LEUKOCYTE DYNAMICS IN MICE AND MEN

Ineke den Braber

ISBN: 978-90-8559-538-0
Printed by Optima Grafische Communicatie, Rotterdam, The Netherlands.

LEUKOCYTE DYNAMICS IN MICE AND MEN

Leukocyten dynamiek in muizen en mensen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
dinsdag 16 juni 2009 des middags te 2.30 uur

door

Apolonia Josiena den Braber

geboren op 2 mei 1979 te Barendrecht

Promotoren:	Prof. dr. R.J. de Boer
	Prof. dr. F. Miedema
Co-promotor:	Dr. K. Tesselaar

The studies in this thesis were financially supported by the Landsteiner Foundation for Blood Transfusion Research (LSBR grant 0210).

… zonder dat een mens het werk, dat God gemaakt heeft,
kan uitvinden, van het begin tot het einde toe.
Prediker 3:11b

Aan mijn ouders

CONTENTS

Chapter 1

Introduction

The ability of the immune system to eliminate a wide spectrum of pathogens is dependent on the presence of a considerable and diverse peripheral T-cell pool. The importance of this is clearly illustrated in patients who suffer from aids, scid or other immunodeficiency syndromes, which make them vulnerable to many kinds of infections, generally resulting in death due to opportunistic infections[1;2]. More insight in the processes that regulate the size and diversity of the immune system can be helpful for the development of T-cell reconstitution approaches.
In adults the number of functional T cells is preserved throughout life by a dynamic self-regulating process, in which cell production is counterbalanced by cell loss[3]. Usually, disruption of T-cell homeostasis by massive expansion of antigen-specific T cells during immune responses is followed by a return to the previous equilibrium. This steady state causes continuous rejuvenation of the immune system and is important for the maintenance of T-cell diversity during life.

Naive T-cell homeostasis

The murine naive T-cell pool is generally considered to exist in two sub-populations: recent thymic emigrants (RTE) that originate in the thymus and resident naive T cells that emanate from peripheral self-renewal (Fig.1). The life span of both subsets is determined by the rate of cell death and differentiation into effector/memory T cells.

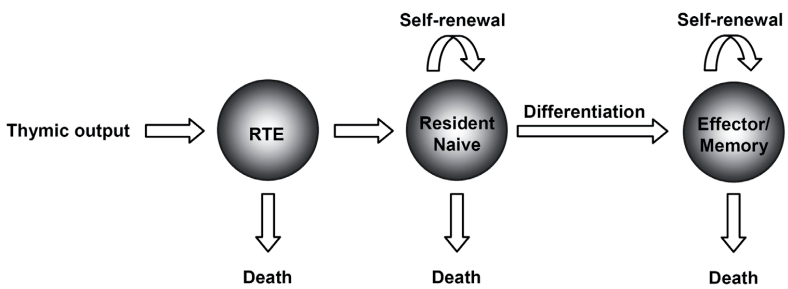


Figure 1 Schematic representation of T-cell dynamics. By definition RTE can only derive from the thymus. These cells are either lost or incorporated in the resident naive T-cell pool. Peripheral self-renewal contributes to the maintenance of both resident naive and effector/memory T cells. Each T-cell subset has its own average life span, which is determined by the rate of cell death and differentiation.

Thymus: Thymic output is the only source of new T-cell specificities. Mature thymocytes leave the thymus after a differentiation process of 3 weeks[4]. Each day approximately 1% of murine total thymocytes (in adult mice corresponding to about 10^6 cells) is exported[5;6]. The importance of the thymus in naive T-cell homeostasis is demonstrated by experiments in which thymic output is modulated: 1. Grafting addi-

tional thymus lobes causes a significant increase in the number of naive T cells in the periphery, proportional to the number of grafted thymi[5]. 2. Thymectomy of adult mice results in 70% reduced naive T-cell counts, suggesting that the majority of the naive T cells is directly dependent on thymus output[7]. 3. Mice with a 100-fold reduced thymic function exhibit only 12- and 23-fold reduced naive $CD4^+$ and naive $CD8^+$ T-cell counts respectively, suggesting either excessive thymus production under normal conditions or the induction of homeostatic compensatory mechanisms at low naive T-cell numbers[8]. In case of reduced or absent thymocyte production, such as in old mice or after thymectomy, not only naive T-cell numbers are declined, but the pool also shows holes in the TCR repertoire as T-cell specificities are ultimately lost in a situation of negligible thymus output[7;9].

Self-renewal: The observation that naive T cells not entirely disappear in the absence of a thymus suggests that peripheral mechanisms contribute to naive T-cell maintenance. In thymectomized mice, naive T cells were indeed shown to take up BrdU label, a drug incorporated into dividing cells, suggesting self-renewal of these cells[10;11]. Under lymphopenic conditions dividing naive T cells acquire a memory phenotype[12-14]. These memory-like T cells eventually can revert back to the naive phenotype[15]. In a rather full T-cell compartment however, self-renewal of naive T cells seems not to change phenotype[10;11], suggesting that naive T-cell proliferation is contributing to naive T-cell maintenance under healthy conditions.

Longevity: In addition to thymic output and self-renewal, the homeostatic regulation of life span contributes to naive T-cell maintenance. Instead of having an intrinsic life span, competition for space or niches may determine how long naive T cells live[7]. Survival of these cells requires both interaction with self-peptide MHC complexes[16;17] and the pro-survival cytokine interleukin 7 (IL-7)[18;19]. At low naive T-cell densities, competition for these factors is low, allowing cells to live longer, whereas at higher densities cells have a shorter life span as consequence of increased competition between the cells[20;21]. The availability of pro-survival cytokines affects the average life span of the naive T-cell pool. Indeed, naive T cells in old mice, and in thymectomized mice, both exhibiting lower cell densities, have a longer average life span than those in young control mice[22-24]. Competition for self-peptide MHC complexes permits small numbers of individual T-cell clones to survive simultaneously in separate niches, thereby maximizing the diversity of the naive T-cell pool[21].

Memory T-cell homeostasis

Differentiation: Due to MHC-specific competition for survival signals, the frequency of peptide-MHC specific naive T cells is kept extremely low, their total number in a mouse being estimated between 20 and 200 cells for naive $CD4^+$ and between 80

and 1200 cells for naive $CD8^+$ T cells[25-28]. Appropriate activation of naive T cells requires peptide-MHC interaction, costimulation and pro-inflammatory cytokines[29;30]. Since considerable numbers of antigen-specific T cells are necessary for a successful immune response, differentiation of naive T cells into effector/memory T cells is accompanied by a programmed massive clonal expansion[31], each naive $CD4^+$ and $CD8^+$ precursor population in the end eventually being amplified about 300- and >50,000-fold, respectively[26;28]. Consequently, up to 10^6 – 10^7 antigen-specific $CD4^+$ and $CD8^+$ cells can be detected at the peak of the immune response[32-34]. Irrespective whether or not the pathogen is cleared 90-95% of these cells die during a programmed contraction phase[35;36]. Whereas memory $CD4^+$ T cells show a gradual attrition over time in the absence of new antigens[32], the clonal size of antigen-specific memory $CD8^+$ T cells remains approximately 1000-fold higher than that of the original naive precursors for long time periods [26;32;37;38]. New immune challenges with heterologous viruses have been described to both qualitatively and quantitatively change pre-existing immunity[38;39] so that the effector/memory T-cell pool remains as diverse as possible. The erosion is however not so absolute as previously thought, since introduction of new effector/memory T-cell specificities leads to only moderate attrition in the number of pre-existing memory T cells[40]. This demonstrates that the size of the effector/memory T-cell compartment grows in size according to the immunological experience of the organism. Due to the ongoing exposure to pathogens during the lifetime of a mouse, the total number of effector/memory T cells indeed gradually accumulates during ageing[41].

Self-renewal and longevity: Although memory T cells provide life-long protection against previously encountered antigens, the individual cells are not long-lived. BrdU labeling experiments revealed that 80% of memory $CD4^+$ and 50% of memory $CD8^+$ T cells acquire label during a five week labeling period[11]. This rapid turnover rate indicate that memory T-cell population is particularly sustained by ongoing self-renewal. In contrast to naive T cells, both memory $CD4^+$ and $CD8^+$ T cells that are formed after acute infection are not maintained *via* MHC contacts[42-45], but rather by cytokine signaling via the common cytokine receptor γ-chain (γ_c), where IL-7 especially stimulates survival and IL-15 predominantly regulates homeostatic proliferation of the memory T cells[46]. The cytokines are exchangeable, as increased levels of IL-7 can overcome the requirement for IL-15 and *vice versa*[47-49]. Only in the absence of both cytokines lymphopenia-induced proliferation is impossible[47;49].

Ageing, chronic immune activation and T-cell homeostasis

Ageing: Due to increased medical opportunities to treat infectious diseases, the life expectancy of people has increased, resulting in a higher proportion of elderly in the world population. It is well-known that old people show a reduced immune respon-

siveness and are hence more susceptible to infections and less protected by vaccinations[50]. This urges the need for investigation how the immune system is maintained in the elderly. *De novo* production of naive T cells by the thymus is clearly reduced in aged mice and is correlated with thymic involution[6]. This multifactorial process involves both a reduced number of bone marrow-derived early T-lineage progenitors and changes in the thymic micro-environment[51-53]. Interestingly, bone marrow precursor cells from aged mice are able to generate functional $CD4^+$ memory T cells[54]. Alterations in the endocrine system contribute to thymic atrophy, as treatment of old mice with growth factors can stimulate thymopoiesis[55;56], whereas sex steroids accelerate thymic involution[57], and surgical castration results in thymic regeneration in old male mice and after bone marrow transplantation in young male mice[58].

Besides the lower production of thymic emigrants, resulting in holes in the naive T-cell repertoire[9], both naive and memory $CD4^+$ T cells show a reduced functionality in aged mice, demonstrated by incomplete differentiation, hypo-responsiveness, diminished cytokine production and poor proliferative responses of the $CD4^+$ T cells[59;60]. Although the frequency of antigen-specific $CD8^+$ T cells is reduced causing a delayed and reduced immune response, the functionality of naive, effector and memory $CD8^+$ T cells is comparable in young and old mice[61-63]. The reduced production of thymic emigrants, the gradual differentiation-induced loss of naive T cells and clonal expansions are thought to be responsible for the reduced repertoire diversity in the murine naive T-cell pool[9;64], whereas the diversity in the memory T-cell pool is compromised by the occurrence of age-associated oligoclonal expansions[65-67].

Chronic immune activation: The effect of antigen persistence and the concomitant presence of pro-inflammatory cytokines on the differentiation rate of naive T cells is largely unknown. During persistent murine cytomegalovirus infection the memory T-cell population is partly maintained by continuous recruitment of naive T cells[68]. As long as this differentiation process is antigen-specific and the pro-inflammatory environment does not impair naive T-cell production, there is no apparent risk that the total naive T-cell pool may shrink via differentiation-induced depletion. However, TCR-dependent differentiation of naive T cells by chronic stimulation via CD70, leads to a progressive depletion of these cells[69]. This suggests that chronic immune activation may result in bystander activation, possibly induced by increased cytokine levels, finally resulting in naive T-cell depletion and premature death.

Maintenance of memory T cells after acute infections when the pathogen is cleared differs essentially from that of chronic infections. Whereas the homeostatic cytokines IL-7 and IL-15 are essential for memory T-cell maintenance after acute infection[46],

memory $CD8^+$ T cells that are generated during pathogen persistence are unable to undergo homeostatic proliferation in the absence of antigen[70;71]. Transfer of virus-specific memory T cells, generated during chronic infections, into naive hosts, leads to disappearance of the memory T cells, demonstrating that the life span of the cells is rather short in the absence of antigen[70;71]. Thymectomy before immunization with LCMV has little impact on the number of LCMV-specific memory T cells over prolonged periods of time[72], despite the observation that naive T cells could be recruited to the memory T-cell pool by low levels of persisting antigen[73]. Although insufficient for maintenance of steady memory T-cell numbers[70], thymic emigrants could contribute to the heterogeneity of the population[73]. Specific antigenic peptide is needed to maintain steady memory T-cell numbers, whereas inflammatory signals alone are insufficient under conditions of chronic immune activation[70]. The presence of persistent antigen leads to extensive antigen-specific proliferation[70], probably resulting in functional exhaustion, characterized by high expression levels of PD-1[74]. *In vivo* blocking of the PD-1/PD-1L pathway leads to a restored T-cell function[75], whereas IL-10 receptor blockade prevents viral persistence and hence the establishment of chronic infections[76;77], suggesting that T-cell unresponsiveness can be reversed.

Scope of this thesis

Mouse data often form the basis for understanding human immunological processes, although extrapolation is often not verified. Here we compared the turnover rates of both naive T cells and granulocytes in humans and (adult and old) mice using stable isotope labeling (Chapter 2, 3 and 5). Since T-cell turnover rates based on labeling approaches differ considerably in the literature, we investigated whether the parameter estimates are dependent on the length of the labeling period (Chapter 4). To quantitate the relative contribution of thymic output, peripheral self-renewal, longevity and differentiation in the maintenance of naive T cells, mathematical comparisons were performed between control and thymectomized wild-type mice (Chapter 5). A similar approach was used in CD70Tg mice that intrinsically show a hyperactive immune system, to study the influence of chronic immune activation on T-cell homeostasis, (Chapter 6). And finally, a general discussion of the preceding findings is presented (Chapter 7).

REFERENCE LIST

1. Cavazzana-Calvo,M. and A.Fischer. 2007. Gene therapy for severe combined immunodeficiency: are we there yet? *J. Clin. Invest* 117:1456-1465.
2. Forsman,A. and R.A.Weiss. 2008. Why is HIV a pathogen? *Trends Microbiol.* 16:555-560.
3. Asquith,B., C.Debacq, D.C.Macallan, L.Willems, and C.R.Bangham. 2002. Lymphocyte kinetics: the interpretation of labeling data. *Trends Immunol.* 23:596-601.
4. Berzins,S.P., R.L.Boyd, and J.F.Miller. 1998. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. *J. Exp. Med.* 187:1839-1848.
5. Berzins,S.P., D.I.Godfrey, J.F.Miller, and R.L.Boyd. 1999. A central role for thymic emigrants in peripheral T cell homeostasis. *Proc. Natl. Acad. Sci. U. S. A* 96:9787-9791.
6. Hale,J.S., T.E.Boursalian, G.L.Turk, and P.J.Fink. 2006. Thymic output in aged mice. *Proc. Natl. Acad. Sci. U. S. A* 103:8447-8452.
7. Di Rosa,F., S.Ramaswamy, J.P.Ridge, and P.Matzinger. 1999. On the life span of virgin T lymphocytes. *J. Immunol.* 163:1253-1257.
8. Almeida,A.R., J.A.Borghans, and A.A.Freitas. 2001. T cell homeostasis: thymus regeneration and peripheral T cell restoration in mice with a reduced fraction of competent precursors. *J. Exp. Med.* 194:591-599.
9. Yager,E.J., M.Ahmed, K.Lanzer, T.D.Randall, D.L.Woodland, and M.A.Blackman. 2008. Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *J. Exp. Med.* 205:711-723.
10. Parretta,E., G.Cassese, A.Santoni, J.Guardiola, A.Vecchio, and F.Di Rosa. 2008. Kinetics of *in vivo* proliferation and death of memory and naive CD8 T cells: parameter estimation based on 5-bromo-2'-deoxyuridine incorporation in spleen, lymph nodes, and bone marrow. *J. Immunol.* 180:7230-7239.
11. Tough,D.F. and J.Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med.* 179:1127-1135.
12. Cho,B.K., V.P.Rao, Q.Ge, H.N.Eisen, and J.Chen. 2000. Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. *J. Exp. Med.* 192: 549-556.
13. Ge,Q., H.Hu, H.N.Eisen, and J.Chen. 2002. Naive to memory T-cell differentiation during homeostasis-driven proliferation. *Microbes. Infect.* 4:555-558.
14. Murali-Krishna,K. and R.Ahmed. 2000. Cutting edge: naive T cells masquerading as memory cells. *J. Immunol.* 165:1733-1737.
15. Goldrath,A.W., L.Y.Bogatzki, and M.J.Bevan. 2000. Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *J. Exp. Med.* 192:557-564.
16. Takeda,S., H.R.Rodewald, H.Arakawa, H.Bluethmann, and T.Shimizu. 1996. MHC class II molecules are not required for survival of newly generated CD4+ T cells, but affect their long-term life span. *Immunity.* 5:217-228.
17. Tanchot,C., F.A.Lemonnier, B.Perarnau, A.A.Freitas, and B.Rocha. 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science* 276:2057-2062.
18. Schluns,K.S., W.C.Kieper, S.C.Jameson, and L.Lefrancois. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells *in vivo*. *Nat. Immunol.* 1:426-432.
19. Tan,J.T., E.Dudl, E.LeRoy, R.Murray, J.Sprent, K.I.Weinberg, and C.D.Surh. 2001. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc. Natl. Acad. Sci. U. S. A* 98:8732-8737.
20. Mahajan,V.S., I.B.Leskov, and J.Z.Chen. 2005. Homeostasis of T cell diversity. *Cell Mol. Immunol.* 2:1-10.
21. Hataye,J., J.J.Moon, A.Khoruts, C.Reilly, and M.K.Jenkins. 2006. Naive and memory CD4+ T cell survival controlled by clonal abundance. *Science* 312:114-116.
22. Jones,S.C., K.Clise-Dwyer, G.Huston, J.Dibble, S.Eaton, L.Haynes, and S.L.Swain. 2008. Impact of post-thymic cellular longevity on the development of age-associated CD4+ T cell defects. *J. Immunol.* 180:4465-4475.
23. Swain,S.L. 2005. Thanks for the memory! *J. Immunol.* 175:4823-4826.

24. von Boehmer,H. and K.Hafen. 1993. The life span of naive alpha/beta T cells in secondary lymphoid organs. *J. Exp. Med.* 177:891-896.
25. Obar,J.J., K.M.Khanna, and L.Lefrancois. 2008. Endogenous naive CD8+ T cell precursor frequency regulates primary and memory responses to infection. *Immunity.* 28:859-869.
26. Blattman,J.N., R.Antia, D.J.Sourdive, X.Wang, S.M.Kaech, K.Murali-Krishna, J.D.Altman, and R.Ahmed. 2002. Estimating the precursor frequency of naive antigen-specific CD8 T cells. *J. Exp. Med.* 195:657-664.
27. Casrouge,A., E.Beaudoing, S.Dalle, C.Pannetier, J.Kanellopoulos, and P.Kourilsky. 2000. Size estimate of the alpha beta TCR repertoire of naive mouse splenocytes. *J. Immunol.* 164:5782-5787.
28. Moon,J.J., H.H.Chu, M.Pepper, S.J.McSorley, S.C.Jameson, R.M.Kedl, and M.K. Jenkins. 2007. Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity.* 27:203-213.
29. Curtsinger,J.M., C.S.Schmidt, A.Mondino, D.C.Lins, R.M.Kedl, M.K.Jenkins, and M.F. Mescher. 1999. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J. Immunol.* 162:3256-3262.
30. Curtsinger,J.M., C.M.Johnson, and M.F.Mescher. 2003. CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine. *J. Immunol.* 171:5165-5171.
31. van Stipdonk,M.J., G.Hardenberg, M.S.Bijker, E.E.Lemmens, N.M.Droin, D.R.Green, and S.P.Schoenberger. 2003. Dynamic programming of CD8+ T lymphocyte responses. *Nat. Immunol.* 4:361-365.
32. Homann,D., L.Teyton, and M.B.Oldstone. 2001. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat. Med.* 7:913-919.
33. Butz,E.A. and M.J.Bevan. 1998. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity.* 8:167-175.
34. Murali-Krishna,K., J.D.Altman, M.Suresh, D.J.Sourdive, A.J.Zajac, J.D.Miller, J.Slansky, and R.Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity.* 8:177-187.
35. Badovinac,V.P., B.B.Porter, and J.T.Harty. 2002. Programmed contraction of CD8(+) T cells after infection. *Nat. Immunol.* 3:619-626.
36. Harty,J.T. and V.P.Badovinac. 2008. Shaping and reshaping CD8+ T-cell memory. *Nat. Rev. Immunol.* 8:107-119.
37. Kaech,S.M., E.J.Wherry, and R.Ahmed. 2002. Effector and memory T-cell differentiation: implications for vaccine development. *Nat. Rev. Immunol.* 2:251-262.
38. Selin,L.K., K.Vergilis, R.M.Welsh, and S.R.Nahill. 1996. Reduction of otherwise remarkably stable virus-specific cytotoxic T lymphocyte memory by heterologous viral infections. *J. Exp. Med.* 183:2489-2499.
39. Selin,L.K., M.Y.Lin, K.A.Kraemer, D.M.Pardoll, J.P.Schneck, S.M.Varga, P.A.Santolucito, A.K.Pinto, and R.M.Welsh. 1999. Attrition of T cell memory: selective loss of LCMV epitope-specific memory CD8 T cells following infections with heterologous viruses. *Immunity.* 11:733-742.
40. Vezys,V., A.Yates, K.A.Casey, G.Lanier, R.Ahmed, R.Antia, and D.Masopust. 2009. Memory CD8 T-cell compartment grows in size with immunological experience. *Nature* 457:196-199.
41. Lerner,A., T.Yamada, and R.A.Miller. 1989. Pgp-1hi T lymphocytes accumulate with age in mice and respond poorly to concanavalin A. *Eur. J. Immunol.* 19:977-982.
42. Leignadier,J., M.P.Hardy, M.Cloutier, J.Rooney, and N.Labrecque. 2008. Memory T-lymphocyte survival does not require T-cell receptor expression. *Proc. Natl. Acad. Sci. U. S. A* 105:20440-20445.
43. Polic,B., D.Kunkel, A.Scheffold, and K.Rajewsky. 2001. How alpha beta T cells deal with induced TCR alpha ablation. *Proc. Natl. Acad. Sci. U. S. A* 98:8744-8749.
44. Murali-Krishna,K., L.L.Lau, S.Sambhara, F.Lemonnier, J.Altman, and R.Ahmed. 1999. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* 286:1377-1381.
45. Swain,S.L., H.Hu, and G.Huston. 1999. Class II-independent generation of CD4 memory T cells from effectors. *Science* 286:1381-1383.

46. Surh,C.D., O.Boyman, J.F.Purton, and J.Sprent. 2006. Homeostasis of memory T cells. *Immunol. Rev.* 211:154-163.
47. Goldrath,A.W., P.V.Sivakumar, M.Glaccum, M.K.Kennedy, M.J.Bevan, C.Benoist, D. Mathis, and E.A.Butz. 2002. Cytokine requirements for acute and basal homeostatic proliferation of naive and memory CD8+ T cells. *J. Exp. Med.* 195:1515-1522.
48. Kieper,W.C., J.T.Tan, B.Bondi-Boyd, L.Gapin, J.Sprent, R.Ceredig, and C.D.Surh. 2002. Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8+ T cells. *J. Exp. Med.* 195:1533-1539.
49. Tan,J.T., B.Ernst, W.C.Kieper, E.LeRoy, J.Sprent, and C.D.Surh. 2002. Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. *J. Exp. Med.* 195:1523-1532.
50. Weinberger,B., D.Herndler-Brandstetter, A.Schwanninger, D.Weiskopf, and B.Grubeck-Loebenstein. 2008. Biology of immune responses to vaccines in elderly persons. *Clin. Infect. Dis.* 46:1078-1084.
51. Aw,D., F.Taylor-Brown, K.Cooper, and D.B.Palmer. 2008. Phenotypical and morphological changes in the thymic microenvironment from ageing mice. *Biogerontology*.
52. Taub,D.D. and D.L.Longo. 2005. Insights into thymic aging and regeneration. *Immunol. Rev.* 205:72-93.
53. Min,H., E.Montecino-Rodriguez, and K.Dorshkind. 2005. Effects of aging on early B- and T-cell development. *Immunol. Rev.* 205:7-17.
54. Eaton,S.M., A.C.Maue, S.L.Swain, and L.Haynes. 2008. Bone marrow precursor cells from aged mice generate CD4 T cells that function well in primary and memory responses. *J. Immunol.* 181:4825-4831.
55. Min,D., A.Panoskaltsis-Mortari, O.Kuro, G.A.Hollander, B.R.Blazar, and K.I.Weinberg. 2007. Sustained thymopoiesis and improvement in functional immunity induced by exogenous KGF administration in murine models of aging. *Blood* 109:2529-2537.
56. Montecino-Rodriguez,E., R.Clark, and K.Dorshkind. 1998. Effects of insulin-like growth factor administration and bone marrow transplantation on thymopoiesis in aged mice. *Endocrinology* 139:4120-4126.
57. Olsen,N.J., S.M.Viselli, J.Fan, and W.J.Kovacs. 1998. Androgens accelerate thymocyte apoptosis. *Endocrinology* 139:748-752.
58. Sutherland,J.S., G.L.Goldberg, M.V.Hammett, A.P.Uldrich, S.P.Berzins, T.S.Heng, B.R.Blazar, J.L.Millar, M.A.Malin, A.P.Chidgey, and R.L.Boyd. 2005. Activation of thymic regeneration in mice and humans following androgen blockade. *J. Immunol.* 175:2741-2753.
59. Linton,P.J., L.Haynes, N.R.Klinman, and S.L.Swain. 1996. Antigen-independent changes in naive CD4 T cells with aging. *J. Exp. Med.* 184:1891-1900.
60. Haynes,L., S.M.Eaton, E.M.Burns, T.D.Randall, and S.L.Swain. 2003. CD4 T cell memory derived from young naive cells functions well into old age, but memory generated from aged naive cells functions poorly. *Proc. Natl. Acad. Sci. U. S. A* 100:15053-15058.
61. Kapasi,Z.F., K.Murali-Krishna, M.L.McRae, and R.Ahmed. 2002. Defective generation but normal maintenance of memory T cells in old mice. *Eur. J. Immunol.* 32:1567-1573.
62. Li,S.P., Z.Cai, W.Shi, A.Brunmark, M.Jackson, and P.J.Linton. 2002. Early antigen-specific response by naive CD8 T cells is not altered with aging. *J. Immunol.* 168:6120-6127.
63. Po,J.L., E.M.Gardner, F.Anaraki, P.D.Katsikis, and D.M.Murasko. 2002. Age-associated decrease in virus-specific CD8+ T lymphocytes during primary influenza infection. *Mech. Ageing Dev.* 123:1167-1181.
64. Ahmed,M., K.G.Lanzer, E.J.Yager, P.S.Adams, L.L.Johnson, and M.A.Blackman. 2009. Clonal expansions and loss of receptor diversity in the naive CD8 T cell repertoire of aged mice. *J. Immunol.* 182:784-792.
65. Messaoudi,I., J.Lemaoult, J.A.Guevara-Patino, B.M.Metzner, and J.Nikolich-Zugich. 2004. Age-related CD8 T cell clonal expansions constrict CD8 T cell repertoire and have the potential to impair immune defense. *J. Exp. Med.* 200:1347-1358.
66. Messaoudi,I., J.Warner, D.Nikolich-Zugich, M.Fischer, and J.Nikolich-Zugich. 2006. Molecular, cellular, and antigen requirements for development of age-associated T cell clonal expansions *in vivo*. *J. Immunol.* 176:301-308.

67. Posnett,D.N., D.Yarilin, J.R.Valiando, F.Li, F.Y.Liew, M.E.Weksler, and P.Szabo. 2003. Oligoclonal expansions of antigen-specific CD8+ T cells in aged mice. *Ann. N. Y. Acad. Sci.* 987:274-279.
68. Snyder,C.M., K.S.Cho, E.L.Bonnett, D.S.van, G.R.Shellam, and A.B.Hill. 2008. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. *Immunity.* 29:650-659.
69. Tesselaar,K., R.Arens, G.M.van Schijndel, P.A.Baars, M.van der Valk, J.Borst, M.H.van Oers, and R.A.van Lier. 2003. Lethal T cell immunodeficiency induced by chronic costimulation via CD27-CD70 interactions. *Nat. Immunol.* 4:49-54.
70. Shin,H., S.D.Blackburn, J.N.Blattman, and E.J.Wherry. 2007. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *J. Exp. Med.* 204:941-949.
71. Wherry,E.J., D.L.Barber, S.M.Kaech, J.N.Blattman, and R.Ahmed. 2004. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc. Natl. Acad. Sci. U. S. A* 101:16004-16009.
72. Miller,N.E., J.R.Bonczyk, Y.Nakayama, and M.Suresh. 2005. Role of thymic output in regulating CD8 T-cell homeostasis during acute and chronic viral infection. *J. Virol.* 79:9419-9429.
73. Vezys,V., D.Masopust, C.C.Kemball, D.L.Barber, L.A.O'Mara, C.P.Larsen, T.C.Pearson, R.Ahmed, and A.E.Lukacher. 2006. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *J. Exp. Med.* 203:2263-2269.
74. Sharpe,A.H., E.J.Wherry, R.Ahmed, and G.J.Freeman. 2007. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat. Immunol.* 8:239-245.
75. Barber,D.L., E.J.Wherry, D.Masopust, B.Zhu, J.P.Allison, A.H.Sharpe, G.J.Freeman, and R.Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439:682-687.
76. Brooks,D.G., M.J.Trifilo, K.H.Edelmann, L.Teyton, D.B.McGavern, and M.B.Oldstone. 2006. Interleukin-10 determines viral clearance or persistence *in vivo*. *Nat. Med.* 12:1301-1309.
77. Ejrnaes,M., C.M.Filippi, M.M.Martinic, E.M.Ling, L.M.Togher, S.Crotty, and M.G.von Herrath. 2006. Resolution of a chronic viral infection after interleukin-10 receptor blockade. *J. Exp. Med.* 203:2461-2472.

Chapter 2

Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool

Nienke Vrisekoop[1,2,*], Ineke den Braber[1,2,*], Anne Bregje de Boer[1,2], An F.C. Ruiter[3], Mariëtte T. Ackermans[3], Saskia N. van der Crabben[4], Elise H.R. Schrijver[1], Gerrit Spierenburg[1], Hans P. Sauerwein[4], Mette D. Hazenberg[1,2], Rob J. de Boer[5], Frank Miedema[1,2], José A.M. Borghans[1,2,5] and Kiki Tesselaar[1,2]

[1] Department of Immunology, University Medical Center, Utrecht, 3508 AB, The Netherlands; [2] Department of Clinical Viro-Immunology, Sanquin Research and Landsteiner Laboratory of the Academic Medical Center; [3] Department of Clinical Chemistry, AMC, Amsterdam, 1100 DD, The Netherlands; [4] Department of Endocrinology and Metabolism, AMC, Amsterdam, 1100 DD, The Netherlands; [5] Department of Theoretical Biology, Utrecht University, Utrecht, 3584 CH, The Netherlands

**These authors contributed equally to this work*

PNAS 2008; 105: 6115-6120

ABSTRACT

In mice recent thymic emigrants (RTE) make up a large part of the naive T-cell pool and have been suggested to be a distinct, short-lived pool. In humans however, the life span and number of RTE are unknown. While $^{2}H_2O$ labeling in young mice showed high thymic-dependent daily naive T-cell production, long term up- and down-labeling with $^{2}H_2O$ in human adults revealed a low daily production of naive T cells. Using mathematical modeling, we estimated human naive $CD4^+$ and $CD8^+$ T-cell half lives of 4.2 and 6.5 years, respectively, while memory $CD4^+$ and $CD8^+$ T cells had half lives of 0.4 and 0.7 years. The estimated half life of recently produced naive T cells was much longer than these average half lives. Thus, our data are not only incompatible with a substantial short-lived RTE population in human adults, but also suggest that the few naive T cells that are newly produced are preferentially incorporated in the peripheral pool.

INTRODUCTION

The role of the thymus in HIV infection is still poorly understood[1;2]. On the one hand, thymic failure has been suggested to play a crucial role in $CD4^+$ T-cell loss during HIV infection[3], and rapid thymic rebound has been proposed to be responsible for T-cell reconstitution during anti-viral treatment[4]. On the other hand, it has been argued that thymic output in adults might be too low to have a large impact on $CD4^+$ T-cell depletion[5]. In general these issues are addressed with estimates of thymic output, and naive and memory T-cell production rates and life spans which are simply extrapolated from observations in mice, monkeys, and lymphopenic or irradiated humans[6-11].

Naive T cells are generally thought to turnover relatively slowly, but it has been suggested that in mice, a considerable part of the naive T-cell pool consists of RTE with relatively rapid turnover[7;11;12]. In humans naive T-cell numbers, T-cell receptor excision circles (TRECs) and expression of CD31 have been used to measure thymic output[9;13;14]. Dion et al[4] observed rapid changes in the Sj/Vbeta TREC ratio within 3 months after infection with HIV, which suggested the presence of a rapidly turning over RTE pool in human adults containing most of the TRECs in the periphery, similar to young rodents and chickens[15;16]. However, because TRECs are long-lived, none of these approaches is specific for T cells that have recently emigrated from the thymus[1;2;5], and therefore they fail to quantify thymic output in humans.

Peripheral T-cell proliferation might also contribute to the maintenance of the naive T-cell pool in human adults, however, it is unclear which fraction of these cells remains in the naive T-cell pool[17]. The contribution of RTE and peripheral T-cell proliferation to the maintenance of the naive T-cell pool can only be determined by studying the fate of newly produced T cells. *In vivo* labeling with stable isotopes in combination with appropriate mathematical analysis of these data provides a way to obtain T-cell decay and production rates, and to follow the fate of recently produced T cells. Data on stable isotope labeling of naive and memory human T cells[18-22] are available, but several short-comings of these studies hamper their interpretation: (i) the short-term labeling period, which did not allow for sufficiently high labeling levels of naive cells[19;20;22], and (ii) the lack of delabeling curves in long-term labeling studies[18], which would have shown whether recently produced T cells contribute to the T-cell pool under investigation, or rapidly disappear by death or activation, and (iii) the frequent use of the precursor-product relationship[18;23;24] leading to underestimation of the extent of T-cell turnover[25]. Using the precursor-product relationship one measures the *net* accrual of label and ignores the possibility that cells were produced and lost during the labeling period, because of a short life span.

Here we combined long term *in vivo* stable-isotope labeling and label-decay studies of T cells, to obtain sufficient levels of labeling and sufficient data points to allow for reliable parameter fitting using a mathematical model to estimate naive T-cell production and loss rates. Our analyses showed a slow accumulation of label within the naive T-cell pool, due to low daily production of naive T cells with a very long half life. These data are incompatible with the presence of a substantial short-lived RTE pool in humans.

RESULTS

Thymic output in mice

Since the main focus of this study was to establish the role of thymic output and peripheral T-cell proliferation in the maintenance of the naive T-cell pool, we first established if thymic output - and more specifically, a rapidly turning over RTE pool - could be detected using the ^2H_2O-labeling technique. It is generally accepted that young rodents have considerable thymic output[26;27]. BrdU-labeling studies, TREC dynamics and thymic engraftment in mice have revealed that the RTE pool of mice has a fast turnover[11;16], with an average life span of only 3 weeks[7;12]. We first investigated whether this rapidly turning over RTE pool can be detected in young male mice using the ^2H_2O-labeling technique, and compared euthymic and thymectomized mice to establish to what extent RTE affect the labeling within the naive T-cell pool.

Thymectomy of 7-week-old mice resulted in a severe and significant reduction in absolute $CD4^+$ and $CD8^+$ T-cell counts in lymph nodes (LNs) and spleen in comparison to both age-matched controls (Fig.1, A) and sham-thymectomized mice (data not shown). Taking into account the natural reduction in T-cell numbers in peripheral lymphoid organs during aging, the fastest decrease in T-cell counts was seen in the first 3 weeks after thymectomy. Between 3 and 8 weeks post-thymectomy, the number of naive $CD4^+$ and $CD8^+$ T cells continued to decline more rapidly than in euthymic mice, while at later time points they decreased at approximately similar rates in thymectomized and euthymic mice. 14-16 weeks after surgery naive $CD4^+$ T cells were reduced by 73% (p=0,029) in spleen and 77% (p=0,029) in LNs, while naive $CD8^+$ T cells were reduced by 67% (p=0,029) in spleen and 70% (p=0,029) in LNs (Fig.1, B; and data not shown).

^2H_2O labeling of young (12-13 week old) euthymic mice for 9-10 weeks resulted in 71 ± 3 % up-labeling for naive $CD4^+$ T cells and 50 ± 1 % for naive $CD8^+$ T cells in the spleen (Fig.1, C). Comparable fractions of labeled cells were found in the LNs (data not shown). This high degree of labeling in T cells could be due to substantial

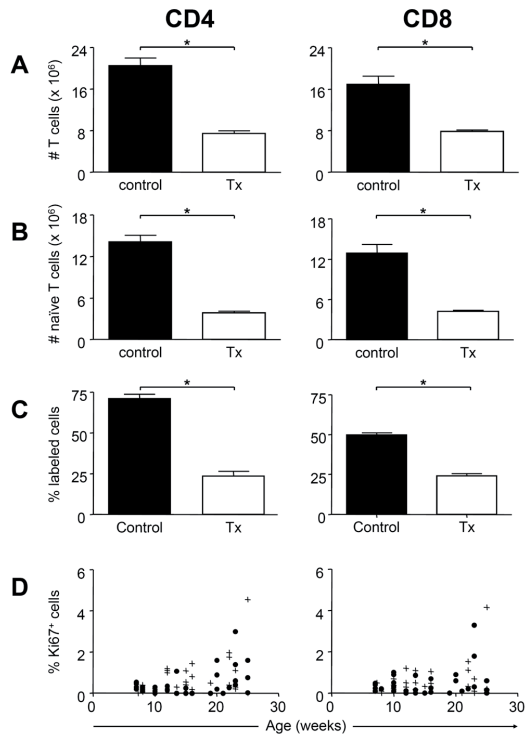


Figure 1 Thymic output is important for the maintenance of the naive T-cell pool in young mice and can be quantitated using ^2H_2O-labeling A,B) Absolute numbers of total (A) and naive (B) $CD4^+$ and $CD8^+$ T cells in spleen of 3 control (black bars) and 4 thymectomized (open bars) animals 14-16 weeks after time of surgery. C) Accumulated ^2H_2O-labeling in naive $CD4^+$ and $CD8^+$ T cells of the same euthymic and athymic mice after a 9-10 week labeling period. Data are displayed as mean ± standard error of the mean (n = 3-4). $p \leq 0.05$ is considered significant (*). D) Percentage of $Ki67^+$ cells within the naive $CD4^+$ and naive $CD8^+$ T-cell pool of control (●) and thymectomized (+) mice at different time points before and after thymectomy.

peripheral proliferation of naive T cells and/or reflect output of labeled thymocytes. To discriminate between these possibilities, mice were thymectomized at the age of 7 weeks and submitted to the long term ^2H_2O-labeling protocol 5 to 6 weeks later. In the absence of thymic output the fraction of labeled naive T cells after 10 weeks up-labeling was 2-3 fold reduced (23 ± 3 % for naive $CD4^+$ T cells; 24 ± 1 % for naive $CD8^+$ T cells, Figure 1C, p=0.05). Interestingly, the fraction of cycling ($Ki67^+$) naive T cells was not altered in athymic mice (Fig.1, D). This indicates that the difference in labeled naive T cells between euthymic and thymectomized mice reflects the amount of label that was acquired while dividing in the thymus. Collectively, these data demonstrate that thymic output can be measured using ^2H_2O labeling. Furthermore, the observation that thymectomy led to a significantly reduced fraction

of labeled cells and a significant loss of naive T cells, confirms that in young mice RTE contribute substantially to the naive T-cell pool.

^2H_2O labeling in human volunteers

Next we aimed to determine the role of thymic output and peripheral T-cell proliferation in the maintenance of the naive T-cell pool, and the turnover rate of the memory T-cell pool in 5 adult humans, aged between 20 and 25 years. During the 9-week up-labeling period and subsequent 16 week down-labeling period blood samples were drawn at 14 time points. Absolute numbers of $CD4^+$ and $CD8^+$ T cells and the fraction of naive $CD45RO^-CD27^+$ and memory $CD45RO^+$ $CD4^+$ and $CD8^+$ T cells were measured and these fractions were sorted for measurement of deuterium enrichment in the DNA. In addition, we measured *ex vivo* proliferation by the expres-

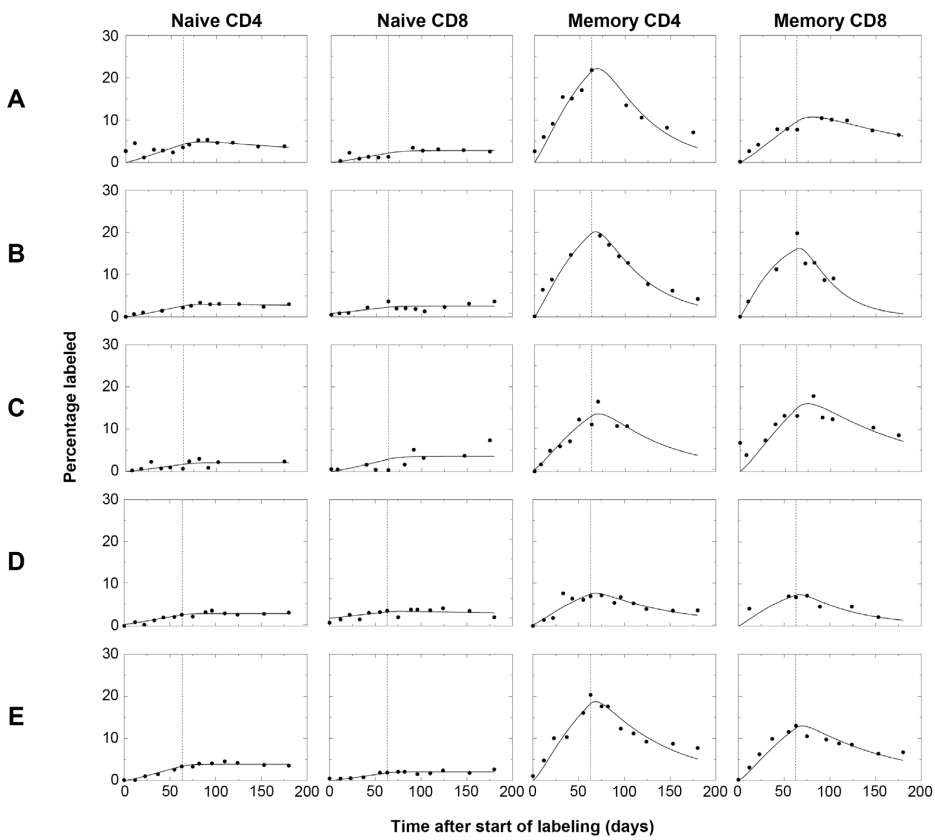


Figure 2 Best fits of the naive and memory $CD4^+$ and $CD8^+$ T-cell enrichment curves. Label enrichment was scaled between 0 and 100% by normalizing for the percentage label obtained in granulocytes (see Methods). In the graph the end of the labeling period is marked by a vertical line.

sion of the proliferation marker Ki67 within these same naive and memory T-cell populations to exclude the possibility that episodes of overt immune activation affected turnover rates. Characteristics of the healthy volunteers are given in supplementary Table S1.

Body water and granulocyte enrichment

As a measure for body water enrichment during up- and down-labeling, we quantified ^2H_2O enrichment in urine at each time point. At the earliest time points after start of ^2H_2O labeling, body water enrichment had not yet reached its maximum level, while shortly after cessation of label body water was still found to be enriched. We therefore modeled the body water enrichment curves (see Methods and supplementary Figure S1) and corrected for these best fits when analyzing the enrichment in the different cell populations. Since granulocytes are known to turn-

Table I Turnover rates (p) and loss rates of labeled cells (d) per day.

		A	B	C	D	E	Median
Naive CD4	p	**0.0009** (0.0007-0.0015)	**0.0005** (0.0004-0.0005)	**0.0003** (0.0002-0.0005)	**0.0004** (0.0003-0.0004)	**0.0006** (0.0005-0.0006)	**0.0005**
	d	**0.0040** (0.0000-0.0106)	**0.0009** (0.0000-0.0029)	**0.0000** (0.0000-0.0083)	**0.0000** (0.0000-0.0026)	**0.0000** (0.0000-0.0010)	**0.0000**
Naive CD8	p	**0.0004** (0.0003-0.0006)	**0.0003** (0.0002-0.0003)	**0.0005** (0.0003-0.0010)	**0.0003** (0.0001-0.0005)	**0.0003** (0.0002-0.0004)	**0.0003**
	d	**0.0000** (0.0000-0.0050)	**0.0000** (0.0000-0.0000)	**0.0000** (0.0000-0.0119)	**0.0024** (0.0000-0.0132)	**0.0000** (0.0000-0.0000)	**0.0000**
Memory CD4	p	**0.0067** (0.0059-0.0081)	**0.0058** (0.0052-0.0068)	**0.0034** (0.0026-0.0044)	**0.0017** (0.0014-0.0022)	**0.0045** (0.0040-0.0052)	**0.0045**
	d	**0.0203** (0.0158-0.0250)	**0.0198** (0.0163-0.0227)	**0.0145** (0.0069-0.0230)	**0.0115** (0.0070-0.0161)	**0.0126** (0.0094-0.0154)	**0.0145**
Memory CD8	p	**0.0021** (0.0018-0.0025)	**0.0060** (0.0043-0.0083)	**0.0034** (0.0029-0.0046)	**0.0019** (0.0014-0.0028)	**0.0028** (0.0025-0.0034)	**0.0028**
	d	**0.0065** (0.0041-0.0096)	**0.0304** (0.0192-0.0436)	**0.0089** (0.0040-0.0133)	**0.0161** (0.0077-0.0262)	**0.0098** (0.0071-0.0127)	**0.0098**

Depicted are turnover rates (p) and loss rates of labeled cells (d) per day as estimated by the mathematical model. The 95%-confidence intervals were determined by bootstrapping[46], resampling the residuals 500 times.

Table II Half lives of naïve and memory $CD4^+$ and $CD8^+$ T cells.

	A	B	C	D	E	Median
naive CD4	801	1517	2374	1899	1187	**1517**
naive CD8	1737	2762	1341	2398	2374	**2374**
memory CD4	104	119	204	402	155	**155**
memory CD8	327	116	202	359	244	**244**

Depicted are half lives (ln2/p) in days derived from the mathematical model.

over rapidly, labeling of the granulocyte population of each individual was measured to estimate the maximal level of label intake that cells could possibly attain (see Methods and supplementary Figure S1). Furthermore, because DNA baseline enrichment is not only determined by naturally occurring extremely low ^2H_2O enrichment, but also by the more abundant naturally occurring heavy carbon atoms, we also longitudinally measured a negative control who did not drink ^2H_2O, which pointed out that background fluctuations were negligible (data not shown).

Turnover of naive and memory $CD4^+$ and $CD8^+$ T cells

First of all, all labeling data of the different T-cell subsets were divided by the estimated maximum granulocyte enrichment of each volunteer (see Methods). The mathematical model was used to fit the corrected data, and to determine the average turnover rate (p) and the average rate at which labeled cells were lost from the population (d). It is important to realize that the accrual of label during label

administration is truly representative of the T-cell population as a whole, while the loss of label after label cessation is only based on those cells that have picked up label by cell division. We therefore based our analyses on a so-called kinetic heterogeneity model in which the average turnover rate of the T-cell population is not necessarily equal to the loss rate of labeled cells[28].

The median turnover rates of naive $CD4^+$ and $CD8^+$ T cells were found to be as low as p = 0.0005 and 0.0003 per day, corresponding to median half lives of 1517 and 2374 days for naive $CD4^+$ and $CD8^+$ T cells, respectively (Fig.2 and Tables I and II). The turnover rates of memory $CD4^+$ and $CD8^+$ T cells were found to be approximately 10-fold higher, i.e. p = 0.0045 and 0.0028 per day, corresponding to half lives of 155 and 244 days for memory $CD4^+$ and $CD8^+$ T cells, respectively.

Using the individual naive $CD4^+$ and $CD8^+$ T-cell counts revealed a median naive $CD4^+$ T-cell production of 8.2 $x10^7$ cells per day and a median naive $CD8^+$ T-cell production of 2.4 $x10^7$ per day (Table III). Since this daily production of new naive T

Table III Total production of naïve T cells per day.

	A	B	C	D	E	Median
CD4	11.50	5.36	2.24	8.19	13.10	**8.19**
CD8	2.53	1.51	2.39	2.07	3.53	**2.39**
Total	14.03	6.87	4.63	10.26	16.63	**10.26**

Total production of naïve T cells per day (*10^7), calculated as *p** [the naive cell count per liter blood] * [5 liter blood] * 50, assuming that 2% of lymphocytes reside in the blood[47].

cells is the sum of thymic output and homeostatic proliferation within the naive T-cell pool, our data provide an upper estimate of daily thymic production of $1.7x10^8$ T cells per day (see Table III).

The median rates at which labeled memory $CD4^+$ and $CD8^+$ T cells were lost from the memory population were found to be 0.0145 and 0.0098 per day, respectively. Interestingly, in none of the individuals did we find a significant loss of labeled naive $CD4^+$ or $CD8^+$ T cells during the 16 weeks after cessation of label (Fig.2 and Table I), indicating that newly produced naive T cells – whether produced by the thymus or by peripheral T-cell proliferation – had a longer expected life span than the average naive T cell. Our data are therefore not compatible with the presence of a substantial short-lived RTE pool in adult healthy humans.

DISCUSSION

By *in vivo* labeling of T-cell subsets using ^2H_2O, and mathematical analysis of label enrichment, our data provide reliable estimates for the average turnover rates of naive and memory $CD4^+$ and $CD8^+$ T cells in healthy adults. Although isotope labeling studies in humans are typically restricted to blood, it has previously been reported that labeling kinetics in human T cells derived from blood and lymphoid tissues are comparable[29]. Label incorporation in T cells derived from mouse peripheral lymph nodes and spleen was also similar (unpublished results). Seemingly there is little difference in labeling of the analyzed T-cell subsets derived from the different lymphoid compartments.

The very low accumulation of label in naive T cells (<5%) that we observed after 9 weeks of up-labeling is compatible with the data reported by Hellerstein et al[18]. Our median estimated half lives between 1517 and 2374 days for naive T cells and between 155 and 244 days for memory T cells are, however, much longer than previous estimates based on stable-isotope labeling, which varied from 112 days to 361 days for naive T cells, and from 14 days to 235 days for memory T cells[25]. The

use of T-cell death rates, which overestimate T-cell turnover because of the bias towards cells that have recently divided[30], and the lack of data points during the uplabeling phase in previous short-term labeling experiments might explain these discrepancies. Michie et al[10] used the presence of T cells with dicentric chromosomes after radiation to measure the half life of naive and memory T cells. They estimated a half life of 182 days for $CD45RO^+$ and 630 days for $CD45RA^+$ T cells. Given the notion that $CD45RA^+$ T cells can contain a substantial fraction of effector ($CD45RA^+CD27^-$) cells we additionally used CD27 expression on $CD45RA^+$ T cells to identify naive T cells. This difference in definition of the naive subset may explain the difference in the estimated life spans between these studies. Furthermore, it is conceivable that the half lives of T cells were affected by radiation.

The origin of variation in the calculated half lives of the adult humans is unknown: no relation was found between this parameter and T-cell counts, Ki67 expression or age. The relative differences in calculated half lives were however in the same range as the differences in the other parameters that we and others have measured[31].

The maximum total daily naive T-cell production in our 5 healthy volunteers of 13.1 x 10^7 $CD4^+$ T cells and 3.5 x 10^7 $CD8^+$ T cells implies that the thymus in human adults is exporting maximally 1.7 x 10^8 T cells per day. Part of the labeling of the naive T-cell population may, however, also be due to peripheral T-cell proliferation. The estimated total daily naive T-cell production is in close agreement with the daily accumulation of 10^8 naive T cells in patients with a depleted T-cell pool[8;32], which suggests that thymic output and naive T-cell proliferation do not homeostatically respond to that level of peripheral T-cell depletion. Still the production of naive T cells in such depleted situations might be underestimated because naive T cells may transit more rapidly to the memory pool.

Our analyses also enabled us to follow the fate of recently produced cells. In the memory T-cell population we found that the decay rate of labeled cells is higher than the average production rate, indicating that the turnover of cells that picked up label was higher than the turnover of the average cell in the memory population. This finding is in line with previous labeling studies, which all showed that the loss of labeled cells exceeded their production[28;30;33]. Unexpectedly, we found that this was not the case for the naive T-cell population, as no significant loss of labeled cells was observed in any of the 5 individuals during the 16 weeks after label cessation. Thus, newly produced (i.e. labeled) naive T cells, whether produced by the thymus or by peripheral proliferation, tended to live longer than the average cell in the naive T-cell population. This implies that newly produced naive T cells were preferentially incorporated into the peripheral naive T-cell pool, which contradicts the notion of a substantial short-lived RTE pool in human adults.

To be sure that administration of ^2H_2O would efficiently label thymic emigrants, we performed ^2H_2O labeling in euthymic and thymectomized mice. Studies have shown that thymic output in young rodents is substantial[26;27], and TREC dynamics, BrdU labeling and thymic engraftment have demonstrated that the RTE pool in mice has a fast turnover[7;11;12;16]. In line with previous studies, we found that thymectomy in young mice gives a considerable reduction in peripheral naive T-cell numbers already within 3 weeks after thymectomy[34;35], indicating an important contribution of RTE to the naive T-cell population in young mice. Thymectomy induced a 2-3 fold decrease in labeled naive T cells, showing that the ^2H_2O protocol successfully labeled RTE in euthymic mice.
In thymectomized mice T-cell numbers were severely reduced. It has been described that under lymphopenic circumstances homeostatic naive T-cell proliferation and conversion to a memory phenotype occurs[36]. Whether reversion of memory T cells to a naive phenotype occurs, is still unclear[37]. Since no increase in the fraction of $Ki67^+$ naive or effector/memory T cells was observed in thymectomized mice, the residual labeling of naive T cells is most likely not due to homeostatic proliferation or reversion. For the human study, we can formally not rule out the possibility of conversion, but since we performed the study in healthy persons with normal T-cell counts, one may expect very low conversion and reversion rates.

Our data shed a different light on thymic output and RTE dynamics. In contrast to the observations by Berzins et al which suggested that in young mice an excess of short-lived RTE is produced, which are displaced three weeks after export from the thymus[12], we found that in human adults RTE are rare and long-lived, and thus remain in the peripheral T-cell pool. The advantage of a short-lived RTE pool has been proposed to be the continuous supply of T cells with a diverse repertoire to the long-lived resident naive T-cell pool[38]. The preferential incorporation of RTE into the resident naive T-cell pool that we here describe for human adults seems a much more efficient way to continuously rejuvenate the naive T-cell pool and repertoire than a daily excess production of RTE with a very short life span. The unfortunate consequence of this sparse production of long-lived naive T cells may be, however, that naive T-cell supply may be limiting in clinical conditions of chronic T-cell depletion, and – due to the increasing life expectancy – even in healthy elderly, which may have severe clinical consequences.
Taken together, our data confirm the presence of a significant RTE pool with rapid turnover in young mice, while in human adults recently produced naive T cells are rare and long-lived.

MATERIALS AND METHODS

Mouse studies

Mice

C57Bl/6 mice were maintained by in-house breeding at the Netherlands Cancer Institute (Amsterdam) under specific pathogen-free conditions in accordance with institutional and national guidelines. To exclude possible gender effects, we only used male mice in this study.

Thymectomy

7-week-old male C57Bl/6 mice were anesthesized by i.p. injection with Hypnorm (0.4 mg/kg fentanyl citrate and 12.5 mg/kg fluanisone, Janssen Animal Health, Buckinghamshire, UK) and Dormicum (6.3 mg/kg, Roche Nederland BV, Woerden, The Netherlands). The mouse was placed in supine position and its limbs and maxilla were taped to a surgical board. The skin was prepped with 70% alcohol. A midline incision was made from the lower cervical region to the level of the fifth rib. The skin was loosened and the salivary glands were pushed laterally. Directly adjacent to the sternum the two upper ribs were cut, thereby exposing the thymus. Vacuum suction was applied to remove the organ. The skin was closed by interrupted sutures and the animals were warmed until recovery from anaesthesia. Post-operative survival was 85%. The completeness of thymectomy was confirmed by visual inspection, both directly after removal of the organ and at the conclusion of the experiment.

^2H_2O labeling protocol

12-13 week old animals obtained one boost injection (i.p.) of 15 ml/kg with 99.8 % ^2H_2O (Cambridge Isotopes, Cambridge, MA) and were subsequently fed with 4% ^2H_2O for 9 or 10 weeks (control and thymectomized mice respectively).

FACS staining and cell sorting

Due to the limited amount of blood that can be drawn from mice, and the amount of cells needed for GC/MS analysis, we were unable to isolate enough cells of each T-cell subset from blood derived from (thymectomized) mice. Therefore, thymus, spleen and (axillary, brachial, inguinal and superficial cervical) LNs were isolated from 21-23 week old thymectomized and control C57Bl/6 mice. Single cell suspensions were obtained by mechanical disruption. Red blood cells were lysed with ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.4). Cells were washed, resuspended in IMDM/7% FCS and counted. 5 10^5 cells/well were seeded in 96 well plates in FACS staining buffer (PBS/1% FCS)

and stained with CD4-PerCP or CD8-PerCP, CD44-APC and CD62L-PE (BD PharMingen, San Diego, CA) in the presence of blocking 2.4G2 mAb (CD16/CD32). Following incubation with Cytofix/cytoperm solution (BD) the cells were incubated with α-Ki67 or α-IgG (BD) in 0.1% saponin (in FACS staining buffer). Cells were analyzed on a LSR II flow cytometer and BD FACSDiva software. For logistical reasons, splenocytes were left overnight at room temperature and stained the next day for CD4-PerCP, CD8-FITC, CD44-APC and CD62L-PE (BD). Cell recovery after overnight storage was usually 77%, preferential loss of particular T-cell subsets was not observed. Naive ($CD62L^+$, $CD44^-$) cells were sorted using a FACSAria cell sorter and FACSDiva software (BD). Purity of the sorted cells was 81-97% for naive $CD4^+$ T cells (average: 96% for control vs. 83% for Tx mice) and 86-99% for naive $CD8^+$ T cells (average: 98% for control vs. 88% for Tx mice). Granulocytes were isolated by density gradient centrifugation of blood using a combination of histopaque-1119 and Ficoll-paque, followed by red blood cell lysis. Granulocytes and thymocytes were frozen until further processed.

Statistical analysis

The Mann-Whitney test was performed to determine differences between mouse groups. All statistical analyses were performed using the software program SPSS 12.0 (SPSS Inc, Chicago, Illinois). Differences with $p \leq 0.05$ were considered significant.

Human studies

Subjects and in vivo ^2H_2O labeling protocol

Five healthy male volunteers (characteristics of the T-cell compartment of these volunteers are given in supplementary Table S1) were submitted to the AMC hospital to receive the initial administration dose of 10 ml ^2H_2O per kg body water in small portions throughout the day. Body water was estimated to be 60% of body weight. As a maintenance dose, the subjects daily drank 1/8 of this initial dose at home for 9 weeks. Blood and urine were collected before labeling and 6 times during the labeling protocol. In addition, during the down-label phase of 16 weeks blood and urine was collected 7 times. All patients were healthy and were asked to answer a questionnaire to exclude (a high risk of) infections and immuno-modulatory medication. This study was approved by the medical ethical committee of the AMC and written informed consent was obtained from all volunteers.

Flow cytometry and cell sorting

Absolute $CD4^+$ and $CD8^+$ T-cell counts were determined by dual-platform flow-cytometry.

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque density gradient centrifugation from heparinized blood and cryopreserved until further processed. Peripheral blood T-cell proliferation in $CD4^+$ and $CD8^+$ T-cell subsets was studied by flow-cytometric measurements of Ki67 nuclear antigen, as described previously[39-41].

To measure the fraction of labeled cells within the naive ($CD45RO^-CD27^+$) and memory ($CD45RO^+$) $CD4^+$ and $CD8^+$ T-cell population, cryopreserved PBMC were thawed and incubated with monoclonal antibodies (mAb) to CD45RO-FITC (Caltag Laboratories, Burlingame, CA), CD27-PE, CD4-PerCP and CD8-APC (BD). The specified cell fractions were isolated by cell sorting on a MoFlow high speed cell sorter or on a FACSAria (BD). Purity of the sorted cells was on average 96% for naive $CD4^+$ and $CD8^+$ T cells, 95% for memory $CD4^+$ T cells and 91% for memory $CD8^+$ T cells.

Measurement of ^2H_2O enrichment in body water and DNA

Deuterium enrichment in urine was measured by a method adopted from Previs et al[42]. The isotopic enrichment of DNA was measured according to the method described by Neese et al[21] with minor modifications. Briefly, DNA was enzymatically hydrolyzed into deoxyribonucleotides after which the deoxyadenosines were purified using a SPE column. The adenosine residue was captured by cation resin and the deoxyribose was derivatized to deoxyribosepentane-tetraacetate (PTA) before injection into the gas chromatograph (6890 series, Agilent Technologies, Palo Alto, CA). The mass of the derivate was measured by positive chemical ionization mass spectrometry (5973 MSD, Agilent Technologies) at m/z 245 (M_0) and 246 (M_1). Since M_1 is known to be concentration-dependent, we first used the peak area at M_0 to determine the suspected natural abundance for each sample[43]. The enrichment (EM_1) was subsequently determined by dividing the peak area at M_1 by the total peak area ($M_1 + M_0$), after subtraction of the corrected natural abundance from the measured M_1 enrichment. We first fitted the urine enrichment data of each individual to a simple label enrichment/decay curve (see Supporting Information, supplementary Figure S1 and supplementary Table S2). The best fits for urine enrichment were incorporated when analyzing the enrichment in the different cell populations. Up- and down-labeling of the granulocyte population of each individual was analyzed mathematically to determine the maximal level of label intake that cells could possibly attain (see Supporting Information, supplementary Figure S1 and supplementary Table S3). The average turnover rate and the loss rate of labeled cells in each T-cell subset were determined after normalization by this granulocyte maximum (see Supporting Information). For ease of the procedure mouse thymocytes were used (if available) to determine the maximal level of label

intake. In thymectomized mice the enrichment of fully turned-over granulocytes was used as indicator, as maximal label enrichment in mouse thymocytes and granulocytes was found to be similar.

Mathematical modeling

Since the fraction of heavy water in body water is similar to that in urine[44], we model the measured up- and down-labeling of the urine enrichment by the following differential equations for normal water, w, and heavy water, h, in the urine

$\frac{dw}{dt} = (1-f)s - \delta w$ and $\frac{dh}{dt} = fs - \delta h$ during label intake ($t \le \tau$), and

$\frac{dw}{dt} = s - \delta w$ and $\frac{dh}{dt} = -\delta h$ after label intake ($t > \tau$),

where f represents the fraction of ^2H_2O in the drinking water, t represents time in days and labeling was stopped at $t = \tau$ days, δ represents the turnover rate of body water per day, and s is the amount of water consumed in liters per day. These equations can be solved analytically, and rewritten in terms of the fraction, $U(t)$, of ^2H_2O in the urine. The baseline urine enrichment, $U(0)=\beta$, that is attained after the boost of label by the end of day 0 determines the initial conditions, i.e., $w(0) = (1-\beta)s/\delta$ and $h(0) = \beta s/\delta$, such that:

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

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

The parameter estimates of the best fits for the urine curves are given in Supplementary Table S2.

To model the label enrichment of adenosine in the DNA of cells we assume identical reaction kinetics of hydrogen and deuterium, and of labeled and unlabeled adenosines. Further, we extended the model of Asquith *et al*.[28] to include the dependence on the actual enrichment of the body water (as estimated by $U(t)$). Since the adenosine deoxyribose (dR) moiety contains seven hydrogen atoms that can be replaced by deuterium, one expects an amplification factor, $c > 1$, in the enrichment of dR in DNA relative to the body ^2H_2O enrichment. At the low levels of body ^2H_2O enrichment that one typically achieves ($U(t) < 2\%$), the likelihood of double labeling is very low[21;45]. Theoretically, the fraction of adenosine dR moieties that have exactly one enriched hydrogen atom is expected to be $c \cong \binom{7}{1} U(t)(1-U(t))^6$. However, because of dilution by the purine nucleoside pathway, one typically measures the amplification factor *c* from the enrichment in dR in the DNA of cells with a rapid turnover, like granulocytes or monocytes[21;45].

Consistent amplification factors of c = 3.5 to c = 4 for body water enrichment levels of 2-3% have thus been reported[21].

Following Asquith *et al.*[28] label enrichment of adenosine in the DNA of a population of cells was modeled by

$$\frac{\mathrm{d}l}{\mathrm{d}t} = pcU(t))A - dl$$

where l is the total amount of labeled adenosine in the DNA, p is the average production rate of that population, c is the amplification factor, A is the total amount of adenosine in the DNA of that population, and d is the death rate of cells carrying labeled adenosine. Basically one writes that each adenosine residue replicates at rate p and will incorporate a deuterium atom with probability $cU(t)$. For naïve T cells this replication may occur both in the periphery and the thymus. Scaling this equation by the total amount of adenosine in the DNA, i.e., defining $L = l/A$, yields

$$\frac{\mathrm{d}L}{\mathrm{d}t} = pcU(t) - dL \qquad \text{(Equation 2)}$$

throughout the labeling and de-labeling period, where *L* represents the fraction of labeled deoxyribose residues of adenosine in DNA. The corresponding analytical solutions for the enrichment of adenosine in DNA are

$$L(t) = \frac{cpf}{\delta - d}\left[\frac{\delta}{d}(1-\mathrm{e}^{-dt}) - (1-\mathrm{e}^{-\delta t}) + \frac{\beta}{f}(\mathrm{e}^{-dt} - \mathrm{e}^{-\delta t})\right] \qquad \text{(Equation 3a)}$$

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

$$L(t) = \frac{cpf}{\delta - d}\left[\frac{\delta}{d}(\mathrm{e}^{-d(t-\tau)} - \mathrm{e}^{-dt}) - (\mathrm{e}^{-\delta(t-\tau)} - \mathrm{e}^{-\delta t}) + \frac{\beta}{f}(\mathrm{e}^{-dt} - \mathrm{e}^{-\delta t})\right] \qquad \text{(Equation 3b)}$$

after label intake ($t > \tau$). Note that the amplification factor and the division rate always appear together, and that pc can therefore only be estimated as a parameter combination.

Equations 3a and 3b were fitted to each individual's granulocyte enrichment data, yielding 0.299 ≤ pc ≤ 0.419 per day, and death rates 0.078 < d < 0.103 per day (see the Supplementary information). Assuming that the granulocytes are fully turned-over, i.e., assuming that $p = d$ for the granulocytes, we estimate amplification factors of c = 4.46, 4.93, 5.15, 3.78 and c = 4.03 for the five individuals (which is indeed lower than the theoretical maximum of 7).

Next, Equations 3a and 3b were applied to calculate the average turnover rate p and the loss rate of labeled cells d in each T-cell population. The parameter p in Equation (2) represents T-cell production resulting from both T-cell proliferation and thymic

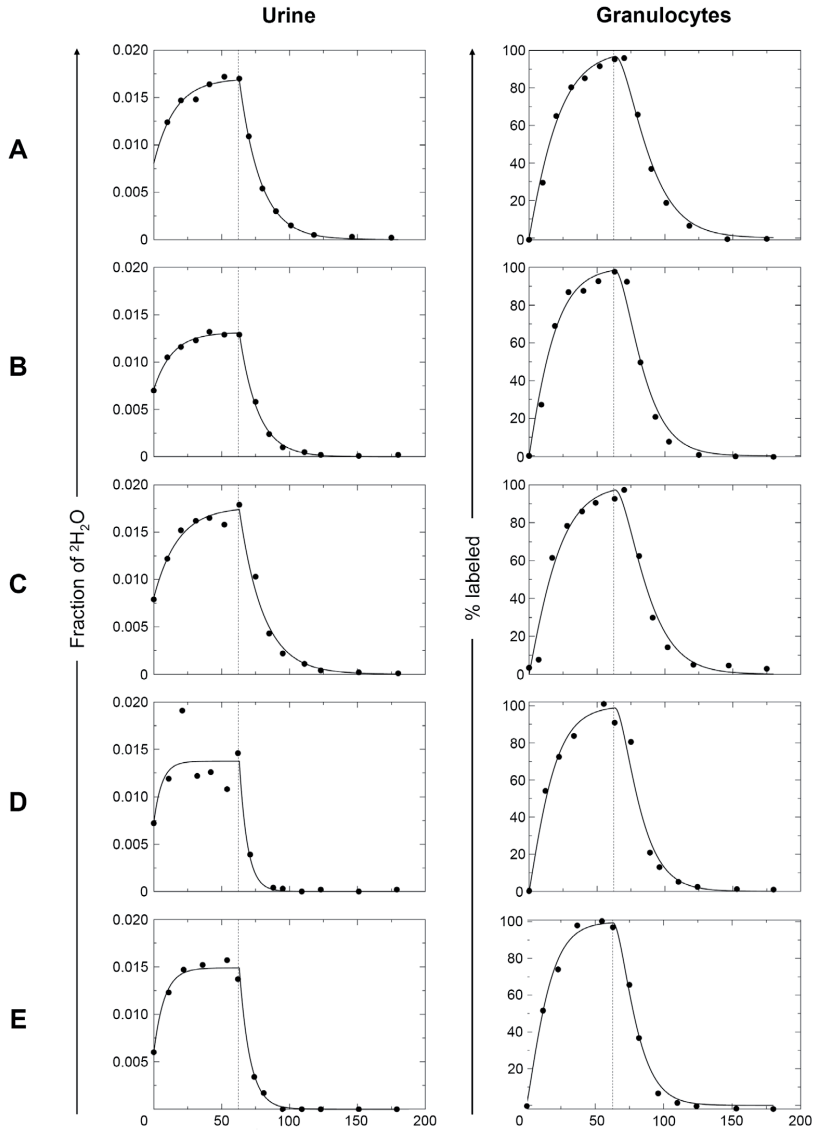
output. Since p determines the average T-cell turnover rate, pN (the average number of naive cells produced per day) provides an upperbound for the number of T cells exported from the thymus per day. To correct for the body water enrichment as measured by $U(t)$ and the amplification factor, c, of each individual person, all data were normalized by dividing by the maximum cf. Doing the same for the granulocyte data the maximum label enrichment in granulocytes was basically scaled to 100%.

Cells that divided during the first days of the labeling period will have incorporated less deuterium than those that divided later. If such cells were to die earlier than cells that divided later, the loss of label during the chase phase need not be exponential because poorly labeled cells would die earlier. However, since we are assuming an exponential distribution of expected life spans in these models, cells are assumed to have death rates that are independent of their age.

ACKNOWLEDGEMENTS

The authors greatly acknowledge Marc Hellerstein and Rich Neese for sharing the ^2H_2O labeling and GC/MS protocols and Loes Rijswijk for improving the thymectomy procedure in mice. This research has been funded by AIDS Fonds Netherlands (grants 4025, 4024, 7010), the Netherlands Organization for Scientific Research (NWO, grants 916.36.003 and 016.048.603), the HFSP (grant RGP0010/2004) and the Landsteiner Foundation for Blood Research (LSBR grant 0210).

SUPPLEMENTARY INFORMATION



Supplementary Figure S1 Best fits of the fraction of ^2H_2O in urine and the percentage of labeled deoxyribose residues of adenosine in granulocytes after scaling by the maximum enrich-ment level $p\pi/d\delta$ of the granulocyte population (see Methods). In the graph the end of the labeling period is marked by a vertical line.

Supplementary Table S1 Characteristics of healthy volunteers.

	A	B	C	D	E
Age at start of the protocol	24	22	25	20	22
$CD4^+$ count per µl blood	890 (810-1040)	690 (663-808)	830 (780-990)	1300 (1080-1730)	1320 (1130-1500)
$CD8^+$ count per µl blood	470 (420-550)	355 (320-413)	500 (460-590)	440 (410-530)	820 (660-910)
% naive CD4	60 (54-66)	68 (65-71)	37 (34-41)	69 (67-73)	68 (63-71)
% memory CD4	40 (34-45)	32 (29-34)	54 (48-56)	31 (27-33)	31 (28-36)
% naive CD8	54 (50-58)	68 (63-70)	37 (31-41)	65 (62-71)	59 (52-62)
% memory CD8	35 (29-40)	17 (14-18)	18 (15-21)	12 (11-14)	20 (19-21)
% $Ki67^+$ within CD4	2.75 (2.33-3.19)	1.86 (1.47-2.25)	2.13 (1.82-2.30)	1.36 (0.59-2.18)	1.61 (1.37-2.12)
% $Ki67^+$ within naïve CD4	0.76 (0.39-1.05)	0.91 (0.65-1.24)	0.77 (0.59-0.99)	0.29 (0.21-1.40)	0.43 (0.33-0.73)
% $Ki67^+$ within memory CD4	5.00 (4.29-5.02)	3.42 (2.93-4.45)	3.18 (2.87-3.52)	1.82 (1.55-2.50)	3.84 (3.05-4.24)
% $Ki67^+$ within CD8	1.65 (1.45-1.88)	1.46 (1.26-1.92)	2.29 (1.68-2.78)	1.26 (0.85-1.72)	1.34 (1.14-1.81)
% $Ki67^+$ within naïve CD8	0.94 (0.57-1.12)	0.73 (0.57-1.13)	0.97 (0.63-1.21)	0.47 (0.22-0.68)	0.50 (0.42-0.65)
% $Ki67^+$ within memory CD8	1.87 (1.61-2.05)	2.35 (1.98-2.97)	NA	1.51 (1.22-2.51)	3.54 (2.46-4.01)

Depicted are median values and interquartile ranges over follow-up.

Supplementary Table S2 Parameter estimates of the urine enrichment curves, where f represents the fraction of ^2H_2O in the drinking water, δ is the turnover rate of body water per day, and β represents the baseline urine enrichment attained after the boost of label by the end of day 0.

Individual	f	δ	β
A	0.0170	0.064	0.0080
B	0.0129	0.088	0.0070
C	0.0173	0.075	0.0082
D	0.0138	0.128	0.0074
E	0.0150	0.119	0.0059

Supplementary Table S3 Parameter estimates of the granulocyte enrichment curves (before scaling), where d represents the loss rate of labeled granulocytes and p combines granulocyte turnover and the maximum number of deuterium atoms that can be incorporated in the deoxyribose residue of adenosine.

Individual	p	d
A	0.384	0.086
B	0.419	0.085
C	0.402	0.078
D	0.299	0.079
E	0.415	0.103

REFERENCE LIST

1. Douek,D. 2004. Thymic output and HIV infection: on the right TREC. *Immunity.* 21:744-745.
2. Hazenberg,M.D., J.A.Borghans, R.J.de Boer, and F.Miedema. 2003. Thymic output: a bad TREC record. *Nat. Immunol.* 4:97-99.
3. Douek,D.C., M.R.Betts, B.J.Hill, S.J.Little, R.Lempicki, J.A.Metcalf, J.Casazza, C.Yoder, J.W.Adelsberger, R.A.Stevens, M.W.Baseler, P.Keiser, D.D.Richman, R.T.Davey, and R.A. Koup. 2001. Evidence for increased T cell turnover and decreased thymic output in HIV infection. *J. Immunol.* 167:6663-6668.
4. Dion,M.L., J.F.Poulin, R.Bordi, M.Sylvestre, R.Corsini, N.Kettaf, A.Dalloul, M.R.Boulassel, P.Debre, J.P.Routy, Z.Grossman, R.P.Sekaly, and R.Cheynier. 2004. HIV infection rapidly induces and maintains a substantial suppression of thymocyte proliferation. *Immunity.* 21:757-768.
5. Hazenberg,M.D., S.A.Otto, J.W.Cohen Stuart, M.C.Verschuren, J.C.Borleffs, C.A.Boucher, R.A.Coutinho, J.M.Lange, T.F.Rinke de Wit, A.Tsegaye, J.J.van Dongen, D.Hamann, R.J.de Boer, and F.Miedema. 2000. 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.* 6:1036-1042.
6. Arron,S.T., R.M.Ribeiro, A.Gettie, R.Bohm, J.Blanchard, J.Yu, A.S.Perelson, D.D.Ho, and L.Zhang. 2005. Impact of thymectomy on the peripheral T cell pool in rhesus macaques before and after infection with simian immunodeficiency virus. *Eur. J. Immunol.* 35:46-55.
7. Berzins,S.P., D.I.Godfrey, J.F.Miller, and R.L.Boyd. 1999. A central role for thymic emigrants in peripheral T cell homeostasis. *Proc. Natl. Acad. Sci. U. S. A* 96:9787-9791.
8. Clark,D.R., R.J.de Boer, K.C.Wolthers, and F.Miedema. 1999. T cell dynamics in HIV-1 infection. *Adv. Immunol.* 73:301-327.
9. Mackall,C.L., T.A.Fleisher, M.R.Brown, M.P.Andrich, C.C.Chen, I.M.Feuerstein, M.E. Horowitz, I.T.Magrath, A.T.Shad, S.M.Steinberg, and . 1995. Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. *N. Engl. J. Med.* 332:143-149.
10. Michie,C.A., A.McLean, C.Alcock, and P.C.Beverley. 1992. Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature* 360:264-265.
11. Tough,D.F. and J.Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med.* 179:1127-1135.
12. Berzins,S.P., R.L.Boyd, and J.F.Miller. 1998. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. *J. Exp. Med.* 187:1839-1848.
13. Douek,D.C., R.D.McFarland, P.H.Keiser, E.A.Gage, J.M.Massey, B.F.Haynes, M.A.Polis, A.T.Haase, M.B.Feinberg, J.L.Sullivan, B.D.Jamieson, J.A.Zack, L.J.Picker, and R.A.Koup. 1998. Changes in thymic function with age and during the treatment of HIV infection. *Nature* 396:690-695.
14. Kimmig,S., G.K.Przybylski, C.A.Schmidt, K.Laurisch, B.Mowes, A.Radbruch, and A.Thiel. 2002. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. *J. Exp. Med.* 195:789-794.
15. Kong,F.K., C.L.Chen, A.Six, R.D.Hockett, and M.D.Cooper. 1999. T cell receptor gene deletion circles identify recent thymic emigrants in the peripheral T cell pool. *Proc. Natl. Acad. Sci. U. S. A* 96:1536-1540.
16. Sempowski,G.D., M.E.Gooding, H.X.Liao, P.T.Le, and B.F.Haynes. 2002. T cell receptor excision circle assessment of thymopoiesis in aging mice. *Mol. Immunol.* 38:841-848.
17. Grossman,Z., M.Meier-Schellersheim, A.E.Sousa, R.M.Victorino, and W.E.Paul. 2002. CD4+ T-cell depletion in HIV infection: are we closer to understanding the cause? *Nat. Med.* 8:319-323.
18. Hellerstein,M.K., R.A.Hoh, M.B.Hanley, D.Cesar, D.Lee, R.A.Neese, and J.M.McCune. 2003. Subpopulations of long-lived and short-lived T cells in advanced HIV-1 infection. *J. Clin. Invest* 112:956-966.
19. Macallan,D.C., B.Asquith, A.J.Irvine, D.L.Wallace, A.Worth, H.Ghattas, Y.Zhang, G.E. Griffin, D.F.Tough, and P.C.Beverley. 2003. Measurement and modeling of human T cell kinetics. *Eur. J. Immunol.* 33:2316-2326.

20. Macallan,D.C., D.Wallace, Y.Zhang, C.De Lara, A.T.Worth, H.Ghattas, G.E.Griffin, P.C. Beverley, and D.F.Tough. 2004. Rapid turnover of effector-memory CD4(+) T cells in healthy humans. *J. Exp. Med.* 200:255-260.
21. Neese,R.A., L.M.Misell, S.Turner, A.Chu, J.Kim, D.Cesar, R.Hoh, F.Antelo, A.Strawford, J.M.McCune, M.Christiansen, and M.K.Hellerstein. 2002. 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* 99:15345-15350.
22. Wallace,D.L., Y.Zhang, H.Ghattas, A.Worth, A.Irvine, A.R.Bennett, G.E.Griffin, P.C. Beverley, D.F.Tough, and D.C.Macallan. 2004. Direct measurement of T cell subset kinetics in vivo in elderly men and women. *J. Immunol.* 173:1787-1794.
23. Hellerstein,M.K. and R.A.Neese. 1992. Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers. *Am. J. Physiol* 263:E988-1001.
24. Macallan,D.C., C.A.Fullerton, R.A.Neese, K.Haddock, S.S.Park, and M.K.Hellerstein. 1998. 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* 95:708-713.
25. Borghans,J.A. and R.J.de Boer. 2007. Quantification of T-cell dynamics: from telomeres to DNA labeling. *Immunol. Rev.* 216:35-47.
26. Scollay,R.G., E.C.Butcher, and I.L.Weissman. 1980. Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur. J. Immunol.* 10:210-218.
27. Weissman,I.L. 1967. Thymus cell migration. *J. Exp. Med.* 126:291-304.
28. Asquith,B., C.Debacq, D.C.Macallan, L.Willems, and C.R.Bangham. 2002. Lymphocyte kinetics: the interpretation of labelling data. *Trends Immunol.* 23:596-601.
29. Kovacs,J.A., R.A.Lempicki, I.A.Sidorov, J.W.Adelsberger, I.Sereti, W.Sachau, G.Kelly, J.A.Metcalf, R.T.Davey, Jr., J.Falloon, M.A.Polis, J.Tavel, R.Stevens, L.Lambert, D.A.Hosack, M.Bosche, H.J.Issaq, S.D.Fox, S.Leitman, M.W.Baseler, H.Masur, M.M.Di, D.S.Dimitrov, and H.C.Lane. 2005. Induction of prolonged survival of CD4+ T lymphocytes by intermittent IL-2 therapy in HIV-infected patients. *J. Clin. Invest* 115:2139-2148.
30. Grossman,Z., R.B.Herberman, S.Dimiter, I.M.Rouzine, M.John, A.S.Perelson, S.Bonhoeffer, H.Mohri, and D.David. 1999. T Cell Turnover in SIV Infection. *Science* 284:555a.
31. Comans-Bitter,W.M., G.R.de, B.R.van den, H.J.Neijens, W.C.Hop, K.Groeneveld, H.Hooijkaas, and J.J.van Dongen. 1997. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J. Pediatr.* 130:388-393.
32. Cohen,S.J., D.Hamann, J.Borleffs, M.Roos, F.Miedema, C.Boucher, and R.J.de Boer. 2002. Reconstitution of naive T cells during antiretroviral treatment of HIV-infected adults is dependent on age. *AIDS* 16:2263-2266.
33. Ribeiro,R.M., H.Mohri, D.D.Ho, and A.S.Perelson. 2002. In vivo dynamics of T cell activation, proliferation, and death in HIV-1 infection: why are CD4+ but not CD8+ T cells depleted? *Proc. Natl. Acad. Sci. U. S. A* 99:15572-15577.
34. Budd,R.C., J.C.Cerottini, C.Horvath, C.Bron, T.Pedrazzini, R.C.Howe, and H.R. MacDonald. 1987. Distinction of virgin and memory T lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. *J. Immunol.* 138:3120-3129.
35. Di Rosa,F., S.Ramaswamy, J.P.Ridge, and P.Matzinger. 1999. On the lifespan of virgin T lymphocytes. *J. Immunol.* 163:1253-1257.
36. Tanchot,C., C.A.Le, B.Martin, S.Leaument, N.Dautigny, and B.Lucas. 2002. Conversion of naive T cells to a memory-like phenotype in lymphopenic hosts is not related to a homeostatic mechanism that fills the peripheral naive T cell pool. *J. Immunol.* 168:5042-5046.
37. Ge,Q., H.Hu, H.N.Eisen, and J.Chen. 2002. Different contributions of thymopoiesis and homeostasis-driven proliferation to the reconstitution of naive and memory T cell compartments. *Proc. Natl. Acad. Sci. U. S. A* 99:2989-2994.
38. Berzins,S.P., A.P.Uldrich, J.S.Sutherland, J.Gill, J.F.Miller, D.I.Godfrey, and R.L.Boyd. 2002. Thymic regeneration: teaching an old immune system new tricks. *Trends Mol. Med.* 8:469-476.
39. Baars,P.A., M.M.Maurice, M.Rep, B.Hooibrink, and R.A.van Lier. 1995. Heterogeneity of the circulating human CD4+ T cell population. Further evidence that the CD4+CD45RA-. *J. Immunol.* 154:17-25.

40. Hamann,D., P.A.Baars, M.H.Rep, B.Hooibrink, S.R.Kerkhof-Garde, M.R.Klein, and R.A.van Lier. 1997. Phenotypic and functional separation of memory and effector human CD8+ T cells. *J. Exp. Med.* 186:1407-1418.
41. Hazenberg,M.D., J.W.Stuart, S.A.Otto, J.C.Borleffs, C.A.Boucher, R.J.de Boer, F.Miedema, and D.Hamann. 2000. 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* 95:249-255.
42. Previs,S.F., J.W.Hazey, F.Diraison, M.Beylot, F.David, and H.Brunengraber. 1996. Assay of the deuterium enrichment of water via acetylene. *J. Mass Spectrom.* 31:639-642.
43. Patterson,B.W., G.Zhao, and S.Klein. 1998. Improved accuracy and precision of gas chromatography/mass spectrometry measurements for metabolic tracers. *Metabolism* 47: 706-712.
44. Ackermans,M.T., A.M.Pereira Arias, P.H.Bisschop, E.Endert, H.P.Sauerwein, and J.A.Romijn. 2001. The quantification of gluconeogenesis in healthy men by (2)H2O and [2-(13)C]glycerol yields different results: rates of gluconeogenesis in healthy men measured with (2)H2O are higher than those measured with [2-(13)C]glycerol. *J. Clin. Endocrinol. Metab* 86:2220-2226.
45. Hellerstein,M.K. and R.A.Neese. 1999. Mass isotopomer distribution analysis at eight years: theoretical, analytic, and experimental considerations. *Am. J. Physiol* 276:E1146-E1170.
46. Henderson,A.R. 2005. The bootstrap: a technique for data-driven statistics. Using computer-intensive analyses to explore experimental data. *Clin. Chim. Acta* 359:1-26.
47. Westermann,J. and R.Pabst. 1990. Lymphocyte subsets in the blood: a diagnostic window on the lymphoid system? *Immunol. Today* 11:406-410.

Chapter 3

Granulocyte life span revisited

Janesh Pillay[1], Ineke den Braber[2], Nienke Vrisekoop[2], Lydia M. Kwast[2], Rob J. de Boer[3], José A.M. Borghans[2,3], Kiki Tesselaar[2] and Leo Koenderman[1]

[1] Department of Respiratory Medicine, University Medical Center Utrecht, Utrecht, The Netherlands; [2] Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands; [3] Department of Theoretical Biology, Utrecht University, Utrecht, The Netherlands

ABSTRACT

The emerging concept for a role of granulocytes in immune regulation is difficult to reconcile with the generally accepted life span of circulating granulocytes of 12-14 hours. Circumventing the artifacts associated with the conventional *ex vivo* labeling, we determined the average granulocyte life span using *in vivo* incorporation of deuterium from $^{2}H_{2}O$ into DNA of proliferating granulocyte precursors. Our study demonstrated an average circulating granulocyte life span of 6.3 days in humans and 21.6 hours in mice, and a transit time through the bone marrow consistent with previous reports. These data are consistent with a role for granulocytes in complex long-term processes such as immune regulation and recirculation.

Granulocytes are terminally differentiated cells of the innate immune system. They comprise up to 60% of circulating white blood cells in humans and are essential for first-line defense against invading micro-organisms. There is an emerging concept that, apart from their well established anti-microbial functions, granulocytes can influence adaptive immune responses through cytokine production, pathogen shuttling to draining lymph nodes, antigen presentation and modulation of Th1/Th2 responses[1-3]. These novel functions do not cohere with the generally accepted circulating life span of 12-14 hours[4], which was determined using transfer of *in vitro* labeled granulocytes. In these transfer experiments possible retention of leukocytes in lungs, liver and spleen, due to activation during *ex vivo* manipulation[5], may have led to underestimation of granulocyte life span. In addition, the relatively short duration (< 3 days) of the experiments precluded detection of granulocytes that recirculate to the blood[6].

To circumvent the complications introduced by *in vitro* labeling, we labeled the granulocyte pool *in vivo* in healthy mice and humans by administration of ^2H_2O. Using a combination of gas chromatography and mass-spectrometry (GC/MS) the fraction of ^2H-labeled adenosine in the DNA of the proliferating granulocyte pool was measured and the kinetics of the granulocyte pool was determined[7]. The disappearance rate of the granulocytes was estimated using a mathematical model that takes into account the availability of ^2H_2O as measured in urine or plasma, for men and mice respectively.

Since granulocytes are solely produced in the bone marrow (BM) in the mitotic phase, the rate at which granulocytes enter the circulation is characterized by i) the rate of division in the mitotic phase, ii) the delay of newly-formed granulocytes entering the post-mitotic pool (PMP), and iii) the delay in mobilization to the blood (Fig.1, D).

To be able to compare granulocyte dynamics in BM and blood we used mice as a model system. We administered ^2H_2O for 7 days and simultaneously measured the accrual and loss of label in plasma, BM-derived and blood-derived granulocytes. Isolation of the BM PMP revealed a transit time of 1.6 days in the PMP, in addition to 0.7 day residence time in the mitotic pool. The resulting combined delay of 2.3 days in the BM was confirmed by mathematical modeling of the blood data, which revealed a similar delay of 2.2 days. The average life span of circulating granulocytes in mice was estimated to be 21.6 hours.

In the human study blood samples were drawn during a 9-week labeling and a 16 week down-labeling period. When the mathematical model was applied to ^2H_2O

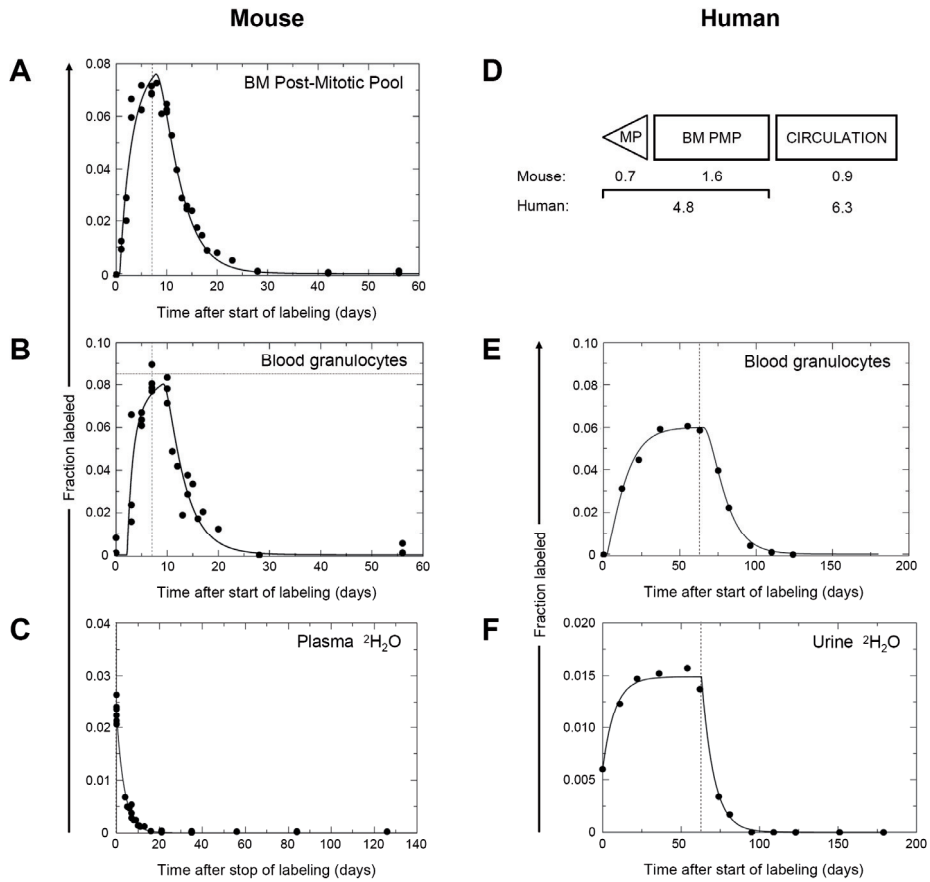


Figure 1 Average granulocyte life spans determined by *in vivo* ^2H_2O labeling. Cross-sectional up- and down-labeling of murine (A) BM PMP and (B) blood granulocytes. (E) Representative example of longitudinal up- and down-labeling of human blood granulocytes (for other examples see Reference 7). Dashed lines indicate the time of label cessation on day 7 (A-C) or day 63 (E, F). Maximum labeling from mice that had been exposed to ^2H_2O from the moment of their conception is indicated by a dotted line (B). Curves were fitted as described in methods and corrected for the enrichment curves in (C) plasma or (F) urine. (D) Model and estimates of average granulocyte life spans in days. MP and BM PMP represent the mitotic and post-mitotic pool in the bone marrow, respectively. Murine estimates in BM and blood could be directly measured, and human data were mathematically modeled from the data obtained from circulating human granulocytes by the model validated in the mouse (see methods).

labeling data from the five healthy individuals[7], we estimated an average life span of 6.3 ± 1.5 days (mean ± SD) for circulating granulocytes, and a delayed exit from the BM PMP of 4.8 ± 1.7 days (mean ± SD). This delay was similar to the transit time through the PMP that was previously reported based on *in vivo* ^{3}H-thymidine labeling of BM in humans[4].

Our BM transit times in mice and humans are in good agreement with previous reports[4;8]. The average granulocyte life span in mice of 21.6 hours is similar to that in earlier findings[8]. The average life span of 6.3 days in humans is 10 times higher than previous estimates, however[4]. This difference is likely due to differences in experimental approach, such as lack of activation induced by the *in vitro* labeling and the extended labeling period, preventing dilution of labeled blood granulocytes by recirculation of unlabeled cells from tissues.
Finally, an average human granulocyte life span of 6.3 days would support the emerging concept that neutrophils participate in complex long-term processes such as immune regulation and recirculation.

METHODS

Human volunteers
Characteristics and experimental procedures of the five male healthy volunteers were described previously[7].

Mice
C57Bl/6 mice were maintained by in-house breeding at the Central Animal Facility of Utrecht University under specific pathogen-free conditions in accordance with institutional and national guidelines. 12-week old mice obtained a boost injection (i.p.) of 16.5 ml/kg of 90% ^2H_2O in PBS (Cambridge Isotopes, Cambridge, MA), followed by subsequent feeding with 4% ^2H_2O in drinking water for one week. To determine the maximum possible amount of labeled DNA under these feeding conditions, mice were labeled by maternal intake of 4% ^2H_2O from preconception to the time of weaning and subsequent administration of 4% ^2H_2O in drinking water of the offspring. Maximally labeled granulocytes were isolated from 5-week old offspring.

Granulocyte isolation
Human granulocytes were isolated by means of a Ficoll-Paque gradient, followed by hypotonic lysis of erythrocytes as previously described[7]. This method yields a granulocyte purity of >95%. Isolation of the murine PMP-BM pool was performed by centrifugation over various Percoll-gradients[9]. Isolation of circulating murine granulocytes was performed with a BD FACS vantage based on their distinct forward and sideward scatter.

Measurement and mathematical modeling of ^2H_2O enrichment in serum and DNA

Deuterium enrichment in DNA, plasma and urine was determined as previously described[7]. Enrichment data were fitted using a previously developed mathematical model[7], which was extended by allowing for a delayed appearance of labeled DNA in the compartment under investigation.

REFERENCE LIST

1. Abadie,V., E.Badell, P.Douillard, D.Ensergueix, P.J.Leenen, M.Tanguy, L.Fiette, S.Saeland, B.Gicquel, and N.Winter. 2005. Neutrophils rapidly migrate via lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood* 106:1843-1850.
2. Maletto,B.A., A.S.Ropolo, D.O.Alignani, M.V.Liscovsky, R.P.Ranocchia, V.G.Moron, and M.C. Pistoresi-Palencia. 2006. Presence of neutrophil-bearing antigen in lymphoid organs of immune mice. *Blood* 108:3094-3102.
3. Tacchini-Cottier,F., C.Zweifel, Y.Belkaid, C.Mukankundiye, M.Vasei, P.Launois, G.Milon, and J.A.Louis. 2000. An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with Leishmania major. *J. Immunol.* 165: 2628-2636.
4. Dancey,J.T., K.A.Deubelbeiss, L.A.Harker, and C.A.Finch. 1976. Neutrophil kinetics in man. *J. Clin. Invest* 58:705-715.
5. Hogg,J.C. and C.M.Doerschuk. 1995. Leukocyte traffic in the lung. *Annu. Rev. Physiol* 57: 97-114.
6. Buckley,C.D., E.A.Ross, H.M.McGettrick, C.E.Osborne, O.Haworth, C.Schmutz, P.C. Stone, M.Salmon, N.M.Matharu, R.K.Vohra, G.B.Nash, and G.E.Rainger. 2006. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *J. Leukoc. Biol.* 79:303-311.
7. Vrisekoop,N., I.den Braber, A.B.de Boer, A.F.Ruiter, M.T.Ackermans, S.N.van der Crabben, E.H.Schrijver, G.Spierenburg, H.P.Sauerwein, M.D.Hazenberg, R.J.de Boer, F.Miedema, J.A.Borghans, and K.Tesselaar. 2008. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. *Proc. Natl. Acad. Sci. U. S. A* 105:6115-6120.
8. Basu,S., G.Hodgson, M.Katz, and A.R.Dunn. 2002. Evaluation of role of G-CSF in the production, survival, and release of neutrophils from bone marrow into circulation. *Blood* 100: 854-861.
9. Mocsai,A., C.L.Abram, Z.Jakus, Y.Hu, L.L.Lanier, and C.A.Lowell. 2006. Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat. Immunol.* 7:1326-1333.
10. We would like to express our gratitude to Dirk Roos for critically reviewing the manuscript.

Chapter 4

The effect of the length of the labeling period on the estimated average T-cell turnover rate

Tendai Mugwagwa[1,*], Ineke den Braber[2,*],Lydia Kwast[2], Elise H.R. Schrijver[2],Gerrit Spierenburg[2], Koos Gaiser[2], An F.C. Ruiter[3], Mariette T. Ackermans[3], Becca Asquith[4], Rob J. de Boer[1,#],Kiki Tesselaar[2,#] and José A.M. Borghans[1,2,#]

[1] Department of Theoretical Biology, Utrecht University, Utrecht, The Netherlands; [2] Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands; [3] Department of Clinical Chemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; [4] Department of Immunology, Wright-Fleming Institute, Imperial College London, London, UK

[, #] These authors contributed equally to this work*

ABSTRACT

Our limited knowledge of the expected life spans of naive and memory T cells during health and disease hampers our understanding of the functioning of the immune system. Life spans have been estimated in mice and men with various labeling approaches, with disturbingly different outcomes. Even results based on stable-isotope labeling, which provides the most reliable tool to measure lymphocyte life spans that is currently available, differ significantly between laboratories. Here we show that variation in the length of the labeling period may be responsible for these differences. When applied to kinetically heterogeneous cell populations, such as the effector/memory T-cell pool in mice, the average life span may be underestimated when labeling periods are long, because sub-populations with the highest rates of turnover may approach maximum label intake during the labeling period. Such problems do not arise in kinetically homogeneous cell populations, such as the naive T-cell pool in mice and men. We introduce a new modeling strategy that can be used to estimate labeling-time-independent average life spans of heterogeneous cell populations.

INTRODUCTION

Knowledge on the average life spans of different leukocyte populations has important consequences for our insights in diverse immunological processes. The long life span of naive T cells for example suggests that priming of a specific naive T cell is a rare event and reconstitution of these cells after depletion, such as in HIV infection, takes considerable time. Leukocyte life spans are generally estimated by *in vivo* labeling experiments of traceable components built into the DNA of proliferating cells. Recently, the non-radioactive stable isotopes $^2H_2$-glucose and ^2H_2O have been introduced for *in vivo* DNA labeling and quantification of leukocyte production and death in humans. The average life spans of naive, memory and total $CD4^+$ and $CD8^+$ T cells that have been estimated using these two compounds, however, differ considerably between different laboratories[1]. The origin of these differences has so far not been explained.

It has been shown before that, if cell populations are kinetically heterogeneous, the rate at which label is lost after label cessation tends to decrease when the length of the labeling period increases. As labeled cells are by definition cells that have recently divided, loss of label may be biased towards cells with relatively rapid turnover. For longer labeling periods, the rate at which label is lost more closely resembles the average turnover rate of the cell population[2]. Importantly, however, the average production rate of the cell population, which is typically estimated during label administration, should not be dependent on the length of the labeling period.

Although part of the discrepancies between laboratories might result from the use of different stable isotopes, we observed a consistent positive correlation between estimated average life spans and the duration of label administration, even when the same stable isotope was used. In line with this, studies based on deuterated glucose – which tends to be administered for shorter periods than deuterated water – consistently reported shorter average life spans than deuterated water studies[1]. We therefore investigated how the length of the labeling period could influence the estimated average life span. We tested the hypothesis that if sub-populations with high rates of turnover could approach maximal label incorporation during the labeling period, longer labeling may underestimate cell turnover, because the subsequent accrual of label is due to cells with relatively slow rates of turnover.

We performed ^2H_2O experiments in mice which only differed in the length of the labeling period, and analyzed the kinetics of naive and effector/memory T cells. We found that effector/memory T cells in mice form a kinetically heterogeneous population, and that using the generally accepted model[2] longer labeling periods resulted in longer estimated average life spans. In contrast, naive T cells in mice were found to be kinetically homogeneous, and their estimated average turnover rate was not

influenced by the length of the labeling period. We propose an alternative modeling strategy, based on a multi-compartment model, which can be used to fit average life spans that are independent of the length of the labeling period even for populations that are kinetically heterogeneous.

RESULTS

Effect of the length of the labeling period on estimated production and disappearance rates

To investigate whether the length of label administration may influence the estimated average life span of T cells, we gave 12-week old C57Bl/6 mice a bolus of ^2H_2O and subsequently 4% ^2H_2O in drinking water for one, four, or eight weeks. Total thymocytes and naive and effector/memory $CD4^+$ and $CD8^+$ T cells were isolated from the spleen during the labeling and down-labeling phases and deuterium enrichment in the DNA was measured. The enrichment curves of naive and effector/memory T cells for the three different labeling periods (Fig.1), were fitted with the mathematical model proposed by Asquith et al[2]. In this model p represents the average turnover (or production) rate and d^* represents the disappearance rate of labeled cells. Production of labeled naive T cells is a combination of thymic output and proliferation, whereas labeled effector/memory T cells emanate from renewal and differentiating naive T cells. Labeled naive and effector/memory T cells disappear by differentiation, migration and/or cell death[3]. The model was extended to correct for the ^2H_2O concentration in the plasma (see Methods). After withdrawal of deuterium from the drinking water the concentration of the label in the plasma dropped with a half-life of several days. We refer to this mathematical model as the extended single-compartment model.

Each of the data sets was fitted separately with the extended single-compartment model (Fig.1). For the effector/memory T cells, both the disappearance rate of labeled cells, d^*, and the average turnover rate, p, decreased significantly when the length of the labeling period increased (Fig.2). The fact that the disappearance rate of labeled effector/memory T cells depended on the length of the labeling period suggest that effector/memory T cells in mice form a kinetically heterogeneous population. The average life span estimated from the one-week labeling experiment was $1/p$ = 15 days for $CD4^+$ and 28 days for $CD8^+$ effector/memory T cells, whereas those estimated from the four and eight-week labeling experiments were $1/p$ = 25 and 45 days, respectively. This difference in average life spans of effector/memory T cells that we estimated by this model has to be an artifact. In contrast, we found no evidence for different average turnover rates, p, and disappearance rates of labeled

cells, d^*, for naive T cells, between the different labeling periods (Fig.2), suggesting that naive T cells in mice form a kinetically homogeneous population. For naive $CD4^+$ and $CD8^+$ T cells, we found expected life spans of $1/p$ = 48 and 83 days cells, respectively.

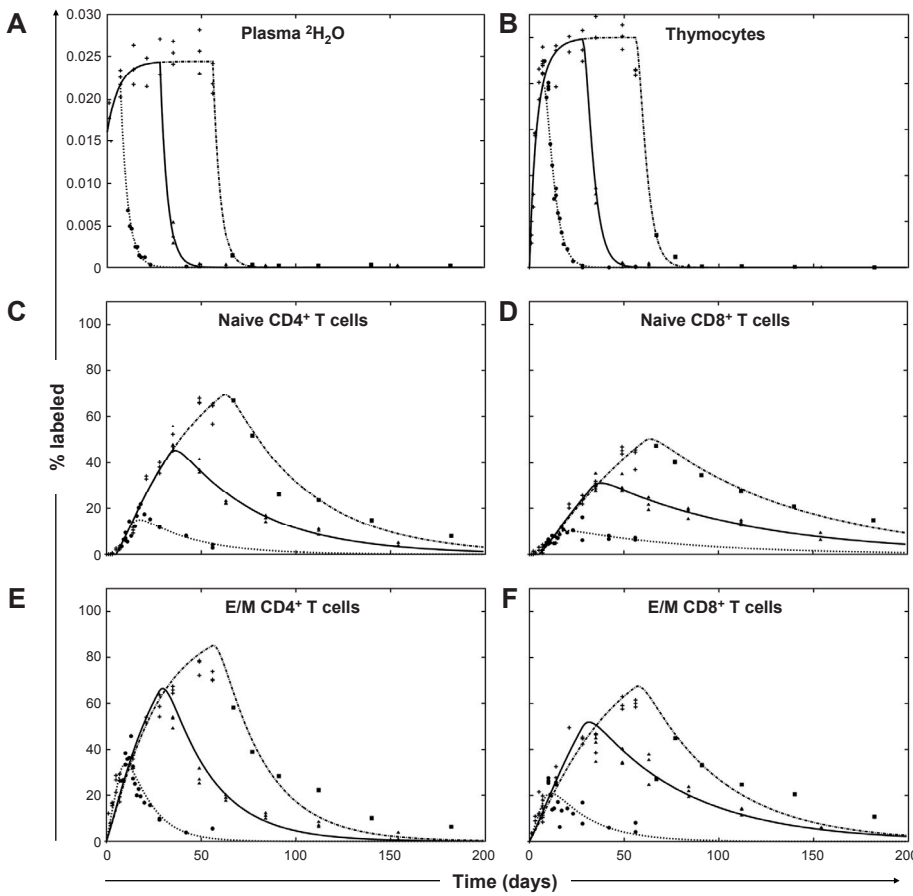


Figure 1 Best fits of the naive and effector/memory T-cell enrichment curves. Label enrichment in T cells was corrected for ^2H_2O enrichment in plasma (A) and scaled between 0 and 100% by normalizing for the percentage label obtained in thymocytes (B). At different time points before, during and after labeling, the percentages of labeled naive $CD4^+$ (C), naive $CD8^+$ (D), effector/memory $CD4^+$ (E) and effector/memory $CD8^+$ T cells (F) in the spleen were determined. Each symbol represents the enrichment in T-cell subsets from one C57Bl/6 mouse during up-labeling (+) and 1- (●), 4- (▲) and 8-week down-labeling (■). Data was fitted with the extended single-compartment model to estimate the average turnover rate of the total population and the disappearance rate of the labeled cell population.

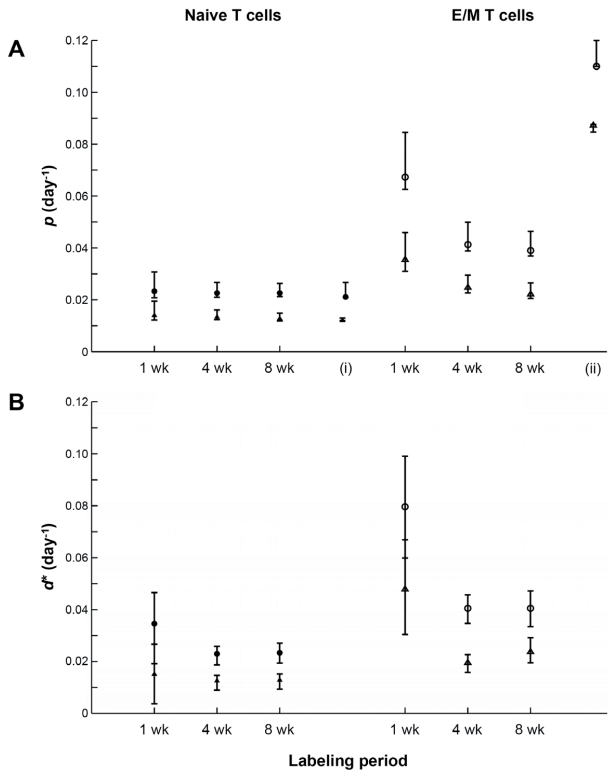


Figure 2 Length of the labeling period does interfere with the turnover and disappearance rates of effector/memory, but not naive, T cells. The estimated average turnover rates, p, of the total populations (A) and the disappearance rates, d*, of the labeled cell populations (B) from the extended single-compartment model. The estimates were based on the best fits through the 1-, 4- and 8-week labeling data of naive $CD4^+$ (●), naive $CD8^+$ (▲), effector/memory $CD4^+$ (○) and effector/memory $CD8^+$ (∆) T cells (Fig.1). The points indicated by (i) and (ii) are the average turnover rates from fitting the data to the two-compartment model (Fig.3).

Introduction of a multi-compartment model

Although the model introduced by Asquith et al.[2] captures kinetic heterogeneity in the sense that the rate at which label accumulates during label administration may differ from the rate at which it is lost after label cessation, both the accrual and loss of label are described by the same single exponential. Such a model may not correctly describe a cell population that consists of many sub-populations with different kinetics. Instead it will seek a compromise between the initial rapid label intake by the most rapidly turning over sub-population, and the subsequent slower label intake by the sub-populations with slower turnover. Such compromises may cause the estimated average turnover rates to become dependent on the length of the labeling period.

Our own experiments described above are illustrative of this problem. Theoretically, the upslopes of the three label experiments should follow exactly the same curve and approach the same asymptote. According to the model by Asquith et al.[2], this asymptote is reached when a fraction p/d^* is labeled. Because short labeling periods lead to high values of the disappearance rate of labeled cells, d^*, the uplabeling curves can only be the same if the estimated average turnover rate, p, is higher for short labeling periods. Hence, the differences in average turnover rates estimated during label intake may actually be imposed by the different downslopes.

To overcome these problems we propose a new modeling approach, which explicitly takes into account kinetic heterogeneity between sub-populations, even during the labeling period, and which yields average turnover rates that are independent of the length of the labeling period. The new model splits the cell population of interest into an arbitrary number of compartments, each with a different turnover rate. Within each compartment there is no kinetic heterogeneity, i.e. all cells are produced and lost at equal rates. Kinetic heterogeneity between sub-populations has indeed been reported. For instance, memory T cells in both mice and men can be phenotypically separated into effector-memory (T_{EM}) and central-memory (T_{CM}) cells[4], with kinetic differences, e.g., the estimated turnover rate of human $CD4^+$ T_{EM} cells is three times faster than that of $CD4^+$ T_{CM} cells[5]. The generalized mathematical model considers n different sub-populations, forming a fraction α_i of the total population, with average turnover rate p_i (where i = 1,2,..n). By systematically fitting the general model to the data and each time increasing the number of sub-populations, we found that the data was best described with maximally two *sub-populations*, with turnover rates p_1 and p_2, and relative sizes of α and (1-α), respectively. We fitted the data of the three labeling experiments, using this new modeling approach, allowing for one single upslope and three different downslopes.

Kinetic heterogeneity in the effector/memory T-cell pool

The effector/memory T-cell data were significantly better described by a two-compartment model compared to a single-compartment model (F-test: $p \approx 0$), reconfirming that both $CD4^+$ and $CD8^+$ effector/memory T-cell pools are kinetically heterogeneous (Fig.3). The description of the three labeling experiments was much better with the new model, because the up-labeling curves were now (forced to be) the same for the different labeling periods, and the down-labeling curves were allowed to be bi-phasic. The description of the naive T-cell data (Fig.3) did not significantly improve by adding a second compartment to the one-compartment model (F-test: p = 0.075 for naive $CD4^+$ T cells and p = 1 for naive $CD8^+$ T cells), further supporting the notion that the naive T-cell pool in mice is kinetically homogeneous.

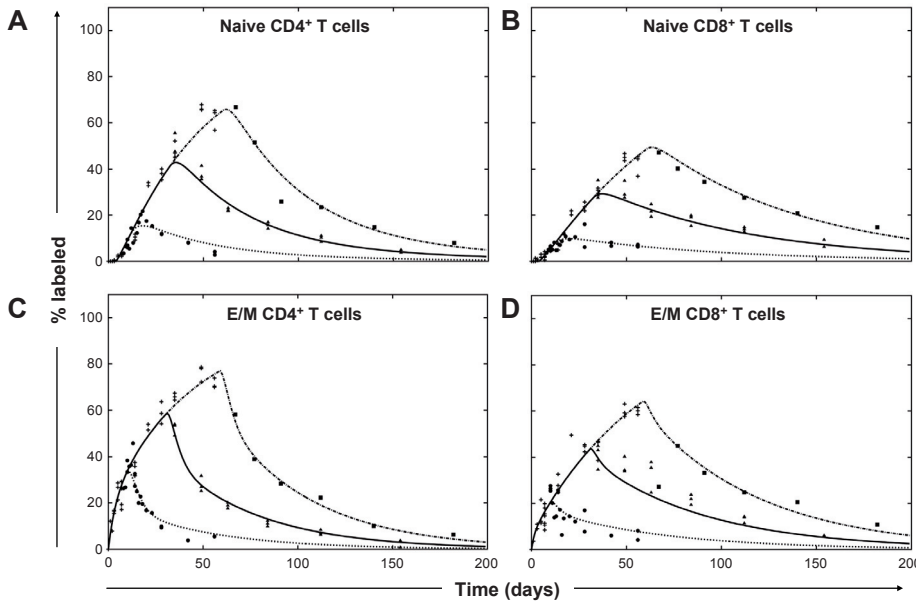


Figure 3 A two-compartment model better describes effector/memory T-cell enrichment data. At different time points before, during and after labeling, the fraction of labeled naive $CD4^+$ (A), naive $CD8^+$ (B), effector/memory $CD4^+$ (C) and effector/memory $CD8^+$ T cells (D) in the spleen were determined. Each symbol represents the enrichment in T-cell subsets from one C57Bl/6 mouse during up-labeling (+) and 1- (●), 4- (▲) and 8-week down-labeling (■). Data was fitted with a two-compartment model to estimate the average turnover rate of the total population and the disappearance rate of the labeled cell population.

According to the best fits using the two-compartment model, the effector/memory $CD4^+$ and $CD8^+$ T-cell pools in mice consist of a small sub-population with rapid turnover, and a larger sub-population with slow turnover. Within the effector/memory $CD4^+$ T-cell population, 25.4 % of the cells had a fast turnover rate of p = 0.368 per day, while the other 74.6% of the cells turned over more slowly at a rate p = 0.021 per day (Table I). The resulting average turnover rate of effector/memory $CD4^+$ T cells was $p = \alpha * p_1 + (1 - \alpha) * p_2$ = 0.109 per day, defining an average life span of $1/p$ = 9 days. Within the effector/memory $CD8^+$ T-cell population, 10.1% of the cells had a fast turnover rate of p = 0.721 per day, while the other 89.9% of the cells turned over at rate p = 0.016 per day (Table I). The average turnover rate of effector/memory $CD8^+$ T cells was thus 0.087 per day, giving an average life span of $1/p$ = 11 days.
By explicitly modeling kinetic heterogeneity, we explained the labeling data independent of the length of the labeling period.

Table I Estimated turnover rates of naive and effector/memory T cells based on one, four or eight weeks labeling using the two-compartment model. Upper and lower confidence intervals are given in parentheses.

		Naive	Effector/memory
CD4	p_1 (day^{-1})	0.025 (0.022 – 0.029)	0.368 (0.272 – 0.558)
	p_2 (day^{-1})	0.009 (0.003 – 0.015)	0.021 (0.011 – 0.041)
	α	0.755 (0.527 – 0.856)	0.254 (0.209 – 0.304)
	average p (day^{-1})	0.021 (0.013 – 0.027)	0.109 (0.066 – 0.198)
CD8	p_1 (day^{-1})	0.012 (0.012 – 0.013)	0.721 (0.337 – 1.000)
	p_2 (day^{-1})	0.012 (0.012 – 0.013)	0.016 (0.005 – 0.047)
	α	1.000 (1.000 – 1.000)	0.101 (0.083 – 0.130)
	average p (day^{-1})	0.012 (0.012 – 0.013)	0.087 (0.033 – 0.171)
Shared	Δ_1 (days)	4.117 (3.774 – 4.243)	0.000 (0.000 – 0.000)
	Δ_2 (days)	0.425 (0.143 – 1.459)	2.707 (1.989 – 3.423)

DISCUSSION

Our study shows that the previously observed differences in estimated average life spans of T cells based on stable-isotope labeling studies[1] may in part be caused by differences in the labeling periods, with longer labeling periods giving rise to longer estimated average life spans. By varying the labeling period from one to eight weeks in one experimental setting, and by fitting effector/memory T-cell labeling data with the widely used model, proposed by Asquith et al.[2], we found that the estimated average turnover rate decreased as the labeling period became longer (Fig.2). This dependence of the estimated average turnover rate on the labeling period was probably caused by kinetic heterogeneity in the cell population under investigation. In the longer labeling experiments the sub-population with the highest rate of turnover could approach saturation in labeling, which led to underestimation of the average turnover rate when fitting the data with a single exponential.

Although at first sight these results seem to suggest that shorter labeling experiments provide more reliable estimates for the average turnover rate, short labeling experiments also have their drawbacks. The average turnover rate of a cell population can only be estimated during the labeling phase; having too few data points during this phase due to the short labeling period thus also limits the reliability of the estimated turnover rates. We therefore suggest to perform long-term labeling experiments with densely sampling during the up-labeling and propose a new modeling strategy which helps the interpretation of such experiments. Our new modeling approach allows for explicit kinetic heterogeneity, is not restricted to single

exponentials describing the up- and downslopes, and yields average life spans independent of the length of the labeling period.

The average life spans that we estimated using our multi-compartment model were even shorter than the average life span estimated in the shortest labeling experiment. A possible explanation is that even during the 1 week labeling experiment, the fastest sub-population approached maximal label intake. Alternatively, our new model may have overestimated the average turnover rate, because it did not account for the possibility that cells that have recently divided may disappear more quickly than cells that have not (Macallan et al, manuscript in preparation). If part of the rapid loss of labeled cells was due to this so called temporal heterogeneity and not to kinetic heterogeneity of the cell population, our model fit could only compensate for this by introducing an extra population with rapid turnover, thereby overestimating the average turnover rate.

It is widely believed that, although phenotypically identical, naive T cells in mice exist in two separate populations with different kinetics: recent thymic emigrants having a turnover of approximately three weeks[6;7] and truly naive T cells, with an estimated life span of several months[8-10]. However, we found no evidence whatsoever for such kinetic heterogeneity in the naive T-cell population: 1) the introduction of a two-compartment model did not improve the quality of the fits of the naive $CD4^+$ and $CD8^+$ T-cell labeling data, and 2) using the original extended single-compartment model revealed very similar disappearance rates of labeled naive T cells after labeling for one, four or eight weeks (even though the naive T-cell population had not fully been labeled after 1 week of label administration). Both observations suggest that the naive T-cell population in mice is kinetically homogeneous.

In summary our analyses show that when fitting labeling data from kinetically heterogeneous cell populations, such as the effector/memory T-cell population in mice, long labeling periods may underestimate the average turnover rate because subpopulations may approach maximal label intake. The best solution to avoid such underestimations is not to label for shorter periods, because that would limit the number of data points, but to sample densely during the labeling phase and use a model that explicitly describes multiple compartments with different kinetics. The fact that the estimated life span of naive T cells in mice was independent of the labeling period suggests that the naive T-cell pool is kinetically homogeneous, which is incompatible with the co-existence of short-lived recent thymic emigrants and long-lived truly naive T cells.

MATERIALS AND METHODS

Mice

C57Bl/6 mice were maintained by in-house breeding at the Central Animal Facility at Utrecht University under specific pathogen-free conditions in accordance with institutional and national guidelines. ^2H_2O labeling was achieved by giving 12-week old mice one boost injection (i.p.) of 16.5 ml/kg of 90% ^2H_2O in PBS (Cambridge Isotopes, Cambridge, MA), followed by subsequent feeding with 4% ^2H_2O in drinking water for 1, 4 or 8 weeks.

Antibodies

FITC-labeled CD8 (clone 53-6.7), PE-conjugated CD62L (clone MEL-14), PerCP-labeled CD4 (clone RM4-5) and APC-labeled CD44 (clone IM7) were purchased from BD Biosciences PharMingen (San Diego, CA).

Cell preparation and flow cytometry

Spleen and thymus were isolated from C57Bl/6 mice at different time points before, during and after ^2H_2O labeling. After mechanical disruption, the cells were prepared and stained as previously described[11]. Cells were analyzed on a LSR II flow cytometer and BD FACSDiva software. Naive ($CD62L^+$, $CD44^-$) and effector/memory ($CD44^+$) T cells were sorted using a FACSAria cell sorter and FACSDiva software (BD). Purity of the sorted cells was comparable between the different labeling experiments. The average purity was: 98.3 ± 1.6 % (naive $CD4^+$, 91.1 – 99.9 %), 97.9 ± 1.4 % (effector/memory $CD4^+$, 92.8 – 99.7 %), 98.4 ± 1.2 (naive $CD8^+$, 92.9 – 100 %) and 96.7 ± 1.7 % (effector/memory $CD8^+$, 89.6 – 99.6 %). Thymocytes and sorted T cells were frozen until further processed.

Measurement of ^2H_2O enrichment in plasma and DNA

Deuterium enrichment in serum was measured as reported by Previs et al[12]. The isotopic enrichment of DNA was determined as previously described[11].

Mathematical modeling *of ^2H_2O data*

We assume that the fraction of heavy water in body water is equal to that in plasma[13], and model the measured up- and down-labeling of the plasma enrichment by the following differential equations

$\frac{dS}{dt} = \pi - \delta S$ during label intake ($t \leq \tau$), and

$\frac{dS}{dt} = -\delta S$ after label intake ($t > \tau$),

where S represents the fraction of ^2H_2O in plasma, t represents time in days and labeling was stopped at $t = \tau$ days, δ represents the turnover rate of body water per day during up-labeling and π is the source of ^2H_2O into body water per day. The plasma enrichment data were fitted to the analytical solutions $S(t)$ of these differential equations with $S(0) = S_0$, representing the baseline plasma enrichment attained after the boost of label by the end of day 0, such that:

$$S(t) = \frac{\pi}{\delta}(1-e^{-\delta t}) + S_0 e^{-\delta t} \text{ during label intake } (t \le \tau), \text{ and} \quad \text{(Equation 1a)}$$

$$S(t) = \left[\frac{\pi}{\delta}(1-e^{-\delta \tau}) + S_0 e^{-\delta \tau}\right] e^{-\delta(t-\tau)} \text{ after label intake } (t > \tau). \quad \text{(Equation 1b)}$$

The best fit for the plasma data is shown in Fig.1A and the parameter estimates are given in Supplementary Table S1.

To model the label enrichment of adenosine in the DNA of cells we assume identical reaction kinetics of hydrogen and deuterium and of labeled and unlabeled adenosines. Further, we extended the model of Asquith *et al*.[2] to include the dependence on the actual enrichment of the body water (as estimated by $S(t)$). Since the adenosine deoxyribose (dR) moiety contains seven hydrogen atoms that can be replaced by deuterium, one expects an amplification factor, c > 1, in the enrichment of dR in DNA relative to the body ^2H_2O enrichment. At the low levels of body ^2H_2O enrichment that one typically achieves ($S(t)$ < 4%), the likelihood of double labeling is very low[2;14]. One typically measures the amplification factor c from the enrichment in dR in the DNA of cells with a rapid turnover, like granulocytes or thymocytes[14;15]. Consistent amplification factors of c = 3.5 to c = 4 for body water enrichment levels of 2-3% have thus been reported[15].

Following Asquith *et al*.[2] the label enrichment of adenosine in the DNA of a population of cells was modeled during up-labeling and down-labeling by the differential equation

$$\frac{dl}{dt} = pcS(t))A - d^* l$$

where l is the total amount of labeled adenosine in the DNA, p is the average production rate of that population, and d* is the disappearance rate of cells carrying labeled adenosine. Basically one writes that each adenosine residue replicates at rate p and will incorporate a deuterium atom with probability $cS(t)$. For naive T cells this replication may occur both in the periphery and the thymus. Scaling this equation by the total amount of adenosine in the DNA, i.e., defining $L = l/A$, yields

$$\frac{dL}{dt} = pcS(t) - d^* L \quad \text{(Equation 2)}$$

throughout the labeling and de-labeling period, L represents the fraction of unlabeled and labeled deoxyribose residues of adenosine in DNA respectively. The corresponding analytical solutions for the enrichment of adenosine in DNA are

$$L(t) = \frac{c}{\delta - d^*}\left[p(S_0 e^{-pt} - S(t)) + \pi(1 - \mathrm{e}^{-pt})\right] \quad \text{(Equation 3a)}$$

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

$$L(t) = \frac{pc}{\delta - d^*}\left[S(\tau)\mathrm{e}^{-d^*(t-\tau)} - S(t)\right] + L(\tau)e^{-d^*(t-\tau)} \quad \text{(Equation 3b)}$$

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

Equations 3a and 3b were fitted to thymocyte enrichment data, yielding pc = 1.288 (1.121 – 1.481) per day, and a disappearance rate d^* = 0.4164 (0.3769 – 0.4684) per day (see Supplementary Table S1). Assuming that the thymocytes are fully labeled, i.e., assuming that $p = d^*$ for the thymocytes, we estimate amplification factors of c = 3.093 (2.974 – 3.161) (which is indeed lower than the theoretical maximum of 7).

Next, Equations 3a and 3b were applied to calculate the average turnover rate p and the loss rate of labeled cells d^* in each T-cell population. The parameter p in Equation (2) represents T-cell production resulting from both T-cell proliferation and thymic output. Since p determines the average T-cell turnover rate, pN (the average number of naive cells produced per day) provides an upper bound for the number of T cells exported from the thymus per day. To correct for the body water enrichment as measured by $S(t)$ and the amplification factor, c, all data were normalized by dividing by $c\pi/\delta$. Doing the same for the thymocyte data the maximum label enrichment in thymocytes was basically scaled to 100%. We observe a lag in appearance of labeled naive T cells in the spleen after start of labeling and a lag in the labeling peak after stop of labeling, suggesting that cells divide in a different compartment such as the thymus and then migrate to the spleen where we measure them. We modified Equations 3a and 3b by introducing 2 time delays, Δ_1 to account for the lag during uplabeling and Δ_2 to allow for a possible additional lag period after stop of label.

$$L(t) = \frac{c}{\delta - p}\left[p(S_0 e^{-p(t-\Delta_1)} - S(t)) + \pi(1 - \mathrm{e}^{-p(t-\Delta_1)})\right] \quad \text{(Equation 4a)}$$

during label intake ($t \le (\tau + \Delta_2)$), and

$$L(t) = \frac{pc}{\delta - d^*}\left[S(\tau)\mathrm{e}^{-d^*(t-\Delta_1-\tau)} - S(t)\right] + L(\tau)e^{-d^*(t-\Delta_1-\tau)} \quad \text{(Equation 4b)}$$

after label intake ($t > (\tau + \Delta_2)$).

Note that if $t \le 0$, $L(t) = 0$. Cells that divided during the first days of the labeling period will have incorporated less deuterium than those that divided later. If such

cells were to die earlier than cells that divided later, the loss of label during the chase phase need not be exponential because poorly labeled cells would die earlier. However, since we are assuming an exponential distribution of expected life spans in these models, cells are assumed to have disappearance rates that are independent of their age.

Statistical analysis. All statistical analyses were performed using the software program SPSS 15.0 (SPSS Inc, Chicago, Illinois). Differences with $p \leq 0.05$ were considered significant.

ACKNOWLEDGEMENTS

The authors acknowledge Joyce Visser and Sabine Versteeg for technical assistance and excellent animal care. This research has been funded by the Landsteiner Foundation for Blood Transfusion Research (LSBR grant 0210) and the Netherlands Organization for Scientific Research (NWO, VICI grant 92750029).

SUPPLEMENTARY INFORMATION

Supplementary Table S1. Parameters values and 95% confidence intervals for the best fit of ^2H_2O enrichment in serum and thymocytes (see Fig.1).

Parameter		Value (confidence intervals)
Serum	S_0	0.015 (0.012 – 0.018)
	δ (day^{-1})	0.261 (0.230 – 0.272)
	π (day^{-1})	0.006 (0.006 – 0.007)
Thymocytes	d^* (day^{-1})	0.416 (0.377 – 0.468)

REFERENCE LIST

1. Borghans,J.A. and R.J.de Boer. 2007. Quantification of T-cell dynamics: from telomeres to DNA labeling. *Immunol. Rev.* 216:35-47.
2. Asquith,B., C.Debacq, D.C.Macallan, L.Willems, and C.R.Bangham. 2002. Lymphocyte kinetics: the interpretation of labelling data. *Trends Immunol.* 23:596-601.
3. Hellerstein,M.K. 1999. Measurement of T-cell kinetics: recent methodologic advances. *Immunol. Today* 20:438-441.
4. Sallusto,F., J.Geginat, and A.Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* 22:745-763.
5. Macallan,D.C., D.Wallace, Y.Zhang, C.De Lara, A.T.Worth, H.Ghattas, G.E.Griffin, P.C. Beverley, and D.F.Tough. 2004. Rapid turnover of effector-memory CD4(+) T cells in healthy humans. *J. Exp. Med.* 200:255-260.
6. Berzins,S.P., R.L.Boyd, and J.F.Miller. 1998. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. *J. Exp. Med.* 187:1839-1848.
7. Berzins,S.P., D.I.Godfrey, J.F.Miller, and R.L.Boyd. 1999. A central role for thymic emigrants in peripheral T cell homeostasis. *Proc. Natl. Acad. Sci. U. S. A* 96:9787-9791.
8. Parretta,E., G.Cassese, A.Santoni, J.Guardiola, A.Vecchio, and F.Di Rosa. 2008. Kinetics of *in vivo* proliferation and death of memory and naive CD8 T cells: parameter estimation based on 5-bromo-2'-deoxyuridine incorporation in spleen, lymph nodes, and bone marrow. *J. Immunol.* 180:7230-7239.
9. Di Rosa,F., S.Ramaswamy, J.P.Ridge, and P.Matzinger. 1999. On the lifespan of virgin T lymphocytes. *J. Immunol.* 163:1253-1257.
10. Tough,D.F. and J.Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med.* 179:1127-1135.
11. Vrisekoop,N., I.den Braber, A.B.de Boer, A.F.Ruiter, M.T.Ackermans, S.N.van der Crabben, E.H.Schrijver, G.Spierenburg, H.P.Sauerwein, M.D.Hazenberg, R.J.de Boer, F.Miedema, J.A.Borghans, and K.Tesselaar. 2008. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. *Proc. Natl. Acad. Sci. U. S. A* 105:6115-6120.
12. Previs,S.F., J.W.Hazey, F.Diraison, M.Beylot, F.David, and H.Brunengraber. 1996. Assay of the deuterium enrichment of water via acetylene. *J. Mass Spectrom.* 31:639-642.
13. Ackermans,M.T., A.M.Pereira Arias, P.H.Bisschop, E.Endert, H.P.Sauerwein, and J.A. Romijn. 2001. The quantification of gluconeogenesis in healthy men by (2)H2O and [2-(13)C]glycerol yields different results: rates of gluconeogenesis in healthy men measured with (2)H2O are higher than those measured with [2-(13)C]glycerol. *J. Clin. Endocrinol. Metab* 86:2220-2226.
14. Hellerstein,M.K. and R.A.Neese. 1999. Mass isotopomer distribution analysis at eight years: theoretical, analytic, and experimental considerations. *Am. J. Physiol* 276:E1146-E1170.
15. Neese,R.A., L.M.Misell, S.Turner, A.Chu, J.Kim, D.Cesar, R.Hoh, F.Antelo, A.Strawford, J.M.McCune, M.Christiansen, and M.K.Hellerstein. 2002. 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* 99:15345-15350.

Chapter 5

Naive T-cell maintenance in mice differs essentially from that in humans

Ineke den Braber[1,*], Tendai Mugwagwa[2,*], Elise H.R. Schrijver[1], Liset Westera[1], Gerrit Spierenburg[1], Koos Gaiser[1], An F.C. Ruiter[3], Mariette T. Ackermans[3], José A.M Borghans[1,2,#], Rob J. de Boer[2,#] and Kiki Tesselaar[1,#]

[1] Department of Immunology, University Medical Center, Utrecht, The Netherlands; [2] Department of Theoretical Biology, Utrecht University, Utrecht, The Netherlands; [3] Department of Clinical Chemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

[, #] These authors contributed equally to this work.*

ABSTRACT

Naive T cells need to be sustained to ensure protection against novel pathogens, even though thymus output decreases dramatically with age. To better understand how naive T-cell numbers are maintained, we here quantified thymus output, peripheral T-cell renewal and cellular longevity of naive $CD4^+$ and $CD8^+$ T cells in C57Bl/6 mice of different ages. T-cell turnover rates and the contribution of the thymus were determined by a combination of long-term deuterium labeling and thymectomy experiments. We found that, irrespective of the age of the mice, production of naive T cells took place almost exclusively in the thymus, while the contribution of peripheral naive T-cell proliferation was negligible. Similar frequencies of T-cell receptor excision circle (TREC)-positive naive T cells in young and old mice confirmed the insignificance of peripheral naive T-cell renewal in mice. These findings contrast earlier findings in humans, demonstrating that peripheral renewal plays a major role in the maintenance of the naive T-cell pool. The principal mechanism of naive T-cell maintenance in mice thus differs fundamentally from those in humans.

INTRODUCTION

A broad T-cell repertoire is essential to mount immune responses to novel pathogens. Naive T cells are generated *via* two different pathways: *de novo* production in the thymus, giving rise to naive T cells with new specificities[1;2], and peripheral cell division, leading to duplication of particular T-cell specificities. Both in mice and men, the daily output of T cells from the thymus declines with age[3;4]. Thymus involution is probably responsible for the gradual decline in naive $CD4^+$ and $CD8^+$ T-cell numbers with age, although naive T-cell numbers in both mice and men decline less dramatically than thymocyte numbers[5;6]. In line with this finding, during aging the peripheral T-cell pool becomes dominated by memory T cells[6;7], coinciding with severe perturbations of the naive T-cell repertoire and impaired immunity at very old age [8-10]. Because of these parallels between T-cell kinetics in mice and men, insights into the role of thymus output and proliferation in the maintenance of the T-cell pool are often extrapolated from mice to men, and *vice versa*. But in fact, it is unknown how the average life spans of $CD4^+$ and $CD8^+$ T cells of mice and men relate to the longevity of the species, and whether the main mechanism of naive T-cell maintenance in the face of decreasing thymus output is similar in mice and men.

TREC analysis can be used to estimate thymic output and peripheral proliferation. In this way it has been shown that peripheral proliferation contributes substantially, as much as 90%, to the maintenance of the human peripheral naive T-cell pool (Vrisekoop et al, manuscript in preparation). The recently introduced technique of stable isotope labeling has paved the way for reliable quantification of lymphocyte turnover. Mathematical interpretation of deuterium labeling data from healthy human adults has revealed that human naive T cells are long-lived, with an average life expectancy of six years for naive $CD4^+$ and nine years for naive $CD8^+$ T cells, and that recently-produced naive T cells live even longer[11].

Using deuterium labeling in adult and old C57Bl/6 mice, we here determined that the average life span of murine naive $CD4^+$ and $CD8^+$ T cells is about 6-7 and 11-17 weeks respectively, and show that these cells undergo similar numbers of rounds-of-turnover during the life of both mice and men. In contrast, we found that naive T-cell production in mice is mainly coming from the thymus, even in aged mice. This shows that the main mechanism *via* which the naive T-cell pool is maintained differs fundamentally between mice and men. It follows that C57Bl/6 mice might not be a good model to study human T-cell homeostasis.

RESULTS

Turnover of naive T cells

To determine the normal turnover rates of naive $CD4^+$ and $CD8^+$ T cells in young adult mice, 12-week old C57Bl/6 mice were labeled by administration of 4% deuterated water (^2H_2O) and deuterium enrichment in the DNA of thymocytes and naive T cells from the spleen was determined for the four weeks up and a subsequent 18 weeks down-labeling period. It took about a week for the first labeled naive T cells to appear in the spleen, and they continued to accumulate for one week after the end of labeling (Fig.1), suggesting that these cells were labeled by cell division in another compartment, probably the thymus.

The cellular turnover rates were estimated by fitting the labeling data to a mathematical model, which distinguishes between an average turnover or production rate, p, and a death rate of labeled cells, d^*. The average turnover rates of naive

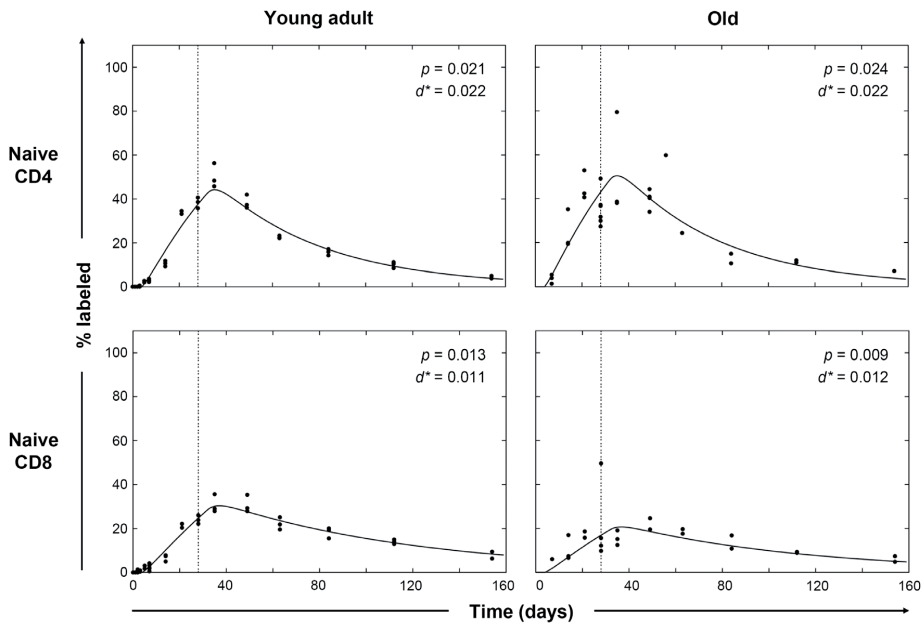


Figure 1 Estimation of average naive CD4+ and CD8+ T-cell turnover rates from deuterium labeling experiments. 12-week old (left graphs) and 85-week old mice (right graphs) were given 4% ^2H_2O for four weeks. Each dot in the graphs represents the percentage of labeled naive $CD4^+$ (upper graphs) or naive $CD8^+$ T cells (lower graphs) in the spleen of one C57BL/6 mouse. The vertical lines mark the end of ^2H_2O administration at four weeks. The level of label enrichment was normalized to that of thymocytes (see Materials and Methods). The data were fitted with a mathematical model described in Materials and Methods. The estimated average turnover rate (p) and death rate of the labeled cells (d^*) are given in each graph.

Table I Estimated average life spans of naive $CD4^+$ and $CD8^+$ T cells in euthymic and thymectomized mice

Method	Age	Average life spans (days)[a]	
		Naive CD4	Naive CD8
^2H_2O	12 weeks	48 (40 – 54)[b]	78 (66 – 95)
	85 weeks	41 (36 – 46)	116 (92 –139)
Cell counts	7 weeks	17 (15 – 20)	56 (50 – 62)
	12 weeks	28 (24 – 34)	59 (53 – 65)
	85 weeks	40 (34 – 50)	88 (83 – 91)
	12 weeks (Tx)	74 (61 – 103)	130 (123 – 132)

[a] The average life spans of young-adult and old mice were based on the estimated turnover rate (life span = $1/p$) from the deuterium labeling experiments (denoted by ^2H_2O), and on the combination of estimated parameters from the homeostatic cell death model and the T-cell count at the indicated age (denoted by Cell counts, and where the average life span is defines as $1/(r+dnN)$, where N is the average number of naive T cells at the indicated age).
[b] 95% confidence limits are given in brackets.

$CD4^+$ and $CD8^+$ T cells in adult mice were found to be 0.021 and 0.013 per day (Fig.1), corresponding to average life spans ($1/p$) of 48 and 78 days, respectively (Table I). In contrast to what is commonly observed in deuterium labeling studies[12], the death rates of labeled cells, d^*, did not significantly differ from the average turnover rates, p (Fig.1), suggesting that naive T cells form a relatively homogeneous population.

Contribution of the thymus

Although deuterium labeling experiments provide the most reliable tool to estimate cellular turnover rates, they fail to distinguish between production of naive T cells in the thymus and their peripheral renewal[13]. In a separate set of experiments we therefore recorded the numbers of single positive (SP) $CD4^+$ and $CD8^+$ thymocytes and naive $CD4^+$ and $CD8^+$ T cells in the spleen and lymph nodes of normal euthymic or sham-thymectomized mice and mice that were thymectomized (Tx) at seven weeks of age (Fig.2). There were no significant differences in thymocyte or naive T-cell numbers between normal mice and mice that were sham-thymectomized at seven weeks of age (data not shown), so that we combined the data from both mice in one euthymic control group. In euthymic mice, the numbers of SP $CD4^+$ and $CD8^+$ thymocytes were found to increase exponentially after birth, peaked at week 6/7, and decreased almost 40% during week seven. Hereafter, thymocyte numbers declined exponentially at a rate of 50% per year (see Methods and Supplementary Figure S1, A). Naive T-cell numbers in spleen and lymph nodes peaked at week 7/8, and subsequently declined more slowly than the thymocyte pool (Fig.2). The decline in naive T-cell numbers after thymectomy was bi-phasic, with an early rapid loss,

followed by a long-lasting slower decline (Fig.2), suggestive for the existence of a homeostatic mechanism.

To distinguish between production of naive T cells in the thymus and by peripheral T-cell proliferation, we devised a mathematical model that was fitted to the naive T-cell counts of euthymic and thymectomized mice of different ages. The model describes thymus output, peripheral renewal, and naive T-cell loss, due to cell death or priming into the memory T-cell pool (see Methods). The model allowed for death rates that decrease when T-cell numbers decline as the mechanism of homeostasis (Fig.S1, B). The naive $CD4^+$ and $CD8^+$ T-cell counts of euthymic and thymectomized mice turned out to be described very well with a simple model that totally lacks peripheral renewal of naive T cells, and in which cellular death rates increase

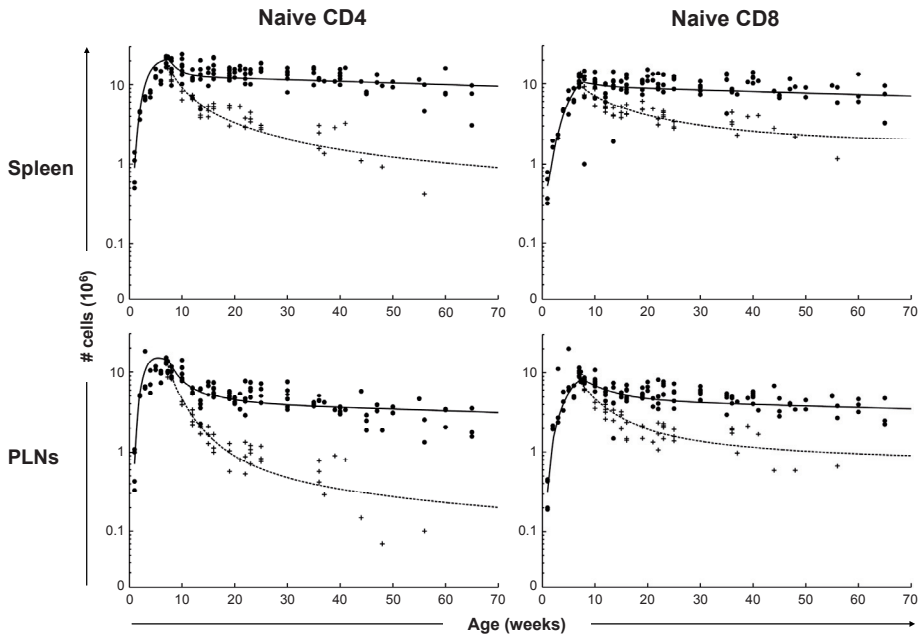


Figure 2 Size of the naive $CD4^+$ and $CD8^+$ T-cell populations over age in euthymic and thymectomized mice. T-cell numbers of naive $CD4^+$ (left panels) and $CD8^+$ (right panels) T cells were determined in euthymic mice (●) and mice which had been thymectomized at week 7 (+) in spleen (upper graphs) and PLNs (lower graphs). The population densities in control and thymectomized mice were fitted to a mathematical model allowing for a source from the thymus that is proportional to the number of SP thymocytes, and a cellular death rate that increases linearly with the population density (Fig. S1 and Materials and Methods). The best fit of the model to the combined data sets of spleen and lymph nodes of normal and thymectomized mice is depicted by the solid and dashed lines. Best fitting parameters are given in Supplementary Tables S1, S2 and S3, and corresponding average life spans are given in Table I.

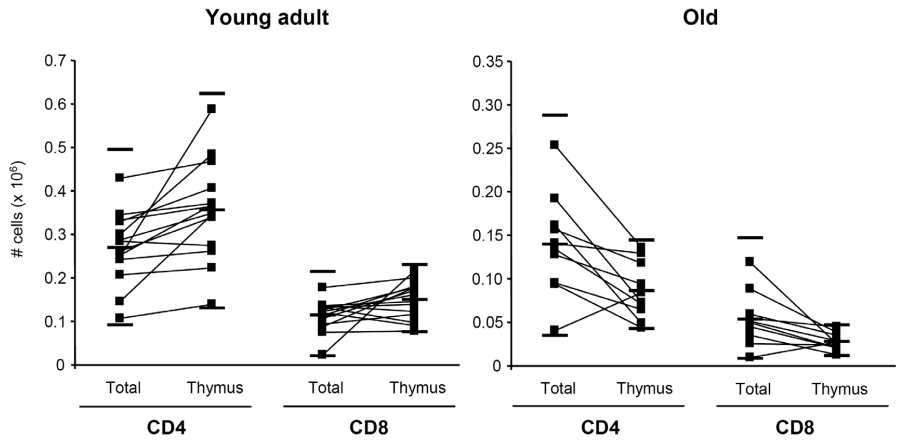


Figure 3 Comparison of total daily production of naive T cells and thymic output. Total daily production of naive T cells in spleens from young-adult (12 weeks) and old mice (85 weeks) was determined by multiplying the estimated average production rates, *p*, from the ^2H_2O-labeling experiment (Fig.1) with the naive T-cell numbers in the spleen of individual mice. Thymic output of the same mice was calculated by multiplying the fraction of emigrating single positive thymocytes (ε = 0.039) with the absolute number of single positive thymocytes of each mouse. Mean of the data is indicated by a horizontal bar. Mean of the data is indicated by a horizontal bar. The upper and lower horizontal bars represent the minimum and maximum values that could be attained (based on the confidence limits of p and ε).

linearly with the T-cell population density (Fig.2). According to this model the average life spans of naive $CD4^+$ and $CD8^+$ T cells in 12-week old mice are 28 and 59 days, respectively, which is in reasonable agreement with the 48 and 78 day expected life spans estimated from the deuterium labeling experiments (Table I). Assuming that the export of naive T cells from the thymus is proportional to the number of SP thymocytes[14] (depicted in Fig.S1, A), we estimated that every day 3.9% (3.6 – 4.3%) of the SP-thymocyte pool emigrates from the thymus to the spleen. In 12-week old mice this corresponds to the emigration of 4.1 10^5 newly produced naive $CD4^+$ and 1.7 10^5 naive $CD8^+$ T cells to the spleen. Calculating the daily production by multiplying the average turnover rates (p) from the ^2H_2O experiments with the actual number of naive T cells in the spleen, yielded numbers of 2.7 10^5 for naive $CD4^+$ T cells and 1.2 10^5 for naive $CD8^+$ T cells. The similarity of this total daily production and daily thymic output shows that naive $CD4^+$ and $CD8^+$ T cells in young adult C57Bl/6 mice are hardly formed by peripheral T-cell proliferation (Fig.3).

Since some studies suggest that decrease in naive T-cell numbers is compensated by increased proliferation[15], we also analyzed the data with an alternative model, in which the rate of proliferation can increase when cell numbers become low (Fig.S1,

C). The fit of this alternative model to the data of control and thymectomized aging mice (Fig.S3) was almost as good as the fit with the model in which T-cell death rates were dependent on population densities (Fig.2). Because the alternative model gives the *maximum* proliferation rate that is still compatible with the data, it yielded a somewhat lower thymus output of 2.2% of the SP thymocytes emigrating to the spleen per day. In normal 12-week old mice, the average time between subsequent divisions was estimated to be 407 and 244 days for naive $CD4^+$ and $CD8^+$ T cells, respectively. Combining the estimated average life spans from the deuterium labeling experiment with this average interdivision time showed that the life spans of the naive T cells were 3 to 8-fold shorter than these interdivision times, reconfirming the finding that most naive T cells in normal mice make hardly any division during their stay in the naive T-cell pool.

Old mice

Knowing that naive T-cell production in young adult mice is almost exclusively due to T-cell production by the thymus, we studied how this changes when mice become old and have much lower thymus output. To this end, we performed deuterium labeling experiments in 85-week old mice (Fig.1). Naive $CD4^+$ T cells in aged mice were found to have an average life span of 41 days, which did not significantly differ from that in 12-week old mice (48 days). The expected life span of naive $CD8^+$ T cells was found to be 116 days, which is nearly 50% higher than that in young adult mice (78 days, Table I). Total daily production (p x N) of naive $CD4^+$ and $CD8^+$ T cells in 85-week old mice was on average 1.4 10^5 and 0.6 10^5 respectively. The mean thymic output, based on the density-dependent death model, was estimated to contribute 1.0 10^5 naive $CD4^+$ and 0.3 10^5 naive $CD8^+$ T cells. Thus, even in very old mice, in which thymus output has dropped significantly, the majority of naive T cells were produced by the thymus, and not by peripheral T-cell proliferation (Fig.3).

Of note, even in thymectomized mice, in which naive T-cell numbers were clearly reduced, naive T cells may hardly be formed by peripheral division. In 12-week old mice that were thymectomized at 7 weeks of age, the numbers of naive $CD4^+$ and $CD8^+$ T cells in the periphery can fully be explained by increased average life spans (of 74 and 130 days, respectively, Table I), due to reduced competition between T cells. Based on these data alone, one cannot exclude, however, that naive cell numbers in thymectomized mice may be partially maintained by peripheral T-cell proliferation. From ^2H_2O data in thymectomized mice we know that this is indeed the case. After 10 weeks of labeling approximately 24% of the naive $CD4^+$ and $CD8^+$ T cells was labeled, corresponding to a proliferation rate of 0.24/70=0.003 per day[11]. As 85-week old mice and 20-week old thymectomized mice have approximately three-fold decreased naive T-cell numbers compared to 20-week old control mice

(data not shown), this suggests that such drop in naive T-cell numbers may also lead to a similar compensatory proliferation rate in old mice. This compensatory proliferation rate is however only a small fraction compared to the total production rates of naive $CD4^+$ and $CD8^+$ T cells in old mice (p = 0.024 and p = 0.009, Fig.1).

TREC dynamics in mice

One important piece of evidence that naive T cells in humans undergo peripheral proliferation while maintaining the naive phenotype comes from the gradual decline in naive T-cell TREC contents with age. TRECs are stable episomal DNA circles that can only be formed in the thymus, and are not replicated during cell division. As a consequence the fraction of $TREC^+$ naive T cells decreases when cells divide[16;17]. An experimental prediction from our modeling results is that – in contrast to humans – in normal aging mice the average TREC content of naive $CD4^+$ and $CD8^+$ T cells should not decrease. We tested this prediction by comparing the average TREC contents of thymocytes and naive T cells from normal euthymic mice of 20-39 and 85 weeks of age. Indeed we found no evidence for TREC dilution in mouse naive T cells (Fig.4), which confirms our main finding that – irrespective of their age – naive $CD4^+$ and $CD8^+$ T cells in euthymic mice are almost exclusively formed by thymus output. Interestingly, even in thymectomized mice the fractions of $TREC^+$ naive T cells were hardly decreased, showing that even under these relatively lymphopenic conditions mouse naive T cells are hardly formed by peripheral T-cell division.

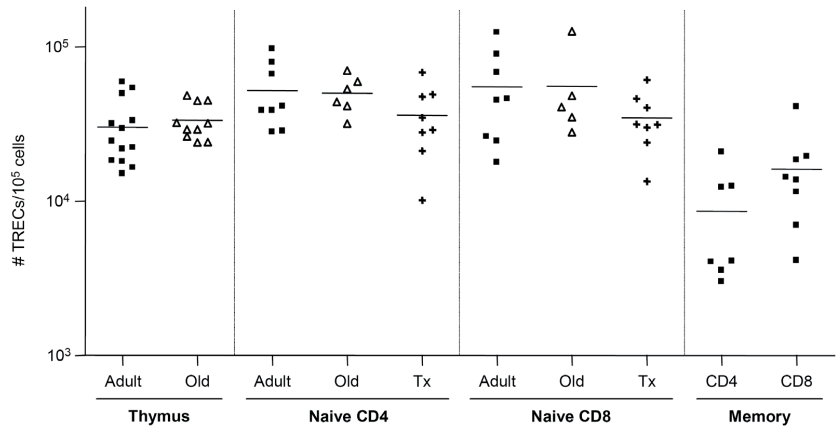


Figure 4 TREC analysis in thymocytes and T-cell subsets. The number of SjTRECs per 10^5 naive T cells were determined by real time quantitative PCR in 20-56 week old mice (control [adult] or thymectomized [Tx] at week 7) and in 80-100 week old mice [old]. Horizontal bars depict median values. As controls TREC contents of memory T cells and thymocytes were included. TREC contents of naive T cells in adult, thymectomized and old mice did not differ significantly from each other.

DISCUSSION

The recent introduction of stable isotope labeling in immunology has finally introduced a reliable way to measure lymphocyte life spans, and has resolved important controversies on leukocyte life spans in the literature[13]. Here we applied stable isotope labeling to C57BL/6 mice to measure the daily production rates of naive $CD4^+$ and $CD8^+$ T cells in young and old mice. In young adult mice, naive $CD4^+$ T cells were found to have an average life span of about 48 days, which did not change when the mice aged, but was estimated to double to 74 days when naive T-cell counts decreased after thymectomy. Naive $CD8^+$ T cells in young adult mice were found to have an average life span of 78 days, and to live somewhat longer when the mice got older (116 days) or were thymectomized (130 days). The 48-day expected life span of naive $CD4^+$ T cells based on deuterium labeling is in good agreement with previous estimates based on BrdU labeling[18]. Parretta et al. recently studied naive $CD8^+$ T-cell dynamics in thymectomized C57BL/6 mice using BrdU labeling[19] and found a 2.5-fold shorter expected life span of 52 days compared to the 130 days that we estimated, but agree that these cells hardly divide. Because they excluded potential cytotoxicity of BrdU[19] the reason for this 2,5-fold difference remains unclear. The expected life spans of naive $CD4^+$ and $CD8^+$ T cells in C57BL/6 mice imply that during the lifetime of their host (approximately 100 weeks), these cells are replaced about 10-15 times. Similarly, the expected life spans of naive $CD4^+$ (6 years) and $CD8^+$ T cells (9 years) in humans that we previously estimated using deuterium labeling[11] amount to 10-15 rounds of replacement during the life span of their human host.

Despite these similarities between mouse and human lymphocyte life spans, we observed a major qualitative difference between naive T-cell dynamics in mice and men. By combining deuterium labeling and thymectomy experiments in mice with mathematical modeling, we found that – in contrast to human naive lymphocytes – mouse naive T cells are hardly formed by peripheral T-cell proliferation. The limited role for peripheral T-cell proliferation in the maintenance of the naive T-cell pool in mice that we report here is fully compatible with results from a classical study by Tough and Sprent[18], which reported 10% $BrdU^+$ naive T cells after five weeks of BrdU-labeling in thymectomized C57BL/6 mice, and with our own previous findings in thymectomized C57BL/6 mice after nine weeks of deuterium labeling[11]. Expressing this percentage of labeled cells in terms of a daily production rate, these data suggest that - at maximum - a fraction p = 0.10/35 = 0.003 of all naive T cells in euthymic mice undergoes peripheral proliferation while maintaining the naive phenotype per day, which is indeed about five to ten times smaller than the total daily

production rates for naive $CD4^+$ and $CD8^+$ T cells respectively, in normal mice that we estimated here. Similar low division rates following thymectomy have recently been reported[19]. Of note, the true contribution of peripheral T-cell proliferation to the maintenance of the naive pool in healthy mice may be even lower, because division rates of naive T cells might be slightly increased after thymectomy, due to low T-cell numbers.

The conclusion that peripheral T-cell proliferation hardly contributes to the maintenance of the naive T-cell pool in mice was confirmed by a lack of TREC dilution in the naive T-cell compartment of ageing mice. In humans, there is ample evidence that naive T-cell TREC contents decline about 10-fold from early adulthood to old age[20-22]. This gradual TREC dilution has been shown to be evidence for peripheral T-cell proliferation within the naive lymphocyte pool of humans[16;17]. A recent study from our own laboratory pointed out that at most 10% of naive T cells in healthy human adults have never divided in the periphery (Vrisekoop et al. manuscript in preparation), showing that peripheral renewal plays a major role in the maintenance of the naive T-cell pool of young human adults.

What could be responsible for these qualitative differences between mouse and human lymphocyte kinetics? In adult humans, the amount of productive thymic tissue decreases approximately 5% per year[3], which corresponds to a decline rate of 0.00014 per day. In adult mice we estimate that the number of SP thymocytes declines at a rate of 0.0014 per day (see Methods), which is 10-fold faster than in humans. Qualitatively, the mouse therefore seems a good model with respect to the regression of the thymus after puberty. However, because humans live about 40-fold longer than C57BL/6 mice, their 10-fold slower loss of productive thymic tissue still means that at old age the relative contribution of the thymus is much smaller than that in mice.

Although we cannot exclude that our results would have been different in different strains of mice (with different thymus output) or in mice exposed to pathogens, our results at least show that one should be extremely careful when applying insights obtained from this widely-used mouse model to the human situation.

It has previously been proposed that the naive T-cell pool consists of two different subsets with different kinetics: a pool of recent thymus emigrants (RTE), with an expected life span of about 3 weeks, and a subset of resident naive T cells with slower turnover[18;19;23;24]. Our previous deuterium labeling studies provided no evidence for such kinetic differences within the naive T-cell pool of young human adults. On the contrary, recently-produced naive T cells in human adults were found

to have a larger life expectancy than resident naive T cells, because the loss rate of labeled cells, d^*, was found to be lower than the average production rate p[11]. In mice, the loss rates of labeled cells, d^*, did not significantly differ from the average production rates, p, suggesting that naive T cells in adult mice form a relatively homogeneous population. Indeed, we here showed that naive T-cell numbers in euthymic and thymectomized mice can perfectly be explained by a model that does not incorporate separate RTE dynamics.

The major implication of this work is that one cannot freely extrapolate naive T-cell kinetic data from young adult (or old) mice to young adult (or old) humans, for the main mechanism by which naive T-cell numbers are maintained is qualitatively different in mice and men.

MATERIALS AND METHODS

Mice

C57Bl/6 mice were maintained by in-house breeding at the Netherlands Cancer Institute in Amsterdam (thymectomy experiment) or the Central Animal Facility at Utrecht University in Utrecht (^2H_2O labeling) under specific pathogen-free conditions in accordance with institutional and national guidelines. Thymectomy was performed as described previously[11]. ^2H_2O labeling was achieved by giving 12-week old mice one boost injection (i.p.) of 15 ml/kg with 99.8% ^2H_2O (Cambridge Isotopes, Cambridge, MA), followed by subsequent feeding with 4% ^2H_2O in drinking water for four weeks.

Antibodies

PE-conjugated antibodies recognizing CD62L (clone MEL-14), PerCP-labeled CD4 (clone RM4-5) and CD8 (clone 53-6.7), APC-labeled CD44 (clone IM7) were purchased from BD Biosciences PharMingen (San Diego, CA).

Cell preparation and flow cytometry

Spleen, (axillary, brachial, inguinal and superficial cervical) PLNs and (if present) thymus were isolated from C57Bl/6 mice of different ages and mechanically disrupted to obtain single cell suspensions. Cell preparation and FACS staining were performed as previously described[11]. Cells were analyzed on a LSR II flow cytometer and BD FACSDiva software. Naive ($CD62L^+$, $CD44^-$) cells were sorted using a FACSAria cell sorter and FACSDiva software (BD). The average purity was: 98.4 ± 1.0% (naive $CD4^+$, 96.3 – 99.7%), 97.2 ± 1.6% (effector/memory $CD4^+$, 92.8 –

99.2%), 98.4 ± 1.1% (naive $CD8^+$, 94.8 – 99.7%) and 96.0 ± 2.1% (effector/memory $CD8^+$, 89.6 – 99.3%). Thymocytes and sorted T cells were frozen until further processed.

Measurement of ^2H_2O enrichment in serum and DNA

Deuterium enrichment in serum was measured as reported by Previs et al[25]. The isotopic enrichment of DNA was determined as previously described[11].

Mathematical modeling *of thymectomy data*

Using a simple mathematical model to quantify naive T-cell dynamics in control and thymectomized mice, we considered that under normal conditions naive T cells are maintained by thymic output and peripheral T-cell proliferation, whereas the cells are lost via differentiation into effector/memory T cells and through cell death. Thymic output was described by a phenomenological function $f(t)$, which was proportional to the number of single positive (SP) thymocytes (explained in the next section). We allowed for a delay Δ of cells circulating from the thymus to the periphery similar to that estimated from the heavy water experiment, Δ = 4.1 days.

Both cell death (d_nN), (Fig.S1, B) and proliferation $\frac{r}{1+\frac{N}{q}}$ (Fig.S1, C) were assumed to be density-dependent, assuming cells to proliferate more and live longer under lymphopenic conditions, when survival signals are more abundant. The number of naive T cells is consequently given by:

$$N' = \varepsilon f(t-\Delta) + \frac{r}{1+\frac{N}{q}}N - d_n N^2$$

To investigate the minimum model required to describe the data we considered two extreme cases of the mathematical model by assuming:

(i). a density-independent proliferation rate keeping death density-dependent:

$N' = \varepsilon f(t-\Delta) + rN - d_n N^2$, and

(ii). a density-independent death rate keeping proliferation density-dependent:

$$N' = \varepsilon f(t-\Delta) + \frac{r}{1+\frac{N}{q}}N - d_n N$$

Of note: since naive T cells do have an intrinsic (limited) lifespan[23], also density-independent death will occur. As proliferation, differentiation and density-independent cell death were all proposed to be constant, a net proliferation rate (r)

was used in model (i). Likewise, in case of model (ii), the net death rate includes differentiation.

Since T cells continuously recirculate around the body, it is reasonable to expect that naive T-cell dynamics in different body organs is similar. Thus we simultaneously modeled dynamics in the spleen and peripheral lymph nodes (PLN), relating the two with a proportionality coefficient $\psi(t)$, dependent on cell densities in the two organs such that $N_{PLN} = \psi(t) N_{spleen}$.

In the first weeks, when $t = \varsigma$, the increase in the Spleen/PLN ratio is described by $\psi(t) = \theta(1 - e^{x_1 t})$ after which the ratio decreases. This second phase is described in control mice by $\psi(t) = \psi(\varsigma)\left(z_1 e^{-x_2(t-\varsigma)} + (1 - z_1) e^{-x_3(t-\varsigma)} \right)$. However in thymectomized mice, from the time of thymectomy, $t = T_{off}$, the spleen/PLN ratio is then described by $\psi(\mathrm{t}) = \psi(T_{off})\left(z_2 e^{-y_2(t-T_{off})} + (1 - z_2) e^{-y_3(t-T_{off})} \right)$.

The proportionality function $\psi(t)$ was fitted to data and the parameter estimates of the best fit (Fig.S2) are given in Supplementary Table S2.

Mathematical modeling *of thymic output*

We describe the dynamics of single positive thymocytes using a function $f(t)$ which is a modification of the thymic involution function described by Steinmann et al[3]. While the generation of $CD4^+$ and $CD8^+$ SP thymocytes takes different routes, the mechanism and a driving force of thymic involution is the same. As such we consider that $CD4^+$ and $CD8^+$ SP thymocytes have different filling up dynamics but have the same thymic involution dynamics.

Thymic output is described by a function that is made up from a sum of exponents with constants v_1, v_2 and v_3 describing the rate at which the thymus becomes populated by thymocytes (v_1) and two rates describing the involution of the thymus. During the first few weeks of life, the thymus fills up at a rate σv_1 per day such that $f(t) = \sigma(1 - e^{v_1 t})$ until time $t = T_{off}$. If there was no thymic involution, σ would be the maximum number of SP thymocytes, but due to the onset of thymic involution at time T_{off}, the maximum is $\sigma(1 - e^{v_1 T_{off}})$ cells.

Thymic involution, starting at T_{off}, is biphasic and is hence described by a sum of two exponents. The constant v_2 describes initial and faster slope and v_3 describes the later and slower slope during thymic involution. These two exponents are weighted by a constant γ such that $f(t) = \sigma(1 - e^{v_1 T_{off}}) \left[\gamma e^{-v_2 (t - T_{off})} + (1 - \gamma) e^{-v_3 (t - T_{off})} \right]$ from time $t = T_{off}$ onwards. In both $CD4^+$ and $CD8^+$ SP thymocytes, involution resulted in a drop

of more than 50% within a week resembling a partial thymectomy. The parameter values of the best fit to the SP thymocytes data are shown in Supplementary Table S3.

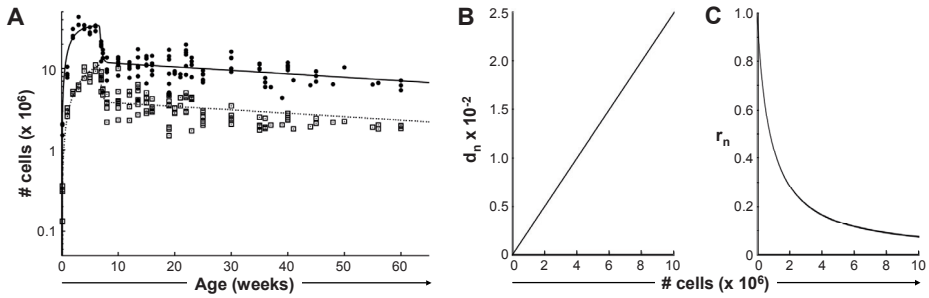
Mathematical modeling *of* ^2H_2O *data*

Enrichment data were fitted using a previously developed mathematical model (Chapter 4). The best fit for the plasma data is shown in Supplementary Figure S4 and the corresponding parameter estimates are given in Supplementary Table S5.

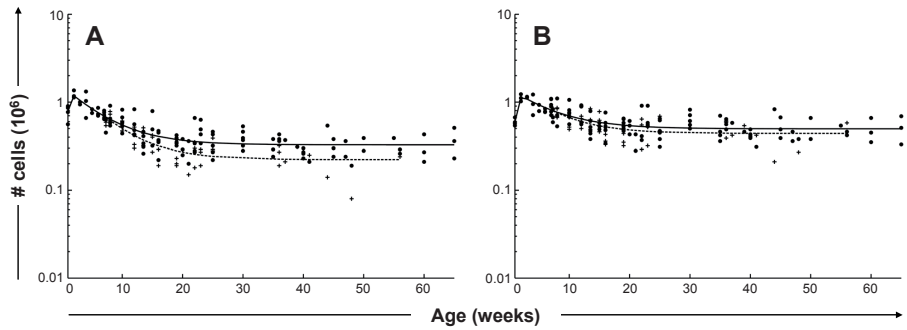
ACKNOWLEDGEMENTS

Our special thanks go to Vitaly Ganusov for the introduction of the density-dependent homeostasis models. We acknowledge Mette Hazenberg for sharing the thymectomy protocol, Loes Rijswijk for improving the technique, and Henk Starrevelt, Sjaak Greeven and Linda Nijdam for technical assistance and excellent animal care. We thank Frank Miedema, Linde Meyaard and Grada van Bleek for stimulating discussions. This research has been funded by the Landsteiner Foundation for Blood Transfusion Research (LSBR grant 0210) and the Netherlands Organization for Scientific Research (NWO, VICI grant 92750029).

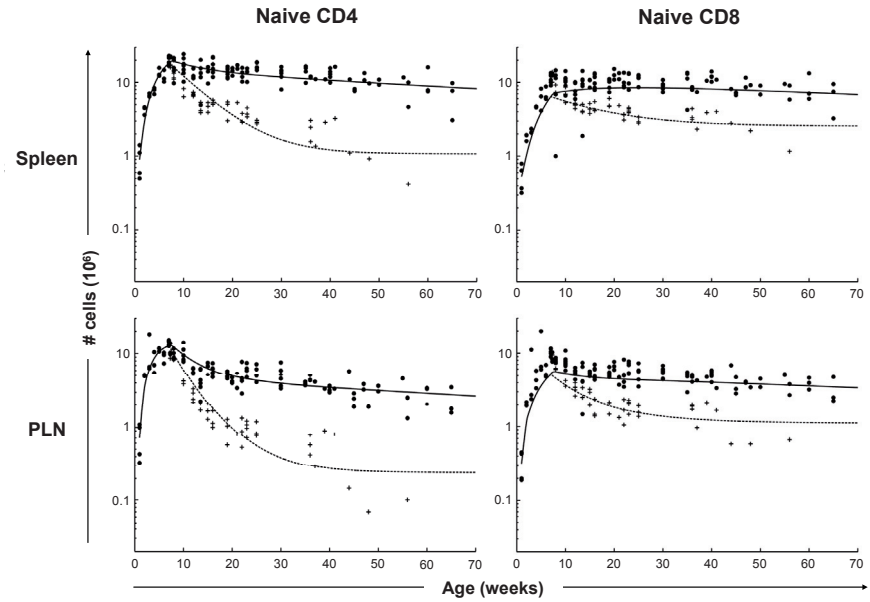
SUPPLEMENTARY INFORMATION



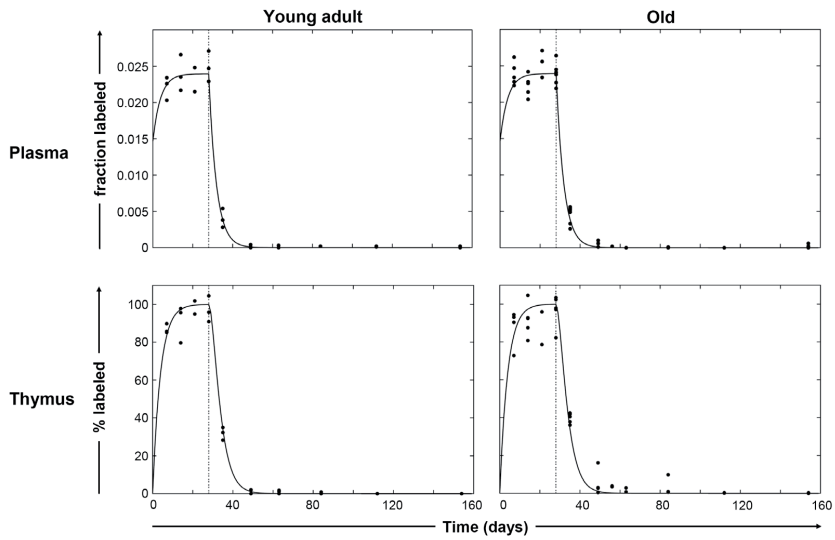
Supplementary Figure S1 Visual representation of model parameters: Age-dependent number of $CD4^+$ (●) and $CD8^+$ (□) single positive thymocytes from C57Bl/6 mice of different ages (A), model parameters visualizing density-dependent death (B), and density-dependent proliferation (C).



Supplementary Figure S2 Proportionality function $\psi(t)$ correlating naive $CD4^+$ (A) and naive $CD8^+$ (B) T-cell dynamics in spleen and peripheral lymph nodes, based on cell densities in the two organs. Solid and dashed lines represent the best fits of the proportionality function to data from control (●) and thymectomized mice (+), respectively. Model parameters are provided in Supplementary Table S2.



Supplementary Figure S3 Massive loss of naive T cells after thymectomy of adult mice. Best fits of density-dependent proliferation model through naive $CD4^+$ and naive $CD8^+$ T-cell counts in spleen and peripheral lymph nodes in control (●) and thymectomized (+) C57Bl/6 mice. The cellular death rates were fixed to the estimates obtained with deuterium labeling (Fig.1). Best fitting parameters are given in Supplementary Tables S2, S3 and S4.



Supplementary Figure S4 Best fits of plasma and thymocyte enrichment in young and old mice. Parameter values are given in Supplementary Table S5.

Supplementary Table S1 Parameter estimates as estimated by the density-dependent death model. The 95%-confidence intervals are given between parentheses and were determined by the bootstrapping method[26].

	Parameter	Value (confidence limits)
CD4	d_n (x 10^{-9} day^{-1})	2.930 (2.536 – 3.333)
	r_n (x -10^{-4} day^{-1})	8.648 (0.419 – 27.85)
CD8	d_n (x 10^{-9} day^{-1})	2.309 (1.835 – 2.812)
	r_n (x 10^{-3} day^{-1})	4.075 (1.257 – 6.799)
Shared	ε (x 10^{-2} day^{-1})	3.930 (3.586 – 4.299)

Supplementary Table S2 Parameters values and 95% confidence limits for the best fit of the function $\Psi(t)$ to $CD4^+$ and $CD8^+$ naive T cells in PLN and spleen for both control and thymectomized mice (see Supplementary Figure S2).

Parameter	Value (confidence limits)	
	CD4	CD8
α	1.578 (1.197 – 2.373)	5.401 (1.399 – 18.58)
x_1	0.098 (0.049 – 0.168)	0.017 (0.004 – 0.096)
x_2	0.024 (0.020 – 0.030)	0.022 (0.015 – 0.030)
x_3	0	0
y_2	0.025 (0.016 – 0.041)	0.024 (0.011 – 0.054)
y_3	0	0
z_1	0.723 (0.692 – 0.750)	0.576 (0.523 – 0.618)
z_2	0.679 (0.597 – 0.784)	0.459 (0.363 – 0.589)
ζ	14.00 (11.56 – 18.16)	14.72 (11.56 – 19.50)

Supplementary Table S3 Parameters values and 95% confidence limits for the best fit of the thymic output function to $CD4^+$ and $CD8^+$ single positive thymocytes (see Supplementary Figure S1, A).

	Parameter	Value (confidence limits)
CD4	σ (x10^7 cells)	3.459 (3.224 – 3.772)
	v_1 (day^{-1})	0.084 (0.065 – 0.106)
CD8	σ (x10^7 cells)	1.679 (1.074 – 9.786)
	v_1 (day^{-1})	0.023 (0.003 – 0.056)
Shared	v_2 (day^{-1})	0.001 (0.001 – 0.002)
	v_3 (day^{-1})	0.524 (0.187 – 2.961)
	T_{off} (days)	46.88 (42.00 – 48.58)
	γ	0.352 (0.313 – 0.384)

Supplementary Table S4 Parameter estimates as estimated by the density-dependent proliferation model. The 95%-confidence intervals are given between parentheses and were determined by the bootstrapping method[26].

	Parameter	Value (confidence limits)
CD4	r_n (day^{-1})	1.000 (1.000 – 1.000)
	k (x 10^4)	2.303 (2.128 – 3.286)
CD8	r_n (day^{-1})	1.000 (1.000 – 1.000)
	k (x 10^4)	3.095 (2.694 – 3.273)
Shared	ε (x 10^{-2} day^{-1})	2.162 (2.112 – 2.270)

Supplementary Table S5 Parameters values and 95% confidence limits for the best fit of the serum and thymocytes heavy water enrichment from young and old mice shown in Supplementary Figure S4.

	Parameter	Value (confidence limits)
Serum	S_0	0.015 (0.012 – 0.018)
	d_s (day^{-1})	0.261 (0.230 – 0.272)
	π (day^{-1})	0.006 (0.006 – 0.007)
Thymocytes	$p_{\text{young mice}}$ (day^{-1})	0.416 (0.377 – 0.468)
	$p_{\text{old mice}}$ (day^{-1})	0.307 (0.254 – 0.470)

REFERENCE LIST

1. Tanchot,C. and B.Rocha. 1997. Peripheral selection of T cell repertoires: the role of continuous thymus output. *J. Exp. Med.* 186:1099-1106.
2. Jones,S.C., K.Clise-Dwyer, G.Huston, J.Dibble, S.Eaton, L.Haynes, and S.L.Swain. 2008. Impact of post-thymic cellular longevity on the development of age-associated CD4+ T cell defects. *J. Immunol.* 180:4465-4475.
3. Steinmann,G.G., B.Klaus, and H.K.Muller-Hermelink. 1985. The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study. *Scand. J. Immunol.* 22:563-575.
4. Hale,J.S., T.E.Boursalian, G.L.Turk, and P.J.Fink. 2006. Thymic output in aged mice. *Proc. Natl. Acad. Sci. U. S. A* 103:8447-8452.
5. Sempowski,G.D., M.E.Gooding, H.X.Liao, P.T.Le, and B.F.Haynes. 2002. T cell receptor excision circle assessment of thymopoiesis in aging mice. *Mol. Immunol.* 38:841-848.
6. Fagnoni,F.F., R.Vescovini, G.Passeri, G.Bologna, M.Pedrazzoni, G.Lavagetto, A.Casti, C.Franceschi, M.Passeri, and P.Sansoni. 2000. Shortage of circulating naive CD8(+) T cells provides new insights on immunodeficiency in aging. *Blood* 95:2860-2868.
7. Lerner,A., T.Yamada, and R.A.Miller. 1989. Pgp-1hi T lymphocytes accumulate with age in mice and respond poorly to concanavalin A. *Eur. J. Immunol.* 19:977-982.
8. Ahmed,M., K.G.Lanzer, E.J.Yager, P.S.Adams, L.L.Johnson, and M.A.Blackman. 2009. Clonal expansions and loss of receptor diversity in the naive CD8 T cell repertoire of aged mice. *J. Immunol.* 182:784-792.
9. Yager,E.J., M.Ahmed, K.Lanzer, T.D.Randall, D.L.Woodland, and M.A.Blackman. 2008. Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *J. Exp. Med.* 205:711-723.
10. Naylor,K., G.Li, A.N.Vallejo, W.W.Lee, K.Koetz, E.Bryl, J.Witkowski, J.Fulbright, C.M. Weyand, and J.J.Goronzy. 2005. The influence of age on T cell generation and TCR diversity. *J. Immunol.* 174:7446-7452.
11. Vrisekoop,N., I.den Braber, A.B.de Boer, A.F.Ruiter, M.T.Ackermans, S.N.van der Crabben, E.H.Schrijver, G.Spierenburg, H.P.Sauerwein, M.D.Hazenberg, R.J.de Boer, F.Miedema, J.A.Borghans, and K.Tesselaar. 2008. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. *Proc. Natl. Acad. Sci. U. S. A* 105:6115-6120.
12. Asquith,B., C.Debacq, D.C.Macallan, L.Willems, and C.R.Bangham. 2002. Lymphocyte kinetics: the interpretation of labeling data. *Trends Immunol.* 23:596-601.
13. Borghans,J.A. and R.J.de Boer. 2007. Quantification of T-cell dynamics: from telomeres to DNA labeling. *Immunol. Rev.* 216:35-47.
14. Berzins,S.P., R.L.Boyd, and J.F.Miller. 1998. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. *J. Exp. Med.* 187: 1839-1848.
15. Surh,C.D. and J.Sprent. 2008. Homeostasis of naive and memory T cells. *Immunity.* 29: 848-862.
16. Dutilh,B.E. and R.J.de Boer. 2003. Decline in excision circles requires homeostatic renewal or homeostatic death of naive T cells. *J. Theor. Biol.* 224:351-358.
17. Hazenberg,M.D., S.A.Otto, J.W.Cohen Stuart, M.C.Verschuren, J.C.Borleffs, C.A.Boucher, R.A.Coutinho, J.M.Lange, T.F.Rinke de Wit, A.Tsegaye, J.J.van Dongen, D.Hamann, R.J.de Boer, and F.Miedema. 2000. 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.* 6:1036-1042.
18. Tough,D.F. and J.Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med.* 179:1127-1135.
19. Parretta,E., G.Cassese, A.Santoni, J.Guardiola, A.Vecchio, and F.Di Rosa. 2008. Kinetics of *in vivo* proliferation and death of memory and naive CD8 T cells: parameter estimation based on 5-bromo-2'-deoxyuridine incorporation in spleen, lymph nodes, and bone marrow. *J. Immunol.* 180:7230-7239.
20. Harris,J.M., M.D.Hazenberg, J.F.Poulin, D.Higuera-Alhino, D.Schmidt, M.Gotway, and J.M.McCune. 2005. Multiparameter evaluation of human thymic function: interpretations and caveats. *Clin. Immunol.* 115:138-146.

21. Jamieson,B.D., D.C.Douek, S.Killian, L.E.Hultin, D.D.Scripture-Adams, J.V.Giorgi, D. Marelli, R.A.Koup, and J.A.Zack. 1999. Generation of functional thymocytes in the human adult. *Immunity.* 10:569-575.
22. Kilpatrick,R.D., T.Rickabaugh, L.E.Hultin, P.Hultin, M.A.Hausner, R.Detels, J.Phair, and B.D.Jamieson. 2008. Homeostasis of the naive CD4+ T cell compartment during aging. *J. Immunol.* 180:1499-1507.
23. Di Rosa,F., S.Ramaswamy, J.P.Ridge, and P.Matzinger. 1999. On the lifespan of virgin T lymphocytes. *J. Immunol.* 163:1253-1257.
24. Berzins,S.P., D.I.Godfrey, J.F.Miller, and R.L.Boyd. 1999. A central role for thymic emigrants in peripheral T cell homeostasis. *Proc. Natl. Acad. Sci. U. S. A* 96:9787-9791.
25. Previs,S.F., J.W.Hazey, F.Diraison, M.Beylot, F.David, and H.Brunengraber. 1996. Assay of the deuterium enrichment of water via acetylene. *J. Mass Spectrom.* 31:639-642.
26. Henderson,A.R. 2005. The bootstrap: a technique for data-driven statistics. Using computer-intensive analyses to explore experimental data. *Clin. Chim. Acta* 359:1-26.

Chapter 6

T-cell exhaustion and depletion in mice with chronic immune activation via CD27/CD70 costimulation

Ineke den Braber[1,*], Tendai Mugwagwa[2,*], Elise H.R. Schrijver[1], Erik Mul[3], Floris van Alphen[3], Rob J. de Boer[2,#], José A.M Borghans[1,2,#], and Kiki Tesselaar[1,#]

[1] Department of Immunology, University Medical Center Utrecht,Utrecht, The Netherlands; [2] Department of Theoretical Biology, Utrecht University, Utrecht, The Netherlands; [3] Department of Clinical Viro-immunology, Sanquin Research, Amsterdam, The Netherlands

[,#] These authors contributed equally to this work*

ABSTRACT

It has recently become generally accepted that chronic immune activation in HIV-1 infection plays an important role in $CD4^+$ T-cell depletion. The role of thymic impairment by HIV-1 is however still debated. We here show that even in the context of substantial thymic output, chronic immune activation can lead to severe naive T-cell depletion. In CD70Tg mice, excessive immune activation via CD27/CD70 results in the accumulation of T cells with an effector/memory phenotype and depletion of naive T cells. We studied T-cell numbers in normal and hyper-immune mice with different amounts of thymic output. Our data show that hyper-activation on its own is sufficient to cause naive T-cell depletion, and reduced thymic output accelerates this. Fitting the data with a mathematical model confirmed the impact of hyperactivation on naive T-cell depletion. Continuous immune activation also induced enhanced PD-1 expression, and administration of IL-7 to the transgenic mice killed the mice within a few days. Administration of immune stimulatory mediators to untreated HIV patients could therefore also have adverse effects.

INTRODUCTION

$CD4^+$ T-cell depletion is the hallmark of HIV-1 infection. Thanks to decades of intensive research it has become accepted that chronic immune activation is one of the main drivers of naive $CD4^+$ T-cell depletion in HIV-1 infection, and that direct killing of naive $CD4^+$ T cells by the virus plays a minor role[1;2]. The topical question addresses the mechanism by which immune activation drives naive T-cell depletion. Continuous priming of naive T cells either by HIV-1 or by an increased microbial pressure might be involved in this process[3;4]. The role the of impairment of *de novo* thymic production of T cells in $CD4^+$ T-cell depletion is still a matter of debate[5-7]. Loss of lymphoid cells and profound stromal damage has been observed in the thymus of HIV-1 infected patients[8]. Since thymic output in human adults contributes only marginally to the maintenance of the peripheral T-cell pool (Vrisekoop et al, manuscript in preparation), the effect of thymus dysfunction on naive T-cell depletion remains questionable however.

Exhaustion or functional impairment of the T-cell compartment also hampers T-cell immunity in HIV-1 infected individuals. Loss of functional capacity, like cytotoxicity, cytokine production and proliferative capacity, is thought to reflect prolonged excessive immune activation and to correlate with disease progression. Enhanced expression and function of inhibitory receptors like CTLA-4 and PD-1 might reflect this dysfunction, and blockade of these receptors improves *in vitro* T-cell function and vaccination responses in SIV-infected macaques[9-13]. Based on these observations PD-1 blockade is being considered as a treatment option in HIV infection.

Upon highly active antiretroviral therapy (HAART) of HIV-infected individuals, the viral load usually decreases and naive T-cell numbers slowly increase[14;15]. Slow T-cell recovery is a problem in some HIV patients, and stimulation of T-cell reconstitution might therefore be beneficial. So far IL-2 and GH treatment have been used in HIV patients. IL-2 treatment typically increased $CD4^+$ regulatory T cells[16;17]; in contrast, GH treatment significantly increased T-cell numbers and had little adverse effects[18]. IL-7 has potent stimulatory effects on T-cell numbers and T-cell diversity in healthy humans[19], but has not yet been tested in HIV patients.

In this study we addressed the effect of chronic immune activation and thymic output on naive T-cell depletion in mice. We studied wild-type mice and two CD70Tg mouse lines in which continuous CD27/CD70 interaction induces chronic immune activation. We used a mathematical model to investigate the main effects of CD27/CD70, eventually leading to T-cell depletion. We found that CD70Tg mice have a reduced but functional thymic output, because further reducing their thymic output caused substantial naive T-cell loss. The hyper-activation in CD70Tg mice is

also responsible for naive T-cell depletion, and induces naive T-cell depletion in mice with normal thymic output. Continuous immune stimulation enhanced PD-1 expression, which might be a protective mechanism against immunopathology by excessive amounts of effector cells, as suggested by the lethal effect of IL-7 administration. In conclusion our data suggest that immune activation by itself may induce severe immune depletion in situations of marginal thymic output, as found in human adults.

RESULTS

Chronic stimulation of murine T cells *via* CD27 in CD70Tg-F13 mice induces excessive differentiation of naive $CD4^+$ and $CD8^+$ T cells into effector/memory T cells and accelerates thymic involution[20]. To investigate to what extent both effects contribute to the observed naive T-cell decline in CD70Tg-F13 mice, we measured thymocyte and T-cell subset numbers in the spleen of control and thymectomized wild-type and CD70Tg-F13 mice, and applied mathematical modeling to interpret the data.
Thymectomy of 7-week old mice resulted in a rapid decline of naive T-cell numbers, both in wild-type C57Bl/6 and CD70Tg-F13 mice (Fig.1), demonstrating that also in transgenic mice thymic output plays an important role in the maintenance of the naive T-cell compartment, despite the observed thymic involution. The loss of naive T cells was however most rapid in thymectomized CD70Tg-F13 mice, and the naive T-cell decline in these mice markedly exceeded the naive T-cell decline in wild-type thymectomized mice, demonstrating that next to accelerated thymic involution, continuous immune activation further decreased naive T-cell numbers in these mice (Fig.1).

To quantify the effect of the decline in thymic output and the increase in immune activation, we fitted the data to a mathematical model. In this model naive T cells are maintained by export of a daily fraction (ε) of the total number of single positive (SP) thymocytes ($f(t)$), and are lost via cell death and a constant rate of cell priming. Since both priming and peripheral renewal are taken to occur at a fixed rate, i.e. are density-independent, the resultant priming rate (r_n) is a net rate, combining both characteristics. Naive T-cell homeostasis is accounted for by introducing density-dependent cell death in the model, where naive T cells are thought to live longer at low T-cell densities when survival factors are more abundant.
Effector/memory T cells are produced by peripheral renewal and naive T-cell priming, followed by subsequent clonal expansion. Effector/memory T cells are lost by density-dependent death. We fitted the wild-type and CD70Tg data simultaneously

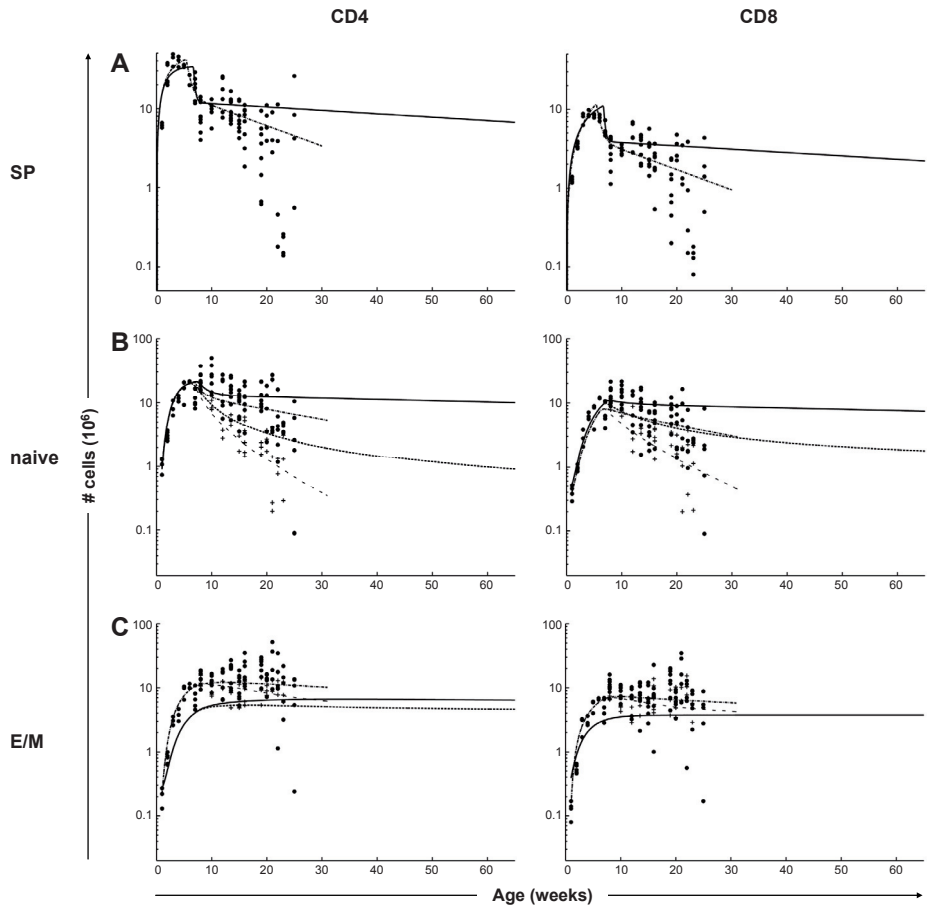


Figure 1 Decline in naive T-cell numbers after thymectomy of C57Bl/6 and CD70Tg-F13 mice. (A) Age-dependent number of single positive (SP) thymocytes in CD70Tg-F13 mice of different ages (●). For clarity only the data points from CD70Tg-F13 mice are shown; data points from C57Bl/6 mice are shown in Chapter 5. The number of SP thymocytes in both mouse strains was described by a phenomenological model (see methods). (B, C) Total number of (B) naive and (C) effector/memory (E/M) $CD4^+$ and $CD8^+$ T cells in the spleen of thymectomized (+) and control (•) CD70Tg-F13 mice. The dashed-dotted and thin dashed lines represent the best fits of control and thymectomized CD70Tg-F13 mice; solid and thick dashed lines the best fits of control and thymectomized C57Bl/6 mice, respectively. Model parameters are provided in Table I.

allowing for different priming rates (r_n) and clonal expansion rate (a) between the two data sets. All other parameters were assumed to remain unaffected in CD70Tg-F13 mice (allowing other parameters to vary, failed to improve the quality of the fits). The estimated parameter values for the best fit of the model to the data are shown in Table I.

Table I. Parameter estimates for C57Bl/6, CD70Tg-F12 and CD70Tg-F13 mice as estimated by the mathematical model. The 95%-confidence intervals are given between parentheses and were determined by the bootstrapping method[21].

		CD4	CD8
C57Bl/6	r_n (day^{-1})	0.000 (-0.003 – 0.002)	-0.003 (-0.006 – 0.000)
	a (day^{-1})	0.012 (0.007 – 0.021)	0.000 (-0.029 – 0.021)
CD70TG-F12	r_n (day^{-1})	0.059 (0.052 – 0.078)	0.021 (0.017 – 0.029)
	a (day^{-1})	0.038 (0.026 – 0.046)	0.089 (0.042 – 0.117)
CD70TG-F13	r_n (day^{-1})	0.015 (0.012 – 0.018)	0.012 (0.010 – 0.015)
	a (day^{-1})	0.080 (0.056 – 0.137)	0.221 (0.149 – 0.616)
Shared	d_n (x 10^{-9} day^{-1})	2.894 (2.501 – 3.385)	2.103 (1.623 – 2.678)
	r_m (day^{-1})	0.057 (0.033 – 0.099)	0.288 (0.224 – 0.537)
	h (x 10^6)	2.008 (0.869 – 4.280)	1.082 (0.264 – 3.320)
	d_m (day^{-1})	0.018 (0.010 – 0.032)	0.065 (0.037 – 0.013)
	ε (day^{-1})	0.041 (0.038 – 0.045)	

By the best fit of the model we estimated that in both wild-type and CD70Tg-F13 mice 4% of the number of SP thymocytes was exported on a daily basis (Table I: ε = 0.041 per day). Since the size of the thymus was different between C57Bl/6 and CD70Tg-F13 mice (Fig.1), the absolute number of emigrating SP thymocytes per day was reduced in CD70Tg-F13 mice (e.g., in 12-week old CD70Tg-F13 mice this amounts to a 15% and 25% reduction for CD4$^+$ and CD8$^+$ RTE, decreased naive T-cell numbers in CD70Tg-F13 mice, but thymic output continues to contribute to the maintenance of the naive T-cell pool in these mice. In CD70Tg-F13 mice we estimated a daily loss of 1,5% and 1,2% of the naive CD4$^+$ and CD8$^+$ T cells, respectively, by priming only (r_n = 0.015 and 0.012; Table I). Indeed the average life spans of naive T cells in 12-week old CD70Tg-F13 mice are 15% to 30% shorter than those in wild type mice (Table II). The increased naive T-cell priming was also reflected in the memory pool. Compared to the wild-type situation in 12-week old mice there were at least 2,5 fold more effector/memory T cells produced by T-cell differentiation. As a result, 60% of the CD4$^+$ and 47% of the CD8$^+$ memory cells in CD70Tg-F13 mice were produced by naive T-cell activation and their subsequent expansion. In contrast, in wild-type mice more than 60% of the memory T cells were formed by renewal. Summarizing, continuous activation in CD70Tg-F13 mice decreased thymic output and increased naive T-cell priming, and both contributed about equally to the naive T-cell depletion in these mice.

Table II. Effects of immune activation on naive $CD4^+$ and $CD8^+$ T-cell life span. Life spans (in days) of naive $CD4^+$ and $CD8^+$ T cells were calculated for 12-week old mice.

	C57Bl/6		CD70Tg-F13	
	Naive CD4	Naive CD8	Naive CD4	Naive CD8
Control	26	56	22	40
Thymectomized	59	104	39	53

Strength of immune activation correlates with the amount of T-cell depletion

For our initial analysis of CD27 function, we generated two CD70Tg lines[22]. The second CD70Tg strain, F12, also constitutively expresses CD70, but at a lower level. The F12 line has more physiological levels of CD70 expression, and these mice have increased fractions of effector/ memory T cells, but less than CD70Tg-F13 mice. Additionally, CD70Tg-F12 mice show no signs of enhanced thymic involution compared to wild-type mice (Fig.2), suggesting a normal thymic output[23]. The main dynamic difference between C57Bl/6 mice and CD70Tg-F12 mice would therefore be the peripheral activation rates. To address the effect of continuous immune activation in the context of a fairly normal thymic output we measured thymocyte and T-cell numbers in CD70Tg-F12 mice and fitted the same mathematical model combining the data sets of all three types of mice. The naive and effector/memory T-cell numbers in CD70Tg-F12 mice can again be explained by an increased priming and clonal expansion rate (Table I). Since thymic output is normal in the F12 mice, this shows that enhanced priming is sufficient to decrease naive T-cell numbers. Comparison of the parameters of CD70Tg-F12 and CD70Tg-F13 mice showed that the clonal expansion rate, a, was highest in CD70Tg-F13 mice, but the priming rate, r_n, was highest in F12 mice. Since the clonal expansian rate, a, combines naive T-cell priming with their subsequent clonal expansion, this suggests a much more extensive clonal expansion in F13 mice.

Increased fraction of PD-1 expressing memory cells in CD70Tg mice

Remarkable differences were observed when evaluating the life span of the different animals: control C57Bl/6 mice lived on average 24 months, CD70Tg-F13 mice lived only 5-6 months, and thymectomy had no influence on these life spans (data not shown). Since thymectomy does lower naive T-cell numbers in CD70Tg-F13 mice, low naive T-cell numbers by themselves fail to explain the short life span of F13 mice. We therefore tested whether continuous immune activation has additional effects on immune-function. Regulatory T cells were measured by the number of $CD4^+$ $CD25^+$ $CD103^+$ $GITR^+$ T cells, and we measured exhaustion of memory cells by analysing the fraction of PD-1 expressing cells within this compartment.

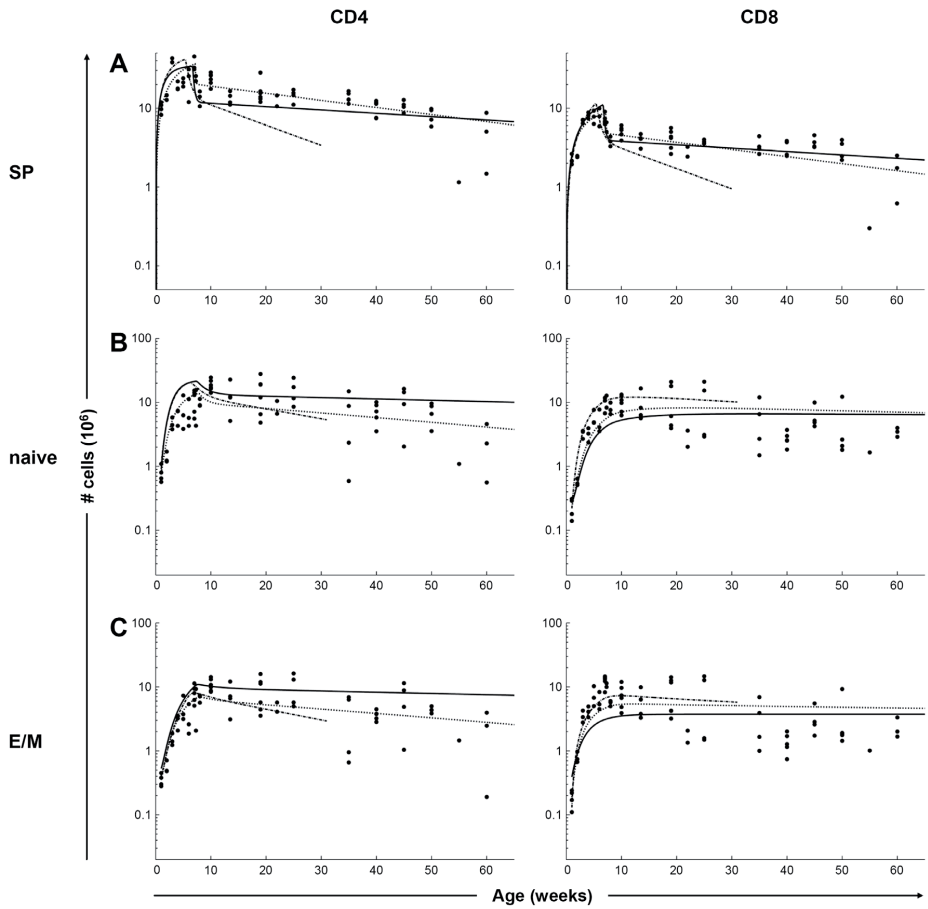


Figure 2. Comparison of thymocyte and T-cell counts in C57Bl/6, CD70Tg-F12 and CD70Tg-F13 mice. Best fits through the number of (A) SP thymocytes, (B) naive and (C) effector/memory T-cell numbers in C57Bl/6 (solid line), CD70Tg-F12 (dotted line) and CD70Tg-F13 mice (dashed-dotted line). Data points from CD70Tg-F12 mice are given as black dots. Model parameters are given in Table I.

Continuous expression of CD70 at young age increased the fraction and the number of regulatory T cells in both spleen (Fig.3, A) and peripheral lymph nodes (data not shown). In older mice this effect seemed to revert, as the number of regulatory T cells was two-fold decreased in old F12 mice. PD-1 expression was increased in the $CD4^+$ and $CD8^+$ memory compartment in the spleen (Fig.3, B) and in the PLN (data not shown). This increase of PD-1 expression was seen in both transgenic lines, but was most pronounced in the spleen of the CD70Tg-F13 line. In both lines the effect was stronger in older mice. This permanently increased expression of PD-1 within

the memory compartment may indicate immune exhaustion and may partially explain the immune dysfunction at old age.

IL-7 treatment is lethal for CD70Tg-F13 but not C57Bl/6 mice

IL-7 administration has been used as treatment for T-cell depleted patients. IL-7 enhances thymic function and restoration of naive T-cell pool[24;25], and IL-7 can down-regulate PD-1 expression[26]. We aimed to treat six C57Bl/6 and six CD70Tg-F13 mice for three weeks with hIL-7 to investigate whether we could improve their T-

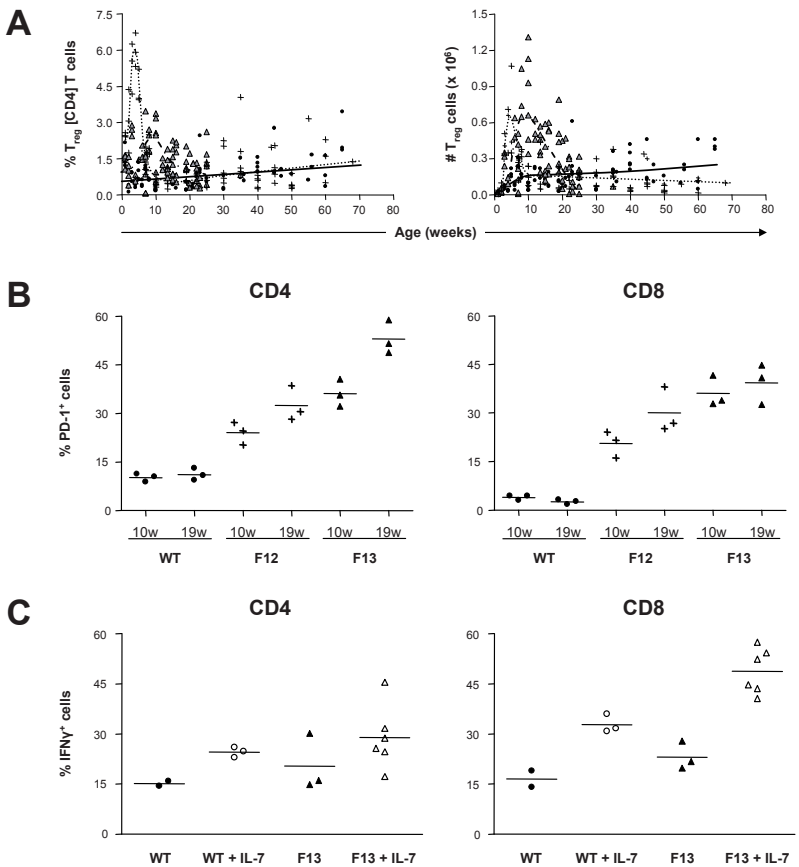


Figure 3. Functional characteristics of CD70Tg mice. (A) The percentage CD25$^+$ CD103$^+$ GITR$^+$ regulatory T cells within the CD4$^+$ T-cell population, the absolute number of regulatory T cells, and (B) the percentage of PD-1$^+$ cells within the CD4$^+$ and CD8$^+$ T-cell pool were determined in the spleen of C57Bl/6 (●), CD70Tg-F12 (+), and CD70Tg-F13 mice (▲) of different ages. Solid, dotted and dashed dotted lines in (A) represent the average trend in C57Bl/6, CD70Tg- F12 and CD70Tg-F13 mice. (C) Splenocytes were isolated from 19-week old control (●) and IL-7 treated C57Bl/6 (○) and control (▲) and IL-7-treated CD70Tg-F13 mice (Δ). The cells were stimulated for 4h with PMA/ionomycin in the presence of Brefeldin A, and subsequently intracellularly stained with IFNγ. The symbols depict the percentage of positive cells within the CD4 or CD8 gated cells.

cell numbers and immunity to enhance their life span. Unexpectedly, all CD70Tg-F13 mice died between two to fifteen days after start of the experiment, while the C57Bl/6 mice remained healthy. Repeating the experiment with a two-day treatment period, we could not find any evidence for an enlarged thymus, or enhanced T-cell numbers. However, despite the short treatment period, hIL-7 appeared to improve T-cell function as the percentage of IFN-γ producing $CD8^+$ T cells in CD70Tg-F13 mice tended to be increased (Fig.3, C).

DISCUSSION

In this study we addressed the effect of continuous immune activation in mice constitutively expressing CD70. Although enhanced immune activation by itself was sufficient to reduce naive T-cell numbers, we only observe severe depletion in young mice in the context of accelerated thymic involution. In these mice thymic output plays an important role in the maintenance of naive T-cell numbers, and in young mice there seems to be an overproduction of naive T cells by the thymus that is compensated for by a transient increase of the death rate (see Chapter 5). Total naive T-cell depletion is therefore difficult to achieve, and seems unfeasible in our model because of the limited number of cells expressing CD70. In human adults with a much lower thymic output, however, even moderately enhanced priming rates may lead to severe T-cell depletion.

The estimated priming rates, r_n, in CD70Tg mice were around 1 to 6% per day, but this might be an underestimation of the true priming rate in CD70Tg mice as in our mathematical model the priming rate is the net result of a positive renewal rate and a negative priming rate. Analysis of Ki-67 expression indeed showed increased fractions of proliferating cells within the naive and memory compartments in CD70Tg mice. This might indicate that in the CD70Tg system increased renewal compensates somewhat for the naive T-cell loss by increased priming. The increased Ki67 expression in the naive compartment is also readily explained by the increased clonal expansion however, i.e., by phenotypically-naive proliferating transitional cells that are on their way to the memory compartment.

Despite an overall similarity, careful analysis of F12 and F13-transgenic mice revealed several differences. In line with our expectations based on the level of CD70 expression, we saw a higher number and more differentiated phenotype of memory cells in F13 mice, an increased fraction of memory cells expressing PD-1, and estimated the largest contribution of clonal expansion on memory T-cell production in CD70Tg-F13 mice. The most striking difference was the difference in life spans of the mice. Both failing immunity and disturbed haematopoiesis could be the

explanation for this difference. At the time of death (at 5-6 months), T-cell numbers are so much reduced in CD70Tg-F13 mice that opportunistic infections are to be expected. This need not be the explanation however, because individuals and mice with extremely low (naive) T-cell numbers can live without clinical complications. Nef-transgenic mice have dramatically reduced T-cell numbers, but have a normal life span, without overt increases in infection incidence[27]. In addition, healthy Ethiopians, that are reported to have several features of chronic immune activation, exhibit reduced proportions of naive T cells and a diminished TREC content, but do not die prematurely as a consequence of aids-like symptoms (Tsegaye et al, manuscript in preparation). Chronic immune activation does lead to exhaustion of the memory pool in HIV-1 infection, and our PD-1 and cytokine data suggest that this is also the case in the transgenic mice. It might thus be that it is the combination of low naive numbers and an exhausted memory compartment causing the immune failure and death in CD70Tg mice. We indeed observed CD70Tg-F13 mice suffering and dying from opportunistic infections[20]. Alternatively, the enormous numbers of IFN-γ producing effector cells in the CD70Tg mice[22] could indirectly cause immunopathology, and the premature death of these mice. Severe anaemia is observed in some old CD70Tg mice, and IFN-γ inhibits erythropoiesis[28]. Enhancement of T-cell numbers producing IFN-γ and TNF-α has been implicated in hematopoietic destruction in aplastic anemia[29].

When we treated CD70Tg-F13 mice with hIL-7, with the idea that this would increase their life span, the result was dramatic. None of the mice survived the treatment. Studies on the role of Fas-induced death in CD70Tg mice have shown that increased numbers of effector cells leads to immunopathology, and an even more premature death of the mice[30]. IL-7 is known to induce $CD8^+$ memory T-cell proliferation, and in combination with TCR triggering, is known to abate PD-1 expression[26]. Administration of this cytokine to the CD70Tg mice could thus have led to a massive increase of functional memory T cells causing the death of the mice. Additionally, if the IL-7 were to down-regulate PD-1 expression these results would suggest that the increased PD-1 expression in CD70Tg mice is a negative feedback mechanism preventing the excessive formation of functional effector T cells. In untreated CD70Tg mice the enhanced PD-1 expression may thus protect the mice from immunopathology and prolongs their life expectancy.

In conclusion we show that enhanced immune activation can drive naive T-cell depletion, especially in the context of low thymic output. IL-7 treatment of CD70Tg mice resulted in their death, possibly caused by immunopathology of large numbers of cytokine producing effector/memory T cells. In analogy, treatment of HIV-1 patients with IL-7 or PD-1 might have adverse effects.

MATERIALS AND METHODS

Mice

C57Bl/6, heterozygous CD70Tg-F12, and hemizygous CD70Tg-F13 mice[22], all on a C57Bl/6 background, were maintained by in-house breeding at the Netherlands Cancer Institute (Amsterdam) or the Central Animal Facility of the Utrecht University (Utrecht) under specific pathogen-free conditions in accordance with institutional and national guidelines. The phenotype of C57Bl/6 and CD70Tg mice was comparable in both animal facilities (data not shown). Thymectomy was performed as described previously[31]. Mice were i.p. injected with 10 µg hIL-7 (Cytheris, Vanves, France) in PBS on a daily basis for 3 weeks.

Antibodies

FITC-labeled antibodies against IFN-γ (clone XMG1.2), Ki-67 (clone B56), CD4 (clone RM4-5)and CD8a (clone 53-6.7), PE-conjugated antibodies recognizing CD43 (clone 1B11) and CD62L (clone MEL-14), PerCP-labeled CD4 (clone RM4-5) and CD8 (clone 53-6.7), APC-labeled CD44 (clone IM7) were purchased from BD Biosciences PharMingen (San Diego, CA). Anti-CD25 (PE) antibodies (clone PC61 5.3) were purchased from Caltag laboratories (Burlingame, CA). FITC-conjugated antibo-dies against CD103 (clone 2E7) and biotinylated GITR (clone DTA-1) were purchased from eBioscience (San Diego, CA).

Cell preparation and flow cytometry

Spleen, (axillary, branchial, inguinal and superficial cervical) lymph nodes and (if present) thymus were isolated from C57Bl/6 and CD70Tg mice of different ages. Single cell suspensions were obtained by mechanical disruption. Red blood cells were lysed with ammonium chloride solution (155 mM NH4Cl, 10 mM KHCO3 and 0.1 mM EDTA, pH 7.4). Cells were washed, resuspended in IMDM/7% FCS and counted. FACS-staining was performed as described previously[31]. Intracellular staining for IFN-γ was performed after 4h stimulation with 10 ng/ml PMA and 100 µg/ml ionomycin in the presence of 1 µg/ml of the protein-secretion inhibitor Brefeldin A (BD Biosciences PharMingen, San Diego, CA).

Mathematical modeling

Using a mathematical model to quantify naive T-cell dynamics in control and thymectomized mice, we consider a naive T-cell pool that is maintained by input from the thymus. We describe thymic output with a phenomenological function $f(t)$ which is proportional to the number of single positive (SP) thymocytes (Fig.2; see Chapter 5 for a detailed description). The rates of thymic involution are the same for $CD4^+$

and CD8$^+$ SP thymocytes, but their population kinetics (σ and v_1) are allowed to be different. Parameter estimates of the best fit of this model to the data are given in Supplementary Table S1.

In addition to an input of naive T cells from the thymus, the mathematical model allows for maintenance of naive T cells by renewal at a rate r_n per day and a density-dependent death rate (d_nN), allowing cells to live longer when the population density is low (see Chapter 5). Naive T-cell dynamics are described by the equation:
$N' = \varepsilon f(t) - r_n N - d_n N^2$.
Memory T cells are produced from either density dependent activation of naive T cells at a rate $\frac{a}{1+\frac{M}{h}}$ or by density dependent renewal at a rate $\frac{r_m}{1+\frac{M}{h}}$, where h is the half saturation constant, i.e. when $m = h$, the production of memory cells is half maximal. Memory T-cell dynamics are described by the equation: $M' = \frac{aN + r_m M}{1+\frac{M}{h}} - d_m M$

Due to their continuous recirculation around the body, naive T-cell dynamics should be averaged over different body organs, and we simultaneously model dynamics in the spleen and peripheral lymph nodes (PLN) by fitting a function, $\psi(t)$, relating the number of cells in PLN to those in the spleen. In the first weeks, when $t = \varsigma$, the ratio of cells in PLN to spleen increases, which is described by $\psi(t) = \theta(1 - e^{x_1 t})$. Hereafter this ratio decreases, which in control mice is described by the equation: $\psi(t) = \psi(\varsigma)\left(z_1 e^{-x_2(t-\varsigma)} + (1-z_1)e^{-x_3(t-\varsigma)}\right)$. At the time of thymectomy the ratio changes and we describe the PLN/spleen ratio by the same function with new parameters, i.e., $\psi(t) = \psi(T_{off})\left(z_2 e^{-y_2(t-T_{off})} + (1-z_2)e^{-y_3(t-T_{off})}\right)$, where $T_{off} > \varsigma$.

The function $\psi(t)$ was fitted to the data of each mouse strain separately, and the parameter estimates of the best fits (Fig.S1) are given in Supplementary Table S2.

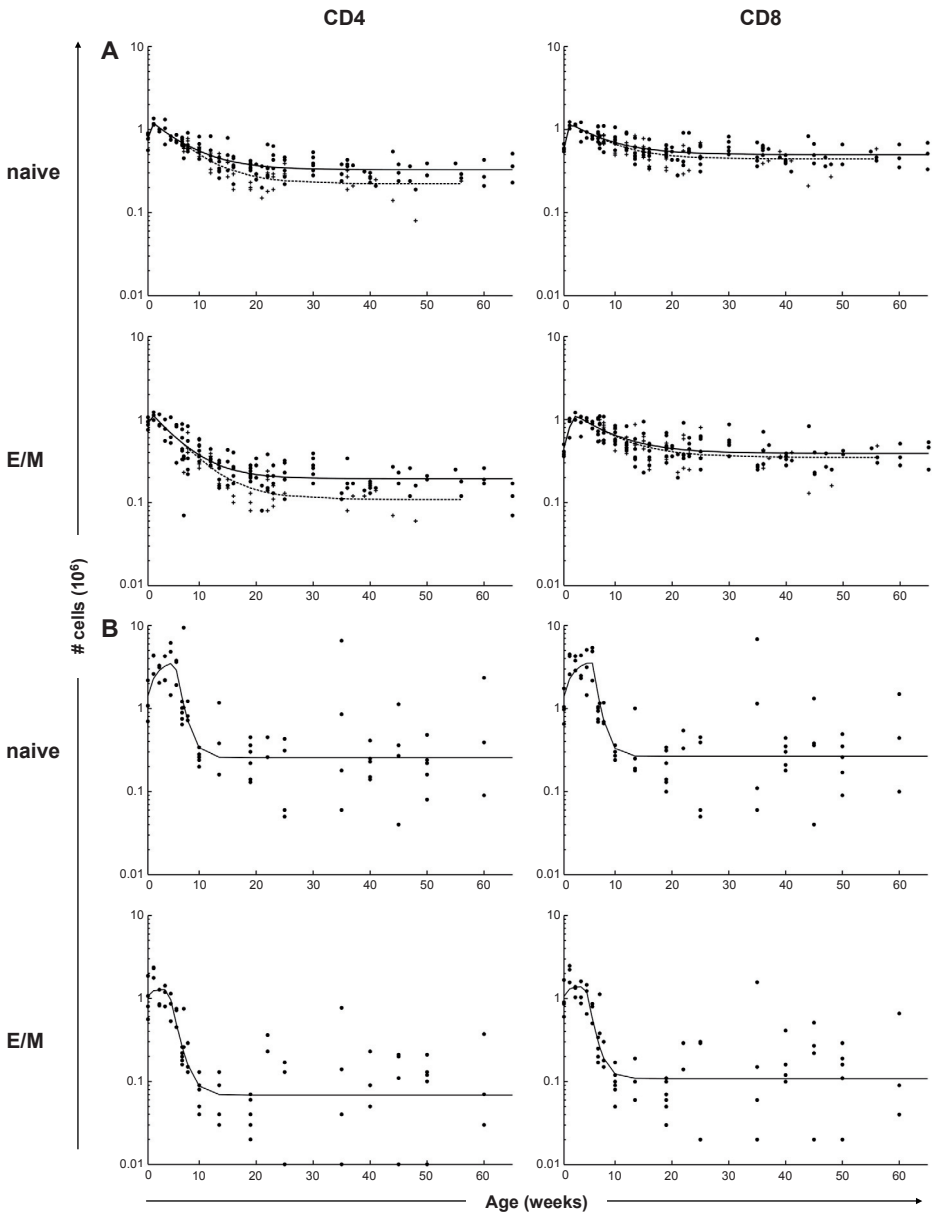
Statistical analysis

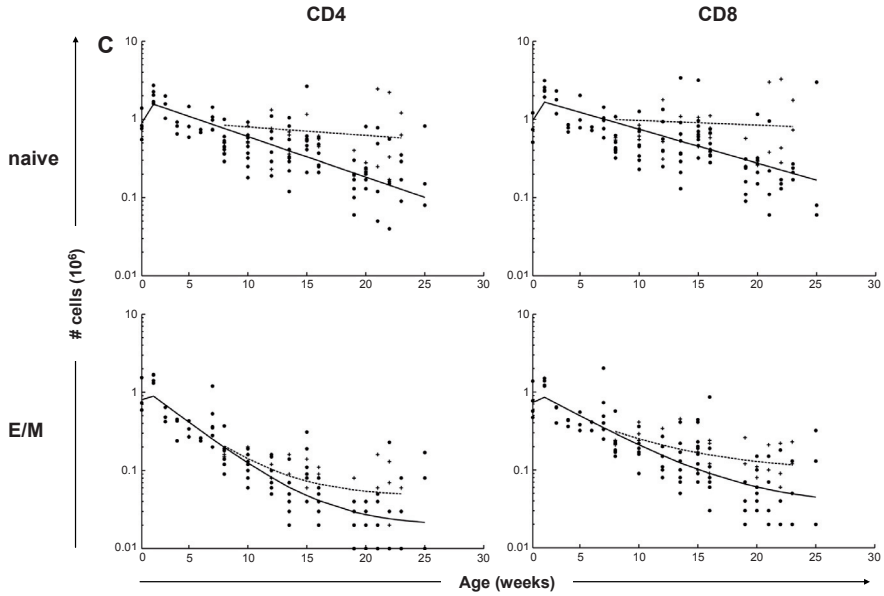
All statistical analyses were performed using the software program SPSS 15.0 (SPSS Inc, Chicago, Illinois). Differences with $p \leq 0.05$ were considered significant.

ACKOWLEDGEMENTS

We thank Sjaak Greeven and Linda Nijdam for biotechnical assistance. This research has been funded by the Landsteiner Foundation for Blood Research (LSBR grant #0210).

SUPPLEMENTARY INFORMATION





Supplementary Figure S1 Proportionality function $\psi(t)$ correlating T-cell dynamics in spleen and peripheral lymph nodes, based on cell densities in the two organs. Proportionality functions of B57Bl/6 mice (A), CD70Tg-F12 mice (B), and CD70Tg-F13 mice (C). Solid and dashed lines represent the best fits of the proportionality function to data from control mice (●) and thymectomized mice (+), respectively. Model parameters are provided in Supplementary Table S2.

Supplementary Table S1 Parameters values and 95% confidence intervals for the best fit of the thymic output function to $CD4^+$ and $CD8^+$ single positive thymocytes from C57Bl/6 mice, CD70Tg-F12 mice and CD70Tg-F13 mice.

	C57Bl/6	CD70Tg-F12	CD70Tg-F13
CD4			
σ (x 10^7 cells)	3.459 (3.224 – 3.772)	4.555 (3.580 – 10.82)	4.741 (3.925 – 6.058)
v_1 (day^{-1})	0.084 (0.065 – 0.106)	0.032 (0.010 – 0.056)	0.056 (0.035 – 0.087)
CD8			
σ (x 10^7 cells)	1.679 (1.074 – 9.786)	0.995 (0.692 – 3.508)	241.8 (0.944 – 697.6)
v_1 (day^{-1})	0.023 (0.003 – 0.056)	0.041 (0.006 – 0.507)	0.000 (0.000 – 0.093)
Shared			
v_2 (day^{-1})	0.001 (0.001 – 0.002)	0.003 (0.002 – 0.004)	0.201 (0.097 – 0.415)
v_3 (day^{-1})	0.524 (0.187 – 2.961)	1.846 (0.132 – 36.71)	0.008 (0.003 – 0.012)
T_{off} (days)	46.88 (42.00 – 48.58)	50.21 (47.85 – 50.97)	37.79 (34.25 – 40.38)
γ	0.352 (0.313 – 0.384)	0.555 (0.437 – 0.652)	0.655 (0.579 – 0.760)

Supplementary Table S2 Parameters values and 95% confidence intervals for the best fit of the function $\psi(t)$ expressing the ratio of $CD4^+$ and $CD8^+$ naive T cells in PLN to those in the spleen for both control and thymectomized C57Bl/6, CD70Tg-F12 and CD70Tg-F13 mice.

	C57Bl/6	CD70Tg-F12	CD70Tg-F13
CD4			
α	1.578 (1.197 – 2.373)	3.880 (2.212 – 22.99)	3.500 (1.425 – 15.84)
x_1	0.098 (0.049 – 0.168)	0.064 (0.006 – 2.915)	0.0422 (0.007 – 0.202)
x_2	0.024 (0.020 – 0.030)	0.125 (0.055 – 0.480)	0
x_3	0	0	0.017 (0.013 – 0.022)
y_2	0.025 (0.016 – 0.0408)	0.025 (0.016 – 0.041)	0.004 (0.000 – 0.007)
y_3	0	0	0
z_1	0.723 (0.692 – 0.750)	0.929 (0.873 – 0.957)	0
z_2	0.679 (0.597 – 0.784)	0	0
ζ	14.00 (11.56 – 18.16)	40.16 (28.00 – 49.00)	14.00 (8.692 – 26.23)
CD8			
α	5.401 (1.399 – 18.58)	3.952 (2.304 – 30.90)	3.427 (1.441 – 13.34)
x_1	0.017 (0.004 – 0.096)	0.062 (0.005 – 2.961)	0.047 (0.009 – 0.322)
x_2	0.022 (0.015 – 0.030)	0.141 (0.066 – 0.810)	0
x_3	0	0	0.014 (0.009 – 0.021)
y_2	0.024 (0.011 – 0.054)	0.024 (0.011 – 0.054)	0.002 (0.000 – 0.006)
y_3	0	0	0
z_1	0.576 (0.523 – 0.618)	0.927 (0.877 – 0.959)	0
z_2	0.459 (0.363 – 0.589)	0	0
ζ	14.72 (11.56 – 19.50)	41.70 (33.98 – 49.00)	14.00 (7.442 – 33.70)

REFERENCE LIST

1. Pandrea,I., D.L.Sodora, G.Silvestri, and C.Apetrei. 2008. Into the wild: simian immunodeficiency virus (SIV) infection in natural hosts. *Trends Immunol.* 29:419-428.
2. Douek,D.C., M.Roederer, and R.A.Koup. 2008. Emerging Concepts in the Immunopathogenesis of AIDS. *Annu. Rev. Med.*
3. Paiardini,M., I.Frank, I.Pandrea, C.Apetrei, and G.Silvestri. 2008. Mucosal immune dysfunction in AIDS pathogenesis. *AIDS Rev.* 10:36-46.
4. Brenchley,J.M., D.A.Price, T.W.Schacker, T.E.Asher, G.Silvestri, S.Rao, Z.Kazzaz, E. Bornstein, O.Lambotte, D.Altmann, B.R.Blazar, B.Rodriguez, L.Teixeira-Johnson, A.Landay, J.N.Martin, F.M.Hecht, L.J.Picker, M.M.Lederman, S.G.Deeks, and D.C.Douek. 2006. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat. Med.* 12:1365-1371.
5. Dion,M.L., J.F.Poulin, R.Bordi, M.Sylvestre, R.Corsini, N.Kettaf, A.Dalloul, M.R.Boulassel, P.Debre, J.P.Routy, Z.Grossman, R.P.Sekaly, and R.Cheynier. 2004. HIV infection rapidly induces and maintains a substantial suppression of thymocyte proliferation. *Immunity.* 21:757-768.
6. Douek,D.C., R.D.McFarland, P.H.Keiser, E.A.Gage, J.M.Massey, B.F.Haynes, M.A.Polis, A.T.Haase, M.B.Feinberg, J.L.Sullivan, B.D.Jamieson, J.A.Zack, L.J.Picker, and R.A.Koup. 1998. Changes in thymic function with age and during the treatment of HIV infection. *Nature* 396:690-695.
7. Ribeiro,R.M. and R.J.de Boer. 2008. The contribution of the thymus to the recovery of peripheral naive T-cell numbers during antiretroviral treatment for HIV infection. *J. Acquir. Immune. Defic. Syndr.* 49:1-8.
8. Ho Tsong,F.R., A.D.Colantonio, and C.H.Uittenbogaart. 2008. The role of the thymus in HIV infection: a 10 year perspective. *AIDS* 22:171-184.
9. Day,C.L., D.E.Kaufmann, P.Kiepiela, J.A.Brown, E.S.Moodley, S.Reddy, E.W.Mackey, J.D. Miller, A.J.Leslie, C.DePierres, Z.Mncube, J.Duraiswamy, B.Zhu, Q.Eichbaum, M.Altfeld, E.J.Wherry, H.M.Coovadia, P.J.Goulder, P.Klenerman, R.Ahmed, G.J.Freeman, and B.D. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443:350-354.
10. Jones,R.B., L.C.Ndhlovu, J.D.Barbour, P.M.Sheth, A.R.Jha, B.R.Long, J.C.Wong, M. Satkunarajah, M.Schweneker, J.M.Chapman, G.Gyenes, B.Vali, M.D.Hyrcza, F.Y.Yue, C.Kovacs, A.Sassi, M.Loutfy, R.Halpenny, D.Persad, G.Spotts, F.M.Hecht, T.W.Chun, J.M. McCune, R.Kaul, J.M.Rini, D.F.Nixon, and M.A.Ostrowski. 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J. Exp. Med.* 205:2763-2779.
11. Kaufmann,D.E., D.G.Kavanagh, F.Pereyra, J.J.Zaunders, E.W.Mackey, T.Miura, S.Palmer, M.Brockman, A.Rathod, A.Piechocka-Trocha, B.Baker, B.Zhu, G.S.Le, M.T.Waring, R.Ahern, K.Moss, A.D.Kelleher, J.M.Coffin, G.J.Freeman, E.S.Rosenberg, and B.D.Walker. 2007. Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction. *Nat. Immunol.* 8:1246-1254.
12. Trautmann,L., L.Janbazian, N.Chomont, E.A.Said, S.Gimmig, B.Bessette, M.R.Boulassel, E.Delwart, H.Sepulveda, R.S.Balderas, J.P.Routy, E.K.Haddad, and R.P.Sekaly. 2006. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat. Med.* 12:1198-1202.
13. Velu,V., K.Titanji, B.Zhu, S.Husain, A.Pladevega, L.Lai, T.H.Vanderford, L.Chennareddi, G.Silvestri, G.J.Freeman, R.Ahmed, and R.R.Amara. 2009. Enhancing SIV-specific immunity *in vivo* by PD-1 blockade. *Nature* 458:206-210.
14. Nottet,H.S., S.J.van Dijk, E.B.Fanoy, I.W.Goedegebuure, D.de Jong, N.Vrisekoop, D.van Baarle, V.Boltz, S.Palmer, J.C.Borleffs, and C.A.Boucher. 2009. HIV-1 can persist in aged memory CD4+ T lymphocytes with minimal signs of evolution after 8.3 years of effective highly active antiretroviral therapy. *J. Acquir. Immune. Defic. Syndr.* 50:345-353.
15. Vrisekoop,N., R.van Gent, A.B.de Boer, S.A.Otto, J.C.Borleffs, R.Steingrover, J.M.Prins, T.W.Kuijpers, T.F.Wolfs, S.P.Geelen, I.Vulto, P.Lansdorp, K.Tesselaar, J.A.Borghans, and F.Miedema. 2008. Restoration of the CD4 T cell compartment after long-term highly active antiretroviral therapy without phenotypical signs of accelerated immunological aging. *J. Immunol.* 181:1573-1581.

16. Kovacs,J.A., M.Baseler, R.J.Dewar, S.Vogel, R.T.Davey, Jr., J.Falloon, M.A.Polis, R.E. Walker, R.Stevens, N.P.Salzman, and . 1995. Increases in CD4 T lymphocytes with intermittent courses of interleukin-2 in patients with human immunodeficiency virus infection. A preliminary study. *N. Engl. J. Med.* 332:567-575.
17. Kovacs,J.A., S.Vogel, J.A.Metcalf, M.Baseler, R.Stevens, J.Adelsberger, R.Lempicki, R.L. Hengel, I.Sereti, L.Lambert, R.L.Dewar, R.T.Davey, Jr., R.E.Walker, J.Falloon, M.A.Polis, H.Masur, and H.C.Lane. 2001. Interleukin-2 induced immune effects in human immunodeficiency virus-infected patients receiving intermittent interleukin-2 immunotherapy. *Eur. J. Immunol.* 31:1351-1360.
18. Napolitano,L.A., D.Schmidt, M.B.Gotway, N.Ameli, E.L.Filbert, M.M.Ng, J.L.Clor, L.Epling, E.Sinclair, P.D.Baum, K.Li, M.L.Killian, P.Bacchetti, and J.M.McCune. 2008. Growth hormone enhances thymic function in HIV-1-infected adults. *J. Clin. Invest* 118:1085-1098.
19. Sportes,C., F.T.Hakim, S.A.Memon, H.Zhang, K.S.Chua, M.R.Brown, T.A.Fleisher, M.C. Krumlauf, R.R.Babb, C.K.Chow, T.J.Fry, J.Engels, R.Buffet, M.Morre, R.J.Amato, D.J.Venzon, R.Korngold, A.Pecora, R.E.Gress, and C.L.Mackall. 2008. Administration of rhIL-7 in humans increases *in vivo* TCR repertoire diversity by preferential expansion of naive T cell subsets. *J. Exp. Med.* 205:1701-1714.
20. Tesselaar,K., R.Arens, G.M.van Schijndel, P.A.Baars, M.van der Valk, J.Borst, M.H.van Oers, and R.A.van Lier. 2003. Lethal T cell immunodeficiency induced by chronic costimulation via CD27-CD70 interactions. *Nat. Immunol.* 4:49-54.
21. Henderson,A.R. 2005. The bootstrap: a technique for data-driven statistics. Using computer-intensive analyses to explore experimental data. *Clin. Chim. Acta* 359:1-26.
22. Arens,R., K.Tesselaar, P.A.Baars, G.M.van Schijndel, J.Hendriks, S.T.Pals, P.Krimpenfort, J.Borst, M.H.van Oers, and R.A.van Lier. 2001. Constitutive CD27/CD70 interaction induces expansion of effector-type T cells and results in IFNgamma-mediated B cell depletion. *Immunity.* 15:801-812.
23. Berzins,S.P., D.I.Godfrey, J.F.Miller, and R.L.Boyd. 1999. A central role for thymic emigrants in peripheral T cell homeostasis. *Proc. Natl. Acad. Sci. U. S. A* 96:9787-9791.
24. Pido-Lopez,J., N.Imami, D.Andrew, and R.Aspinall. 2002. Molecular quantitation of thymic output in mice and the effect of IL-7. *Eur. J. Immunol.* 32:2827-2836.
25. Sempowski,G.D., M.E.Gooding, H.X.Liao, P.T.Le, and B.F.Haynes. 2002. T cell receptor excision circle assessment of thymopoiesis in aging mice. *Mol. Immunol.* 38:841-848.
26. Bennett,F., D.Luxenberg, V.Ling, I.M.Wang, K.Marquette, D.Lowe, N.Khan, G.Veldman, K.A.Jacobs, V.E.Valge-Archer, M.Collins, and B.M.Carreno. 2003. Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokine-driven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses. *J. Immunol.* 170:711-718.
27. Koenen,P.G., F.M.Hofhuis, M.A.Oosterwegel, and K.Tesselaar. 2007. T cell activation and proliferation characteristic for HIV-Nef transgenic mice is lymphopenia induced. *J. Immunol.* 178:5762-5768.
28. Felli,N., F.Pedini, A.Zeuner, E.Petrucci, U.Testa, C.Conticello, M.Biffoni, A.Di Cataldo, J.A.Winkles, C.Peschle, and R.De Maria. 2005. Multiple members of the TNF superfamily contribute to IFN-gamma-mediated inhibition of erythropoiesis. *J. Immunol.* 175:1464-1472.
29. Dufour,C., A.Corcione, J.Svahn, R.Haupt, V.Poggi, A.N.Beka'ssy, R.Scime, A.Pistorio, and V.Pistoia. 2003. TNF-alpha and IFN-gamma are overexpressed in the bone marrow of Fanconi anemia patients and TNF-alpha suppresses erythropoiesis *in vitro*. *Blood* 102: 2053-2059.
30. Arens,R., P.A.Baars, M.Jak, K.Tesselaar, M.van der Valk, M.H.van Oers, and R.A.van Lier. 2005. Cutting edge: CD95 maintains effector T cell homeostasis in chronic immune activation. *J. Immunol.* 174:5915-5920.
31. Vrisekoop,N., I.den Braber, A.B.de Boer, A.F.Ruiter, M.T.Ackermans, S.N.van der Crabben, E.H.Schrijver, G.Spierenburg, H.P.Sauerwein, M.D.Hazenberg, R.J.de Boer, F.Miedema, J.A.Borghans, and K.Tesselaar. 2008. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. *Proc. Natl. Acad. Sci. U. S. A* 105:6115-6120.

Chapter 7

General discussion

Knowledge about the life span and production rate of immune cells gives insight into the mechanisms by which these cells are maintained in healthy situations, and how infections may perturb the balance between production and death. In this thesis we investigated T-cell dynamics in mice of young and old age, and under conditions of chronic immune activation. In addition, the turnover of human T cells and granulocytes was estimated and compared with those in mice. The two most intriguing results are: 1. recent thymic emigrants may not exist as a separate population with its own dynamics apart from the resident naive T-cell pool, and 2. the maintenance of naive T cells is fundamentally different between mice and men. Here we discuss these findings and their possible implications in a broader context.

RTE characteristics

Since the establishment of thymus function in the early 1960s, maintenance of the naive T-cell pool has been regarded to occur via two mechanisms: thymic output of new naive T cells and self-renewal of already existing naive T cells in the periphery[1;2]. In case of haematopoietic stem cell transplantation, highly active antiretroviral therapy (HAART) of HIV-infected patients and other clinical situations of T-cell reconstitution, measurement of thymic output is useful as thymic output is the only source capable to generate a diverse T-cell repertoire. Quantification of thymic output can be performed by the detection of the number of newly exported T cells from the thymus that have not yet divided in the periphery, referred to as recent thymic emigrants (RTE). Frequencies of human RTE have been assessed by detection of T-cell receptor excision circles (TRECs), the stable episomal DNA circles that are formed during T-cell receptor (TCR) rearrangement[3]. However, as TRECs are long-lived and do not replicate upon peripheral self-renewal of naive T cells, the average TREC content of naive T cells is strongly influenced by proliferation and does not indicate how many cells have recently emigrated from the thymus[4;5]. Phenotypic identification of RTE would overcome this problem, as long as the proposed markers are down-regulated upon peripheral cell division of the thymic emigrants. Human $CD31^+$ $CD4^+$ naive, and human and murine $CD103^+$ $CD8^+$ naive T cells have been proposed to be enriched in RTE, as these cells have been shown to disappear after thymectomy and have a higher TREC content and/or TCR diversity compared to $CD31^-$ $CD4^+$ naive and $CD103^-$ $CD8^+$ naive T cells[6-8]. However, unique phenotypic markers to discriminate between RTE and resident naive T cells are only available in rats and chickens[9;10].

Characteristics of murine RTE could therefore only be acquired via invasive intrathymic injection with fluorescent dyes such as FITC and CFSE[11-14], and non-invasive detection of green fluorescent protein expressing thymic emigrants in Rag2p-GFP transgenic mice[15;16]. In this way it was demonstrated that murine RTE

efficiently home to secondary lymphoid organs and the small intestine[12]. In the periphery they undergo phenotypic maturation, increasing the surface expression of Qa2, IL-7Rα and CD28 and decreasing the expression of CD3 and CD24[15;16]. Moreover, RTE undergo functional maturation as the cells proliferate poorly upon *in vitro* stimulation, partially due to significantly lower IL-2 production than resident naive T cells[15;16].

The strongest argument for the existence of two separate populations within the naive T-cell pool came from thymus transplantation studies that suggested kinetic differences between RTE and resident naive T cells[13]. Engraftment of Ly5.2 mice with two neonatal Ly5.1 thymic lobes enabled long-term detection of thymic emigrants in the periphery. Comparison of the actual number of peripheral Ly5.1 RTE with the estimated number of exported thymocytes revealed that thymic emigrants that enter the full peripheral pool persist for at least three weeks[13]. After this period host Ly5.2 bone marrow cells reconstituted the graft thymus, which resulted in replacement of peripheral Ly5.1 thymic emigrants, most probably by new RTE. As the replacement rate of Ly5.1 cells was faster than that of Ly5.2 cells, this indicated that the average life span of RTE was shorter than that of resident naive T cells[13]. Transplantation of mice with six thymic lobes resulted in increased peripheral T-cell numbers that could be explained with the number of thymic emigrants exported per lobe in three weeks[17]. These data also suggested that the life span of RTE is only three weeks, considerably shorter than the average life span of naive $CD8^+$ T cells that was estimated to be two months[18], whereas naive T cells even can survive up to six months after thymectomy[19].

Thus, based on the phenotypic, functional and kinetic differences between RTE and resident naive T cells, the existence of two separate subpopulations within the murine naive T-cell pool became generally accepted.

RTE do not exhibit a different turnover rate compared to resident naive T cells

Searching for additional evidence for two naive T-cell populations with separate dynamics, we tested whether different ^2H_2O labeling periods reflected the kinetic heterogeneity in the naive T-cell pool, predicted by Asquith et al for heterogeneous populations[20]. According to this model, the labeled subpopulation does not reflect a representative sample of the total cell population, because it should be biased towards the subset with the fastest turnover. As a consequence, the estimated death rate of the labeled cell population (d^*) should depend on the length of the labeling period[20]. The longer the labeling period, the more slowly-turning-over cells will have picked up label, and hence the longer the estimated half-life ($1/d^*$) of the labeled cells. We found however, that short- and long-term labeling resulted in similar

estimated death rates of murine naive T cells, suggesting that this pool is homogeneous in a kinetic sense (Chapter 4).

In addition, despite the fact that only part of the total naive T-cell pool was labeled, the death rates of labeled naive T cells were similar to their average production rates, *p* (Chapter 4 and 5), again suggesting that naive T cells form a homogeneous population.

In contrast, the biphasic loss of naive T cells after thymectomy of adult mice seems consistent with the existence of both a fast and a slow-turning over population. However, these data can readily be explained without separate RTE dynamics, simply by the introduction of density-dependent death, increasing the death rate at high cell densities (Chapter 5). If we use our density-dependent death model lacking separate RTE dynamics to simulate Berzins' transplantation studies[13;17], and use the parameter estimates obtained in Chapter 5, we can perfectly predict the observed increase in peripheral T cells after engraftment with two, six or nine ectopic thymus lobes (data not shown).

In line with the existence of two separate populations, our estimated life span of the average population, differed from the predicted life span of RTE. Based on ^2H_2O labeling, murine naive $CD4^+$ and naive $CD8^+$ T cells were estimated to live respectively 48 and 83 days on average (Chapter 4), and RTE have been proposed to live only 3 weeks[13;17]. However, we showed that proliferation of naive T cells is negligible in healthy mice[21] (Chapter 5), what suggests that the vast majority of labeled naive T cells represents thymic emigrants. We indeed found that ^2H_2O enrichment in thymectomized mice was significantly reduced as compared to age-matched euthymic controls (Chapter 2). The different estimated RTE life spans that were obtained via thymus engraftment[13] and ^2H_2O labeling (Chapter 4) could be due to experimental approach. The observed short life span of RTE could be age dependent as thymus transplantation was performed at the age of six weeks[13;17] at the peak of T-cell densities when the death rate could be maximal (Chapter 5). We therefore predict that if thymus transplantations were performed at a later time point with lower T-cell numbers in the recipient mice, the estimated average life span of RTE would be longer (Chapter 5). Alternatively, isolation of the neonatal thymic lobes in combination with temporal deficiency of blood supply might have reduced the life span of the resultant Ly5.1 thymic emigrants, erroneously interpreted as evidence for a fast turning over RTE pool compared to the non-manipulated peripheral naive T cells.

Based on the kinetic homogeneity of the murine naive T-cell pool, and the negligible self-renewal of these cells, we postulate that the average survival probabilities of RTE and resident naive T cells are similar. Since naive T-cell survival is not only dependent on interleukin-7 (IL-7)[22;23], but also on self-peptide MHC complexes[24;25] this age-independent substitution of peripheral naive T cells by thymic emigrants

allows competition for clone-specific survival signals which would maximize TCR diversity of the whole repertoire.

Human naive T-cell dynamics is essentially different from that in mice

In C57Bl/6 mice almost all naive T cells represent thymic emigrants (Chapter 5). The observation that these newly produced cells live as long as the average murine naive T cell in the periphery differs from the situation in human adults were newly produced naive T cells tend to live longer than the average naive T cell (Chapter 2). This suggests that homeostatic mechanisms of naive T cells differ between adult mice and men.

In both murine (Chapter 2 and data not shown) and human newborns, proliferation of naive T cells plays a role in filling the developing naive T-cell pool, as the percentage of $Ki67^+$ cells within the naive T-cell population in neonates is up to 10-fold higher than that in young adults[26]. Despite the increase in total body T-cell numbers from birth to puberty, the absolute TREC number is stable in healthy children from eight months until 16 year of age, suggesting that expansion of the naive T-cell pool after birth is not only due to thymic production, but also highly dependent on self-renewal of peripheral naive T cells[27]. However, whereas the fraction of proliferating cells rapidly decreases and is negligible in adult mice (Chapter 5), peripheral proliferation of human naive T cells seems essential in human adults, demonstrated by an age-dependent decline in TREC content and telomere shortening in the human ($CD31^+$) naive T-cell pool between 20 and 70 years of age[28;29].

Thymic involution in humans starts one year after birth, as demonstrated by an overall decrease in cellularity of the organ and a replacement of the perivascular spaces with fat[30-32]. Nevertheless, several lines of evidence suggest that the human thymus remains active to some extent during adult life: i. The frequency of thymocyte subsets in middle-aged individuals is comparable to that in young persons, suggesting normal thymocyte differentiation[30]. ii. Treatment of HIV patients with HAART causes an age-dependent increase in naive T cells[33]. In addition, long-term HAART results in normalized total $CD4^+$ T-cell TREC numbers, suggesting that also in adults thymic output is the source of the observed increase in naive T-cell counts after therapy[34;35]. iii. Autologous stem cell transplantation results in a progressive increase of $CD31^+$ naive $CD4^+$ T cells, coinciding with an increased TREC content[36]. iv. Thymectomy of adults results in reduced TREC contents[3].

Despite the fact that no unambiguous RTE marker is present, an upper estimate for thymic output in humans was calculated by Vrisekoop et al. by measuring the TREC content in $CD31^+$ naive $CD4^+$ T cells in individuals of different ages. In this way it was calculated that at most 10% of naive $CD4^+$ T cells is derived from thymic output in adulthood (Vrisekoop et al, manuscript in preparation). Recently protein tyrosine

kinase 7 (PTK7) was proposed as a possible marker for human $CD4^+$ RTE as $PTK7^+$ $CD31^+$ naive $CD4^+$ T cells show constant TREC contents during ageing and disappear rapidly after thymectomy[37]. Perfectly matching with the calculated maximal percentage RTE in the human naive T-cell pool by Vrisekoop et al, $PTK7^+$ $CD31^+$ $CD4^+$ naive T cells constitute only 10% of the adult $CD4^+$ naive T-cell pool in young adults. As the stability of PTK7 expression is unknown, but the marker is lost upon cell division, this suggests that maximally 90% of the naive T-cell pool has undergone cell division[37].

The important role for self-renewal in the maintenance of the human naive T-cell population is in sharp contrast to our finding that thymic output in mice accounts for almost all naive T-cell production, whereas proliferation of murine naive T cells is negligible (Chapter 5 and Fig.1).

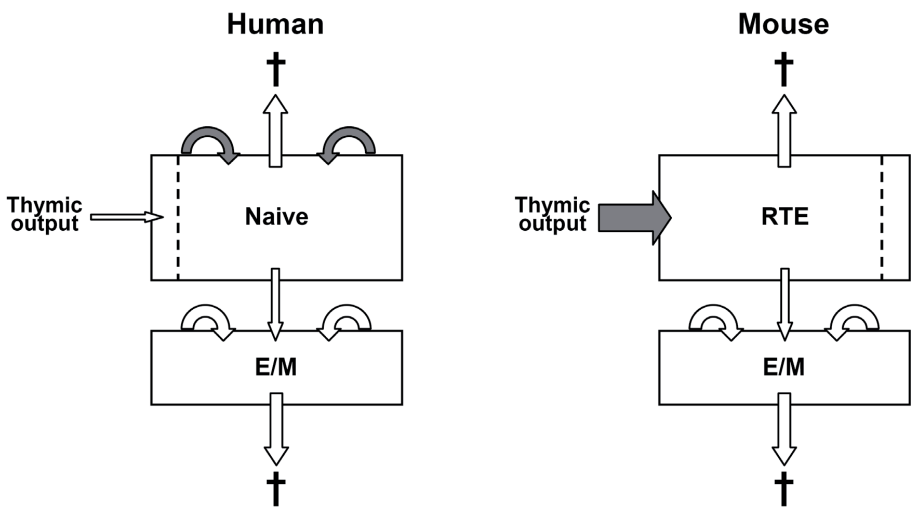


Figure 1 General model of T-cell maintenance in adult mice and men. The majority of the human naive T-cell pool is maintained via peripheral proliferation. In contrast, the size of the murine naive T-cell pool is largely determined by the level of thymic output, and self-renewal of naive T cells is negligible. In humans recently produced naive T cells tend to live longer than the average naive T cell in the peripheral pool. Death rates of RTE and resident naive T cells are however equal in mice, suggesting a comparable chance to die or being activated to differentiate into effector/memory (E/M) T cells. Maintenance of the effector/memory T-cell population is in both cases mainly dependent on peripheral self-renewal.

Possible reasons for different mechanisms of naive T-cell homeostasis between mice and men

Why is the mechanism of naive T-cell maintenance fundamentally different between the two species? Is it due to a scaling effect from mice to men? Reflects it a

functional advantage in either species? Or would the difference be due to a difference between clean laboratory mice and men living in natural conditions?

Scaling: Body size is associated with numerous variables, such as heart beat, capillary volume, lung function, metabolic rate, life span, that all scale differently between species[38;39]. As cellular life span, thymic involution and peripheral self-renewal of naive T cells all scale in a different way from mice to men (Table I), this will automatically result in qualitative differences in mechanisms of naive T-cell maintenance, due to the distinct life span of the cells or the species. For example: thymic involution in humans is four times faster than in mice, if scaled to life span differences between mice and men.
The 40-fold increased turnover of naive T cells in humans, one to one scaled with the average life span of mice and men, is in marked contrast with the observation that estimated granulocyte life span is only 7-fold higher in men compared to mice (Chapter 3). It is reasonable that there is a minimal life span for a cell to be able to exert its function, but the reason of the relatively short survival time of human granulocytes is unknown. Apparently, every cell type scales in a unique way.

Table I Comparison of estimated parameters in adult mice and men.

		Human	Mouse	Scaling (H/M)	Relative scaling[b]
Thymic involution		5% / year	50% / year	0.1-fold	4-fold
Cell division	Naive CD4	unknown[a]	<0.9 / year	–	–
	Naive CD8	unknown	<1.5 / year	–	–
Average life span	Naive CD4	2189 days	48 days	46-fold	1.1-fold
	Naive CD8	3425 days	83 days	41-fold	1.0-fold
	Granulocytes	9 days	1.2 days	7-fold	0.2-fold
	Species	80 years	2 years	40-fold	–

[a] Dutilh and De Boer calculated that only one cell division in 50% of the naive T cells was sufficient to account for the observed decline in TREC content of human naive T cells during ageing[28].
[b] Scaling relative to the average life span of the species.

Functional advantage: From an efficiency point of view, naive T-cell production by the thymus is relatively "expensive" as more than 90% of the formed thymocytes die during the process of positive and negative selection[40]. On the other hand, thymopoiesis is the only mechanism of creating new TCR specificities, whereas peripheral self-renewal can only increase the clonal size of specific T-cell specificities. Mice have a 14-fold lower TCR-diversity as compared to humans (Table II). As there is no evidence that small species are exposed to less pathogens than big organisms, continued thymic output in mice may compensate for this reduced diversity.

Table II Estimated T-cell counts, αβ-TCR diversity and clonal size in adult mice and men.

		Cell counts[a 41]	# αβ TCRs[41;42]	Clone size[41;42]	Life span (days)[b 43]
Human	Naive CD4	$2\ 10^{11}$	$\geq 2.5\ 10^{7}$	10^{3}	2189
	E/M CD4		$2\ 10^{5}$	variable	224
	Naive CD8	$1\ 10^{11}$	$\geq 2.5\ 10^{7}$	10^{3}	3425
	E/M CD8		$2\ 10^{5}$	variable	352
Murine	Naive CD4	$75\ 10^{6}$	$1.8\ 10^{6}$	<50	48
	E/M CD4	$30\ 10^{6}$	–	variable	9-28
	Naive CD8	$50\ 10^{6}$	$1.8\ 10^{6}$	<50	83
	E/M CD8	$20\ 10^{6}$	–	variable	11-45

[a] Murine cell counts = 5 x T-cell counts from Chapter 5, assuming that 20% of total T cells reside in the spleen[42].
[b] Life span estimates derived from Chapter 2 and 4.

Interestingly, more than 75% of the TCRβ chains of naive $CD8^+$ T cells have been shown to be unique in genetically identical non-immunized mice[44], suggesting that the potential repertoire is much larger than that present in the murine peripheral T-cell pool. This implies that a daily thymus output of $0.54\ 10^6$ cells in mice (Chapter 5) might considerably influence the actual repertoire that is available at any time point. Whether the total potential repertoire is present in the human peripheral T-cell pool is unknown. It would be interesting to test if the repertoire of an individual at different ages changes during aging, or whether identical twins have unique TRCs. In any case thymic output in mice might add to the TCR diversity that is present during the course of infection. However, the duration of antigen presentation during infection with *Listeria monocytogenes* is restricted to three days, despite the continued persistence of viable bacteria[45]. Influenza-derived antigen presentation persists for more than three weeks after viral clearance, recruiting antigen-specific naive $CD4^+$ T cells to the effector/memory compartment [46]. These divergent results suggest that the duration of antigen-presentation might be pathogen-specific. Even in case of a limited priming time of three days, approximately $3 \times 0.6\ 10^6 = 1.8\ 10^6$ RTE can be produced (Chapter 5). Based on the 75% unique TCRs that are present in genetically identical mice[44], this would correspond to $0.75 \times 1.8\ 10^6 = 1.4\ 10^6$ additional T-cell specificities, that might contribute to the immune response.

Pathogen pressure: One could argue that the difference in the mechanism of naive T-cell maintenance might be artificial, since laboratory mice that are kept under specific pathogen free conditions were compared with healthy humans that are exposed to a high pathogen pressure. Increasing the activation status using transgenic mice, might partly solve this issue. However, after introduction of chronic

immune activation in CD70Tg mice, where peripheral self-renewal of the T cells was increased, proliferation of activated naive T cells resulted in effector/memory formation instead of maintenance of the murine naive T-cell pool (Chapter 6).
Thus, because in both young, old, thymectomized and chronically immune activated mice, self-renewal plays only an insignificant role (Chapter 4, 5 and 6), laboratory mice, that are worldwide used as a model to study naive T cell dynamics in men, are not a good model to study naive T-cell homeostasis in humans.

REFERENCE LIST

1. Almeida,A.R., J.A.Borghans, and A.A.Freitas. 2001. T cell homeostasis: thymus regeneration and peripheral T cell restoration in mice with a reduced fraction of competent precursors. *J. Exp. Med.* 194:591-599.
2. Miller,J.F. 2004. Events that led to the discovery of T-cell development and function--a personal recollection. *Tissue Antigens* 63:509-517.
3. Douek,D.C., R.D.McFarland, P.H.Keiser, E.A.Gage, J.M.Massey, B.F.Haynes, M.A.Polis, A.T.Haase, M.B.Feinberg, J.L.Sullivan, B.D.Jamieson, J.A.Zack, L.J.Picker, and R.A.Koup. 1998. Changes in thymic function with age and during the treatment of HIV infection. *Nature* 396:690-695.
4. Hazenberg,M.D., S.A.Otto, J.W.Cohen Stuart, M.C.Verschuren, J.C.Borleffs, C.A.Boucher, R.A.Coutinho, J.M.Lange, T.F.Rinke de Wit, A.Tsegaye, J.J.van Dongen, D.Hamann, R.J.de Boer, and F.Miedema. 2000. 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.* 6:1036-1042.
5. Hazenberg,M.D., J.A.Borghans, R.J.de Boer, and F.Miedema. 2003. Thymic output: a bad TREC record. *Nat. Immunol.* 4:97-99.
6. Kimmig,S., G.K.Przybylski, C.A.Schmidt, K.Laurisch, B.Mowes, A.Radbruch, and A.Thiel. 2002. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. *J. Exp. Med.* 195:789-794.
7. McFarland,R.D., D.C.Douek, R.A.Koup, and L.J.Picker. 2000. Identification of a human recent thymic emigrant phenotype. *Proc. Natl. Acad. Sci. U. S. A* 97:4215-4220.
8. Staton,T.L., B.Johnston, E.C.Butcher, and D.J.Campbell. 2004. Murine CD8+ recent thymic emigrants are alphaE integrin-positive and CC chemokine ligand 25 responsive. *J. Immunol.* 172:7282-7288.
9. Hosseinzadeh,H. and I.Goldschneider. 1993. Recent thymic emigrants in the rat express a unique antigenic phenotype and undergo post-thymic maturation in peripheral lymphoid tissues. *J. Immunol.* 150:1670-1679.
10. Kong,F., C.H.Chen, and M.D.Cooper. 1998. Thymic function can be accurately monitored by the level of recent T cell emigrants in the circulation. *Immunity.* 8:97-104.
11. Scollay,R., W.F.Chen, and K.Shortman. 1984. The functional capabilities of cells leaving the thymus. *J. Immunol.* 132:25-30.
12. Staton,T.L., A.Habtezion, M.M.Winslow, T.Sato, P.E.Love, and E.C.Butcher. 2006. CD8+ recent thymic emigrants home to and efficiently repopulate the small intestine epithelium. *Nat. Immunol.* 7:482-488.
13. Berzins,S.P., R.L.Boyd, and J.F.Miller. 1998. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. *J. Exp. Med.* 187:1839-1848.
14. Graziano,M., Y.St-Pierre, C.Beauchemin, M.Desrosiers, and E.F.Potworowski. 1998. The fate of thymocytes labeled *in vivo* with CFSE. *Exp. Cell Res.* 240:75-85.
15. Boursalian,T.E., J.Golob, D.M.Soper, C.J.Cooper, and P.J.Fink. 2004. Continued maturation of thymic emigrants in the periphery. *Nat. Immunol.* 5:418-425.
16. Hale,J.S., T.E.Boursalian, G.L.Turk, and P.J.Fink. 2006. Thymic output in aged mice. *Proc. Natl. Acad. Sci. U. S. A* 103:8447-8452.
17. Berzins,S.P., D.I.Godfrey, J.F.Miller, and R.L.Boyd. 1999. A central role for thymic emigrants in peripheral T cell homeostasis. *Proc. Natl. Acad. Sci. U. S. A* 96:9787-9791.
18. von Boehmer,H. and K.Hafen. 1993. The life span of naive alpha/beta T cells in secondary lymphoid organs. *J. Exp. Med.* 177:891-896.
19. Di Rosa,F., S.Ramaswamy, J.P.Ridge, and P.Matzinger. 1999. On the life span of virgin T lymphocytes. *J. Immunol.* 163:1253-1257.
20. Asquith,B., C.Debacq, D.C.Macallan, L.Willems, and C.R.Bangham. 2002. Lymphocyte kinetics: the interpretation of labeling data. *Trends Immunol.* 23:596-601.
21. Parretta,E., G.Cassese, A.Santoni, J.Guardiola, A.Vecchio, and F.Di Rosa. 2008. Kinetics of *in vivo* proliferation and death of memory and naive CD8 T cells: parameter estimation based on 5-bromo-2'-deoxyuridine incorporation in spleen, lymph nodes, and bone marrow. *J. Immunol.* 180:7230-7239.

22. Schluns,K.S., W.C.Kieper, S.C.Jameson, and L.Lefrancois. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells *in vivo*. *Nat. Immunol.* 1:426-432.
23. Tan,J.T., E.Dudl, E.LeRoy, R.Murray, J.Sprent, K.I.Weinberg, and C.D.Surh. 2001. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc. Natl. Acad. Sci. U. S. A* 98:8732-8737.
24. Takeda,S., H.R.Rodewald, H.Arakawa, H.Bluethmann, and T.Shimizu. 1996. MHC class II molecules are not required for survival of newly generated CD4+ T cells, but affect their long-term life span. *Immunity.* 5:217-228.
25. Tanchot,C., F.A.Lemonnier, B.Perarnau, A.A.Freitas, and B.Rocha. 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science* 276: 2057-2062.
26. Schonland,S.O., J.K.Zimmer, C.M.Lopez-Benitez, T.Widmann, K.D.Ramin, J.J.Goronzy, and C.M.Weyand. 2003. Homeostatic control of T-cell generation in neonates. *Blood* 102:1428-1434.
27. Hazenberg,M.D., S.A.Otto, A.M.van Rossum, H.J.Scherpbier, R.de Groot, T.W.Kuijpers, J.M.Lange, D.Hamann, R.J.de Boer, J.A.Borghans, and F.Miedema. 2004. Establishment of the CD4+ T-cell pool in healthy children and untreated children infected with HIV-1. *Blood* 104:3513-3519.
28. Dutilh,B.E. and R.J.de Boer. 2003. Decline in excision circles requires homeostatic renewal or homeostatic death of naive T cells. *J. Theor. Biol.* 224:351-358.
29. Kilpatrick,R.D., T.Rickabaugh, L.E.Hultin, P.Hultin, M.A.Hausner, R.Detels, J.Phair, and B.D.Jamieson. 2008. Homeostasis of the naive CD4+ T cell compartment during aging. *J. Immunol.* 180:1499-1507.
30. Bertho,J.M., C.Demarquay, N.Moulian, M.A.Van der, S.Berrih-Aknin, and P.Gourmelon. 1997. Phenotypic and immunohistological analyses of the human adult thymus: evidence for an active thymus during adult life. *Cell Immunol.* 179:30-40.
31. Weerkamp,F., E.F.de Haas, B.A.Naber, W.M.Comans-Bitter, A.J.Bogers, J.J.van Dongen, and F.J.Staal. 2005. Age-related changes in the cellular composition of the thymus in children. *J. Allergy Clin. Immunol.* 115:834-840.
32. Steinmann,G.G., B.Klaus, and H.K.Muller-Hermelink. 1985. The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study. *Scand. J. Immunol.* 22:563-575.
33. Cohen,S.J., D.Hamann, J.Borleffs, M.Roos, F.Miedema, C.Boucher, and R.J.de Boer. 2002. Reconstitution of naive T cells during antiretroviral treatment of HIV-infected adults is dependent on age. *AIDS* 16:2263-2266.
34. Ribeiro,R.M. and R.J.de Boer. 2008. The contribution of the thymus to the recovery of peripheral naive T-cell numbers during antiretroviral treatment for HIV infection. *J. Acquir. Immune. Defic. Syndr.* 49:1-8.
35. Vrisekoop,N., R.van Gent, A.B.de Boer, S.A.Otto, J.C.Borleffs, R.Steingrover, J.M.Prins, T.W.Kuijpers, T.F.Wolfs, S.P.Geelen, I.Vulto, P.Lansdorp, K.Tesselaar, J.A.Borghans, and F.Miedema. 2008. Restoration of the CD4 T cell compartment after long-term highly active antiretroviral therapy without phenotypical signs of accelerated immunological aging. *J. Immunol.* 181:1573-1581.
36. Thiel,A., T.Alexander, C.A.Schmidt, G.K.Przybylski, S.Kimmig, S.Kohler, H.Radtke, E. Gromnica-Ihle, G.Massenkeil, A.Radbruch, R.Arnold, and F.Hiepe. 2008. Direct assessment of thymic reactivation after autologous stem cell transplantation. *Acta Haematol.* 119:22-27.
37. Haines,C.J., T.D.Giffon, L.S.Lu, X.Lu, M.Tessier-Lavigne, D.T.Ross, and D.B.Lewis. 2009. Human CD4+ T cell recent thymic emigrants are identified by protein tyrosine kinase 7 and have reduced immune function. *J. Exp. Med.* 206:275-285.
38. West,G.B., J.H.Brown, and B.J.Enquist. 1997. A general model for the origin of allometric scaling laws in biology. *Science* 276:122-126.
39. Nevill,A.M., S.Bate, and R.L.Holder. 2005. Modeling physiological and anthropometric variables known to vary with body size and other confounding variables. *Am. J. Phys. Anthropol.* Suppl 41:141-153.
40. Scollay,R.G., E.C.Butcher, and I.L.Weissman. 1980. Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur. J. Immunol.* 10:210-218.

41. Arstila,T.P., A.Casrouge, V.Baron, J.Even, J.Kanellopoulos, and P.Kourilsky. 1999. A direct estimate of the human alphabeta T cell receptor diversity. *Science* 286:958-961.
42. Casrouge,A., E.Beaudoing, S.Dalle, C.Pannetier, J.Kanellopoulos, and P.Kourilsky. 2000. Size estimate of the alpha beta TCR repertoire of naive mouse splenocytes. *J. Immunol.* 164:5782-5787.
43. Vrisekoop,N., I.den Braber, A.B.de Boer, A.F.Ruiter, M.T.Ackermans, S.N.van der Crabben, E.H.Schrijver, G.Spierenburg, H.P.Sauerwein, M.D.Hazenberg, R.J.de Boer, F.Miedema, J.A.Borghans, and K.Tesselaar. 2008. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. *Proc. Natl. Acad. Sci. U. S. A* 105:6115-6120.
44. Bousso,P., A.Casrouge, J.D.Altman, M.Haury, J.Kanellopoulos, J.P.Abastado, and P. Kourilsky. 1998. Individual variations in the murine T cell response to a specific peptide reflect variability in naive repertoires. *Immunity.* 9:169-178.
45. Wong,P. and E.G.Pamer. 2003. Feedback regulation of pathogen-specific T cell priming. *Immunity.* 18:499-511.
46. Jelley-Gibbs,D.M., D.M.Brown, J.P.Dibble, L.Haynes, S.M.Eaton, and S.L.Swain. 2005. Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. *J. Exp. Med.* 202:697-706.

Samenvatting voor niet-ingewijden

Het immuunsysteem: het leger en zijn onderdelen

Het lichaam van elk individu kan vergeleken worden met een land dat elke dag, gewild of ongewild, vreemden binnenlaat. Deze binnendringers kunnen goede of kwade bedoelingen hebben. Zo kunnen virussen, bacteriën, parasieten en schimmels ziekten veroorzaken, terwijl ons dagelijkse voedsel juist zorgt voor instandhouding van ons lichaam. De huid fungeert als eerste verdedigingslinie om de ziekteverwekkers (pathogenen) buiten het lichaam te houden. Wordt deze linie echter doorbroken, dan is het de taak van het afweersysteem (immuunsysteem) om de binnengedrongen ziekteverwekkers onschadelijk te maken. Voor het uitvoeren van deze taak zijn twee verschillende legers aan cellen beschikbaar. Het eerste leger (het innate immuunsysteem) slaat er direct min of meer onbesuisd op los, zodra het een vermeende afwijking ziet. Dit leger baant de weg voor het tweede leger (het adaptieve immuunsysteem). Dit adaptieve immuunsysteem wordt aangestuurd door het innate systeem (het eerste leger) en is enorm specifiek: het onderzoekt op grond van de signalen van de eerste leger welke strategie het beste op de vijand los gelaten kan worden en slaat dan (enige dagen later) ongenadig toe. Beide legers zijn dus nauw aan elkaar verbonden en door hun verschillende manier van aanpak wordt het lichaam optimaal beschermd.

Naast deze grove indeling kan het afweersysteem nog verder onderverdeeld worden. Zoals een nationaal leger bestaat uit onderscheiden legereenheden, zo bestaat ook het immuunsysteem uit verschillende divisies, die elk hun eigen functie hebben: spionnen, generaals, tankdivisies en hulptroepen zijn allemaal vertegenwoordigd. In een gezonde situatie worden er geen hele grote gevechten geleverd, en bestaat de belangrijkste taak van het leger om door het lichaam te patrouilleren, om te controleren of alles in het land nog onder controle is. Ziekteverwekkers die gesnapt worden, worden opgegeten en afgebroken door vuilnismannen (granulocyten), veelvraten (macrofagen) en spionnen (dendritische cellen): opgeruimd staat netjes. Dendritische cellen hebben daarnaast nog de functie om het adaptieve afweersysteem (het tweede leger) te activeren. Binnen in de cellen worden de ziekteverwekkers in mootjes (peptiden) gehakt. Het zo ontstane restafval wordt op houdertjes geplakt (de zogeheten MHCs) die vervolgens op het celopppervlak geëxposeerd worden. Behalve lichaamsvreemde elementen, worden ook lichaamseigen elementen in stukjes geknipt, in de MHC-houdertjes gestopt en op het celoppervlak getoond. Via de MHC-houdertjes kan een cel dus aan de buitenkant laten zien wat hij in huis heeft. Controleurs (T cellen) controleren cellen voortdurend op afwijkingen. Voor deze controle maken ze gebruik van een voelhoorn (T-cel receptor) die voor elke T cel anders is. Als de cel alleen lichaamseigen mootjes laat zien, is er niets aan de hand en wordt de cel met rust gelaten. Maar zodra de controleurs afwijkende cellen (geïnfecteerde cellen of

tumorcellen) ontdekt hebben, komt er een afweerreactie op gang en wordt de cel vernietigd.

De thymus: T cellen in opleiding

Om hun controlerende taak goed te kunnen uitvoeren, krijgen T cellen een gedegen opleiding in de thymus (zwezerik). Om te slagen voor de opleiding krijgen de studenten T cellen (thymocyten) één opdracht: maak een voelhoorn (T cel receptor) waarmee onderscheid gemaakt kan worden tussen lichaamsvreemde en lichaamseigen bouwstenen, zodat je weet wie aangevallen moet vallen en wie niet. Het volbrengen van deze opdracht is een zaak van leven en dood. Als de voelhoorn van de studenten T cellen namelijk niet aan het beoordelingscriterium voldoet, krijgen de cellen geen herkansing, maar worden ze direct gedood. Het belang van deze opdracht is duidelijk: een T cel die geen onderscheid kan maken tussen lichaamseigen en lichaamsvreemd, zou het eigen lichaam aanvallen in plaats van ziekteverwekkers. Dit is het geval bij autoimmuunziekten, waarbij het lichaam zijn eigen cellen afbreekt.

Voor het maken van de voelhoorn (T cel receptor) krijgen de studenten T cellen (thymocyten) een grote bouwdoos, die gevuld is met voelhoornonderdelen, een schaar en plakmiddel. Hiermee kan naar hartelust geknipt en geplakt worden. De onderdelen zijn allemaal aan elkaar geplakt en netjes gerangschikt op V-, D- en J-onderdelen. Iedere student T cel kan een voelhoorn maken door een willekeurige V, een willekeurige D en een willekeurige J aan elkaar plakken en de tussenliggende gedeelten eruit te knippen. Als de student de T cel receptor stap voor stap gemaakt heeft en in veelvoud op zijn oppervlak geplaatst heeft, kan de voelhoorn, en daarmee dus de student T cel (thymocyt), getest worden op zijn bruikbaarheid.

De selectie van geschikte kandidaten vindt plaats in twee fasen. Allereerst is er een positieve selectie, waarbij de voelhoorn moet kunnen binden met lichaamseigen bouwstenen (MHC) op thymuscellen. De studenten T cellen (thymocyten) die hier niet toe in staat zijn, gaan dood, de anderen gaan door naar de tweede ronde. Bij de tweede selectieronde (negatieve selectie) wordt getest of de cellen niet té sterk reageren als hun voelhoorn bindt aan het lichaamseigen bouwstenen (MHC). De workaholics die hyperreactief zijn (en dus het gevaar lopen om té snel alarm te slaan en het eigen lichaam aan te vallen), worden eveneens uitgeschakeld en sterven een vroegtijdige dood. Slechts ongeveer 2-5% van de cellen voldoet aan de gestelde eisen en komt als een rijpe T cel in de bloedbaan terecht. Deze manier van selecteren lijkt erg inefficiënt, maar is van essentieel belang. Omdat elke persoon zijn eigen set aan lichaamseigen bouwstenen (MHC-houdertjes) heeft, kan op deze wijze namelijk voorkomen worden dat de T cellen gezonde cellen aanvallen. Daarbij komt dat als er compleet nieuwe virussen ontstaan gedurende de geschiedenis

(denk aan HIV en SARS), de mogelijkheid er is om ook tegen deze nieuwe ziekteverwekkers een afweerreactie op gang te brengen.

Circulatie: T cellen op patrouille

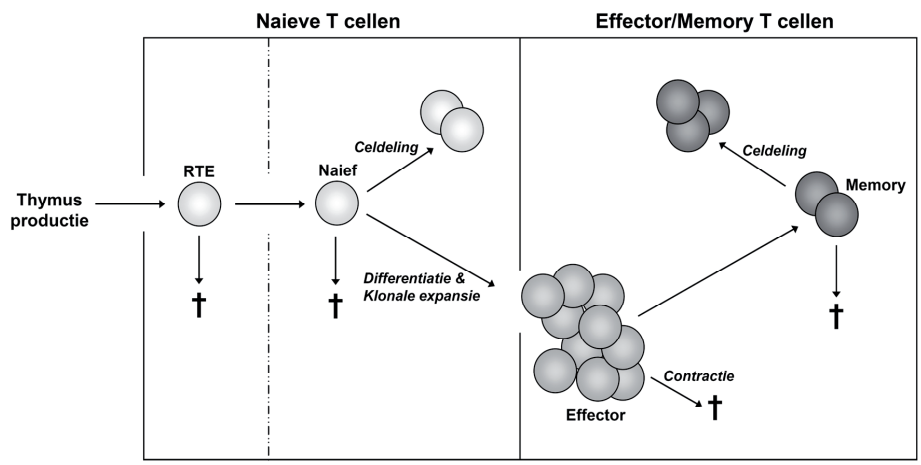
De T cellen die de vijand nog nooit herkend hebben, worden naïeve T cellen genoemd. Een gedeelte van deze cellen heeft kortgeleden de thymus verlaten, en wordt daarom ook nog afzonderlijk aangeduid als Recente Thymus Emigranten (RTE) genoemd (Fig. 1). De naïeve T cellen patrouilleren via het bloed en de lymfe door het lichaam: op zoek naar de vijand. In plaats dat een T cel verschillende voelhoorns (T celreceptoren) op zijn oppervlakte heeft, en dus verschillende ziekteverwekkers kan herkennen, heeft iedere afzonderlijke T cel zijn eigen specifieke voelhoorn op het oppervlak. Als gevolg daarvan kan elke T cel slechts één onderdeeltje van een ziekteverwekker herkennen. Met andere woorden: een T cel die een stukje van het griepvirus herkent, zal geen onderdelen van het mazelenvirus herkennen. Om alle bestaande ziekteverwekkers te kunnen herkennen, moeten er dus miljoenen T cel varianten zijn (T cel diversiteit). Omdat er een beperkte ruimte beschikbaar is in het lichaam, bestaat er van elke T cel variant slechts een relatief klein aantal cellen. Hoe vinden die minuscuul kleine cellen hun specifieke vijand in een land dat miljoenen keren groter is? De vraag dringt temeer gezien het feit dat ziekteverwekkers het lichaam kunnen binnendringen via verschillende routes en overal een infectie kunnen veroorzaken, terwijl T cellen normaal alleen in het bloed, de lymfe en de lymfoïde organen (milt, lymfeknopen) voorkomen. De oplossing is even eenvoudig als doeltreffend: dendritische cellen (spionnen) verhuizen van de plaats van infectie via de lymfevaten naar de lymfeknopen, waar ze vastgehouden worden. Tijdens de reis, die ongeveer een dag duurt, promoveren de dendritische cellen tot generaals, zodat ze het leger optimaal kunnen activeren en aansturen. Ook T cellen verplaatsen zich continu door het lichaam en komen via de bloedbaan eveneens in de lymfeknopen terecht. Zodoende hoeven de T cellen alleen deze strategische posities te scannen om de gezondheid van het lichaam te controleren en wordt de trefkans tussen de T cel en zijn specifieke ziekteverwekker enorm vergroot. In de lymfeknopen zoeken de T cellen gedurende enige uren vele dendritische cellen (generaals) op, om te kijken of er een dendritische cel is met een MHC houdertje met restafval dat precies past op de voelhoorn van de T cel (zoals een sleutel past in een slot). Is dat niet het geval, dan verlaten de T cellen de lymfeknopen en circuleren ze verder om na enige minuten weer in de volgende lymfeknoop aan te landen, waar het zoekproces vervolgd wordt.

De immuunrespons: Ten aanval!

Als een naïeve T cel zijn specifieke vijand-onderdeeltje vindt, wordt de naïeve T cel, die tot dan toe op non-actief stond, geactiveerd. Om te zorgen dat alle neuzen dezelfde kant opstaan, communiceren de generaals (dendritische cellen) en de T cellen met elkaar en met andere legerdivisies via signaalstofjes (cytokines) die fungeren als postduiven, morse-tekens of e-mailtjes. Zodoende verloopt het gevecht volgens één duidelijke strategie en zitten de divisies elkaar niet in het vaarwater.

De grote diversiteit aan T cellen zorgt ervoor dat er allicht een aantal varianten aanwezig zijn die een binnengedrongen bacterie of virus zullen herkennen. Het aantal van die betreffende T cellen is echter gering en zeker onvoldoende om de ziekteverwekker uit te schakelen. Om zeker te zijn van de overwinning moet dus een groot specifiek leger op de been gebracht worden. De specifieke T cellen vermenigvuldigen zichzelf door flink te delen (klonale expansie), zodat binnen 4-6 dagen de hoeveelheid ziekteverwekker-specifieke T cellen in de miljoenen loopt (Fig.1). Tijdens deze vermenigvuldiging worden de cellen bewapend en promoveren (differentiëren) ze tot zogenoemde effector T cellen (Fig.1).

Sommige effector T cellen, de hulptroepen ($CD4^+$ helper T cellen), blijven in de lymfeknoop en zijn betrokken bij de activatie van andere immuuncellen, zoals B cellen. De B cellen produceren antistoffen, waarmee ze bacteriën onschadelijk



Figuur 1 Studenten T cellen (thymocyten) die geslaagd zijn voor het examen verlaten de thymus als Recente Thymus Emigranten (RTE). Samen met andere naïeve T cellen, die ook nog steeds hun doelwit-vijand niet gevonden hebben, gaan deze cellen op patrouille. Als er een ziekteverwekker gedetecteerd wordt, worden de naïeve T cellen geactiveerd, zodat ze differentiëren en enorm veel delen (klonale expansie). Het grote leger aan effector T cellen wat daardoor ontstaat, vernietigt de vijand en pleegt vervolgens zelfmoord (contractie). Een klein deel van de cellen blijft voortbestaan als memory (geheugen) T cellen, die direct actief worden als dezelfde vijand opnieuw aan de macht probeert te komen.

maken. Bijkomend effect is dat de met antistoffen omgeven bacteriën een heerlijk hapje vormen voor de vuilnismannen en veelvraten (granulocyten en macrofagen), zodat al het afval ook nog eens netjes afgevoerd wordt.
Andere effector T cellen verlaten de lymfeknopen als tanks ($CD8^+$ cytotoxische T cellen) en gaan op weg naar geïnfecteerde cellen, om die te bombarderen met hun afweergeschut. Dit afweergeschut bestaat o.a. uit perforines, die zoals de naam al doet vermoeden, gaten in de geïnfecteerde cellen perforeert, waardoor vervolgens afbraakenzymen (granzymes) geduwd worden. Het gevolg laat zich raden…
Nadat de binnendringers uitgeschakeld zijn, gaan de meeste effector T cellen dood (contractie), maar een aantal blijft in leven als geheugen T cellen (Fig.1). Deze memory T cellen vormen de basis van het immunologisch geheugen dat er voor zorgt dat als dezelfde ziekteverwekker opnieuw het lichaam binnendringt, de memory T cellen onmiddellijk opnieuw geactiveerd worden. Op deze manier kan er sneller een effectieve afweerreactie op gang komen zodat de persoon in kwestie niet ziek wordt. Op dit immunologisch geheugen-principe zijn vaccinaties gebaseerd.

T cel homeostase: Onderhoud

Een groot en divers T cellenleger is essentieel om een scala aan ziekteverwekkers te vernietigen. Door de gevoerde oorlogen sneuvelen er nogal wat T cellen; en anders leggen bejaarde T cellen alsnog op hun oude dag het loodje. Dit verlies wordt gecompenseerd door de continue productie van naïeve T cellen door de thymus, waardoor er steeds weer nieuwe T cel varianten ontstaan. Dit is, zoals reeds eerder gezegd, een vrij kostbare productiemethode met een hoge energierekening, omdat slechts 2-5% van de geproduceerde cellen in de circulatie (bloed, lymfe) terecht komt. Een goedkopere en snellere manier om het aantal T cellen te laten toenemen is door celdeling, waarbij een cel zich splitst in twee identieke dochtercellen. Keerzijde van celdeling is dat er geen nieuwe T cel varianten ontstaan: het bestaande repertoire blijft even groot; alleen het aantal T cellen met een specifieke voelhoorn neemt toe.
In het lichaam bestaan naïeve (non-actieve), effector (aanvals-) en memory (geheugen) T cellen naast elkaar. Naïeve T cellen zijn namelijk belangrijk om de diversiteit van de T cellen populatie te waarborgen; effector T cellen om de vijand te ontmaskeren en uit te schakelen; en memory T cellen om snel ziekteverwekkers te vernietigen, die opnieuw een poging ondernemen om de macht over te nemen. Het totale aantal T cellen blijft in een volwassen individu ongeveer gelijk: er is sprake van een evenwicht tussen T cel productie en T cel sterfte. Tijdens de veroudering van mensen en dieren, gebeuren er twee belangrijke dingen in het lichaam: 1. de productie van nieuwe T cellen door de thymus vermindert (thymusinvolutie) en 2. de populatie memory (geheugen) T cellen wordt steeds groter, omdat er gedurende het

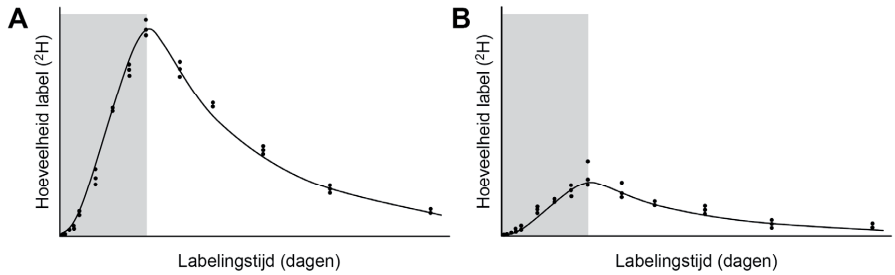
leven vele gevechten hebben plaatsgevonden om griep, mazelen, rode hond, waterpokken, voedselvergiftiging, enzovoorts te boven te komen. Gezien de beperkte hoeveelheid ruimte die er voor T cellen is, is het logische gevolg van beide processen dat het aantal naïeve (non-actieve) T cellen afneemt bij het ouder worden. Omdat daarmee ook de diversiteit van het T cellen leger afneemt, hebben ouderen een verhoogde kans op infecties.

Dit proefschrift: metingen aan het T cellen leger

De centrale vraag waar wij ons in dit proefschrift mee hebben bezig gehouden is: hoe wordt het T cellen leger in stand gehouden? Om dit te onderzoeken worden er veel studies uitgevoerd in proefdieren (voornamelijk muizen). Het bouwplan van muizen en mensen lijkt namelijk erg veel op elkaar, zodat kennis die opgedaan wordt in muizen vaak overgedragen kan worden op de situatie in mensen. Ondanks het vele onderzoek dat al gedurende tientallen jaren uitgevoerd is, bestaat zelfs op eenvoudige vragen niet altijd een duidelijk antwoord. Als je aan een paar willekeurige immunologen zou vragen: hoe lang leeft een naïeve (non-actieve) T cel? Dan kun je antwoorden verwachten die variëren van een paar weken tot een paar jaar. Dit komt omdat de levensduur van een T cel moeilijk te meten is, maar daarnaast zou een deel van de verwarring ook kunnen ontstaan doordat de ene wetenschapper het heeft over naïeve T cellen in muizen en de andere over de situatie in mensen. Waarom is het eigenlijk überhaupt belangrijk om te weten hoelang een T cel leeft? Deze kennis levert belangrijke inzichten op voor verschillende immunologische processen: een lange levensduur van naïeve T cellen suggereert bijvoorbeeld dat activatie en differentiatie (promotie tot effector T cellen) zelden voorkomt, en dat de heropbouw (reconstitutie) van het afweersysteem na chemotherapie of bestraling vrij lang duurt.

Zwaar water

Om de vraag hoelang cellen leven op te lossen, hebben we gebruik gemaakt van zogenaamd zwaar water (^2H_2O). Zwaar water kun je zien als gewoon water, maar dan met een “label” eraan. Water wordt gebruikt als bouwsteen voor de productie van allerlei celonderdelen (eiwitten, vetten, DNA) die nodig zijn als er een nieuwe cel geproduceerd wordt. Door gedurende enige tijd zwaar water te geven aan mensen of muizen, zal dat label (^2H) ingebouwd worden in die nieuwe onderdelen (Fig.2). De hoeveelheid label in het DNA tijdens de toediening van zwaar water (oplabeling), is dus een maat voor de hoeveelheid geproduceerde cellen (productiesnelheid). Als je dan stopt met het geven van zwaar water, kun je meten hoe snel het label uit de cellen verdwijnt (down-labeling). Dit geeft aan hoe snel cellen doodgaan (sterftesnelheid).



Figuur 2 Als de hoeveelheid label in cellen snel toeneemt tijdens de labelingsperiode (aangegeven door het grijze vlak), is de productiesnelheid van die celpopulatie hoog (A). Als er nauwelijks label wordt opgenomen door cellen, worden er nauwelijks nieuwe cellen geproduceerd (B). De snelheid waarmee de cellen het label verliezen, geeft aan hoe snel de cellen dood gaan. Met behulp van wiskundige formules kunnen de levensduur van cellen, productie- en sterftesnelheden exact berekend worden.

Flowcytometrie

Als je wilt bepalen hoelang een naïeve (non-actieve) T cel leeft, moet je die populatie apart isoleren van effector (aanvals-) en memory (geheugen) T cellen. Dit doen we met behulp van een Sorter. Op het oppervlak van cellen zitten allerlei verschillende eiwitten, en die eiwitten zijn voor elke celpopulatie net weer iets anders. Aan een mix van cellen (afkomstig uit het bloed van gezonde vrijwilligers of uit de milt van muizen), voegen we vlaggetjes toe, die aan die eiwitten binden. Vervolgens worden miljoenen cellen door de Sorter gemeten en in verschillende populaties gesplitst op grond van hun grootte en de kleur van de gebonden vlaggetjes. Op deze manier kunnen populaties verkregen worden met een zuiverheid van gemiddeld 98%. Uit de gesorteerde celpopulaties wordt het DNA geïsoleerd, wat vervolgens verder opgewerkt en gemeten wordt.

Behalve het splitsen van cellen, kan met deze techniek ook gekeken worden naar verschillende celeigenschappen. Door gebruik te maken van verschillende vlaggetjes kan gemeten worden of een cel op non-actief staat, zojuist of al langer geleden geactiveerd is, deelt, dood aan het gaan is, enzovoorts. Door deze informatie van gezonde vrijwilligers en verschillende groepen patiënten met elkaar te vergelijken, kan informatie verkregen worden over het werkingsmechanisme en het verloop van ziekteprocessen.

Bevindingen

Wat hebben we nu eigenlijk ontdekt in de afgelopen jaren? In gezonde, volwassen mensen worden er gemiddeld 82 miljoen nieuwe naïeve $CD4^+$ (helpers) en 24 miljoen naïeve $CD8^+$ T cellen (tanks) per dag geproduceerd (Hoofdstuk 2). Dat zijn

grote aantallen, maar als je het vergelijkt met het totale aantal T cellen dat in het menselijk lichaam aanwezig is, dan wordt slechts ongeveer 0.1% van het totale T cellenleger per dag vervangen. Naïeve (non-actieve) $CD4^+$ en $CD8^+$ T cellen leven 6 en 9 jaar in mensen, terwijl memory (geheugen) $CD4^+$ en $CD8^+$ T cellen veel korter leven: 7 en 12 maanden. (Ter vergelijking: de vuilnismannen van het afweersysteem [granulocyten] leven slechts 6 dagen: Hoofdstuk 3). Opvallend is dat niet alle naïeve T cellen een evengrote overlevingskans hebben: cellen die net geproduceerd zijn, hebben de langste levensverwachting. Dit lijkt heel logisch: baby's hebben in het algemeen meer jaren voor de boeg te verwachten dan volwassenen en bejaarden, maar in de T cel wereld was het algemene idee (op grond van muizenexperimenten), dat net geproduceerde cellen die uit de thymus komen (recente thymus emigranten) een kortere levensverwachting hebben dan cellen die al een tijdje meedraaien. De spaarzamenlijk geproduceerde nieuwe naïeve T cellen krijgen dus een overlevings-voorkeurbehandeling.

Nu zou je denken: klaar is Kees. Maar zo gemakkelijk was het niet. Toen we onze resultaten vergeleken met de uitkomsten van andere laboratoria, zagen we dat onze geschatte levensduur van T cellen tien keer zo hoog was als die van anderen. De vraag is dan natuurlijk: wie heeft er nu het juiste antwoord gevonden? Om daar duidelijkheid over te krijgen hebben we onderzocht hoe er zo'n verschillende uitkomst verkregen kan worden als er min of meer vergelijkbare technieken gebruikt worden. Variatie in de lengte van zwaar water toediening bleek de boosdoener te zijn (Hoofdstuk 4). Een nieuw voorgesteld wiskundig model zorgt ervoor dat de uitslag niet meer afhankelijk is van de labelingsduur.

Een tweede resultaat uit deze studie was dat T cellen die recent uit de thymus komen, in muizen dezelfde levensverwachting hebben als alle andere naïeve T cellen: 7 en 12 weken voor naïeve $CD4^+$ en $CD8^+$ T cellen. Recente thymus emigranten in muizen hoeven wat dat betreft dus niet gezien te worden als een aparte populatie binnen de naïeve T cel populatie; een bevinding die lijnrecht ingaat tegen de heersende opvatting dat recente thymus emigranten korter leven dan de overige naïeve T cellen in muizen.
Een grappige bevinding is dat de levensverwachting van naïeve T cellen in muizen perfect schaalt met de levensverwachting van de soort: naïeve T cellen in muizen leven ongeveer 40x korter dan die in mensen, terwijl ook de levensverwachting 40x korter is (een muis leeft gemiddeld 2 jaar, een mens 80 jaar: zie Fig.3).

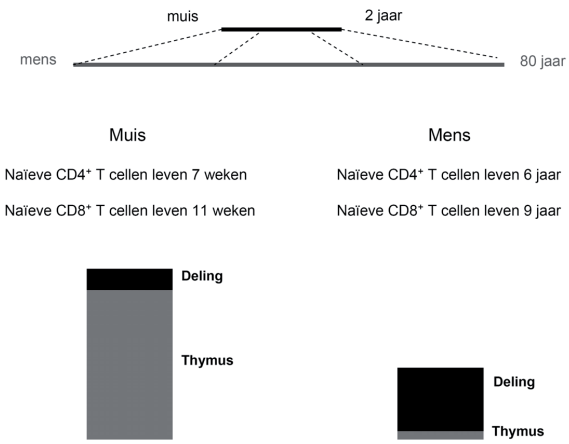
Ondanks dat studies met zwaar water labeling het meest betrouwbaar zijn om productie en sterftesnelheden te bepalen, kan daarmee geen onderscheid gemaakt

worden tussen productie door de thymus en door celdeling. Om de relatieve bijdrage van beide processen te bepalen hebben we een nieuwe serie experimenten uitgevoerd. In volwassen muizen hebben we de thymus operatief verwijderd (thymectomie), waarna we vervolgens gekeken hebben naar het effect daarvan op het aantal naïeve T cellen in het lichaam (Hoofdstuk 5). Kort na thymectomie neemt het aantal naïeve T cellen sterk af; daarna is de afname meer gradueel. Blijkbaar is er een compensatie mechanisme aan het werk als er weinig T cellen aanwezig zijn. Er zijn twee mogelijkheden: 1. de levensduur van cellen kan variëren naar mate er meer of minder concurrentie om voedsel (survival factoren) is, en/of 2. cellen delen meer als er weinig T cellen zijn. Beide mechanismen kunnen de gevonden resultaten verklaren. Welke verklaring ook gekozen wordt, uit berekeningen blijkt dat slechts 12% van de naïeve $CD4^+$ en 33% van de naïeve $CD8^+$ T cellen delen. Zelfs in oude muizen, waar de thymus sterk geslonken is, wordt de meerderheid van naïeve T cellen geproduceerd door de thymus. De waarneming dat de thymus de belangrijkste producent is van naïeve T cellen in muizen, staat in sterk contrast met de situatie in volwassen mensen. Daar wordt namelijk 90% van alle naïeve T cellen gevormd door celdeling. Hoewel de levensduur van naïeve T cellen in muizen en mensen prima overeenkomt, als je rekening houdt met de levensduur van beide soorten, is er dus een belangrijk kwalitatief verschil tussen T cel handhaving in muis en mens (Fig.3). Consequentie van dit alles is dat men resultaten van muizenstudies (helaas) niet klakkeloos over kan brengen op mensen.

Tenslotte vroegen we ons af wat er precies gebeurt in het geval dat het afweersysteem chronisch geactiveerd wordt (zoals dat bijvoorbeeld het geval is bij HIV geïnfecteerde patiënten). Om dat te onderzoeken hebben we gebruik gemaakt van muizen die genetisch iets anders waren dan de standaard labmuizen (transgene muizen). In deze muizen verdwijnen de naïeve (non-actieve) T cellen veel sneller dan normaal en gaan de dieren vroegtijdig dood aan aids-achtige verschijnselen (Hoofdstuk 6). Zelfs als de thymus veel nieuwe T cellen produceert, leidt chronische activatie tot ernstige vermindering van het aantal naïeve T cellen, omdat deze tot effector/memory T cel promoveren.

Samenvattend, zijn de conclusies van dit proefschrift: 1. In vergelijking met muizen worden er in mensen weinig naïeve T cellen geproduceerd (Fig.3), 2. De net gevormde naïeve T cellen hebben een langere levensduur dan de gemiddelde naïeve T cel in mensen, maar een evengrote overlevingskans dan de andere naïeve T cellen in muizen, 3. De thymus genereert de meeste naïeve T cellen in muizen, terwijl celdeling de belangrijkste component is in de handhaving van de naïeve T cel populatie in mensen, 4.Overstimulatie van het afweersysteem leidt tot ernstige

reductie van het aantal naïeve T cellen in muizen met en zonder thymus-output, 5. Granulocyten in mensen leven zes dagen: 10x langer dan tot nog toe werd aangenomen, en 6. Met behulp van een nieuw wiskundig model kan de levensduur van cellen onafhankelijk van de labelingsduur geschat worden.



Figuur 3 Hoewel de levensduur van naïeve (non-actieve) T cellen relatief gezien overeenkomt in muizen en mensen, is de wijze waarop ze geproduceerd worden totaal verschillend. Naïeve T cellen in muizen worden vooral door de thymus gemaakt, terwijl de overgrote meerderheid van naïeve T cellen in mensen door celdeling ontstaat.

Dankwoord
Curriculum Vitae

DANKWOORD

Na 5.5 jaar wetenschap is het nu hoog tijd om iedereen te bedanken voor alle hulp, gezelligheid, bezinning en ontspanning. Dit boekje is eigenlijk het resultaat van de inspanning van alle hieronder genoemde personen: zonder jullie was dit niet gelukt!

Kiki, het begon met het lezen van je CD70Tg muizenpaper, dat ik dacht: daar zou ik ook aan willen werken. Gelukkig zag jij dat ook wel zitten en begonnen we met het opstarten van een muizenpoot van HIV onderzoek bij Sanquin. Langzamerhand verschoof het blikveld naar T cel homeostase in gezonde situatie, wat het onderzoek alleen maar interessanter maakte. Ik heb me er blijvend over verbaasd hoe je na al die jaren nog steeds stevig met twee benen in de lab-praktijk staat. Je enthousiasme, behulpzaamheid (stug doorfacsen tot middernacht), nuchterheid en je vele praktische adviezen waren erg waardevol. Enorm bedankt daarvoor!
José en Rob, een wiskundige kijk op de immunologie bood vaak verrassende perspectieven. De waarde van modelleren is me zeker duidelijk geworden! Eerst doen, dan denken, blijkt in de wetenschappelijke praktijk erg goed te werken. Dank voor jullie constructieve bijdragen! Frank, je bijdrage aan discussies en papers was altijd waardevol. Tendai, thanks a lot for your mathematical contributions! Again and again new models were formulated and tested in a surprisingly good mood. Thanks to you, the experiments resulted in a couple of fantastic papers!

Gerrit, Erik, Koos en Floris, petje af voor de miljarden gesorte cellen! Het leek een eindeloos verhaal, maar het resultaat mag er wezen! An en Mariëtte, de tijd op het AMC was gezellig en productief. De resultaten zijn terug te vinden in vier van de vijf hoofdstukken. Bedankt voor het wegwijs maken in de GC/MS-wereld en de bereidheid om mee te denken! Elise, geen experiment was te groot en je bleef altijd het overzicht houden: top! Mette, zonder jou zou de thymectomie niet van de grond gekomen zijn. Dank voor het aanleren van de techniek!

Het was altijd erg gezellig in het muizenhuis van het NKI! Wat was ik blij dat ik onder jouw vleugels kwam, Loes! Dankzij jouw chirurgische expertise kwam het helemaal goed met de overleving van de muizen na thymectomie: die vacuümpomp was een gouden greep! Ook daarna was je altijd beschikbaar voor advies of een gezellig praatje: enorm bedankt voor alles! Martin, proefdierexpert bij uitstek en meedenkende vraagbaak. Je nuchtere kijk op dingen leverden altijd weer eye-openers op: ik heb het erg gewaardeerd! Henk Starrevelt, met z'n tweeën ging het thymectomeren toch echt een stuk sneller: dank voor alle muizenhechtingen! En voor de vele

injecties, muizenverzorging en overige praktische hulp waren daar altijd Sjaak, Brechtje, Dick, Henk G, Louis, Arnoud, Linda en Merel: geweldig!

Misschien is het muizenhuis-eigen, maar ook in het GDL heerste er altijd een goede sfeer en gezelligheid. Helma, Willeke, Kees en Harry: bedankt voor jullie constructieve meedenken! Joyce en Sabine, fijn dat de vele injecties nooit een probleem waren! Annemieke, Manuela en de overige dierverzorgers: het was altijd fantastisch voor elkaar!

Lydia, wat was ik blij dat er iemand kwam om muizendiscussies mee aan te gaan tijdens de werkbespreking en in de wandelgangen. Dat intuberen laten we maar aan anderen over. Veel succes met je nieuwe onderzoek! Liset, dat waren verrassende TREC-resultaten die je boekte! Nu zelf AIO: dat gaat wel goed komen! Ik ben benieuwd naar de resultaten. Thomas, het was leuk om met je samen te werken! Janesh, altijd wat te leuteren onder het prutsen in het muizenhuis. Het was gezellig!

Ex-KVI-virologen, terwijl iedereen naar Utrecht verhuisde, bleef ik gedeeltelijk in Amsterdam hangen. Gesprekjes op de gang, het beschikbaar stellen van een complete werkplek en allerhande andere zaken, hebben ervoor gezorgd dat ik met veel plezier nog twee jaar langer met jullie ben opgetrokken. Hanneke, bedankt voor de mogelijkheid om mijn experimenten te kunnen afronden.

Wat zou het werk zijn geweest zonder de gezelligheid, tips, humor, steun, discussies en betrokkenheid van mijn (ex-)kamergenoten? Nening, Christine, Bregje, Eva, Nienke, Marieke, Ana, Tomek en Leonie: bedankt voor alles!

Edith, Steve, Gerben en Johanna, mijn eerste stappen op het wetenschappelijke pad werden door jullie begeleid. Het legde de basis voor mijn AIO-onderzoek. Dank voor de wetenschappelijke vorming die jullie me hebben meegegeven!

Er zijn nog veel meer mensen die door hun belangstelling op het werk of privé mij altijd gesteund hebben. Het voert te ver om iedereen hier te noemen, maar ook vanaf deze plaats: bedankt!

Kerkfamilie in Spijk, ik ben dankbaar dat ik een onderdeel van jullie mag zijn. De wekelijkse bezinning zet alles in het leven weer in het juiste perspectief.

Familieleden door het hele land, maar vooral Oma den Braber: bedankt voor de interesse die er altijd was!

Anna-Marie, Corrie, Jenine, Jennie, Marlies, Mirjam, Rianne en echtgenoten, bedankt voor de gezelligheid buiten het lab! Vrienden van de middelbare school moet je koesteren!

Jeannet, je inbreng bleef niet beperkt tot meedenken in praktische zaken. ’s Avonds nog even helpen met meten, getallen invoeren of bestanden doorlezen: het kon altijd. Bedankt voor je vriendschap, steun en geduld. Ik ben blij dat jij mijn paranimf wilt zijn!

Lieve Aline en Roel, Jaap en Henrieke, Lieneke en Arie: petje af voor jullie! Ik voel me gezegend met zo’n familie! Met Wilco, Jonieke, Lidia, Art, Susanne, Sanne, Gerdiene en twee kleintjes op komst, breidt de familie gestaag uit. Het wordt er alleen maar gezelliger op!

Lieve papa en mama, heel erg bedankt voor alle onvoorwaardelijke liefde, steun en vertrouwen. Uit de grond van mijn hart: ik hou van jullie! Mama, de onzichtbare motor achter het hele gezin: ik ben blij dat u 16 juni ook letterlijk achter mij staat als paranimf!

Boven dit alles gaat mijn dank uit naar de Heere God, de Schepper van al het leven, Die al die jaren wijsheid en kracht gegeven heeft, en Die nog steeds spreekt door Zijn Woord en Geest. Soli Deo Gloria!

Dat hemel, aard’ en zee en berg en dal,
Hoever men ook Zijn scepter ziet regeren,
Nu Zijnen naam en grote deugden eren;
En gij, mijn ziel, loof gij Hem bovenal.

CURRICULUM VITAE

Ineke den Braber werd op 2 mei 1979 geboren te Barendrecht. Na het behalen van het VWO diploma aan het Van Lodensteincollege te Amersfoort, begon zij in 1997 aan de studie Biologie aan de Universiteit Utrecht. Tijdens deze opleiding heeft zij stage gelopen bij de afdeling Veterinaire Farmacologie, Farmacie en Toxicologie van bovengenoemde universiteit onder supervisie van dr. G.J. Schaaf en prof. dr. J. Fink-Gremmels. Vervolgens liep zij stage bij de afdeling Cellular Immunology-3 van het La Jolla Institute for Allergy and Immunology (San Diego, USA) onder supervisie van dr. E.M. Janssen en dr. S.P. Schoenberger. In november 2002 werd het doctoraal examen cum laude gehaald. Vanaf juni 2003 werkte zij als assistent in opleiding bij de afdeling Klinische Viroimmunologie van Sanquin Research (Amsterdam) en, na verhuizing van de vakgroep, bij de afdeling Immunologie van het Universitair Medisch Centrum Utrecht, onder begeleiding van dr. K. Tesselaar, prof. dr. R.J. de Boer en prof. dr. F. Miedema. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Vanaf juni 2009 zal zij werkzaam zijn als post-doc binnen de groep van prof. dr. W. van Eden, op de afdeling Infectieziekten en Immunologie van de Universiteit Utrecht.