

# Establishment of the CD4<sup>+</sup> T-cell pool in healthy children and untreated children infected with HIV-1

Mette D. Hazenberg, Sigrid A. Otto, Annemarie M.C. van Rossum, Henriëtte J. Scherpbier, Ronald de Groot, Taco W. Kuijpers, Joep M.A. Lange, Dörte Hamann, Rob J. de Boer, José A.M. Borghans, and Frank Miedema

**Current understanding of how the T-cell pool is established in children and how this is affected by HIV infection is limited. It is widely believed that the thymus is the main source for T cells during childhood. Here we show, however, that healthy children had an age-related increase in total body numbers of naive and memory T cells, whereas absolute numbers of T-cell receptor excision circles (TRECs) did not increase. This suggests that expansion of**

**the naive T-cell pool after birth is more dependent on T-cell proliferation than was previously recognized. Indeed, the proportion of dividing naive T cells was high, especially in younger children, which is consistent with expansion through proliferation, in addition to antigen-mediated naive T-cell activation leading to formation of the memory T-cell pool. In untreated children infected with HIV-1, total body numbers of T cells and TRECs were**

**low and stable, whereas T-cell division levels were significantly higher than in healthy children. We postulate that in children infected with HIV, similar to adults infected with HIV, continuous activation of naive T cells leads to erosion of the naive T-cell pool and may be a major factor in lowering CD4<sup>+</sup> T-cell numbers. (Blood. 2004;104:3513-3519)**

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## Introduction

To date, it is not completely understood how HIV infection influences T-cell dynamics in children. This is partially due to the fact that little is known about the generation of the T-cell pool in healthy children. Under healthy circumstances, peripheral blood naive T-cell numbers are high in newborns and decline with increasing age, whereas memory T-cell numbers seem less dependent on age. Autopsy studies in children and adults negative for HIV have shown that thymic epithelium involutes from birth onward.<sup>1</sup> This has led to the suggestion that thymic output declines with increasing age. However, estimating thymic output of naive T cells has been difficult because no methods are available to reliably discriminate cells that recently emigrated from the thymus (recent thymic emigrants, RTEs) from older naive T cells. In the absence of a more reliable marker, the age-related decline in naive T-cell numbers per blood volume has been taken as evidence for deteriorating thymic function. However, as body size and blood volume increase significantly until adulthood, total body naive T-cell numbers and thereby naive T-cell production during infancy may have been underestimated.

It has been hypothesized that in adults, HIV-1 infection could interfere with naive T-cell production by the thymus, thereby contributing to the CD4<sup>+</sup> T-cell decline that characterizes HIV-1 infection.<sup>2-6</sup> Indeed, with the use of the severe combined immunodeficiency (SCID)-hu mouse model it was demonstrated that

HIV-1 is capable of infecting maturing thymocytes.<sup>3,5</sup> Many studies have tried to address the impact of HIV-1 infection on thymic function and T-cell numbers in children, using for example computed tomography (CT) scanning of thymic size and analysis of T-cell receptor excision circle (TREC) frequencies of peripheral blood mononuclear cells (PBMCs) or purified T-cell subsets.<sup>7-10</sup> However, these techniques only allow indirect estimations of thymic function. In case of CT imaging one has to assume that size is a proper correlate of thymic function, and it is now well established that, especially in the setting of HIV-induced chronic immune stimulation, TREC frequencies are more dependent on cell division than on thymic function.<sup>11</sup> Because it is not known to what extent HIV infection in children affects T-cell division rates, analysis of TREC frequencies as a measure for thymic output should be interpreted with caution when applied to children.

Other factors apart from thymic dysfunction may play a role in HIV-induced CD4<sup>+</sup> T-cell decline. It has been hypothesized that in adults chronic immune activation through persistent HIV-1 infection could also contribute to CD4<sup>+</sup> T-cell loss.<sup>12,13</sup> Continuous activation of naive T cells may lead to gradual erosion of the naive T-cell pool, as naive T cells are difficult to replace, especially if HIV impairs thymic output. As it is not known how T-cell activation levels are affected by HIV infection in children, it is not clear to what extent immune hyperactivation could also play a role in pediatric HIV pathogenesis.

From the Department of Clinical Viro-Immunology, Sanquin Research at CLB, Amsterdam, the Netherlands; the Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands; the Department of Pediatrics, Sophia Children's Hospital/Erasmus University Medical Center, Rotterdam, the Netherlands; the Department of Pediatrics, Emma's Children's Hospital/Academic Medical Center, University of Amsterdam, the Netherlands; the Division of Infectious Diseases, Tropical Medicine and AIDS, the National AIDS Therapy Evaluation Center (NATEC), the Department of Internal Medicine, Academic Medical Center, University of Amsterdam, the Netherlands; the Department of Theoretical Biology, Utrecht University, the Netherlands; and the Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, the Netherlands.

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**Reprints:** Frank Miedema, Department of Immunology, Rm KC02.085.2, University Medical Center Utrecht, Lundlaan 6, 3584 EA Utrecht, The Netherlands; e-mail: f.miedema@umcutrecht.nl.

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In the present study we measured basic parameters of T-cell dynamics in healthy children, and the effect of HIV-1 infection thereon, by analyzing total, naive, and memory CD4<sup>+</sup> T-cell numbers, TREC numbers and total, naive, and memory CD4<sup>+</sup> T-cell division rates in the blood of healthy children and children infected with HIV-1 that had not received highly active antiretroviral therapy (HAART). Analysis of total body T-cell numbers instead of CD4<sup>+</sup> T-cell numbers per volume of blood showed that in healthy children, naive CD4<sup>+</sup> T-cell numbers increased at least until the age of 5 years, and that peripheral proliferation played a larger role in this expansion than expected. Chronic immune activation induced by HIV-1 infection interfered with the establishment of the CD4<sup>+</sup> T-cell pool, mainly affecting the naive T-cell compartment.

## Patients, materials, and methods

### Patients

Cryopreserved peripheral blood samples from 16 untreated children infected with HIV-1 and 25 healthy subjects of comparable ages (Table 1) were analyzed. Healthy controls were children visiting the Academic Medical Center outpatient clinic for various conditions that were not related to immunopathologic or infectious diseases. All except 1 of the children positive for HIV were vertically infected; this 1 child (aged 8.5 years in Figures 1-3) did not differ from the other children in T-cell numbers, Ki67 expression, or TREC dynamics. On study entry, institutional review board-approved informed consent was obtained as appropriate.

### Phenotypic analysis

Peripheral blood T-cell division was studied by flow cytometric measurements of Ki67 nuclear antigen expression on naive (CD27<sup>+</sup>/CD45RO<sup>-</sup>), CD27<sup>+</sup> memory, and CD27<sup>-</sup> memory (CD27<sup>+</sup> or CD27<sup>-</sup>CD45RO<sup>+</sup>) CD4<sup>+</sup> T cells, as described previously.<sup>14-16</sup> PBMCs were thawed and incubated with CD4-PerCP (peridinin chlorophyll protein) monoclonal antibodies (mAb), CD45RO-PE (phycoerythrin; Becton Dickinson [BD], San Jose, CA) and biotinylated CD27 mAb (CLB, Amsterdam, The Netherlands), washed, and incubated with Streptavidin-APC (allophycocyanin; BD). After fixation and permeabilization with FACS (fluorescence-activated cell sorting) Lysing Solution and FACS Permeabilization Buffer (BD), respectively, lymphocytes were stained intracellularly with Ki67-FITC (fluorescein isothiocyanate) mAb (Immunotech, Marseille, France), fixed using Cellfix (BD), and analyzed on a FACSCalibur (BD) with Cellquest software.

### Cell separation

After 15 minutes of incubation with 20  $\mu$ L CD4-conjugated magnetic beads per 10<sup>7</sup> cells, CD4<sup>+</sup> T cells were isolated from PBMCs by positive selection over MiniMACS separation columns, yielding more than 90% purity (Miltenyi Biotec, Sunnyvale, CA).

**Table 1. Baseline characteristics of untreated children infected with HIV-1 and healthy children**

	Healthy, n = 25	HIV-1 infected, n = 16	P
Age, y	5.5 (0.7-16.0)	4.1 (0.3-12.9)	NS
CD4 <sup>+</sup> T cells/ $\mu$ L	1561 (740-3110)	701 (50-2650)	.001
Number of TRECs/ $\mu$ L	155.8 (22.5-1423.4)	43.5 (0.5-268.5)	.005
Ki67 <sup>+</sup> CD4 <sup>+</sup> T cells, %	2.6 (0.9-12.4)	10.2 (3.1-16.7)	< .001
Plasma HIV RNA, copies/mL	NA	6 $\times$ 10 <sup>4</sup> (410-1.4 $\times$ 10 <sup>6</sup> )	NA

Median values (and range) are depicted;  $P < .05$  was considered statistically significant (Mann-Whitney  $U$  test). NS indicates not significant; NA, not applicable.

### TREC analysis

DNA was purified from CD4<sup>+</sup> T cells using the QIAamp Blood Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Signal joint (Sj) TREC frequency of these fractions was quantified by using a real-time polymerase chain reaction (PCR) method as described previously.<sup>11</sup> To normalize for input DNA, the C $\alpha$  constant region that remains present on T-cell receptor (TCR) genes despite TCR rearrangement processes was amplified in every sample tested. The number of Sj copies present in a given cell population was calculated by including dilution series of a standard that was created by cloning the Sj fragment in the *Hind*III site of a pUC-19 vector in each PCR experiment. By applying the Ct-value (the minimal number of cycles necessary to exceed threshold values) to the standardization curve, the Sj TREC frequency could be calculated for each sample. TREC frequency was expressed as the number of TREC copies per CD4<sup>+</sup> T cell, assuming that 1  $\mu$ g DNA represents 150 000 T cells.

### Total body numbers of T cells and TRECs

The number of TRECs per microliter blood was calculated by multiplying the TREC frequency of purified CD4<sup>+</sup> T cells with the number of peripheral blood CD4<sup>+</sup> T cells (per microliter). For each child, T-cell numbers and TREC numbers were adjusted for the age-related increase in total blood volume by multiplying peripheral blood values (per microliter blood) with each individual's body weight at the time of blood draw and with the average blood volume per kilogram body weight. The latter remains constant at about 80 mL/kg despite changes in body weight with age.<sup>17</sup> Total body T-cell and TREC numbers were calculated by multiplying the adjusted peripheral blood values with a factor of 50 for healthy children or 100 for children infected with HIV, assuming that in the healthy situation only 2%, and in HIV infection, only 1% of the lymphocytes reside in the circulation<sup>18</sup> (total body T-cell or TREC number = [number of T cells or TRECs/ $\mu$ L blood]  $\times$  10<sup>3</sup>  $\times$  80  $\times$  [body weight in kg]  $\times$  (50 or 100)). It should be noted that patients infected with HIV with lower levels of immune activation may have less sequestration of T cells in lymphoid tissues than patients with higher levels of immune activation. For the former patients the calculated total body T-cell and TREC numbers may thus represent an overestimation, and differences with healthy children may actually be even more pronounced than reported here.

### Mathematical model

The model used here is an extension of our previously developed mathematical model, describing the dynamics of naive and memory T-cell numbers and TRECs.<sup>11</sup> The size of the naive T cell pool ( $N$ ) increases by thymic output ( $\sigma(t)$ ) and naive T-cell proliferation and decreases by cell death and activation of naive T cells that acquire a memory phenotype<sup>18,19</sup>:  $dN/dt = \sigma(t) + p_n N - aN - d_n N$ . Here,  $d_n$  represents the death rate of naive T cells,  $a$  is the rate of activation of naive T cells toward the memory pool, and  $p_n = p_{0n}/(1 + N/h)$  represents the naive T-cell proliferation rate. Thymic output at time  $t$  was modeled as an exponentially decaying function:  $\sigma(t) = \sigma_0 \exp[-vt]$ , where  $\sigma_0$  is the newborn thymus production of naive T cells, and  $v$  is the involution rate of the thymus.

The memory pool ( $M$ ) changes by T-cell activation, proliferation, and cell death:  $dM/dt = raN + p_m M - d_m M$ , where  $r$  is the clone size resulting from activation of a single naive T cell,  $d_m$  is the death rate of memory T cells, and  $p_m = p_{0m}/(1 + M/h)$  represents the memory T-cell proliferation rate. The dynamics of total TRECs in the naive and memory T-cell compartments are given by the following 2 equations:  $dT_N/dt = c\sigma(t) - (d_n + a)T_N$  and  $dT_M/dt = aT_N - d_m T_M$  where  $c$  is the number of TRECs per thymocyte, and  $T_N$  and  $T_M$  are the total number of TRECs in the naive and memory T-cell pool, respectively. See the legend of Figure 4 for parameters.

### Statistical analysis

Group characteristics of patients and healthy children were compared with the nonparametric Mann-Whitney  $U$  test. To test correlations between age

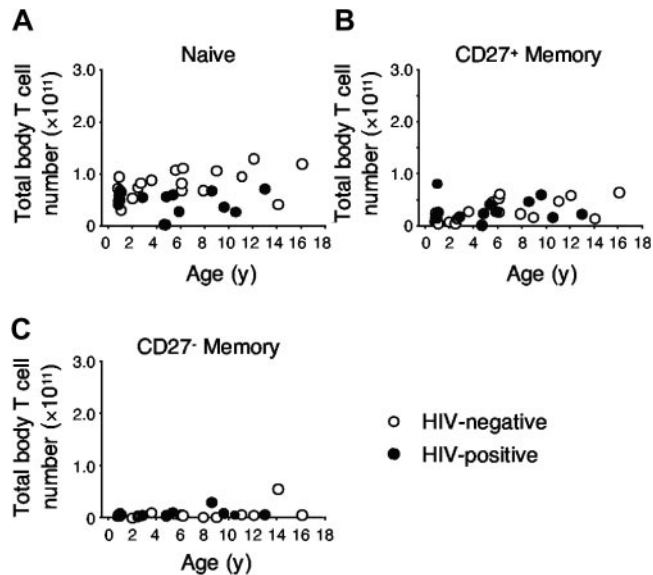
and total body T-cell numbers, TREC numbers and Ki67 expression, nonparametric Spearman coefficients were calculated.

## Results

### In healthy children, total body CD4<sup>+</sup> T-cell numbers increase with age

In our cohort of healthy children, CD4<sup>+</sup> T-cell numbers per microliter blood declined with increasing age (data not shown). However, from birth to adolescence, body weight increases dramatically, which is associated with a 15- to 20-fold increment in blood volume. Thus, in children, T-cell numbers measured per volume of blood do not adequately reflect the total body number of T cells. We therefore adjusted T-cell numbers per microliter blood for the weight of each child and found that total body CD4<sup>+</sup> T-cell numbers increased with age, at least until 5 years of age (Figure 1A). This was related to an increase in both naive and memory CD4<sup>+</sup> T-cell numbers (Figure 2).

A number of groups have reported estimates of thymic output in children and adults using quantitative PCR techniques to measure TREC frequencies.<sup>9,10,20</sup> TRECs are episomal products that are formed during T-cell receptor gene rearrangements that characterize intrathymic T-cell development. Because TRECs are diluted with cell division, and in humans TRECs and naive T cells are assumed to be long lived,<sup>18,21</sup> neither TREC frequencies (the number of TRECs per T cell) nor total TREC numbers in the blood reflect the actual thymic function.<sup>11,22</sup> However, TCR gene rearrangements and formation of TRECs are thought to occur in the thymus only<sup>20</sup>; therefore, we measured total body numbers of TRECs to determine the source of naive T cells. In our cohort of healthy children, total body TREC numbers did not change with age, while the naive CD4<sup>+</sup> T-cell pool was still being established (Figure 1A; Table 2). The observed combination of increasing naive and memory CD4<sup>+</sup> T-cell numbers without an increase of absolute TREC numbers suggests that a few months after birth peripheral T-cell division may have a much greater contribution to the establishment of the naive T-cell pool than was previously recognized. To assess levels of peripheral T-cell division in healthy children of different ages, we measured Ki67 expression on T cells in the blood of these children. Interestingly, the proportion of dividing Ki67<sup>+</sup> CD4<sup>+</sup> T cells was highest in infants and declined with increasing age. This was observed in naive and memory T cell subsets (Figure 3A; Table 2). These data are compatible with the concept that peripheral naive and memory T-cell division may

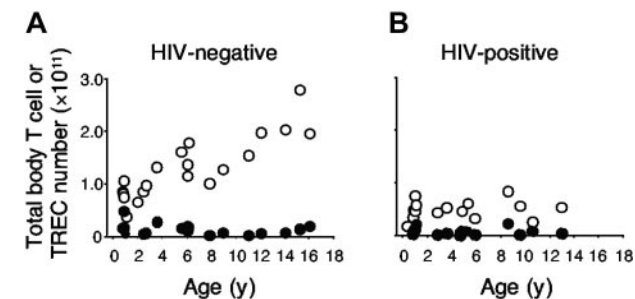


**Figure 2. Reduced total body naive and memory CD4<sup>+</sup> T-cell numbers in HIV infection.** (A) In healthy children (○), total body naive T-cell numbers increased over time, concomitant with an increase in CD27<sup>+</sup> and CD27<sup>-</sup> memory CD4<sup>+</sup> T cells (B-C). In children infected with HIV (●) expansion of the naive (A) and memory (B-C) CD4<sup>+</sup> T-cell pools was significantly reduced. See Table 2 for nonparametric Spearman correlation coefficients of all parameters with age.

contribute significantly to the establishment of the T-cell pool in young children.

### Mathematical model analysis of the establishment of the naive T-cell pool

Thus, experimental data obtained from healthy children suggested that peripheral proliferation of naive T cells is important in the postnatal expansion of the naive T-cell pool. Because it is difficult to test the likelihood of these findings experimentally, we chose an alternative approach by extending our previously developed mathematical model,<sup>11</sup> as described in “Patients, materials, and methods” (Figure 4A). The parameters used in this model were as much as possible based on experimental estimates (see the legend of Figure 4). We used the model as a “proof of principle” to test whether T-cell and TREC dynamics observed experimentally in healthy children could be mimicked in the presence of significant

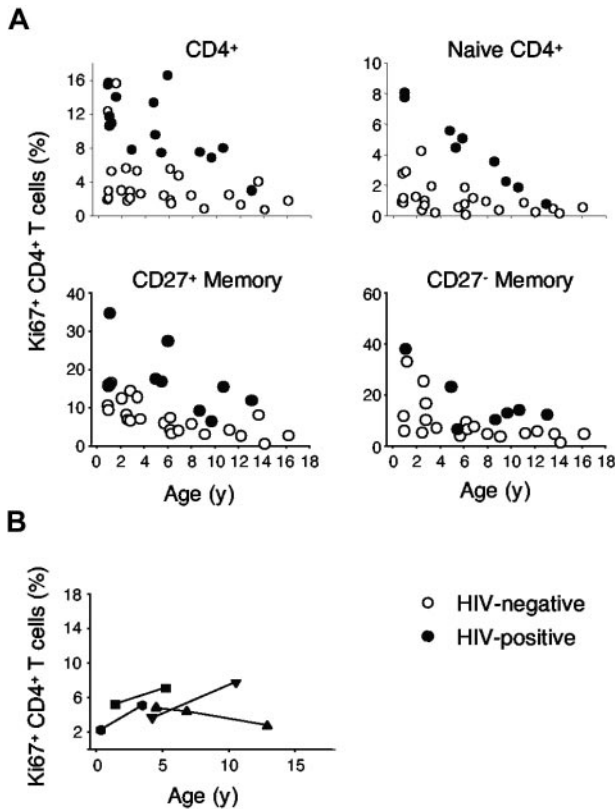


**Figure 1. Expansion of the CD4<sup>+</sup> T-cell pool while TREC numbers remained stable in healthy children.** (A) In healthy children, total body CD4<sup>+</sup> T-cell numbers (○) increased with age, but total body TREC numbers (●) did not change over time. (B) HIV-1 infection was associated with low and stable numbers of total body CD4<sup>+</sup> T cells (○) and TRECs (●).

**Table 2. Correlations between age and T-cell numbers, TRECs, or Ki67 expression**

	Healthy		HIV-1	
	r	P	r	P
Total body CD4 <sup>+</sup> T-cell number	0.853	<.001	0.190	.05
Naive CD4 <sup>+</sup>	0.487	.029	0.009	NS
CD27 <sup>+</sup> memory CD4 <sup>+</sup>	0.696	<.001	0.354	NS
CD27 <sup>-</sup> memory CD4 <sup>+</sup>	0.553	.011	0.165	NS
Ki67 <sup>+</sup> CD4 <sup>+</sup> T cells, %	-0.434	.034	-0.667	.009
Ki67 <sup>+</sup> naive CD4 <sup>+</sup>	-0.554	.005	-0.979	<.001
Ki67 <sup>+</sup> CD27 <sup>+</sup> memory CD4 <sup>+</sup>	-0.828	<.001	-0.686	.041
Ki67 <sup>+</sup> CD27 <sup>-</sup> memory CD4 <sup>+</sup>	-0.704	.001	-0.393	NS
Total body TREC number	-0.110	NS	-0.084	NS
Plasma HIV-1 RNA	NA	NA	-0.596	.019

Depicted are Spearman correlation coefficients (r) and P values for the correlation of each parameter with age in children HIV<sup>+</sup> (n = 16) and HIV<sup>-</sup> (n = 25). The data are shown in Figures 1 to 3 (except plasma HIV-1 RNA). NS indicates not significant; NA, not applicable.



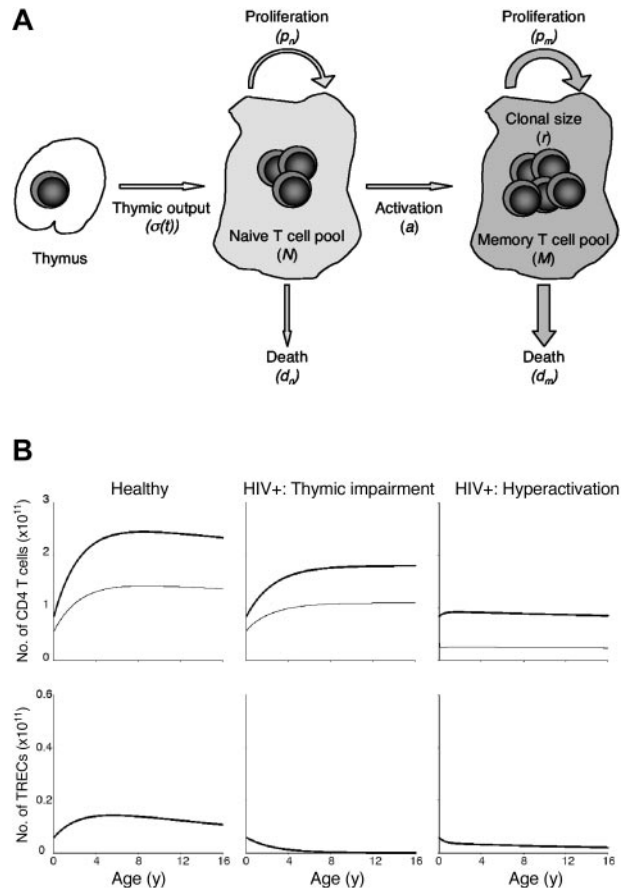
**Figure 3. Increased T-cell division in healthy children and children infected with HIV.** (A) The proportion of dividing naive and memory CD4<sup>+</sup> T cells was high in healthy infants (○) and declined with increasing age. In children infected with HIV (●), the proportion of dividing T cells was significantly higher than in healthy children. (B) The observed age-related decline in Ki67 expression in children infected with HIV most likely reflects a selection bias. Longitudinal analysis in a subset of older patients of whom cryopreserved samples of earlier time points were available showed that Ki67 expression by CD4<sup>+</sup> T cells was already low at those earlier time points. See Table 2 for nonparametric Spearman correlation coefficients.

naive T-cell proliferation. Indeed, in the model slowly increasing total body naive T-cell numbers until the age of 5, concomitant with a rise in the number of memory T cells and stable total TREC numbers over time (compare Figures 1 and 2 with Figure 4B, left panels) required that, quantitatively, the contribution of proliferation to the establishment of the T-cell pool was at least as large as the contribution of thymic output, supporting our hypothesis.

**HIV-1 infection interfered with expansion of the CD4<sup>+</sup> T-cell pool**

We then calculated total body CD4<sup>+</sup> T-cell numbers in a group of untreated children infected with HIV-1, based on the children’s body weights and T-cell numbers per microliter blood. Contrary to what we observed in healthy children, total body CD4<sup>+</sup> T-cell counts were low and stable in children of different ages positive for HIV (Figure 1B). Total (median [range] HIV<sup>-</sup>, 1.2 × 10<sup>11</sup> [3.8 × 10<sup>10</sup>-2.8 × 10<sup>11</sup>]; HIV<sup>+</sup>, 4.7 × 10<sup>10</sup> [3.5 × 10<sup>9</sup>-8.4 × 10<sup>10</sup>]) and naive (HIV<sup>-</sup>, 8.4 × 10<sup>10</sup> [3.2 × 10<sup>10</sup>-1.3 × 10<sup>11</sup>]; HIV<sup>+</sup>, 5.5 × 10<sup>10</sup> [4.1 × 10<sup>9</sup>-7.2 × 10<sup>10</sup>]) CD4<sup>+</sup> T-cell numbers and total body TREC numbers (HIV<sup>-</sup>, 1.4 × 10<sup>10</sup> [3.4 × 10<sup>9</sup>-4.9 × 10<sup>10</sup>]; HIV<sup>+</sup>, 6.2 × 10<sup>9</sup> [7.1 × 10<sup>7</sup>-2.2 × 10<sup>10</sup>]) were significantly reduced compared with healthy controls (*P* < .001, *P* = .001, and *P* = .046, respectively). CD27<sup>-</sup> memory CD4<sup>+</sup> T-cell numbers were significantly increased compared with healthy controls (HIV<sup>-</sup>, 3.0 × 10<sup>9</sup> [2.1 × 10<sup>8</sup>-5.5 × 10<sup>10</sup>]; HIV<sup>+</sup>, 6.4 × 10<sup>9</sup> [2.2 × 10<sup>9</sup>-

3.0 × 10<sup>10</sup>]; *P* = .012) and CD27<sup>+</sup> memory CD4<sup>+</sup> T-cell numbers were not affected by HIV infection (HIV<sup>-</sup>, 2.0 × 10<sup>10</sup> [5.1 × 10<sup>9</sup>-6.5 × 10<sup>10</sup>]; HIV<sup>+</sup>, 2.4 × 10<sup>10</sup> [2.1 × 10<sup>9</sup>-8.2 × 10<sup>10</sup>]; *P* = .48) (Figures 1-2). It should be noted that the scales of Figures 1 and 2 were deliberately set at a maximum of 3.0 × 10<sup>11</sup>, such that naive, memory, and TREC numbers are shown relative to total CD4<sup>+</sup> T-cell numbers and the contribution of each to the total CD4<sup>+</sup> T-cell pool size can be easily appreciated (Figures 1-2).



**Figure 4. Model analysis of the impact of thymic dysfunction or chronic immune hyperactivation on T-cell numbers.** (A) Cartoon representing the model as explained in “Patients, materials, and methods.” Briefly, the size of the naive T-cell pool (*N*) increases with thymic production of naive T cells (σ(*t*)) and naive T-cell proliferation (ρ<sub>*n*</sub>), and decreases by cell death (d<sub>*n*</sub>) and activation of naive T cells that acquire a memory phenotype (*a*). The memory T-cell pool size (*M*) increases by T-cell activation (*r* × *a*; *r* is the clonal size resulting from activation of a single naive T cell) and proliferation of memory T cells (ρ<sub>*m*</sub>) and decreases by memory T-cell death (d<sub>*m*</sub>). (B, left panels) The age-related changes in total body naive and memory T-cell and TREC dynamics in healthy children as described by the model accurately imitate changes observed experimentally (see Figures 1 and 2 for comparison). Parameters describing naive T-cell dynamics were adopted from Hazenberg et al<sup>11</sup> and are based on experimental data: d<sub>*n*</sub> = 0.001 per day, and ρ<sub>*n*</sub> = 0.1 per day. The number of TRECs per mature thymocyte was set to *c* = 0.2, to reach realistic total TREC numbers. On the basis of experimental estimates,<sup>1</sup> the rate of thymic involution was set to 4% (ie, *v* = 0.0001 per day). By setting σ<sub>0</sub> = 10<sup>9</sup> cells/day, the model reveals a thymic output of 4 × 10<sup>7</sup> cells per day for a 25-year-old individual, which seems appropriate.<sup>18,19</sup> Memory cells were assumed to proliferate and die several fold faster than naive T cells<sup>18,19</sup>: ρ<sub>*m*</sub> = 0.2 per day, d<sub>*m*</sub> = 0.02 per day, *a* = 0.0001 per day, and *r* = 100 cells. The parameter *h* was set to 2 × 10<sup>9</sup> cells, to scale the total peripheral T-cell pool to 2 to 3 × 10<sup>11</sup> cells.<sup>18</sup> The effect of HIV infection was simulated by reducing thymic output 100-fold (ie, σ<sub>0</sub> = 10<sup>6</sup> cells/day; middle panels) or by increasing T-cell activation and death rates (ρ<sub>*n*</sub> = 0.2, d<sub>*n*</sub> = 0.005, ρ<sub>*m*</sub> = 0.4, d<sub>*m*</sub> = 0.1, and *a* = 0.0002 per day; right panels). In the upper panels, thick lines represent naive T cells and thin lines the memory T cells. Modeling immune hyperactivation led to a better description of the experimentally observed reduced expansion of the naive and memory T-cell pools during HIV infection than thymic impairment.

Ki67 expression by CD4<sup>+</sup> T cells in infants infected with HIV was significantly higher than in healthy children (Table 1; Figure 3A). In fact, proportions of Ki67<sup>+</sup> CD4<sup>+</sup> T cells were even higher than previously reported for adults infected with HIV<sup>16</sup> (median for untreated HIV<sup>+</sup> adults, 8.1%; interquartile range [IQR], 2.5). In children infected with HIV the age-related decline in the proportion of Ki67<sup>+</sup> CD4<sup>+</sup> T cells was much more pronounced than in healthy children (Figure 3A). Plasma HIV-1 RNA levels also declined with age (Table 2) but did not correlate with Ki67 expression ( $r = 0.226$ ,  $P = .46$ ). Indeed, when controlling for plasma HIV-1 RNA, CD4<sup>+</sup> T cell Ki67 expression remained significantly associated with age ( $r = -0.664$ ,  $P = .018$ ). This is a cross-sectional study, however, and because all children (except 1, see “Patients, materials, and methods”) were infected in utero or at birth, children older than 8 years are long-term nonprogressors. As the level of CD4<sup>+</sup> T-cell activation has been shown to predict AIDS-free survival,<sup>23,24</sup> the observation of low T-cell activation in the older children infected with HIV most likely reflects a survival bias. Indeed, Ki67 expression in the older children was not considerably different from healthy control values, even when measured early in infection (Figure 3B).

#### Mathematical model analysis of the effect of thymic impairment and immune hyperactivation on the size of the T-cell pool

It has been hypothesized that naive T-cell depletion in HIV-1 infection may be caused by reduced naive T-cell production by the HIV-infected thymus and/or increased immune activation inducing erosion of the naive T-cell pool. We tested the effect of thymic impairment versus chronic immune activation on total body naive and memory T-cell numbers and TREC numbers in children infected with HIV by varying the values for the relevant parameters of the model. First, we studied HIV-induced impairment of the thymus by lowering thymic naive T-cell production as much as 100-fold, which resulted in a slower increase in total body naive and memory T-cell numbers with age compared with the healthy situation (Figure 3B, middle panels). However, the experimentally observed increase in the T-cell pool size in children infected with HIV was even more reduced. Therefore, we also studied the effect of increasing immune activation levels by increasing the naive and memory proliferation and activation rates 2-fold and their death rate 5-fold. Interestingly, this led to severe reductions in growth of the total body naive and memory CD4<sup>+</sup> T-cell pools, comparable to what was observed experimentally (Figure 4B, right panels).

## Discussion

To better understand the impact of HIV infection on T-cell dynamics in children one first needs to understand how the T-cell pool is established in healthy children. Whereas T-cell numbers per blood volume decrease with age, we found that total body CD4 T-cell numbers (T-cell numbers per blood volume corrected for the age-related increase in body mass and blood volume) actually increased with age, despite the well-described concomitant decline in thymic size.<sup>1</sup> Interestingly, total body numbers of TRECs remained stable over time. TRECs are the by-products of  $\alpha\beta$ T-cell receptor gene rearrangements that are thought to occur exclusively in the thymus. However, neither T-cell phenotype nor TREC analysis allows for reliable quantification of recent thymic emigrants and thereby actual thymic function, because naive TREC-

containing T cells seem to be long lived in humans,<sup>18,21</sup> making it difficult to separate recently produced thymic emigrants from long-lived “older” naive T cells. Total body TREC numbers rather reflect the cumulative result of past and ongoing thymic T-cell production and death of TREC<sup>+</sup> T cells. Thus, taking into account the high frequency of dividing naive and memory T cells, especially in infants, compared with healthy adults,<sup>16</sup> the dichotomy that we observed between changes of naive CD4<sup>+</sup> T-cell numbers (increasing) and TREC numbers (stable) in healthy children may be best explained by peripheral expansion of naive T cells. These data suggest that peripheral expansion may be an important factor in the establishment of the naive and memory T-cell pools.

Also in mice, T-cell division levels were found to be increased after birth and to decline with increasing age.<sup>25</sup> Because neonatal mice had very low T-cell numbers per spleen, this increased T-cell division was attributed to homeostatic mechanisms in response to low T-cell numbers.<sup>26</sup> However, when expressed per (kilo)gram of body weight, both murine and human neonates actually have large numbers of T cells, suggesting that their T-cell compartments are not “empty” at all. Thus, alternatively, increased T-cell division in young mice and children is more likely to be driven by high interleukin 7 (IL-7) levels,<sup>27</sup> by other growth factors, and by de novo antigen exposure in the first years of life. Indeed, it has recently been shown that even during fetal life, mature T-cell responses can be elicited by viral and parasite infections,<sup>28-30</sup> suggesting that the immune system of newborns and infants is more robust than previously assumed.

The data presented here refine conclusions by Mackall et al<sup>31</sup> that in T-cell-depleted children, immune reconstitution is mainly dependent on thymic function. In that study the relative contribution of the thymus to T-cell reconstitution was estimated based on recovery of phenotypically naive T-cell numbers, assuming that naive T cells are only produced by the thymus. Whereas the T-cell pool can expand only when the thymus has provided an initial critical number, our data suggest that in young healthy children, the contribution of peripheral expansion to the establishment of the naive T-cell pool is at least of the same order of magnitude as the contribution of the thymus. The extent to which peripheral expansion of naive T cells, driven by, for example, IL-7,<sup>32</sup> plays a role in the recovery of the naive T-cell pool in T-cell-depleted children remains to be determined.

In our cohort of children infected with HIV, total body T-cell and TREC numbers were significantly lower compared with healthy children. Interestingly, T-cell division levels were very high, in naive and memory T-cell subsets. In adults, it has been well documented that HIV-1 infection is characterized by persistently increased immune activation, the level of which is highly predictive of disease progression, independent of plasma HIV-1 RNA or CD4<sup>+</sup> T-cell numbers.<sup>33,34</sup> It has been postulated that because in adults naive T cells are difficult to replace once they are lost, continuous activation and recruitment of naive T cells (eg, by HIV-1 infection but also in non-HIV-related chronic immune activation<sup>35</sup>), may lead to erosion of the naive T-cell pool.<sup>12,13</sup> In children that have been T cell depleted, recovery of T-cell numbers is faster than in adults,<sup>31</sup> but when compared with age-matched control subjects, normal values were seldom reached.<sup>36,37</sup> Because these data suggest that even in children naive T-cell loss cannot be compensated for, we hypothesized that chronic immune activation in children positive for HIV could play an equally important role in naive T-cell depletion as observed for adults infected with HIV. To

illustrate this, we increased T-cell division, activation, and death rates several fold in our mathematical model. This led to a significant decline in total body naive and memory T-cell and TREC numbers, comparable to what was observed experimentally. In contrast, even a 100-fold reduction of thymic output at a young age affected total body naive T-cell and TREC numbers to a much lesser degree. Indeed, children with long-term nonprogressing HIV (those older than 8 years of age) had consistently low levels of immune activation, independent of viral load, supporting our hypothesis.

These data in the present cross-sectional study were obtained from a relatively small group of healthy children and children infected with HIV and, therefore, have to be interpreted with caution, both when significant and nonsignificant effects were observed. However, with these limitations in mind, our study offers novel insights in the dynamics of the peripheral T-cell pool in children of different ages and warrants further studies on the contribution of thymic emigrants to the formation of the T-cell pool in healthy and T-cell-depleted children.

Taken together, our data suggest that in healthy children, increased naive T-cell division leads to expansion of the naive T-cell pool, whereas in untreated children infected with HIV-1, even higher increased naive T-cell division leads to exhaustion of the naive T-cell pool. This paradox can be explained if in the healthy situation, naive T-cell division is driven by cytokines such as IL-7 or growth factors such as growth hormone, the levels of which are negatively correlated with age.<sup>38</sup> Indeed, it has been shown that non-antigen-mediated naive T-cell division induces

naive T-cell expansion without transition of naive T cells to the memory T-cell pool.<sup>39,40</sup> Nevertheless, some of the naive T-cell division may be transitional, reflecting antigen-mediated activation of naive T cells and resulting in differentiation into memory T cells. In untreated HIV-1 infection, it is assumed that most of the increased naive T-cell division is antigen-mediated, leading to increased consumption of naive T cells and erosion of the naive T-cell pool instead of expansion.<sup>12,13,40</sup>

In conclusion, these data suggest that in healthy children, peripheral expansion of naive T cells plays a more important role in the establishment of the naive T-cell pool than previously anticipated, and that in children infected with HIV chronic immune activation can lead to low total body naive and memory CD4<sup>+</sup> T-cell numbers. It has been shown recently that when compared with age-matched control values, in most children infected with HIV on HAART who were older than 2 years of age, naive T-cell numbers increased but never reached completely normal levels.<sup>36</sup> This suggests that the generation of a normal size T-cell pool in children is critical, and that early interference with establishment of the T-cell pool by HIV-1 may be difficult to overcome.

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