributed to the faster turnover rate of the aged naive $CD8^+$ T-cell pool, we measured the expression of the cell-cycle marker Ki-67 in the $CD95^-$ and $CD95^+$ fractions of the naive $CD4^+$ and $CD8^+$ T-cell pools. Indeed, the percentage of Ki-67 expressing cells was significantly higher among $CD95^+$ compared to $CD95^-$ cells (p-value=0.03 for both $CD4^+$ and $CD8^+$; Figure 3D). Thus, the larger fraction of rapidly proliferating $CD95^+$ cells could explain the observed increase in turnover of the "naive" $CD8^+$ T-cell pools of the elderly individuals.

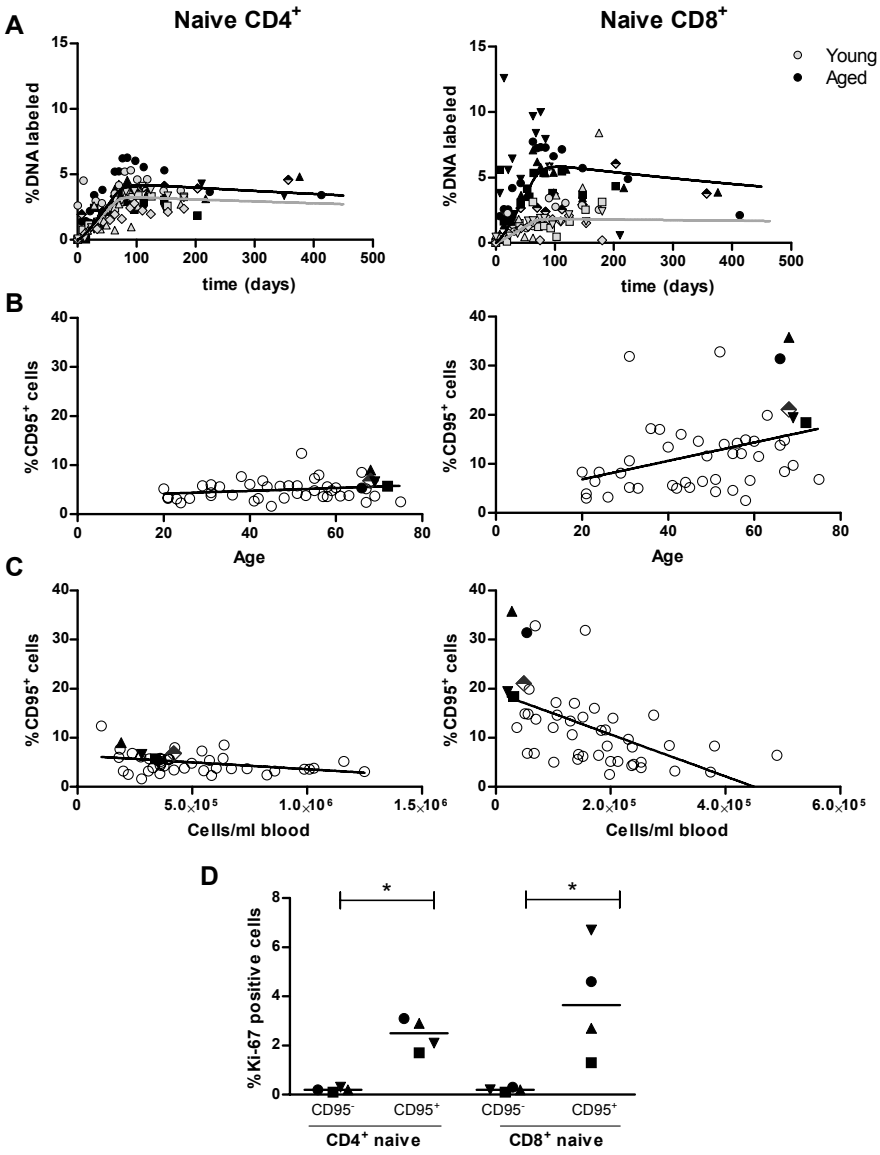

Figure 3. Analysis of ^{2}H enrichment and CD95 expression within the naive T-cell pools. (A) Best fits of the mixed effect multi-exponential model (see Supplemental Information) to ^{2}H enrichment in the DNA of naive $CD4^{+}$ and $CD8^{+}$ T cells from young[27], gray symbols and curve) and elderly individuals (black symbols and curve). Label enrichment was scaled between 0 and 100% by normalizing for the maximum enrichment in granulocytes. (B,C) The expression of CD95 on naive $CD4^{+}$ (left) and $CD8^{+}$ (right) T cells was determined in elderly males (black symbols) of the $^{2}H_{2}O$ labeling study and in other healthy donors of varying ages (open symbols, n=41). The percentage of $CD95^{+}$ naive T cells was plotted against age (B) and against the number of naive $CD4^{+}$ or $CD8^{+}$ T cells per ml blood (C). The elderly male who tested seropositive for CMV is depicted by a semi-filled diamond (B+C). The lines in panels C and D represent linear regression analyses. (D) Ki-67 expression was measured within the $CD95^{-}$ and $CD95^{+}$ fractions of naive $CD4^{+}$ and $CD8^{+}$ T cells in elderly males (n=4). The median is represented by a horizontal line. Different symbols indicate different individuals. Asterisks mark significant differences (p-value<0.05).

No peripheral homeostatic compensation in the naive CD4⁺ T-cell pool, despite decreasing thymic output

Because the thymus involutes with age[8], and deuterium is incorporated by new naive T cells that are produced in both the thymus and the periphery, the similar turnover rates of naive CD4⁺ T cells in our young and elderly individuals could be an indication for compensatory increases in peripheral T-cell division in the elderly. Therefore, we quantified the contribution of thymic T-cell production and peripheral T-cell division to the daily turnover of naive CD4⁺ T cells in young and elderly subjects. We previously demonstrated that daily thymic output can be deduced from the average turnover rate, the absolute cell number, and the TREC content of naive T cells[26]. Using this approach (see Supplemental Information), we estimated that thymic output declined significantly from 16 million cells per day in young individuals to less than one million cells per day in elderly individuals (p-value=0.02; Figure 4A), a change that is well in line with the previously estimated 10-fold decrease in thymic output during adulthood[8]. By subtracting this estimated daily thymic output from the total daily production of naive CD4⁺ T cells (Figure 4B), we deduced the average naive CD4⁺ peripheral T-cell division rate in young and elderly subjects (see Supporting Information). Remarkably, despite the tenfold decrease in thymic output, peripheral naive CD4⁺ T-cell division rates were not significantly higher in the elderly (Figure 4C), suggesting that peripheral homeostatic compensation for loss of thymic output did either not occur or was negligible.

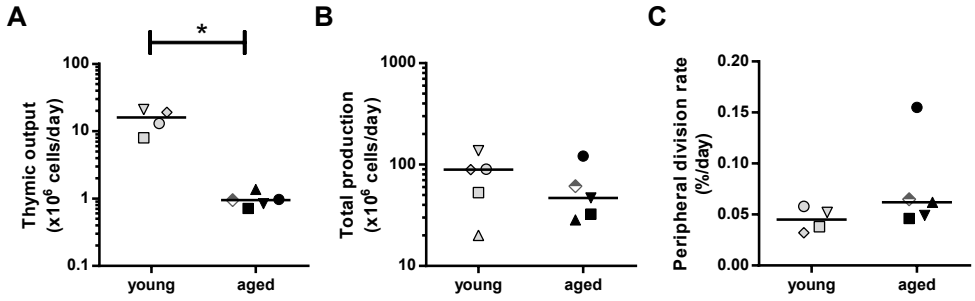

Figure 4. Contributions of daily thymic output and peripheral T-cell division to total naive CD4⁺ T-cell production. (A) Estimated daily thymic output (cells/day) in young and aged individuals, calculated by multiplying the total daily naive CD4⁺ T-cell production by the normalized naive CD4⁺ T-cell TREC content (normalized using TREC contents of single positive CD4⁺ thymocytes), as described previously[26]. The asterisk marks a significant difference (p-value<0.05) in daily thymic output between young and aged individuals. (B) Total production of naive CD4⁺ T cells per day in young and aged individuals, calculated as (the average turnover rate *p*) x (the absolute number of naive CD4⁺ T cells per liter blood) x (5 liter blood) x 50, assuming that 2% of lymphocytes reside in the blood[47]. (C) Estimated daily peripheral division rate per cell, calculated by dividing the estimated total peripheral T-cell division (in supplemental Figure 7B) by the total number of naive CD4⁺ T cells present in the periphery. The normalized TREC content of naive CD4⁺ T cells in young individuals was previously reported in den Braber *et al.*[26]; as no TREC measurements were obtained for individual C, this individual was not included in the analysis. TREC contents are shown in supplemental Figure 7A. Different symbols indicate different individuals. The elderly male who tested seropositive for CMV is depicted by a semi-filled diamond.

Discussion

Using long-term *in vivo* ^2H_2O labeling in healthy young and elderly individuals, we observed neither age-related differences in population dynamics nor signs of compensatory mechanisms for the population maintenance of different B-cell and T-cell subsets. Our data convincingly point out that maintenance of lymphocyte populations during healthy aging does not require substantial alterations in lymphocyte turnover. These results are consistent with a previous deuterated-glucose labeling study which reported no significant age-related difference in the turnover of total B cells[30], and we extend these insights by showing that also within the different B-cell subsets as well as for γδ T cells, turnover rates do not change during healthy aging.

Although lymphopenia-induced T-cell proliferation is clearly triggered in rodents with low T-cell numbers[20-22], there is no unambiguous evidence for the occurrence of homeostatic T-cell proliferation in primates and humans. Increased percentages of Ki-67-expressing T cells have been observed in different clinical conditions of lymphopenia including in HIV infection, after SCT, and post-thymectomy[13;31-33], and also under more physiological circumstances in aging rhesus macaques and humans[24;25;34]. In the rhesus macaque model of immune senescence, fractions of Ki-67$^+$ naive T cells were found to correlate positively with age, and negatively with the percentage of naive cells in the CD4$^+$ and CD8$^+$ T-cell pools and with TCR diversity[34]. Naive T-cell turnover rates increased exponentially when the percentage of naive T cells in the CD8$^+$ T-cell pool dropped below 4%[34], supporting the idea that a certain pool size threshold may exist below which compensatory mechanisms get activated. In humans, Naylor et al. reported an increase in CD4$^+$ T-cell division rates after the age of 70[24], and Sauce et al. observed a direct association between decreased naive T-cell numbers and increased frequencies of Ki-67$^+$ naive T cells in healthy elderly individuals aged 76 and older[25]. Although such correlations may be suggestive for the occurrence of homeostatic proliferation, it is in fact not clear whether increased cell division rates are induced by low cell numbers. What may be interpreted as a favorable homeostatic response to low cell numbers may alternatively reflect a different, perhaps even maleficent proliferative process. In fact, a third factor (related to aging) may induce both cell loss and increased lymphocyte turnover, or increased lymphocyte proliferation could even be *driving* cell loss. Increased levels of proliferation observed in HIV and SCT patients, for example, turned out to be related to immune activation or clinical events, rather than to reflect a homeostatic response to low cell numbers[32;33]. Likewise, a chronic inflammatory state associated with aging[35] may drive increased lymphocyte proliferation and lymphocyte loss.

We found a reduced pool size and an increased turnover rate of naive CD8$^+$ T cells in the aged, which was accompanied by the relative abundance of cycling CD95$^+$ T cells. As expression of CD95 has been shown to be upregulated in response to IL-7 *in vitro*[36], and IL-7 is known to play a key role in regulating proliferative responses *in vivo*[37], these CD95$^+$ cells

could in theory reflect homeostatically dividing naive $CD8^+$ T cells. However, this idea is not supported by the observation that almost all $CD95^+$ cells expressed the IL-7 receptor (> 90% $CD127^+$), which is typically downregulated upon IL-7 binding. Phenotype analyses indicated that the $CD95^+$ ($CD27^+CD45RO^-$) $CD8^+$ T-cell population contained both memory stem cells[38], expressing CCR7 and CD28, and effector-like ($CCR7^-CD28^{+/-}$) cells (for representative density dot plots, see supplemental Figure 6). These results stress that the age-related increase in naive $CD8^+$ T-cell turnover should not be interpreted as evidence for homeostatically increased naive $CD8^+$ T-cell division at old age.

Deuterium-labeling data of the naive $CD4^+$ T-cell pool gave more straightforward insights into the possible role of homeostatic compensation during healthy aging, as this population did not contain high levels of $CD95^+$ T cells at any age. We found that the average turnover rate of CD45RA+CD27+ naive $CD4^+$ T cells did not change during healthy aging, which is in line with a previous deuterated-glucose labeling study (Wallace *et al.*, 2004) which reported similar dynamics of $CD45RA^+$ $CD4^+$ T cells in healthy young and aged individuals. Remarkably, despite the significant loss of thymic output that we estimated between the 3rd and the 7th decade of life, peripheral naive $CD4^+$ T-cell division rates were not increased. Naive $CD4^+$ T-cell numbers tended to be reduced in the elderly, but this was not significant (Figure 1C). The significant drop in naive $CD8^+$ T-cell numbers and the non-significant change in naive $CD4^+$ T-cell numbers over age are perfectly in line with observations from a recent large cross-sectional study, in which aging correlated with a decline in the naive $CD4^+$ count in CMV-positive individuals, but not in CMV-negative individuals[7]. In this respect, it is important to note that 9 out of 10 of the healthy elderly individuals in our study were CMV-negative (the only CMV-positive elderly individual is marked in all figures by a semi-filled diamond). Loss of thymic output was also not compensated for by increased cell survival, as this should have been reflected by reduced naive T-cell turnover rates. Hence, our data show no signs of homeostatic compensation for reduced thymic output in the naive $CD4^+$ T-cell pool during healthy aging.

Since deuterium-labeling studies are always limited to relatively small sample sizes, one may wonder whether our study had the power to detect compensatory responses in lymphocyte turnover during healthy aging. Indeed, statements of statistical non-significance should be interpreted with caution for such small sample sizes. Our study was, however, able to detect 2-fold changes in lymphocyte turnover with a power larger than 80%, and 1.5-fold changes with a power larger than 70%. We show that the most likely explanation for our findings is that thymic output contributes so little to the total production of naive T cells even in young adults (supplemental Figure 7C), that a compensatory response for the further decline in thymic output with age is simply not required or too small to be measured. Importantly, the disadvantage of small numbers of individuals in deuterium-labeling studies is counterbalanced by the advantage that they provide very reliable quantitative estimates. This is firstly owing to the frequent sampling per individual during labeling and de-labeling

phases, and secondly because in long-term labeling studies the information on cell turnover is recorded over a period of several weeks, thereby providing a turnover estimate that is considerably less sensitive to fluctuations in cell turnover than for example a "snapshot" measurement of Ki-67 expression.

The lack of correlation between naive $CD4^+$ T-cell numbers and turnover rates that we found (supplemental Figure 8) contrasts the previously observed correlations between the fraction of Ki-67^+ naive T cells and the naive T-cell pool size in elderly rhesus macaques and humans[24;25;34]. Although we cannot formally exclude the possibility that such a correlation may have gone unnoticed because of our relatively small group sizes, we think that other reasons underlie this difference. One option is that age differences explain the contrast between the studies, as our elderly individuals were slightly younger than the subjects in the previous human aging studies[24;25]. However, we think it is more likely that other factors related to immune status underlie the previously observed correlation between T-cell counts and proliferation rates at high age. Because Ki-67 can be expressed by homeostatically dividing cells but also by naive T cells that proliferate to become memory cells, the increased Ki-67 levels observed in some elderly might reflect increased immune activation, for example due to persistent infection with CMV which has a high prevalence in the elderly, or to other factors that increase inflammation with age[35]. The increased naive T-cell turnover rates observed in aging rhesus macaques[34] are compatible with this scenario, as all monkeys turned out to be CMV-positive (dr. J. Nikolich-Zugich, personal communication). Although the number of CMV-positive elderly individuals was too low to investigate the effect of CMV in our elderly cohort, the low frequency of CMV-positive individuals among our elderly subjects is at least suggestive that CMV may play a role. Future studies among CMV-positive and CMV-negative individuals are needed to address the role of CMV in truly healthy aging. Finally, although the largely CMV-negative group of elderly individuals included in this study may not be representative for the elderly population, it provided us with the unique opportunity to study whether homeostatic mechanisms are evident in truly healthy aging in the absence of CMV as a possible confounder. We conclude that neither major age-related differences in lymphocyte turnover, nor signs of compensatory mechanisms for population maintenance are present in truly healthy aging.

Thanks to the combination of deuterium-labeling data and TREC analyses we were also able to calculate how the *absolute* number of cells produced by the thymus per day changed during healthy aging. We found that thymic output declined from 16 million cells per day in young adults to less than one million cells per day in elderly individuals, in line with the previously estimated 10-fold decrease in thymic output based on histological studies[8]. Previously, Bains *et al*.[39] also combined different techniques to estimate daily thymic output in young adults, by analyzing TREC contents and Ki-67 expression data. Remarkably, with roughly 350 million newly produced naive $CD4^+$ T cells per day, their estimate of thymic output was an order of magnitude higher than our estimated 16 million cells per day. Recent work suggests

that Bains et al. may have overestimated the fraction of dividing cells by measurement of Ki-67 expression, which appears to remain elevated for days after completion of cell division[40;41] and may thereby have indirectly overestimated daily thymic output.

In summary, we have provided reliable estimates of the average turnover rates of various B-cell and T-cell subsets in healthy young and elderly individuals, and found no signs of homeostatic compensation during *truly* healthy aging, e.g., for reduced thymic output. Our insights will aid the interpretation of past, current, and future investigations in a variety of interventions and diseases, which may reveal, for example, whether increased cell division rates in certain lymphopenic conditions reflect a favorable compensatory mechanism or rather the detrimental effect of inflammation.

Acknowledgements

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References

1. Goronzy JJ, Weyand CM. Understanding immunosenescence to improve responses to vaccines. Nat. Immunol. 2013;14:428-436.
2. Montecino-Rodriguez E, Berent-Maoz B, Dorshkind K. Causes, consequences, and reversal of immune system aging. J.Clin.Invest 2013;123:958-965.
3. Fagnoni FF, Vescovini R, Passeri G et al. Shortage of circulating naive CD8(+) T cells provides new insights on immunodeficiency in aging. Blood 2000;95:2860-2868.
4. Goronzy JJ, Lee WW, Weyand CM. Aging and T-cell diversity. Exp.Gerontol. 2007;42:400-406.
5. Lazuardi L, Jenewein B, Wolf AM et al. Age-related loss of naive T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes. Immunology 2005;114:37-43.
6. Saule P, Trauet J, Dutriez V et al. Accumulation of memory T cells from childhood to old age: central and effector memory cells in CD4(+) versus effector memory and terminally differentiated memory cells in CD8(+) compartment. Mech.Ageing Dev. 2006;127:274-281.
7. Wertheimer AM, Bennett MS, Park B et al. Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T cell subsets in humans. J Immunol 2014;192:2143-2155.
8. Steinmann GG, Klaus B, Muller-Hermelink HK. The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study. Scand.J.Immunol. 1985;22:563-575.
9. Argentati K, Re F, Donnini A et al. Numerical and functional alterations of circulating gammadelta T lymphocytes in aged people and centenarians. J.Leukoc.Biol. 2002;72:65-71.
10. Michishita Y, Hirokawa M, Guo YM et al. Age-associated alteration of gammadelta T-cell repertoire and different profiles of activation-induced death of Vdelta1 and Vdelta2 T cells. Int.J.Hematol. 2011;94:230-240.
11. Re F, Poccia F, Donnini A et al. Skewed representation of functionally distinct populations of Vgamma9Vdelta2 T lymphocytes in aging. Exp.Gerontol. 2005;40:59-66.
12. Roux A, Mourin G, Larsen M et al. Differential impact of age and cytomegalovirus infection on the gammadelta T cell compartment. J.Immunol. 2013;191:1300-1306.
13. van Gent R, Schadenberg AW, Otto SA et al. Long-term restoration of the human T-cell compartment after thymectomy during infancy: a role for thymic regeneration? Blood 2011;118:627-634.
14. Kogut I, Scholz JL, Cancro MP, Cambier JC. B cell maintenance and function in aging. Semin.Immunol 2012;24:342-349.
15. Ademokun A, Wu YC, Dunn-Walters D. The ageing B cell population: composition and function. Biogerontology. 2010;11:125-137.
16. Kuranda K, Vargaftig J, de la Rochere P et al. Age-related changes in human hematopoietic stem/progenitor cells. Aging Cell 2011;10:542-546.
17. McKenna RW, Washington LT, Aquino DB, Picker LJ, Kroft SH. Immunophenotypic analysis of hematogones (B-lymphocyte precursors) in 662 consecutive bone marrow specimens by 4-color flow cytometry. Blood 2001;98:2498-2507.
18. Ogawa T, Kitagawa M, Hirokawa K. Age-related changes of human bone marrow: a histometric estimation of proliferative cells, apoptotic cells, T cells, B cells and macrophages. Mech.Ageing Dev. 2000;117:57-68.
19. Dunn-Walters DK, Ademokun AA. B cell repertoire and ageing. Curr.Opin.Immunol. 2010;22:514-520.
20. Bell EB, Sparshott SM, Drayson MT, Ford WL. The stable and permanent expansion of functional T lymphocytes in athymic nude rats after a single injection of mature T cells. J.Immunol. 1987;139:1379-1384.
21. Miller RA, Stutman O. T cell repopulation from functionally restricted splenic progenitors: 10,000-fold expansion documented by using limiting dilution analyses. J.Immunol. 1984;133:2925-2932.

22. Freitas AA, Rocha B. Population biology of lymphocytes: the flight for survival. Annu.Rev.Immunol. 2000;18:83-111.
23. Fry TJ, Mackall CL. Interleukin-7: master regulator of peripheral T-cell homeostasis? Trends Immunol. 2001;22:564-571.
24. Naylor K, Li G, Vallejo AN et al. The influence of age on T cell generation and TCR diversity. J.Immunol. 2005;174:7446-7452.
25. Sauce D, Larsen M, Fastenackels S et al. Lymphopenia-driven homeostatic regulation of naive T cells in elderly and thymectomized young adults. J.Immunol. 2012;189:5541-5548.
26. den Braber I, Mugwagwa T, Vrisekoop N et al. Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. Immunity. 2012;36:288-297.
27. Vrisekoop N, den Braber I, de Boer AB et al. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. Proc.Natl.Acad.Sci.U.S.A 2008;105:6115-6120.
28. Ganusov VV, Borghans JA, de Boer RJ. Explicit kinetic heterogeneity: mathematical models for interpretation of deuterium labeling of heterogeneous cell populations. PLoS.Comput.Biol. 2010;6:e1000666.
29. Westera L, Drylewicz J, den Braber I et al. Closing the gap between T-cell life span estimates from stable isotope-labeling studies in mice and humans. Blood 2013;122:2205-2212.
30. Macallan DC, Wallace DL, Zhang Y et al. B-cell kinetics in humans: rapid turnover of peripheral blood memory cells. Blood 2005;105:3633-3640.
31. Borghans JA, Bredius RG, Hazenberg MD et al. Early determinants of long-term T-cell reconstitution after hematopoietic stem cell transplantation for severe combined immunodeficiency. Blood 2006;108:763-769.
32. Hazenberg MD, Stuart JW, Otto SA et al. T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). Blood 2000;95:249-255.
33. Hazenberg MD, Otto SA, de Pauw ES et al. T-cell receptor excision circle and T-cell dynamics after allogeneic stem cell transplantation are related to clinical events. Blood 2002;99:3449-3453.
34. Cicin-Sain L, Messaoudi I, Park B et al. Dramatic increase in naive T cell turnover is linked to loss of naive T cells from old primates. Proc.Natl.Acad.Sci.U.S.A 2007;104:19960-19965.
35. Macaulay R, Akbar AN, Henson SM. The role of the T cell in age-related inflammation. Age (Dordr.) 2013;35:563-572.
36. Cimbro R, Vassena L, Arthos J et al. IL-7 induces expression and activation of integrin alpha4beta7 promoting naive T-cell homing to the intestinal mucosa. Blood 2012;120:2610-2619.
37. Takada K, Jameson SC. Naive T cell homeostasis: from awareness of space to a sense of place. Nat.Rev. Immunol. 2009;9:823-832.
38. Gattinoni L, Lugli E, Ji Y et al. A human memory T cell subset with stem cell-like properties. Nat.Med. 2011;17:1290-1297.
39. Bains I, Thiebaut R, Yates AJ, Callard R. Quantifying thymic export: combining models of naive T cell proliferation and TCR excision circle dynamics gives an explicit measure of thymic output. J.Immunol. 2009;183:4329-4336.
40. Hogan T, Shuvaev A, Commenges D et al. Clonally diverse T cell homeostasis is maintained by a common program of cell-cycle control. J.Immunol. 2013;190:3985-3993.
41. de Boer RJ, Perelson AS. Quantifying T lymphocyte turnover. J.Theor.Biol. 2013;327:45-87.
42. Watson PE, Watson ID, Batt RD. Total body water volumes for adult males and females estimated from simple anthropometric measurements. Am.J Clin.Nutr. 1980;33:27-39.
43. Hazenberg MD, Otto SA, Cohen Stuart JW et al. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. Nat.Med. 2000;6:1036-1042.

44. Busch R, Neese RA, Awada M, Hayes GM, Hellerstein MK. Measurement of cell proliferation by heavy water labeling. Nat.Protoc. 2007;2:3045-3057.

45. Westera L, Zhang Y, Tesselaar K, Borghans JA, Macallan DC. Quantitating lymphocyte homeostasis in vivo in humans using stable isotope tracers. Methods Mol.Biol. 2013;979:107-131.

46. Patterson BW, Zhao G, Klein S. Improved accuracy and precision of gas chromatography/mass spectrometry measurements for metabolic tracers. Metabolism 1998;47:706-712.

47. Westermann J, Pabst R. Lymphocyte subsets in the blood: a diagnostic window on the lymphoid system? Immunol.Today 1990;11:406-410.

Supplemental Information

Mathematical modeling. To control for changing levels of ^2H in body water over the course of the experiment, ^2H enrichment in urine was fitted with a simple label enrichment/decay curve for each individual:

during label intake ($t \leq \tau$): $U(t) = f(1-e^{-\delta t}) + \beta e^{-\delta t}$ (Equation 1a)

after label intake ($t > \tau$): $U(t) = \left[f(1-e^{-\delta \tau}) + \beta e^{-\delta \tau}\right] e^{-\delta(t-\tau)}$ (Equation 1b)

as described previously[27], where U(t) represents the fraction of ^2H_2O in plasma at time t (in days), f is the fraction of ^2H_2O in the drinking water, labeling was stopped at $t = \tau$ days, δ represents the turnover rate of body water per day, and β is the plasma enrichment attained after the boost of label by the end of day 0. We incorporated these best fits when analyzing the enrichment in the different cell populations. Up- and delabeling of the granulocyte population of each individual was analyzed as described previously[27], to estimate the maximum level of label intake that cells could possibly attain. The label enrichment data of all cell subsets were subsequently scaled by the granulocyte asymptote of each individual.

Labeling data of the different leukocyte subsets were fitted with a mathematical model that allowed for kinetic heterogeneity between cells of the same population. Each kinetic subpopulation i was modelled to contain a fraction α_i of cells with turnover rate p_i. Because the population sizes hardly changed, we considered a steady state for each kinetic subpopulation (i.e., production equals loss), and label enrichment of adenosine in the DNA of each subpopulation i was modelled by the following differential equation:

$$\frac{dl_i}{dt} = p_i c U(t) \alpha_i A - p_i l_i \quad \text{(Equation 2a)}$$

where l_i is the total amount of labeled adenosine in the DNA of subpopulation i and A is the total amount of adenosine in the cell population under investigation, c is an amplification factor that needs to be introduced because the adenosine deoxyribose (dR) moiety contains multiple hydrogen atoms that can be replaced by deuterium[27], and p_i is the average turnover rate of subpopulation i. Basically, labeled adenosines in subpopulation i are gained when a deuterium atom is incorporated with probability cU(t) in the DNA of cells that replicate at rate p_i, and they are lost when cells of subpopulation i are lost at rate p_i. For naive T cells this replication may occur both in the periphery and in the thymus. Scaling this equation by the total amount of adenosine in the DNA of subpopulation i, i.e., defining $L_i = l_i/(\alpha_i A)$, yields

$$\frac{dL_i}{dt} = p_i c U(t) - p_i L_i \quad \text{(Equation 2b)}$$

throughout the up- and delabeling period, where L_i represents the fraction of labeled adenosine dR moieties in the DNA of subpopulation i. The corresponding analytical solutions are

$$L_i(t) = \frac{c}{\delta - p_i}\left[\delta f(1-e^{-p_i t}) - p_i f(1-e^{-\delta t}) + \beta\, p_i(e^{-p_i t} - e^{-\delta t})\right] \quad \text{(Equation 3a)}$$

during label intake ($t \leq \tau$), and

$$L_i(t) = \frac{c}{\delta - p_i}\left[\delta f(e^{-p_i(t-\tau)} - e^{-p_i t}) - p_i f(e^{-\delta(t-\tau)} - e^{-\delta t}) + \beta\, p_i(e^{-p_i t} - e^{-\delta t})\right] \quad \text{(Equation 3b)}$$

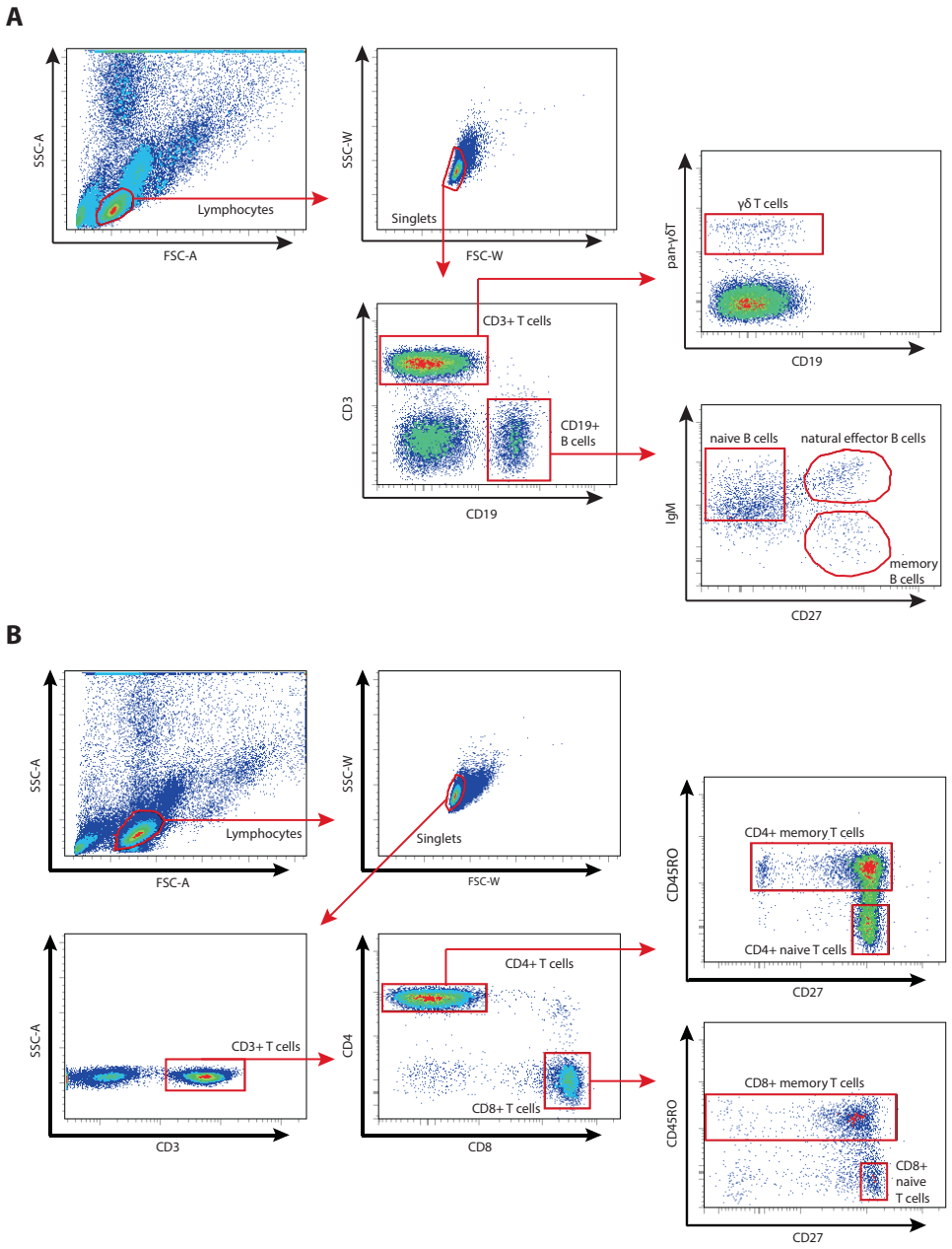
after label intake ($t > \tau$).

The fraction of labeled DNA in the total T-cell population under investigation was subsequently derived from $L(t) = \sum \alpha_i L_i(t)$, and the average turnover rate p was calculated from $p = \sum \alpha_i p_i$.

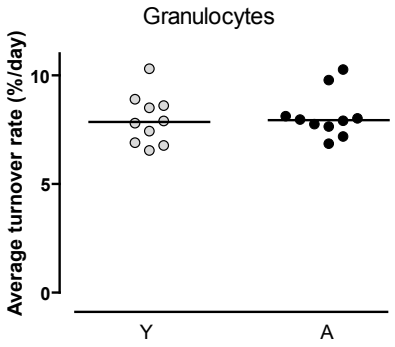
Because all enrichment data were expressed as fractions, labeling data were arcsin(sqrt) transformed before the mathematical model was fitted to the data. We followed a stepwise selection procedure to determine the number of kinetically different subpopulations to include in the model, adding a new kinetically different subpopulation into the model until the average turnover rate was no longer significantly changed[29]. For populations that appeared to behave kinetically homogeneously, the fitting procedure set the contribution of the extra subpopulation(s) to zero. Average turnover rates p of different leukocyte populations were estimated by fitting the enrichment data for each individual and average lifespans were calculated as 1/p. The enrichment data were also fitted using mixed-effects models to illustrate the difference in dynamics at the group level (Figure 3A). The average turnover rates estimated using mixed-effects models are not reported in the manuscript and did not differ from the ones we estimated by doing individual fitting. The labeling curves of memory $CD4^+$ and $CD8^+$ T-cells, as well of memory and natural effector B-cells were significantly better described by a model including two kinetically different subpopulations while the other leukocyte populations required only one.

Daily thymic output was calculated as described previously[26], based on a model describing naive T-cells and TREC dynamics in the periphery at a *cellular level* described by Hazenberg *et al.*[43]. Briefly, the total production of naive $CD4^+$T cells per day was first calculated as (the average turnover rate *p*) x (the absolute number of naive $CD4^+$T cells per liter blood) x (5 liter blood) x 50, assuming that 2% of lymphocytes reside in the blood[47]. The total daily naive $CD4^+$T-cell production was multiplied by the normalized naive $CD4^+$ T-cell TREC content (normalized using TREC contents of single positive $CD4^+$ thymocytes) to estimate the daily thymic output in cells/day. The peripheral T-cell division rate per day was obtained by subtracting daily thymic output from total daily production and dividing this value by the absolute number of naive $CD4^+$T cells. The model used to calculate T-cell turnover (from deuterium labeling data)

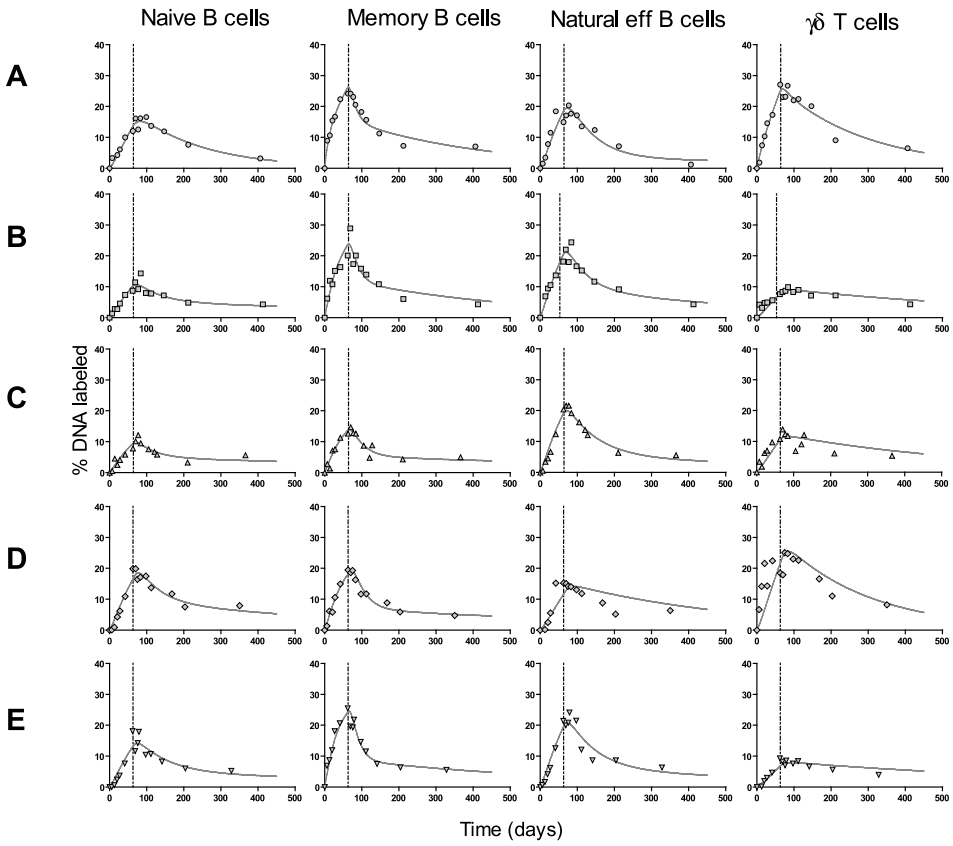
is based on the same "basic" model describing naive T-cell dynamics in the periphery but it has a different structure than the TREC model. Firstly, it describes incorporation and loss of deuterium at the *DNA level.* Secondly, it does not contain an explicit term for the thymic output because at the DNA level, one cannot distinguish between deuterium incorporated during cell division in the thymus or during peripheral T-cell division. Therefore, the model used to calculate T-cell turnover describes the dynamics of the sum of thymic output and peripheral T-cell division. Because both models are derived from the same "basic model" one can use the combined results to calculate peripheral T-cell division.



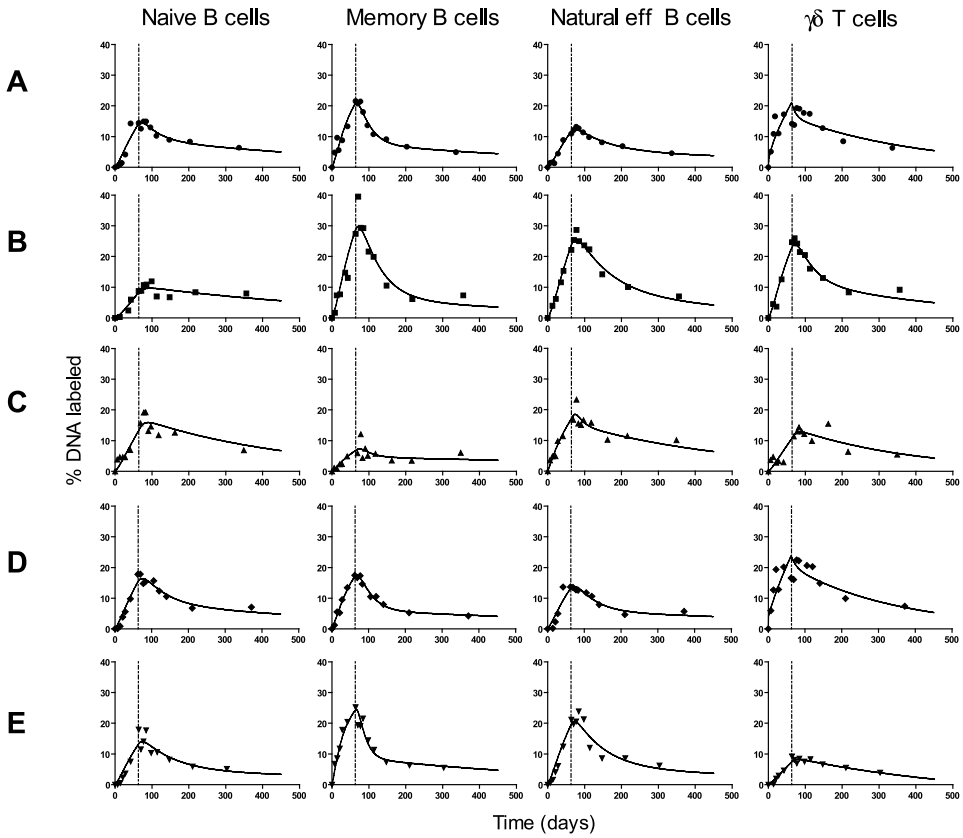
Supplemental Figure 1. Sort gating strategy. Strategy for sorting of B cells and γδ T cells (A) and for naive and memory $CD4^+$ and $CD8^+$ T cells (B).



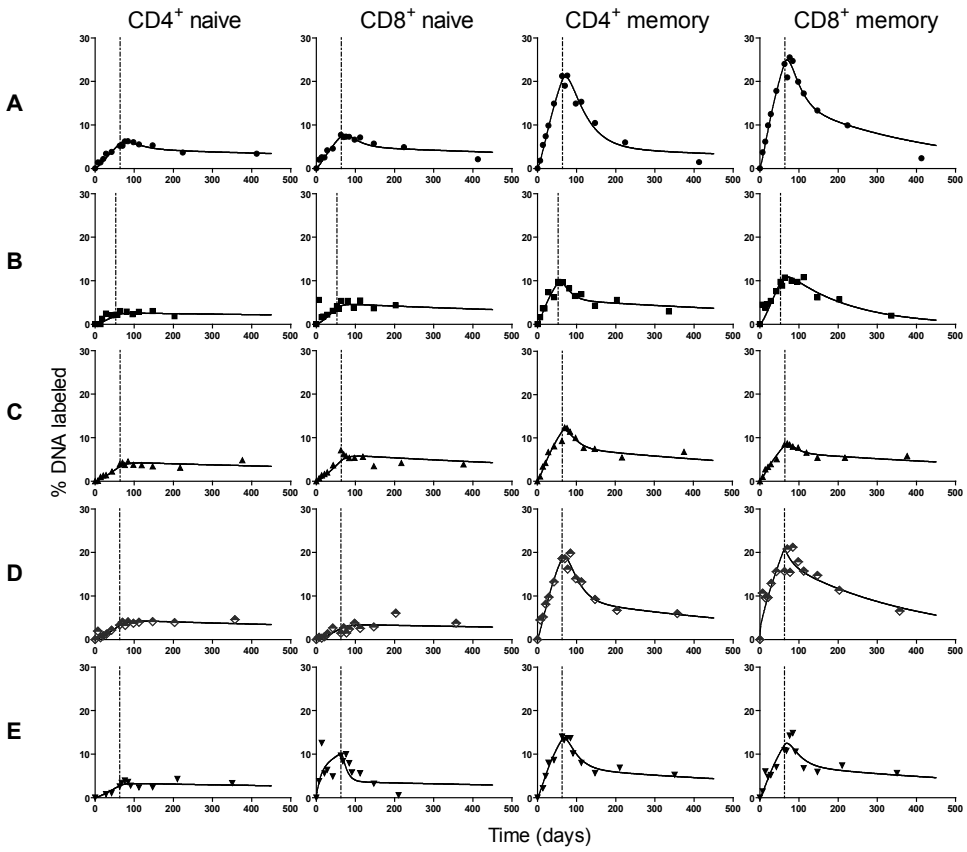
Supplemental Figure 2. Estimated average turnover rates of granulocytes in young and elderly individuals. Estimates of the average turnover rate of granulocytes in young (gray symbols) and aged (black symbols) individuals. Horizontal lines represent median values.



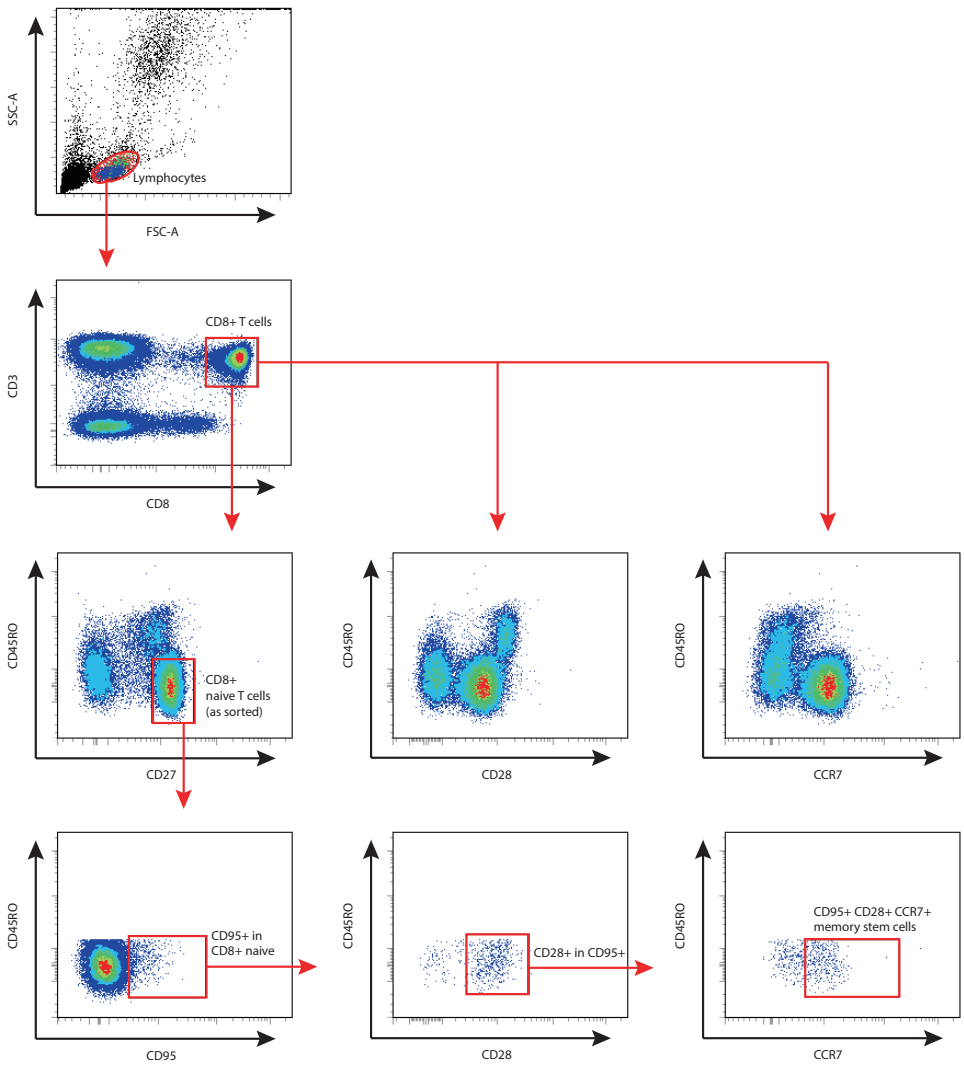
Supplemental Figure 3. **Best fits of ^2H enrichment in B-cell subsets and in γδ T cells in young individuals.** Best fits of the multi-exponential model to the enrichment in naive, memory, and natural effector (Natural eff) B cells, and γδ T cells in the five young individuals (A-E). Label enrichment in the DNA was scaled between 0 and 100% by normalizing for the maximum enrichment in granulocytes (see Supplemental Information). The end of ^2H_2O administration is marked by a dashed vertical line.



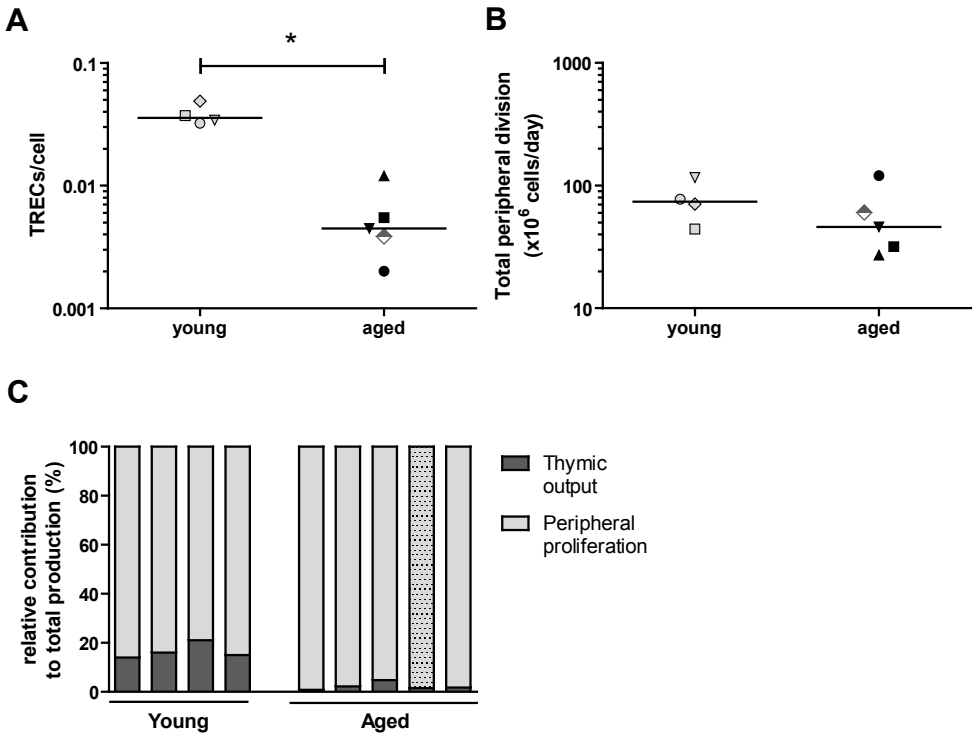
Supplemental Figure 4. Best fits of ^2H enrichment in B-cell subsets and in γδ T cells in elderly individuals. Best fits of the multi-exponential model to the enrichment in naive, memory, and natural effector (Natural eff) B cells, and γδ T cells in the five aged individuals (A-E). Label enrichment in the DNA was scaled between 0 and 100% by normalizing for the maximum enrichment in granulocytes (see Supplemental Information). The end of ^2H_2O administration is marked by a dashed vertical line.



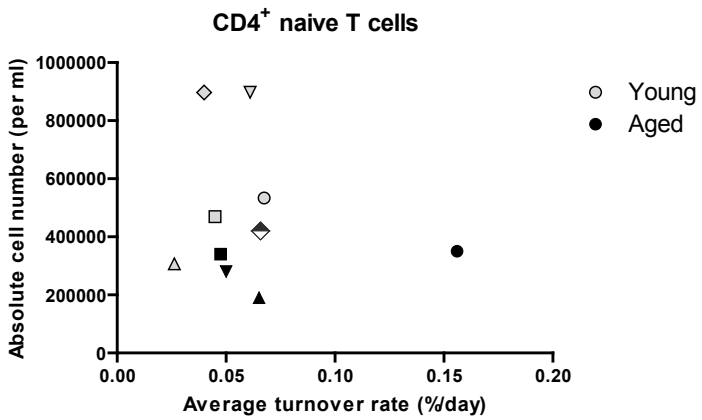
Supplemental Figure 5. Best fits of ^2H enrichment in T-cell subsets in elderly individuals. Best fits of the multi-exponential model to the enrichment in naive and memory $CD4^+$ and $CD8^+$ T cells in the five aged individuals. Label enrichment in the DNA was scaled between 0 and 100% by normalizing for the maximum enrichment in granulocytes (see Supplemental Information). The end of ^2H_2O administration is marked by a dashed vertical line. Enrichment data of the corresponding subsets in young individuals were previously published[27;29]. (D) This elderly male tested seropositive for CMV.



Supplemental Figure 6. Composition of the CD95⁺ fraction of the naive CD8⁺ T-cell pool. Representative density dot plots of flow cytometric analysis of the CD95⁺ fraction present in sorted naive CD8⁺ T cells. This specific analysis was performed for 3 young and 8 aged participants of the heavy water labeling study.



Supplemental Figure 7. TREC content and total peripheral division of naive CD4⁺ T cells in young and elderly individuals. (A) The number of TRECs/cell was determined in naive CD4⁺ cells of the five elderly individuals enrolled in this study for analysis of T cell subsets, and compared with the TREC content that was determined for four young individuals in the previous T-cell ^2H_2O study[26]. Due to limited material, TREC contents could not be reliably measured for naive CD8⁺ T cells. The asterisk marks a significant difference (p-value<0.05). (B) Estimated total peripheral division in cells per day, obtained by subtracting the estimated daily thymic output from the total naive CD4⁺ T-cell production. (A+B) The elderly male who tested seropositive for CMV is depicted by a semi-filled diamond. (C) Relative contributions of thymic output (dark gray bars) and peripheral T-cell division (light gray bars) to the total production of naive CD4⁺ T cells. The elderly male who tested seropositive for CMV is indicated by a dotted bar.



Supplemental Figure 8. No correlation between the absolute number and the turnover rate of CD4⁺ naive T cells. Absolute CD4⁺ naive T-cell counts from young and aged individuals (Figure 1C) plotted against the corresponding CD4⁺ naive T-cell turnover rate (Figure 2C). Within the naive CD4⁺ T-cell population, there is no significant correlation between the absolute cell count and average turnover rate (p-value=0.95). The elderly male who tested seropositive for CMV is indicated by a semi-filled diamond.

4

T-cell and B-cell turnover rates are homeostatically increased in response to lymphopenia after autologous stem cell transplantation

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Abstract

Based on findings in mice, it is generally thought that maintenance of lymphocyte populations relies on peripheral homeostatic mechanisms through which lymphocyte production and survival rates are increased when lymphocyte numbers are low. In fact, there is little evidence for the existence of such homeostatic mechanisms in humans. In healthy aging, we did not find any signs of homeostatic compensation for the age-related decline in thymic output, and increased T-cell proliferation after allogeneic hematopoietic stem cell transplantation (SCT) has been associated with clinical events rather than with low cell numbers. Here, we used *in vivo* $^{2}H_{2}O$ labeling to quantify the turnover of different B-cell and T-cell subsets in patients who underwent an autologous SCT, and had no signs of infectious complications. After a reconstitution period of 10-13 months, absolute numbers of naive ($CD27^{+}CD45RO^{-}$) and memory ($CD45RO^{+}$) $CD4^{+}$ T cells and memory ($IgM^{-}CD27^{+}$) and natural effector ($IgM^{+}CD27^{+}$) B cells were still low in these patients. $^{2}H_{2}O$ labeling showed that the production rates of naive and memory $CD4^{+}$ T cells as well as memory B cells in SCT patients were significantly increased. Likewise, the average TREC content of naive $CD4^{+}$ T cells in these patients was higher than in healthy controls. Using a mathematical model we demonstrate that this could be fully explained by the emptiness of the lymphocyte pool, which causes recent thymic emigrants (with high TREC contents) to be overrepresented among peripheral naive T cells, and is thus no evidence that thymus output is increased. Taken together, this work suggests that despite the slow reconstitution of lymphocytes, peripheral lymphocyte proliferation rates are in fact homeostatically increased.

Introduction

Under healthy conditions, the peripheral B-cell and T-cell populations are maintained at relatively stable numbers throughout life. It is generally thought that this is due to the action of homeostatic mechanisms, which regulate peripheral lymphocyte division or survival rates when cell numbers are subject to change, e.g. in conditions of decreased lymphopoiesis or increased cell loss. Indeed, rodent studies have suggested that in response to severe lymphopenic conditions, peripheral lymphocyte division and survival rates are increased by a homeostatic mechanism. For example, fast expansion and extended survival of B cells was observed after adoptive transfer into B-cell deficient hosts, and was related to the peripheral B-cell density of the host[1]. For T cells, adoptive cell transfer into severely lymphocyte-depleted animals has been shown to induce robust peripheral cell expansion, a phenomenon called lymphopenia-induced proliferation (LIP)[2-4].

In humans, severe lymphopenic conditions caused by various lymphocyte-depleting therapies have been associated with significantly elevated percentages of proliferating (Ki-67$^+$) CD4$^+$ and CD8$^+$ naive and memory T cells compared to non-depleted controls[5-7]. It is unclear, however, to what extent this T-cell expansion reflects a homeostatic response to low lymphocyte numbers, or an immune response triggered by therapy-related tissue damage or infectious complications. Temporal T-cell expansions occurring during the first year after allogeneic stem cell transplantation (alloSCT) were shown to be associated with clinical events that cause immune activation, such as viral infections and graft-versus-host disease (GVHD)[7]. Moreover, the percentage of Ki-67$^+$ lymphocytes was found to decline as soon as 1-3 months after rabbit anti-thymocyte globulin (rATG) treatment or alloSCT, when patients were still lymphopenic[6;7]. Hence, the occurrence of a lymphopenia-induced homeostatic response in humans is still disputable.

We have recently shown that despite a decrease in naive T-cell numbers and a declining thymic output with age, lymphocyte turnover rates do not change during healthy aging[8]. In another study, an increase in naive and memory T-cell turnover was only found after the age of 65, which was proposed to reflect increased T-cell activation rather than a homeostatic response[9]. Collectively, these studies question the existence of peripheral homeostatic mechanisms in humans. This may also explain why the reconstitution of human lymphocyte populations post-SCT is generally very slow[10-12]. In the autologous setting, in which GVHD does not occur, B-cell and CD8$^+$ T-cell numbers reach normal levels within approximately 6 months post-SCT, whereas the recovery of naive and memory CD4$^+$ T cells typically takes at least 2-3 years[13-19]. After alloSCT, the occurrence of GVHD was reported to slow down reconstitution even further, probably because of the GVHD-related damage to the bone marrow and thymic tissue[10;20]. In a recent heavy water (^2H_2O) labeling study, we have shown that in healthy individuals the renewal rate of the naive T-cell population is less than 0.1% per day, corresponding to an average naive T-cell lifespan of 6-9 years[8;21]. The slow reconstitution

of naive T cells in lymphocyte-depleted humans may thus in fact reflect the normal, slow production of naive T cells, in absence of a homeostatic response.

To elucidate whether under lymphopenic conditions, and in the absence of clinically-manifested immune activation, lymphocytes have increased rates of cell production or survival, we used *in vivo* heavy water (^2H_2O) labeling to quantify the turnover of different T-cell and B-cell subsets in patients who received an *autologous* stem cell transplantation (autoSCT) and had no signs of infectious complications. One year after autoSCT, absolute numbers of memory and natural effector B cells and $CD4^+$ T cells in these patients were still significantly lower than in healthy controls of similar age, which coincided with a significantly higher average production rate of most lymphocyte subsets. The average TREC contents of naive $CD4^+$ T cells in these patients were clearly increased, which could be fully explained by the overrepresentation of recent thymic emigrants in the relatively empty pool of these patients. Since Ki-67-expression levels were increased, our data suggest that peripheral proliferation rates are homeostatically increased when peripheral cell counts are low.

Materials and Methods

Patient characteristics and in vivo ^2H_2O labeling. Three male patients (age 54-61y) who received an autoSCT for the treatment of a hematologic malignancy were enrolled in the study after having provided written informed consent (for pre-transplant conditioning and other patient characteristics, see Table 1). Following repeated subcutaneous injections with granulocyte-colony stimulating factor (G-CSF), stem cells were obtained by leukapheresis of peripheral blood. Lymphocyte populations were not depleted from the graft prior to transplantation. Patients had reconstituted to lymphocyte levels sufficient for deuterium labeling purposes (i.e., >20 cells/μl, $CD4^+$ naive T cells in particular) and had no signs of transplantation-related complications, severe infections, inadequate liver or kidney function, or cardiovascular disease before and during the study. Use of medication during the study was unrelated to the malignancy and the SCT (Table 1). The median age of these patients was slightly younger (57 years) than that of the healthy controls that were used for comparison (68 years). This will not have affected the results of our study, since lymphocyte dynamics are hardly affected by age[8]. *In vivo* ^2H_2O labeling was performed as previously described[8] with small adaptations. Briefly, patients received an oral ramp-up dose of 7.5 ml of ^2H_2O (99.8% enriched, Cambridge Isotope Laboratories) per kg body water on the first day of the study, and drank a daily portion of 1.25 ml ^2H_2O per kg body water for 6 weeks at home. Urine samples were collected during the first 10 weeks of the study, and blood was withdrawn 4 times during the labeling period, and 5 times in the ~25 weeks thereafter. This study was approved by the medical ethical committee of the University Medical Center Utrecht and conducted in accordance with the Helsinki Declaration of 1975, revised in 2008.

Table 1: Patient characteristics

Patient	Age at start ^2H_2O	Symbol	Malignancy	Induction therapy	Conditioning Regimen	Time since SCT at start ^2H_2O (days)	Medication usage during study
L01	61	●	NHL (Mantle Cell Lymphoma)	R-CHOP*, Ara-C	BEAM**	287	Triamterene, Simvastatin
L02	54	●	MM	Thalidomide, Dexamethasone, Carfilzomib	Melphalan	420	Tamsulosin
L03	57	●	MM	Thalidomide, Dexamethasone, Adriamycin	Melphalan	358	Omeprazole

*R-CHOP = Rituximab, Cyclophosphamide, Adriamycin, Vincristin, Prednisone
**BEAM = Carmustine, Etoposide, Ara-C (cytarabin), Melphalan

Cell isolation, flow cytometry and cell sorting. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Paque (GE Healthcare) density gradient centrifugation from heparinized blood. Granulocytes were obtained by 2 cycles of erythrocyte lysis of the granulocyte/erythrocyte pellet. To determine baseline enrichment, an additional sample of total PBMCs was frozen on the first day of the study, prior to ^2H_2O intake. Absolute peripheral cell numbers were determined using TruCOUNT tubes (BD), in which whole blood was stained using CD45-PerCP, CD3-FITC (BioLegend), CD4-APC-eF780, CD19-eFluor450 (eBioscience) and CD8-V500 (BD) antibodies. After erythrocyte lysis with FACS Lysing Solution (BD) samples were instantly analyzed.

For sorting of T-cell and B-cell subsets, PBMCs were incubated with IgM-FITC (SouthernBiotech), CD3-eFluor450, CD4 APC-eFluor780, CD27-APC (eBioscience), CD8-PE, CD19-PerCP (Biolegend), and CD45RO-PE-Cy7 (BD). $CD3^+CD4^+$ and $CD3^+CD8^+$ naive ($CD27^+CD45RO^-$) and memory ($CD45RO^+$) T cells and $CD19^+$ naive (IgM^+CD27^-), memory (IgM^-CD27^+) and natural effector (IgM^+CD27^+) B cells were sorted on a FACSAria II cell sorter (BD). Peripheral proliferation of T cells and B cells was studied by analyzing the expression of Ki-67 nuclear antigen. To distinguish various T-cell and B-cell subsets, cells were first stained extracellularly with antibodies against the markers that were used for sorting. Subsequently, cells were fixed and permeabilized (Cytofix/Cytoperm, BD), and stained intracellularly with Ki-67-FITC (DAKO). Washing steps were carried out using Perm/Wash buffer (BD). All cells were analyzed on an LSR-II flow cytometer (BD) using FACSDiva software (BD). Flow cytometric analyses and cell sorting were always performed on freshly isolated material.

Measurement of ^2H enrichment in DNA and body water. DNA was isolated from PBMCs, sorted cell subsets, and granulocytes using the Reliaprep Blood gDNA miniprep system (Promega), and stored at -20°C until further processing. Deuterium enrichment in DNA samples was measured according to the GC/MS method described by Busch et al.[22] with minor modifications as previously described[8]. Briefly, DNA was enzymatically hydrolyzed into

deoxyribonucleotides and derivatized to penta-fluoro-triacetate (PFTA) before injection (DB-17MS column, Agilent Technologies) into the gas chromatograph (7890A GC System, Agilent Technologies). PFTA was analyzed by negative chemical ionization mass spectrometry (5975C inert XL EI/CI MSD with Triple-Axis Detector, Agilent Technologies) measuring ions m/z 435 and m/z 436.

After collection, urine samples were stored at -20°C until further processing. Deuterium enrichment in urine was analyzed on the same GC/MS system (using a PoraPLOT Q 25x0.32 column, Varian) by electron impact ionization as previously described[23].

Quantification of leukocyte turnover by mathematical modeling of urine and DNA enrichment data. Mathematical models were fitted to the urine and DNA enrichment data as previously described[8]. Briefly, a simple label enrichment/decay curve was fitted to the urine enrichment levels for each individual, and was incorporated in the mathematical model describing deuterium enrichments in the DNA of the different cell populations. The estimated maximum level of ^{2}H enrichment in the granulocyte population of each patient was considered to be the maximum level of label incorporation that cells could possibly attain, and was used to scale the enrichment data of the other cell subsets. The enrichment data of the different lymphocyte subsets were fitted using a multi-exponential model, in which each subpopulation *i* contains a fraction α_i of cells with turnover rate p_i per day[24]. The average turnover rate *p* of each subpopulation was subsequently calculated as $p = \sum_i \alpha_i p_i$. Total daily production of T-cell and B-cell subsets was calculated as (the average turnover rate *p*) x (the absolute number of naive T cells per liter blood) x (5 liter blood) x 50, assuming that 2% of lymphocytes reside in the blood[25].

The model that was fitted assumed constant cell numbers over the study period (i.e. equal cellular production and loss). Although this assumption is in line with our observation that there were no significant changes in cell numbers over the study period, in SCT patients one would in fact expect cell production to exceed cellular loss as long as cell subsets are reconstituting. The fact that we did not see any significant increases in cell numbers in any of the analyzed subsets is probably caused by the notoriously large variation in cell-count measurements. Importantly, our finding that the average production rates of some cell populations were increased after autoSCT is not affected by this assumption. As has previously been shown, even for cell populations that are not in steady state, this deuterium enrichment model correctly estimates the average production rate *p*[26]. Ideally, if one would have reliable estimates of the changes in cell numbers over time, one could subsequently derive the average loss rate *d* directly from the cell numbers, to study whether cell survival is also affected after autoSCT.

TREC analysis. In naive $CD4^+$ T-cell samples sorted on the first day of the study, signal joint TREC numbers and DNA input were quantified with a ViiA™ 7 Real-Time PCR System (Applied

Biosystems) as previously described[27]. The mathematical model used to interpret TREC contents is explained in the Supplemental Information.

Statistical analyses. Deuterium enrichment data were fitted with R using the nlm function[28]. The number of kinetically different subpopulations needed to describe the labeling data of each cell population was determined using the F-test. 95% confidence limits were determined by bootstrap analysis on the residuals. Simulations were performed with the software Berkeley Madonna™ (Macey & Oster, University of California, Berkeley, USA). Median values between autoSCT patients and healthy controls of similar age[8] were compared using Mann-Whitney tests (GraphPad Software, Inc). Differences with a p-value<0.05 were considered significant.

Results

Patient follow-up

To investigate whether homeostatic regulation of human lymphocyte dynamics occurs during periods of lymphopenia, we studied the turnover rate of various B-cell and T-cell subsets in 3 patients who received an autoSCT for the treatment of a hematological malignancy (for patient characteristics, see Table 1). Patients were included approximately one year after autoSCT, they received deuterated water (^2H_2O) for six weeks, and during the one year follow-up period peripheral blood samples were collected to measure the deuterium enrichment in the DNA of their granulocytes, $CD4^+$ and $CD8^+$ naive and memory T cells, and naive, memory and natural effector B cells. To quantify the average turnover rate of the different lymphocyte populations, we fitted a multi-exponential model to the deuterium enrichment data[24]. This model takes into account that cell populations can contain subpopulations with different turnover rates and corrects for possible effects of the duration of label administration, which was 6 weeks for the autoSCT patients studied here, and 9 weeks for the previously studied healthy controls[8]. For the 3 autoSCT patients, individual deuterium enrichment data and the best fits of the multi-exponential model are shown in supplemental Figure 1 for B-cell subsets and supplemental Figure 2 for T-cell subsets.

Deuterium enrichment in granulocytes

As peripheral granulocyte counts were previously shown to normalize during the first weeks post-SCT[29], we expected that deuterium incorporation in granulocytes one year post-SCT would be similar to that observed in healthy individuals. The absolute number of peripheral granulocytes was not different between autoSCT patients and healthy controls (Figure 1A). Analysis of deuterium enrichment in the DNA of granulocytes showed that label accrual and subsequent loss were indeed similar for the autoSCT patients and healthy controls (Figure 2A).

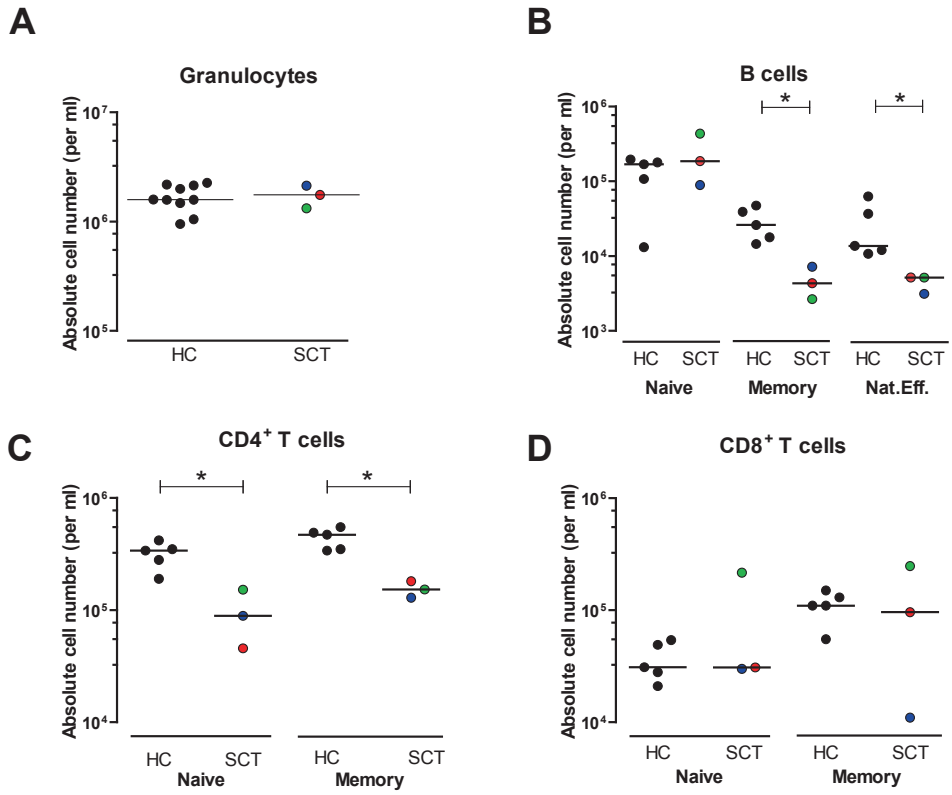

Figure 1. Absolute numbers of granulocytes, B cells, and T cells. Absolute numbers of (A) granulocytes, (B) naive, memory and natural effector (Nat.Eff.) B cells, (C) naive and memory $CD4^+$ T cells and (D) naive and memory $CD8^+$ T cells per ml of peripheral blood, in SCT patients (colored symbols) and healthy controls (HC) (black symbols). Horizontal lines represent median values. Asterisks mark significant differences (p-value<0.05) between autoSCT patients and healthy controls. Data from healthy controls were obtained from the study by Westera et al[8]. For the autoSCT data, the blue dot corresponds to patient L01, the red dot to L02, and the green dot to L03. Median values of frequent measurements performed during the ^2H_2O labeling studies were compared between healthy controls and SCT patients. For all subjects, the absolute counts of all subsets did not significantly change during the study protocol (not shown).

Increased B-cell production rates 1 year post-SCT

At approximately 1 year post-SCT, the naive B-cell subset (IgM^+CD27^-) had reached normal numbers in the blood (Figure 1B). Numbers of memory and natural effector B cells were still significantly lower than in healthy controls (p-value = 0.04 for both subsets, Figure 1B). To investigate whether these low cell counts were associated with altered B-cell dynamics, we compared the deuterium enrichment the in DNA of the B-cell subsets between autoSCT patients and healthy controls, and estimated their average turnover rates, i.e., the average fraction of each B-cell subset that is renewed per day, by the sum of bone marrow output and peripheral cell division (see supplemental Figure 1 for individual fits). In autoSCT patients, the maximum level of deuterium enrichment that was attained in most of the B-cell subsets

was higher than that in healthy individuals (Figure 2B-D), despite the fact that the label period was shorter in autoSCT patients. For memory B cells, the higher enrichment level corresponded with a 5-fold higher estimated production rate compared to healthy controls (p-value = 0.04, Figure 3A, left panel). For naive and natural effector B cells, production rates seemed to be slightly increased, but these differences were not significant (Figure 3A, left panel). These results suggest that when B-cell numbers are low, B-cell production is increased by an increase in bone marrow output, and/or in peripheral B-cell division.

Increased $CD4^+$ T-cell production rates but normal $CD8^+$ T-cell dynamics 1 year post-SCT

We next investigated whether the lymphopenic condition following autoSCT also influenced T-cell dynamics. Whereas $CD8^+$ naive and memory T-cell numbers were not significantly different between autoSCT patients and healthy individuals, $CD4^+$ naive and memory T-cell counts in the autoSCT patients were approximately 3-4 fold lower than in healthy controls (p-value = 0.04 for both subsets, Figure 1C,D). Hence, if a lymphopenia-induced homeostatic response exists in the human T-cell pool at one year post-SCT, we would most likely find it in the $CD4^+$ T-cell compartment. In line with the relatively high incorporation of deuterium by $CD4^+$ T cells in autoSCT patients (Figure 2E,F), the average production rates of naive and memory $CD4^+$ T cells were, respectively, 5-fold and 3-fold higher than in healthy controls (p-value = 0.04 for both subsets, Figure 3A, left panel; see supplemental Figure 2 for individual fits).

In the $CD8^+$ T-cell pool, naive T cells also tended to reach higher enrichment levels in autoSCT patients compared to healthy controls, while the enrichment levels of memory $CD8^+$ T cells were largely overlapping (Figure 2G,H). The estimated average production rates of $CD8^+$ T cells were, however, not significantly different between the two groups, and interindividual differences within each group were rather large (Figure 3A, right panel; see supplemental Figure 2 for individual fits). Thus, in contrast to what was observed in the $CD4^+$ T-cell pool, both cell numbers and production rates in the $CD8^+$ T-cell pool tended to be normalized after a 1-year reconstitution period.

Total daily lymphocyte production in autoSCT patients is at least as high as in healthy controls

We next used the estimated turnover rates and the median absolute cell counts per individual to calculate, for each lymphocyte subset, the total number of cells produced per day (see Materials and Methods). This estimate of *total daily production* is the sum of cells produced by the bone marrow or thymus and by peripheral cell division, the latter being dependent on the availability of cells with proliferation potential. Despite their low cell densities, total daily lymphocyte production in autoSCT patients was not significantly different from that in healthy controls for almost every lymphocyte subset, and was even significantly increased for naive B cells (Figure 3B).

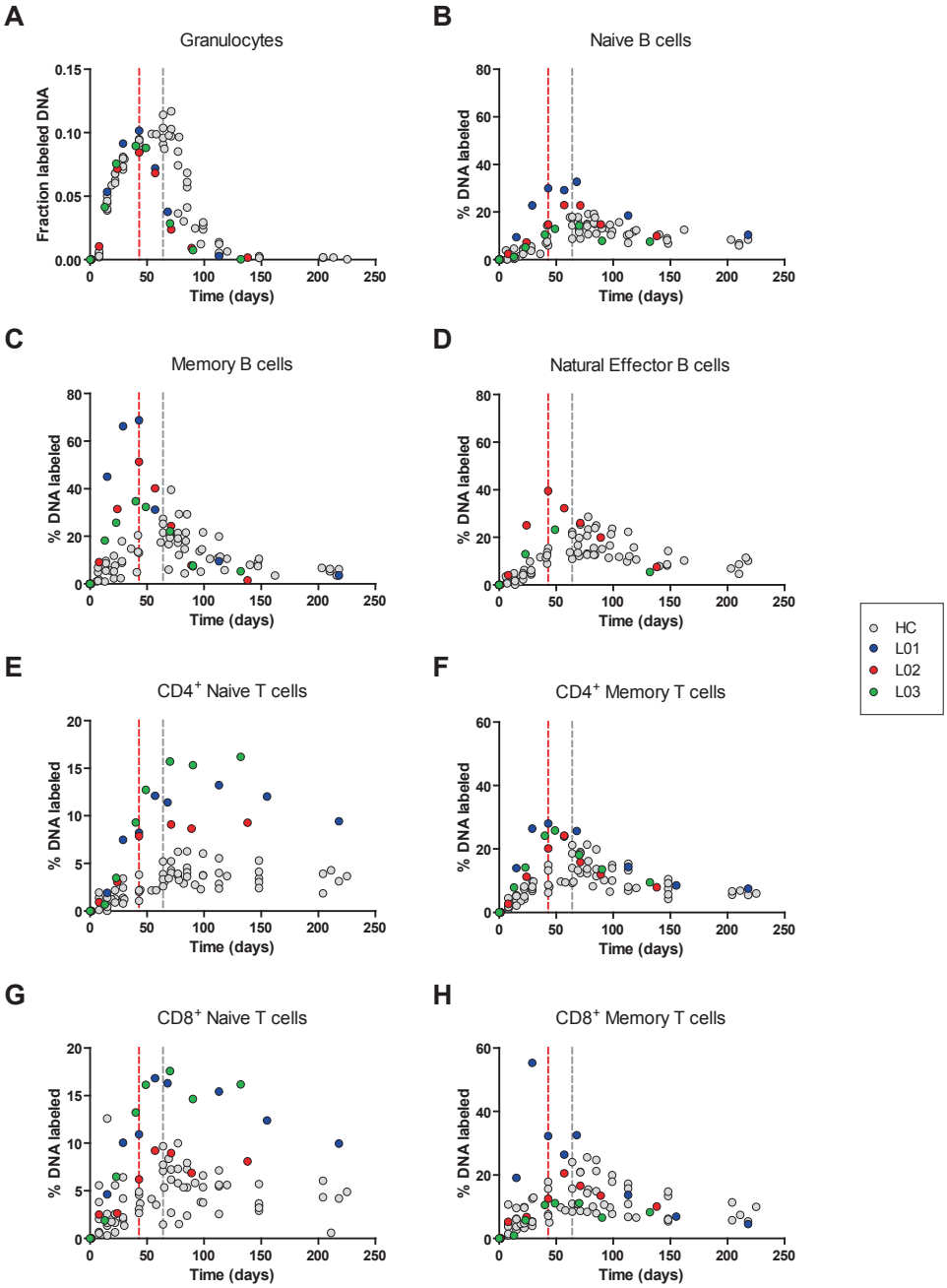

Figure 2. Deuterium enrichment in granulocytes, B-cell and T-cell subsets. Deuterium enrichment in the DNA of granulocytes (A); naive (B), natural effector (C), and memory B cells (D); and naive $CD4^+$ (E), memory $CD4^+$ (F), naive $CD8^+$ (G), and memory $CD8^+$ (H) T cells. Colored symbols indicate SCT patients and gray symbols indicate healthy controls of similar age (n=5)[8]. In patient L01, cell numbers were too low to measure deuterium enrichment in natural effector B cells. Note that for SCT patients the labeling period ended after 6 weeks (red vertical line), whereas it ended after 9 weeks (gray vertical line) for the healthy controls. Average turnover rates estimated from these curves are shown in Figure 3.

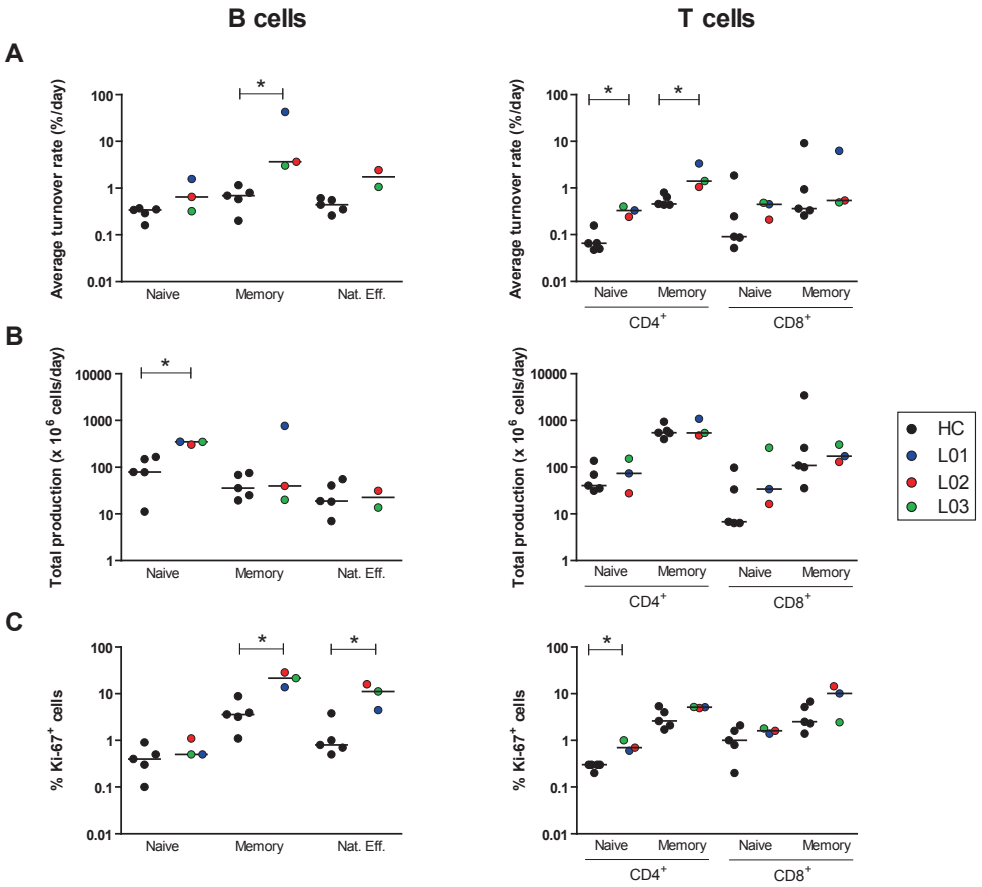

Figure 3. Summary of average turnover rates, daily production levels, and percentages of Ki-67 expression of B-cell and T-cell subsets. (A) Average turnover rates of B-cell and T-cell subsets were obtained by fitting the multi-compartment model to the enrichment curves shown in supplemental Figures 1 and 2. Deuterium enrichment levels were not measured in natural effector B cells of patient L01 due to insufficient sort yields. (B) Total daily production levels of B-cell and T-cell subsets were calculated as (the average turnover rate *p*) x (the absolute number of naive T cells per liter blood) x (5 liter blood) x 50, assuming that 2% of lymphocytes reside in the blood[25]. (C) The percentage of cells expressing Ki-67 in B-cell and T-cell subsets as determined by flow cytometry. For all panels, healthy control data were obtained from the study by Westera et al[8]. Horizontal lines represent median values. Asterisks mark significant differences (p-value<0.05) between SCT patients and healthy controls.

The contribution of thymic output and peripheral cell division

Since deuterium labeling does not distinguish between *de novo* lymphocyte production and peripheral cell division, we used additional markers to study the origin of the increased lymphocyte production in autoSCT patients. The relative Ki-67 expression level of the different lymphocyte subsets largely paralleled the estimated production rates based on deuterium labeling (Figure 3A,C), suggesting that the increased turnover rates were at least in part due to a higher rate of peripheral lymphocyte division. For T cells, we also investigated whether there was any evidence for altered thymus output in autoSCT patients.

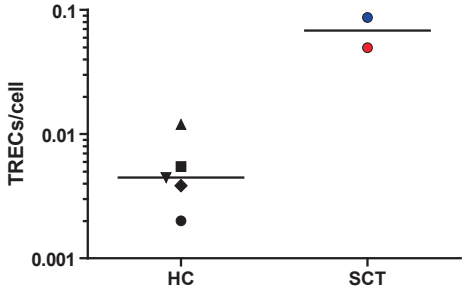

Figure 4. Average TREC content of naive CD4+ T cells. The number of TRECs/cell in $CD4^+$ naive T cells was determined as previously described[27]. For patient L03, TREC contents were not successfully measured due to limited material.

To this end, we measured the average T-cell receptor excision circle (TREC) content of $CD4^+$ naive T cells. TREC contents were 10-fold higher in autoSCT patients compared to healthy controls (Figure 4) in line with what has previously been reported for autoSCT patients[13], but not for alloSCT patients[7]. Although such increased TREC contents may be suggestive for increased thymus output, these results are difficult to interpret, because recent thymic emigrants, rich in TRECs, may be overrepresented in the peripheral T-cell pool post-SCT when lymphocyte numbers have not yet normalized[27].

We therefore used a mathematical model to interpret the differences in naive T-cell numbers and their TREC contents between autoSCT patients and healthy controls. In this model, naive T cells are produced by the thymus in an age-dependent manner $\sigma(t)$ and by peripheral T-cell division at a rate ρ per day, and are lost at a rate δ per day (see Supplemental Information). With this model, we tested whether increased thymic output after autoSCT was necessary to explain the data. Therefore we predicted the dynamics of naive $CD4^+$ T-cell numbers and their TREC contents after autoSCT, making two conservative assumptions: (*i*) daily thymus output is not affected by autoSCT, (*ii*) based on the observed increases in deuterium enrichment and Ki-67 expression, we assume that the peripheral T-cell division rate ρ is increased (which by itself is expected to have a TREC-diluting effect) while the loss rate of naive T cells remains unaffected (see Supplemental Information). Under these assumptions, the model predicted the dynamics of naive T cells and their TREC contents very well (Figure 5A,B). The reconstitution of naive T cells was predicted to occur very slowly, while their average TREC content increased very quickly after autoSCT, reaching supra-normal levels, after which they slowly decreased over time. The increased TREC contents shortly after autoSCT are most likely caused by the emptiness of the naive T cell pool and the resulting overrepresentation of recent thymic emigrants[27] (Figure 5C). Once cell numbers increase and peripheral T-cell proliferation starts to contribute significantly to the reconstitution of the T-cell pool, the average TREC content gradually decreases to the level observed in age-matched healthy controls. The predicted naive T-cell production rate (sum of renewal via the thymus and via peripheral cell division) was very high shortly after SCT and the contribution

of thymic output to newly formed naive T cells dropped from 25% to 10% during the first year post-SCT (Figure 5C). About two years post-SCT, the predicted turnover rate tended to stabilize at a level similar to our deuterium estimates (0.003 for predictions vs. 0.005 based on deuterium labeling, Figure 3A and 5D). Thus, the observed changes in both T-cell turnover and TRECs post autoSCT are compatible with a scenario in which thymic output is not affected and peripheral lymphocyte division rates are increased when cell numbers are low.

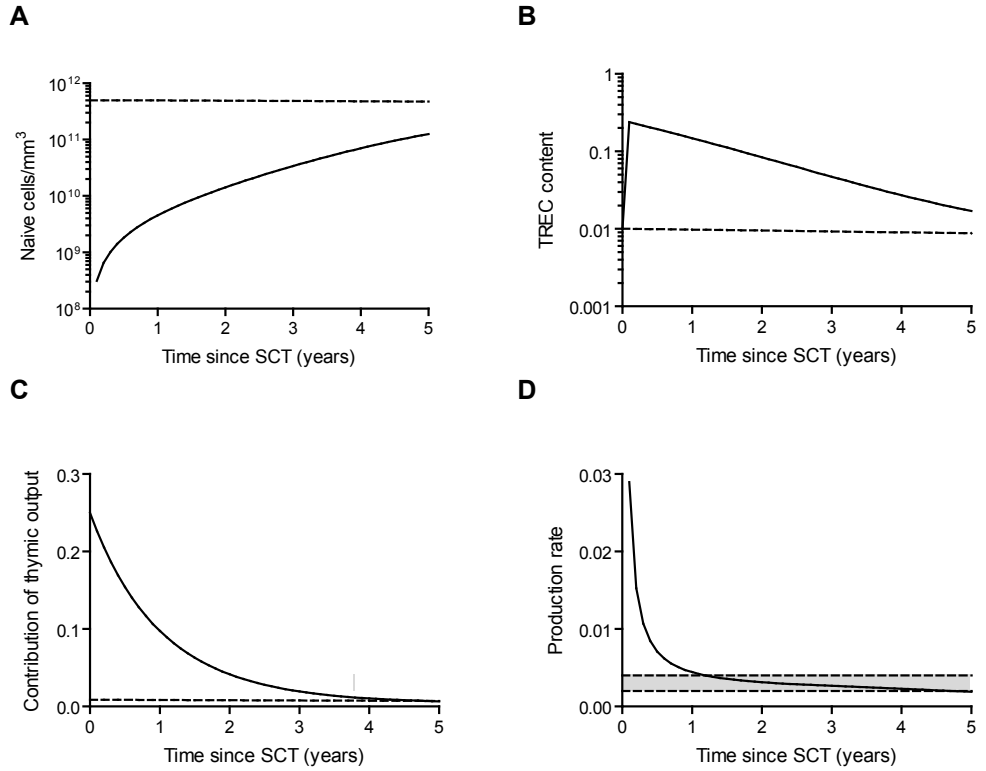

Figure 5. Simulated naive T-cell numbers and TREC dynamics after SCT. Predictions of (A) naive T-cell dynamics, (B) the average naive T-cell TREC content, (C) the relative contribution of thymic output, and (D) the average naive T-cell production rate, after autoSCT (solid lines) and in healthy individuals (dashed lines). These predictions were based on the mathematical model described in the Supplemental Information. The grey area in panel D represents the range of values measured in deuterium labeling experiments among healthy controls[8]. The average production rate plotted in D was calculated as the sum of daily thymic output $\sigma(t)$ and total peripheral T-cell division $\rho(N)N$ divided by the naive T-cell count N. Parameters: σ_0=3.65×10^{10} cells/year, h=3.2×10^{11}, δ =0.0005day^{-1}, c=0.25 and v=0.05/year.

Discussion

Following an autologous stem cell transplantation, cell counts of lymphocyte subsets, and of $CD4^+$ T cells in particular, can remain well below normal values for over one year [13;15-19;30;31]. This exceedingly slow reconstitution of lymphocytes raised the question whether the human immune system is at all capable of responding to situations of lymphopenia, e.g., by a homeostatic increase in lymphocyte production or survival rates. To answer this question, we here performed *in vivo* deuterium labeling in 3 hematology patients one year after autoSCT. After a recovery period of one year, the numbers of memory and effector B cells, and $CD4^+$ naive and memory T cells were still significantly lower than in healthy controls (Figure 1), while $CD8^+$ T-cell and naive B-cell counts had normalized. AutoSCT patients had a significantly higher turnover rate of $CD27^+IgM^-$ memory B cells and naive and memory $CD4^+$ T cells, and a tendency for a higher turnover rate of most other lymphocyte subsets when compared to healthy subjects. Mathematical analysis of the combined data of TRECs, deuterium enrichment and Ki-67 expression suggests that in response to lymphopenia, peripheral lymphocyte proliferation rates are homeostatically increased. Our finding that the cell count and dynamics of the naive B-cell pool had normalized one year post-SCT is in line with the idea that B cells are generated in excess in the bone marrow, and that peripheral B-cell survival depends on competition for the survival cytokine BAFF (B-cell activating factor, also called B lymphocyte stimulator, BLys)[32]. Whereas under lymphocyte-replete conditions most transitional B cells (B cells that have just left the bone marrow) die due to a low availability of BAFF, high levels of BAFF *per cell* observed after lymphocyte depletion have been correlated with increased cell survival[33;34]. A high but normal level of daily B-cell production, and a relatively long B-cell survival may thus explain the rapid reconstitution of transitional and naive B cells observed after lymphocyte depletion[33;35]. In contrast, we and others[29;36;37] found memory B cells to remain deficient for months. Others have attributed the delayed reconstitution of memory B cells to treatment-related damage to secondary lymphoid organs, which may hamper the formation of germinal centers that are essential for somatic hypermutation and isotype switching[37;38]. We found that the production rate of memory and natural effector B cells were in fact similar to or even higher than in healthy subjects, also reflected by the significantly higher percentages of $Ki\text{-}67^+$ cells in autoSCT patients. This suggests that even one year after transplantation, a homeostatic response is still ongoing in the B-cell compartment, which may be beneficial for the further recovery of memory and effector B cell numbers.

In the human T-cell pool, enhanced frequencies of Ki-67 expressing cells that were observed previously in lymphodeficient conditions[7;9;39-42], often appeared to coincide with clinical events and immune activation[5-7;9;30;31;39]. We here show that in autoSCT patients, the turnover rates of naive and memory $CD4^+$ T cells are also increased in absence of (clinically manifested) immune activation, suggesting that a homeostatic response does occur in response to lymphopenia. Although Hazenberg et al. concluded that the increased T-cell

division rates observed after alloSCT were related to immune activation[7], SCT patients who did not suffer from clinical events also had higher percentages of Ki-67$^+$ cells compared to healthy controls, which is in support of a lymphopenia-induced homeostatic response. The fact that we did not find a homeostatic T-cell response in healthy aging[8] may be explained by the relatively small difference in naive T-cell numbers between young and elderly individuals. Possibly, a population size threshold exists below which the cell number needs to drop before a homeostatic response is triggered. Alternatively, the difference in turnover between healthy young and elderly individuals may have been too subtle to detect.

We previously estimated that in healthy elderly individuals, the contribution of thymic output to naive T-cell production is only as little as 1% and because of the extreme longevity of naive T cells and the stability of TREC this may even be an overestimate[8]. The elevated TREC contents that we observed in autoSCT patients as compared to age-matched healthy controls demonstrate that despite their advanced age and the possible damaging effect of chemotherapy treatment, the thymus still contributed substantially to naive T-cell reconstitution in these patients. Elevated TREC contents are not per definition related to an increased thymic output. In lymphopenic conditions, TRECs may be relatively enriched due to the abundance of TREC-bearing recent thymic emigrants, while on the other hand they may be diluted due to the lymphopenia-induced proliferative response[27]. Using a mathematical model that includes the occurrence of homeostatic T-cell proliferation (as observed in autoSCT patients), a small contribution of thymic output and the T-cell death rates that we previously measured in healthy controls[8], we here show that the supranormal TREC contents at 1 year post-SCT may simply be a reflection of normal thymic output adding recent thymic emigrants to an empty pool. Once peripheral T-cell numbers increase, and peripheral division contributes more to T-cell production, TREC contents start to decline. Our prediction shows that even without an increase in thymic output, and in the presence of a homeostatically increased peripheral T-cell division rate, TRECs may remain elevated for over 5 years post-SCT (Figure 5B).

Despite the fact that in response to lymphopenia, the turnover rates of most B-cell and T-cell subsets are increased, the rate of T-cell and B-cell reconstitution is still remarkably slow. This slow reconstitution may partially be a reflection of the very low turnover of these cells under healthy conditions; indeed the average turnover rate of, for example, naive T cells is as low as 0.03-0.09% per day in healthy adults[8;21]. The rate of reconstitution must however also be affected by something else than basal turnover rates because the subsets with the highest basal turnover rates were not always the ones the reconstituted most quickly. Of the different B-cell subsets, for example, naive B cells have the slowest turnover rate in healthy individuals[8], while in autoSCT patients it is the only subset that normalizes within 6 months post-transplantation (Figure 1B). For the T-cell compartment, CD4$^+$ and CD8$^+$ naive T cells have a much lower turnover than their memory counterparts[8;21], but while CD4$^+$ naive and memory T cells are still deficient, CD8$^+$ naive and memory T cells have reached normal cell

counts by 6 months post-transplantation (Figure 1C,D). It could be that shortly (i.e., less than 2 months) after transplantation, naive B cells and $CD8^+$ T cells respond more robustly to the lymphopenic situation than e.g. $CD4^+$ T cells do, for example because they are more sensitive to alterations in the availability of survival molecules, like IL-7, IL-15, and BAFF. To answer this question, one would also need to investigate the turnover rates of lymphocyte subsets earlier after transplantation.

In summary, we here show that following autoSCT, and in the absence of clinically manifested immune activation, the turnover rates of numerically deficient B-cell and T-cell subsets are higher than in healthy controls, suggesting that lymphocyte production rates are homeostatically increased in response to lymphopenic conditions. Although this accelerates numerical recovery of the lymphocyte pools, recovery of some B-cell and T-cell subsets still takes months to years, leading to long-term immunodeficiency and an increased susceptibility to infections and cancer relapse[43]. Hence, to improve the outcome of hematopoietic stem cell transplantations, it may be necessary to boost immune reconstitution therapeutically.

Acknowledgments

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References

1. Gaudin E, Rosado M, Agenes F, McLean A, Freitas AA. B-cell homeostasis, competition, resources, and positive selection by self-antigens. Immunol.Rev. 2004;197:102-115.
2. Bell EB, Sparshott SM, Drayson MT, Ford WL. The stable and permanent expansion of functional T lymphocytes in athymic nude rats after a single injection of mature T cells. J.Immunol. 1987;139:1379-1384.
3. Miller RA, Stutman O. T cell repopulation from functionally restricted splenic progenitors: 10,000-fold expansion documented by using limiting dilution analyses. J.Immunol. 1984;133:2925-2932.
4. Freitas AA, Rocha B. Population biology of lymphocytes: the flight for survival. Annu.Rev.Immunol. 2000;18:83-111.
5. Jones JL, Thompson SA, Loh P et al. Human autoimmunity after lymphocyte depletion is caused by homeostatic T-cell proliferation. Proc.Natl.Acad.Sci.U.S.A 2013;110:20200-20205.
6. Bouvy AP, Kho MM, Klepper M et al. Kinetics of homeostatic proliferation and thymopoiesis after rATG induction therapy in kidney transplant patients. Transplantation 2013;96:904-913.
7. Hazenberg MD, Otto SA, de Pauw ES et al. T-cell receptor excision circle and T-cell dynamics after allogeneic stem cell transplantation are related to clinical events. Blood 2002;99:3449-3453.
8. Westera L, van Hoeven V, Drylewicz J et al. Lymphocyte maintenance during healthy aging requires no substantial alterations in cellular turnover. Aging Cell 2015;14:219-227.
9. Naylor K, Li G, Vallejo AN et al. The influence of age on T cell generation and TCR diversity. J.Immunol. 2005;174:7446-7452.
10. Bosch M, Khan FM, Storek J. Immune reconstitution after hematopoietic cell transplantation. Curr. Opin.Hematol. 2012;19:324-335.
11. Williams KM, Hakim FT, Gress RE. T cell immune reconstitution following lymphodepletion. Semin. Immunol. 2007;19:318-330.
12. Bemark M, Holmqvist J, Abrahamsson J, Mellgren K. Translational Mini-Review Series on B cell subsets in disease. Reconstitution after haematopoietic stem cell transplantation- revelation of B cell developmental pathways and lineage phenotypes. Clin.Exp.Immunol. 2012;167:15-25.
13. Douek DC, Vescio RA, Betts MR et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. Lancet 2000;355:1875-1881.
14. Rutella S, Pierelli L, Bonanno G et al. Immune reconstitution after autologous peripheral blood progenitor cell transplantation: effect of interleukin-15 on T-cell survival and effector functions. Exp. Hematol. 2001;29:1503-1516.
15. Malphettes M, Carcelain G, Saint-Mezard P et al. Evidence for naive T-cell repopulation despite thymus irradiation after autologous transplantation in adults with multiple myeloma: role of ex vivo CD34+ selection and age. Blood 2003;101:1891-1897.
16. Simonelli C, Zanussi S, Pratesi C et al. Immune recovery after autologous stem cell transplantation is not different for HIV-infected versus HIV-uninfected patients with relapsed or refractory lymphoma. Clin.Infect.Dis. 2010;50:1672-1679.
17. Tsukamoto H, Nagafuji K, Horiuchi T et al. Analysis of immune reconstitution after autologous CD34+ stem/progenitor cell transplantation for systemic sclerosis: predominant reconstitution of Th1 CD4+ T cells. Rheumatology.(Oxford) 2011;50:944-952.
18. Parrado A, Casares S, Prieto J et al. Repopulation of circulating T, B and NK lymphocytes following bone marrow and blood stem cell transplantation. Hematol.Cell Ther. 1997;39:301-306.
19. Reimer P, Kunzmann V, Wilhelm M et al. Cellular and humoral immune reconstitution after autologous peripheral blood stem cell transplantation (PBSCT). Ann.Hematol. 2003;82:263-270.
20. Seggewiss R, Einsele H. Immune reconstitution after allogeneic transplantation and expanding options for immunomodulation: an update. Blood 2010;115:3861-3868.

21. Vrisekoop N, den Braber I, de Boer AB et al. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. Proc.Natl.Acad.Sci.U.S.A 2008;105:6115-6120.
22. Busch R, Neese RA, Awada M, Hayes GM, Hellerstein MK. Measurement of cell proliferation by heavy water labeling. Nat.Protoc. 2007;2:3045-3057.
23. Westera L, Zhang Y, Tesselaar K, Borghans JA, Macallan DC. Quantitating lymphocyte homeostasis in vivo in humans using stable isotope tracers. Methods Mol.Biol. 2013;979:107-131.
24. Westera L, Drylewicz J, den Braber I et al. Closing the gap between T-cell life span estimates from stable isotope-labeling studies in mice and humans. Blood 2013;122:2205-2212.
25. Westermann J, Pabst R. Lymphocyte subsets in the blood: a diagnostic window on the lymphoid system? Immunol.Today 1990;11:406-410.
26. de Boer RJ, Perelson AS. Quantifying T lymphocyte turnover. J.Theor.Biol. 2013;327:45-87.
27. Hazenberg MD, Otto SA, Cohen Stuart JW et al. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. Nat.Med. 2000;6:1036-1042.
28. R Development Core Team: A language and environment for statistical computing. 2010. Ref Type: Generic
29. Storek J, Geddes M, Khan F et al. Reconstitution of the immune system after hematopoietic stem cell transplantation in humans. Semin.Immunopathol. 2008;30:425-437.
30. Zhang Y, Wallace DL, de Lara CM et al. In vivo kinetics of human natural killer cells: the effects of ageing and acute and chronic viral infection. Immunology 2007;121:258-265.
31. Zhang Y, de LC, Worth A et al. Accelerated in vivo proliferation of memory phenotype CD4+ T-cells in human HIV-1 infection irrespective of viral chemokine co-receptor tropism. PLoS.Pathog. 2013;9:e1003310.
32. Woodland RT, Schmidt MR, Thompson CB. BLyS and B cell homeostasis. Semin.Immunol. 2006;18:318-326.
33. Thompson SA, Jones JL, Cox AL, Compston DA, Coles AJ. B-cell reconstitution and BAFF after alemtuzumab (Campath-1H) treatment of multiple sclerosis. J.Clin.Immunol. 2010;30:99-105.
34. Tchao NK, Turka LA. Lymphodepletion and homeostatic proliferation: implications for transplantation. Am.J.Transplant. 2012;12:1079-1090.
35. Marie-Cardine A, Divay F, Dutot I et al. Transitional B cells in humans: characterization and insight from B lymphocyte reconstitution after hematopoietic stem cell transplantation. Clin.Immunol. 2008;127:14-25.
36. Suzuki I, Milner EC, Glas AM et al. Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: lack of somatic mutation indicates a maturational arrest. Blood 1996;87:1873-1880.
37. Avanzini MA, Locatelli F, Dos SC et al. B lymphocyte reconstitution after hematopoietic stem cell transplantation: functional immaturity and slow recovery of memory CD27+ B cells. Exp.Hematol. 2005;33:480-486.
38. Storek J, Witherspoon RP, Webb D, Storb R. Lack of B cells precursors in marrow transplant recipients with chronic graft-versus-host disease. Am.J.Hematol. 1996;52:82-89.
39. Hazenberg MD, Stuart JW, Otto SA et al. T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). Blood 2000;95:249-255.
40. Sauce D, Larsen M, Fastenackels S et al. Lymphopenia-driven homeostatic regulation of naive T cells in elderly and thymectomized young adults. J.Immunol. 2012;189:5541-5548.
41. van Gent R, Schadenberg AW, Otto SA et al. Long-term restoration of the human T-cell compartment after thymectomy during infancy: a role for thymic regeneration? Blood 2011;118:627-634.

42. Borghans JA, Bredius RG, Hazenberg MD et al. Early determinants of long-term T-cell reconstitution after hematopoietic stem cell transplantation for severe combined immunodeficiency. Blood 2006;108:763-769.
43. Cavazzana-Calvo M, Andre-Schmutz I, Dal CL et al. Immune reconstitution after haematopoietic stem cell transplantation: obstacles and anticipated progress. Curr.Opin.Immunol. 2009;21:544-548.
44. Steinmann GG, Klaus B, Muller-Hermelink HK. The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study. Scand.J.Immunol. 1985;22:563-575.
45. den Braber I, Mugwagwa T, Vrisekoop N et al. Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. Immunity. 2012;36:288-297.

Supplemental Information

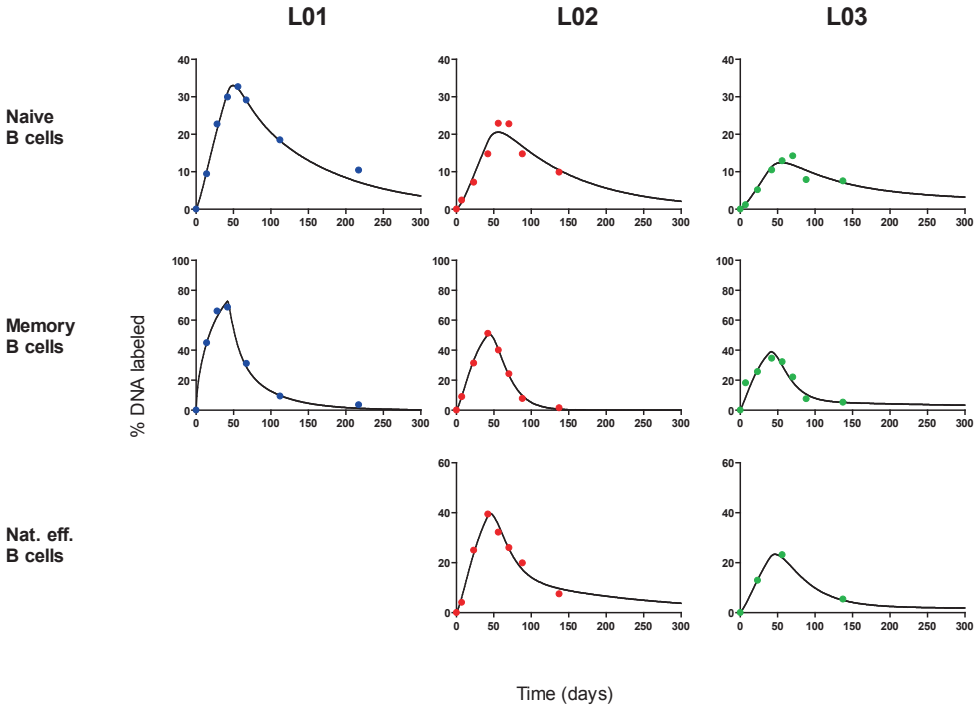
Mathematical modeling of naive T-cell and TREC dynamics. Naive T-cell and TREC dynamics were investigated using a previously developed mathematical model[27]. In this model, *N* is the total number of naive $CD4^+$ T cells and *T* the total amount of TRECs in the naive T-cell population, *σ(t)* is the time-dependent source of naive T cells from the thymus where *t* is the age of the individual in years, *c* is the average number of TRECs per recent thymic emigrant, *δ* the rate of naive T-cell loss through cell death and priming of naive into memory cells, and *ρ* the rate of naive T-cell division. The model can be written as follows:

$$\frac{dT}{dt} = c\sigma(t) - \delta T$$

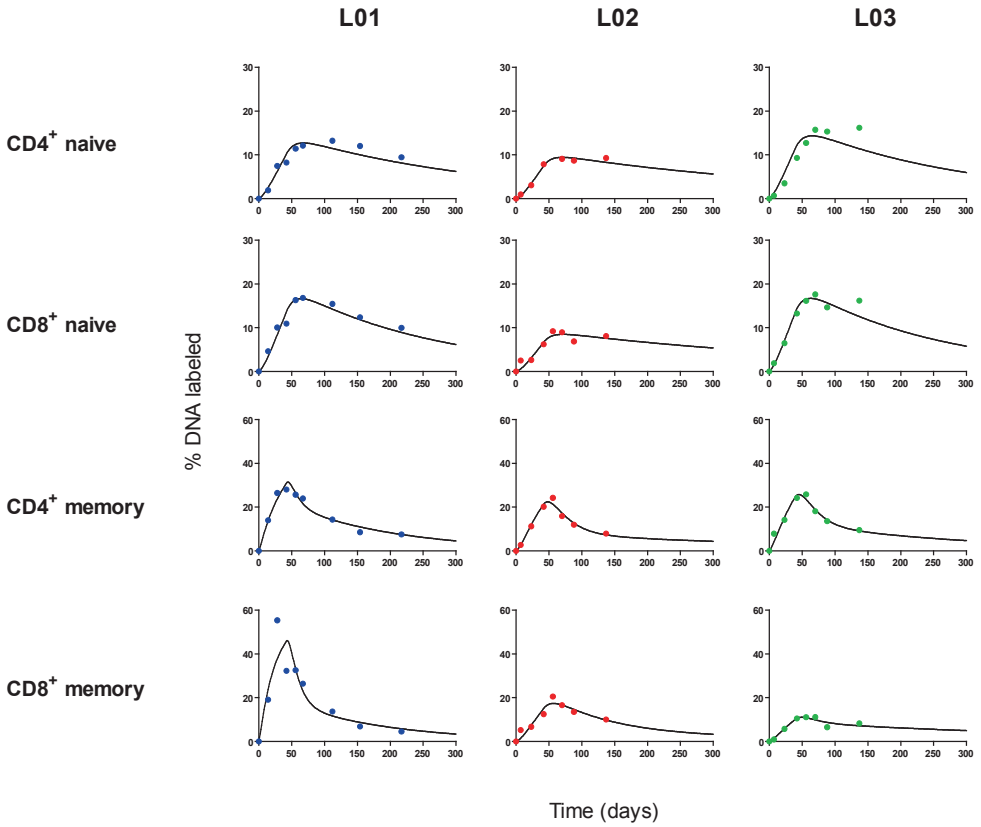
$$\frac{dN}{dt} = \sigma(t) + \rho(N)N - \delta N$$

where $\sigma(t) = \sigma_0 e^{-\upsilon t}$. Thymic output is assumed to decay exponentially at rate *υ* per year and σ_0 represents thymus production of naive T cells at birth. Since TRECs can only be produced in the thymus, the total number of TRECs is not affected by peripheral T-cell proliferation. The average TREC content of naive T cells is defined as *A=T/N*. Both the T-cell loss rate *δ* and the division rate *ρ* may depend on cell densities. We included a density-dependent T-cell division rate $\rho(N) = e^{-N/h}$ and fixed the T-cell loss rates, *δ,* to turnover estimates obtained from our previous deuterium labeling studies in healthy individuals[8].

To simulate naive T-cell numbers and their average TREC contents after SCT, we considered the average TREC content of a 50 year old healthy individual as starting point and dramatically reduced the total number of naive T cells. The rate of naive T-cell loss, *δ,* was set to the average turnover estimates obtained from our deuterium labeling studies among healthy individuals[8]. The parameter *h* was fixed to the value used to describe naive T-cell numbers and their TREC content over age in a healthy individual. The involution rate of the thymus was fixed at 0.05/year according to a previous study[8;44], and the TREC content was scaled such that $c = 0.25$[45].



Supplemental Figure 1. Individual data and best fits of ^{2}H enrichment in B-cell subsets of auto SCT patients. Best fits of the multi-exponential model to the deuterium enrichment in naive, memory and natural effector B cells in 3 autoSCT patients. The labeling curves of all B cell subsets were significantly better described by a model including two kinetically different compartments than by a single-compartment model. Label enrichment in the DNA was scaled between 0 and 100% by normalizing for the maximum enrichment in granulocytes (see Materials and Methods).



Supplemental Figure 2. **Individual data and best fits of ^{2}H enrichment in T-cell subsets of auto SCT patients.** Best fits of the multi-exponential model to the deuterium enrichment in naive and memory $CD4^{+}$ and $CD8^{+}$ T cells in 3 autoSCT patients. The labeling curves of memory $CD4^{+}$ and $CD8^{+}$ T-cells were significantly better described by a model including two kinetically different subpopulations while the naive T-cell subsets required only one. Label enrichment in the DNA was scaled between 0 and 100% by normalizing for the maximum enrichment in granulocytes (see Materials and Methods).

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The effect of ATG and maintenance immunosuppressive drug therapy on T-cell dynamics in renal transplant patients

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Abstract

Immunosuppressive drug therapy, which often includes lymphocyte-depleting antibody therapy with for example anti-thymocyte globulin (ATG), is widely applied to prevent or treat rejection of allogeneic solid organ transplants. However, these therapies adversely compromise the immune system of allograft recipients. It is still unclear to what extent immunosuppressive drugs impair normal T-cell dynamics in lymphoreplete and lymphocyte-depleted transplantation patients. Using *in vivo* stable-isotope labeling and Ki-67 expression analysis, we studied the turnover of T-cell subsets in renal transplant recipients, who were either treated with maintenance immunosuppressive agents only, or were additionally lymphocyte-depleted by ATG administration because of a humoral rejection response in the first two weeks after transplantation. The use of maintenance immunosuppressive drug therapy was associated with relatively low naive and memory T-cell numbers, but had no significant effect on T-cell turnover rates. ATG therapy caused a severe depletion and persistent deficiency of in particular $CD4^+$ T cells. Nevertheless, T-cell turnover rates were hardly affected in the ATG-treated patients. These results suggest that immunosuppressive agents might not substantially reduce T-cell turnover rates in patients with a full lymphocyte pool, and that in the presence of these drugs, homeostatic proliferative responses might not be induced despite a severe lymphopenic condition following ATG therapy.

Introduction

Solid-organ transplantations are often complicated by the development of alloreactive immune responses that lead to rejection of the graft. To prevent acute and chronic graft rejection, allograft recipients are treated with immunosuppressive induction and maintenance regimens that generally consist of corticosteroids, CD25 antagonizing antibodies, calcineurin inhibitors, and mycophenolate mofetil (MMF) [1]. While MMF inhibits T-cell proliferation, the other agents target signaling through the interleukin 2 (IL-2) receptor and T-cell receptor (TCR), thereby inhibiting IL-2-mediated and antigen-mediated T-cell responses[1-5]. Lymphocyte-depleting antibody therapies form a widely applied additional line of therapy, especially for patients at high risk of developing an acute allograft response and for patients with severe or corticosteroid-resistant rejection episodes. Anti-thymocyte globulin (ATG), a mixture of rabbit or horse-derived antibodies directed against a broad range of T-cell and non T-cell antigens[6], is currently one of the most commonly used lymphocyte depletion therapy after kidney transplantation[7-9]. ATG therapy causes a rapid depletion of the majority of the T-cell population, with a more thorough depletion of $CD4^+$ T cells compared to $CD8^+$ T cells, and a deeper depletion of the naive T-cell subsets compared to the central memory and effector T-cell subsets[10-12].

By diminishing the T-cell response to the allogeneic graft, ATG and immunosuppressive maintenance therapy substantially reduce the incidence of graft rejection[7;13]. However, because of the non-specific action of these drugs, immuno-suppressive treatment comes with many side-effects, ranging from hypertension, diabetes, and nephrotoxicity, to impaired wound healing and susceptibility to infection[14;15]. ATG treatment causes persistent T-cell deficiency[16], which has also been associated with increased susceptibility to infectious diseases and malignancies[17-19].

Multiple studies have consistently shown that following ATG, $CD8^+$ T-cell numbers normalize within 6 months, while $CD4^+$ T-cell numbers remain below baseline levels for years[10-12;19;20]. The influence of ATG treatment and maintenance immunosuppressive therapy on T-cell dynamics is still poorly understood. In general, the rate of T-cell reconstitution depends on (*i*) the rate at which new T cells are produced via thymus-dependent T-cell development and peripheral T-cell division, and (*ii*) the survival capacity of the newly produced T cells. In long-term heavy water labeling studies we have shown that in healthy adults the renewal rate of both the $CD4^+$ and $CD8^+$ naive T-cell population is less than 0.1% per day[21;22], which may explain why T-cell reconstitution is a relatively slow process. We have recently shown that in severely lymphocyte depleted stem-cell transplantation patients, $CD4^+$ T-cell turnover rates were increased (chapter 4 of this thesis), suggesting that very low peripheral cell numbers can induce a homeostatic increase in T-cell production. The observation that in rhesus macaques lymphocyte depletion by either total body irradiation or ATG treatment resulted in increased percentages of proliferating (Ki-67^+) T cells[23], supports that this is a more generally

occurring mechanism. It is unclear whether a similar change in T-cell turnover occurs following ATG treatment in solid organ transplanted patients. Since the T-cell compartment needs to reconstitute in the presence of immunosuppressive agents, aimed at preventing the outgrowth of graft-responsive T cells, a lymphopenia-induced T-cell proliferative response may be repressed in these patients.

Using *in vivo* stable-isotope labeling and Ki-67 expression analysis, we here investigated the long-term effects of immunosuppressive maintenance therapy and ATG by studying the T-cell dynamics of ATG-treated and non-ATG treated renal transplant recipients one year post-transplantation. Despite the pronounced and long-lasting depletion of in particular $CD4^+$ peripheral T-cell numbers in ATG-treated individuals, and the immunosuppressive therapy in all renal transplant patients, we did not find differences in the turnover rates of naive and memory T-cells between these patients and healthy controls. These results suggests that the immunosuppressive agents might not substantially decrease the T-cell turnover rates in the patients not treated with ATG, and that in the presence of these drugs proliferative responses might not be induced despite the lymphopenic condition in ATG-treated patients.

Materials and Methods

Subjects. Five renal transplant recipients and two age-matched healthy donors (see Table 1 for patient characteristics) were enrolled in a deuterated water (^2H_2O) labeling study after having provided written informed consent. All transplant recipients received basic immunosuppressive therapy from the day of transplantation, consisting of induction therapy with (non-depleting) anti-CD25 monoclonal antibody (Simulect®, intravenous administration of 20 mg just before surgery and 20 mg at day 4 after transplantation), and maintenance therapy consisting of oral prednisolone 10 mg daily, oral tacrolimus aimed at serum levels of 8-14 ng/ml and 1 gram oral mycophenolate mofetil (MMF) twice daily. None of the patients had been treated with immunosuppressive drugs prior to transplantation. Two transplant recipients received rATG (rabbit-derived Anti-thymocyte globulin) because of acute humoral rejection. The rATG regimen consisted of a starting dose of 5mg/kg administered within two weeks post-transplantation, and three to five repetitive doses in the following 14 days, to attain a blood lymphocyte count below 150 $x10^6$/l. The dosage of repetitive administrations was based on the total lymphocyte count after each administration (> $300x10^6$/l: dose 5mg/kg; >$200x10^6$/l but <$300x10^6$/l: dose 3mg/kg; >$150x10^6$/l but <200 $x10^6$/l: dose 2mg/kg; <$150x10^6$/l: no administration). CMV-seropositive recipients were pre-emptively treated with valganciclovir at a therapeutic dose, adjusted to renal function. None of the patients got symptomatic CMV infection or reactivation. The study was approved by the local medical ethics committee.

Table 1.Patient characteristics

Study ID	Symbol	Age	Sex	Graft #	Donor	Lymphocyte depletion treatment	CMV status D/R	Time post-Tx at start ^2H_2O (days)
ATG-764	●	41	F	1st	LRD	ATG	+/+	418
ATG-793	■	59	F	1st	PM	ATG	+/-	364
No ATG-750	○	43	M	2nd	LURD	-	-/+	371
No ATG-803	□	67	M	1st	LURD	-	+/+	371
No ATG-824	△	51	M	1st	LRD	-	+/-	387
HD1	○	60	F	n/a	n/a	n/a	n/a	n/a
HD2	□	41	F	n/a	n/a	n/a	n/a	n/a

LRD, living related donor; LURD, living unrelated donor; PM, post-mortal. All patients received aCD25 induction therapy and maintenance immunosuppressive therapy consisting of prednisolone, tacrolimus, mycophenolate mofetil (see Materials and Methods). For none of the patients CMV reactivation was detected.

^2H_2O labeling. On the first day of the study, study subjects drank a starting dose of 10 ml of ^2H_2O (99.8% enriched, Cambridge Isotope Laboratories, Tewksbury, MA, USA) per kilogram of body water (60% total bodyweight) in small doses throughout the day. Because of possible side effects at higher doses, subjects were admitted to the hospital for observation during administration of the starting dose. As a maintenance dose, subjects drank 1/8 of the initial dose per day for a period of 9 weeks. Most subjects experienced transient vertigo that was limited to the first day of the study. Blood and urine samples were collected just before the start of ^2H_2O labeling, and at week 2, 5, 9, 11, 14, 18 and 30 after the start of labeling. Additional urine samples were collected at week 4, 7, 13 and 25.

Cell isolation, flow cytometry, and sorting of T-cell subsets. Peripheral blood mononuclear cells (PBMCs) were isolated using standard density gradient centrifugation and were subsequently cryopreserved until analysis. Granulocytes were enriched from the granulocyte/erythrocyte pellet by lysis of erythrocytes. For isolation of $CD4^+$ and $CD8^+$ naive ($CD45RA^+$ $CD27^+$), memory ($CD45RA^-$ $CD27^+$) and effector ($CD27^-$) T cells, we labeled PBMCs with αCD3-PE (eBioscience Inc, San Diego, CA, USA), αCD8-PerCP-Cy5.5 (BD, San Diego, CA, USA), αCD4-FITC (BD), αCD27 APC (eBioscience) and αCD45RA PE-Cy7 (BD). Cells were sorted on a FACs ARIA (BD). Purity of the sorted cells was at least 97 %. Absolute cell counts in whole blood were determined within 2 hours after withdrawal using the Coulter Counter (Beckman Coulter, Woerden, The Netherlands). To determine T-cell subset distribution and cell cycling, 5×10^5 PBMCs were stained with αCD3APC (eBioscience), αCD4-AlexaFluor 700 (BD), αCD8 PE Texas Red (Life Technologies Europe BV, Bleiswijk, Netherlands), αCD45RA PE-Cy7 (BD), αCD27 APC-eFluor780 (eBioscience), and αKi67-PE (BD). Samples were acquired on a FACSCanto (BD). Analysis was performed using FlowJo Mac (FlowJo Ashland, OR, USA).

Viral diagnostics. Quantitative PCR and serology for cytomegalovirus (CMV) were analyzed as previously described[24;25]. Reactivation of viral infection was defined as positive viral PCR in a seropositive patient as previously described.

DNA isolation. DNA was isolated from sorted T-cell subsets using the QIAamp DNA Blood Mini Kit (if sort yield > 50.000 cells) or the QIAamp DNA Micro Kit (if sort yield <50.000 cells) according to manufacturer's instructions. Samples were stored at -20°C until further processing.

Measurement of ^{2}H enrichment in DNA and urine samples. Deuterium enrichment in DNA samples was measured using the GC/MS method described by Busch et al.[26] with minor modifications as previously described[22]. Briefly, DNA was enzymatically hydrolyzed and derivatized to penta-fluoro-triacetate (PFTA) before injection into the gas chromatograph (DB-17MS column, 7890A GC System, Agilent Technologies). PFTA was analyzed by negative chemical ionization mass spectrometry (5975C inert XL EI/CI MSD with Triple-Axis Detector, Agilent Technologies) measuring ions m/z 435 and m/z 436. Urine samples were stored at -20°C until further processing. Deuterium enrichment in urine was analyzed on the same GC/MS system (using a PoraPLOT Q 25x0.32 column, Varian) by electron impact ionization as previously described[27].

Quantification of T-cell turnover rates by mathematical modeling of urine and DNA enrichment data. A mathematical model was fitted to the urine and DNA enrichment data as previously described[22]. Briefly, urine enrichment levels were fitted with a simple label enrichment/decay curve and subsequently incorporated into the mathematical analysis of the enrichment in the different cell populations. The ^{2}H enrichment in the granulocyte population of each patient was considered to be the maximum level of label incorporation that cells could possibly attain. A multi-exponential model[28] was fitted to the enrichment data of the different leukocyte subsets and scaled to the maximum enrichment asymptote of the granulocyte population. Average turnover rates p of different lymphocyte populations were estimated by fitting the enrichment curves for each individual separately. Deuterium enrichment data were fitted with R using the nlm function[29]; 95% confidence limits were determined by bootstrap analysis on the residuals.

Results

Subjects and ^2H_2O labeling

To investigate the effect of immunosuppressive therapy and ATG-mediated lymphocyte depletion on T-cell dynamics, we included five renal transplant recipients on immunosuppressive therapy – two of whom also received ATG – and two healthy age-matched volunteers in a heavy water (^2H_2O) labeling study (for subject characteristics, see Table 1). The standard immunosuppressive treatment consisted of induction therapy with anti-CD25 antibodies and maintenance immunosuppression with corticosteroids (Prednisolone), calcineurin inhibitor (Tacrolimus), and an anti-proliferative agent (mycophenolate mofetil, MMF). ATG treatment was started within 2 weeks post-transplantation and administered in 3-5 repetitive doses, depleting the lymphocyte population to a level below $150x10^6$ cells/l.

Graft recipients started the ^2H_2O-labeling protocol approximately 1 year after their kidney transplantation. All subjects drank ^2H_2O for a period of 9 weeks and were followed-up for another 21 weeks thereafter. During the whole study period, urine and blood samples were frequently collected for the measurement of deuterium enrichment in body water, granulocytes, and T-cell subsets. T-cell turnover rates were determined using mathematical modeling as described in detail in Materials and Methods.

The effect of immunosuppressive therapy and ATG on peripheral T-cell numbers

We first investigated how induction- and maintenance immunosuppressive therapy affected peripheral T-cell numbers, by analyzing total lymphocyte counts and T-cell numbers of all renal transplant patients in the first year following kidney transplantation, before the start of the ^2H_2O labeling study. While total lymphocyte counts and $CD8^+$ T-cell numbers of non-ATG treated patients were mostly in the lower range of the healthy reference values (Figure 1A, D, gray symbols), their total $CD3^+$ and $CD4^+$ T-cell numbers were just below the lower limit of normal (Figure 1B-C, gray symbols). ATG treatment had a pronounced additional effect, causing a rapid depletion of both $CD4^+$ and $CD8^+$ T cells, reflected by low total lymphocyte counts and $CD3^+$ T-cell numbers from two weeks post-transplantation onward (Figure 1A-D, black symbols). In the ATG-treated individuals, $CD3^+$ T-cell numbers remained far below normal levels for the entire first year following ATG therapy, which was mostly due to the slow reconstitution of $CD4^+$ T cells (Figure 1B, C, black symbols). After one year of reconstitution, $CD8^+$ T-cell counts in ATG-treated patients were close to the lower limit of normal (Figure 1D, black symbols).

From the start of the ^2H_2O labeling study, we determined the absolute numbers of total lymphocytes, $CD3^+$, $CD4^+$ and $CD8^+$ T cells, and naive ($CD27^+CD45RA^+$), memory ($CD27^+CD45RA^-$) and effector ($CD27^-$) T cells, at every study-related blood withdrawal. During the ^2H_2O labeling study, i.e. in the second year after kidney transplantation, we observed a further reconstitution of absolute lymphocytes, and $CD3^+$, $CD4^+$ and $CD8^+$ T-cell numbers in

the ATG-treated subjects, although CD4+ T-cell numbers remained deficient until the end of the study (Figure 1A-D, black symbols from 'Start ^2H_2O'). Absolute cell counts in the non-ATG treated patients were variable but on average remained constant throughout the second year after transplantation (Figure 1A-D, gray symbols from 'Start ^2H_2O'). Except for non-ATG treated patient 803 (gray square), absolute lymphocyte, and CD3+ and CD4+ T-cell numbers remained relatively low in all renal transplant recipients. Naive and memory T-cell counts, but not the effector T-cell subsets, tended to be lower in renal transplant recipients compared to the healthy controls, with perhaps an additional effect of ATG only visible in the CD4+ T-cell compartment (Figure 2).

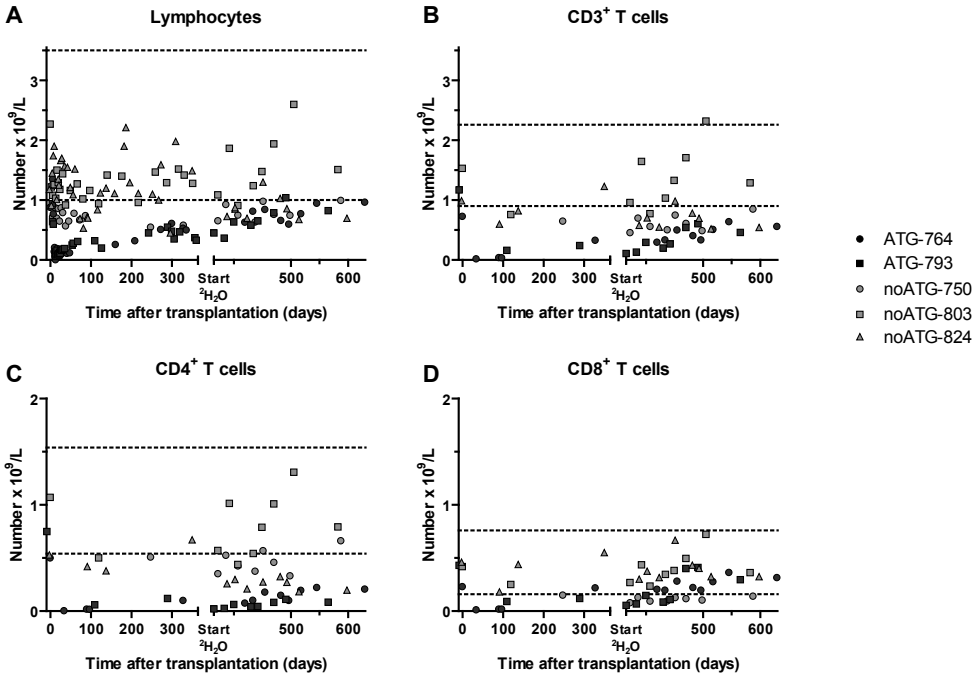


Figure 1. Lymphocyte and T-cell counts in the first year post-transplantation. Absolute peripheral blood counts of total lymphocytes (A), CD3+ (B), CD4+ (C), and CD8+ T cells (D) in the first year post transplantation and during the ^2H_2O labeling study. In the first year post-transplantation, cell counts were determined by diagnostic procedures that were unrelated to the ^2H_2O labeling study. During the study, cell counts were determined with every blood withdrawal in the research laboratory. Dark gray symbols represent the ATG-treated patients. Every individual is depicted with a unique symbol. The area between the dotted lines indicates the range of normal lymphocyte and T-cell counts in healthy adults[41].

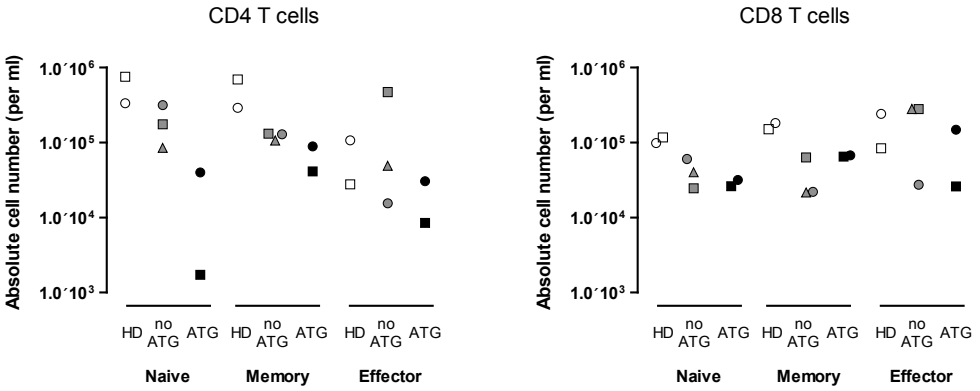


Figure 2. Absolute counts of T-cell subsets during ^2H_2O-labeling study. Absolute numbers of $CD4^+$ and $CD8^+$ naive, memory, and effector T cells in peripheral blood were determined at every blood withdrawal (n=8) during the ^2H_2O-labeling study. Median values for each individual are shown, depicted with a unique symbol for each individual (see Table 1). HD, healthy donors (open symbols); no ATG, graft recipients not treated with ATG (gray symbols); ATG, graft recipients treated with ATG (black symbols).

The effect of immunosuppressive therapy and ATG on T-cell turnover rates

In stem-cell transplanted individuals, in whom the lymphocyte compartment was severely depleted by chemotherapy treatment, we have previously observed increased levels of T-cell turnover, suggesting the occurrence of a lymphopenia-induced proliferative response (chapter 4 of this thesis). We investigated whether reduced T-cell numbers in renal transplant patients similarly triggered an increase in T-cell turnover rates, or whether such a homeostatic response was repressed by the immunosuppressive maintenance therapy.

To examine how immunosuppression and ATG therapy affected T-cell dynamics after kidney transplantation, we analyzed deuterium enrichment levels in renal transplant recipients and age-matched healthy donors (for individual deuterium enrichment data, see Figure 3). The average turnover rates of all T-cell subsets, which were determined using mathematical modeling, did not seem to be different between the study groups (Figure 4), which may be related to the limited number of subjects. Only the turnover of $CD8^+$ naive T cells tended to be higher in the ATG-treated patients compared to the non-ATG treated patients and healthy controls. Note that for effector T cells, the material was too limited to compare deuterium enrichment data between the study groups. In the few subjects for whom we could sort sufficient numbers of effector T cells, deuterium enrichment levels were comparable to those in the corresponding memory T-cell subsets (data not shown). Altogether, the deuterium labeling data suggest that while immunosuppressive therapy hardly influenced T-cell proliferation rates in non-ATG patients, substantial proliferative responses did not occur in ATG treated patients in the presence of immunosuppressive drugs, despite their severe lymphopenic condition.

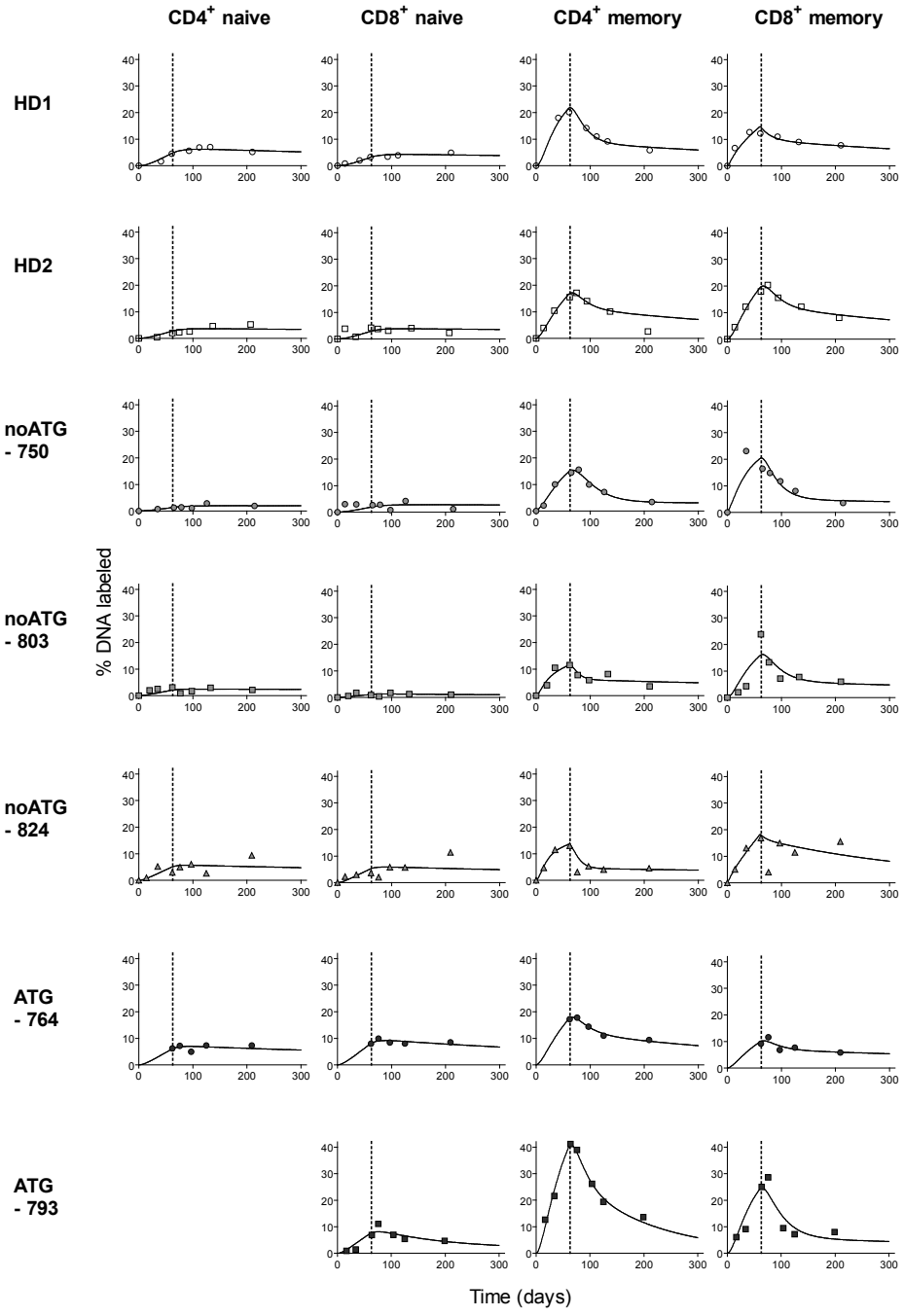


Figure 3. Individual data and best fits of ^{2}H enrichment in T-cell subsets in renal transplant recipients and healthy controls. Best fits of the multi-exponential model to the enrichment in naive and memory T cells in five renal transplant recipients and two healthy controls. The labeling curves of the memory T-cell subsets were significantly better described by a model including two kinetically different

subpopulations while the labeling curves of the naive T-cell subsets required only one compartment. Label enrichment in the DNA was scaled between 0 and 100% by normalizing for the maximum enrichment in granulocytes (see Methods). The end of ^2H_2O administration is marked by the dashed lines. For patient 764, enrichment data were limited to the down-labeling phase due to technical complications. For patient 793, the enrichment in CD4$^+$ naive T cells could not be measured due to too low cell numbers. For effector T cells, the material was too limited to compare deuterium enrichment data between the study groups. In the few subjects for whom we could sort sufficient numbers of effector T cells, deuterium enrichment levels were comparable to those in the corresponding memory T-cell subsets (data not shown).

Ki-67 expression levels are not substantially changed by immunosuppressive therapy

Although MMF, an important component of the immunosuppressive regimen, is known to be a potent inhibitor of lymphocyte proliferation by inhibiting the *de novo* synthesis pathway of guanine[5], little is known about the extent to which MMF inhibits T-cell proliferation *in vivo*. Different techniques used to study the effects of MMF have provided diverging insights; while *in vitro* T-cell proliferation in response to αCD3/αCD28 was found to be almost completely impaired in the presence of MMF, Ki-67 expression measured directly *ex vivo* was hardly affected[30]. Because of these differences in MMF-effects observed with different techniques, we also measured Ki-67-expression on CD4$^+$ and CD8$^+$ naive, memory, and effector T cells. The percentage of cells expressing Ki-67 did not seem different for any of the T-cell subsets between the non-ATG treated patients and healthy controls (Figure 5), consistent with the results from our deuterium labeling study. Only for the ATG-treated patient whose lymphocyte numbers were most severely depleted (ATG-793, black squares, Figure 5), the percentages of Ki-67$^+$ T cells were increased. Both the deuterium enrichment data and the Ki-67-expression data thus suggest that T-cell turnover rates are not substantially affected by immunosuppressive therapy under lymphoreplete conditions.

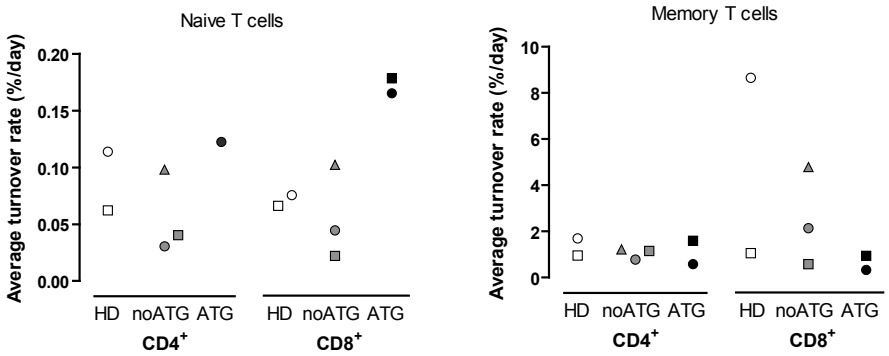


Figure 4. Average turnover rates of CD4$^+$ and CD8$^+$ naive and memory T cells. All average turnover rates were estimates by fitting the multi-exponential model to the individual data sets as described in Fig 3. Data of each individual are displayed with unique symbols (see Table 1); HD, healthy donor (open symbols); no ATG, graft recipients not treated with ATG (gray symbols); ATG, graft recipients treated with ATG within 2 weeks post-transplantation (black symbols). For ATG-patient 793 the turnover rate of CD4$^+$ naive T cells is missing due to low peripheral cell counts and limited material. For ATG-patient 764 (black dot), turnover rates were estimated only on deuterium enrichments measured after the stop of label administration (see Figure 3).

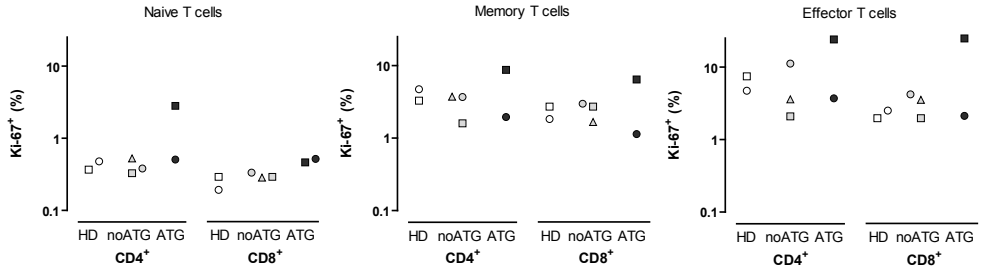


Figure 5. Ki67 expression in CD4⁺ and CD8⁺ naive, memory and effector T cells. The percentage of CD4⁺ and CD8⁺ naive, memory and effector T cells expressing the cell cycle marker Ki-67 was determined by flow cytometry. Symbols indicate median values of measurements performed throughout the study. Data of each individual are displayed with unique symbols (see Table 1); HD, healthy donor (open symbols); no ATG, graft recipients not treated with ATG (gray symbols); ATG, graft recipients treated with ATG within 2 weeks post-transplantation (black symbols).

Discussion

In an *in vivo* ^2H_2O-labeling study we investigated the impact of maintenance immunosuppressive therapy and ATG-mediated lymphocyte depletion on absolute T-cell numbers and T-cell dynamics in renal transplant recipients. Even though treatment with standard induction and maintenance immunosuppressive therapy was associated with relatively low peripheral T-cell numbers, we observed no differences in the *in vivo* turnover rates of various T-cell subsets compared to healthy age-matched controls. Although ATG therapy caused a rapid and robust depletion of CD4⁺ and CD8⁺ T cells, and resulted in a persistent deficiency of CD4⁺ T cells in particular, the turnover rates of CD4⁺ and CD8⁺ naive and memory T-cell subsets were hardly affected one year after ATG treatment.

While treatment with immunosuppressive drugs is necessary to prevent graft rejection, it adversely causes a life-long immunocompromised state of the allograft recipient, which at least in part may be related to an impaired maintenance of the T-cell pool. The various components of immunosuppressive regimens each have a different effect on the T-cell compartment. Corticosteroids have been reported to induce apoptosis in double positive thymocytes, to suppress the production of many (pro-inflammatory) cytokines, and to down-regulate adhesion molecules, thereby inhibiting T-cell migration, proliferation and inflammation[31;32]. Anti-CD25 monoclonal antibodies suppress IL-2-mediated proliferation by blocking signaling through the high-affinity IL-2 receptor[3], and calcineurin inhibitors inhibit T-cell proliferation by interfering with signaling downstream of the T-cell receptor[4]. Finally, MMF inhibits the *de novo* synthesis of purines, which mainly affects proliferation of lymphocytes[5]. Despite these effects that have been described for the immunosuppressive drugs, we found little evidence for decreased T-cell turnover rates in renal transplant patients on immunosuppressive therapy, and although T-cell numbers were lower than in healthy age-matched controls, they were still in the normal range. These results suggest that despite

the presence of immunosuppressive drugs, a certain level of T-cell proliferation is sustained to maintain the peripheral T-cell pool. A possible explanation for these findings is that immunosuppressive therapy primarily targets T-cell proliferation induced by antigen (via the TCR) and immune activation (via IL-2), but hardly affects homeostatic proliferation. Similarly, blocking of the IL-2 receptor alpha chain by anti-CD25 antibodies *in vitro* has been shown to inhibit the IL-2-induced proliferation of resting (naive) and activated T cells, but to enhance IL-7-induced proliferation[33].

Reports on the *in vivo* effects of MMF as inhibitor of T-cell proliferation are ambiguous. While activation-induced T-cell proliferation was previously shown to be almost completely impaired in the presence of MMF *in vitro* [30;34], we and others have found that *ex vivo* Ki-67 expression was hardly affected by MMF treatment[5;30;35]. Vrisekoop et al. previously proposed that this discrepancy between the effects of MMF on *in vitro* T-cell proliferation and *ex vivo* Ki-67 expression could be explained if Ki-67 expression is induced before the stage of the cell cycle that is inhibited by MMF. If cells can express Ki-67 without completing proliferation, Ki-67-expression levels may thus overestimate T-cell proliferation[30]. Although this could in principle explain why Ki-67 is not lower in MMF-treated patients compared to healthy controls, our deuterium enrichment data also suggest that T-cell turnover is not substantially inhibited by MMF. Alternatively, discrepancies between the effect of MMF on *ex vivo* Ki-67 expression and T-cell responses to *in vitro* stimulation may be related to the subpopulation of cells that is proliferating. It has been reported that the *de novo* purine synthesis pathway, which is inhibited by MMF, is essential for proliferation of activated T cells, while resting T cells and thymocytes also rely on the salvage pathway[36]. Moreover, the type II isoform of inosine 5'-monophosphate dehydrogenase, that is most actively inhibited by MMF, is mainly expressed by activated T cells[37]. Thus, while activation-induced expansion (such as the *in vitro* response to stimulation) may be efficiently blocked by MMF, homeostatic proliferation of T cells (as measured by *ex vivo* Ki-67 expression) may still occur. This may also explain why T-cell turnover rates are hardly affected in MMF-treated renal transplant recipients, despite the drastic effects of MMF in in vitro stimulation assays.

Changes in T-cell turnover rates may be more evident in ATG-treated renal transplant patients, who still had a severely depleted $CD4^+$ T-cell pool at the start of the deuterium labeling study. In autologous stem cell transplantation (autoSCT) patients, whose lymphocyte compartment was depleted by chemotherapy, we recently found that the turnover rate of $CD4^+$ naive and memory T cells was significantly higher compared to healthy controls (chapter 4 of this thesis), suggesting that lymphopenia induces an increased T-cell proliferative response. Since one year after kidney transplantation, the $CD4^+$ T-cell compartments of ATG-treated renal transplant patients were still as depleted as those of autoSCT patients, and given the small effect of MMF on the turnover rates of non-ATG treated patients, we would have expected an increase in $CD4^+$ T-cell turnover following ATG therapy. The fact that we did not find this, suggests that the maintenance treatment with immunosuppressive agents

counteracted the occurrence of a lymphopenia-induced proliferative T-cell response. Thus, while immunosuppressive therapy hardly affects T-cell turnover rates in lymphoreplete patients, it seems to prevent an increase in T-cell proliferation in ATG-treated lymphopenic patients.

A possible explanation for these observations is that the immunosuppressive agents inhibit a form of peripheral expansion that is not essential to maintain the T-cell compartment under lymphoreplete conditions, but does play an important role during immune reconstitution. Lymphopenia-induced homeostatic proliferation is thought to be driven by cytokines (like IL-7, IL-15, and IL-21) resulting in relatively slow proliferation, and by cognate antigen-MHC interactions that result in more rapid cell expansion[38]. It is plausible that both mechanisms contribute to immune reconstitution, and are responsible for the increased T-cell turnover observed in autoSCT patients (chapter 4 of this thesis). Immunosuppressive drugs possibly inhibit cognate antigen-stimulated proliferation more effectively than, for example, IL-7 induced proliferation, an idea that is compatible with their modes of action (see above). Interestingly, when the deuterium enrichment data of all subjects were plotted in a single figure per T-cell subset, we observed that deuterium enrichment levels of naive $CD4^+$ and $CD8^+$ T cells and memory $CD4^+$ T cells tended to be lower in the non-ATG treated patient group, and higher in the ATG-treated patients when compared to the healthy controls (supplemental Figure 1). Although these observations should be interpreted with caution because of the small group sizes, they suggest that (homeostatic) T-cell proliferation may also be slightly inhibited in non-depleted patients, and that lymphopenia-induced proliferation may not be completely inhibited by the immunosuppressive therapy. Findings from two recent studies are in line with this hypothesis, showing increased percentages of $CD4^+$ and $CD8^+$ T cells expressing the cell cycle marker Ki-67 in the first months following ATG administration, despite treatment with immunosuppressive drugs[12;39].

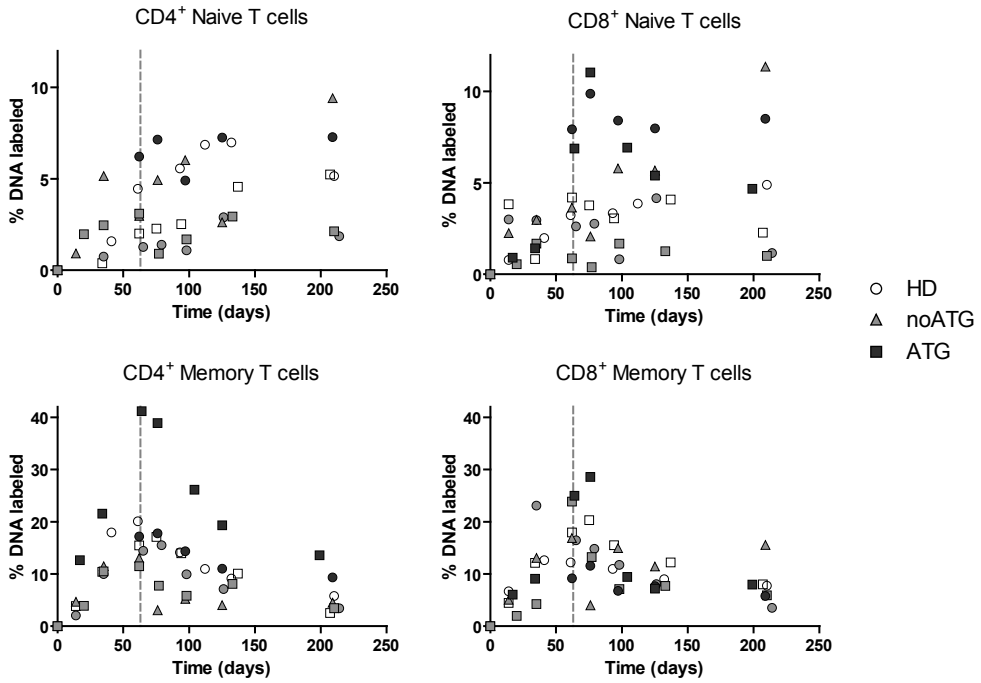
In summary, our findings suggest that while immunosuppressive maintenance therapy does not have a major impact on T-cell maintenance, it effectively inhibits lymphopenia-induced proliferation, and may thereby delay reconstitution following ATG-mediated lymphocyte depletion. Although a proliferative response would be favorable in view of immune reconstitution, it may also have adverse effects in allograft recipients. For example, studies in mice have shown that T cells that have undergone homeostatic proliferation become resistant to tolerance induction, and drive rejection of cardiac allografts[40]. On the other hand, long-term immune deficiency also negatively affects the immune competence of allograft recipients. Hence, to improve the clinical condition after solid organ transplantation, it is important to circumvent the need of non-specific, total lymphocyte depletion, for example by discovering ways to specifically target alloreactive lymphocytes.

References

1. van Sandwijk MS, Bemelman FJ, Ten Berge IJ. Immunosuppressive drugs after solid organ transplantation. Neth.J.Med. 2013;71:281-289.
2. Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. N.Engl.J.Med. 2005;353:1711-1723.
3. Tkaczuk J, Yu CL, Baksh S et al. Effect of anti-IL-2Ralpha antibody on IL-2-induced Jak/STAT signaling. Am.J.Transplant. 2002;2:31-40.
4. Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature 1992;357:695-697.
5. Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. Immunopharmacology 2000;47:85-118.
6. Bonnefoy-Berard N, Vincent C, Revillard JP. Antibodies against functional leukocyte surface molecules in polyclonal antilymphocyte and antithymocyte globulins. Transplantation 1991;51:669-673.
7. Brennan DC, Daller JA, Lake KD, Cibrik D, Del CD. Rabbit antithymocyte globulin versus basiliximab in renal transplantation. N.Engl.J.Med. 2006;355:1967-1977.
8. Mourad G, Morelon E, Noel C, Glotz D, Lebranchu Y. The role of Thymoglobulin induction in kidney transplantation: an update. Clin.Transplant. 2012;26:E450-E464.
9. Mohty M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. Leukemia 2007;21:1387-1394.
10. Muller TF, Grebe SO, Neumann MC et al. Persistent long-term changes in lymphocyte subsets induced by polyclonal antibodies. Transplantation 1997;64:1432-1437.
11. Louis S, Audrain M, Cantarovich D et al. Long-term cell monitoring of kidney recipients after an antilymphocyte globulin induction with and without steroids. Transplantation 2007;83:712-721.
12. Havenith SH, Remmerswaal EB, Bemelman FJ et al. Rapid T cell repopulation after rabbit anti-thymocyte globulin (rATG) treatment is driven mainly by cytomegalovirus. Clin.Exp.Immunol. 2012;169:292-301.
13. Preville X, Flacher M, LeMauff B et al. Mechanisms involved in antithymocyte globulin immunosuppressive activity in a nonhuman primate model. Transplantation 2001;71:460-468.
14. Schaefer SM, Susal C, Sommerer C, Zeier M, Morath C. Current pharmacotherapeutical options for the prevention of kidney transplant rejection. Expert.Opin.Pharmacother. 2013;14:1029-1041.
15. Zaza G, Tomei P, Granata S, Boschiero L, Lupo A. Monoclonal antibody therapy and renal transplantation: focus on adverse effects. Toxins.(Basel) 2014;6:869-891.
16. Waller EK, Langston AA, Lonial S et al. Pharmacokinetics and pharmacodynamics of anti-thymocyte globulin in recipients of partially HLA-matched blood hematopoietic progenitor cell transplantation. Biol.Blood Marrow Transplant. 2003;9:460-471.
17. Issa NC, Fishman JA. Infectious complications of antilymphocyte therapies in solid organ transplantation. Clin.Infect.Dis. 2009;48:772-786.
18. Ducloux D, Bourrinet E, Motte G, Chalopin JM. Antiphospholipid antibodies as a risk factor for atherosclerotic events in renal transplant recipients. Kidney Int. 2003;64:1065-1070.
19. Thibaudin D, Alamartine E, Mariat C, Absi L, Berthoux F. Long-term kinetic of T-lymphocyte subsets in kidney-transplant recipients: influence of anti-T-cell antibodies and association with posttransplant malignancies. Transplantation 2005;80:1514-1517.
20. Gurkan S, Luan Y, Dhillon N et al. Immune reconstitution following rabbit antithymocyte globulin. Am.J.Transplant. 2010;10:2132-2141.
21. Vrisekoop N, den Braber I, de Boer AB et al. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. Proc.Natl.Acad.Sci.U.S.A 2008;105:6115-6120.
22. Westera L, van Hoeven V, Drylewicz J et al. Lymphocyte maintenance during healthy aging requires no substantial alterations in cellular turnover. Aging Cell 2015;14:219-227.

23. Meyer C, Walker J, Dewane J et al. Impact of irradiation and immunosuppressive agents on immune system homeostasis in rhesus macaques. Clin.Exp.Immunol. 2015
24. van de Berg PJ, Heutinck KM, Raabe R et al. Human cytomegalovirus induces systemic immune activation characterized by a type 1 cytokine signature. J.Infect.Dis. 2010;202:690-699.
25. Boom R, Sol C, Weel J et al. A highly sensitive assay for detection and quantitation of human cytomegalovirus DNA in serum and plasma by PCR and electrochemiluminescence. J.Clin.Microbiol. 1999;37:1489-1497.
26. Busch R, Neese RA, Awada M, Hayes GM, Hellerstein MK. Measurement of cell proliferation by heavy water labeling. Nat.Protoc. 2007;2:3045-3057.
27. Westera L, Zhang Y, Tesselaar K, Borghans JA, Macallan DC. Quantitating lymphocyte homeostasis in vivo in humans using stable isotope tracers. Methods Mol.Biol. 2013;979:107-131.
28. Westera L, Drylewicz J, den Braber I et al. Closing the gap between T-cell life span estimates from stable isotope-labeling studies in mice and humans. Blood 2013;122:2205-2212.
29. R Development Core Team: A language and environment for statistical computing. 2010. Ref Type: Generic
30. Vrisekoop N, Sankatsing SU, Jansen CA et al. Short communication: No detrimental immunological effects of mycophenolate mofetil and HAART in treatment-naive acute and chronic HIV-1-infected patients. AIDS Res.Hum.Retroviruses 2005;21:991-996.
31. Ashwell JD, Lu FW, Vacchio MS. Glucocorticoids in T cell development and function*. Annu.Rev. Immunol. 2000;18:309-345.
32. Coutinho AE, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol.Cell Endocrinol. 2011;335:2-13.
33. Monti P, Brigatti C, Heninger AK, Scirpoli M, Bonifacio E. Disengaging the IL-2 receptor with daclizumab enhances IL-7-mediated proliferation of CD4(+) and CD8(+) T cells. Am.J.Transplant. 2009;9:2727-2735.
34. Sankatsing SU, Prins JM, Yong SL et al. Mycophenolate mofetil inhibits T-cell proliferation in kidney transplant recipients without lowering intracellular dGTP and GTP. Transpl.Int. 2008;21:1066-1071.
35. Garcia F, Plana M, Arnedo M et al. Effect of mycophenolate mofetil on immune response and plasma and lymphatic tissue viral load during and after interruption of highly active antiretroviral therapy for patients with chronic HIV infection: a randomized pilot study. J.Acquir.Immune.Defic.Syndr. 2004;36:823-830.
36. Fairbanks LD, Bofill M, Ruckemann K, Simmonds HA. Importance of ribonucleotide availability to proliferating T-lymphocytes from healthy humans. Disproportionate expansion of pyrimidine pools and contrasting effects of de novo synthesis inhibitors. J.Biol.Chem. 1995;270:29682-29689.
37. Carr SF, Papp E, Wu JC, Natsumeda Y. Characterization of human type I and type II IMP dehydrogenases. J.Biol.Chem. 1993;268:27286-27290.
38. Williams KM, Hakim FT, Gress RE. T cell immune reconstitution following lymphodepletion. Semin. Immunol. 2007;19:318-330.
39. Bouvy AP, Kho MM, Klepper M et al. Kinetics of homeostatic proliferation and thymopoiesis after rATG induction therapy in kidney transplant patients. Transplantation 2013;96:904-913.
40. Wu Z, Bensinger SJ, Zhang J et al. Homeostatic proliferation is a barrier to transplantation tolerance. Nat.Med. 2004;10:87-92.
41. Zorginstituut Nederland. Farmacotherapeutisch Kompas. Ref Type: Generic

Supplemental Figures



Supplemental Figure 1. Overlay of deuterium enrichment data in healthy donors and renal transplant recipients. Deuterium enrichment was measured in the DNA of sorted naive and memory T-cell subsets as described in Materials and Methods. Data of each individual are displayed with unique symbols (see Table 1); HD, healthy donor (open symbols); no ATG, graft recipients not treated with ATG (gray symbols); ATG, graft recipients treated with ATG (black symbols). The dotted line represents the end of the labeling period. For ATG-patient 793 enrichment data in $CD4^+$ naive T cells is missing due to low peripheral cell counts and limited material.

6

General discussion

6

The studies described in this thesis were focused on the investigation of lymphocyte dynamics in healthy and in lymphopenic conditions. To be able to understand how for example aging, chronic infections, hematopoietic malignancies and lymphocyte-depleting interventions affect the lymphocyte compartment, it is essential to understand how lymphocytes are produced and maintained in the healthy situation. In our studies we analyzed the absolute and relative abundance of lymphocyte subsets, and in addition used deuterium labeling; a unique technique that allowed us to investigate the production rate and lifespan of lymphocyte subsets *in vivo*. Our main findings are that (i) during healthy aging, lymphocyte turnover rates do not substantially change despite a tenfold decline in thymic output, while (ii) in the severe lymphopenic condition following chemotherapy and stem-cell transplantation, turnover rates of deficient lymphocyte subsets are homeostatically increased, promoting their reconstitution. In this chapter our findings will be discussed in a broader perspective.

Recent thymic emigrants in mice and men

The production of new T cells occurs via T-cell development in the thymus and via division of mature T cells in the periphery. The unique aspect of thymus-mediated T-cell development is that it generates new T-cell receptor (TCR) specificities, and therefore thymic output is essential for the establishment of a broad TCR repertoire. Although many immunologists have tried to estimate human thymus function and the relative contribution of the thymus to T-cell production in various conditions, thymic output remains very difficult to measure. A common limitation of the current approaches, i.e., measuring T-cell receptor excision circles (TRECs) or the expression of the cell surface markers CD31 or PTK7, is that they are not uniquely expressed by recent thymic emigrants, and hence do not directly represent thymic output[1-4].

To assess the relationship between thymic output, the peripheral RTE population, and naive T-cell maintenance, it is not only important to phenotypically define RTE, but also to have insights into the dynamics of RTE, and to know whether they are kinetically different from the resident, mature naive (MN) T-cell population. Because of all the limitations in studying human RTE biology, insights into RTE dynamics are mostly derived from studies in mice. Following thymectomy, the number of naive T cells was observed to decline in a biphasic manner[2], which hints towards the existence of a relatively short-lived RTE and longer-lived MN T-cell pool. Alternatively, these observations could be explained by a peripheral density-dependent death rate, and hence these thymectomy experiments do not give a definite answer on the dynamics of RTE. Studies that were specifically designed to investigate RTE dynamics have mainly used either RAG2p-GFP transgenic mice in which RTE temporarily express GFP, or mice transplanted with a neonatal, congenic thymus graft[5]. However, these studies reported conflicting findings, suggesting that (*i*) RTE are exempt from peripheral T-cell homeostasis for a period of 3 weeks[6;7], (*ii*) RTE are relatively short-lived[8] or (*iii*) RTE are relatively long-lived cells in the naive T-cell pool[9]. Biological arguments could support either findings. RTE

may be relatively long-lived just because they are the youngest cells that still have a long life-expectance. On the other hand, it has been suggested that RTE leave the thymus in an immature state[10] and the need for continued peripheral maturation and selection may result in a relatively short lifespan for RTE.

The controversial findings on the relative longevity of RTE have been partly ascribed to differences in the age of the mice whose T cells were studied[9] (chapter 2). Firstly, the peripheral T-cell density, which is known to change rapidly in the first weeks of life[2;11] (chapter 2), may affect T-cell lifespans. Secondly, in adoptive transfer experiments, the expected lifespan of the donor-derived naive T cells was shown to increase with the age of the donor mouse[12], suggesting that cell-intrinsic age may influence T-cell lifespan as well. To exclude peripheral T-cell density as a possible confounder, we therefore studied RTE dynamics in comparison to the average naive T-cell dynamics in mice of at least 12 weeks old, as at that age the size of the peripheral T-cell pool remains stable throughout adulthood. Moreover, the unique combination of thymus transplantations, deuterium labeling studies and mathematical modeling also allowed us to compare the average lifespan of naive T cells (i.e., RTE+MN T cells) from adult mice to the RTE lifespan estimates from both neonatal-graft derived RTE and adult RTE. The data from our studies consistently indicate that RTE are relatively short-lived cells in the naive T-cell pool. Interestingly, the loss of thymus-graft derived RTE in our experiments was slower than reported by another thymus transplantation study in younger mice, suggesting that (age-related) differences in peripheral T-cell density may indeed affect RTE lifespan (chapter 2).

Knowing that the contribution of the thymus to naive T-cell maintenance differs considerably between mice and men[2] (see below), it remains challenging to extrapolate our findings to the human situation. On the other hand, dynamic aspects of the RTE compartment could be analogous in both species, as is also the case for e.g. age-related thymus involution. After stem-cell transplantation in humans, we and others have observed relatively high TREC contents in the naive T-cell pool[13-15] (chapter 4). Although some have interpreted these findings as an indication for thymic rebound[13;14], high TREC contents can alternatively be explained by the over-representation of RTE in a relatively empty naive T-cell pool[15] (chapter 4). Moreover, the lifespan of RTE may be increased in lymphopenic conditions. While the two key murine studies were conflicting on the relative survival of RTE in a lymphoreplete environment, they consistently reported that RTE have a superior survival over resident naive T cells in lymphopenic mice[8;9]. Thus, emptiness of the peripheral naive T-cell pool may induce an increase in the longevity of RTE, which would biologically be very relevant in in conditions that require replenishment of the naive T-cell pool and its TCR repertoire.

Thymic output in aging humans

As the thymus starts degenerating as soon as a few years after birth[16], the role of the thymus throughout human life is much debated. Fundamental insights into the age-related involution of the thymus come from studies by Steinmann et al., who reported that with age functional thymic tissue is progressively replaced by fat[17]. To understand the consequences of thymus involution, we have estimated the absolute daily production of T cells by the thymus in young and elderly healthy volunteers using TREC content analysis, stable-isotope labeling, and mathematical modeling (chapter 3). Our estimates demonstrate that between the 3rd and 7th decade of life, thymic output still declines significantly, from approximately 16 million to hardly 1 million naive T cells per day. This decline in thymic output is comparable with the estimated 5% yearly decline that was based on the histological observations by Steinmann et al[17].

Aging is generally associated with an increased susceptibility to, for example, infectious diseases and cancer, and a decreased protective response to vaccination. Because a decline in the percentage and number of naive T cells has been observed with age and a reduction in the TCR repertoire diversity has been observed in elderly over 75 years of age[18-21], it is generally believed that thymus involution is partially responsible for this decline in immune function[16;22]. In mice, the severely diminished size and diversity of the naive T-cell pool observed at old age[23-25] is indeed likely to be related to thymus involution, knowing that in mice the majority of new T cells come from the thymus. In humans however, the relative contribution of the thymus to T-cell production is only about 15% in young adults, and as small as 1% in elderly individuals aged between 65 and 75 years[2] (chapter 3). Such low levels of thymic output, in combination with a certain level of peripheral T-cell division, are apparently sufficient in humans to stay healthy until at least the age of 65. Peripheral T-cell division and the profound longevity of naive T cells probably sustain a sufficient size and diversity of the naive T-cell compartment, provided that T-cell survival and division generally do not select for certain TCR specificities. In line with this idea, we found that $CD4^+$ naive T-cell numbers were only marginally lower in the elderly subjects of our healthy cohort (chapter 3). Moreover, other studies have shown that TCR repertoire diversity of the $CD4^+$ naive T-cell compartment is in fact similar in young adults and 65-year old individuals[21;26].

Changes in the $CD4^+$ naive T-cell pool with age: an important role for CMV

The finding that in some elderly individuals naive $CD4^+$ T cell numbers and repertoire diversity can be maintained despite a pronounced age-related loss in thymic output raises the question whether changes in the $CD4^+$ T-cell count and repertoire diversity observed in other aged individuals are caused by thymus involution. In recent literature, it was reported that changes in the composition and repertoire diversity of the $CD4^+$ naive T-cell pool were more pronounced in elderly who were seropositive for cytomegalovirus (CMV)[26-28]. CMV infection is notorious for its tremendous effect on expansion and repertoire skewing in the effector/

memory T-cell pools[29-31]. Since a large majority of the Western population is infected with CMV, it is remarkable that 9 out of 10 elderly in our healthy aging study appeared to be CMV seronegative (chapter 3). Because of this, we were able to study how aging affects T-cell dynamics in absence of CMV infection as a possible confounder. Although an effect of CMV was not observable in our single CMV$^+$ aged individual, our data do support the idea that the CD4$^+$ naive T-cell pool need not be affected by the age-related decline in thymic output.

How important is the thymus?

If such a small contribution of the thymus suffices for the maintenance of a stable and diverse T-cell pool into old age, one may wonder how essential thymic output actually is after the T-cell pool has been established in neonatal life. Removal of the thymus because of cardiac surgery in early childhood was shown to induce a severe reduction in TREC contents, and in the number and diversity of naive T cells during the first years after surgery[32-35], a condition that resembles observations in some elderly. To date, it is however unclear whether thymectomy results in any clinical complications related to immune incompetence[36]. Remarkably, only in a minority of thymectomized individuals the TREC content and composition of the T-cell compartment remained disturbed until 2-3 decades post-thymectomy[32;35;37] and in one study, CMV infection was found to be associated with more persistent changes after thymectomy. Normalization of the TREC contents and the T-cell composition in the majority of thymectomized individuals may be related to an increase in the peripheral T-cell division and the extreme longevity of naive T cells. However, signs of thymus regrowth have also been observed in some patients[35], suggesting that thymic T-cell development may not have been completely abolished. Thus, studies in thymectomized individuals do no give a definite answer on the role of the thymus, since little residual thymus function may be sufficient and essential for maintaining immune function into adulthood.

Altogether, the findings in elderly and thymectomized individuals suggest that peripheral T-cell division, in combination with the long lifespan of naive T cells, maintain a healthy naive T-cell pool with a diverse TCR repertoire for several decades, as long as the immune system is not chronically triggered by, for example, persistent infections like CMV. However, it is possible that restricted thymic output will manifest in some degree of immunodeficiency on the longer term. While we and others have not found substantial disturbances of the T-cell pool until the age of 70, a substantial reduction in naive T-cell numbers and TCR diversity seems to be evident in even older subjects[18;21;38]. It is very unlikely that this is merely due to the further decline in thymic output. Rather, in the presence of certain (chronic) antigenic stimuli like CMV infection, sustained immune activation, T-cell proliferation, and differentiation may eventually cause exhaustion of the naive T-cell pool and reduce its repertoire diversity. Perhaps, the observed differences between the elderly of our study and the even older individuals in other studies are related to the very stringent criteria that we used to select our healthy individuals. Whether thymectomy leads to an accelerated failure of naive T-cell

homeostasis remains unknown, as the longest follow-up of thymectomized subjects currently reported is about 30 years and signs of accelerated aging may manifest only later in life.

Finally, it is important to note that the thymus is essential for the establishment of the naive T-cell compartment, which is illustrated by an interesting case report study by Heitger et al[39]. In this study, reconstitution of the naive T-cell compartment after bone marrow transplantation (BMT) in a previously thymectomized child was compared to the reconstitution in thymus-bearing hosts. Reconstitution of naive $CD4^+$ T cells was severely impaired in the thymectomized patient even up to two years post-BMT, while in the thymus-bearing hosts effective outgrowth of naive $CD4^+$ T cells was observed[39]. The importance of the thymus in immune reconstitution is further supported by the fact that the rate of naive T-cell reconstitution after lymphocyte depletion and hematopoietic transplantation is slower in adults compared to children[40]. In chapter 3, we estimated that in healthy elderly the thymus still produces roughly 1 million cells per day based on measured TREC contents in $CD4^+$ naive T cells. As naive T cells are very long-lived cells, TREC-containing cells may in fact have been in the periphery for years, and hence - strictly speaking - our data do not provide evidence for a functional thymus at the age of 65. However, in all autologous stem-cell transplanted patients, aged 54-61 years, we measured significantly higher TREC contents compared to age-matched healthy controls at 1 year post-SCT, demonstrating that the thymus was still functional in these patients (chapter 4). Taken together, our data support that the thymus is still functional at older age, that it contributes only little to daily naive T-cell production in healthy individuals, but significantly to immune reconstitution in lymphopenic patients.

Enhanced peripheral T-cell division: the solution for thymus involution?

As severe lymphopenia in the mouse has been shown to induce an increase in peripheral T-cell division, a phenomenon called lymphopenia-induced proliferation, it is generally thought that in humans the peripheral division rate is similarly influenced by the peripheral T-cell density. An inverse correlation between the absolute number of naive T cells and the percentage of cells expressing Ki-67 has been reported in elderly, thymectomized young adults, and HIV patients[41], suggesting that low or declining cell numbers may indeed induce increased proliferation in humans. Then, is an enhanced level of peripheral proliferation, driven by a homeostatic response, responsible for the maintenance of the naive T-cell pool during advanced aging? In our group of particularly healthy elderly, we did not find signs of increased peripheral T-cell division in response to the profound decline in thymic output, suggesting that a homeostatic response either did not occur, or was too small to be measured (chapter 3). In contrast, increased proportions of Ki-67-expressing naive T cells in elderly humans and monkeys have been observed by others[21;41;42]. Several reasons may explain why we did not find this correlation in our healthy subjects. If differences in peripheral division rates are very small, we cannot exclude that we may have missed the correlation because of our small sample size. On the other hand, the elderly volunteers selected for our

study were exceptionally healthy for their age. In these individuals, a substantial change in peripheral T-cell division was apparently *not needed* to maintain a healthy immune system. Whether the reported correlation between increased percentages of Ki-67 and low cell numbers solely reflects a beneficial homeostatic response, aimed at maintaining the T-cell compartment is disputable. In HIV-infected individuals and patients treated with allogeneic stem cell transplantation, increased percentages of Ki-67-expressing T cells were shown not to be directly linked to the lymphopenic state, but were instead associated with immune activation[15;43]. Therefore, increased Ki-67-levels observed in the aged individuals of the other studies may have been the result of increased immune activation, which may even *drive* the decline in naive T cells through increased naive T-cell death and differentiation into the memory T-cell compartment. In view of the major impact that CMV has on the composition of the T-cell compartment (see the section on the role of CMV), it would be interesting to investigate whether CMV is an important confounder in studies on homeostatic proliferation.

Lymphopenia-induced T-cell proliferation occurs after therapeutic lymphocyte depletion

Although we did not find indications for a homeostatic increase in T-cell division in aged individuals to compensate for thymus involution (chapter 3), our results do not exclude that a homeostatic response does occur in, for example, severe lymphopenic conditions. In chapter 4 we studied whether lymphocyte turnover rates are altered after chemotherapy-mediated lymphocyte depletion in combination with hematopoietic stem cell transplantation. By including patients that were treated with an autologous stem cell transplantation (autoSCT), we excluded the risk of graft-versus-host disease (GVHD), which was previously shown to be a confounding driver of T-cell division[15]. We found that one year after transplantation, naive and memory $CD4^+$ T cells, and memory B cells, were still significantly lower in peripheral cell count compared to healthy controls. Interestingly, these exact subsets displayed a significantly higher lymphocyte production rate, which is suggestive for an association between peripheral cell number and lymphocyte production.

To assess whether a correlation indeed exists between the average turnover rate and peripheral cell count, we combined the naive T-cell data of all deuterium labeling studies of this thesis and plotted these in a single figure (Figure 1). Although the peripheral cell counts of $CD4^+$ and $CD8^+$ naive T cells are clearly variable in healthy individuals (open and gray-filled circles), there is no clear inverse association between cell counts and turnover. Regardless of the peripheral cell count, naive T-cell turnover is generally very low in all healthy individuals. Within these healthy subjects, a higher turnover rate can only be observed for the $CD8^+$ naive T-cell pool of two elderly individuals, but this is not clearly related to their low cell count. In chapter 3 we explain that this finding is most likely the result of a rapidly-dividing $CD95^+$ subfraction that is more abundant in the elderly.

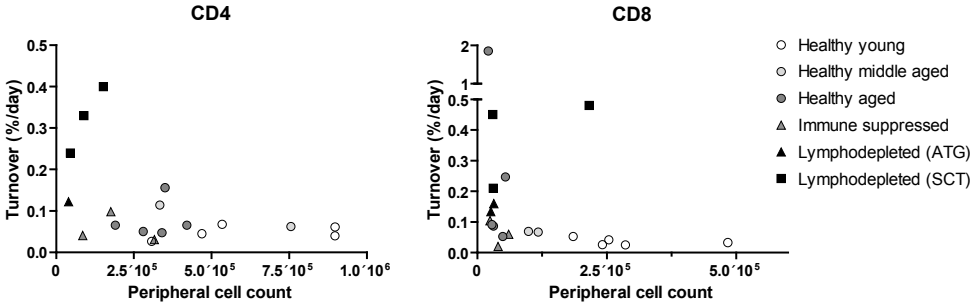


Figure 1. The turnover rate of naive T cells in relation to their peripheral cell counts. $CD4^+$ (left) and $CD8^+$ (right) naive T-cell turnover rates determined in healthy volunteers and patient groups using deuterium labeling (chapters 3-5) plotted against the corresponding peripheral cell count per individual. The healthy young (20-25y) and healthy aged (65-75y) were involved in the healthy aging study (chapter 3), while the heathy middle aged individuals (41y and 60y) served as healthy controls for the kidney graft recipients, whose T-cell dynamics were studied for the effect of ATG-mediated lymphocyte depletion and immunosuppressive therapy (chapter 5). In all studies, naive T cells were defined as $CD27^+$ and either expression of CD45RA or lack of CD45RO expression.

Of the non-healthy individuals, only the SCT patients - whose peripheral counts are also among the lowest - consistently displayed higher turnover rates in the $CD4^+$ T-cell compartment. It is possible that the peripheral naive T-cell population needs to be depleted below a certain threshold, before a homeostatic proliferative response is triggered. In a recent study in rhesus macaques, lymphocyte depletion by ATG-treatment or total body irradiation was reported to induce an increase in T-cell proliferation (increased percentage of $Ki\text{-}67^+$ cells) suggesting that lymphopenia-induced proliferation is general phenomenon and is not dependent on the method of lymphocyte depletion. While treatment of renal transplant recipients with anti-thymocyte globulin (ATG) did result in severe lymphopenia beyond one year post-depletion, increased turnover rates were not observed in these patients. It is likely that treatment with immunosuppressive agents in these patients counteracted the induction of a homeostatic proliferative response (chapter 5).

Overall, we do not find a clear correlation between peripheral naive T-cell numbers and their turnover rates. For the naive $CD4^+$ T-cell compartment we do find signs of a homeostatic response when cell numbers are very low. It is unclear whether a similar response occurs for naive $CD8^+$ T cells. As following lymphocyte depletion, $CD8^+$ T cells generally reconstitute faster, it would be informative to additionally study $CD8^+$ T-cell turnover within the first months after lymphocyte depletion.

Maintenance of the human B-cell compartment

In addition to T-cell subsets, we have also studied the maintenance and reconstitution of B-cell subsets in healthy aging and after stem cell transplantation respectively (chapters 3 and 4). Similar to our findings for the T-cell compartment, we observed no substantial differences in the turnover rate of B-cell subsets between young and aged individuals, and peripheral

B-cell counts were also similar in both groups. The absence of substantial differences in B-cell numbers and their turnover rates does not exclude the possibility that changes in bone marrow output or peripheral division occur with aging. Although literature is conflicting on how age affects the bone marrow and peripheral B-cell compartment[22;44;45], some studies have reported a decline in B-cell progenitors with age[46-48] and a loss of B-cell repertoire diversity[49], which could be suggestive for an age-related reduction in bone-marrow output.

Like thymic output, bone marrow output cannot easily be measured in peripheral blood. About a decade ago, van Dongen and colleagues introduced the use of a Kappa-deleting recombination excision circles (KREC) assay to determine the replication history of peripheral B-cell subsets[50]. Similar to TRECs, KRECs are stable non-replicating DNA circles formed during *de novo* development, and upon every cell division KREC contents are twofold diluted. The KREC content in B-cell subsets is therefore informative on the level of cell division that has taken place since development in the bone marrow. To find out if the relative contribution of bone marrow output and peripheral cell division is different between young and elderly healthy individuals, KREC assays were performed with DNA samples of naive, memory, and natural effector B-cell subsets. In agreement with the findings in healthy donor material by van Zelm et al.[50], naive B cells have a history of only 1-3 divisions, while natural effector and memory B cells have divided much more since their formation in the bone marrow (7-8 divisions on average, Figure 2). Although some interindividual differences can be observed, the average replication history of all B-cell subsets was not different between the healthy young and aged subjects. Thus, these data do not provide any support for the idea that bone marrow output declines with age, or that the level of peripheral B-cell division is different between healthy young and elderly individuals. Instead, our data suggest that the contribution of bone marrow output to naive B-cell maintenance remains substantial throughout adulthood.

It is unknown whether B-cell development in the bone marrow is affected by lymphocyte-depleting therapies. One year after autoSCT, naive B cells were fully reconstituted and the turnover rate of naive B cells was not different between SCT patients and healthy controls (chapter 4). At that time, the contribution of bone marrow output and peripheral division does not seem to be different between SCT patients and healthy controls, as deduced from the similar replication histories of their naive B cells (Figure 2). As we did find an increased turnover rate for memory and natural effector B cells in SCT patients, it is possible that these subsets have relatively low KREC contents compared to healthy individuals. Because of limited DNA material, we were however not able to analyze the KREC contents of these subsets.

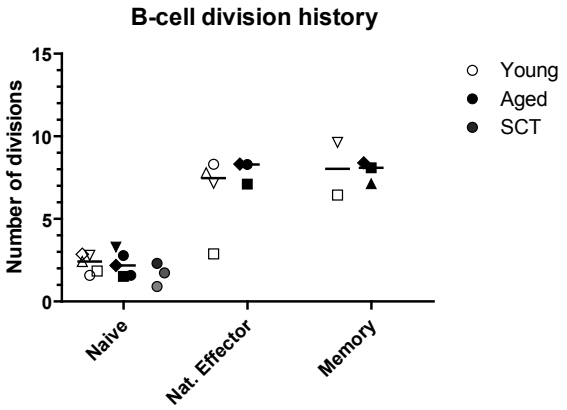


Figure 2. Replication history of B-cell subsets in healthy young and aged volunteers and SCT patients. KREC assays were performed by van Zelm and colleagues, following the procedure previously described[50]. DNA of naive B cells was available for KREC analysis for all healthy "B-cell" individuals and all SCT patients described in chapter 3 and 4 respectively. DNA material of the other subsets was limited and replication history could only be measured for natural effector B cells in 4 young and 3 elderly individuals, and memory B cell data is limited to 2 young and 3 elderly healthy individuals.

What determines the rate of lymphocyte reconstitution?

In this thesis we investigated lymphocyte reconstitution in two settings; following autologous stem cell transplantation in patients treated for a hematological malignancy with high-dose chemotherapy, and in renal allograft recipients in whom lymphocytes were depleted by anti-thymocyte globulin (ATG) treatment (chapters 4 and 5). An important difference between these conditions is that during reconstitution, allograft recipients were treated with immunosuppressive drugs to prevent graft rejection, while autoSCT patients were not. Despite this contrast, the patterns of $CD4^+$ and $CD8^+$ T-cell reconstitution were remarkably similar in both patient groups. We analyzed peripheral cell counts approximately one year after lymphocyte depletion, and at that time, total $CD8^+$ T-cells had reconstituted to a level in the range of normal for healthy adults, while $CD4^+$ T-cell numbers were still substantially diminished in both patient groups (chapters 4 and 5).

For the ATG-treated patients, one could argue that immunosuppression may have delayed immune reconstitution by suppressing T-cell proliferation. Although T-cell turnover was indeed not increased in the ATG-treated patients, the autoSCT patients also still had subnormal $CD4^+$ T-cell counts despite the occurrence of a proliferative homeostatic response. Moreover, immunosuppressive therapy does not explain the difference in reconstitution between $CD4^+$ and $CD8^+$ T cells observed in both groups. Differences in baseline turnover between $CD4^+$ and $CD8^+$ naive T cells do not provide an explanation, as in healthy individuals $CD8^+$ naive and memory T cells turnover more slowly than their $CD4^+$ counterparts[51] (chapter 3). Perhaps a robust proliferative response shortly after lymphocyte depletion drives faster reconstitution of $CD8^+$ T cells, but as cell counts have normalized by one year after lymphocyte depletion, the proliferative response may have waned by the time we measured T-cell

turnover. Proliferation may be more easily triggered in $CD8^+$ T cells, for example, because MHC class I molecules are more widely expressed compared to the restricted expression of MHC class II. Alternatively, $CD8^+$ T cells may be more responsive to survival cytokines like IL-7, and IL-15, which are more freely available under lymphopenic conditions. It has been reported that the $CD8^+$ naive T cells proliferating most robustly in response to IL-7 and IL-15 *in vitro*, contain high proportions of clones that are also most abundant in the peripheral blood[52], suggesting that cytokine-responsiveness plays an important role in shaping the $CD8^+$ naive T-cell pool. Additionally, $CD8^+$ naive T cells display a higher degree of clonality than their $CD4^+$ counterparts, which may suggest that the rate of peripheral $CD8^+$ naive T-cell division is higher than of $CD4^+$ naive T cells[52]. Thus, following lymphocyte depletion, reconstitution of naive $CD4^+$ T cells may in fact be more dependent on thymic output.

The pros and cons of homeostatic proliferation

The slow reconstitution of (in particular) naive T cells causes a long-lasting immunodeficient state in patients that have been therapeutically lymphocyte depleted. Since prolonged immune incompetence has been associated with an increased risk of infectious diseases, relapses, and malignancies[53], improving the rate of reconstitution may seem a desirable clinical strategy. However, is faster reconstitution a true advantage?

The results described in chapter 4 suggest that T-cell production is increased for the subsets that are still deficient in peripheral cell count. Although we cannot exclude that thymic rebound may also play a role, it is likely that increased division of peripheral T cells is mainly responsible for the higher production rates in these patients. If lymphopenia-induced proliferation (LIP) occurs randomly and the TCR repertoire diversity is well maintained, it is naturally of benefit for immune reconstitution. However, if cell division occurs more selectively by T-cell clones with certain TCR specificities, LIP may cause serious problems.

In the allogeneic transplantation and autoimmune disease settings, therapy-induced lymphopenia has been associated with clinical complications that were related to the process of immune reconstitution. Enhanced T-cell division after allogeneic stem cell transplantation was observed to coincide with the development of graft-versus-host disease[15]. In a mouse-model of solid organ transplantation, homeostatic proliferation was shown to render T cells resistant to tolerance induction and to cause allograft rejection[54]. Studies in mice and humans have associated lymphopenia with the development of a variety of autoimmune diseases[55]. Increased T-cell proliferation and reduced thymic output were recently proposed as risk factors for the development of autoimmune disease after lymphocyte-depletion with the monoclonal antibody Alemtuzumab[56].

Boosting T-cell reconstitution may hence not be beneficial for some lymphopenic conditions, in which alloreactive or autoreactive T cells need to remain suppressed. For those conditions, future research should aim at preventing generalized lymphocyte depletion, and specific targeting of T-cell specificities that cause disease.

Deuterium labeling versus analysis of Ki-67 expression

The large majority of the research that is described in this thesis is based on deuterium labeling studies. The unique aspect of deuterium labeling is that it allows to study the turnover of cell populations *in vivo*, with no influence on physiological conditions. Yet, deuterium labeling is not a widely applied technique. Deuterium labeling studies are laborious and expensive, measurement of deuterium enrichment requires specific laboratory equipment and expertise, and the *in vivo* human studies are of relatively high burden for study participants, mainly because of frequent study visits and substantial time investment. These are obvious reasons why deuterium labeling studies are generally limited to relatively small sample sizes. An alternative and more frequently used approach to investigate cell turnover is the *ex vivo* measurement of the cell cycle marker Ki-67. Do these techniques lead to different outcomes, and what are the advantages of using deuterium labeling?

An important difference between the techniques is the time frame of the measurement. While deuterium labeling provides a turnover estimate that is based on a period of up-labeling and down-labeling (typically a total period of several months), Ki-67-expression reflects a snapshot of the percentage of cells in cell cycle at the moment of blood withdrawal. Secondly, a turnover rate estimated from deuterium-labeling data represents a truly quantitative estimate, while Ki-67-expression data are difficult to translate into daily lymphocyte production rates. Thirdly, incorporation of deuterium labeling occurs both during *de novo* lymphocyte production in the thymus and the bone marrow and during peripheral cell division, whereas Ki-67 is assumed to be more a read out for peripheral cell division. Because of these technical differences, deuterium labeling and Ki-67 analysis may lead to contradicting results. Finally, for cell subsets with a very low turnover rate, such as naive T cells, Ki-67-expression may be an unreliable measure, because the percentage of cells that express the cell cycle marker is generally below 1%. As deuterium is generally administered for several weeks, it is more likely that sufficient cell production has taken place in this time period to be able to reliably measure the turnover rate, even for cell populations with a turnover rate as low as that of naive T cells.

To test whether Ki-67-expression and turnover rates give diverging outcomes, or if a relationship exists between these measurements, we plotted, for all data reported in this thesis, the turnover rates estimated with deuterium labeling, against the corresponding percentage of Ki-67$^+$ cells (Figure 3). For the combined data of CD4$^+$ and CD8$^+$ naive and memory T-cell data, and naive, memory and natural effector B cells from healthy controls and patient groups, we observed a clear correlation between the turnover rate and percentage of Ki-67 expression. To evaluate whether a relationship exists within subsets, we highlighted the data obtained for naive T cells, memory T cells, CD27$^-$ B cells and CD27$^+$ B cells in separate figure panels. While for memory T-cells (Figure 3B) and CD27$^+$ B-cells (Figure 3D) (the subsets with a relatively high turnover) turnover rates and Ki-67 expression are related, this is not the case for naive T-cells (Figure 3A) and not very convincing for naive B-cells (Figure 3C), which generally have much lower turnover rates.

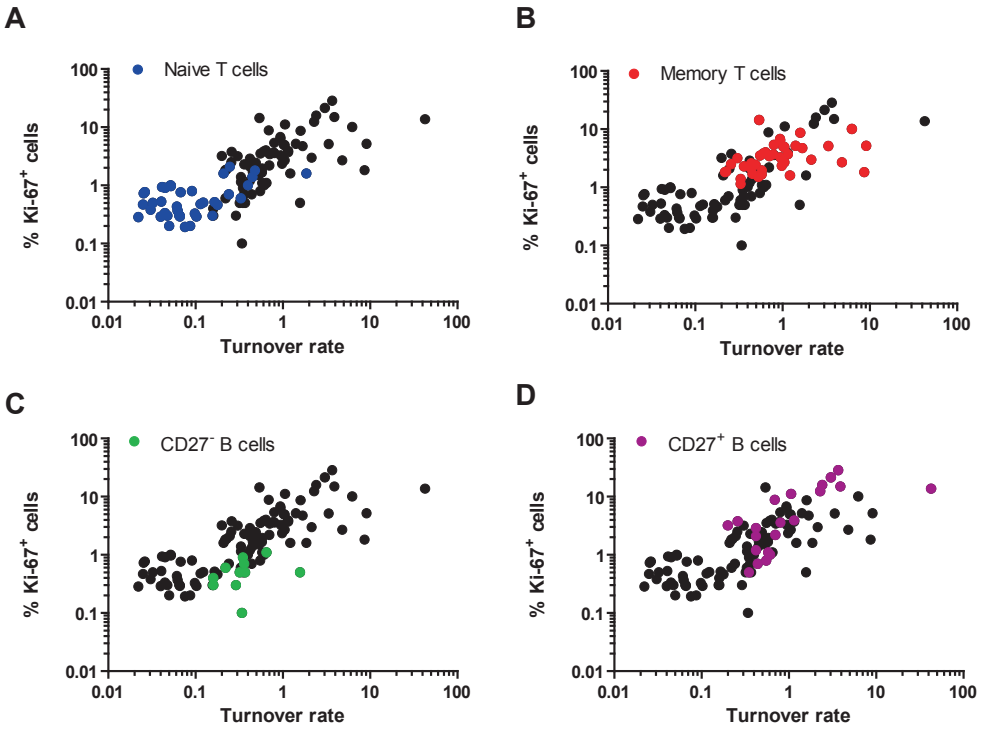


Figure 3. Correlations between turnover rate based on deuterium labeling and percentage of Ki-67$^+$ cells. All turnover rates estimated in chapters 3-5 are plotted against the corresponding percentage of Ki-67-expressing cells. Estimates are derived from healthy young and aged individuals, from autoSCT patients, from renal transplant recipients who were treated with immunosuppressive maintenance therapy and from ATG-treated renal transplant recipients who were also treated with immunosuppressive drugs. All panels show the same data, but the highlighted populations are different: (A) naive T cells, (B) memory T cells, (C) CD27$^-$ naive B cells, (D) CD27$^+$ natural effector and memory B cells. Panels A and B highlight both CD4$^+$ and CD8$^+$ T cells, as no distinct pattern was observed for the separate subsets.

Two other studies have also reported a relationship between the turnover rate based on deuterium labeling and the percentage of Ki-67 expression[57;58]. Based on data of CD4 and CD8 T cells, the percentages of Ki-67 expression tend to be about 4-fold higher than the corresponding turnover rates based on deuterium labeling[58], while in a small dataset of B cells, a 10-fold difference was observed[57]. These observations that Ki-67-expression gives a higher estimate than the turnover rate may have several reasons. It was recently suggested that Ki-67 remains expressed for several days after cell division has completed[58;59]. Moreover, Ki-67-expression was shown to be initiated in an early phase in the cell cycle[60]. *In vitro* studies with the proliferation inhibitor MMF have suggested that Ki-67 may be expressed by cells that enter, but do not complete the cell cycle[61]. Ki-67 expression analysis may thus overestimate cellular production rates.

In conclusion, once the exact relationship between turnover rates based on deuterium labeling and Ki-67 expression is known, snapshot measurements of Ki-67 expression may allow for estimation of average turnover rates for cell populations that turnover at significant rates. For cell populations that turnover very slowly, in contrast, deuterium labeling remains the best alternative. Extended studies on the relationship between Ki-67-expression and quantitative turnover rates - in various cell subsets and various study groups - are however needed to confirm that average turnover rates can be deduced from the more simple Ki-67-expression analysis for other cell subsets.

References

1. Bains I, Yates AJ, Callard RE. Heterogeneity in thymic emigrants: implications for thymectomy and immunosenescence. PLoS.One. 2013;8:e49554.
2. den Braber I, Mugwagwa T, Vrisekoop N et al. Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. Immunity. 2012;36:288-297.
3. Hazenberg MD, Borghans JA, de Boer RJ, Miedema F. Thymic output: a bad TREC record. Nat.Immunol. 2003;4:97-99.
4. Kilpatrick RD, Rickabaugh T, Hultin LE et al. Homeostasis of the naive CD4+ T cell compartment during aging. J.Immunol. 2008;180:1499-1507.
5. Fink PJ, Hendricks DW. Post-thymic maturation: young T cells assert their individuality. Nat.Rev. Immunol. 2011;11:544-549.
6. Berzins SP, Boyd RL, Miller JF. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. J.Exp.Med. 1998;187:1839-1848.
7. Berzins SP, Godfrey DI, Miller JF, Boyd RL. A central role for thymic emigrants in peripheral T cell homeostasis. Proc.Natl.Acad.Sci.U.S.A 1999;96:9787-9791.
8. Houston EG, Jr., Higdon LE, Fink PJ. Recent thymic emigrants are preferentially incorporated only into the depleted T-cell pool. Proc.Natl.Acad.Sci.U.S.A 2011;108:5366-5371.
9. Dong J, Chen Y, Xu X et al. Homeostatic properties and phenotypic maturation of murine CD4+ pre-thymic emigrants in the thymus. PLoS.One. 2013;8:e56378.
10. Fink PJ. The biology of recent thymic emigrants. Annu.Rev.Immunol. 2013;31:31-50.
11. Sempowski GD, Gooding ME, Liao HX, Le PT, Haynes BF. T cell receptor excision circle assessment of thymopoiesis in aging mice. Mol.Immunol. 2002;38:841-848.
12. Tsukamoto H, Clise-Dwyer K, Huston GE et al. Age-associated increase in lifespan of naive CD4 T cells contributes to T-cell homeostasis but facilitates development of functional defects. Proc.Natl.Acad. Sci.U.S.A 2009
13. Douek DC, Vescio RA, Betts MR et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. Lancet 2000;355:1875-1881.
14. Hakim FT, Memon SA, Cepeda R et al. Age-dependent incidence, time course, and consequences of thymic renewal in adults. J.Clin.Invest 2005;115:930-939.
15. Hazenberg MD, Otto SA, de Pauw ES et al. T-cell receptor excision circle and T-cell dynamics after allogeneic stem cell transplantation are related to clinical events. Blood 2002;99:3449-3453.
16. Palmer DB. The effect of age on thymic function. Front Immunol. 2013;4:316.
17. Steinmann GG, Klaus B, Muller-Hermelink HK. The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study. Scand.J.Immunol. 1985;22:563-575.
18. Fagnoni FF, Vescovini R, Passeri G et al. Shortage of circulating naive CD8(+) T cells provides new insights on immunodeficiency in aging. Blood 2000;95:2860-2868.
19. Lazuardi L, Jenewein B, Wolf AM et al. Age-related loss of naive T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes. Immunology 2005;114:37-43.
20. Goronzy JJ, Lee WW, Weyand CM. Aging and T-cell diversity. Exp.Gerontol. 2007;42:400-406.
21. Naylor K, Li G, Vallejo AN et al. The influence of age on T cell generation and TCR diversity. J.Immunol. 2005;174:7446-7452.
22. Chinn IK, Blackburn CC, Manley NR, Sempowski GD. Changes in primary lymphoid organs with aging. Semin.Immunol. 2012;24:309-320.
23. Linton PJ, Dorshkind K. Age-related changes in lymphocyte development and function. Nat.Immunol. 2004;5:133-139.
24. Yager EJ, Ahmed M, Lanzer K et al. Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. J.Exp.Med. 2008;205:711-723.

25. Hale JS, Boursalian TE, Turk GL, Fink PJ. Thymic output in aged mice. Proc.Natl.Acad.Sci.U.S.A 2006;103:8447-8452.
26. Goronzy JJ, Fang F, Cavanagh MM, Qi Q, Weyand CM. Naive T cell maintenance and function in human aging. J.Immunol. 2015;194:4073-4080.
27. Wertheimer AM, Bennett MS, Park B et al. Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T cell subsets in humans. J.Immunol. 2014;192:2143-2155.
28. Litjens NH, de Wit EA, Betjes MG. Differential effects of age, cytomegalovirus-seropositivity and end-stage renal disease (ESRD) on circulating T lymphocyte subsets. Immun.Ageing 2011;8:2.
29. Nikolich-Zugich J. Aging of the T cell compartment in mice and humans: from no naive expectations to foggy memories. J.Immunol. 2014;193:2622-2629.
30. Looney RJ, Falsey A, Campbell D et al. Role of cytomegalovirus in the T cell changes seen in elderly individuals. Clin.Immunol. 1999;90:213-219.
31. Chidrawar S, Khan N, Wei W et al. Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals. Clin.Exp.Immunol. 2009;155:423-432.
32. Sauce D, Larsen M, Fastenackels S et al. Evidence of premature immune aging in patients thymectomized during early childhood. J.Clin.Invest 2009;119:3070-3078.
33. Halnon NJ, Jamieson B, Plunkett M et al. Thymic function and impaired maintenance of peripheral T cell populations in children with congenital heart disease and surgical thymectomy. Pediatr.Res. 2005;57:42-48.
34. Prelog M, Keller M, Geiger R et al. Thymectomy in early childhood: significant alterations of the CD4(+) CD45RA(+)CD62L(+) T cell compartment in later life. Clin.Immunol. 2009;130:123-132.
35. van GR, Schadenberg AW, Otto SA et al. Long-term restoration of the human T-cell compartment after thymectomy during infancy: a role for thymic regeneration? Blood 2011;118:627-634.
36. Appay V, Sauce D, Prelog M. The role of the thymus in immunosenescence: lessons from the study of thymectomized individuals. Aging (Albany.NY) 2010;2:78-81.
37. Elder RW, George RP, McCabe NM et al. Immunologic Aging in Adults with Congenital Heart Disease: Does Infant Sternotomy Matter? Pediatr.Cardiol. 2015
38. Hirokawa K, Utsuyama M, Kasai M et al. Understanding the mechanism of the age-change of thymic function to promote T cell differentiation. Immunol.Lett. 1994;40:269-277.
39. Heitger A, Neu N, Kern H et al. Essential role of the thymus to reconstitute naive (CD45RA+) T-helper cells after human allogeneic bone marrow transplantation. Blood 1997;90:850-857.
40. Williams KM, Hakim FT, Gress RE. T cell immune reconstitution following lymphodepletion. Semin. Immunol. 2007;19:318-330.
41. Sauce D, Larsen M, Fastenackels S et al. Lymphopenia-driven homeostatic regulation of naive T cells in elderly and thymectomized young adults. J.Immunol. 2012;189:5541-5548.
42. Cicin-Sain L, Messaoudi I, Park B et al. Dramatic increase in naive T cell turnover is linked to loss of naive T cells from old primates. Proc.Natl.Acad.Sci.U.S.A 2007;104:19960-19965.
43. Hazenberg MD, Stuart JW, Otto SA et al. T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). Blood 2000;95:249-255.
44. Ademokun A, Wu YC, Dunn-Walters D. The ageing B cell population: composition and function. Biogerontology. 2010;11:125-137.
45. Kogut I, Scholz JL, Cancro MP, Cambier JC. B cell maintenance and function in aging. Semin.Immunol. 2012;24:342-349.
46. Ogawa T, Kitagawa M, Hirokawa K. Age-related changes of human bone marrow: a histometric estimation of proliferative cells, apoptotic cells, T cells, B cells and macrophages. Mech.Ageing Dev. 2000;117:57-68.

47. McKenna RW, Washington LT, Aquino DB, Picker LJ, Kroft SH. Immunophenotypic analysis of hematogones (B-lymphocyte precursors) in 662 consecutive bone marrow specimens by 4-color flow cytometry. Blood 2001;98:2498-2507.
48. Kuranda K, Vargaftig J, de la Rochere P et al. Age-related changes in human hematopoietic stem/progenitor cells. Aging Cell 2011;10:542-546.
49. Dunn-Walters DK, Ademokun AA. B cell repertoire and ageing. Curr.Opin.Immunol. 2010;22:514-520.
50. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J.Exp.Med. 2007;204:645-655.
51. Vrisekoop N, den Braber I, de Boer AB et al. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. Proc.Natl.Acad.Sci.U.S.A 2008;105:6115-6120.
52. Qi Q, Liu Y, Cheng Y et al. Diversity and clonal selection in the human T-cell repertoire. Proc.Natl.Acad.Sci.U.S.A 2014;111:13139-13144.
53. Bosch M, Khan FM, Storek J. Immune reconstitution after hematopoietic cell transplantation. Curr.Opin.Hematol. 2012;19:324-335.
54. Wu Z, Bensinger SJ, Zhang J et al. Homeostatic proliferation is a barrier to transplantation tolerance. Nat.Med. 2004;10:87-92.
55. Khoruts A, Fraser JM. A causal link between lymphopenia and autoimmunity. Immunol.Lett. 2005;98:23-31.
56. Jones JL, Thompson SA, Loh P et al. Human autoimmunity after lymphocyte depletion is caused by homeostatic T-cell proliferation. Proc.Natl.Acad.Sci.U.S.A 2013;110:20200-20205.
57. van GR, Kater AP, Otto SA et al. In vivo dynamics of stable chronic lymphocytic leukemia inversely correlate with somatic hypermutation levels and suggest no major leukemic turnover in bone marrow. Cancer Res. 2008;68:10137-10144.
58. de Boer RJ, Perelson AS. Quantifying T lymphocyte turnover. J.Theor.Biol. 2013;327:45-87.
59. Hogan T, Shuvaev A, Commenges D et al. Clonally diverse T cell homeostasis is maintained by a common program of cell-cycle control. J.Immunol. 2013;190:3985-3993.
60. Gerdes J, Lemke H, Baisch H et al. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J.Immunol. 1984;133:1710-1715.
61. Vrisekoop N, Sankatsing SU, Jansen CA et al. Short communication: No detrimental immunological effects of mycophenolate mofetil and HAART in treatment-naive acute and chronic HIV-1-infected patients. AIDS Res.Hum.Retroviruses 2005;21:991-996.

6

Appendices

Nederlandse samenvatting voor niet-ingewijden

De dynamiek van lymfocyten in gezonde en lymfopene condities

In het kort…

In dit proefschrift laten wij zien dat wanneer lymfocyten (een type afweercellen) worden vernietigd ten gevolge van chemotherapie, de aanmaaksnelheid van nieuwe lymfocyten van nature is verhoogd, om zo hun herstel te bevorderen. Lymfocyten zijn essentieel voor een goed functionerend afweersysteem, en aantasting van deze celpopulatie leidt tot een verhoogde kans op infecties en het (opnieuw) ontwikkelen van kanker, en daardoor een verhoogde kans op overlijden. Na het verlies van lymfocyten kan het echter maanden tot jaren duren voordat hun aantallen weer een gezond niveau bereiken. Om te kunnen begrijpen waarom de lymfocytenpopulatie langdurig verstoord blijft, onderzochten wij de aanmaaksnelheid en levensduur van deze cellen in gezonde vrijwilligers, en hun dynamiek in patiënten met lymfopenie (een drastisch verlaagd lymfocytenaantal). Ondanks het zeer langzame herstel van sommige lymfocytenpopulaties na chemotherapie en stamceltransplantatie, vonden we dat de aanmaaksnelheid van deze cellen wel degelijk verhoogd is. Dit bleek niet het geval in lymfopene niertransplantatiepatiënten, waarschijnlijk doordat zij behandeld werden met afweeronderdrukkende medicijnen. Ook onderzochten wij de bijdrage van de thymus (zwezerik) en van celdeling in de lymfeknopen aan het behoud en herstel van de lymfocytenpopulatie. Hoewel thymusproductie een zeer kleine en met de leeftijd afnemende rol speelt, draagt het ook in patiënten op leeftijd substantieel bij aan het lymfocytenherstel. Deze basale inzichten in de lymfocytendynamiek vormen de basis voor het ontwikkelen van betere behandelmethoden in situaties waarbij lymfocytenpopulaties zijn verstoord, die ervoor zorgen dat het afweersysteem ofwel minder wordt aangetast, ofwel sneller kan herstellen.

Het afweersysteem

Ons lichaam is uitgerust met een goed ontwikkeld afweersysteem dat ons beschermt tegen allerlei schadelijke invloeden van buitenaf (zoals ziekmakende bacteriën, virussen en schimmels) en van binnenuit (zoals kankercellen, die in feite lichaamseigen, ontspoorde cellen zijn). Het afweersysteem bestaat uit twee takken: de aangeboren afweer en de adaptieve, specifieke afweer. De aangeboren afweer herkent vaste, gemeenschappelijke patronen op ziekmakende indringers en op 'ontspoorde' lichaamseigen cellen en kan ze op een snelle manier onschadelijk maken. De adaptieve afweer werkt beduidend langzamer, maar herkent op een heel specifieke manier stukjes van lichaamsvreemde eiwitten, de zogenaamde *antigenen*, en is in staat om een geheugen te ontwikkelen voor de ziekteverwekkers waarmee het in aanraking komt.

Lymfocyten

B-cellen en T-cellen, die gezamenlijk ook wel *lymfocyten* worden genoemd, zijn de witte bloedcellen die behoren tot het adaptieve afweersysteem. Lymfocyten vormen een essentieel onderdeel van ons afweersysteem. Dat wordt vooral duidelijk wanneer er een gebrek is aan deze cellen, bijvoorbeeld in bepaalde aangeboren afwijkingen of na een allesvernietigende chemotherapie ter behandeling van bloedkanker. Patiënten met een drastisch verlaagd lymfocytenaantal hebben een verhoogde kans op allerlei infecties en het (opnieuw) ontwikkelen van kanker en daardoor een verhoogde kans op overlijden.

Kenmerkend voor lymfocyten is dat elke cel een unieke antigeenreceptor op zijn oppervlakte heeft, waarmee specifieke antigenen worden herkend. B-cellen en T-cellen hebben een verschillend werkingsmechanisme. De voornaamste functie van B-cellen is het produceren van specifieke antistoffen die vrij circuleren in het bloed. Deze antistoffen kunnen bijvoorbeeld binden aan virussen, en zo voorkomen dat het virusdeeltje een cel infecteert. Wanneer een cel toch geïnfecteerd is geraakt kunnen T-cellen uitkomst bieden. Als een T-cel met zijn specifieke receptor een antigeen herkent, kan hij namelijk direct celdood opwekken in de zieke cel en zo voorkomen dat de infectie zich verder verspreidt.

De ontwikkeling van lymfocyten

B-cellen en T-cellen ontwikkelen vanuit een gemeenschappelijke bloedstamcel, die zich bevindt in het beenmerg. De ontwikkeling van nieuwe B-cellen vindt volledig plaats in het beenmerg, terwijl een groot deel van de T-celontwikkeling gebeurt in de *thymus* (zwezerik). Tijdens deze ontwikkeling verkrijgen B-cellen en T-cellen hun unieke antigeenreceptor, die ook wel respectievelijk B-cel- of T-celreceptor wordt genoemd. Om een brede herkenning van allerlei ziekteverwekkers te waarborgen heeft ieder individu miljoenen verschillende antigeenreceptoren, die ontwikkelen volgens een complex proces. Voordat een ontwikkelde B-cel of T-cel de bloedbaan in gaat vindt eerst een strenge controle plaats of de cel wel een functionele antigeenreceptor heeft, die bovendien géén lichaamseigen eiwitten herkent. Zo

wordt over het algemeen voorkomen dat er een afweerreactie tegen eigen, gezonde cellen optreedt. Als er lymfocyten zijn die aan deze controle ontsnappen, kan dit leiden tot een auto-immuunziekte, bijvoorbeeld type 1 diabetes.

Lymfocyten die na hun ontwikkeling gaan circuleren in de bloedbaan, de milt en de lymfeknopen (samen ook wel de *periferie* genoemd) zijn in eerste instantie nog *naïeve* cellen; dat wil zeggen dat zij hun specifieke antigeen nog niet zijn tegengekomen. Lymfocyten kunnen jarenlang circuleren en vervolgens doodgaan zonder dat ze ooit hun antigeen tegenkomen. Als een naïeve lymfocyt zijn specifieke antigeen herkent in de periferie en hierdoor wordt geactiveerd, zal deze gaan vermeerderen door celdeling en zich verder ontwikkelen tot *effector* cel, om meer functionele eigenschappen te verwerven die nodig zijn voor een goede afweerreactie. Tijdens deze reactie worden ook geheugen of *memory* cellen gevormd: bij een nieuwe blootstelling aan hetzelfde antigeen zorgen zij voor een snellere en effectievere afweerreactie.

Lymfocyten worden dus niet alleen in het beenmerg en in de thymus geproduceerd. Ook celdeling in de periferie speelt een belangrijke rol bij de lymfocytenproductie.

Behoud van lymfocytenpopulaties: homeostase

Omdat lymfocyten zo belangrijk zijn voor onze afweer, is het essentieel dat hun aantallen in balans of *in homeostase* worden gehouden. Ook is het belangrijk dat er een evenwichtige verdeling blijft van de verschillende subtypen of *populaties* B-cellen en T-cellen (denk aan de naïeve, memory en effector subtypen). In alle lymfocytenpopulaties vindt er een continue *turnover* plaats, dat wil zeggen, bestaande cellen worden continu vervangen door nieuwe cellen. Om het aantal cellen binnen een populatie stabiel te houden, moet er dus een goede balans zijn tussen de mate van celproductie (door beenmerg, thymus en perifere celdeling) en celdood. De snelheid waarmee een celpopulatie continu vernieuwd wordt, wordt ook wel *turnoversnelheid* genoemd.

In het algemeen is deze aanmaak en dood van cellen goed gereguleerd en worden de lymfocytenpopulaties gedurende het leven goed onderhouden. Bij bepaalde situaties, zoals chronische infecties (bv. HIV), kanker, of ten gevolge van de behandeling van bijvoorbeeld leukemie, kunnen de lymfocytenpopulaties echter ernstig verstoord raken. Een extreem voorbeeld is de situatie na zware chemotherapie en/of bestralingstherapie, waarna vaak een stamceltransplantatie volgt. Een dergelijke behandeling vernietigt de complete lymfocytenpopulatie en het kan voor sommige subtypen, de naïeve T-cellen in het bijzonder, maanden tot jaren duren voordat hun aantallen weer een gezond niveau bereiken. In zulke situaties zouden veranderingen in de lymfocytendynamiek, bijvoorbeeld een verhoogde aanmaak of een verlengde levensduur, gunstig kunnen zijn voor het herstel van de lymfocytenpopulaties. Het is echter onduidelijk of zulke regulatie van de lymfocytendynamiek plaatsvindt wanneer hun aantallen sterk zijn aangetast.

Doel van dit proefschrift

Om te kunnen begrijpen hoe de lymfocytenpopulaties door ziekte of therapieën worden verstoord, en hoe ze daar weer van herstellen, is het eerst belangrijk om te weten hoe de aantallen lymfocyten in gezondheid in stand gehouden worden. In de literatuur beschreven schattingen van de productie en celdood binnen lymfocytenpopulaties variëren echter tot wel 100-voudig. Bovendien is het nog onduidelijk welke rol het beenmerg en de thymus spelen in het behoud van B-cel en T-celpopulaties, ten opzichte van celdeling in de periferie. In dit proefschrift hebben we de celproductie en celdood binnen lymfocytenpopulaties onderzocht in gezonde jonge en oudere mensen (**hoofdstuk 3**), in patiënten die chemotherapie en een stamceltransplantatie hebben gehad (**hoofdstuk 4**), en in mensen die vanwege een niertransplantatie met afweer-onderdrukkende medicijnen (immuunsuppressiva) worden behandeld (**hoofdstuk 5**). Het doel van deze studies was (i) het bepalen van de *turnoversnelheid* van B-cel en T-celpopulaties in gezondheid, en of deze dynamiek tijdens *gezonde veroudering* verandert, (ii) onderzoeken of in situaties waarbij het lymfocytenaantal drastisch is verlaagd (*lymfopenie*) de lymfocytendynamiek is veranderd en wat het effect is van immuunsuppressiva, (iii) de bijdrage van de thymus aan het behoud van de naïeve T-celpopulatie te onderzoeken in verschillende condities. Ook hebben we de samenstelling en turnoversnelheid van de naïeve T-celpopulatie in meer detail onderzocht in de muis, één van de meest gebruikte modelsystemen binnen de immunologie (**hoofdstuk 2**).

Zwaar water labeling: een methode om de dynamiek van lymfocyten te meten

Het merendeel van het onderzoek dat beschreven wordt in dit proefschrift is uitgevoerd met een speciale techniek; *zwaar water labeling*. Met behulp van deze methode is het mogelijk om in het lichaam, zowel van muizen als van mensen, te meten hoe snel verschillende typen lymfocyten aangemaakt worden, zonder dat het normale functioneren van het lichaam daarbij wordt beïnvloed. Water (H_2O) is een molecuul dat bestaat uit een zuurstofatoom (O) en twee waterstofatomen (H). Water is een hele belangrijke bouwstof in ons lichaam. Bij iedere cel die wordt gemaakt, wordt water als bron gebruikt om onder andere het genetische materiaal, het DNA, te vermenigvuldigen. Bij zwaar water labelen maken we gebruik van *zwaar water* (^2H_2O). Dit water is letterlijk zwaarder, in plaats van waterstofatomen bevat het namelijk 2 *deuteriumatomen* (^2H), die twee keer zo zwaar zijn als een normaal waterstofatoom. In onze studies dronken de proefpersonen gedurende 6-9 weken iedere dag een beetje zwaar water. In deze periode, kwamen er dus deuteriumatomen terecht in hun lichaam, die onder andere als bron gebruikt werden om nieuw DNA te maken bij de celdeling. Tijdens en na de periode waarin de vrijwilligers zwaar water dronken namen wij regelmatig bloed bij hen af. Hierdoor konden wij in het laboratorium meten hoe snel er deuterium werd ingebouwd in het DNA van hun verschillende B-cel en T-celpopulaties; een maat voor hoe snel er nieuwe cellen worden geproduceerd. Ook volgden wij hoe snel dit deuterium weer verloren ging nadat er gestopt was met het drinken van zwaar water; een maat voor hoe lang de cellen in leven bleven.

Door alle verkregen gegevens samen te voegen, en met behulp van wiskundige modellen te analyseren, konden wij de turnoversnelheid van verschillende lymfocytenpopulaties in deze proefpersonen bepalen.

TRECs & KRECs: inzicht in de bijdrage van de thymus en het beenmerg

Een beperking van de zwaar water techniek is dat we niet kunnen onderscheiden waar de nieuwe cellen worden gemaakt, in de thymus of het beenmerg, of in de periferie. Om meer inzicht te krijgen in de rol van de thymus en het beenmerg hebben we zwaar water labeling gecombineerd met "TREC" of "KREC" analyse. TRECs, of T-celreceptor excisiecirkels, zijn cirkelvormige stukjes DNA die tijdens de T-celontwikkeling in de thymus worden gemaakt. Deze cirkeltjes worden *alléén* in de thymus gemaakt; wanneer een cel deelt in de periferie zal de TREC niet worden gekopieerd. Elke TREC is dus oorspronkelijk in de thymus gemaakt. KRECs zijn soortgelijke DNA cirkels, maar dan specifiek voor B-cellen en zij worden alleen in het beenmerg gemaakt. De hoeveelheid TRECs of KRECs die respectievelijk de T-cel en B-celpopulaties bevatten (ten opzichte van het totale aantal T-cellen en B-cellen) kunnen we meten in het laboratorium, en dit geeft ons informatie over wat de bijdrage van de thymus en het beenmerg is bij de T-cel en B-celproductie. De interpretatie van TREC/KREC analyses is echter niet eenvoudig. Als we bijvoorbeeld een verlaagde TREC-inhoud vinden in naïeve T-cellen, wil dat niet zeggen dat de thymus minder T-cellen maakt. Het TREC-gehalte is namelijk ook erg afhankelijk van de mate van celdeling en celsterfte in de periferie. Voor de interpretatie van onze resultaten combineren we daarom de gegevens die we met zwaar water labeling en TREC/KREC analyse genereren en maken we gebruik van wiskundige modellen.

Kleine en afnemende rol van de thymus tijdens gezonde veroudering

In **hoofdstuk 3** hebben wij onderzocht wat de invloed is van gezonde veroudering op de B-cel en T-celpopulaties. Het ouder worden gaat vaak gepaard met een toenemende gevoeligheid voor infecties en het ontwikkelen van kanker. Een mogelijke reden hiervoor zou kunnen zijn dat de B-cel en T-celpopulaties niet meer goed in stand gehouden worden op latere leeftijd. Het is bekend dat de T-celproductie door de thymus, ook wel *thymus output* genoemd, afneemt met de leeftijd, maar het is onduidelijk hoeveel consequenties dat heeft voor de T-celpopulaties. Er zijn ook resultaten gerapporteerd die erop wijzen dat *beenmerg output* vermindert tijdens veroudering.

De resultaten beschreven in hoofdstuk 3 laten zien dat de thymus output in gezonde vijfenzestigplussers (minder dan 1 miljoen cellen per dag) inderdaad veel lager is dan in twintigers (ongeveer 16 miljoen cellen per dag). We vinden echter ook dat de thymus zelfs in twintigers voor maar een kleine fractie van de naïeve T-celproductie zorgt namelijk; minder dan 20%. De overige ruim 80% van de nieuwe naïeve T-cellen wordt dus gemaakt door T-celdeling in de periferie.

In tegenstelling tot de thymus output, hebben we geen aanwijzingen gevonden dat de functionaliteit van het beenmerg lager is in ouderen. Daarnaast lijkt het beenmerg zowel in jongeren als ouderen een substantiële bijdrage te leveren aan de productie van naïeve B cellen (**hoofdstuk 3 en 6**).

Geen grote verschillen in de dynamiek van lymfocyten tussen jong en oud

Ondanks het feit dat de thymus output drastisch afneemt met de leeftijd, is er slechts een lichte daling in het aantal naïeve T cellen waarneembaar in de gezonde ouderen die wij onderzochten. De *turnoversnelheid* van de T-cel en B-celpopulaties bleek vergelijkbaar in jongvolwassenen en ouderen. Er wordt vaak gedacht dat voor behoud van de T-celpopulatie in ouderen, het gebrek aan thymus output gecompenseerd moet worden met een verhoogde celdeling in de periferie. Wij vinden hiervoor geen bewijs. Waarschijnlijk is de rol van de thymus al zo klein in jongvolwassenen, dat een verhoogde deling in ouderen simpelweg niet nodig is om de T-celpopulatie in stand te houden.

Toch kunnen we uit onze resultaten niet concluderen dat de afnemende thymus output geen gevolgen heeft voor het functioneren van ons afweersysteem. De thymus is uniek in het vormen van nieuwe specifieke antigeenreceptoren, en op den duur kan een gebrek aan thymus output leiden tot een gebrekkige diversiteit binnen de T-celpopulatie, en dus een toenemende gevoeligheid voor ziekteverwekkers.

Versnelde aanmaak van lymfocyten wanneer hun aantallen verlaagd zijn

In gezonde veroudering hebben we dus geen aanwijzingen kunnen vinden dat de celdeling omhoog gaat bij een verlaging van de thymus output en een (lichte) verlaging van het aantal naïeve T-cellen. Of een dergelijke terugkoppeling, die ook wel *homeostatische reactie* wordt genoemd, überhaupt bestaat in de mens is al langere tijd een punt van discussie in de literatuur. Sommige studies hebben wel een verband gerapporteerd tussen verlaagde celaantallen en een verhoogde celdeling, maar dit werd waarschijnlijk deels veroorzaakt door het optreden van afstotingsreacties of infecties. Om te testen of een homeostatische reactie kan optreden, hebben wij de lymfocytendynamiek onderzocht in patiënten die voor bloedkanker waren behandeld met hoge doses chemotherapie en een stamceltransplantatie, waarbij de stamcellen van hunzelf afkomstig waren en dus geen afstoting konden opwekken (**hoofdstuk 4**). In deze patiënten waren ruim een jaar na de stamceltransplantatie enkele lymfocytenpopulaties nog drastisch verlaagd in celaantallen. Een interessante bevinding is dat binnen deze lymfocytenpopulaties, de productiesnelheid beduidend hoger was dan in de gezonde vrijwilligers beschreven in hoofdstuk 3, wat betekent dat het lichaam van nature probeert om de celaantallen zo snel mogelijk te laten herstellen. Hoewel de patiënten een leeftijd hadden van boven de 50 jaar, bleek dat ook de thymus nog een substantiële bijdrage leverde aan het herstel van hun T-cellen.

Het effect van afweer-onderdrukkende medicijnen: lymfocytenproductie op een laag pitje
Ook patiënten die een niertransplantatie hebben ondergaan kampen vaak voor lange tijd met ernstig verlaagde lymfocytenaantallen. Dit is niet direct het gevolg van de transplantatie, maar wordt veroorzaakt doordat zij behandeld worden met afweer-onderdrukkende medicijnen, om te voorkomen dat de donornier wordt afgestoten. Patiënten met een donornier moeten levenslang deze medicijnen slikken (als onderhoudstherapie) die de lymfocytenproductie remmen. Sommige patiënten, bij wie de kans op afstoting hoog is, krijgen daarnaast nog een eenmalige behandeling met een medicijn dat de lymfocyten zelfs vernietigt. Hoewel dit gunstig is voor het behoud van de donornier, gaat de behandeling ook gepaard met een verhoogde kans op infectieziekten en kanker. Wij hebben in niertransplantatiepatiënten gekeken naar het effect van afweer-onderdrukkende therapie op lymfocytenaantallen en - turnoversnelheid (**hoofdstuk 5**). De patiënten die onderhoudstherapie kregen, maar van wie de lymfocyten niet waren vernietigd, hadden wel relatief lage celaantallen, maar de levensduur van hun lymfocyten bleef onveranderd. Patiënten die wel een lymfocyt-vernietigende behandeling hadden gehad, hadden zelfs een jaar na deze behandeling nog een ernstig verlaagd T-celaantal. In tegenstelling tot onze bevindingen in patiënten na stamceltransplantatie (**hoofdstuk 4**), hadden deze nierpatiënten geen verhoogde lymfocytenproductie. Dit suggereert dat de homeostatische reactie wordt onderdrukt tijdens behandeling met afweer-onderdrukkende medicatie, waardoor niertransplantatiepatiënten mogelijk nog langer lijden aan een lymfocytentekort.

De dynamiek van (naïeve) T-cellen in meer detail: cellen geproduceerd door de thymus vormen een populatie van relatief kortlevende cellen
Tot slot hebben we in **hoofdstuk 2** de naïeve T-celpopulatie in meer detail onderzocht. Er wordt vaak gedacht dat de naïeve T-celpopulatie is onderverdeeld in twee subpopulaties, namelijk (*i*) de recente thymusemigranten (RTE), cellen die kortgeleden door de thymus zijn geproduceerd, en (*ii*) de *mature* naïeve (MN) T cellen die al langere tijd in de periferie verblijven en daar kunnen vermeerderen door celdeling. In het algemeen wordt er aangenomen dat de RTE relatief kortlevende cellen zijn. Daarom worden ze door sommige wetenschappers verantwoordelijk gehouden voor grote en snelle veranderingen in het aantal naïeve T cellen, die bijvoorbeeld optreden tijdens HIV infectie. Inzicht in de dynamiek van RTE is dus belangrijk om veranderingen in de naïeve T-celpopulatie te kunnen verklaren. Daarnaast zijn RTE de directe afstammelingen van thymus output, en geeft inzicht in de RTE-populatie ook inzicht in de werking van de thymus.

Omdat het in de mens erg moeilijk is om onderscheid te maken tussen RTE en MN T-cellen, komen bestaande inzichten in de eigenschappen van RTE voornamelijk uit de muis. Over de levensduur van RTE (in de muis) bestaat echter onenigheid; sommige onderzoekers beweren dat ze relatief kortlevend zijn, terwijl in een recente publicatie wordt gesuggereerd dat ze juist relatief langlevend zijn. Wij hadden het vermoeden dat dit verschil in conclusies

werd veroorzaakt door o.a. een verschil in de leeftijd van de bestudeerde muizen. Daarom hebben wij de levensduur van RTE onderzocht met een combinatie van zwaar water labeling en *thymustransplantaties* in muizen van dezelfde leeftijd. Thymustransplantaties zijn erg waardevol voor het verkrijgen van RTE-inzichten. Cellen uit de donorthymus zijn in de ontvangende muis eenvoudig te onderscheiden van de andere cellen. Bovendien weten we dat alle donorcellen die we in bloed, lymfeknopen en milt terugvinden na de thymustransplantatie, pas kortgeleden uit de thymus zijn gekomen en dus RTE zijn. De resultaten van zowel onze transplantatie-experimenten als onze zwaar water labeling experimenten suggereren dat RTE relatief *kortlevende* cellen zijn. Aanvullende studies zullen moeten uitwijzen in hoeverre de situatie in de mens vergelijkbaar is met onze bevindingen in de muis.

Conclusie

Het onderzoek dat beschreven staat in dit proefschrift geeft fundamentele inzichten over hoe verschillende lymfocytenpopulaties in stand gehouden worden tijdens gezonde veroudering en hoe het lichaam reageert op condities waarin er een ernstig tekort is aan deze cellen. Ook hebben we de rol van de thymus bij het behoud van de T-celpopulatie bestudeerd. Belangrijke bevindingen van ons onderzoek zijn (*i*) een zeer kleine en afnemende rol voor thymus output in volwassenen, die in lymfopene situaties toch substantieel bijdraagt aan het herstel van de T-celpopulatie, (*ii*) de afwezigheid van verhoogde celdeling in de periferie tijdens gezonde veroudering om de afname in thymus output te compenseren, (*iii*) het bestaan van een homeostatisch mechanisme dat zorgt voor een verhoogde celproductie ten tijde van lymfopenie, wat van nature het herstelproces bevordert. Deze inzichten zijn van belang voor verder onderzoek naar- en het begrip van- uiteenlopende ziektebeelden waarbij lymfocyten zijn verstoord, en voor de ontwikkeling van effectieve behandelmethoden.

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Curriculum Vitae

Vera van Hoeven was born on December 27, 1984 in Leusden, The Netherlands. After finishing secondary school at Grotius College in Heerlen in 2003, she started her bachelor studies in Biomedical Sciences at Utrecht University. She obtained her bachelor's degree in 2006, and continued her education with the master's program Immunity & Infection at Utrecht University. As a part of this program, she performed a 9-month internship in the group of dr. Tuna Mutis at the Laboratory of Clinical Chemistry and Hematology of the University Medical Center Utrecht. Here, she studied T-cell responses directed against tumor-associated antigens in Multiple Myeloma supervised by Robbert Spaapen and Michael van der Veer. In 2008, Vera went to the Burnet Institute in Melbourne, Australia for her second, 6-month internship. Under supervision of dr. Johnson Mak and dr. Marcel Hijnen, she investigated the structural and biochemical properties of the HIV Gag and Gag-Pol proteins. After obtaining her master's degree in Biomedical Sciences in 2009, she started her PhD at the department of Immunology (currently the Laboratory of Translational Immunology) in the University Medical Center Utrecht, under the supervision of dr. José Borghans and dr. Kiki Tesselaar (copromotors), and prof. dr. Frank Miedema and prof. dr. Rob de Boer (promotors). During her PhD, she studied the dynamics of lymphocyte populations in healthy and lymphopenic conditions, and the results of this work are described in this thesis.

Vera is currently working as a postdoc in the Academic Medical Center in Amsterdam, where she investigates the protective role of innate lymphoid cells after hematopoietic stem cell transplantation in the group of Mette Hazenberg.

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