Lymphocyte dynamics in health and disease

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Introduction

Lymphocyte development

The immune system is comprised of an extensive array of cell types and mediators which combine their efforts to resolve infections caused by pathogens like viruses and bacteria. Of the various immune cell populations, T cells and B cells are the most specialized cells in the sense that each cell has unique antigen-specificity. B cells develop in the bone marrow and produce specific sets of immunoglobulins which are expressed on the cell surface. T-cell development starts in the bone marrow and is finished in the thymus. Here, T-cell progenitors enter as $CD4^-CD8^-$ T cells which can develop into functional T cells expressing either αβ or γδ T-cell receptors (TCRs). T cells bearing αβTCRs have matured into $CD4^+CD8^+$ cells and are selected for their ability to recognize self-peptide presenting major histocompatibility complexes (MHC) expressed on thymic stromal cells. Successful interaction of the αβTCR and CD8 with MHC class I generates $CD8^+$ T cells, whereas appropriate interaction of the αβTCR and CD4 with MHC class II results in the generation of $CD4^+$ T cells (1).

T-cell subsets

After completing their maturation, $CD4^+$ and $CD8^+$ T cells egress from the thymus and are called naive T cells since they have not recognized any foreign antigen yet. Naive T cells are long-lived cells which can circulate for years and may die before ultimately encountering their specific antigen (2-4). Upon antigen encounter, naive T cells become activated, proliferate and differentiate into memory and effector T cells. $CD8^+$ T cells have direct cytotoxic reactivity once activated and can kill infected cells directly by production of cytotoxins such as perforins and granzymes (5). Activated $CD4^+$ T cells 'help' macrophages and B cells during these responses by supplying cytokines which enable the maturation of these cells (6).

Naive T-cell homeostasis

Naive T-cell counts remain relatively stable during life (7-9). This naive T-cell homeostasis is a result of the balance between the production of naive T cells and their differentiation and death. Beside production by the thymus, new naive T cells can be generated by proliferation of naive T cells in the periphery (10,11). Loss of naive T cells can occur by death or by differentiation of naive T cells into memory/effector T cells. Thymic output declines significantly with age (12-15). Therefore, the contribu-

tion of the thymus to naive T-cell homeostasis wanes during life, which suggests that homeostasis of the naive T-cell compartment in adults may rely mostly on peripheral T-cell proliferation and prolonged survival (longevity) of naive T cells.

Naive and memory T cells continually need signals to survive. Studies with knock-out mice have shown that both survival and proliferation of naive $CD4^+$ and $CD8^+$ T cells are largely dependent on the interaction of these cells with self-peptide-presenting MHC class II and I molecules respectively (16-18), and on binding of interleukin-7 (IL-7) (17,19,20). Based on these studies it was proposed that the size of the naive T-cell compartment is dependent on the availability of these survival signals. In a normal, physiologically "full" T-cell compartment the likelihood of naive T cells to interact with IL-7, and perhaps MHC molecules, is limited. In contrast, during lymphocyte depletion, self-peptide-presenting MHC molecules and IL-7 are abundantly present for the few circulating T cells (17,20). The multitude of signals that naive T cells receive, result not only in survival but also in proliferation of naive T cells.

Memory $CD4^+$ T cells also require interaction with MHC class II and IL-7 for their survival and proliferation (21). Memory $CD8^+$ T cells in contrast are not dependent on the interaction with self-peptide-presenting MHC class I molecules, but their survival and proliferation relies on interaction with IL-7 and IL-15 (22).

Studies in humans have confirmed that IL-7 is essential in T-cell homeostasis. IL-7 is produced by stromal cells in bone marrow and lymph nodes, as well as by thymic and intestinal epithelial cells (23,24). *In vitro* studies have shown that low levels of IL-7 are required for survival of naive T cells, whereas higher levels are needed for naive T cells to proliferate (25,26). The fact that IL-7 levels in blood of healthy individuals are lower than in lymphopenic patients suggests that naive and memory T cells actually consume IL-7 and compete for the available IL-7 in order to survive (27).

Thymic output

It has been difficult to directly determine the contribution of thymic naive T-cell production to the establishment and maintenance of the naive T-cell compartment. The main reason is that it is hard to distinguish between naive T cells that have recently been produced by the thymus (recent thymic emigrants: RTE) and cells that have already been in the periphery for some time. Several methods are used to assess thymic output or to identify RTE. The most widely used tool to determine the level of thymic output is the measurement of T-cell receptor excision circles (TRECs). These epichromosomal, circular DNA remnants are formed during VDJ

gene rearrangements of the αβTCR (13,28). Since TRECs cannot be replicated, they are diluted upon each cell division. Consequently, memory T cells carry on average fewer TRECs than naive T cells, since they have a longer proliferative history. Hence, TREC contents (the average number of TREC per T cell) reflect a combination of thymic output and peripheral T-cell proliferation in the periphery, whereas the total number of TRECs per µl blood relates to thymic naive T-cell production (29). TRECs can nevertheless not be used to estimate RTE levels. Firstly, even in naive T cells that have divided multiple times TRECs can still be present. Secondly, TRECs can reside in naive T cells long after these cells have been produced by the thymus.

Another way to identify thymic progeny is by measuring the expression of CD31 (or PECAM-1). The fraction of CD31-expressing naive $CD4^+$ T cells declines during aging (30). Therefore, it was suggested that naive $CD4^+$ T cells may lose expression of CD31 upon division. Evidence for this hypothesis was obtained by the observation that the TREC content of $CD31^-$ naive $CD4^+$ T cells was in general 8-fold lower than that of $CD31^+$ naive $CD4^+$ T cells (31). Based on these data it was proposed that once $CD31^+$ naive $CD4^+$ T cells proliferate, they become $CD31^-$ naive $CD4^+$ T cells and have diluted their TRECs. In addition, this would implicate that $CD31^+$ naive $CD4^+$ T cells are CD4 RTE. However, it was later shown that the TREC content of $CD31^+$ naive $CD4^+$ T cells also declines during aging, which implies that CD31 expression is not always lost upon naive $CD4^+$ T cell division and that CD31 is better considered a thymic proximity marker (since $CD31^+$ naive $CD4^+$ T cells have higher TREC contents than $CD31^-$ naive $CD4^+$ T cells) than a RTE marker (32).

Ex vivo assessment of T-cell dynamics

Although the use of CD31 and TRECs provides no precise estimate of thymic naive T-cell production, these markers are still useful to obtain insight into the origin and proliferative history of the naive T-cell compartment. Both tools have been used to describe T-cell dynamics in healthy individuals and immune-compromised individuals which reconstitute the T-cell compartment after lymphocyte depletion following chemotherapy, stem cell transplantation (SCT) or HIV-1 infection. Interpretation of TREC and CD31 data has to be done with caution, however. Following T-cell depletion after SCT (33) and at the start of treatment for HIV-1 infection (13), increases in TREC content of $CD4^+$ and $CD8^+$ T cells have been observed. Intuitively, it can be argued that increased thymic naive T-cell production is responsible for this phenomenon as newly produced thymic naive T cells are enriched for TRECs. However, a more likely explanation for the rise in TREC content of $CD4^+$ and $CD8^+$ T cells is that the entrance of normal numbers of TREC-enriched cells into a relatively empty

T-cell pool results in an overall TREC-enrichment of the T-cell compartment (34,35). Although thymic output may increase after T-cell depletion - as was suggested in a study on adults by the increase in thymic mass after SCT (36) - increasing TREC provide no evidence in this direction.

In vivo assessment of T-cell dynamics

In addition to these indirect *ex vivo* measures, T-cell dynamics can also be studied *in vivo*, for example by administration of the stable isotopes $^{2}H_2$-glucose or $^{2}H_2O$. The advantage of the use of $^{2}H_2O$ is that it can easily be administered orally for relatively long time periods (e.g. several weeks), thereby allowing for labeling of cells with relatively slow kinetics such as naive T cells. Both $^{2}H_2O$ and $^{2}H_2$-glucose have been applied in studies on T-cell dynamics in healthy and HIV-1 infected individuals (37). Both labeling with $^{2}H_2O$ and $^{2}H_2$-glucose have shown that in healthy young adults hardly any label is incorporated into the DNA of naive T cells (2-4). Since naive T cells can acquire label either during proliferation in the periphery or by division during their maturation in the thymus, these data show that the maximum level of naive T-cell production by the thymus is approximately 0.1% of the total naive T-cell compartment per day and that the average naive T cell has a lifespan of several years (4,37). In addition, no loss of label in naive T cells was observed during half a year after cessation of label intake, suggesting that newly-produced naive T cells are not rapidly lost but are preferentially incorporated into the naive T-cell compartment (4). In HIV-1 infection, turnover of both naive and memory T cells was found to be increased, but normalized after initiation of highly active antiretroviral therapy (HAART) (38-40).

Scope of this thesis

Naive T-cell homeostasis is maintained by a balance of production, differentiation and death of naive T cells. New naive T cells can be produced by both thymic output and peripheral T-cell proliferation, and it has been shown that especially during early childhood both mechanisms are responsible for the establishment of the naive T-cell compartment (10,11). As the thymus atrophies during life, thymic naive T-cell production in adults becomes limited. Recovery of the naive T-cell compartment following immune depletion was shown to be age-dependent, which hints at an important role for the thymus in naive T-cell reconstitution (36,41,42). The role of peripheral T-cell proliferation in the recovery of the naive T-cell com-

partment is however still poorly understood. Early after initiating HAART in HIV-1 infected adults, naive T-cell proliferation drops to normal levels despite incomplete naive T-cell recovery (38). In contrast, in early childhood naive T-cell proliferation levels have been found elevated (10,11). Hence in children, naive T-cell proliferation may also account for recovery of the naive T-cell compartment following immune depletion. As was shown in mice, severe immune depletion resulted in enhanced levels of naive T-cell proliferation as a homeostatic response (16,17,19). Whether this phenomenon is similar in humans and whether the level of peripheral naive T-cell proliferation is dependent on the degree of naive T-cell depletion is unknown.

The main objective of the research described in this thesis is to enhance our understanding of the versatile nature of naive T-cell homeostasis and lymphocyte dynamics in health, disease and during reconstitution after immune depletion. In the first part of this thesis, the establishment and maintenance of the T-cell compartment is assessed in health and during immune reconstitution. In chapter 2, the establishment of the T-cell compartment in healthy children is described, whereas in chapter 3 T-cell homeostasis is assessed in the absence of thymic output resulting from thymectomy. The role of thymic output and peripheral proliferation in the reconstitution of the naive T-cell compartment is studied in children who recover from chemotherapy treatment for leukemia or lymphoma (chapter 4). In children and adults who are treated with HAART for chronic HIV-1 infection, the importance of both mechanisms of naive T-cell production is described in chapter 5. In chapter 6, a new method to identify a subset of CD4 RTE has been developed. This method, which is based on the incomplete commitment of CD4 RTE to the CD4 lineage, might offer an alternative to the use of TRECs or CD31 in the assessment of thymic output.

In the second part of this thesis, lymphocyte dynamics *in vivo* are studied by $^{2}H_{2}O$ labeling. Although it was shown that naive and memory T-cell turnover in HIV-1 infection is increased, it remains uncertain whether this turnover is a consequence of the chronic immune activation which relentlessly tries to eradicate HIV-1, or of T-cell homeostasis which acts to replenish the gradually depleting T-cell compartment (chapter 7). In chapter 8, the $^{2}H_{2}O$ labeling technique has been applied to determine if stable B-cell chronic lymphocyte leukemia (B-CLL) is characterized by a pool of slowly accumulating leukemic cells, or whether the leukemic compartment displays a considerable degree of heterogeneity and *in vivo* turnover.

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Part I

2

Refined characterization and reference values of the pediatric T- and B-cell compartments

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Abstract

Work in the past years has led to a refined phenotypical description of functionally distinct T- and B-cell subsets. As a consequence, since both lymphocyte compartments are established and undergo dramatic changes during childhood, redefined pediatric reference values of both compartments are needed. In a cohort of 145 healthy children, aged 0-18 years, the relative and absolute numbers of the various T- and B-cell subsets were determined. We found that beside thymic output, naive T-cell proliferation contributed significantly to the establishment of the naive T-cell compartment. At birth, regulatory T cells (Tregs) mainly had a naive phenotype whereas 'memory-like' Tregs accumulated slowly during childhood. Beside the $CD27^{+}IgM^{+}IgD^{+}$ memory B-cell population, the recently identified $CD27^{-}IgG^{+}$ and $CD27^{-}IgA^{+}$ memory B-cell populations were already present at birth. These data provide reference values of the T- and B-cell compartment during childhood for studies of immunological disorders or immune reconstitution in children.

Introduction

Previous studies, mostly originating from the late 1990s, have characterized the composition of the T- and B-cell compartments by flowcytometry and have shown that these lymphocyte compartments undergo dramatic changes in both their composition and cell numbers during childhood (1-5). These studies provided age-related immunological reference values that are needed to aid clinicians in the treatment of hematological or immunological diseases.

However, new insights into the composition and establishment of these compartments have been gained over the last years. It has been shown that naive T cells are not generated exclusively by the thymus but also by proliferation of naive T cells within the periphery (6). Furthermore, the existence of a specialized T-cell subset with regulatory function has become generally accepted (7). These regulatory T cells (Tregs) are characterized as $CD127^{-}CD25^{+}$ $CD4^{+}$ T cells expressing the transcription factor Foxp3 (8-10), and are thought to function as crucial regulators in controlling immune responses and maintaining self-tolerance (11).

Particularly for the peripheral B-cell compartment, novel phenotypically distinct subsets have recently been described. B-cell development takes place in the bone marrow where $sIgM^{+}sIgD^{+}$ B cells exit as immature, transitional B cells. These recent bone marrow emigrants (RBE) have been characterized as $CD38^{+}IgM^{+}IgD^{+}CD27^{-}CD10^{+}$ B cells (12,13). Subsequently, RBE develop into functionally mature, $CD38^{-}IgM^{+}IgD^{+}CD27^{-}CD10^{-}$ naive B cells (14-19). Naive B cells can differentiate into plasma or memory B cells after binding of antigen by specific immunoglobulin (Ig) receptors (18,20).

CD27 has been established as a marker for peripheral memory B cells. The $CD27^{+}$ memory B-cell population, which can be divided into different subsets including $CD27^{+}IgM^{+}IgD^{+}$ (IgM^{+} memory B cells) and isotype-switched $CD27^{+}IgG^{+}$ and $CD27^{+}IgA^{+}$ cells, represent 30-40% of total peripheral B cells in adults (14-17,19,21). Isotype-switched memory B cells are formed in germinal centers of peripheral lymphoid organs in response to T-cell dependent antigens after appropriate T-cell help. Peripheral $CD27^{+}IgM^{+}IgD^{+}$ memory B cells are recognized as counterparts of splenic marginal zone B cells and are supposed to be dedicated to immune responses to T-cell independent antigens (14,15,21,22).

In this study, we provide an overview of the changes within the T- and B-cell compartments during childhood and present reference values of various components of these lymphocyte compartments. We investigated the relative contribution of thymic output and T-cell proliferation in the establishment of the naive T-cell compartment during childhood. In addition, the development of the Treg compartment was assessed by measuring the levels of $CD127^{-}CD25^{+}CD4^{+}$ T cells and we assessed whether

these Tregs had a naive or memory Treg phenotype. The composition and changes within the B-cell compartment during childhood were analyzed by determining the levels of the different B-cell subsets, including RBE, naive B cells and the various memory B-cell populations.

Materials and methods

Study population

Whole EDTA-anticoagulated blood samples were obtained by venipuncture from 145 healthy children, aged 0-18 years. All children visited the University Medical Center in Utrecht in order to undergo an elective urological or plastic surgery. The children were considered healthy as they did not have any history of infectious diseases or hematological and immunological disorders. Cord blood samples were obtained from healthy full-term neonates directly after delivery. The study was approved by the medical ethical committee and written informed consent was obtained from all study participants or their legal guardians in agreement with the Helsinki Declaration of 1975, revised in 1983.

Flow cytometry

To assess the composition of the B-cell compartment by flow cytometry, 200µl of whole blood was washed thrice with phosphate-buffered saline (PBS). Subsequently, cells were stained with monoclonal antibodies to CD19-PerCP-Cy5.5, CD27-APC, CD38-FITC, CD38-APC, CD10-PE, IgM-FITC, IgG-FITC, IgD-FITC, IgD-PE and IgA-FITC (Becton Dickinson) for 20', followed by a 10' erythrocyte lysis step using FACS™ Lysis Solution (Becton Dickinson). After washing once with PBS, cells were analyzed on a FACSCalibur (Becton Dickinson). Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque density gradient centrifugation and were stored in liquid nitrogen until further processing. Cryopreservation was performed using a computerized freezing device that results in optimal quality of frozen cells (23). Extensive characterization of the T-cell compartment was performed on thawed cryopreserved PBMC. PBMC were incubated with mAb to CD4-Pacific Blue, CD4-FITC, CD8-PerCP-Cy5.5, CD8-APC-Cy7, CD25-APC-Cy7, CD27-APC, CD31-PE, CD38-PerCP-Cy5.5, CD45RO-PE, CD45RO-PE-Cy7, HLA-DR-FITC (Becton Dickinson) or CD127-PE (Immunotech). To measure T-cell proliferation, PBMC were stained intracellularly with Ki67-FITC (Monosan) after fixation and permeabilization with Cytofix/Cytoperm™ and Perm/Wash™ according to manufacturer's instructions (Becton Dickinson). After washing with PBS, cells were analyzed on a LSRII (Becton Dickinson).

Absolute lymphocyte numbers were determined with a Cell-Dyn Sapphire™ Hematology Analyzer (Abbott Diagnostics) and were used to calculate absolute numbers of T and B cells.

Population definitions

Naive ($CD27^+CD45RO^-$)(N), central memory ($CD27^+CD45RO^+$)(CM), effector memory ($CD27^-CD45RO^+$)(EM) and (terminally-differentiated) effector ($CD27^-CD45RO^-$)(TEMRA) subsets were identified within the $CD4^+$ and $CD8^+$ T-cell compartments as depicted in Figure 1A (24,25). From several of these subsets, expression of CD25, CD31, CD38, CD127, HLA-DR and Ki67 was determined. Tregs were characterized as $CD127^-CD25^+CD4^+$ T cells (Figure 1B).

Definitions of B-cell subsets are depicted in Figure 1C,D. Recent bone marrow emigrants (RBE) were defined as $CD38^+IgD^+CD10^+$ B cells, which are also $CD27^-$ and IgM^+ (12,13). Naive B cells and RBE constitute the $CD27^-IgM^+IgD^+$ B cells in blood (14-17,19). To calculate naive B-cell levels, RBE levels were subtracted from

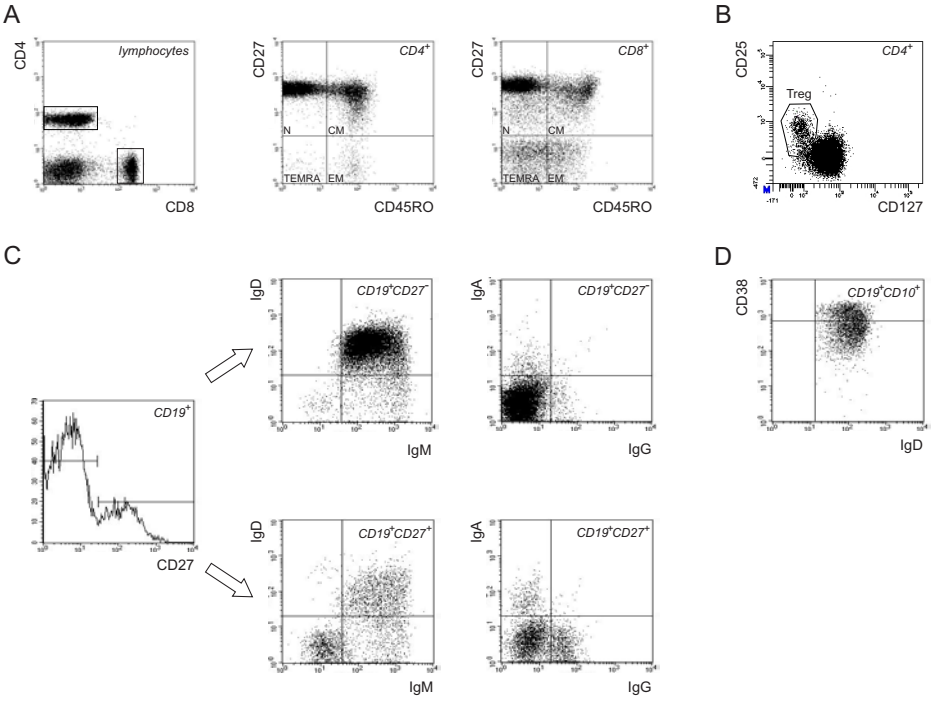

Figure 1. Characterization of T- and B-cell subsets

Population definitions as described in Materials and methods. A) T-cell subsets were defined by CD27 and CD45RO expression. B) Treg were identified as $CD127^-CD25^+CD4^+$ T cells. C) Based on CD27 expression, $CD19^+$ B cells were analyzed for IgM, IgD, IgA and IgG expression. D) RBE were identified as CD10-expressing $CD38^+IgD^+$ B cells.

$CD27^-IgM^+IgD^+$ B-cell levels. This percentage corresponded with an alternative gating strategy for naive B cells based on $CD27^-CD38^-IgD^+$ B cells (18,26), which validates this gating strategy.

Class-switched and non-class-switched memory B cells were identified based on surface Ig and CD27 expression. Non-class-switched memory B cells were defined as $CD27^+IgM^+IgD^+$ B cells (IgM^+ memory B cells) (14,15,21,22), whereas $CD27^+$ class-switched memory B cells were defined as $CD27^+IgM^-IgD^-$ B cells which constitute the $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B-cell populations (14-16,19). The presence of $CD27^+IgM^+IgD^-$ memory B cells (21) as well as the recently identified $CD27^-IgG^+$ and $CD27^-IgA^+$ memory B-cell subpopulations was also determined (27).

MACS cell separation

To measure the total number of sjTRECs and sjTREC content of $CD4^+$ and $CD8^+$ T cells, these subsets were purified from thawed PBMC by magnetic-bead separation using the MiniMACS multisort kit according to manufacturer's instructions (Miltenyi Biotec Inc).

sjTREC analysis

DNA was isolated using the QIAamp Blood Kit according to manufacturer's instructions (Qiagen). Signal joint T-cell receptor excision circle (sjTREC) numbers were quantified by real-time polymerase chain reaction (PCR) as described previously (28,29). sjTREC content per T cell was calculated by dividing the sjTREC content by 150.000 (assuming that 1µg DNA corresponds with 150.000 T cells).

Total body numbers of naive T cells and sjTRECs

Total body naive $CD4^+$ and $CD8^+$ T-cell counts and total body $CD4^+$ and $CD8^+$ sjTREC numbers were calculated as described previously (6). In brief, total body counts were calculated by multiplying the counts per ml blood by 80 × 50 × [body weight in kg] assuming a blood volume of 80 ml/kg of body weight, and 2% of lymphocytes residing in the blood of healthy individuals.

Results

As lymphocyte counts per µl blood decrease and alterations in various T- and B-cell subsets occur especially during early childhood, the cohort of 145 healthy children, ranging from 0-18 years of age, was divided into 10 age strata, which were narrowest at early childhood and consisted of at least six individuals per stratum. The data per stratum is shown in Tables 1-5, whereas individual data points are depicted in Suppl. Figures 1-6.

T-cell compartment

Naive, memory and effector T-cell subsets

Changes within the CD4$^+$ T-cell compartment were most obvious during the first six years of life (Tables 1-3; Suppl. Figures 1,2). In this period, CD4$^+$ T-cell counts gradually decreased. In contrast, CD8$^+$ T-cell counts decreased mainly after the age of six (Table 2).

Based on the expression of CD27 and CD45RO, the CD4$^+$ and CD8$^+$ T-cell compartments were divided into naive (N), central memory (CM), effector memory (EM) and (terminally-differentiated) effector (also called TEMRA) T cells (Figure 1A). Almost all T cells were naive in cord blood (Table 1; Figure 2A,B; Suppl. Figure 1). Memory T-cell percentages and counts (predominantly central memory T cells) gradually increased until the age of six, causing a shift in the composition of both the CD4$^+$ and CD8$^+$ T-cell compartments (Tables 1,2; Figure 2A,B; Suppl. Figures 1,2). From six years onward, naive/memory ratios in both T-cell compartments were relatively stable (Table 3).

Regulatory T cells

Regulatory T cells (Treg) are identified by intracellular staining for Foxp3 expression (8). Recently, it was shown that these cells can also be identified as CD127$^-$CD25$^+$CD4$^+$ T cells (9,10). By identifying Tregs with the latter phenotypical markers (Figure 1B), the percentage of these cells within the CD4$^+$ T-cell compartment was found to remain fairly constant throughout childhood, although absolute numbers per µl blood declined (Tables 1,2; Suppl. Figures 1,2). In adults, Tregs have been shown to have either a naive (thymically-derived, naturally occurring) or memory (activation-

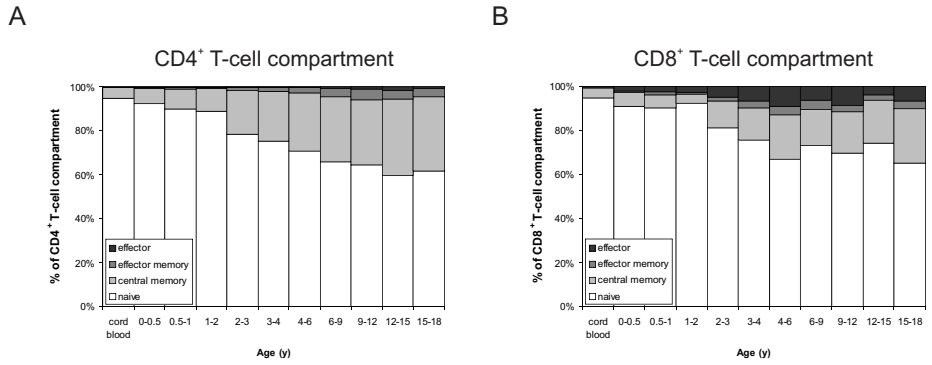

Figure 2. CD4$^+$ and CD8$^+$ T-cell subset distribution during childhood

The relative contribution of the naive, central memory, effector memory and effector subpopulations in the A) CD4$^+$ and B) CD8$^+$ T-cell compartments during childhood.

Table 1. Percentages of total T cells and T-cell subsets

		no.	cord blood	0-6 months	6-12 months	1-2 years	2-3 years	3-4 years	4-6 years	6-9 years	9-12 years	12-15 years	15-18 years
lymphocytes (of leukocytes)		*98*	**nd**	**63.1 (50.1-70.1)**	**58.2 (48.4-76.5)**	**63.4 (43.0-65.2)**	**47.7 (33.4-62.8)**	**52.0 (36.3-60.5)**	**39.9 (30.2-59.2)**	**46.3 (26.0-49.6)**	**42.0 (34.5-48.2)**	**40.8 (24.2-51.3)**	**34.3 (27.0-44.1)**
total CD4$^+$ T cells		*109*	**46.7 (40.2-61.9)**	**43.2 (37.6-46.4)**	**43.5 (29.8-63.4)**	**42.4 (33.0-55.0)**	**34.5 (28.1-43.2)**	**37.1 (18.3-42.7)**	**38.2 (26.8-42.3)**	**31.5 (26.5-41.4)**	**36.2 (28.4-44.4)**	**35.0 (30.4-52.9)**	**38.2 (29.3-52.9)**
%	*Ki67$^+$*	*83*	1.8 (0.8-2.5)	1.4 (0.8-2.1)	0.6 (0.2-1.5)	0.5 (0.1-1.9)	1.1 (0.5-1.7)	0.9 (0.4-1.3)	1.0 (0.6-1.6)	0.8 (0.4-1.5)	0.6 (0.4-1.1)	1.0 (0.5-1.8)	0.9 (0.6-1.4)
%	*CD25$^+$CD127$^-$CD27$^+$CD45RO$^-$*	*80*	4.6 (3.1-6.3)	5.7 (4.1-8.8)	4.5 (3.2-7.5)	3.7 (2.9-5.7)	3.0 (1.9-5.5)	3.6 (2.0-4.1)	2.9 (1.5-3.8)	3.1 (1.3-4.8)	2.4 (1.0-3.4)	2.2 (1.2-4.6)	1.7 (0.8-2.6)
%	*CD25$^+$CD127$^-$CD45RO$^+$*	*80*	0.8 (0.3-1.2)	0.9 (0.7-1.2)	1.0 (0.4-1.5)	1.0 (0.3-1.3)	1.7 (1.0-2.1)	1.3 (0.8-3.0)	1.9 (1.0-2.7)	2.0 (1.0-3.3)	1.6 (1.3-2.6)	2.3 (1.4-3.6)	1.3 (0.9-2.2)
%	*CD31$^+$*	*81*	76.2 (69.7-79.1)	67.1 (55.7-76.7)	66.5 (54.1-75.3)	60.3 (47.2-71.2)	55.9 (53.2-67.5)	64.7 (53.9-75.3)	61.3 (59.3-74.8)	54.7 (44.3-68.4)	53.0 (41.4-56.7)	40.9 (35.7-59.4)	46.8 (33.4-61.8)
%	*CD31$^+$CD27$^+$CD45RO$^-$*	*81*	73.1 (68.1-75.9)	67.4 (56.3-76.4)	65.8 (54.8-73.5)	60.4 (45.5-70.7)	54.4 (52.3-66.6)	62.9 (52.7-73.9)	59.5 (56.8-72.8)	51.9 (43.9-66.4)	49.7 (40.1-54.8)	38.8 (33.5-58.2)	42.8 (32.7-60.9)
%	*CD38$^+$*	*85*	98.2 (97.2-98.7)	95.4 (91.1-96.5)	95.3 (93.2-96.6)	91.1 (84.5-94.9)	87.4 (82.4-91.9)	84.0 (80.8-90.0)	82.7 (78.5-89.6)	77.3 (72.8-85.4)	69.1 (66.3-80.2)	70.0 (51.3-80.4)	65.2 (50.9-71.8)
%	*HLA-DR$^+$*	*85*	0.3 (0.2-0.5)	0.8 (0.6-1.0)	0.7 (0.5-1.1)	0.8 (0.4-2.0)	1.4 (0.9-2.0)	1.9 (0.6-2.6)	1.6 (0.6-4.1)	1.7 (0.5-2.9)	2.0 (0.6-3.4)	1.4 (1.1-5.0)	1.4 (0.8-2.2)
%	*CD38$^+$HLA-DR$^+$*	*85*	0.3 (0.2-0.4)	0.7 (0.5-0.8)	0.6 (0.4-1.0)	0.6 (0.3-1.3)	0.7 (0.6-1.2)	1.1 (0.4-1.5)	0.8 (0.3-2.5)	1.0 (0.3-1.8)	0.9 (0.3-2.0)	0.8 (0.5-1.6)	0.6 (0.3-1.0)
%	*CD127$^+$*	*82*	89.7 (88.3-94.6)	78.0 (68.7-86.1)	74.3 (65.8-86.6)	78.0 (70.7-84.5)	80.0 (71.4-91.6)	70.6 (63.9-61.9)	80.0 (61.9-84.6)	76.3 (59.2-84.6)	81.7 (71.5-89.5)	81.1 (65.8-89.6)	79.1 (70.3-87.3)
regulatory T cells (CD127$^-$CD25$^+$CD4$^+$)		*81*	**5.2 (3.5-7.0)**	**6.8 (4.9-9.8)**	**5.5 (3.6-9.0)**	**5.1 (3.7-6.7)**	**5.0 (2.9-7.4)**	**4.9 (3.3-6.8)**	**4.9 (3.1-6.1)**	**5.4 (2.3-7.7)**	**4.0 (2.6-6.1)**	**4.7 (2.8-7.2)**	**3.1 (2.2-4.1)**
%	*CD27$^+$CD45RO$^-$*	*80*	85.1 (77.0-94.3)	87.2 (82.6-89.7)	84.2 (80.7-90.0)	80.7 (71.6-92.7)	65.7 (59.5-75.0)	70.5 (51.8-82.3)	59.3 (44.0-75.2)	59.1 (49.5-71.0)	53.6 (37.8-69.6)	52.5 (36.8-66.4)	54.0 (34.8-70.3)
%	*CD45RO$^+$*	*80*	14.9 (5.7-23.0)	12.6 (10.3-16.9)	15.8 (9.8-19.3)	19.0 (7.1-28.3)	34.2 (24.8-40.1)	29.4 (17.7-46.8)	40.6 (24.2-55.5)	40.8 (28.9-50.3)	44.2 (30.2-62.2)	46.9 (33.4-62.9)	45.9 (29.6-62.8)
naive CD4$^+$ T cells		*109*	**94.5 (91.9-98.1)**	**92.6 (89.6-94.6)**	**89.7 (83.5-94.7)**	**87.4 (82.3-95.1)**	**78.8 (71.5-84.2)**	**74.8 (64.5-83.8)**	**72.7 (57.8-81.0)**	**65.1 (55.6-75.8)**	**62.7 (53.4-74.7)**	**60.2 (49.3-72.0)**	**62.0 (49.4-71.9)**
%	*Ki67$^+$*	*82*	1.5 (0.7-2.1)	0.7 (0.3-1.2)	0.2 (0.1-0.5)	0.2 (0.1-0.4)	0.2 (0.0-0.3)	0.2 (0.1-0.3)	0.1 (0.1-0.1)	0.2 (0.1-0.3)	0.1 (0.1-0.1)	0.1 (0.1-0.2)	0.2 (0.1-0.4)
%	*CD25$^+$*	*81*	5.5 (4.0-7.4)	4.7 (2.6-7.6)	4.1 (1.4-6.3)	2.4 (1.3-3.8)	2.6 (1.0-3.7)	1.8 (0.5-4.2)	1.7 (0.8-3.0)	1.8 (0.7-4.5)	1.2 (0.6-3.0)	1.4 (0.8-2.9)	1.9 (0.5-3.2)
%	*CD31$^+$*	*81*	78.1 (71.7-81.3)	71.3 (60.5-81.8)	71.5 (58.8-83.0)	66.0 (48.4-76.3)	69.3 (65.0-79.5)	79.1 (65.2-83.4)	79.3 (71.6-85.3)	76.0 (61.0-84.2)	67.1 (57.9-80.5)	69.8 (50.7-78.9)	63.9 (51.4-79.1)
%	*CD127$^+$*	*82*	91.3 (90.1-95.7)	82.1 (71.7-88.6)	79.6 (73.3-85.5)	79.5 (74.3-90.3)	83.9 (78.3-95.2)	72.5 (66.1-85.8)	81.1 (69.4-89.2)	81.0 (64.6-88.8)	85.2 (75.1-91.3)	85.5 (80.8-93.3)	84.1 (77.6-91.9)
central memory CD4$^+$ T cells		*109*	**5.1 (1.9-8.0)**	**6.9 (5.1-9.3)**	**9.5 (4.8-13.3)**	**12.1 (4.1-16.5)**	**19.3 (14.9-25.5)**	**23.4 (14.3-33.0)**	**23.7 (17.9-39.0)**	**30.2 (22.5-37.0)**	**30.7 (21.4-40.3)**	**35.2 (24.5-44.4)**	**33.8 (24.3-42.7)**
%	*Ki67$^+$*	*76*	nd	14.5 (8.3-21.9)	5.8 (3.9-9.3)	5.5 (1.3-11.7)	3.9 (3.3-5.8)	3.9 (1.4-5.8)	3.8 (2.5-7.9)	2.8 (1.3-4.3)	1.5 (1.2-2.3)	2.1 (1.6-3.4)	2.4 (1.7-3.8)
%	*CD25$^+$*	*64*	nd	nd	9.6 (6.3-13.1)	7.2 (3.1-10.8)	6.5 (4.1-10.1)	5.9 (2.4-10.1)	6.8 (3.6-8.4)	5.7 (3.3-13.4)	4.7 (3.3-13.1)	5.5 (3.0-9.1)	5.7 (3.2-9.0)
%	*CD31$^+$*	*74*	nd	13.4 (11.1-17.0)	15.2 (10.0-22.0)	8.0 (6.0-11.6)	10.6 (6.1-15.1)	10.1 (7.3-11.3)	11.0 (6.6-20.5)	9.4 (5.1-14.0)	6.7 (5.4-22.2)	8.1 (2.0-11.4)	5.1 (2.2-11.2)
%	*CD127$^+$*	*75*	nd	48.9 (41.8-69.4)	49.9 (44.6-61.5)	57.8 (46.7-62.8)	65.8 (44.4-84.4)	59.2 (55.0-69.8)	63.5 (56.3-84.3)	69.5 (62.3-79.5)	74.0 (66.7-82.8)	77.7 (67.0-84.3)	82.4 (74.8-91.7)
effector memory CD4$^+$ T cells		*109*	**0.0 (0.0-0.1)**	**0.3 (0.1-0.8)**	**0.4 (0.2-2.7)**	**0.4 (0.2-0.8)**	**1.4 (0.5-2.2)**	**1.4 (0.4-3.4)**	**2.1 (0.9-5.1)**	**3.2 (1.5-9.7)**	**5.7 (2.6-6.7)**	**3.9 (2.1-5.5)**	**3.2 (2.1-7.4)**
effector CD4$^+$ T cells		*109*	**0.0 (0.0-0.9)**	**0.3 (0.0-0.5)**	**0.2 (0.1-1.5)**	**0.4 (0.1-0.5)**	**0.2 (0.0-0.9)**	**0.4 (0.1-1.1)**	**0.2 (0.1-0.5)**	**0.4 (0.1-1.3)**	**0.9 (0.1-2.9)**	**0.3 (0.1-8.5)**	**0.3 (0.1-2.2)**

Table 1. continued

		no.	cord blood	0-6 months	6-12 months	1-2 years	2-3 years	3-4 years	4-6 years	6-9 years	9-12 years	12-15 years	15-18 years
total memory CD4⁺ (CD45RO⁺) T cells		*109*	**5.1 (1.9-8.1)**	**7.0 (5.3-10.1)**	**10.3 (5.1-15.2)**	**12.3 (4.6-17.3)**	**21.0 (15.6-27.5)**	**24.5 (15.6-35.4)**	**26.9 (18.9-42.1)**	**34.3 (24.0-43.4)**	**33.9 (24.5-46.4)**	**39.5 (27.9-48.8)**	**37.5 (27.3-49.8)**
%	*Ki67⁺*	*76*	nd	16.3 (9.1-21.5)	5.7 (4.7-9.9)	5.8 (1.7-12.3)	4.3 (3.7-6.4)	3.9 (1.6-6.1)	4.0 (2.9-7.2)	2.8 (1.4-4.2)	1.6 (1.4-2.5)	2.3 (1.7-3.8)	2.7 (1.7-3.8)
total CD8⁺ T cells		*109*	**16.3 (14.3-21.3)**	**14.9 (7.7-22.0)**	**14.2 (11.0-17.8)**	**15.5 (14.0-26.0)**	**16.7 (12.2-22.6)**	**21.3 (14.7-27.2)**	**24.5 (17.5-31.1)**	**20.4 (13.8-28.8)**	**22.4 (16.4-36.2)**	**18.7 (13.6-23.5)**	**18.8 (13.5-22.0)**
%	*Ki67⁺*	*82*	3.9 (1.9-5.0)	2.0 (0.9-4.0)	1.0 (0.5-2.2)	1.2 (0.2-2.7)	1.5 (0.8-6.1)	1.2 (0.5-2.8)	1.2 (0.8-4.2)	0.9 (0.5-2.2)	0.7 (0.6-1.5)	0.8 (0.3-1.5)	1.0 (0.5-1.6)
%	*CD31⁺*	*81*	99.3 (97.4-99.7)	93.5 (79.7-97.6)	93.6 (81.7-96.9)	85.4 (73.9-95.3)	73.1 (53.4-85.5)	79.7 (67.1-89.7)	82.4 (51.1-90.3)	75.5 (54.4-88.3)	71.4 (60.1-82.2)	75.6 (57.5-89.4)	81.1 (50.2-86.6)
%	*CD38⁺*	*85*	97.6 (93.5-98.6)	90.9 (84.9-96.6)	90.2 (85.2-96.2)	71.4 (65.8-86.1)	78.2 (67.5-88.1)	69.5 (51.5-82.6)	66.2 (56.2-81.4)	59.4 (49.1-65.3)	47.1 (39.1-61.6)	50.6 (37.0-68.7)	32.4 (20.8-48.2)
%	*HLA-DR⁺*	*83*	0.2 (0.1-0.3)	0.8 (0.5-4.5)	0.7 (0.6-2.1)	1.7 (0.4-5.8)	4.8 (1.4-9.2)	4.1 (1.4-18.5)	2.2 (0.7-12.8)	3.0 (1.5-4.2)	2.3 (1.4-5.8)	1.6 (0.7-7.4)	3.6 (1.2-8.1)
%	*CD38⁺HLA-DR⁺*	*83*	0.2 (0.1-0.3)	0.8 (0.5-4.1)	0.7 (0.6-2.0)	1.7 (0.4-4.4)	4.1 (1.3-8.8)	3.3 (1.1-16.0)	1.4 (0.5-10.7)	1.9 (1.1-3.2)	1.5 (0.9-3.6)	0.8 (0.4-3.7)	2.2 (0.9-4.2)
%	*CD127⁺*	*82*	93.5 (86.7-97.7)	79.0 (68.3-92.5)	84.9 (61.3-88.2)	78.9 (59.6-85.5)	69.4 (50.1-87.0)	59.5 (42.6-76.0)	67.3 (39.8-83.7)	72.5 (41.7-87.3)	72.9 (45.5-83.7)	81.2 (62.3-89.4)	69.4 (64.2-82.1)
naive CD8⁺ T cells		*109*	**95.4 (90.4-98.1)**	**94.9 (78.5-97.1)**	**94.4 (68.7-96.6)**	**95.7 (77.5-98.5)**	**86.7 (57.1-91.4)**	**74.7 (54.6-90.6)**	**64.6 (53.2-87.2)**	**75.8 (57.0-83.7)**	**74.0 (49.2-82.7)**	**74.7 (62.3-86.3)**	**64.3 (48.6-87.5)**
%	*Ki67⁺*	*82*	3.6 (1.8-4.6)	1.4 (0.7-3.4)	0.7 (0.3-1.5)	0.3 (0.1-1.5)	0.5 (0.3-3.0)	0.3 (0.2-1.6)	0.4 (0.2-2.2)	0.3 (0.1-0.8)	0.2 (0.1-0.8)	0.2 (0.1-0.3)	0.4 (0.2-0.5)
%	*CD25⁺*	*80*	14.3 (3.1-16.4)	1.0 (0.3-6.6)	0.6 (0.3-1.5)	1.0 (0.3-1.7)	0.6 (0.1-0.9)	0.3 (0.2-3.0)	0.5 (0.2-1.0)	0.4 (0.0-3.0)	0.2 (0.2-1.3)	0.2 (0.0-0.7)	0.6 (0.2-1.6)
%	*CD31⁺*	*81*	99.7 (98.7-99.9)	96.4 (80.4-98.7)	94.4 (92.6-97.4)	89.8 (81.2-95.2)	80.6 (76.5-90.1)	88.3 (79.5-97.4)	89.6 (61.4-95.0)	86.4 (72.0-96.3)	86.7 (68.6-91.3)	84.2 (65.2-94.0)	85.5 (64.8-93.7)
%	*CD127⁺*	*82*	96.0 (91.8-98.9)	86.6 (78.6-94.4)	92.0 (82.8-94.2)	89.1 (71.7-92.4)	88.7 (78.6-93.0)	74.6 (58.8-94.1)	85.0 (76.8-93.8)	83.9 (76.2-95.6)	91.6 (74.0-96.4)	90.6 (81.4-96.2)	84.6 (77.2-92.0)
central memory CD8⁺ T cells		*109*	**4.2 (1.7-8.8)**	**3.7 (2.6-14.6)**	**5.2 (3.1-11.6)**	**4.1 (1.0-8.3)**	**10.5 (5.9-22.3)**	**14.2 (5.5-23.3)**	**16.6 (8.5-38.9)**	**16.6 (9.2-22.6)**	**18.3 (11.5-30.7)**	**19.1 (12.2-27.2)**	**24.2 (9.8-37.6)**
%	*Ki67⁺*	*52*	nd	nd	nd	nd	8.4 (3.1-24.5)	7.2 (3.5-17.0)	5.6 (3.6-12.8)	2.8 (1.8-6.9)	2.7 (1.4-3.7)	3.6 (0.8-6.7)	3.4 (1.6-12.6)
%	*CD31⁺*	*73*	nd	76.7 (62.1-89.3)	70.6 (53.2-89.7)	65.8 (56.9-73.3)	62.0 (50.9-64.7)	69.3 (60.8-78.6)	75.5 (54.2-84.2)	62.3 (43.6-75.5)	57.9 (51.1-77.0)	58.9 (36.4-62.2)	75.7 (51.2-85.7)
%	*CD127⁺*	*54*	nd	nd	nd	nd	32.1 (14.8-54.4)	36.6 (23.0-42.6)	39.5 (14.3-58.2)	57.2 (25.8-76.3)	62.8 (16.6-80.2)	76.2 (48.5-83.7)	70.5 (56.3-93.7)
effector memory CD8⁺ T cells		*109*	**0.1 (0.0-0.1)**	**0.1 (0.0-3.1)**	**0.1 (0.1-7.1)**	**0.1 (0.0-2.8)**	**0.8 (0.3-6.8)**	**1.3 (0.4-12.7)**	**4.3 (0.4-6.0)**	**2.3 (0.7-14.0)**	**1.6 (0.7-7.5)**	**1.2 (0.8-6.2)**	**3.7 (0.2-6.9)**
effector CD8⁺ T cells		*109*	**0.7 (0.0-1.1)**	**0.3 (0.1-6.7)**	**0.3 (0.2-12.7)**	**0.6 (0.1-11.6)**	**2.5 (0.6-16.4)**	**2.8 (0.8-21.7)**	**4.3 (0.6-28.9)**	**3.8 (0.9-17.9)**	**6.1 (1.5-20.8)**	**1.4 (0.8-13.2)**	**6.6 (0.8-14.0)**
total memory CD8⁺ (CD45RO⁺) T cells		*109*	**4.3 (1.7-8.8)**	**3.8 (2.7-15.6)**	**5.3 (3.1-18.7)**	**4.1 (1.0-11.1)**	**11.4 (6.4-28.7)**	**17.4 (7.4-30.0)**	**20.8 (10.6-40.6)**	**19.6 (13.4-29.9)**	**19.1 (13.3-33.7)**	**21.3 (13.0-29.3)**	**28.0 (11.7-42.9)**
%	*Ki67⁺*	*41*	nd	nd	nd	nd	16.5 (4.1-27.4)	4.7 (2.4-16.3)	5.9 (3.4-12.4)	2.7 (1.9-7.7)	2.1 (1.6-4.4)	2.9 (1.1-5.4)	3.2 (1.5-11.7)

Values are presented as medians (5^{th}-95^{th} percentiles). Total CD4 and CD8 T cells (percentage of lymphocytes) and subsets (percentage of total CD4 or CD8 T cells) in bold face. Expression of specific markers within these cell populations in italics. no. = number of individuals; nd = not determined.

Table 2. Counts of total T cells and T-cell subsets

	no.	cord blood	0-6 months	6-12 months	1-2 years	2-3 years	3-4 years	4-6 years	6-9 years	9-12 years	12-15 years	15-18 years
leukocytes (x10^9/L)	*98*	nd	10.8 (4.7-14.8)	12.1 (6.6-13.6)	8.7 (7.4-14.3)	9.5 (6.6-12.9)	8.5 (5.0-11.2)	8.4 (6.7-11.9)	7.0 (4.0-12.5)	6.0 (5.3-11.2)	5.9 (4.1-8.3)	5.9 (4.8-7.4)
lymphocytes (x10^9/L)	*98*	nd	5.2 (3.2-9.8)	6.4 (3.8-9.3)	5.6 (4.6-6.0)	4.4 (2.8-6.4)	3.7 (2.2-5.9)	3.4 (2.8-4.8)	3.0 (1.8-5.0)	2.7 (2.1-4.0)	2.4 (1.5-2.8)	2.1 (1.5-2.7)
total CD4$^+$ T cells	*98*	nd	1985 (1294-4012)	2721 (1327-4455)	2073 (1902-2977)	1469 (925-2477)	1044 (646-2331)	1186 (1000-1931)	992 (641-1453)	970 (695-1473)	904 (608-1217)	809 (560-1067)
regulatory T cells (CD127$^-$CD25$^+$CD4$^+$)	*73*	nd	208 (64-282)	136 (74-280)	107 (79-162)	63 (42-69)	78 (30-126)	58 (41-86)	47 (18-86)	36 (23-89)	41 (25-64)	26 (19-41)
CD27$^+$CD45RO$^-$	*73*	nd	175 (55-251)	123 (60-237)	100 (57-147)	41 (27-49)	58 (16-93)	33 (21-58)	28 (10-61)	25 (9-52)	20 (10-40)	16 (7-23)
CD45RO$^+$	*73*	nd	21 (9-36)	25 (11-43)	18 (9-26)	20 (13-27)	20 (10-38)	26 (11-31)	20 (8-30)	20 (10-36)	19 (12-32)	11 (8-23)
naive CD4$^+$ T cells	*98*	nd	1856 (1164-3712)	2512 (1134-4204)	1797 (1565-2794)	1153 (685-2055)	882 (430-1871)	894 (630-1414)	661 (375-1096)	596 (441-1109)	523 (311-781)	469 (335-725)
CD31$^+$	*73*	nd	1684 (781-2695)	1920 (788-2564)	1188 (961-1597)	824 (438-1037)	908 (296-1446)	644 (493-1054)	460 (244-894)	442 (313-785)	304 (239-477)	325 (207-585)
central memory CD4$^+$ T cells	*98*	nd	130 (110-299)	230 (119-355)	241 (112-314)	293 (210-442)	236 (155-476)	357 (222-502)	266 (195-427)	262 (215-489)	328 (232-373)	242 (201-419)
effector memory CD4$^+$ T cells	*98*	nd	6 (1-17)	9 (5-67)	12 (4-18)	16 (10-34)	16 (5-51)	39 (11-50)	28 (14-97)	45 (26-75)	29 (19-59)	25 (15-78)
effector CD4$^+$ T cells	*98*	nd	7 (1-14)	4 (3-37)	8 (3-11)	3 (1-12)	4 (2-10)	3 (1-7)	3 (1-10)	10 (0-28)	2 (1-101)	2 (1-21)
total memory CD4$^+$ (CD45RO$^+$) T cells	*98*	nd	139 (111-315)	240 (126-378)	245 (121-329)	311 (224-456)	251 (167-499)	399 (234-534)	304 (216-497)	322 (246-564)	357 (252-417)	273 (216-490)
total CD8$^+$ T cells	*98*	nd	747 (394-1865)	850 (593-1517)	892 (667-1473)	733 (394-1197)	868 (365-1255)	852 (602-1203)	628 (249-1440)	652 (426-991)	447 (228-577)	399 (216-499)
naive CD8$^+$ T cells	*98*	nd	669 (345-1635)	789 (499-1423)	834 (637-1113)	526 (336-927)	673 (248-999)	573 (442-733)	426 (203-961)	448 (267-683)	325 (165-424)	257 (118-312)
central memory CD8$^+$T cells	*98*	nd	28 (14-260)	40 (26-127)	34 (8-127)	66 (38-239)	105 (32-228)	156 (62-402)	103 (35-256)	100 (72-262)	82 (32-153)	96 (30-166)
effector memory CD8$^+$ T cells	*98*	nd	1 (0-18)	1 (0-67)	1 (0-47)	5 (2-77)	18 (2-106)	36 (3-67)	16 (2-109)	18 (3-51)	5 (3-26)	10 (1-26)
effector CD8$^+$ T cells	*98*	nd	5 (0-50)	3 (1-123)	5 (1-193)	15 (5-174)	25 (7-218)	40 (5-310)	20 (3-198)	50 (8-155)	5 (3-59)	20 (3-65)
total memory CD8$^+$ (CD45RO$^+$) T cells	*98*	nd	29 (14-268)	41 (26-177)	35 (8-174)	74 (41-314)	151 (36-246)	196 (79-425)	134 (38-296)	123 (77-303)	89 (35-163)	111 (33-189)

Absolute counts x 10^6 per liter blood, unless stated differently. Values are presented as medians (5^{th}-95^{th} percentiles). Counts of T-cell subsets in bold face, whereas counts of specific cell populations within these T-cell subsets in italics. no. = number of individuals; nd = not determined.

Table 3. sjTRECs and T-cell ratios

	no.	cord blood	0-6 months	6-12 months	1-2 years	2-3 years	3-4 years	4-6 years	6-9 years	9-12 years	12-15 years	15-18 years
$CD4^+$ T-cell sjTREC content (per cell)	*78*	0.098 (0.080-0.173)	0.180 (0.083-0.343)	0.087 (0.035-0.317)	0.225 (0.047-0.251)	0.130 (0.018-0.377)	0.093 (0.016-0.299)	0.057 (0.023-0.342)	0.032 (0.014-0.190)	0.030 (0.013-0.102)	0.045 (0.007-0.108)	0.037 (0.017-0.058)
$CD8^+$ T-cell sjTREC content (per cell)	*77*	0.112 (0.096-0.172)	0.145 (0.060-0.222)	0.084 (0.028-0.341)	0.151 (0.043-0.206)	0.078 (0.022-0.285)	0.085 (0.021-0.239)	0.036 (0.015-0.289)	0.032 (0.011-0.152)	0.034 (0.016-0.104)	0.047 (0.009-0.120)	0.031 (0.012-0.055)
$CD4^+$ T-cell sjTREC number	*71*	nd	484 (188-1206)	384 (50-953)	436 (105-626)	142 (25-585)	102 (36-625)	71 (24-433)	33 (13-289)	22 (19-118)	36 (5-94)	24 (14-56)
$CD8^+$ T-cell sjTREC number	*70*	nd	131 (41-345)	69 (17-551)	113 (39-145)	65 (12-267)	54 (19-293)	32 (13-181)	17 (5-219)	18 (8-85)	10 (3-68)	13 (4-26)
total $CD4^+$/$CD8^+$ T-cell ratio	*109*	2.8 (2.2-4.3)	2.6 (1.9-5.5)	2.7 (1.9-5.4)	2.7 (1.4-3.7)	2.4 (1.3-3.0)	1.6 (0.9-2.7)	1.6 (0.9-2.3)	1.6 (1.0-3.0)	1.6 (0.9-2.5)	2.2 (1.5-3.7)	2.2 (1.4-4.0)
naive $CD4^+$/$CD8^+$ T-cell ratio	*109*	2.9 (2.2-4.3)	2.8 (1.9-5.5)	2.9 (1.8-5.4)	2.7 (1.5-3.5)	2.2 (1.4-2.8)	1.7 (0.9-2.5)	1.7 (1.0-2.3)	1.5 (0.9-2.6)	1.6 (0.8-2.1)	1.7 (1.1-3.1)	1.7 (1.3-4.6)
naive/memory $CD4^+$ T-cell ratio	*109*	18.4 (11.8-61.9)	13.2 (9.0-18.6)	8.7 (5.5-18.6)	7.1 (4.8-22.8)	3.8 (2.6-5.4)	3.1 (1.9-5.4)	2.8 (1.4-4.3)	1.9 (1.3-3.2)	1.9 (1.2-3.1)	1.5 (1.0-2.7)	1.7 (1.0-2.7)
naive/memory $CD8^+$ T-cell ratio	*109*	22.2 (11.5-71.8)	25.0 (5.1-36.3)	17.8 (3.7-31.3)	23.4 (8.4-116.1)	7.6 (2.3-14.4)	4.6 (2.0-15.9)	3.1 (1.4-9.2)	3.8 (2.0-6.5)	3.7 (1.5-6.4)	3.6 (2.3-7.8)	2.4 (1.1-8.6)
naive/memory regulatory T-cell ratio	*80*	5.7 (3.4-18.1)	6.9 (4.9-8.8)	5.3 (4.2-9.2)	4.2 (2.5-13.2)	1.9 (1.5-3.0)	2.4 (1.1-5.0)	1.5 (0.8-3.1)	1.5 (1.0-2.5)	1.2 (0.6-2.4)	1.1 (0.6-2.0)	1.2 (0.6-2.4)

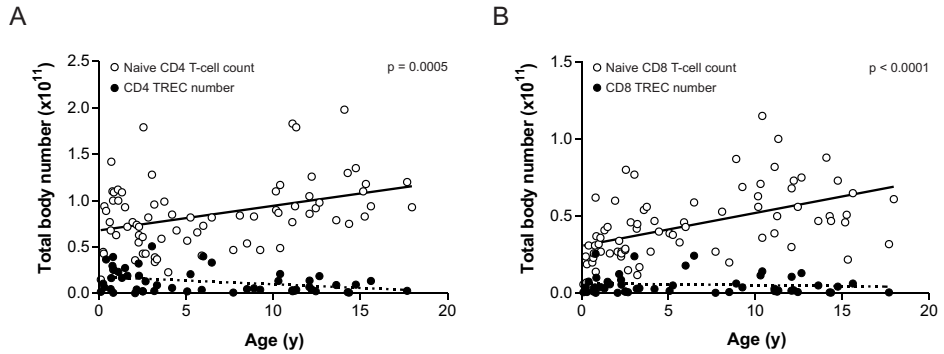

Figure 3. Total body sjTREC numbers and naive T-cell counts during childhood

Total body naive $CD4^+$ and $CD8^+$ T-cell counts and total body $CD4^+$ and $CD8^+$ sjTREC numbers were calculated as described in Materials and methods. The slopes of the linear regression lines were statistically different ($p<0.05$) as assessed by Analysis of Covariance.

induced, inducible) phenotype and are generally present in a 1:2 ratio (9). However, in cord blood, 85.1% of the Treg-compartment consisted of naive ($CD27^+CD45RO^-$) Tregs (Table 1; Suppl. Figure 1). In contrast to memory ($CD45RO^+$) Treg counts per µl blood, which remained stable during childhood, the number of naive Treg declined with age (Table 2; Suppl. Figure 2). Consequently, the naive/memory Treg ratio of about 6:1 during the first years of life decreased to approximately 1:1 in later childhood (Table 3).

Naive T-cell production: thymic output and peripheral proliferation

To assess the relative contribution of thymic output and peripheral proliferation in the establishment of the T-cell compartment, the levels and numbers of T-cell receptor excision circles (sjTRECs), and Ki67 and CD31 expression levels of naive T cells were determined.

Although T-cell counts per µl blood declined from birth to adolescence (Table 2; Suppl. Figure 2), these cell numbers do not reflect total body T-cell counts as the total blood volume increases in a growing child. When T-cell counts per µl blood were adjusted for body weight (Materials and methods), we found that total body naive $CD4^+$ (Figure 3A) and $CD8^+$ T-cell counts (Figure 3B) increased gradually during childhood. If only thymic export would have contributed to the establishment of the naive T-cell compartment during childhood, total body $CD4^+$ and $CD8^+$ sjTREC numbers would have to increase with a similar slope as total body naive $CD4^+$ and $CD8^+$ T-cell counts (6). We found however, that total body sjTREC numbers remained relatively stable while total body T-cell counts increased (Figure 3, difference between the slopes: $p\leq0.0005$). These data imply that besides thymic naive T-cell production, also naive T-cell proliferation contributes to the establishment of the T-cell compartment in children. This is in line with the decreasing level of

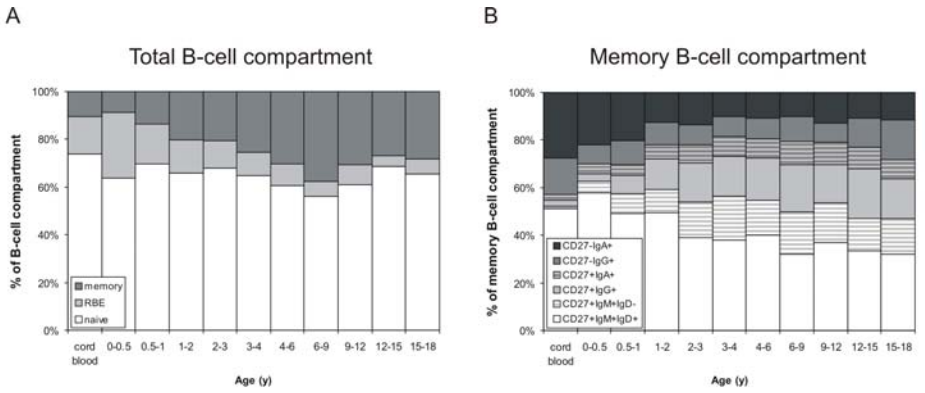

Figure 4. B-cell subset distribution during childhood

A) The relative contribution of the RBE, naive and total memory subpopulations in the B-cell compartment, and B) the contribution of the six memory B-cell subpopulations in the total memory B-cell population during childhood.

$CD31^+CD27^+CD45RO^-$ $CD4^+$ T cells (Table 1; Suppl. Figure 3B), representing naive $CD4^+$ T cells that are most proximal to the thymus (30,31)(Vrisekoop et al, manuscript in preparation). In the first half year of life the median fraction of $Ki67^+$ naive $CD4^+$ and $CD8^+$ T cells were elevated compared to the fractions beyond that age (Table 1; Suppl. Figure 4A). Naive $CD8^+$ T-cell proliferation levels were approximately 2-fold higher in comparison to proliferation levels of naive $CD4^+$ T cells in early childhood (Table 1). The naive $CD4^+/CD8^+$ ratio was 2.9 at birth and dropped to 1.7 in the following five years (Table 3), which is most likely caused by a larger expansion of naive $CD8^+$ T cells compared to naive $CD4^+$ T cells early in life.

T-cell activation

The combined expression of CD38 and HLA-DR on the cell surface is often used to describe (virally) activated T cells. In our study population of healthy children hardly any activated $CD4^+$ or $CD8^+$ T cells were observed (Table 1; Suppl. Figure 4B).

B-cell compartment

Total $CD19^+$ B cells

In agreement with previous publications (1,2) absolute numbers of $CD19^+$ B cells increased shortly after birth to a maximum at 6-12 months and decreased afterwards, reaching stable numbers at the end of puberty (Tables 4,5; Suppl. Figure 6).

Recent bone marrow emigrants (RBE) and naive B cells

Naive B cells constituted the largest peripheral B-cell subset throughout childhood (Figure 4; Table 4; Suppl. Figure 5). Recent bone marrow emigrants (RBE) are pro-

Table 4. Percentages of total B cells and B-cell subsets

	no.	cord blood	0-6 months	6-12 months	1-2 years	2-3 years	3-4 years	4-6 years	6-9 years	9-12 years	12-15 years	15-18 years
total B cells	*90*	11.5 (7.6-15.5)	31.3 (20.5-40.9)	26.1 (11.1-45.4)	21.1 (16.3-26.8)	25.7 (17.3-30.3)	20.0 (14.4-25.1)	17.9 (7.9-22.5)	16.5 (8.5-20.2)	13.1 (4.3-18.2)	12.3 (7.8-23.7)	11.5 (7.8-15.1)
RBE B cells	*83*	15.5 (5.6-30.7)	27.6 (17.1-32.2)	15.4 (9.3-26.7)	14.8 (9.7-17.9)	10.2 (3.6-21.4)	9.1 (6.4-13.9)	9.3 (6.8-11.5)	5.8 (3.4-9.0)	6.4 (2.9-23.8)	4.8 (1.5-7.3)	5.2 (1.5-19.1)
naive B cells	*83*	75.9 (55.6-81.3)	60.2 (55.2-73.9)	73.7 (57.5-84.7)	65.7 (61.5-68.7)	68.7 (50.4-78.5)	66.5 (49.7-77.1)	59.1 (52.3-72.1)	52.6 (47.8-69.8)	57.2 (51.5-73.3)	70.6 (64.6-80.1)	64.7 (59.0-81.1)
$CD27^+IgM^+IgD^+$ memory B cells	*145*	4.6 (1.3-11.5)	4.5 (1.6-10.1)	5.9 (3.4-14.0)	10.2 (4.6-15.0)	7.6 (5.1-12.3)	8.3 (4.8-16.1)	11.4 (6.7-18.1)	10.4 (6.3-22.0)	10.3 (6.5-22.2)	9.0 (4.7-15.5)	7.6 (4.6-16.8)
$CD27^+IgM^+IgD^-$ memory B cells	*145*	0.1 (0.0-0.3)	0.4 (0.1-0.7)	1.1 (0.4-2.6)	1.9 (1.6-2.6)	2.3 (1.6-6.6)	4.5 (0.9-9.3)	4.3 (2.0-9.5)	6.9 (2.0-11.8)	2.9 (1.8-13.3)	2.8 (1.6-11.3)	3.8 (0.9-9.1)
$CD27^+IgG^+$ memory B cells	*100*	0.2 (0.1-0.5)	0.3 (0.1-0.4)	1.0 (0.4-1.7)	2.3 (1.5-4.2)	3.3 (0.8-6.3)	4.3 (1.8-6.2)	5.6 (0.8-10.3)	6.1 (2.7-14.0)	4.7 (1.5-8.8)	5.4 (2.1-9.4)	4.7 (0.8-11.7)
$CD27^+IgA^+$ memory B cells	*100*	0.2 (0.1-0.6)	0.4 (0.2-0.5)	0.7 (0.3-1.0)	1.3 (0.8-1.7)	1.4 (0.7-2.9)	1.8 (1.0-3.5)	2.6 (1.0-3.9)	3.5 (1.1-6.1)	2.4 (1.3-6.1)	2.6 (1.2-3.8)	2.6 (0.2-4.6)
$CD27^-IgG^+$ memory B cells	*100*	1.4 (1.1-2.3)	0.7 (0.5-0.8)	1.2 (0.4-2.9)	2.1 (0.7-2.6)	1.7 (0.5-3.4)	1.5 (1.1-3.9)	2.4 (0.7-6.8)	4.1 (1.8-6.5)	2.6 (0.7-4.4)	3.1 (1.1-8.0)	2.6 (0.6-28.4)
$CD27^-IgA^+$ memory B cells	*100*	2.5 (2.2-4.7)	1.8 (1.4-2.4)	2.4 (1.4-4.4)	2.5 (1.0-4.3)	2.1 (1.3-5.8)	2.5 (1.3-4.9)	3.3 (1.6-4.9)	4.1 (1.1-5.7)	2.6 (1.2-10.3)	3.1 (1.1-5.3)	4.0 (0.8-5.6)

Total B cells as percentage of lymphocytes. B-cell subsets as percentage of total B cells. Values as medians (5^{th}-95^{th} percentiles). no. = number of individuals

Table 5. Counts of total B cells and B-cell subsets

	no.	cord blood	0-6 months	6-12 months	1-2 years	2-3 years	3-4 years	4-6 years	6-9 years	9-12 years	12-15 years	15-18 years
total B cells	*90*	858 (559-1054)	1623 (961-3679)	1717 (571-3680)	1115 (871-1553)	1157 (686-1732)	709 (359-1552)	593 (278-1022)	418 (296-784)	338 (116-555)	284 (119-578)	210 (114-436)
RBE B cells	*73*	89 (72-164)	459 (164-1184)	214 (70-821)	143 (109-278)	143 (41-248)	72 (23-95)	41 (24-98)	33 (13-63)	24 (12-35)	14 (2-41)	10 (6-41)
naive B cells	*73*	698 (439-800)	988 (627-2136)	1378 (420-2181)	738 (586-955)	757 (346-1356)	470 (244-724)	344 (149-618)	263 (154-413)	233 (60-300)	193 (83-398)	153 (72-257)
$CD27^+IgM^+IgD^+$ memory B cells	*90*	25 (24-34)	61 (49-179)	71 (30-396)	116 (40-177)	87 (50-148)	62 (27-210)	62 (32-164)	48 (24-135)	35 (9-109)	21 (10-74)	20 (10-39)
$CD27^+IgM^+IgD^-$ memory B cells	*90*	0 (0-2)	10 (6-19)	16 (2-42)	21 (16-34)	33 (19-53)	27 (6-113)	24 (7-98)	32 (7-65)	9 (3-34)	7 (3-39)	10 (2-40)
$CD27^+IgG^+$ memory B cells	*90*	2 (1-3)	4 (1-15)	13 (9-44)	28 (13-55)	38 (6-57)	29 (13-95)	32 (4-73)	26 (13-74)	16 (4-49)	11 (7-32)	8 (2-51)
$CD27^+IgA^+$ memory B cells	*90*	1 (1-1)	5 (3-10)	69 (21-106)	16 (8-20)	17 (8-26)	10 (6-54)	13 (4-26)	13 (5-35)	9 (3-26)	7 (4-13)	4 (1-20)
$CD27^-IgG^+$ memory B cells	*90*	12 (12-16)	12 (5-20)	22 (8-48)	22 (8-35)	20 (4-51)	18 (4-27)	15 (4-28)	17 (8-35)	7 (4-20)	8 (3-22)	5 (1-49)
$CD27^-IgA^+$ memory B cells	*90*	21 (14-24)	30 (17-50)	51 (15-85)	26 (11-48)	31 (10-70)	18 (5-75)	17 (6-42)	18 (5-34)	10 (7-35)	10 (1-17)	10 (2-17)

Absolute counts x 10^6 per liter blood. Values are presented as medians (5^{th}-95^{th} percentiles). no. = number of individuals

genitors of naive B cells and they formed the second largest B-cell population until the age of four (Table 4; Suppl. Figure 5). After peak levels in the first six months of life, absolute numbers and percentages of RBE and naive B cells both decreased gradually during early childhood and stabilized during puberty (Tables 4,5; Figure 4; Suppl. Figures 5,6), reaching numbers similar to adults (12-14,16,19).

Memory B cells

The memory B-cell compartment consists of six subpopulations: $CD27^+IgM^+IgD^+$ memory B cells, $CD27^+$ class-switched (IgG^+ and IgA^+) memory B cells, $CD27^+IgM^+IgD^-$ memory B cells and $CD27^-$ class-switched (IgG^+ and IgA^+) memory B cells were identified (15,16,19,21,22,27).

Despite the relatively large variation in absolute numbers and percentages of cells within each subset, there were several novel observations (Tables 4,5; Suppl. Figures 5,6). Firstly, in contrast to $CD27^+$ class-switched (IgG^+ and IgA^+) and $CD27^+IgM^+IgD^-$ memory B cells, $CD27^+IgM^+IgD^+$ memory B cells and $CD27^-$ class-switched (IgG^+ and IgA^+) B cells were already present in ample amounts in cord blood (Figure 4A,B; Suppl. Figure 5). Secondly, from birth on, $CD27^+IgM^+IgD^+$ memory B cells constituted the largest subpopulation in the memory B-cell compartment (Figure 4B; Suppl. Figures 5,6). Thirdly, all memory B-cell subpopulations increased in size during the first years of life. However, the subsets already present at birth reached their peak earlier (1-2 years) compared to the other ($CD27^+$ class-switched and $CD27^+IgM^+IgD^-$ memory B cells) subsets (2-3 years). In later childhood the numbers of all memory B-cell subsets declined to values found in adults (Table 5; Suppl. Figure 6).

Discussion

New insights into the composition of the T- and B-cell compartments have been gained over the last years. In this study, we have thoroughly characterized the T- and B-cell compartment of children aged 0-18 years. We found that besides naive T-cell generation by the thymus also proliferation within the periphery provides the growing infant with the necessary (naive) T cells, since total body naive $CD4^+$ and $CD8^+$ T-cell counts gradually increased during childhood while total body $CD4^+$ and $CD8^+$ sjTREC numbers remained relatively stable. These findings corroborate and extend our previously published data (6). Naive T-cell proliferation was found to be highest during the first half year of life. We hypothesize that this may be due to interleukin-7 (IL-7), a cytokine that has been shown to promote naive T-cell survival and, at higher concentrations, naive T-cell proliferation (32). IL-7 levels at young age have been found to be relatively high (33) and naive T cells at young age are intrinsically more

sensitive to IL-7-induced proliferation (34-36). Together, this may contribute to the observed elevated proliferation levels of naive T cells in early childhood.

Total Treg levels were relatively stable during childhood. We found that Tregs at birth mostly had a naive phenotype, which suggests that they are thymically-derived and therefore may be designated as naturally occurring Tregs. During childhood, a relative increase in the levels of Tregs having a memory phenotype was observed at the expense of naive Tregs. This likely reflects an accumulation of activation-induced Tregs during childhood. The relative enrichment of these so-called inducible Tregs in the Treg compartment apparently progresses with age as the naive/memory Treg ratio in adults was described to be 1:2 whereas we found this ratio to be 1:1 in late childhood (9).

Our detailed outline of the composition of the peripheral B-cell compartment during childhood pointed out that the absolute numbers of RBE increased directly after birth and reached a maximum at 0-6 months of age, while naive B-cell numbers reach a maximum at 6-12 months after birth. This temporal discrepancy between RBE and naive B cells fits with a model of increased bone marrow output of RBE immediately after birth, which then differentiate into naive B cells.

$CD27^+IgM^+IgD^+$ memory B cells, (IgM^+ memory B cells), constitute the majority of memory B cells during childhood. In adults, $CD27^+IgM^+IgD^+$ memory B cells carry mutated Ig receptors and are supposed to play an important role in the control of infections with encapsulated bacteria (16,21). During the first years of life the $CD27^+IgM^+IgD^+$ memory B cells slowly accumulate mutations in their Ig receptors (21), which might explain the transient immaturity of the antibody response against T-cell independent antigens, including bacterial capsular polysaccharide antigens, during this period of life (37,38). Recent data suggest that $CD27^+IgM^+IgD^+$ memory B cells do not develop by antigen-driven immune responses (39,40), which suggests that signaling independent of Ig receptors might be implicated in the diversification of the B-cell repertoire and generation of $CD27^+IgM^+IgD^+$ memory B cells.

During aging the number and percentage of $CD27^+$ class-switched memory B cells slowly increases. These memory B cells carry mutated Ig receptors and are generated in germinal centers during immune responses to T-cell dependent antigens (41). We show for the first time that, in contrast to these $CD27^+$ class-switched memory B cells, $CD27^-IgG^+$ and $CD27^-IgA^+$ memory B cells were already present in cord blood and during infancy. In adults these B cells also express mutated Ig and seem to participate in antigen-driven recall responses (27). IgG2-deficiency has been found to correlate with increased susceptibility to infections with encapsulated bacteria in adults, which stresses the importance of this isotype in the defense against these pathogens. The observation that IgG2 gene expression is lower in $CD27^-IgG^+$ compared to $CD27^+IgG^+$ memory B cells may therefore partly explain the inability to

mount a protective response against encapsulated bacteria during infancy due to the relatively larger abundance of $CD27^{-}IgG^{+}$ memory B cells at these ages (27).

This extensive study provides reference values of the T- and B-cell compartment in children from birth to 18 years of age and can assist in clinical studies in which reconstitution of the lymphocyte compartment is monitored or to identify hematological or immunological deficiencies or diseases. In addition, these data provide new insights into the phenotypical development of the T- and B-cell compartments.

Acknowledgments

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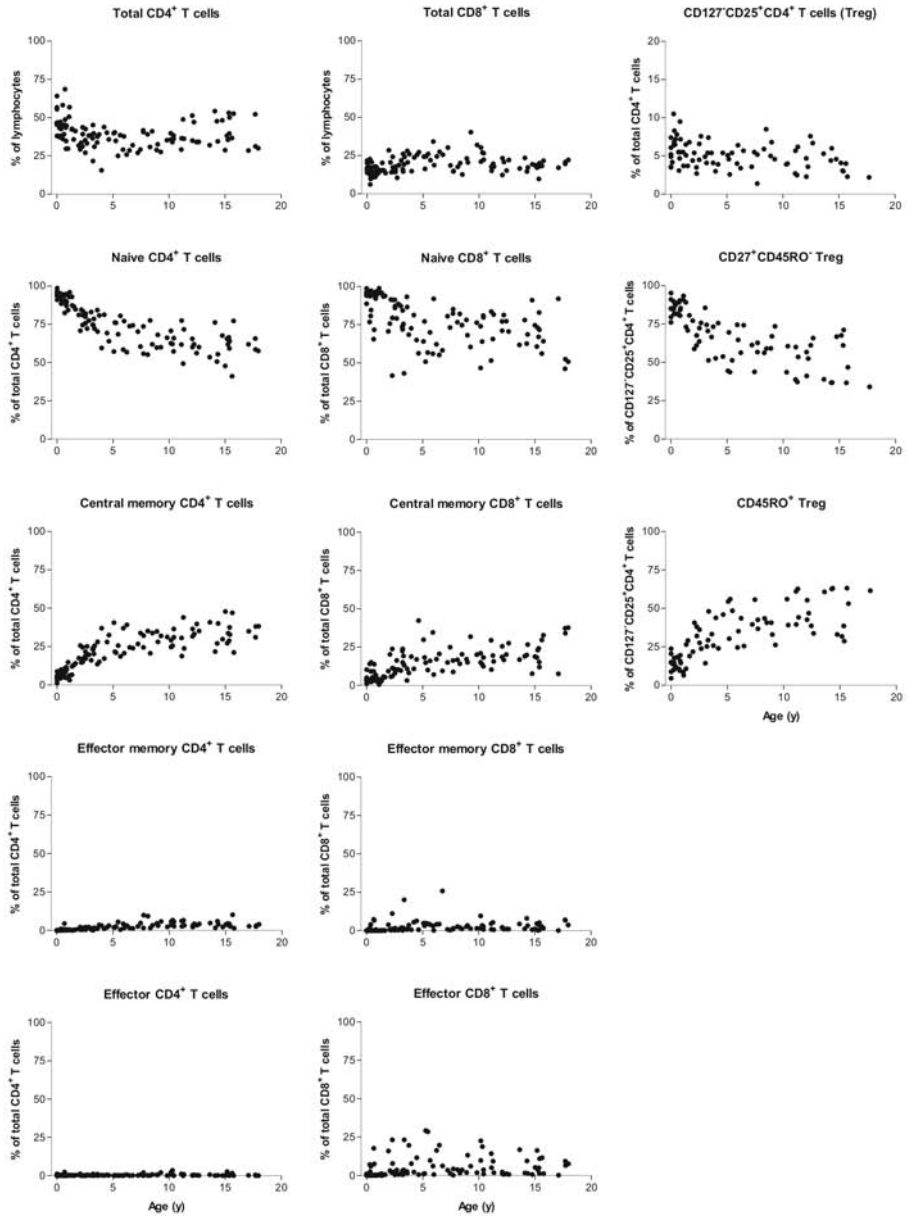
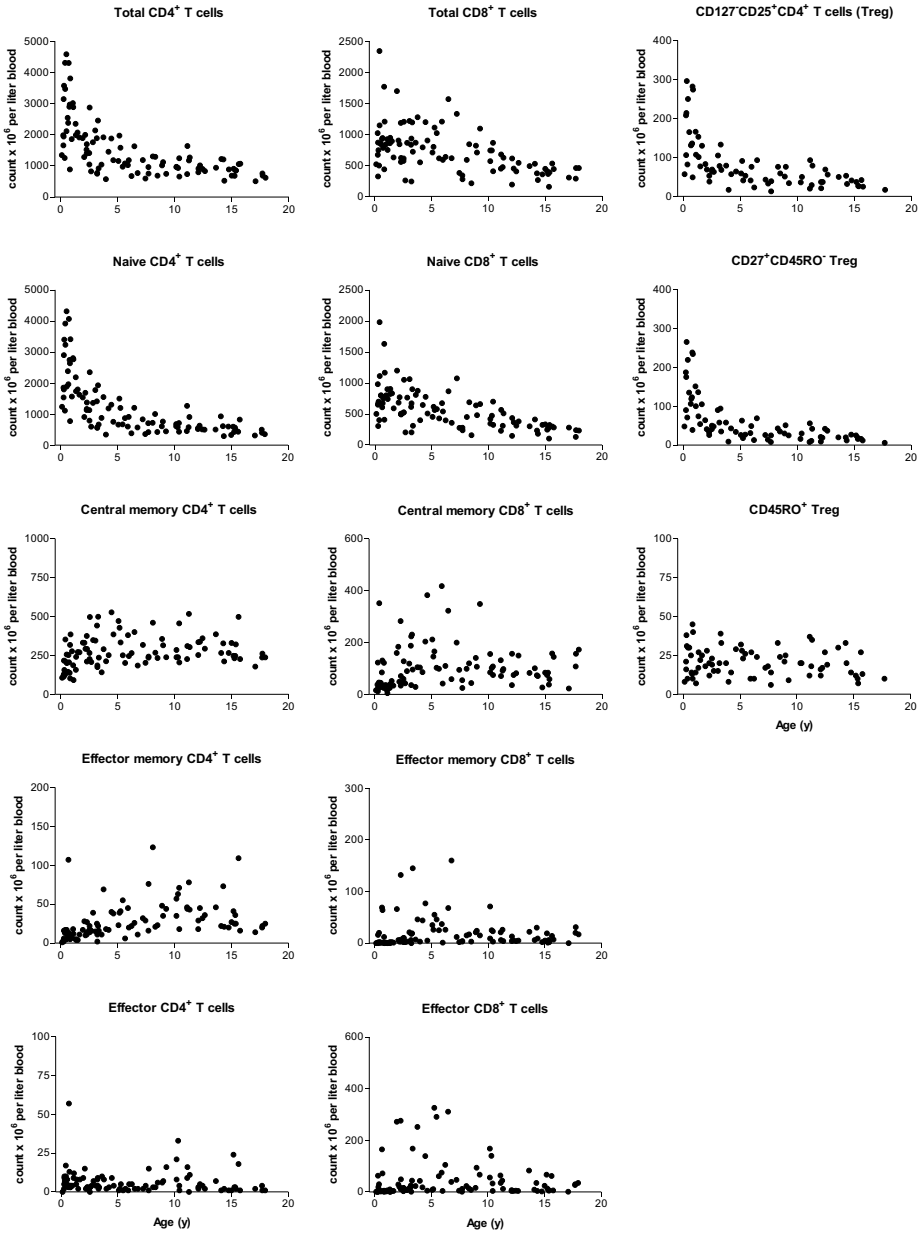


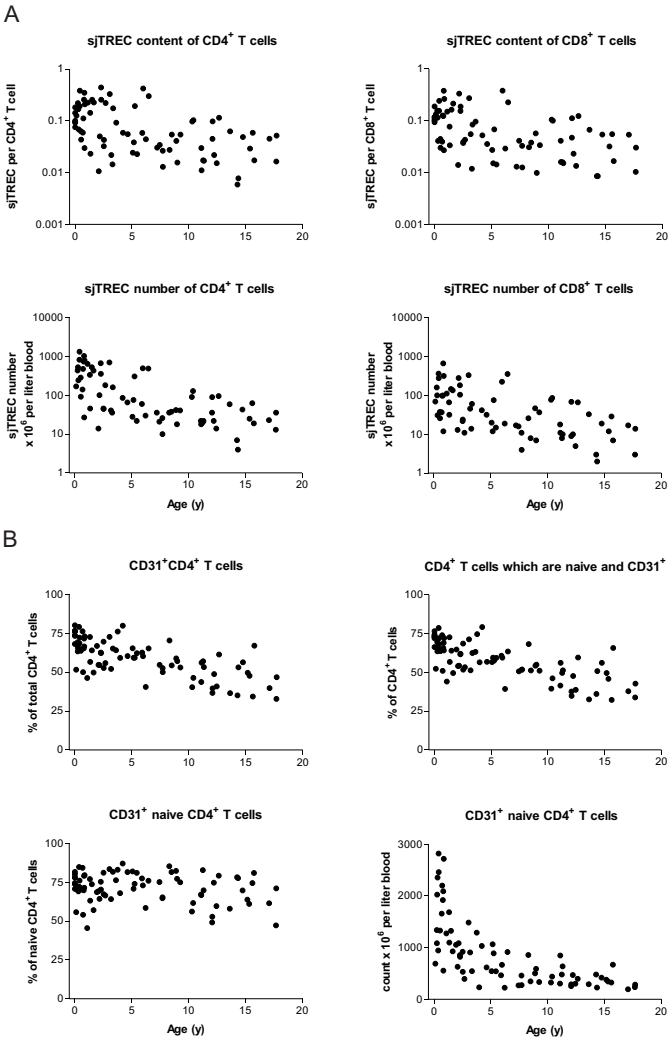
Supplementary Figure 1. Percentages of total T cells and T-cell subsets

Percentages of total $CD4^+$ and $CD8^+$ T cells in lymphocytes and subsets in the total $CD4^+$ and $CD8^+$ T-cell compartment during childhood. Each dot represents a single individual. Population definitions as described in Materials and methods.



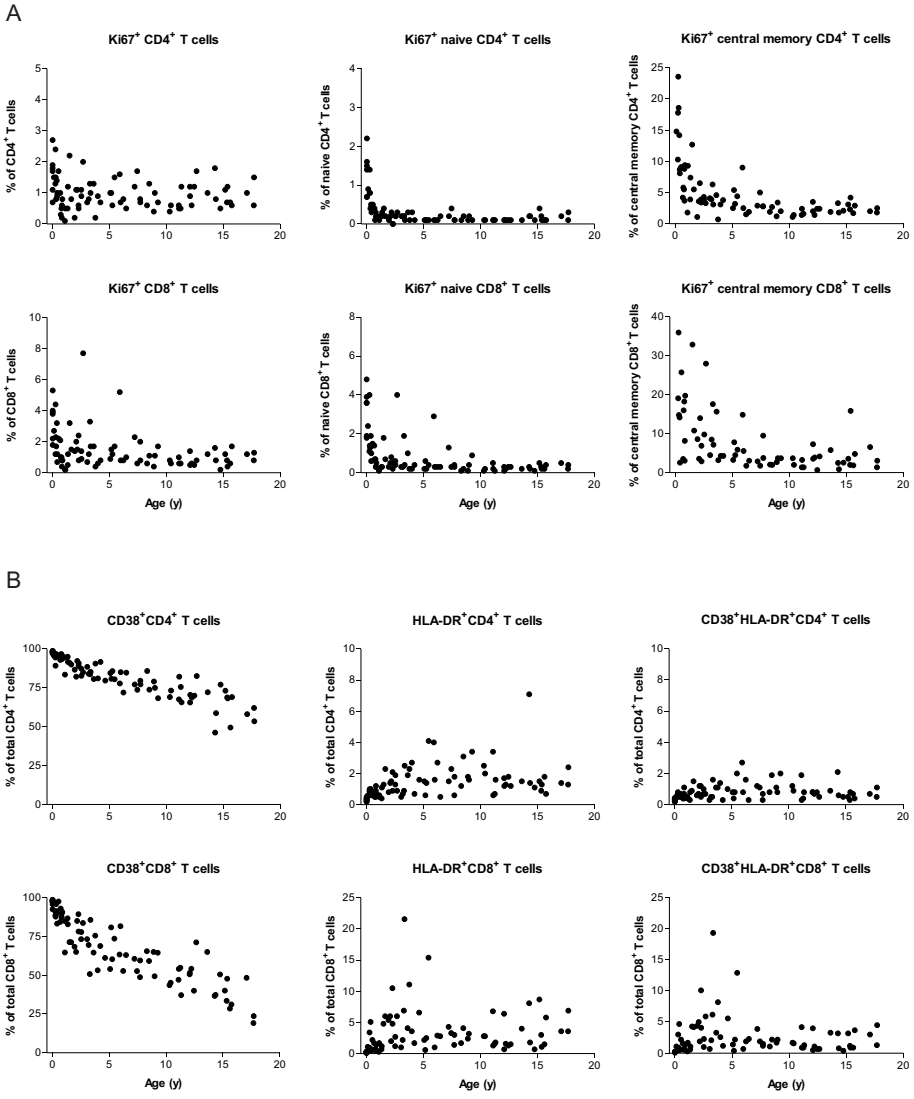
Supplementary Figure 2. Counts of total T cells and T-cell subsets

Counts of total T cells and T-cell subsets in blood during childhood. Each dot represents a single individual. Population definitions as described in Materials and methods.



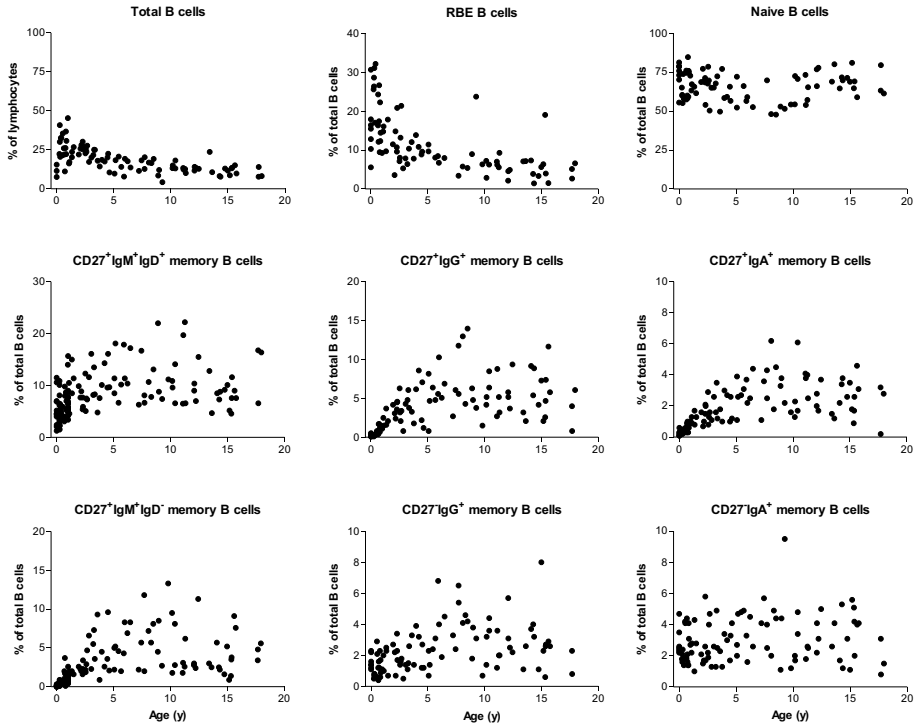
Supplementary Figure 3. sjTRECs and CD31 expression

A) Total number of sjTRECs and sjTREC content of $CD4^+$ and $CD8^+$ T cells. B) Expression of CD31 on total and naive CD4 T cells. Each dot represents a single individual.



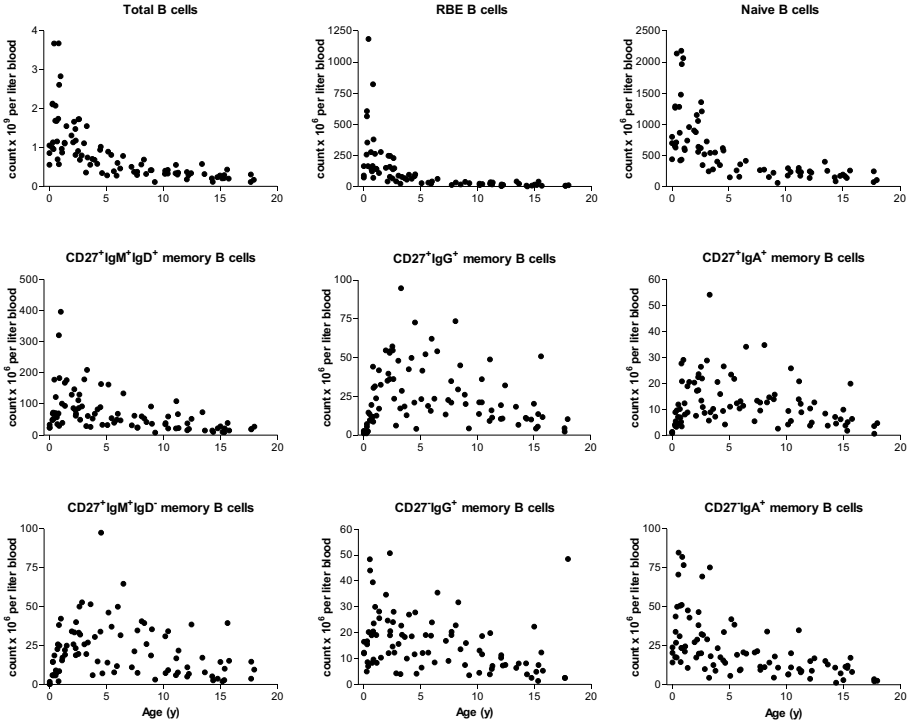
Supplementary Figure 4. CD38, HLA-DR and Ki67 expression of T cells

Expression of A) Ki67 in total CD4$^+$ and CD8$^+$ T cells and T-cell subsets, and of B) CD38 and HLA-DR on total CD4$^+$ and CD8$^+$ T cells during childhood. Each dot represents a single individual. Population definitions as described in Materials and methods.



Supplementary Figure 5. Percentages of total B cells and B-cell subsets

Percentages of total B cells in lymphocytes and subsets in the total B-cell compartment during childhood. Each dot represents a single individual. Population definitions as described in Materials and methods.



Supplementary Figure 6. Counts of total B cells and B-cell subsets

Counts of total B cells and B-cell subsets in blood during childhood. Each dot represents a single individual. Population definitions as described in Materials and methods.

3

Short- and long-term effects of neonatal thymectomy on the peripheral T-cell compartment

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Abstract

Both peripheral naive T-cell proliferation and thymic naive T-cell production contribute to the establishment of the naive T-cell compartment during childhood. Here, we studied the short- and long-term effects of neonatal thymectomy on the establishment and maintenance of the naive T-cell compartment in humans. We monitored the increase of total body naive T-cell counts in healthy children and assessed the impact of thymectomy on the establishment of the naive T-cell compartment. In the first three years after neonatal thymectomy the $CD4^+$ and $CD8^+$ T-cell compartments were contracted. Naive T-cell subsets were most severely affected. In contrast to healthy children, in which total body naive T-cell counts increase during the first year of life, total body naive T-cell counts in thymectomized children remained relatively stable up to three years after thymectomy. We found no evidence for homeostatically increased levels of naive T-cell proliferation to compensate for the absence of thymic naive T-cell production in thymectomized children. Long term (6.7-31.5 years) after thymectomy, the size and composition of the T-cell compartment was found to be normal. Analyses of total body $CD4^+$ T-cell TREC numbers, and MRI scans of mediastinum of three participants, long term after thymectomy opened up the possibility that naive T cells were most likely produced from thymic origin. Collectively, these data suggest that the thymus is of pivotal importance for the establishment of the naive T-cell compartment in early childhood, and that the lack of output from the thymus is not compensated for by increased levels of T-cell proliferation. The fact that the T-cell compartment nevertheless normalized long-term after thymectomy suggests that thymic tissue may regenerate even in patients who have been fully thymectomized.

Introduction

Naive T cells are produced by the thymus. Although the perivascular spaces in the thymus are progressively replaced with fat and thymic naive T-cell production declines with age, the adult thymus is still able to produce new naive T cells as all stages of thymocyte development have been observed in thymic tissue derived from adults (1-4), and newly produced thymic naive T cells were observed in adults following antiretroviral treatment for HIV-1 infection (5,6). Despite the decrease in thymic output during life, naive T-cell counts remain relatively stable during adult life (7). In childhood, both thymic output and peripheral proliferation contribute to the establishment and maintenance of the naive T-cell compartment (8). In adulthood, the contribution of the thymus is more limited and homeostasis of the naive T-cell compartment is believed to be maintained by production of new naive T cells by proliferation in the periphery and prolonged survival (longevity) of naive T cells (9).

Studies in mice have shown that after thymectomy at 7 weeks of age, naive T-cell numbers in spleen and lymph nodes reduced rapidly, and that the size of the recent thymic emigrant (RTE) pool in mice is therefore substantial (Den Braber et al. manuscript in preparation). A combination of $^{2}H_{2}O$ labeling and thymectomy experiments have shown that the establishment and maintenance of the naive T-cell compartment in mice is largely dependent on naive T-cell production by the thymus (Den Braber et al. manuscript in preparation).

The effect of thymectomy on the T-cell compartment in humans is very different from that in mice. In human adults, the effect of thymectomy has mainly been studied in individuals who suffer from myasthenia gravis (MG). No alterations of the numbers and percentages of total T cells and T-cell subsets were observed after thymectomy in adult MG patients (10). In contrast, T-cell receptor excision circle (TREC) content in peripheral blood mononuclear cells (PBMC) declined in these patients following thymectomy, suggesting that peripheral naive T-cell proliferation, and perhaps increased longevity were responsible for maintenance of naive T-cell counts. However, studying the effect of thymectomy on the T-cell compartment in MG is confounded by the autoimmune features of the disease and/or the immunosuppressive treatment (10,11). Nonetheless, it seems that thymectomy performed in adult life has no major effects on the established T-cell compartment.

Thymectomy is also performed in early human life. In cardiac surgery of children born with congenital heart disease removal of the thymus is often standard procedure. A number of studies have described the impact of neonatal thymectomy on the T-cell compartment during childhood and have shown that numbers of $CD4^{+}$ and $CD8^{+}$ T cells were decreased already within the first year following thymectomy, but

was sustained almost two decades thereafter (12-16). As expected, mainly naive T-cell counts were decreased (15,16). This suggests that removal of the thymus early in life may affect the naive T-cell compartment, and that the naive T-cell compartment cannot be fully restored. However, the naive T-cell compartment of thymectomized children was never depleted to the same extent as observed in thymectomized mice. Since TREC contents of $CD4^+$ T cells were found mostly low or undetectable after 1-18 years following neonatal thymectomy (15), it was suggested that peripheral homeostatic mechanisms play a role in maintenance of naive T-cell numbers in the absence of thymic output.

In this study, we evaluated the establishment of the naive T-cell compartment in healthy and neonatally thymectomized individuals. By use of TRECs and the proliferation marker Ki67, we evaluated the mechanism of T-cell generation and maintenance following thymectomy, and assessed whether loss of thymic naive T-cell production is homeostatically compensated for by higher levels of naive T-cell proliferation. In addition, we addressed the short- and long-term effects of neonatal thymectomy on the T-cell compartment.

Materials and methods

Study population and blood specimens

Whole blood samples were obtained by venipuncture from healthy and thymectomized individuals. Thymectomy was performed mainly in the first months of life as a consequence of cardiac surgery for congenital heart disease. Characteristics of the 20 thymectomized participants are shown in Table 1. Blood was drawn once or twice, either prior to thymectomy or 0.2-31.5 years after thymectomy. All 102 healthy children, aged 0.1-18.0 years, visited the University Medical Center in Utrecht in order to undergo an elective urological or plastic surgery. The children were considered healthy as they did not have any history of infectious diseases or hematological and immunological disorders. Cord blood samples were obtained from healthy full-term neonates directly after delivery. Adult blood samples were collected from 67 healthy volunteers, aged 21.0-39.7 years. The study was approved by the medical ethical committee and written informed consent was obtained from all study participants or their legal guardians in agreement with the Helsinki Declaration of 1975, revised in 1983.

Cell preparation and flow cytometry

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque density gradient centrifugation and were stored in liquid nitrogen until further processing.

Table 1. Study group

early postTx group	gender	age (y) at Tx	age (y) at sample 1	age (y) at sample 2	thymic tissue present
T01	m	0.0	0.0	1.9	-
T02	f	0.0	0.0	1.3	-
T03	m	0.0	0.0	2.3	-
T04	m	0.0	0.0	0.2	-
T05	m	0.1	1.0	-	-
T06	m	0.1	2.0	-	-
T07	f	0.0	0.6	2.0	-
T08	m	0.0	0.3	-	-
T09	m	0.0	2.1	-	no thymus upon opening chest
T10	m	0.0	0.7	-	no thymus upon opening chest
T11	m	0.3	0.9	-	-
late postTx group	**gender**	**age (y) at Tx**	**age (y) at sample 1**	**age (y) at sample 2**	**thymic tissue present**
T12	f	0.6	6.7	-	no thymus upon opening chest
T13	m	0.0	7.6	-	-
T14	f	0.0	8.0	-	-
T15	m	0.0	15.6	-	-
T16	m	0.0	18.6	-	tissue observed on MRI scan
T17	m	0.0	21.8	-	-
T18	f	0.6	24.7	-	tissue observed on MRI scan
T19	m	0.6	25.1	-	-
T20	f	1.1	31.5	-	tissue observed on MRI scan

Tx = thymectomy

Cryopreservation was performed using a computerized freezing device that results in optimal quality of frozen cells (17).

Characterization of the T-cell compartment was performed on thawed cryopreserved PBMC, which were incubated with mAb to CD4-Pacific Blue, CD8-AmCyan, CD8-PerCP-Cy5.5, CD27-APC, CD45RO-PE (Becton Dickinson), CD3-Pacific Blue, CD4-APC-AF750, CD8-APC-AF750, CD45RO-PE-Cy7 (eBioscience). Within the $CD4^+$ T-cell compartment, naive ($CD27^+CD45RO^-$) and memory ($CD45RO^+$) subsets were identified, whereas in $CD8^+$ T-cell compartment an additional subset, the effector ($CD27^-CD45RO^-$) subset, was identified (18,19). To measure T-cell proliferation, thawed PBMC were stained intracellularly with Ki67-FITC (Monosan) after fixation and permeabilization with Cytofix/Cytoperm™ and Perm/Wash™ according to manufacturer's instructions (Becton Dickinson). After washing with PBS, cells were analyzed on a LSRII (Becton Dickinson) and analyzed by FACSDiva software (Becton Dickinson).

Absolute lymphocyte numbers were determined with a Cell-Dyn Sapphire™ Hematology Analyzer (Abbott Diagnostics) and were used to calculate absolute numbers of T cells and T-cell subsets.

MACS cell separation

To measure the total number of TRECs and TREC content of $CD4^+$ T cells, these subsets were purified from thawed PBMC by magnetic-bead separation using the MiniMACS multisort kit according to manufacturer's instructions (Miltenyi Biotec Inc).

TREC analysis

DNA was isolated using the QIAamp Blood Kit according to manufacturer's instructions (Qiagen). Signal joint T-cell receptor excision circle (TREC) numbers were quantified by real-time polymerase chain reaction (PCR) as described previously (20,21). TREC content per T cell was calculated by dividing the TREC content by 150.000 (assuming that 1µg DNA corresponds with 150.000 T cells).

Total body numbers of naive T cells and TRECs

Total body naive $CD4^+$ and $CD8^+$ T-cell counts numbers were calculated as described previously (8). In brief, total body counts were calculated by multiplying the T-cell counts per ml blood by 80 × 50 × [body weight in kg] assuming a blood volume of 80ml/kg of body weight, and 2% of lymphocytes residing in the blood of healthy individuals.

Results

The effect of neonatal thymectomy on the composition of the T-cell compartment was assessed in 20 individuals 0.2-31.5 years after thymectomy. To study the short- and long-term effects of neonatal thymectomy, these individuals were divided into two groups according to time after thymectomy: 11 individuals were monitored within the first three years after neonatal thymectomy (early postTx group) and 9 individuals were included more than six years following thymectomy (late postTx group). Characteristics of the participants are shown in Table 1.

Early impact on the $CD4^+$ and $CD8^+$ T-cell compartment after thymectomy

At the time of thymectomy, the counts of total, naive and memory $CD4^+$ T cells and total, naive, memory and effector $CD8^+$ T-cell subsets in the early postTx group were similar to those of age-matched controls (open triangles in Figure 1A,B). In healthy individuals, $CD4^+$ and $CD8^+$ T-cell counts per microliter blood gradually declined during childhood (Figure 1A,B). Following thymectomy, $CD4^+$ and $CD8^+$ T-cell counts in individuals in the early postTx group declined to levels below those of age-matched controls (Figures 1A,B). The faster declines in $CD4^+$ and $CD8^+$ T-cell

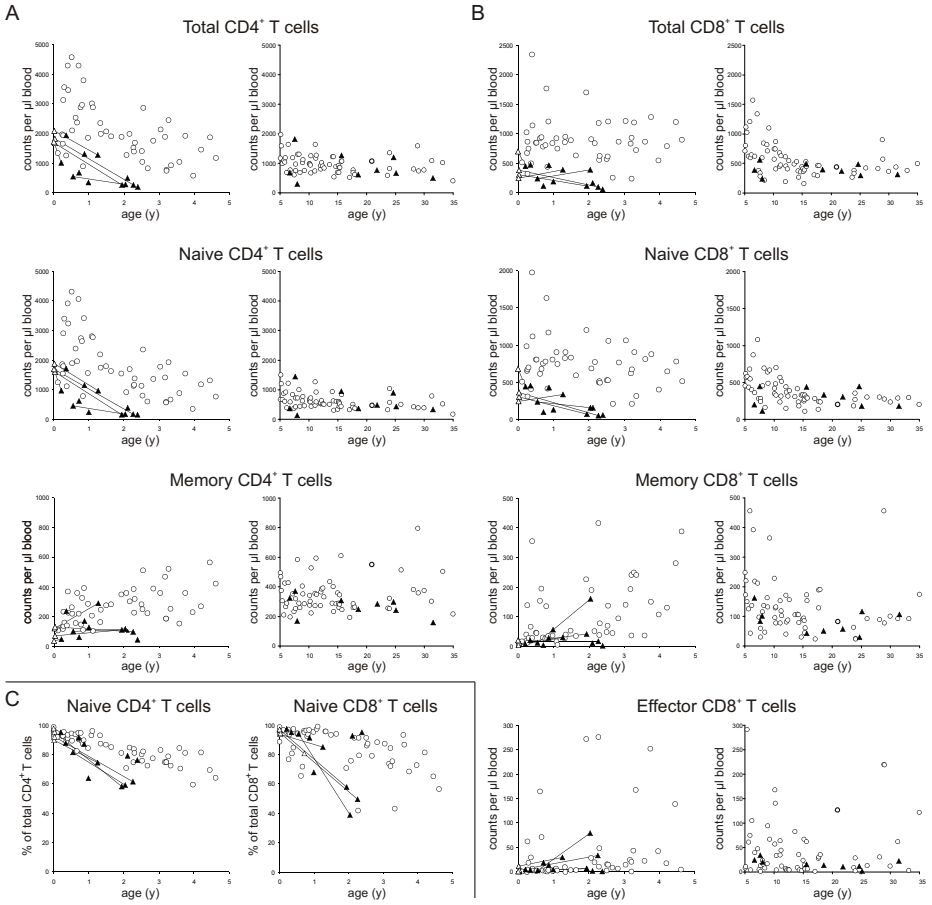

Figure 1. Total T cells and T-cell subset counts. A) Total, naive and memory CD4+ T-cell counts, and B) total, naive, memory and effector CD8+ T-cell counts per µl blood. Left panel: early postTx group; right panel: late postTx group. C) Percentage of naive CD4+ and naive CD8+ T cells in the early postTx group. White dots represent controls; white triangles indicate values prior to thymectomy; black triangles indicate values post thymectomy. Solid lines connect data points from a single individual.

counts per microliter blood in thymectomized children were mainly due to a more rapid decline of naive CD4+ and CD8+ T-cell counts (Figure 1A,B). Indeed, the percentage of naive CD4+ and CD8+ T cells in the early postTx group was clearly lower than control values (Figure 1C). Effector CD8+ T-cell counts in the early postTx group were within the normal range (Figure 1B). Taken together, naive CD4+ and CD8+ T-cell counts per microliter blood are clearly affected in the first years following neonatal thymectomy.

No expansion of the naive T-cell compartment following neonatal thymectomy

The decline in the number of T cells per microliter blood which is typically observed during childhood is partially due to growth of the body and a concomitant growth of the vasculature, lymphatic system and lymphoid organs. In fact, the total number of T cells in the body in healthy individuals increases during childhood (8). In healthy children, we found that the total body number of naive $CD4^+$ and $CD8^+$ T cells mainly increased in the first year of life and remained at relatively constant levels in subsequent years (Figure 2A,B). We studied the changes in the total body number of naive T cells in the absence of thymic naive T-cell production. Following thymectomy, total body naive T-cell numbers did not increase and in some of the thymectomized individuals even declined (Figure 2A,B). Hence, these data suggest that the expansion of the naive T-cell compartment in the first year of life does not occur in the absence of thymic naive T-cell production.

No increased level of homeostatic naive T-cell proliferation following thymectomy

We assessed whether in the absence of thymic output naive T-cell counts were maintained by naive T-cell proliferation, or solely by longevity. We found that the TREC content of $CD4^+$ T cells in early postTx group were in the lower range of normal values (Figure 2C). In addition, we found that total body TREC numbers were decreased compared to control values (Figure 2D). These data suggest that after thymectomy naive T cells had proliferated and diluted their TRECs. In addition, TREC-containing naive T cells had been lost.

Subsequently, we studied whether there was any evidence for increased homeostatic naive T-cell proliferation to compensate for the loss of thymic naive T-cell production. Therefore, we assessed the fraction of naive $CD4^+$ and $CD8^+$ T cells expressing the proliferation marker Ki67 after neonatal thymectomy. As shown in Figure 3A, naive $CD4^+$ T-cell proliferation levels were not elevated compared to age-matched controls, except for one participant: T07. This participant was one of two individuals who also demonstrated increased naive $CD8^+$ T-cell proliferation levels (Figure 3B). It is likely that these elevated naive T-cell proliferation levels were a result of an infection, since T07 also showed increased levels of activated ($CD38^+HLA\text{-}DR^+$) $CD8^+$ T cells (data not shown). In general, an increase in the levels of naive T-cell proliferation was not observed in the first three years after thymectomy, which suggests that there is no proliferative compensation for the loss of thymic output.

Normal T-cell compartment after at least six years following neonatal thymectomy

In the absence of thymic naive T-cell production, maintenance of the naive T-cell compartment relies on T-cell longevity and proliferation. As the $CD4^+$ and $CD8^+$ T-cell

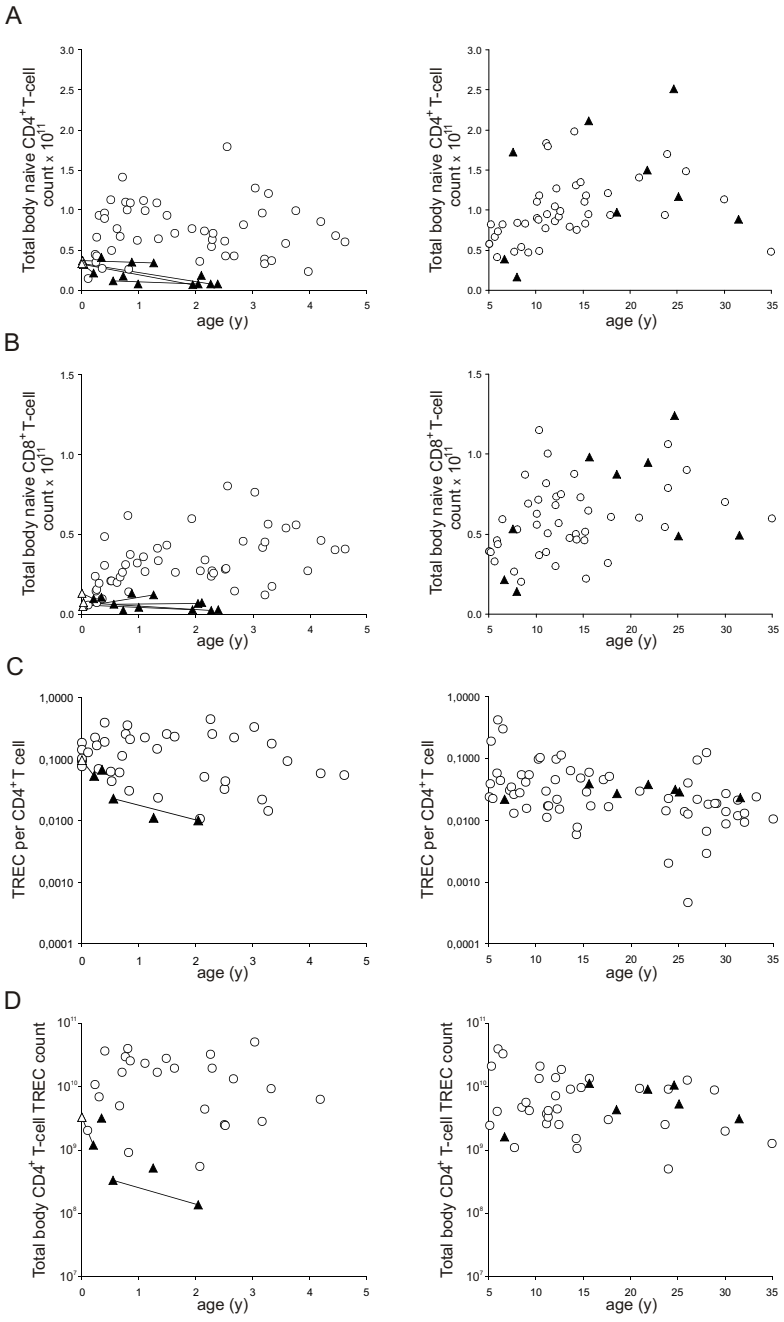

Figure 2. Total body naive T-cell counts and TRECs. A) Total body number of naive CD4$^+$ T cells, B) total body number of naive CD8$^+$ T cells, C) total body CD4$^+$ T-cell TREC number and D) TREC content of CD4$^+$ T cells. Left panel: early postTx group; right panel: late postTx group. White dots represent controls; white triangles indicate values prior to thymectomy; black triangles indicate values post thymectomy. Solid lines connect data points from a single individual.

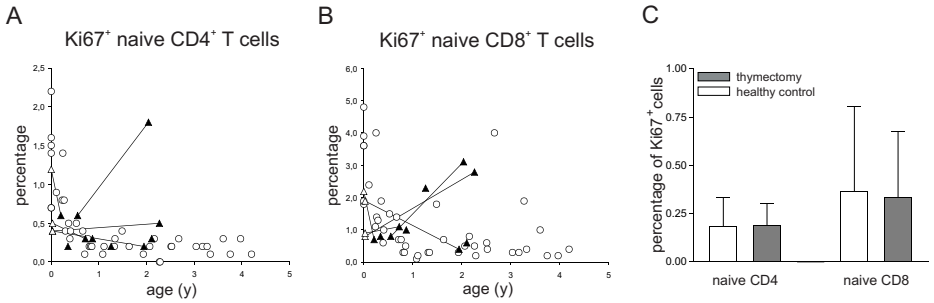

Figure 3. Naive T-cell proliferation following thymectomy. The percentage of Ki67-expressing A) naive $CD4^+$ T cells and B) naive $CD8^+$ T cells was assessed in the early postTx group. White dots represent controls; white triangles indicate values prior to thymectomy; black triangles indicate values post thymectomy. Solid lines connect data points from a single individual. C) The percentage of Ki67-expressing naive $CD4^+$ and naive $CD8^+$ T cells in the late postTx group. Whiskers represent standard deviations.

compartments were affected in the first three years following neonatal thymectomy, we investigated the effects of thymectomy in the long run. We studied the T-cell compartments of a late postTx group consisting of 5 individuals who underwent a neonatal thymectomy and 4 individuals who had been thymectomized at 0.6-1.1 years of age. Analysis was performed between 6.7 and 31.5 years of age (Table 1). In general, all individuals in the late postTx group had relatively normal numbers of total $CD4^+$ and $CD8^+$ T cells and $CD4^+$ and $CD8^+$ T-cell subsets, although total, naive and memory $CD4^+$ T-cell counts of T14 were lower than age-matched controls (see the right hand panels in Figure 1A,B). Total body naive $CD4^+$ and $CD8^+$ T-cell counts were also within normal ranges (see the right hand panels in Figure 2A,B). Remarkably, levels of total body naive T cells in the late postTx group were higher than in the early postTx group (Figure 2A,B). These data suggest that production of new naive T cells had occurred after the first three years following thymectomy in these individuals.

Surprisingly, we found that the $CD4^+$ T-cell TREC content (Figure 2C) and total body $CD4^+$ T-cell TREC numbers (Figure 2D) in the late postTx group were similar to age-matched control values, and somewhat higher than observed in individuals of the early postTx group. Proliferation levels (measured by Ki67 expression) of naive $CD4^+$ and naive $CD8^+$ T cells of the individuals of the late postTx group were within normal ranges (Figure 3C). These data therefore suggest that in the thymectomized individuals of the late postTx group new TREC containing naive T cells had been produced.

From three participants (T16, T18 and T20), MRI scans of the mediastinum were performed at the age of 18. Despite the fact that all these individuals had reportedly undergone a full thymectomy, on these scans tissue was observed at the anatomical location of the thymus (Figure 4). Although we cannot ascertain the identity of this

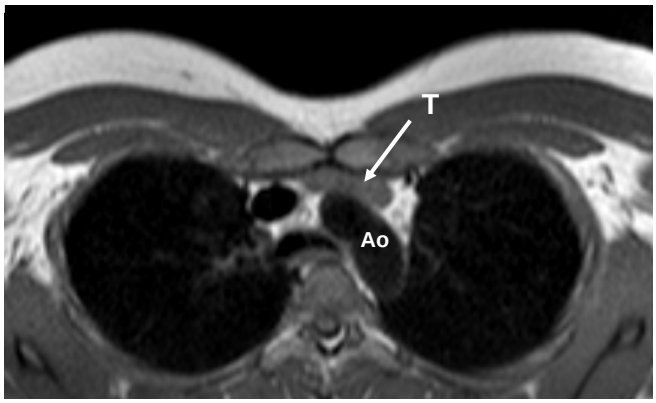

Figure 4. MRI scan. MRI scan of the mediastinum of participant T18. T = tissue present at the anatomical location of the thymus; Ao = aorta.

tissue it is likely that in the light of the data collected from the late postTx group, this body mass indeed consists (in part) of thymic tissue in which T-cell development is occurring.

Discussion

A number of studies have addressed the impact of neonatal thymectomy on the T-cell compartment. Although decreased numbers of naive T cells were observed (15,16), it is still not known how the naive T-cell compartment is maintained following neonatal thymectomy. Since peripheral naive T-cell proliferation is the only source of new naive T cells in the absence of thymic output, and it has been shown to contribute to the establishment of the naive T-cell compartment during childhood (8,22), we hypothesized that peripheral naive T-cell proliferation accounts for the maintenance of the naive T-cell compartment and may even be homeostatically elevated to compensate for the loss of thymic naive T-cell production.

In the first year of life, a rapid increase in the number of total body naive T cells is observed in healthy individuals. Beside thymic output, this increase is attributed for a substantial part to peripheral naive T-cell proliferation (8). In absence of thymic output, we had expected that total body naive T-cell counts would increase, although perhaps not to the levels in healthy individuals. We found, however, that total body naive T-cell counts remained stable or even decreased in the first three years after neonatal thymectomy. Apparently, naive T-cell proliferation can only expand naive T-cell numbers in the presence of thymic naive T-cell production. This implies that recently produced naive T cells by the thymus proliferate and contribute to the establishment of the naive T-cell compartment during childhood. Therefore, it seems

likely that the thymus plays a determining role in the expansion of the naive T-cell compartment in the first year of life.

Despite the fact that total body naive T-cell counts in young children did not increase after thymectomy, naive T-cell proliferation may be homeostatically elevated to maintain naive T-cell counts. Alternatively, naive T cells in thymectomized children may have acquired prolonged longevity as competition for interaction with self-peptide-presenting MHC molecules and IL-7 with naive T cells generated by the thymus is reduced. The observation that the TREC content of $CD4^+$ T cells was within the lower range of normal values may indeed suggest that there has been either a short period, or a constant but moderately higher level of increased naive T-cell proliferation following thymectomy. However, even when levels of naive T-cell proliferation are normal in the absence of thymic output, the TREC content of T cells in thymectomized individuals is expected to show a larger age-related decline than healthy individuals. Since no new TREC-containing naive T cells are produced in the absence of the thymus, the TREC content of total T-cell population declines more rapidly in athymic individuals during aging than in individuals with thymic naive T-cell production. Moreover, we did not find increased levels of naive T-cell proliferation (as measured by $Ki67^+$ expression) in the absence of thymic output. These data show that there is no evidence for a compensatory role of homeostatic naive T-cell proliferation in the establishment and maintenance of the naive T-cell compartment in the first three years following neonatal thymectomy.

Studies in mice have demonstrated that survival and proliferation of naive T cells is dependent on the interaction with interleukin-7 (IL-7) and/or major histocompatibility complex (MHC) molecules (23,24). In a normal physiological "full" T-cell compartment, naive T cells likely compete for these survival signals, but after T-cell depletion these signals might induce homeostatic naive T-cell proliferation as they are abundantly present for the few circulating naive T cells (25,26). It is uncertain whether this mechanism of naive T-cell survival and proliferation is similar in humans, although a potential role for IL-7 in naive T-cell homeostasis has been observed (27-29). Neonatal thymectomy may not immediately result in a substantial degree of naive T-cell depletion, but in the long-term absence of thymic output it would be expected that levels of naive T-cell proliferation increase as a homeostatic response to maintain sufficient numbers of naive T cells.

So why did we not observe increased naive T-cell proliferation levels as a compensatory homeostatic response for the T-cell depletion after thymectomy? If indeed increased availability of IL-7 is a prerequisite for compensatory proliferation this suggests that with removal of the thymus not only a source of naive T cells, but also a source of IL-7 has been removed. Alternatively, only naive T cells recently produced by the thymus may be responsive to IL-7 induced cell division. After thymectomy,

the remaining naive T-cell pool will already be partly unresponsive to IL-7 induced proliferation, and will gradually become even more so as the compartment becomes older.

After a least six years following neonatal thymectomy, naive $CD4^+$ and $CD8^+$ T-cell numbers were similar to those in healthy age-matched controls and higher than in children shortly after thymectomy. Of three participants, magnetic resonance imaging (MRI) scans at the age of 18 were available. These scans showed that there was tissue present at the anatomical site which is normally occupied by the thymus. Unfortunately, this type of imaging does not allow for discrimination between thymic and adipose tissue. But since we observed that total body $CD4^+$ T-cell TREC numbers in the oldest individuals of the late postTx group were comparable to those of healthy controls and higher than in the early postTx group, this suggests that new thymic tissue was formed over the years despite the fact that these individuals had reportedly been fully thymectomized.

If indeed thymic tissue regenerates, this process is slow as it does not seem to happen within the first few years after thymectomy. Upon reopening of the chest of participant T12, who had low total body naive T-cell and TREC numbers, at an additional cardiac surgery 6.7 years after thymectomy, no thymic tissue could be observed. The fact that we found profound perturbations in the T-cell compartment of children in the early postTx group supports this observation. There are a few reports which have shown enlargement of thymic mass after cessation of chemotherapy or following stem cell transplantation (30,31), but to date no evidence exists of formation of new thymic tissue at the anatomical location of the thymus after its complete removal. Alternatively, T-cell and TREC numbers in the late postTx may have normalized by production of TREC-bearing naive T cells at other sites, like the intestine (32,33). However, based on two case reports on thymectomized individuals who received a bone marrow transplant following chemotherapy, no evidence for this hypothesis exists (34,35).

In summary, we have shown that the thymus is important for the establishment of the naive T-cell compartment in the first years of life. Even though naive T-cell proliferation contributes significantly to the establishment and maintenance of the naive T-cell compartment in young children, it is unable to compensate for the loss of thymic naive T-cell production in the first years after neonatal thymectomy.

Acknowledgments

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4

Rejuvenation of the naive CD4$^+$ and CD8$^+$ T-cell compartments after cessation of chemotherapy in children

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Abstract

In healthy children, the naive T-cell compartment is established by a combination of thymic naive T-cell production and peripheral T-cell proliferation. Here, we studied the contribution of these two routes of naive T-cell production in 31 children during T-cell recovery from chemotherapy treatment for childhood leukemia or lymphoma. We found that at the moment of stop of chemotherapy the $CD4^+$ T-cell compartment was more depleted than the $CD8^+$ T-cell compartment. Both T-cell compartments recovered to normal age-matched values within six months after cessation of chemotherapy. This recovery led to rejuvenation of the T-cell compartment, because memory T-cell counts remained decreased while naive T-cell counts even reached supranormal levels. In agreement, assessment of T-cell receptor excision circles (TREC) showed that an important component of naive T-cell regeneration was due to T-cell production by the thymus. Even during the relatively short period in which $CD4^+$ T-cell numbers were still severely depleted, we did not find any evidence for enhanced levels of lymphopenia-driven T-cell proliferation.

Introduction

To avoid opportunistic or life-threatening infections, it is pivotal that reconstitution of the T-cell compartment is accomplished rapidly following immune depletion. Previous studies have shown that the speed of T-cell reconstitution after lymphocyte depletion is dependent on the ability of the thymus to produce new naive T cells (1-6). Since thymic T-cell production has been shown to decline with age (7-9), it is perhaps not surprising that children are able to reconstitute the naive T-cell compartment faster than adults after intensive cytotoxic chemotherapy, stem cell transplantation (SCT) or antiretroviral treatment for HIV-1 infection (3,10-12). Nevertheless, even adolescents and young adults restored their naive $CD4^+$ T-cell compartment completely almost two decades after cessation of chemotherapy, while in these patients naive $CD8^+$ T-cell counts even recovered to supranormal levels (13). Similar observations were made in our study on HIV-1 infected adults receiving long-term highly active antiretroviral therapy (HAART)(14). In adults with moderate $CD4^+$ T-cell depletion at start of HAART, reconstitution of naive $CD4^+$ T-cell counts was complete after at least seven years of treatment. Collectively, these data imply that recovery of the naive T-cell compartment can be accomplished in individuals of all ages, although it may take longer with progressing age and in cases of more severe immune depletion.

Reconstitution of the $CD4^+$ T-cell compartment in adults after chemotherapy is in general slower than reconstitution of the $CD8^+$ T-cell compartment, which suggested that thymic regeneration does not proportionally account for recovery of both compartments (15). However, for $CD8^+$ T cells the rise in counts in the first months of reconstitution can mainly be attributed to expansions of effector-type $CD8^+$ T cells. Recovery of naive $CD4^+$ and naive $CD8^+$ T-cell counts was proportional, suggesting that their production seems to come from a common origin (15,16).

Another mechanism of naive T-cell production is peripheral naive T-cell proliferation. During the establishment of the naive T-cell compartment in healthy children, both mechanisms, thymic production and peripheral proliferation, are involved in generation of naive T cells (17,18). Peripheral proliferation is especially high in the first year of life when there is a large growth of T-cell numbers, suggesting that this process may be (partially) homeostatically driven. Whether naive T-cell proliferation is homeostatically elevated during reconstitution following T-cell depletion is still uncertain. After initiation of HAART in HIV-1 infected adults, naive T-cell proliferation can be normalized, even when $CD4^+$ T-cell numbers are still reduced (19). In a detailed study on T-cell reconstitution after stem cell transplantation, naive T-cell proliferation levels were higher than in healthy controls (20). However, the increased levels of naive T-cell proliferation coincided with the occurrence of Graft-versus-

Host Disease (GvHD) or infections; in individuals without GvHD or infections the level of naive T-cell proliferation was found to be normal, even when T-cell numbers had not yet normalized (20).

In this study, we examined T-cell reconstitution after cessation of chemotherapy in 31 children who were treated for childhood leukemia or lymphoma. By measuring T-cell receptor excision circles (TRECs) in combination with the thymic proximity marker CD31 and the proliferation marker Ki67, we analyzed the role of naive T-cell production by the thymus and the role of peripheral T-cell proliferation during recovery of the naive T-cell compartment. By comparing the data with our previous analyses in HIV-1 infected children on HAART, we investigated whether the same mechanism of T-cell generation dictate reconstitution of the $CD4^+$ T-cell compartment in children in different lymphocyte-depleted situations.

Materials and methods

Study population

Thirty-one children were included who underwent intensive cytotoxic chemotherapy for treatment of acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), anaplastic lymphoma (AL), Non-Hodgkin lymphoma (NHL) or Hodgkin lymphoma (HL). Patient characteristics are shown in Table 1. Only patients who had no severe infections and did not demonstrate any signs of relapse of the leukemia or lymphoma were included. From these children, whole EDTA- or heparinized anticoagulated blood samples were obtained to assess the reconstitution of the T-cell compartment. Blood was withdrawn between one to eight times during follow-up. The time point post-chemotherapy at which the patients were included varied. Patients were either

Table 1. Study group

	directly at cessation	long-term after cessation
no. of individuals	8	23
mean age at inclusion (range in years)	10.3 (4.6-16.3)	9.1 (3.7-16.4)
time since stop chemotherapy of first sampling (range in years)	0.0-0.2	0.3-3.2
time since stop chemotherapy of last sampling (range in years)	1.3-2.2	1.7-5.1
Acute lymphatic leukemia	7	14
Acute myeloid leukemia	0	4
Hodgkin lymphoma	0	3
Non-Hodgkin lymphoma	0	2
Anaplastic lymphoma	1	0

monitored directly at the end of treatment or were included up to 3 years after stop of chemotherapy.

To study T-cell reconstitution in a different immune-depleted setting, we previously analyzed T-cell reconstitution among 13 HIV-1 infected children on HAART (14). These children attained undetectable viral loads within 3 months after initiation of treatment, after which loads remained undetectable. As a control group, we analyzed EDTA- or heparinized anticoagulated blood samples of 102 healthy children, aged 0-18 years undergoing elective urological or plastic surgery (Van Gent et al. submitted). These children were considered healthy as they did not have any history of infectious diseases or hematological and immunological disorders.

The study was approved by the medical ethical committee and written informed consent was obtained from all study participants or their legal guardians in agreement with the Helsinki Declaration of 1975, revised in 1983.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque density gradient centrifugation and were stored in liquid nitrogen until further processing. Cryopreservation was performed using a computerized freezing device that results in optimal quality of frozen cells (21). Characterization of the T-cell compartment was performed on thawed cryopreserved PBMC. PBMC were incubated with mAb to CD4-Pacific Blue, CD4-FITC, CD8-PerCP-Cy5.5, CD8-APC-Cy7, CD27-APC, CD38-PerCP-Cy5.5, CD45RO-PE, CD45RO-PE-Cy7, HLA-DR-FITC (Becton Dickinson). PBMC were stained intracellularly with Ki67-FITC (Monosan) after fixation and permeabilization with Cytofix/Cytoperm™ and Perm/Wash™ according to manufacturer's instructions (Becton Dickinson). Stained cells were analyzed on a LSRII (Becton Dickinson).

Calculating absolute T-cell counts during childhood

Absolute lymphocyte numbers were determined with a Cell-Dyn Sapphire™ Hematology Analyzer (Abbott Diagnostics) and were used to calculate numbers of $CD4^+$ and $CD8^+$ T cells and their subsets. Because T-cell counts decrease during childhood, total and subset T-cell counts of the healthy controls were stratified into ten age-groups and used to normalize T-cell counts of the children monitored after cessation of chemotherapy and the HIV-1 infected children during HAART. These age-groups were respectively: 0-0.5, 0.5-1.0, 1.0-2.0, 2.0-3.0, 3.0-4.0, 4.0-6.0, 6.0-9.0, 9.0-12.0, 12.0-15.0 and 15.0-18.0 years. Each group consisted of at least seven children.

Population definitions

Naive ($CD27^+CD45RO^-$), memory ($CD45RO^+$) and effector ($CD27^-CD45RO^-$ [only in the $CD8^+$ T-cell compartment]) subsets were identified within the $CD4^+$ and $CD8^+$

T-cell compartments (22,23). Activated $CD4^+$ and $CD8^+$ T cells were identified by combined expression of CD38 and HLA-DR. Expression of the thymic proximity marker CD31 (24) was assessed on naive $CD4^+$ T cells. The level of proliferation within each of the T-cell subsets was determined by Ki67 expression (19).

MACS cell separation

To measure the total number of TRECs and TREC content of $CD4^+$ and $CD8^+$ T cells, these subsets were purified from thawed PBMC by magnetic-bead separation using the MiniMACS multisort kit according to manufacturer's instructions (Miltenyi Biotec Inc).

TREC analysis

DNA was isolated using the QIAamp Blood Kit according to manufacturer's instructions (Qiagen). Signal joint T-cell receptor excision circle (TREC) numbers were quantified by real-time polymerase chain reaction (PCR) as described previously (25,26). TREC content per T cell was calculated by dividing the TREC content by 150.000 (assuming that 1µg DNA corresponds with 150.000 T cells).

Results

Study population

The reconstitution of the T-cell compartment after cessation of chemotherapy treatment was assessed in 31 pediatric patients who were diagnosed with childhood leukemia or lymphoma (ALL, AML, NHL, HL and AL). Since the treatment protocols specific for each of these diseases did not differentially impact T-cell reconstitution we did not distinguish between the underlying disease when analyzing T-cell reconstitution in these 31 patients. All patients were longitudinally followed for at least six months. The patients were, however, included at various time points after cessation of chemotherapy. We therefore separately analyzed a group of eight patients (grey symbols) who were included directly (0.0-0.2 years) after cessation of chemotherapy, and a group of twenty-three children (black symbols) who were included later (0.3-3.2 years) after stop of chemotherapy (Table 1).

Reconstitution of the $CD4^+$ and $CD8^+$ T-cell compartments

To evaluate the degree of T-cell depletion and recovery, we assessed the total number of T cells and their subsets after chemotherapy. T-cell counts per µl blood were expressed as a percentage of age-matched control values, since these counts decrease during childhood. In Figure 1, the reconstitution of total $CD4^+$ and $CD8^+$

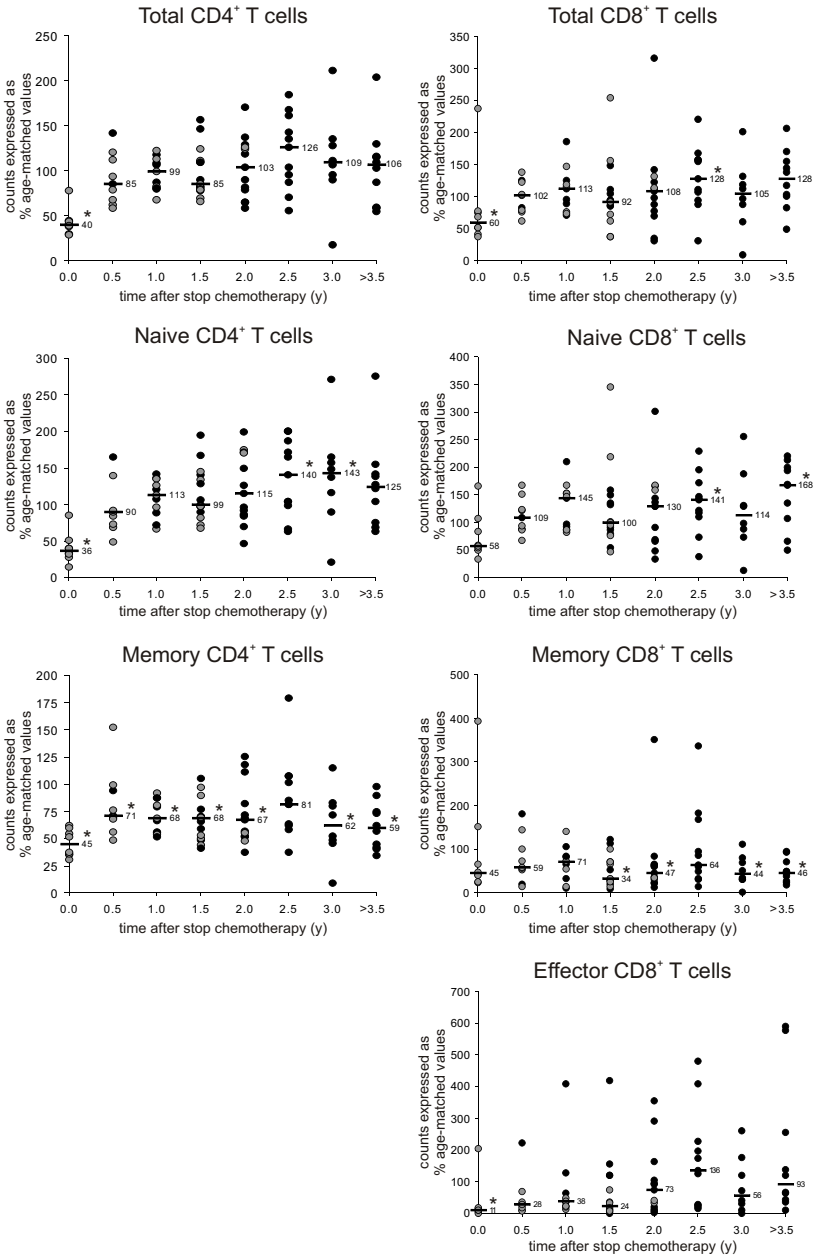

Figure 1. T-cell recovery after stop of chemotherapy. Recovery of the total number of CD4+ and CD8+ T cells and CD4+ and CD8+ T-cell subsets after cessation of chemotherapy. Cell counts are expressed as percentage of age-matched control values. Gray dots represent individual data points from children included directly (0.0-0.2 years) after cessation of chemotherapy; black dots represent individual data points from children included 0.3-3.2 years after stop of chemotherapy. Horizontal lines and numbers indicate median values. Statistical significance (compared with healthy controls) was determined by the non-parametric Mann-Whitney *U* test for unpaired data. *, $p < 0.05$

T cells and their subsets is shown for all 31 children. At stop of chemotherapy, $CD4^+$ T-cell counts were significantly reduced to a median of 40% of normal values ($p<0.001$). Within the first six months, $CD4^+$ T-cell counts recovered to normal levels and remained within normal range thereafter. Counts of both naive ($p<0.001$) and memory ($p<0.001$) $CD4^+$ T-cell subsets were decreased at cessation of chemotherapy. Naive $CD4^+$ T-cell counts recovered to normal levels within six months, and even significantly higher than normal levels were observed at 2.5 and 3.0 years after stop of chemotherapy. Memory $CD4^+$ T-cell counts also raised in the first six months post-chemotherapy, but remained significantly lower than age-matched values during almost the entire follow-up.

The degree of T cell depletion was less for the $CD8^+$ T-cell compartment than the $CD4^+$ T-cell compartment directly after cessation of chemotherapy ($p=0.05$). Total $CD8^+$ T-cell counts were significantly decreased ($p=0.018$), but regained normal levels within the first six months following chemotherapy and remained relatively constant thereafter (Figure 1). Naive $CD8^+$ T-cell counts were higher than naive $CD4^+$ T cells ($p=0.021$), and were not significantly decreased at cessation of chemotherapy ($p=0.068$). Levels of naive $CD8^+$ T cells increased subsequently and supranormal levels were observed after at least 2.5 years post-chemotherapy. Memory $CD8^+$ T-cell counts remained low during reconstitution while numbers of effector $CD8^+$ T cells gradually increased after a significant reduction at cessation of chemotherapy ($p=0.008$).

In the light of age-dependent thymic involution, we evaluated the rate of T-cell recovery over age, but age did not influence the rate of recovery of the T-cell compartment, as in all prospectively analyzed individuals (age range at cessation of chemotherapy: 4.6 – 16.3 years) normal T-cell numbers were attained within six months after stop of chemotherapy.

Collectively, these data demonstrate that the $CD4^+$ T-cell compartment was more affected by chemotherapy than the $CD8^+$ T-cell compartment. Reconstitution to normal levels was accomplished within the first six months after cessation of chemotherapy. Moreover, a tendency towards enrichment of naive T cells was observed in both the $CD4^+$ and $CD8^+$ T-cell compartments.

The speed of $CD4^+$ T-cell recovery is similar as in HIV-1 infected children on HAART

The speed of T-cell recovery may be dependent on the cause of the T-cell depletion, for example because the thymus may be differently affected in different diseases. In addition, in HIV-1 infection, the size of the $CD8^+$ T-cell compartment is in general less affected than the $CD4^+$ T-cell compartment. Therefore, we investigated whether $CD4^+$ T-cell reconstitution in children treated with HAART was comparable to that in children following chemotherapy. We compared our results with those obtained

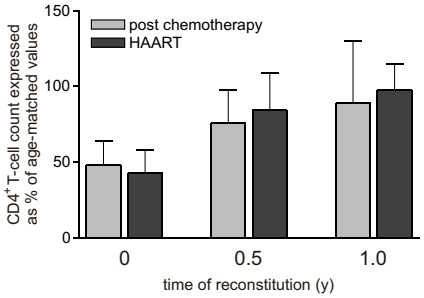

Figure 2. CD4⁺ T-cell reconstitution in different immune depleted settings. CD4⁺ T-cell recovery was monitored at six months and a year after cessation of chemotherapy (8 children; mean age 10.3 years), and after initiation of HAART for HIV-1 infection (13 children; mean age 5.0 years). Both groups had a similar level of initial CD4⁺ T-cell depletion. Cell counts are expressed as percentage of age-matched control values. Whiskers represent standard deviations.

from 13 HIV-1 infected children, who had a similar degree of CD4⁺ T-cell depletion at initiation of HAART as the eight children who were monitored directly after cessation of chemotherapy (Figure 2; p=0.47, and reference 14). In both groups, CD4⁺ T-cell counts increased to normal levels with similar kinetics during the first year of reconstitution (Figure 2). Hence, this suggests that in these different immune depleted settings the speed of CD4⁺ T-cell reconstitution is similar.

Thymic output contributes to naive T-cell reconstitution

After cessation of chemotherapy, naive T-cell counts rapidly restored to (supra) normal levels. To address the role of thymic output in this rapid recovery, we measured total CD4⁺ T-cell TREC numbers per µl blood in the eight patients who were monitored directly after cessation of chemotherapy. We found that the CD4⁺ T-cell TREC number increased concomitant with the number of naive CD4⁺ T cells after cessation of chemotherapy (Figure 3). These data suggest that in children the thymus contributes to recovery of the naive T-cell compartment following chemotherapy treatment.

No homeostatically increased naive T-cell proliferation during reconstitution

To address whether the T-cell depletion induced increased peripheral T-cell proliferation, we analyzed the expression level of the proliferation marker Ki67 in naive CD4⁺ T cells of the eight individuals who were prospectively monitored directly after stop of chemotherapy and studied whether naive T-cell proliferation levels were related to the level of naive CD4⁺ T-cell depletion. We found the highest levels of naive T-cell proliferation directly after cessation of chemotherapy, especially in individuals with the most severe level of naive CD4⁺ T-cell depletion. Nevertheless, by far not all individuals with a severely depleted naive CD4⁺ T-cell compartment

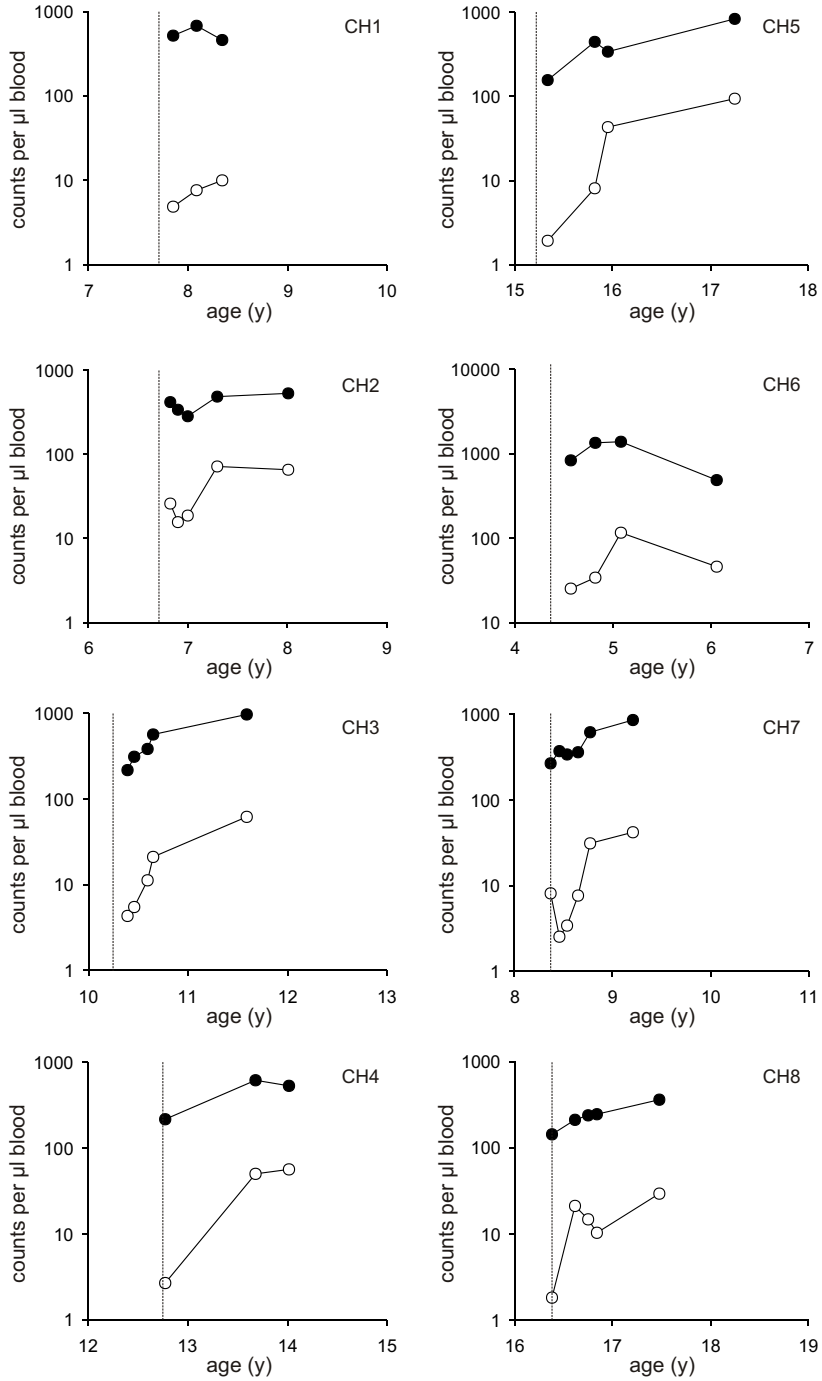

Figure 3. Thymic contribution to naive T-cell recovery. Dynamics of naive CD4+ T-cell counts (black dots) and CD4+ T-cell TREC numbers (white dots) per µl blood in 8 patients included directly (0.0-0.2 years) after cessation of chemotherapy. Vertical dashed line indicates time point of stop of chemotherapy.

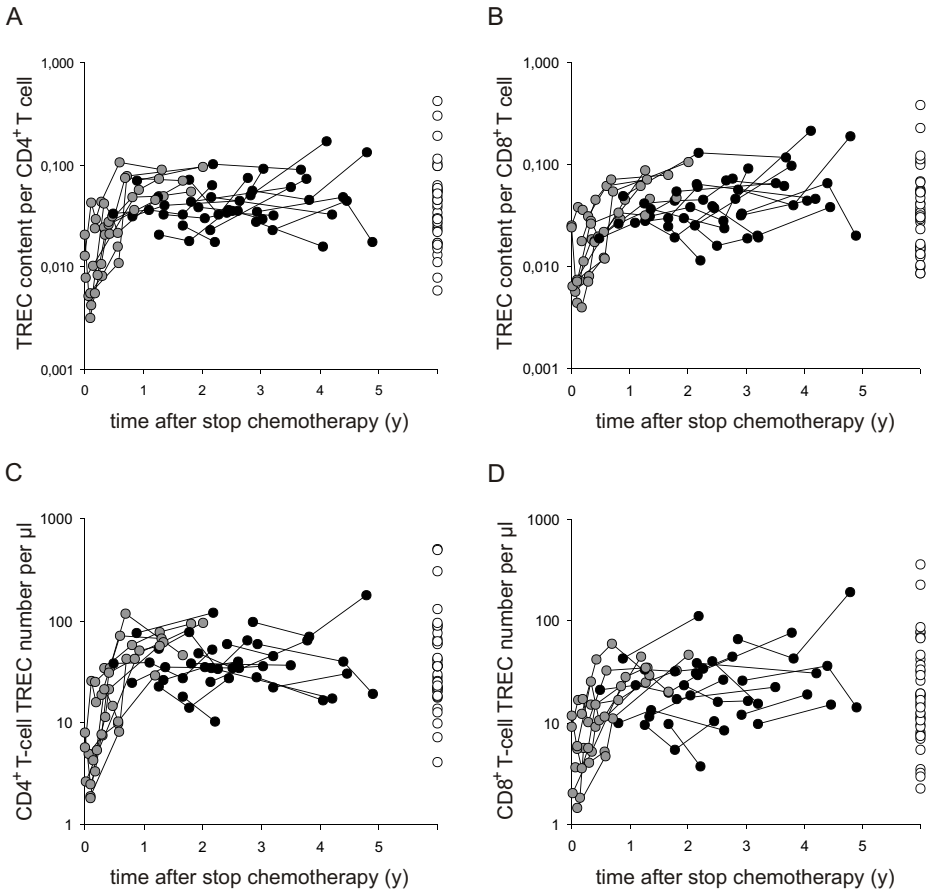

Figure 4. Dynamics of T-cell reconstitution. TREC content in A) $CD4^+$ and B) $CD8^+$ T cells, and the number of TRECs per µl blood in C) $CD4^+$ and D) $CD8^+$ T cells after cessation of chemotherapy. Gray dots represent individual data points from children monitored directly (0.0-0.2 years) after cessation of chemotherapy; black dots represent individual data points from children included 0.3-3.2 years after stop of chemotherapy. White dots indicate control values. Lines connect data points from a single individual.

displayed increased proliferation levels (Figure 5A). At 2 to 3 months after cessation of chemotherapy, naive T-cell proliferation levels in these patients were only moderately increased while naive $CD4^+$ T-cell counts had hardly restored to normal levels (Figure 5A). At six months after stop of chemotherapy, when naive $CD4^+$ T-cell counts had almost normalized, naive $CD4^+$ T-cell proliferation levels were within the normal range (Figure 5A). Results for naive $CD8^+$ T-cell recovery were less evident, since naive $CD8^+$ T-cell numbers were much less depleted than $CD4^+$ T-cell numbers, and $CD8^+$ T-cell proliferation levels were hardly increased above normal values (Figure 5C). Collectively, these data show that peripheral naive T-cell proliferation may be enhanced in the severest cases of naive T-cell depletion, but that also normal

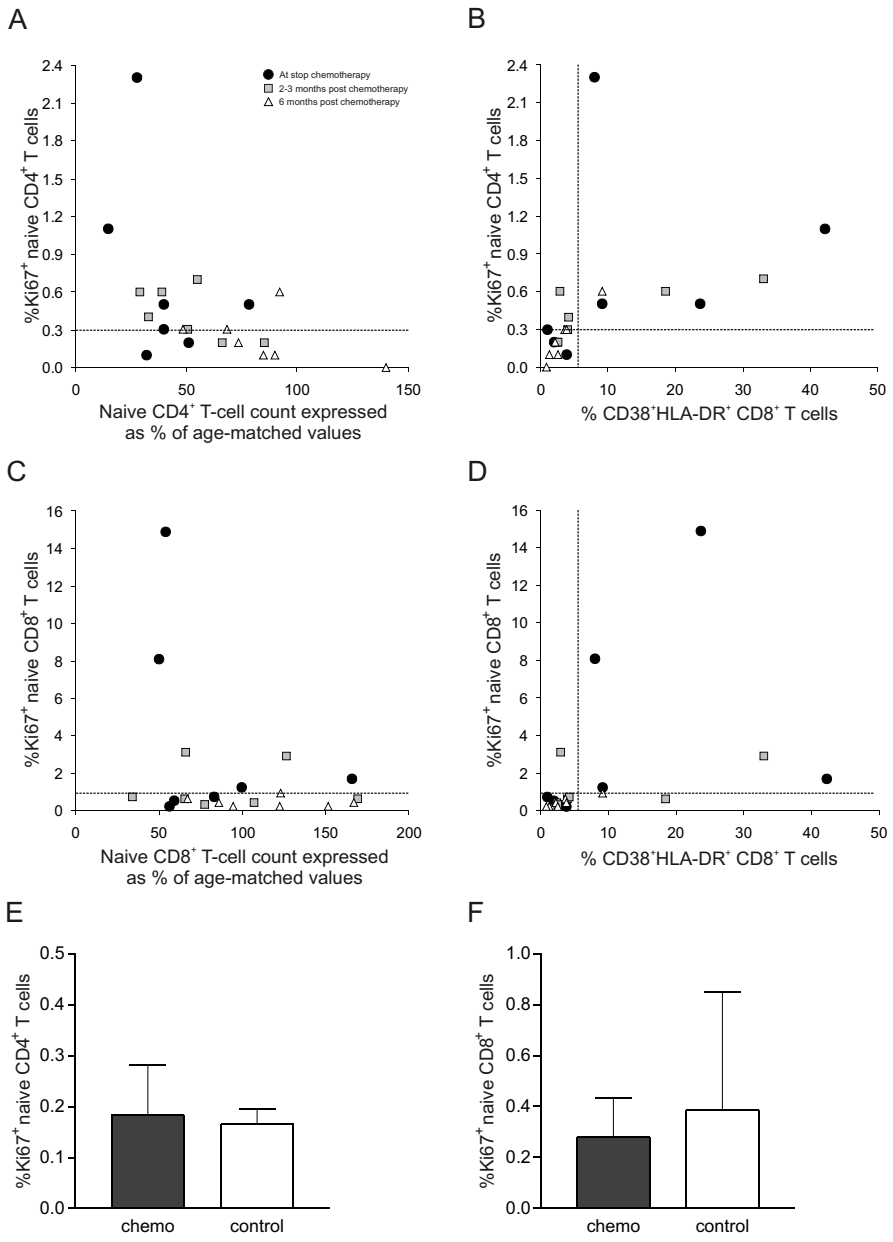

Figure 5. Naive CD4⁺ T-cell proliferation and T-cell activation. Relationship between A+C) the level of Ki67⁺ naive CD4⁺ or CD8⁺ T cells and naive CD4⁺ or CD8⁺ T-cell counts, and B+D) the fraction of Ki67⁺ naive CD4⁺ or CD8⁺ T cells and the level of activated (CD38⁺HLA-DR⁺) CD8⁺ T cells in the eight individuals who were prospectively monitored directly after cessation of chemotherapy. Each symbol represented a single individual at a given time, the type of symbol represented the elapsed time after cessation of chemotherapy. Dotted lines represent 95th percentile of normal values. E) Average proliferation levels of naive CD4⁺ and CD8⁺ T cells at the latest included time point after cessation of chemotherapy. Whiskers indicate standard deviations.

levels of naive T-cell proliferation were observed despite considerable naive T-cell depletion.

Increased naive T-cell proliferation levels may be due to episodes of T-cell activation. Levels of $CD38^+HLA\text{-}DR^+$ $CD8^+$ T cells provide a good indication for the level of T-cell activation *in vivo*. To analyze whether elevated levels of naive T-cell proliferation coincided with elevated levels of T-cell activation we measured the expression of $CD38^+HLA\text{-}DR^+$ $CD8^+$ T cells, and found that naive $CD4^+$ and $CD8^+$ T-cell proliferation was associated with T-cell activation (Figure 5B,D). Taken together, the elevated naive T-cell proliferation levels after chemotherapy seem to be more related to T-cell activation than to reflect a homeostatic response to the induced lymphodepletion.

Naive T-cell maintenance long-term after chemotherapy is similar to healthy individuals

Knowing that the naive T-cell compartment tended to recover to normal levels relatively fast after cessation of chemotherapy, we expected that also the relative contribution of thymic output and peripheral proliferation in the maintenance of T-cell numbers would be normalized long term after cessation of chemotherapy. We therefore prospectively monitored TRECs and Ki67 expression in the 23 patients who were included 0.3-3.2 years after cessation of chemotherapy. Both $CD4^+$ and $CD8^+$ T-cell TREC contents and numbers remained stable and within the normal range (Figure 4). Proliferation levels of naive $CD4^+$ and naive $CD8^+$ T cells were also normal long-term after chemotherapy (Figure 5E,F). Collectively, these data suggest that after attaining complete naive T-cell reconstitution following chemotherapy in children, the balance between naive T-cell generation by thymic production and peripheral proliferation was similar to that in healthy children.

Discussion

In this study, we investigated the quantitative T-cell recovery in children who were treated with chemotherapy for leukemia or lymphoma. After cessation of chemotherapy, naive T cells in both $CD4^+$ and $CD8^+$ T-cell compartments restored rapidly and attained normal levels within six months. These findings were similar to observations in a previous study on T-cell reconstitution after chemotherapy in children (27). Others found full recovery of $CD4^+$ T-cell counts to take 6-12 months (3,15,28), but $CD4^+$ T-cell counts were more severely affected at cessation of chemotherapy. The speed of reconstitution in these studies seemed to be related to the degree of T-cell depletion. In our study, the degree of T-cell depletion was less substantial, which may explain why complete T-cell recovery was already observed within six months after cessation of chemotherapy.

We found that chemotherapy affected the naive and memory $CD4^+$ T-cell subsets equally. In contrast, naive $CD4^+$ T cells recovered to normal or even supranormal levels within six months, while memory $CD4^+$ T-cell levels remained low during almost the entire follow-up. Naive $CD8^+$ T cells were initially less affected than memory $CD8^+$ T cells. Similar to what was observed for the $CD4^+$ T-cell compartment, also the $CD8^+$ T-cell compartment contained higher than normal levels of naive cells after stop of chemotherapy. Apparently, effective T-cell reconstitution in children can reset the naive/memory composition of the T-cell compartment to a composition normally present at a younger age. Whether this rejuvenation of the T-cell compartment persists in the long run will have to be examined.

New naive T cells were generated by the thymus during reconstitution. Although naive T cells may also have been produced by peripheral naive T-cell proliferation, no homeostatically elevated levels were observed at any stage of T-cell depletion in our patient group. In mice, two types of naive T-cell proliferation have been described: spontaneous proliferation which is rapid and induces a naive-to-memory phenotypical shift, and homeostatic proliferation which is slow and dependent on the concentration of IL-7 in the microenvironment (29). Whether both types of proliferation also exist in humans is uncertain. Because spontaneous proliferation is described mainly to occur after severe lymphopenia (29), the degree of lymphodepletion after cessation of chemotherapy in our study group may not have been severe enough for spontaneous proliferation to occur. Therefore, naive T-cell proliferation may have indeed contributed to naive T-cell recovery, but it was not lymphopenia-driven.

There are several reasons why the kinetics of $CD4^+$ T-cell reconstitution in children treated with chemotherapy or HAART may differ. Firstly, HIV-1 will be suppressed but never fully cleared by antiretroviral therapy and low levels of residual virus might interfere with T-cell reconstitution. Secondly, chemotherapy has a depleting effect on all $CD4^+$ and $CD8^+$ T-cell subsets, whereas in HIV-1 infection expansions of memory and effector $CD8^+$ T cells are typically observed, which may counteract the reconstitution of naive $CD4^+$ and $CD8^+$ T cells. Lastly, it is unknown whether and to what extent HIV-1 and chemotherapy differentially affect thymic function.

Nevertheless, we found that in children with a similar degree of $CD4^+$ T-cell depletion, the speed of $CD4^+$ T-cell recovery after cessation of chemotherapy was similar to the recovery after initiation of HAART for HIV-1 infection. This suggests that the higher levels of $CD8^+$ T cells in HIV-1 infection do not negatively influence the effectiveness of $CD4^+$ T-cell reconstitution. Unfortunately, we did not have any data on reconstitution of $CD4^+$ T-cell subsets in the HIV-1 infected children on HAART. In these children, the rise in $CD4^+$ T-cell counts may be partly accounted for by redistribution of memory $CD4^+$ T cells from lymphoid tissues to the blood which occurs within the first weeks after initiation of treatment (30). In various settings

of immune depletion following stem cell transplantation in children, it was found that T-cell recovery could take a year or even longer (31). Since we showed that recovery following chemotherapy and after initiation of HAART are very similar and accomplished within one year, this suggests that in SCT poor early grafting may be responsible for delayed T-cell recovery (32). In addition, it may imply that chemotherapy treatment and HIV-1 infection do not substantially affect the maturation of T-cell progenitors in bone marrow.

In conclusion, we have shown that after stop of chemotherapy children are able to restore their $CD4^+$ and $CD8^+$ T-cell compartments within six months. Recovery led to rejuvenation of the T-cell compartment, because memory T-cell counts remained decreased while naive T-cell counts even reached supranormal levels. Increased levels of naive T-cell proliferation during reconstitution did not seem to be driven by homeostasis, but were linked to events of T-cell activation.

Acknowledgments

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5

Restoration of the CD4 T cell compartment after long-term highly active antiretroviral therapy without phenotypical signs of accelerated immunological aging

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Abstract

It remains uncertain whether full T-cell reconstitution can be established in HIV-infected children and adults with long-term sustained virological control by highly active antiretroviral therapy (HAART). In this study, we comprehensively analyzed various phenotypical markers of CD4 T-cell recovery. In addition to measuring T-cell activation and proliferation markers, CD4 T-cell generation and aging of the CD4 T-cell compartment were assessed by measuring T-cell receptor excision circles (TREC) and the fraction of CD31 expressing naive CD4 T cells. In all children and in adults with relatively high CD4 T-cell counts at start of therapy (>200 cells/μl), total CD4 T-cell numbers normalized within one year of therapy. After long-term HAART (4.4-9.6 years), naive CD4 T-cell counts had normalized in both groups. Although in adults with low baseline CD4 T-cell counts (<200 cells/μl) total CD4 T-cell numbers normalized eventually after at least seven years of HAART, naive CD4 T-cell counts had still not recovered. TREC data showed that thymic T-cell production contributed to naive T-cell recovery at all ages. The fraction of CD31 expressing naive CD4 T cells was found to be normal, suggesting that the CD4 T-cell repertoire was diverse after long-term HAART. Hence, under sustained viral suppression during long-term HAART the T-cell compartment has the potential to fully recover by generating new naive T cells both in children, and in adults with high baseline CD4 T-cells counts. Irrespective of baseline CD4 T-cell counts, reconstitution occurred without a significant effect on T-cell aging as reflected by markers for replicative history.

Introduction

Since the introduction of highly active antiretroviral therapy (HAART) as treatment for HIV-1 infection, many studies have investigated its effect on the reconstitution of the CD4 T-cell compartment. Analyses of reconstitution in HIV cohorts, with a large variation in treatment protocols and degree of viral suppression, have shown that normalization of the T-cell compartment is generally not achieved. Only few studies have analyzed reconstitution in patient groups with sustained viral suppression. These studies have shown that in the majority of adult patients CD4 T-cell counts gradually increase (1,2). Some studies report CD4 T-cell counts to reach a plateau after three to five years of HAART (2,3); others however found continuous CD4 T-cell gains throughout four years of therapy (1). Total CD4 T-cell numbers in adult patients with low baseline (pre-therapy) CD4 T-cell counts were found to remain low throughout the reconstitution period and failed to normalize within four to five years of HAART (1,2). However, the net yearly increase in CD4 T-cell counts was shown to be independent of baseline CD4 T-cell counts (1).

Because thymic output decreases from early childhood, one would expect that immune reconstitution is less effective with increasing age (4). Indeed, increases in total and naive CD4 T-cell numbers were found to be higher in younger HIV-infected children compared to older HIV-infected children on HAART (5,6) and higher in HIV-infected children compared to adults (7). However, when normalized for naive and total T-cell numbers in age-matched controls the relative gain was age-independent (6,8).

The importance of thymic output during reconstitution has been suggested by the association between poor CD4 T-cell reconstitution during HAART and age (1,2,9-11), low absolute numbers of naive CD4 and CD8 T cells (10,12), less thymic tissue (10,11,13-15) and low absolute numbers of T-cell receptor excision circles (TRECs)(16). TRECs are by-products of T-cell receptor rearrangements which are not duplicated upon cell division (17,18). Consequently, absolute numbers of TRECs per ml blood can provide information on thymic output, whereas the replicative history of the T-cell compartment, either by activation or homeostatic proliferation, can be determined by measuring the average TREC content per cell in conjunction with T-cell numbers.

In addition to thymic output, reconstitution of the T-cell compartment can be achieved by peripheral T-cell proliferation. Studies in HIV-infected adults have shown that although T-cell proliferation declined after initiation of therapy, it was still increased compared to healthy individuals up to two years after treatment initiation (19). Furthermore, CD4 and CD8 T-cell activation markers had not normalized after two to six years of therapy (19-22). Similarly, HIV-infected children who had been on

HAART for three to four years had significantly higher levels of activated ($CD38^+HLA\text{-}DR^+$) CD4 and CD8 T cells than age-matched controls (12). These increased levels of T-cell proliferation and activation during HAART may represent a homeostatic response to fill up the empty T-cell compartment. However, since T-cell proliferation declines after HAART initiation even though the CD4 T-cell compartment is still very much depleted (19,23), it can be argued that residual activation rather than a homeostatic response drives T-cell proliferation during HAART. In addition, the observations that low TREC contents (10-12,16,24,25), short telomeres (10) and high levels of activated $CD38^+HLA\text{-}DR^+$ T cells (12,19,20,26,27) and proliferating ($Ki67^+$) naive CD4 and CD8 T cells (27) are related to poor CD4 T-cell reconstitution support the notion that residual activation causes the increased levels of T-cell proliferation.

Based on immune reconstitution studies after stem cell transplantation (SCT) in which inadequate long-term T-cell reconstitution, especially in adults, has repeatedly been reported (28-31), normalization of the T-cell compartment within seven years of HAART is not to be expected. However, the incomplete T-cell reconstitution after SCT may be explained by the inclusion of a subset of SCT patients with poor early engrafting (32) or graft-versus-host disease (33,34) rather than by long-term immune failure caused by accelerated loss of thymic output in these patients. Recently, another study showed successful T-cell reconstitution 17 years after chemotherapy-induced lymphopenia (35). Moreover, Dion et al. showed that efficient thymopoiesis contributed to T-cell recovery during HAART (36). Therefore we hypothesized that adequate T-cell reconstitution may be possible in HIV-1 infected individuals on continuous treatment with HAART.

The introduction of HAART has resulted in sustained virological suppression, i.e., viral loads below the detection threshold during the course of therapy, in many HIV-infected individuals. This gave us the opportunity to study whether under sustained virological suppression during the entire treatment, full T-cell reconstitution in HIV-1 infected children and adults can in principle be achieved during long-term (4.4-9.6 years) HAART, and whether this T-cell reconstitution results in accelerated aging of the T-cell compartment in the long run. Analysis of quantitative naive and memory CD4 T-cell recovery was combined with measurements of activation and proliferation markers in order to establish to what extent long-term HAART had normalized these parameters. Furthermore, we assessed the replicative and post-thymic history of T cells by analyzing their TREC contents and the fraction of CD31 expressing naive CD4 T cells. If the increase in CD4 T-cell counts observed during HAART would mainly be due to T-cell proliferation, this would lead to increased aging of the peripheral T-cell pool, i.e. in dilution of the T-cell TREC content and low fractions of $CD31^+$ naive CD4 T cells. Conversely, decreasing levels of T-cell proliferation in concert with the entrance of thymic emigrants into a virtually empty peripheral

T-cell compartment could in theory lead to rejuvenation of the peripheral T-cell compartment. We show that in children and even in adults with more than 200 CD4 T cells/µl blood at start of therapy, the CD4 T-cell compartment has the potential to fully recover during long-term successful HAART. Thymic naive T-cell generation and possibly also peripheral naive T-cell proliferation contributed to this recovery in such a way that reconstitution did not have a significant effect on T-cell aging as evaluated by CD31 expression and TREC analyses.

Materials and Methods

Study population

To avoid unwanted patient selection bias, we included all HIV-1 infected children and adults (21 and 26, respectively) from the patient population of the UMC Utrecht and AMC Amsterdam who matched our selection criterions (see Table 1 for patient characteristics). To be eligible they had to be treated with long-term continuous HAART (range children: 4.4-9.6 years; range adults: 7.0-9.2 years) and had to have adequate viral suppression for the complete period of follow-up, with a minimum detection threshold of 50 copies HIV-1 RNA per ml. Individuals with an occasional appearance of plasma HIV-1 RNA above the detection threshold were included in the analyses, but only if the load returned to undetectable levels within 3 months upon detection and without modifications to HAART regimens. Thirteen adults and nine children had, on one or more occasions, plasma HIV-1 RNA copies above the detection threshold. Patient A04 had three blips, all in the first two years of treatment. HAART was defined either as a combination of at least three drugs from at least two different drug classes (which are: protease inhibitors (PI), nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI) and the nucleotide reverse transcriptase inhibitor tenofovir) or as a combination of three nucleoside reverse transcriptase inhibitors including abacavir. No patient selection was performed on immunological parameters (Table 2).

All control values of the various immunological parameters were provided by a cohort of healthy volunteers, consisting of 92 children (age range: 0.1-18.0 years) and 107 adults (age range: 19.0-65.0 years). Because T-cell counts decrease during childhood, total and subset T-cell counts of the healthy controls were stratified into ten age-groups in order to normalize T-cell counts of children during HAART. These age-groups were respectively: 0-0.5, 0.5-1.0, 1.0-2.0, 2.0-3.0, 3.0-4.0, 4.0-6.0, 6.0-9.0, 9.0-12.0, 12.0-15.0 and 15.0-18.0 years. Each group consisted of at least seven children.

Table 1. Patient characteristics

Partici-pant	Age in years start HAART	Age in years long-term HAART	Duration in years of HAART	CD4 T-cell count start HAART	CD4 T-cell count long-term HAART	% of normal CD4 T-cell count start HAART	% of normal CD4 T-cell count long-term HAART	No. of drug classes during HAART	no. of blips	maximum load at blip copies HIV-1 RNA per ml plasma
C 01	11.7	17.6	5.9	475	827	48.5	98.5	1 or 2	0	-
C 02	4.0	11.6	7.6	695	860	50.4	87.8	2	0	-
C 03	4.8	9.9	5.2	920	1306	66.7	133.3	2	2	119
C 04	0.4	4.8	4.4	2274	1859	79.8	134.7	2	0	-
C 05	5.4	13.5	8.1	469	620	34.0	73.8	1 or 2	0	-
C 06	7.1	13.7	6.6	262	593	26.7	70.6	1 or 2	2	65
C 07	5.6	12.9	7.3	397	670	28.8	79.8	2	2	110
C 08	3.1	10.2	7.1	787	684	57.0	69.8	1 or 2	0	-
C 09	0.4	5.4	5.0	1270	731	44.6	53.0	2 or 3	0	-
C 10	0.3	5.5	5.1	1990	1221	69.8	88.5	2 or 3	0	-
C 11	5.8	10.9	5.1	448	1172	32.5	119.6	2	1	120
C 12	10.0	15.0	5.0	370	682	37.8	81.2	2	1	56
C 13	5.3	12.2	6.9	850	1180	61.6	140.5	2	3	84
C 14	4.8	12.3	7.5	860	910	62.3	92.9	2 or 3	0	-
C 15	0.9	7.3	6.4	930	1370	43.1	139.8	2 or 3	1	190
C 16	9.5	16.5	7.0	410	870	41.8	103.6	2 or 3	2	78
C 17	0.8	10.4	9.6	193	1162	7.2	118.6	1 or 2	0	-
C 18	0.4	6.9	6.5	194	1340	6.8	136.7	1 or 2	0	-
C 19	0.8	8.7	7.8	442	1320	16.6	134.7	1 or 2	1	485
C 20	13.2	21.3	8.1	50	1044	6.0	124.3	1 or 2	0	-
C 21	2.2	7.3	5.1	160	1099	7.4	112.1	2	0	-
mean	*4.6*	*11.1*	*6.5*	*688*	*1025*	*39.5*	*104.5*			
range	*0.3 - 13.2*	*4.8 - 21.3*	*4.4 - 9.6*	*50 - 2274*	*593 - 1859*	*6.0 - 79.8*	*53.0 - 140.5*			

Table 1. continued

Partici-pant	Age in years start HAART	Age in years long-term HAART	Duration in years of HAART	CD4 T-cell count start HAART	CD4 T-cell count long-term HAART	% of normal CD4 T-cell count start HAART	% of normal CD4 T-cell count long-term HAART	No. of drug classes during HAART	no. of blips	maximum load at blip copies HIV-1 RNA per ml plasma
A 01	28.4	35.6	7.3	370	1430	46.3	178.8	2	0	-
A 02	35.2	43.0	7.8	410	1330	51.3	166.3	2	0	-
A 03	36.7	43.8	7.1	440	1010	55.0	126.3	1 or 2	0	-
A 04	45.1	52.2	7.1	290	960	36.3	120.0	2	3	1350
A 05	41.3	49.0	7.7	320	1140	40.0	142.5	2	1	123
A 06	31.2	39.4	8.2	504	810	63.0	101.3	2	0	-
A 07	30.9	39.7	8.8	380	1320	47.5	165.0	2	0	-
A 08	39.8	48.6	8.8	560	1470	70.0	183.8	2	0	-
A 09	40.0	47.8	7.8	240	600	30.0	75.0	2 or 3	0	-
A 10	42.3	51.4	9.1	320	620	40.0	77.5	2 or 3	2	115
A 11	37.2	45.2	8.0	350	410	43.8	51.3	2	1	69
A 12	38.7	46.7	8.0	19	570	2.4	71.3	2	0	-
A 13	50.8	58.8	8.0	160	760	20.0	95.0	2	0	-
A 14	39.3	46.8	7.5	60	870	7.5	108.8	2	0	-
A 15	34.9	43.5	8.7	174	500	21.8	62.5	2	2	66
A 16	36.5	44.8	8.3	50	610	6.3	76.3	2	0	-
A 17	42.1	49.1	7.0	170	880	21.3	110.0	2	1	67
A 18	36.6	44.3	7.7	100	430	12.5	53.8	2	1	123
A 19	33.9	42.6	8.7	50	420	6.3	52.5	2	1	63
A 20	43.2	51.6	8.4	180	350	22.5	43.8	2	1	807
A 21	42.0	49.6	7.6	30	510	3.8	63.8	2 or 3	1	453
A 22	31.1	39.1	8.0	30	1340	3.8	167.5	2 or 3	3	691
A 23	35.4	44.1	8.6	20	530	2.5	66.3	2 or 3	2	157
A 24	31.4	40.7	9.2	30	490	3.8	61.3	2 or 3	0	-
A 25	29.2	37.8	8.6	50	1380	6.3	172.5	2	2	217
A 26	34.5	43.1	8.6	40	620	5.0	77.5	2 or 3	0	-
mean	*37.2*	*45.3*	*8.1*	*206*	*822*	*25.7*	*102.7*			
range	*28.4 - 50.8*	*35.6 - 58.8*	*7.0 - 9.2*	*19 - 560*	*350 - 1470*	*2.4 - 70*	*43.8 - 183.8*			

Table 2. Patient inclusion

patient group	total patient population virological responders long-term HAART	loss of patients to emigration	PBMC available at baseline	PBMC available at long-term HAART	no participation	in study
children UMC	18	1	0	17	0	17
children AMC	4	0	0	4	0	4
adults UMC	18	0	0	14	4	14
adults AMC	52	0	19	19	7	12

This study was approved by the medical ethical committee and written informed consent was obtained from all study participants or their legal guardians in agreement with the Helsinki Declaration of 1975, revised in 1983.

Flow cytometry and cell sorting

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque density gradient centrifugation from heparinized blood and were cryopreserved until further use. Absolute counts and maturational status of CD4 T cells and immune activation status of CD4 and CD8 T cells were routinely measured. Absolute CD4 T-cell counts were determined by dual-platform flow cytometry. Naive ($CD27^+CD45RO^-$) and memory ($CD45RO^+$) CD4 T-cell fractions and activated ($CD38^+HLA\text{-}DR^+$) CD4 and CD8 T cells were assessed by flow cytometry. To measure the fraction of $CD31^+$ cells within the naive CD4 T-cell population and peripheral blood T-cell proliferation in CD4 T-cell subsets, cryopreserved PBMC were thawed and incubated with mAb to CD45RO-FITC (Caltag) or CD45RO-PE, CD31-PE, CD4-PerCP (Becton Dickinson (BD), San Jose, California, USA) and biotinylated CD27 (Sanquin Reagents). After washing, cells were incubated with anti-Streptavidin-APC (BD). To measure T-cell proliferation, cells were fixed (FACS Lysing Solution, BD), permeabilized (FACS Permeabilisation Buffer, BD) and stained intracellularly with Ki67-FITC (Monosan, The Netherlands), after which cells were resuspended in Cellfix (BD) and were analyzed on a FACSCalibur (BD) with CellQuest software.

To measure the TREC content within the naive ($CD27^+CD45RO^-$) CD4 T-cell population, PBMC were incubated with mAb to CD45RO-FITC (Caltag Laboratories, Burlingame, CA, USA), CD27-PE and CD4-PerCP (BD). The specified cell fractions were isolated by cell sorting on a FACSAria (BD). To measure the TREC content within CD4 T cells, these subsets were purified from thawed PBMC by magnetic-bead separation using the MiniMACS multisort kit according to manufacturer's instructions (Miltenyi Biotec Inc, Sunnyvale, California, USA).

TREC analysis

DNA was isolated using the QIAamp Blood Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Signal joint T-cell receptor excision circle (TREC) numbers were quantified using real-time PCR as described previously (18,37).

Mathematical model

To study the effect of HAART on the dynamics of T-cell TREC contents, we used a previously developed mathematical model for TREC decline with age (18), described by the following two differential equations:

$$dN/dt = \alpha + pN - dN$$
$$dT/dt = \alpha - dT$$

where N is the number of CD4 T cells, T is the total number of TRECs, $\alpha = a\ e^{-vt}$ represents thymic output which declines at rate v per year, and T cells proliferate at rate p and die at rate d per day. The TREC content of the CD4 T-cell population was calculated as T/N. It has previously been shown that the average T-cell TREC content does not decrease with age if there is no homeostatic regulation of T-cell numbers (38). We therefore modeled the rate of T-cell proliferation as:

$$p = p_{biv} + p_0 / (1 + N^2 / h^2)$$

where p_0 represents the maximum proliferation rate in healthy individuals (which is attained when T-cell numbers are very low), h is the T-cell count at which the division rate is half-maximal in healthy individuals, and p_{biv} represents the extra T-cell proliferation induced by HIV infection.

Results

Recovery of CD4 T-cell counts in children and adults during long-term HAART

We aimed to determine if, during sustained viral suppression, CD4 T-cell counts normalized in children and adults on long-term (4.4-9.6 years) HAART, and whether children, as they may have better reconstituting capabilities, demonstrated enhanced CD4 T-cell recovery compared to adults. The mean CD4 T-cell count at start of HAART (baseline) was 688 cells per µl blood (range: 50-2274) for children and 206 cells per µl blood (range: 19-560) for adults. Since T-cell counts per µl blood decrease with age and stabilize at the start of adolescence, total CD4 T-cell counts, as well as counts of CD4 T-cell subsets were normalized for age-matched control values,

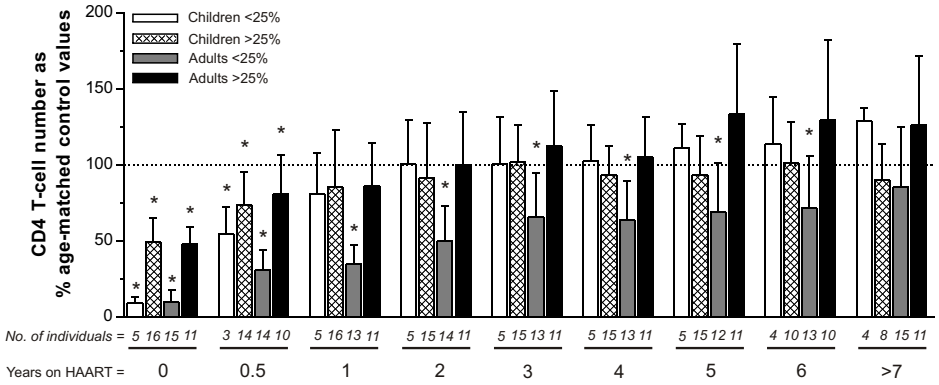

Figure 1. CD4 T-cell recovery in children and adults on HAART. CD4 T-cell counts as a percentage of age-matched control values (denoted by dotted horizontal lines) are illustrated from start of HAART and thereafter. Children and adults were subdivided into two groups according to a baseline CD4 T-cell number of 25% of control values. Data are shown as mean + standard deviation (SD). Statistical significance from 100% was determined by a one-sample t-test (* $p<0.05$), given the assumption that the groups are derived from a population with a normal distribution, as was confirmed by the non-parametric One-Sample Kolmogorov-Smirnov Test.

which were derived from our cohort of healthy volunteers and were similar to values published by others (39,40). The mean normalized CD4 T-cell number at start of HAART was higher for children than for adults (39.5% and 25.7% of the age-matched control value, respectively). To make a comparison between T-cell reconstitution in adults and children, and to evaluate the influence of baseline cell numbers on T-cell reconstitution, we separately analyzed pediatric and adult patients with low and high CD4 T-cell numbers at start of treatment. The value to discriminate these groups was chosen to be 25% of age-matched control values, which is equal to 200 CD4 T cells per µl blood in adults and delineates stages 1&2 versus 3 of the CDC classification system. In children with CD4 T-cell counts lower than 25% of their age-matched value at baseline, CD4 T-cell counts reached similar levels within one year of HAART as in children who had higher baseline CD4 T-cell counts ($p=0.80$, Figure 1) and in both groups CD4 T-cell counts were not significantly different from age-matched values from one year of HAART onward. In contrast, in adults the difference in CD4 T-cell counts between the two groups remained throughout follow-up. After one year of HAART, CD4 T-cell counts did not significantly differ from those of controls ($p=0.136$) in adults with high baseline CD4 T-cell counts. CD4 T-cell counts in adults with low baseline CD4 T-cell counts persistently lagged behind, but eventually after at least seven years of HAART, CD4 T-cell counts were attained which did not differ from control values ($p=0.176$).

CD4 T-cell subsets are perturbed in adults after long-term HAART

Although total CD4 T-cell counts of children and adults had returned to normal levels after long-term HAART with sustained viral suppression, we questioned if the naive T-cell compartment had restored to normal levels and whether expansions within the memory T-cell compartment had contracted. Because differences in naive and memory CD4 T-cell subsets between children with high and low baseline CD4 T-cell counts were not observed after long-term HAART (data not shown), the data are no longer presented separately for the two groups of children. In children, numbers of naive ($CD27^+CD45RO^-$) CD4 T cells had returned to normal levels after long-term HAART, whereas numbers of memory ($CD45RO^+$) CD4 T cells were significantly lower than in healthy age-matched controls (p=0.004, Figure 2). Adults with low baseline CD4 T-cell counts had normal numbers of memory CD4 T cells and lower naive CD4 T-cell counts after long-term HAART compared to healthy controls (p=0.001, Figure 2). In contrast, in adults with high baseline CD4 T-cell counts, numbers of memory CD4 T cells were higher than in healthy controls (p=0.022), and the naive CD4 T-cell compartment had regained normal numbers (Figure 2).

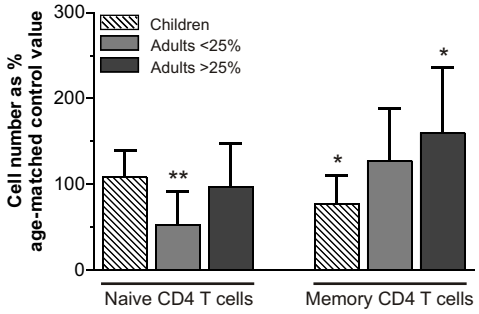

Figure 2. Recovery of CD4 T-cell subsets in children and adults after long-term HAART. Numbers of naive ($CD27^+CD45RO^-$) and memory ($CD45RO^+$) CD4 T cells after long-term HAART (4.4-9.6 years) expressed as percentage of age-matched control values in HIV-infected children, and adults with low or high baseline CD4 T-cell counts. Adults were subdivided into two groups according to a baseline CD4 T-cell number of 25% of control values. Data are shown as mean + SD. Statistical significance was determined by the non-parametric Mann-Whitney *U* test: * p<0.05; ** p<0.01.

Normal CD4 T-cell TREC contents and numbers after long-term HAART

Next we studied the origin of the newly-produced naive T cells during HAART and whether CD4 T-cell recovery was associated with altered aging of the T-cell compartment. On the one hand, increased peripheral T-cell proliferation could lead to accelerated aging and reduced diversity of the T-cell compartment in terms of diluted T-cell TREC contents and low fractions of CD31 expressing naive CD4 T cells. On the other hand, if new T cells were only derived from thymic output, their

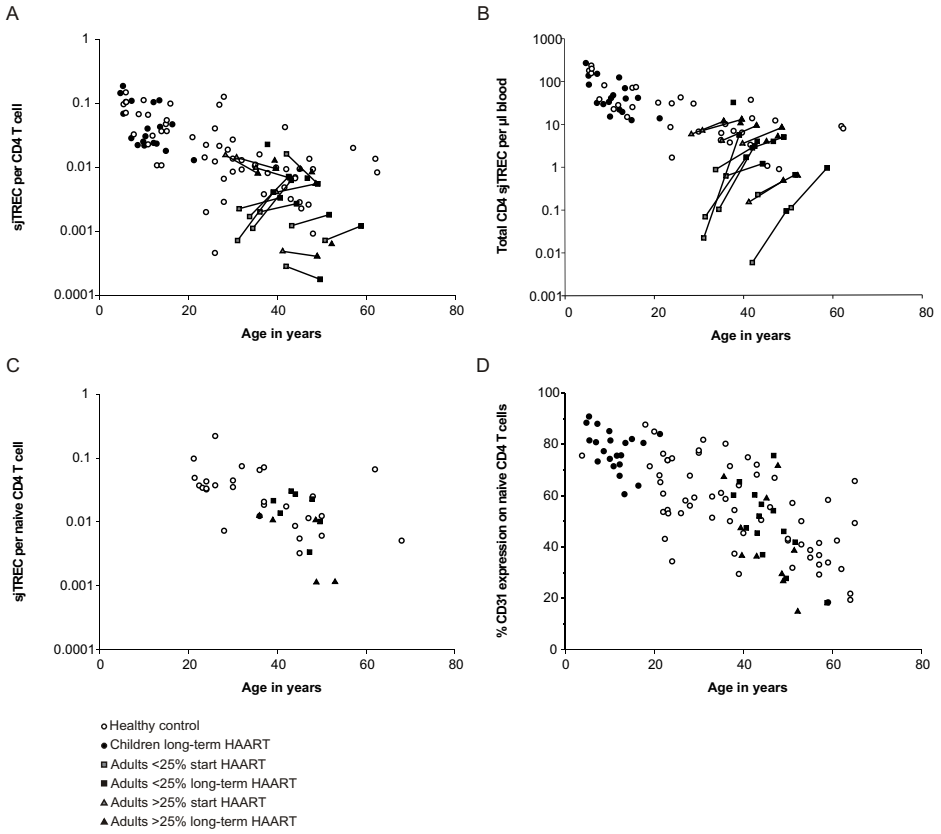

Figure 3. Recovery of various immune parameters after long-term HAART. A) TREC content per CD4 T cell. B) Total CD4 T-cell TREC numbers per µl blood. C) TREC content of naive CD4 T cells. D) Percentage of $CD31^+$ cells in the naive CD4 T-cell compartment. Adults were subdivided into two groups according to a baseline CD4 T-cell number of 25% of control values. Lines connect data points at start of HAART and after long-term HAART (4.4-9.6 years) within one individual. Whenever only one point is given it represents the value after long-term HAART.

appearance in a virtually empty T-cell pool may lead to higher TREC contents and higher levels of $CD31^+$ naive CD4 T cells compared to healthy age-matched controls (33). After long-term HAART, all children and most adults had similar TREC contents in CD4 T cells as age-matched controls (Figure 3A). In general, total CD4 T-cell TREC numbers (Figure 3B) as well as TREC contents of sorted naive CD4 T cells (Figure 3C) after long-term HAART did not differ from control values either. From a number of adults baseline CD4 T-cell TREC contents and numbers were available. In adults with low CD4 T-cell TREC contents at start of HAART an increase was observed during long-term HAART reaching (the lower end of) normal TREC contents, with the exception of two individuals who still had low TREC contents (Figure 3A). However, all these adults showed an increase in total CD4 T-cell TREC numbers during

HAART (Figure 3A,B). This suggests that thymic T-cell production contributed to the recovery of the naive CD4 T-cell compartment. Thus, in terms of TREC contents, the CD4 T-cell compartment did not seem to have undergone accelerated aging during long-term HAART.

Normal fraction of CD31 expressing naive CD4 T cells after long-term HAART

$CD31^+$ naive CD4 T cells are most proximal to the thymus (41) and have been described to have a much more diverse T-cell receptor repertoire than $CD31^-$ naive CD4 T cells (41,42). During aging the percentage of CD31 expressing naive CD4 T cells has been shown to decrease (41), raising the question whether the fraction of CD31 expressing naive CD4 T cells, and thereby normal levels of naive T-cell repertoire diversity, can be attained during immune reconstitution. To determine whether CD4 T-cell recovery was associated with accelerated aging of the naive T-cell compartment, the fraction of $CD31^+$ cells was determined within the naive CD4 T-cell compartment after long-term HAART. The fraction of $CD31^+$ naive CD4 T cells after long-term HAART in both adults and children was comparable to age-matched values (Figure 3D) which corroborates the finding that the extensive increases in CD4 T-cell counts during HAART had not caused accelerated aging of the naive CD4 T-cell subset.

Mathematical model explaining the observed T-cell recovery during long-term HAART

At first glance, it may seem counter-intuitive that the T-cell compartment shows no signs of rejuvenation or aging, in terms of abnormal TREC values and CD31 percentages, after reconstitution of a substantially depleted CD4 T-cell compartment. However, the use of a simple previously developed mathematical model for TREC dynamics (Materials and Methods)(18) shows that in fact this is exactly what is to be expected. In healthy individuals, T-cell TREC contents reflect an equilibrium between the levels of proliferation and thymic output characteristic for the age of the individual. At higher age, when there is less thymic output, the higher level of T-cell proliferation that is required to keep T-cell numbers relatively constant causes the TREC content of the T-cell population to be reduced. This TREC dilution effect is accelerated during HIV infection, which is known to considerably increase T-cell proliferation levels (Figure 4, age 20)(18). When long-term HAART causes the level of immune activation to return to nearly normal levels, the CD4 T-cell TREC content reverts to its normal age-matched control level, representing the equilibrium between the level of T-cell proliferation and thymic output characteristic for the age of the individual (Figure 4, age 30).

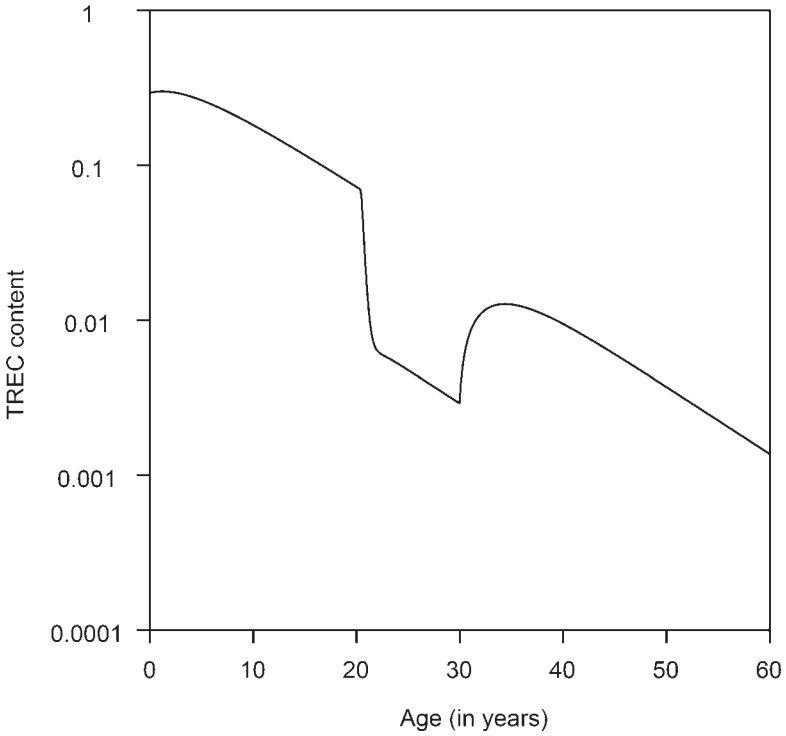

Figure 4. TREC dynamics according to the mathematical model. The change in CD4 T-cell TREC content with age was simulated for a healthy individual who got infected with HIV at the age of 20, and started HAART at 30 years of age. HIV infection was simulated by increasing the T-cell proliferation and death rate at the age of 20. As a result, the CD4 T-cell TREC content decreased dramatically, and subsequently followed the age-dependent decline that was also observed in healthy individuals. At the age of 30, when HAART reduced the level of T-cell proliferation and death to normal levels, the CD4 T-cell TREC content reverted to its normal age-matched control level, representing the equilibrium between the level of T-cell proliferation and thymic output characteristic for the age of the individual. Parameters: a = 10^8 cells/day, υ = 0.1/year, h = 2.5x10^{10} cells, p_0 = 0.1/day, and d = 0.001/day. The extra T-cell proliferation induced by HIV was modeled by p_{hiv} = 0.008/day and a concomitant increased T-cell death rate of d = 0.01/day (18).

Increased T-cell division rates in adults after long-term HAART

Because the T-cell compartment had recovered to normal levels after long-term HAART, we expected T-cell division to have returned to normal levels. To assess this, the expression of the proliferation marker Ki67 was measured within CD4 and CD8 T-cell subsets after long-term HAART. In children, naive ($CD27^+CD45RO^-$) and memory ($CD45RO^+$) CD4 T cells after long-term HAART indeed expressed similar levels of Ki67 as age-matched controls (Figure 5A,B). In both adult groups, in contrast, Ki67 expression within naive ($p<0.001$) and memory ($p<0.01$) CD4 T cells after long-term HAART was still increased compared to age-matched controls. No significant differences between the two adult groups were observed in terms of Ki67 expression in any of the CD4 T-cell subsets after long-term HAART (Figure 5A,B).

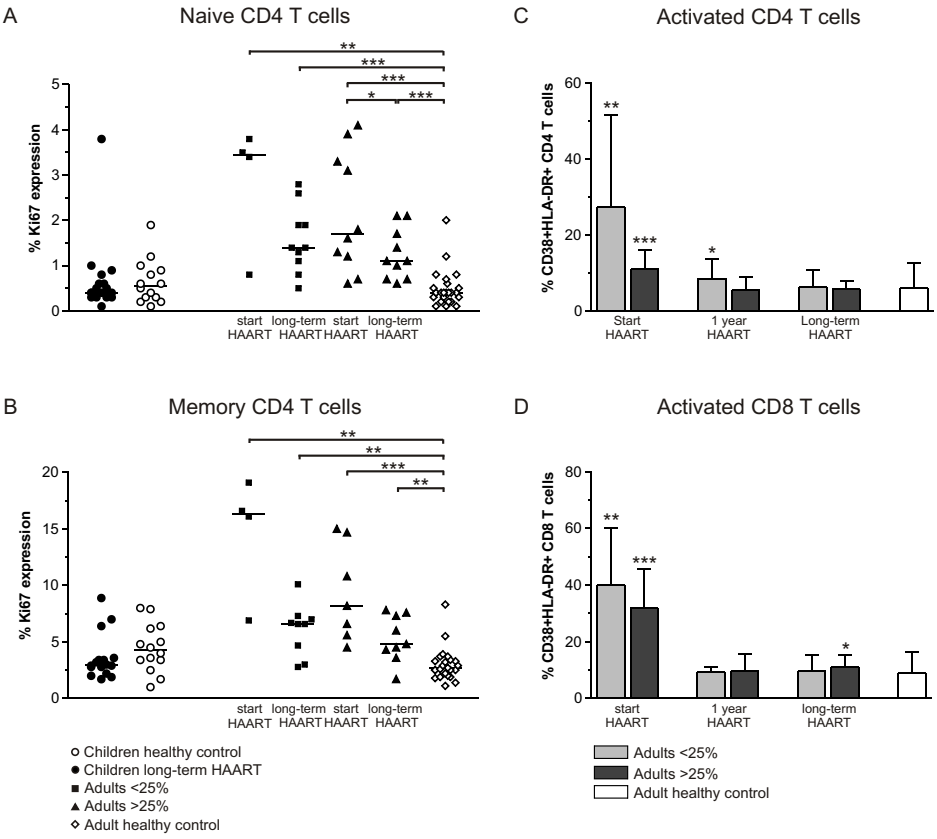

Figure 5. T-cell proliferation and activation levels during long-term HAART. Proliferation of naive A) and memory B) CD4 T cells after long-term HAART. Horizontal lines depict median Ki67 expression levels of each T-cell subset. The percentage of C) activated ($CD38^+HLA\text{-}DR^+$) CD4 and D) CD8 T cells during long-term HAART. Adults were subdivided into two groups according to a baseline CD4 T-cell number of 25% of control values. Data are shown as mean + SD. Statistical significance (compared to healthy controls) was determined by the nonparametric Mann-Whitney U test for unpaired data, whereas the nonparametric Wilcoxon Signed-Ranked Test was used for paired data: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Nevertheless, Ki67 expression in both adult groups had declined in all T-cell subsets during HAART, although only significantly within naive CD4 T cells of adults with high baseline CD4 T-cell counts (p=0.05; Figure 5A).

Increased levels of Ki67 expression are not related to T-cell activation

Although viral levels were below the threshold of detection, it cannot be ruled out that T-cell activation by residual viral replication could cause the increased levels of proliferation observed within the CD4 T-cell subsets in adults after long-term HAART. Therefore, the expression of the T-cell activation markers CD38 and HLA-DR was determined on CD4 and CD8 T cells from adults. The expression of these activation

markers had already normalized one year after initiation of HAART, except on CD4 T cells from adults with low baseline CD4 T-cell counts, and remained relatively stable at normal levels during follow-up (Figure 5C,D). Only adults with high baseline CD4 T-cell counts showed a slightly elevated expression level of activation markers on CD8 T cells after long-term HAART (p=0.013; Figure 5D), although after one year of HAART normalization was observed. No relation between the levels of activation and Ki67 expression of CD4 T cells (p=0.216 low baseline CD4 T cells; p=0.686 high baseline CD4 T cells; p=0.154 grouped, data not shown) or CD8 T cells (p=0.125 low baseline CD4 T cells; p=0.793 high baseline CD4 T cells; p=0.168 grouped, data not shown) was observed, suggesting that the elevated levels of T-cell proliferation were not clearly associated with T-cell activation. Expression levels of CD38 and HLA-DR on CD4 and CD8 T cells of children were normal after long term HAART (data not shown).

Discussion

Contrary to the common belief, our analyses show that both in HIV-1 infected children and adults, which were selected for sustained viral suppression and long follow-up, reconstitution of the CD4 T-cell compartment during long-term HAART can be achieved. Although it was shown recently that after seven years of continuous HAART CD4 T-cell counts in adults can reach levels of 800 cells per µl blood (43), most previously published studies failed to point out to what extent normalization of the T-cell compartment occurs during successful HAART. In these studies concerning immune reconstitution during HAART, a selection was made for immunological responders and/or non-responders (8,10-12,16,25,27,44,45), age-matched controls were not included (5,7,10,11,25,27,46-48) and mainly relatively short-term effects were studied (5-7,10,19,47-50). In many of the pediatric studies, patients had been pretreated (5,6,12,24,25,48,49) and residual viral load often persisted (7,11,12,24,25,48).

The fact that parameters like the fraction and absolute numbers of CD31 expressing naive CD4 T cells, and T-cell TREC content decrease with age suggests that even during aging of healthy individuals the immune system is not able to keep T-cell numbers stable without generation of T cells by peripheral proliferation. During reconstitution after severe T-cell depletion one may therefore intuitively expect to see signs of accelerated aging of the T-cell compartment. We found, however, that after long-term HAART the T-cell TREC content and the fraction of CD31 expressing naive CD4 T cells, had all returned to the level of age-matched controls, even in adults with baseline CD4 T-cell counts below 200 cells/µl. The increases in these immune parameters during HAART demonstrate that thymic output was a source

of new naive T cells in children, but also in adults. This supports the data from a previous report by Dion et al. (36). In healthy individuals, the TREC content of naive CD4 T cells has been found to decline during aging (17). It was shown that density-dependent peripheral naive T-cell proliferation is most likely causing this decline (38,51). If during HAART the thymus would be the only source of naive T-cell production, one would expect that after long-term HAART TREC contents would be higher than those of age-matched healthy individuals (33). Since the TREC content in naive CD4 T cells was found to be similar to that of age-matched healthy individuals, our data suggest that in addition to thymic naive T-cell production also peripheral naive T-cell proliferation contributed to the recovery of the naive T-cell compartment during HAART.

The observation that the fraction of CD31 expressing naive CD4 T cells, which are cells most proximal to the thymus (42) and are characterized by a diverse T-cell receptor repertoire (41) had normalized, suggests that after long-term HAART the naive T-cell repertoire may be as diverse as in healthy age-matched individuals. Furthermore, we found that telomere length of memory CD45RA$^-$ T cells had normalized during long-term therapy (data not shown). The restricted use of antibodies in the flow-FISH assay does not allow for staining with CD27 mAbs. Consequently, telomere length of naive T cells could not be measured as CD45RA$^+$ T cells may be contaminated by the presence of terminally-differentiated effector (CD45RA$^+$CD27$^-$) CD8 T cells. However, since the average telomere length of memory cells is highly linked to the telomere length of naive T cells (52), it is most unlikely that the telomere length of naive T cells was significantly shortened after long-term HAART, because memory T cells were found to have normal telomere lengths.

Although all of our data suggest successful recovery of the CD4 T-cell compartment after long-term HAART, our analyses focused on quantitative reconstitution only. Functional assays will have to be performed in future research in order to ascertain whether the quantitative recovery of the CD4 T-cell compartment that we observed by phenotypical measures also leads to qualitative reconstitution of T-cell function after long-term HAART.

In agreement with previous observations by others, who studied T-cell reconstitution after 18 months of treatment (48), we found that children with low baseline CD4 T-cell counts recovered to similar levels of CD4 T cells as children with higher baseline counts within one year of HAART, and that from one year of therapy onward both groups had no significantly lower CD4 T-cell counts compared to healthy age-matched values. Furthermore, numbers of naive CD4 T cells had normalized in children on long-term HAART. Hence, these data suggest that postponing the initiation of treatment of HIV-infected children, which may be beneficial to avoid unwanted side-effects of HAART, may not interfere with T-cell reconstitution during HAART.

Although patient numbers are limited in this study, CD4 T-cell counts after long-term HAART normalized in both adults with low and high baseline CD4 T-cell counts. Apparently, the occurrence of one or two viral blips observed during the entire follow-up in a number of patients did not seem to interfere with the recovery of CD4 T-cell counts. However, the number of naive CD4 T cells after long-term HAART did not normalize in adults with low baseline CD4 T-cell counts and full phenotypical CD4 T-cell reconstitution is therefore not observed. It remains to be determined whether naive CD4 T-cell reconstitution is indeed hampered in individuals who have low CD4 T-cell counts at start of therapy or whether this group will eventually reach naive CD4 T-cell levels similar to those in the group with higher baselines in a few more years.

Strikingly, the proliferation marker Ki67 was still slightly elevated in adults after seven years of HAART, even though CD4 T-cell counts had normalized. Although we found no correlation between T-cell activation markers and the fraction of $Ki67^+$ T cells, residual viral activity, which for the most part does not exceed the detection threshold, may be responsible for the observed proliferation levels in the various T-cell subsets.

Taken together, these data demonstrate an unanticipated capability of the immune system to eventually recover from severe T-cell depletion, provided that HIV viremia is properly controlled. HIV-infected children were able to reconstitute the T-cell compartment well at any stage of lympho-depletion, whereas adults with low baseline CD4 T-cell counts took longer to normalize CD4 T-cell counts, but they did not have normalized naive T-cell fractions after long-term HAART. On a phenotypical basis, in children, but unexpectedly also in adults with more than 200 CD4 T cells/μl blood at start of therapy, naive T cells were generated such that reconstitution of the immune system did not lead to accelerated aging of the T-cell compartment.

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6

Phenotypical reversion of peripheral naive CD4$^+$ T cells: a measure for CD4 recent thymic emigrants

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Abstract

Thymic function is an important factor for naive T-cell production and has a substantial role in T-cell recovery in immune-depleted settings. Therefore, assessment of thymic output is essential in clinical practice. Common methods to determine naive T-cell generation, such as T-cell receptor excision circles (TRECs) and CD31 expression on naive $CD4^+$ T cells provide insight into the balance of naive T-cell production by the thymus and peripheral T-cell proliferation. These methods do not allow for identification of recently-produced naive T cells by the thymus at the cellular level, however. In mice, it was shown that IL-7 promotes CD8 lineage commitment in the thymus, and we investigated the idea that (a subset of) CD4 recent thymic emigrants (RTE) may not yet have been fully committed to the CD4 lineage after exit from the thymus and still be susceptible for IL-7 induced redirection towards the CD8 lineage. Indeed, a fraction of cord blood naive $CD4^+$ T cells cultured in the presence of IL-7 re-expressed low levels of CD8αα and/or CD8αβ. With age the fraction of naive $CD4^+CD8^{lo}$ T cells that were generated decreased and in PBMC from thymectomized individuals cultured with IL-7 these cells were hardly generated. A higher fraction of naive $CD4^+CD8^{lo}$ T cells compared to naive $CD4^+CD8^-$ T cells expressed CD31, suggesting that naive $CD4^+CD8^{lo}$ T cells were more proximal to the thymus. CD4 single positive (SP) thymocytes, the direct precursors of CD4 RTE, were also capable of undergoing IL-7 induced redirection towards the CD8 lineage, although this plasticity gradually decreased during intrathymic CD4SP maturation. We conclude that IL-7 induced re-expression of CD8 is a property of a subset of naive $CD4^+$ T cells which have recently left the thymus. Hence, we provide a novel way to measure the presence of CD4 RTE.

Introduction

During adult life naive T-cell numbers remain relatively stable (1). Three factors influence the homeostasis of the naive T-cell compartment: production, activation and longevity/death of naive T cells (2,3). New naive T cells are produced by the thymus but also by naive T-cell division in the periphery (4-7). As involution of the thymus starts during childhood, thymic output decreases with age, and although the adult thymus is still able to produce naive T cells this implies that maintenance of the naive T-cell compartment in adults depends mainly on naive T-cell longevity and proliferation (8-10).

After immune depletion following e.g. stem cell transplantation, chemotherapy or during highly active antiretroviral treatment (HAART) for HIV-1 infection, the naive T-cell compartment reconstitutes. It has been shown that thymic output is important for naive T-cell reconstitution, and that naive T-cell counts in children recover faster than in adults (11,12). Recent thymic emigrants (RTE) are the peripheral counterparts of newly produced thymic T cells and measurement of the number of RTE would be useful to determine the level of thymic output during aging and immune reconstitution.

T-cell receptor excision circles (TRECs) are now widely used to estimate thymic naive T-cell production. These epichromosomal circular DNA remnants are formed during recombination of the T-cell receptor in developing thymocytes and their level can be measured by RT-PCR (5,13). TRECs cannot be replicated and are diluted upon cell division. Hence, the TREC content per T cell provides a measure of the replicative history of a T cell, whereas the total T-cell TREC number estimates the number of T cells which have egressed from the thymus (14). Although TRECs have proven useful in providing insight into the balance between thymic output and peripheral proliferation in health and disease, they cannot be used to directly identify RTE. Firstly, a TREC-containing naive T cell is not necessarily a RTE, since a naive T cell which has divided can still contain a TREC. Secondly, TRECs describe naive T-cell dynamics at the population level instead of identifying RTE at the cellular level.

CD31 (or PECAM-1) is another marker which has been used to determine the level of RTE. Approximately 85% of naive $CD4^+$ T cells in cord blood are $CD31^+$, and the percentage of $CD31^+$ naive $CD4^+$ T cells decreases with age (15). Furthermore, $CD31^-$ naive $CD4^+$ T cells have an approximately 8-fold lower TREC content and a less diverse Vβ-repertoire than $CD31^+$ naive $CD4^+$ T cells (15,16). These data suggest that CD31 expression on naive $CD4^+$ T cells is lost upon naive $CD4^+$ T-cell division and that $CD31^+$ naive $CD4^+$ T cells represent RTE. Opposing this notion is the finding that the TREC content of $CD31^+$ naive $CD4^+$ T cells declines with age, implying that (a fraction of) $CD31^+$ naive $CD4^+$ T cells divide and retain CD31 expression (17). As

a consequence, CD31 can be used a as thymic proximity marker, rather than a RTE marker.

The maximum level of thymic output in healthy young adults has been determined *in vivo* by heavy water labeling studies (3,18). These studies showed that thymic naive T-cell production is maximally 0.1% of the naive T-cell compartment per day and that these newly generated cells are very long-lived as their half-life exceeds the average naive T-cell half-life of 4-6 years (3). Although these data have provided the first adequate estimate for the level of RTE in early adulthood, an easy unanimous assay to determine the level of RTE at a given age is currently still lacking.

The problem of the currently used methods (CD31 and TRECs) to identify RTE is that they are based on cell division. Since considerable time may pass between egress from the thymus and naive T-cell division, it would be better to characterize RTE by a marker which is lost or gained within a short timeframe after a naive T cell has egressed from the thymus. Therefore, RTE may be better identified on the basis of an intrinsic feature or functional capacity which distinguishes them from non-RTE (mature) naive T cells.

In this study, we have exploited the latter possibility. In mice, it has been shown that IL-7 promotes CD8 lineage commitment in the thymus (19,20), and we investigated the idea that (a subset of) CD4 RTE may not yet have been fully committed to the CD4 lineage after exit from the thymus and stay susceptible to IL-7 induced redirection towards the CD8 lineage. We show that in humans IL-7 induced re-expression of CD8 is a property of a subset of naive $CD4^+$ T cells which have recently left the thymus. Hence, we introduce CD4/CD8 lineage plasticity as a novel method to identify CD4 RTE.

Materials and methods

Study population and blood and thymus specimens

Whole EDTA-anticoagulated blood samples were obtained by venipuncture from healthy and thymectomized individuals. All healthy children, aged 0.5-16.8 years, visited the University Medical Center in Utrecht in order to undergo an elective urological or plastic surgery. The children were considered healthy as they did not have any history of infectious diseases or hematological and immunological disorders. Adult blood samples were collected from healthy volunteers. Cord blood samples were obtained from healthy full-term neonates directly after delivery. Blood samples from thymectomized individuals were collected from children who underwent or had undergone cardiac surgery because of congenital heart disease in the first months of life. Thymocytes were isolated from the thymus lobes that were removed

during these surgeries. The study was approved by the medical ethical committee and written informed consent was obtained from all study participants or their legal guardians in agreement with the Helsinki Declaration of 1975, revised in 1983.

Cell preparation and cultures

Cord blood (CBMC) and peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque density gradient centrifugation. Thymic lobes were mechanically dissociated through a 70µm cell strainer (BD Falcon) and thymocytes were collected in PBS. CBMC, PBMC and FACS-sorted naive T-cell and thymocyte subsets were cultured with or without a final concentration of 10ng/ml IL-7 (Sigma) in RH10: RPMI1640 supplemented with L-Glutamate and 25mM HEPES (Gibco), containing

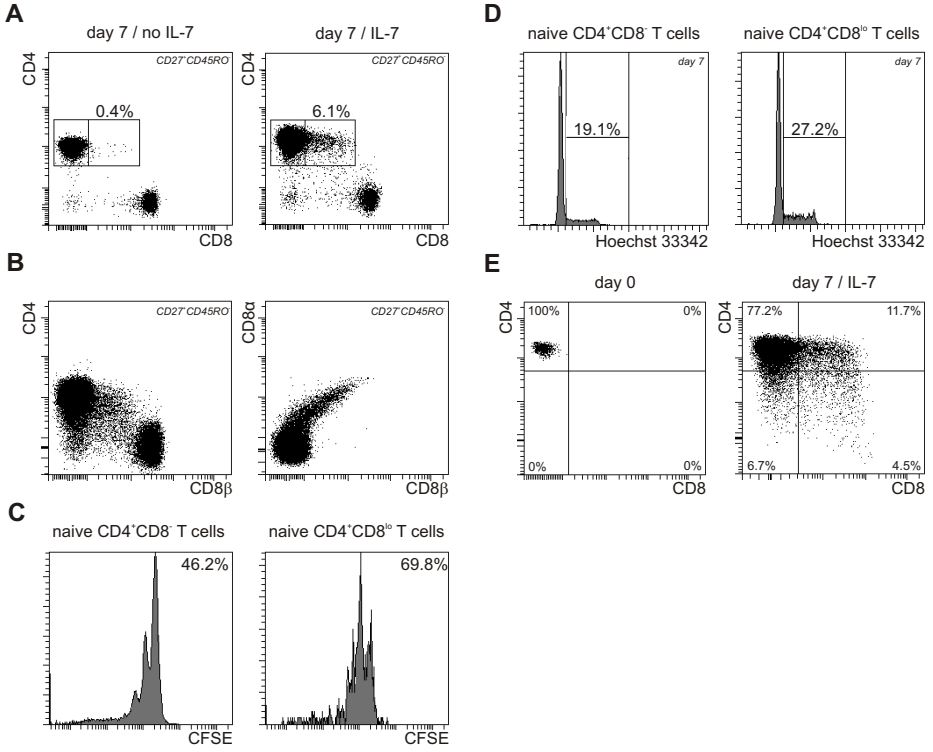

Figure 1. IL-7 induced generation of naive CD4$^+$CD8^{lo} T cells. CBMC were cultured without or with IL-7 (10ng/ml) for 7 days. A) IL-7 induced expression of CD8 on naive CD4$^+$ T cells. Values in plots represent the percentage of naive CD4$^+$ T cells expressing CD8. Cells in plots are phenotypically naive (CD27$^+$CD45RO$^-$) cells. B) Naive CD4$^+$CD8^{lo} T cells express CD8αα and/or CD8αβ at day 7. C) Naive CD4$^+$CD8^{lo} T cells show more CFSE dilution than naive CD4$^+$CD8$^-$ T cells during 7-day culture with IL-7. Values in plots represent the percentage of divided cells. D) More naive CD4$^+$CD8^{lo} T cells are in cell cycle as measured by Hoechst 33342 than naive CD4$^+$CD8$^-$ T cells on day 7 of culture with IL-7. E) Sorted cord blood naive CD4$^+$ T cells were able to attain CD8 and/or lose CD4 expression upon culture with IL-7.

10% Human Pooled Serum (HPS) (Sanquin), Penicillin/Streptomycin (100 U/ml) (Invitrogen) and 6.0 x 10^{-5}M β-mercaptoethanol (v/v) (Calbiochem). For thymocyte cultures, culture medium was replenished every 3 days.

Naive $CD4^+$ T cells were defined as $CD27^+CD45RO^-CD4^+$ T cells. The level of naive $CD4^+CD8^{lo}$ T cells in CBMC and PBMC was determined by assessing the level of naive $CD4^+$ T cells which expressed low levels of CD8 (Figure 1A; right panel) after 7-day culture with IL-7, subtracted by the level of naive $CD4^+CD8^{lo}$ T cells (background level) which were observed in cultures without addition of IL-7 (Figure 1A; left panel).

Flow cytometry

CBMC, PBMC or FACS-sorted cells were incubated with mAb to CD3-Pacific Blue, CD4-PE-Cy7, CD4-APC-AlexaFluor750, CD8-APC-AlexaFluor (eBioscience), CD4-Pacific Blue, CD8-PerCP-Cy5.5, CD8β-PE, CD27-APC, CD31-PE, CD45RO-PE, CD45RO-PE-Cy7, CD127-PE (Becton Dickinson). Naive $CD4^+$ T cells from CBMC or PBMC, CD4SP and CD8SP thymocytes, and CD4SP thymocyte subsets were purified by cell sorting on a FACSAria (Becton Dickinson). Purity of cells was >97%. To measure the level of CBMC, PBMC or FACS-sorted cells in cell cycle during culture, cells were stained intracellularly with Hoechst 33342 (4μg/ml) (Sigma) after fixation and permeabilization with Cytofix/Cytoperm™ and Perm/Wash™ according to manufacturer's instructions (Becton Dickinson). The level of proliferating cells (DNA content of >2N per cell) was determined after doublet discrimination on a LSRII (Becton Dickinson).

CFSE labeling

CBMC, PBMC and FACS-sorted naive T-cell and thymocyte subsets were pelleted and resuspended at a final concentration of $10x10^6$ cells/ml. Cells were labeled in 1μM CFSE in PBS for 10 min at 37°C. Labeling was blocked by addition of 2ml HPS (Sanquin). After washing thrice with PBS, cells were cultured in RH10 with or without a final concentration of 10ng/ml IL-7 (Sigma) at 37°C in 5% CO_2 atmosphere.

To determine the number of precursor cells which had divided in culture we used the following equation:

$$p_d = 100 \cdot \left[\frac{\sum_{n=1}^{\infty} \left(\frac{c_n}{2^n} \right)}{\sum_{n=1}^{\infty} \left(\frac{c_n}{2^n} \right) + c_0} \right]$$

where p_d is the percentage of cells which had divided in culture, c_n represents the number of cells which underwent n divisions and c_0 represents the number of cells which did not divide in culture.

Results

During naive T-cell development in the thymus, $CD4^+CD8^+$ (DP) thymocytes differentiate via the intermediate $CD4^+CD8^{lo}$ stage into $CD4^+$ MHC class II-restricted single positive (SP) thymocytes, after which they exit the thymus as naive $CD4^+$ T cells or CD4 RTE (21,22). We studied whether (all) CD4 RTE had completely achieved CD4 lineage commitment upon thymic egress. In mice, IL-7 promotes CD8 lineage commitment in the thymus. We hypothesized that if CD4 RTE are not yet fully committed after egress from the thymus, they might be redirected towards the CD8 lineage by high levels of IL-7 (19,20).

CBMC were cultured in the presence of IL-7 (10ng/ml). After 7 days in culture, the level of CD8 expression on naive ($CD27^+CD45RO^-$) $CD4^+$ T cells was evaluated and we found that between 4.5-15.6% of naive $CD4^+$ T cells expressed low levels of CD8 (Figures 1A, 2A). These naive $CD4^+CD8^{lo}$ T cells expressed the CD8αβ heterodimer and/or the CD8αα homodimer (Figure 1B). Naive $CD4^+CD8^{lo}$ T cells were detectable from day 3 in culture and initially expressed only CD8αα (data not shown), which suggests that transcription of *Cd8a* precedes that of *Cd8b*.

Naive $CD4^+CD8^{lo}$ T cells were more susceptible for IL-7 induced proliferation than naive $CD4^+CD8^-$ T cells: they had divided more in culture (Figure 1C) and relatively more $CD4^+CD8^{lo}$ T cells were in the S- or G_2/M-phase of the cell cycle at day 7 (Figure 1D).

Naive $CD4^+CD8^{lo}$ T cells are derived from naive $CD4^+CD8^-$ T cells

It is likely that the naive $CD4^+CD8^{lo}$ T cells originate from naive $CD4^+CD8^-$ T cells and had attained CD8 expression during culture. However, since naive $CD4^+CD8^{lo}$ T cells have proliferated more than naive $CD4^+CD8^-$ T cells (Figures 1C,D) it could also be that these cells were derived from naive $CD4^+CD8^{lo}$ T cells that were present at low levels at the start of culture with IL-7 and selectively grew out. Indeed, naive $CD4^+CD8^{lo}$ T cells were detected at the start of culture at levels varying between 0.0-1.1% (data not shown). To determine from which cell population the naive $CD4^+CD8^{lo}$ T cells were derived, naive $CD4^+CD8^-$ and $CD4^+CD8^{lo}$ T cells present in CBMC were sorted and cultured for seven days in the presence of IL-7. Naive $CD4^+CD8^-$ T cells gave rise to $CD8^{lo}$-expressing naive $CD4^+$ T cells, but also to cells with $CD4^-CD8^-$ or $CD4^-CD8^+$ phenotypes (Figure 1E). Unfortunately, we were unable to obtain pure

naive $CD4^+CD8^{lo}$ T cells. Therefore, we cannot rule out that the naive $CD4^+CD8^{lo}$ T cells observed during culture with IL-7 may to some extent originate from cells with a similar phenotype at the start of culture. Nevertheless, we provide evidence that naive $CD4^+CD8^{lo}$ T cells were generated from naive $CD4^+CD8^-$ T cells during culture with IL-7.

Naive $CD4^+CD8^{lo}$ T cells are CD4 RTE

We assumed that the naive T cells which became $CD4^+CD8^{lo}$ T cells upon culture with IL-7 were (a subset of) CD4 RTE that had not fully committed to the CD4 lineage and were redirected towards the CD8 lineage by IL-7. Since thymic output declines during life (23), we expected that the percentage of naive $CD4^+CD8^{lo}$ T cells that would be generated in culture should decrease with age. Indeed, when PBMC from donors of various age were cultured with IL-7, fewer naive $CD4^+CD8^{lo}$ T cells were generated with advancing age and beyond the age of 20 hardly any naive $CD4^+CD8^{lo}$ T cells were observed in culture (Figure 2A). In addition, we determined whether after 7-day culture in the presence of IL-7, the expression of the thymic proximity marker CD31 differed between naive $CD4^+CD8^{lo}$ T cells and naive $CD4^+CD8^-$ T cells. Of the naive $CD4^+CD8^-$ T cells, 75.8% was $CD31^+$, whereas 97.5% of naive $CD4^+CD8^{lo}$ T cells expressed CD31 (Figure 2B). Collectively, these data are compatible with the idea that the naive $CD4^+CD8^{lo}$ T cells which are generated in culture with IL-7 represent (a subset of) CD4 RTE.

Naive $CD4^+CD8^{lo}$ T cells are hardly generated in thymectomized individuals

Since we think that naive $CD4^+CD8^{lo}$ T cells are formed from (a subset of) CD4 RTE, we expect that they cannot be generated in culture with IL-7 in the absence of thymic output. Therefore, we cultured PBMC from individuals between 0.2-8.0 years old who, in the first months of life, underwent cardiac surgery for congenital heart disease and were thymectomized during this procedure. In all of these individuals hardly any naive $CD4^+CD8^{lo}$ T cells were observed after 7 days in culture with IL-7 (Figure 2A). From one individual, pre- and post-surgery PBMC were available and subsequently cultured with IL-7. Hardly any naive $CD4^+CD8^{lo}$ T cells were observed when cells obtained 2.5 months after removal of the thymus were cultured with IL-7, whereas cells obtained prior to thymectomy generated abundant numbers of naive $CD4^+CD8^{lo}$ T cells upon culture with IL-7 (Figure 2C). These results demonstrate that (this subset of) naive $CD4^+$ T cells complete CD4 lineage commitment within at least 2.5 months after egress from the thymus.

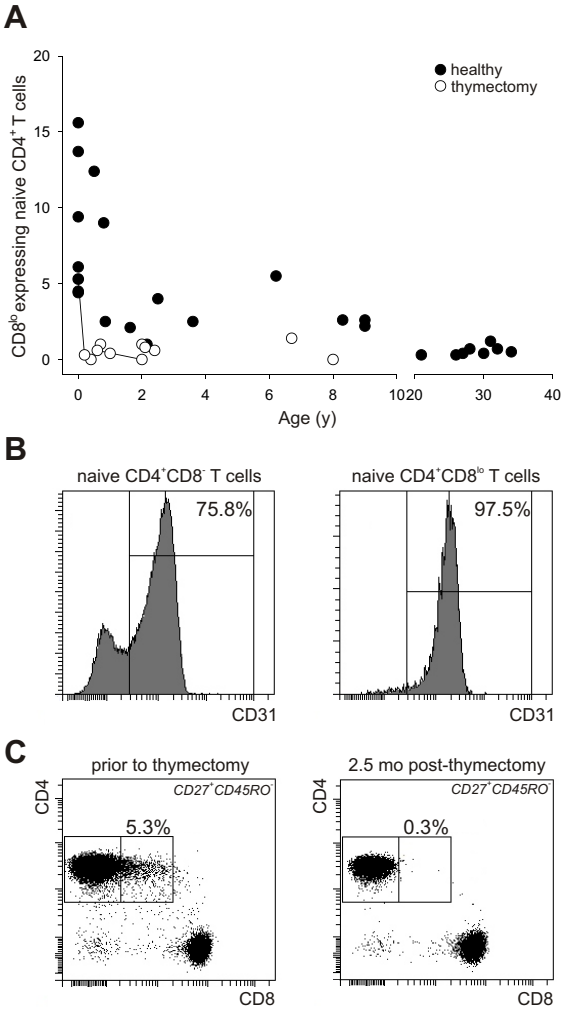

Figure 2. Naive CD4⁺CD8^{lo} T cells resemble CD4 RTE. A) PBMC/CBMC from healthy and thymectomized individuals were cultured with IL-7 (10ng/ml). The level of CD8^{lo}-expressing naive (CD27⁺CD45RO⁻) CD4⁺ T cells was assessed after 7 days. Solid lines connect longitudinal data points. B) Expression of CD31 on naive CD4⁺CD8⁻ and naive CD4⁺CD8^{lo} T cells in PBMC after 7-day culture with IL-7 (10ng/ml). C) Expression of CD8 on naive CD4⁺ T cells in PBMC prior to and 2.5 mo after thymectomy. Values in plots represent the percentage of naive CD4⁺ T cells expressing CD8.

Not all CD4SP thymocytes undergo IL-7 mediated redirection towards the CD8 lineage

We questioned whether the ability to re-express CD8 was a feature of all CD4 RTE, or that only a fraction of CD4 RTE was not yet fully committed to the CD4 lineage. Therefore, we tested the ability of CD4SP thymocytes, the progenitors of CD4 RTE, to re-express CD8 in the presence of IL-7. CD4SP thymocytes were sorted and cultured with IL-7 for 12 days. After 6 days, 37.0% of CD4SP thymocytes had obtained CD8

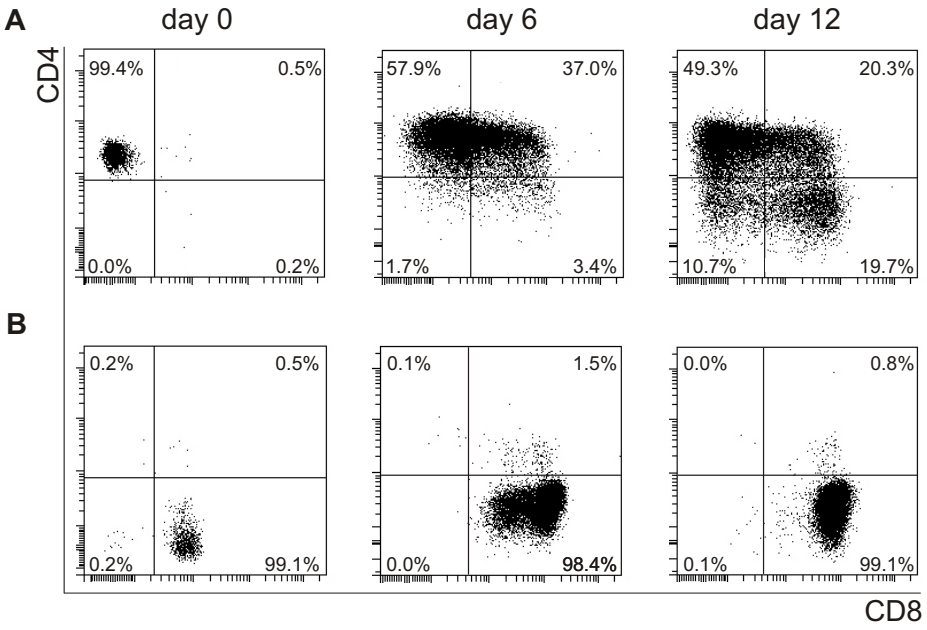

Figure 3. Plasticity of CD4SP and CD8SP thymocytes. A) CD4SP ($CD3^+CD4^+CD8^-$) thymocytes and B) CD8SP ($CD3^+CD4^-CD8^+$) thymocytes were sorted and cultured with IL-7 (10ng/ml) for 12 days. Culture medium was replenished every 3 days.

expression (Figure 3A). At day 12, 19.7% of cells had even further progressed towards the CD8 lineage and had become $CD4^-CD8^+$ (Figure 3A). As a control, we sorted and cultured CD8SP thymocytes similarly. Upon culture no major changes in CD4 and CD8 expression were observed, although a small number of CD8SP thymocytes (1.5% at day 6) were CD4-positive (Figure 3B). Collectively, these data imply firstly that a fraction of the CD4SP thymocytes has not finished full commitment to the CD4 lineage and can be redirected to the CD8 lineage upon culture with IL-7. Secondly, since not all CD4SP thymocytes were redirected towards the CD8 lineage, this suggests that the naive $CD4^+CD8^{lo}$ T cells generated in PBMC or from sorted naive $CD4^+$ T cells represent a fraction of all CD4 RTE.

The capability to redirect towards the CD8 lineage declines during CD4SP thymocyte maturation

Since not all CD4SP thymocytes demonstrated the ability of IL-7 induced redirection towards the CD8 lineage, we investigated which fraction of the total CD4 RTE pool the progenitors of the naive $CD4^+CD8^{lo}$ T cells represent. Based on the expression of CD27 and CD45RO (Figure 4A), the CD4SP thymocyte population was divided into three subpopulations: immature ($CD27^-CD45RO^+$), intermediate ($CD27^+CD45RO^+$) and mature ($CD27^+CD45RO^-$) CD4SP thymocytes. Expression of IL-7Rα (CD127) on the cell surface varied between the subpopulations and increased upon CD4SP

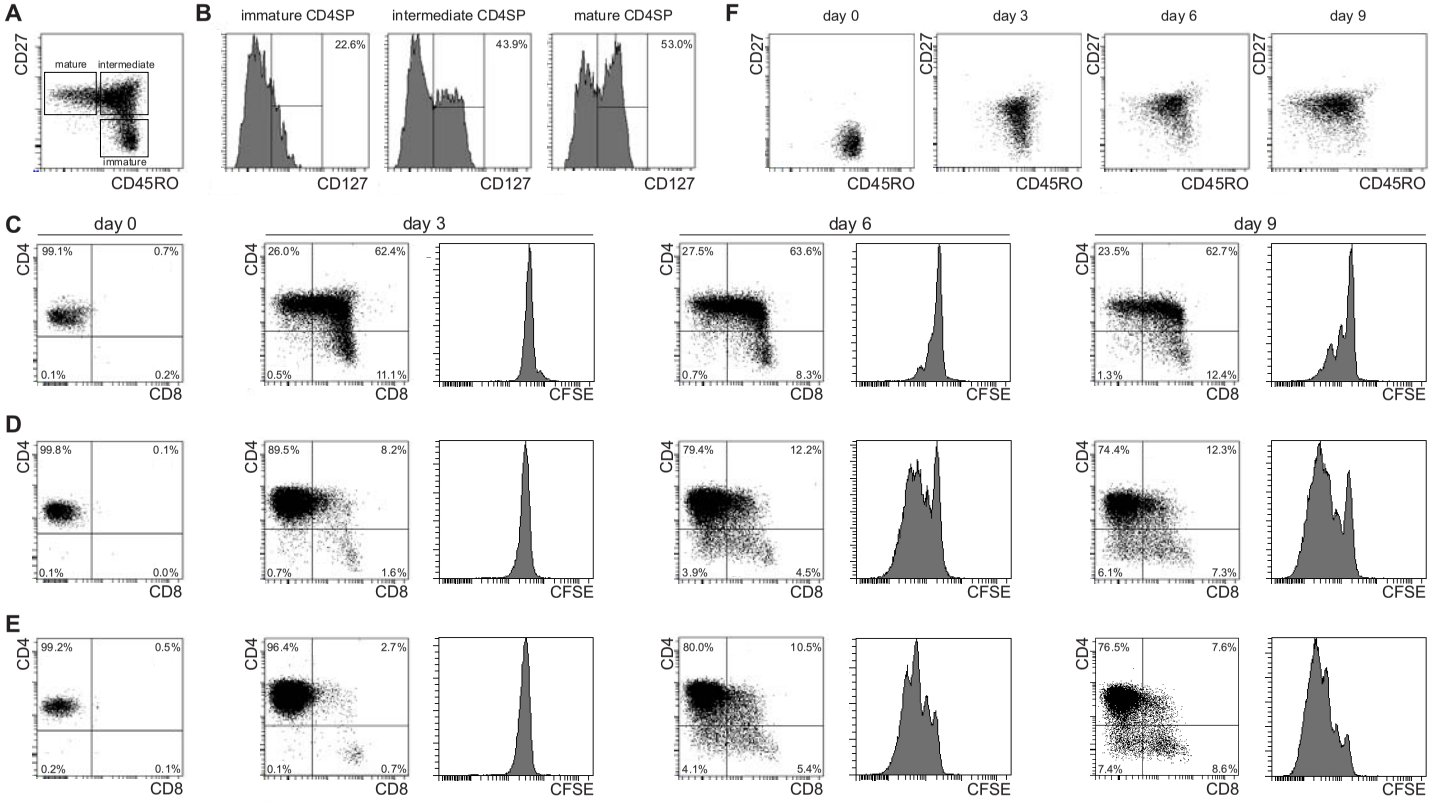

Figure 4. IL-7 induced plasticity of CD4SP thymocyte subsets. A) Three developmental CD4SP ($CD3^+CD4^+CD8^-$) thymocytes subsets were defined based on the expression of CD27 and CD45RO. B) Expression of IL-7Rα (CD127) on CD4SP thymocyte subsets. C) Immature, D) intermediate and E) mature CD4SP thymocytes were sorted, labeled with CFSE and cultured for 9 days with IL-7 (10ng/ml). Culture medium was replenished every 3 days. F) Immature CD4SP thymocytes cultured with IL-7 develop into intermediate and mature CD4SP thymocytes.

thymocyte maturation (Figure 4B). Each subpopulation was sorted, labeled with CFSE and cultured with IL-7 for 9 days (Figure 4C-E). Approximately 75% (at day 9) of immature CD4SP thymocytes was redirected towards the CD8 lineage. Redirection was already apparent at day 3 (Figure 4C). Surprisingly, even without the addition of IL-7, 12% of immature CD4SP thymocytes was able to redirect towards the CD8 lineage (data not shown). Intermediate and mature CD4SP thymocytes cultured with IL-7 also progressed towards the CD8 lineage, but with delayed kinetics (Figure 4D,E). Mature CD4SP thymocytes displayed the highest level of IL-7 induced cell division in culture (Figure 4C-E). Loss of CD4 expression was accompanied by relatively more division compared to acquisition of CD8 expression (Figure 5). These data suggest that during maturation of CD4SP thymocytes in the thymus, the IL-7 induced ability to redirect towards the CD8 lineage declines as more cells are likely to have completed the program of CD4 lineage commitment. In contrast, the ability of cells to divide in culture with IL-7 increases during thymocyte maturation and could be related to expression levels of the IL-7Rα in these subsets (Figure 4B). Indeed, immature CD4SP thymocytes differentiated towards an intermediate and mature CD4SP thymocyte stage during the 9-day culture and showed delayed proliferation kinetics (Figure 4C,F).

Similar to the naive $CD4^+CD8^{lo}$ T cells generated from naive $CD4^+$ T cells in cord blood and PBMC (Figure 1B), expression of CD8 on the cell surface of CD4SP thymocyte subsets consisted of CD8αα and/or CD8αβ (data not shown).

The progenitors of naive $CD4^+CD8^{lo}$ T cells account for 20% of all CD4 RTE

Mature CD4SP thymocytes exit the thymus as naive $CD4^+$ T cells and therefore are the progenitors of CD4 RTE. Since mature CD4SP thymocytes in culture with

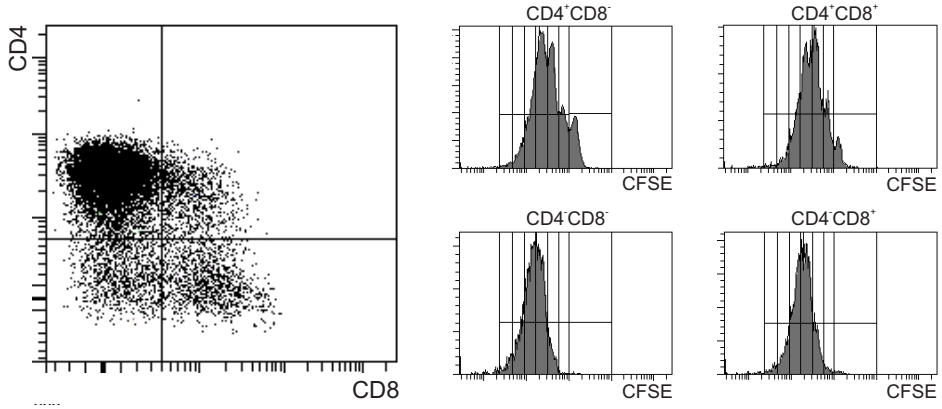

Figure 5. Proliferation and differentiation of mature CD4SP thymocytes. CFSE labeled, mature CD4SP thymocytes at day 9 of culture with IL-7 (10ng/ml) had differentiated into $CD4^+CD8^+$, $CD4^-CD8^-$ and $CD4^-CD8^+$ cells. Each of these populations had different CFSE profiles. Culture medium was replenished every 3 days.

IL-7 differentiated into $CD4^+CD8^+$, $CD4^-CD8^-$ and $CD4^-CD8^+$ cells and each of these populations had different CFSE profiles (Figure 5), we were able to calculate (Materials and methods) the original percentage of mature CD4SP thymocytes which eventually differentiated during culture. On average, 20% of mature CD4SP thymocytes was able to differentiate during culture with IL-7 (Figure 5). This implies that the naive $CD4^+CD8^{lo}$ T cells generated in culture from cord blood or PBMC comprise at maximum 20% of all CD4 RTE.

Discussion

For years, researchers have sought for an ideal method to identify RTE. To date, the measurements of TREC levels in $CD31^+$ and $CD31^-$ naive $CD4^+$ T cells provided the best estimates to identify and calculate CD4 RTE levels at a given age (Vrisekoop et al. manuscript in preparation). The rationale behind this methodology is the link of RTE to division. A naive T cell will lose the predicate RTE once it has divided, since the original naive T cell which has once exited the thymus has been substituted for two new naive T cells generated in the periphery. Although this idea is valid, it has a major drawback. Naive T cells have relatively long life spans and can live for many years (3). Because naive T cells presumably hardly proliferate, no distinction can be made between TREC-containing naive $CD31^+$ $CD4^+$ T cells which have indeed recently left the thymus and those that have been circulating for years without dividing in the periphery. Although both these cells are thymic emigrants, there is no legitimate reason to designate the older ones as RTE. Therefore, the quest for an ideal method to identify RTE has been ongoing.

In this study, we propose such a method. Instead of identifying a cell-specific marker already present on CD4 RTE, we identified a subset of naive $CD4^+$ T cells which, at the time of thymic egress, have retained a characteristic of CD4SP thymocytes. These naive $CD4^+$ T cells could re-express CD8 in the presence of high levels of IL-7. It has been shown in mice that lineage decision is dependent on the duration of the signal mediated through the TCR and CD4 or CD8 after recognition of MHC class II or I, respectively (24,25). This signal leads to downregulation of CD8 expression and consequently lasts shorter in thymocytes that have been selected for MHC class-I restricted TCRs. At the thymic $CD4^+CD8^{lo}$ stage TCR-mediated signaling in these thymocytes eventually ceases. These cells are then 'rescued' by IL-7, as this cytokine has been shown to promote survival and differentiation of cells towards the CD8 lineage after signaling through the TCR has waned (19,20). Apparently, excess levels of IL-7 in culture can result in the redirection of a peripheral subset of $CD4^+$ MHC class II-restricted T cells towards the CD8 lineage as these cells have not yet

finished the program of CD4 T-cell commitment. We show that CD4SP thymocytes can already be fully committed to the CD4 lineage at an early stage of CD4SP thymocyte development, but also that full commitment may not be achieved before egress from the thymus. Apparently, there is a large temporal window in which full commitment to the CD4 lineage (and IL-7 induced redirection towards the CD8 lineage) can occur. The molecular basis for this temporal window is unclear, but may involve the regulation of the transcription factors ThPOK and Runx3, which are key determinants in CD4/CD8 lineage decision (26-28).

During CD4SP thymocyte maturation the capability to redirect towards the CD8 lineage by IL-7 decreased. Immature CD4SP thymocytes demonstrated the greatest capability to be redirected towards the CD8 lineage. Nevertheless, approximately 25% of immature CD4SP thymocytes was already irreversibly committed to the CD4 lineage and this may even be an underestimation. Thymocytes selected for MHC class I- and class II-restricted TCRs initially downregulate CD8 expression and become $CD4^+CD8^{lo}$ cells that differentiate into either CD8SP or CD4SP thymocytes, respectively (19,20). We found that immature CD4SP thymocytes could re-express CD8 in the absence of IL-7. Therefore, it seems that CD8 expression can be lost completely on thymocytes selected for MHC class I-restricted TCRs and that both $CD4^+CD8^{lo}$ and immature CD4SP thymocyte populations contain cells which will ultimately commit to the CD8 lineage.

Our interpretation that naive $CD4^+CD8^{lo}$ T cells generated from naive $CD4^+$ T cells in culture with IL-7 represent CD4 RTE is supported by the observation that the capacity for their generation decreased with age, which is compatible with a decrease of thymic output over the years. The observation that naive $CD4^+CD8^{lo}$ T cells have a higher percentage of CD31 expression and are thus presumably more proximal to the thymus than naive $CD4^+CD8^-$ T cells substantiates this concept. Finally, naive $CD4^+CD8^{lo}$ T cells were hardly generated in PBMC from thymectomized individuals cultured with IL-7. In fact, we would have expected to find no generation of naive $CD4^+CD8^{lo}$ T cells at all upon culture of PBMC from thymectomized patients with IL-7. The few RTE that were observed may have originated from residual thymus output due to incomplete removal of the thymus. Alternatively, new naive T cells might be produced by another source. Although a small second thymus has been found in some strains of mice, it is currently unclear whether it is also present in humans (29). If so, the low levels of naive $CD4^+CD8^{lo}$ T cells which were generated in culture may represent thymic progeny from this secondary thymus. Nevertheless, compared to healthy individuals these levels were much lower.

Now it is possible to determine the level of CD4 RTE at a given age by determining the percentage of $CD8^{lo}$ expressing naive $CD4^+$ T cells in PBMC after a 7-day culture with IL-7. In this way, a proper indication of thymic function can be obtained,

even when, like in samples from immunocompromised individuals, cell numbers are limited.

Taken together, we present a novel method to identify a subset of CD4 RTE. The rationale of this method is not based on naive T-cell division, which complicates the use of CD31 and TRECs as RTE markers. We have shown that these CD4 RTE are able to re-express CD8 when cultured with IL-7 *in vitro*, an ability that is lost within at least 2.5 months after thymic egress. Because of its simplicity, this method is highly suitable to assess thymic naive T-cell production in clinical practice.

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Part II

7

Newly-produced naive and memory T cells are rapidly lost during HIV infection

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Abstract

The cause of the progressive decline of $CD4^+$ T-cell numbers, the hallmark of human immunodeficiency virus (HIV) infection, remains debated. Several studies have convincingly shown that there is increased production of $CD4^+$ T cells during HIV infection. This increased T-cell production could (i) reflect a homeostatic response to the progressive loss of $CD4^+$ T cells, or (ii) be due to chronic immune activation, causing naive T cells to divide, differentiate, and exit the naive T-cell pool. Because of the limited capacity to replenish naive T cells, this would eventually lead to depletion of the naive T-cell pool. Using long-term *in vivo* ^2H_2O labeling, we here show for the first time that – in contrast to naive T cells in healthy subjects – newly-produced naive $CD4^+$ and $CD8^+$ T cells in HIV-infected individuals are preferentially lost from the naive T-cell pool. Even in the memory T-cell compartment, recently-produced T cells of HIV-infected individuals tend to be lost. These data suggest that the increased levels of T-cell production during HIV infection are not a homeostatic response to the loss of $CD4^+$ T cells; they seem to be a cause rather than a consequence of the chronic loss of $CD4^+$ T cells.

Introduction

Previous studies have shown that the production rates of $CD4^+$ and $CD8^+$ T cells during HIV-infection are increased (1-6). These increased rates of T-cell production are not merely due to shifts in the percentages of naive and memory T cells (7), because deuterium incorporation studies in separated naive and memory T-cell populations have pointed out that both naive and memory $CD4^+$ and $CD8^+$ T cells are turning over more rapidly in HIV-infected individuals (8).

The elevated levels of T-cell production in HIV-infected patients have been proposed to reflect either a homeostatic response to compensate for the progressive loss of $CD4^+$ T cells (9,10), or to be driven by immune activation (11,12). It has previously been shown that HIV-infected patients suffering from AIDS have increased levels of IL-7 production in lymphoid tissue (13), and that naive T cells can divide in response to IL-7 while retaining the naive phenotype (14,15), suggesting that homeostatic proliferation may play a role in HIV infection. On the other hand, the observation that HAART strongly decreases the percentage of Ki67-expressing $CD4^+$ T cells long before $CD4^+$ T-cell numbers have recovered to normal values argues against a homeostatic response driving the increased turnover of $CD4^+$ T cells (11).

Explaining the increased level of T-cell production during HIV-infection by homeostasis or by immune activation has different implications for the loss of recently-produced T cells, and for the mechanism of $CD4^+$ T-cell depletion. If increased T-cell turnover during HIV-infection is due to a homeostatic response to the progressive loss of $CD4^+$ T cells, recently-produced T cells would not be expected to be lost rapidly, in order to contribute to the peripheral T-cell pool. On the other hand, if increased T-cell turnover reflects a state of immune activation, this could lead to increased loss rates of naive and memory T cells and a preferential loss of recently-produced T cells. To discriminate between these two possibilities, the fate of newly-produced T cells needs to be determined. Unfortunately, such data are still lacking, because the loss of isotope-labeled naive $CD4^+$ and $CD8^+$ T cells after long-term label administration has never been followed in HIV-infected patients. To obtain these data, we performed long-term *in vivo* labeling with deuterated water (^2H_2O) in 4 HIV patients, and followed the fate of newly-produced naive and memory $CD4^+$ and $CD8^+$ T cells during a 16-week down-labeling period.

Results and Discussion

A steady-state model for label enrichment

Absolute $CD4^+$ T-cell counts and fractions of naive $CD4^+$ and $CD8^+$ T cells remained fairly constant in all HIV patients during the entire protocol (data not shown), and were low compared to 5 healthy volunteers in whom we previously measured T-lymphocyte turnover using the same protocol (16) (Table 1). Fractions of $Ki67^+$ $CD4^+$ and $CD8^+$ T cells in the HIV patients were also constant during the execution of the study and tended to be high compared to healthy controls (Table 1). Because naive and memory $CD4^+$ and $CD8^+$ T-cell counts did not change during the analyses, we fitted the label enrichment data to a steady-state mathematical model describing the label enrichment in urine, granulocytes and naive and memory $CD4^+$ and $CD8^+$ T cells (see Methods and Figure 1), to determine the average lymphocyte turnover rate (p) and the average rate at which labeled cells were lost from the specific cell

Table 1. Characteristics of HIV-infected individuals

	A	B	C	D	Healthy[a]
Age at start protocol (yrs)	47	63	54	25	22
$CD4^+$ count (cells/µl blood)	306[b] (283-354)	182 (165-221)	189 (165-243)	450 (425-503)	890
$CD8^+$ count (cells/µl blood)	667 (558-816)	1612 (1574-1798)	296 (259-384)	532 (489-548)	470
% naive $CD4^+$	52.8 (51.0-64.5)	23.7 (20.3-26.9)	23.9 (22.4-24.4)	49 (45.6-56.5)	68
% memory $CD4^+$	42.7 (33.9-46.2)	74.7 (71.6-78.8)	74.6 (74.0-76.6)	42.6 (35.0-45.9)	32
% naive $CD8^+$	17.3 (15.4-17.4)	8.1 (6.9-10.2)	14 (13.0-14.6)	25.6 (22.8-29.2)	59
% memory $CD8^+$	29.6 (28.4-35.5)	67 (65.1-69.1)	58.5 (57.2-63.0)	24.9 (20.2-26.7)	18
% $Ki67^+$ in $CD4^+$	4.7 (3.6-6.4)	7.2 (6.3-8.4)	6.4 (4.7-8.3)	2.8 (2.0-3.6)	1.9
% $Ki67^+$ in naive $CD4^+$	1.3 (0.8-1.6)	3.8 (3.1-4.3)	4.1 (2.9-4.8)	1.4 (0.7-2.0)	0.8
% $Ki67^+$ in memory $CD4^+$	7 (6.2-10.2)	7.7 (6.9-9.7)	6.4 (4.7-7.9)	4.6 (3.0-6.5)	3.4
% $Ki67^+$ in $CD8^+$	2.4 (1.4-3.6)	2.6 (2.5-3.4)	8.3 (6.3-10.2)	4.1 (3.4-6.4)	1.5
% $Ki67^+$ in naive $CD8^+$	1.9 (0.9-2.3)	2.2 (2.1-3.0)	4.7 (4.0-5.6)	4.4 (2.2-6.9)	0.7
% $Ki67^+$ in memory $CD8^+$	3.8 (2.1-5.9)	2.8 (2.5-3.7)	9.4 (6.8-11.1)	10.8 (8.2-13.9)	2.1

[a] Median values from healthy individuals described in Vrisekoop et al. 2008 (reference 16).

[b] Depicted are median values and interquartile ranges during the entire follow-up.

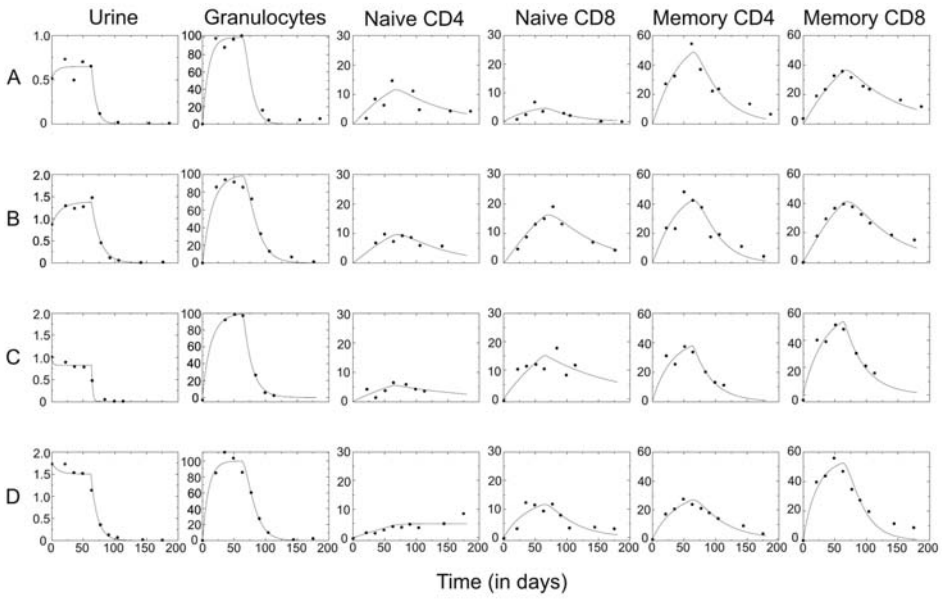

Figure 1. Fits of the ^2H_2O fraction in urine and the enrichment of T cells and granulocytes. Best fits of the fraction of ^2H_2O in urine and of the enrichment curves of granulocytes and naive and memory $CD4^+$ and $CD8^+$ T cells of 4 HIV-infected individuals. Label enrichment in the DNA of the different cell populations was scaled between 0 and 100% by normalizing for the estimated maximum percentage label obtained in granulocytes (see Methods).

population (*d*) (16). In the model that we used, *p* is not necessarily equal to *d* even when T-cell numbers are in steady state, because the loss of label after label cessation is only based on those cells that have recently divided, while the accrual of label during label administration is truly representative of the T-cell subpopulation as a whole (17). In other words, the model allows for different turnover rates of T cells that have recently divided and T cells that have not.

Production rates of $CD4^+$ and $CD8^+$ T cells in HIV infection

After 9 weeks of ^2H_2O administration, the granulocytes of our HIV-infected study subjects reached similar enrichment levels as previously-measured healthy volunteers (16). These data were used to estimate the maximum level of label intake that cells could possibly attain. Using this estimated maximum enrichment level as a reference, HIV-infected patients reached labeling levels of about 5-20% for naive $CD4^+$ and $CD8^+$ T cells, and 30-50% for memory $CD4^+$ and $CD8^+$ T cells, respectively, in line with previous data from HIV-infected subjects (8). When our labeling data were fitted to the mathematical model (Figure 1), the average turnover rates (*p*) of both naive and memory $CD4^+$ and $CD8^+$ T cells appeared to be increased in HIV-infected individuals compared to healthy volunteers (Figure 2). The median

Table 2. Average turnover rates (p) and loss rates of labeled lymphocytes (d) represented as a fraction of the cell population size per day

		A	B	C	D	HIV[a]	Healthy[b]
Naive CD4$^+$	***p***	0.003 (0.0017-0.0038)[c]	0.002 (0.0018-0.0032)	0.001 (0.0006-0.0017)	0.001 (0.0005-0.0008)	**0.002**	**0.001**
	d	0.012 (0.005-0.023)	0.014 (0.008-0.023)	0.007 (0.000-0.020)	0.000 (0.000-0.000)	**0.010**	**0.000**
Naive CD8$^+$	***p***	0.002 (0.0008-0.0030)	0.004 (0.0033-0.0047)	0.003 (0.0019-0.0046)	0.004 (0.0024-0.0051)	**0.003**	**0.000**
	d	0.025 (0.009-0.054)	0.014 (0.010-0.018)	0.008 (0.000-0.018)	0.024 (0.013-0.038)	**0.019**	**0.000**
Memory CD4$^+$	***p***	0.015 (0.0123-0.0197)	0.018 (0.0126-0.0244)	0.014 (0.0110-0.0186)	0.008 (0.0069-0.0098)	**0.015**	**0.005**
	d	0.025 (0.018-0.032)	0.035 (0.023-0.052)	0.033 (0.022-0.044)	0.024 (0.018-0.029)	**0.029**	**0.015**
Memory CD8$^+$	***p***	0.008 (0.0076-0.0097)	0.011 (0.0097-0.0122)	0.019 (0.0161-0.0230)	0.023 (0.0183-0.0299)	**0.015**	**0.003**
	d	0.013 (0.010-0.015)	0.016 (0.013-0.019)	0.030 (0.023-0.037)	0.041 (0.030-0.053)	**0.023**	**0.010**

[a] Median values of the four HIV-infected individuals

[b] Median values of healthy volunteers as described in Vrisekoop et al. 2008 (reference 16)

[c] 95%-confidence intervals (given in parentheses) were determined by a bootstrap method (reference 31)

production rates of naive CD4$^+$ and CD8$^+$ T cells were 0.18% and 0.33% of the naive CD4$^+$ or CD8$^+$ T-cell pool per day, corresponding to median half-lives of 385 and 210 days, respectively, which is 4-11 times shorter than in healthy volunteers. The median production rate of both memory CD4$^+$ and CD8$^+$ T cells was 1.5% of the memory CD4$^+$ or CD8$^+$ T-cell pool per day, corresponding to a median half-life of 46 days, i.e. 3-5 times shorter than in uninfected individuals (Table 2). These observed differences in T-cell turnover in healthy and HIV-infected individuals cannot be explained by age differences, because T-cell turnover rates have been shown to be hardly influenced by age (18).

The fate of recently-produced CD4$^+$ and CD8$^+$ T cells in HIV infection

When the fate of labeled naive and memory CD4$^+$ and CD8$^+$ T cells was followed after label cessation, the disappearance rate of recently-produced (i.e. labeled) memory CD4$^+$ and CD8$^+$ T cells turned out to be about 2-fold increased in HIV-infected individuals compared to healthy controls, reaching d = 2.9% and d = 2.3% of the labeled cell population per day for memory CD4$^+$ and CD8$^+$ T cells, respectively (Table 2). In stark contrast to the dynamics of naive CD4$^+$ and CD8$^+$ T cells in healthy volunteers, for which we have previously shown that recently-produced cells are preferentially

incorporated into the long-lived naive T-cell pool (16), recently-produced naive $CD4^+$ and $CD8^+$ T cells in HIV-infected individuals tended to be lost at rates of d = 1% and 2% of the labeled cell population per day, respectively (Table 2). It is unlikely that redistribution accounts for loss of labeled cells since label enrichment of $CD4^+$ and $CD8^+$ T cells has been found to be similar in lymph nodes and blood (4,19).

These data provide the first formal and *in vivo* evidence that newly-produced naive and memory T cells in HIV-infection are short-lived, and do not tend to be maintained in the naive and memory peripheral T-cell pools. Newly-produced naive T cells were even found to be preferentially lost from the naive T-cell pool, as their loss rate (d) exceeded the loss rate of the average naive T cell (p). It has previously been described that under lymphopenic conditions, naive T cells may proliferate and acquire a memory phenotype, a process known as lymphopenia-induced proliferation (LIP) (20). The preferential loss of newly-generated naive T cells in HIV-infected patients that we observed may in part be due to LIP, driving naive T cells into the memory T-cell pool. Even in the memory T-cell pool, however, newly-generated T cells were lost at higher rates than in healthy volunteers. Taken together, these data therefore strongly suggest that the increased turnover rate of $CD4^+$ T cells in HIV-infected individuals does not reflect a homeostatic response to compensate for the chronic loss of $CD4^+$ T cells.

Immune activation as the driver of increased T-cell turnover in HIV

Our data are fully in line with the hypothesis that the increased levels of T-cell production in HIV-infected individuals are to a large extent due to chronic immune activation, leading to increased priming of peripheral naive T cells into the memory compartment. In the memory compartment, T cells have an intrinsically shorter half-life that is further decreased by HIV-infection (Table 2). Together, this may explain the progressive loss of $CD4^+$ T cells during HIV-infection (12).

Previous indirect evidence for the critical role of immune activation in the progressive $CD4^+$ T-cell loss during HIV-infection came from the findings that i) the level of T-cell activation is the strongest prognostic factor for disease progression, even independent of plasma viral load (21-23), and that ii) both SIV-infected sooty mangabeys, which do not develop AIDS despite high HIV loads (24), and rare, long-term asymptomatic HIV-infected patients with high plasma loads of pathogenic HIV (25) were shown to have low levels of immune activation. It has recently been suggested that the acute, drastic loss of memory $CD4^+$ T cells from the gastro-intestinal tract causes the translocation of microbial products from the gut into the blood in HIV-infected individuals, and could thereby be the driver of systemic immune activation during HIV-infection (26).

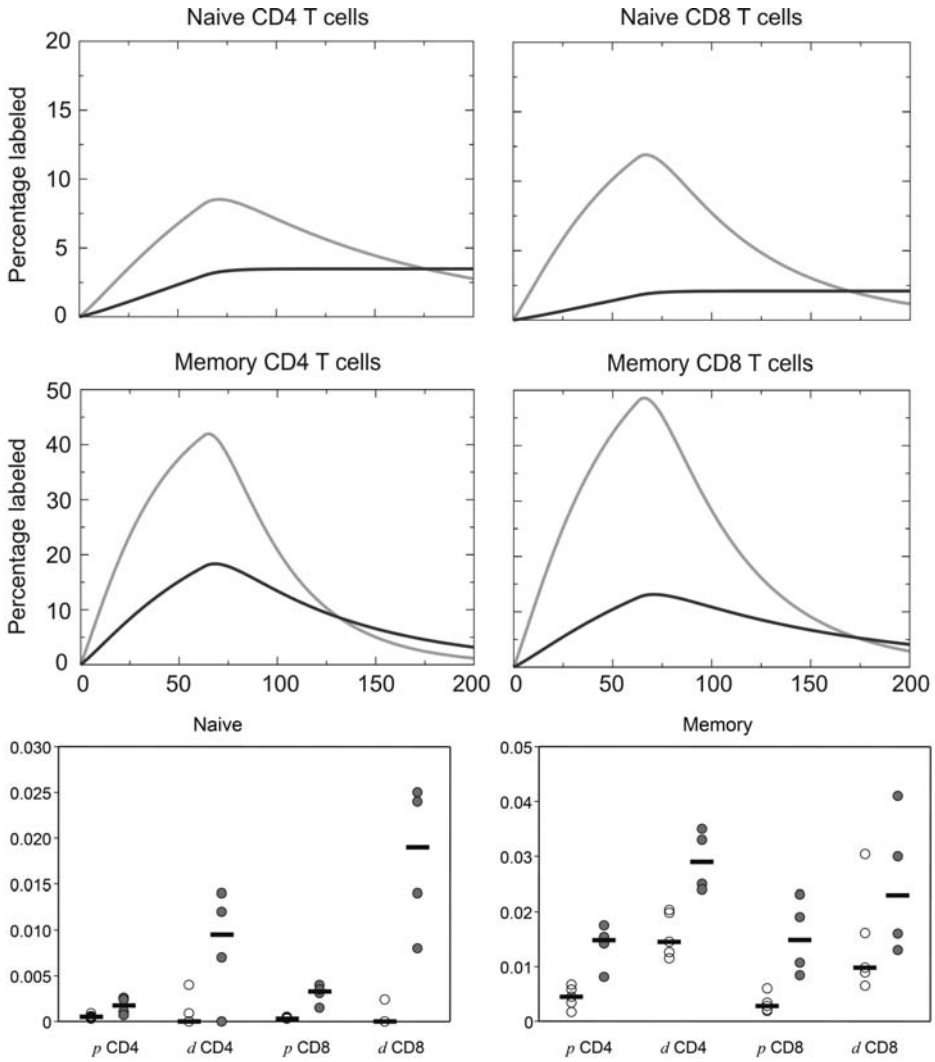

Figure 2. T-cell turnover and loss of recently-produced T cells in healthy and HIV-infected individuals. A) Comparisons of the enrichment curves of HIV-infected and healthy individuals, based on the median T-cell turnover rate (*p*) and median loss rate of labeled cells (*d*) of naive and memory $CD4^+$ and $CD8^+$ T cells of HIV-infected (upper curves) and healthy (lower curves) individuals. B) The estimated average T-cell turnover rates (*p*) and loss rates of labeled lymphocytes (*d*) per day in HIV-infected individuals and healthy volunteers.

Why are only $CD4^+$ T cells lost in HIV infection?

If cell loss is largely due to immune activation it remains puzzling why only $CD4^+$ T cells, and not also $CD8^+$ T cells, NK cells and B cells (27), are progressively lost during HIV-infection. Based on short-term deuterated glucose labeling data, it has previously been suggested that this is due to preferential destruction (3) or pref-

erential activation (28) of $CD4^+$ T cells in HIV-infected individuals. Our data show very similar production and death rates for $CD4^+$ and $CD8^+$ T cells. In line with this, both naive $CD4^+$ T cells and naive $CD8^+$ T cells have been shown to be gradually lost during HIV-infection (7,22). However, as long as mathematical models assume steady state T-cell numbers, none of the previous models (3,28) neither the current model could by definition mimic a situation in which $CD4^+$ and not $CD8^+$ T cells are gradually lost during HIV-infection. The gradual loss of about 800 $CD4^+$ T cells/μl blood in 10 years, i.e. a loss of 38 $CD4^+$ T cells/μl blood during the protocol of 25 weeks, may be too small to be picked up in the daily turnover rates.

Summarizing, the long-term administration of ^2H_2O and follow-up of recently-produced T cells after label cessation enabled us to reliably measure T-cell turnover even in the naive T-cell pool of HIV-infected individuals. Our data suggest that the increased levels of naive and memory $CD4^+$ T-cell production during HIV infection do not reflect a homeostatic response to compensate for the loss of $CD4^+$ T cells. Instead, the increased levels of T-cell production are counteracted by increased loss rates of newly-produced T cells. This finding is fully in line with the paradigm that increased T-cell production in HIV-infected individuals is caused by chronic immune activation, reducing the lifespan of naive and memory T cells. Increased T-cell production therefore seems to be a cause rather than a consequence of the progressive loss of $CD4^+$ T cells during chronic HIV infection.

Materials and Methods

Subjects and in vivo ^2H_2O labeling protocol

Four HIV-infected male volunteers were admitted to the AMC hospital, Amsterdam, the Netherlands to receive the initial 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 drank 1/8 of their initial dose daily for nine weeks. Blood and urine were collected before labeling, at the end of the first labeling day, four times during the rest of the nine-week labeling phase and five times during the down-label phase of 16 weeks. All patients were untreated at inclusion and during the whole protocol (CDC class A). Patient B experienced bronchitis (diagnosed and treated by the general practitioner) which started a few days prior to the second visit at day 22. Patient C withdrew from the protocol from day 113 onward, because he was advised to start treatment, and developed a disseminated Varicella shortly after withdrawal. Patient D developed gastro-enteritis (light fever and diarrhea) a few days prior to the visit at day 63. Details about the HIV-infected volunteers are shown

in Table 1. This study was approved by the medical ethical committee of the AMC and written informed consent was obtained from all volunteers. The results of five healthy volunteers who followed a similar protocol have been described previously (16).

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. T-cell proliferation in $CD4^+$ and $CD8^+$ T-cell subsets was studied by flow-cytometric measurements of the Ki67 nuclear antigen, as described previously (11). To measure the fraction of labeled cells within the naive ($CD45RO^-CD27^+$) and memory ($CD45RO^+$) $CD4^+$ and $CD8^+$ T-cell population, these subsets were isolated by cell sorting on a FACSAria (BD) as previously described (16). Purity of the sorted cells was on average 99.2% for naive $CD4^+$, 98.7% for naive $CD8^+$ T cells, 98.1% for memory $CD4^+$ T cells and 97.1% for memory $CD8^+$ T cells.

Measurement of ^2H_2O enrichment in body water and DNA and mathematical modeling

Deuterium enrichment in urine was measured by a method adopted from Previs et al.(29). The isotopic enrichment of DNA was measured according to the method described by Neese et al. (30) with minor modifications (16). We first fitted the urine enrichment data of each individual to a simple label enrichment/decay curve as described previously (16) (see Figure 1) and incorporated these best fits when analyzing the enrichment in the different cell populations. Up- and down-labeling of the granulocyte population of each individual was analyzed mathematically to estimate the maximum level of label intake that cells could possibly attain. The label enrichment data of all cell subsets were subsequently scaled by the granulocyte asymptote of each individual (16). Half-lives were calculated from the average turnover rates as $\ln 2 / p$.

Supplemental Material

Supplementary Tables S1 and S2 give the estimated parameters for urine and granulocytes, respectively.

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Supplementary Table S1. 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 (see reference 16)

Individual	*f*	δ	β
A	0.006	0.133	0.005
B	0.014	0.071	0.009
C	0.008	0.544	0.010
D	0.015	0.097	0.018

Supplementary Table S2. Parameter estimates of the granulocyte enrichment curves (before scaling), where *p* represents the average production rate of granulocytes, *c* is the amplification factor, and *d* represents the loss rate of labeled granulocytes (see reference 16)

Individual	*pc*	*d*
A	0.639	0.125
B	0.408	0.098
C	0.308	0.073
D	0.395	0.089

8

In vivo dynamics of stable chronic lymphocytic leukemia inversely correlate with somatic hypermutation levels and suggest no major leukemic turnover in bone marrow

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Abstract

Although accumulating evidence indicates that chronic lymphocytic leukemia (CLL) is a disease with appreciable cell dynamics, it remains uncertain whether this also applies to patients with stable disease. In this study, $^{2}H_{2}O$ was administered to a clinically homogeneous cohort of nine stable, untreated CLL patients. CLL dynamics in blood and bone marrow were determined and compared to normal B cell dynamics in blood from five healthy individuals who underwent a similar $^{2}H_{2}O$ labeling protocol. Average CLL turnover rates (0.08%-0.35% of the clone per day) were approximately 2-fold lower than average B cell turnover rates from healthy individuals (0.34%-0.89%), while the rate at which labeled CLL cells in blood disappeared (0.00%-0.39% of B cells per day) was approximately 10-fold lower compared to labeled B cells from healthy individuals (1.57%-4.24% per day). Leukemic cell turnover parameters inversely correlated with the level of somatic hypermutation of the CLL clone (IgVH mutations). Although CLL cells in bone marrow had a higher level of label enrichment than CLL cells in blood, no difference between proliferation rates and pro- and anti-apoptotic profiles of CLL cells from these compartments was observed. These data suggest that in stable disease there is a biological relationship between the degree of somatic hypermutation of the CLL clone and its dynamics *in vivo*. Furthermore, in contrast to lymph nodes, the bone marrow does not seem to be a major CLL proliferation site.

Introduction

For several decades chronic lymphocytic leukemia (CLL) has been regarded as a homogeneous accumulative disease in which intrinsic apoptotic defects of clonal $CD5^+CD19^+$ B cells account for the etiology and progression of the disease. However, in recent years evidence has been gathered which challenged this paradigm. Although the balance between pro- and anti-apoptotic regulators in CLL is shifted towards an anti-apoptotic profile as compared to normal B cells (1), an intrinsic defect in apoptosis has not been found. Also, it has become clear that the disease is rather heterogeneous, both in its clinical behavior and its biology.

Based upon IgVH gene mutations (2-4), specific chromosomal abnormalities (5,6), and expression of the surface marker CD38 (4,7,8) and the intracellular protein ZAP-70 (9-13), prognostic subgroups can be defined in which cases with unmutated IgVH genes, deletion of the short arm of chromosome 17 or the long arm of chromosome 11, and expression of CD38 and/or ZAP-70 have an inferior clinical outcome (4-8).

CLL cells receive signals from the microenvironment which provide them with the means to enter the cell cycle and to circumvent apoptosis, resulting in accumulation of the total tumor clone. These survival signals are hypothesized to be induced by cell-cell interactions with SDF-producing nurselike cells (14,15), bone marrow stromal cells (16,17) CD40L-expressing $CD4^+$ T cells (18,19) and stimuli from chemokines or growth factors such as VEGF (20), SDF-1 (15), BAFF (21) and APRIL (21). The interaction with these micro-environmental stimuli is thought to occur predominantly in specific tissues like lymph nodes, spleen and bone marrow (22). Indeed, in a comparative study between CLL cells derived from lymph nodes and peripheral blood, we recently confirmed not only increased expression of the cell cycle markers Ki67 and survivin, but also a strong overexpression of anti-apoptotic regulators in lymph node-derived CLL cells (1,17). These observations imply that CLL may not simply result from an accumulation of leukemic cells, but may be a dynamic disease where cells proliferate and die with various rates in different compartments.

'Heavy water' labeling experiments can be used to study cell dynamics *in vivo*. Administration of deuterated water (^2H_2O) is a safe and reliable method for labeling of dividing cells *in vivo* for long time spans, and has resolved the issues of label toxicity which have hampered previous labeling studies (23,24). During intake of ^2H_2O, deuterium (^2H) is incorporated into the deoxyribose moiety of newly synthesized DNA, and thereby labels dividing cells. The presence of deuterium can be detected by gas chromatography and mass spectrometry (GC/MS) (25). Up- and downlabeling profiles of cells can be analyzed mathematically, providing cell-specific turnover characteristics for each individual.

Recently, Messmer et al applied ^2H_2O labeling to a clinically heterogeneous CLL patient cohort. They showed that there is substantial leukemic cell turnover (production) in CLL, with proliferation rates ranging from 0.1% to 1.0% of the entire clone per day (26), which strongly supports the concept of CLL as a dynamic disease. Nevertheless, a number of issues remain unresolved. Firstly, it remains to be elucidated whether the observed dynamic state also applies to patients with untreated clinically stable disease and whether the dynamic state is related to the level of IgVH gene mutations as has been suggested by *in vitro* telomere length analyses (27). Secondly, it is unclear whether recently divided leukemic cells tend to contribute to the maintenance of the total CLL clone or tend to be lost rapidly. Thirdly, most leukemic cells do not reside in the blood, but mainly in lymph nodes and bone marrow. These sites may provide CLL cells with a number of micro-environmental stimuli. However, it still has to be determined whether leukemic cells preferentially proliferate at these sites *in vivo*.

The aim of the present study was to determine *in vivo* leukemic cell dynamics in stable CLL patients by measuring not only CLL turnover in both blood and bone marrow, but also by measuring disappearance rates of recently divided cells during an extended downlabeling period of approximately one year. In this cohort of clinically stable CLL patients, an inverse correlation between the level of mutations in the IgVH gene and both the average CLL turnover rate and the disappearance rate of recently proliferated CLL cells was found. Furthermore, based on labeling data of leukemic cells in blood and bone marrow in concert with the pro- and anti-apoptotic profile at the RNA- and protein level, we show that the bone marrow does not seem to be a major site of CLL proliferation.

Materials and methods

Patients and in vivo ^2H_2O labeling protocol

One female and nine male participants, who were diagnosed with CLL by established criteria (28), and who had stable disease (stable leukemic cell counts) for at least one year without any need for treatment were enrolled in the study after having provided written informed consent (Table 1). All patients were admitted for one day at the Academic Medical Center, Amsterdam (AMC) where they received an initial bolus of 10 ml ^2H_2O (99.8% enriched) per kg body water in small portions. Blood was withdrawn prior to the first portion and urine was collected after the last portion. Body water was estimated to be 60% of body weight for males, and 50% of body weight for females. As a maintenance dose, the subjects drank 12.5% of the initial dose at home daily during a nine-week labeling period. Blood and urine were collected five

Table 1. Characteristics of CLL patients

patient	age at day 0 years	gender	RAI stage	time since diagnosis years	IgVH mutation %	no. of lymphocytes 10^9 per ml		CD38* %	cytogenetic abnormalities†
						start of study	end of study		
CLL01	75.3	M	2	5	2.8	55.7	61.1	71.4	11q- (21%)
CLL02	77.1	M	0	6	11.2	42.2	42.9	0.9	none
CLL03	58.6	M	2	6	4.0	57.8	47.0	85.0	trisomy 12 (58%)
CLL05	72.5	M	0	2	3.0	44.4	39.0	0.3	13q- (75%)
CLL06	71.1	M	0	2	6.6	5.8	6.3	0.7	none
CLL07	71.0	F	0	3	1.3	14.0	18.9	0.7	13q- (48%)
CLL08	50.9	M	0	1	0.4	57.6	64.0	1.0	trisomy 12 (52%)
CLL09	66.1	M	4	15	3.0	121.3	113.0	0.1	13q- (56%)
CLL10	41.7	M	0	1	8.9	37.1	46.2	0.4	13q- (17%)

M indicates male; F, female

*Expressed as the median CD38 expression level on CD5+CD19+ cells during study follow-up

†As was detected by FISH analysis

or six times at regular intervals during the labeling period and eight times during the downlabeling period of about one year. From CLL04 no data were obtained as this participant dropped out of the study at day 14 because of personal reasons. Six participants, (CLL01, CLL02, CLL07, CLL08, CLL09 and CLL10), underwent a bone marrow puncture at the end of the labeling period. To minimize contamination of the harvested bone marrow with peripheral blood, only the first syringe of aspiration was used. Microscopic analysis of the aspirates confirmed high quality bone marrow-derived cells as indicated by the presence of stromal cells, nucleated precursor red cells and megakaryocytes in all cases. In addition, three patients (CLL01, CLL03 and CLL08) underwent a bone marrow biopsy.

Next to the CLL participants, five healthy participants (for individual details, see Vrisekoop et al (29)) were enrolled in this study. They underwent a similar ^2H_2O labeling protocol as described above, with the exception that the downlabeling period consisted of 150 days in which blood and urine were collected seven times. This study was conducted in accordance with the ethical standards of our institutional medical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, revised in 1983.

Flow cytometry and cell sorting

Peripheral blood (PBMC) or bone marrow mononuclear cells were obtained by Ficoll-Paque density gradient centrifugation from heparinized blood and were stained with a combination of monoclonal antibodies (mAb) (CD5-PE, CD19-PerCP and CD38-APC (BD)). Proliferation of $CD5^+CD19^+$ CLL cells in blood and bone marrow was studied by measuring expression of Ki67 nuclear antigen. Briefly, cells were fixed (FACS Lysing Solution, BD), permeabilized (FACS Permeabilizing Solution 2,

BD) and stained intracellularly with Ki67-FITC (Monosan, The Netherlands). Cells were analyzed on a FACSCalibur (BD) with CellQuest software.

Bone marrow mononuclear cells from CLL patients were stained with CD5-PE and CD19-APC mAb (BD). After washing, $CD5^+CD19^+$ cells were isolated by cell sorting on a FACSAria (BD) to determine label incorporation of bone marrow leukemic cells. Purity of sorted cells was at least 97%.

MACS cell separation

Granulocytes were obtained following lysis of erythrocytes from the cell pellet after Ficoll-Paque density gradient centrifugation. Since the granulocyte fractions of CLL patients were often contaminated with leukemic cells, these cells were purified by cell separation over MACS Separation Columns (Miltenyi Biotec GmbH, Germany) using CD16-conjugated magnetic beads, according to the manufacturer's instructions. $CD19^+$ cells from healthy participants and from CLL patients were purified from PBMC in a similar fashion. Purity of all MACS-sorted cell populations was >90%. $CD19^+$ cells from CLL patients mainly consisted of CLL cells as the percentage of $CD19^+$ cells expressing CD5 was >93.6%. From CLL03 no pure $CD16^+$ fractions could be obtained. Genomic DNA was isolated from each cell population using the Blood Quick Pure kit (Bioké Nucleospin, Macherey Nagel GmbH & Co., Germany), according to the manufacturer's instructions.

Measurement of ^2H_2O enrichment in body water and DNA

Deuterium enrichment in urine was measured by a method adopted from Previs et al (30). The isotopic enrichment of DNA isolated from granulocytes and $CD19^+$ cells was measured according to the method described by Neese et al (25) with minor modifications (29).

Mathematical modeling

The label enrichment in urine, granulocytes and $CD19^+$ cells was fitted to a steady-state kinetic heterogeneity model, which was described previously (29,31). This model takes into account the fact that the kinetics of cells that have recently divided (and hence picked up label) may be intrinsically different from those that have not. E.g., the labeled fraction may be biased towards cells with relatively rapid turnover. The model thereby allows the average rate of cell production (determined during uplabeling) to differ from the rate of disappearance of labeled cells after label cessation (due to death, maturation or migration), even under steady state conditions.

Labeling data were fitted according to the method we described previously (29). In brief, we first fitted the urine enrichment data, as a measure of body water enrichment (Supplemental Figure 1), to determine the fraction f of ^2H_2O in the drinking water,

the baseline urine enrichment β attained after the boost of label by the end of day 0, and the turnover rate δ of body water, for each individual (Supplemental Table 1). These parameters were incorporated when fitting the enrichment in granulocytes

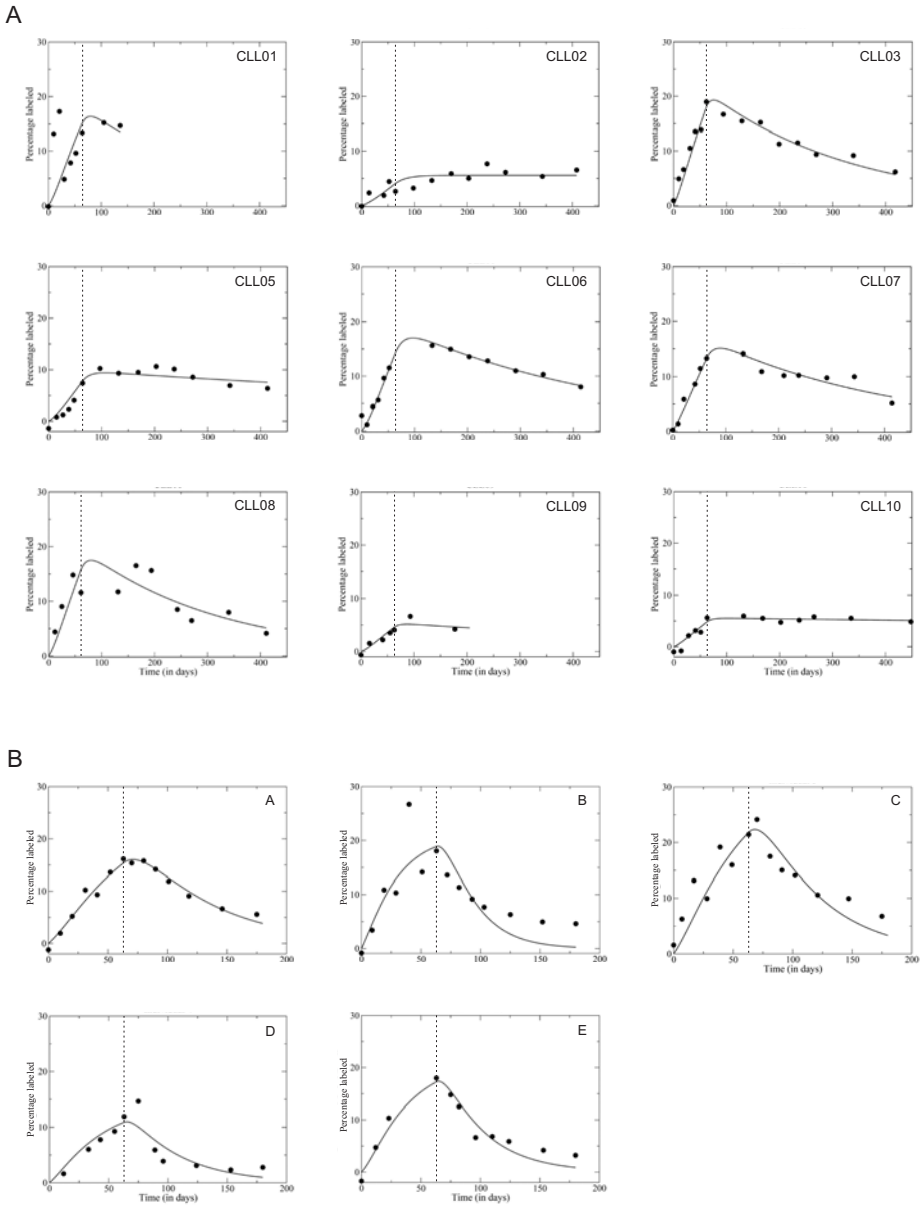

Figure 1. In vivo cell dynamics. ^{2}H-enrichment of deoxyadenosines in the DNA of peripheral blood A) CLL cells and B) B cells from healthy individuals. Label enrichment was scaled between 0 and 100% by normalizing for the maximum percentage of label obtained in granulocytes. The dotted line represents the time point of label cessation. Labeling data were fitted as described in Materials and methods.

Table 2. Average turnover rates and disappearance rates of labeled cells in blood

	CLL kinetics according to the model	
CLL patient	**turnover rate (p) % per day**	**disappearance rate (d) % per day**
CLL01	0.30	unr.
CLL02	0.08	0.00
CLL03*	0.35	0.37
CLL05	0.15	0.07
CLL06	0.29	0.24
CLL07	0.25	0.28
CLL08	0.33	0.39
CLL09	0.08	unr.
CLL10	0.08	0.02
median	0.25	0.24

	normal B-cell kinetics according to the model	
healthy participant	**turnover rate (p) % per day**	**disappearance rate (d) % per day**
A	0.43	1.57
B	0.89	4.24
C	0.65	1.97
D	0.34	2.28
E	0.61	2.81
median	0.61	2.28

unr. Indicates unreliable, because of too few datapoints

*the average turnover rate (p) was calculated by normalizing by the median of the granulocyte enrichment maxima of the other CLL participants

(Supplemental Figure 1), CLL cells (Figure 1A) and B cells from healthy individuals (Figure 1B). Assuming that granulocytes undergo complete turnover, the enrichment of granulocytes of each individual was analyzed mathematically to determine the maximal level of label enrichment *cf* that cells could possibly attain (Supplemental Table 2). This maximum is not only determined by the fraction *f* of $^{2}H_{2}O$ in the drinking water, but also by an amplification factor *c*, because the derivative that was analyzed contains multiple hydrogen atoms that can be replaced by deuterium. At each time point the percentage of labeled DNA of CLL cells in blood and bone marrow and of labeled DNA of normal B cells in blood was normalized by dividing by this maximum *cf* for each individual (29).

RNA isolation and reverse transcription–multiplex ligation-dependent probe amplification assay

Total RNA was isolated using the RNeasy mini kit (Qiagen). A reverse transcription-multiplex ligation-dependent probe amplification assay (RT-MLPA) procedure was performed as described previously (32,33) using 100 ng total RNA as input. Results were analyzed using the programs Genescan analysis and Genotyper (Applied

Biosystems), and further analyzed with Excel spreadsheet software (Microsoft, Redman, WA). Data were normalized by setting the sum of all signals at 100% and expressing individual peaks relative to the 100% value.

In vitro CD40 stimulation and Western blotting

In vitro CD40 stimulation of CLL cells followed by western blotting of lysates was performed as described (1,33). Blots were probed with the following antisera: polyclonal Mcl-1 (catalog no. 554103; Pharmingen, BD Biosciences), monoclonal anti-Noxa (clone 114C307.1; Imgenex, San Diego, CA), polyclonal anti-Bcl-XL (catalog no. 610211; Pharmingen, BD Biosciences), polyclonal anti-Bcl2 (catalog no. ALX-210-701-C100, Alexis) and antiserum to ß-actin (clone I-19; Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical analyses

Correlations were performed using the non-parametric Spearman's Rank Correlation Coefficient. Differences in Ki67 expression and ^2H-enrichment data between blood and bone marrow CLL cells were analyzed by the non-parametric Wilcoxon Signed Rank Test for paired data. Differences in mRNA expression of pro- and anti-apoptotic molecules were determined using the Student's *t* test.

Results

Patient follow-up

In order to assess leukemic cell dynamics in stable disease, ten untreated CLL patients with stable disease activity were enrolled in the ^2H_2O labeling study. One patient (CLL04) dropped out at day 14. Data of the remaining nine patients were analyzed. All patients had stable lymphocyte numbers during follow-up (Table 1). Participants did not receive any disease-related treatment prior to and during the study, except CLL09 who had received two cycles of chlorambucil treatment 10 years prior to the start of the study. This participant died after 205 days of follow-up, due to uncontrolled sepsis following pneumonia. CLL01 was monitored until day 136 because of cardiac surgery.

From each participant, the ^2H_2O-enrichment in urine (Supplemental Figure 1) and ^2H-enrichment in the DNA of granulocytes (Supplemental Figure 1) were fitted to the mathematical model (Materials and methods), as well as ^2H-enrichment in the DNA of CLL cells (Figure 1A) or B cells from healthy individuals (Figure 1B). The strength of this model is that it allows for discrimination between the average turnover rate (*p*) of the total leukemic or normal B cell compartment in blood, and the rate (*d*) at

which recently divided cells (which have taken up label) disappear from the population, by death, maturation or migration to another body compartment (31).

Since granulocytes presumably undergo complete turnover, label enrichment of these cells was assessed in order to estimate the maximum level of label intake that cells could possibly attain (Supplemental Figure 1) (25,34).

Low leukemic cell turnover in blood of stable CLL patients

Labeling data was fitted to the mathematical model (Materials and methods) to determine kinetic parameters of leukemic cells (Figure 1A) or B cells from healthy individuals (Figure 1B) in blood. The average turnover rate of the total leukemic population (*p*) and the disappearance rate (*d*) of labeled leukemic cells in the blood for each participant are given in Table 2. For patients CLL01 and CLL09, we only report *p,* and not *d*, because only a limited number of downlabeling data could be collected from these patients. As purification of granulocytes from CLL03 proved unsuccessful, the kinetic parameters of the leukemic clone of this patient were calculated using the median of all granulocyte enrichment maxima from the other CLL participants.

Average CLL turnover rates varied between 0.08%-0.35% of the CLL clone per day. In a subset of patients (CLL03, CLL06, CLL07 and CLL08), these turnover rates were balanced by similar disappearance rates of labeled leukemic cells (0.24%-0.39% per day), whereas in others (CLL02, CLL05 and CLL10) hardly any or no labeled leukemic cells disappeared (0.00%-0.07% per day) during the downlabeling period. The latter group of patients also had the lowest average CLL turnover rates (0.08%-0.15% per day). Turnover rates of CLL cells were approximately 2-fold lower compared to turnover rates of B cells from healthy individuals (0.34%-0.89% of the B cell population per day). The largest difference between the dynamics of CLL cells and B cells from healthy individuals was the disappearance rate of recently proliferated cells: labeled B cells from healthy individuals were lost at almost 10-fold higher rates (1.57%-4.24% of the B-cell population per day) compared to CLL cells in patients (0.00%-0.39% of the CLL clone per day).

The IgVH mutation level correlates negatively with leukemic cell turnover

As expected in a cohort of patients selected for stable disease activity, the majority of patients (seven out of nine) had leukemic cells with mutated IgVH genes (≥2% mutations compared to germline sequence) with a range of 0.4% to 11.2% (Table 1). Since prior studies found a robust correlation between the level of mutations and telomere lengths (used as surrogate parameter for cell turnover) (27), we assessed whether the level of IgVH mutations correlated with the dynamics of the CLL clone. We found a significant inverse correlation between the level of IgVH mutations and

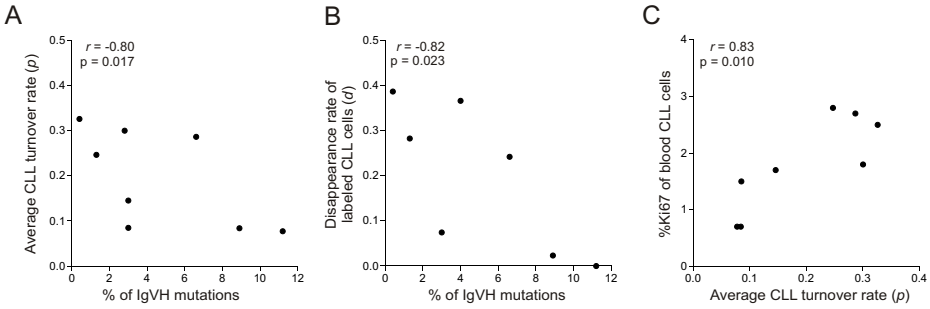

Figure 2. Relation of the level of IgVH mutations with the calculated parameters. A) Inverse correlation between the average CLL turnover rate in blood and the percentage of IgVH mutations. B) The disappearance rate of labeled cells from the blood is inversely correlated with the percentage of IgVH mutations. C) Average Ki67 expression of blood CLL cells is positively correlated with the average CLL turnover rate in blood. Statistical significance ($p<0.05$) was determined by the non-parametric Spearman's Rank Correlation Coefficient. The turnover rate of CLL03 was not included in the analyses of A) and C) as this value was calculated using the median of the maximum granulocyte enrichment of the other patients.

1) the level of average leukemic cell turnover *p* (Figure 2A; p=0.017; *r*=-0.80), and 2) the disappearance rate of labeled cells *d* (Figure 2B; p=0.023; *r*=-0.82). In addition, we found a strong correlation between the average leukemic cell turnover rate *p* and expression of the proliferation marker Ki67 (which is expressed in late G_1 phase of the cell cycle) in leukemic cells residing in blood (Figure 2C; p=0.010; *r*=0.83). Collectively, these data suggest that in this stable CLL patient cohort, there is a biological link between the level of somatic hypermutation and leukemic cell dynamics.

No increased proliferation of leukemic cells in the bone marrow compared to blood

We have previously shown (17) that lymph node CLL cells are characterized by a strong expression of the proliferation markers Ki67 and *survivin* (a molecule that is only expressed during the G_2/M phase in proliferating cells (35)) compared to blood CLL cells. These findings suggest that the level of ^2H-incorporation in the DNA of CLL cells in the blood mainly reflects proliferation at other sites in the body. To assess the contribution of leukemic cell turnover in the bone marrow, six patients underwent a bone marrow puncture at the end of the labeling period. The level of ^2H-incorporation in the DNA of bone marrow CLL cells was compared to that in blood CLL cells (Figure 3A). Bone marrow CLL cells were found to have significantly higher levels of ^2H-incorporation than CLL cells in blood (p=0.046). When we performed a paired analysis between the average level of Ki67 expression of CLL cells in blood and bone marrow, however, no statistical difference was observed (p=0.574, Figure 3B). Proliferation levels were also assessed by mRNA expression levels of *survivin* (measured by MLPA). *Survivin* was hardly expressed both in blood and bone marrow CLL cells (Figure 3C). *Survivin* levels measured by MLPA in lymph

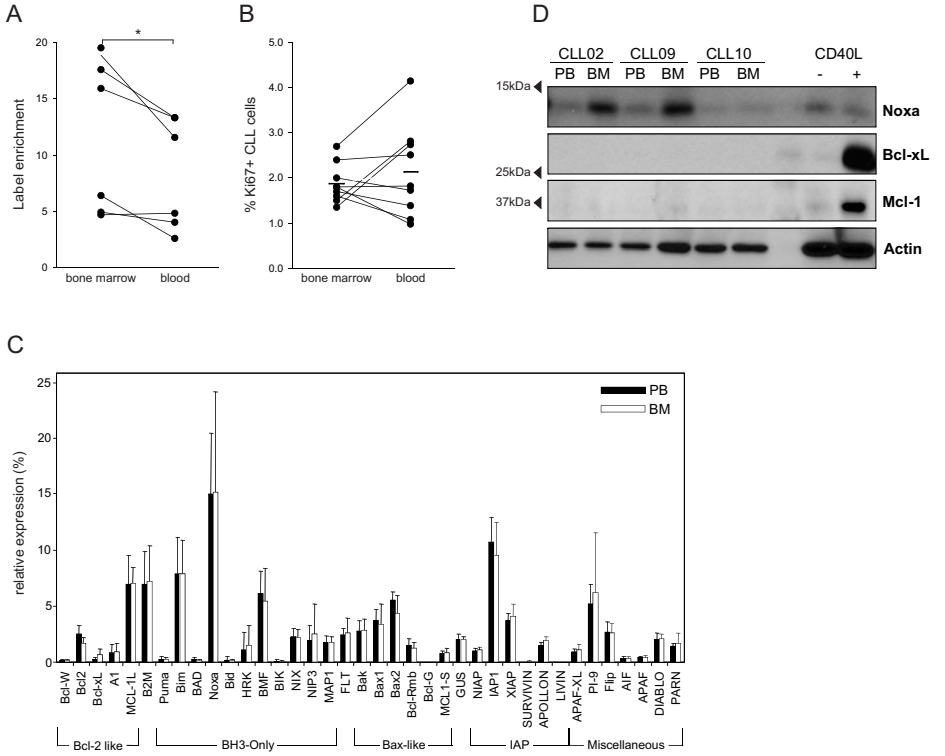

Figure 3. Comparison of proliferation and survival of blood and bone marrow CLL cells. A) ^{2}H-enrichment in DNA of bone marrow and blood CLL cells at the end of the labeling period. B) The average percentage of Ki67 expression of blood CLL cells compared to the percentage of Ki67 expression of bone marrow CLL cells. Statistical significance ($p<0.05$) was determined by the non-parametric Wilcoxon Signed Rank Test for paired data and is denoted by an asterix. C) Relative expression of 34 apoptosis regulators was investigated of six paired blood (black bars) and bone marrow (white bars) samples. Results of individual apoptosis regulatory genes are shown as the median expression of the samples relative to the total signal in the samples, with standard deviation. Non-apoptosis genes included as housekeeping genes are β2-microglobulin (B2M), Ferritin Light chain (FLT), β-glucoronidase (GUS), and poly(A)-specific ribonuclease (PARN). Statistical significance ($p<0.05$) was determined using the Student's *t* test. (D) Protein lysates of three paired blood and bone marrow samples were subjected to western blot analyses. Blots were stained with antibodies directed against Noxa, Mcl-1 or Bcl-XL, and reprobed with an antibody against β-actin as a loading control. As a control, CLL cells were stimulated for 3 days with irradiated untransfected or CD40L-transfected fibroblasts, mimicking the expression pattern of lymph node CLL cells.

node samples of CLL patients in the aforementioned study (17) were approximately 10-fold higher (relative expression in lymph nodes: 0.60±0.35 (n=9); bone marrow: 0.06±0.09, Figure 3C). Although only the first syringe of bone marrow aspirate was used for further analysis it cannot be excluded that the aspirates were slightly contaminated with peripheral blood CLL cells which could result in an underestimation of the actual number of proliferating cells in the bone marrow. Therefore, we also analyzed bone marrow biopsies from three patients (CLL01, CLL03 and CLL08). High

power field microscopy and immunohistochemical staining showed an average of 65% bone marrow infiltration with small $CD5^+CD20^+$ cells. Staining of these samples with Ki67 revealed only very sparse (≤1%) $Ki67^+$ cells within leukemic cell fields, without the presence of clear proliferation centers (data not shown). Collectively, these data suggest that the higher level of ^2H-incorporation in the DNA of CLL cells from the bone marrow is due to migration of CLL cells that have proliferated elsewhere into the bone marrow.

Similar apoptotic profile of leukemic cells in blood and bone marrow

The expression levels of apoptosis-regulating genes of lymph node CLL cells have also been shown to differ from those in the blood (higher expression of the anti-apoptotic proteins Bcl-XL and Mcl-1, and lower expression of the pro-apoptotic protein Noxa) (17). We examined whether the apoptotic profile of circulating CLL cells in the blood differed from CLL cells in the bone marrow. A paired comparison by MLPA was performed to determine mRNA levels of a large panel of apoptosis regulators in CLL cells in these two compartments. A shift in the balance between survival and apoptosis genes could not be observed (Figure 3C). A subset of these are post-transcriptionally regulated, and therefore protein levels of molecules with known altered expression in lymph node CLL cells (17) were measured in paired samples from patients CLL02, CLL09 and CLL10 by western blot analyses (Figure 3D). Since prolonged CD40 activation of CLL cells results in expression profiles of apoptosis-regulatory genes quite similar to lymph node CLL cells, CD40-stimulated peripheral blood CLL cells were used as a control, and these showed the expected induction of Bcl-XL and Mcl-1, and decreased expression of Noxa (Figure 3D). In sharp contrast to lymph node CLL cells and CD40-stimulated blood CLL cells no expression of Bcl-XL and Mcl-1 could be detected in either blood or bone marrow CLL cells. In two of the three patients increased expression of Noxa was found in bone marrow leukemic cells as compared to CLL cells from peripheral blood. Hence, besides similar expression of proliferation markers of CLL cells in blood and bone marrow, these data suggest that in bone marrow pro-survival micro-environmental stimuli, like CD40 activation (18,19), are not a dominant feature.

Discussion

The dogma that CLL is a static disease of a leukemic clone that is slowly accumulating because of impaired apoptosis is gradually replaced by the view that proliferation and death within the leukemic clone is taking place at substantial levels. In a prior study by Messmer and colleagues considerable production rates of leukemic clones

were observed in a heterogeneous group of CLL patients (26). We extend their findings and add new insights at three levels. Firstly, we assessed leukemic cell dynamics in a homogeneous patient group with stable disease activity and determined whether the level of IgVH mutations correlated with the observed dynamics. Secondly, with a substantial downlabeling period and the use of our mathematical model, we were able to evaluate the fate of labeled cells, which thus provided the means to identify whether cells that had recently proliferated contributed to the maintenance of the leukemic compartment or had a smaller survival chance than undivided leukemic cells. Finally, we studied not only CLL dynamics in blood, but also evaluated the role of the bone marrow as a potential site for leukemic cell production.

In the $^{2}H_{2}O$-labeling study performed by Messmer et al it was found that patients with average CLL turnover rates higher than 0.35% of the CLL clone per day, were more likely to exhibit or develop active disease (26). These findings fit very well with the fact that daily average turnover rates of CLL cells in our small cohort of patients with stable disease activity were at maximum 0.35% of the CLL clone per day. We showed that the *in vivo* CLL dynamics differed to a large extent from B cell dynamics in healthy individuals: average turnover rates and disappearance rates of recently labeled cells were respectively 2-fold and 10-fold lower in CLL patients compared to healthy individuals. Two factors complicate this comparison. Firstly, normal B cell dynamics were assessed in individuals who were younger than the CLL patients. A comparison of these two groups nevertheless seems legitimate, since in a 6,6-$^{2}H_{2}$-glucose labeling study hardly any differences in B cell dynamics were observed between individuals younger than 35 and older than 65 years of age (36). Secondly, it is not straight forward to which cell population the dynamics of $CD5^{+}CD19^{+}$ CLL cells should be compared. Based on microarray data it has been suggested that CLL cells bare most resemblance with memory B cells (37), suggesting that the most fair comparison would be to compare CLL dynamics with memory B cell dynamics in healthy individuals. 6,6-$^{2}H_{2}$-glucose labeling studies have pointed out that the turnover rate of $CD27^{+}$ memory B cells is higher than the average turnover rate of the overall B cell compartment (36). If we had compared CLL dynamics with healthy memory B cell dynamics, the low turnover rates and in particular the low disappearance rates of CLL cells would thus most likely only have become more apparent.

Examination of CLL dynamics between individuals revealed that differences exist between patients concerning average turnover rates and the loss of label after label cessation. In CLL03, CLL06, CLL07 and CLL08 labeled cells were lost with similar rates as cells acquired label during the labeling phase. In contrast, in CLL02, CLL05 and CLL10 average turnover rates were lower and hardly any labeled cells were lost during the downlabeling phase. Yet, the average number of circulating tumor cells remained stable in all patients. Although we measured only a small number of patients, these

data suggest that CLL dynamics as measured in blood can differ between patients. Furthermore, the CLL clone within an individual patient may consist either of a single population, or of multiple populations each having different dynamics. What causes these differences in CLL dynamics between and in individual patients is not known, but may involve differential expression of site-specific homingmarkers or variations in retention and release of CLL cells from tissues like bone marrow, spleen, lymph nodes, etc. Moreover, whether the labeling patterns we observed in our patient cohort are similar to those in CLL patients with aggressive disease is not known but could be of interest and should be studied in a larger cohort of patients.

CLL dynamics inversely correlated with the level of mutations in the IgVH gene. This association suggests that *in vivo* a biological link exists between the level of somatic hypermutation and proliferation and death of the CLL clone. It has been shown that the prognosis is poor in the subset of patients with less than 2% of mutations (unmutated CLL), but also within the group with more than 2% of mutations (mutated CLL) the prognosis differs with the best prognosis for the subset of patients with the highest degree of somatic hypermutation (38). In addition, it was shown that within a group of patients with mutated CLL, telomere length of the leukemic cells correlated with overall survival (39). The well established difference in prognosis based on the division between less or equal/more than 2% IgVH mutations led to the hypothesis that CLL comprises two different disease entities: one in which the oncogenic hit occurred early in a germinal center reaction and one in which the oncogenic hit took place after a germinal center reaction (40). *In vitro* studies have shown that unmutated CLL cells become activated by B cell receptor (BCR) triggering which possibly results in increased proliferation and more aggressive behavior of the CLL clone, while the majority of cases of mutated CLL are more or less anergic to BCR signaling (41). Damle et al found a positive correlation between telomere length and the percentage of IgVH mutations and an inverse correlation between the level of IgVH mutations and telomerase activity (27). In agreement, in preliminary observations we found an inverse correlation between the expression of Ki67 in freshly isolated blood CLL cells and the level of IgVH mutations ($p=0.01$) in 18 randomly selected CLL patients (unpublished data). Collectively, these findings suggest that the level of mutations, rather than the mutational status, is indicative for the proliferative capacity of the leukemic clone. A biological rationale for this concept was provided in a recent study, which showed that the normal B cell response is a highly dynamic process based on the (re-) entrance of memory B cell clones in multiple germinal centers, resulting in increasing percentages of mutations following each germinal center round (42). Our data that CLL dynamics inversely correlated with the percentage of mutations in the IgVH gene hint at a model in which susceptibility of CLL cells to BCR triggering does not depend on the mutation status *per se*, but rather

on the number of germinal center rounds the cells went through prior to the (final) oncogenic hit.

The turnover of CLL cells has been hypothesized to occur primarily in lymph nodes and in bone marrow (43), but the assumed influence of micro-environmental stimuli on the maintenance of the leukemic clone within these compartments is largely based on *in vitro* studies (16,18,19,44-46). Recently, examination of lymph node CLL cells provided additional support to this hypothesis as these cells were shown to have an anti-apoptotic expression profile with upregulation of Mcl-1, Bcl-XL, and survivin and concomitant downregulation of Noxa when compared to CLL cells in blood (17). Insights in proliferation centers in bone marrow are limited as these are almost exclusively seen in cases with extensive marrow involvement (47). We showed that in our patient cohort with stable disease the large majority of bone marrow CLL cells did not proliferate and, in general, did not possess an altered apoptotic regulator profile as compared to peripheral blood CLL cells. The higher level of labeled CLL cells in bone marrow compared to blood may be explained by the possible migration of leukemic cells which have divided at other sites in the body into the bone marrow. Taken together, our data suggest that, at least in stable disease and in contrast to the findings for lymph nodes, the bone marrow is not a major CLL proliferation site.

In conclusion, we have shown that also in stable CLL, the leukemic population is maintained by a balance of on-going proliferation and cell death. A better understanding of cell kinetics of the leukemic clone will hopefully result in a further tailoring of therapeutic approaches for the different subtypes of this still incurable disease.

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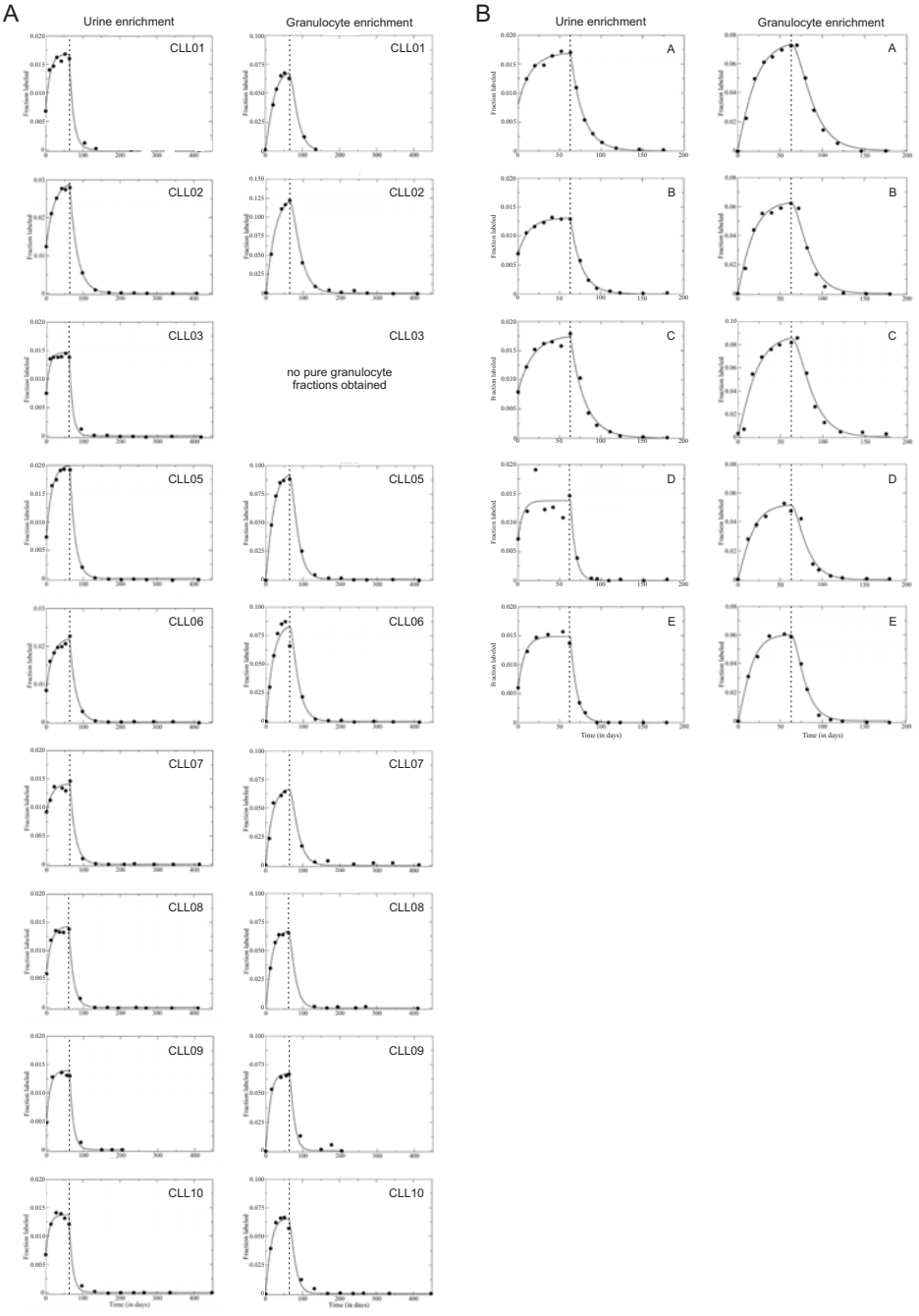
Supplementary Table 1. Parameter estimates of the urine enrichment curves, where *f* represents the fraction of ^2H_2O in the drinking water, δ is the turnover rate of body water per day, and β represents the baseline urine enrichment attained after the boost of label by the end of day 0

CLL patient	*f*	*δ*	*β*
CLL01	0.017	0.086	0.007
CLL02	0.030	0.048	0.013
CLL03	0.015	0.109	0.008
CLL05	0.020	0.068	0.008
CLL06	0.022	0.057	0.009
CLL07	0.014	0.066	0.009
CLL08	0.014	0.078	0.006
CLL09	0.014	0.100	0.005
CLL10	0.014	0.103	0.007
healthy participant	***f***	***δ***	***β***
A	0.017	0.064	0.008
B	0.013	0.088	0.007
C	0.017	0.075	0.008
D	0.014	0.128	0.007
E	0.015	0.119	0.006

Supplementary Table 2. Parameter estimates of the granulocyte enrichment curves (before scaling), where *p* represents the average production rate of granulocytes, *c* is the amplification factor, and *d* represents the loss rate of labeled granulocytes

CLL patient	*pc*	*d*	*c*
CLL01	0.288	0.070	4.11
CLL02	0.393	0.090	4.37
CLL05	0.470	0.100	4.70
CLL06	0.488	0.127	3.84
CLL07	0.412	0.086	4.79
CLL08	0.625	0.132	4.73
CLL09	0.930	0.193	4.82
CLL10	0.526	0.110	4.78
healthy participant	***pc***	***d***	***c***
A	0.384	0.086	4.47
B	0.419	0.085	4.93
C	0.402	0.078	5.15
D	0.299	0.079	3.78
E	0.415	0.103	4.03



Supplemental Figure 1. Label enrichment in urine and granulocytes. ^2H_2O-enrichment in urine and ^2H-enrichment of deoxyadenosines in the DNA of granulocytes during follow-up in A) CLL patients and B) healthy individuals. The dotted line represents the time point of label cessation. Labeling data were fitted as described in Materials and methods.

9

Discussion

In this thesis the roles of thymic output and peripheral naive T-cell proliferation are studied during the establishment and maintenance of the naive T-cell compartment in health, disease and during immune reconstitution. Here these findings are put in a general perspective.

Establishment of the T-cell compartment during childhood

In chapter 2, we assessed the role of thymic output and peripheral proliferation in the establishment of the $CD4^+$ and $CD8^+$ T-cell compartment in healthy children. It has previously been shown that total body T-cell counts increase during childhood (1). As the body, and concomitantly the vasculature, lymphatic system and lymphoid organs steadily grow during childhood, the "niche" which T cells can occupy steadily expands. Studies in mice have shown that there is homeostasis regarding the number of naive and memory $CD4^+$ and naive $CD8^+$ T cells, and the availability of self-peptide-presenting major histocompatibility complex (MHC) ligands and interleukin-7 (IL-7). It has been proposed that in a fully established T-cell compartment *in vivo*, the availability of MHC ligands and IL-7 is limiting and all circulating naive and memory $CD4^+$ T cells and naive $CD8^+$ T cells compete for these factors in their struggle to survive (2-4). However, during growth gradually more MHC ligands and IL-7 may become available. As a consequence, the number of T cells during childhood may increase homeostatically. Because new antigens are encountered continuously, the memory $CD4^+$ T-cell compartment expands and this expanded memory compartment may compete with and limits the expansion of naive $CD4^+$ and $CD8^+$ T cells during childhood (Figure 1C).

In chapter 2, we found that naive T-cell proliferation levels in healthy children are low and relatively constant after one year of age. As thymic naive T-cell production gradually declines after birth, it is likely that the average lifespan of naive T cells is prolonged, as there is less competition with new thymically-produced naive T cells for available MHC ligands and IL-7. Consequently, the likelihood that naive T cells will divide during their life increases. As a result, total body naive T-cell numbers may gradually increase during childhood, despite a continuously decreasing contribution of thymic output.

The increase in total body naive T-cell counts at young age in healthy children was previously shown to be accompanied by constant total body TREC numbers (1). We confirmed these findings in our much larger cohort of healthy children (see chapter 2 and Figure 1). Collectively, these data show that there is a substantial contribution of peripheral T-cell proliferation to the establishment of the naive T-cell compartment during childhood (1). Nevertheless, we found in chapter 3 that in children who had

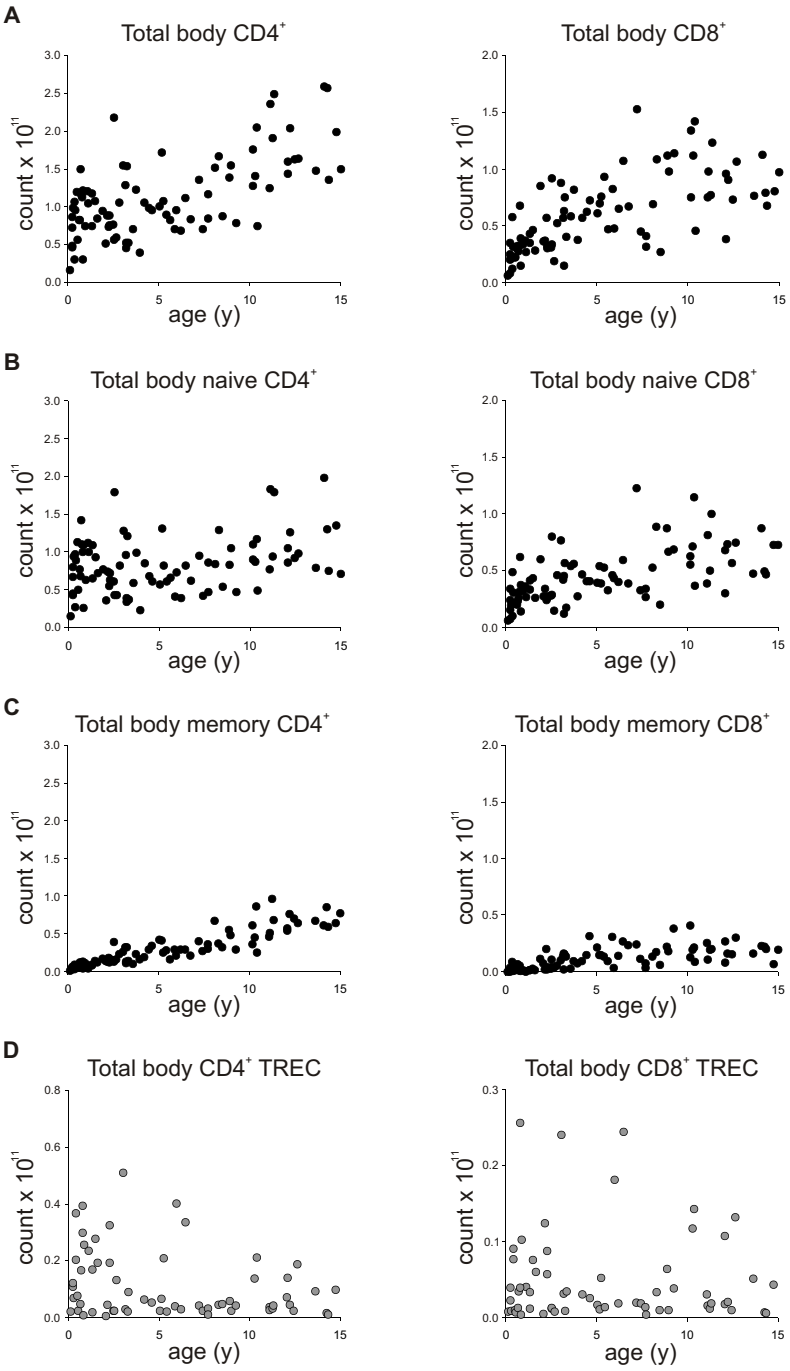


Figure 1. Total body T-cell counts and total body TREC numbers. The numbers of A) total, B) naive and C) memory $CD4^+$ and $CD8^+$ T cells in the body during childhood. D) The numbers of total body TRECs in $CD4^+$ and $CD8^+$ T cells during childhood. Each dot represents a single individual.

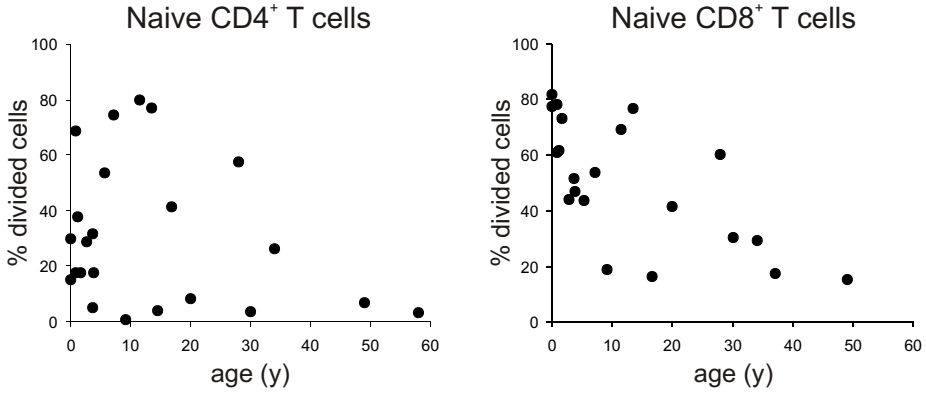


Figure 2. Naive T-cell responsiveness to IL-7. Naive CD4+ and CD8+ T cells were sorted from PBMC of donors with various ages. Cells were subsequently labeled with CFSE and cultured for 7 days in the presence of IL-7 (10ng/ml). The percentage of divided cells represents the percentage of cells which had diluted CFSE.

undergone neonatal thymectomy, total body naive T-cell counts did not increase during the first three years of life. This suggests that in healthy children naive T cells that have recently been produced by the thymus proliferate in the periphery. By removing the thymus, not only the cells that are normally produced by the thymus, but also their progeny that is normally formed in the periphery is lacking. Several studies have suggested that naive T cells in young children are more responsive to IL-7 induced proliferation than naive T cells at older age (5-7) (Figure 2). One possibility is that T cells that have recently been generated by the thymus have an intrinsically higher responsiveness to IL-7 induced proliferation, which may account for the relatively high levels of naive T-cell proliferation at young age. Concomitant with a gradual decrease in thymic output during childhood, or a more drastic loss of thymic output in thymectomized children, this intrinsic responsiveness to IL-7 will wane.

We found that total body TREC numbers in healthy individuals were high until 1 to 2 years of age and were at lower but relatively constant levels thereafter (Figure 1D). One might therefore even speculate that below two years of age the naive T-cell production rate by the thymus is too high and the available space in the periphery is limiting. This is supported by the observation that thymic cellularity increases from birth until 9 months of age (8). As a consequence of the high levels of naive T cells in the circulation, the total body TREC number increases. It may be proposed that naive T cells will remain in the circulation for only a limited time span, as they are outcompeted by other naive T cells. The finding that TREC contents of CD4+ and CD8+ T cells are higher in the first two years of life may support this hypothesis. Of note, these data do not imply that recently produced naive T cells by the thymus

have shorter half-lives than naive T cells which have remained for a longer time in the periphery, but that all naive T cells may have a short half-life. Although it has been found that naive T-cell proliferation is increased in the first six months of life (1,5), the contribution of peripheral proliferation to the establishment of the naive T-cell compartment in case of high levels of thymic naive T-cell production may be small: even if naive T cells proliferate, they are rapidly lost in the face of high levels of thymic naive T-cell production.

Reconstitution of the T-cell compartment during childhood

The mechanisms by which naive T cells regenerate after severe lymphocyte depletion in humans remain largely unclear. It was found that younger children are able to produce more naive T cells than older children, but when naive T-cell counts were expressed as percentage of age-matched values it seemed that during childhood the capability to reconstitute the naive T-cell compartment was independent of age (9). Comparison of recovery of the naive T-cell compartment in children and adults after intensive cytotoxic chemotherapy, showed faster recovery in children than in adults (10,11). Similar results were found for naive T-cell reconstitution following bone marrow or stem cell transplantation (12-14) or during antiviral treatment for HIV-1 infection (15). Since thymic output decreases with age, these data suggest that the speed of naive T-cell recovery after severe lymphocyte depletion is to a large extent determined by thymic output. However, as discussed above, also the capacity of T cells to proliferate, e.g. in response to IL-7, changes drastically with age. In chapters 3-5, we investigated the relative roles of thymus output and peripheral T-cell proliferation to T-cell regeneration in different immune-depleted settings.

The role of naive T-cell proliferation during immune reconstitution

Studies in mice have shown that proliferation levels of naive and memory T cells are typically increased during severe lymphopenia (16,17), and that this so-called lymphopenia-induced proliferation (LIP) is accompanied by a phenotypical shift from naive-to-memory T cells. Further investigation revealed that there are actually two types of LIP: spontaneous and homeostatic (18). Homeostatic LIP is slow and dependent on IL-7. In contrast, spontaneous LIP is rapid, independent of IL-7 and results in the conversion of naive-to-memory/effector-like T cells. Self-peptide/MHC ligand stimulation is involved in both forms of LIP (18). In humans, LIP has hardly been studied. It has been shown that severe depletion of T cells following stem cell transplantation initially results in expansion of $CD45RO^+$ T cells and a delayed emergence of $CD45RA^+$ T cells in the circulation (11,19). This may imply that in

humans, thymically-produced new naive T cells which enter a severely depleted environment rapidly start to proliferate and acquire a memory phenotype.

In chapter 3, we found that in the first three years after neonatal thymectomy there was no evidence for increased levels of naive T-cell proliferation as a homeostatic response to the loss of thymic output. Naive T-cell homeostasis may nevertheless be affected by neonatal thymectomy. The loss of thymic naive T-cell production may result in prolonged survival of the already circulating naive T cells. Similarly, in chapter 4, we found that after cessation of chemotherapy in children, naive T-cell proliferation levels were not always increased, and if they were, they were related to events of T-cell activation, rather than to a homeostatic compensatory response. Previous studies have also suggested that naive T-cell proliferation levels in lymphocyte-depleted HIV-1 infected individuals do not respond homeostatically (20), and that increased levels of naive T-cell proliferation in SCT patients were related to clinical events or graft-versus-host disease rather than homeostatically driven (21).

What could explain the lack of homeostatically regulated levels of naive T-cell proliferation during naive T-cell reconstitution in children? One option is that the well-established phenomenon of LIP in mice simply does not occur in humans. Alternatively, in our studies, the degree of T-cell depletion may not have been severe enough to induce LIP. At the time of cessation of chemotherapy (chapter 4) circulating (naive) $CD4^+$ and $CD8^+$ T cell numbers were reduced to approximately 40% and 60% of normal values, respectively. However, findings on $CD4^+$ T-cell recovery in severely-depleted HIV-1 infected adults do not support the latter possibility. In these patients, $CD4^+$ T-cell proliferation levels returned to normal after start of HAART, even when T-cell counts were still severely depleted (20).

We therefore conclude that there is no evidence that in humans naive T-cell proliferation is driven by lymphopenia. Rather, in addition to thymic output, constant low levels of naive T-cell proliferation maintain a fully established naive T-cell compartment and contribute to recovery during lymphodepletion.

The role of the thymus during immune reconstitution

Since thymic output decreases with age, it has been questioned whether adults can completely reconstitute naive T-cell counts after T-cell depletion. Studies addressing the recovery of the T-cell compartment in HIV-1 infected patients treated for 4-5 years with HAART demonstrated that numbers of $CD4^+$ T cells increased from start of therapy but did not fully normalize (22,23). In chapter 5, we investigated whether adults could restore their T-cell compartments to normal levels after prolonged therapy, and to what extent their T-cell regeneration differed from that in children. After at least seven years of successful HAART, we found in all adults that total $CD4^+$

T-cell counts had recovered. The speed of recovery of the $CD4^+$ T-cel compartment was dependent on the degree of T-cell depletion at start of therapy. Naive $CD4^+$ T-cell counts did not recover to normal levels in severely (<25% of normal values) $CD4^+$ T-cell depleted adults, but normalized in patients who were less severely (>25% of normal values) depleted at start of therapy. Recovery of naive T-cell counts was dependent on thymic function as well as naive T-cell division. Despite the fact that thymic output in adults is limited, it clearly contributed to naive T-cell reconstitution, because total TREC numbers per µl blood increased over time even in adults. Thus, long-term successful treatment with HAART may eventually lead to complete recovery of the naive $CD4^+$ T-cell compartment even in severely T-cell depleted adults. Strikingly, recovery of T-cell counts in HIV-1 infected children on HAART was not dependent on the degree of T-cell depletion at start of treatment. Within one year, $CD4^+$ T-cell counts restored to normal levels. In contrast, after long-term (4.4-9.6 years) HAART, memory T-cell counts were significantly lower than in age-matched controls. This suggests that the $CD4^+$ T-cell compartment "rejuvenates" after reconstitution from severe T-cell depletion. Also in children recovering from chemotherapy-induced lymphocyte depletion (chapter 4), we found that after cessation of treatment the $CD4^+$ and $CD8^+$ T-cell compartments were relatively enriched for naive T cells compared to healthy age-matched controls.

Collectively, these data suggest that reconstitution of the naive T-cell compartment is to a large extent dependent on thymic naive T-cell production even in adults with relatively small thymic output.

How to improve naive T-cell reconstitution?

Rapid T-cell recovery is necessary to avoid life-threatening opportunistic infections. Strategies to boost naive T-cell reconstitution in children and adults can be directed towards enhancement of thymic naive T-cell production or peripheral expansion of naive T cells, or a combination of both.

IL-7 could be a good candidate to improve naive T-cell generation. In a recent study, short-term administration of recombinant human IL-7 (rhIL-7) to 16 non-lymphodepleted cancer patients revealed IL-7-induced expansion of naive and memory T cells in an already fully established T-cell compartment (24). Elevated proliferation of peripheral naive and memory T cells was observed and there was no evidence for enhanced thymopoiesis (24). The diversity of the T-cell compartment was increased, as a result of the relatively large contribution of naive T cells to the total T-cell compartment. In contrast, the diversity of the naive T-cell compartment was not increased, presumably because thymic naive T-cell production was not enhanced

and more naive T cells bearing TCRs with the same antigen-specificity were generated by peripheral proliferation. It remains unclear whether the T-cell expansions persist in the long run, because after cessation of rhIL-7 administration the available IL-7 will become less abundant and may become more limiting for the expanded number of cells. It can even be argued that in the long run rhIL-7 administration may be disadvantageous, because contraction of the T-cell compartment following stop of treatment may lead to loss of certain T-cell specificities as more cells have to compete for limiting levels of self-peptide-presenting MHC ligands and IL-7.

To date, administration of IL-7 in immune depleted settings in humans has not been performed. In mice it has been shown that administration of IL-7 following bone marrow transplantation does not result in rapid recovery of naive T cells, although memory T cell numbers increased significantly. These increased numbers of memory T cells did not exacerbate graft-versus-host disease (25). In baboons treated with IL-7 for 6-10 weeks after stem cell transplantation, peripheral T-cell proliferation increased both $CD4^+$ and $CD8^+$ T-cell counts (26). Hence, although the use of IL-7 in lymphodepleted situations may expand the number of circulating T cells, its inability to enhance thymic naive T-cell production may in the long run render this treatment unsatisfactory.

It has been recognized that thymic involution is associated with a decline in the levels of growth hormone (GH) and a rise in the levels of sex steroids (estrogen and testosterone) during aging (27). Interfering with these hormones may therefore provide alternative means to improve naive T-cell production. Castration in mice and rats has indeed been shown to result in increased thymopoiesis, reformation of thymic architecture and an increase in the number of circulating peripheral T cells (28). A recent study addressed whether the daily administration of GH during 6-12 months could enhance naive T-cell production in HIV-1 infected adults (29). Indeed, naive T-cell counts increased dramatically as a result of both enhanced thymic output and peripheral T-cell proliferation. However it remains unclear whether after discontinuation of GH administration the increased naive T-cell counts are sustained (30). In contrast to what has been reported for IL-7 administration, however, the increase in the number of thymically-derived naive T cells following GH administration might lead to rejuvenation of the T-cell compartment. Although GH may cause mild toxicity (oedema, arthralgia) it may prove to be the best option to revert thymic involution or to increase thymic function during reconstitution. Further studies will have to exemplify whether GH administration at lower doses may be effective in enhancing naive T-cell production during lymphocyte depletion. Moreover, other hormones like ghrelin and keratinocyte growth factor (KGF) may offer suitable alternatives to enhance thymic naive T-cell production in humans suffering from lymphocyte depletion (31-33).

The concept of recent thymic emigrants revisited

Quantification of naive T-cell production by the thymus would allow clinicians to evaluate thymic function in health, disease and during reconstitution. To date, no ideal method is available to directly identify recent thymic emigrants (RTE), i.e naive T cells which have recently left the thymus. Measurement of T-cell receptor excision circles (TRECs) and CD31 expression on naive $CD4^+$ T cells are commonly-used methods to gain insight into the balance between thymic naive T-cell production and proliferation of naive T cells in the periphery. However, the interpretation of these methods does not allow for a direct assessment of current thymic output, but is based on changes of these markers within a timeframe, usually of months. The other main reason why the interpretation of TREC data and CD31 expression has proved unsatisfactory to determine RTE levels, is the fundamental link of these methods to cell division. Upon naive T-cell division, two daughter cells are generated in the periphery and the original thymic emigrant is lost. However, the status of the original thymically produced naive T cell as RTE can be questioned. Although this cell is originally a thymic emigrant, to consider it as "recent" if it had circulated for years in the periphery prior to cell division seems inappropriate.

The main problem of identifying RTE is one of semantics: what is a RTE? How recent does a RTE have to be? And how do we define "recent" anyway? Most will agree that a property that identifies RTE from non-RTE naive T cells has to include a *time-dependency*, because any RTE will eventually become a non-RTE at some point in time. Although this type of characterization may be intuitively correct, the question remains at which time point RTE will not be "recent" anymore.

RTE may also be distinguished from non-RTE naive T cells by *intrinsic* differences. Intuitively, "recent" may therefore apply to a functional or phenotypical difference between these two types of naive T cells. This intrinsic aspect is time-dependent, because such a hypothetical functional or phenotypical property is lost at the time of transition from RTE to non-RTE. We hypothesized that the intrinsic property which distinguishes RTE may be a feature that RTE share with single positive thymocytes (their progenitors) but not with non-RTE naive T cells. CCR9 was proposed as a likely candidate to identify RTE on functional means (34). Since its ligand, thymus-expressed chemokine (TECK), is produced in the thymus but not in the periphery (except in the small intestine), $CCR9^+$ naive T cells expressing cells were considered RTE. Although CCR9 expression on naive T cells was lost during aging and hardly any $CCR9^+$ naive T cells were observed following neonatal thymectomy, the fraction of $CCR9^+$ naive T cells was found to increase after thymectomy, suggesting that CCR9 expression is not restricted to RTE (34).

The identification of a subset of CD4 RTE as described in chapter 6 may prove the best option thus far. This method identifies RTE on functional and temporal characteristics as we found that a subset (~20%) of CD4 RTE still contains some degree of CD4/CD8 lineage plasticity. These cells re-expressed CD8 in the presence of IL-7, suggesting that they were redirected towards the CD8 lineage. Full commitment to the CD4 lineage seems to be accomplished within 2.5 months after thymic egress. Hence, we propose a new marker to identify RTE based on a thymic feature these cells still bear. As this feature is lost within only a few months in the periphery, this method to identify RTE may be appropriate and satisfactory. However, this definition will imply that the original paradigm that the progeny of a dividing RTE are no longer RTE will have to be dropped. If RTE would functionally resemble single positive thymocytes and these features are retained despite proliferation *in vivo* or *in vitro*, then it would be still legitimate to call naive T cells RTE even if they had proliferated *in vivo*.

Now that we can identify RTE, the next step is to determine not only the level of thymic output in healthy individuals, but also in immune depleted settings, like after cessation of chemotherapy, following stem cell transplantation or in HIV-1 infection.

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Nederlandse samenvatting

Het afweersysteem

Het afweersysteem bestaat uit een groot aantal celtypen en mediatoren die het lichaam beschermen tegen een scala aan pathogenen, zoals virussen, bacteriën, parasieten en schimmels. De herkenning van pathogenen door het afweersysteem is gebaseerd op het onderscheid tussen lichaamseigen en lichaamsvreemde structuren (antigenen). Een belangrijke component van het afweersysteem om geïnfecteerde cellen te herkennen en op te ruimen zijn de T-cellen. Iedere T-cel bezit een T-cel receptor (TCR), waarvan er twee typen bestaan: αβTCR en γδTCR. In dit proefschrift worden αβTCR T-cellen bestudeerd en daarvan bestaan twee typen: $CD4^+$ T-cellen en $CD8^+$ T-cellen. Omdat iedere TCR een unieke antigeenspecificiteit heeft, is het mogelijk om een grote variëteit aan antigenen te herkennen. Deze antigenen worden gepresenteerd via major histocompatibility (MHC) moleculen, welke aanwezig zijn op de meeste lichaamscellen. Als lichaamseigen antigenen worden gepresenteerd zal er geen afweerreactie volgen, terwijl presentatie van lichaamsvreemde antigenen doorgaans resulteert in een immuunreactie.

De eerste ontwikkelingsstadia van T-cellen vinden plaats in het beenmerg, waarna de voorloper T-cellen naar de thymus (zwezerik) migreren. In de thymus verkrijgen de ontwikkelende T-cellen de antigeenspecifieke TCR. Ook wordt de keuze gemaakt of de volwassen T-cel CD4 (nodig voor MHC klasse II herkenning) of CD8 (nodig voor MHC klasse I herkenning) tot expressie zal brengen. Slechts een fractie van de ontwikkelende T-cellen zal de thymus verlaten, omdat T-cellen met TCRs die een interactie aangaan met lichaamseigen antigenen of geheel geen specificiteit bezitten worden vernietigd. Zodra een T-cel de thymus verlaat noemt men deze 'naief' omdat in de periferie nog geen interactie met een specifiek antigeen heeft plaatsgevonden. Naieve T-cellen kunnen jarenlang circuleren alvorens een lichaamsvreemd antigeen te herkennen, of zelfs sterven zonder er één te herkennen. Echter, zodra een specifiek antigeen herkend wordt, wordt de naieve T-cel geactiveerd en vermenigvuldigd deze zich door celdeling. Effector $CD8^+$ T-cellen die bij dit proces ontstaan, verkrijgen receptoren om naar de plaats van de infectie te migreren en bezitten de capaciteiten om ter plaatse de pathogene invasie, voornamelijk van virale aard, een halt toe te roepen door geïnfecteerde cellen te herkennen en op te ruimen. Geactiveerde $CD4^+$ T-cellen 'helpen' andere afweercellen, zoals macrofagen en B-cellen, tijdens de afweerreactie. Naast effector T-cellen worden er memory T-cellen gevormd. Deze cellen kunnen tijdens een nieuwe infectie van hetzelfde pathogeen sneller en adequaat reageren.

Homeostase van het naieve T-cel compartiment

Er wordt gedacht dat de grootte van het naieve T-cel compartiment (de verzameling van alle naieve T-cellen in het lichaam) homeostatisch gereguleerd is, d.w.z. dat er een balans is tussen productie en levensduur van naieve T-cellen enerzijds en dood en differentiatie van naieve T-cellen anderzijds. Naieve T-cellen worden geproduceerd in de thymus, maar ook door deling van naieve T-cellen in de periferie. Beide mechanismen zijn in mensen belangrijk voor de opbouw van het naieve T-cel compartiment in de kinderjaren. De grootte van de thymus neemt af tijdens het leven en het wordt daarom gedacht dat de productie van naieve T-cellen met gelijke tred afneemt. Om naieve T-cel aantallen in stand te houden wordt aangenomen dat het gradueel wegvallen van de productie door de thymus wordt gecompenseerd door zowel verlengde levensduur van naieve T-cellen als door naieve T-cel deling.

Doel van dit proefschrift

In bepaalde klinische situaties waarin het T-cel compartiment is verstoord, zoals na stamcel transplantatie, chemotherapie of HIV-1 infectie, is het voor de patiënt van belang om het naieve T-cel compartiment snel weer op te bouwen tot een normaal niveau, om de kans op ongewenste en zelfs levensbedreigende infecties te minimaliseren. Met name in volwassenen kan volledige reconstitutie van naieve T-cellen een aantal jaren in beslag nemen en het is daarom belangrijk om de reconstitutie te versnellen. Daarvoor dient bestudeerd te worden welke mechanismen in gezonde kinderen een rol spelen terwijl het T-cel compartiment zich opbouwt, maar ook welke mechanismen in kinderen verantwoordelijk zijn voor de snelle reconstitutie van het T-cel compartiment tijdens immuundepletie. In dit proefschrift wordt de dynamiek van het naieve T-cel compartiment onderzocht, zowel in gezonde kinderen als in kinderen en volwassenen waarin het T-cel compartiment gedepleteerd is. Door een begrip te verkrijgen hoe de dynamiek van het T-cel compartiment gereguleerd is, kunnen mogelijk therapieën geïnitieerd worden om reconstitutie van het naieve T-cel compartiment te bespoedigen.

Opbouw van het naieve T-cel compartiment in kinderen

In **hoofdstuk 2** onderzochten we de opbouw van het T-cel compartiment in kinderen. We bevestigden dat de opbouw van het naieve T-cel compartiment bewerkstelligd wordt door zowel productie van naieve T-cellen door de thymus als naieve T-cel

proliferatie in de periferie. In tegenstelling tot eerder verricht onderzoek waarin gevonden is dat na vijf jaar het naieve T-cel compartiment zijn volledige grootte heeft bereikt, vonden we dat ook na het vijfde levensjaar het naieve T-cel compartiment langzaam in grootte toeneemt.

In **hoofdstuk 3** bestudeerden we de opbouw van het naieve T-cel compartiment in kinderen bij wie, als gevolg van een hartoperatie, in de eerste maanden van het leven de thymus wordt verwijderd (een thymectomie). We vonden dat in de eerste drie levensjaren na thymectomie het aantal naieve T-cellen in het lichaam niet toeneemt, terwijl we in **hoofdstuk 2** concludeerden dat proliferatie mede verantwoordelijk is voor de opbouw van het naieve T-cel compartiment. Hieruit kunnen we opmaken dat juist de naieve T-cellen die geproduceerd worden door de thymus de capaciteit bezitten om te delen in de periferie.

Waarom verschillen recent door de thymus geproduceerde naieve T-cellen van oudere naieve T-cellen? Als gevolg van de thymectomie valt de bijdrage van naieve T-cel productie door de thymus weg waardoor het naieve T-cel compartiment verouderd. In **hoofdstuk 9** vonden we dat in gezonde individuen de veroudering van het naieve T-cel compartiment leidt tot een verminderde intrinsieke gevoeligheid van naieve T-cellen om te delen na interactie met het cytokine IL-7, een belangrijke component in naieve T-cel homeostase. De capaciteit om te delen na interactie met IL-7 lijkt daarom een eigenschap van jonge naieve T-cellen te zijn. Omdat na thymectomie het naieve T-cel compartiment als geheel verouderd verdwijnt mogelijk de capaciteit van naieve T-cellen om te delen na interactie met IL-7. Welke factoren de gevoeligheid van naieve T-cellen voor IL-7 bepalen is echter nog onduidelijk.

T-cel reconstitutie

Om de heropbouw van het naieve T-cel compartiment na een periode van immuundepletie te kunnen versnellen, is het van belang de mechanismen verantwoordelijk voor het herstel van het naieve T-cel compartiment te identificeren. Dit herstel verloopt in kinderen over het algemeen sneller dan in volwassenen. Het is geopperd dat de grotere thymus in kinderen zorgt voor een grotere naieve T-cel productie en verantwoordelijk is voor de snelheid van reconstitutie. Aangezien naieve T-cel proliferatie een belangrijke rol speelt in de opbouw van het naieve T-cel compartiment in de kinderjaren bestaat de mogelijkheid dat proliferatie ook bijdraagt tot een sneller herstel van het naieve T-cel compartiment. In **hoofdstuk 4** onderzochten we hoe naieve T-cel reconstitutie in kinderen tot stand kwam na beëindiging van chemotherapie als behandeling voor leukemie of een lymfoom. Herstel van naieve T-cel aantallen werd bewerkstelligd binnen zes maanden na het stoppen van de

chemotherapie. De thymus was inderdaad een bron van naieve T-cellen tijdens reconstitutie, omdat TREC aantallen (een maat voor naieve T-cel productie door de thymus) na het beëindigen van de chemotherapie toenamen. Proliferatie van naieve T-cellen in de periferie was echter minimaal. In een aantal patiënten vonden we verhoogde naieve T-cel proliferatie, maar dit leek geïnduceerd door activatie, waarschijnlijk veroorzaakt door een infectie of als gevolg van de chemotherapie. We hebben daarom geen aanwijzingen dat depletie van het naieve T-cel compartiment resulteert in homeostatisch verhoogde naieve T-cel proliferatie om het compartiment op te vullen. Dit is in overeenstemming met de bevindingen in **hoofdstuk 3**, waarbij we in een gedepleteerd naief T-cel compartiment na thymectomie geen verhoogde delingsactiviteit van naieve T-cellen vonden.

Aangezien de productie van naieve T-cellen door de thymus in volwassenen lager is dan in kinderen, wilden we de reconstitutie capaciteit van het $CD4^+$ T-cel compartiment bestuderen in kinderen en volwassenen. Hiervoor bestudeerden we individuen behandeld met highly active antiretroviral therapy (HAART) voor chronische HIV-1 infectie (**hoofdstuk 5**). HAART is tot dusver de meest effectieve therapie om de infectie van HIV-1 te onderdrukken. In tegenstelling tot volwassenen bleek in kinderen de mate van $CD4^+$ T-cel depletie bij aanvang van de therapie niet bepalend voor de snelheid van $CD4^+$ T-cel reconstitutie. In kinderen herstelden $CD4^+$ T-cel aantallen binnen een jaar na aanvang van therapie tot fysiologisch normale waarden. In volwassenen met $CD4^+$ T-cel aantallen hoger dan 25% van normaalwaarden (>200 $CD4^+$ T-cellen per µl bloed) herstelden $CD4^+$ T-cel aantallen eveneens binnen een jaar, maar in volwassenen met $CD4^+$ T-cel aantallen lager dan 25% van normaalwaarden (<200 $CD4^+$ T-cellen per µl bloed) duurde het minstens 7 jaar alvorens normale aantallen bereikt werden. Naieve $CD4^+$ T-cel aantallen in deze groep volwassenen waren na 7 jaar therapie nog steeds verlaagd. De thymus was een bron van nieuwe naieve T-cellen omdat TREC aantallen na langdurig HAART hoger waren dan tijdens de start van de therapie. Blijkbaar is er in zowel kinderen als volwassenen een belangrijke rol weggelegd voor de thymus in de reconstitutie van het naieve T-cel compartiment tijdens behandeling met HAART. Omdat het herstel in de HIV-1 geïnfecteerde volwassenen met de laagste $CD4^+$ T-cel aantallen tijdens de start van de therapie vele jaren in beslag nam, lijkt er tijdens reconstitutie geen belangrijke rol weggelegd voor naieve T-cel proliferatie in het herstel van naieve T-cel aantallen.

Concluderend kunnen we stellen dat naieve T-cel productie door de thymus bepalend is voor de reconstitutie snelheid van het naieve T-cel compartiment. Proliferatie draagt bij aan de reconstitutie van het naieve T-cel compartiment maar er waren geen aanwijzingen dat proliferatie homeostatisch verhoogd is tijdens T-cel depletie. Om naieve T-cel reconstitutie te versnellen is het daarom raadzaam om methoden

te ontwikkelen die naieve T-cel productie door de thymus bevorderen, omdat de recentelijk door de thymus geproduceerde naieve T-cellen ook de capaciteit bezitten om te delen in de periferie. Mogelijke opties om naieve T-cel productie door de thymus te bevorderen worden belicht in **hoofdstuk 9**.

Nieuwe methode ter bepaling van naieve T-cel productie door de thymus

Het bepalen van naieve T-cel productie door de thymus zou een ideaal middel zijn om de bijdrage van de thymus in naieve T-cel reconstitutie in immuungedepleteerde individuen te bepalen. Het is tot op heden moeilijk gebleken om naieve T-cellen die net uit de thymus gekomen zijn (recent thymic emigrants (RTE)) te onderscheiden van naieve T-cellen die enige tijd in circulatie zijn. Gangbare methoden om de productie van naieve T-cellen door de thymus te bepalen, zoals TRECs and CD31 expressie, bieden inzicht in de wijze van opbouw en reconstitutie van het T-cel compartiment, maar kunnen het aantal RTE niet direct kwantificeren. In **hoofdstuk 6** beschrijven we een nieuwe methode om een subset van CD4 RTE aan te tonen. Deze cellen kunnen worden onderscheiden van niet-RTE omdat ze enige mate van plasticiteit bezitten. Door het toevoegen van IL-7 kunnen deze cellen "terugdifferentiëren" naar het fenotype van een voorloper stadium van $CD4^+$ T-cel ontwikkeling in de thymus: het $CD4^+CD8^{lo}$ stadium. Na thymectomie vonden we dat naieve $CD4^+$ T-cellen niet/nauwelijks "terugdifferentiëren" naar $CD4^+CD8^{lo}$ T-cellen, waarschijnlijk omdat ze verouderd zijn en de plasticiteit hebben verloren. Een volgende stap zal zijn om deze techniek te gebruiken om naieve T-cel productie door de thymus te bepalen in gezonde individuen over de leeftijd, in patiënten die naieve T-cel aantallen herstellen na immuundepletie of tijdens chronische HIV-1 infectie.

Dynamiek van lymphocyten in vivo

Chronische HIV-1 infectie zorgt voor een langzame depletie van naieve $CD4^+$ en $CD8^+$ T-cellen *in vivo* (d.w.z. in het lichaam). Daarnaast wordt de infectie gekenmerkt door verhoogde proliferatie van T-cellen, maar de oorzaak hiervan is nog niet geheel duidelijk. Enerzijds kan de verhoogde proliferatie een gevolg zijn van een homeostatische, maar niettemin tekortschietende respons om T-cel aantallen op peil te houden. Anderzijds kan de verhoogde proliferatie veroorzaakt worden door chronische activatie van T-cellen door de persisterende aanwezigheid van het voortdurend muterende HIV-1. Hierdoor worden continu naieve T-cellen geactiveerd waardoor het naieve T-cel compartiment langzaam leegloopt. Om tussen deze

twee hypotheses onderscheid te maken bestudeerden we in **hoofdstuk 7** de T-cel dynamiek *in vivo* van vier HIV-1 geïnfecteerde individuen m.b.v. zwaar water (^2H_2O) labeling. Omdat deuterium (^2H) ingebouwd wordt in nieuw gesynthetiseerd DNA kan met deze techniek productie en celdood *in vivo* worden bestudeerd. We vonden dat proliferatie van T-cellen in HIV-1 infectie hoofdzakelijk veroorzaakt wordt door chronische immuunactivatie. Recent geproduceerde naieve T-cellen (door de thymus of door proliferatie in de periferie) blijven in gezonde individuen lang leven, terwijl deze cellen in de HIV-1 geïnfecteerde individuen verdwijnen (door sterfte of differentiatie naar memory/effector T-cellen). Deze data ondersteunen wederom dat naieve T-cel proliferatie niet homeostatisch verhoogd is tijdens immuundepletie.

In **hoofdstuk 8** werd de dynamiek van stabiele B-cel chronische lymphocytaire leukemie (B-CLL) *in vivo* bestudeerd m.b.v. ^2H_2O labeling. Oorspronkelijk werd gedacht dat deze leukemie wordt veroorzaakt door een ophoping van geactiveerde B-cellen die langzaam delen en niet doodgaan. Er zijn echter steeds meer aanwijzingen dat er aanzienlijke productie en celdood van leukemische cellen plaatsvindt. We vonden dat zelfs in stabiele B-CLL er een aanzienlijke dynamiek van B-CLL cellen is waarbij de balans tussen proliferatie en celdood de grootte van de leukemische kloon in stand houdt. In tegenstelling tot wat voorheen gedacht werd, lijkt deze dynamiek zich niet af te spelen in het beenmerg, maar vindt voornamelijk plaats in lymfeknopen.

Dankwoord

Curriculum Vitae

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Dankwoord

Wetenschap bedrijven doe je niet alleen. Dit proefschrift is daarom met hulp en inzet van velen tot stand gekomen. Ik heb het voorrecht gehad om in een uitstekende wetenschappelijke onderzoeksgroep terecht te komen. Tijdens de vrijdagmorgen werkbespreking werden verhitte wetenschappelijke discussies afgewisseld met veel humor en plezier. Het is ogenschijnlijk mogelijk om bepaalde aspecten vanuit minstens drie verschillende perspectieven te aanschouwen. Kiki, José en Frank, bedankt voor alle input en wijsheden, van jullie heb ik veel geleerd. Kiki, ik kreeg van jou veel onderzoeksvrijheid en kon altijd terecht voor advies, zowel op theoretisch als technisch vlak. José, jouw enthousiasme werkt aanstekelijk. Je hebt me meerdere malen verbaasd met allerlei wiskundige wonderbaarlijkheden en je vond altijd mogelijkheden om een goed stuk nog beter te maken. Frank, door de zaken om te draaien wist je altijd onverwachte resultaten in een juist daglicht te zetten. Ook heb je me laten zien hoe wetenschap nu echt werkt in de praktijk. Veel succes met je nieuwe uitdaging in de Raad van Bestuur!

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Curriculum Vitae

Rogier van Gent werd op 31 maart 1980 geboren te Geldermalsen. Na het behalen van het VWO diploma aan het GSG Lingecollege te Tiel is hij in september 1998 gestart met de studie Medische Biologie aan de Universiteit Utrecht. Stages werden uitgevoerd bij de afdeling Experimentele Neurologie (onder begeleiding van prof. dr. P.R. Bär en drs. L.A.B. Wisman) en de afdeling pathologie (onder begeleiding van dr. M. Tilanus en drs. J. Reinders) in het UMC Utrecht. Na het behalen van het doctoraal examen in juni 2003 verwierf hij een studiebeurs van de Radboudstichting om een jaar Filosofie te studeren aan de Katholieke Universiteit Nijmegen. In januari 2005 startte hij als promovendus bij de afdeling Immunologie van het UMC Utrecht, onder begeleiding van dr. José A.M. Borghans en dr. Kiki Tesselaar in de groep van prof. dr. Frank Miedema. De resultaten van dat onderzoek zijn beschreven in dit proefschrift.

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