T-cell dynamics in healthy and HIV-infected individuals

Nienke Vrisekoop

COLOFON

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Introduction

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Chapter **1**

The unusual occurrence of opportunistic infections and malignancies in young gay men, now known as acquired immune deficiency syndrome (AIDS), was first recognized in the United States in 1981 [1,2]. The virus that caused this new syndrome was isolated in 1983 and termed human immunodeficiency virus (HIV) [3-7]. At the end of 2006, an estimated 39.5 million people were living with HIV and 2.9 million people died of AIDS in that same year (UNAIDS report 2006).

Acute HIV infection is characterized by a peak in viral load and concomitant immune activation as measured by T-cell proliferation [8,9]. It is now clear that a major part of the memory $CD4^+$ T-cell pool in the gut is irreversibly lost in the first weeks of infection [10,11]. The subsequent chronic stage of infection is accompanied by a stable viral load and gradually declining $CD4^+$ T-cell numbers. Although HIV viral load is relatively stable in this period, the virus has a short half-life and is persistently replicating at very high levels [12]. The loss of $CD4^+$ T cells eventually leads to susceptibility to opportunistic infections and ultimately death.

Although the progressive decline in $CD4^+$ T-cell numbers is the hallmark of HIV infection, the mechanisms behind this depletion remain controversial. Currently the most prevailing ideas include direct HIV-induced cytopathicity [10,11], thymic impairment [13-15] and chronic immune activation [16]. The scope of the first part of this thesis was to further delineate the importance of these different mechanisms in $CD4^+$ T-cell depletion.

Since the introduction of highly active antiretroviral therapy (HAART) many studies have investigated the effect of treatment on the reconstitution of the $CD4^+$ T-cell pool. After 3 to 5 years of HAART, $CD4^+$ T-cell counts had not reached normal levels in the majority of patients [17-19], raising the question whether full T-cell reconstitution is in principle possible during long-term HAART and, if so, whether this recovery can be sustained during aging. Both thymic output and peripheral T-cell proliferation can contribute to T-cell reconstitution. The source of the newly produced T cells that are formed during reconstitution might have a strong effect on the replicative history, and thereby on the "age" of the T-cell pool. In the second part of this thesis we studied the effects of long-term HAART on $CD4^+$ T-cell reconstitution and the composition of the T-cell pool in HIV-infected adults and children.

In chickens and mice it has been shown that recent thymic emigrants (RTE) form a substantial pool of short-lived naive T cells [20,21]. In humans however, the lifespan and number of RTE are unknown. Currently there is no unambiguous marker for cells that have recently emigrated from the thymus. Therefore, the contribution of the thymus to the maintenance of the T-cell pool during aging, the role of thymic impairment in HIV-related $CD4^+$ T-cell depletion and the role of thymic output during reconstitution following HAART are difficult to assess.

T-cell receptor excision circles (TRECs) are formed during T-cell receptor rearrangement in the thymus and have been used as a surrogate marker for thymic output. Since TRECs are not duplicated upon cell division and are divided over the daughter cells, the density of

TRECs in a T-cell population gives information on the replicative history of that T-cell population. Because CD31 expressing naive $CD4^+$ T cells were shown to have higher TREC contents than CD31 negative naive T cells, it was postulated that CD31 could discriminate between RTE and naive T cells that have undergone peripheral proliferation [22]. The second part of this thesis is continued with investigations on the potential use of CD31 as a RTE marker, and studies on CD31 and TREC dynamics during aging and over HIV seroconversion. To determine whether a short-lived RTE pool contributes significantly to the human naive T-cell pool, as has been shown in mice, we used *in vivo* heavy water labeling of T cells from young healthy adults, and determined the number of naive and memory $CD4^+$ and $CD8^+$ T cells that are newly produced per day, and followed the survival of these recently produced T cells in the peripheral T-cell compartment.

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Part I

Immune activation

T-cell dynamics and the role of apoptosis in HIV infection

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Chapter 2

Introduction

$CD4^+$ T-lymphocyte loss is one of the hallmarks of human immunodeficiency virus (HIV) infection. Because the percentage of T cells undergoing apoptosis is dramatically increased during HIV infection, it may not seem surprising that $CD4^+$ T cells are progressively lost during HIV infection. There is no consensus, however, on the mechanisms behind increased T-cell apoptosis in HIV infection and on the reason why HIV induces an instant loss of $CD4^+$ T cells while $CD8^+$ T-cell numbers decline only at late stages of infection. Even though our insights into the different effects of HIV on the immune system have improved a lot during the last decades, many open questions remain.

Ameisen and Capron [1] were the first to propose that $CD4^+$ T-cell loss in HIV infection might be due to inappropriate induction of programmed T-cell death. They proposed that HIV primes $CD4^+$ T cells to a state in which they become very sensitive to T-cell apoptosis upon further stimulation. Factors proposed to be involved in HIV-induced inappropriate apoptosis may include direct cytopathicity of HIV in productively infected $CD4^+$ T cells, and apoptosis of $CD4^+$ T cells via CD4 cross-linking by viral proteins. Later it was argued that $CD4^+$ T cells are eventually lost in HIV infection, because the immune system gets exhausted by continuously trying to replenish the $CD4^+$ T-cell pool [2], while others have argued that the key problem in HIV infection is interference with T-cell renewal, because HIV induces increased apoptosis in the thymus [3-5]. In contrast, we and others have proposed that apoptosis in HIV infection is a natural result of increased immune activation induced by the virus, and that it is this state of chronic immune activation that leads to the progressive loss of $CD4^+$ T cells in HIV infection [6,7]. In this chapter, the strengths and weaknesses of the different explanations for increased T-cell apoptosis and its relation to $CD4^+$ T-cell loss during HIV-infection are reviewed.

Inappropriate T-cell apoptosis: direct HIV-induced cytopathicity and CD4 cross-linking

The idea that $CD4^+$ T-cell loss in HIV infection is due to direct killing of infected $CD4^+$ T cells by the virus was fueled by *in vitro* studies in which $CD4^+$ T cells that were infected in culture were shown to undergo apoptosis [8], and by the well-described positive correlation between HIV viral load and the rate of disease progression [9,10]. A number of experimental observations cannot, however, easily be reconciled with this hypothesis.

First, if most $CD4^+$ T-cell loss were to be due to direct killing of HIV-infected cells, one would expect a strong correlation between the number of infected $CD4^+$ T cells and disease progression. Surprisingly, however, the number of infected cells appeared to correlate poorly with plasma viral RNA load [11], which is known to be one of the strongest predictors of disease progression. Additionally, the level of *in vitro* cytopathicity of HIV appeared not to correlate with the extent of $CD4^+$ T-cell depletion, as no difference in $CD4^+$ T-cell loss could be found between SCID-hu mice infected with noncytopathic or cytopathic HIV isolates [12]. Also in simian immunodeficiency virus (SIV)-infected primates, viral cytopathicity did not correlate with disease progression. *In vitro*, SIV is equally cytopathic to rhesus macaque and

sooty mangabey lymphocytes. However, SIV infection in rhesus macaques is associated with $CD4^+$ T-cell loss and progression to AIDS while $CD4^+$ T-cell numbers in sooty mangabeys are not affected by SIV infection [13].

Second, several investigators argued that the number of productively infected $CD4^+$ T cells is too low to account for all the $CD4^+$ T-cell loss occurring during HIV infection. Hardly any (<0.01%) plasma virus was found to be infectious [10], and replication-competent integrated HIV-1 DNA appeared to be present in only a minor (<0.05%) fraction of susceptible T cells [11]. However, if infected cells are lost rapidly, low numbers of infected cells measured at any moment in time may still add up to a large total number of infected cells. Nevertheless, the T-cell subset undergoing apoptosis seems much larger and easier to detect than the subset of T cells that are productively infected, suggesting that T-cell apoptosis is not confined to the relatively small number of productively infected T cells. Indeed, when lymph node biopsies from HIV-infected children or SIV-infected macaques were analyzed *in situ*, only a minor fraction of apoptotic lymphocytes were found to be productively infected with HIV and, vice versa, only few productively infected cells turned out to undergo apoptosis [14]. Hence, the bulk of $CD4^+$ T-cell loss cannot be explained by direct cytopathicity of the virus.

Thus, direct cytopathicity alone does not account for the increased apoptosis levels observed in HIV infection. *In vitro* studies suggested that the viral protein gp120 can induce cross-linking of CD4 molecules on the T-cell surface, which, in the absence of T-cell receptor triggering, leads to $CD4^+$ T-cell apoptosis [15-17]. Similarly, the viral proteins Tat and Nef have been shown to exert apoptotic effects on $CD4^+$ T cells [18]. Indeed, the latter mechanisms of HIV-induced $CD4^+$ T-cell apoptosis may play a larger role in $CD4^+$ T-cell depletion *in vivo* than direct cytopathicity, as they are not confined to the relatively small fraction of T cells that are productively infected by HIV.

Interestingly, however, data from our laboratory and from others showed that T-cell apoptosis in HIV infection is not limited to $CD4^+$ T cells. In fact, the highest percentage of cells undergoing apoptosis during HIV infection is typically observed in the $CD8^+$ T-cell population [17,19-21]. Thus, although the different mechanisms described above offer an explanation as to why levels of apoptosis are increased in the $CD4^+$ T-cell pool, they fail to explain most of the T-cell apoptosis, i.e. that occurring in the $CD8^+$ T-cell pool, during HIV infection.

T-cell exhaustion induced by continuous high T-cell turnover

A seminal paper from David Ho's laboratory changed the insights into HIV dynamics altogether by showing that the viral population in infected individuals is highly dynamic, even during the asymptomatic phase of HIV infection, in which the total viral load is known to be relatively stable. Ho and collaborators showed that after initiation of highly active antiretroviral treatment (HAART), the HIV viral load decreases very rapidly. The authors argued that before treatment was started, the virus must have turned over just as rapidly,

but that the rapid loss of viral particles pretreatment remained invisible, because it was balanced by high virus production. When HAART prevented the generation of new virus, the rapid decay of the virus suddenly became evident [2]. In the same paper, Ho et al. reported a very rapid increase in $CD4^+$ T-cell counts after the initiation of HAART, and argued along the same lines that $CD4^+$ T cells were apparently destroyed and replaced at a rapid pace during untreated HIV infection. It was proposed that $CD4^+$ T cells are eventually lost during HIV infection, because the immune system cannot cope with the persistent high $CD4^+$ T-cell turnover that is required to keep the T-cell pool at its homeostatic level [2].

We studied if one could find any evidence for such high turnover-induced exhaustion of the $CD4^+$ T-lymphocyte pool in HIV-infection. To this end, we have measured $CD4^+$ T-cell telomeres, which are known to shorten upon T-cell division, in HIV-infected individuals and age-matched healthy controls. A long history of increased $CD4^+$ T-cell proliferation in HIV infection should be apparent by a shortened average telomere length in HIV-infected individuals compared to controls. Surprisingly, however, we found no reduction in the average $CD4^+$ T-cell telomere length, but did find a significant shortening of the $CD8^+$ T-cell telomeres in HIV patients compared to healthy controls [22]. Thus, if anything, the $CD8^+$ T-lymphocyte pool in HIV^+ individuals seemed to have a more extensive history of increased T-cell division than the $CD4^+$ T-cell pool. In addition, studies from our laboratory and other laboratories showed that the initial steep rise in $CD4^+$ T-cell counts following the start of HAART is most likely caused by redistribution of lymphocytes from the lymphoid tissues to the peripheral blood rather than by rapid production of new $CD4^+$ T cells. Immune reconstitution in HIV-infected adults on HAART appeared to occur biphasically due to rapid redistribution of memory T cells and a gradual increase of naive T cells, and this seldom leads to normalization of the $CD4^+$ T-cell count [23-25]. The rapid increase in $CD4^+$ T-cell numbers upon initiation of HAART thus does not imply that $CD4^+$ T cells have a short half-life during untreated HIV infection. We and others estimated $CD4^+$ T-cell dynamics during HIV infection by measuring Ki67 expression and using BrdU labeling, and came to the conclusion that $CD4^+$ T-cell turnover during HIV infection is increased maximally only three- to fivefold [26-29]. Interestingly, $CD8^+$ T-cell turnover is also increased severalfold during untreated HIV infection [30]. The reason for this increased T-cell turnover remains controversial.

Several researchers proposed that $CD4^+$ T-cell proliferation rates in HIV infection are increased because of homeostatic mechanisms that are compensating for $CD4^+$ T-cell loss [2,31]. We refer to this hypothesis as the "pull hypothesis": the immune system is trying to keep the total T-cell number at a fixed homeostatic level, and when many cells are lost due to HIV infection, lots of new cells have to be generated. In contrast, we and others have proposed that increased T-cell proliferation in HIV infection is directly caused by the virus, which is chronically stimulating the immune system, and that T-cell loss may be a result of this chronic immune stimulation [7] The latter hypothesis, referred to as the "push hypothesis," will be later discussed.

If increased T-cell turnover rates in HIV infection would reflect a homeostatic response to low numbers of $CD4^+$ T cells, one would expect T-cell turnover to remain increased as long as $CD4^+$ T-cell numbers are below the normal homeostatic level. We showed, however, that the increased T-cell turnover characteristic of HIV infection is rapidly lost during HAART, despite sustained low $CD4^+$ T-cell numbers [30]. This, and the observation that $CD8^+$ T-cell division rates are also increased during untreated infection, argues against the idea that increased T-cell turnover during HIV infection is due to a homeostatic response.

These data also show that T-cell proliferation may hardly be increased during a phase of immune recovery. It is generally assumed that during a phase of T-cell depletion homeostatic responses (e.g., increases in thymic function and/or peripheral T-cell proliferation) drive T-cell recovery. However, in a study of 22 lymphopenic patients with hematological malignancies who received allogeneic HLA-matched peripheral stem cell transplantation, we found no evidence for increased thymic function. T-cell division was increased only in a subset of lymphopenic patients, was specifically related to clinical events, such as graft versus host disease or episodes of infectious diseases, and proportions of dividing cells declined rapidly after transplantation despite still very low T-cell numbers [32]. Together, these studies cast serious doubts on the natural homeostatic capacities of the immune system during lymphopenia.

Interference with thymic function

Maintenance of a full peripheral $CD4^+$ T-cell pool may not only be hampered by HIV-induced peripheral T-cell death, but also by a lack of *de novo* T-cell generation [33]. Because thymopoiesis is generally thought to be the main source of T-cell generation, it has been proposed that HIV-induced apoptosis of thymocytes in the presence of slightly increased – or even normal [4] – peripheral T-cell death might explain why $CD4^+$ T-cell numbers are gradually lost during asymptomatic HIV infection [3,5,34]. Interference with thymic function would also explain why HIV infection is associated with a gradual decline in both naive $CD4^+$ and naive $CD8^+$ T-cells [35,36]. However, current data do not unequivocally show that thymic output is severely decreased throughout HIV infection, and even if it was, recent data has brought into question the conclusion that this could explain a severe loss of $CD4^+$ T cells in the periphery.

Experiments in mice and data from humans do suggest that HIV is able to infect the thymus. To analyze the effect of HIV infection on thymopoiesis in vivo, McCune and collaborators used the SCID-hu mouse model, in which human fetal liver and thymus are implanted under the kidney capsule of immunodeficient SCID mice [37,38]. These mice show T lymphopoiesis for profound periods of time, and the implanted thymus is permissive for infection with HIV [39,40]. Upon intrathymic HIV injection, infected cells and increased programmed cell death could be seen throughout the thymus, leading to a loss of $CD4^+CD8^+$ double-positive thymocytes, a decrease of the $CD4^+$:$CD8^+$ thymocyte ratio, and an overall reduction of thymocyte cellularity [3]. Importantly, HIV infection in the SCID-hu mouse may give rise to more severe effects on the thymus than during natural HIV

infection, because HIV in this model system is typically injected directly into the thymus. Evidence for HIV-induced thymocyte loss is, however, not restricted to the SCID-hu mouse model, as it has also been observed in some fetuses aborted from HIV-infected mothers [41], in thymus biopsies from HIV-infected children [42,43], in thymic tissue studied at autopsy from individuals who died of HIV infection [44], and in SIV-infected rhesus monkeys [45]. It should be noted, however, that so far mostly syncytium-inducing (SI) HIV isolates were shown to interfere with thymopoiesis. When SCID-hu mice were infected with non-SI (NSI) HIV variants, changes in thymus composition occurred much more slowly [46,47], suggesting that loss of thymic output may only play a role in peripheral $CD4^+$ T-cell loss relatively late during disease progression. In a rhesus monkey model of HIV infection, loss of thymic output could also only be observed at late stages of disease, just before the animals developed AIDS-like symptoms [48]. However, because of a lack of direct tools to measure ongoing thymic function *in vivo*, it remains to be determined how abrogation of thymic function affects peripheral blood T-cell numbers.

To study more directly if HIV infection impairs thymic output in humans, an *ex vivo* marker of recent thymic emigrants would be helpful. Measuring the number of T cells with a naive phenotype does not suffice to measure thymic output because naive T cells are relatively long-lived and may retain their naive phenotype upon homeostatic proliferation. Recently, several laboratories tried to quantify thymic output more directly by measuring T-cell receptor excision circles (TRECs), the DNA by-products of T-cell receptor gene rearrangements [49]. As TRECs are extrachromosomal DNA circles, they are not copied during T-cell division and are, therefore, only produced upon *de novo* T-cell generation in the thymus. The fact that the average TREC content (i.e. the average number of TRECs per T cell) of $CD4^+$ and $CD8^+$ T cells decreases with age was taken to reflect the age-related involution of the thymus, and thereby was taken as evidence that T-cell TREC contents can be used as a reliable measure of thymic output [5,50]. We showed, however, that TREC data need to be interpreted with great caution, because not only thymic output, but also T-cell death and proliferation influence the average TREC content of a T-cell population [51,52].

In HIV-infected individuals the average TREC content of the naive $CD4^+$ and $CD8^+$ T-cell population is typically decreased as compared to healthy controls. Taking the TREC content of the naive T-cell population as a direct measure of thymic output, it was argued that thymic output is severely reduced even during the asymptomatic phase of HIV infection. Using a mathematical model, we showed, however, that decreased TREC contents in HIV-infected individuals are much more likely due to increased T-cell proliferation than to thymic impairment. In fact, because of the longevity of naive T cells, a decrease in thymic output hardly affects the TREC content of the naive T-cell population [51]. The TREC contents of peripheral T cells from thymectomized individuals indeed start to decrease only years after thymectomy [53]. Because there is ample evidence for increased T-cell proliferation during HIV infection, decreased TREC contents in HIV-infected individuals should thus not be taken as evidence for decreased thymic output.

It remains to be determined to what extent infection of the thymus contributes to HIV disease progression. In fact, even if HIV infection of the thymus was to decrease the effective thymic output, it is not known to what extent that would affect the peripheral T-cell population. In a small group of HIV-infected individuals who were thymectomized for myasthenia gravis before HIV infection, thymectomy did not preclude long-term survival. Increases in $CD4^+$ T-cell numbers when those thymectomized individuals were treated with HAART turned out to be similar to those in non-thymectomized HIV-infected individuals on HAART [44].

Thymic output in adults was estimated to be low, in the order of 10^7 to 10^8 naive T cells per day [29]. Because of this, naive T-cell reconstitution following T-cell depletion of various causes such as bone marrow/stem cell transplantation, monoclonal antibody treatment [54-56], and HIV infection [25], is very slow, and thymectomy in HIV-negative individuals does not significantly affect peripheral blood T-cell numbers [53]. It can thus be expected that in adults, HIV-induced abrogation of thymic function will not significantly change peripheral blood T-cell numbers. However, in children, in whom thymic function is optimal, HIV infection of the thymus may contribute significantly to HIV-induced naive T-cell loss, but only if thymic function plays a major role in daily T-cell homeostasis. To study this, we followed changes in total body T-cell numbers and TREC numbers with age in a group of healthy children of different ages. We studied total body TREC numbers instead of TREC contents, because total TRECs are not influenced by T-cell proliferation and are thus a more direct measure of thymic output than TREC contents [52]. If the major source of T cells in young children was to be the thymus, one would expect the dynamics of T-cell and TREC numbers to be similar. Instead, we found large discrepancies between the two. While total body naive T-cell numbers increased during the first years of life, total TREC numbers remained stable during this period, suggesting that the increase in naive T-cell numbers was largely due to peripheral T-cell proliferation [57]. Thus, even in children, thymic output seems to be less critical in maintaining or increasing peripheral blood naive T-cell numbers, and HIV-related impairment of thymic function may, therefore, not have a significant impact on peripheral blood T-cell numbers. Even complete abrogation of thymic output in juvenile macaques who were thymectomized was shown to have very little impact on the peripheral T-cell compartment, both in healthy and in SIV-infected macaques [58,59].

Together, these data suggest that inappropriate apoptosis of maturing T cells in the HIV-infected thymus may hamper normal thymic function, but that its effect on peripheral blood T-cell numbers may not be significant, both in children and in adults.

Appropriate T-cell death: chronic immune activation in HIV pathogenesis

Because the moderate increase in $CD4^+$ and $CD8^+$ T-cell turnover in HIV infection is not likely to be a homeostatic response to compensate for $CD4^+$ T-cell loss (earlier referred to as the "pull" hypothesis), the alternative "push" hypothesis was put forward. As already mentioned, we and others proposed that the increased levels of T-cell proliferation and the preferential loss of naive T cells during HIV-1 infection are caused by chronic immune

activation in response to the persistently active virus, and hypothesized that $CD4^+$ T-cell loss may be an "appropriate" result of this chronic immune stimulation [6,7,30,60].

One of the strongest arguments for the idea that HIV induces increased T-cell proliferation is that shortly after initiation of HAART, when viral reproduction is already prevented but $CD4^+$ T-cell numbers are still low, T-cell proliferation decreases dramatically [30]. Moreover, increases in the expression of the proliferation marker Ki67 during HIV-1 infection are not confined to the $CD4^+$ T-cell pool and are, in fact, even more profound in the $CD8^+$ T-cell pool, which becomes depleted only in the final stages of HIV infection [26,27,29,30]. In line with this observation, HIV-1 induced telomere shortening [22], T-cell apoptosis [17,20,21,61] and TREC dilution [51] were found to be most pronounced in the $CD8^+$ T-cell population.

If chronic immune activation is a key player in HIV-1 pathogenesis, one would expect the level of immune activation post-seroconversion to be associated with disease progression. High proportions of proliferating $Ki67^+CD4^+$ and $Ki67^+CD8^+$ T cells post-seroconversion, and high levels of the activation markers CD70, CD38, and HLA-DR expressed by $CD4^+$ and $CD8^+$ T cells were indeed found to be associated with rapid disease progression [62-64]. In fact, the level of immune activation turned out to be an even better predictor for disease progression than HIV viral load [63,64]. Interestingly, $CD8^+$ T-cell activation was found to correlate positively with HIV viral load [63,65,66], which is strongly associated with the rate of disease progression. Because $CD8^+$ T cells play an important role in controlling viral replication, in principle, such a positive correlation would not be expected, unless chronic T-cell activation is the key problem in HIV infection.

Interestingly, we found that increased levels of immune activation before seroconversion are also predictive for disease progression after an individual becomes infected with HIV. In a study of homosexual men, we showed that low $CD4^+$ T-cell counts and high CD70 expression by $CD4^+$ T cells before seroconversion were associated with fast disease progression upon HIV infection [64]. Similarly, HIV-1-infected injecting drug users with low $CD4^+$ TREC contents pre-seroconversion showed a steeper decline in $CD4^+$ T-cell count throughout infection than those with high pre-seroconversion $CD4^+$ TREC contents [67]. Thus, the level of immune activation prior to HIV-1 infection seems to affect disease progression.

Also during HAART the level of immune activation was shown to be correlated with changes in $CD4^+$ T cell counts [66,68]. HAART causes a rapid decrease in plasma virus load, thereby alleviating chronic immune activation, leading to an immediate decrease in T-cell proliferation and apoptosis levels. High residual levels of immune activation during HAART turned out to be associated with poor immune reconstitution, again underscoring the central role of immune activation in HIV infection [66].

Based on these data, the alternative paradigm emerged that hyperactivation of the immune system is the key factor in HIV pathogenesis, and that increased levels of T-cell apoptosis are a natural result of this hyperactivated state of the immune system [7,60]. High levels of

apoptosis were not only described for HIV infection but also, for example, during the acute phase of immune response in mice infected with acute lymphocytic choriomeningitis virus (LCMV) [69] and humans infected with Epstein-Barr virus [70,71] or cytomegalovirus [72]. Meanwhile many additional data have been found to support this view.

In vivo labeling studies

Recent T-cell labeling studies using BrdU, deuterated glucose and deuterated water confirmed that T-cell turnover is increased in both the $CD4^+$ and $CD8^+$ T-cell population of HIV-infected individuals [28,73-76]. Upon half an hour *in vivo* pulse-labeling of T cells from HIV-infected individuals with BrdU, Kovacs et al. [75] identified at least two distinct T-cell subpopulations, one characterized by a slow and the other by a faster rate of T-cell turnover. HIV infection turned out to increase the average turnover of $CD4^+$ and $CD8^+$ T lymphocytes by activating resting cells and driving these cells into the rapidly proliferating subpopulation. Interestingly, however, HIV did not affect the turnover of either of the two subpopulations, and HAART decreased the proportion of labeled cells in the rapidly proliferating pool but did not affect the decay rate of labeled cells.

Ribeiro et al. [76] reported that after a 7-day infusion of deuterated glucose, an increased transition of $CD4^+$ T cells from the resting to the activated compartment could be measured in HIV-infected individuals compared to healthy controls. In contrast to Kovacs et al. [75], Ribeiro et al. found an HIV-related increase in proliferation and death rates of activated $CD4^+$ T cells [76]. This discrepancy between the two studies may be due to the different labeling techniques. Whereas cessation of label administration leads to an immediate decay of deuterium labeled DNA, BrdU-labeled cells show much slower decay kinetics. Because BrdU measures the proportion of positive cells rather than label-positive DNA strands (as in the case of deuterium studies), cell division in the absence of label infusion can actually increase the amount of $BrdU^+$ cells, because the label is divided equally over the daughter cells, rendering them $BrdU^+$. Thus, the net decay rate of $BrdU^+$ cells in the presence of continuous cell division will be lower than the actual death rate [77].

A general problem of *in vivo* labeling techniques such as those described here, is that they are biased towards measuring the dynamics of the T-cell population with the highest turnover. Whereas the cells that pick up the label are truly representative of the whole T-cell population, including rapidly and slowly dividing T-cell populations, measuring the rate at which the label is lost upon label retraction is biased because of the dominance of the specific population of T cells with rapid turnover [60,78]. It is, therefore, not surprising that such division-dependent labeling techniques consistently predicted higher T-cell death rates than proliferation rates. The longer the label is administered, the more closely the estimated T cell death rate will get to the true average death rate of the whole T-cell population [78]. In this respect, the recent introduction of deuterated water labeling is a big step ahead, as it provides a non-toxic alternative for BrdU labeling, and can more easily be administered for prolonged periods of time compared to deuterated glucose.

Despite the above-mentioned shortcomings of these labeling techniques, these studies, which are the first to show *in vivo* T-cell kinetics, confirmed the hypothesis that T cells that recently divided and, hence, picked up the label, tend to have a relatively short life span [7,60]. The fact that recently divided cells in HIV-infected patients are prone to die argues against a homeostatic response to $CD4^+$ T-cell depletion and is consistent with the idea that T-cell division and death is driven by immune activation.

HIV-infected individuals with discordant responses to antiviral therapy

Another argument in favor of the idea that immune activation is the key player in HIV infection comes from HIV-infected individuals who show discordant responses to antiviral therapy. Most patients treated with antiretroviral therapy show a significant decline in viral load concomitant with an increase in peripheral blood T-cell numbers. However, some patients are only virologically responsive to antiviral therapy, but do not show immunologic improvement ("virologic responders") while others are only responsive in immunological terms, showing increases in peripheral blood T-cell numbers despite detectable viremia ("immunologic responders"). Immunologic responders have lower levels of T-cell activation, proliferation, and apoptosis compared to untreated HIV-infected individuals, despite the presence of detectable virus, and show sustained $CD4^+$ T-cell gains [68,79,80]. Thus, antiviral therapy can have beneficial effects in terms of $CD4^+$ T-cell counts even in the presence of detectable plasma viremia, most likely because it reduces the level of immune activation. On the other hand, in patients with poor immunologic outcome despite good virologic responses, high *ex vivo* rates of apoptosis have been found [81]. In general, immunologic discordant responders have a favorable clinical outcome compared to virologic discordant responders [82,83], suggesting that controlling the level of immune activation is more important than controlling HIV viral load.

HIV-2

Differences in the natural course of HIV-1 and HIV-2 infection were also taken as evidence for a central role of immune activation in HIV infection. In HIV-2 infected patients, the asymptomatic phase takes much longer compared to HIV-1 infection, because $CD4^+$ T cells decline much more slowly. HIV-2-infected patients have lower levels of T-cell activation, proliferation, and apoptosis, and a lower plasma viral load than HIV-1 infected individuals. When HIV-1 and HIV-2 infected individuals were stratified on the basis of $CD4^+$ T-cell counts, the levels of T-cell activation and proliferation were shown to be similar despite large differences in plasma viral load [65,84]. Sousa et al. interpreted this close association between immune activation and the $CD4^+$ T-cell count as evidence that the level of immune activation, and not viral load, determines disease outcome [65]. One could, however, also reason the other way round, namely that the *rate* at which $CD4^+$ T cells are lost, which is known to be higher in HIV-1 compared to HIV-2 infection, should apparently be due to differences in HIV viral load, because it is the only apparent difference between HIV-1- and HIV-2-infected individuals with similar $CD4^+$ T-cell counts.

SIV

If in HIV infection high levels of immune activation are associated with a bad prognosis, one would also expect the reverse, namely that low levels of immune activation are associated with a good prognosis. The latter situation seems to be the case in some SIV-infected primates. In sooty mangabeys, one of the natural hosts of SIV, $CD4^+$ T cells are typically not depleted, despite high viral loads. Both immune activation and T-cell apoptosis were shown to be unaffected in these animals, when compared to non-infected sooty mangabeys. Sooty mangabeys may have been selected in the presence of SIV to give only a limited immune response against the virus. Transmission of SIV to other primate species that are not natural hosts to SIV, such as macaques, is known to induce $CD4^+$ T-cell decline and progression to AIDS. SIV infection of rhesus macaques is characterized by increased levels of immune activation [13]. *In vivo* labeling of dividing T cells using BrdU confirmed these findings by showing that in SIV-infected macaques, the turnover of both $CD4^+$ and $CD8^+$ T cells was increased compared to uninfected macaques [77]. These data support the view that low levels of T-cell activation are associated with a good prognosis, regardless of the presence of high levels of virus load, again underscoring the central role of chronic immune activation in HIV and SIV pathogenesis.

Collectively, these data changed our viewpoint on HIV infection drastically. Where once it was thought that "inappropriate apoptosis" was the main problem in HIV infection, and increased T-cell proliferation a response to cope with this loss of cells, much research is now based on the opposite idea that chronic T-cell activation is driving T-cell loss through a natural process of "appropriate apoptosis" of activated T-cell clones.

Is chronic immune activation alone enough to induce $CD4^+$ T-cell loss?

To study if chronic immune activation could induce $CD4^+$ T-cell loss directly, Tesselaar et al. [85] studied T-cell dynamics in other situations of chronic immune stimulation independent of HIV infection. A transgenic mouse model was developed in which CD70 is constitutively expressed on the cell surface of B cells. This leads to continuous stimulation of the T-lymphocyte pool via interaction with CD27 on the T-cell surface. Remarkably, these mice developed a phenotype reminiscent of HIV infection, in that their peripheral T-cell pool was progressively depleted and the mice eventually died of opportunistic infections [85].

A similar HIV-independent disturbance of the T-cell pool reminiscent of HIV infection was observed in a large group of healthy Ethiopians analyzed in the Ethio-Netherlands AIDS Research Project (ENARP). HIV-negative Ethiopians were found to have highly increased levels of immune activation, compared to HIV-negative Dutch individuals, probably related to differences in domestic antigen exposure and the high antigenic burden by common parasitic infections in Ethiopia [86,87]. In fact, these healthy individuals have low $CD4^+$ T-cell counts, low percentages of $CD4^+$ and $CD8^+$ naive T cells, expansion of $CD8^+$ memory and effector T-cell subsets, and decreased TREC contents, comparable to HIV-infected Dutch individuals [51,86,87]. Changes in the peripheral T-cell pool of healthy Ethiopians

appeared to occur at very early ages. Whereas cord blood samples from healthy Ethiopians and Dutch individuals were virtually identical with respect to TREC contents and T-cell subsets, within a few years of childhood considerable differences were observed (Tsegaye et al. submitted) [88]. Thus, also in this situation, chronic antigen exposure and chronically increased immune activation were associated with progressive $CD4^+$ T-cell decline.

The fact that a considerable T-cell loss can be observed in situations of chronic immune activation independent of HIV infection suggests that $CD4^+$ T-cell loss in HIV infection may be a direct result of chronic stimulation induced by HIV. Of note, even in healthy individuals, the total number of T cells, including the number of naive $CD4^+$ and $CD8^+$ T cells declines with increasing age [89,90].

Why are only $CD4^+$ and not $CD8^+$ T cells lost in HIV infection?

Although many of the data discussed in this chapter underline the importance of chronic immune activation in HIV pathogenesis, one question remains to be answered: If chronic immune activation causes $CD4^+$ T cells to decline, and if both $CD4^+$ and $CD8^+$ T-cell turnover are increased during HIV infection, why then is the $CD8^+$ T-cell pool not progressively depleted? As already mentioned, increases in T-cell proliferation and apoptosis, telomere shortening, and TREC dilution in HIV-infected individuals are even more pronounced in $CD8^+$ T cells than in $CD4^+$ T cells.

To answer this question, it is important to note that although $CD4^+$ T-cell depletion is the main characteristic of HIV infection, other changes in T-cell subsets typically occur during HIV infection. After an initial increase due to peripheral expansion, the $CD4^+$ memory T-cell population is progressively depleted. Because of a massive expansion of the memory $CD8^+$ T-cell pool in response to HIV infection, total numbers of $CD8^+$ T cells are typically increased throughout the asymptomatic phase of infection. However, depletion of the naive T-cell compartment is not restricted to $CD4^+$ T cells, as naive $CD8^+$ T cells begin to decline shortly after infection as well [35,36,64]. Eventually, shortly preceding AIDS diagnosis, even total $CD8^+$ T-cell numbers start to decline.

We argue that naive $CD8^+$ T-cell loss might be as important as naive $CD4^+$ T-cell loss, and is driven by similar mechanisms. Thus, based on data outlined in this chapter, we hypothesized that HIV induces continuous activation of naive $CD4^+$ and naive $CD8^+$ T cells that are recruited to become memory and effector T cells and are thereby lost from the naive T-cell pools. Because naive T cells are difficult to replace, continuous naive T-cell recruitment will lead to naive T-cell depletion [7]. In addition, intrinsic differences in cell survival kinetics between activated $CD4^+$ and $CD8^+$ T cells are likely to be the cause of differences between total $CD4^+$ and $CD8^+$ T-cell numbers in the blood of HIV-infected individuals.

Differences in $CD4^+$ and $CD8^+$ T-cell dynamics upon antigen-induced T-cell activation are not specific for HIV infection. Given the different physiological tasks of $CD4^+$ T cells (giving help to induce other immune responses) and $CD8^+$ T cells (creating effector cells to actively kill

infected cells), it may not be surprising that the $CD8^+$ T-cell population is more prone to induce and maintain large clones of effector cells than the $CD4^+$ T-helper population. Indeed, in mice infected with LCMV, LCMV-specific $CD8^+$ T cells expanded about 20-fold more than LCMV-specific $CD4^+$ T cells. Moreover, after the contraction phase of the immune response, when the antigen was cleared, the size of the antigen-specific $CD8^+$ T-cell pool remained stable while LCMV-specific $CD4^+$ T cells continued to decrease over time [91]. A similar decline in antigen-specific $CD4^+$ T-cell memory cells was found in mice upon infection with *Listeria monocytogenes* [92]. Furthermore, Ahmed et al. recently showed that murine memory $CD8^+$ T cells are less sensitive to apoptosis than naive $CD8^+$ T cells [93]. In contrast, when primed T-cell receptor transgenic $CD4^+$ T cells were rechallenged after transfer to normal mice, the transgenic cells initially expanded but progressively declined due to apoptosis after 2 days [94].

These results indicate that the size of the memory $CD4^+$ T-cell pool diminishes with time, while expanded $CD8^+$ T-cell clones show a much longer survival, also during other infections. Interestingly, as indicated above, HIV-negative Ethiopians with chronically increased levels of immune activation also show a selective loss of naive $CD4^+$, naive $CD8^+$ T cells, and memory $CD4^+$ T cells, but an expansion of memory/effector $CD8^+$ T cells, supporting the idea that the $CD4^+$ T-cell population is generally more prone to be depleted upon chronic immune activation than the $CD8^+$ T-cell population.

In HIV-infected individuals, labeling studies with deuterated glucose confirmed differences in T-cell dynamics between $CD4^+$ and $CD8^+$ T cells. After a 7-day labeling period Mohri et al. [74] observed that $CD4^+$ and $CD8^+$ T-cell proliferation were increased in HIV-1 infected humans as compared to uninfected individuals. Remarkably, however, T-cell death rates were only increased in the $CD4^+$ T-cell population, which led them to conclude that $CD4^+$ T-cell depletion in HIV infection is caused by selectively increased $CD4^+$ T-cell destruction. More recently, the same data have been analyzed using a two-compartment model, describing a resting T-cell pool and an activated pool. When the labeling dynamics of $CD4^+$ and $CD8^+$ T cells in HIV-infected individuals were compared to those of healthy controls, it was found that $CD4^+$ T cells, but not $CD8^+$ T cells, entered the activated compartment faster during HIV infection. $CD4^+$ T cells in the activated pool had increased proliferation and death rates, but HIV infection did not affect the size of the activated pool. In contrast, the activated $CD8^+$ T-cell compartment became larger during HIV infection, due to diminished reversion of activated cells into the resting pool, while proliferation and death rates remained unaffected by HIV infection [76]. The fact that $CD8^+$ T cells in HIV infection have shorter telomeres and more severely diluted TRECs than $CD4^+$ T cells also suggests that activated $CD4^+$ T cells are more prone to die than activated $CD8^+$ T cells.

Conclusion: an integrated view

Taken together, HIV infection is associated with an increased level of T-cell apoptosis, which, by itself, is not inappropriate, because it is part of the physiological response to immune activation. However, the continuously increased levels of apoptosis characteristic of

HIV infection reflect the continuous presence of a very active virus, causing chronic activation of the immune system that, because lost T cells are difficult to replace, leads to depletion of the naive $CD4^+$ and naive $CD8^+$ T-cell pools, as well as the memory $CD4^+$ T-cell pool (Figure 1). Thus, by wearing the immune system out, in case of HIV-1 infection, an appropriate immune response ultimately may lead to unsuitable immune deficiency.

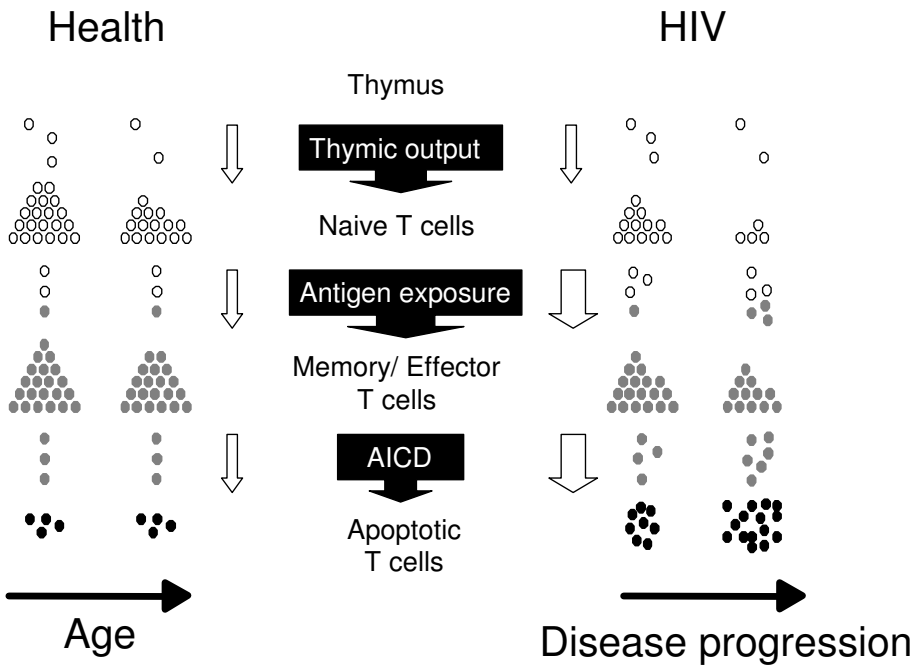

Figure 1. Increased naive T cell consumption due to immune hyperactivation. Thymic output of T cells to the naive compartment is low and constant in healthy as well as HIV-infected adults. During age, upon encountering infections, naive cells will enter the memory/ effector T cell pool, leading to a gradual decrease in the naive T cell compartment. Upon infection activated and expanded cells will die of activation-induced cell death (AICD). During HIV infection the same process occurs only at a faster rate due to chronic immune activation by the virus. (Adapted from Hazenberg MD et al., *Nat Immunol* 2000).

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Low immune activation despite high levels of pathogenic HIV-1 results in long-term asymptomatic disease

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Chapter 3

Abstract

Long-term asymptomatic HIV-infected individuals (LTA) usually have low viral load and low immune activation. To discern whether viral load or immune activation is dominant in determining progression to AIDS, we studied three exceptional LTA with high viral load. HIV-1 isolates from these LTA were as pathogenic as viruses from progressors in organ culture. Despite high viral load, these LTA had low levels of proliferating and activated T cells compared to progressors, like other LTA. In contrast to progressors, HIV-specific $CD4^+$ T-cell responses in these LTA were maintained. Thus, low immune activation despite high viral load preserves HIV-specific T-cell responses and resulted in a long-term asymptomatic phenotype.

Both viral load and immune activation have been shown to be associated with progression to AIDS [1-7]. Since immune activation and viral load are highly correlated, it is hard to determine which factor is dominant in determining disease progression. To further elucidate the importance of immune activation relative to viral load in HIV disease progression, we studied three rare long-term asymptomatic HIV-infected individuals (LTA) from the Amsterdam Cohort Studies on HIV infection and AIDS that have a high viral load (LTA-HVL) equal to the viral load seen in progressors (Figure 1).

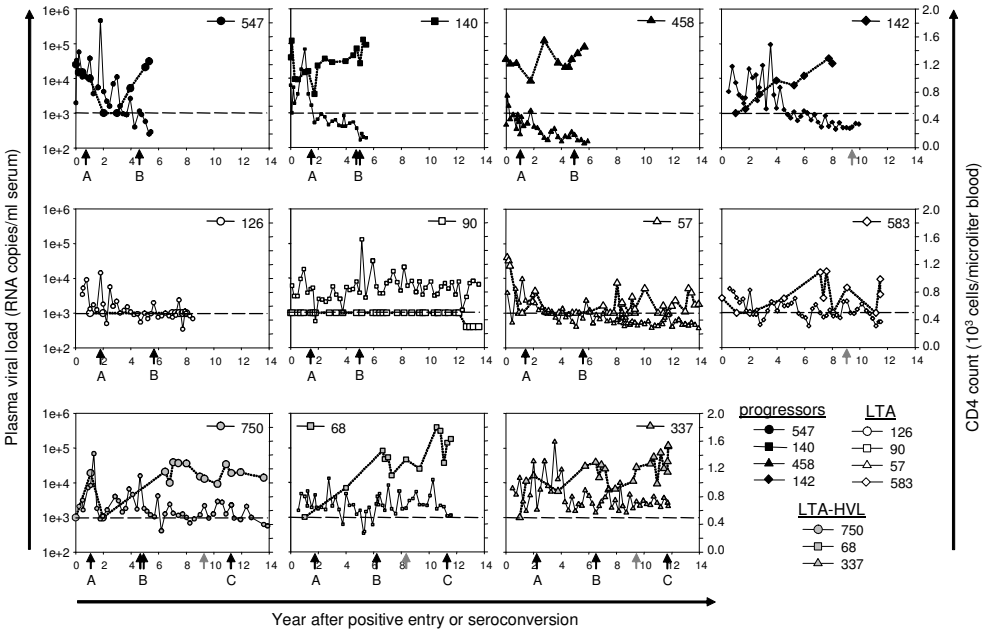

Figure 1. Longitudinal analysis of CD4$^+$ T-cell numbers in blood and HIV-1 RNA copies in plasma of the patients used in this study. Plasma viral RNA copies per ml (left axes, large symbols and dashed lines) and CD4$^+$ T cells per µl blood (right axes, small symbols and solid lines) against time in years after seroconversion or after seropositive entry into the cohort. The horizontal dashed lines represent the detection limit of 1000 RNA copies/ml. Gray arrows along the x-axes indicate the times of isolation of HIV-1 clones used in Figures 2 and 3 while black arrows with letters refer to timepoints of T-cell isolation for analyses shown in Figure 4.

Although the high viral load of these LTA-HVL was consistent with a high replicative capacity of isolated virus clones *in vitro* [8], slow disease progression despite high viral load may be caused by a decreased pathogenic phenotype of the virus. We tested the cytopathic effects (CPE) of these viruses in human fetal thymic organ culture (FTOC) as described previously [9]. The time-points of viral isolation used in these assays are depicted by gray arrows in Figure 1. Eight mm^3 pieces of thymic tissue were infected with 8x10^3 $TCID_{50}$ of HIV-1 clones

derived from progressors, LTA or LTA-HVL. Viral replication and percentages of $CD4^+$ and $CD8^+$ thymocytes were assayed over a three week time-period by p24 ELISA and flow cytometry with CD4-PE and CD8-PerCP MAb respectively. In mock-infected FTOC, approximately, 78% of light scatter-gated thymocytes expressed CD4 at 14 and 21 days post infection (Figure 2A).

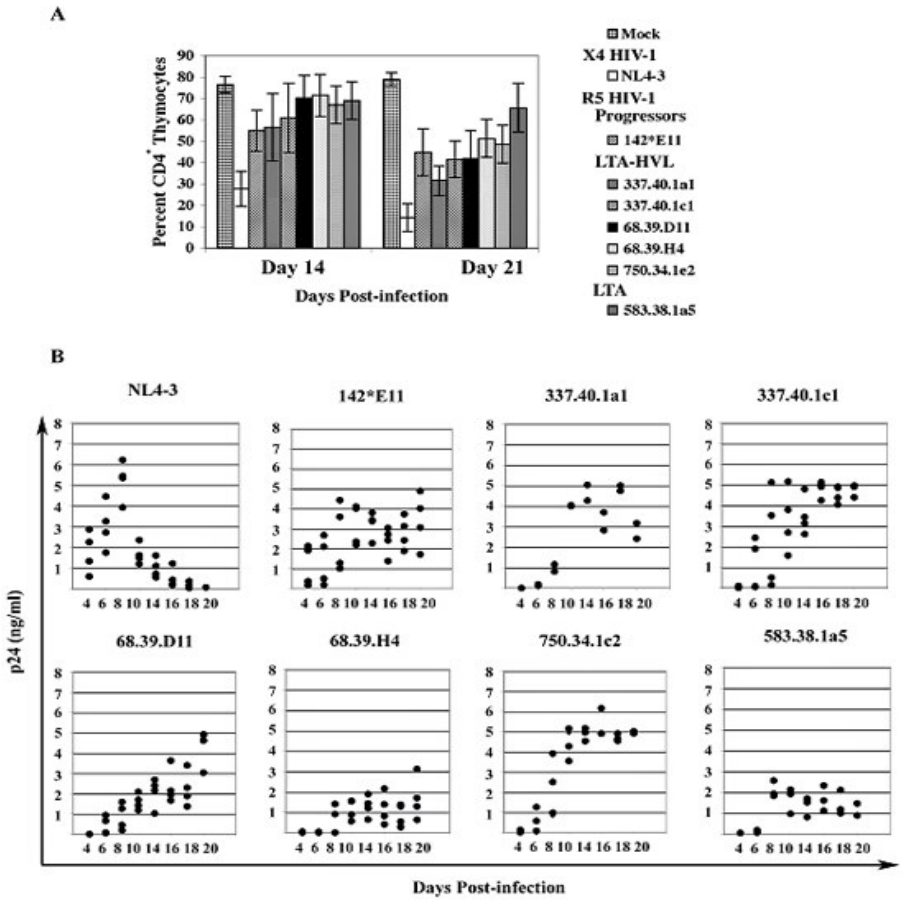

Figure 2. R5 HIV-1 clones from long-term asymptomatic individuals with high viral load (LTA-HVL) replicated to high levels and depleted $CD4^+$ thymocytes in FTOC. FTOC were infected with the indicated HIV-1 clones. (A) The bars represent average percentages of $CD4^+$ thymocytes (CD4SP and DP) in FTOC with error bars indicating standard deviations. The number of samples used to obtain data on day 14 and day 21 post-infection, respectively, are as follows: n=9 for mock in five experiments; n=4 for NL4-3 in two experiments; n=8 for ACH 142*E11 in five experiments; n=3 for ACH 337.40.1a1 in two experiments; n=5 for ACH 337.40.1c1, ACH 68.39.D11, ACH 68.39.H4, ACH 750.34.1e2, ACH 583.38.1a5 in three experiments. (B) Viral replication was quantified by measuring HIV-1 capsid antigen (p24) concentration in FTOC medium on day 4, 6, 8, 10, 14, 16, 18, and 20 post-infection with a commercial ELISA kit (NEN Life Science Products). Each dot represents the medium from an individual FTOC.

Both progressor and LTA-HVL derived R5-HIV-1 clones caused depletion of more than 35% of $CD4^+$ thymocytes when compared to mock infection at 21 days post infection ($p<0.001$; statistical significance was determined by analysis of variance with Tukey's test for all pairwise comparisons). Likewise, both progressor and LTA-HVL derived R5-HIV-1 clones replicated to similar levels except clones from LTA-HVL patient 68, in which replication peaked later in the course of infection (Figure 2B). In contrast, R5-HIV-1 clones derived from LTA with low viral load failed to exhibit significant CPE and replicated poorly.

Next we tested the ability of progressor and LTA-HVL derived R5-HIV-1 clones to deplete $CD4^+$ T cells in secondary lymphoid tissue histoculture. Lymphocytes were isolated from human spleen histoculture on days 16 and 21 post-infection with HIV-1 clones derived from progressors or LTA-HVL, stained with CD4-PE and CD8-PerCP MAbs, and analyzed by flow cytometry with a lymphocyte gate based on 90^o and low angle light scatter. Again, progressor and LTA-HVL R5-HIV-1 clones depleted $CD4^+$ T cells significantly ($P<0.0001$), compared to mock infected tissue on both day 16 and day 21, as measured by the ratio of $CD4^+$ to $CD8^+$ cells (Figure 3A). No statistical difference in CPE was observed between progressor and LTA-HVL clones in FTOC or in spleen histoculture.

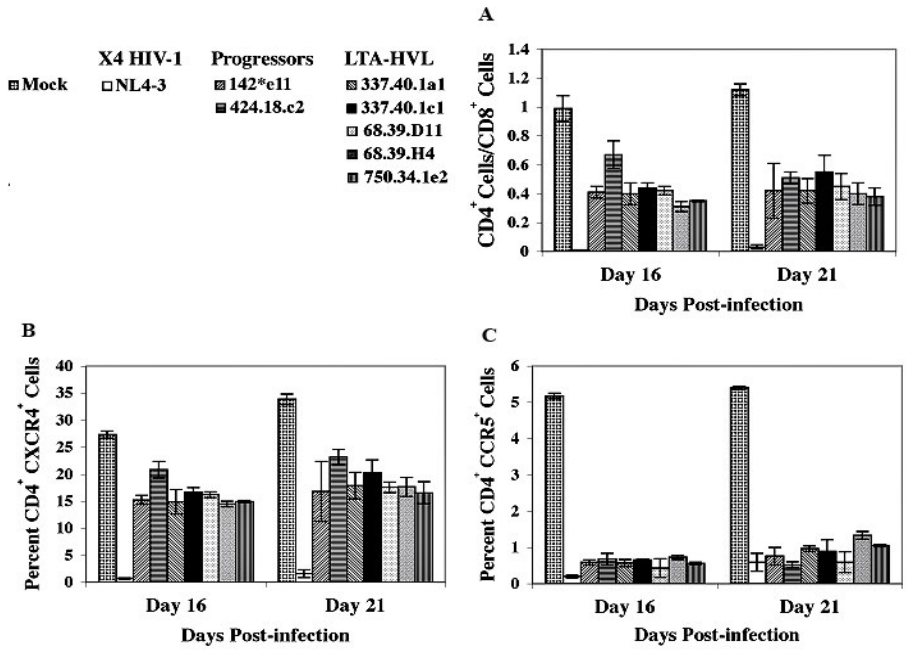

Figure 3. Depletion of $CD4^+$ T cells in human spleen histoculture infected *ex vivo* with HIV-1. Spleen histocultures were infected with the indicated HIV-1 clones. (A) Depletion of $CD4^+$ lymphocytes measured by change in the ratio of $CD4^+$ to $CD8^+$ T cells. (B) Depletion of $CD4^+CXCR4^+$ T lymphocytes. (C) Depletion of $CD4^+CCR5^+$ T lymphocytes. Data shown are representative of two experiments done in duplicate with error bars indicating standard deviations.

As expected, the R5-HIV-1 clones preferentially depleted $CD4^+CCR5^+$ T cells, while the X4-HIV-1 molecular clone NL4-3 depleted the $CD4^+CXCR4^+$ subset of T cells more efficiently (Figure 3B, 3C). These results show that R5-HIV-1 clones obtained from LTA-HVL were equally replication competent and cytopathic as progressor derived clones. Therefore, the replication and CPE of these R5-HIV-1 clones in primary and secondary lymphoid tissue organ culture were not reflective of the clinical status of the patients from which they were derived.

We hypothesized that the LTA-HVL showed slow disease progression due to low level immune activation despite high viral load, since high levels of activated and proliferating ($Ki67^+$) $CD4^+$ and $CD8^+$ T cells during HIV infection are better prognostic factors than viral load [4,6,7] and even pre-seroconversion immune activation has been shown to be predictive of disease progression [6,10,11]. Moreover, patients that fail virologically on HAART, but have low levels of T-cell activation, proliferation and apoptosis show a continuous increase in $CD4^+$ T cells [12-14]. Similarly, during natural SIV infection, sooty mangabeys do not develop disease despite high viral load and their immune activation is as low as uninfected animals. In contrast, pathogenic SIV infection in macaques induces strong immune activation, $CD4^+$ T-cell loss and disease progression [15]. Collectively these data implicate immune activation as a driving force of HIV pathogenesis.

To test this hypothesis we longitudinally followed the immune activation status (measured as previously described [6]) of the three LTA-HVL and compared these values with progressors matched for HIV viral load and LTA with low viral load (Figure 1). Time-points measured were approximately 1 year, 5 years and for LTA-HVL additionally 11 years after seroconversion or seropositive entry into the cohort. The time-points used for analysis are depicted by black arrows in Figure 1.

Progressors showed high percentages of activated $HLA\text{-}DR^+CD38^+$ $CD4^+$ and $CD8^+$ T cells within 5 years of HIV-1 infection. In contrast, the LTA-HVL had levels of activated T cells comparable to LTA with low viral load even after more than 10 years of HIV-1 infection. A similar trend was found for proliferating, Ki67-expressing, $CD4^+$ and $CD8^+$ T cells (Figure 4A).

Jansen et al. [16] have shown that HIV-specific $CD4^+$ T-cell responses had no predictive power for disease progression in a prospective cohort study in a multivariate analysis including immune activation. HIV-specific $CD4^+$ T-cell responses were lost over time in progressors compared to LTA. To further elucidate whether loss of HIV-specific $CD4^+$ T-cell responses was due to high viral load or to high level immune activation, we determined absolute numbers of Gag-specific IFN-γ and IL-2 producing $CD4^+$ T cells (measured as previously described [16]). Gag-specific IFN-γ and IL-2 producing $CD4^+$ T cells were readily detected early after infection (Figure 4B). These responses, while preserved in LTA with both high and low viral load, tended to decrease over time in progressors. The effect was most pronounced for absolute numbers of IL-2 producing $CD4^+$ T cells (Figure 4B). This

suggests that it is not high viral load alone, but instead chronic immune activation with high viral load that causes loss of functional Gag-specific $CD4^+$ T cells over time.

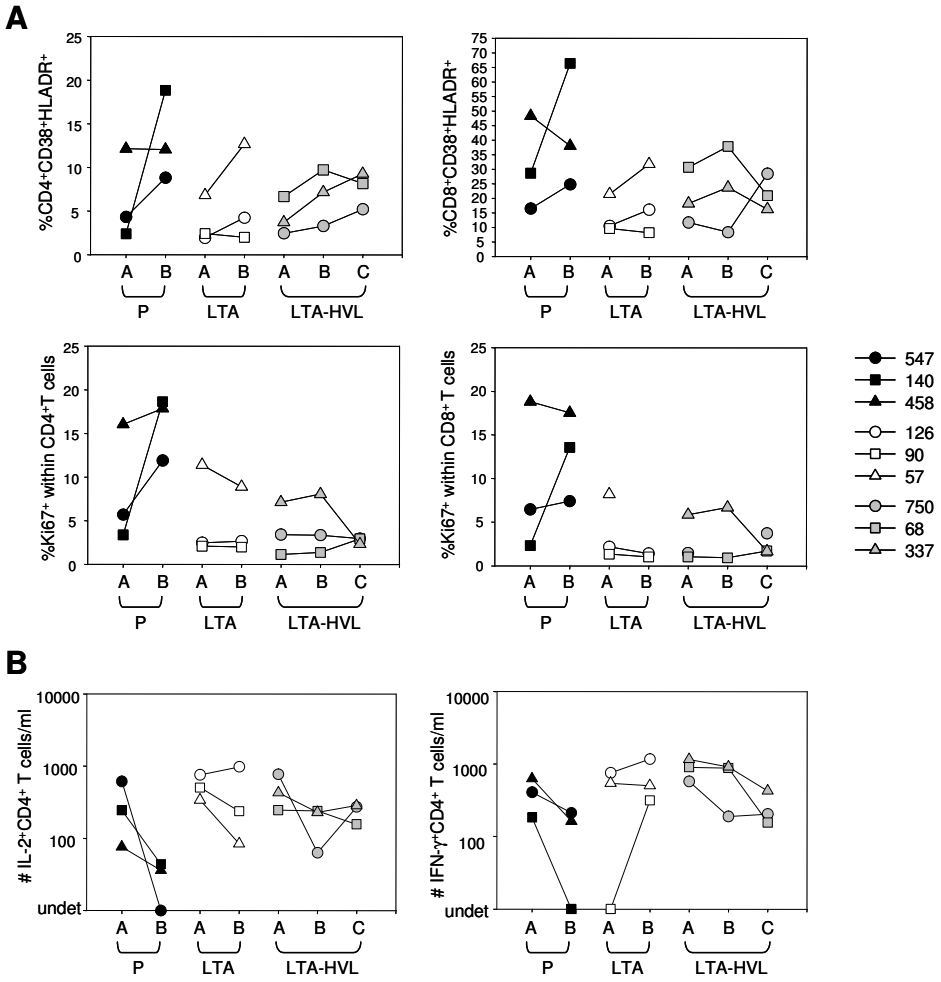

Figure 4. LTA-HVL had low immune activation and preserved HIV-specific responses.
(A) Percentage activated ($CD38^+HLA\text{-}DR^+$) and proliferating ($Ki67^+$) $CD4^+$ and $CD8^+$ T cells. (B) Absolute numbers of HIV-specific IL-2 (upper panel) and IFN-γ producing (lower panel) $CD4^+$ T cells. Time points measured in Figure A and B correspond to black arrows in Figure 1. Black symbols depict progressors, white symbols LTA with low viral load and grey symbols LTA with high viral load.

In summary, despite high viral load HIV-1 infection did not evoke high levels of immune activation or proliferation in LTA-HVL. Although few patients could be studied, these data provide additional support for the idea that chronic immune activation is an important driving force for $CD4^+$ T-cell decline and loss of HIV-specific $CD4^+$ T-cell responses in HIV-1 infected individuals.

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Immune abnormalities in healthy Ethiopians are induced by chronic immune activation and start at early age

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Chapter 4

Abstract

Healthy Ethiopians show several immune characteristics reminiscent of HIV infection, including low total and naive $CD4^+$ T-cell numbers, and elevated levels of activated T cells. These atypical immune characteristics have been proposed to reflect chronic immune activation by environmental antigens in Ethiopians. Here we studied changes in T-cell receptor excision circles (TRECs), CD31 expression on naive $CD4^+$ T cells and T-cell telomere lengths with age in healthy Dutch and Ethiopian individuals. At birth, $CD4^+$ TREC contents and naive T-cell numbers in Ethiopians were found to be comparable to those in Dutch neonates. At very young age, however, both $CD4^+$ T-cell TREC contents and naive T-cell numbers fell dramatically in Ethiopian, but not in Dutch children. These differences between Ethiopian and Dutch individuals remained well into adulthood, because $CD4^+$ T-cell TREC contents and naive T-cell numbers decreased at similar rates with age in Ethiopian and Dutch individuals. Using a simple mathematical model, we show that these parallel declines in TREC and naive T-cell numbers during aging are consistent with a relatively high basal level of immune activation in Ethiopian individuals. $CD4^+$ and $CD8^+$ T-cell telomere length and proportions of $CD31^+$ cells in the naive $CD4^+$ T-cell pool were not found to be significantly reduced in Ethiopian compared to Dutch adults. Taken together, these data support the idea that immunological differences between healthy Ethiopian and Dutch individuals are caused by chronic immune activation in Ethiopians, and suggest that many immune features characteristic for HIV infection can be the direct result of chronic activation induced by the virus.

Introduction

HIV-1 infection is characterized by low total and naive $CD4^+$ T-cell numbers, increased $CD8^+$ T-cell numbers, elevated levels of activated $CD4^+$ and $CD8^+$ T cells, and a decreased T-cell receptor excision circle (TREC) content of both $CD4^+$ and $CD8^+$ T cells. There is increasing evidence that these characteristics of HIV-infection are, to a large extent, due to chronic immune activation induced by the virus [1-5]. The level of immune activation during HIV-infection as measured by Ki67-expression is one of the strongest predictors of $CD4^+$ T-cell loss and progression to AIDS, independent of viral load [6,7]. Remarkably, a number of immune characteristics that are typically observed during HIV-infection have also been found in healthy, HIV-negative Ethiopians, including low naive and total $CD4^+$ T-cell numbers, and increased levels of the T-cell activation markers CD38 and HLA-DR [1,3,8-17] compared to other Africans and Caucasians [8-15,17-23]. These immune characteristics have previously been shown to be related to chronic helminth infection [10]. The observed similarities between HIV-infected individuals and healthy Ethiopians suggest that chronic immune activation by itself may induce a severe loss of $CD4^+$ T cells.

To investigate whether chronic immune activation independent of HIV-infection is indeed responsible for the atypical immune characteristics of healthy Ethiopians, we performed a detailed characterization of different immune parameters associated with immune activation in healthy Ethiopian and Dutch individuals of different ages, varying from neonates to children and adults. Measurements included $CD4^+$ T-cell TRECs, CD31 expression of naive $CD4^+$ T cells, and $CD4^+$ and $CD8^+$ T-cell telomeres lengths. TRECs are extra-chromosomal by-products of the T-cell receptor rearrangement process in the thymus. Because TRECs are not replicated during mitosis, they are diluted with each cellular division [29,30], and therefore serve as a cumulative marker for T-cell proliferation. Similarly, T-cell telomere length, which shortens with each round of cell division, was measured to follow the replicative history of T cells [31]. $CD31^+$ naive $CD4^+$ T cells are most proximal to the thymus and have a more diverse T-cell receptor repertoire than $CD31^-$ naive $CD4^+$ T cells. During aging the fraction and absolute number of $CD31^+$ naive $CD4^+$ T cells decrease [28,49].

While at birth the immune parameters that were measured in Ethiopians were found to be similar to those in Dutch neonates, already at young age significant differences between Ethiopian and Dutch children could be observed. Using a mathematical model we show that the temporal differences between Ethiopian and Dutch individuals are consistent with a higher baseline level of immune activation in Ethiopians, which is most likely caused by the high antigenic burden to which Ethiopians become exposed shortly after birth.

Materials and Methods

Subjects

A total of 116 subjects between 0 and 40 years of age were included in this cross-sectional study. Neonates and children aged 5-16 years were recruited from Wonji, a town about 114 km South east of the capital Addis Ababa. The children under 5 years of age are residents from in and around Addis Ababa participating as control subjects of a separate project at

EHNRI. Adults are participants of a long-term cohort study on HIV-1 infection and progression in Ethiopians performed by the Ethiopian Netherlands AIDS Research Project in Wonji and Akaki, a suburb of the capital Addis Ababa. Individuals included in the present study were negative for intestinal parasites (using direct and formol ether concentration techniques) and HIV-1 antibodies. Cord blood (CB) samples were obtained from full term normal deliveries. Detailed description of study subjects has been reported previously [17]. The study was conducted in accordance with the ethical guidelines of EHNRI and the Ethiopian Science and Technology Commission. Informed consent was obtained from all subjects or guardians as necessary. Dutch controls included in the present study were ten full term neonates with normal deliveries, eight 1-5 years old children, twelve 5-16 years old children and 29 adults aged 18-45 years. Cord blood was collected after delivery, according to the guidelines of Eurocord Nederland Foundation (Leiden, The Netherlands).

Peripheral blood mononuclear cell (PBMC) and cord blood mononuclear cell (CBMC) isolation

Whole blood was collected in EDTA vacutainer tubes and processed on the same day. PBMCs/CBMCs were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation and viably frozen using a computerized freezing machine (KRYO 10, Biomedical Series II). Frozen cells were stored in liquid nitrogen until analyzed. All laboratory markers for both Ethiopian and Dutch individuals were analyzed at the department of Clinical Viro-immunology, Sanquin Research at CLB and Landsteiner Laboratory, Amsterdam, the Netherlands.

Flow cytometry

To measure the percentage naive ($CD45RO^-CD27^+$) $CD4^+$ and $CD8^+$ T cells and the expression of CD31 on naive $CD4^+$ T cells, cryopreserved PBMCs/CBMCs were thawed and incubated with PerCP-conjugated CD4 or CD8 monoclonal antibodies (mAb) (Becton Dickinson (BD); San Jose, CA), PE-conjugated CD31 (BD), FITC-conjugated CD45RO (Caltag) or CD27 (BD), APC-conjugated CD45RO (BD) and/or biotinylated CD27 (Sanquin, Amsterdam, The Netherlands) mAb. If biotinylated CD27 was used, cells were washed and additionally incubated with anti-Streptavidin-APC (BD). After 20 minutes incubation at 4^oC, cells were washed with PBA, fixed with Cellfix (BD), and analyzed on a FACS Calibur (BD) with Cellquest software.

Measurement of T-cell receptor excision circles (TRECs) in purified $CD4^+$ and $CD8^+$ T cells

$CD4^+$ and $CD8^+$ T cells were purified from thawed PBMC using magnetic beads (Miltenyi Biotec GmbH, Germany) according to manufacturer's instructions. In brief, after 15 min incubation with CD4 or CD8 conjugated magnetic beads, $CD4^+$ and $CD8^+$ T cells were sorted from PBMC by positive selection over MACS Separation Columns (Miltenyi Biotec GmbH, Germany). Genomic DNA was isolated from the purified $CD4^+$ and $CD8^+$ cell fractions using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), as recommended by the manufacturer. Quantification of signal joint TRECs was performed by real-time quantitative

PCR using a Perkin Elmer TaqMan machine (Perkin Elmer Biosystems, The Netherlands). The PCR reaction was performed on 5 µl of DNA samples (equivalent to 150,000 cells) with the probes and primers (Perkin Elmer Biosystems) that have previously been described for signal joint TRECs.[1] Each PCR reaction was performed in a 50 µl solution containing 0.25 µl Taq polymerase, 5µl 10xTaqman buffer, 10µl 25µM $MgCl_2$, 1 µl dNTP, 4.5 µl of each of the forward and reverse primers, and 2 µl probe (all from Perkin Elmer Biosystems). As an internal control for the amount of input DNA used, the Cα constant region that remains intact on the TCR genes despite rearrangement processes was amplified in every sample tested. PCR conditions were 50°C for 2 min, 95°C for 10 min, after which 50 cycles of amplification were carried out (95°C for 15 sec, 60°C for 1 min). To determine the number of copies of excision circles in a defined amount of sample DNA, cloned signal joint sequences were used as a standard. A serial dilution of these standards was included in each experiment. A standard curve was then plotted and TREC values were calculated. Samples were analyzed in duplicates.

Telomere length determination using Flow-FISH

Telomere length was determined in a subgroup of adult subjects from whom enough cells were available (n=20), by the fluorescent *in situ* hybridisation assay using flow cytometry (Flow-FISH). In brief, cells were thawed and stained with biotinylated CD4 or CD8 mAbs (Sanquin Research at CLB and Landsteiner Laboratory, The Netherlands) for 15 min at 4°C. After washing with PBA, cells were incubated with Streptavidin Cy-5 for 15-20 min, and washed with PBS. Then 100 µl BS3 solution [Bis(sulfosuccinimidyl) suberate] (Pierce, Rockford, IL) was added for fixation, and cells were kept at 4°C for 30 min. Samples were first washed with PBS and then with 1 ml hybridisation solution (Formamide, 1M Tris pH 7.2, 4% BSA, 5M NaCl). After adding FITC labelled peptide nucleic acid (PNA) probe [(C3TA2)3 PNA, Applied Biosystems, Bedford, MA] to all tubes except the no-probe (unstained control), DNA denaturation was done at 82°C waterbath for 10 min. Cells were rapidly cooled on ice and the telomere probe was hybridized for 60-90 min at RT in the dark. Excess telomere probe was removed by repeated washing with a post hybridisation wash solution (Formamide, 1M Tris pH 7.2, 4% BSA, 5M NaCl, 10% Tween). After washing with PBA, Cellfix (BD) was added and data were acquired with the FACSCalibur using cellquest software. Tubes containing all reagents except the telomere probe were used to set a negative marker. For each sample, both the no-probe (unstained control) and with-telomere probe tubes were analyzed in duplicate.

Mathematical model

To study the effect of increased activation of peripheral T cells on the age-dependent decline in TREC contents, we used a previously developed mathematical model for TREC decline with age [1], described by the following two differential equations:

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

$dT/dt = c\alpha - dT$

in which N is the number of $CD4^+$ or $CD8^+$ T cells, $\alpha = a\,e^{-vt}$ represents thymic output which declines at rate v with time, T cells proliferate at rate p and die at rate d per day. The TREC content of the $CD4^+$ or $CD8^+$ T-cell population is subsequently calculated by T/N. It has previously been shown that TREC content does not decrease with age if there is no homeostatic regulation of T-cell numbers. We therefore model the rate of T-cell proliferation as:

$p = p_e + p_0 / (1 + N^2/h^2)$

where p_0 represents the maximum proliferation rate in healthy Dutch 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, and p_e represents the extra proliferation due to environmental triggers to which Ethiopians are exposed.

Statistical analysis

Data were analyzed using STATA statistical software (Stata 6.0, Stata corporation, College Station, Texas, USA). The distribution of the different markers was compared between age categories (at birth, in children, and in adults between 18-40 years of age) using the non-parametric Wilcoxon rank-sum test and between population groups (Ethiopians versus Dutch) using the non-parametric Mann-Whitney U test. P-values <0.05 were considered significant. Loss of TRECs with age in both Ethiopians and Dutch was estimated using linear regression, excluding the data from cord blood. We tested if there was a significant difference between the rate of TREC loss in Ethiopians and Dutch individuals, using a linear model including an interaction term between age and group (Ethiopian vs. Dutch). Correlations were calculated using Spearman's correlation coefficients.

Results

$CD4^+$ and $CD8^+$ TREC content changes with age in Ethiopian and Dutch individuals

We measured the average number of TRECs per $CD4^+$ and $CD8^+$ T cell in 105 Ethiopian subjects of different ages: 10 neonates, 8 children between 1-5 (median 3) years of age; 28 children between 5-16 (median 11) years of age, and 59 adults aged 20-40 (median 31). An age-related decline in TREC content was observed in both $CD4^+$ and $CD8^+$ T cells (Figure 1). TREC contents of $CD4^+$ T cells were highest at birth (median value: 26.8 $x10^3$/µg DNA), and subsequently significantly declined to 4.8 and 1.8 $x10^3$/µg DNA in children between 1-5 and 5-16 years old, respectively, and to 1.1 $x10^3$/µg DNA in adults. Median $CD8^+$ TREC contents decreased from 24.0, to 3.5, 1.6, and 0.08 $x10^3$/µg DNA for the respective age groups of 0, 1-5 years, 5-16 years, and adults. In contrast to the $CD4^+$ TREC contents, the $CD8^+$ TREC contents of individuals between 5 and 16 years of age were not significantly different from those of adults ($p=0.37$).

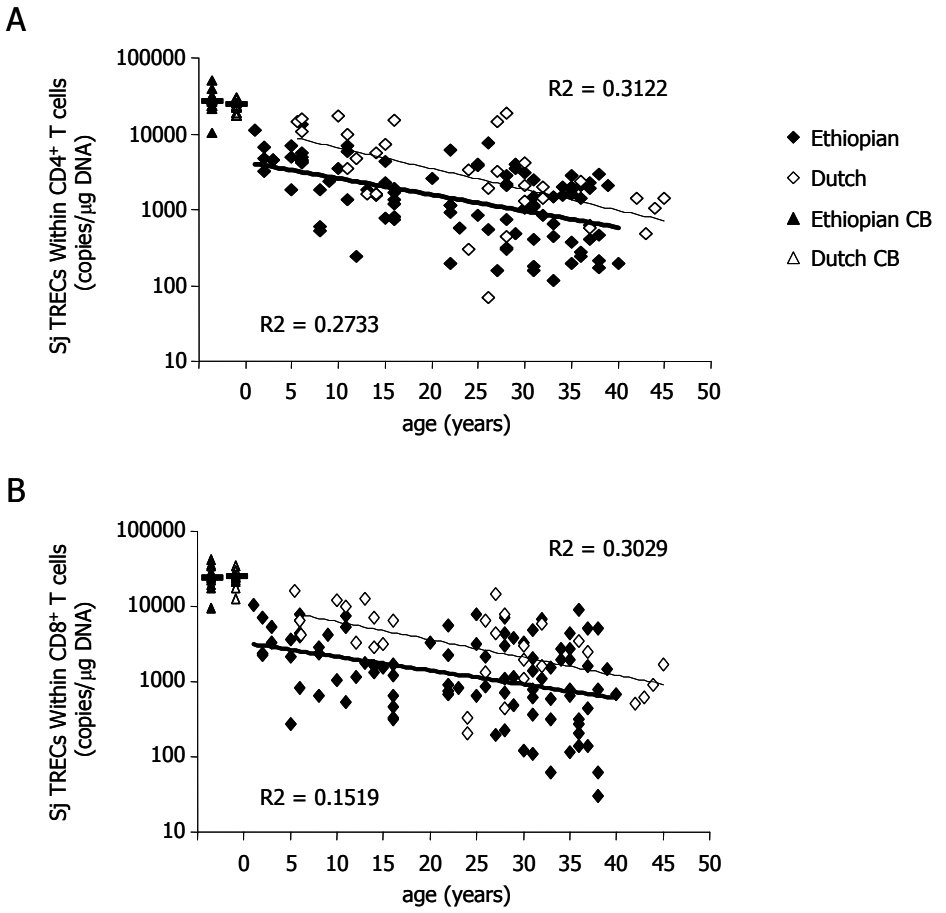

Figure 1. Changes in $CD4^+$ and $CD8^+$ T-cell TREC contents of Ethiopians versus Dutch with age. Ethiopian (filled) and Dutch (open) TREC values in cord blood are denoted by triangles, while diamonds denote TREC values in children and adults. $CD4^+$ (A) and $CD8^+$ (B) T-cell TREC contents were similar for Ethiopians and Dutch at birth, but dropped dramatically shortly after birth in Ethiopians. Horizontal bars show median values for Ethiopian and Dutch cord blood TREC contents. Regression lines were made by excluding cord blood samples.

These Ethiopian TREC data were compared to those obtained from Dutch control subjects of comparable age groups: at birth (n=10), 5-16 (median 11.5) years (n=12), and 18-45 (median 30) years (n=19). At birth, Ethiopian and Dutch individuals had comparable TREC contents of both $CD4^+$ (Figure 1A) and $CD8^+$ T cells (Figure 1B). Soon after birth, however, clear differences between $CD4^+$ and $CD8^+$ TREC contents of Ethiopian and Dutch children became apparent: for the majority of Ethiopians across the age groups $CD4^+$ and $CD8^+$ T-cell TREC contents were lower compared to Dutch age-matched controls. This difference was due to a considerable drop in TREC contents at very early age in Ethiopian children,

which was not observed in Dutch subjects. Indeed, both $CD4^+$ and $CD8^+$ TREC contents in Ethiopian children between 5-16 years of age were significantly lower compared to age-matched Dutch controls (Figure 2 $p<0.001$ for both $CD4^+$ and $CD8^+$ TREC content). On average, $CD4^+$ and $CD8^+$ TREC contents of Ethiopian children were more than 4 times lower than their Dutch counterparts ($p<0.001$). After this initial difference in TREC decline at very early age, TREC contents in Ethiopians and Dutch individuals declined at similar rates. Indeed the slopes of the regression lines through the TREC data of Ethiopian and Dutch subjects were not significantly different when data on cord blood samples were excluded ($p=0.44$ for $CD4^+$ and $p=0.55$ for $CD8^+$).

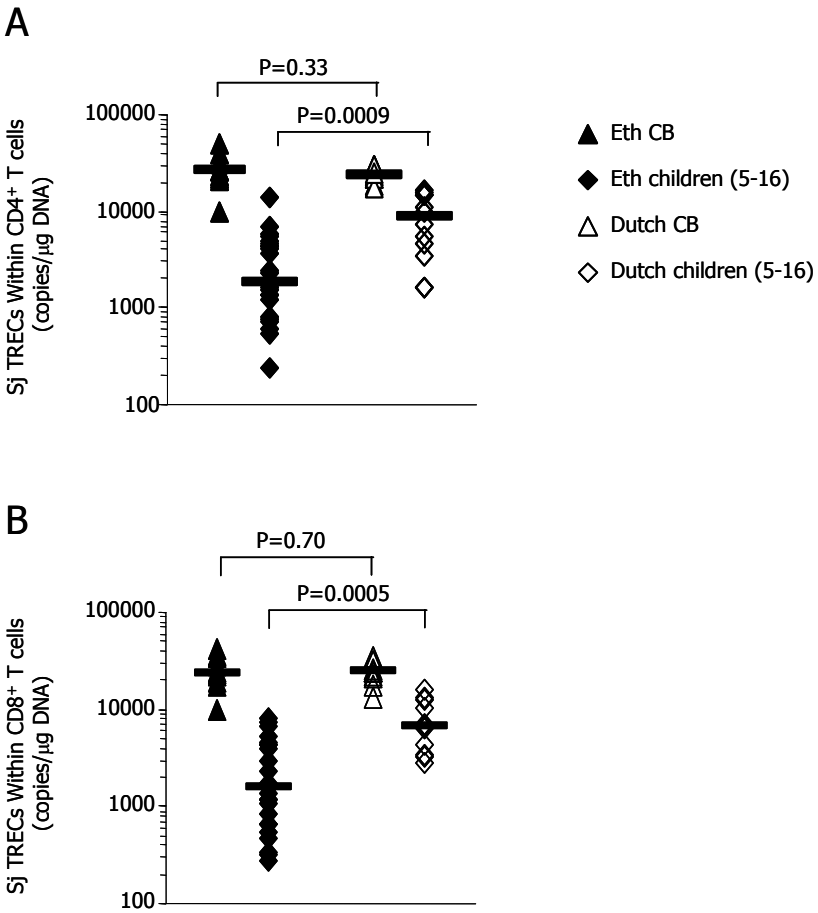

Figure 2. TREC contents in Ethiopian and Dutch neonates and 5-16 years old children. The median TREC content in $CD4^+$ (A) and $CD8^+$ (B) T cells of Ethiopian cord blood (filled triangles, n=10) were not significantly different from Dutch control cord blood (open triangles, n=10). The median $CD4^+$ (upper panel) and $CD8^+$ (lower panel) T-cell TREC contents of Ethiopian children (filled diamonds, n=28) were significantly lower than those of Dutch children (open diamonds, n=12). Horizontal bars show median values. P values were calculated using the non-parametric Mann-Whitney U test.

Early TREC decline in Ethiopian children coincides with a loss of naive T cells

It is well known that in healthy individuals the percentage of naive T cells declines progressively with age. We studied if the decline in $CD4^+$ TREC contents that we observed in young Ethiopian children coincided with a loss of naive $CD4^+$ T cells. Indeed, there was a strong correlation between the percentage of naive $CD4^+$ T cells and $CD4^+$ TREC contents in Ethiopian children ($r=0.70$, $p<0.0001$). As shown in Figure 3, the percentage of naive $CD4^+$ T cells in Ethiopian neonates was very high (median >90%, see also Tsegaye et al. 2003 [17]) and comparable to that in Dutch neonates. Shortly after birth, however, the percentage of naive $CD4^+$ T cells dropped much more dramatically in Ethiopian children than in Dutch age-matched controls ($p<0.0001$). The median percentage of naive $CD4^+$ T cells dropped from 53.6% in children under five years old to 32% in 5-16 years old Ethiopian children, while in Dutch children it dropped from 82.9% to 62.1%. Differences in naive $CD4^+$ T-cell percentages between Ethiopian and Dutch children for both age categories were statistically significant ($p<0.01$).

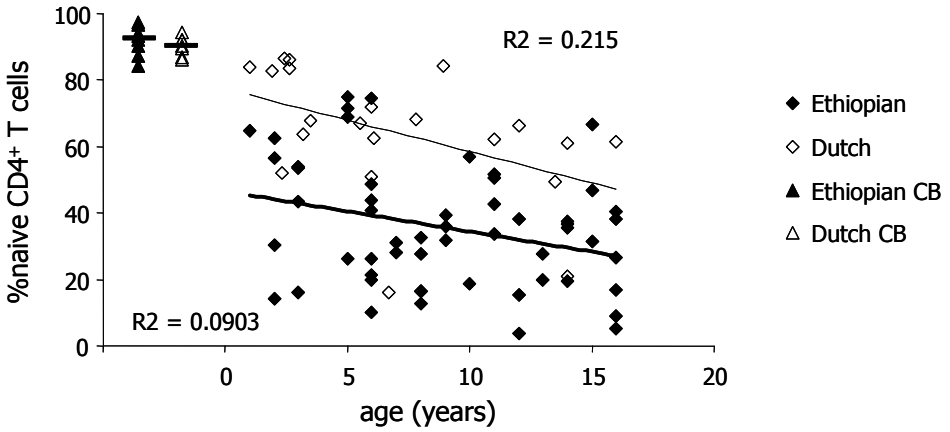

Figure 3. Proportions of naive $CD4^+$ T cells in Ethiopians and Dutch 1-16 years old children. Ethiopian (filled) and Dutch (open) naive ($CD45RO^-CD27^+$) proportions in cord blood are denoted by triangles, while diamonds denote naive proportions in children and adults. The percentages of naive $CD4^+$ T cells were comparable in Ethiopian and Dutch cord blood (age 0), but declined more rapidly in Ethiopian children compared to Dutch children. Horizontal bars indicate median values. Regression lines were made by excluding cord blood samples.

Biphasic TREC dynamics in healthy Ethiopians are caused by chronic immune activation

We used a previously developed mathematical model for TREC dynamics [1] to study whether the observed differences in TREC dynamics with age between Ethiopian and Dutch individuals could be due to a higher basal level of T-cell proliferation in Ethiopians, caused by chronic immune activation. Indeed, when the rate at which T cells proliferate in Ethiopians was set to a higher (fixed) value than the value that was previously used for

Caucasians, the model showed a biphasic TREC decline with age. The higher T-cell proliferation rate in Ethiopians caused a rapid drop in TREC content directly after birth. Even though the T-cell proliferation rate for Ethiopians was continuously higher than that of the Dutch, the model showed that the slopes at which TREC contents subsequently decline with age are similar (Figure 4).

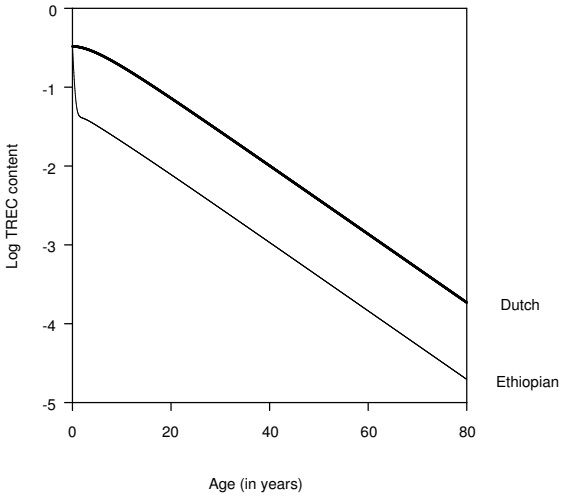

Figure 4. Simulation results of the mathematical model. Ethiopian (thin line) and Dutch (thick line) TREC content dynamics with age (x-axes depicted in log scale). Parameters: α=10^8 cells/day, v=0.1/year, c=1, h=2.5x10^{10} (ref 1), p_0=0.1/day, d=0.001/day. The extra T-cell proliferation due to environmental triggers to which Ethiopians are exposed are represented by p_e=0.008 and a concomitant increased T-cell death d=0.01/day.

$CD4^+$ and $CD8^+$ telomere length in Ethiopian and Dutch adults

To further study differences in the replicative history of T cells in Ethiopian and Dutch adults, we measured T-cell telomere lengths using Flow-FISH in a subgroup of adult Ethiopians aged 20-36 (median 29.5) years (n=20), and in ten Dutch age-matched controls aged 22-36 (median 28) years. As shown in Figure 5, the median telomere lengths of both $CD4^+$ and $CD8^+$ T cells tended to be somewhat higher in Dutch compared to Ethiopian healthy adults, but differences were not significant (p=0.23 for $CD4^+$ and p=0.82 for $CD8^+$). The respective $CD4^+$ and $CD8^+$ telomere length (median fluorescence) values for Ethiopians versus Dutch were: 78.6 and 73.9 versus 83.0 and 78.4. However, the variation in $CD4^+$ T-cell telomere lengths in Ethiopian adults (range: 46.9-102) was much larger than in Dutch controls (range: 68.4-100), and approximately 40% of the Ethiopian study population had fluorescence values below the 2.5th percentile limit of the Dutch controls.

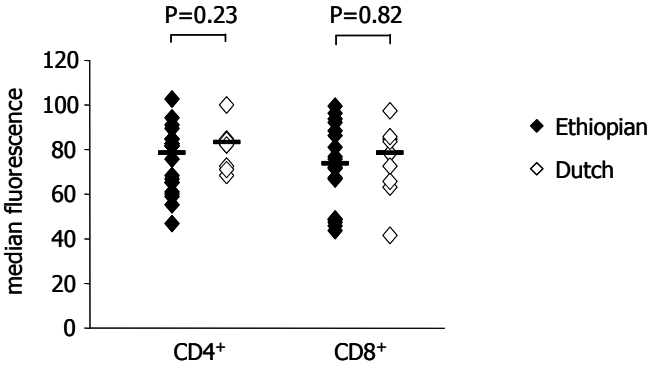

Figure 5. Replicative history of T cells as measured by telomere length in Ethiopian and Dutch adults. Telomere length of T cells was measured by FLOW-FISH and results are expressed in median fluorescence. Median values for neither $CD4^+$ nor $CD8^+$ T cells were significantly different between the two populations. However, a large proportion of Ethiopians showed shortened $CD4^+$ T-cell telomeres. Horizontal bars indicate median values. P values were calculated using the non-parametric Mann-Whitney U test.

The fraction of $CD31^+$ naive $CD4^+$ T cells is comparable between adult Ethiopian and adult Dutch individuals

CD31 has recently been described as a qualitative thymic proximity marker [28,49] (Vrisekoop et al. submitted). In addition to 34 Ethiopian adults aged 20-40 years (median age 31 yrs), we determined the fraction of $CD31^+$ cells in the naive $CD4^+$ T-cell pool of 10 children aged 6-16 years (median age 12.5 yrs) and of 10 cord blood samples. As has been found for Caucasians [28], the proportion of naive $CD31^+$ cells in the naive $CD4^+$ T-cell pool declined significantly with age in Ethiopians. The respective median percentages $CD31^+$ naive $CD4^+$ T cells for Ethiopian children and adults were 73.5% and 61.5% (p=0.002). When Ethiopians were compared to age-matched Dutch controls, neither significant differences in the fraction $CD31^+$ naive $CD4^+$ T cells could be detected at birth (86.0% and 79.6% respectively, p=0.115) nor in adults (61.5% and 59.7% respectively, p=0.844)(Figure 6).

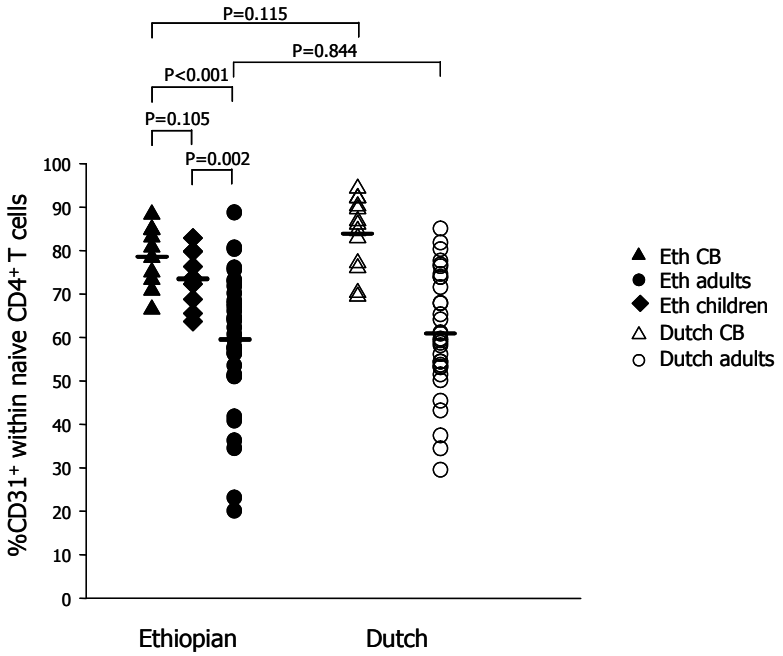

Figure 6. No differences in CD31 expression on naive CD4$^+$ T cells between Ethiopian and Dutch individuals at birth and during adulthood. The percentages CD31$^+$ cells within the naive CD4$^+$ T-cell pool significantly declined during age in Ethiopians. If Ethiopian and Dutch individuals were compared, neither significantly differences at birth (triangles) nor in adulthood (circles) were observed. Horizontal bars indicate median values. P values were calculated using the non-parametric Mann-Whitney U test.

Discussion

There is increasing evidence that chronic immune activation plays a major role in HIV-1 [1,2,5,32-35] and SIV [36] pathogenesis. For example, the level of immune activation induced by HIV has been shown to be one of the strongest predictors of CD4$^+$ T-cell decline [6,7]. CD4$^+$ T-cell depletion has also been described in other situations of chronic immune activation independent of HIV-infection, suggesting that chronic immune stimulation by itself could induce such a loss. Adult HIV-negative Ethiopians were reported to have low CD4$^+$ T-cell counts and multiple features of chronic immune activation, based on CD38 and HLA-DR T-cell activation markers and immunophenotyping by CD27 and CD45RO, as compared to Caucasians [1,8-15,17]. These differences have been proposed to be due to persistent immune activation induced by the large pathogenic burden in Ethiopia.

In the present study, we compared TREC contents, CD31 expression and T-cell telomere length to investigate whether immunological differences between Ethiopian and Dutch individuals are already evident at birth or develop upon exposure to environmental antigens. We found no differences in the measured immune parameters between Ethiopian and Dutch

neonates. Differences between Ethiopian and Dutch individuals appeared to develop during the first few years of life. Healthy Ethiopian children showed remarkable declines in $CD4^+$ and $CD8^+$ TREC contents shortly after birth, which was not observed in the Dutch control group. After the initially more rapid decrease in these parameters in Ethiopians, TREC contents declined in a parallel fashion in Dutch and Ethiopian individuals. The observed age-dependent declines in T-cell TREC contents are in line with previous studies in Caucasians [30,38-40]. Our model showed that the observed biphasic TREC dynamics observed in Ethiopians can be explained by an increased level of T-cell proliferation that sets in shortly after birth and is maintained throughout adulthood. Similarly, the early erosion of the naive T-cell pool in Ethiopians, which parallels an increasing proportion of cells with a more differentiated ($CD27^-$ memory) phenotype [17], differed from the age related changes in naive T-cell percentages in the Dutch counterparts, and could be explained by a continuous larger fraction of T cells that become activated in Ethiopians versus Dutch individuals. Such high levels of T-cell proliferation in Ethiopians are most likely caused by the high antigenic burden to which Ethiopians are exposed [1]. Our model results confirm an increased rate of T-cell activation due to exposure to a high antigenic burden is expected to have its major impact at early age, and does not lead to a faster rate of TREC decline during adulthood.

The fact that at birth TREC contents of Ethiopian and Dutch individuals were similar, supports the notion that the low $CD4^+$ T-cell counts and TREC contents of Ethiopians are to a large extent environmentally driven [17]. It has previously been shown that T-cell numbers and activation status are similar in Israelis and Ethiopian immigrant Jews after some years of stay in Israel [10], underscoring the hypothesis of an environmental cause [43]. In line, if we mimic the situation of discontinuing immune activation in the mathematical model, TREC content values in Ethiopians are predicted to increase to Dutch age-matched values (data not shown). It remains to be elucidated, however, why healthy adult Ethiopians do and other Africans, for instance Ugandans in whom the phenomenon of chronic immune activation has been demonstrated [43], do not have low $CD4^+$ T-cell counts [18,23].

In line with previous studies in Caucasians [28], the frequency of CD31-expressing naive $CD4^+$ T cells declined significantly with age in healthy Ethiopians. CD31 has been proposed as a marker to distinguish naive $CD4^+$ T cells that are most proximal to the thymus and have a diverse T-cell receptor repertoire [28,49] (Vrisekoop et al. submitted). While the percentage naive $CD4^+$ T cells was significantly reduced in adult Ethiopian compared to adult Dutch individuals, the proportion of cells expressing CD31 within the naive $CD4^+$ T-cell pool was comparable in Dutch and Ethiopian adults. Likewise, we have previously found (Vrisekoop et al. submitted) that although HIV-infection caused a continuous depletion of absolute naive $CD4^+$ T-cell numbers, the fraction of $CD31^+$ cells within the remaining naive $CD4^+$ T-cells was hardly affected. Together these data indicate that during chronic immune activation $CD31^+$ and $CD31^-$ naive $CD4^+$ T cells are lost to a similar extent.

Although the median telomere length of Ethiopian adults was not significantly shorter than that of Dutch controls, about 40% of the Ethiopian subjects did show evidence of shortened $CD4^+$ T-cell telomeres. In some of the Ethiopian subjects, however, $CD4^+$ and $CD8^+$ TREC

content were reduced, but nevertheless normal telomere lengths were observed. This is not necessarily contradictory, since small increases in the division rate are thought to have more pronounced effects on TREC dilution [1] than on telomere length [44]. Of note, telomerase activity was not found to be enhanced in these Ethiopian subjects (data not shown).

Our earlier studies and the present results in HIV-negative Ethiopians show that many immunological features of HIV infection, i.e. low total and naive $CD4^+$ T-cell counts, high T-cell proliferation rates, and decreased TREC contents, also occur in situations of chronic immune activation independent of HIV infection, and may thus in case of HIV infection be a direct consequence of the immune activating effect of the virus. While it has repeatedly been suggested that low TREC contents in HIV infection reflect impaired thymic output [30,45], we have argued that the speed at which TRECs are reduced in HIV infection suggests that it results from chronic immune activation induced by HIV [1,3,31]. In this study, we lend further support to this argument by showing a remarkable and rapid dilution of TRECs in non-HIV mediated chronically immune activated subjects at very early age.

Although we identified many immunological characteristics of HIV infection in a non-HIV context, and showed that they are a natural result of immune activation in Ethiopians, it remains to be explained why healthy Ethiopians do not develop AIDS-like symptoms. Possible factors that may be involved are continuous presentation of HIV antigenic variants, selective and progressive removal of TCR specificities, evolution of SI (syncytium inducing) HIV variants that are capable of infecting not only memory T lymphocytes but also naive $CD4^+$ T cells and thymocytes, and irreversible impairment of T-cell functions. Reversal of impaired immune function in situations of other chronic infections has been demonstrated. Antihelminthic treatment, for instance, has been shown to reverse both quantitative and qualitative defects of T lymphocytes [10,14,16,46-48]. Our results also suggest that additional interventions against non-HIV related chronic diseases, that drive the activation of the immune system, could have beneficial effects on the response to vaccines [48] in general or to antiretroviral therapy (ART) to HIV when subjects with such an immune background get infected with HIV.

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Slow $CD4^+$ T-cell decline in Ethiopian compared to Dutch HIV-infected individuals is due to lower T-cell proliferation rates

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Chapter 5

Abstract

It has recently been shown that HIV-infected Ethiopians have a slower rate of $CD4^+$ T-cell decline than Dutch HIV-infected individuals. This was unexpected, because healthy Ethiopians have a higher basal level of immune activation than healthy Dutch individuals and high immune activation is one of the best predictors for HIV-disease progression. Here we found that the percentages proliferating $Ki67^+$ cells within the naive and memory $CD4^+$ and $CD8^+$ T-cell subsets were lower in HIV^+ Ethiopians compared to Dutch HIV-infected patients matched for $CD4^+$ T-cell count. Thus, the slower $CD4^+$ T-cell decline in HIV^+ Ethiopians might be explained by lower levels of immune activation.

Introduction

It has previously been found that T-cell activation as determined by expression of HLA-DR [1] and T-cell proliferation as measured by Ki67 expression [2] are increased in healthy Ethiopians compared to Caucasians. This high level of immune activation in healthy Ethiopians is most likely caused by exposure to environmental pathogens and is accompanied by low numbers of total and naive CD4$^+$ T cells [1-4]. Similarly, persistent immune activation has been proposed to cause CD4$^+$ T-cell depletion in HIV infection [5]. Indeed, the presence of high T-cell immune activation and proliferation levels was found to be the best predictor for HIV-disease progression [6-8].

Because of the basal persistent immune activation in healthy Ethiopians, one would expect that upon HIV infection Ethiopians might also have higher levels of immune activation than Dutch HIV$^+$ individuals, and concomitantly might show a faster rate of CD4$^+$ T-cell decline and progression to AIDS. Surprisingly, Mekonnen et al. [9] found that the rate of CD4$^+$ T-cell decline was in fact significantly lower in HIV$^+$ Ethiopians compared to HIV$^+$ Dutch patients. The rate of CD4$^+$ T-cell decline was strongly dependent on the CD4$^+$ T-cell count in both Dutch and Ethiopian HIV-infected individuals, and since HIV$^+$ Ethiopians have lower baseline CD4$^+$ T-cell counts, this could explain their lower loss rates. However, even when the data were stratified according to CD4$^+$ T-cell counts, the loss rate of CD4$^+$ T cells remained lower in HIV$^+$ Ethiopians [9]. Differences in viral load or the lack of syncytium-inducing (SI), CXCR4 tropic virus variants amongst Ethiopian HIV-infected individuals [10], also did not explain the faster CD4$^+$ T-cell decline in Dutch HIV-infected individuals [9], suggesting other factors play a role.

Here we analyzed the fraction of proliferating T cells in Ethiopian and Dutch HIV-infected individuals. It turned out that despite their higher baseline levels of immune activation, Ethiopians had lower fractions of Ki67$^+$ T cells upon HIV-infection compared to Dutch HIV-infected individuals. These observations are in line with the earlier finding that immune activation levels are one of the strongest predictors of HIV disease progression [6-8].

Materials and Methods

Study population

Ethiopian HIV-infected individuals (n=19) were recruited from (i) The All African Leprosy Research and Training Centre (ALERT) and Higher 23 Health Centre, and (ii) from two cohort sites of the Ethio-Netherlands AIDS Research Project (ENARP). Dutch HIV-infected individuals (n=19) were included from the Amsterdam Cohort Studies on HIV infection and AIDS and were matched to the HIV$^+$ Ethiopians for CD4$^+$ T-cell counts. All studied HIV-infected individuals were naive to antiretroviral therapy and had CD4$^+$ T-cell numbers above 200 cells/µl blood. The study protocol was approved by ethical clearance committees and informed consent was obtained from all study participants.

Flow cytometry

As a measure for immune activation we determined the percentage proliferating $Ki67^+$ T cells (measured as previously described (Tegbaru et al., submitted)) in total, naive ($CD45RO^-CD27^+$), memory ($CD45RO^+CD27^+$), effector/memory ($CD45RO^+CD27^-$) and ($CD45RO^-CD27^-$) effector $CD4^+$ and $CD8^+$ T cells by FACS analysis.

Statistical analysis

Differences between Dutch and Ethiopian HIV-infected individuals were analyzed by the non-parametric Mann-Whitney test using the software program SPSS 12.0.1 (SPSS Inc., Chicago, Illinois, USA). Of note, if we were unable to measure Ki67 expression in a specific cell subset due to limited cell availability, the Ki67 expression of that particular T-cell subset in the individual with matched $CD4^+$ T-cell count was also omitted for analysis.

Results

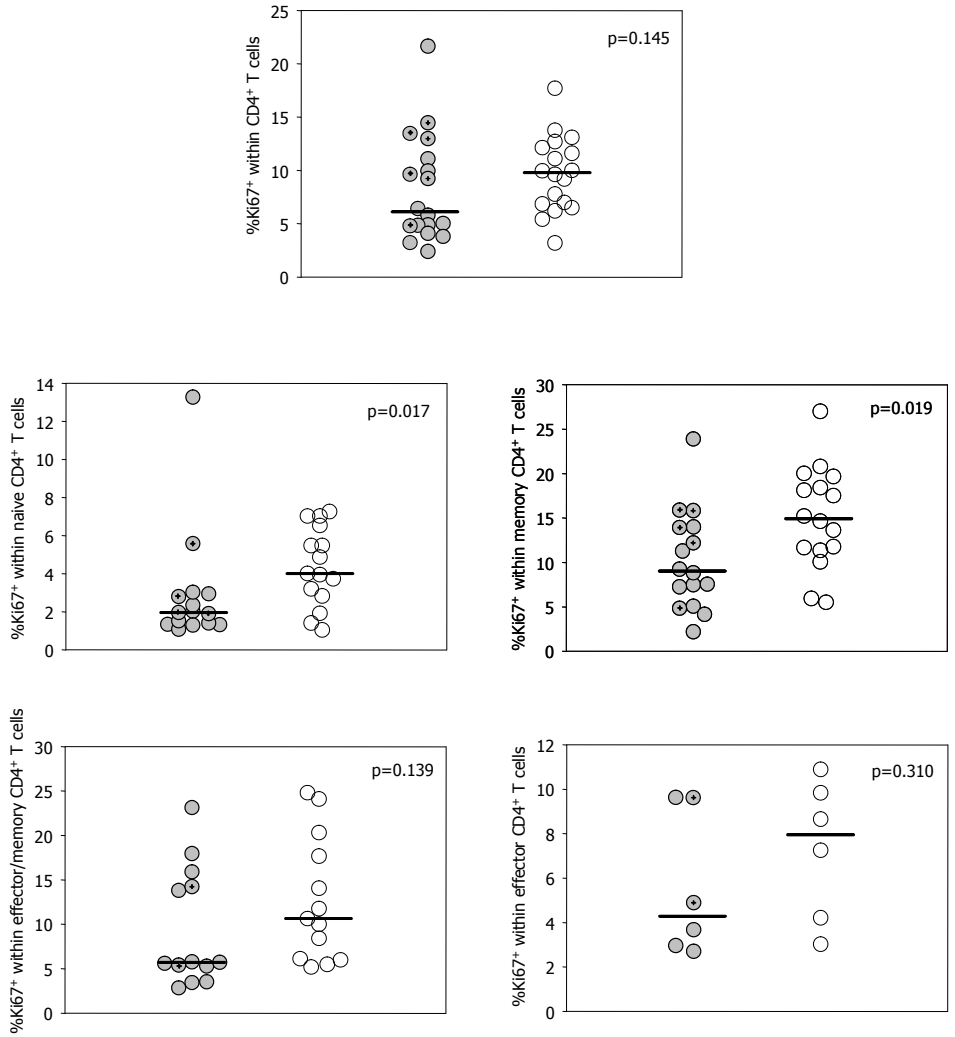
Since the rate of $CD4^+$ T-cell decline is dependent on $CD4^+$ T-cell numbers [9] and because the fraction of proliferating ($Ki67^+$) T cells increases during disease progression [6], every HIV^+ Ethiopian individual was matched with a Dutch HIV^+ individual according to $CD4^+$ T-cell count. Given that SI virus variants are rare amongst Ethiopian HIV-infected individuals, we only included Dutch HIV-infected individuals who exclusively harbored NSI (CCR5-using) virus variants at the time of analysis. The characteristics of the HIV^+ Ethiopian and HIV^+ Dutch groups are depicted in Table 1. Age and viral RNA load in plasma were not significantly different between Ethiopian and Dutch HIV-infected individuals. However, only in 10 out of 19 Dutch HIV^+ individuals viral load was determined at the time-point at which PBMC were analyzed. If we included the mean plasma viral loads of adjacent time-points for the other 8 patients (for one patient plasma viral load was not available) the median load of the Dutch HIV-infected individuals and statistical significance were unaffected. As reported before [11], $CD8^+$ T-cell counts were significantly higher in the HIV-infected Ethiopian compared to HIV-infected Dutch individuals ($p=0.002$).

In all analyzed $CD4^+$ and $CD8^+$ T-cell subsets a similar trend of increased $Ki67^+$ fractions in Dutch compared to Ethiopian HIV-infected individuals could be observed. However, probably due to the small sample size, statistically significant differences were not reached for every T-cell subset. The median percentage $Ki67^+$ within total $CD4^+$ T cells was 9.79% in HIV^+ Dutch and 6.11% in HIV^+ Ethiopian individuals ($p=0.145$). Median percentages of $Ki67^+$ were 4.01% versus 1.96% within naive ($p=0.017$), 14.93% versus 9.03% in memory ($p=0.019$) and 10.64% versus 5.71% in effector/memory ($p=0.139$) $CD4^+$ T cells in HIV-infected Dutch and Ethiopian individuals, respectively (Figure 1A). Although the $CD4^+$ effector population is usually very small in healthy individuals, this population of $CD4^+$ T cells tends to be enlarged during chronic immune activation [12]. We could measure Ki67 expression in the $CD4^+$ effector population in 6 Dutch and Ethiopian HIV-infected individuals and $Ki67^+$ fractions were 7.95% versus 4.28% ($p=0.310$), correspondingly.

The median percentage Ki67+ within total CD8+ T cells was 8.77% in Dutch versus 5.78% in Ethiopian HIV-infected individuals (p=0.030). Differences in median Ki67+ fractions between HIV+ Dutch and Ethiopian individuals in CD8+ T cells subsets were 4.46% versus 3.59% within naive (p=0.239), 13.99% versus 8.44% in memory (p=0.050), 10.62% versus 5.55% in effector/memory (p=0.034) and 5.08% versus 3.47% in effector (p=0.034) CD8+ T cells, respectively (Figure 1B).

Interestingly, seven out of nineteen HIV+ Ethiopians suffered from *Mycobacterium tuberculosis* (TB) (Figure 1, +-marked grey circles), which we previously showed to result in increased T-cell proliferation (Tegbaru et al., submitted). Thus in spite of TB co-infection in 7 HIV+ Ethiopians, Dutch HIV+ individuals appeared to have significantly higher fractions of Ki67+ T cells.

A



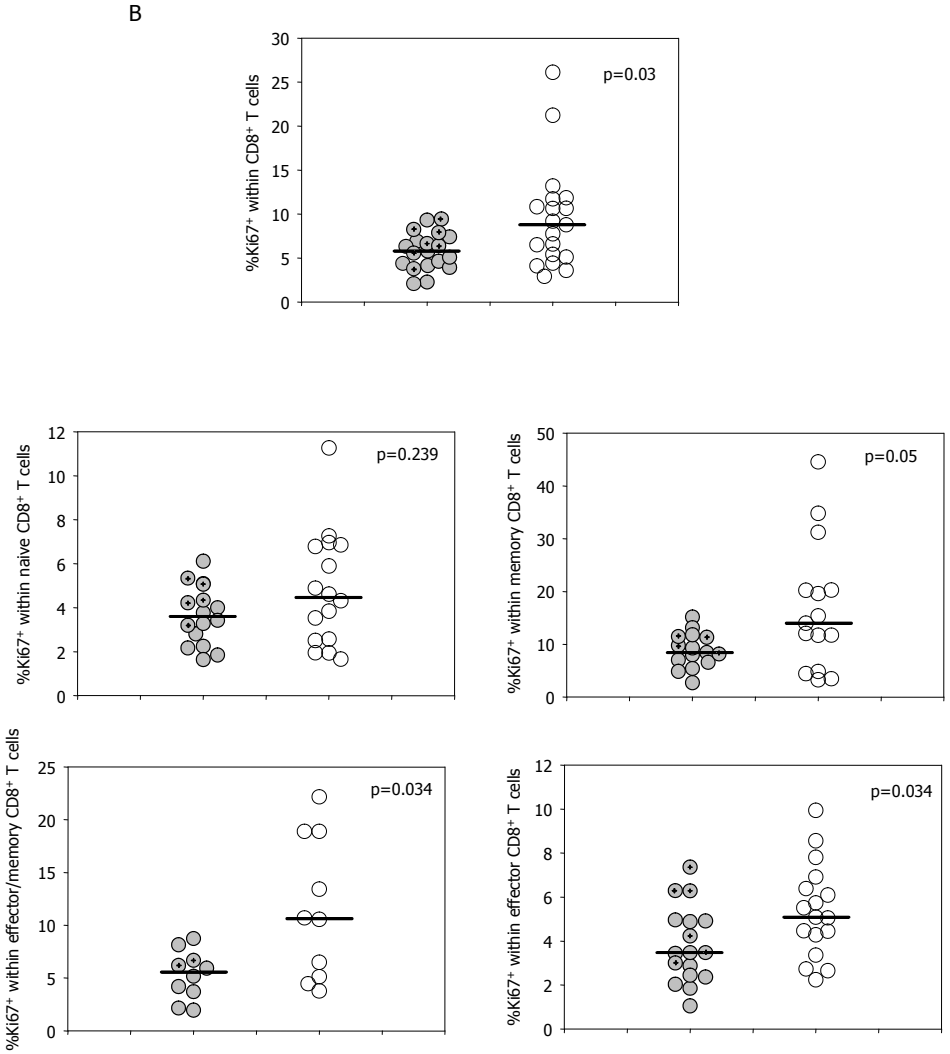


Figure 1. Lower fractions of Ki67$^+$ T cells in HIV$^+$ Ethiopian compared to HIV$^+$ Dutch individuals. Percentage Ki67$^+$ T cells within total, naïve, memory, effector/memory and effector CD4$^+$ (A) and CD8$^+$ (B) T cells of HIV$^+$ Dutch and Ethiopian individuals. Grey circles denote HIV$^+$ Ethiopians, +-marked grey circles HIV$^+$ Ethiopians co-infected with TB, and open circles represent HIV$^+$ Dutch individuals. Differences between Dutch and Ethiopian HIV-infected individuals were analyzed by the non-parametric Mann-Whitney test. Medians and p-values are indicated in the figure.

Discussion

Since immune activation is regarded as the driving force for HIV-disease progression [5], the pre-existing higher immune activation levels in healthy Ethiopians suggest that, upon HIV infection, Ethiopians might have a faster rate of CD4$^+$ T-cell decline compared to HIV$^+$ Dutch individuals. Unexpectedly, it has previously been found that HIV$^+$ Ethiopians have a slower rate of CD4$^+$ T-cell decline compared to HIV$^+$ Dutch individuals [9]. In line with this slower loss of CD4$^+$ T cells in HIV$^+$ Ethiopians we here find that, despite their higher basal levels of immune activation, Ethiopians had lower fractions of Ki67$^+$ T cells upon HIV-infection compared to Dutch HIV-infected individuals. The fact that Dutch HIV-infected individuals tended to have higher Ki67$^+$ fractions, even though 7 out of 19 HIV$^+$ Ethiopians were co-infected with TB, which we have shown to increase Ki67$^+$ T-cell fractions in Ethiopian HIV-infected individuals (Tegbaru et al., submitted), is in support of our conclusion.

To exclude transient high fractions of Ki67$^+$ T cells due to opportunistic infections we studied HIV-infected individuals who had CD4$^+$ T-cell numbers above 200 cells/µl blood and all HIV$^+$ Dutch individuals had not progressed further than CDC group III. Higher levels of T-cell proliferation in Dutch HIV$^+$ individuals could not be explained by syncytium inducing, CXCR4-using HIV-1 variants because we only included HIV$^+$ Dutch individuals that exclusively harbored NSI virus variants.

Although high T-cell immune activation was found to be the best predictor for HIV disease progression, viral load is correlated with immune activation and has similarly been shown to be related to progression to AIDS [6-8]. HIV viral load is typically lower in Ethiopians early after HIV infection [11]. This is mainly due to Ethiopians infected with HIV-1 subtype C, who have a lower viral load than Ethiopians with the predominant subtype C' early in infection [11]. During HIV infection, however, viral load in Ethiopians with subtype C is known to increase, and 2 years after seroconversion the viral load of Ethiopians was found to be similar to that of Dutch clade B HIV-infected individuals [11]. As mentioned above, there was no evidence for lower viral load in Ethiopians compared to Dutch HIV-infected individuals in our study group (Table 1) that could explain the lower Ki67$^+$ fractions in HIV$^+$ Ethiopians. Our results could also not be explained by including 8 (out of 19) women in the HIV$^+$ Ethiopian group, who are known to have a lower viral load than men [13], since Ki67$^+$ fractions were not significantly lower in HIV-infected women compared to men in any of the T-cell subsets.

HIV infection has been reported to increase the fractions of proliferating Ki67$^+$ T cells 3 to 4-fold in Ethiopians (Tegbaru et al., submitted) [14]. Thus although HIV infection increases the fraction of proliferating cells in both Ethiopian and Dutch HIV-infected individuals, apparently HIV infection results in less proliferation in Ethiopians. It has been proposed previously that Ethiopians might have been evolutionarily selected to respond less to environmental pathogens, to minimize accelerated aging of the immune system as a consequence of continuous high level exposure to pathogens [9]. Alternatively, parasitic helminths in Ethiopians might create an anti-inflammatory environment [15]. It remains to

be determined whether the lower Ki67$^+$ fractions within T-cell subsets of Ethiopians are indeed determined by host factors or are related to HIV-1 subtype C.

Table 1. Characteristics of Ethiopian and Dutch HIV$^+$ individuals

	Ethiopian (n=19)	Dutch (n=19)
Gender (F/M)	8/11	0/19
TB co-infected	7	0
Age (median, range)	31 (22-50)	31 (24-50)
CD4$^+$ T cell count in cells/µl (median, range)	326 (212-493)	330 (210-490)
CD8$^+$ T cell count in cells/µl (median, range)*	1128 (315-3231)	800 (400-1430)
Viral RNA load in plasma in copies/ml (median, range)	46,000 (5,100-840,000) n=18	39,000 (1,000-260,000) n=10

* significantly different between the two groups (p=0.002)

Acknowledgements

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No detrimental immunological effects of mycophenolate mofetil and HAART in treatment-naive acute and chronic HIV-1-infected patients

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Chapter 6

Abstract

Mycophenolate mofetil has been proposed for HIV-1 therapy because of its guanine-depleting effect, which is expected to interfere with HIV-1 replication directly by hampering reverse transcription and indirectly via inhibition of $CD4^+$ T-cell proliferation. However, treatment with mycophenolate mofetil might also compromise lymphocyte reconstitution and HIV-specific immunity. Therefore we longitudinally studied the effects of mycophenolate mofetil in combination with HAART on T-cell proliferation, lymphocyte reconstitution and HIV-specific $CD4^+$ and $CD8^+$ T-cell responses in six therapy-naive, acute or chronic HIV-1-infected patients, as compared to eight patients treated with HAART alone. T-cell proliferation in whole blood cultures of patients treated with mycophenolate mofetil was inhibited. Strikingly, *ex vivo* Ki67 expression within T cells was not influenced by treatment with mycophenolate mofetil. *In vitro* studies showed that Ki67 expression occurs at an early step of the cell cycle and was not inhibited by guanine depletion. When treatment with mycophenolate mofetil was stopped a transient increase in apoptosis and Ki67-expressing T cells was detected. This observation together with near complete inhibition of T-cell proliferation in whole blood cultures during treatment with mycophenolate mofetil indicated that T-cell proliferation was inhibited in patients treated with mycophenolate mofetil. Still, there was no evidence for detrimental effects of treatment with mycophenolate mofetil in addition to HAART on $CD4^+$ T-cell reconstitution or HIV-specific immunity.

It has proven impossible to eradicate HIV-1 with currently available antiretroviral therapies. Beside a latent viral reservoir that is maintained in non-replicating cells [1-4], a low level of viral replication is sustained even in individuals in whom highly active antiretroviral therapy (HAART) has decreased the plasma viremia to undetectable levels [5-10]. A logical approach to improve treatment of HIV-1 is to directly or indirectly interfere with the availability of target cells and target cell characteristics necessary for completion of virus replication. Although T-cell proliferation, assessed by the expression of the proliferation marker Ki67, decreases by HAART, it remains elevated compared to normal values throughout 48 weeks of treatment [11]. To reduce residual replication and reseeding of the latent reservoir even further [6], lymphocyte proliferation is thus a likely target for treatment.

The immunosuppressant mycophenolate mofetil (MMF) is a prodrug of mycophenolic acid (MPA), its active metabolite, which selectively suppresses lymphocyte proliferation. MPA suppresses lymphocyte proliferation through noncompetitive, reversible inhibition of inosine 5'-monophosphate dehydrogenase (IMPDH), a key enzyme in the *de novo* synthetic pathway of the purine guanine [12,13]. Inhibiting IMPDH results in a decrease of available guanine with the result that lymphocytes fail to progress to the S phase of the cell cycle [14-16]. Whereas T and B lymphocytes strongly depend on *de novo* synthesis of purines for their proliferation, other cell types are able to make use of both *de novo* synthesis and the salvage pathway. Therefore, MPA is believed to have a more potent cytostatic effect on lymphocytes than on other cell types.

MMF has been proposed for HIV-1 therapy, since guanine depletion is expected to prevent HIV-1 replication indirectly via inhibition of $CD4^+$ T-cell division and because MPA suppressed HIV-1 replication directly by hampering reverse transcription *in vitro* [17]. Furthermore, the efficacy of the guanosine analogue reverse transcriptase inhibitor abacavir increases in the presence of MPA [18]. Indeed Chapuis et al. [19] demonstrated that the addition of MMF to HAART in HIV-1-infected patients with a plasma HIV-1 RNA of less than 5 copies/ml for over 36 weeks resulted in a decrease of the number of latently infected $CD4^+$ T cells. Moreover, they reported that treatment with MMF resulted in a minor decrease in the percentage of dividing $CD4^+$ and $CD8^+$ T cells as measured by Ki67 expression. On the other hand, inhibiting lymphocyte proliferation by MPA might also inhibit the immune response against HIV-1, causing decreased clearance of the virus. In addition, lymphocyte counts could be negatively influenced by MPA, with the risk of reducing or preventing lymphocyte reconstitution. Here we report the immunological effects of the immunosuppressive drug MMF, which interferes with *de novo* guanine synthesis and HIV-1 replication, during antiretroviral treatment in HIV-1-infected patients.

Fourteen antiretroviral-naive men, seven with an acute infection (treatment started within 2 months after onset of symptoms of acute HIV infection) and seven with a chronic infection (treatment started when $CD4^+$ T-cell numbers were <350 cells/µl or when plasma HIV-1 RNA was >30,000 copies/ml) were recruited. A detailed description and baseline characteristics have been reported elsewhere [20,21]. All patients started with a triple class, five-drug regimen consisting of didanosine, abacavir, lamivudine, indinavir (boosted with

ritonavir) and nevirapine. Patients were randomized to one group with MMF and one group without MMF. Overall, no side effects were seen in patients using MMF and no significant additional effects of MMF were observed compared to HAART alone on HIV-1 virological parameters [20].

Profound inhibition of T-cell proliferation in cultures of diluted whole blood of patients treated with MMF (measured as previously described [22]), as well as *in vitro* inhibition of lymphocyte proliferation by plasma from a patient treated with MMF, suggested a strong *in vivo* effect of MPA. However, *ex vivo* Ki67 expression by naive, memory and effector $CD4^+$ and $CD8^+$ T cells [23] appeared not to be lower when MMF was administered concomitantly with HAART (Figure 1 shows results on Ki67 expression in total $CD4^+$ T cells) as has been reported recently by Garcia et al.

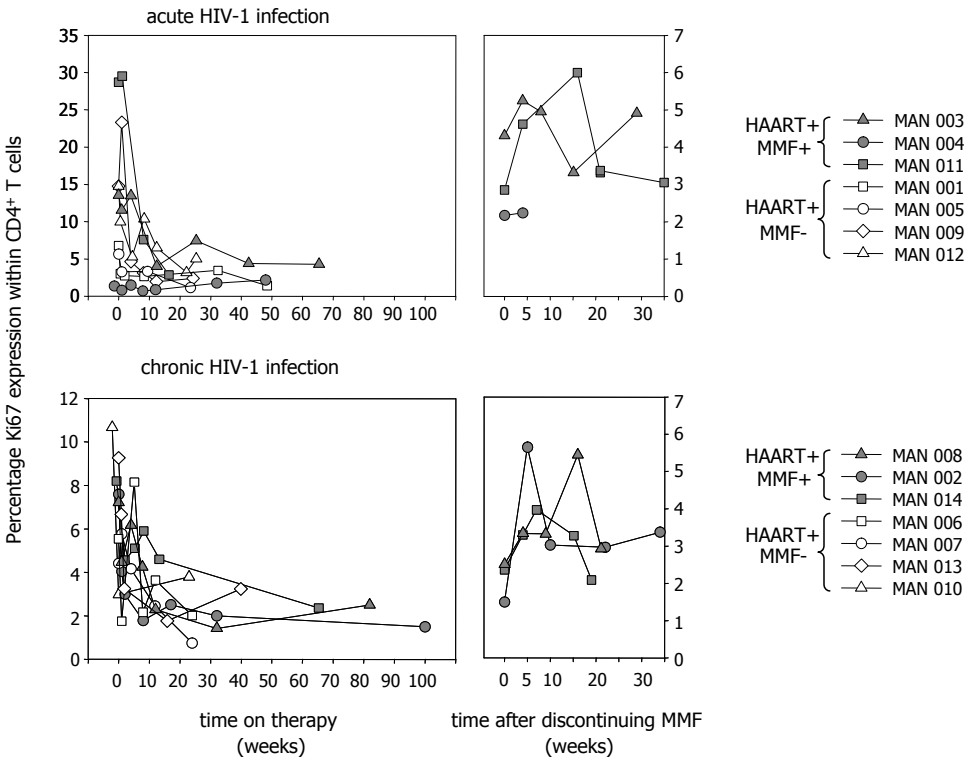

Figure 1. *Ex vivo* Ki67 expression by $CD4^+$ T cells. Percentage Ki67 expression within total $CD4^+$ T cells in acute (upper panel) and chronic (lower panel) HIV-1 infection during treatment. In the left panels patients treated with mycophenolate mofetil (gray symbols) are compared to patients treated with HAART alone (white symbols). Right panels illustrate Ki67 expression when treatment with mycophenolate mofetil is discontinued, time point zero being the last time point indicated in the left panel.

[24] This apparent discrepancy can in part be explained by our *in vitro* studies which showed that Ki67 is expressed at a step of the cell cycle prior to the inhibition of MPA. MPA treatment (3 µg/ml, Gibco BRL, Grand Island, NY) was indeed able to inhibit thymidine incorporation for at least 8 days (Figure 2) and to inhibit CFSE dilution at least up to 6 days of stimulation (1 µg/ml αCD3 and αCD28). Ki67 expression in the presence of MPA, however, was elevated at day 4, its expression being even more pronounced at day 6 (this increased Ki67 was found in both $CD4^+$ and $CD8^+$ T cells and is shown for $CD8^+$ T cells in Figure 2).

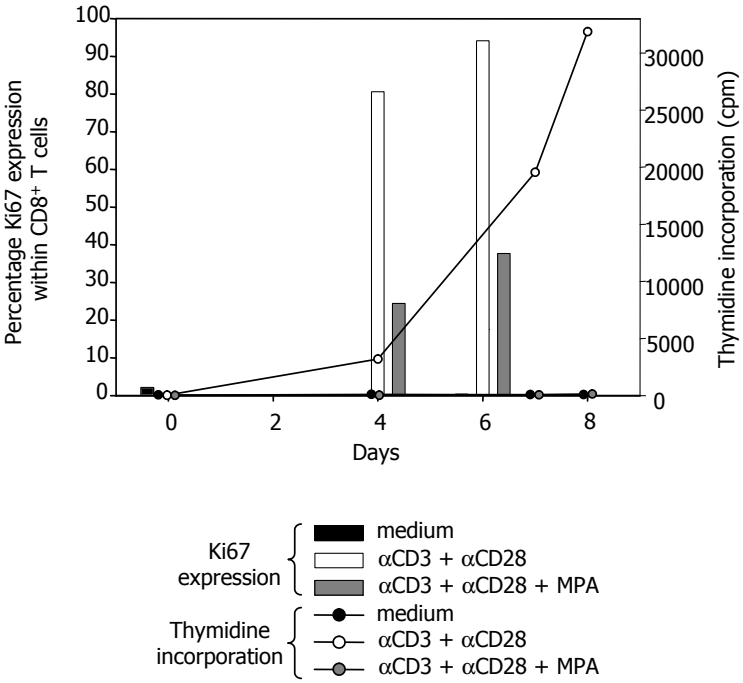

Figure 2. Representative *in vitro* experiment comparing thymidine incorporation with Ki67 expression in $CD8^+$ T cells after stimulation with αCD3 and αCD28. Thymidine incorporation (right axes) in stimulated mycophenolic acid-treated cells (gray dots) is compared to stimulated control cells (white dots) and unstimulated control cells (black dots) up to 8 days of culture. After 4 and 6 days of culture Ki67 expression in $CD8^+$ T cells (left axes) in mycophenolic acid-treated cells (gray bars) is compared to unstimulated control cells (black bars) and stimulated control cells (white bars).

Since thymidine incorporation was still inhibited 4 days after the elevation of Ki67-expressing T cells this excluded the possibility that MPA had lost its inhibitory effect with time of culture. Therefore these experiments confirm that Ki67 expression occurs at an early step of the cell cycle (G1 phase) [25] that is not inhibited by MPA. Thus measuring Ki67 in MMF-treated individuals gives an overestimation of the number of cells that will actually finish their cycle. However, since proliferating cells clonally expand in response to antigen

and Ki67 is expressed in every stage of the cell cycle of proliferating cells [25] one can envisage that MPA will at least cause a decrease in the percentage of $Ki67^+$ T cells due to the lack of Ki67-expressing progeny. Indeed in our *in vitro* experiments the percentage of Ki67-expressing cells does appear to be decreased in stimulated cells treated with MPA (Figure 2) and in the clinical trial with MMF reported by Chapuis et al. [19], a minor decrease in the fraction of proliferating T cells was found. At first glance, these results seem inconsistent with our data and the findings of Garcia et al. [24], which do not show a decrease in Ki67-expressing lymphocytes in patients additionally treated with MMF. The considerable effect of potent HAART alone could mask an effect of MMF, since in our study design a five drug regimen was used as opposed to only two drugs in the study reported by Chapuis et al. [19]. High variability between the persons in this small study group could also conceal an effect of MPA and might explain the discrepancy. Chapuis et al. [19] determined the additional effect of treatment with MMF after each individual's set point during treatment with HAART alone was reached. Instead, we monitored the patients when MMF treatment was withdrawn to investigate the effect of MPA on Ki67 expression at an individual level. Indeed, after discontinuation of treatment with MMF, we found a temporary increase in Ki67 in five of six patients (Figure 1 right panel). The only exception was patient MAN004, who had exceptionally high $CD4^+$ T-cell counts and low Ki67 expression before treatment and who turned out to serorevert during treatment [26]. The transient increase in the proportion of dividing cells after discontinuing treatment with MMF may be a reflection of ongoing T-cell activation during treatment with HAART. MPA will prevent completion of the cell cycle in these activated cells. On a daily basis this supposedly is a small proportion of T cells in a HAART-treated person, but since the lifespan of short-lived activated memory/effector cells is estimated to be 2-3 weeks [27], they may still accumulate to a measurable number in a person additionally treated with MMF. When treatment with MMF is discontinued these proliferation-arrested cells will go through cell cycle in a rather synchronized way, causing a temporary elevation in the percentage of Ki67-expressing T cells. Interestingly, a similar transient increase was noticed for the number of latently HIV-1-infected resting HLA-DR^- $CD4^+$ T cells in these patients [20]. In accordance, after discontinuation of treatment with MMF an increase in apoptosis was found (measured by FACS analysis using Annexin V and Propidium Iodide staining, Bender MedSystems, Vienna, Austria), which probably reflects activation-induced cell death of the proliferating cells.

Lymphocyte reconstitution during treatment with HAART is generally biphasic with an initial steep rise in $CD4^+$ T-cell count during the first 3 weeks of treatment, reflecting redistribution from the lymphoid tissues, followed by a more gradual increase [28]. Given that MPA inhibits lymphocyte proliferation, MPA might hamper lymphocyte reconstitution or could even reduce lymphocyte counts. Therefore, we compared the kinetics of total and naive $CD4^+$ T-cell counts defined as described previously [29,30]. To consider the effects of redistribution and to account for fluctuations, pre-treatment levels were compared to mean counts of week 4, 8 and 12 and to mean counts of week 48, 56 and 64 during treatment. Additional treatment with MMF had no effect on the increase in total $CD4^+$ T-cell numbers over time (Figure 3).

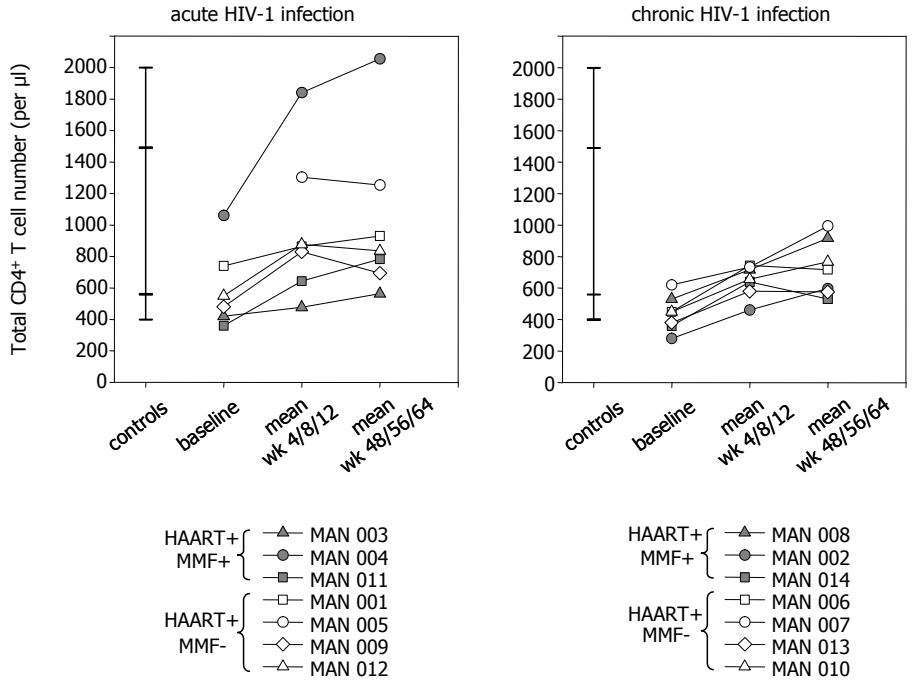

Figure 3. Total CD4$^+$ T-cell counts (per µl blood) pretreatment compared to the mean of weeks 4/8/12 and weeks 48/56/64 during treatment. Patients with additional mycophenolate mofetil treatment (gray symbols) were compared to patients treated with HAART alone (white symbols) and to healthy controls (5th to 95th and 1st to 99th percentiles). Left panel; patients with acute HIV-1 infection. Right panel; patients with chronic HIV-1 infection.

Even though variability between patients was high, reconstitution of naive CD4$^+$ T-cell numbers was also not affected by MMF treatment. Hence, we confirm that the increase in total and naive numbers of CD4$^+$ T cells is not affected by MPA in HIV-1-infected individuals [19,24,31,32]. This may be explained by the fact that MPA acts differentially on thymocytes, resting and activated T cells, and that these subsets contribute differentially to the T-cell pool. The key enzyme in the *de novo* pathway inhibited by MPA, IMPDH, exists in two isoforms. One isoform (type II), mainly used by activated lymphocytes, is much more actively inhibited by MPA than the other isoform (type I) used by resting cells [33,34]. Since activated T cells are prone to die by activation-induced cell death, proliferation within this population presumably does not contribute to maintain the T-cell pool. Furthermore, the *de novo* pathway might be of less importance for thymocytes, since enzymes of the salvage pathway are more active or present in higher levels in thymocytes compared to mature T cells [35,36]. Indeed, MPA has been reported to have a limited inhibitory effect on proliferation of thymocytes in mice [37].

Inhibition of T-cell proliferation might dampen the specific T-cell response against HIV-1. To monitor a potential negative effect of MPA on HIV-1-specific T-cell reactivity, we longitudinally measured the proportion of $CD4^+$ and $CD8^+$ T cells that produce interferon (IFN)-γ after stimulation with HIV-1-specific antigens. To measure IFN-γ-producing HIV-1-specific $CD4^+$ and $CD8^+$ T cells, $CD8^+$ T cells were stimulated with HLA-specific peptides and $CD4^+$ T cells were stimulated with either gag or nef peptide pools in the presence of costimulation, after which cells were analyzed using a FACSCalibur flow cytometer [38,39]. IFN-γ-producing HIV-1-specific $CD8^+$ T cells were enumerated using IFN-γ-specific ELIspot assays for patients with HLA-A3 and HLA-B7, as previously described [4,6,40]. We were unable to look at HIV-1-specific immunity in two patients in the HAART-alone arm since they both had HLA-types that did not match the specific peptides available. Before initiating therapy HIV-1-specific IFN-γ-producing $CD4^+$ lymphocytes could be detected in three of six patients within the MMF arm and in five of six patients treated with HAART alone. HIV-1-specific IFN-γ-producing $CD8^+$ lymphocytes prior to therapy were present in three of six patients treated with MMF and four of six patients within the HAART alone arm. Remarkably, HIV-1-specific IFN-γ-producing $CD4^+$ T cells (one patient) and $CD8^+$ T cells (two patients) were detected during MMF treatment in patients in whom no response could be measured before therapy. In those patients in whom a response could be measured before therapy a response could still be detected while they received MMF. Although there was a high variability of IFN-γ production in response to therapy between all patients, at least no obvious decreases in the percentage of IFN-γ-producing cells could be detected in patients additionally treated with MMF compared to patients treated with HAART alone (data not shown). However, since the number of HIV-1-specific IFN-γ-producing cells is measured after *in vitro* stimulation of PBMC, and thus in the absence of MPA, it remains hard to translate these results to the *in vivo* situation in the presence of MPA, especially since *in vitro* cultures have shown that MPA inhibits IFN-γ production by stimulated PBMC [41,42]. Still, at least HIV-1-specific T cells remain present during treatment with MMF.

This pilot study adds to the data suggesting that no obvious toxicities emerge during MMF treatment in HIV-1-infected patients. Our data indicate that T-cell proliferation is indeed inhibited in patients treated with MMF and this did not seem to have evident detrimental immunological effects. However, the virological effects of MMF in addition to potent antiretroviral therapy appeared to be minimal [20]. MMF has been postulated for antiviral treatment of multi-drug resistant HIV-1 infection [31]. Furthermore, since treatment with MMF causes minimal adverse effects, MMF might be promising for therapy-naive patients not yet eligible for HAART. By inhibiting lymphocyte proliferation and decreasing the amount of infected cells, the rate of $CD4^+$ T-cell loss might be diminished, thereby delaying requirement for antiretroviral agents. Indeed, recent studies showed that stopping HAART in the presence of MMF resulted in improved control of virus replication and a lower increase in Ki67-expressing $CD4^+$ T cells compared to patients without MMF [24,43,44].

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Part II

T-cell dynamics

Analysis of CD31 as a T-cell thymic proximity marker

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Chapter 7

Abstract

CD31 has been proposed as a marker that discriminates between ($CD31^+$) recent thymic emigrants and ($CD31^-$) naive $CD4^+$ T cells that have undergone peripheral proliferation. Consistent with this idea, it has been found that the percentage of $CD31^+$ T cells in the naive $CD4^+$ T-cell pool declines with age, and that the TREC content of $CD31^+$ naive $CD4^+$ T cells is consistently higher than that of their $CD31^-$ counterparts. Here we more thoroughly address the potential use of CD31 as a marker for recent thymic emigrants. First we established whether $CD31^+$ naive $CD8^+$ T cells similarly reflect the part of the T-cell pool that is most proximal to the thymus. Indeed, sorted $CD31^+$ naive $CD8^+$ T cells had a higher TREC content than sorted $CD31^-$ naive $CD8^+$ T cells. Although less pronounced than in naive $CD4^+$ T cells, the percentage of $CD31^+$ naive $CD8^+$ T cells similarly shows an age-dependent decline. We found, however, that the TREC content of $CD31^+$ naive $CD4^+$ and $CD8^+$ T cells declined with age, indicating that $CD31^+$ naive T cells are at least in part generated by peripheral proliferation. We investigated whether chronic immune activation by HIV-infection altered the fraction of $CD31^+$ T cells within the naive $CD4^+$ T-cell pool. Although absolute numbers of $CD31^+$ naive $CD4^+$ T cells decreased during HIV-infection, the fraction of $CD31^+$ T cells within the naive $CD4^+$ T-cell pool was not significantly lower than in healthy controls. To conclude, $CD4^+$ and $CD8^+$ naive $CD31^+$ T cells are most proximal to the thymus, but CD31 expression on naive T cells is not restricted to recent thymic emigrants.

Introduction

The contribution of the thymus to the maintenance of the T-lymphocyte pool during ageing, and to immune reconstitution in T-cell depleted patients continues to be debated. This is largely due to the fact that there is still no reliable and unambiguous marker for thymic output. Although thymic CT scans have shown that thymic volume correlates with naive $CD4^+$ T-cell counts in healthy and HIV-infected individuals [1], they do not provide a quantitative means to measure thymic output and cannot be used for frequent or large scale longitudinal monitoring. Alternatively, increases in naive T-cell numbers during immune reconstitution have been used to measure thymic output. However, since naive T cells can divide while retaining their naive phenotype [2,3], increases in naive T-cell numbers may reflect peripheral proliferation rather than thymic output.

Another method that is widely used to determine thymic output is the measurement of T-cell receptor excision circles (TRECs). Since these T-cell receptor excision products are only formed during T-cell receptor rearrangement in the thymus, they have been interpreted as a direct marker for thymic output [4]. However, since TRECs are not duplicated during T-cell proliferation, the average number of TRECs per T cell (referred to as TREC content) declines upon T-cell division. Although measuring increases in absolute numbers of TRECs per ml blood in a depleted situation is a useful indication for thymic output, the interpretation of absolute TREC numbers is hampered by T-cell death and the longevity of T cells. Thus caution should be taken when using TRECs as a direct marker for thymic output [5,6]. Furthermore, additional phenotypic and functional analysis of $TREC^+$ T cells is impossible.

Recently Kimmig et al. [2] proposed CD31 to be a marker that can be used to discriminate between recent thymic emigrants and naive $CD4^+$ T cells that have undergone peripheral T-cell proliferation. Since CD31 is known to be down-regulated upon $CD4^+$ and Jurkat T-cell stimulation [7,8], it was suggested that upon interaction with self-peptides presented by MHC, $CD31^+$ naive $CD4^+$ T cells divide and down-regulate CD31 expression while retaining the naive phenotype**.** Indeed, $CD31^-$ T cells had a gene expression profile characteristic of recent TCR engagement and the TREC content of $CD31^-$ naive $CD4^+$ T cells was found to be consistently reduced compared to that of $CD31^+$ naive $CD4^+$ T cells [2]. Furthermore, $CD31^+$ naive $CD4^+$ T cells were shown to have a polyclonal T-cell receptor Vβ repertoire compared to the more oligoclonal repertoire of $CD31^-$ naive $CD4^+$ T cells [9].

In contrast to $CD4^+$ T cells, CD31 is not down-regulated on memory $CD8^+$ T cells [10]. Nevertheless, CD31 could be used as a thymic proximity marker for naive CD8 T cells if the TREC content of $CD31^+$ naive CD8 T cells exceeds that of $CD31^-$ naive CD8 T cells.

CD31, also known as platelet-endothelial cell adhesion molecule (PECAM-1), is a transmembrane glycoprotein and a member of the Ig superfamily [11]. Homophilic as well as heterophilic interactions (with CD38 and integrins) have been described for CD31 [12,13]. CD31 contains an ITIM motif on the intracellular part which has been proposed to suppress apoptosis of T cells [14] and to dampen TCR signalling through the ITAM motif on the TCR [15,16]. Other proposed functions of CD31 are related to cellular adhesion [12,17,18]. On T cells, CD31 is expressed from the double positive thymocyte stage onward

[10,19]. With age, both the fraction of $CD31^+$ cells within the naive $CD4^+$ T-cell pool and the absolute number of $CD31^+$ naive $CD4^+$ T cells decrease [2,9].
If $CD31^+$ naive T cells are indeed exclusively thymus derived, CD31 could finally be the tool to unambiguously assess the role of thymic output. Indeed recent studies have begun to use CD31 to determine the role of the thymus in long-term immune reconstitution after hematopoietic stem cell transplantation [20] and to assess thymus function in relapsing-remitting multiple sclerosis [21] and immuno-suppressed kidney transplant patients [22]. An additional advantage of CD31 as a marker for recent thymic emigrants would be that sorting of $CD31^+$ T cells would allow for additional functional analyses of purified recent thymic emigrants.
Although the lower TREC content of the naive $CD31^-$ $CD4^+$ T cell subset shows that these cells have gone through more rounds of division since they emigrated from the thymus than $CD31^+$ T cells, this does not imply that naive $CD31^+$ T cells are exclusively formed by the thymus. We therefore investigated in further detail the robustness of CD31 as a marker for thymic output. The second goal of this study was to investigate the dynamics of the $CD31^+$ and $CD31^-$ naive $CD4^+$ T-cell pools during progressing stages of HIV infection. We hypothesized that chronic immune activation would lead to accelerated maturation of the naive T-cell pool and thereby to a reduction in the fraction of $CD31^+$ cells within the naive $CD4^+$ T-cell pool.
Together, these studies on the dynamics of $CD31^+$ naive T cells provide new insights into the nature of this naive T cell population and into the use of CD31 as a recent thymic emigrant marker. Although $CD4^+$ and $CD8^+$ naive $CD31^+$ T cells are most proximal to the thymus, $CD31^+$ naive T cells divide without the loss of CD31 expression and therefore do not strictly represent recent thymic emigrants.

Materials and methods

Blood samples

Peripheral blood mononuclear cells (PBMC) were acquired by Ficoll-Paque density gradient centrifugation from heparinized blood or buffycoats. Blood bank donors of different ages were used as controls. Samples from HIV-infected patients were partly derived from the Amsterdam Cohort Studies on HIV and AIDS and from patients who were treated at the Amsterdam Medical Center [30]. Fractions $CD31^+$ within naive CD4 T cells were measured in 61 HIV-infected individuals and 15 individuals who had progressed to AIDS. AIDS was defined as a CD4 T-cell count level below 200 cells/µl. Furthermore, we longitudinally determined the fraction $CD31^+$ within naive CD4 T cells in 11 individuals over seroconversion and in 18 patients longitudinally during HIV disease progression. None of the HIV-infected patients had ever been treated at the time of sampling.

Flow cytometry and cell sorting

To measure the fraction of $CD31^+$ T cells within the naive $CD4^+$ and $CD8^+$ T-cell population, cryopreserved PBMC were thawed and incubated with monoclonal antibodies (mAb) to CD45RO-FITC (Caltag), CD31-PE, CD4- or CD8-PERCP (BD) and biotinylated CD27 (Sanquin Reagents). After washing, cells were incubated with anti-Streptavidin-APC (BD), after which cells were fixed using Cellfix (BD) and analyzed on a FACSCalibur (BD) with Cellquest software. For Annexin V staining and cell sorting CD27 was omitted, in which case cells were stained as described above in a parallel sample to check for the absence of $CD27^-$ T cells within the $CD4^+CD45RO^-$ population. Apoptosis was measured by FACS analysis using mAb to CD31-PE, CD4-PERCP and CD45RO-APC (BD), after which cells were washed and stained with Annexin V-FITC (Bender MedSystems, Vienna, Austria) according to manufacturer's protocol. To purify $CD4^+CD45RO^-CD31^+$ and $CD4^+CD45RO^-CD31^-$ cells, cryopreserved PBMC were thawed and stained with mAb to CD45RO-FITC, CD31-PE and CD4-PERCP. To purify $CD8^+$ $CD45RO^-CD27^+CD31^+$/ $CD45RO^-CD27^+CD31^-$/ $CD45RO^+CD31^+$ and $CD45RO^+CD31^-$ cells, cryopreserved PBMC were thawed and stained with mAb to CD45RO-FITC, CD31-PE, CD8-PERCP and CD27-APC. The specified cell fractions were isolated by cell sorting on a MoFlow high speed cell sorter or a FACSAria (BD).

TREC analysis

After cell sorting, 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 previously described [5,31]. The number of Sj TREC copies present in a given cell population was calculated by including a dilution series of a Sj standard [5] 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 content could be calculated for each sample. To normalize for input DNA, the number of Cα constant regions that remain present on the TCR genome despite TCR rearrangements was determined in every sample tested. From the average TREC content as measured per μg DNA, the TREC content per cell was calculated by dividing the TREC content by 150.000 (assuming that 1 μg DNA corresponds to 150.000 cells).

Mathematical model of $CD31^+$ and $CD31^-$ naive $CD4^+$ T cell population dynamics

In the model, $CD31^+$ naive T cells (N_p) are generated by thymic output, which decreases exponentially with age ($\alpha e^{-\nu t}$), and disappear by density-dependent death (at rate d), by transition to the $CD31^-$ naive T-cell population (at rate m) and by direct activation into the memory T-cell pool (at rate a_p). Since our TREC analyses (Figure 1B) show that $CD31^+$ naive T cells do divide and remain $CD31^+$, $CD31^+$ T cells in the model are also formed by T-cell proliferation (at rate p_p). $CD31^-$ naive T cells are generated when $CD31^+$ T cells lose their CD31 marker (at rate m) and by T-cell proliferation within the $CD31^-$ T cell pool (at rate p_n), and are lost by density-dependent death (at rate d) and by activation into the memory pool (at rate a_n). The total numbers of TRECs in the $CD31^+$ (T_p) and the $CD31^-$ (T_n) naive T-cell

pools change by the same processes except T-cell proliferation. Assuming that $CD31^-$ T cells cannot revert to $CD31^+$, the model is described by the following differential equations:

$dN_p/dt = \alpha e^{-vt} + p_p N_p - dN_p - mN_p - a_p N_p$

$dN_n/dt = mN_p + p_n N_n - dN_n - a_n N_n$

$dT_p/dt = c\alpha e^{-vt} - dT_p - mT_p - a_p T_p$

$dT_n/dt = mT_p - dT_n - a_n T_n$

where c is the average number of TRECs per recent thymic emigrant, and density-dependent death is modelled by a simple linear increase of the death rate: $d = \varepsilon(N_p+N_n)$. A homeostatic term is required to obtain a TREC decline with age [28]. Since T-cell proliferation rates do not change with age [32], we chose for a density-dependent death term. TREC contents were calculated by dividing the total number of TRECs in the $CD31^+$ (T_p) or $CD31^-$ (T_n) naive T-cell pool by the number of $CD31^+$ (N_p) or $CD31^-$ (N_n) naive T cells.

Statistical analysis

Normality of the data was tested using the Shapiro-Wilk *W* test for normality. Based on the outcome of this test, correlations were calculated using Pearson's (R_p) or Spearman's rank correlation coefficients (R_s). The Mann-Whitney U test was used to determine differences between group characteristics. Differences between the TREC content within $CD31^+$ and $CD31^-$ naive $CD4^+$ T cells were analysed using the Wilcoxon signed rank test. We tested if there was a significant difference between the rate of TREC loss in $CD31^+$ and $CD31^-$ naive T cells using a linear model including an interaction term between age and group ($CD31^+$ vs. $CD31^-$).

Results

Characteristics of $CD31^+$ naive $CD8^+$ T cells

In line with Kimmig et al. [2], we found that the fraction of $CD31^+$ T cells within the naive $CD4^+$ T-cell pool decreased significantly with age in healthy individuals (Figure 1A, R_p=-0.714, p<0.001), and that $CD31^+$ naive $CD4^+$ T cells in healthy individuals had a higher TREC content than their $CD31^-$ counterparts (p<0.001). The percentage $CD31^+$ in naive $CD8^+$ T cells also declined during aging, although to a lesser extent than found in the naive $CD4^+$ T cell pool (Figure 1B, R_s=-0.726, p=0.001). Similar to CD4 T cells, sorted $CD31^+$ naive CD8 T cells always had a higher TREC content than $CD31^-$ naive $CD8^+$ T cells (Figure 2, p=0.008). We also determined if the difference in TREC content was sustained in sorted $CD31^+$ and $CD31^-CD45RO^+$ memory $CD8^+$ T cells, this was however not the case (Figure 2, p=0.625).

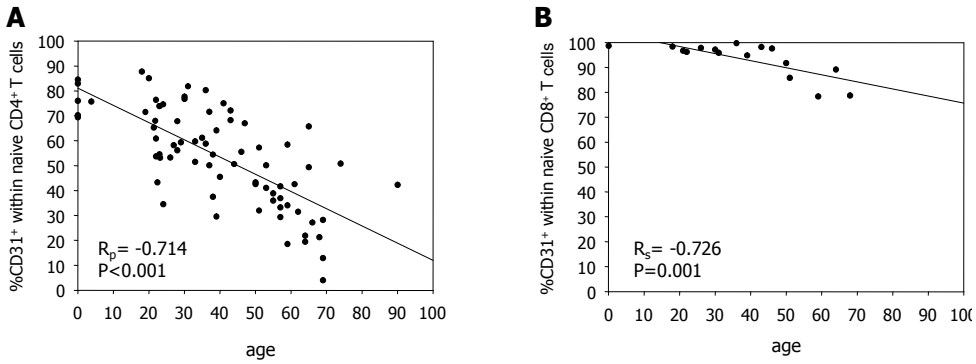


Figure 1. The fractions of CD31$^+$ cells within the naive CD4$^+$ and CD8$^+$ T cell pools decline with age. In healthy controls, a negative correlation between age and the proportion of CD31$^+$ cells within the naive CD4$^+$ (A, Rs= -0.795) and CD8$^+$ T-cell pool (B, Rs= -0.810) was found.

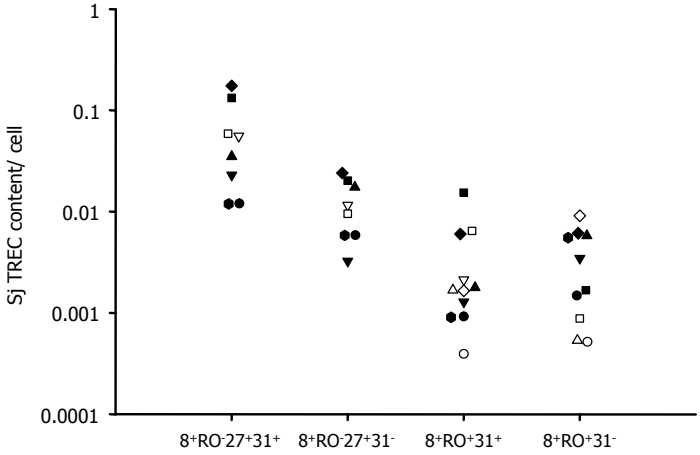


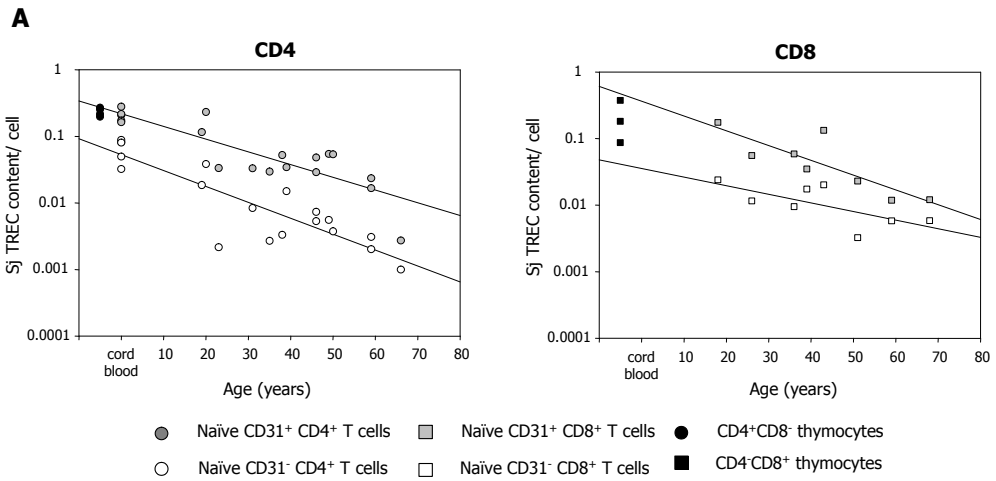
Figure 2. TREC contents of CD31$^+$ and CD31$^-$ naive and memory CD8$^+$ T cells. Depicted are TREC contents within sorted naive (CD45RO$^-$CD27$^+$) and memory (CD45RO$^+$) CD31$^+$ and CD31$^-$ CD8$^+$ T cells. Each symbol represents a different healthy donor.

The TREC content of CD31$^+$ naive CD4$^+$ and CD8$^+$ T cells decreases with age

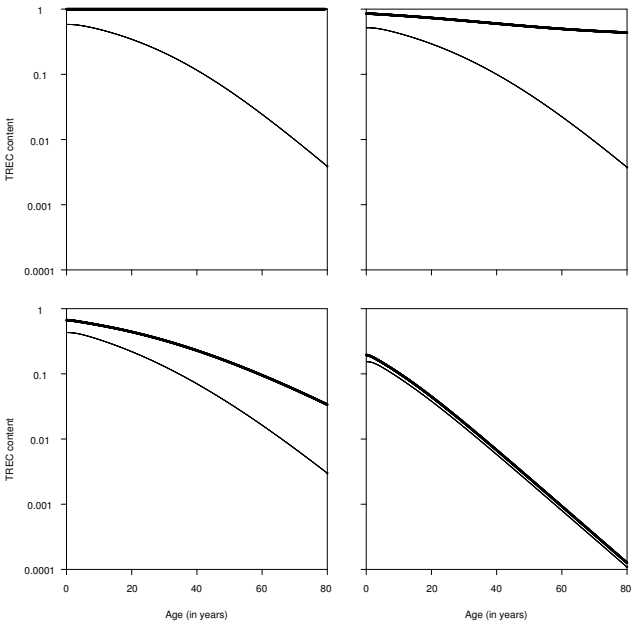
Although higher TREC contents and longer telomeres (as measured by flow-FISH [23] in 3 healthy donors, data not shown) of naive CD31$^+$ T cells indicated that these cells have gone through fewer rounds of division than CD31$^-$ T cells, this does not mean that CD31$^+$ T cells are exclusively of thymic origin. To study whether peripheral T-cell division contributes to the CD31$^+$ naive T-cell population, we measured the TREC content of the CD31$^+$ naive T-cell population during aging. Since the TREC content of thymocytes has been reported to be constant with age [24], and TRECs are extremely stable [4], we argued that if CD31 would

be a true marker for thymic output and every $CD31^+$ T cell that divides becomes $CD31^-$, the TREC content of $CD31^+$ naive T cells should remain constant with age.

The TREC content of $CD31^+$ naive $CD4^+$ T cells in cord blood was found to be in the same range of TREC contents of $CD4^+CD8^-$ single-positive thymocytes (p=0.556), suggesting that cord blood $CD31^+$ naive $CD4^+$ T cells had not markedly proliferated since they emerged from the thymus. However with age, the TREC contents of $CD31^+$ naive $CD4^+$ T cells (Figure 3A left panel, R_s=-0.795, p<0.001), as well as $CD31^+$ naive $CD8^+$ T cells (Figure 3A right panel, R_s=-0.810, p=0.015) declined significantly. The slopes of the TREC content decline with age were not statistically different between $CD31^-$ and $CD31^+$ naive T cells (p=0.270 and p=0.262, respectively for $CD4^+$ and $CD8^+$ T cells). To investigate the implications of this parallel decline for the dynamical properties of $CD31^+$ and $CD31^-$ naive T cells, we extended a previously developed model for T-cell and TREC dynamics [5], now distinguishing $CD31^+$ and $CD31^-$ T cells (see materials and methods). The model demonstrates that a parallel decline in TREC contents of $CD31^+$ and $CD31^-$ T cells, as was experimentally observed, puts a lower bound on the proliferation rate of $CD31^+$ T cells: the larger their proliferation rate, the more parallel the TREC declines of $CD31^+$ and $CD31^-$ T cells (Figure 3B, upper and lower left panels). If we consider the extreme situation where $CD31^+$ naive T cells proliferate much faster than $CD31^-$ naive T cells, the TREC declines are comparable, but the higher TREC content in $CD31^+$ compared to $CD31^-$ naive T cells which we consistently found, is lost (Figure 3B, lower right panel). As we observed nearly parallel declines, these analyses thus show that T-cell proliferation adds significantly to the size of the $CD31^+$ T-cell pool, implying that CD31 cannot be used to reliably measure thymic output. Using a similar proliferation rate for $CD31^+$ and $CD31^-$ naive T cells (upper right panel from Figure B), the model mimics the decline in the fraction of $CD31^+$ cells within the naive $CD4^+$ and $CD8^+$ T-cell pools with age (Figure 3C) provided that thymic output declines with age. Of note, the higher fraction $CD31^+$ naive $CD8^+$ T cells was attained by decreasing the fraction $CD31^+$ T cells that transit to the $CD31^-$ naive T-cell population, which resulted in slightly lower $CD31^+$ and $CD31^-$ naive CD8 T cells TREC contents compared to $CD31^+$ and $CD31^-$ naive $CD4^+$ T cells in the model.



B


TREC content
1
0.1
0.01
0.001
0.0001
0
20
40
60
80
Age (in years)

C

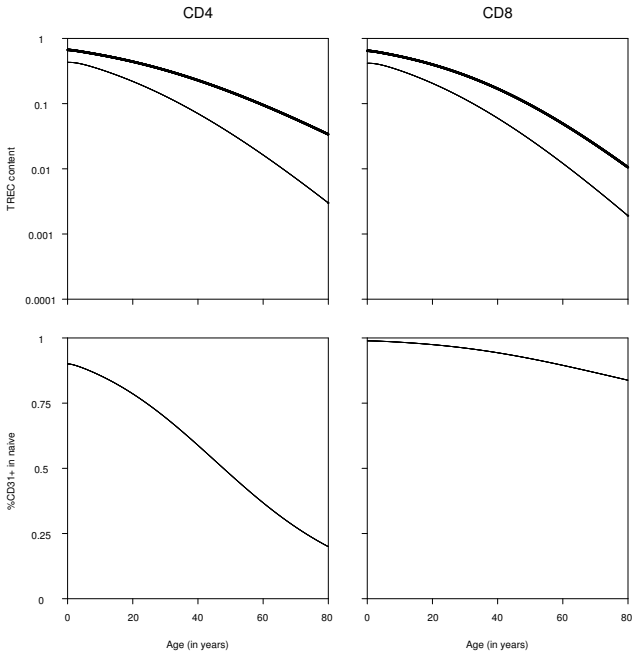

CD4
CD8
TREC content
%CD31+ in naive
1
0.75
0.5
0.25
0
0.1
0.01
0.001
0.0001
0
20
40
60
80
Age (in years)

Figure 3. CD31$^+$ naive T cells are not exclusively of thymic origin. (A) TREC contents of sorted CD31$^+$ (grey circles, Rs=-0.795 and p<0.001) and CD31$^-$ (white circles, Rs=-0.816 and p<0.001) naive CD4$^+$ T cells as well as TREC contents of sorted CD31$^+$ (grey squares, Rs=-0.810 and p=0.015) and CD31$^-$ (white squares, Rs=0.690 and p=0.058) naive CD8$^+$ T cells from healthy blood bank donors decreased with aging. Single positive CD4$^+$CD8$^-$ thymocytes (black circles) had comparable TREC contents as sorted CD31$^+$ cord blood CD4$^+$ T cells (p=0.556). Black squares denote single positive CD4$^-$ CD8$^+$ thymocytes. (B) Simulation results of the mathematical model for the dynamics of CD31$^+$ and CD31$^-$ TREC contents with age. Upper left panel: TREC decline of CD31$^+$ (thick line) and CD31$^-$ (thin line) naive T cells with age in healthy individuals without proliferation within the CD31$^+$ naive T cells. Parameters: $\alpha = 10^9$ cells/day, $v = 0.1$/year, $c = 1$, $\varepsilon = 9.1 \cdot 10^{-16}$ (ref 5), $p_p = 0$, $p_n = 0.0005$/day, $m = 0.0001$/day, $a_p = a_n = 0.0004$/day. Upper right panel: Insufficient proliferation of CD31$^+$ T cells causes deviations between the TREC declines of CD31$^+$ and CD31$^-$ naive T cells. Parameters as in upper left panel, except for $p_p = 0.0002$/day. Lower left panel: If there is sufficient proliferation of CD31$^+$ T cells, the TREC contents of CD31$^+$ and CD31$^-$ naive T cells decline in a parallel fashion. Parameters as in upper left panel, except for $p_p = p_n = 0.0005$/day. Lower right panel: If we introduce higher proliferation of CD31$^+$ than CD31$^-$ naive T cells, TREC declines run parallel, but the TREC content of CD31$^+$ naive T cells is as low as the TREC content of CD31$^-$ naive CD4$^+$ T cells. Parameters as in upper left panel, except for $p_p = 0.002$/day. (C) If we consider the parameters from the lower left panel in B, which best described the data, the model predicted a loss of fraction CD31$^+$ within naive CD4$^+$ T cells with age (lower left panel), provided that thymic output declines with age. The higher fraction CD31$^+$ naive CD8$^+$ T cells (lower right panel) was achieved by decreasing the fraction CD31$^+$ T cells that transit to the CD31$^-$ naive T-cell population (m=0.00001) and resulted in slightly lower CD31$^+$ and CD31$^-$ naive CD8 T-cell TREC contents (upper right panel).

Dynamics of CD31$^+$ and CD31$^-$ naive CD4$^+$ T cells during HIV infection

During HIV infection the percentage of proliferating naive T cells is increased [5,25]. We studied whether this was reflected in the percentage of CD31$^+$ naive CD4$^+$ T cells in HIV infection and during progression to AIDS. Cross-sectional analysis however showed that HIV-infected individuals did not have significantly lower fractions of CD31$^+$ cells within the naive CD4$^+$ T cell pool compared to age-matched healthy individuals (Figure 4A, p=0.84). In patients with AIDS, the fraction of CD31$^+$ T cells in the naive CD4$^+$ T-cell pool did have the tendency to be reduced compared to healthy adults (Figure 4A, p=0.069). As the fraction of CD31$^+$ cells in the naive CD4$^+$ T-cell population appeared to change in the course of HIV infection, we performed longitudinal analyses both pre- and post-seroconversion, including 2 patients who developed AIDS (Figure 4B). Although the fraction of CD31$^+$ cells within the naive CD4$^+$ T-cell pool was found to significantly decrease over seroconversion (p=0.042), only six out of the eleven patients studied showed a clear decline. No statistical difference in the fraction CD31$^+$ naive CD4$^+$ T cells was found during chronic HIV-infection (p=0.530). Absolute numbers of CD31$^+$ naive T cells did consistently decrease during HIV disease progression. Thus, although CD31$^+$ naive CD4 T cells are depleted during HIV infection, this depletion does not seem to be subset specific.

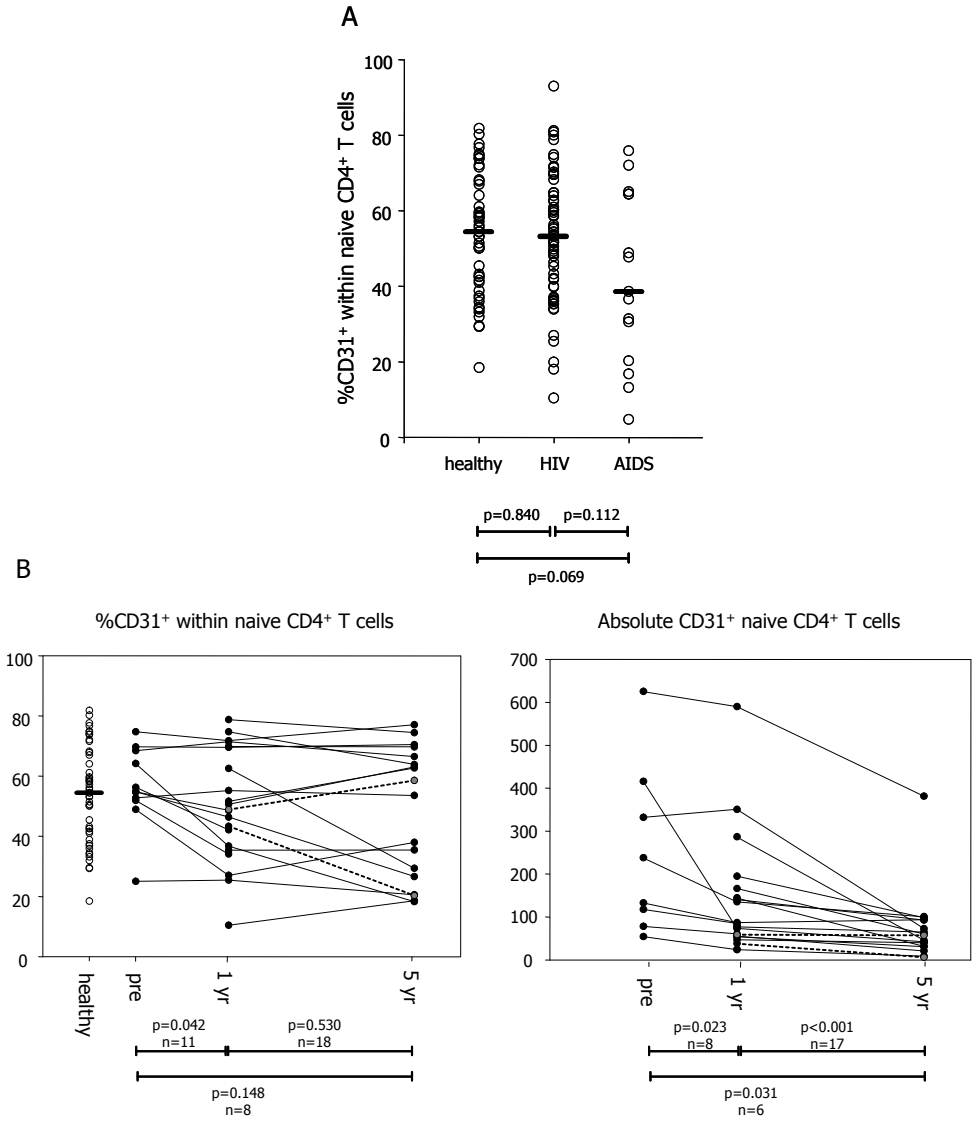


Figure 4. CD31$^+$ T cells within the naive CD4$^+$ T-cell pool during HIV infection. (A) The proportion of CD31$^+$ cells within the naive CD4$^+$ T-cell pool of HIV-infected patients and individuals with AIDS are compared to healthy age-matched individuals. (B) The fraction CD31$^+$ cells within the naive CD4$^+$ T-cell pool and absolute numbers of CD31$^+$ naive CD4$^+$ T cells measured longitudinally before and 1 and 5 years after HIV seroconversion (black circles). Grey circles and dotted lines denote 2 patients suffering from AIDS. Healthy age-matched controls are depicted by white circles.

Discussion

CD31 has recently been used as a marker to identify recent thymic emigrants within the naive CD4$^+$ T-cell pool [2,20-22]. Here we show that in analogy to CD4$^+$ T cells, CD31$^+$

naive $CD8^+$ T cells have a higher TREC content than $CD31^-$ naive $CD8^+$ T cells and that the fraction of $CD31^+$ cells within the naive $CD8^+$ T cell pool declines with age. Our data show, however, that the TREC content of both $CD31^+$ naive $CD4^+$ and $CD8^+$ T cells declines with age, implying that these cells are not exclusively formed by thymic output, but also by peripheral T-cell proliferation. The rates at which TREC contents of $CD31^+$ naive T cells, $CD31^-$ naive T cells, and total T cells (not shown) decline with age were found to be similar. It is highly unlikely that the observed TREC decline in $CD31^+$ naive $CD4^+$ and $CD8^+$ T cells is due to changes in TREC content of recent thymic emigrants (RTE) with age, because thymocytes from individuals of very different ages have been shown to have comparable TREC contents [24] and TREC contents of single positive thymocytes are more likely to increase than decrease with age [26,27]. The observed TREC decline in $CD31^+$ naive $CD4^+$ T cells can also not be accounted for by a fixed number of divisions that RTE may undergo shortly after emigration from the thymus into the periphery, because that would result in an age-independent TREC content difference between thymocytes and peripheral $CD31^+$ naive $CD4^+$ T cells. The equal TREC contents of single positive $CD4^+CD8^-$ thymocytes and $CD31^+$ naive $CD4^+$ cord blood cells also rules out such early post-thymic T- cell proliferation.

In our model, the TREC loss in the naive $CD31^+$ T cell pool with age was explained by a homeostatic response to the decline in thymic output [28]. An alternative explanation could however be, that the differences in $CD31^+$ and $CD31^-$ naive T cell TREC contents are caused by the pool of RTE that resides in the $CD31^+$ naive T cell population, containing most of the TRECs, which declines steadily with age. Although we did not include an RTE pool in our model, both interpretations yield essentially the same conclusions, namely (i) that $CD31^+$ naive T cells are not only thymus-derived but also formed by peripheral T-cell proliferation, and (ii) that $CD31^+$ naive T cells are more proximal to the thymus than $CD31^-$ naive T cells.

The assumed homeostatic proliferation of naive $CD31^+$ T cells might be cytokine dependent. Indeed *in vitro* experiments have shown cytokine-induced proliferation of $CD31^+$ naive CD4 T cells, without down-regulation of CD31 [9].

We analyzed whether HIV infection alters the fraction of $CD31^+$ cells within the naive $CD4^+$ T-cell population. Although HIV-infection caused a continuous depletion of absolute numbers of $CD31^+$ naive $CD4^+$ T cells, the fraction of $CD31^+$ cells within the naive $CD4^+$ T-cell population was found to be much less affected, indicating that $CD31^+$ and $CD31^-$ naive $CD4^+$ T cells were generally lost to a similar extent.

Summarizing, our data suggest that peripheral division is an important source of $CD31^+$ T cells. Although CD31 expression is to some degree associated with thymic proximity, it can thus not reliably be used as a direct marker for thymic output. Both CD31 expression and TREC measurements are influenced by peripheral T-cell proliferation and thus have similar drawbacks as markers of thymic proximity [5,6], mainly because both appear to be influenced by changes in peripheral T-cell proliferation. It remains to be investigated whether CD31 can at least provide a more user-friendly alternative to TREC analyses, or whether the combination of CD31 and TREC analyses may help to unravel the different components that determine the intricate dynamics of the T-cell population. In view of a recent paper suggesting that the age of naive $CD4^+$ T cells is involved in age-related defects

of the immune system [29], CD31 might be useful to identify the naive T-cell subset that contains the youngest subpopulation of the naive T-cell pool.

Acknowledgements

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Biphasic CD4$^+$ T-cell TREC dynamics during HIV infection

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Chapter 8

Abstract

HIV-1 infection has frequently been shown to be associated with reduced numbers of T-cell receptor excision circles (TREC). However, most TREC data come from cross-sectional studies, and little is known about the course of the changes in TRECs during HIV disease progression. We have obtained longitudinal data on $CD4^+$ T-cell TRECs and T-cell counts in human subjects before and after HIV seroconversion. The data demonstrate that $CD4^+$ T-cell TREC dynamics during HIV infection are biphasic, with a rapid TREC loss during the first year and a slow loss during the chronic phase of infection. During the first year of HIV infection, the total number of TRECs in the blood is at least halved, and the loss of TRECs exceeds the loss of naive $CD4^+$ T cells. During later stages of HIV infection, $CD4^+$ T-cell TREC contents remain fairly constant because total $CD4^+$ T-cell TREC loss parallels the loss of naive and effector/memory $CD4^+$ T cells. These data are interpreted using mathematical models, which show that recruitment of a large fraction of naive $CD4^+$ T cells to the effector/memory compartment during acute HIV infection is sufficient to explain the biphasic dynamics of TREC numbers and content.

Introduction

HIV-1 infection is associated with reduced total T-cell receptor excision circle (TREC) numbers and reduced TREC levels per peripheral T cell [1-3]. The interpretation of these observations is difficult because various mechanisms may play a role, including shifts in the naive/memory T-cell ratio, TREC dilution by increased T-cell division, increased death and priming of naive T cells, and impaired export of T cells from the HIV-infected thymus.

Vbeta and Sj TRECs are formed sequentially during thymic TCR rearrangement and since peripheral proliferation would dilute both TRECs equally, a change in the Sj/Vbeta TREC ratio should reflect intrathymic events. Recently Dion et al. [4] described relatively rapid changes in Sj/Vbeta TREC ratios within 3 months post HIV infection, which they attributed to the rapid dynamics of recent thymic emigrants (RTE) containing most of the TRECs, as has been shown in mice [5] and chickens [6]. However, our own recent work has indicated that in human adults recently produced naive T cells are rare and long-lived (Vrisekoop et al. submitted), which questions the existence of a short-lived RTE pool in humans. Moreover, the effect of SIV infection on TRECs in sooty mangabeys was shown to exceed the effect of thymectomy [7]. Thus the rapid TREC dynamics observed in HIV infection cannot fully be explained by interference with RTEs.

Most TREC data come from cross-sectional studies, and little is known about the course of the changes in TRECs during HIV disease progression, especially because of the large inter-individual differences in TREC of both healthy and HIV-infected subjects. Indeed, a considerable overlap between TREC content in $CD4^+$ T cells from healthy and HIV-infected individuals has been reported [3,8-10]. Such inter-individual differences have hampered the interpretation of cross-sectional TREC data. For example, Nobile et al. [9] reported that HIV-infected individuals with high $CD4^+$ T-cell counts had increased $CD4^+$ T-cell TREC contents, while patients with low $CD4^+$ T-cell counts had lower $CD4^+$ T-cell TREC contents than age-matched controls. Longitudinal interpretation of these cross-sectional data would suggest that $CD4^+$ T-cell TREC contents increase over HIV seroconversion and progressively decrease during HIV disease progression. Alternatively, the observed high TREC contents in patients with high $CD4^+$ T-cell counts may be due to a selection bias towards individuals with high $CD4^+$ TREC contents pre-seroconverion due to e.g. low basal peripheral T-cell proliferation and/or high levels of thymic output. Therefore, extrapolation of cross-sectional data to longitudinal interpretation is prone to be erroneous.

The longitudinal studies on TREC dynamics in HIV infection that are available measured TRECs in PBMC [3,8,11,12]. In only half of the patients the TREC content of PBMC was found to decrease over seroconversion, whereas in the other half they stayed stable [3]. When measured longitudinally from around 6 years after seroconversion onward the TREC content in PBMC longitudinally declined [8,12]. More specifically, Chattopadhyay et al. [11] found that the average slope of TREC content in PBMC before onset of AIDS was not significantly different from zero, however, at the time T-cell counts began to decline the PBMC TREC content steeply declined. Because these studies all measured TREC contents in PBMC, these results may not be representative for the TREC content of $CD4^+$ T cells, since

the fraction of $CD4^+$ T cells declines during disease progression and the TREC content of $CD8^+$ T cells has been found to be lower than the TREC content of $CD4^+$ T cells [9,10].
Summarizing, during early stages of HIV infection cross-sectional $CD4^+$ T-cell TREC content data suggest either an increased [9] or unaffected TREC content [10], whereas longitudinal data in PBMC suggest a decline in TREC content in half of the patients (Zhang). During chronic infection, declining [12] and stable [11] TREC contents have been described longitudinally in PBMC, while cross-sectional data have suggested a decrease in $CD4^+$ T-cell TREC content [9]. To further elucidate the dynamics of the $CD4^+$ T-cell TREC content during HIV-infection, we performed a longitudinal analysis of $CD4^+$ T-cell counts, naive/memory $CD4^+$ T-cell ratios and $CD4^+$ T-cell TREC dynamics over HIV seroconversion and during the subsequent chronic phase of infection. Rather than interpreting the group as a whole, the parameters of each individual were analyzed separately with the help of a mathematical model.

Materials and Methods

Patient groups

Patient samples for the longitudinal study and part of the samples for the cross-sectional data were derived from the Amsterdam Cohort Studies on HIV infection and AIDS. The cross-sectional data was extended with data obtained from previous studies (Vrisekoop et al. submitted) [13]. None of the HIV-infected patients had been treated at the time of sampling. Individuals were called acute HIV-infected patients when sampling took place within 2 months after onset of symptoms of acute HIV infection. Age-matched blood bank donors were used as healthy controls.

Flow cytometry and cell sorting

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque density gradient centrifugation from heparinized blood and viably frozen until further processed. Absolute $CD4^+$ and $CD8^+$ T-cell counts were determined by dual-platform flow cytometry. Effector/memory ($CD27^+CD45RA^-$, $CD27^-CD45RA^-$ and $CD27^-CD45RA^+$) and naive ($CD27^+CD45RA^+$) $CD4^+$ and $CD8^+$ T-cell fractions were assessed by flow cytometry as described previously and analyzed on a FACSCalibur with CellQuest software (Becton Dickinson (BD), San Jose, California) [14].
To measure the TREC content within naive ($CD45RO^-CD27^+$) $CD4^+$ T cells, PBMC were incubated with the monoclonal antibodies CD45RO-FITC (Caltag Laboratories, Burlingame, CA), CD27-PE, CD4-PerCP and CD8-APC (BD) and naive $CD4^+$ T cells were isolated by cell sorting on a MoFlow or a FACSAria (BD). In 14 out of 32 healthy individuals $CD45RA^+$ $CD4^+$ T cells were sorted by magnetic beads. Because the fraction $CD45RA^+CD27^-$ effector $CD4^+$ T cells is virtually absent in healthy individuals (≤1.5% in 10 controls in whom we used magnetic beads and we were able to measure the effector subset), this fraction represents $CD45RA^+CD27^-$ naive $CD4^+$ T cells. To measure the TREC content within $CD4^+$ T cells, this subset was purified from thawed PBMC by magnetic bead separation, using the MiniMACS

multisort kit according to manufacturer's instructions (Miltenyi Biotec Inc, Sunnyvale, California).

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 by using a real-time PCR method as previously described [15].

Mathematical model

We modeled the dynamics of both naive (N) and effector/memory (M) $CD4^+$ T cells by the following two differential equations:

$$N' = \sigma e^{-vt} + \alpha V(t)N - (d_n + s + aV(t))N$$

$$M' = (c_1 aV(t) + c_2 s)N + \frac{r}{1 + M/k}M - (d_m + \beta V(t))M$$

In the mathematical model σe^{-vt} represents daily thymic output which declines with age at rate v. For simplicity we omitted a naive T-cell renewal term; in the absence of virus, naive cells are maintained only by thymic output, die at rate d_n per day, and are recruited into the memory pool at rate s per day. Effector/memory $CD4^+$ T cells are maintained by constant activation and clonal expansion of naive T cells ($c_2 s$) as well as by density-dependent renewal ($r/(1+M/k)$) and they die at rate d_m per day. $V(t)$ is a function that represents the dynamics of the virus. The function increases to a peak at around day 10, after which it decreases to a low viral set point at week 3.

Upon HIV infection, more naive cells are recruited into the effector/memory pool dependent on the amount of virus present ($c_1 aV(t)$). The effector/memory cells' death rate is also increased in a virus dependent way ($\beta V(t)$), either through direct killing by the virus, or by conversion to an activated T-cell population with high turnover. The third effect of the presence of virus is an increase in the rate of cell division within the naive T-cell population.

The number of TRECs within the naive and effector/memory T-cell populations are modeled by the following differential equations:

$$T_N' = q\sigma e^{-vt} - (d_n + s + aV(t))T_N$$

$$T_M' = (aV(t) + s)T_N - (d_m + \beta V(t))T_M$$

where q denotes the fraction of naive cells coming from the thymus that contain a TREC.

Statistics

The non-parametric Mann-Whitney U test was performed for group comparisons. Differences between paired data during longitudinal follow-up were tested using the Wilcoxon signed ranks test.

Results

TREC dynamics in CD4$^+$ T cells over seroconversion and during HIV infection

We performed a longitudinal analysis of TREC contents per CD4$^+$ T cell over HIV-seroconversion in 13 individuals. The median time before seroconversion was 4.8 years (range 1.8-11.4 years) and the median time after seroconversion was 1.0 year (range 0.8-1.3 years). This period was defined as phase I. Seven of these 13 patients and an additional 5 patients were followed during chronic HIV infection. The median time points analyzed for these 12 patients were 1.0 (range 0.8-1.5) year and 5.0 (range 3.5-5.2) years since seroconversion. This stage is referred to as phase II. Absolute CD4$^+$ T-cell counts and fractions of naive and effector/memory CD4$^+$ T cells were also determined for all patients.

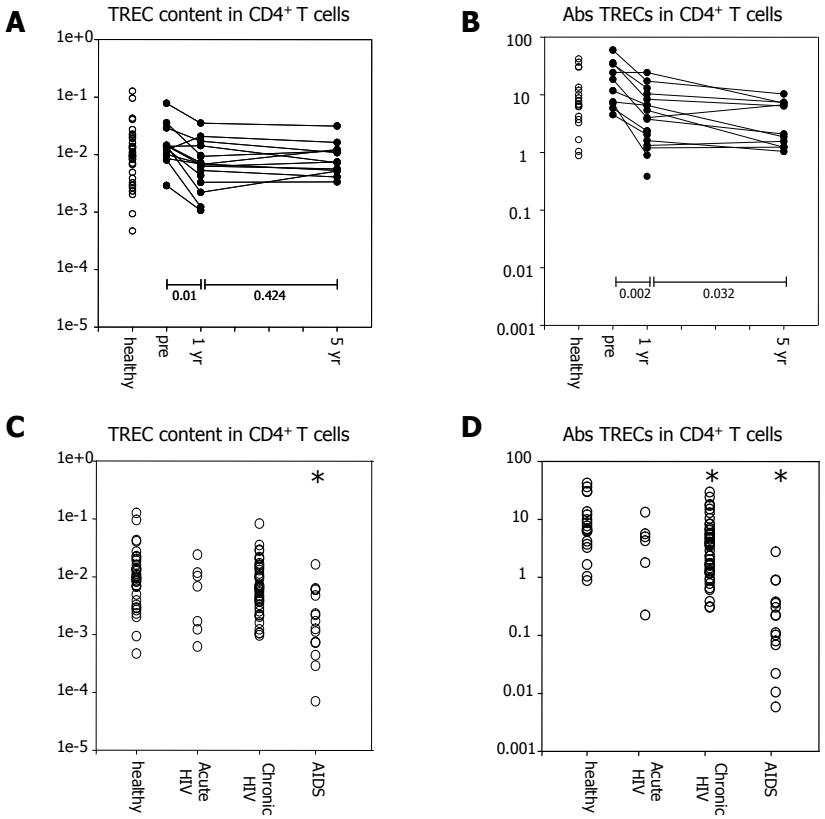

Figure 1. CD4$^+$ T-cell TREC dynamics over seroconversion and during HIV infection. TREC content per CD4$^+$ T cell (A) and total CD4$^+$ T-cell TREC numbers per µl blood (B) measured longitudinally over seroconversion and during chronic HIV infection are depicted in the upper panels. P-values are indicated in the figure. In the lower panels, cross-sectional data on TREC content per CD4$^+$ T cell (C) and total CD4$^+$ T-cell TREC numbers per µl blood (D) during acute and chronic HIV infection as well as during progression to AIDS are compared to age-matched controls. P-values <0.05 are indicated by an asterisk.

Longitudinal $CD4^+$ T-cell TREC contents showed biphasic dynamics during HIV disease progression, consisting of a rapid decline in $CD4^+$ T-cell TREC content over seroconversion (p=0.01), and a rather stable TREC content during the chronic phase (p=0.424, Figure 1A). Absolute TRECs in the $CD4^+$ T-cell population also showed a steep decline over seroconversion (p=0.002), but continued to decline, albeit to a lesser extent, during chronic infection (p=0.032, Figure 1B).
Despite these clear longitudinal $CD4^+$ T-cell TREC dynamics, we did not find any significant differences in a cross-sectional study comparing $CD4^+$ T-cell TREC contents from 7 HIV-infected individuals during acute infection, 46 chronic HIV-infected individuals, and 38 healthy age-matched controls. Only HIV-infected individuals who had progressed to AIDS (n=16) had a significantly lower $CD4^+$ T-cell TREC content compared to healthy individuals (Figure 1C), underlining the importance of longitudinal follow-up in TREC analyses. Absolute numbers of TRECs within $CD4^+$ T cells were significantly reduced in chronic HIV-infected individuals and AIDS patients (Figure 1D).

In-depth analysis of TREC and $CD4^+$ T-cell kinetics during HIV disease progression

To understand the mechanism behind the early rapid and late slow decay of $CD4^+$ T-cell TREC contents, we performed more in-depth analysis of the data by calculating the fractional changes per year in naive and effector/memory $CD4^+$ T-cell numbers and absolute TREC counts within the $CD4^+$ T-cell pool during the different phases of infection. We found that during Phase I, the fractional loss of TRECs in $CD4^+$ T cells was higher than the loss of naive $CD4^+$ T cells, resulting in a decrease in the $CD4^+$ T-cell TREC content. In 4 out of 11 patients the number of naive $CD4^+$ T cells even increased despite decreased $CD4^+$ T-cell TRECs (see Figure 2A). Although effector/memory $CD4^+$ T-cell numbers showed a general tendency to decrease during Phase I, 2 out of 11 patients showed an increase in effector/memory $CD4^+$ T-cell numbers during this phase, which was not correlated with the change in naive $CD4^+$ T-cell numbers (see circles Figure 2B). If we translate these results to naive/memory $CD4^+$ T-cell ratios, no general trend could be observed in phase I (Figure 2C). The fractional changes of all the variables in phase II were less steep compared to phase I. The general trend was that the fractional loss of total TRECs in $CD4^+$ T cells, naive $CD4^+$ T cells and effector/memory $CD4^+$ T cells was similar during phase II (see diamonds Figure 2A and B). Indeed, naive/memory $CD4^+$ T-cell ratios were mostly stable in this phase (Figure 2C) and the proportional loss of TRECs and naive $CD4^+$ T cells was reflected in a stable TREC content in the $CD4^+$ T-cell pool.

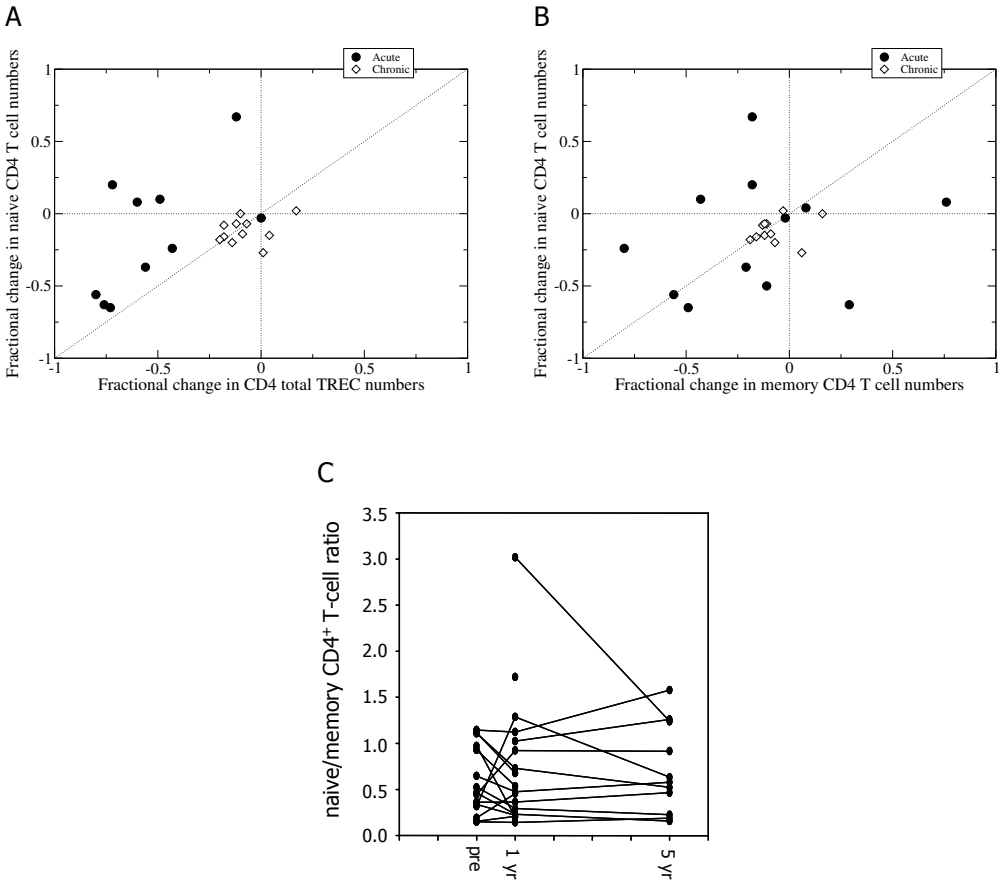

Figure 2. Fractional loss of naive and effector/memory CD4$^+$ T cells and total CD4$^+$ T-cell TRECs. The fractional loss of naive CD4$^+$ T cells is compared to the fractional loss of total TREC numbers in CD4$^+$ T cells (A) and to the fractional loss of effector/memory CD4$^+$ T cells (B). Black circles represent the fraction lost during the first year of infection (phase I) and white diamonds denote the loss during the chronic phase (phase II) of HIV infection. Panel (C) depicts longitudinal changes in the naive/memory CD4$^+$ T-cell ratios.

Explaining the biphasic CD4$^+$ T-cell TREC kinetics during HIV disease progression

The rapid decline in CD4$^+$ T-cell TREC content during phase I cannot merely be attributed to TREC dilution by peripheral T-cell proliferation, because absolute TREC numbers were also lost from the pool (Figure 1B). In fact, in all but three individuals CD4$^+$ T-cell TREC numbers declined more than 50 percent in phase I (Figure 2A). The decrease in CD4$^+$ T-cell TREC content in phase I was not related to time before seroconversion. An alternative explanation for the rapid decline in CD4$^+$ T-cell TREC content during phase I would be that naive CD4$^+$ T cells are lost more rapidly from the blood than memory CD4$^+$ T cells, resulting in a

decreased naive/memory $CD4^+$ T-cell ratio. However, as shown in Figure 2C, naive/memory $CD4^+$ T-cell ratios both increased and decreased during phase I.

The early rapid decrease in total $CD4^+$ T-cell TREC numbers and TREC content together with the smaller loss of absolute naive $CD4^+$ T cells during the first year of HIV-infection seems in good agreement with losing a small RTE pool containing most of the TRECs in the periphery. Recent ^2H_2O studies from our laboratory have indicated, however, that newly produced naive T cells in young human adults are long-lived, and are preferentially incorporated into the peripheral naive T-cell pool, which is not compatible with the existence of a substantial short-lived RTE pool in human adults (Vrisekoop et al. submitted). We therefore searched for alternative explanations for the current set of longitudinal $CD4^+$ T-cell TREC and subset data during the different stages of HIV infection.

We developed a novel mathematical model for the dynamics of TRECs, naive and effector/memory $CD4^+$ T cells (see Methods and Figure 3A). In the model, naive $CD4^+$ T cells are maintained by thymic output, while effector/memory $CD4^+$ T cells are maintained by activation (and subsequent clonal expansion) of naive $CD4^+$ T cells and by density-dependent renewal. Viral load was modeled by a function $V(t)$ which peaks at day 10.5 and subsequently decreases to reach a lower viral setpoint at week 3. The activation of naive $CD4^+$ T cells into the effector/memory compartment as well as the loss of effector/memory $CD4^+$ T cells are directly dependent on viral load. To achieve more TREC loss than naive $CD4^+$ T-cell loss during the first year of HIV infection, we modeled naive $CD4^+$ T-cell renewal to be dependent on viral load ($V(t)$), resulting in a predominant increase in division of naive $CD4^+$ T cells during the early phase of infection. Since the amount of naive $CD4^+$ T cells that is recruited to the memory pool is also viral load dependent, the division within the naive $CD4^+$ T-cell pool could be triggered as a result of the large number of naive $CD4^+$ T cells lost by transfer to the memory pool, for instance by IL-7 accessibility [16,17]. For simplicity, a renewal term for naive $CD4^+$ T cells was omitted for the healthy situation.

Simulations of the model could mimic the experimentally observed $CD4^+$ T-cell and TREC dynamics. We found that a loss of $CD4^+$ T cells, TREC numbers and TREC content could be achieved if a large fraction of naive $CD4^+$ T cells is recruited into the effector/memory compartment during the first year of HIV infection. These cells and their TRECs are rapidly lost as a result of the relatively short natural lifespan of effector/memory $CD4^+$ T cells, which might be further shortened by virus-mediated killing of effector/memory $CD4^+$ T cells. During the chronic stage of HIV infection $CD4^+$ T-cell TREC contents remain fairly constant because total TREC loss remains in pace with the loss of naive and effector/memory $CD4^+$ T cells.

The model results show that adding increased $CD4^+$ T-cell proliferation during early HIV infection suffices to mimic the experimental observations (Figure 3B), including a 50% loss of total TRECs from the $CD4^+$ T-cell pool concomitant with a smaller loss of naive $CD4^+$ T cells during the early phase of infection.

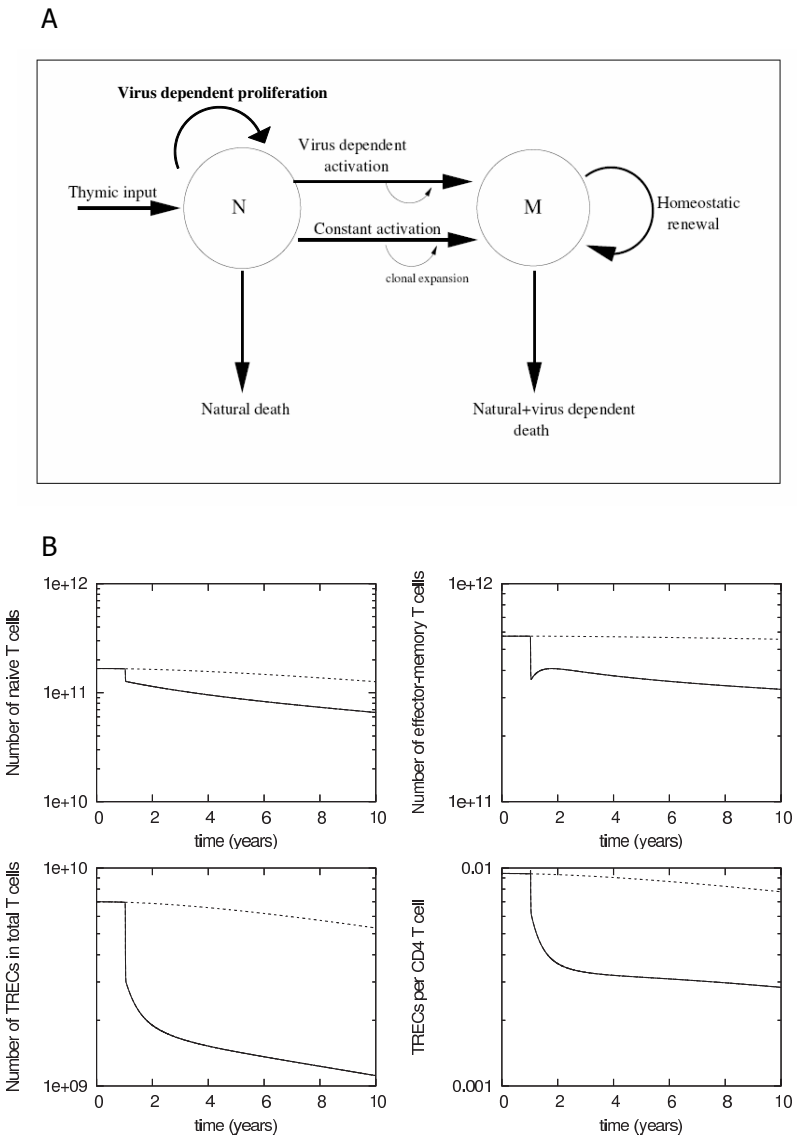

Figure 3. T-cell and TREC dynamics during HIV infection according to the mathematical model. (A) A schematic representation of the mathematical model. (B) Simulation result for the model with viral load dependent increases in activation and naive $CD4^+$ T-cell division (solid line) compared to healthy dynamics during the same period (dotted line).

TREC dynamics in naive $CD4^+$ T cells during HIV infection

The naive $CD4^+$ T-cell dynamics predicted by the model display a similar biphasic pattern as observed for total $CD4^+$ T-cell TREC contents (Figure 4A). Due to limited cell availability, sorted naive $CD4^+$ T-cell TREC data were unfortunately not available for Phase I. We were

able to determine whether stable TREC contents in FACS-sorted naive $CD4^+$ T cells could indeed be observed during chronic infection (Phase II) in 7 patients followed longitudinally. As predicted, the naive $CD4^+$ T-cell TREC content did not decrease during follow-up even when patients progressed to AIDS or got infected with CXCR4-using virus variants capable of infecting naive $CD4^+$ T cells (p=0.469 if first and last time points are compared, Figure 4B). No significant difference could be found between healthy age-matched controls and HIV-infected individuals (Figure 4B). However, as was the case for our cross-sectional study between $CD4^+$ TREC contents in healthy and HIV-infected individuals, the initial decrease could easily be masked by the large inter-individual variation in TREC contents.

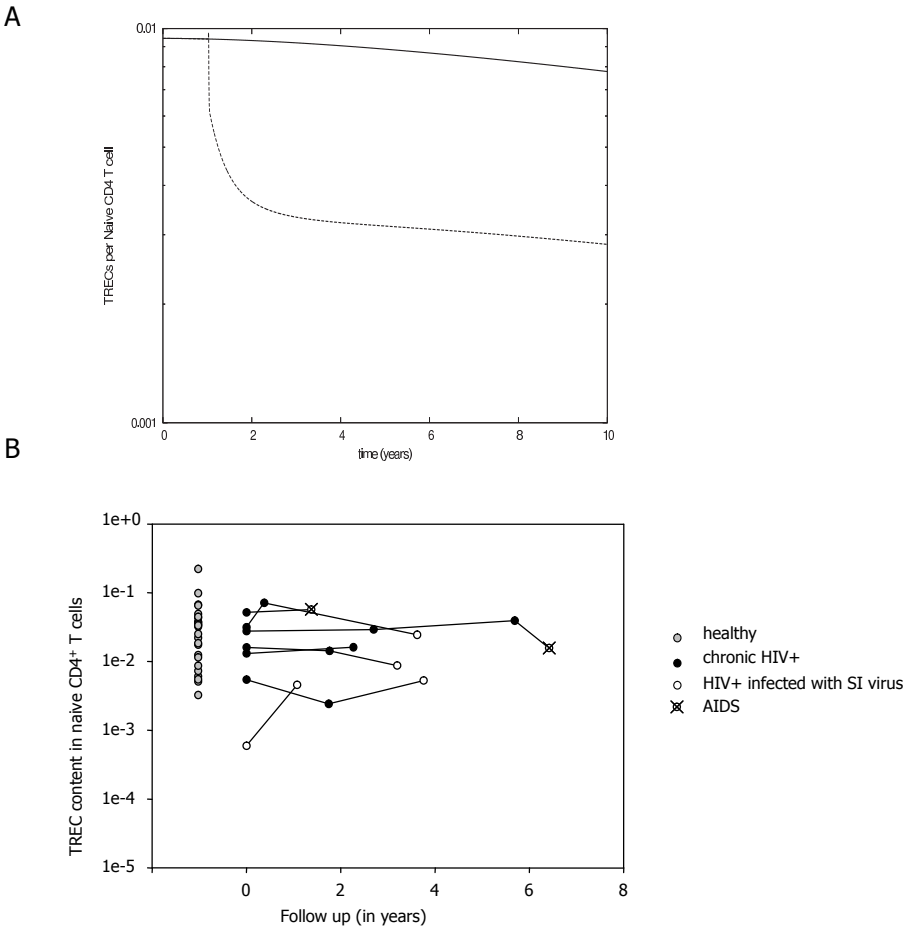

Figure 4. Naive $CD4^+$ T-cell TREC content dynamics during chronic HIV infection. (A) Simulation results for the model with viral load dependent increases in activation and naive $CD4^+$ T-cell division (dotted line) compared to healthy dynamics during the same period (solid line). (B) Longitudinal TREC content per naive $CD4^+$ T cell during chronic HIV-infection (black circles), infection with CXCR4-using/ SI virus variants (white circles) and progression to AIDS (x-marked circles) compared to healthy controls (grey circles).

Discussion

The comparison of longitudinal and cross-sectional TREC data made clear that evident dynamics can easily be masked by large inter-individual differences in TREC measurements. In line with our own cross-sectional data, Sempowski et al. [10] were also not able to detect differences in $CD4^+$ T-cell TREC contents during acute infection in their cross-sectional study. They concluded that TRECs were not affected during acute HIV infection, however, we demonstrate that conclusions from cross-sectional data should be taken with caution. We now show that longitudinal $CD4^+$ T-cell TREC dynamics during HIV infection are biphasic, with a rapid early phase and a slow late phase. The sharp decline in $CD4^+$ T-cell TREC content in the first year of HIV infection was the result of a more rapid loss of total $CD4^+$ T-cell TRECs than naive cells. Remarkably, in the majority of patients as much as 50% of the total $CD4^+$ T-cell TRECs was lost during Phase I. During the chronic phase of HIV infection $CD4^+$ T-cell TREC contents remained fairly constant because absolute TREC loss was accompanied by a similar loss of naive and effector/memory $CD4^+$ T cells.

At first glance, these observations seem in good agreement with the loss of a short-lived RTE pool upon HIV infection, as thymectomy has been shown to cause a similar drop in TREC content in mice [5] and chickens [6]. Since RTE in mice and chickens are short-lived and contain most of the TRECs in the peripheral pool, loss of thymic output would be expected to result in a fast decline of TRECs and a smaller loss of naive T cells from the peripheral pool. However, heavy water labeling studies revealed that recently produced naive $CD4^+$ T cells in young human adults have a very long half-life exceeding at least 4 years (Vrisekoop et al. submitted), indicating that there is no short-lived RTE pool in human adults. Moreover, although thymectomy in chickens [6] has been shown to result in a biphasic sharp decrease in TREC content, thymectomy in sooty mangabeys [18] did not result in a decline in TREC content even 7 months after thymectomy. We therefore presented an alternative model, in which a large fraction of naive $CD4^+$ T cells is transferred into the effector/memory $CD4^+$ T-cell compartment during the first year of HIV infection, which was shown to be consistent with the longitudinal TREC and T-cell dynamics that we observed. The viral load dependent activation that we used in the model is in line with the high viral load and concomitant high $Ki67^+$ $CD4^+$ T cells found during acute HIV infection in humans [10,19]. Furthermore, it has been found that during interruption of successful HAART in humans, viral load and $CD4^+$ T-cell proliferation increase, with a concomitant decrease of the $CD4^+$ T-cell TREC content, which is also in good agreement with the increased level of $CD4^+$ T-cell recruitment of naive $CD4^+$ T cells during the early phase of infection in our model [20]. Of note, not all naive $CD4^+$ T cells transferred to the effector/memory pool need to be HIV specific. Bystander activation by cytokines or other pathogens e.g. due to a compromised gastrointestinal mucosal surface might even play the dominant role in chronic immune activation during HIV infection [21].

Although our model presents one way to explain the T-cell and TREC dynamics without the need for a short-lived RTE pool, we do not exclude other mechanisms that may play a role. For example, increasing naive cell death rather than increasing naive T-cell recruitment to the effector/memory pool upon HIV infection might explain the data equally well.

Furthermore, we did not take redistribution of T cells to the lymphoid organs into account. Upon HIV infection, the percentage naive $CD4^+$ T cells was found to be increased in lymphoid tissues and TREC losses were found to be less pronounced in lymphoid tissues than in the periphery. While in PBMC an inverse correlation between TREC and cellular viral load was found, there was a positive relation between TREC and cellular viral load in lymphoid tissue. These data therefore might suggest that TREC bearing cells are selectively trapped in response to high viral load [22].

To conclude, we find that in the first year of HIV infection total TRECs in the $CD4^+$ T-cell pool are lost more rapidly than naive cells, resulting in a decline in $CD4^+$ T-cell TREC content, while during later stages of HIV infection $CD4^+$ T-cell TREC contents remain fairly constant because total $CD4^+$ T-cell TREC loss remains in pace with the loss of naive and memory $CD4^+$ T cells.

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

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Chapter 9

Abstract

In mice a large part of the naive T-cell pool consists of short-lived recent thymic emigrants (RTE). In humans however, the lifespan and number of RTE are unknown. While ^2H_2O labeling in mice showed high thymic-dependent daily naive T-cell production, long term up- and down-labeling with ^2H_2O in young human adults revealed a low daily production of naive T cells. Using mathematical modeling, we estimated human naive $CD4^+$ and $CD8^+$ T-cell half-lives of 4.2 and 6.6 years, respectively, while memory $CD4^+$ and $CD8^+$ T cells had half-lives of 0.4 and 0.7 years. The estimated half-life of recently produced naive T cells was much longer than these average half-lives. Thus, our data are not only incompatible with a substantial short-lived RTE population in human adults, but also suggest that the few naive T cells that are newly produced are preferentially incorporated in the peripheral pool.

Introduction

Treatment-induced T-cell depletion in clinical situations, such as stem cell transplantation (SCT), chemotherapy and radiotherapy, is occurring more and more frequently, even involving older patients, prompting the need to understand the processes responsible for T-cell reconstitution. Although T-cell reconstitution is generally thought to be dependent on both thymic output and peripheral T-cell proliferation, there is still controversy on the capacities of both processes. Long term failure of immune reconstitution after SCT has been thought to be related to exhaustion of thymic output [1,2], but a recent study in transplanted SCID patients pointed out that immune failure resulted from poor early grafting rather than thymic exhaustion [3]. In HIV infection in particular, the role of the thymus is still poorly understood [4,5]. On the one hand, thymic failure has been suggested to play a crucial role in $CD4^+$ T-cell loss during HIV infection [6], and rapid thymic rebound has been proposed to be responsible for T-cell reconstitution during anti-viral treatment [7]. On the other hand, it has been argued that thymic output in adults might be too low to have a large impact on $CD4^+$ T-cell depletion [8]. In general these issues are addressed with estimates of thymic output, and naive and memory T-cell production rates and life spans which are simply extrapolated from observations in mice, monkeys, and lymphopenic or irradiated humans [9-14]. Reliable estimates of these variables are still missing for healthy human adults.

Naive T cells are generally thought to turnover relatively slowly, but it has been suggested that in mice, a considerable part of the naive T-cell pool consists of recent thymic emigrants (RTE) with relatively rapid turnover [12,13,15]. In humans naive T-cell numbers, T-cell receptor excision circles (TRECs) and expression of CD31 have been used to measure thymic output [10,16,17]. Dion et al. [18] used the ratio of two types of TRECs in the peripheral T-cell pool, Vbeta and Sj TRECs, which are formed sequentially during thymic TCR rearrangement as a signature for RTE. They observed rapid changes in the Sj/Vbeta TREC ratio within 3 months after infection with HIV, which suggested the presence of a rapidly turning over RTE pool in human adults containing most of the TRECs in the periphery, similar to rodents and chickens [19,20]. However, none of these approaches are specific for T cells that have recently emigrated from the thymus, and therefore they fail to quantify thymic output in humans.

Peripheral T-cell proliferation might also contribute to the maintenance of the naive T-cell pool in human adults, however, it is unclear which fraction of these cells remains in the naive T-cell pool [21]. The contribution of RTE and peripheral T-cell proliferation, to the maintenance of the naive T-cell pool can only be determined by studying the fate of newly produced T cells. Long term *in vivo* labeling with stable isotopes in combination with appropriate mathematical analysis of these data provides one way to obtain reliable T-cell decay and production rates, and to follow the fate of recently produced T cells. Data on stable isotope labeling of naive and memory human T cells [22-26] are available, but several short-comings of these studies hamper their interpretation: (i) the short-term labeling period in most studies [23,25,26], resulting in little contribution of cells with a slow turnover [27-29] (ii) the frequent use of the precursor-product relationship [24,30,31] leading to

underestimation of the extent of T-cell turnover, because it measures the *net* accrual of label, and ignores the possibility that more cells were produced, which were lost during the labeling period because of their short life-span [32] (iii) the lack of delabeling curves in long-term labeling studies [24], which would have provided information on the decay rate of labeled cells and would thereby have pointed out whether recently produced T cells contribute to the T-cell pool under investigation, or rapidly disappear by death or activation. Here we combined long term *in vivo* stable-isotope labeling and label-decay studies of naive and memory T cells with mathematical analyses that allowed for the distinction between T-cell production rates and decay rates of labeled T cells, in young healthy adults [28]. Our analyses showed a slow accumulation of label within the naive T-cell pool, due to low daily production of naive T cells with a very long half-life. These data are incompatible with the presence of a substantial short-lived RTE pool in humans.

Materials and Methods

Mouse studies

Mice

C57Bl/6 mice were maintained by in-house breeding at the Netherlands Cancer Institute (Amsterdam) under specific pathogen-free conditions in accordance with institutional and national guidelines. Animal experiments were carried out in accordance with institutional and national guidelines and approved by the Experimental Animal Committee of the Netherlands Cancer Institute.

Thymectomy

7-wk-old male C57Bl/6 mice were anesthesized by intraperitoneal (i.p.) injection with Hypnorm (0.4 mg/kg fentanyl citrate and 12.5 mg/kg fluanisone, Janssen Animal Health, Buckinghamshire, UK) and Dormicum (6.3 mg/kg, Roche Nederland BV, Woerden, The Netherlands). The mouse was placed in supine position and its limbs and maxilla were taped to a surgical board. The skin was prepped with 70% alcohol. A midline incision was made from the lower cervical region to the level of the fifth rib. The skin was loosened and the salivary glands were pushed laterally. Directly adjacent to the sternum the 2 upper ribs were cut, thereby exposing the thymus. Vacuum suction was applied to remove the organ. The skin was closed by interrupted sutures and the animals were warmed until recovery from anaesthesia. Post-operative survival was 85%. The completeness of thymectomy was confirmed by visual inspection, both directly after removal of the organ and at the conclusion of the experiment. Only fully thymectomized animals were included in this study.

^2H_2O labeling protocol

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

FACS staining and cell sorting

Thymus, spleen and (axillary, branchial, inguinal and superficial cervical) lymph nodes were isolated from 21-23 week old C57Bl/6 mice. Single cell suspensions were obtained by mechanical disruption. Red blood cells were lysed with ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.4). Cells were washed, resuspended in IMDM/7% FCS and counted. Cells/well were seeded in 96 well plates in FACS staining buffer (PBS/1% FCS) and stained with CD4-PerCP or CD8-PerCP in combination with CD44-APC and CD62L-PE (all from BD PharMingen, San Diego, CA) in the presence of blocking 2.4G2 mAb. Following incubation with Cytofix/cytoperm solution (BD PharMingen) the cells were incubated with α-Ki67 or α-IgG (BD PharMingen) in 0.1% saponin (in FACS staining buffer). Cells were analyzed on a LSR II flow cytometer and BD FACSDiva software. The next day splenocytes were stained for CD4-PerCP, CD8-FITC, CD44-APC and CD62L-PE (BD PharMingen), and naive ($CD62L^+$, $CD44^-$) cells were sorted using a FACSAria cell sorter and FACSDiva software (BD). Purity of the sorted cells was 81-97% for naive $CD4^+$ T cells (average: 96% for control vs. 83% for ATx mice) and 86-99% for naive $CD8^+$ T cells (average: 98% for control vs. 88% for ATx mice). Granulocytes were isolated by density gradient centrifugation of blood using a combination of histopaque-1119 and Ficoll-paque, followed by red blood cell lysis. Granulocytes and thymocytes were frozen until further processed.

Statistical analysis

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

Human studies

Subjects and *in vivo* ^2H_2O labeling protocol

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

Flow cytometry and cell sorting

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

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque density gradient centrifugation from heparinized blood and cryopreserved until further processed. Peripheral blood T-cell proliferation in $CD4^+$ and $CD8^+$ T-cell subsets was studied by flow-cytometric measurements of Ki67 nuclear antigen, as described previously [42-44].
To measure the fraction of labeled cells within the naive ($CD45RO^-CD27^+$) and memory ($CD45RO^+$) $CD4^+$ and $CD8^+$ T-cell population, cryopreserved PBMC were thawed and incubated with monoclonal antibodies (mAb) to CD45RO-FITC (Caltag Laboratories, Burlingame, CA), CD27-PE, CD4-PerCP and CD8-APC (BD). The specified cell fractions were isolated by cell sorting on a MoFlow high speed cell sorter or on a FACSAria (BD). Purity of the sorted cells was on average 96% for naive $CD4^+$ and $CD8^+$ T cells, 95% for memory $CD4^+$ T cells and 91% for memory $CD8^+$ T cells.

Measurement of ^2H_2O enrichment in body water and DNA

Deuterium enrichment in urine was measured by a method adopted from Previs et al. [45].
The isotopic enrichment of DNA was measured according to the method described by Neese et al. [22] with minor modifications. Briefly, DNA was enzymatically hydrolyzed into deoxyribonucleotides after which the deoxyadenosines were purified using a SPE column. The adenosine residue was captured by cation resin and the deoxyribose was derivatized to deoxyribosepentane-tetraacetate (PTA) before injection into the gas chromatograph (6890 series, Agilent Technologies, Palo Alto, CA). The mass of the derivate was measured by positive chemical ionization mass spectrometry (5973 MSD, Agilent Technologies) at m/z 245 (M_0) and 246 (M_1). Since M_1 is known to be concentration-dependent, we first used the peak area at M_0 to determine the suspected natural abundance for each sample [46]. The enrichment (EM_1) was subsequently determined by dividing the peak area at M_1 by the total peak area (M_1 + M_0), after subtraction of the corrected natural abundance from the measured M_1 enrichment. We first fitted the urine enrichment data of each individual to a simple label enrichment/decay curve (see below and Supplementary 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 determine the maximal level of label intake that cells could possibly attain. In mice the enrichment of fully turned-over granulocytes (thymectomized mice) or thymocytes (euthymic mice) after 9-10 weeks of labeling were used to determine this maximal level of label intake.

Mathematical modeling

Up- and down-labeling of the urine enrichment data was modelled by the following differential equations:

$\frac{\mathrm{d}U}{\mathrm{d}t} = \pi - \delta U$ during label intake ($t \leq \tau$), and

$$\frac{dU}{dt} = -\delta U \quad \text{after label intake } (t > \tau),$$

where U represents the fraction of ^2H_2O in urine, t represents time in days and labeling was stopped at $t = \tau = 63$ days, δ represents the turnover rate of body water per day, and π is the source of ^2H_2O into body water per day. The urine enrichment data of each individual were fitted to the analytical solutions $U(t)$ of these differential equations with $U(0) = \beta$, representing the baseline urine enrichment attained after the boost of label by the end of day 0, such that:

$$U(t) = \frac{\pi}{\delta}(1-e^{-\delta t}) + \beta e^{-\delta t} \quad \text{during label intake } (t \leq \tau), \text{ and} \qquad \text{(Equation 1a)}$$

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

The parameter estimates of the best fits for the urine curves are given in Supplementary Table 1.

Label enrichment in the granulocyte population was modelled by the following differential equation:

$$\frac{dL}{dt} = pU(t) - dL \qquad \text{(Equation 2)}$$

throughout the labeling and de-labeling period, where L represents the fraction of labeled deoxyribose residues of adenosine in granulocytes, d represents the loss rate of labeled granulocytes and p combines granulocyte turnover and the maximum number of deuterium atoms that can be incorporated in the deoxyribose residue of adenosine. The corresponding analytical solutions for the fraction of labeled granulocytes:

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

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

$$L(t) = \frac{p\pi}{\delta - d}\left[\frac{1}{d}(e^{-d(t-\tau)} - e^{-dt}) - \frac{1}{\delta}(e^{-\delta(t-\tau)} - e^{-\delta t}) + \frac{\beta}{\pi}(e^{-dt} - e^{-\delta t})\right] \qquad \text{(Equation 3b)}$$

after label intake ($t > \tau$), were fitted to each individual's granulocyte enrichment data.

Equations 3a and 3b were also applied to calculate the average turnover rate p and the loss rate of labeled cells d in each cell population, where L now represents the fraction of labeled deoxyribose in the specific cell population. The parameter p in Equation (2) represents T-cell production resulting from both T-cell proliferation and thymic output. Since p determines the average T-cell turnover rate, pN (the average number of naive cells produced per day) provides an upperbound for the number of T cells exported from the thymus per day.

Since even after correction for body water enrichment, the asymptotes of granulocyte enrichment were lower than 100%, the asymptotes of all lymphocyte subsets were scaled by dividing by the granulocyte asymptote p/d of each individual. In the figures all data and fits were normalized by the maximum label enrichment of granulocytes, i.e. by dividing by $\frac{p\pi}{d\delta}$ of the granulocytes of the individual, such that the maximum label enrichment in granulocytes was scaled to 100%.

Results

Thymic output in mice

Since the main focus of this study was to establish the role of thymic output and peripheral T-cell proliferation in the maintenance of the naive T-cell pool, we first established if thymic output - and more specifically, a rapidly turning over RTE pool - could be detected using the ^2H_2O labeling technique. It is generally accepted that young rodents have considerable thymic output [33,34]. BrdU labeling studies, TREC dynamics and thymic engraftment in mice have revealed that the RTE pool of mice has a fast turnover [13,35], with an average life span of only 3 weeks [12,15] We first investigated whether this rapidly turning over RTE pool can be detected in mice using the ^2H_2O labeling technique, and compared euthymic and thymectomized mice to establish to what extent RTE affect the labeling within the naive T-cell pool.

Thymectomy of 7-week-old mice resulted in a severe and significant reduction in absolute $CD4^+$ and $CD8^+$ T-cell counts in lymph nodes and spleen in comparison to both age-matched controls (Figure 1A) and sham-thymectomized mice (data not shown). Taking into account the natural reduction in T-cell numbers in peripheral lymphoid organs during aging, the fastest decrease in T-cell counts was seen in the first 3 weeks after thymectomy. Naive $CD4^+$ T cells were reduced by 57% (p=0.022) in spleen and 59% (p=0.006) in lymph nodes, while naive $CD8^+$ T cells were reduced by 34% (p=0.024) in spleen and 50% (p=0.006) in lymph nodes, 3 weeks post-thymectomy (data not shown). Between 3 and 8 weeks post-thymectomy, the number of naive $CD4^+$ and $CD8^+$ T cells continued to decline more rapidly than in euthymic mice, while at later time points they decreased at approximately similar rates in thymectomized and euthymic mice.

^2H_2O labeling of young adult (12-13 week old) euthymic mice for 9-10 weeks resulted in 71 ± 3 % up-labeling for naive $CD4^+$ T cells and 50 ± 1 % for naive $CD8^+$ T cells in the spleen (Figure 1B). Comparable fractions of labeled cells were found in the lymph nodes (data not shown). This high degree of labeling in T cells could be due to substantial peripheral proliferation of naive T cells and/or reflect output of labeled thymocytes. To discriminate between these possibilities, mice were thymectomized at the age of 7 weeks and submitted to the long term ^2H_2O labeling protocol 5 to 6 weeks later. In the absence of thymic output the fraction of labeled naive T cells after 10 weeks up-labeling was 2-3 fold reduced (23 ± 3 % for naive $CD4^+$ T cells; 24 ± 1 % for naive $CD8^+$ T cells, Figure 1B, p=0.05).

Interestingly, the fraction of cycling (Ki67$^+$) naive T cells was not reduced in athymic mice, indicating that RTE had picked up label in the thymus rather than in the periphery (Figure 1C). Collectively, these data demonstrate that thymic output can be measured using ^2H_2O labeling. Furthermore, the observation that thymectomy led to a significantly reduced fraction of labeled cells and a significant loss of naive T cells, confirms that in young adult mice RTE contribute substantially to the naive T-cell pool.

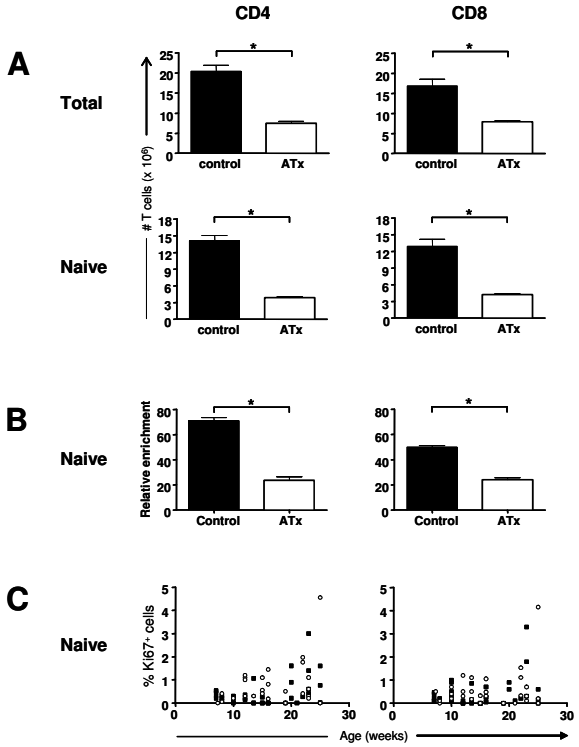

Figure 1. Thymic output is important for the maintenance of the naive T-cell pool in adult mice and can be quantitated using ^2H_2O-labeling. (A) Absolute numbers of total and naive CD4$^+$ and CD8$^+$ T cells in spleen of thymectomized and control animals 14-16 weeks after time of surgery. (B) Accumulated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

^2H_2O labeling in human volunteers

Next we aimed to determine the role of thymic output and peripheral T-cell proliferation in the maintenance of the naive T-cell pool, and the turnover rate of the memory T-cell pool in 5 young adult humans, aged between 20 and 25 years. During the 9 week up-labeling period and subsequent 16 week down-labeling period blood samples were drawn at 14 time points. Absolute numbers of CD4$^+$ and CD8$^+$ T cells and the fraction of naive CD45RO$^-$CD27$^+$ and memory CD45RO$^+$ CD4$^+$ and CD8$^+$ T cells were measured and these fractions were sorted for measurement of deuterium enrichment in the DNA. In addition, we measured *ex vivo* proliferation by the expression of the proliferation marker Ki67 within these same naive and memory T-cell populations to exclude the possibility that episodes of immune activation affected turnover rates. Characteristics of the healthy volunteers are given in Table 1.

Table 1. Characteristics of healthy volunteers

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

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

Body water and granulocyte enrichment

As a measure for body water enrichment during up- and down-labeling, we quantified ^2H_2O enrichment in urine at each time point. At the earliest time points after start of ^2H_2O labeling, body water enrichment had not yet reached its maximum level, while shortly after cessation of label body water was still found to be enriched. We therefore modeled the body water enrichment curves (see Methods and Supplementary Figure 1) and corrected for these best fits when analyzing the enrichment in the different cell populations. Since granulocytes are known to turnover rapidly, labeling of the granulocyte population of each individual was measured to estimate the maximal level of label intake that cells could possibly attain (see Methods and Supplementary Figure 1). Furthermore, because DNA baseline enrichment is not only determined by naturally occurring extremely low ^2H_2O enrichment, but also by more abundant naturally occurring heavy carbon molecules, we also longitudinally measured a negative control who did not drink ^2H_2O, which pointed out that background fluctuations were negligible (data not shown).

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

First of all, all labeling data of the different T-cell subsets were divided by the estimated maximum granulocyte enrichment of each volunteer (see Methods). The mathematical model was used to fit the corrected data, and to determine the average turnover rate (p) and the average rate at which labeled cells were lost from the population (d). It is important to realize that the accrual of label during label administration is truly representative of the T-cell population as a whole, while the loss of label after label cessation is only based on those cells that have picked up label by cell division. We therefore based our analyses on a so-called kinetic heterogeneity model in which the average turnover rate of the T-cell population is not necessarily equal to the loss rate of labeled cells [28].

The median turnover rates of naive CD4$^+$ and CD8$^+$ T cells were found to be as low as p=0.0005 and 0.0003 per day, corresponding to median half-lives of 1517 and 2398 days for naive CD4$^+$ and CD8$^+$ T cells, respectively (Figure 2 and Tables 2 and 3).

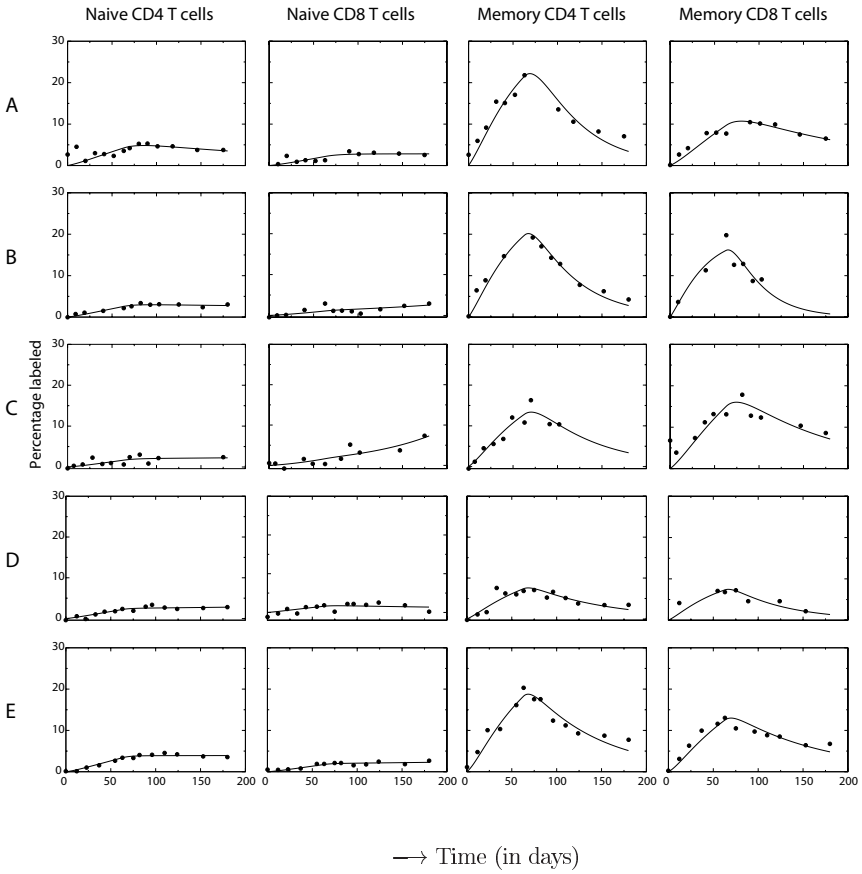

Figure 2. Best fits of the naive and memory CD4$^+$ and CD8$^+$ T-cell enrichment curves. Label enrichment was scaled between 0 and 100% by normalizing for the percentage label obtained in granulocytes (see Methods).

Table 2. Turnover rates (*p*) and loss rates of labeled cells (*d*) per day

		A	B	C	D	E	Median
Naive CD4$^+$	***p***	**0.0009** (0.0007-0.0014)	**0.0005** (0.0004-0.0005)	**0.0003** (0.0002-0.0005)	**0.0004** (0.0003-0.0004)	**0.0006** (0.0005-0.0006)	**0.0005**
	d	**0.0040** (-0.0014-0.0104)	**0.0009** (-0.0007-0.0028)	**-0.0007** (-0.0076-0.0097)	**-0.0009** (-0.0033-0.0014)	**-0.0001** (-0.0016-0.0013)	**-0.0001**
Naive CD8$^+$	***p***	**0.0004** (0.0003-0.0006)	**0.0002** (0.0001-0.0003)	**0.0002** (0.0001-0.0004)	**0.0003** (0.0001-0.0005)	**0.0003** (0.0002-0.0004)	**0.0003**
	d	**-0.0000** (-0.0061-0.0051)	**-0.0060** (-0.0145-0.0004)	**-0.0115** (-0.0181--0.0062)	**0.0024** (-0.0055-0.0141)	**-0.0011** (-0.0034-0.0015)	**-0.0011**
Memory CD4$^+$	***p***	**0.0067** (0.0059-0.0081)	**0.0058** (0.0052-0.0068)	**0.0034** (0.0026-0.0044)	**0.0017** (0.0014-0.0022)	**0.0045** (0.0040-0.0052)	**0.0045**
	d	**0.0203** (0.0158-0.0250)	**0.0198** (0.0163-0.0227)	**0.0145** (0.0069-0.0230)	**0.0115** (0.0070-0.0161)	**0.0126** (0.0094-0.0154)	**0.0145**
Memory CD8$^+$	***p***	**0.0021** (0.0018-0.0025)	**0.0060** (0.0043-0.0083)	**0.0034** (0.0029-0.0046)	**0.0019** (0.0014-0.0028)	**0.0028** (0.0025-0.0034)	**0.0028**
	d	**0.0065** (0.0041-0.0096)	**0.0304** (0.0192-0.0436)	**0.0089** (0.0040-0.0133)	**0.0161** (0.0077-0.0262)	**0.0098** (0.0071-0.0127)	**0.0098**

Depicted are turnover rates (*p*) and loss rates of labeled cells (*d*) per day as estimated by the mathematical model (underlined if not significantly different from 0). The 95%-confidence intervals were determined by a bootstrap method.

Table 3. Half-lives of naive and memory CD4$^+$ and CD8$^+$ T cells

	A	B	C	D	E	Median
naive CD4$^+$	801	1517	2374	1899	1187	1517
naive CD8$^+$	1737	4415	3349	2398	2374	2398
memory CD4$^+$	104	119	204	402	155	155
memory CD8$^+$	327	116	202	359	244	244

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

The turnover rates of memory $CD4^+$ and $CD8^+$ T cells were found to be approximately 10-fold higher, i.e. p=0.0045 and 0.0028 per day, corresponding to half-lives of 155 and 244 days for memory $CD4^+$ and $CD8^+$ T cells, respectively. Using the individual naive $CD4^+$ and $CD8^+$ T-cell counts revealed a median naive $CD4^+$ T-cell production of 8.2 $x10^7$ cells per day and a median naive $CD8^+$ T-cell production of 2.1 $x10^7$ per day (Table 4). Since this daily production of new naive T cells is the sum of thymic output and homeostatic proliferation within the naive T-cell pool, our data provide an upper estimate of daily thymic production of 1.7$x10^8$ T cells per day (see Table 4).

Table 4. Total production of naive T cells per day

	A	B	C	D	E	Median
Naive $CD4^+$ (*10^7)	11.50	5.36	2.24	8.19	13.10	8.19
Naive $CD8^+$ (*10^7)	2.53	0.95	0.96	2.07	3.53	2.07
Total naive (*10^7)	14.03	6.31	3.20	10.26	16.63	10.26

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

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

Discussion

By *in vivo* labeling of T-cell subsets using ^2H_2O, and mathematical analysis of label enrichment, our data provide reliable estimates for the average turnover rates of naive and memory $CD4^+$ and $CD8^+$ T cells in healthy adults. Although isotope labeling studies in humans are typically restricted to blood, it has previously been reported that labeling kinetics in human T cells derived from blood and lymphoid tissues are comparable [36]. The very low accumulation of label in naive T cells (<5%) that we observed after 9 weeks of up-labeling is compatible with the data reported by Hellerstein et al. [24] Our estimated half-lives between 1517 and 2398 days for naive T cells and between 155 and 244 days for memory T cells are, however, much longer than previous estimates based on stable-isotope labeling, which varied from 112 days to 361 days for naive T cells, and from 14 days to 235

days for memory T cells [32]. Differences in the labeling-period [28], and use of T-cell death rates, which overestimate T-cell turnover because of the bias towards cells that have recently divided [27], might explain these discrepancies. Michie et al. [9] used the presence of T cells with dicentric chromosomes after radiation to measure the half-life of naive and memory T cells. They estimated a half-life of 182 days for $CD45RO^+$ and 630 days for $CD45RA^+$ T cells. Given the notion that $CD45RA^+$ T cells can contain a substantial fraction of effector ($CD45RA^+CD27^-$) cells we additionally used CD27 expression on $CD45RA^+$ T cells to identify naive T cells. This difference in definition of the naive subset may explain the difference in the estimated life spans between these studies. Furthermore, it is conceivable that the half-lives of T cells are affected by radiation.

The maximum total daily naive T-cell production in our 5 healthy volunteers of 13.1 $x10^7$ $CD4^+$ T cells and 3.5 $x10^7$ $CD8^+$ T cells implies that the thymus in young human adults is exporting maximally $1.7x10^8$ T cells per day. Part of the labeling of the naive T-cell population may, however, also be due to peripheral T-cell proliferation. The estimated total daily naive T-cell production is in close agreement with the daily accumulation of 10^8 naive T cells in patients with a depleted T-cell pool [11,37]. This suggests that thymic output and naive T-cell proliferation do not homeostatically respond to peripheral T-cell depletion. Still the production of naive T cells in such depleted situations might be underestimated because naive cells may transit more rapidly to the memory pool.

Our analyses also enabled us to follow the fate of recently produced cells. In the memory T-cell population we found that the decay rate of labeled cells was higher than the average production rate, indicating that the turnover of cells that picked up label was higher than the turnover of the average cell in the memory population. This finding is in line with previous labeling studies, which all showed that the loss of labeled cells exceeded the production [27-29]. Unexpectedly, we found that this was not the case for the naive T-cell population, as no significant loss of labeled cells was observed in any of the 5 individuals during the 16 weeks after label cessation. Thus, newly produced (i.e. labeled) naive T cells, whether produced by the thymus or by peripheral proliferation, tended to live longer than the average cell in the naive T-cell population. This implies that newly produced naive T cells were preferentially incorporated into the peripheral naive T-cell pool, which contradicts the notion of a substantial short-lived RTE pool in young human adults.

In order to be sure that administration of ^2H_2O would efficiently label short-lived RTE, we performed ^2H_2O labeling in euthymic and thymectomized mice. Previous studies have shown that thymic output in young adult rodents is substantial [33,34], and TREC dynamics, BrdU labeling and thymic engraftment have demonstrated that this RTE pool in mice has a fast turnover [12,13,15,38]. In line with previous studies, we found that thymectomy in mice gives a considerable reduction in peripheral naive T-cell numbers already within 3 weeks after thymectomy [39,40], indicating an important contribution of short-lived RTE to the naive T-cell population in mice. Thymectomy induced a 2-3 fold decrease in labeled naive T cells, showing that the ^2H_2O protocol successfully labeled RTE in euthymic mice.

Our data shed a very different light on thymic output and RTE dynamics. In contrast to the currently prevailing idea that RTE are short-lived and do not enter the pool of resident naive

cells , we conclude that in human adults RTE are rare and long lived, and remain in the peripheral T-cell pool [15]. The advantage of a short-lived RTE pool has been proposed to be the continuous supply of T cells with a diverse repertoire to the long-lived resident naive T-cell pool [41]. The preferential incorporation of RTE into the resident naive T-cell pool that we here describe for human adults seems a much more efficient way to continuously rejuvenate the naive T-cell pool and repertoire than a daily excess production of RTE with a very short life span. The unfortunate consequence of this sparse production of long-lived naive T cells may be, however, that naive T-cell supply may be limiting in clinical conditions of chronic T-cell depletion, and -- due to the increasing life expectancy -- even in healthy elderly, which may have severe clinical consequences.
Taken together, our data confirm the presence of a significant RTE pool with rapid turnover in mice, while in young human adults recently produced naive T cells are rare and long-lived.

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Supplementary Table 1.

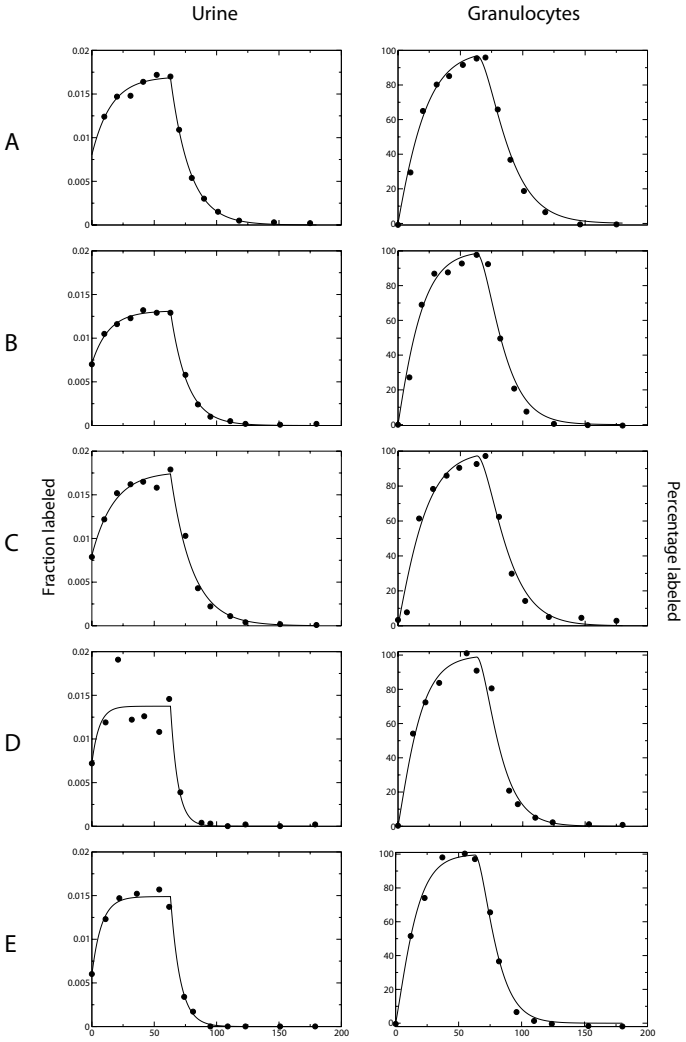
Parameter estimates of the urine enrichment curves, where π represents the source of ^2H_2O into the body water per day, δ is the turnover rate of body water per day, and β represents the baseline urine enrichment attained after the boost of label by the end of day 0.

Individual	π	δ	β
A	0.00109	0.064	0.0080
B	0.00114	0.088	0.0070
C	0.00130	0.075	0.0082
D	0.00177	0.128	0.0074
E	0.00178	0.119	0.0059

Supplementary Table 2.

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

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



Supplementary Figure 1. Best fits of the fraction of ^2H_2O in urine and the percentage of labeled deoxyribose residues of adenosine in granulocytes after scaling by the maximum enrichment level $\frac{p\pi}{d\delta}$ of the granulocyte population (see Methods).

Full T-cell reconstitution after long-term HAART without signs of accelerated aging of the T-cell pool in adults and children

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Chapter 10

Abstract

We studied whether full T-cell reconstitution can in principle be achieved in HIV-infected adults and children with complete long-term virological control by HAART. To study quantitative and qualitative T-cell recovery a comprehensive analysis was performed, including analyses of naive, memory and effector $CD4^+$ and $CD8^+$ T-cell recovery, activation and proliferation markers, CD31 expression, TREC numbers and telomere lengths. $CD4^+$ T-cell counts in children recovered to age-matched control values within 1 year, even when HAART was initiated at an extremely lymphopenic stage. In adults with $CD4^+$ T-cell counts >200 cells/µl at HAART initiation $CD4^+$ T-cell counts recovered within 1 year of therapy. Adults with lower $CD4^+$ T-cell numbers at HAART initiation persistently sustained lower counts than adults with high pre-therapy $CD4^+$ T-cell counts throughout follow-up, but nevertheless normalized $CD4^+$ T-cell counts after 7 years of HAART. Absolute numbers of naive $CD4^+$ T cells normalized in all children and in adults with high baseline $CD4^+$ T-cell counts, whereas naive $CD4^+$ T-cell counts in adults with low baseline $CD4^+$ T-cell numbers lagged behind. After long-term HAART the TREC content and TREC numbers in adults and children had returned to the levels of age-matched controls. Restoration of the nearly empty T-cell pool apparently did not interfere with T-cell aging, because evidence neither for accelerated aging nor for rejuvenation of the T-cell pool was obtained. Strikingly, the proliferation marker Ki67 had normalized after long-term HAART in children, but remained elevated in HIV-infected adults after long-term treatment despite relatively low levels of T-cell activation.

Introduction

Since the introduction of highly active antiretroviral therapy (HAART) many studies have investigated the effect of treatment on the reconstitution of the $CD4^+$ T-cell pool. It has now been generally accepted that $CD4^+$ T-cell counts gradually increase in the majority of adult patients in whom the virus is successfully suppressed [1,2]. Some studies report a plateau effect in $CD4^+$ T-cell count after 3 to 5 years of HAART [2,3], whereas Hunt et al. [1] found continuous $CD4^+$ T-cell gains throughout 4 years of therapy. $CD4^+$ T-cell counts in adult patients with low nadirs pre-therapy were found to remain low throughout the reconstitution period and failed to normalize after 4 to 5 years of HAART [1,2]. However, the net yearly increase in $CD4^+$ T-cell counts was shown to be independent of nadir $CD4^+$ T-cell counts [1].

Immune reconstitution depends on thymic output and peripheral proliferation. Based on the higher thymic output in children, one would expect that immune reconstitution is more effective in children than in adults [4]. Indeed in HIV-infected children $CD4^+$ T-cell counts tend to normalize after 12 to 18 months of treatment [5,6] and increases in total and naive $CD4^+$ T cells are higher in young HIV-infected children on HAART [5,7]. However, the norms children have to meet are also more demanding. Consequently, when studied as a percentage of age-matched values, increases in naive $CD4^+$ T-cell counts in HIV-infected children during HAART are age independent [5]. Similarly, although net increases in naive T cells after initiation of HAART are higher in HIV^+ children than in HIV^+ adults [8], when corrected for age-matched values, net naive $CD4^+$ T-cell recovery was found to be comparable [9].

The importance of thymic output during reconstitution is suggested by the association of poor $CD4^+$ T-cell reconstitution during HAART with age [1,2,10-12], low absolute numbers of naive $CD4^+$ and $CD8^+$ T cells [11,13], less thymic tissue [11,12,14-16] and low absolute numbers of T-cell receptor excision circles (TRECs) [17]. TRECs are by-products of T-cell receptor rearrangement and are not duplicated upon cell division [18,19]. Consequently, absolute numbers of TRECs can provide information on thymic output. Alternatively, because TRECs are diluted upon cell division, the TREC content of the T-cell pool is a measure for replicative history [19].

Besides thymic output, reconstitution of the T-cell pool can be achieved by peripheral T-cell proliferation. During untreated HIV infection, however, T-cell proliferation is a consequence of high immune activation rather than a homeostatic mechanism to $CD4^+$ T-cell depletion [20] and is a prognostic marker for progression to AIDS [21]. Concomitant accelerated aging of the T-cell pool during HIV infection is reflected by a decrease in TREC content [18,19,22,23]. Increased T-cell proliferation during HAART can theoretically either be attributed to residual immune activation or to a homeostatic increase to fill up the empty T-cell pool. Studies in HIV-infected adults, have shown that 2 years of therapy did not normalize T-cell proliferation levels [24] and $CD4^+$ and $CD8^+$ T-cell activation markers were not normalized up to 2 to 6 years of therapy [24-27]. Similarly, HIV-infected children followed on HAART for 3-4 years had significantly higher levels of activated ($CD38^+HLA\text{-}DR^+$) $CD4^+$ and $CD8^+$ T cells than age-matched controls [13]. The fact that low TREC

contents [11-13,17,23,28], shorter telomeres [11] and high levels of activated $CD38^+HLA\text{-}DR^+$ T cells [13,24,25,29,30] and proliferating ($Ki67^+$) naive $CD4^+$ and $CD8^+$ T cells [30] are related to poor $CD4^+$ T-cell reconstitution, argues for residual activation rather than homeostatically induced proliferation.

During HAART, decreasing T-cell proliferation in concert with entrance of recent thymic emigrants into a virtually empty peripheral T-cell pool, could in theory rapidly rejuvenate the peripheral T-cell pool. Conversely, if the $CD4^+$ T-cell count increase would be mainly due to extensive proliferation, this would dilute the TREC content and shorten telomeres even further. Although it has been reported that the TREC content normalizes in HIV-infected children on HAART to the lower normal range [9,13,22,23,31], studies on TREC content during HAART in HIV-infected adults are inconclusive, reporting lower, higher or normalized TREC content [9,32-34].

In general, most studies concerning immune reconstitution during HAART selected for immunological responders and/or non-responders [9,11-13,17,23,30,35,36], or did not include age-matched controls [7,8,11,12,23,30,34,37,38], thereby failing to study to what extent normalization had occurred. In addition, mainly relatively short-term effects were studied [5,7,8,11,22,24,31,34,38]. In many of the studies in children, patients had been pre-treated [5,7,13,23,28,31,38] and there often was residual viral load [8,12,13,23,28,38].

Here, we addressed the question whether T-cell reconstitution can in principle be achieved and therefore focused on patients with complete long-term (7-9 years) virological control by HAART. We assessed the relative contributions of peripheral T-cell division and thymic T-cell production to quantitative and qualitative long-term restoration of the T-cell pool in both adults and children. We combined analysis of quantitative naive, memory and effector $CD4^+$ and $CD8^+$ T-cell recovery with measurements of activation markers and proliferation markers to establish to what extent long-term HAART has normalized these parameters. Now that treated HIV-infected individuals have a relatively long life expectancy, it is essential to understand whether recovery of the T-cell compartment during long-term HAART in HIV-infected individuals might result in accelerated aging of the T-cell pool in the long run. To investigate accelerated aging of the T-cell pool and to determine the source of repopulating cells, we analyzed their replicative history and 'post-thymic history' by telomere length, TREC content and by the fraction $CD31^+$ T cells within the naive T-cell pool. $CD31^+$ naive $CD4^+$ T cells are most proximal to the thymus [39] (Vrisekoop et al. submitted) and have been described to have a much more diverse T-cell receptor repertoire compared to $CD31^-$ naive $CD4^+$ T cells [39,40]. During aging the percentage $CD31^+$ naive $CD4^+$ T cells has been shown to decrease [39], posing the question whether these levels can be attained during immune reconstitution. Identification of factors that truly contribute to functional, long-lasting immune recovery will help to improve and evaluate therapeutic approaches of HIV-1 infection.

Material and methods

Study population

Twenty-one HIV-1 infected children (mean age: 5.6 years; age range: 0.3-13.2 years) and twenty-six HIV-1 infected adults (mean age: 37.2 years; age range: 28.4-50.8 years) were selected from the patient population of the UMC, Utrecht and AMC, Amsterdam. They were treated with long-term continuous HAART (range children: 4.4-9.6 years; range adults: 7.0-9.2 years) and had adequate viral suppression for the complete period of follow-up, with a minimum detection threshold of 50 copies per ml. However, an occasional appearance of plasma HIV-1 RNA above the detection threshold, after which the load returned to undetectable levels within 3 months after detection, was allowed. Of the selected patients 13 adults and ten children had one or more occasions of plasma HIV-1 RNA appearance above the detection threshold during long-term follow-up, with a median of 200 copies per ml. All children were vertically infected with HIV-1, except one child who was infected by transmission via blood transfusion at the age of 13. This study was approved by the medical ethical committee and written informed consent was obtained from all study participants or their legal guardians.

Flow cytometry and cell sorting

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque density gradient centrifugation from heparinized blood and cryopreserved until further processed. Absolute counts, maturation status and immune activation status of $CD4^+$ and $CD8^+$ T cells were routinely measured. Absolute $CD4^+$ and $CD8^+$ T-cell counts were determined by dual-platform flow cytometry. Naive ($CD27^+CD45RA^+$), central memory ($CD27^+CD45RA^-$), effector/memory ($CD27^-CD45RA^-$) and effector ($CD27^-CD45RA^+$) $CD4^+$ and $CD8^+$ T-cell fractions and activated ($CD38^+$HLA-DR^+) $CD4^+$ and $CD8^+$ T cells were assessed by flow cytometry. Of note, sometimes CD45RO rather than CD45RA was used to designate naive cells. To measure the fraction $CD31^+$ cells within the naive $CD4^+$ T-cell population and peripheral blood T-cell proliferation in $CD4^+$ and $CD8^+$ T-cell subsets, cryopreserved PBMC were thawed and incubated with monoclonal antibodies (mAb) to CD45RO-FITC (Caltag) or CD45RO-PE, CD31-PE, CD4- or CD8- PerCP (Becton Dickinson (BD), San Jose, California) and biotinylated CD27 (Sanquin Reagents). After washing, cells were incubated with anti-Streptavidin-APC (BD). For measuring T-cell proliferation, cells were fixated (FACS Lysing Solution, BD), permeabilized (FACS Permeabilisation Buffer, BD) and stained intracellularly with Ki67-FITC (Monosan, The Netherlands), after which cells were fixed using Cellfix (BD) and analyzed on a FACSCalibur (BD) with CellQuest software.
To measure the TREC content within the naive ($CD27^+CD45RO^-$) $CD4^+$ and $CD8^+$ T-cell population, PBMC were incubated with monoclonal antibodies (mAb) to CD45RO-FITC (Caltag Laboratories, Burlingame, CA), CD27-PE, CD4-PerCP and CD8-APC (BD). The specified cell fractions were isolated by cell sorting on a FACSAria (BD). To measure the TREC content within $CD4^+$ 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, Sunnyvale, California).

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 by using a real-time PCR method as previously described [19,41].

Measuring telomere length by Flow-FISH

The average length of telomere repeats at chromosome ends in memory ($CD45RA^-$) T cells was measured by flow-FISH as previously described [42-44]. Briefly, bovine thymocytes were added to the PBMC as an internal control and cells were hybridized with or without 0.3 µg/ml telomere specific FITC conjugated $(C_3TA_2)_3$ PNA probe. Next, cells were washed and counterstained with 0.01 µg/ml LDS 751 (Exciton Chemical Co. Inc.; Dayton, Ohio), to be able to distinguish the thymocytes from the PBMC. The fluorescence was acquired on a FACSCalibur (BD) and analyzed with CellQuest software (BD). The specific fluorescence was calculated by subtracting the autofluorescence of unstained cells from the fluorescence measured in cells hybridized with the FITC-labeled telomere PNA probe. To convert the specific fluorescence into kb of telomere repeats, we added bovine thymocytes with a known telomere length to each sample as an internal standard [44].

Results

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

We aimed to determine if $CD4^+$ T-cell counts normalized in children and adults and whether children demonstrated enhanced $CD4^+$ T-cell recovery compared to adults as they may have better reconstituting capabilities. 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 numbers per µl blood decrease during growth and stabilize at the start of adolescence, $CD4^+$ T-cell numbers were normalized for age-matched control values [45] in order to assess whether full $CD4^+$ T-cell recovery had been accomplished. For adults a mean $CD4^+$ T-cell number of 800 per µl blood was used as reference value [46]. The mean normalized $CD4^+$ T-cell number at start of HAART was higher for children than for adults (39.5% and 29.4% of the age-matched control value, respectively). To make a fair comparison between T-cell reconstitution in adults and children, and in patients with low or high $CD4^+$ T-cell numbers at HAART initiation, we separately analyzed patients with low and high $CD4^+$ T-cell number at start of treatment. The value for separation was chosen to be 25% of age-matched controls, 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 numbers lower than 25% of their age-matched control value at baseline, $CD4^+$ T-cell numbers reached similar levels as children who had higher baseline $CD4^+$ T-cell counts within 1 year of HAART ($p=0.80$, Figure 1A) and both groups were not significantly different

from age-matched controls from 1 year onward. In contrast, in adults the difference in $CD4^+$ T-cell counts between the two groups remained throughout the follow-up. In adults with high baseline $CD4^+$ T-cell numbers, the largest increase in $CD4^+$ T-cell numbers was observed during the first half year of HAART. After 1 year of HAART, no statistical difference was found with age-matched control (p=0.136). $CD4^+$ T-cell counts in adults with low nadir $CD4^+$ T-cell numbers persistently lagged behind, but ultimately, after at least 7 years of HAART, reached numbers which did not differ from age-matched control values (p=0.176). Significantly elevated numbers of $CD8^+$ T cells were found after long-term HAART in adults (p<0.001 for both groups).

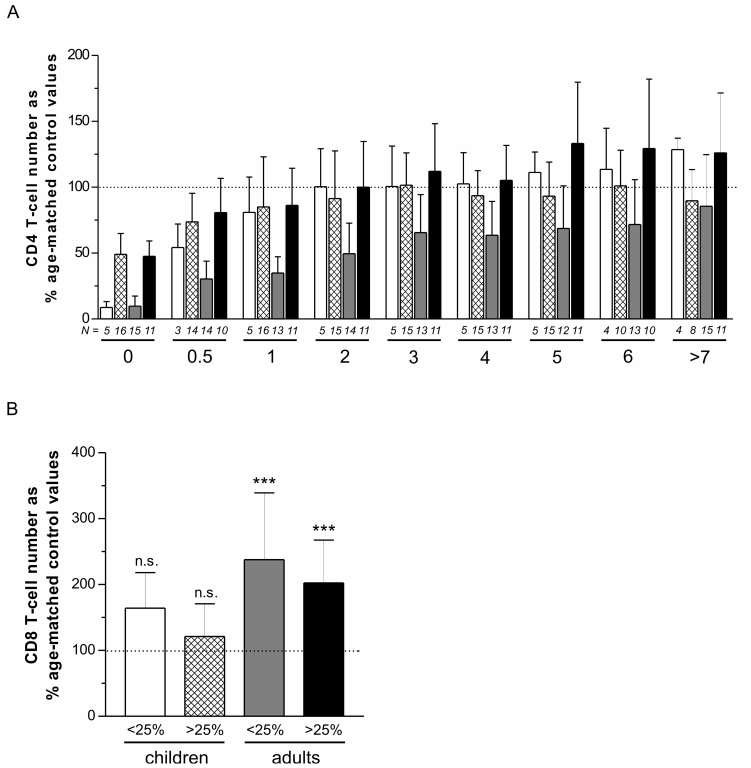

Figure 1. $CD4^+$ and $CD8^+$ T-cell recovery in children and adults on long-term HAART. (A) $CD4^+$ T-cell numbers, corrected for age, are increased from start of HAART and both children groups as well as adults with high baseline $CD4^+$ T-cell number reach numbers not significantly different from healthy individuals after one year of treatment. Adults with low baseline $CD4^+$ T-cell numbers reach normal numbers after at least 7 years of treatment. (B) Age-corrected $CD8^+$ T-cell numbers are significantly increased in both adult groups after long-term HAART, whereas $CD8^+$ T-cell numbers in children are not significantly elevated. Bars in A represent the groups as depicted in B. Data are shown as mean + SD. *** p<0.001; n.s. = not significant. *N* = no. per group. Statistical significance from 100% is determined by a one-sample t-test.

These numbers were not significantly different from baseline numbers (low baseline $CD4^+$ T cells: p=0.173 and high baseline $CD4^+$ T cells: p=0.789, data not shown), indicating that the $CD8^+$ T-cell accumulation in the period of untreated HIV infection had still not resolved. In children $CD8^+$ T-cell expansions were no longer observed after long-term HAART, although baseline $CD8^+$ T-cell numbers were unknown (Figure 1B).

$CD4^+$ and $CD8^+$ T-cell subsets are disturbed in adults after long-term HAART

Although $CD4^+$ T-cell numbers of children and adults had returned to normal levels after long-term HAART, we questioned if naive and memory subsets had also normalized completely. Because no differences in naive and memory $CD4^+$ and $CD8^+$ T-cell subsets were observed after long-term HAART between children with high and low baseline $CD4^+$ T-cell count (data not shown), we no longer presented the data separately for children. In children, percentages and numbers of naive ($CD27^+CD45RO^-$) and effector memory ($CD27^-CD45RO^+$) $CD4^+$ T-cells had returned to normal levels after long-term HAART, whereas central memory ($CD27^+CD45RO^+$) $CD4^+$ T-cell percentages were significantly decreased (p<0.001). Adults with low baseline $CD4^+$ T-cell numbers had significantly higher fractions of central memory $CD4^+$ T cells (p<0.001), concomitant with lower percentages (p<0.001) and absolute numbers (p=0.002; data not shown) of naive $CD4^+$ T cells after long-term HAART compared to healthy controls. In contrast, in adults with high baseline $CD4^+$ T-cell numbers, percentages of naive $CD4^+$ T cells were slightly decreased, which was borderline significant (p=0.055), but numbers had normalized. Central memory $CD4^+$ T-cell percentages (p=0.028) and numbers (p=0.04; data not shown) were increased.

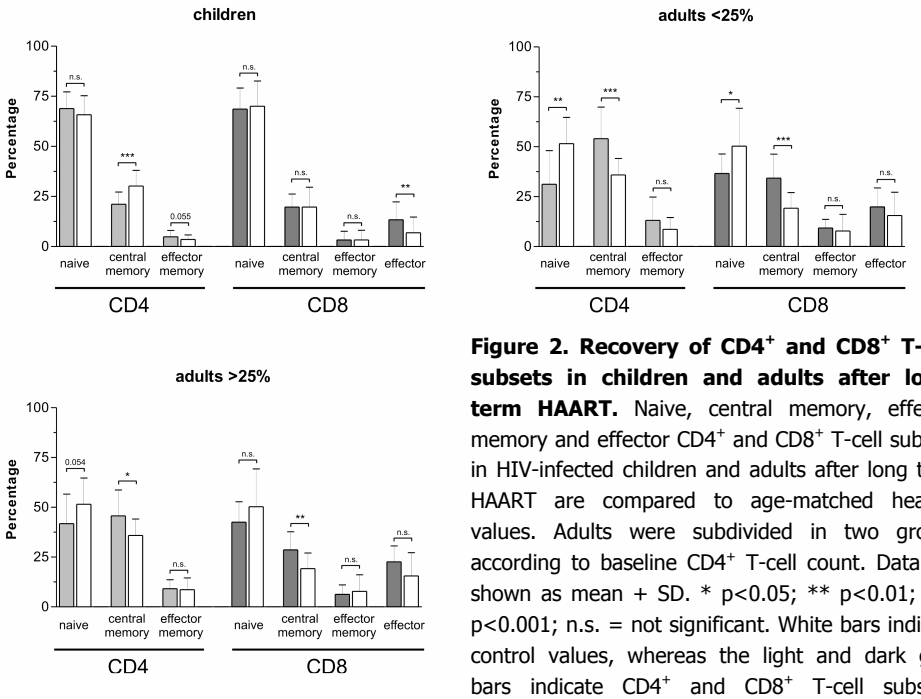

Figure 2. Recovery of $CD4^+$ and $CD8^+$ T-cell subsets in children and adults after long-term HAART. Naive, central memory, effector memory and effector $CD4^+$ and $CD8^+$ T-cell subsets in HIV-infected children and adults after long term HAART are compared to age-matched healthy values. Adults were subdivided in two groups according to baseline $CD4^+$ T-cell count. Data are shown as mean + SD. * p<0.05; ** p<0.01; *** p<0.001; n.s. = not significant. White bars indicate control values, whereas the light and dark grey bars indicate $CD4^+$ and $CD8^+$ T-cell subsets, respectively. Statistical significance is determined by the nonparametric Mann-Whitney *U* test.

In children, percentages of naive, central memory and effector memory $CD8^+$ T cells were not different from age-matched control values, although effector $CD8^+$ T cells ($CD27^-$ $CD45RO^-$) were increased (p=0.004) after long-term HAART (Figure 2). No significant differences were observed between the two groups of adults with regard to the $CD8^+$ T-cell subset distribution. Increased central memory $CD8^+$ T-cell numbers ($p<0.01$ for both adult groups) contributed to the observed higher numbers of $CD8^+$ T cells after long-term HAART in adults (Figure 1B). Of note, numbers of naive $CD8^+$ T cells were significantly enhanced compared to normal values (low baseline $CD4^+$ T cells: p=0.003; high baseline $CD4^+$ T cells: p=0.001), although naive $CD8^+$ T-cell percentages were significantly decreased in adults with low baseline $CD4^+$ T-cell numbers (p=0.015) after long-term HAART.

$CD4^+$ T-cell recovery is accomplished by both thymic output and peripheral proliferation and is not associated with accelerated aging of the T-cell compartment

We next studied the origin of the newly produced naive T cells during HAART and whether $CD4^+$ T-cell recovery was associated with accelerated aging of the T-cell compartment. One might envisage that excess peripheral proliferation could dilute TRECs and result in a T-cell population with shortened telomeres. On the contrary, if new T cells were mainly derived from thymic output a relatively high TREC content and long telomeres might be the result.

a. $CD4^+$ T-cell TREC content and numbers are similar compared to age-matched values in adults and children

After long-term HAART, both children and adults had similar TREC contents in $CD4^+$ T cells as age-matched control values (Figure 3A). Also absolute $CD4^+$ T-cell TREC numbers per µl blood did not differ from control values (Figure 3B). For a number of adults baseline $CD4^+$ T-cell TREC contents and numbers were available. In all these adults an increase in $CD4^+$ T-cell TREC number was observed during HAART, showing a contribution of thymic T-cell production to the recovery of the naive $CD4^+$ T-cell compartment (Figure 3A). In most adults with decreased $CD4^+$ T-cell TREC contents at start of HAART an increase was observed to levels of healthy controls during long-term HAART, whereas adults who already had normal TREC contents at initiation of HAART demonstrated a similar decline as age-matched control values.

In two adults $CD4^+$ T-cell TREC contents at initiation of HAART were low and did not recover to age-matched control values. Both individuals demonstrated reduced percentages and numbers of naive $CD4^+$ T cells, although they showed an increase in $CD4^+$ T-cell TREC number per µl blood after long-term HAART. Because the decrease in TREC content within $CD4^+$ T cells is strongly affected by memory T-cell expansions, we additionally studied the naive T-cell proliferative history by determining TRECs in FACS-sorted naive $CD4^+$ T cells (Figure 3C). One of the two individuals with low $CD4^+$ T-cell TREC content displayed normal naive $CD4^+$ T-cell TREC content, suggesting that the increased number of memory $CD4^+$ T cells observed in this individual caused the decrease in $CD4^+$ T-cell TREC content. The other individual displayed a decreased TREC content per naive $CD4^+$ T-cell, implying that extensive naive T-cell proliferation had taken place.

b. The expression of CD31$^+$ on naive CD4$^+$ T cells after long-term HAART is comparable to age-matched control values in adults and children

To determine whether the origin of the CD4$^+$ T-cell recovery was associated with accelerated aging of the naive T-cell pool we determined the fraction of CD31$^+$ cells within the naive CD4$^+$ T-cell pool after long-term HAART. The extensive increases in CD4$^+$ T-cell counts had not caused accelerated aging of the naive CD4$^+$ T-cell subset since the fraction of CD31$^+$ naive CD4$^+$ T cells after long-term HAART in both adults and children was comparable to age-matched control values (Figure 3D).

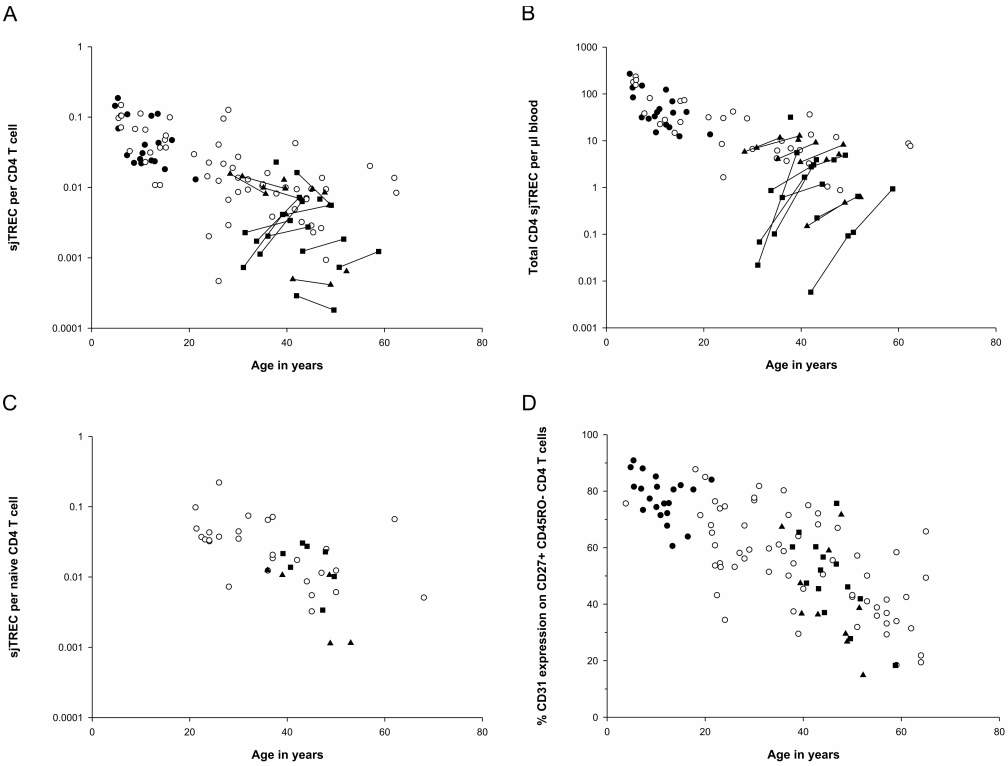

Figure 3. Recovery of the CD4$^+$ T-cell compartment by thymic output and peripheral proliferation after long-term HAART. (A) TREC content per CD4$^+$ T cell. (B) Absolute TREC numbers per µl blood. (C) TREC content in naive CD4$^+$ T cells. (D) Percentages of CD31$^+$ naive T cells. ● children; ■ adults with low baseline CD4$^+$ T-cell numbers; ▲ adults with high baseline CD4$^+$ T-cell numbers; ○ control values. Lines connect data points at start and after long-term HAART within one individual.

c. The telomere length of memory T cells did not normalize in all adults

To assess the replicative history of T cells after long-term HAART, shortening of the mean telomere length was determined in adults by FlowFISH. Because this method is restricted in the use of antibodies, we could only measure the telomere length for memory ($CD45RA^-$) T cells. Due to limited cell availability, the telomere length of T cells could not be assessed in children on long-term HAART. Seven of 25 adults demonstrated telomere lengths shorter than the 10^{th} percentile for healthy individuals, indicating extensive division of the memory T-cell compartment (data not shown). Interestingly, these 7 adults all belonged to the group with low baseline $CD4^+$ T-cell numbers at start of HAART. Compared to the other adults in this group, who had normal telomere-lengths, they displayed significantly decreased percentages of naive $CD4^+$ T cells ($p=0.020$) and significantly increased percentages of memory ($CD45RO^+$) T cells ($p=0.024$). Furthermore the percentage $CD8^+$ T cells was significantly increased in individuals with shorter telomeres ($p=0.037$). Since telomeres are reported to be shorter in $CD8^+$ T cells during HIV infection [47], this higher fraction $CD8^+$ T cells might explain the shorter telomeres in these individuals.
Of eight adults longitudinal measurements were performed. Similar to the dynamics of the $CD4^+$ T-cell TREC content, no major alterations were observed during treatment of adults 6 adults who already had normal telomere-lengths at HAART initiation. The other two adults followed longitudinally displayed short telomeres at baseline and both demonstrated an increase in length during long-term HAART, however, only one of those two regained normal telomere-lengths.

Persisting increased division rates of T cells in adults on long-term HAART

To evaluate the effect of HAART on the rate of T-cell division, the expression of the proliferation marker Ki67 was measured within $CD4^+$ and $CD8^+$ T-cell subsets after long-term HAART. In children, Ki67 expression in the $CD4^+$ T-cell subsets could only be measured after long-term HAART. Naive and central memory $CD4^+$ T cells in children expressed similar levels of Ki67 compared to age-matched controls, whereas proliferation within the effector memory compartment was significantly decreased ($p=0.019$) (Figure 4).
In adults, initial mean baseline Ki67 expression in each $CD4^+$ T-cell subset was significantly higher than control values. After long-term HAART, in all subsets the expression declined, though not always significantly, but not to normal values (Figure 4). No significant differences between the two adult groups were observed in Ki67 expression in any of the $CD4^+$ T-cell subsets. After long-term HAART, in both adult groups increased Ki67 expression was observed within naive ($p<0.001$) and central memory ($p<0.01$) $CD4^+$ T cells compared to age-matched controls, whereas only increased proliferation within effector memory $CD4^+$ T cells was observed in adults with low baseline $CD4^+$ T-cell numbers ($p=0.015$).
Increased proliferation was also observed within the $CD8^+$ T-cell compartment. After long-term HAART, adults with low baseline $CD4^+$ T-cell numbers showed increased proliferation of naive ($p<0.001$) and effector memory ($p<0.001$) $CD8^+$ T cells, whereas elevated Ki67 expression was found in naive ($p<0.001$) and central memory ($p=0.019$) $CD8^+$ T cells of adults with high baseline $CD4^+$ T-cell numbers. Expression of Ki67 within the $CD8^+$ T-cell

compartment of children on long-term HAART could not be determined because of limited cell availability.

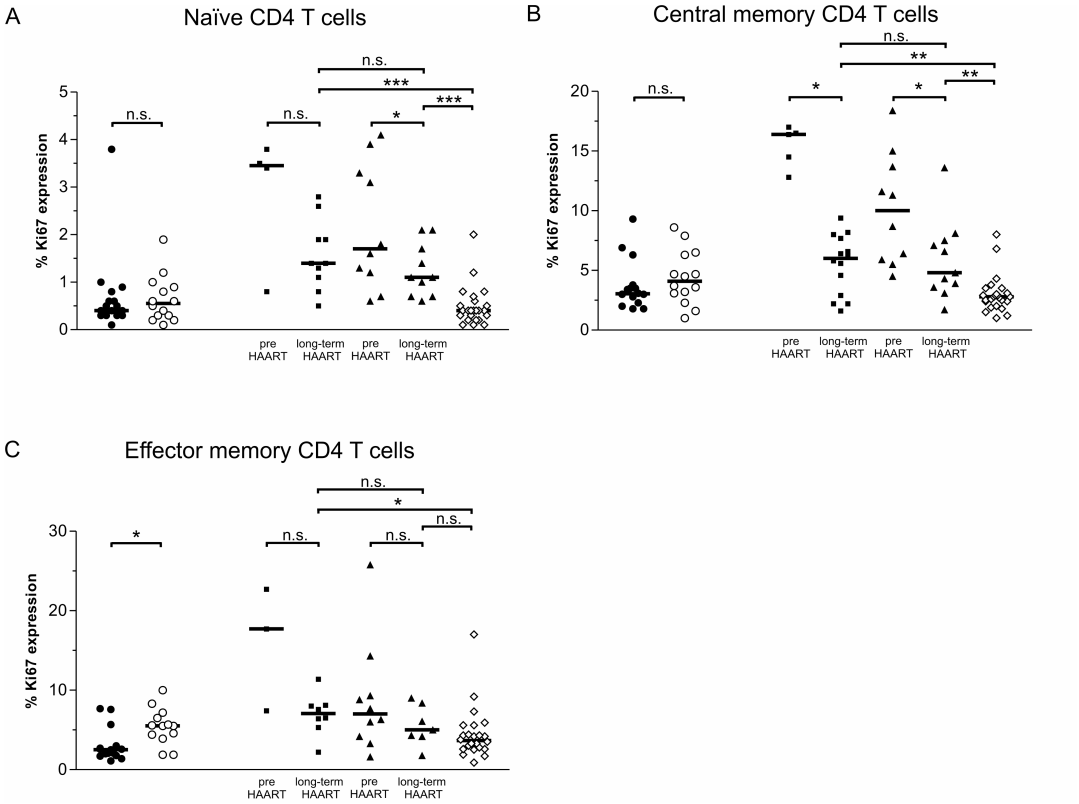

Figure 4. Proliferation within the CD4$^+$ T-cell compartment after long-term HAART.
Proliferation within naive (A), central memory (B) and effector memory (C) CD4$^+$ T cells.
● children; ♦ adults with low nadir CD4$^+$ T-cell numbers; ▲ adults with high nadir CD4$^+$ T-cell numbers; ○ children control values; ◇ adult control values. Horizontal lines depict median Ki67 expression. Statistical significance is determined by the nonparametric Mann-Whitney *U* test: * p<0.05; ** p<0.01; *** p<0.001; n.s. = not significant.

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

Although viral levels were below the threshold of detection, we wondered if T-cell activation by residual viral replication could cause increased proliferation observed within the CD4$^+$ and CD8$^+$ T-cell subsets in adults after long-term HAART. Therefore, the expression of T-cell activation markers CD38 and HLA-DR was determined on CD4$^+$ and CD8$^+$ T cells from adults. Normalization of the expression of these activation markers was, except on CD4$^+$ T cells for adults with low baseline CD4$^+$ T-cell numbers, already apparent one year after initiation of HAART and remained relatively stable at normal levels during follow-up (Figure 5).

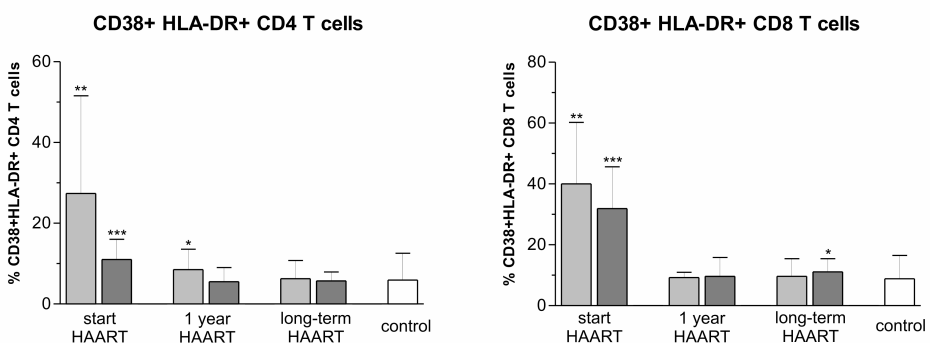

Figure 5. Activation of CD4$^+$ and CD8$^+$ T cells of adults during long-term HAART. Percentages of activated (CD38$^+$HLA-DR$^+$) CD4$^+$ and CD8$^+$ T cells. White bars indicate control values, whereas the light and dark grey bars indicate adults with low and high baseline CD4$^+$ T-cell number, respectively. Data are shown as mean + SD. Statistical significance is determined by the nonparametric Mann-Whitney *U* test: * p<0.05; ** p<0.01; *** p<0.001

Adults with high baseline CD4$^+$ T-cell numbers were an exception and demonstrated elevated expression of activation markers on CD8$^+$ T cells after long-term HAART (p=0.013), although after one year of HAART normalization was observed. Strikingly, no relation between activation and levels of Ki67 expression within 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 residual T-cell proliferation is not related to 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

Our analysis showed that both in children and in adults with complete viral suppression during HAART, full reconstitution of the CD4$^+$ T-cell compartment during long-term HAART was achieved. In agreement with what has been described before, though after 18 months of treatment [38], children with low baseline CD4$^+$ T-cell counts recovered to similar levels of CD4$^+$ T cells as children with higher baseline counts within 1 year of HAART, and from 1 year of therapy onward both groups had no significantly lower CD4$^+$ T-cell count compared to healthy age-matched control values. Furthermore, both the fraction and absolute numbers of naive CD4$^+$ T-cells had normalized in children. Although absolute CD4$^+$ T-cell counts were not significantly lower than healthy controls in adults with low and high baseline CD4$^+$ T-cell count, the difference between adults with a low and high CD4$^+$ T-cell count at treatment initiation was sustained throughout follow-up. In agreement, in adults with high baseline CD4$^+$ T-cell numbers fractions and numbers of naive CD4$^+$ T cells after long-term HAART had normalized, whereas naive CD4$^+$ T-cell numbers after long-term HAART in adults with low baseline CD4$^+$ T-cell numbers lagged behind. It remains to be

determined whether the group with low $CD4^+$ T-cell baselines will eventually reach $CD4^+$ T-cell levels similar to those in the group with higher baselines, or whether this difference reflects natural variation in $CD4^+$ T-cell count. The variation in $CD4^+$ T-cell count is considerable in healthy controls (5^{th} and 95^{th} percentile of 560 and 1490 cells/μl in our data set), and it is conceivable that individuals who start with lower $CD4^+$ T-cell counts before HIV infection, end up with lower $CD4^+$ T-cell nadirs upon disease progression.

The finding that most adults had normalized $CD4^+$ T-cell counts within 7 years on HAART is unexpected. In contrast to the good immune reconstitution found in this study, inadequate long-term T-cell reconstitution has been reported after bone marrow transplantation (BMT) [48-51]. This discrepancy may be explained by inclusion of a subset of patients with poor early grafting [52], or experiencing graft versus host disease [53,54] rather than long-term immune failure caused by accelerated loss of thymic output in these patient groups. Recently, another study showed successful reconstitution 17 years after chemotherapy induced lymphopenia [55].

Next, we addressed the issue to what extent the observed increase in $CD4^+$ T-cell numbers was generated by thymic output or peripheral T-cell proliferation. The source of the newly produced T cells that are formed during reconstitution might have a strong effect on the replicative history, the age, of the T-cell pool. On the one hand, one could envisage that reconstituting a virtually empty T-cell pool with newly produced thymus-derived naive cells might lead to rejuvenation of the T-cell pool. Indeed an overshoot in TREC content during reconstitution of an empty pool has been reported previously [33,34]. On the other hand, peripheral proliferation may go at the expense of TREC content and telomere length, cumulative markers for T-cell replication. The fact that parameters like the fraction and absolute numbers of $CD31^+$ naive T cells, TREC content and telomere length decrease with age suggests that the immune system is not able to keep up without generation of T cells by peripheral proliferation during aging of healthy individuals, let alone during reconstitution.

Surprisingly, our analyses of the different markers showed no evidence for accelerated aging or rejuvenation of the T-cell pool. With respect to TREC content, telomere length and the expression of $CD31^+$ on naive $CD4^+$ T cells, parameters for thymic proximity and replicative history, the T-cell pool had returned to the level of age-matched controls. We have previously used a mathematical model to explain the decline in TREC content during immune activation [19]. If we extend this model and mimic the situation of discontinuing immune activation, indeed TREC content values appeared to increase to normal age-matched values (Tsegaye et al. submitted). Normal fractions of $CD31^+$ naive $CD4^+$ T cells, which are cells most proximal to the thymus [40] (Vrisekoop et al. submitted) and are characterized by a diverse T-cell receptor repertoire [39], suggests that the naive T-cell pool after long-term HAART is as diverse in repertoire as observed in healthy age-matched individuals. Furthermore, increases in absolute numbers of TRECs in adults suggest thymic output to be a source of new naive T cells. Taken together, these data show that $CD4^+$ T-cell recovery most likely is derived from production of naive $CD4^+$ T cells generated by both the thymus and by peripheral T-cell proliferation. Even in adults with $CD4^+$ T-cell counts

below 200 cells/µl, reconstitution did not seem to wear out the T-cell pool, since the TREC content of naive and total $CD4^+$ T cells were normal in almost all patients on long-term HAART. In accordance, telomeres in memory T cells were not shorter compared to age-matched controls in 18 out of 25 adults on HAART. Hence, these data demonstrate an unanticipated capability of the immune system in adults to eventually recover from severe depletion when viremia is properly controlled.

Strikingly, the proliferation marker Ki67 was still elevated in adults even after 7 years of HAART despite mostly normalized activation markers already at 1 year after therapy. Although the percentages of activated cells in our study are not very different from previous studies, it was previously reported that ongoing therapy for 2 to 6 years did not normalize $CD4^+$ and $CD8^+$ T-cell activation markers [13,24,25,27,29,30,34]. This discrepancy is attributable to the values of activation markers found in healthy controls. Whereas other studies used 8 to 59 healthy controls, we used 182 healthy controls tested in parallel as reference values and found median percentages of $CD38^+HLA\text{-}DR^+$ cells of 4% and 7% for $CD4^+$ and $CD8^+$ T cells respectively. The relatively high control values used in comparison with other studies, together with the significant increased level of activated $CD8^+$ T cells after long-term HAART in the group with high baseline $CD4^+$ T-cell count, could suggest that T-cell activation levels are still marginally increased in these HIV-infected adults. On the other hand, the lack of a correlation between T-cell activation markers and the fraction of $Ki67^+$ T cells might suggest that proliferation in late stage HAART may not be merely attributable to increased T-cell activation.

To conclude, whereas children were able to reconstitute well at any stage of lympho-depletion caused by the infection, adults with low baseline $CD4^+$ T-cell count took longer to normalize $CD4^+$ T-cell counts and had not fully normalized naive T-cell fractions. Our data also suggest that in both children and adults, naive T-cells are generated in the thymus and by peripheral division, and that therefore reconstitution of the immune system does not lead to accelerated aging of the T-cell pool.

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Discussion

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Chapter 11

In this thesis T-cell turnover and thymic output are studied in healthy humans, individuals suffering from HIV or non-HIV induced chronic immune activation, and during T-cell reconstitution following HAART. Here these findings are discussed in a broader context.

Chronic immune activation as a cause for T-cell depletion

There is no consensus with regard to the mechanism behind $CD4^+$ T-cell depletion by HIV infection (Chapter 2). Currently the most prevailing ideas are direct HIV-induced cytopathicity, including rapid and profound depletion of $CCR5^+$ $CD4^+$ T cells in the gut of patients during acute infection [1,2], thymic impairment [3-5] and chronic immune activation [6].

Chronic immune activation on its own (in healthy Ethiopians) was shown to be sufficient to induce similar characteristics as observed in HIV-infected individuals, including low $CD4^+$ T-cell counts, increased levels of T-cell activation and proliferation, low fractions of naive $CD4^+$ T cells and a decreased $CD4^+$ and $CD8^+$ T-cell TREC content (Chapter 4) [7-10].

$CD4^+$ T-cell TREC dynamics upon immune activation in Ethiopians and upon HIV infection display many similarities. TREC contents of $CD4^+$ T cells from cord blood of Ethiopian neonates are similar to values of healthy Dutch neonates (Chapter 4). Over HIV seroconversion and during early childhood in healthy Ethiopians immune activation is increased, causing a rapid decline in $CD4^+$ T-cell TREC content followed by a quasi-equilibrium (Chapter 4 and 8). Treatment with HAART reduces immune activation in HIV-infected individuals with a concomitant normalization of absolute total and naive $CD4^+$ T-cell counts as well as $CD4^+$ T-cell TREC content (Chapter 10). Likewise, following migration of Ethiopians to Israel, T-cell activation is reduced and absolute $CD4^+$ T-cell counts and the fraction of naive T cells tend to improve [7].

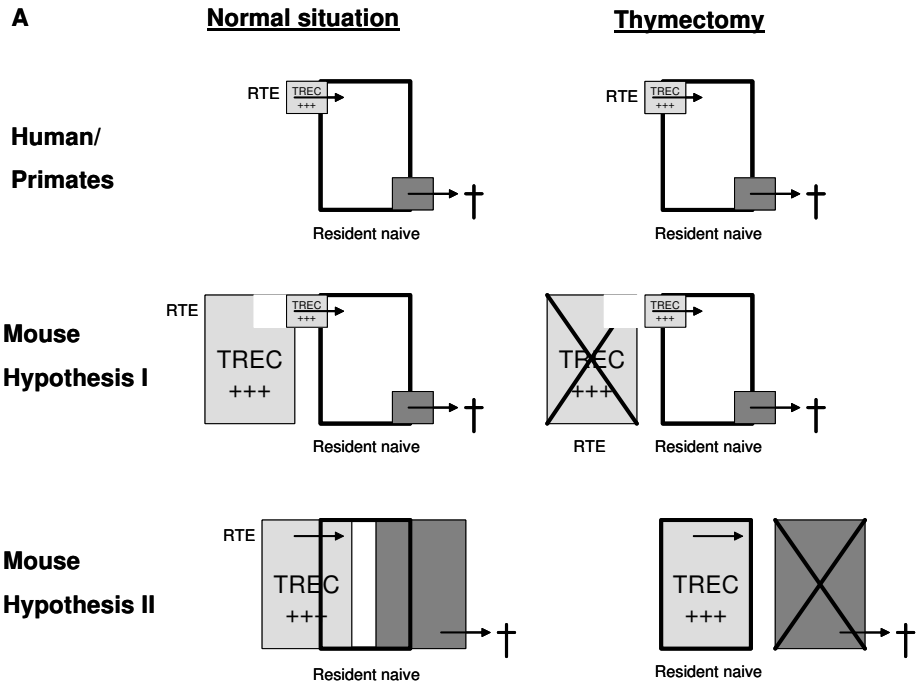
In Chapter 3 we described a small series of unique HIV-infected individuals with low immune activation who have prolonged stable $CD4^+$ T-cell counts despite high viral load. The virus clones of these patients were shown to be highly replicative and induced as much viral pathogenicity in organ cultures as virus isolates from individuals who progressed to AIDS. The absence of HIV disease progression in these patients, like in SIV-infected sooty mangabeys [11], suggests that HIV-induced immune activation is more important than direct HIV cytopathicity in the course of HIV disease progression.

Thymic output in mice and men

It is generally assumed that the dynamics of recent thymic emigrants (RTE) are conserved in evolution, and similar in mice, chickens, humans and primates. The currently most prevailing idea is that RTE in mice and chickens are short-lived, which seems in sheer contrast with the preferential survival of newly produced naive T cells in humans (Chapter 9). Although a short-lived RTE pool could imply rapid death or fast transition of RTE to the resident T-cell pool, at the moment the first implication is favored. The idea of a short-lived,

rapidly dying, RTE pool in mice comes from a combination of interpretations from multiple studies.

The sharp decline in naive T-cell numbers within 3 weeks after thymectomy in mice and the subsequent stabilizing T-cell counts (Chapter 9) are suggestive for a rapid loss of short-lived RTE followed by a slow decline in long-lived resident naive T cells (Figure 1A, hypothesis I). However, based on these data alone, it could equally well be that the RTE themselves are not preferentially lost, but compete with resident naive T cells for homeostatic signals, which will lead to random death of old RTE and resident naive T cells (Figure 1A, hypothesis II). Following TREC dynamics after thymectomy could distinguish between these options. If RTE are preferentially lost, this would lead to a drastic reduction of the TREC content within 3 weeks, since RTE are relatively TREC rich and the left-over resident naive T cells are expected to have a lower TREC content. However, if loss of naive T cells would occur randomly, TREC bearing T cells would be lost at a similar rate as TREC negative T cells, which would not result in such a drastic reduction of the TREC content of the peripheral T-cell pool. The $CD4^+$ T-cell TREC content was found to decline 3.4-fold within 11 weeks after thymectomy in mice [12], suggesting that a lot of TREC rich T cells are preferentially lost in a relatively short time frame, which would be compatible with a preferential loss of RTE. Collectively these data suggest that the RTE pool is completely replaced by new RTE and the old RTE are dying rather than incorporated into the naive T-cell pool (Figure 1A, hypothesis I). However, Broers et al. [13] reported a far less impressive decline of 2.3 fold 28 weeks post-thymectomy in mice, which questions the selective death of TREC rich RTE.



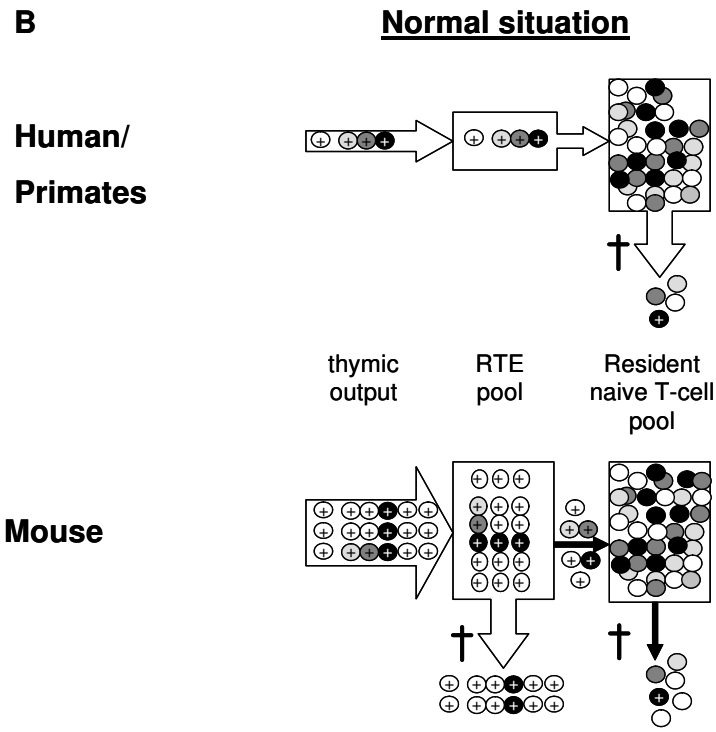


Figure 1 (A) Upper panel: In humans newly produced cells, probably including RTE, have a longer life span than the average naive T-cell in the pool. In mice RTE are being replaced every 3 weeks. However, it is unclear whether they are lost before (middle panel) or after incorporation in the resident naive T-cell pool (lower panel). (B) Abundance of T-cell receptor specificity (designated by different colors) may play a pivotal role in survival, acting on both RTE and resident naive T cells. Because RTE over-represent certain specificities [17] they are preferentially lost. If thymic output is reduced, there will be less competition for homeostatic signals in the periphery, which would increase the life-span of RTE. The + denotes TREC bearing cells, which for simplicity are only RTE in this figure.

Berzins et al. [14] engrafted multiple thymi into adult mice and showed that the increase in peripheral naive T-cell numbers almost equaled the amount of thymic export of the previous 3 weeks. This suggests that the RTE pool is turned over within 3 weeks. Furthermore, the first paper published on thymic engraftment by Berzins et al. [15] showed that when a Ly5.2 thymus was grafted into a Ly5.1 mouse, the Ly5.2 cells emigrating from the thymus were exempt from death for 3 weeks and were subsequently progressively replaced by host Ly5.1 cells that had inhabited the grafted thymus. Between 4 and 8 weeks after thymic engraftment, more peripheral Ly5.2 T cells had been lost than would be expected if Ly5.2 cells would be randomly replaced by new Ly5.1 RTE, indicating that the average life-span of RTE is shorter than that of resident naive T cells (Figure 1A, hypothesis I).

The fact that RTE are short-lived in mice seems to contradict the preferential survival of newly produced cells in humans (Chapter 9). However, these discrepant observations between men and mice may still be reconciled, if we consider that RTE and naive T cells compete for T-cell receptor dependent survival signals. Low clonal abundance in a polyclonal repertoire favors the survival of naive T cells and could therefore explain the occurrence of both long-lived and short-lived naive T cells in the same pool [16]. In other words, if a cell has a rare T-cell receptor specificity it may undergo less competition for homeostatic signals and live relatively long. It is conceivable that the resident naive T-cell pool has already been shaped according to T-cell receptor specificity and that this pool therefore has an advantage in acquiring survival signals compared to the RTE pool. Indeed it has been shown that thymic selection produces a repertoire with marked overrepresentation of a subset of CDR3 sequences and that this post-selection repertoire is noticeably remolded in the periphery [17]. If thymic output is reduced, there will be less competition for homeostatic signals, which would increase the life-span of RTE. The large difference in thymic contribution to the naive T-cell pool of 20 year old human volunteers (Chapter 9) versus 8 weeks old mice [15] could then explain the long life-span of RTE in humans (Figure 1B).

Calculating thymic output

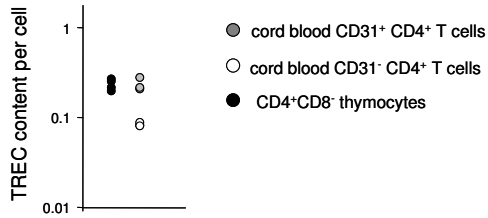
Studies on the influence of thymic impairment on HIV-induced $CD4^+$ T-cell depletion and the contribution of thymic output to the reconstitution of a lymphopenic T-cell pool are hampered by the lack of an unambiguous marker for RTE. The recently proposed use of CD31 as a marker for RTE was found to have similar drawbacks as other naive T-cell markers and TRECs, namely that T-cell division can occur without loss of its expression (Chapter 7).

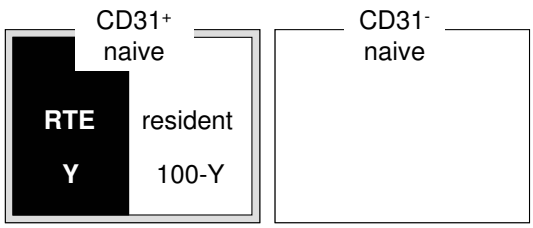
The TREC content in $CD31^+$ cord blood $CD4^+$ T cells and single positive $CD4^+CD8^-$ thymocytes was found to be comparable (Figure 2A and Chapter 7), implying that $CD31^+$ T cells have not divided since they left the thymus. Thus, $CD31^+$ naive $CD4^+$ T cells in new-borns may be considered RTE. Now that we know the TREC content of RTE, the TREC content of $CD31^+$ T cells can be used to estimate the RTE fraction in the naive T-cell pool at any age. Based on the claim that $CD31^-$ naive T cells are derived from $CD31^+$ naive $CD4^+$ T cells [18] we reason that the TREC content of non-RTE "resident" $CD31^+$ naive $CD4^+$ T cells will be at least as high as the TREC content of $CD31^-$ naive $CD4^+$ T cells. At every age, the TREC content measured in $CD31^+$ naive T cells is a composite of the TREC content of non-RTE naive $CD31^+$ T cells (which is assumed to be at least as high as the TREC content of $CD31^-$ naive $CD4^+$ T cells) and the TREC content of RTE. Together, this information provides an upper-estimate for the fraction of RTE in $CD31^+$ naive T cells. Using the percentage of $CD31^+$ naive $CD4^+$ T cells in the total naive T-cell pool, one can then obtain an upper-estimate for the fraction of RTE in the naive T-cell pool (see Figure 2A). This estimated fraction is indeed only an upper-estimate, because non-RTE naive $CD31^+$ $CD4^+$ T cells might in reality have a higher TREC content than $CD31^-$ naive $CD4^+$ T cells. Figure 2B (gray line)

depicts the decline of the upper-estimate of the percentage of RTE in the naive T-cell pool with age.

Similar calculations can be made based on the TREC content of naive $CD4^+$ T cells rather than $CD31^+$ naive $CD4^+$ T cells, by assuming that all TRECs are contained in the RTE pool. This assumption makes the estimates less precise, however, because the TRECs that are actually present in the $CD31^-$ and $CD31^+$ non-RTE resident naive $CD4^+$ T cells are mistakenly attributed to RTE. Since the RTE pool declines with age, the TREC content in non-RTE will increasingly contribute to the TREC content of the naive T-cell pool. As a consequence, with increasing age an increasing proportion of TRECs in the periphery would mistakenly be assigned to be RTE, leading to a higher estimated fraction of RTE in the naive T-cell pool. than obtained when using TREC contents of $CD31^+$ and $CD31^-$ naive T cells (Figure 2B).

A





$$(y)\ \text{(TREC content RTE)} + (100\text{-}y)\ \text{(TREC content CD31}^-\text{ at x yrs)} = \textbf{TREC content CD31}^+\textbf{ at x yrs}$$

$$\text{over } 100$$

y = upper estimate of percentage RTE in CD31+ naive T-cell pool
z = upper estimate of percentage RTE in total naive T-cell pool = y * fraction CD31+ in the naive T-cell pool

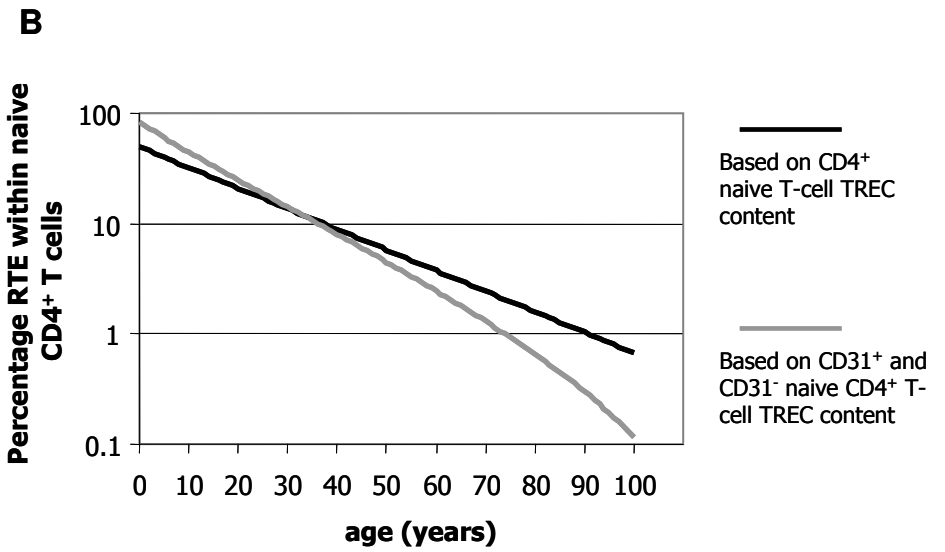


Figure 2 (A) Because $CD31^+$ cord blood cells have a similar TREC content as single positive thymocytes (upper panel), we assume RTE to have a similar TREC content. Furthermore, we assume that non-RTE $CD31^+$ naive $CD4^+$ T cells have a similar TREC contents as $CD31^-$ naive $CD4^+$ T cells. In the lower panel the calculations to attain the percentage RTE within the naive T-cell pool are described. (B) The percentage RTE within naive T cells with age, when calculated as described in (A), is depicted in gray. Black denotes the percentage RTE within naive $CD4^+$ T cells with age calculated by using the TREC content in naive $CD4^+$ T cells, assuming none of the TRECs reside in the non-RTE resident naive T-cell pool.

In Chapter 9 we used the death rate of naive T cells and the size of the naive T-cell population to obtain a median upper estimate of thymic output of 1.7×10^8 T cells per day. Now that we also have an upper estimate for the fraction of RTE in the naive T-cell population, we can obtain an even tighter estimate for daily thymic output (s), because the absolute number of RTE in the body (N) equals the daily input from the source (s) divided by the daily disappearance rate of RTE (d) ($N = s/d$). From 4 of the 5 healthy volunteers in whom we labeled naive T cells we also measured the TREC content in the naive T-cell pool, which can be used to estimate the fraction RTE as mentioned above. Since we know the absolute naive T-cell number per ml blood, we can estimate the absolute number of naive RTE in the body. In Chapter 9 we showed that in humans recently produced naive T cells live longer than the average naive T-cell in the pool. Using the disappearance rate of 1/1500 days found for the average naive $CD4^+$ T cell in the pool, overestimates the disappearance rate of RTE and thereby yields an overestimate for RTE production. In Table 1 the total daily naive T-cell production, the estimated daily production by the thymus and the contribution of peripheral T-cell proliferation are given. Using this method we were able to narrow down the upper estimate for thymic output by 3- to 4-fold.

Table 1. Production of naive CD4$^+$ T cells per day

	A	B	D	E
Total naive CD4$^+$ (*10^7)	11.50	5.36	8.19	13.10
Thymic naive CD4$^+$ (*10^7) Using life span Chapter 9	2.48	1.10	2.67	3.10
Peripheral naive CD4$^+$ (*10^7) Using life span Chapter 9	9.02	4.26	5.52	10.00
Thymic naive CD4$^+$ (*10^7) Using life span Dion et al. [5]	22.05	18.58	56.29	40.94

We concluded in Chapter 9 that newly produced naive CD4$^+$ T cells are long lived. Could it be that the fraction of labeled naive T cells with a long life span was predominantly derived from peripheral T-cell proliferation and that the contribution of short-lived RTE was too low to be picked up by the ^2H_2O labeling technique? The amount of label acquired by naive T cells after 9 weeks of labeling was barely higher than the detection limit of the technique, therefore it seems plausible that the 4 times lower estimates of thymic output desribed above might have been missed. However, if RTE have a shorter life span, as Dion et al. suggested [5], more cells would have to be produced per day to reach the same fraction of RTE in the naive T-cell pool. In the paper by Dion et al. [5] changes in TREC contents occurred within 3 months. If we use a life span of 3 months instead of 1500 days in the $N=s/d$ equation (as described above), the amount of naive CD4$^+$ T cells that would have to be produced per day in order to maintain the estimated number of RTE would by far exceed the number of newly produced naive T cells per day that we estimated using heavy water labeling (Chapter 9 and Table 1). Therefore, in case of a short-lived RTE pool all labeled naive T cells we found in our healthy volunteers should have been RTE and we should have seen a fast decay of labeled naive T cells after label cessation.

Reconstitution

It is of interest to know whether the estimated daily naive T-cell production (Chapter 9) suffices to explain the increases in naive T-cell numbers found after 7 years of HAART in HIV-infected adults (Chapter 10). In Table 2 the minimal, maximal and median estimates of naive T-cell production from the ^2H_2O labeling study were used to determine the time in years that is needed to explain the median net increase of 230 cells per µl blood and the maximum increase of 493 per µl blood during 7 years of HAART in adults. The median naive CD4$^+$ T-cell increase after 7 years of HAART can readily be explained, even by the lowest estimate of naive T-cell production. However, this is a crude estimate since naive T cells may have been recruited into the memory pool and death of naive CD4$^+$ T cells is not taken into account. Thus many more cells might need to be produced in order to fill up the T-cell

pool. Furthermore, the HIV-infected individuals on HAART are older compared to the volunteers of the heavy water labeling studies, and therefore probably have an even lower production of naive T cells. To establish whether the T-cell pool is able to homeostatically respond to lympho-depletion by increasing T-cell production and/or life span, it will be interesting to conduct ^2H_2O labeling studies in HIV-infected individuals on long-term HAART, especially in light of the still elevated levels of $Ki67^+$ expressing T cells found after 7 years of effective HAART.

Table 2. Estimated time (in years) needed to achieve the net increase in naive $CD4^+$ T cells observed after 7 years of HAART in HIV-infected adults

	Time (years) needed to achieve the median increase in abs naive $CD4^+$ of 230 cells/µl	Time (years) needed to achieve the maximum increase in abs naive $CD4^+$ of 493 cells/µl
Using max estimate daily total naive T-cell production	1.20	2.58
Using median estimate daily total naive T-cell production	1.92	4.12
Using min estimate daily total naive T-cell production	7.04	15.07

In this era of HIV, HAART and stem cell transplantation, insight into the dynamics of human T cells is of extreme importance. Although generally considered text-book immunology, in fact extremely little is known about the dynamics and life-span of T cells in healthy humans. While classical studies on T-cell turnover in humans are typically based on data from unnatural situations, *e.g.* T-cell reconstitution after severe T-cell depletion [19], or loss of T cells with chromosome damage [20], we here measured T-cell turnover rates under normal physiological conditions thereby providing better estimates of T-cell turnover in healthy humans than are currently available. Our results are not only incompatible with a substantial short-lived RTE population in human adults, but to the contrary suggest that the few naive T cells that are newly produced are preferentially incorporated in the peripheral T-cell pool.

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Summary

This thesis focuses on T-cell dynamics in healthy and both treated and untreated HIV-infected individuals. The first part considers the mechanisms of $CD4^+$ T-cell depletion during HIV infection and the role of immune activation in particular. In the second part different surrogate markers for thymic output are addressed, the life spans of naive and memory $CD4^+$ and $CD8^+$ T cells are assessed by heavy water labeling and the role of thymic output and peripheral proliferation on T-cell reconstitution during highly active antiretroviral treatment (HAART) in HIV-infected adults and children is investigated.

In Chapter 2 an overview is given on the different mechanisms that have been postulated as the cause for HIV-induced $CD4^+$ T-cell depletion. Two of these mechanisms, namely direct HIV-induced cytopathicity and chronic immune activation, were addressed in three exceptional long term non progressors with high viral load in chapter 3. Virus isolates from these patients were highly pathogenic in organ cultures implying that direct virus kill is insufficient to fully explain $CD4^+$ T-cell depletion. Despite high viral load, chronic immune activation was low in these patients, indicating that the lack of immune activation might be responsible for the absence of disease progression.

If chronic immune activation indeed is the main cause for $CD4^+$ T-cell depletion in HIV-infected individuals, other situations of chronic immune activation should induce similar characteristics as found in HIV infection. To investigate the effects of non-HIV related chronic immune activation, we studied healthy Ethiopians who are known to have increased exposure to environmental pathogens in chapter 4. While immune characteristics of Ethiopian and Dutch neonates are similar, and therefore differences are not genetic, the $CD4^+$ T-cell TREC content and fraction naive $CD4^+$ T cells decrease at very early childhood in Ethiopia, reaching a new equilibrium thereafter.

Assuming that the higher baseline activation in Ethiopians is added up to the immune activation upon HIV infection, the reported slower $CD4^+$ T-cell decline in Ethiopians was unexpected and argued against chronic immune activation as a major cause for the $CD4^+$ T-cell decline in HIV infection. However, in chapter 5 we surprisingly found that when Dutch and Ethiopian HIV-infected patients were matched for $CD4^+$ T-cell count, the percentages proliferating $Ki67^+$ cells within the $CD4^+$ and $CD8^+$ T-cell subsets were lower in Ethiopians. Thus, the slower $CD4^+$ T-cell decline in HIV^+ Ethiopians could again be explained by lower levels of immune activation.

Residual T-cell activation and proliferation are also related to reduced $CD4^+$ T-cell gains during HAART. Therefore, inhibiting T-cell proliferation by mycophenolate mofetil (MMF) during HAART might benefit $CD4^+$ T-cell reconstitution. On the other hand, proliferation might also contribute to the recovery of $CD4^+$ T cells upon treatment. To study the effects of inhibiting T-cell proliferation on lymphocyte reconstitution, we longitudinally followed 6 patients treated with MMF in combination with HAART, as compared to 8 patients treated with HAART alone in Chapter 6. Inhibiting proliferation during HAART by MMF treatment for 1 year did not lead to impaired T-cell reconstitution, implicating that peripheral proliferation is not essential for short-term immune reconstitution.

Kimmig et al. have proposed CD31 to be a new unambiguous marker for thymic output. This paper has attracted much attention since studies on the role of thymic output during aging and in situations of T-cell depletion (i.e. due to bone marrow transplantation, HIV infection or chemotherapy) are hampered by the lack of a marker for recent thymic emigrants. In Chapter 7, we first established whether $CD31^+$ naive $CD8^+$ T cells similarly reflect a T-cell pool that is more proximal to the thymus. Indeed, sorted $CD31^+$ naive $CD8^+$ T cells had a higher TREC content than sorted $CD31^-$ naive $CD8^+$ T cells. Although less pronounced than in naive $CD4^+$ T cells, the percentage of $CD31^+$ naive $CD8^+$ T cells similarly shows an age-dependent decline. Next, we investigated in further detail the potential of CD31 as a marker for thymic output. We found, that the TREC content of sorted $CD31^+$ naive $CD4^+$ T-cells declined with age, indicating that $CD31^+$ naive $CD4^+$ T-cells are at least in part generated by peripheral proliferation. Thus, our data suggest that peripheral T-cell proliferation is an important source of $CD31^+$ T cells, and that CD31 cannot be used as a reliable marker for thymic output.

However, CD31 is still useful to identify the naive T-cell subset that is most proximal to the thymus and contains most TCR diversity. Therefore we decided to look at the dynamics of $CD31^+$ naive T cells during HIV infection. Since chronic immune activation is thought to cause accelerated aging of the immune system, one would expect to find a decreased fraction of $CD31^+$ T cells within the naive $CD4^+$ T-cell pool during HIV infection. However, although absolute numbers of $CD31^+$ naive $CD4^+$ T cells declined during HIV progression, the fraction $CD31^+$ cells within the naive T-cell pool was not significantly different from healthy controls. Taken together, this chapter illustrates the limitations and possibilities of the use of CD31 as a thymic proximity marker.

Although measuring TREC content can give valuable information on T-cell proliferation and thymic output, studies on TREC content during HIV infection have yielded contradictory results. Increased, decreased and equal TREC content within $CD4^+$ T cells of HIV^+ individuals have been reported. All these studies were performed cross-sectionally and the discrepancies between the studies may be due to a selection bias and or a large interindividual variation. The few longitudinal studies available measured TREC content in PBMC, which might not be representative for $CD4^+$ T-cell TREC dynamics. In Chapter 8 we performed longitudinal TREC analysis pre-and post-seroconversion in longitudinal samples from the Amsterdam Cohort Studies to shed more light on TREC dynamics during HIV infection. During the first year of HIV infection, the absolute number of TRECs is at least halved, and exceeds the loss of naive $CD4^+$ T cells, resulting in a decline in TREC content over seroconversion. A parallel loss of absolute $CD4^+$ TREC numbers and naive and effector/memory $CD4^+$ T cells resulted in stable $CD4^+$ T-cell TREC contents during chronic infection. These data are interpreted using mathematical models, which show that the transfer of a large fraction of naive $CD4^+$ T cells to the effector/memory compartment during acute HIV infection is sufficient to explain the biphasic dynamics of TREC numbers and content. Ultimately, upon the development of AIDS this equilibrium is disturbed and the TREC content within $CD4^+$ T cells decreases further. This is the first study where a

longitudinal analysis of TREC content is performed and it helps to resolve previous apparently conflicting studies.

In mice it has been shown that RTE form a substantial pool of short-lived naive T cells. In humans however, the lifespan and number of RTE are unknown and the existence of an independent RTE pool is still controversial. In Chapter 9 the recently developed technology of heavy water labeling was used to estimate T-cell turnover rates in healthy men and mice. The use of both the up- and down-labeling phase together with novel in-depth mathematical modeling permitted us to very accurately estimate the life span of not only the average T-cell population but especially of the recently produced naive T-cell population in healthy humans. Our data confirm the existence of a large short-lived RTE pool in mice. In humans, however, we show that recently produced naive T cells are long-lived and even preferentially incorporate into the naive T-cell pool. Our data thereby provide the first conclusive experimental evidence that in healthy humans with a full immune system there is no substantial population of short-lived RTE.

HAART treated HIV-infected adults and children with adequate suppression of virus replication and long-term follow up were studied in Chapter 10. HIV-infected children and adults were capable of fully reconstituting their $CD4^+$ T-cell compartment to age-matched values during long term HAART. Although children could recover to normal levels within 1 year after treatment even when HAART was initiated at an extremely lymphopenic stage, adults with a $CD4^+$ T-cell nadir below 200 cells/μl persistently sustained lower $CD4^+$ T-cell counts than adults with high pre-therapy $CD4^+$ T-cell counts throughout follow-up, but nevertheless normalized $CD4^+$ T-cell counts after 7 years of HAART. Absolute numbers of naive $CD4^+$ T cells normalized in all children and in adults with high baseline $CD4^+$ T-cell counts, whereas naive $CD4^+$ T-cell counts in adults with low $CD4^+$ T-cell nadirs lagged behind. We more thoroughly addressed whether increases in $CD4^+$ T-cell numbers were generated by proliferation or thymic output and whether the extensive regeneration would result in accelerated aging of the T-cell pool. Strikingly, the proliferation marker Ki67 had normalized in children, but remained elevated in adults despite relatively low levels of activation. Even in adults with $CD4^+$ T-cell counts below 200 cells/μl, reconstitution did not seem to wear out the T-cell pool, since the TREC content in naive and total $CD4^+$ T cells and telomere length in T lymphocytes were normal in most patients on long term HAART. Thus the reconstituted peripheral T-cell pool of adults and children did not seem to have more 'proliferative history' as compared to age-matched controls.

Finally, the studies in this thesis are discussed in a broader context in Chapter 11. Chronic immune activation during HIV infection is compared to non-HIV related immune activation, demonstrating a considerable overlap. Furthermore, differences and similarities between recent thymic emigrants in humans, monkeys, chicken and mice are addressed. The estimates of naive T-cell production are related to the increases in naive $CD4^+$ T-cell numbers found during HAART in HIV-infected adults and studies on CD31 as a marker for thymic output are linked to heavy water labelling data to estimate an even lower maximum thymic output in healthy humans.

Samenvatting voor niet-ingewijden

Het immuun systeem

Het immuun systeem is erg complex en vele celtypes dragen bij aan de bescherming tegen virussen en bacteriën. Voor het begrip van dit proefschrift zijn vooral de T cellen van belang. Deze cellen worden geproduceerd in de thymus en T cellen die net uit de thymus komen worden logischerwijs recente thymus emigranten genoemd. De T cellen zijn onder te verdelen in $CD4^+$ 'helper' T cellen en $CD8^+$ 'killer' T cellen. Wanneer een T cel in het bloed of de lymf knopen nog nooit zijn ziekteverwekker is tegengekomen wordt deze naïef genoemd. Na herkenning van de ziekteverwekker gaan de naïeve T cellen aanzienlijk delen en krijgen ze bepaalde eigenschappen om de strijd aan te kunnen gaan (effector T cellen). Vanwege de destructieve eigenschappen en om te zorgen dat T cellen die andere ziekteverwekkers herkennen niet worden ondergesneeuwd, krimpt deze geëxpandeerde pool na enige tijd en blijven de 'memory' $CD4^+$ en $CD8^+$ T cellen over. Deze cellen vormen het geheugen van het immuun systeem en zorgen dat de afweer paraat staat als de ziekteverwekker nogmaals voorbij komt.

HIV infectie

Het humaan immunodeficiënte virus (HIV) werd begin jaren 80 ontdekt. Infectie met HIV wordt gekarakteriseerd door een continue aanwezigheid van het virus en een afname van $CD4^+$ T cellen. Dit verlies van $CD4^+$ T cellen leidt uiteindelijk tot vatbaarheid voor infecties met virussen, bacteriën en schimmels die in de gezonde situatie makkelijk controleerbaar zijn. Dit stadium wordt AIDS (acquired immunodeficiency syndrome) genoemd en heeft de dood tot gevolg. Aan het eind van 2006, werd het aantal met HIV besmette personen geschat op 39,5 miljoen en in datzelfde jaar stierven 2,9 miljoen mensen aan AIDS (UNAIDS report 2006).

Sinds 1996 is er, althans voor westerse HIV-geïnfecteerde individuen, antivirale therapie beschikbaar. Hoewel deze therapie de hoeveelheid virus enorm doet verminderen is de therapie niet in staat het virus volledig uit te roeien.

Omdat HIV de $CD4^+$ T cellen infecteert leek het verlies van deze cellen tijdens HIV infectie gemakkelijk te verklaren. Echter, de meeste $CD4^+$ T cellen die dood gaan tijdens HIV infectie bleken niet geïnfecteerd met HIV. Hoewel het $CD4^+$ T-cel verlies het kenmerk is van HIV infectie, blijft het mechanisme van deze depletie controversieel. De meest voorkomende hypotheses zijn directe schade door HIV infectie, verminderde T-cel productie door de thymus, en chronische immuun activatie.

Dit proefschrift

In het eerste deel van dit proefschrift is het belang onderzocht van de verschillende mechanismen die zijn geopperd om $CD4^+$ T-cel depletie te verklaren. Twee van deze mechanismen, namelijk directe HIV-geïnduceerde schade en chronische immuun activatie, hebben we bestudeerd in 3 zeldzame HIV-geïnfecteerde individuen die een hoge virale load hadden maar weinig $CD4^+$ T-cel depletie. De directe schade van het virus van deze

patiënten op $CD4^+$ T cellen en thymocyten was vergelijkbaar met andere patiënten, maar opvallend genoeg was de immuun activatie veel lager dan verwacht. Oftewel veel virusdeeltjes alleen, zonder bijkomende chronische immuun activatie, was onvoldoende om $CD4^+$ T-cel verlies te induceren.

Als chronische immuun activatie de hoofdoorzaak is van de $CD4^+$ T-cel depletie zou je verwachten dat andere situaties van chronische immuun activatie dezelfde karakteristieken als HIV infectie teweeg zouden moeten brengen. Dit hebben we bestudeerd in gezonde Ethiopiërs. Zij staan bekend om chronische activatie van hun immuun systeem, bijvoorbeeld gerelateerd aan infectie met parasieten. Hoewel het immuun systeem van pasgeborenen in Ethiopië en Nederland vergelijkbaar was, was het immuun systeem van Ethiopische kinderen en volwassenen in vele opzichten verstoord zoals we dat bij HIV-geïnfecteerde patiënten ook zien.

Als je er vanuit gaat dat in een HIV-geïnfecteerde Ethiopiër deze hoge achtergrond immuun activatie nog opgeteld wordt bij de immuun activatie door HIV, zou je verwachten dat HIV-geïnfecteerde Ethiopiërs nog sneller hun $CD4^+$ T cellen zouden verliezen dan westerse individuen met HIV. Het omgekeerde is echter eerder gerapporteerd, westerse HIV-geïnfecteerde individuen verliezen hun $CD4^+$ T cellen sneller dan HIV-geïnfecteerde Ethiopiërs. Dit leek tegen de immuun activatie hypothese te pleiten, maar toen we de immuun activatie in HIV-geïnfecteerde Ethiopiërs bestudeerden bleek onverwachts dat HIV minder immuun activatie in Ethiopiërs induceerde dan in Nederlanders en dat deze lagere immuun activatie dus wederom gerelateerd was aan een minder snel $CD4^+$ T-cel verlies.

Onze hypothese is dat de continue activatie, expansie en dood van $CD4^+$ T cellen langzaam tot uitdunning van de $CD4^+$ T-cel pool leidt, omdat het overmatige gebruik van $CD4^+$ T cellen niet kan worden gecompenseerd door meer productie vanuit de thymus. Het afremmen van de chronische immuun activatie door bijvoorbeeld T-cel deling te verhinderen zou theoretisch een manier zijn om de HIV-geïnduceerde $CD4^+$ T-cel depletie tegen te gaan. Hoewel immuun activatie tijdens HIV de voornaamste oorzaak lijkt van het $CD4^+$ T-cel verlies, kan het echter ook zo zijn dat het remmen van T-cel deling tevens nieuwe T-cel productie remt en zo een nog groter $CD4^+$ T cel verlies veroorzaakt. In een pilot studie hebben we de celdeling geremd met behulp van MMF tijdens start van antivirale therapie. MMF had geen negatief effect op de toename van $CD4^+$ T cellen tijdens therapie en zal in de toekomst potentieel gebruikt kunnen worden om de HIV-geïnduceerde $CD4^+$ T-cel depletie tegen te gaan in onbehandelde HIV-geïnfecteerde individuen en zo wellicht de noodzaak voor antivirale therapie uit te stellen.

In het tweede deel van dit proefschrift werd CD31, een potentiële marker voor recente thymus emigranten (RTE), bestudeerd. Een marker voor RTE, is essentieel om de rol van de thymus tijdens het ouder worden en tijdens HIV infectie te bestuderen. Hoewel naïeve $CD4^+$ T cellen die CD31 tot expressie brengen relatief jong zijn, tonen we aan dat ze ook geproduceerd worden door deling buiten de thymus. Met andere woorden, het aantal naïeve T cellen dat CD31 tot expressie brengt bleek geen maat voor de productie van de thymus.

De productie en levensduur van RTE en naïeve en memory $CD4^+$ en $CD8^+$ T cellen van mensen was nog onbekend. Deze informatie is bijvoorbeeld van belang als je wilt weten hoe snel de $CD4^+$ T-cel pool weer kan herstellen tijdens de behandeling van HIV. Met behulp van zwaar water, dat in het DNA wordt ingebouwd van nieuw geproduceerde cellen, hebben we de productie en levensduur van de verschillende celtypes bepaald in jong volwassen mensen. In tegenstelling tot muizen zijn recent geproduceerde naïeve T cellen in de mens langlevend. De gemiddelde levensduur van naïeve $CD4^+$ en $CD8^+$ T cellen is 4.2 en 6.6 jaar en van memory $CD4^+$ en $CD8^+$ T cellen 0.4 en 0.7 jaar.

Sinds de introductie van therapieën tegen HIV, hebben veel studies het herstel van de $CD4^+$ T cellen gevolgd. Drie tot vijf jaar na therapie was dit $CD4^+$ T-cel aantal nog steeds niet genormaliseerd, wat de vraag opriep of HIV-geïnfecteerde mensen überhaupt nog in staat waren tot volledig herstel. T cellen kunnen door de thymus of door deling geproduceerd worden. Omdat deling alleen maar 'meer van hetzelfde' geeft en bovendien veroudering van de T cel pool tot gevolg heeft, wordt nieuwe productie in de thymus als kwalitatief beter gezien. In dit proefschrift wordt het $CD4^+$ T-cel herstel bestudeerd na 7 jaar therapie in zowel kinderen als volwassenen. Waarschijnlijk omdat de thymus productie van kinderen hoger is dan volwassenen herstelden de $CD4^+$ T-cel aantallen van kinderen met HIV al binnen 1 jaar therapie. Geheel tegen onze verwachting waren zelfs volwassenen in staat om normale $CD4^+$ T-cel aantallen te bereiken na 7 jaar therapie. Zowel thymus productie als celdeling speelden een rol bij de productie van nieuwe T cellen. Het herstel had zelfs in volwassenen geen veroudering van de T-cel pool tot gevolg.

Samenvattend zijn de conclusies van dit proefschrift dat chronische immuun activatie een hoofdrol speelt in het $CD4^+$ T cel verlies tijdens HIV infectie. Andere belangrijke bevindingen zijn de gemiddelde levensduur van naïeve $CD4^+$ en $CD8^+$ T cellen van 4.2 en 6.6 jaar en van memory $CD4^+$ en $CD8^+$ T cellen van 0.4 en 0.7 jaar en het feit dat recent geproduceerde naïeve T cellen in jong volwassen mensen langlevend zijn en preferentieel in de T-cel pool worden geïncorporeerd. Tot slot hebben we gevonden dat het immuun systeem na T-cel depletie de capaciteit heeft te herstellen zonder veroudering van de T-cel pool tot gevolg te hebben.

Curriculum vitae

Nienke Vrisekoop werd op 2 juli 1979 geboren te Zaanstad. In 1997 behaalde zij het VWO diploma aan het Saenredam College te Zaanstad, waarna zij begon aan haar studie medische biologie aan de Vrije Universiteit te Amsterdam. Als onderdeel van haar studie liep zij onderzoeksstages op de afdeling Geneeskundige Oncologie aan bovengenoemde universiteit en de afdeling Klinische Viro-Immunologie van Sanquin Research. Na het behalen van het doctoraal examen in augustus 2001, trad zij in dienst bij de afdeling Klinische Viro-Immunologie van Sanquin Research als onderzoeker in opleiding. De resultaten van dit onderzoek, uitgevoerd onder begeleiding van prof. dr. F. Miedema, dr. K Tesselaar en dr. J.A.M. Borghans, zijn samengevat in dit proefschrift. Vanaf 2007 zal zij werkzaam zijn bij de Lymphocyte Biology Section, Laboratory of Immunology at the National Institutes of Health bij dr. R.N. Germain, alwaar zij als post-doctoral fellow het immuun systeem zal bestuderen met behulp van imaging technieken in muizen.

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Dankwoord

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