# HIV and the immune system during highly active antiretroviral therapy

J.W.T. Cohen Stuart

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# HIV AND THE IMMUNE SYSTEM DURING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

## HIV en het afweersysteem tijdens krachtige antiretrovirale therapie

**(met een samenvatting in het Nederlands)**

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**JAMES WILLEM THEODOOR COHEN STUART**
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Promotor Prof. Dr. I.M. Hoepelman[1]

Co-promotores Dr. J.C.C. Borleffs[1]
Dr. C.A.B. Boucher[2]

[1] Afdeling Interne Geneeskunde, onderafdeling Infectieziekten & AIDS, Universitair Medisch Centrum Utrecht
[2] Laboratorium voor Medische Microbiologie, Eijkman-Winkler Instituut, Universitair Medisch Centrum Utrecht

Beoordelingscommissie Prof. Dr. H.C. Clevers
Prof. Dr. J.L.L. Kimpen
Prof. Dr. J.M.A. Lange
Prof. Dr. J. Verhoef
Dr. H. Schuitemaker
Prof. Dr. B.J.M. Zeegers

*La vie est un sourire errant,*
*Miracle d'aimer ce qui meurt.*

Albert Camus

*Aan de liefde*

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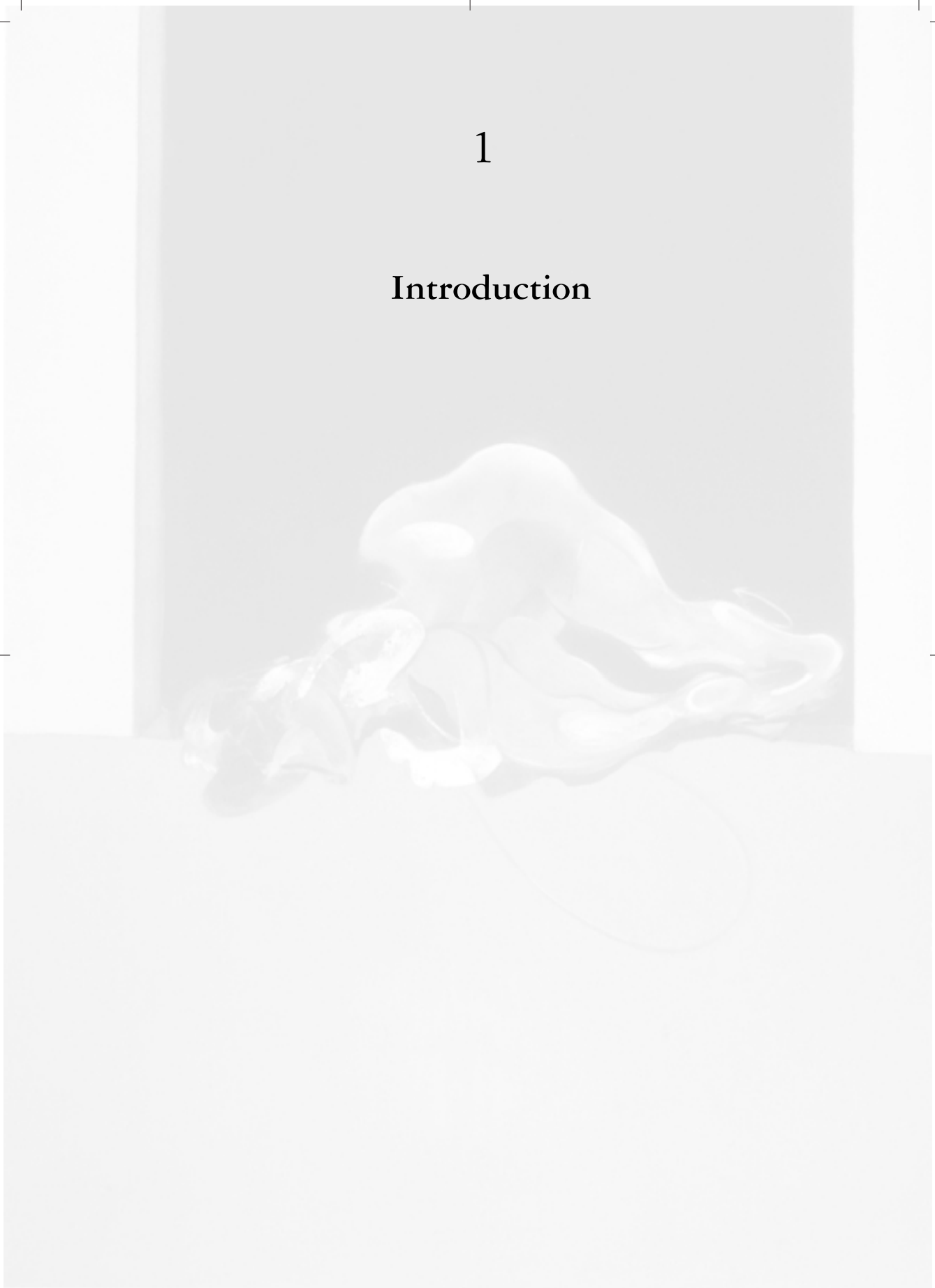
# 1

# Introduction



## 1.1 General Introduction

The human immunodeficiency viruses HIV-1 and HIV-2 are the causative agents of the acquired immunodeficiency syndrome (AIDS). HIV-1 and HIV-2 are retroviruses and members of the family of lentiviruses. The hallmark of HIV infection is a reduction of the number of $CD4^+$ T lymphocytes, resulting in a progressive loss of cellular immunity, the development of AIDS, and ultimately death. The three major transmission routes of HIV are by sexual contact, by blood or blood products, or vertically through maternal-fetal transmission. As of now, in June 2000, it is estimated that 34.3 million individuals worldwide are infected with HIV (1). In 1999, 2.8 million people died from AIDS and the infection rate was 5.4 million people per year (1). More than 70% of the people with HIV and AIDS live in developing countries.

## 1.2 Clinical Manifestations Of HIV Infection

The course of an HIV infection can be divided into three stages: the acute stage, the clinical latency stage and the clinical manifest or AIDS stage. Primary or acute HIV infection is defined as the period from initial infection to complete seroconversion. The incubation period of primary HIV infection is around 4 weeks. Primary HIV infection is symptomatic in up to 70% of HIV infected individuals and is associated with fever, arthralgia, adenopathy, myalgia, truncal (and facial) maculopapular rash and neurologic symptoms, including neuritis, myelopathy and aseptic meningo-encephalitis (2-5). Patients with symptomatic primary infection have a more rapid progression to AIDS than asymptomatic seroconverters (6,7).

Primary HIV infection is followed by an asymptomatic carrier state of variable duration with a median of 10 years, after which most patients develop AIDS (8-12). The asymptomatic phase of HIV infection is frequently characterized by a persistent generalized lymphadenopathy (PGL) (40,41,45).

**Table 1.** AIDS defining illnesses (revised CDC classification 1993)

| |
|---|
| CD4 count < 200 cells/mm$^{3*}$ |
| Candidiasis: esophageal, trachea, bronchi |
| Coccidioidomycosis, extrapulmonary |
| Cryptococcosis, extrapulmonary |
| Cervical cancer |
| Cryptosporidiosis, chronic intestinal (> 1 month) |
| CMV retinitis, or CMV in other than liver, spleen, lymph nodes |
| HIV encephalopathy |
| Herpes Simplex with mucocutaneous ulcer, bronchitis, pneumonia |
| Histoplasmosis: disseminated, extrapulmonary |
| Isosporiasis, chronic, > 1 month |
| Kaposi's sarcoma |
| Lymphoma: Burkitt's, immunoblastic, primary in brain |
| *M. avium* or *M.Kansassi*i, extrapulmonary |
| *M. tuberculosis*, pulmonary or extrapulmonary |
| *Pneumocystis carini*i pneumonia |
| Pneumonia, bacterial, recurrent (2 or more episodes in 1 year) |
| Progressive multifocal leukoencephalopathy |
| *Salmonella* bacteremia, recurrent |
| Toxoplasmosis, cerebral |
| Wasting syndrome due to HIV |

*Not an AIDS defining criterium in the Netherlands.

The AIDS diagnosis is defined by the occurrence of one or more opportunistic infections and/or development of opportunistic malignancies (Table 1). In addition, several organs may be directly affected by the HIV virus including the gastro-intestinal tract (13-15), bone marrow (16-18) and the central and peripheral nervous system (19,20) resulting in e.g. wasting, thrombocytopenia, AIDS dementia, and neuropathy. Without potent antiretroviral treatment, the average survival time after AIDS diagnosis was 2 years (21).

## 1.3 Pathogenesis Of HIV Infection

Primary HIV infection is characterized by high plasma HIV RNA levels, high titers of HIV infected PBMC's and transient depletion of $CD4^+$ T lymphocytes from the blood (Figure 1). During this stage, HIV is disseminated throughout the body and virus is trapped on the protrusions of follicular dendritic cells in the lymphoid tissue (22-27).
Following the acute stage of HIV infection, plasma HIV RNA levels decrease and the CD4 count increases, although it remains lower than in healthy individuals (Figure 1). During the asymptomatic phase, a gradual decline of $CD4^+$ T lymphocytes is paralleled by a gradual increase of plasma HIV RNA levels (28-39). On histological examination, lymph nodes of asymptomatic HIV carriers with PGL show follicular hyperplasia with endothelial cell proliferation and HIV is found in large amounts on the follicular dendritic cells (40-45).
Initially it was believed that during the asymptomatic or clinically latent phase the amount of viral replication would be very low and the amount of virus present in the body minimal. It has been shown over the last couple of years that this concept was seriously flawed. By inhibiting HIV

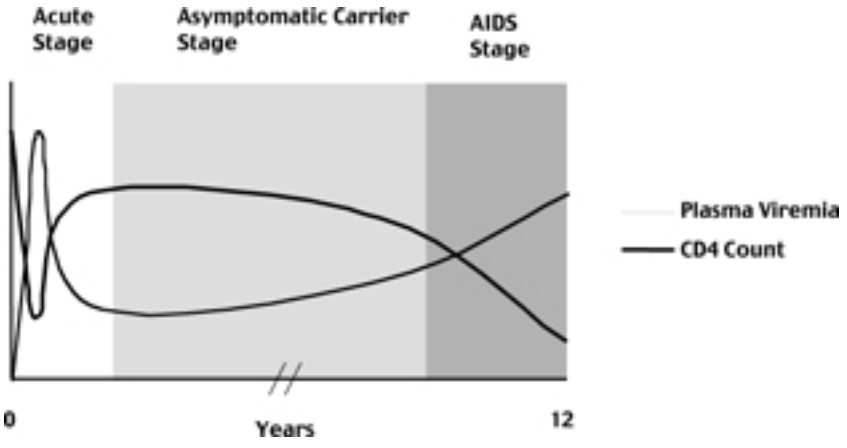



**Figure 1.** *Pathogenesis of HIV infection*

replication with potent antiretroviral drugs and by measuring the decline in the amount of HIV RNA in plasma, the magnitude of viral replication before start of therapy was estimated (46,47). Using a mathematical model, it was estimated that the average lifespan of free HIV virions in plasma was 0.3 days. This corresponds to a half-life (t½) of ~ 6 hours. The average lifespan of productively infected cells was calculated to be 2.2 days (t½ ~ 1.6 days). It was estimated that approximately $10^{10}$ virions are produced and destroyed each day (48). Thus, the asymptomatic phase is a virologically extremely dynamic process.
Clinical progression towards AIDS is reflected by a rapid decrease of $CD4^+$ lymphocytes, increasing plasma HIV RNA levels (Figure 1)(38,39) and deterioration of the lymph node architecture, from follicular hyperplasia to follicular involution and follicular depletion (43).

## 1.4 Mechanisms Underlying $CD4^+$ T-Lymphocyte Depletion

The exact mechanism causing $CD4^+$ T-cell depletion in HIV infection has not yet been unraveled. The decline of $CD4^+$ T lymphocytes during HIV infection is due to an imbalance between production and destruction, i.e. due to increased destruction and/or a reduced regeneration capacity. Several mechanisms of $CD4^+$ T-cell destruction have been proposed (reviewed in 49). First, HIV-infected $CD4^+$ T-cells could be destroyed due to the cythopathic effect of the virus, analogous to observations of HIV killing of $CD4^+$ T cells in vitro. Other proposed mechanisms include dysregulation of cytokine production, auto-immunity, apoptosis due to non specific activation and destruction of infected $CD4^+$ T cells by cytotoxic T-lymphocytes (CTL's). It has been hypothesized that CTL's may also kill uninfected $CD4^+$ T cells (innocent bystander theory) (49).
Evidence has been provided that T-cell renewal may be compromised in HIV infected individuals. First, it was shown that $CD34^+$ hematopoietic progenitor cells are susceptible to HIV infection (50), and have impaired survival and clonogenic capacity during HIV infection (51,52). Second, it was demonstrated that the thymic tissue is affected in individuals with AIDS (53). In studies with SCID-hu mice it was shown that HIV infection

results in depletion of $CD4^+/CD8^+$ thymocytes and reduction of the number of $CD4^+$ T-lymphocytes from the thymic implants (54-46). Furthermore, measuring T-cell receptor excised circles (TREC's) to identify recent thymic emigrants showed that the number of TREC positive T cells is decreased as compared to HIV negative individuals, which was taken as evidence for a reduced export of new T cells from the thymus in HIV infection. Finally, it has been proposed that infection of naive $CD4^+$ T cells by syncytium-inducing HIV variants, via the CXCR4 co-receptor, may interfere with $CD4^+$ T-cell production (166).

It has also been hypothesized, that infection with HIV causes an increased daily production and destruction rate, resulting in the exhaustion of the capacity to regenerate new $CD4^+$ T cells. This hypothesis was based on two studies investigating the repopulation of $CD4^+$ T cells in blood following initiation of potent antiretroviral therapy (46,47). Assuming that the rapid increase of $CD4^+$ T cells during therapy reflects the pretreatment rate of $CD4^+$ T-cell production, it was estimated that the turnover $CD4^+$ T cells is around $2 \times 10^9$ cells/day. However, it has been suggested by others that the initial rise of $CD4^+$ T lymphocytes in the blood after start of antiretroviral therapy could also be due to redistribution of $CD4^+$ T cells from lymphoid tissue to peripheral blood, rather than by de novo production of cells (57).

In addition, several findings argue against a substantial increase in the turnover of T cells. First, by determining the fraction of dividing cells via the expression of the nuclear antigen Ki67 (58), it was shown that T-cell proliferation rate is increased maximally two- to three fold in the $CD4^+$ population, and 6-7 fold in the CD8 population (59-62). This limited increase in the division rate is consistent with studies measuring the replicative history of T cells by the average telomere lengths (63,64). Secondly, using deuterated glucose to label DNA in vivo, showed that the turnover of $CD4^+$ and $CD8^+$ T cells in HIV infected patients is ~3 times higher than that of healthy individuals (65). A 2 to 6 fold increased turnover of $CD4^+$ lymphocytes has also been observed in SIV infected macaques using BrdU to label DNA in vivo (66).

Thus, since T-cell turnover in HIV infection is only slightly increased (2 to 6 fold), it seems that a limitation in the capacity to produce $CD4^+$ T cells plays an important role in the $CD4^+$ T cell depletion during HIV

infections, rather than a high turnover of $CD4^+$ T cells and exhaustion of the immune system.

## 1.5 T-Cell Abnormalities In HIV Infection

The most prominent feature of HIV infection is the depletion and dysfunction of $CD4^+$ T lymphocytes. In addition, HIV causes numerous other disturbances of both the cellular and the humoral immune system. This thesis focuses on the HIV induced changes of the T-cell population and the restoration of the T-cell population during potent antiretroviral therapy.

### 1.5.1. The T-lymphocyte Population

Based on phenotypic and functional properties, the T-cell population can be divided in two subsets, helper cells and cytotoxic/suppressor cells. The helper T cells express CD4, recognize antigen together with major histocompatibiltiy complex (MHC) II and play a crucial role in initiating and orchestrating the specific immune response, such as activation of B cells, helping $CD8^+$ cytotoxic effector cells and activation of macrophages. The cytotoxic/suppressor T cells express CD8, recognize antigen together with MHC I, and play a role in host defense by cytokine release, lysing of target cells or suppressing immune responses by eliminating antigen presenting cells (APC's).

The $CD4^+$ and $CD8^+$ T-cell populations can again be subdivided in naive and memory cells. Naive cells have not yet encountered their specific antigen. To distinguish naive T cells from memory T cells, the high (RA) and low (R0) molecular weight isoform of the CD45 cell surface antigen can be used.

Naïve T cells express CD45RA, memory T cells express CD45R0. However, because memory cells may revert from the CD45R0 to the CD45RA phenotype during cell differentiation (67,68), additional markers are used to identify naive and memory cells, such as CD62L and CD27. T cells co-expressing CD45RA and CD27 can be considered truly naive T cells (or T cells co-expressing CD45RA and CD62L). T cells with

the $CD45R0^+$ and the $CD45RA^+/CD27^-$ phenotype (or $CD45RA^+/CD62L^-$) are considered memory/effector cells (69-71).

**1.5.2 Kinetics Of T-Cell Subsets In HIV Infection**

Infection with HIV causes progressive depletion of both naive and memory $CD4^+$ T cells. The CD8 count of HIV infected patients is usually increased until shortly before the onset of AIDS. This increase is due to expansion of the memory $CD8^+$ T-cell population. Naive $CD8^+$ T-cell counts decrease gradually from early in HIV infection (69,72). The loss of naive $CD4^+$ and $CD8^+$ T cells implies a defect in the capacity to respond to new antigens, which may include new HIV variants that are continuously generated due to the high mutation rate of HIV.

Besides changes in the absolute numbers of T cell subsets, HIV also induces a perturbation of T-cell receptor repertoire of $CD4^+$ and $CD8^+$ T-cell population. Drastic restrictions in $CD8^+$ T-cell repertoire usage were found at all stages of HIV infection (73). In contrast, significant $CD4^+$ T-cell repertoire perturbations are not found in early stages of infection but are most prevalent in patients with the lowest $CD4^+$ T-cell counts (73,74).

**1.5.3 Dysfunctions Of T-lymphocytes In HIV Infection**

HIV induces severe functional immunologic abnormalities in T cells, B cells and APC's early in infection before the number of $CD4^+$ T cells starts to decline (75-78).

In vitro, the T lymphocytes of HIV infected individuals have a diminished or absent proliferative response to aspecific stimuli such as pokeweed mitogen (PWM), phytohemagglutinin (PHA), or by monoclonal antibodies (mAb) to CD3, and combined CD3 plus CD28 mAb's (75,77-82). It was shown that low T-cell responses to PWM and to CD3 mAb with or without CD28 mAb's is predictive for progression to AIDS (80,82). In addition, the in vitro response of T cells of HIV patients to specific stimuli such as Candida antigens, CMV antigens, tuberculin and tetanus toxoid is decreased (81, 83-86).

**1.5.4 The Response Of T cells To HIV Infection**

HIV infection results in a HIV specific response of $CD4^+$ and $CD8^+$ T lymphocytes, which may play an important role in the control of HIV

replication (87-98). However, in most patients, the T-cell responses cannot prevent an increase of the viral load and deterioration of the immune system.

It was shown that in individuals who control viremia in the absence of antiviral therapy, polyclonal, persistent, and vigorous HIV specific $CD4^+$ T-cell proliferative responses were present. The HIV specific proliferative responses of $CD4^+$ T cells to p24 antigen were inversely related to viral load (93). Lymphoproliferative responses of helper T cells to p24 gag, but not p17 gag or gp160 env, can be detected in long term non progressors (LTNPs), whereas they are totally absent in patients progressing to AIDS (94).

The frequency of HIV gag, pol and env specific CTL's is higher in asymptomatic individuals than in people progressing to AIDS, and the HIV specific CTL precursor frequency correlates inversely with the plasma HIV RNA levels (87-92,97,98). A positive correlation has been found between the cytotoxic T-cell response and the T helper response, suggesting that $CD4^+$ T cell help is critical for an effective cytotoxic T-cell response (95,96).

Besides a role in suppressing replication of HIV, it has been proposed that HIV specific CTL's may derange the immune response to HIV by eliminating $CD4^+$ T cells, APC's and by destroying the lymphoid tissue architecture (49).

### 1.5.5 T-Cell Hyperactivation In HIV Infection

HIV infections are characterized by a general activation of the immune system (114), as is evidenced by elevated serum levels of TNF-alpha, soluble IL-2, neopterin and Beta-2-microglobulin (99-101). The B cell compartment is also activated, resulting in hypergamma globulinemia (102). The $CD4^+$ and $CD8^+$ T lymphocytes of HIV infected individuals have increased expression of the activation markers HLA-DR and CD38 and the proliferation marker Ki67 (103-113).

Expression of CD38 on $CD8^+$ T cells was shown to be a strong predictor of progression to AIDS (115). The origin of the increased activation and proliferation of T cells in HIV infection will be discussed in chapters 7 and 8.

## 1.6 Antiretroviral Therapy

Progress in the field of antiretroviral therapy in the past 5 years has been dramatic. This is due to several reasons, including increased knowledge about viral dynamics and the development of reliable techniques for monitoring antiviral response, such as quantitative plasma HIV RNA measurements. In addition, the number of approved antiretroviral agents has increased greatly. In particular, the introduction of the class of protease inhibitors was a crucial step towards a more effective suppression of HIV replication. Presently, 3 classes of antiretroviral drugs are available for the clinical practice: the nucleoside analogue reverse transcriptase inhibitors (NRTI's), the non-nucleoside reverse transcriptase inhibitors (NNRTI's) and the protease inhibitors.

### 1.6.1 Highly Active Antiretroviral Therapy

A combination of 3 or more antiretroviral agents has proven the most effective approach to treat HIV disease (116). Compared to mono or dual therapy, the so-called triple therapy, consisting of two NRTI's and one protease inhibitor, showed a more durable suppression of plasma HIV RNA levels and a more pronounced rise of the CD4 count. Moreover, introduction of the highly active antiretroviral therapy (HAART), consisting of 3 or more antiretroviral drugs, has resulted in a significant reduction in mortality and morbidity among patients with advanced HIV infection in Western Europe and the USA (117-121).

The rationale for the use of combination therapy encompasses 4 arguments. First, to provide additive or synergistic antiviral effect. Second, to prevent the emergence of drug resistant HIV variants. In previously untreated patients, the probability is very small that HIV variants are present with mutations simultaneously conferring resistance to 3 different drugs. In addition, such multidrug resistant viruses may have severely compromised replication capacity. Third, to minimize the toxicity per drug. Fourth, to provide antiretroviral activity in different anatomic and cellular compartments.

The current recommended standard of initial antiretroviral treatment is a combination of two NRTI's and one protease inhibitor or two NRTI's and

one NNRTI (122). Regimens of three NRTI's and regimens including drugs from all three classes are also being evaluated.

Despite the success of HAART, several practical limitations and concerns should be recognized. In a significant number of patients, failure of HAART occurs, as defined by an increase of the plasma HIV RNA levels during treatment. Two studies investigating the virologic response to HAART in clinical practice showed that after 1 year, plasma HIV RNA levels remain below 500 copies/ml in 37% to 60% of the patients (123,124).

Therapeutic failure may be caused by drug resistance or by low active drug levels due to non-adherence, interactions with other drugs or diminished absorption as a result of concomitant intestinal infections. Due to cross-resistance, regimens instituted after failure of the first regimen have been less effective, with a lower percentage of patients achieving maximal suppression for shorter periods of time.

Besides the threat of therapy failure and multidrug-resistance, many other problems have been identified that reduce the quality of life for patients on HAART, such as a high pill burden, multiple daily dosing and dietary constraints. In addition, antiretroviral drugs have numerous adverse effects. NNRTI's may cause skin rash (125-129). NRTI's may cause mitochondrial toxicity, resulting in anemia, myopathy, peripheral neuropathy, pancreatitis or lactic acidosis (130,131). The toxicity associated with the class of protease inhibitors includes diarrhea, nausea, vomiting and fatigue and several laboratory abnormalities such as increased levels of aminotransferases, hyperglycemia, hypertriglyceridemia and hypercholesterolemia. A long-term serious side effect associated with the use of protease inhibitors is lipodystrophy, a syndrome of peripheral lipoatrophy with central adiposity (132-135). The exact role of protease inhibitors in the pathogenesis of lipodystrophy has not been unraveled and it has been hypothesized that NRTI's may also contribute to lipodystrophy (130,131).

### 1.6.2 The Effect Of HAART On HIV Replication

In a majority of patients, HAART results in a sustained suppression of plasma HIV RNA levels to below 50 copies/ml for up to four years. In addition, HAART significantly reduced the amount of HIV RNA in

lymphoid tissue (136-138), where most of the HIV replication and virus shedding occurs. The HIV RNA load is also decreased by HAART in other compartments such as the cerebrospinal fluid (139) and semen (140). The decline of HIV RNA levels in plasma is biphasic. It was hypothesized that the two phases reflect the decline of two different pools of HIV producing cells with different decay rates (141). The plasma HIV RNA level drops by approximately 99% in the first two weeks of treatment owing to the rapid elimination of free virus with a half-life (t½) of approximately 6 hours and loss of productively infected cells with a t½ of 1.6 days. This initial decrease is followed by a slower second-phase decay of plasma viremia with a t½ of 1-4 weeks, which may reflect the decline of long-lived infected cells (141).
Based on these decay characteristics, the possibility of eradication of HIV from all anatomical compartments was proposed. Assuming that the suppression of viral replication was complete and that the t½ of chronically infected cells was 10-14 days, it was estimated that eradication could be possible within 2.3-3.1 years (141).
Newer data however question the feasibility of HIV eradication. Evidence has been provided suggesting that low level of ongoing replication may occur despite a plasma HIV RNA levels < 50 copies/ml during HAART (142-145). In addition, HIV can persist in a latent form, as proviral DNA in resting memory $CD4^+$ T cells. In patients on HAART with prolonged suppression of plasma viremia for up to 30 months, infectious virus could be recovered from latently infected cells (146-149). The average half-life of these latently infected long-lived cells is calculated to be 43.9 months (150). Assuming that the latent reservoir consists of $1x10^5$ cells, eradication would take as long as 60 years (150). Thus, with HAART alone, eradication of HIV does not seem feasible at present.

### 1.6.3 Immune Reconstitution During HAART

In most patients, highly active antiretroviral therapy (HAART) leads to a substantial rise of the CD4 count, primarily due to an increase of memory $CD4^+$ T cells (151-153). The effect of HAART on the CD8 count varies per study from a slight increase (152,154,155) to a decrease (151,156,157). Naive $CD4^+$ and naive $CD8^+$ T cells increase slowly during HAART (151,152,158).

During HAART, proliferative lymphocyte responses to recall antigens and mitogens are enhanced over time (151,155,158-160), while T-lymphocyte activation is largely reduced (151,154,161-163) and T-cell receptor (TCR) repertoires are partly restored (73,74). Proliferative lymphocyte responses specific to HIV-1 antigens, in contrast, remain weak (86,160,164). In addition, it was shown that treatment, initiated even at advanced stages of HIV-1 disease, is capable of reversing the pathological changes in the FDC network (165). A complete normalization of HIV-1 associated immunological alterations has not been reported so far, but the observation period of subjects on potent antiretroviral therapies is still relatively short.

## Focus Of This Thesis

This thesis focuses on the effect of potent antiretroviral combination therapy on HIV replication and on the immune system. The majority of the work (**Chapters 2,3,5,6,7 and 8**) is based on the CHEESE study. This was a randomized, open label, multicenter study in the Netherlands comparing the efficacy and tolerability of saquinavir soft-gelatin-capsules (SGC) versus indinavir, both given as part of a triple therapy containing zidovudine and lamivudine. The results of the first 24 weeks of treatment are presented in **Chapter 2**.

The objective of the study in **Chapter 3** was to compare the plasma exposure of saquinavir-SGC relative to saquinavir hard-gelatin-capsules (HGC), and to determine whether there is a relation between saquinavir plasma exposure and the virological response in patients from the CHEESE study.

In **Chapters 4 and 5**, the influence of age on the regeneration of naive T cells during HAART was investigated in children and adults, respectively. We hypothesized that if it were mainly dependent on thymic function, the T-cell regeneration rate should decrease with age, due to natural involution of the thymus. To understand the mechanism of naive T-cell reconstitution during HAART is important because thymus dependent regeneration of T cells is required for recovery of the HIV induced deletions in the T-cell repertoire.

In **Chapter 6**, we investigated whether thymic output is decreased in HIV infected individuals. It has been proposed that thymic output can be measured by quantifying the number of recent thymic emigrants, which can be identified by T-Cell Receptor Excision Circles (TRECs). Infection with HIV results in a decline of the number of TREC positive T cells. Using a mathematical model, we investigated whether the low number of TREC positive T cells in HIV infection is best explained by increased division rates of naïve T cells or by thymic impairment.

In **Chapters 7 and 8**, the expression of proliferation and activation markers on T cells was investigated in untreated HIV-infected patients and during HAART. Two models have been proposed to explain the hyperactivation and increased proliferation of T cells in HIV-1 infection. One model contends that T-lymphocyte activation in HIV infection is due to a generalized immune activation driven by antigens from HIV and/or from other pathogens (19, 36, 38). Alternatively, increased activation and proliferation of $CD4^+$ T cells may be a homeostatic response to compensate for the loss of $CD4^+$ T cells that are killed by HIV (12,13). The aim of the studies in **Chapter 7 and 8** was to distinguish between these two models.

The objective of the study in **Chapter 9** was to determine whether in patients who achieved plasma HIV RNA levels below 50 copies/ml during HAART, transient relapses ("blips") of the plasma HIV RNA are associated with resistance development and virologic treatment failure.

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# 2

# Randomized Trial Comparing Saquinavir Soft Gelatin Capsules Versus Indinavir As Part Of Triple Therapy (CHEESE study)

James W.T. Cohen Stuart[1,2]
Rob Schuurman[2]
David M. Burger[3]
Peter P. Koopmans[3]
Herman G. Sprenger[4]
Job R. Juttmann[5]
C. Richter[6]
Pieter L. Meenhorst[7]
Richard M.W. Hoetelmans[7]
Frank P. Kroon[8]
Bert Bravenboer[9]
Dörte Hamann[10]
Charles A.B. Boucher[2]
Jan C.C. Borleffs[1]

1 Department of Internal Medicine, University Hospital Utrecht
2 Department of Virology, Eijkman Winkler Institute, University Hospital Utrecht
3 University Hospital Nijmegen
4 University Hospital Groningen
5 Sint Elisabeth Hospital Tilburg
6 Hospital Rijnstate Arnhem
7 Slotervaart Hospital Amsterdam
8 University Hospital Leiden
9 Catharina Hospital Eindhoven
10 Department of Viro-Immunology, Central Laboratory for Blood Transfusion Service, Amsterdam, the Netherlands

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## Abstract

*Objective*: To compare efficacy and tolerability of saquinavir soft gelatin capsule formulation (SQV-SGC) and indinavir, both given as part of a triple drug regimen containing zidovudine and lamivudine, in HIV-1-infected individuals.
*Design*: Randomized, open label, multicenter study.
*Patients*: In total 70 patients were included who were antiretroviral-naive and who had a CD4 cells count <500/µL and/or >10.000 HIV RNA copies/ml plasma and/or HIV-related symptoms. Subjects were assigned randomly to zidovudine 200 mg t.i.d. plus lamivudine 150 mg b.i.d. plus either SQV-SGC 1200 mg t.i.d. (SQV-SGC group) or indinavir 800 mg t.i.d. (indinavir group). Data are presented for all patients up to week 24.
*Results*: Mean baseline CD4 counts (± SE) were 301±29 cells/µL and 310±43 cells/µL in the SQV-SGC and indinavir groups, respectively. The log10 median baseline HIV RNA load was 5.00 copies/ml in the SQV-SGC group and 4.98 copies/ml in the indinavir group. No difference in antiretroviral effect between the treatment arms could be demonstrated. Intention-to-treat analysis (LOCF) at week 24 revealed that RNA levels decreased to < 50 copies/ml in 74.3% of patients in the SQV-SGC group and in 71.4% of the patients in the indinavir group (p=0.78). In the on-treatment analysis the proportion of patients < 50 copies/ml at week 24 was 88.0% in the SQV-SGC group and 84.6% in the indinavir group (p=0.725). Intriguingly, the mean increase of CD4 cells (ITT) in the first 24 weeks was 162±20 cells/µL in the SQV-SGC group and 89±21 cells/µL in the indinavir group (p=0.01), but preliminary data indicate that this difference in CD4 gain may disappear after 24 weeks of treatment. Both regimens were generally well tolerated.
*Conclusion*: During the first 24 weeks of the study, we found no difference in antiviral potency between the indinavir group and the SQV-SGC group. A significantly higher CD4 response in the SQV-SGC group was observed.

**Keywords:** clinical trials, combination therapy, indinavir, saquinavir-SGC

## Introduction

Saquinavir is a potent and specific inhibitor of the protease of human immunodeficiency virus (HIV) types 1 and 2 in vitro. Saquinavir was initially formulated as a hard-gelatin-capsule (SQV-HGC) which, as part of a combined antiretroviral treatment has been shown to be clinically effective (1). However, SQV-HGC has limited oral bioavailability, resulting in relatively low plasma levels. The recently developed soft-gelatin-capsule formulation of saquinavir (SQV-SGC) has improved oral bioavailability over SQV-HGC. At a dosage of 1200mg t.i.d., SQV-SGC achieves an approximately eight-fold higher plasma exposure to saquinavir compared with SQV-HGC (600mg t.i.d.) (2). It has been shown that SQV-SGC provides significantly more reduction of plasma HIV RNA levels in antiretroviral naive patients than SQV-HGC (3), consistent with the observation that the antiviral activity of saquinavir is positively correlated to plasma drug levels (4). Preliminary data from several clinical trials have demonstrated the antiviral potency of SQV-SGC in combination with two reverse transcriptase (RT) inhibitors (5,6).

No previous randomized clinical trial has compared two protease inhibitors in triple therapy background with the same RT inhibitors. The objective of this study was to compare the antiviral efficacy and tolerability of SQV-SGC with indinavir, as part of a triple therapy containing zidovudine and lamivudine in antiretroviral-naive HIV-1-infected individuals.

# Methods

**Study design**

This was a randomized, parallel arm, open label, multi center study comparing antiviral efficacy and safety of zidovudine plus lamivudine plus SQV-SGC or zidovudine plus lamivudine plus indinavir, in HIV-1 infected patients. A stratified randomization procedure was used. Patients were stratified according to plasma HIV RNA level (either > 100,000 or < 100,000 copies/ml). Data up to week 24 of all patients are presented. The study was approved by the internal review boards at each site and all participating patients gave written informed consent.

**Study patients**

Patients of 18 years and older who were seropositive for HIV type 1 and who were antiretroviral naive (with exception of zidovudine use for less than 12 months), were screened for enrollment at eight locations in The Netherlands. Patients were eligible for study treatment if, at the moment of screening plasma HIV RNA levels were at least 10,000 copies/ml (Amplicor HIV monitor test, Roche Diagnostics) and/or if CD4 counts were less than 500 cells / µL and/or if they had a history of HIV related symptoms (CDC stage B or C).

**Exclusion Criteria**

Criteria for exclusion were a hemoglobin level less than 7.0 mmol/l (in males) or 6.5 mmol/l (in females), a platelet count of less than 25 x $10^9$ / l, a neutrophil count of less than 0.75 x $10^9$ / l, serum creatinine level exceeding 1.5 fold the upper normal limit or levels of hepatic aminotransferases (ASAT, ALAT) exceeding 5 fold the upper normal limit. In addition, patients were excluded if they required acute therapy for an active opportunistic infection or if they required systemic antineoplastic chemotherapy and/or radiotherapy. Also excluded were patients with malabsorbtion or inadequate oral intake or patients with unexplained, chronic diarrhea persisting for 14 days or more. Pregnant or breastfeeding women were also excluded as were patients who participated in other investigational studies within 30 days prior to screening.

**Treatment Regimens**

Eligible patients were assigned randomly to either: (i) indinavir (Crixivan, Merck, West Point, Pa) 800mg t.i.d. plus zidovudine (Retrovir, Glaxo-Wellcome, Research Triangle Park, N.C.) 200mg t.i.d. plus lamivudine (Epivir, Glaxo-Wellcome, Research Triangle Park, N.C.) 150 mg b.i.d. or : (ii) Saquinavir Soft-Gelatin-Capsules (Fortovase, Hoffmann-La Roche, Inc., Nutley, New Jersey) 1200mg t.i.d. plus zidovudine 200mg t.i.d. plus lamivudine 150 mg b.i.d..

**Assessments**

The patients were assessed every 4 weeks through week 24. At screening, baseline and every visit, medical history was reviewed and standard biochemical and hematological tests were conducted. Plasma concentrations of the protease inhibitors were determined each study visit.

**Virologic and Immunologic Studies**

At each site, plasma (EDTA) was processed, stored at -70°C and assayed later for HIV RNA by a quantitative reverse transcriptase polymerase chain reaction assay (Roche Amplicor Monitor Standard Assay). The lower limit of detection was 400 HIV RNA copies/ml in this assay. An investigational version of an ultrasensitive RT-PCR assay with a lower limit of detection of 50 copies/ml (Roche Amplicor Monitor) was performed at a central laboratory.

Absolute numbers of $CD4^+$ and $CD8^+$ T-lymphocytes were determined performed at a central laboratory by flowcytometry.

**Statistical Analysis**

The primary parameter of antiretroviral efficacy was HIV RNA (log transformed to the base 10). Secondary parameters were CD4 counts, improvement in or progression to AIDS defining illnesses, the incidence of adverse events and biochemical and haematological safety parameters. Baseline values of HIV RNA levels and CD4 counts were calculated as the geometric mean of two consecutive pretreatment determinations, the first between day -14 and day -7 and the second on day 0.

Analyses of the variables pertaining to efficacy (plasma HIV RNA and

CD4 response) were performed on an intention-to-treat basis that included data of all randomized patients who have received at least one dose of study medication. The last observation was carried forward as the imputation method. On treatment analyses, representing all those patients for whom data were available at that time point, are also presented.
A repeated measures analysis-of-variance-model was used to detect differences between the two treatment arms in area under the curve of plasma HIV RNA levels and CD4 counts with repeated equally spaced observations within the patients. In the analyses of the proportions of patients below limit of detection and incidence of adverse events, the treatments were compared by Fisher's exact tests. All reported P values are two-sided.

## Results

### Study Patients

Seventy patients were enrolled in the study from January 1997 to February 1998, 35 in each treatment group. The baseline characteristics of the patients were not different between treatment groups, as is shown in Table 1. Ten patients discontinued the study after 4-24 weeks for the following reasons: an adverse event (5) (table 2), lost to follow up (1), consent withdrawn (1), receiving of erroneous study medication (1), protocol violation (2). The number of discontinuations was the same between the two treatment groups.

### HIV RNA

Over the initial 24 weeks, plasma HIV RNA levels declined (median) 2.40 log10 in the SQV-SGC group and 2.38 log10 in the indinavir group (Figure 1). No significant differences were observed between the treatment groups in the decrease of plasma HIV RNA levels.
Figure 2A shows the proportion of patients who achieved plasma HIV RNA levels below 400 copies/ml, according to treatment group. At week 24, using an intention-to-treat analysis (ITT), 82.9% (29/35) in the SQV-SGC-arm and 85.7% (30/35) in the indinavir arm had plasma HIV RNA

**Table 1.** *Base-line Characteristics of the study patients*

| Characteristic | SQV-SGC Group (n=35) | indinavir Group (n=35) | P value |
|---|---|---|---|
| Gender - no. of patients (%) | | | |
| male | 32 (91.4) | 31 (88.6) | p=0.99 § |
| female | 3 (8.6) | 4 (11.4) | |
| Mean age (±SD) - yr | 38 (± 8.46) | 37 (± 9.02) | p=0.45¶ |
| Race (ethnic group) - no of patients (%) | | | |
| Caucasian | 30 (85.7) | 33 (94.3) | p=0.42 § |
| Afro-european | 4 (11.4) | 1(2.9) | p=0.35 § |
| Oriental | 1 (2.9) | 1(2.9) | n.a |
| Prior zidovudine therapy - no patients (%) | 2 (5.7) | 1 (2.9) | p=0.99 § |
| Prior AIDS defining illness - no of patients (%) | 7 (20) | 10 (28.6) | p=0.58 § |
| CD4 count - cells / μL | | | |
| Mean | 301 | 310 | p=0.775¶ |
| Range | 10-750 | 30-1075 | |
| Plasma HIV RNA ($log_{10}$ copies/ml) | | | |
| Median | 5.00 | 4.98 | p=0.668¶ |
| Range | 3.41- 6.87 | 3.10 - 6.23 | |

¶: Independent-samples t-test
§: Fisher's exact test
n.a.: not applicable

**Table 2.** *Adverse events According To treatment Group*

| | SQV-SGC Group no. of patients | indinavir Group no. of patients | Fishers's Exact test |
|---|---|---|---|
| Moderate or severe Adverse Events: | | | |
| Clinical nephrolithiasis | 0 | 2 | N.S. |
| Diarrhoea | 5 | 1¶ | N.S. |
| Nausea | 5 | 5 | N.S. |
| Serious Adverse Events: | | | |
| Gastritis | 0 | 1 | N.S. |
| Hematemesis | 1 | 0 | N.S. |
| Urine bladder polyp | 0 | 1 | N.S. |
| Grade 4 Anemia | 0 | 1¶ | N.S. |
| CDC Events: | | | |
| Non Hodgkin lymphoma | 1¶,§ | 1¶,§ | N.S. |
| PCP | 0 | 1¶,§ | N.S. |
| Herpes Zoster | 2 | 3 | N.S. |
| Total CDC Events | 3 | 5 | N.S. |

¶ Resulted in study discontinuation

§ Resulted in death

levels below 400 copies/ml (p=0.74). In the on-treatment (OT) analysis, the proportion of patients < 400 copies/ml at week 24 was 90.3% (28/30) in the SQV-SGC arm and 96.5% (28/29) in the indinavir arm (p=0.57). Figure 2B shows the proportion of patients with plasma HIV RNA levels below 50 copies/ml. At week 24, ITT analysis showed that 74.3% (26/35) in the SQV-SGC-arm and 71.4% (25/35) in the indinavir arm had plasma HIV RNA levels below 50 copies/ml (p=0.78). In the OT analysis, the proportion of patients < 50 copies/ml at week 24 was 88.0% (22/25) in the SQV-SGC arm and 84.6% (22/26) in the indinavir arm (p=0.73).

**CD4 Cell Counts**

During the initial 24 weeks of the study, mean numbers of $CD4^+$ T cells in the peripheral blood (±SEM) increased 162±20 cells / µL and 89±21 cells / µL (Figure 3) in the SQV-SGC group and the indinavir group, respectively (ITT). The increase of $CD4^+$ T lymphocytes was significantly greater in the group assigned to the SQV-SGC containing regimen (repeated measures analysis, p=0.01). In each group, CD4 count response pattern was biphasic with a higher rate of increase in the first four weeks of treatment as compared to the later four-week intervals.

**Adverse Events and AIDS defining events**

Both study treatments were generally well tolerated (Table 2). Five patients withdrew from the study because of an adverse event: In two patients a non-Hodgkin lymphoma was diagnosed, requiring systemic antineoplastic chemotherapy. One patient developed severe anemia, one patient was intolerant to AZT (nausea) and one patient had *Pneumocystis carinii* pneumonia. There were three deaths among the study patients (Table 2).

The incidence of adverse events with moderate or severe intensity shown in table 2. The most common adverse event experienced by the patients was

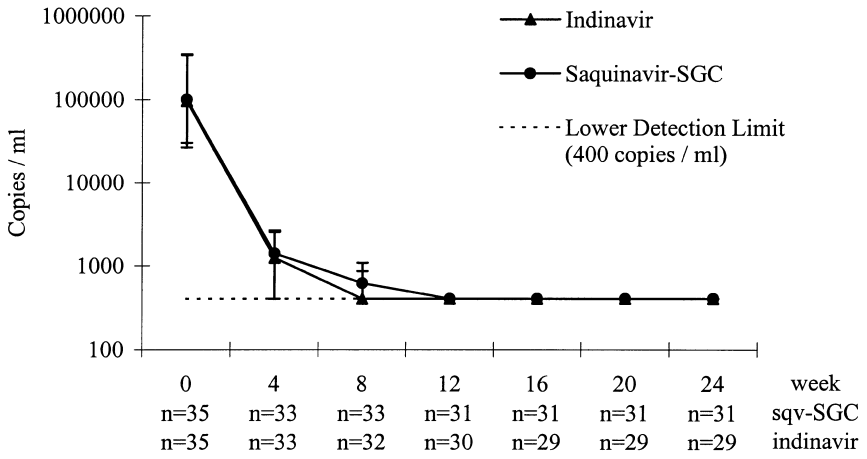


**Figure 1.** *Median plasma HIV RNA levels during the initial 24 weeks of the study. Median values are shown. Bars are 25th and 75th percentiles.*

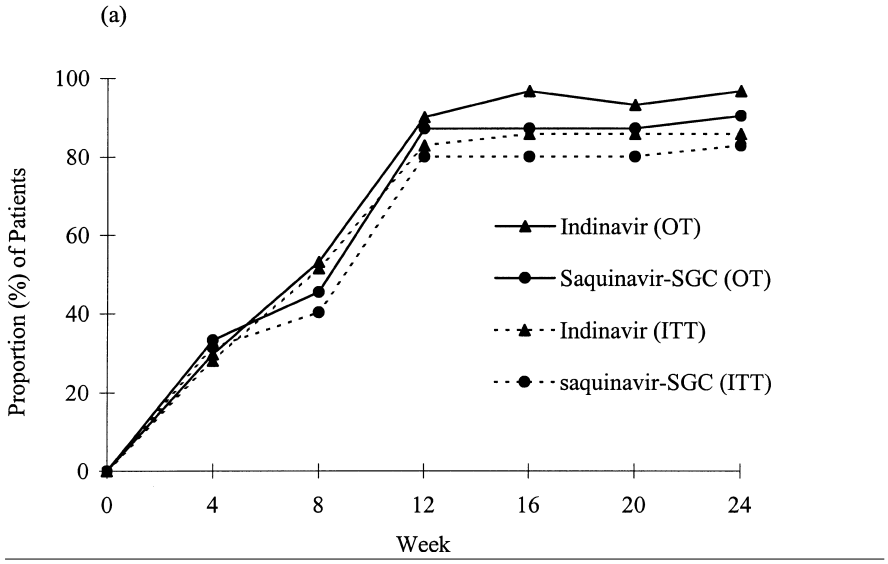


**Figure 2A.** *Proportion of patients with plasma HIV RNA levels of less than 400 copies / milliliter.*

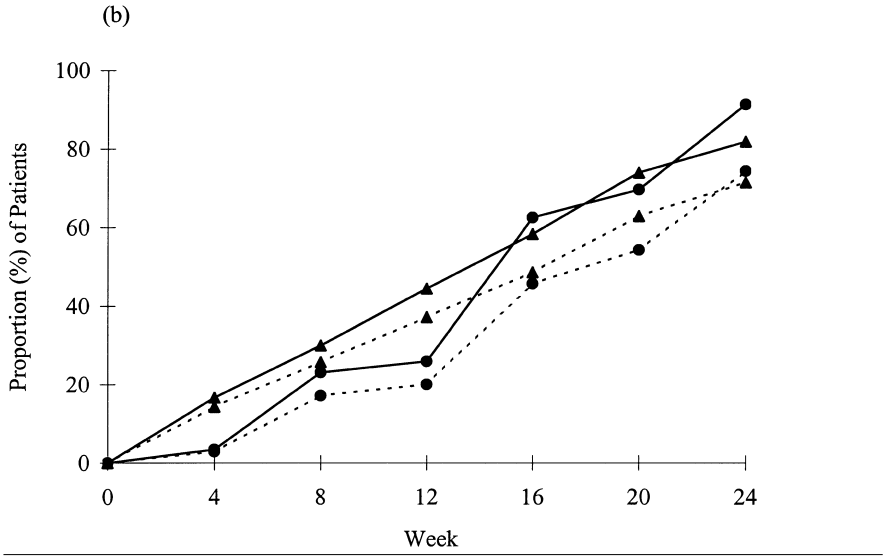


**Figure 2B.** *Proportion of patients with plasma HIV RNA levels of less than 50 copies / milliliter.*

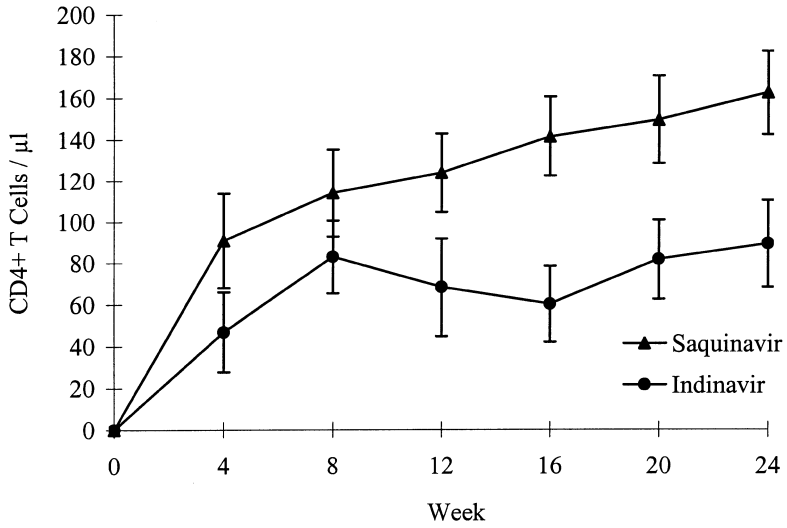


**Figure 3.** *Changes from baseline in the CD4 cell count during the initial 24 weeks of the study. Mean values are shown intend to treat analysis. Bars are standard error of the mean.*

mild-moderate gastro-intestinal discomfort (dyspepsia, nausea, flatulence, abdominal pain or diarrhoea) with no difference in the reporting of symptoms between the two treatment groups.

There were 8 new AIDS defining events during the first 24 weeks of the study, with no difference in the incidence between treatment groups (p=0.45)(table 2).

**Virologic Failures**

In four patients treatment failure was observed (one patient receiving indinavir, three receiving SQV-SGC). Treatment failure was defined as absence of virologic response or having rebound of HIV RNA levels above 400 copies/ml on two or more consecutive study visits following a virological response. Virological response was defined as achieving HIV RNA levels < 400 copies/ml.

One patient from the indinavir-arm with rebound of plasma HIV RNA levels reported intermittent adherence to the study regimen. Later on, this patient adhered to the study regimen as recommended and again achieved plasma HIV RNA levels < 400 copies/ml.

Two patients from the SQV-SGC arm with rebound of plasma HIV RNA levels had low saquinavir plasma levels on several study visits although patient report and pill count did not show low adherence to study medications. On several occasions, estimated saquinavir trough levels in these two patients were less than 50 ng / ml, the calculated EC90 of saquinavir in vivo (28). In one of these patients, malabsorption of SQV-SGC was confirmed by recording a full eight-hour pharmacokinetic curve after observed intake of SQV-SGC with meal.
One patient in the SQV-SGC arm did not achieve plasma HIV RNA levels below 400 copies/ml at any time during the initial 24 weeks of treatment. Adequate saquinavir plasma levels could be detected in this patient. To determine to what extent drug resistance contributed to the treatment failure, genotypic resistance measurements were performed on plasma samples from baseline and week 18. At baseline, no drug resistance mutations were observed in the reverse transcriptase (RT) gene or the protease gene of the HIV RNA population derived from this patient's plasma. At week 18, the HIV RT gene harbored the M184V mutation, conferring high level resistance to 3TC. The absence of a virological response in this patient may be best explained by the high HIV RNA plasma levels before initiation of therapy (day -14: 3,200,000 copies/ml and day 0: 11,500,000 copies/ml).

## Discussion

This trial has a unique design: it is the first randomized prospective trial in HIV infected patients comparing two different protease inhibitors given as part of a triple therapy with the same RT-inhibitor background. Both regimens displayed a powerful antiretroviral effect. We observed no significant difference in antiretroviral activity between the SQV-SGC group and the indinavir group during the initial 24 weeks of the study. At week 24, 74.3% of the intention-to-treat population in the SQV-SGC group and 71.4% in the indinavir group had plasma HIV RNA levels below 50 copies/ml. Eight AIDS defining illnesses were diagnosed with no difference in incidence between the two treatment groups.

Surprisingly, a significantly higher CD4 cell response during the first 24 weeks was observed in the SQV-SGC group as compared with the indinavir group, but preliminary data from the post 24 week period show that this difference may disappear at week 32 of treatment. At week 32, mean CD4 change from baseline (±SEM) was 168±24 cells / µL in the SQV-SGC group (n=23) and 169±25 cells / µL in the indinavir group (n=26).

The explanation for this difference in immunologic response during the first 24 weeks remains unclear. Low baseline CD4 count and large magnitude of viral load reduction during treatment are two determinants of good initial CD4 response during HAART (7,8,9). However, neither baseline CD4 count nor virus load reduction differed between the two treatment groups and therefore these parameters do not explain the difference in CD4 cell response. The rise of $CD4^+$ T cells in blood during HAART has been explained by two mechanisms: proliferation of $CD4^+$ T lymphocytes (7) and/or the recirculation of CD4 T lymphocytes which resided in lymphoid tissue prior to therapy (9). Alternatively, the CD4 count increase during HAART may be explained by a direct influence of antiretroviral agents on recirculation or proliferation of lymphocytes. In needlestick-injured personnel given AZT prophylaxis for one month increases of CD4 counts have been reported (10,11). However, administration of indinavir in two healthy volunteers did not result in a rise of CD4 counts (12). We hypothesize that each antiretroviral drug may have a direct pharmacologic effect on either lymphocyte proliferation and/or trafficking and that saquinavir-SGC may be different from indinavir in this aspect, which may explain the initial difference of the $CD4^+$ T lymphocyte response.

Although the majority of patients achieved plasma HIV RNA levels below 400 copies/ml in both treatment groups, three patients (one receiving indinavir, two receiving SQV-SGC) had rebound of HIV RNA levels above 400 copies/ml on two or more consecutive study visits. Non compliance explains the viral rebound in the patient from the indinavir-arm. In two patients from the SQV-SGC arm, the viral rebound may be explained by low saquinavir plasma levels. In one of these two patients, malabsorption of SQV-SGC was observed.

One patient (receiving SQV-SGC) did not achieve plasma HIV RNA levels below 400 copies/ml, probably due to the extremely high baseline HIV RNA plasma levels. The potency of AZT/3TC/SQV-SGC was not sufficient to prevent residual HIV replication and emergence of resistant virus variants in this patient with a high HIV replication level. In several studies it was demonstrated that high baseline viral loads are associated with less efficient viral suppression (13,14) and a slower rate of HIV clearance from plasma (15). As it has been shown that a five drug regimen provides improved suppression of HIV replication over triple therapy (16), five (or four) drug regimens may be required to attain a durable antiviral response in patients with high pretreatment plasma HIV RNA levels.

In summary, we showed by head-to-head comparison that during the initial 24 weeks of treatment saquinavir soft gelatin capsule formulation shows equivalent antiretroviral efficacy to indinavir, when given as part of a triple therapy containing zidovudine and lamivudine, in antiretroviral-naive HIV-1-infected patients. SQV-SGC therefore is an appropriate choice of protease inhibitor for first line therapy. SQV-SGC is generally well tolerated, which is crucial for long term adherence to antiretroviral regimens. Prolongation of follow up is required to determine whether over the long term SQV-SGC and indinavir may differ with respect to toxicity, antiviral potency or immunologic effect. Studies on a large population may be performed to confirm our findings.

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# 3

# The plasma exposure to saquinavir is not related to the virological response to triple therapy in antiretroviral naive HIV-1-infected patients in the CHEESE Study

Rolf P.G. van Heeswijk[1]
James W.T. Cohen Stuart[2,3]
Charles A.B. Boucher[2]
Jan C.C. Borleffs[3]
Pieter L. Meenhorst[4]
Peter P. Koopmans[5]
Herman G. Sprenger[6]
Job R. Juttmann[7]
C. Richter[8]
Frank P. Kroon[9]
Bert Bravenboer[10]
David M. Burger[11]
Jos H. Beijnen[1]
Richard M.W. Hoetelmans[1]
on behalf of the CHEESE Study Group

1 Dept. of Pharmacy and Pharmacology, Slotervaart Hospital, Amsterdam
2 Dept. of Virology, Eijkman Winkler Institute, University Hospital Utrecht
3 Dept. of Internal Medicine, University Hospital Utrecht
4 Dept. of Internal Medicine, Slotervaart Hospital, Amsterdam
5 Dept. of Internal medicine, University Hospital Nijmegen
6 Dept. of Internal medicine, University Hospital Groningen
7 Dept. of Internal medicine, Sint Elisabeth Hospital Tilburg
8 Dept. of Internal medicine, Hospital Rijnstate, Arnhem
9 Dept. of Internal medicine, University Hospital Leiden
10 Dept. of Internal medicine, Catharina Hospital, Eindhoven
11 Clinical Pharmacy, University Hospital Nijmegen, The Netherlands

*(Submitted for publication)*

## Abstract

*Objective*: To evaluate the exposure to saquinavir when used as soft-gelatin-capsules (SGC) relative to hard-gelatin-capsules (HGC) and to explore the relation between SQV exposure and the virological response in 29 antiretroviral naive HIV-1-infected patients.
*Methods*: Patients were treated with SQV-SGC 1,200mg *tid* plus zidovudine plus lamivudine. Blood samples were obtained at regular intervals during 48 weeks of treatment. As a measure of SQV plasma exposure, the concentration ratio (CR) was used of the observed SQV concentration and the time adjusted average concentration from a reference population of 20 patients using SQV-HGC 1,200mg *tid*.
*Results*: The median CR of all samples was 0.59 (n=208). No relation could be observed between SQV plasma exposure and the plasma HIV-1 RNA decrease during week 0-4 of therapy, nor to plasma HIV RNA clearance rate during week 4-12 ($p \geq 0.62$), nor to the time to achieve plasma HIV-1 RNA <50 copies/ml ($p=0.39$). The CR was higher in patients with plasma HIV-1 RNA <50 copies/ml at week 48 than in patients with detectable plasma HIV-1 RNA at week 48, although not significantly ($p \geq 0.06$). Despite the low SQV plasma exposure, 19/22 patients (84%) had plasma HIV-1 RNA levels <50 copies/ml at week 48 (OT analysis). Interestingly, the median CR of SQV decreased from 0.7 at week 4 to 0.55 at week 48 ($p=0.04$).
*Conclusion*: Higher bioavailability of SQV-SGC as compared to SQV-HCG could not be confirmed. Furthermore, our data suggest that the plasma SQV exposure may not be related to the virological response during 48 weeks in antiretroviral naive HIV-1-infected patients.


## Introduction

Saquinavir is a potent and specific inhibitor of the protease of the human immunodeficiency virus type 1 (HIV-1) and was initially formulated as hard gelatin capsules (SQV-HGC) (1). Due to the limited oral

bioavailability of SQV-HGC, saquinavir plasma concentrations are generally low which may predispose for the development of resistant viral strains and subsequent treatment failure (2). It is therefore recommended not to use SQV-HGC as a single protease inhibitor in highly active antiretroviral therapy (HAART) (3). To overcome these pharmacokinetic limitations, saquinavir has been formulated as soft gelatin capsules (SQV-SGC), with a bioavailability of 331% as compared to SQV-HGC (1). Therefore, SQV-SGC may be considered as a single protease inhibitor in HAART in a dose of 1,200 mg *tid* (3). The antiviral potency of SQV-SGC in combination with two nucleoside analogue reverse transcriptase inhibitors (NRTIs) has been established in several clinical trials (1). In the CHEESE Study, there was no difference in antiretroviral efficacy between SQV-SGC 1,200 mg *tid* (n=35) and indinavir 800 mg *tid* (n=35), both in combination with two NRTIs, in antiretroviral naive HIV-1-infected patients after 48 weeks of treatment (4). However, a slight difference in the response of $CD4^+$ cells was observed in favour of the SQV-SGC group, but this difference was not statistically significant (4).

Several studies previously reported relationships between the exposure to saquinavir and both the decrease in plasma HIV-1 RNA concentration and the increase in $CD4^+$ cell counts (5-7). Furthermore, patients with an estimated plasma saquinavir trough concentration above 50 ng/mL had a significantly higher likelihood to have a sustained decline in HIV-1 RNA of more than 2 $\log_{10}$ units below baseline after 48 week of therapy, in a retrospective study in 130 mainly NRTI-pre-treated HIV-1-infected patients (8). In the current study, we explored if the reported relationships between the exposure to saquinavir and the virological and immunological response also apply to antiretroviral naive HIV-1-infected patients treated with SQV-SGC in combination with two NRTIs. Furthermore, by comparison of the saquinavir plasma concentrations as observed in the CHEESE study with the concentrations of saquinavir in a reference population of 20 HIV-1-infected patients using SQV-HGC 1,200 mg *tid*, we explored differences in the exposure to saquinavir between the two dosage forms.

## Methods

**Study design**

In this study, the relationship between saquinavir plasma exposure and the virological and immunological response to therapy was investigated in 29 patients from the previously described CHEESE Study. Briefly, the CHEESE study was a randomised, parallel arm, open label, multicenter study comparing the antiviral efficacy and safety of zidovudine (Retrovir; Glaxo-Wellcome, Research Triangle Park, North Carolina, USA) plus lamivudine (Epivir; Glaxo-Wellcome) plus SQV-SGC (Fortovase; Hoffmann-La Roche, Inc., Nutley, New Jersey, USA) or zidovudine plus lamivudine plus indinavir (Crixivan; Merck, West Point, Pasadena, USA), in HIV-1 infected patients. Study visits were scheduled every four weeks until week 24 and every eight weeks up to week 48. At screening, baseline and every visit, samples for the quantification of the plasma HIV-1 RNA concentration, the number of $CD4^+$ cells and the plasma saquinavir concentration were collected. The study was approved by the Institutional Review Boards at each site and all participating patients gave written informed consent.

**Study patients**

Patients of 18 years and older who were seropositive for HIV type 1 and who were antiretroviral naive (with exception of zidovudine use for less than 12 months), were screened for enrolment at eight locations in the Netherlands. Patients were eligible for the CHEESE Study if at the moment of screening plasma HIV RNA levels were at least 10,000 copies/mL (Amplicor HIV monitor test, Roche Diagnostics), and/or if CD4 counts were less than 500 cells/μL, and/or if they had a history of HIV related symptoms (CDC stage B or C). The exclusion criteria have been described before (9).

Patients eligible for the present substudy were those assigned to treatment with SQV-SGC 1,200 mg *tid* plus zidovudine 200 mg *tid* plus lamivudine 150 mg *bid*. Patients were included if they had 3 or more evaluable plasma saquinavir concentrations. Plasma saquinavir concentrations were considered evaluable if the time of ingestion of the last saquinavir dose and

the time of blood sampling were known. One or more evaluable samples had to be from week 4-12.

**Pharmacokinetic analysis**
Plasma concentrations of saquinavir were determined at each study visit. The time of ingestion of the last dose of saquinavir and when a blood sample was drawn was recorded. Plasma concentrations of saquinavir were determined with a sensitive and validated reversed-phase high-performance liquid chromatographic assay with a lower limit of quantification of 25 ng/mL at a central laboratory (10). The saquinavir concentrations were compared to the time-adjusted (rounded to half hours) average saquinavir concentration as measured (in the same laboratory) in a reference population of 20 HIV-1-infected patients using SQV-HGC 1,200 mg *tid* plus 2 NRTI's. The ratio of the observed concentration and the reference concentration (concentration ratio; CR) was used as a measure of the exposure to saquinavir for analysis of pharmacokinetic-pharmacodynamic (PK-PD) relationships.

**Virologic and immunologic analysis.**
At each site, plasma (EDTA) was processed, stored at -70 °C and assayed for HIV-1 RNA by an ultrasensitive reverse transcription-PCR assay with a lower limit of detection of 50 copies/mL (Roche Amplicor Monitor) at a central laboratory. Absolute numbers of $CD4^+$ and $CD8^+$ T lymphocytes were determined at a central laboratory by flow cytometry.

For the analysis of PK-PD relationships the following parameters of virologic response were defined: (a) the decrease of the plasma HIV-1 RNA concentration after 4 weeks of therapy, (b) the slope of the ln-transformed (natural logarithm) HIV-1 RNA decline from week 4 to week 12 as estimated by least squares linear regression analysis (i.e. the elimination rate constant, assuming mono-exponential decay between week 4-12), (c) the time to achieve a plasma HIV-1 RNA concentration < 50 copies/mL, and (d) the plasma HIV-1 RNA concentration at 48 weeks follow-up.
The $CD4^+$ increase after 48 weeks was used as a measure of immunological response.

**Statistical Analysis**

Baseline values of plasma $\log_{10}$ HIV-1 RNA concentrations and $CD4^+$ cell counts were calculated as the mean of two consecutive pre-treatment determinations, the first between day -14 and day -7 and the second on day 0. Analysis of the plasma HIV-1 RNA concentration was performed on an intention-to-treat (ITT) basis counting missing values either as failures (ITT-MF) and carrying the last observation forward (ITT-LOCF). On treatment (OT) analyses, representing all those patients for whom data were available at 48 weeks, are also presented.

For the analysis of PK-PD relationships, the median, minimum and maximum CRs of saquinavir from week 4 to 12, and during 48 weeks were calculated for each patient. Linear regression analysis was used to test the association between PK and PD parameters. For statistical calculations, the Statistical Product and Service Solutions (SPSS) for Windows was used (version 6.1, SPSS Inc., Chicago, IL, USA). A p-value of 0.05 or less was considered statistically significant.

## Results

**Patients**

In total, 35 patients were enrolled in the SQV-SGC treatment arm of the CHEESE Study from January 1997 to February 1998. Twenty-nine patients (Table 1) were included in the present study. The mean duration of follow-up was 45 weeks (range 16 to 48 weeks). Twenty-two patients completed 48 weeks of follow-up.

**Saquinavir pharmacokinetics**

A total of 208 evaluable plasma saquinavir concentrations were obtained from 29 patients. The saquinavir concentrations ranged from undetectable (< 25 ng/mL) to 4,306 ng/mL (median 152 ng/mL) and the time post ingestion of the last dose of saquinavir ranged from 0 to 9 hours (median 4.5 h). A scatter plot of all evaluable saquinavir concentrations is shown in Figure 1. The median CR of all samples was 0.59 (interquartile range 0.26 - 1.49, range 0.00 - 21.35). The intra- and interpatient variability of the

**Table 1.** *Baseline characteristics of the study patients (n=29)*

| Characteristic | Values |
|---|---|
| Gender - no. of patients (%) | |
| male | 27 (93.1) |
| female | 2 (6.9) |
| Mean age (±SD) - yr | 39 (± 7.58) |
| Race (ethnic group) - no of patients (%) | |
| Caucasian | 25 (86.2) |
| Afro-european | 3 (10.3) |
| Oriental | 1 (3.5) |
| Prior AIDS defining illness - no of patients (%) | 4 (13.8) |
| CD4 count - cells / µL | |
| Mean | 300 |
| Range | 10-750 |
| Plasma HIV RNA ($log_{10}$ copies/mL) | |
| Median | 5.00 |
| Range | 3.41- 6.87 |

saquinavir CR was 133% and 104%, respectively (analysis of variance; ANOVA).

Three patients had relatively high plasma saquinavir concentrations which were occasionally above 2,000 ng/mL (Fig.1 ). Except for one CR, these three patients had CRs which were above 1.0 during the whole study period. The reason for the relatively high exposure to saquinavir these patients is unclear (the patients did not use co-medication known to interfere with saquinavir metabolism and had no signs of abnormal liver function).

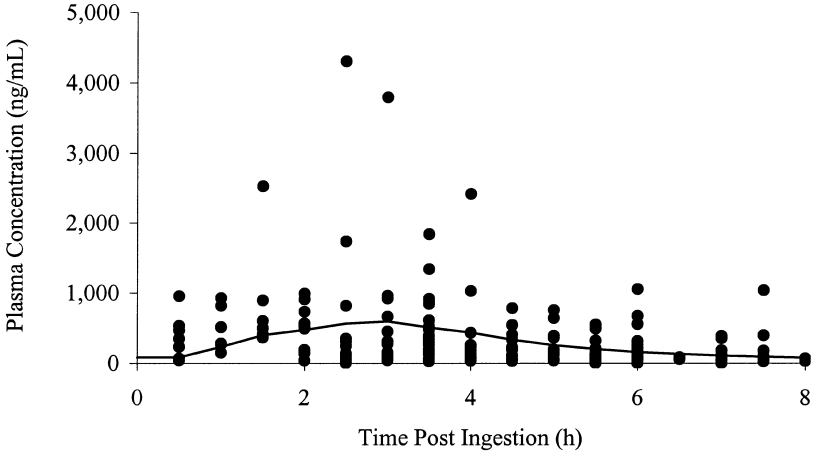


**Figure 1.** *Scatter plot of the saquinavir plasma concentrations (n=208) obtained from 29 HIV-1-infected patients at regular intervals during a 48-week period. The patients used saquinavir soft gelatin capsules 1,200 mg tid plus zidovudine and lamivudine. The solid line represents the average steady-state saquinavir plasma concentration versus time curve during a dosing interval as measured in 20 HIV-1-infected patients using saquinavir hard gelatin capsules 1,200 mg tid.*

Interestingly, the median CR of saquinavir decreased from 0.70 at week 4 (n=28) to 0.55 at week 48 (n=12) (Figure 2, p=0.04, Pearson´s correlation). If only the data from the 12 patients of whom CRs were available at week 48 of therapy were included in this analysis, a similar relationships was observed (p=0.06, $r^2$ = -0.64, Pearson´s correlation).

**Virological and immunological results**

The median $\log_{10}$ decline of the plasma HIV-1 RNA concentration after 4 weeks of therapy was 2.15 (range 0.89 - 3.26). The median elimination rate constant of plasma HIV-1 RNA from week 4 to 12 was 0.21 $week^{-1}$ (range -0.60 - 0.51). The median time to achieve a plasma HIV-1 RNA concentration < 50 copies/mL was 16 weeks, ranging from 4 weeks to > 48 weeks. In an OT analysis 19/22 patients (86.4%) had a plasma HIV-1 RNA concentration < 50 copies/mL after 48 weeks follow-up. In an ITT-MF and ITT-LOCF analysis 19/29 (65.5%) and 22/29 (75.9%) patients

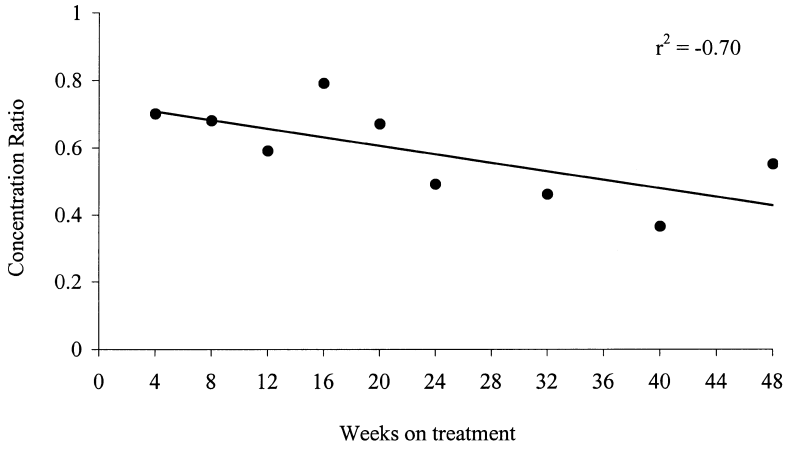


**Figure 2.** *Relationship between the median concentration ratio of saquinavir (see text for explanation) and the duration of treatment as measured in 208 plasma samples obtained from 29 HIV-1-infected patients using saquinavir soft gelatin capsules 1200 mg tid plus zidovudine and lamivudine (p=0.04, Pearson´s correlation).*

had a plasma HIV-1 RNA concentration < 50 copies/mL after 48 weeks follow-up, respectively. During 48 weeks of treatment, the median number of $CD4^+$ cells in peripheral blood increased by 150 cells/μL (range -180 to 730 cells/μL).

**Relationships with saquinavir pharmacokinetics**

Since it is currently unknown which pharmacokinetic parameter of the protease inhibitors is best related to the response to therapy, we used the median, minimum and maximum CR of saquinavir as observed for each individual patient to explore exposure-response relationships. The median, minimum and maximum CR of saquinavir during week 4 to 12 were not related to the decrease in plasma HIV-1 RNA concentration after 4 weeks of therapy nor to the plasma HIV-1 RNA decay rate during 4 to 12 ($p \geq 0.62$).

The median, minimum and maximum CRs of saquinavir during 48 weeks were not related to the time to achieve a plasma HIV-1 RNA

concentration < 50 copies/mL ($p \geq 0.39$). The maximum and minimum CRs (median) for patients with less than 50 HIV-1 RNA copies/mL at week 48 were 2.42 and 0.21, respectively, as compared to 0.99 and 0.11, respectively, for those with a higher plasma viral load > 50 copies/mL at week 48 ($p=0.09$ and $p=0.06$, respectively, Mann-Whitney U test, OT analysis). Patients with a median CR higher than the median CR of all samples did not have a higher chance to achieve an undetectable plasma viral load at 48 weeks ($p \geq 0.14$, Fisher's exact test).

To explore if plasma saquinavir concentrations during the initial three months of antiretroviral therapy (when plasma HIV-1 RNA concentrations are high) have more influence on virologic outcome at week 48, than the overall exposure to saquinavir during 48 weeks, we evaluated the relationships between CR's from week 4-12 and the plasma HIV RNA load at week 48. In an OT analysis, the maximal CR during week 4-12 was higher for patients with a plasma HIV-1-RNA concentration below 50 copies/mL at week 48 as compared to those with a viral load > 50 copies/ml at week 48, although not significantly. (maximal CR values (medians) were 1.41 and 0.49, respectively, for those with a plasma viral load below and above 50 copies/mL at 48 weeks of therapy, $p=0.053$, Mann-Whitney U test). This difference was less clear in an ITT-MS or ITT-LOCF analysis ($p \geq 0.22$).

Of note, the baseline plasma $\log_{10}$ HIV-1 RNA concentration was positively and significantly correlated with the $\log_{10}$ decrease of the plasma HIV-1 RNA concentration after 4 weeks ($p=0.02$, $r^2=0.44$, Pearson´s correlation) and with the time to achieve a plasma HIV-1 RNA concentration below 50 copies/mL ($p<0.01$, $r^2=0.67$, Pearson´s correlation, after exclusion of patients not achieving an undetectable plasma viral load before week 48). Furthermore, patients with an HIV-1 RNA concentration of less than 50 copies/mL at 48 week of therapy had a lower baseline $\log_{10}$ HIV-1 RNA concentration as compared to those with a detectable plasma viral load at 48 weeks, in all analyses ($p \geq 0.08$, Mann-Whitney U test).

The regimen was generally well tolerated, with diarrhoea (n=11) and nausea (n=9) as the most frequently reported side-effects. The exposure to saquinavir was not different between patients with and without side-effects (data not shown). No relationship was observed between the plasma exposure to saquinavir and the immunological response to therapy (data not shown).

## Discussion

The soft gelatine capsule of saquinavir, which was used in this study, was developed to improve the limited oral bioavailability of saquinavir (i.e approximately 4%) when used as the initially developed hard gelatin capsules (1). The oral bioavailability of SQV-SGC has been shown to be 331% relative to SQV-HGC (1). However, a higher bioavailability of SQV-SGC as compared to SQV-HGC could not be confirmed in our study. In contrast, the overall median concentration is 41% lower as compared to the concentrations observed in a reference population of 20 HIV-1-infected patients using SQV-HGC 1,200 mg *tid*.

Several causes may have contributed to the observed low saquinavir concentrations in the CHEESE Study. The plasma saquinavir concentration versus time curves as measured in the reference population were obtained after an observed ingestion of 1200 mg SQV-HGC together with a meal, since it is well known that saquinavir should be ingested with food to ensure adequate absorption (1). In the CHEESE Study, however, the medication was ingested at home (unobserved) and incorrect ingestion (e.g. without an adequate meal) may therefore be an important factor explaining the relative low plasma saquinavir concentrations, despite the use of SQV-SGC.
To exclude the possibility of inaccuracy of our bioanalytical assay as the cause for the low saquinavir concentrations, 40 samples from the CHEESE study were re-analysed by a commercial contract laboratory (using a radioimmunoassay). Cross-analysis of these samples with saquinavir concentrations ranging from 29 to 1,737 ng/mL (median 237 ng/mL)

resulted in a median difference in the saquinavir concentrations of 2.2% (interquartile range -7.4 - 10.8%), which confirmed our results.

During the course of the study the exposure to saquinavir decreased from a CR of 0.70 at week 4 to a CR of 0.55 at week 48. A similar observation was recently reported for HIV-1-infected patients using saquinavir 400 mg *bid* in combination with ritonavir 400 mg *bid* (11). In this study, the plasma pharmacokinetics of saquinavir and ritonavir were assessed in 6 HIV-1-infected patients on 2 occasions, 9-15 months apart. On the second study day, the area under the plasma concentration versus time curve (AUC) of saquinavir was decreased by 33% (median value) while the AUC of ritonavir was unaffected (11). Induction of the expression or activity of P-glycoprotein (Pgp), a multidrug transporting protein which is present in, amongst others, the gastrointestinal tract may contribute to this observation (12). Induction of the expression of Pgp has recently been reported to occur in response to the exposure to Pi's in cell culture (13). Furthermore, changes of the intestinal function due to the exposure to saquinavir may play a role in this respect (14,15). It was recently shown that saquinavir, ritonavir and nelfinavir but not indinavir impair the epithelial barrier in a human intestinal cell line (15). This might explain our observations in the CHEESE Study since the exposure to saquinavir decreased over time while the exposure to indinavir did not appear to change (16). Long-term effects of the exposure to saquinavir on its metabolism or altered patient adherence to the therapeutic regimen over time should also be considered.

Several studies have shown relationships between the exposure to protease inhibitors and their activity and toxicity (5-8, 17,18). These findings, in combination with the wide inter-individual variability in the pharmacokinetics of these drugs, have resulted in a growing interest in therapeutic drug monitoring (TDM) of antiretroviral drugs as an additional tool in the management of HIV-1-infected patients. In the current study, however, no such relationships were observed between the exposure to saquinavir and the virological and immunological response to triple therapy with SQV-SGC 1,200 mg *tid*, zidovudine and lamivudine in antiretroviral naive HIV-1-infected patients during a 48 week follow-up.

Our findings are in contrast with previously reported positive relationships between the exposure to saquinavir and the virological and immunological response. However, the patients in these studies were treated with saquinavir monotherapy (5,6) or were NRTI-experienced (7,8). Consequently, the triple drug regimen which was used by antiretroviral naive patients in the current study is expected to result in a more potent suppression of viral replication, which may reduce the influence of variation in saquinavir exposure on the virologic response. Furthermore, the relative small sample size and the marked intrapatient variability in the exposure to saquinavir may have hampered the detection of PK-PD relationships in our study.

Despite the relatively low plasma saquinavir concentrations in the CHEESE Study, 65.5% of the patients had a plasma HIV-1 RNA concentration < 50 copies/mL at 48 weeks of therapy (ITT-MF) with a median increase in the $CD4^+$ cell count of 150 cells/μL, which was not different from the control arm consisting of indinavir 800 mg *tid* plus zidovudine and lamivudine (16). Of note, the median CR of indinavir in the CHEESE Study was 1.08 suggesting that the exposure to indinavir was similar as in a reference population (16). Seventeen patients in our study continued the combination of SQV-SGC 1200 mg *tid* plus zidovudine and lamivudine after 48 weeks. After a median follow-up of 94 weeks (range 62 to 106 weeks), 14/17 (82%) and 17/17 patients (OT) had a plasma HIV-1-RNA concentration below 50 and 400 copies/mL, respectively. The overall median CR of saquinavir in these 17 patients during the whole treatment period was 0.52. The median overall CR in these 17 patients between week 48 and week 106 was 0.29, suggesting that the observed decrease in the exposure to saquinavir from week 4 to week 48 continues thereafter.

The strong antiviral response despite low saquinavir plasma concentration may be explained by intracellular pharmacokinetics of saquinavir. It was recently shown that the protease inhibitors have distinct intracellular pharmacokinetic properties *in vitro* (19,20). Interestingly, saquinavir showed more intracellular accumulation as compared to indinavir or ritonavir (19,20). Furthermore, the intracellular half-life of saquinavir was

estimated to be approximately 8 h, as compared to a plasma elimination half-life of 1-2 h (20). As a result, production of infectious HIV-1 was suppressed for up to 24 h after removal of extracellular saquinavir, depending on the concentration with which cells were treated initially (20). Accumulation of saquinavir at the site of HIV-1 replication (i.e. intracellular) may have contributed to the sustained virological response, despite relatively low plasma saquinavir concentrations. It was recently shown that patients with subtherapeutic plasma indinavir concentrations may have intracellular concentrations within acceptable limits (21). The relationship between the plasma- and intracellular pharmacokinetics of the protease inhibitors *in vivo* is currently unknown and should be investigated to understand the PK-PD relationships of the protease inhibitors.

In conclusion, a higher bioavailability of saquinavir when used as SQV-SGC versus hard gelatine capsules was not confirmed in this study. Furthermore, there is a trend of decreasing exposure to saquinavir over time. In contrast with previous findings, our data suggest that the plasma exposure to saquinavir may not be related to the suppression of viral replication in antiretroviral naive HIV-1-infected patients over a period of 48 weeks when used with a backbone of two NRTIs. Despite the relatively low plasma saquinavir concentrations in our study, a good virological and immunological response was observed which was maintained up to 106 weeks of follow-up. These findings warrant further evaluation of monitoring plasma drug concentrations during the management of HIV-1-infected patients.

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# 4

# Early recovery of CD4$^+$ T lymphocytes in children on highly active antiretroviral therapy

J.W.T. Cohen Stuart[1]
W.A.T. Slieker[2]
G.T. Rijkers[3]
A.Noest[4]
C.A.B. Boucher[1]
M.H. Suur[6]
R. de Boer[4]
S.P.M. Geelen[3]
H.J. Scherpbier[5]
N.G. Hartwig[6]
H. Hooijkaas[2]
M. Th. Roos[7]
E.R. de Graeff-Meeder[3]
R. de Groot[6]

1 Dept. of Virology, Eijkman-Winkler Institute, University Hospital Utrecht
2 Dept. of Immunology, Erasmus University University Hospital Rotterdam
3 Dept of Pediatrics, University Children's Hospital, Utrecht
4 Dept of Theoretical Biology, University Utrecht
5 Dept of Pediatrics, Academic Medical Center, Amsterdam
6 Dept. Of Pediatrics, University Children's Hospital, Rotterdam
7 Department of Clinical Viro-Immunology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, The Netherlands

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## Abstract

*Introduction*: Regeneration of CD4+ T lymphocytes has been shown to be thymus-dependent in bone marrow transplant recipients and after intensive chemotherapy. The rate of CD4+ T-cell regeneration positively correlates with enlargement of the thymus on radiographs and higher rates of CD4+ T-lymphocyte regeneration were observed in children as compared to adults, consistent with diminishing thymic function with age.

We hypothesized that in HIV infected patients CD4+ T-cell recovery during highly active antiretroviral therapy (HAART) may also be thymus dependent. Therefore, repopulation of naive (CD45RA+), memory (CD45RO+) and total CD4+ T lymphocytes and total CD8+ T lymphocytes in peripheral blood was assessed in 13 HIV infected children during the initial 3 months of HAART.

*Results*: Significantly higher recovery rates of naive, memory and total CD4+ T cells in children below the age of three years were observed as compared to older children. Kinetics of total CD8+ T cells showed no relation to age. Moreover, recovery rates of naive CD4+ T cells in patients below 3 years of age were 10-40 fold higher as compared to previously reported naive CD4+ T-cell recovery rates in adults on HAART.

*Conclusions*: High recovery rates of naive, memory and total CD4+ T cells can be achieved in children below 3 years of age. Changes of CD8 counts did not correlate with age. These results indicate that regeneration of CD4+ T cells during HAART may be a thymus dependent process.


**Keywords:** CD4, CD8, Pediatrics, Antiretroviral therapy, Immune Reconstitution.

## Introduction

Treatment of HIV infected adults with highly active antiretroviral treatment (HAART) results in a decline of plasma HIV RNA levels and an increase of circulating $CD4^+$ T cells. However, because of slow $CD4^+$ T-cell regeneration in adults, complete restoration of $CD4^+$ T-cell numbers and function, if possible at all, will take considerable time. The reconstitution pattern of $CD4^+$ T cells in peripheral blood during HAART is biphasic: an initial rapid rise of $CD4^+$ T cells in the first month of treatment is followed by a much lower rate of $CD4^+$ T-cell increase. The initial rapid CD4 cell rise is predominantly due to an increase of circulating memory $CD4^+$ T cells. Recovery rates of naive $CD4^+$ T cells are low throughout the course of treatment (1,2).

In humans, the capacity to regenerate $CD4^+$ T cells declines with age. In children who received antineoplastic chemotherapy or bone marrow transplantation, the rate of $CD4^+$ T-cell regeneration was higher as compared to adults due to production of $CD45RA^+$ (naive) CD4 cells (3,4,5). In these children, increased production of $CD45RA^+/CD4^+$ T cells is associated with thymic enlargement on radiographs. On the other hand, in adults, sustained depletion of $CD4^+$ T cells has been witnessed after intensive antineoplastic chemotherapies, BMT or CD4 depletion therapy for multiple sclerosis or rheumatoid arthritis using anti CD4 antibodies (3-9). The difference in $CD4^+$ T-cell recovery rates between children and adults suggest that the pathway of $CD4^+$ T-cell regeneration is thymus dependent (10) and that the capacity for $CD4^+$ T-cell regeneration diminishes with age as a result of thymic involution.

In contrast, thymus independent pathways exist for regeneration of $CD8^+$ T cells, via peripheral expansion of mature $CD8^+$ T cells in the lymphoid organs (11,12).

The thymus-dependence of $CD4^+$ T-cell regeneration leads one to expect a more pronounced increase of $CD4^+$ T cells following onset of potent antiretroviral therapy in children as compared to HIV infected adults. In this study we assessed repopulation in blood of $CD4^+$ and $CD8^+$ T cells and naive and memory $CD4^+$ T cells in HIV infected children on HAART.

## Methods

Plasma HIV RNA levels and kinetics of $CD4^+$ and $CD8^+$ T-cell subsets were analyzed as part of a study investigating the efficacy and safety of combination therapy with zidovudine, lamivudine and indinavir in HIV infected pediatric patients. Dosages of each drug were as follows; Zidovudine: 360 mg/$mm^2$/day (t.i.d.); Lamivudine: > 30kg: 150mg (b.i.d.), < 30 kg: 8mg/kg/day (b.i.d.); Indinavir 100-200 mg/day/kg metabolic weight (t.i.d.). Blood samples were obtained at baseline and after two weeks, 4 weeks, 3 months and 6 months from the onset of therapy. Compliance regarding medication intake was assessed during each visit.

Plasma HIV RNA levels were measured using a quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay (Amplicor HIV-1 Monitor, Roche Diagnostic Systems). The lower limit of detection was 400 copies / ml.

Lymphocyte immuno-phenotyping for $CD4^+$ and $CD8^+$ T cells was determined in whole blood by two color flow cytometry, using anti CD3, anti CD4, anti CD8, anti CD45RA and anti CD45RO monoclonal antibodies. $CD4^+$ T lymphocytes expressing the CD45RA surface antigen were considered thymic emigrants and naive cells, $CD4^+$ T lymphocytes expressing the CD45RO surface antigen were considered memory type cells.

In the analysis, data were included from patients who were at least 90 days on therapy and of whom immuno-phenotyping data was available from at least three time points: At baseline, one at 90 days and at least one in between (at week 2 and / or week 4 after initiation of therapy). Data from patients with reported non compliance regarding intake of medication, were excluded from analysis.

At the moment of this analysis, 26 patients had entered the cohort. Data from thirteen children could not be used for analysis due to the following reasons: Of 8 children (0.95-14.9 years old) no blood samples from week 12 were available for immuno-phenotyping and for 2 children (3.1 and 7.3 years old) no blood samples were available from baseline. Two patients (1.1 and 2.5 years old) were non compliant regarding intake of medication. For

**Table 1.** *Baseline characteristics*

| patient | age (years) | plasma HIV RNA (copies / ml) | CD4 (cells / µl) |
|---|---|---|---|
| a | 0.80 | 682000 | 1720 |
| b | 0.88 | 761500 | 1154 |
| c | 2.88 | 51220 | 153 |
| d | 5.39 | 145273 | 195 |
| e | 5.40 | 194300 | 192 |
| f | 6.99 | 32580 | 4 |
| g | 7.70 | 65700 | 65 |
| h | 9.62 | 16800 | 284 |
| i | 10.55 | 18400 | 202 |
| j | 11.17 | 201700 | 4 |
| k | 13.16 | 393420 | 48 |
| l | 13.23 | 1345 | 233 |
| m | 16.33 | 1910 | 11 |

one patient (7.3 years old), no blood samples at all were collected for immuno-phenotyping. The median age of the group excluded from analysis was 3.7 years (range 0.95-14.9 years), whereas the median age of the included group was 7.7 years (range 0.8-16.3 years)). Median CD4 count at baseline of the excluded group was: 502 cells / µl (range 293-1864 cells / µl). In the included group the median CD4 baseline count was 202 cells / µl (range 4-1720 cells / µl) (table 1).

## Results

Thirteen children were included in the analysis of whom the baseline characteristics are shown in table 1. Median plasma HIV RNA level at baseline was 69625 copies / ml.

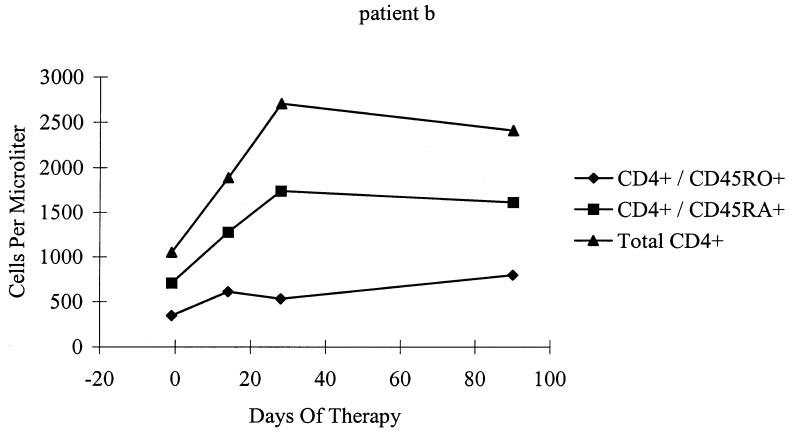


**Figure 1A.** *(Patient b) Responses of circulating numbers of naive and memory and total CD4+ T-lymphocytes during highly active antiretroviral therapy.*

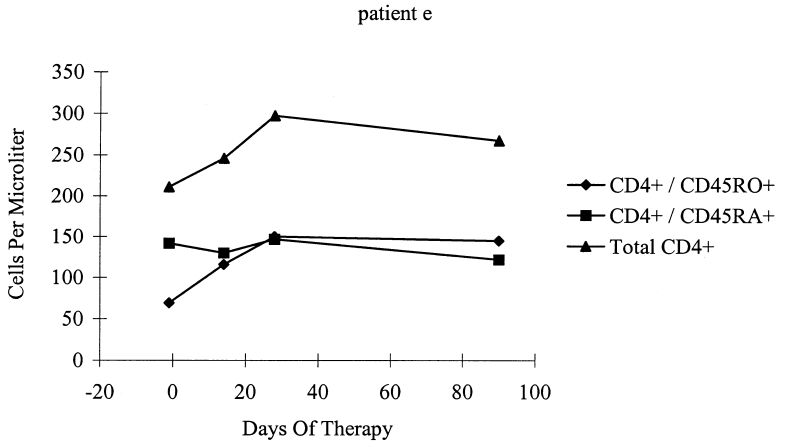


**Figure 1B.** *(Patient e) Responses of circulating numbers of naive and memory and total CD4+ T-lymphocytes during highly active antiretroviral therapy.*

**Table 2.** *Rates of lymphocyte recovery and viral load reduction during the initial three months of therapy.*

| patient | age (years) | recovery rate (cells/µl/day) $CD4^+/CD45RA^+$ | recovery rate (cells/µl/day) $CD4^+/CD45RO^+$ | recovery rate (cells/µl/day) total $CD4^+$ | recovery rate (cells/µl/day) total $CD8^+$ | viral load reduction (10 log) |
|---|---|---|---|---|---|---|
| a | 0.80 | 9.30 | 7.13 | 16.4 | 0.17 | 2.99 |
| b | 0.88 | 8.58 | 4.10 | 12.7 | 16.2 | 3.18 |
| c | 2.88 | 3.20 | 1.90 | 5.8 | 3.2 | 2.11 |
| d | 5.39 | -0.33 | -0.45 | -0.78 | -1.4 | 2.56 |
| e | 5.40 | -0.18 | 0.64 | 0.47 | -6.75 | 2.59 |
| f | 6.99 | 0.44 | 1.11 | 1.54 | 18.4 | 1.06 |
| g | 7.70 | 0.57 | -0.21 | 0.36 | 1.16 | 1.79 |
| h | 9.62 | 1.00 | 0.42 | 1.42 | -1.52 | 1.53 |
| i | 10.55 | 0.65 | 0.05 | 0.69 | -0.02 | 1.57 |
| j | 11.17 | -0.02 | 0.02 | 0.004 | 7.28 | 0.12 |
| k | 13.16 | 1.29 | 0.52 | 1.81 | 1.82 | 3.11 |
| l | 13.23 | 0.30 | 0.08 | 0.38 | -0.8 | 0.43 |
| m | 16.33 | 0.01 | 0.08 | 0.09 | -0.98 | 0.58 |

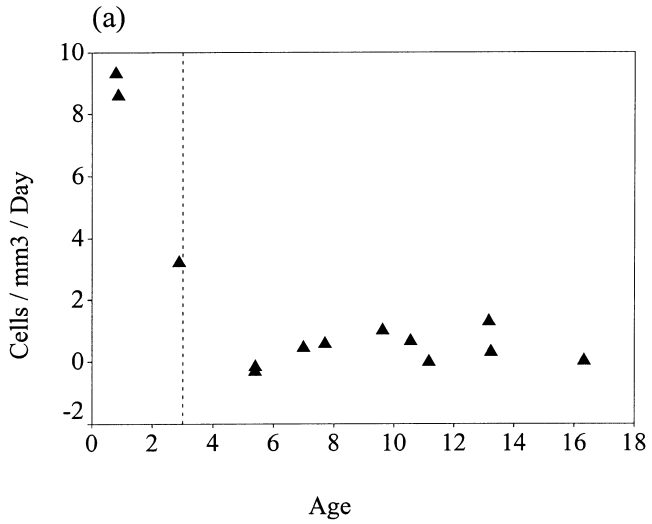


**Figure 2A.** *Recovery rates of CD4+/CD45RA+ T lymphocytes (cells/μl/day) versus age (years).*

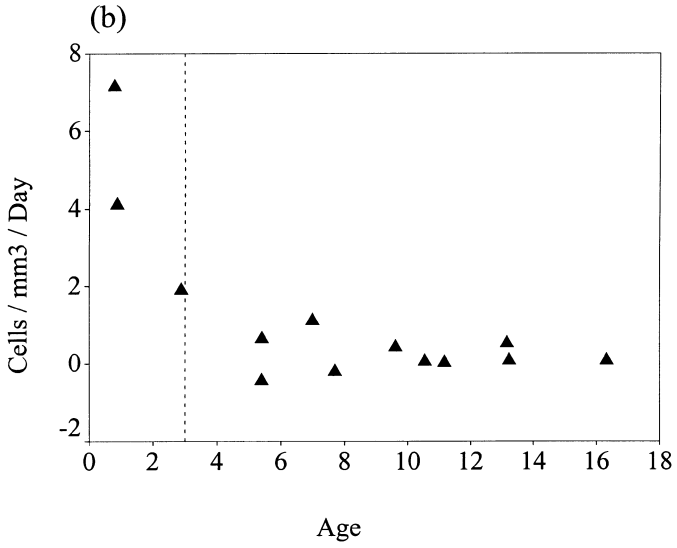


**Figure 2B.** *Recovery rates of CD4+/CD45RO+ T lymphocytes (cells/μl/day) versus age (years).*

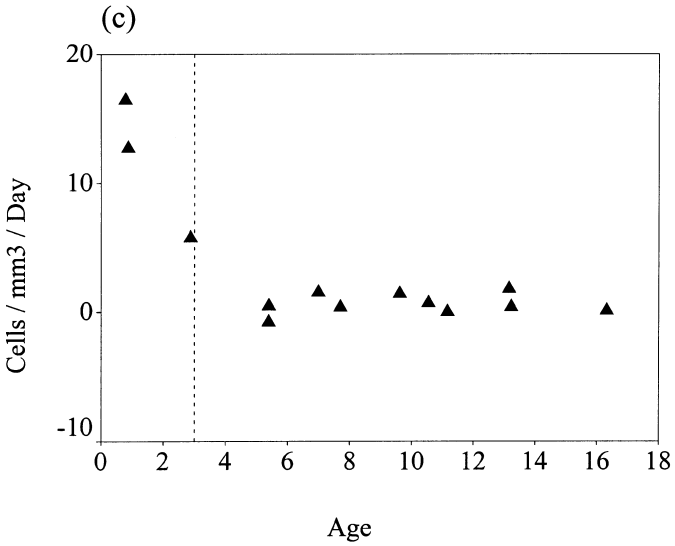


**Figure 2C.** *Recovery rates of total CD4⁺T-lymphocytes (cells/μl/day) versus age (years).*

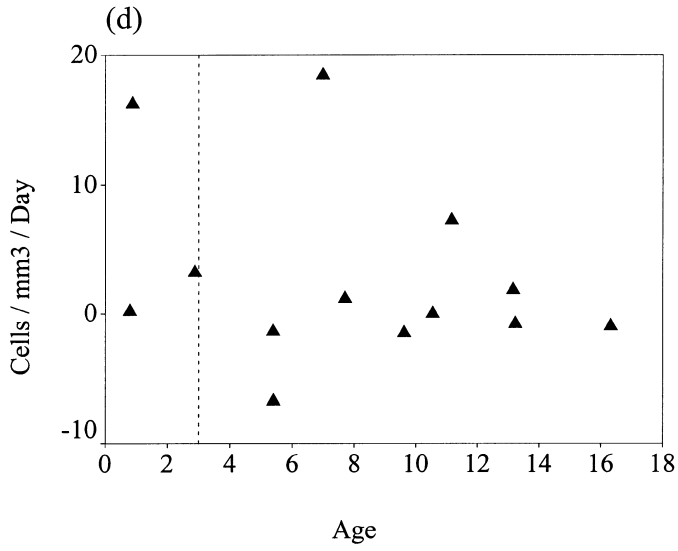


**Figure 2D.** *Recovery rates of total CD8⁺ T-lymphocytes (cells/μl/day) versus age (years).*

During the initial three months of therapy median HIV RNA levels decreased significantly to 500 copies / ml. Median total $CD4^+$ T cells rose from 202 (range 4-1864) cells / µl to 327 (range 11-2150) cells / µl. Median total $CD8^+$ T cells decreased from 936 to 880 cells / µl.
Responses of naive and memory and total $CD4^+$ T cells of two representative patients (b and e) are shown in Figure (1A and 1B). Linear regression analysis was used to calculate recovery rates in blood of total CD4 and CD8 cells and memory and naive CD4 cells during the first 90 days of treatment. Recovery rates were expressed as the change in the absolute number of cells per µl blood per day, as is shown in table 2.
Recovery rates of total $CD4^+$ and total $CD8^+$ T cells and memory and naive CD4 cells were plotted as a function of age at the start of therapy (Figure 2A-2D). A significant difference in recovery rates of naive, memory and total $CD4^+$ cells was observed between children younger then 3 years of age and older children ($p<0.02$, Wald-Wolfowitz test)(Figure 2A,2B,2C). None of the patients above the age of 3 years had $CD4^+$ T-cell recovery rates (total, naive or memory) higher than any of the patients below 3 years of age. In contrast, recovery rates of $CD8^+$ T cells did not show any relation to the age of the children (figure 2D).

## Discussion

We observed that recovery rates of naive ($CD45RA^+$), memory ($CD45RO^+$) and total $CD4^+$ T cells were higher in patients < 3 years in comparison to older children. In three children < 3 years we observed recovery rates of naive $CD4^+$ T cells of 3.20, 8.58 and 9.30 cells/µl/day, which is 10-40 fold higher as compared to recovery rates of naive $CD4^+$ T cells in adults (0.2-0.4 cells/µl/day)(1)(Cohen Stuart et al., unpublished data). Thus, although the number of patients in this analysis is limited, our data clearly demonstrates the possibility of rapid recovery of naive $CD4^+$ T cells in young infants below three years of age.
These observations may be explained in several ways. Because the patients in this study were horizontally infected, except for patient h, k and m (9.6, 13,2 and 16.3 years, respectively) the age of the patient generally equals

the duration of HIV infection. Therefore, the shorter duration of HIV infection in the youngest children may explain the high CD4+ T-cell recovery rates. An age dependent capacity to redistribute T lymphocytes from lymphoid tissues into the circulation after start of HAART could also account for differences of CD4 cell recovery rates. However, such explanation is inconsistent with the absence of any relation between age and CD8 cell kinetics during HAART in this set of children.

When comparing the three youngest patients (a-c) with the others (d-m), it should be noted that patients a and b had higher baseline CD4 counts in comparison to the others. This is probably related to their age. Babies often have high numbers of circulating lymphocytes. It may be argued that high $CD4^+$ T-cell recovery rates in these young children (< 3 years) may result from high $CD4^+$ T-cell baseline values (table 1). Secondly, patient a and b had higher plasma viral loads at baseline in comparison to the other patients. However, in adults, it has been demonstrated that CD4 recovery does not depend on either baseline plasma HIV RNA or baseline CD4 count values (13). Furthermore, no significant difference in the magnitude of viral load reduction was found between the group of youngest (a-c) and the group of older patients (d-m)(Wald-Wolfowitz test: $p=0.71$).

Theoretically, CD45 isotype switching from CD45RO to CD45RA phenotype may also account for increase of $CD45RA^+/CD4^+$ T lymphocytes (15-17). However, direct evidence for reversion from memory to naive phenotype in CD4 T lymphocytes has exclusively been found in experimental murine models. Data on reversion of memory to naive phenotype are conflicting (12) and it is unclear to what extend this phenomenon plays a role in humans in vivo. Moreover, a rapid increase of $CD45RA^+$ $CD4^+$ T cells occurred in patients who also had a rapid increase of total $CD4^+$ T cells. Therefore, if thymus input of $CD45RA^+$ cells would play a minor role and peripheral CD4 T-cell expansion would account for CD4 T-cell increase, the reversion from CD45R0 to CD45RA would have to occur at a substantial level in dividing CD4 cells. This is inconsistent with data demonstrating CD45R0 expression in dividing T-cell populations (18).

We conclude that high recovery rates of naive $CD45RA^+/CD4^+$ T cells in patients <3 years of age during HAART may be best explained by the

presence of a functional thymus. Recently, it was reported that regeneration of CD45RA+/CD4+ T cells and expansion of thymus volume are positively correlated in HIV infected children during HAART (14).
After antineoplastic chemotherapy, the rate of CD4 cell recovery gradually diminishes with age (3,4). In children on HAART, we did not find a gradual decrease of the rate of CD4 recovery but in subjects older than 3 years we observed CD4 recovery rates comparable to the rates of adult HIV infected patients on HAART. This may be explained by precocious involution of the thymus, as a result of HIV mediated destruction of thymic parenchyma.
In summary, we demonstrated that high recovery rates of naive, memory and total CD4+ T cells can be achieved in children below the age of 3 years on HAART, whereas CD8+ T-cell kinetics showed no relation to age. This indicates that regeneration of CD4+ T cells during HAART uses a thymus-dependent pathway.

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# 5

# Reconstitution of naive T cells during antiretroviral treatment of HIV infected adults is dependent on age

James Cohen Stuart[1,2]
Dörte Hamann[3]
Jan Borleffs[2]
Marijke Roos[3]
Frank Miedema[3]
Rob de Boer[4]
Charles Boucher[1]

1 Department of Virology, Eijkman- Winkler Institute, University Medical Center Utrecht
2 Department of Internal Medicine, University Medical Center Utrecht
3 Department of Clinical Viro-Immunology, CLB, and the Laboratory for Experimental and Clinical Immunology Department of Human Retrovirology, Academic Medical Center, University of Amsterdam
4 Theoretical Biology, University Utrecht, the Netherlands.

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## Abstract

*Introduction*: The mechanism of regeneration of naive $CD4^+$ and $CD8^+$ T cells during highly active antiretroviral therapy (HAART) is unclear. If it were mainly dependent on thymic function, the regeneration rate should decrease with age, due to thymic involution.
*Objective*: To determine the influence of age on the regeneration rate of naive and memory T cells in 45 adults on HAART.
*Methods*: The number of naive and memory T cells was determined in whole blood. Naive cells were defined as $CD45RA^+CD27^+$. Cells negative for CD45RA and/or CD27 were considered memory type cells.
*Results*: The age of the patients ranged from 25 to 57 years. On average (±SEM), the regeneration rates of naive $CD4^+$ and $CD8^+$ T cells were $0.34\pm0.04$ and $0.36\pm0.04$ cells/$mm^3$/day, respectively, which is not significantly different ($p=0.5$). The recovery rates of naive $CD4^+$ and $CD8^+$ T cells were negatively correlated to age ($r= -0.41$ and $r= -0.47$, respectively). The recovery rate of memory T cells showed no relation to age. The regeneration rate of naive $CD4^+$ T cells during HAART is similar to previously reported regeneration rates in adults following cytotoxic chemotherapy or CD4 mAb therapy. This suggests a little, or at least reversible, impact of HIV on naive T-cell production.
*Conculusion*: The equal regeneration rate of naive $CD4^+$ and $CD8^+$ T-cells during HAART, and the inverse correlation between age and the recovery rate of naive T cells, both suggest that the thymus contributes considerably to the regeneration of naive T cells in adults on HAART.


**Keywords:** Antiretroviral therapy, CD4, CD8, Naive/memory, Immune Reconstitution

## Introduction

Infection with HIV causes progressive depletion of both naive and memory $CD4^+$ T cells. The CD8 count of HIV infected patients is usually increased until shortly before the onset of AIDS. This increase is due to expansion of the memory $CD8^+$ T-cell population. Naive $CD8^+$ T-cell counts decrease gradually from early after seroconversion (1,2).

In most patients, highly active antiretroviral therapy (HAART) leads to a substantial rise of the CD4 count, primarily due to an increase of memory $CD4^+$ T cells (3-5). The effect of HAART on the CD8 count varies per study from a slight increase (4,6,7) to a decrease (3,8,9). Naive $CD4^+$ and naive $CD8^+$ T cells increase slowly during HAART (3,4,10).

The mechanism of regeneration of the naive T cells during HAART is unclear. It has been attributed to thymus dependent production (10-13), proliferation of naive T cells (11,12,14,15) and reversion from the memory to the naive phenotype (15). To understand the mechanism of naive T-cell regeneration is important because HIV induced deletions in the T-cell receptor repertoire (16,17) can exclusively be restored via thymus dependent production of naive T cells.

The generation and production of T cells is primarily located in the thymus. Because of thymic involution with increasing age (18,19), the capacity to regenerate T cells after depletion diminishes with age. It has been demonstrated that the recovery rate of peripheral $CD4^+$ T cells after antineoplastic chemotherapy decreased with the age of the patients. In adults over 20 years of age, $CD4^+$ T-cell recovery after depletion is slower than in children and appears to be predominantly due to thymus-independent peripheral proliferation (20-24).

Here, we tested whether in HIV infected adults the speed of regeneration of naive T cells during HAART decreases with age. Therefore, we determined the influence of age on the rate of reconstitution of naive and memory $CD4^+$ and $CD8^+$ T cells in 45 adults with successful suppression of plasma HIV RNA levels during HAART.

## Material and methods

**Study population**

The recovery of naive and memory T cells was analyzed in all patients from the previously described CHEESE study cohort (25) with a sustained plasma HIV RNA response to less than 50 copies/ml (n=45). Briefly, this is a randomized study comparing antiviral efficacy of zidovudine (Retrovir, Glaxo-Wellcome, Research Triangle Park, N.C.) plus lamivudine (Epivir, Glaxo-Wellcome, Research Triangle Park, N.C.) plus saquinavir-soft-gelatin-capsules (SQV-SGC, Fortovase, Hoffmann-La Roche, Inc., Nutley, New Jersey) versus zidovudine plus lamivudine plus indinavir (Crixivan, Merck, West Point, Pa), in HIV-1 infected patients. Antiretroviral naive patients were eligible for study treatment if at the moment of screening plasma HIV RNA levels were at least 10,000 copies/ml and/or if CD4 counts were less than 500 cells/$mm^3$ and/or if they had a history of HIV related symptoms (CDC stage B or C). During 48 weeks of treatment, the virologic and the CD4 count response was not different between the two treatment arms (26). Of the selected patients, 23 were from the indinavir arm and 22 from the SQV-SGC arm.

**Blood sampling**

Blood samples were obtained at week -2, at week 0 and every 4 weeks through week 24 and every 8 weeks from week 24 through week 48 of treatment.

**Plasma Viral Load**

Plasma HIV RNA levels were measured using an investigational version of the ultra sensitive quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay (Amplicor HIV-1 Monitor, Roche Diagnostic Systems). The lower limit of detection was 50 copies / ml.

**Monoclonal antibodies**

Peridinin chlorophyll protein (PerCP)-labeled CD4, PerCP-labeled CD8 and phycoerythrin (PE)-labeled CD45RA Mab's were obtained from Becton Dickinson (San Jose, CA). FITC labeled CD27 Mab's were obtained

from CLB (Amsterdam, the Netherlands).*Flow Cytometry.* The fraction of naive and memory (CD45RA- and $CD45RA^+/CD27^-$) T cells was determined by three color FACS analysis using monoclonal antibodies against CD4 (or CD8), CD45RA, and CD27 on EDTA anticoagulated venous blood (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA).

**Statistical Analysis**

Parametric Pearson's correlation coefficients were computed to measure bivariate correlations. All variables used to compute Pearson's correlation coefficients were normally distributed, as tested by the Kolmogorov-Smirnov one-sample test. The paired-samples t test for two related samples was used to detect a difference between the recovery rates of naive $CD4^+$ and $CD8^+$ T cells. All reported p-values are two-sided. All statistical analyses were performed using SPSS for Windows (8.0.0).

## Results

Because immune recovery during HAART is dependent on virus suppression to levels below the limit of detection (27), we investigated the reconstitution of T cells during 48 weeks of therapy in a group of patients who all achieved plasma HIV RNA levels below 50 copies/ml. The baseline characteristics of the patients were as shown in Table 1. The mean age of the patients at baseline was 37.2 years (range 25-57 years). The median plasma HIV RNA level at baseline was 72,000 copies/ml. The patients achieved plasma HIV RNA levels below 50 copies/ml within a median period of 16 weeks (range 4-44).

In the analysis of T-cell reconstitution, T cells co-expressing CD45RA and CD27 were considered truly naive cells (28,29). Memory T cells were defined as $CD45RA^+/CD27^-$ and as $CD45RA^-$ (both $CD45RA^-/CD27^+$ and $CD45RA^-/CD27^-$). During the initial 48 weeks of therapy, the mean (±SEM) total, naive and memory $CD4^+$ T-cell count in peripheral blood increased with 206±34, 112±17 and 53±23 cells/$mm^3$, respectively (Figure 1). The total and memory $CD8^+$ T-cell count decreased with

**Table 1,** *Patient Characteristics At Baseline And Week 48 (N=45)*

| | BASELINE | WEEK 48 |
|---|---|---|
| Age (years ± SD) | 37.2 ± 8.3 | N.A. |
| Plasma HIV RNA levels (copies /ml) | | |
| Median | 72000 | <50 |
| Range | 1630-2,310,000 | N.A. |
| Mean T-cell counts (cells/$mm^3$ ± S.E.M): | | |
| Total $CD4^+$ | 301 ± 28 | 507 ± 40 |
| Naive $CD4^+$ | 98 ± 13 | 210 ± 21 |
| Memory $CD4^+$ | 193 ± 18 | 286 ± 27 |
| Total $CD8^+$ | 1010 ± 80 | 872 ± 43 |
| Naive $CD8^+$ | 131 ± 14 | 257 ± 17 |
| Memory $CD8^+$ | 826 ± 67 | 586 ± 43 |

140±60 and 240±55 cells/$mm^3$ in 48 weeks. The number of circulating naive $CD8^+$ T cells increased with 126±16 cells/$mm^3$ during 48 weeks of therapy.

To estimate the average recovery rate of naive and memory T cells in the blood during the initial 48 weeks of HAART, linear regression analysis was used. The mean (± S.E.M.) recovery rates of naive $CD4^+$ and $CD8^+$ T cells were 0.34±0.04 and 0.36±0.04 cells/$mm^3$/day, respectively. These rates are similar to previously reported recovery rates of naive T cells during HAART in adults (3,4,13). The recovery rates of naive $CD4^+$ and naive $CD8^+$ T cells were not significantly different (paired samples t test, $p=0.5$) and correlated positively ($r = 0.7$, $p<0.001$; Figure 2). The recovery of memory $CD4^+$ T cells was biphasic, with a rapid recovery rate in the first 4 weeks of therapy (1.46±0.4 cells/$mm^3$/day) and a slow recovery rate between week 4 and week 48 (0.17±0.06 cells/$mm^3$/day), consistent with findings of others (4). The mean recovery rate of memory $CD4^+$ T cells over the whole study period (week 0-week 48) was 0.22±0.05

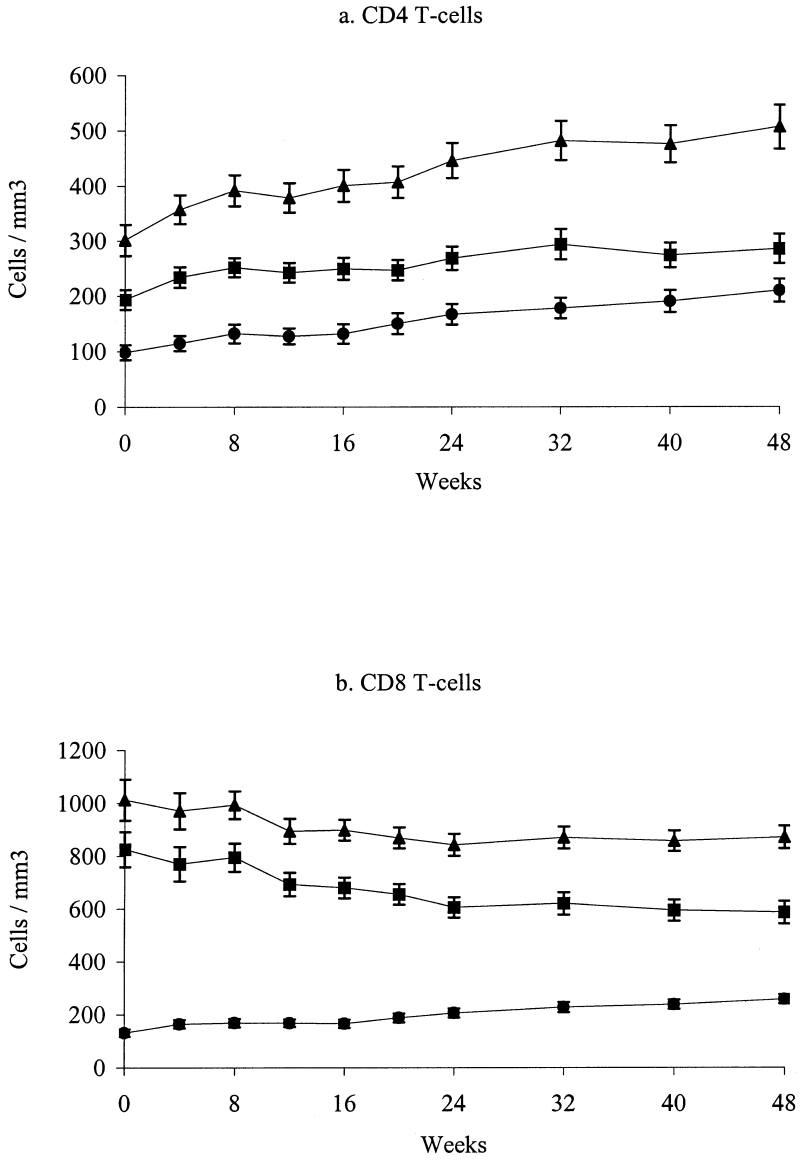


**Figure 1a.** *Reconstitution of absolute number of circulating (a) CD4⁺ and (b) CD8⁺ T cells during 48 weeks of HAART: total count (▲), memory cells (■) and naive cells (●).*

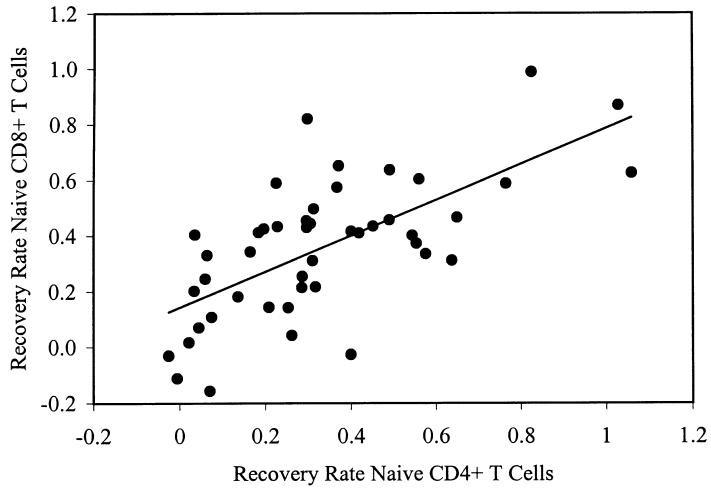


**Figure 2.** *Correlation between the regeneration rate of naive CD4⁺ T cells and naive CD8⁺ T cells (r = 0.7, p<0.001).*

cells/mm$^3$/day. The daily decrease of memory CD8⁺ T cells was 0.72±0.13 cells/mm$^3$/day. No differences of recovery rates were observed between the two treatment groups (data not shown).

To eliminate the contribution of lymphocyte redistribution on the recovery of naive T cells in the blood, the recovery rates were also estimated for the period when plasma HIV RNA levels were below 50 copies/ml. Redistribution of lymphocytes from lymphoid tissue to the blood predominantly occurs during the first weeks of HAART, due to decreased inflammation in the lymphoid tissue (4). The recovery rates of naive CD4⁺ and CD8⁺ T cells in the period after the patients achieved plasma HIV RNA levels <50 copies/ml were 0.34±0.06 and 0.35±0.05 cells/mm$^3$/day, respectively. These recovery rates were not significantly different from the recovery rates based on the entire 48 weeks of treatment (p=0.99 and p=0.7, respectively), suggesting that redistribution had no significant influence on our estimates of the recovery rate of naive T cells.

Because thymic function diminishes with increasing age, we determined the influence of age on the speed of naive T-cell regeneration. The recovery rates of the naive CD4⁺ and CD8⁺ T cells correlated negatively with the age of the patients (r = -0.41, p=0.005 and r = -0.47, p=0.001,

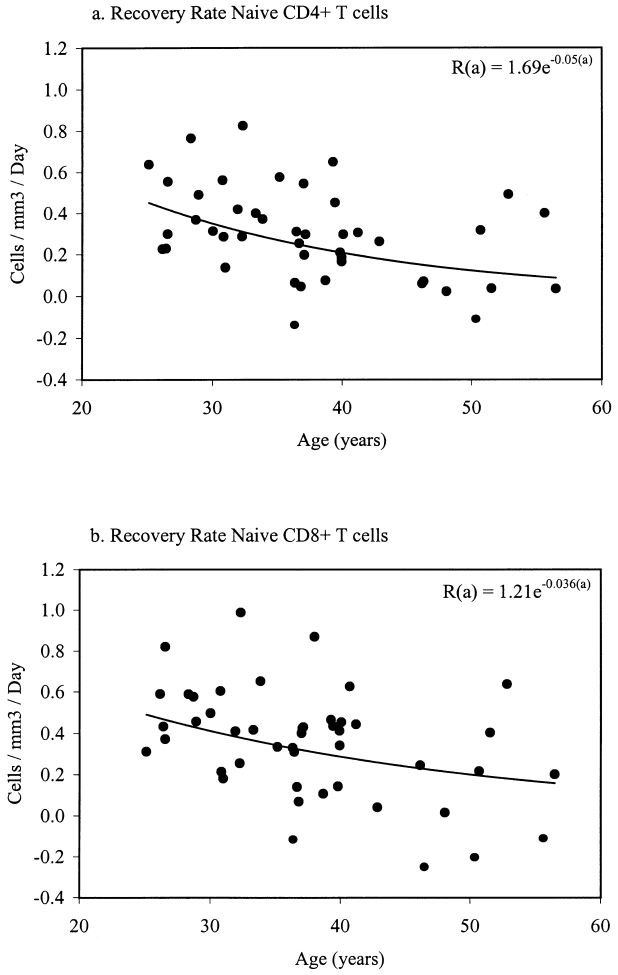


**Figure 3.** *Correlations between age and recovery rates of T-cell subsets. (a) Correlation between age and the recovery rate of naive CD4⁺ T cells (r = -0.41, p=0.005). The decrease of the recovery rate of naive T cells with increasing age fitted to an exponential function: R(a) = $p1e^{p2(a)}$, where R(a) is the recovery rate and a is the age. The parameters p1 and p2 (± S.D.), found by non-linear regression analysis (excluding 2 negative recovery rates) were as follows: p1=1.7 (±1.8), p2= -0.050 (±0.016), corresponding to a decay rate of 5.0% per year.*
*(b) Correlation between the age and the recovery rate of naive CD8⁺ T cells (r = -0.47, p=0.001). The parameters of the R(a) function (excluding 4 negative recovery rates) were: p1=1.2 (±1.7), p2= -0.036 (±0.016), corresponding to a decay rate of 3.6% per year.*

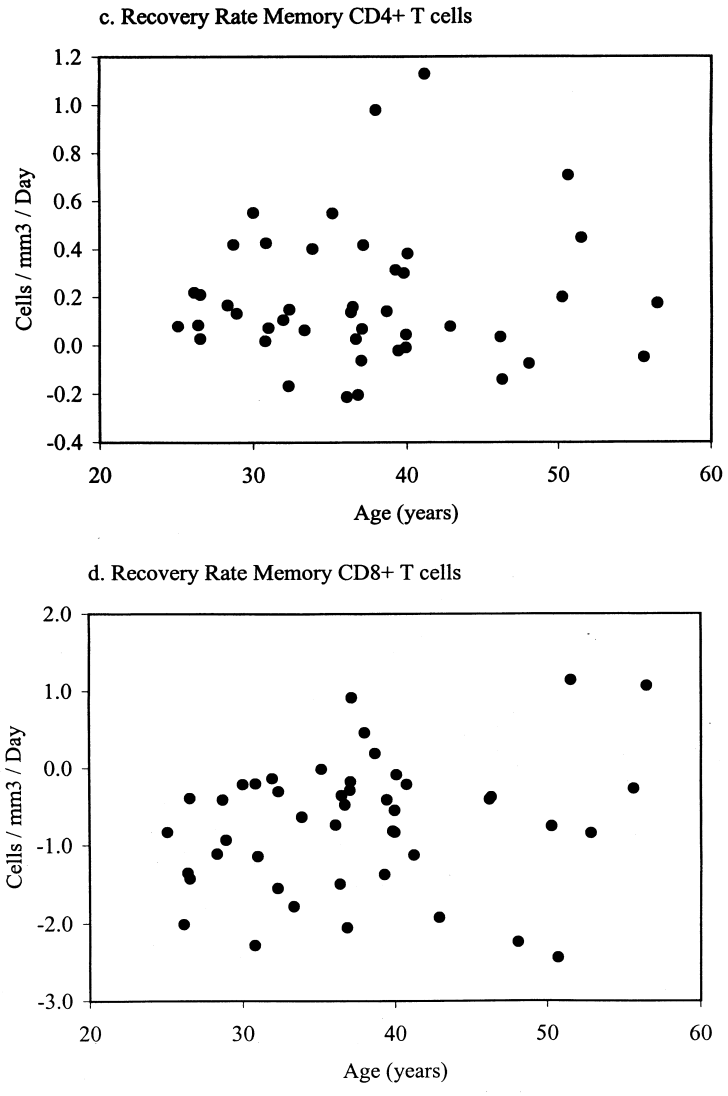


**Figure 3.** *Correlations between age and recovery rates of T-cell subsets.*
*(c) Correlation between the age and the recovery rate of memory CD4⁺ T cells (r = -0.07, p=0.6).*
*(d) Correlation between the age and the decrease rate of memory CD8⁺ T cells (r=0.1, p=0.2).*

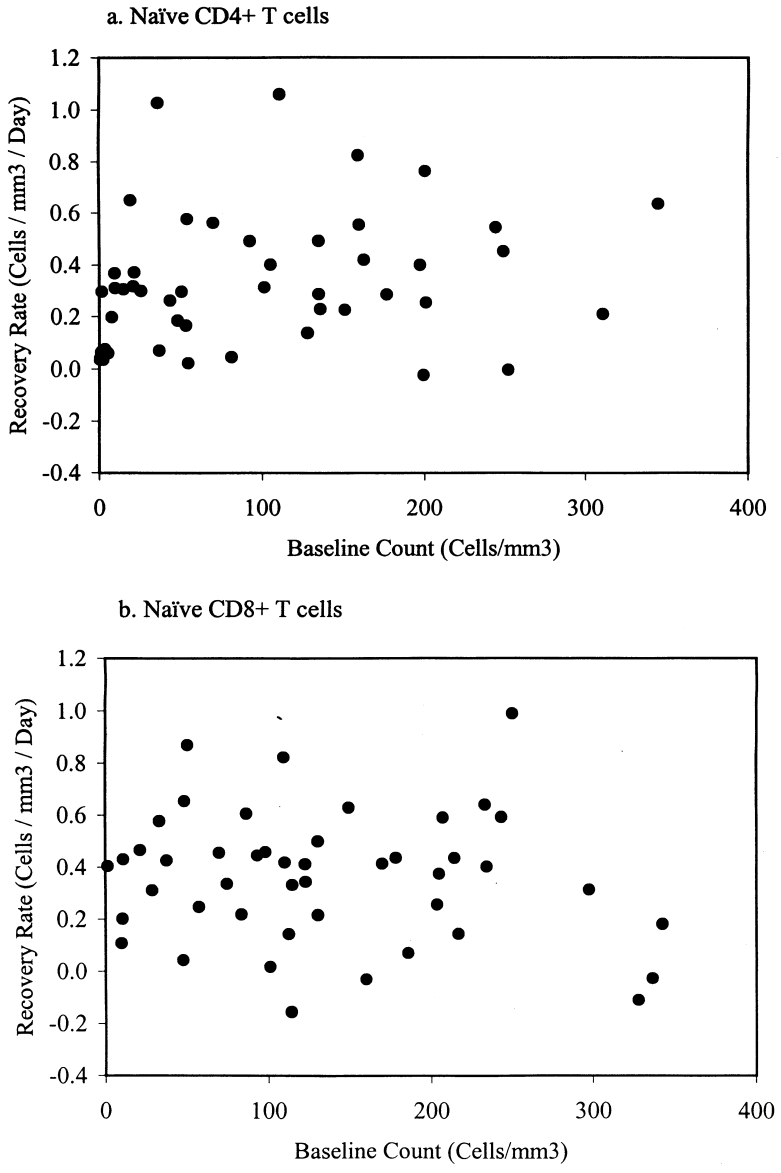


**Figure 4.** *Correlation between baseline naive T-cell counts and the recovery rate of naive T cells during HAART. Figure 4a. Naive CD4⁺ T cells. Figure 4b. Naive CD8⁺ T cells.*

respectively; Figure 3). The decrease of the recovery rates with increasing age fitted best to an exponential function (Figure 3a, 3b). The recovery rates of naive $CD4^+$ and $CD8^+$ T cells decreased 5.0 % per year (p=0.003) and 3.6% per year (p=0.02), respectively. The recovery rate of memory $CD4^+$ T cells was not correlated to the age (r=-0.07, p=0.6), nor was the daily decrease of memory $CD8^+$ T cells (r=0.1, p=0.2). Thus, our results indicate that the age of the patients is an important factor in naive T-cell regeneration.

To investigate whether the regeneration rate of naive T cells depends on the number of pre-existent naive T cells, we determined the relation between pretreatment counts of naive $CD4^+$ and $CD8^+$ T cells and the recovery rate of the respective T-cell subsets. No significant correlations were observed between naive T cell baseline counts and recovery rates (r = 0.2 p=0.2 and r = -0.1, p=0.4, respectively, Figure 4). This suggests that peripheral expansion plays a limited role in the regeneration of naive T cells.

Next, we determined the contribution of other factors on the speed of regeneration of naive T cells. First, the reduction of plasma HIV RNA levels during 48 weeks of HAART did not correlate to the recovery rate of the naive $CD4^+$ and $CD8^+$ T cells (r = 0.1, p=0.3 and r = 0.07, p=0.6, respectively). Secondly, no correlation was found between the rate of naive T-cell recovery and surrogate markers for the stage of HIV disease at baseline, total CD4 count and HIV RNA plasma levels (each p value > 0.3). Thus, baseline naive T-cell counts, viral load reduction during therapy and disease stage prior to therapy, do not contribute in a major way to the regeneration rate of naive T cells during HAART.

## Discussion

In this study, we investigated the changes in the blood of the numbers of phenotypically naive and memory T cells during HAART. For the first time, we demonstrate that the recovery rate of naive $CD4^+$ and $CD8^+$ T cells during HAART is inversely correlated to the age of the patients (figure 3). The rate of increase in memory $CD4^+$ T cells and the decrease

rate of memory $CD8^+$ T cells showed no relation to the age of the patients. Regeneration of naive T cells during HAART may involve a thymus dependent and a thymus independent pathway. Although an influence of age on the thymic independent pathway cannot be excluded, the most plausible explanation for the inverse correlation between age and the regeneration rate of naive T cells is that the thymus dependent pathway plays an important role in naive T-cell regeneration, because thymic function diminishes with age (18,19). Moreover, the exponential decrease of the regeneration rates during aging (5.0% and 3.6% per year for naive $CD4^+$ and $CD8^+$ T cells, respectively, Figure 3) is in close agreement with the reported exponential decay of the volume of the thymic epithelial space at a rate of 4.1% per year in adults above 20 years of age (18). Apparently, the amount of thymic parenchyma plays a rate limiting role in the regeneration of naive T cells (13).

The inverse correlation between age and the recovery rate of naive T cells should be considered with one caveat, because the older patients may also have the longest duration of HIV infection. The duration of HIV infection was unknown in this study. However, laboratory markers of duration of infection, baseline CD4 count and plasma HIV RNA levels, did not correlate with either age nor with the recovery rates of naive T cells. The duration of infection is therefore not likely to be a confounding factor.

The finding that the recovery rates of naive $CD4^+$ and $CD8^+$ T cells during HAART are not significantly different ($p=0.5$) appears in contrast with previous observations that naive $CD8^+$ T-cell regeneration is faster than naive $CD4^+$ T-cell regeneration after antineoplastic chemotherapy (21,24). The higher regeneration rate of naive $CD8^+$ T cells was attributed to extrathymic production of naive $CD8^+$ T cells. However, these studies used CD45RA expression as the marker of naive $CD8^+$ T cells, which seems inaccurate because the $CD45RA^+/CD8^+$ T-cell population contains besides naive cells also fully differentiated effector cells (28,29). The very similar regeneration rate of truly naive $CD4^+$ and $CD8^+$ T-cells ($CD45RA^+/CD27^+$) in our patients suggests that generation of both cell types is under control of a common mechanism, consistent with thymic production of naive T cells.

Interestingly, the rate of increase of naive $CD4^+$ T cells in our patients is similar to the regeneration rates observed in HIV seronegative adults with

iatrogenic $CD4^+$ T-cell depletion due to either antineoplastic chemotherapy or treatment for rheumatoid arthritis with CD4 monoclonal antibodies (20,22,23,24,30). In our patients, the mean rate of increase of naive $CD4^+$ was 0.34 cells/$mm^3$/day, corresponding to an 38 year old subject in Figure 3. After iatrogenic T-cell depletion, the regeneration rates of naive $CD4^+$ T cells vary from 0.005 to 1.1 cells/$mm^3$/day. For the total body, the mean regeneration rate of naive $CD4^+$ T cells in our patients is 0.85 x $10^8$ cells/day, assuming that the total blood volume is 5 liters and that 2% of the lymphocytes are in the blood compartment. This number is in close agreement with estimates of a total body production rate of naive $CD4^+$ T cells of ~$10^8$ cells/day (31), which was based on the decay of lymphocytes with stable chromosome damage after radiation. Thus, in HIV infected patients, the capacity to regenerate naive T cells during HAART is comparable to the situation after iatrogenic T-cell depletion.

There is considerable evidence that the thymus dependent pathway to develop new naive T cells from bone marrow progenitors is downregulated during HIV infection. First, it was shown that $CD34^+$ hematopoietic progenitor cells are susceptible to HIV infection (32), and have impaired survival and clonogenic capacity during HIV infection (33). Second, it was demonstrated that the thymic tissue is affected in individuals with AIDS (34). In studies with SCID-hu mice it was shown that HIV infection results in depletion of $CD4^+$/$CD8^+$ thymocytes and reduction of the number of $CD4^+$ T-lymphocytes from the thymic implants (35-37). Finally, measuring T-cell receptor excised circles (TREC's) to identify recent thymic emigrants showed that the number of TREC positive T cells is decreased as compared to HIV negative individuals, which was taken as evidence for a reduced export of new T cells from the thymus in HIV infection (11,12). However, the decreased number of TREC positive T cells in HIV infected individuals may also be explained by the high division rate of T cells due to HIV infection (38,39). Our finding that the regeneration rate of naive T cells during HAART is comparable to that after iatrogenic T-cell depletion suggests that the HIV-induced dysfunctions of progenitor cells or thymus are either of minor importance or they are rapidly reversed after introduction of HAART.

The relatively low correlation coefficients between age and naive T-cell

recovery rates (Figure 3) may be explained in several ways. First, it may be due to a large variation of thymic output between individuals, in agreement with large differences in the numbers of TREC positive T cells found in healthy individuals (11,12). Secondly, several studies have suggested that thymus independent pathways of naive T-cell regeneration also play a role during HAART.

In one study, it was reported that the number of circulating naive ($CD45RA^+/CD62L^+$) $CD4^+$ T cells increased during HAART in a thymectomized patient (14). However, the increase of naive $CD4^+$ T cells was exclusively observed in the first 12 weeks of HAART and the number remained constant thereafter. The increase of naive $CD4^+$ T cells during 95 weeks of HAART was approximately 25 cells/$mm^3$ (0.038 cells/$mm^3$/day). In our study, and in studies by others with thymus-bearing patients, naive T-cell counts increased continuously throughout the complete course of HAART with a ~10 fold higher rate. The initial rise of naive $CD4^+$ T cells in the thymectomized patient (14) could therefore be explained by a redistribution of naive T cells from (lymphoid) tissue to blood, which typically occurs in the first weeks of HAART (4).

In two other studies, TRECs were measured to identify recent thymic emigrants during HAART (11,12). It was shown that the increases in TREC-positive T cells during HAART were numerically insufficient to account for the increases in phenotypically naive ($CD45RA^+/CD62L^+$) T cells. This suggests that naive T cells may proliferate without converting to the memory phenotype. Increased expression of the proliferation marker Ki67 on naive T cells has been observed in untreated HIV-1 infected patients with less than 100 naive T cells/$mm^3$ (40). However, Ki67 expression on naive T cells was shown to decrease rapidly during the first weeks of HAART (40), whereas in our study the increase of naive T cells is relatively constant, suggesting that proliferation plays a limited role in the regeneration of naive T cells. The absence of a positive correlation between baseline naive T-cell counts and the regeneration rates (Figure 4) also argues against peripheral expansion as a mechanism of naive T-cell regeneration.

Theoretically, reversion of memory cells may also contribute to the recovery of naive T cells during HAART (12,15). It has been suggested that $CD45R0^+$ (memory) T cells may revert to the CD45RA (naive)

isoform (41,42). However, using a combination of CD27 and CD45RA Mab's we were able to distinguish between truly naive T cells ($CD45RA^+/CD27^+$) and memory/effector T cells of the $CD45RA^+/CD27^-$ phenotype, that may have developed via the $CD45R0^+$ stage (28,29). It is therefore unlikely that reversion of memory cells contributes to the recovery of truly naive ($CD45RA^+/CD27^+$) T cells in our study.

In conclusion, the similar regeneration rate of naive $CD4^+$ and $CD8^+$ T cells during HAART, and the inverse correlation between age and recovery rate of naive T cells, suggest that the thymus plays a considerable role in the regeneration of naive T cells in adults during HAART. In addition, our observation that the regeneration rate of naive $CD4^+$ T cells is similar to the rates observed after iatrogenic T-cell depletion, suggests that HIV-induced dysfunctions of progenitor cells and the thymus are either of minor importance or they are rapidly reversed after initiation of HAART.

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# 6

# Increased Cell Division But Not Thymic Dysfunction Rapidly Affects the TREC Content of the Naive T Cell Population in HIV-1 Infection

Mette D. Hazenberg[1]
Sigrid A. Otto[1]
James W.T. Cohen Stuart[2,3]
Martie C.M. Verschuren[4]
Jan C.C. Borleffs[2]
Charles A.B. Boucher[3]
Roel A. Coutinho[5]
Joep M.A. Lange[6]
Tobias F. Rinke de Wit[7]
Aster Tsegaye[7]
Jacques J.M. van Dongen[4]
Dörte Hamann[1]
Rob J. de Boer[8]
Frank Miedema[1,9]

1 Department of Clinical Viro-Immunology, CLB, and the Laboratory for Experimental and Clinical Immunology, Academic Medical Center, University of Amsterdam, Plesmanlaan 125, 1066CX, Amsterdam, The Netherlands
2 Department of Internal Medicine, University Medical Center, Heidelberglaan 100, 3584 CX, Utrecht, The Netherlands
3 Department of Virology, Eijkman-Winkler Institute, University Medical Center, Heidelberglaan 100, 3584 CX, Utrecht, The Netherlands
4 Department of Immunology, Erasmus University, P.O. Box 1738, 3000 DR, Rotterdam, The Netherlands
5 Department of Public Health and Environment, Municipal Health Service, P.O. Box 2200, 1000 CE, Amsterdam, The Netherlands
6 Division of Infectious Diseases, Tropical medicine and AIDS and the National AIDS Therapy Evaluation Center (NATEC), Department of Internal Medicine, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ, The Netherlands
7 Ethiopian-Netherlands AIDS Research Project (ENARP) at the Ethiopian Health and Nutrition Research Institute (EHNRI), P.O. Box 1242, Addis Ababa, Ethiopia
8 Theoretical Biology, Utrecht University, Padualaan 8, 3584 CH, The Netherlands
9 Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

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## Abstract

Recent thymic emigrants can be identified by T Cell Receptor Excision Circles (TRECs) formed during T-cell receptor rearrangement. Decreasing numbers of TRECs have been observed with aging and in HIV-1 infected individuals, suggestive for thymic impairment. Here, we show that in healthy individuals, declining thymic output will affect the TREC content only when accompanied by naive T-cell division. The rapid decline in TRECs observed during HIV-1 infection and the increase following HAART are best explained not by thymic impairment, but by changes in peripheral T-cell division rates. Our data indicate that TREC content in healthy individuals is only indirectly related to thymic output, and in HIV-1 infection is mainly affected by immune activation.


## Introduction

It was postulated that HIV-1 induced $CD4^+$ T-cell depletion could at least partially be due to interference of HIV with *de novo* production of T cells at the level of T-cell progenitor function or thymic output. Several studies have focused on the influence of HIV-1 infection on thymic function, however, results were inconclusive (1,2). Recently, a new method has been reported by Douek and colleagues (3), that may allow a more direct estimation of thymic T-cell production. Formation of a productive T-cell Receptor (TCR)α- gene requires deletion of the TCRδ-gene, which is positioned within the TCRα-locus (4). The TCRδ-gene is flanked by two TCRδ- deleting elements, δRec and (J-α, which preferentially rearrange to each other, thereby deleting the TCRδ locus (5-7). The deleted TCRδ-gene remains present as an extrachromosomal circular excision product, the so-called Signal joint T-Cell Receptor Excision Circle (Sj TREC or TREC) (8,9). These circles are episomal, do not replicate during mitosis and are thus diluted during cell division (4,8). With quantitative PCR, TRECs can be detected and quantified, thereby offering a tool to identify recent thymic emigrants and to estimate thymic output. Using TREC

measurements, it was suggested that, although thymic function declines with age, limited thymic output was still measurable at old age (3,10-12). In HIV-1 infected individuals, low TREC contents in T cells and PBMC were reported in a large percentage of patients studied (3,10,13). TREC content increased during highly active anti-retroviral therapy (HAART), suggestive for improvement of pre-treatment impairment of thymic function (3,10). However, the effect of dilution of these episomal circles, resulting from repeated episodes of pathogen induced cell division during life, or from a chronically activated state of the immune system such as observed in HIV-1 infection (14-16), has not been taken into account in analyses and interpretation of these data. Here we combined measurements of TRECs and cell division rates in naive T cells of healthy and HIV-1 infected individuals with mathematical modeling, to come to a better interpretation of TREC measurements with respect to thymic output.

**Sj TRECs in healthy individuals**

First, to validate the assay, we measured the TREC content of $CD4^+$ and $CD8^+$ T cells of 49 HIV-1 negative individuals, from 0 (cord blood) to 84 years of age. The number of TREC copies declined with increasing age ($P_s < 0.001$, $R_s = -0.800$, data not shown). To study whether TRECs were diluted by cell proliferation, we cultured cord blood peripheral mononuclear cells (PBMC) from HIV-1 negative donors with IL-7 (10 ng/ml) and phytohemagglutinin (PHA, 1 μg/ml) for seven days. Sequentially, the number of PBMC, the proportion of cell death, and TRECs were measured. As expected, *in vitro* cell division led to dilution of TRECs (data not shown). We recently demonstrated that during HIV-1 infection peripheral T-cell division rates are differentially increased in all lymphocyte subsets, including the naive $CD4^+$ and naive $CD8^+$ T-cell pools (14). This may confound the interpretation of TREC analysis when measured in total PBMC or unseparated $CD4^+$ and $CD8^+$ T cells. We have found that the number of TRECs in the naive $CD4^+$ T-cell pool (FACS-sorted purified $CD27^+$ $CD45RA^+$ cells) is relatively high, although significant numbers of TRECs were also detected in the memory population (12) (data not shown). Because of practical limitations, TREC analysis could only be performed on purified $CD45RA^+$ T cells and not on 'truly naive' fractions defined by co-expression of CD27. Therefore,

**Table 1.** *Characteristics of healthy individuals and HIV-1 infected patients. Depicted are median and range. Statistical significance was calculated with the Mann-Whitney U test; p < 0.05 was considered statistically significant.*

| | $CD4^+$ T cells | | | $CD8^+$ T cells | | |
|---|---|---|---|---|---|---|
| | $HIV^-$ (*n* = 17) | $HIV^+$ (*n* = 33) | *P*-value | $HIV^-$ (*n* = 14) | $HIV^+$ (*n* = 7) | *P*-value |
| Age (years) | 37 (24-62) | 41 (24-57) | ns | 40 (24-62) | 42 (27-54) | ns |
| T cells (per µl) | 880 (620-1430) | 400 (160-1350) | < 0.001 | 405 (210-640) | 1570 (770-2720) | < 0.001 |
| Naive T cells (per µl) | 494 (276-1027) | 150 (22-756) | < 0.001 | 248 (149-354) | 218 (126-440) | ns |
| Ki-$67^+$ naive T cells (%) | 0.4 (0.1-0.8) | 3.1 (0.7-5.7) | < 0.001 | 0.3 (0.1-0.9) | 3.4 (1.7-30.7) | < 0.001 |
| Sj TRECs ($x10^{-3}$ copies/ CD45RA+ cell) | 25.1 (5.5-221.4) | 12.1 (0.08-57.1) | < 0.05 | 31.4 (4.2-219.1) | 2.5 (0.13-7.8) | < 0.001 |

subsequent TREC analysis was performed exclusively in purified $CD45RA^+$ $CD4^+$ and $CD45RA^+$ $CD8^+$ T-lymphocyte subsets, in parallel with assessment of peripheral cell division rates in the $CD27^+$ $CD45RA^+$ subsets using Ki-67 expression (17) (see Methods). Throughout the text, the term 'naive' denotes truly naive T cells, as being $CD45RA^+$ and $CD27^+$, otherwise the term $CD45RA^+$ is used.

**Sj TRECs during HIV-1 infection**

Table 1 shows characteristics of HIV-1 infected patients and age matched healthy individuals that were included in our study. HIV-1 infected

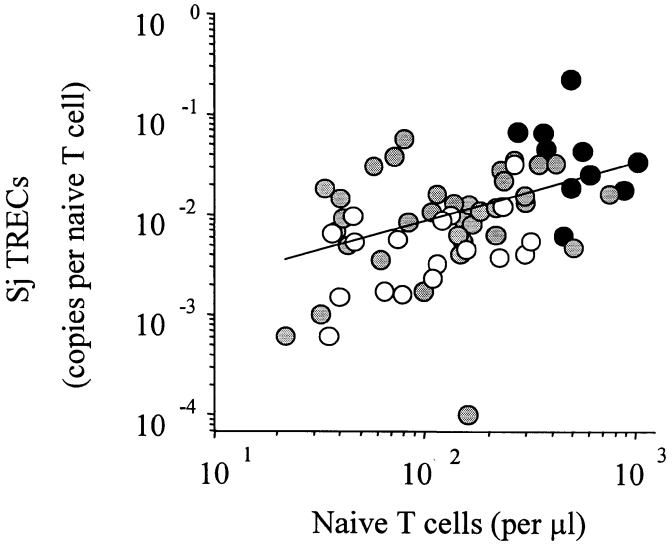


**Figure 1.** *Correlation between TRECs and naive T cell numbers. In HIV-1 infected Dutch patients (gray dots) and non-infected individuals (black dots), the number of TRECs per CD45RA$^+$ CD4$^+$ T cell and the size of this subset are correlated ($R_p$ = 0.427, $P_p$ = 0.003). White dots represent HIV-negative Ethiopian individuals (see text).*

patients had lower numbers of CD4$^+$, and higher numbers of CD8$^+$ T cells, compared to healthy individuals. The number of naive CD4+, but not naive CD8$^+$ T cells, was significantly reduced. Percentages of Ki-67$^+$ naive T cells were increased in these subsets. The percentage of Ki-67$^+$ naive CD4$^+$ T cells was negatively correlated with the number of naive CD4$^+$ T lymphocytes, as reported previously (14) ($R_s$ = - 0.843, $P_s$ < 0.001; data not shown). HIV-1 infected patients had a significantly lower TREC content of CD45RA$^+$ CD4$^+$ and CD45RA$^+$ CD8$^+$ T lymphocytes compared to healthy subjects. The number of TRECs per CD45RA$^+$ T cell correlated with the size of the naive CD4$^+$ T-cell pool, such that patients with high naive T-cell numbers had a high CD45RA$^+$ CD4$^+$ T-cell TREC content ($R_p$ = 0.427, $P_p$ = 0.003; Fig. 1).

**Effects of thymic output and cell division**

The TREC content of a naive T-cell population may depend to various extents on thymic production, cell death and cell division, priming of naive T cells to become memory lymphocytes, and intracellular degradation of TRECs (18). We therefore developed a mathematical model to interpret our data.

In this model, $N$ is the total number of naive ($CD4^+$ or $CD8^+$) T cells and $T$ the total amount of TRECs in the naive T-cell population, $\sigma(a)$ is the age ($a$)-dependent source of naive T cells from the thymus, $\alpha$ the rate of naive T-cell division, $\delta$ the rate of cell death and priming of naive cells, and $c$ is the amount of TRECs in recent thymic emigrants (RTEs). Changes in the number of naive T cells can be described as

$$dN/dt = \sigma(a) + \alpha N - \delta N. \quad \text{(Equation 1)}$$

The total amount of TRECs in this population increases proportionally to thymic production, and decreases when naive T cells die, become primed, or possibly because of intracellular degradation of the TRECs ($\delta_I$), and can be expressed as

$$dT/dt = c\sigma(a) - \delta T - \delta_i T. \quad \text{(Eq. 2)}$$

Note that the *total* amount of TRECs in the naive T-cell population does not decrease by division ($\alpha$). Division only affects the *average* TREC content per naive T cell. By the "quotient-rule" of differentiation, this average, defined as $A = T/N$, should change according to

$$dA/dt = \sigma(a)\,(c - A) / N - \delta_i A - \alpha A \quad \text{(Eq. 3)}$$

The quasi steady state is at

$$\hat{A} = c / [1 + N\,(\delta_I + \alpha) / \sigma(a)]. \quad \text{(Eq. 4)}$$

In the absence of division and degradation, when $\delta_i + \alpha = 0$, the steady state is $\hat{A} = c$. In other words, lower thymic output is only reflected by a reduction in TREC content of a naive cell when there is division of naive T

cells ($\alpha > 0$), and/or intracellular degradation ($\delta_i > 0$). Moreover, if the number of naive T cells $N$ in our model remains proportional to thymic production $\sigma(a)$, the $\sigma(a)$ term cancels from Equation 4. Thus, in that case the TREC content per naive T cell is essentially a measure of division and/or degradation, not of thymic output.

To validate our model, we studied quantitatively how the different parameters are expected to influence the average TREC content of a naive T cell. In the following equation thymus production is assumed to decay exponentially and division to be density dependent:

$$\sigma(a) = \sigma_0 \exp[-va] \quad \text{(5a) and } \alpha = \alpha_0 / (1 + N^2 / h^2), \quad \text{(Eq. 5b)}$$

where $\sigma_0$ is the newborn thymus production of naive T cells, $v$ the involution rate of the thymus, $\alpha_0$ is the maximum division rate of naive T cells (which is approached at the lowest naive T-cell counts), and $h$ the naive T-cell count at which the division is half maximal. This model is in good agreement with the data obtained from healthy individuals (12) (Fig.2). In all three panels, the thymic output decreases exponentially, but the naive T-cell population is maintained by only modest homeostatic changes in either the division ($\alpha$) or the death ($\delta$) rate (see equation 5c). In the case of a density dependent increase in division rate ($\alpha$), the average number of TRECs per naive T cell remains high until puberty, and then decreases several orders of a magnitude (Fig. 2*a*). Alternatively, the life span of naive T lymphocytes could prolong with the decrease in T-cell density that is associated with age-related decline in thymic output. Therefore, we included density dependent death of naive T cells, described as

$$\delta = \delta_0 + \varepsilon N \quad \text{(Eq. 5c)}$$

in our model. In this equation, $\varepsilon N$ allows for a density dependent increase in death rate. The average TREC content also declines orders of magnitude when there is a density dependent decrease in the death rate ($\varepsilon > 0$) and a constant naive T-cell division rate ($\alpha = \alpha_0$, Fig. 2b). However, when $\alpha = 0$ and $\delta$ is density dependent, the average TREC content remains constant during life (Fig. 2c).

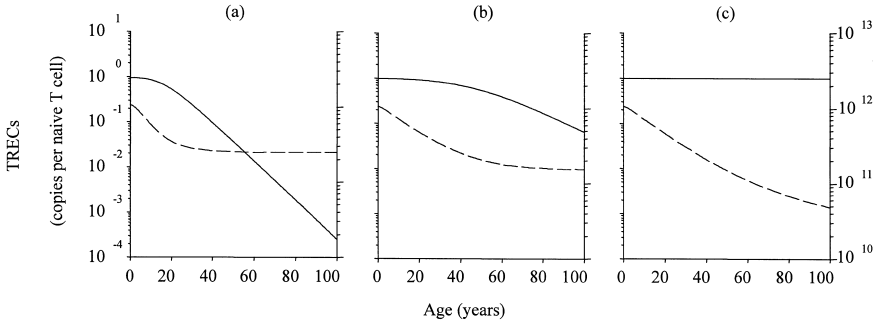


**Figure 2.** *The model behavior as a function of age. Thymic output declines exponentially, and the naive T cell population is maintained by (**a**) a modest increase in cell division ($\alpha$), (**b**) a density dependent decrease in death rate ($\delta$) and a constant cell division rate ($\alpha = 10^{-3}$), or (**c**) a density dependent decrease in death rate ($\delta$) in the absence of cell division ($\alpha_0=0$). Solid lines represent TREC content per naive T cell, dashed lines the total number of naive T cells. **a**, $\alpha_0 = 0.1$/day, $\delta = 10^{-3}$/day and $\varepsilon = 0$. **b**, $\alpha = 10^{-3}$, $\delta_0 = 0$/day, and $\varepsilon = 9.1\times10^{-16}$. **c**, $\alpha_0 = \delta = 0$/day, and $\varepsilon = 8.9\times10^{-16}$. Parameters: $c = 1$, $\delta_i = 0$, $h = 2.5\times10^{10}$ cells, $\sigma_0 = 10^9$ cells/day, and $v = 0.1$/year.*

When TRECs are measured in separated CD45RA$^+$ T-cell population, rather then in 'truly naive' T cells, reversion of CD45RA$^-$ memory cells into this population could also affect the TREC content. Reversion can be included in our model by allowing for a "source" of revertants, $R$, in Eq.1. Because revertant memory cells should contain little or no TRECs, Eq.2 should remain the same. The $R$ term reflects the number of memory cells reverting to a naive phenotype on a daily basis. We have studied the extended model numerically and found that reversion can only have an observable effect on the TREC content when more than half of the total production of naive T cells is due to reversion (i.e., when $R > \sigma(a) + \alpha N$). Although there might be reversion of some memory cells (19-21), it seems unlikely that this occurs at such high rate in healthy people (20). Thus, in our model it is the increase in division rate around puberty that sets the decrease in TREC content after puberty. Furthermore, CD45RA$^-$ CD45RO$^+$ memory cells are thought to revert to a CD45RA$^+$ CD45RO$^-$ phenotype only when in a resting state, i.e. in the absence of their specific

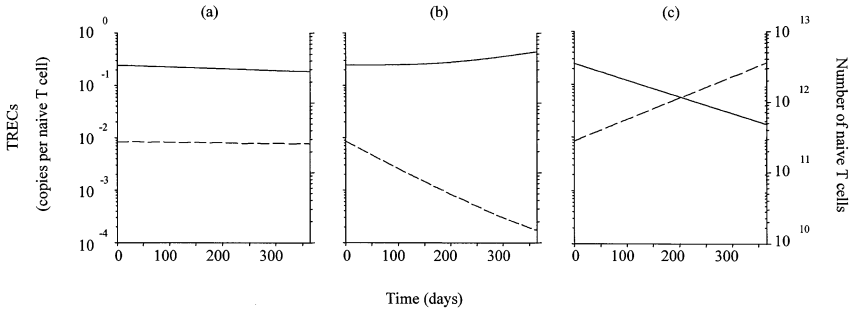


**Figure 3.** *The model behavior of an HIV-1 infected, 30-year old individual over a time period of one year.* ***a****, Blocking thymic output, i.e. setting* $\sigma = 0$*, hardly influences the average TREC content (solid line) or naive T cell numbers (dashed line).* ***b****, Increasing the death and priming rate (*$\delta$*) 10-fold leads to an increase in TREC content and a decrease in the number of naive T cells.* ***c****, Elevating the division rate (*$\alpha$*) 10-fold leads to a decrease in TREC content and an increase in naive T cell numbers. Parameters:* $\alpha = 7.9x10^{-4}$*/day or* $7.9x10^{-3}$*/day,* $c = 1$*,* $\delta = 10^{-3}$*/day,* $\delta_i = 0$*/day, and* $\sigma = 5x10^{7}$*cells/day.*

antigen. In HIV-1 infection, given the hyperactivation, this is probably a rare event. If a substantial reversion rate does exist, it just enhances the decreasing effect of naive T-cell division on TREC content. In any case, peripheral cell division is required (assuming no intracellular decay), and is an important factor, for decreasing TREC content.

**Effects of HIV-1 infection on TREC content**

HIV-1 infection could influence TRECs the naive T-cell compartment in various ways: 1) direct infection of the thymus could lead to abolishment of thymic production ($\sigma$), 2) HIV-mediated killing of naive cells or an increase in the rate of priming into memory cells could lead to an increasing loss of naive T cells ($\delta$), or 3) direct activation of naive T cells, or virus-induced depletion of the naive T-cell compartment, inducing a homeostatic response, could lead to a rise in naive T-cell division rates ($\alpha$). We investigated these effects separately, by choosing a 30-year old individual from Fig. 2 and studying the impact of the above mentioned possibilities during one year of HIV-1 infection (Fig. 3). Blocking thymus

**Table 2.** *Characteristics of HIV-1 negative Ethiopian individuals. Depicted are median and range. Values were compared to the Dutch HIV-negative ($HIV^-$; n = 17) and HIV-1 infected ($HIV^+$; n = 33) subjects described in Table 1. Statistical significance was calculated with the Mann-Whitney U test; P < 0.05 was considered statistically significant.*

| | Ethiopians ($n$ = 18) | Comparison to HIV$^-$ | Comparison to HIV$^+$ |
|---|---|---|---|
| Age (years) | 42 (35-45) | ns | ns |
| CD4 T cells (per μl) | 748 (427-1354) | ns | $P < 0.001$ |
| Naive CD4 (per μl) | 112 (36-316) | $P < 0.001$ | ns |
| Ki67$^+$ naive CD4 (%) | 0.9 (0.2-6.2) | $P < 0.05$ | $P < 0.005$ |
| Sj TRECs (x10$^{-3}$ copies/ CD45RA$^+$ cell) | 4.9 (0.56-31.9) | $P < 0.001$ | $P < 0.01$ |

production ($\sigma$ = 0) hardly influenced the average TREC content or naive T-cell numbers (Fig. 3*a*). This is explained by the longevity of these cells. Increasing the death and priming rate $\delta$ 10-fold led to a decrease in the number of naive T cells and an increase in the TREC content of naive T cells (Fig. 3*b*). The latter is due to the fact that by increasing the death rate, the average naive T cell will become younger. Thus, a larger fraction of all naive T cells will be a recent thymic emigrant, and will hence have a higher average TREC content. Finally, increasing the division rate $\alpha$ 10-fold had a strong and rapid effect on the TRECs (Fig. 3c). Various values of $\alpha$ were tested, and even when naive T-cell division was increased only three-fold compared to normal rates, the TREC content of the naive T-cell

population did reduce (data not shown). Theoretically, increasing the intracellular degradation rate $\delta_I$ 10-fold could lead to a similar decrease in TRECs, however, the normal lifespan of a TREC is not known, and it seems unlikely that HIV-1 infection could affect TRECs in this manner.
Thus, our model shows that the decrease in TRECs during the relatively short period of HIV-1 infection (3 to 7 years) is most simply explained by increased division rates in the naive T-cell compartment. We have recently shown that this is mostly driven by immune activation rather than by homeostatic mechanisms (14). This explanation is supported by our observation that the proportion of Ki-67$^+$ naive T cells was negatively correlated with the number of TRECs per CD45RA$^+$ T cell ($R_s$ = -0.556, $P_s$ = 0.001 and $R_p$ = -0.74, $P_p$ = 0.001 for CD4$^+$ and CD8$^+$ T-cell subsets respectively, Fig. 4*a* and Fig. 4*b*). Furthermore, compared with the group of healthy individuals, HIV-infected patients had a significantly decreased TREC content of the CD45RA$^+$ CD8$^+$ T-cell pool, but did not have lower numbers of naive CD8$^+$ T cells (Table 1). This is in agreement with our previous results, demonstrating that cell division rates in the naive CD8$^+$ T-cell compartment were increased in patients with normal numbers of naive CD8$^+$ T cells (14). Finally, although naive T-cell division is the simplest explanation, it does not exclude the possibility that reversion of memory cells from the CD45RA$^-$ to the CD45RA$^+$ phenotype contributes to the observed decrease in TREC content, especially for the CD8$^+$ T cells.

**Dilution of TRECs by non-HIV mediated immune activation**

To determine whether a decline in TRECs could be observed independently of HIV-1 infection, we studied HIV-negative Ethiopians who have a chronically activated immune system, probably due to the increased pathogen exposure in the domestic Ethiopian environment (22). Compared to HIV-negative Dutch individuals, Ethiopians have lower numbers of naive CD4$^+$ and naive CD8$^+$ T cells, concomitant with an expansion of the primed CD45RO$^+$ CD27$^-$ T-cell compartment (22). If the decline in TREC content of the CD45RA$^+$ T-cell population in HIV-1 infection were indeed related to peripheral dilution caused by cell division, one would expect low TREC contents in T cells from this group of Ethiopian individuals. Eighteen HIV-negative Ethiopians, participating in a cohort study performed in Akaki, Ethiopia, as part of the Ethiopian-

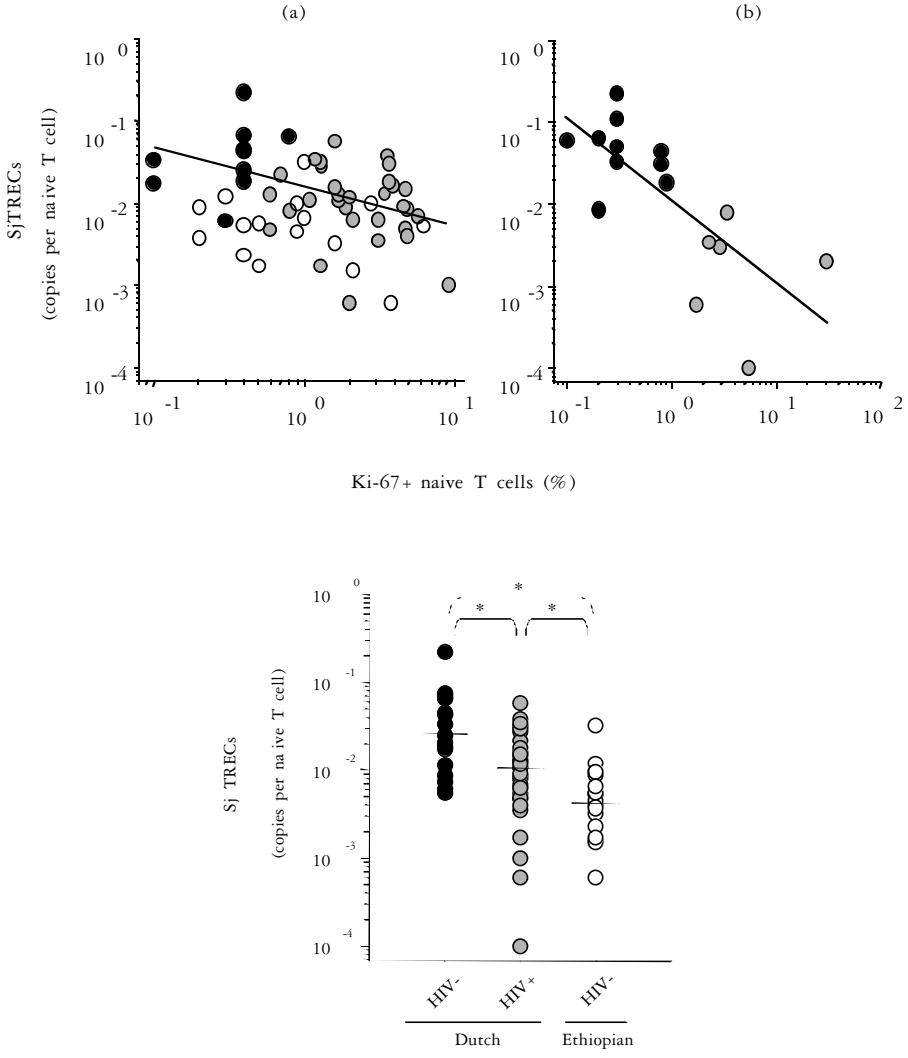


**Figure 4.** *In vivo dilution of TRECs. In HIV-1 infected Dutch patients (gray dots), and in non-infected Dutch individuals (black dots) the number of TRECs declines with increasing percentages of* ***a****, dividing naive CD4⁺ T cells ($R_s$ = -0.556, $P_s$ = 0.001) and* ***b****, dividing naive CD8⁺ T cells ($R_p$ = -0.740, $P_p$ = 0.001). White dots represent HIV-negative Ethiopian individuals (see text).* ***c****, HIV-negative Ethiopian individuals have an even lower TREC content per CD45RA⁺ CD4⁺ T cell compared with HIV-negative and HIV-infected Dutch subjects (* P < 0.05).*

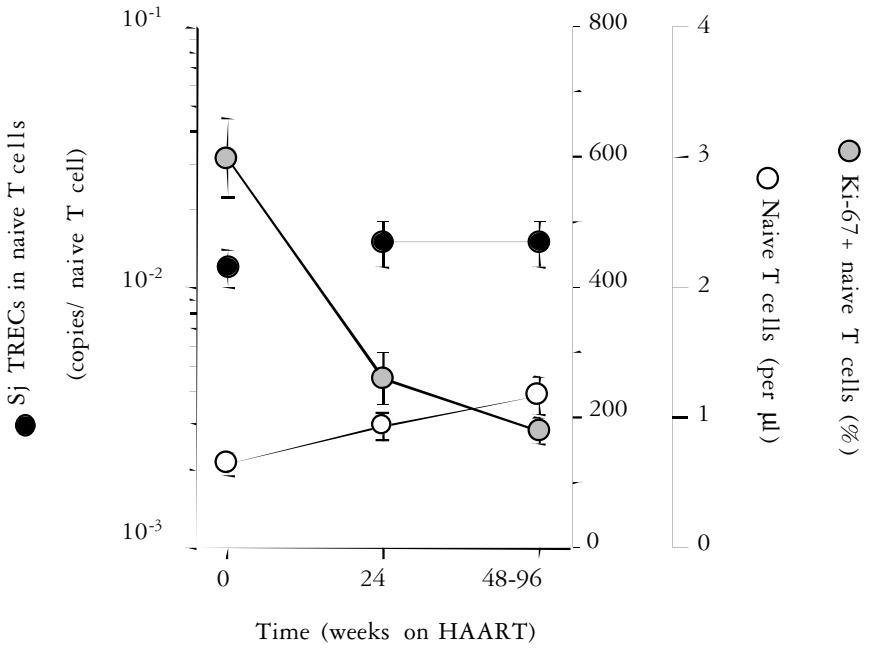


**Figure 5.** *Effect of HAART on TREC content. The number of naive CD4⁺ T cells (white dots), the percentage of Ki-67⁺ naive CD4⁺ T cells (gray dots) and the TREC content per CD45RA⁺ CD4⁺ T cell (black dots) were measured in 33 HIV-1 infected individuals during untreated HIV-1 infection, after 6 months of HAART and after 1 to 2 years of HAART. At all timepoints, the number of naive cells, Ki-67 expression in these cells and the number of TRECs were significantly different from control values (Mann Whitney U test, P < 0.01).*

Netherlands AIDS Research Project (ENARP), were selected based on their seronegative status. Compared with HIV-negative Dutch subjects, Ethiopian individuals had reduced numbers of naive CD4⁺ T cells, increased Ki-67⁺ naive CD4⁺ T cells and decreased CD45RA⁺ T cell TREC contents (Table 2). The number of TRECs per CD45RA⁺ CD4⁺ T cell in these subjects was even lower than in the HIV-1 infected Dutch patients included in our study (Fig. 4*c*). Even though Ki-67 expression is a measure for ongoing cell division and not for the replicative history of the naive cells, it does correlate with low TREC content in these individuals (Fig. 4*a*).

**Effect of Highly Active Anti-Retroviral Therapy on TRECs**

In the above described group of HIV-1 infected Dutch individuals, one to two years of HAART led to a rise in peripheral naive T-cell numbers, a steep reduction in the proportion of dividing cells in all subsets, and a small increase in the TREC content (Fig. 5; data not shown for $CD8^+$ T cells). None of these parameters reached normal levels ($P < 0.01$ for all time points, all parameters, compared with healthy subjects). In eight patients, we analyzed recovery of TRECs shortly after introduction of HAART in more detail. Immediate increase of TREC content in the $CD45RA^+$ T-cell pool was only observed during the first three months of HAART and coincided with a decline in naive $CD4^+$ T-cell division rates, but not with improvement of naive $CD4^+$ T-cell numbers. Analysis of the naive $CD8^+$ T-cell pool revealed similar dynamics (data not shown).

## Discussion

Elevated cell division rates could obscure the interpretation of TREC data. Because many $CD45RA^-$ 'memory' T cells, particularly, may have a history of very rapid cell division in individuals with chronically activated immune systems (16), lowering the average TREC content (12), we measured Sj TRECs in purified $CD45RA^+CD4^+$ and $CD45RA^+CD8^+$ T-cell subsets in parallel with peripheral cell division rates in the naive populations. We compared data obtained from healthy individuals with those from subjects that were shown to have a persistently activated immune system, including HIV-1 infected patients (14,16) and a group of HIV-negative Ethiopian factory workers. Mathematical modeling was used to describe the relation between the various parameters that affect TREC content. This showed that when in healthy individuals the net thymic output decreases, for example because of age-related involution of thymic tissue, the number of TRECs per produced naive T cell does not significantly decrease (see equation 3), as has been experimentally shown (11). The mere fact that the TREC content of naive T cells declines with age (12), according to our model suggests increased naive peripheral T-cell division that accompanies lower thymic T-cell production.

It is now well established that HIV-1 infection leads to chronic immune activation reflected in elevated division rates in all T-cell subsets, including even the naive $CD4^+$ and $CD8^+$ T-cell compartment (14,16,23). This immune activation is observed immediately following HIV-1 infection, and is most pronounced in $CD8^+$ T cells (24,25). We measured cell division in truly naive T cells that were characterized by expression of CD45RA and CD27 antigens. Contamination of this population by memory cells that reverted to a naive phenotype is thereby excluded, since expression of the CD27 antigen is lost upon prolonged antigen-specific stimulation and this loss is irreversible (26). Naive T cells can be activated by stimulatory cytokines, independent of antigen, without losing their naive phenotype (27-29).

According to the model, naive T-cell division would have a rapid and strong effect on the average TREC content. Even a two- to three-fold increase in cell division rates, which is not reflected by changes in telomere lengths (30,31), can induce an observable dilution of TRECs. Indeed, TREC content is low in HIV-1 infection and acute HIV-1 infection leads to a significant reduction in the number of TRECs already after ninety days (10), an observation that our model cannot explain by an immediate fall in thymic output (Fig. 3*a*). It has been estimated that in adults, thymic output is in the order of $10^7$ to $10^8$ lymphocytes per day (32). Even if HIV-1 infection would immediately and completely abolish thymic output, this alone could not lead to such a rapid fall in cellular TREC content (Fig. 3*a*). Elevated rates of T-cell division of naive cells are the most likely cause of the reduced TREC content we observed. Our hypothesis is further supported by the finding that non-HIV mediated persistent immune activation in healthy Ethiopians also leads to a significant loss of TRECs. However, because mixing with low-TREC revertant memory cells could also contribute to the observed reduction in TREC content, it will be important to obtain information on TREC contents of sorted naive T cells. Indeed, preliminary data from our lab obtained in a small group of HIV-1 infected patients showed lower TREC content of sorted 'truly naive' $CD27^+CD45RA^+CD4^+$ T cells compared with healthy age-matched individuals (n=5 and n=3, medians $1.3 \times 10^{-3}$ and $7.2 \times 10^{-3}$ Sj TREC copies per $CD27^+CD45RA^+CD4^+$ T cell, respectively).

Anti-retroviral treatment resulted in improvement of the TREC content of CD45RA$^+$ T cells which correlated with declining naive T-cell division rates, but not with increasing naive T lymphocyte numbers. This is in agreement with recent findings of Zhang *et al.* (10), who found significant HAART- induced recovery of TREC content only in patients with low pre-treatment numbers of TRECs, which did not correlate with recovery of the naive T-cell pool. Rather, the increase in TREC content during HAART most likely is a composite of normalizing naive T-cell peripheral death and division rates (14), some redistribution of naive T cells to the blood (33), and a continuous but low, possibly normal, thymic lymphocyte production.

Taken together, loss of TRECs in HIV-1 infection may primarily be caused by continuous hyperactivation of the immune system as reflected by increased cell division even of naive T cells. Although interference by HIV with thymic output could contribute to CD4$^+$ T-cell depletion (3,30,32,34), measurements of TREC content in CD45RA$^+$ T cells fail to provide experimental evidence for such thymic impairment. Alternatively, it may be that the intrinsically low thymic output in adults cannot compensate for the continuous loss of naive cells incurred by the increased priming of naive cells due to persistent immune activation, resulting in gradual depletion of CD4$^+$ T cells without in fact exhaustion of thymic output (35).

## Methods

### Subjects

Cryopreserved peripheral blood samples from thirty-three HIV-1 infected patients and thirty-five non-infected healthy subjects were analyzed. Cryopreservation was performed using a computerized freezing device that results in optimal quality of frozen cells for functional studies (36). Frozen blood samples were stored in liquid nitrogen. Patient blood samples were obtained from HIV-1 infected individuals participating in three separate trials: the Amsterdam Cohort Study on HIV-1 Infection in Homosexual Men ($n$ = 7), the BMS-50 trial ($n$ = 18) and the CHEESE study ($n$ = 8).

Samples were obtained before and during treatment with triple therapy regimens, containing two reverse transcriptase inhibitors (nucleoside analogues) and one protease inhibitor. As controls, HIV-1 negative Dutch laboratory personnel ($n$ = 17) and Ethiopian factory workers ($n$ = 18) were included. HIV-1 negative individuals were age-matched with the HIV-1 infected patients. In 14 out of 17 healthy Dutch individuals, and 7 out of 33 HIV-infected subjects, enough material was available to perform measurements of TRECs in both the $CD45RA^+$ $CD4^+$ and $CD45RA^+$ $CD8^+$ T-cell compartment. Selection of HIV-negative Ethiopian factory workers was based on their numbers of peripheral blood $CD4^+$ T lymphocytes, varying from less than 500 to more than 1000 cells per µl. No correlation was found between the TREC content of $CD45RA^+$ $CD4^+$ T cells and the total number of $CD4^+$ T cells in that population. It is of notice that although total $CD4^+$ T-cell numbers varied in this study population, the number of naive $CD4^+$ T cells was significantly lower compared with HIV-negative Dutch individuals, even when total $CD4^+$ T-cell numbers were comparable (Table 2).

**Cell proliferation**

Peripheral blood T-cell proliferation was studied by flow cytometric measurements of Ki-67 nuclear antigen expression on naive $CD27^+$ $CD45RO^-$ $CD4^+$ and naive $CD27^+$ $CD45RO^-$ $CD8^+$ T cells, as described previously (14,37,38). Measurements of cell division rates with this method yield similar results as have been obtained previously by in vivo labeling of dividing cells with deuterated glucose (39). Peripheral blood mononuclear cells were thawed and incubated with CD4- or CD8-PerCP mAb, CD45RO-PE (Becton Dickinson, San Jose, California) and biotinylated CD27 mAb (CLB, Amsterdam, The Netherlands). After washing, cells were incubated with Streptavidin-APC (Becton Dickinson). Cells were then fixated and permeabilized with FACS Lysing Solution and FACS Permeabilization Buffer (Becton Dickinson), respectively. Lymphocytes were stained intracellular with Ki-67-FITC mAb (Immunotech, Marseille, France), after which cells were fixed using Cellfix (Becton Dickinson) and analyzed on a FACSCalibur (Becton Dickinson) with Cellquest software.

**Cell separation**

CD45RA$^+$ CD4$^+$ and CD45RA$^+$ CD8$^+$ T cells were purified from thawed PBMC by magnetic separation over columns, using the MiniMACS multisort kit according to manufacturers instructions (Miltenyi Biotec Inc, Sunnyvale, California). Briefly, after 15 minutes incubation with 20 µl CD4 or CD8 conjugated magnetic beads per $10^7$ cells, CD4$^+$ and CD8$^+$ T cells were isolated from PBMC by positive selection over MiniMACS separation columns. Magnetic beads were then released, cells were incubated with CD45RA-conjugated magnetic beads and passed over columns. With this technique, at least 90% purity of the fractions was achieved, such that less then 10% of CD4$^+$ or CD8$^+$ T-cell fractions were contaminated with CD8$^+$ or CD4$^+$ T cells, respectively, or with CD45RA$^-$ T cells. As outlined above, truly naive T cells are defined by co-expression of CD45RA and CD27, however, due to practical reasons, TREC analysis was performed in purified CD45RA$^+$ T cells. Contamination of purified CD45RA$^+$ T cells with CD27$^-$ CD45RA$^+$ cells such that it will affect TREC contents cannot be excluded, especially in the CD8$^+$ T-cell subset of HIV infected individuals (26). It is less likely to occur in CD4$^+$ T cells, since the population of CD4$^+$ CD27$^-$ CD45RA$^+$ T cells is limited in size (22).

**Real-time PCR**

DNA was purified from CD45RA$^+$ CD4$^+$ and CD45RA$^+$ CD8$^+$ T-cell fractions using the QIAamp Blood Kit according to manufacturers instructions (Qiagen, Hilden, Germany). In order to detect Sj TRECs, a real-time quantitative PCR method was used (40). In this PCR, annealing of primers with a site-specific probe, containing a quencher and a reporter dye, results in AmpliTaq Gold DNA Polymerase mediated cleavage of the probe and subsequent separation of the quencher from the reporter, thereby inducing fluorescence of the reporter dye. Each PCR reaction was performed in a 50 µl solution containing 100-200 ng DNA of cell suspension of interest, 1.0 x TaqMan Buffer A (Perkin Elmer Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), 5.0 mM $MgCl_2$, 200 µM dNTPs, 900 nM forward and reverse primer, 200 nM probe, and 1.25 U AmpliTaq Gold (PE Biosystems). The sequences of the utilized primers and probe are the following: forward primer 5'-CCATGCTGACA

CCTCTGGTT-3', reverse primer 5'-TCGTGAGAACGGTGAATGAAG-3' and the probe 5'-CACGGTGATGCATAGGCACCTGC-3'. As an internal control measurement, to normalize for input DNA, the Cα constant region that remains present on TCR genes despite rearrangement processes was amplified in every sample tested (forward primer 5'-CCTGATCCTCTTGTCCCACAG-3'; reverse primer 5'-GGATTTAGA GTCTCTCAGCTGGTACA-3' and probe 5'-ATCCAGAACCCTGACC CTGCCG-3'). Using these primer/probe combinations (PE Biosystems), sequences of 131 basepairs (bp) and 70 bp, respectively, were amplified. A standard was created, by cloning the Sj fragment in the Hind III site of a pUC-19 vector, and the number of Sj copies present in a given cell population was calculated by including a dilution series of this standard in each PCR experiment.
PCR was performed under the following conditions: 50 °C for 2 minutes followed by 95 °C for 10 minutes, after which 50 cycles of amplification were carried out (95 °C for 15 seconds, 60 °C for 1 minute). For each sample the Ct-value, defined as the minimal number of cycles necessary to exceed threshold values, was measured and applied to the standardization curve created from the dilution series described above.

**Statistical analysis**
Group characteristics were compared with the Mann-Whitney U test. Normality of groups was tested using the Shapiro-Wilk *W* Test for normality. Based on the outcome of this test, correlations were calculated using either Spearman's rank correlation coefficient ($R_s$) or Pearson's correlation coefficient ($R_p$).

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# 7

# T-Cell Division in Human Immunodeficiency Virus (HIV)-1 Infection is Mainly Due to Immune Activation: a Longitudinal Analysis in Patients Before and During Highly Active Anti-Retroviral Therapy (HAART)

Mette D. Hazenberg[1]
James W.T. Cohen Stuart[2,3]
Sigrid A. Otto[1]
Jan C.C. Borleffs[2]
Charles A.B. Boucher[3]
Rob J. de Boer[4]
Frank Miedema[1]
Dörte Hamann[1]

1 From the Department of Clinical Viro-Immunology, CLB, and the Laboratory for Experimental and Clinical Immunology and Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, Amsterdam
2 the Department of Internal Medicine, University Hospital Utrecht
3 the Department of Virology, Eijkman-Winkler Institute, University Hospital Utrecht
4 the Department of Theoretical Biology, Utrecht University, Utrecht, The Netherlands.

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## Abstract

In HIV-1 infection, highly increased T-cell turnover was proposed to cause exhaustion of lymphocyte production and consequently development of AIDS. Here, we investigated cell proliferation, as measured by expression of the Ki-67 nuclear antigen, in peripheral blood $CD4^+$ and $CD8^+$ lymphocyte subpopulations before and during HAART. In untreated HIV-1 infection, both the percentage and number of Ki-$67^+$ $CD4^+$ and $CD8^+$ lymphocytes were significantly increased, as compared to values obtained from healthy individuals. A more then 10-fold increase in the percentage of dividing naive $CD4^+$ T cells in the blood was found when the number of these cells were below 100 per $mm^3$ . HAART induced an immediate decline in Ki-67 antigen expression despite often very low $CD4^+$ T-cell numbers, arguing against increased proliferation being a homeostatic response. After approximately 24 weeks of HAART treatment, a transient increase in the number of proliferating cells was seen, but only in the $CD4^+$ $CD27^+$ memory pool. In the $CD8^+$ T-cell compartment, the number of dividing cells was elevated 20- to 25-fold. This increase was most notable in the $CD27^+$ and $CD27^-$ $CD45RO^+$ memory $CD8^+$ T-cell pool, corresponding with the degree of expansion of these subsets. Reduction of plasma HIV-RNA load by HAART was accompanied by a decrease in numbers and percentages of dividing cells in all $CD8^+$ T-cell subsets. Taken together, our results indicate that peripheral T-cell proliferation is a consequence of generalized immune activation.


## Introduction

Several hypotheses have been presented to explain the loss of $CD4^+$ T lymphocytes, the major hallmark of HIV-1 infection that leads to severe immune depletion and ultimately AIDS and death. The model of high lymphocyte turnover has received considerable attention over the past few years (1,2). Based on the observation that during the first few weeks of highly active anti-retroviral therapy (HAART) the number of $CD4^+$ T

cells increases, it was postulated that HIV-1 infection leads to a rapid turnover of lymphocytes, reflecting a new balance between production and death of lymphocytes. The increased production rate would eventually lead to exhaustion of the $CD4^+$ T-cell renewal capacity and result in $CD4^+$ lymphocyte depletion. Indeed, in SIV infected macaques increased T-cell production was observed (3), however, there was a parallel rise in the turnover of B cells and NK cells (4). Moreover, the highest increase was observed in $CD8^+$ T cells, the compartment that is initially expanded and does not get depleted until very late in the course of HIV-1 infection (5-9). HIV-induced lymphocyte depletion and subsequent recovery after HAART appeared to be variable when studied at the level of T-cell subsets. Infection with HIV-1 induces an early decline in the number of naive $CD4^+$ and $CD8^+$ and memory $CD4^+$ T lymphocytes. Conversely, the memory and activated $CD8^+$ T-cell compartments expand initially. Only shortly preceding progression to AIDS, the numbers of these latter cell types fall as well (10-12). HAART induces an early rise of memory $CD4^+$ and $CD8^+$ T-cell numbers, accompanied by a slow increase in naive $CD4^+$ and $CD8^+$ T-cell numbers (13,14). From this, it was concluded that different mechanisms are involved in the dynamics of depletion and recovery of the various T lymphocyte subsets. These mechanisms include interference of HIV with *de novo* T-cell production capacity (15) (D.R.Clark *et al*, submitted for publication) and enhanced sequestration of cells in lymphoid tissues (14;16-18). Recently several groups have reported on cross-sectional studies of T-cell production levels. Most of these reports studied total $CD4^+$ and $CD8^+$ T-cell populations, and did not discriminate between naive and memory subsets (3;7;8;19;20).

To answer the question whether increased T-cell proliferation could be involved in $CD4^+$ lymphocyte depletion we measured expression of the Ki-67 antigen in naive and memory $CD4^+$ T-cell subsets in the blood. Furthermore, we investigated the role of T-cell proliferation in the expansion and depletion of naive, memory and effector $CD8^+$ T-lymphocyte subsets. Our approach was longitudinal: cell division rates were studied from the stage of chronic untreated HIV-1 infection up to a year of treatment with HAART.

## Materials and Methods

**Patients**

To study T-cell turnover before and during anti-retroviral treatment, sequential cryopreserved peripheral blood samples from HIV-1 infected participants of the CHEESE study were used. Cryopreservation was performed using a computerized freezing device that results in optimal quality of viably frozen cells for functional studies (21). Frozen blood samples were stored in liquid nitrogen. No differences in Ki-67 expression were found between freshly isolated or frozen cells in a pilot experiment (data not shown). In the multicenter CHEESE study patients received either zidovudine (Retrovir; 200 mg, three times/day), lamivudine (Epivir; 150 mg, twice/day) and saquinavir soft gel capsules (Fortovase; 1200 mg, three times/day) or zidovudine, lamivudine and indinavir (Crixivan; 800 mg, 3 times/day). The inclusion criteria for this trial were no previous treatment with anti-retroviral therapy except for AZT less than 12 months, $CD4^+$ T-cell count < 500 per µl, and/or HIV RNA > 10000 copies per ml, and/or CDC stage B or C (22). Sixteen out of sixty participants were selected based on baseline $CD4^+$ T-cell numbers and the availability of frozen peripheral blood mononuclear cell (PBMC) samples. Patients were equally distributed among both therapy arms. Expression of the Ki-67 antigen was analyzed at five timepoints: during untreated HIV infection (t=0) and after four (t=4), twelve (t=12), twenty-four (t=24) and forty-eight (t=48) weeks of therapy. As controls, cryopreserved PBMC from five HIV-negative blood bank donors were used.

**Monoclonal antibodies**

CD4-PerCP, CD8-PerCP, CD45RO-PE mAb, and Streptavidin-APC were obtained from Becton Dickinson (San Jose, CA). Biotinylated CD27 mAb was purchased from the CLB (Amsterdam, The Netherlands), and FITC-labeled Ki-67 mAb was purchased from Immunotech (Marseille, France).

**Flow Cytometry**

$CD4^+$ and $CD8^+$ T cells were subdivided into naive ($CD45RO^-$/ $CD27^+$), $CD27^+$ memory ($CD45RO^+$/$CD27^+$), $CD27^-$ memory ($CD45RO^+$/$CD27^-$)

*Number of Ki-67 antigen expressing cells within the total CD4$^+$ (1) and CD8$^+$ (2) T cell population and T cell subsets of untreated HIV-1 infected patients and healthy individuals. Median values for the groups, ranges and the x-fold increase observed in HIV-1 infection compared to the healthy situation are shown. Asterix ($^*$) represents significant difference with control values ($p<0.05$; Mann-Whitney U Test).*

**Table 1.** *Number of Ki-67$^+$ CD4$^+$ T cells (per ml)*

| | Naive | CD27$^+$ memory | CD27$^-$ memory | Total |
|---|---|---|---|---|
| HIV+ | 1.6$^*$ (0.3-3.5) | 13.6$^*$ (3.2-26) | 5.9$^*$ (1-17) | 22.8$^*$ (4.7-42) |
| HIV- | 0.8 (0.6-1.4) | 5.5 (4.3-7.3) | 2.7 (2.1-3.1) | 9.0 (7.5-12) |
| x-fold increase | 2 | 2.5 | 2.2 | 2.5 |

**Table 2.** *Number of Ki-67$^+$ CD8$^+$ T cells (per ml)*

| | Naive | CD27$^+$ memory | CD27$^-$ memory | CD27$^-$ effector | Total |
|---|---|---|---|---|---|
| HIV+ | 6.9$^*$ (1.2-101) | 33.9$^*$ (8-534) | 27.8$^*$ (4.1-515) | 11.3$^*$ (1-195) | 98.3$^*$ (14-1297) |
| HIV- | 0.4 (0.3-1.5) | 0.7 (0.5-2.1) | 0.5 (0.1-1.4) | 0.5 (0.2-1.1) | 2.6 (1.5-5.1) |
| x-fold increase | 17.3 | 48.4 | 55.6 | 22.6 | 37.8 |

and CD27$^-$ effector (CD45RO$^-$/CD27$^-$) cells, as previously described (23;24). In contrast to these earlier studies, CD45RA mAb was replaced by CD45RO mAb and the definition of the T-cell subsets was adjusted to the used mAb combination. Cell proliferation was studied by measuring

expression of the Ki-67 antigen, which is expressed by cells in late G1, S, G2 and M phase of the cell division circle (25-28).
Cryopreserved peripheral blood mononuclear cells were thawed and incubated with $CD4^-$ or $CD8^-$PerCP mAb, $CD45RO^-$PE and biotinylated CD27 mAb. After washing with PBS/0,5 % BSA, cells were incubated with Streptavidin-APC. Red blood cells were lysed and lymphocytes fixated with Facs Lysing Solution (Becton Dickinson). Subsequent permeabilisation was performed by incubating cells with FACS Permeabilisation Buffer (Becton Dickinson), after which cells were stained intracellularly with Ki-67-FITC mAb. Cells were fixed, using Cellfix (Becton Dickinson), and analyzed on a FACSCalibur (Becton Dickinson) with Cellquest software. All incubation steps were performed at 4 °C for 20 minutes; for fixation and permeabilization samples were kept at room temperature for 10 minutes.

**Viral load**
Plasma viral load was determined using RT-PCR detecting HIV-1 RNA (Roche Amplicor Monitor Standard Assay, Roche Diagnostics, Branchburg, New Jersey, USA).

**Statistical analysis**
Patient characteristics at baseline and after various timepoints during treatment with HAART were compared using Wilcoxon Signed Ranks Test and Mann-Whitney-U Test for comparison with healthy control values. Correlations were calculated using Spearman's correlation coefficients.

## Results

**Ki-67 antigen expression in chronic HIV-1 infection**
During chronic untreated HIV-1 infection, both the percentage and the absolute number of $Ki\text{-}67^+$ $CD4^+$ and $CD8^+$ T cells was significantly increased (figure 1, table 1 and 2; $p=0.001$), as compared to healthy controls. The percentage of Ki-67 expression was significantly elevated in

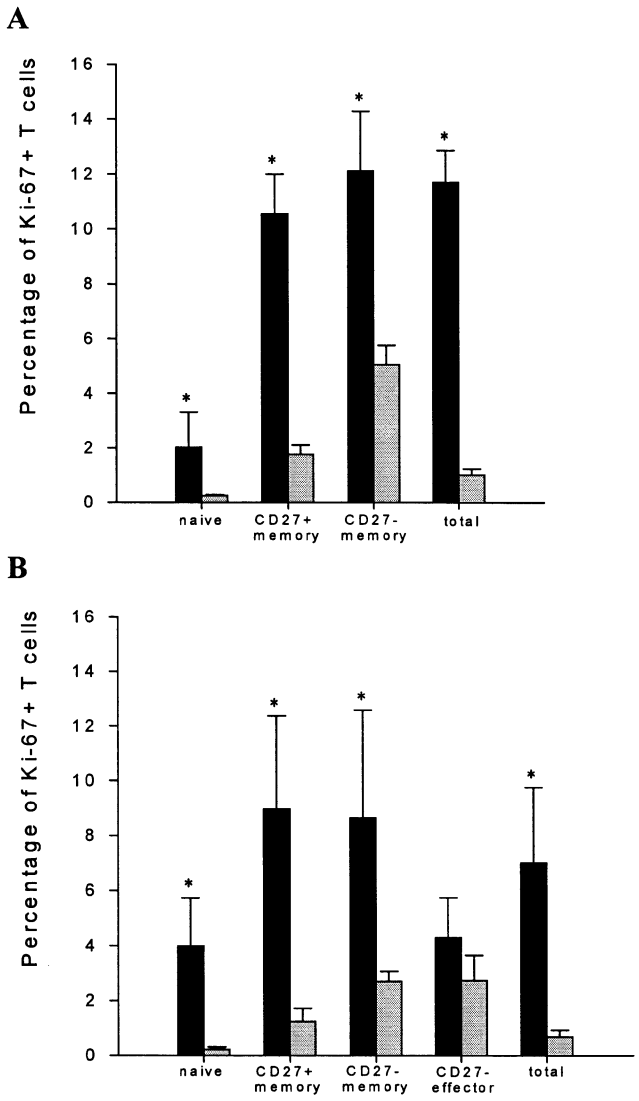



**Figure 1.** *Percentage of Ki-67⁺ T lymphocytes within the CD4⁺ (A) and CD8⁺ (B) peripheral blood T cell populations of healthy individuals (gray bars) and untreated HIV-infected patients (black bars). Asterix (*) represents statistical difference compared to control value ($p<0.005$; Mann-Whitney U Test).*

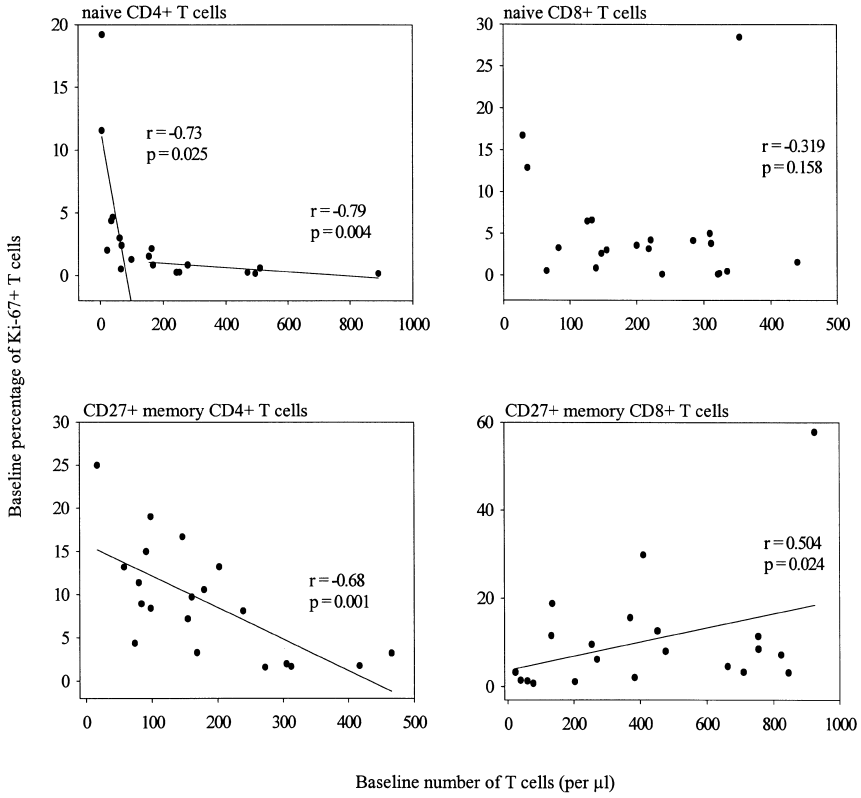



**Figure 2.** *Correlation between the number of CD4⁺ and CD8⁺ naive or CD27⁺ memory T cells and the percentage of Ki-67⁺ naive or CD27⁺ memory cells of untreated HIV-infected and non-infected individuals (Spearman's correlation coefficients).*

all subsets ($p<0.005$), except for the $CD8^+$ $CD27^-$ effector T-cell population (figure 1). Naive T cells showed a ten- (CD4) to twenty- (CD8) fold elevation in Ki-$67^+$ T-cell percentage, and in the memory and effector T-lymphocyte compartment Ki-67 expression was increased up to seven-fold (figure 1). When absolute numbers instead of percentages of dividing cells were analyzed, cell proliferation in the $CD4^+$ T-cell subsets was elevated less than three-fold, whereas in the $CD8^+$ compartment, a twenty- to more then fifty-fold rise was observed (table 1 and 2). This increase was

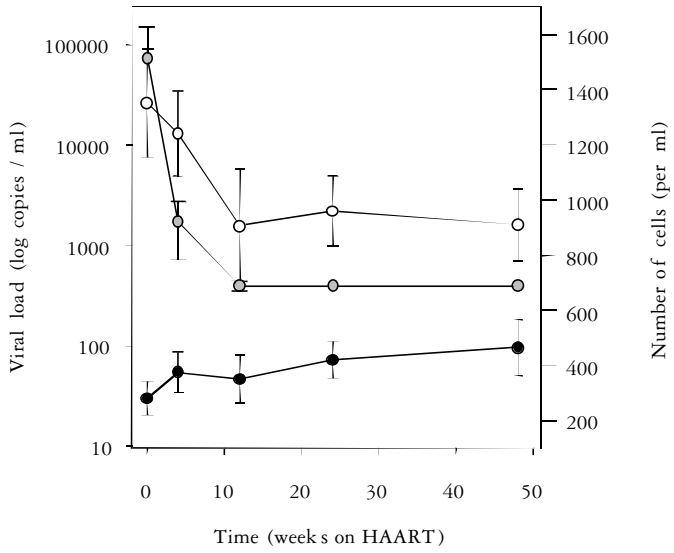
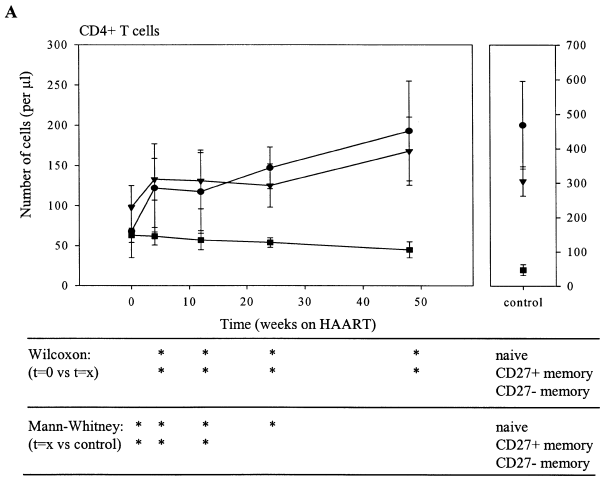



**Figure 3.** *Longitudinal analysis of lymphocyte recovery and plasma HIV-RNA load decline. White dots represent HIV-RNA load, black dots CD4⁺ T cells and gray dots CD8⁺ T cells.*

most notable in the CD27⁺ memory and CD27⁻ memory lymphocyte population (see t=0 figure 4B).

The percentage of naive Ki-67⁺ CD4⁺ T lymphocytes was inversely correlated with the number of naive cells (figure 2), but did not correlate with plasma viral load (data not shown). Strikingly, only when the number of naive CD4⁺ T cells dropped below 100 cells per µl, the proportion of Ki-67 expressing cells in this subset exceeded two percent (naive CD4 count > 100/µl: r = -0.79, p = 0.004; naive CD4 count < 100/µl: r = -0.73, p = 0.025; stratification arbitrary). The same overall trend, but not a significant correlation, was observed for the naive CD8 T-cell compartment (figure 2). The size of the CD27⁺ memory CD4⁺ T-cell pool was negatively correlated with the percentage of proliferating cells in this compartment (figure 2; r = -0.68, p = 0.001). In the CD8⁺ T-cell compartment the opposite was observed. Here, an increase in the percentage of Ki-67 expressing cells correlated positively with the size of



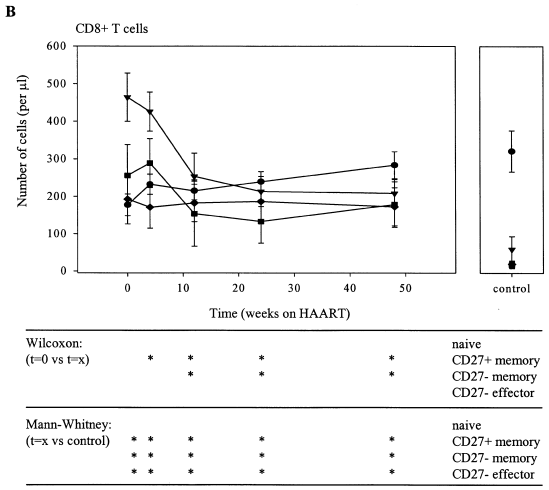




**Figure 4.** *$CD4^+$ and $CD8^+$ T cell subset recovery. Naive (circles), $CD27^+$ memory (triangles), $CD27^-$ memory (squares) and CD27- effector (diamonds) lymphocytes within the $CD4^+$ (A) and $CD8^+$ (B) T cell compartments are shown. Sequential patient values were compared using the Wilcoxon Signed Ranks Test, and the Mann-Whitney U Test was used for comparison with healthy individuals (*: $p<0.05$).*

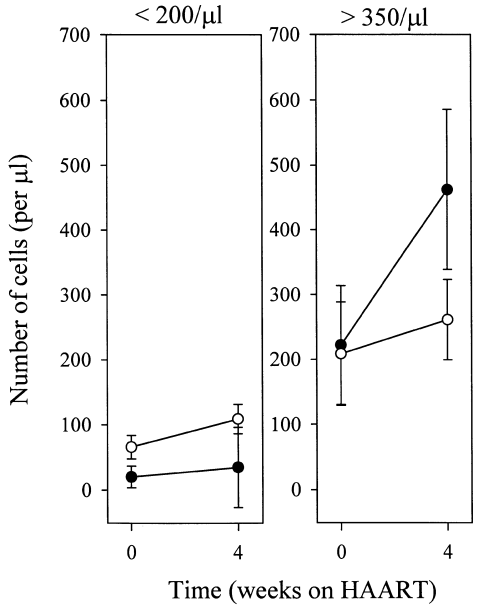



**Figure 5.** *Early recovery of CD4⁺ naive and CD27⁺ memory T lymphocytes. Patients were stratified according to baseline naive T cell values. Black and white dots represent naive and CD27⁺ memory cells, respectively.*

the $CD27^+$ memory T lymphocyte subset (figure 2; r = 0.50, p = 0.024) and the size of the $CD27^-$ memory T-cell subset (data not shown; r = 0.52, p = 0.016). No correlation was found between the same parameters in the $CD4^+$ $CD27^-$ memory or the $CD8^+$ $CD27^-$ effector T-cell population, or between Ki-67 antigen expression and viral load (data not shown).

**Longitudinal analysis of immune recovery during HAART**

Infection with HIV-1 resulted in $CD4^+$ T-cell depletion and expansion of the $CD8^+$ T-cell pool as has been described above (t=0 in figure 4A and 4B). Treatment with anti-retroviral therapy resulted in a significant decrease of plasma HIV-RNA to levels below the limits of detection (lower limit of detection 400 RNA copies/ml). In parallel, total numbers of $CD4^+$ T cells increased, and a fall in total numbers of $CD8^+$ T lymphocytes was observed (figure 3). Figure 4A shows that the therapy-induced rise in

$CD4^+$ T cells observed in the first four weeks of HAART was accounted for by an increase of both $CD27^+$ memory and naive T lymphocytes. Stratification of patients into two groups based on their baseline naive $CD4^+$ T-cell counts revealed that the HAART-induced restoration of the naive subset was better in patients with higher baseline naive $CD4^+$ T-cell values (figure 5). After one year of treatment, numbers of naive and $CD27^+$ memory $CD4^+$ T cells had risen significantly compared to pre-treatment levels, but were still lower compared to control levels (figure 4A, $p<0.05$). In the same period, as depicted in figure 4B, the number of naive $CD8^+$ T cells increased. The $CD27^+$ and $CD27^-$ memory and $CD27^-$ effector $CD8^+$ T lymphocyte subsets remained significantly expanded compared to control values ($p<0.05$), despite an initial decrease in cell number that was significant for $CD27^+$ and $CD27^-$ memory T cells after respectively one and three months ($p<0.05$).

**Ki-67 antigen expression during HAART-induced immune recovery**

Immediately upon reduction of plasma HIV-RNA load by HAART, the total number (figure 6A and 6B) and the percentage (data not shown) of $CD4^+$ and $CD8^+$ Ki-$67^+$ T cells and of Ki-$67^+$ T lymphocytes in all subsets declined. In the $CD4^+$ T-cell compartment, this decline was relatively slow when compared to the rapid decline observed in the $CD8^+$ T-cell population. Furthermore, after six months of HAART, a temporary increase in the number of Ki-$67^+$ lymphocytes was observed in the total $CD4^+$ T-cell population. After one year of treatment numbers of dividing $CD4^+$ T cells had returned to normal. Interestingly, the percentage of proliferating $CD4^+$ naive and $CD27^+$ memory T lymphocytes was still significantly elevated at that time point ($p<0.05$; data not shown). In the $CD8^+$ T-cell compartment, the steepest decline in the number of Ki-$67^+$ T cells was observed in the first four weeks of treatment (figure 6B; $p<0.05$ for $t=0$ vs. $t=4$ in total $CD8^+$ T cells and in naive, $CD27^+$ and $CD27^-$ memory T-cell subsets). After one year, the percentage (data not shown), but not the absolute number of Ki-$67^+$ $CD27^+$ and $CD27^-$ memory and Ki-$67^+$ $CD27^-$ effector T lymphocytes had returned to normal values ($p<0.008$).

Despite the severe depletion of the naive T-cell pool, the number of Ki-$67^+$ naive T lymphocytes fell immediately upon introduction of HAART

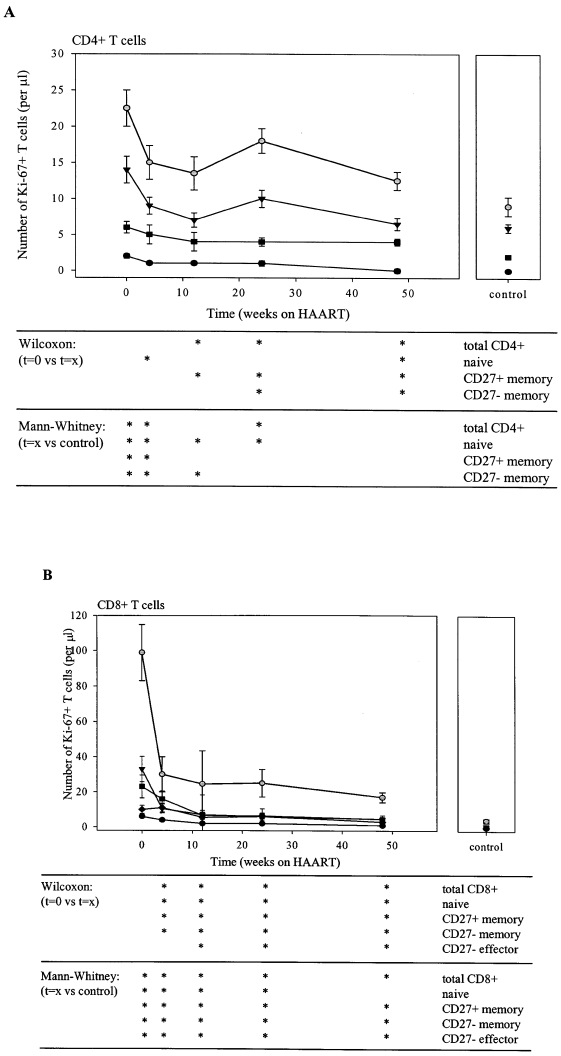



**Figure 6.** *Longitudinal analysis of Ki-67 antigen expression in CD4+ (A) and CD8⁺ (B) T lymphocyte subsets and total populations. Decline of the number of total (gray circles), naive (black circles), CD27⁺ memory (black triangles), CD27⁻ memory (black squares) and CD27⁻ effector (black diamonds) Ki-67⁺ T cells is shown. Sequential patient values were compared using the Wilcoxon Signed Ranks Test, and the Mann-Whitney U Test was used for comparison with healthy individuals (*: p<0.05).*

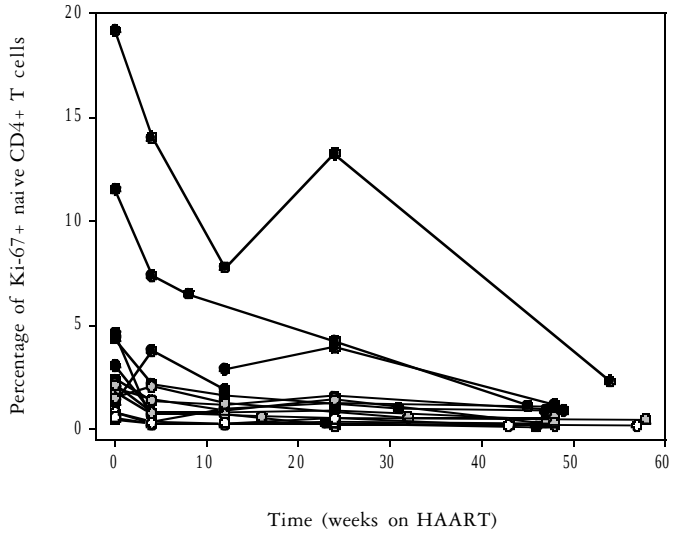



**Figure 7.** *HAART-induced decline in the percentage of proliferating Ki-67⁺ naive CD4⁺ T cells. The courses of individual patients are shown. Black dots represent patients with baseline naive CD4⁺ T cell numbers < 300/µl (n=9), gray dots represent individuals with baseline naive CD4⁺ T cell counts > 300/µl but < 400/µl (n=5) and white dots represent patients with baseline naive CD4⁺ T cell count > 600/µl (n=2).*

(figure 6A and 6B). Figure 7 depicts data of the individual Ki-67 decline in naive $CD4^+$ T lymphocytes. Reduction of Ki-67 expression was relatively slow in two patients who had less then 100 naive $CD4^+$ T cells per µl and the highest percentage of dividing naive $CD4^+$ T cells at baseline. However, as is shown in figure 8A, no correlation was found between early naive, $CD27^+$ memory or $CD27^-$ memory (data not shown) $CD4^+$ T-cell recovery with the baseline size of these subsets or with the number of dividing lymphocytes. In the $CD8^+$ T-cell compartment (figure 8B), recovery of the naive subset correlated negatively with its baseline size and with the number of dividing cells within this pool ($r = -0.511$, $p= 0.043$ and $r = -0.743$, $p = 0.001$ respectively). Finally, patients with the most pronounced reduction in size of the $CD27^+$ or $CD27^-$ memory T-cell subset had highest numbers of $CD27^+$ or $CD27^-$ memory $CD8^+$

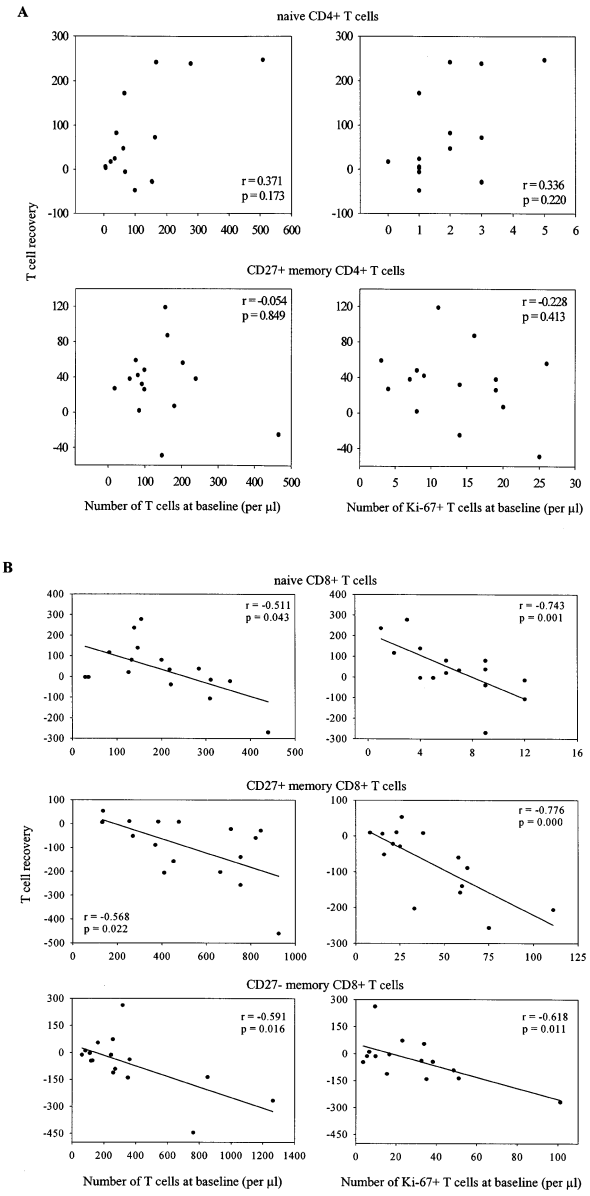



**Figure 8.** *Early recovery of the naive and CD27$^+$ memory CD4$^+$ (A) and naive, CD27$^+$ memory and CD27$^-$ memory CD8$^+$ (B) T cell pool. Correlations are shown between T cell subset recovery in the first four weeks of HAART (delta t=0 versus t=4) and baseline size of subset or number of Ki-67$^+$ cells within each subset (Spearman's correlation coefficients).*

T lymphocytes (r = -0.568, p = 0.022 and r = -0.591, p = 0.016), and highest numbers of dividing cells (r = -0.776, p = 0.000 and r = -0.618, p = 0.011) at baseline. A similar trend, albeit not significant, was observed for the CD27- effector T-cell subset (data not shown).

## Discussion

In this longitudinal study, we measured expression of the Ki-67 antigen, which is taken as a measurement for proliferating cells, in peripheral blood lymphocyte subsets of HIV-1 infected patients. In untreated HIV-1 infection, both the percentage and number of Ki-67$^+$ CD4$^+$ and CD8$^+$ lymphocytes were significantly increased, as compared to values obtained from healthy individuals. These data confirm previous (mainly cross-sectional) studies (5;7;8;29), showing that T-cell turnover is increased maximally two- to three- fold in the CD4$^+$, and six- to seven- fold in the CD8$^+$ T-cell population. However, when investigating the role of T-cell proliferation in the pathogenesis of HIV-1 infection, lymphocyte subsets rather then total CD4$^+$ or CD8$^+$ T-cell pools should be studied, as naive, memory and effector T lymphocytes follow different courses of depletion or expansion. We therefore analyzed changes in expression of the Ki-67 antigen within naive, CD27$^+$ and CD27$^-$ memory and CD27$^-$ effector CD4$^+$ and CD8$^+$ lymphocyte populations before and during the course of HAART.

The finding that in untreated HIV-1 infection, the number of proliferating cells was elevated two to three times in all CD4$^+$ T-cell subsets is in good agreement with earlier results demonstrating that the number of apoptotic cells within the CD4 population was equally distributed over all subsets (10). Together these data show that in general, HIV-1 infection affects both naive and memory peripheral blood CD4$^+$ T cells to a similar degree. The early decrease in the number of naive CD4$^+$ T cells could therefore reflect interference of the virus with T-cell renewal capacity rather than with peripheral production of these cells.

The median percentage of dividing naive CD4$^+$ T cells was elevated ten-fold. Higher increases in percentages of Ki-67$^+$ CD4$^+$ naive T cells were

observed only when the number of naive cells fell below 100 cells per µl. When depletion of lymphocytes would be the driving force for the increase in peripheral naive $CD4^+$ T-cell production, one would expect the high rate of cell division to be maintained during treatment with HAART until the number of cells would be normalized. We observed, however, an immediate decline in the number and percentage of Ki-$67^+$ naive $CD4^+$ lymphocytes, despite the fact that $CD4^+$ T cells were still severely depleted. From this we conclude that increased Ki-67 expression in the naive $CD4^+$ T-cell subset was caused by generalized immune activation rather then being a T-cell homeostatic response to compensate for T-cell depletion.

Furthermore, our data show that the early therapy-induced increase in $CD4^+$ T cells is accounted for by a rise in the number of both naive and $CD27^+$ memory T cells. Our finding that in both subsets the percentage and number of dividing lymphocytes decreased during anti-retroviral treatment points towards other mechanisms than peripheral expansion involved in recovery of the $CD4^+$ T-cell pool. It confirms previous reports indicating that in the first four weeks of HAART, redistribution of trapped $CD27^+$ cells significantly adds to therapy-induced increase in peripheral blood $CD4^+$ T-cell numbers (14;16;17).

Whereas early restoration of the $CD27^+$ memory $CD4^+$ T lymphocyte compartment was fairly constant between patients, recovery of the naive $CD4^+$ T-cell pool was variable. In patients with less severe depletion of naive $CD4^+$ T cells, a relatively steep increase in the number of naive lymphocytes was observed during the first four weeks of HAART, confirming recently published data by Hengel *et al* (31). After this first steep rise, replenishment occurred at a slower rate. Several factors may contribute to restoration of the naive lymphocyte compartment, such as reappearance of pre-existing naive T cells in the peripheral blood, as well as *de novo* T-cell production from a thymic source. The relative importance of each mechanism in recovery of the immune system needs to be established. Recently, it was reported that thymic production of T cells increased substantially during HAART. Its dependence on disease progression remains to be studied (15).

In contrast to the changes occurring in the $CD4^+$ T-cell compartment, in the naive $CD8^+$ T-cell pool, percentages of dividing cells were elevated

regardless of the size of this subset. This points towards a distinct regulation of peripheral T-cell production rates in $CD4^+$ and $CD8^+$ cells. The high proliferation rate in the latter population most likely reflects antigen-induced expansion. The degree of expansion of the $CD8^+$ T-cell subset correlated with the degree of cell proliferation during chronic HIV-1 infection observed in that particular subset. Ongoing antigenic pressure during untreated HIV-1 infection could lead to continuous recruitment of naive $CD8^+$ T cells, thereby maintaining increased percentages of Ki-67 antigen expression in this subset. During HAART both the percentage and the total numbers of dividing cells declined, suggesting that activation related cell division was the driving force for expansion of the primed subset during chronic HIV-1 infection. The early therapy-induced drop in the number of primed $CD8^+$ T cells reached a plateau after six months, at which the $CD27^+$ or $CD27^-$ $CD45RO^+$ memory and CD27-CD45RO- effector $CD8^+$ lymphocyte pool remained significantly expanded. Although the absolute number of dividing primed $CD8^+$ T cells remained elevated, the percentage of Ki-$67^+$ T cells within this subset returned to normal values. Using tetrameric HLA-peptide complexes, it has been shown that HAART reduced the frequency of HIV-1 specific CTLs several fold (32). The early decline in primed $CD8^+$ T cells that we observed could therefore reflect apoptosis of HIV specific CTLs.

The numbers of dividing cells in healthy individuals found in this study correspond well to the range of earlier results obtained with Ki-67 staining (7;8) and with deuterated glucose labeling of dividing cells (19). However, when studying HIV-1 infected patients, in these reports, the average total peripheral blood production of proliferating $CD4^+$ T cells was 5.2 x $10^7$ (range 2.7-8.3 x $10^7$) and 10.8 x $10^7$ (range 7.1-12.9 x $10^7$) of $CD8^+$ T cells. These values are lower then those reported in our study (11.4x$10^7$ (range 2.4-21.0 x$10^7$) and 49.2x$10^7$ (range 7.0-648.5 x$10^7$), respectively), most likely due to the more advanced disease stage of our patients (see Table 3).

Following the initial HAART-induced decline, we observed a transient rise in the number of proliferating $CD4^+$ T cells after six months of therapy, after which these numbers declined further. Since this rise was only due to increased numbers of proliferating $CD27^+$ memory cells, and coincided with normalization of the size of this subset, it could reflect improvement of the functional capacity of these cells. Indeed, T-cell function, as

**Table 3.** *Baseline numbers of CD4⁺ and CD8⁺ T lymphocytes in HIV-1 infected patients and healthy individuals. Median values and ranges are shown.*

| | CD4⁺ T cells | CD8⁺ T cells |
|---|---|---|
| HIV- | 824 | 391 |
| (n = 5) | (466-1391) | (101-598) |
| HIV+ | 280 | 1350 |
| (n = 16) | (50-1060) | (490-3160) |

measured by the *in vitro* proliferative response to CD3 and CD28 mAb, improved and reached a plateau phase after six months of HAART (data not shown). Whereas in our study the rise in T-cell proliferation did not exceed pretreatment values, in a study reported by Fleury *et al.*, a clear increase in cell production was seen over baseline after six months of HAART (7). In that report, the baseline CD4⁺ T-cell counts were relatively high, on average over 600 cells per µl. We found the transient rise in numbers of dividing cells to be more pronounced in patients with higher baseline CD4⁺ T-cell counts (data not shown), pointing towards variances in the stage of HIV-1 infection as the cause of the observed differences.

Taken together, our data are compatible with the idea that most of the elevated cell division and cell death in HIV-1 infection is related to strong persistent immune activation (30;33), rather than a response to, or the cause of lymphocyte depletion. Therefore, alternative factors leading to T-cell decline must be involved in HIV-1 pathogenesis. As has been proposed previously, CD4⁺ T-cell depletion may be due to a diminished capacity for T-cell renewal as a direct or indirect result of HIV-1 infection or could be related to an intrinsically low capacity for renewal in adults which can not even compensate for a slightly enhanced T-cell death due to HIV-1 infection and generalized immune activation (34-37). Furthermore, we observed a clear distinction between CD4⁺ and CD8⁺ T cells with respect to proliferation rates. A similar dichotomy was found before, when much more cell activation induced death and shortened telomeres were found in

the $CD8^+$ T-cell fraction (5;38). This may fit well with differential requirements for clonal expansion to exercise $CD4^+$ helper and $CD8^+$ effector cell functions. Since cell division diminished rapidly with HAART even when T-cell numbers were still very low, our results suggest that the increased peripheral lymphocyte proliferation is not a homeostatic response to T-cell depletion.

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# 8

# The dominant source of CD4+ and CD8+ T-cell activation in HIV infection is antigenic stimulation

James W.T. Cohen Stuart[1,3]
Mette D. Hazenberg[2]
Dörte Hamann[2]
Sigrid A. Otto[2]
Jan C.C. Borleffs[3]
Frank Miedema[3]
Charles A.B. Boucher[1]
Rob J. de Boer[4]

1 Department of Virology, Eijkman-Winkler Institute, University Medical Center Utrecht
2 Department of Clinical Viro-Immunology, CLB, and the Laboratory for Experimental and Clinical Immunology and Department of Human Retrovirology, Academic Medical Center, University of Amsterdam
3 Department of Internal Medicine, University Medical Center Utrecht
4 Department of Theoretical Biology, University Utrecht, the Netherlands.

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## Abstract

To distinguish between antigenic stimulation and $CD4^+$ T-cell homeostasis as the cause of T-cell hyperactivation in HIV infection, we studied T-cell activation in 47 patients before and during HAART. We show that expression of HLA-DR, CD38 and Ki67 on T cells decreased during HAART but remained elevated over normal values until week 48 of therapy. We confirm previous reports that T-cell activation correlates positively with plasma HIV RNA levels (suggesting antigenic stimulation), and negatively with CD4 count (suggesting $CD4^+$ T-cell homeostasis). However, these correlations may be spurious, i.e. misleading, due to the well-established negative correlation between CD4 count and plasma HIV RNA levels. To resolve this, we computed partial correlation coefficients. Correcting for CD4 counts, we show that plasma HIV RNA levels contributed to T-cell hyperactivation. Correcting for plasma HIV RNA levels, we show that $CD4^+$ T-cell depletion contributed to T-cell activation. Correcting for both, the activation of $CD4^+$ and $CD8^+$ T cells remained positively correlated. Since this suggests that the the $CD4^+$ and $CD8^+$ T-cell activation is caused by a common additional factor, we conclude that antigenic stimulation by HIV or other (opportunistic) infections is the most parsimonious explanation for T-cell activation in HIV infection. Persistence of HIV antigens would explain why T-cell activation fails to revert to levels of healthy individuals after 48 weeks of therapy.


**Keywords**: Activation, Proliferation, T lymphocytes, CD4, CD8, Antiretroviral therapy.

## Introduction

The T lymphocytes of HIV infected individuals have increased expression of activation markers HLA-DR and CD38 (1-11) and increased proliferation rates. The latter has been demonstrated using two different techniques. First, by determining the fraction of dividing cells via the expression of the nuclear antigen Ki67 (12), it was shown that T-cell proliferation rate is increased maximally two- to three fold in the $CD4^+$ population, and 6-7 fold in the CD8 population (13-16). This limited increase in the division rate is consistent with studies measuring the replicative history of T cells by the average telomere lengths (17,18). The second technique, using deuterated glucose to label DNA in vivo, showed that the turnover of $CD4^+$ and $CD8^+$ T cells in HIV infected patients is ~3 times higher than that of healthy individuals (19). Increased turnover of $CD4^+$ and $CD8^+$ T lymphocytes has also been observed in SIV infected macaques using BrdU to label DNA in vivo (20).

Two models have been proposed to explain the hyperactivation and increased proliferation of T cells in HIV-1 infection. One model contends that T-lymphocyte activation in HIV infection is driven by antigens from HIV and/or from other pathogens (15, 21,22). Alternatively, increased production of $CD4^+$ T cells may be a homeostatic response to compensate for the loss of $CD4^+$ T cells that are killed by HIV (23,24). The goal of this study was to determine which of these to mechanisms best explains the T-lymphocyte activation in HIV infection. We therefore performed a cross-sectional and a longitudinal analysis of the activation status of T lymphocytes, T-lymphocyte population density, and plasma HIV RNA levels in a large cohort of HIV infected patients before and during HAART.

## Materials and methods

**Study population**

The activation and proliferation status of T cells was analyzed in 47 patients from the previously described CHEESE study cohort (26) with a sustained plasma HIV RNA response to levels < 50 copies/ml. Briefly, this is a randomized study comparing antiviral efficacy of zidovudine (Retrovir, Glaxo-Wellcome, Research Triangle Park, N.C.) plus lamivudine (Epivir, Glaxo-Wellcome, Research Triangle Park, N.C.) plus saquinavir-soft-gelatin-capsules (SQV-SGC, Fortovase, Hoffmann-La Roche, Inc., Nutley, New Jersey) versus zidovudine plus lamivudine plus indinavir (Crixivan, Merck, West Point, Pa), in HIV-1 infected patients. Antiretroviral naive patients were eligible for study treatment if at the moment of screening plasma HIV RNA levels were at least 10,000 copies/ml and/or if CD4 counts were less than 500 cells / μL and/or if they had a history of HIV related symptoms (CDC stage B or C). During 48 weeks of treatment, the virologic and the CD4 count response was not different between the two treatment arms (data not shown). Of the selected patients, 25 were from the indinavir arm and 22 from the SQV-SGC arm.

**Healthy Controls**

As controls for the expression of $Ki67^+$ on T lymphocytes, cryopreserved PBMC's from five HIV-seronegative blood bank donors were used. As controls for expression of CD38 and HLA-DR on T cells, freshly isolated PBMC's from 12 healthy HIV-seronegative donors were used.

**Blood sampling**

Blood samples were obtained at baseline, and every 4 weeks through week 24, and every 8 weeks from week 24 through week 48 of treatment.

**Plasma Viral Load**

Plasma HIV RNA levels were measured using an investigational version of the ultra sensitive quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay (Amplicor HIV-1 Monitor, Roche Diagnostic Systems). The lower limit of detection was 50 copies / ml.

**Table 1.** *Correlation between viral load and T-cell activation/proliferation.*

| Activation Marker | r | Controlled for CD4 |
|---|---|---|
| %Ki67 (CD4) | 0.47 § | 0.46 § |
| %HLA-DR (CD4) | 0.22 § | 0.20 ¶ |
| %Ki67 (CD8) | 0.47 § | 0.54 § |
| %HLA-DR (CD8) | 0.27 § | 0.26 § |
| %CD38 (CD8) | 0.64 § | 0.60 § |

¶:p<0.05
§:p<0.001

**Table 2.** *Correlation between CD4 count and T-cell activation/proliferation.*

| Activation Marker | r | Controlled for CD4 |
|---|---|---|
| %Ki67 (CD4) | -0.58 § | -0.55 § |
| %HLA-DR (CD4) | -0.46 § | -0.43 § |
| %Ki67 (CD8) | -0.30 ¶ | -0.12, p=0.3 |
| %HLA-DR (CD8) | -0.12 ¶ | -0.03, p=0.3 |
| %CD38 (CD8) | -0.42 § | -0.32 § |

¶:p<0.05
§:p<0.001

**Table 3.** *Correlation between activation/proliferation in CD4⁺ and CD8⁺ T-cell subsets.*

| $CD8^+$ T cells | $CD4^+$ T cells | r | Controlled for CD4 | Controlled for Viral Load | Controlled for CD4 and Viral Load |
|---|---|---|---|---|---|
| %HLA-DR | %HLA-DR | 0.58 § | 0.62 § | 0.56 § | 0.62 § |
| % CD38 | %HLA-DR | 0.39 § | 0.24 § | 0.33 § | 0.23 § |
| %Ki67 | % Ki67 | 0.62 § | 0.49 § | 0.28 § | 0.32 ¶ |

¶:p<0.05
§:p<0.001

**Monoclonal antibodies**

Peridinin chlorophyll protein (PerCP)-labeled CD4, PerCP-labeled CD8 and phycoerythrin (PE)-labeled HLA-DR monoclonal antibodies were obtained from Becton Dickinson (San Jose, CA). Fluorescein isothiocyanate (FITC) labeled CD38 and FITC labeled Ki67 monoclonal antibodies were obtained from Immunotech (Marseille, France).

**Flow Cytometry**

The fraction of activated $CD4^+$ and $CD8^+$ T cells was determined by three color FACS analysis using monoclonal antibodies against CD4 (or CD8), CD38 and HLA-DR on heparin-anticoagulated venous blood (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA). In 16 patients, the fraction of proliferating T cells was determined before therapy and at week 4,12, 24 and week 48 of HAART by measuring the expression of the nuclear antigen Ki67 on cryopreserved PBMC's.

**Statistical Analysis**

The non-parametric Mann-Whitney-U-Test (Wilcoxon-Rank-Sum-W Tests) was used to compare patients with controls. Longitudinal changes of patient characteristics were tested using the non-parametric Wilcoxon

Matched Pairs Signed-Rank Test. Pearson's correlation coefficients were computed to measure bivariate correlations. Partial correlations were calculated to analyze the correlation that remains between two variables after removing the correlation that is due to their mutual association with a third variable (51). Correlations were computed for pooled data of all time points (week 0-48) and for data of baseline only. Similar correlations were found for baseline and for the pooled data, although the p-values were generally higher in the baseline correlations probably due to a smaller sample size (Table 1-3). Reported p-values are two-sided. All statistical analyses were performed using SPSS for Windows (8.0.0). Non linear regression analysis was performed using Mathematica, version 2.1.

## Results

**Expression of activation markers before HAART**

T cells expressing Ki67 were considered to be proliferating. Ki67 is a protein expressed by cells in the late G1 and the S, G2 and M phase of the cell cycle (12). T cells expressing HLA-DR were considered to be activated cells. The $CD8^+$ T cells expressing CD38 were also to be considered activated (2,4,6-11). We confirm previous reports (1-11) that, prior to the start of HAART, the expression of HLA-DR and Ki67 on $CD4^+$ T lymphocytes, and the expression of HLA-DR, CD38 and Ki67 on $CD8^+$ T lymphocytes, is higher in HIV-1 infected patients as compared with healthy controls (see Figure 1).

**Effect of HAART on plasma viral load, CD4 count and CD8 count**

The median plasma viral load decreased from 40.000 copies/ml to <50 copies/ml in 16 weeks ($p<0.001$). The CD4 count increased from 301±28 at baseline to 507±40 cells/µl at week 48 ($p<0.001$). The increase of CD4 count during the first 4 weeks of therapy was higher (2.0 cells/$mm^3$/day) as compared with the mean CD4 count rise during later 4 week intervals (0.38 cells/$mm^3$/day), in agreement with a biphasic response pattern of the $CD4^+$ T cells to HAART (27). The CD8 count decreased from 1050±70 cells/$mm^3$ at baseline to 870±60 cells/$mm^3$ at week 48 ($p=0.023$).

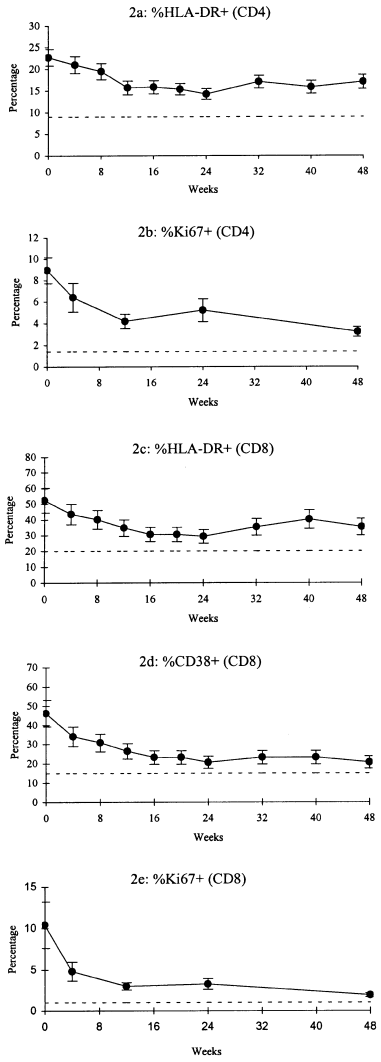


**Figure 1.** *The effect of HAART on the expression of activation antigens on T lymphocytes. Mean values are shown. Bars represent standard error of the mean. The dotted lines represent mean expression levels in healthy HIV-seronegative controls. Figure 2a and 2b show the expression of HLA-DR and Ki67 on CD4⁺ T cells, respectively. Figure 2c, 2d and 2e show the expression of HLA-DR, CD38 and Ki67 on CD8⁺ T cells, respectively. At week 48, the mean expression levels of the activation markers on T cells were still significantly higher than in healthy controls ($p<0.05$ each comparison of patients vs controls (Mann-U-Whitney test)).*

**Effect of HAART on expression of HLA-DR, CD38 and Ki67 on T lymphocytes**

The expression of all activation markers on $CD4^+$ and $CD8^+$ T cells gradually decreased during HAART (all p values < 0.005). At week 48 however, the mean expression levels were still significantly higher than in healthy controls, even though all patients had plasma HIV RNA levels below 50 copies / ml for a median period of 32 (range 0-44) weeks (Figure 1), consistent with the findings of others (28-32).

**Correlation between CD4 count, plasma viral load and percentage of activated and proliferating T cells**

It has been established before that T-cell activation markers are positively correlated with the plasma viral load, and negatively with CD4 counts (11,14). For all 3 activation markers, we report similar correlations (Table 1 and 2). However, these correlations may be spurious, due to the indirect effect of the negative correlation between CD4 count and plasma HIV RNA levels (at baseline r = -0.4, p=0.04). We therefore corrected for the negative correlation between CD4 count and plasma HIV RNA by computing partial correlations. The positive correlations that were observed between the plasma viral load and the expression of activation markers on T lymphocytes (Table 1) are hardly affected by controlling for the indirect effect of the CD4 count. Apparently, independent of homeostatic effects via the CD4 count, the plasma HIV RNA level has a true contribution to the T-cell hyperactivation. This supports the model that antigenic stimulation plays a role in T-cell hyperactivation during HIV infection. Similarly, the negative correlation between the CD4 count and activation of $CD4^+$ T cells, persists after controlling for the plasma HIV RNA load. This suggests that there is a true additional role for CD4 homeostatic effects on $CD4^+$ T-cell activation. Thus, in the $CD4^+$ T-cell compartment, both mechanism seem to play a role. The negative correlation between the CD8 activation markers and the CD4 count however largely disappears (HLA-DR, Ki67, Table 2) when controlling for plasma HIV RNA. Apparently, activation of $CD8^+$ T cells is largely due to plasma HIV RNA levels, and is not directly related to the CD4 count.

In contrast to the inverse relation between CD4 count and the percentage of activated $CD4^+$ T cells, which suggests homeostasis (Table 2), no

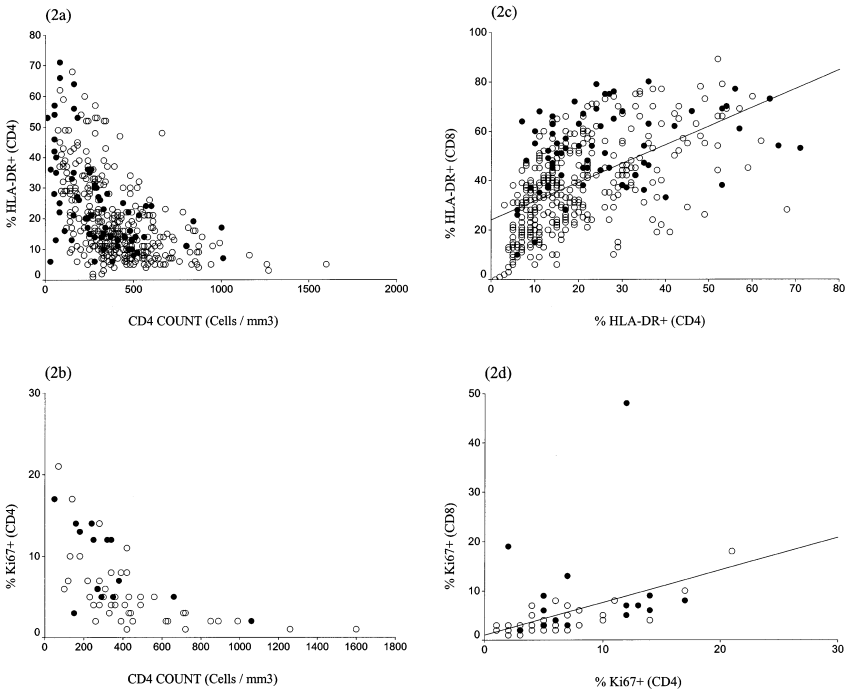


**Figure 2.** *Figure 2a and 2b show the negative correlation between CD4 count and expression of HLA-DR and Ki67 on CD4⁺ T cells, respectively, suggesting homeostatic effects in the CD4⁺ T-cell population. Black dots represent baseline data, white dots represent data from week 4 through week 48 of therapy. Figure 2c shows the correlation between expression of HLA-DR on CD4⁺ T cells and on CD8⁺ T cells. Figure 2d shows the correlation between expression of Ki67 on CD4⁺ T cells and on CD8⁺ T cells. Figure 2a and 2c: n = 461; Figure 2b and 2d: n = 79.*

association was observed between the CD8 count and percentage of CD8⁺ T cells expressing HLA-DR or CD38 (r =0.12 and r = -0.01, respectively, p>0.05). This seems natural as homeostasis is not expected to play a role in the expanded CD8 population. A weak positive correlation between CD8 count, and the percentage of Ki67 expressing CD8 cells was observed (r = 0.22, p=0.047).

**The rate of decay of T-cell activation markers during HAART**

To determine whether a relation exists between decreasing plasma HIV RNA levels and T-cell activation during HAART, we estimated the second phase slope (week 4-48) of HIV RNA from plasma for each patient by linear regression analysis. In addition, the decay rates of the activation markers on T cells were estimated assuming that the percentage of activated T cells at baseline is *a*, and that T-cell activation decreases with rate per day *c* to a level of healthy individuals *b*. We estimated *a, b* and *c* by fitting equation $y = b + a[\exp(-ct)]$ to the measurements of T-cell activation, where *y* is the percentage of activated T cells and *t* is time. No significant correlations were found between the rate of decline of plasma HIV RNA and the decay rates *c* of HLA-DR and Ki67 expression on $CD4^+$ T cells, or the decay rates of HLA-DR, CD38 and Ki67 expression on $CD8^+$ T cells (each absolute Pearson's coefficient < 0.37, each p value >0.24). These findings indicate that the daily decrease of plasma HIV RNA plays a limited role in the decay rate of T-cell activation.

To determine the influence of increasing CD4 counts on $CD4^+$ T-cell deactivation during HAART, we also estimated the daily increase of the CD4 count during HAART for each patient. Because of the biphasic pattern of CD4 count increase, the speed of increase was estimated for the first phase (week 0-4), and the second phase (week 4-48), using linear regression analysis. During both phases, no significant correlations were observed between the daily increase in CD4 count and the decay rates of expression of HLA-DR and Ki67 on $CD4^+$ T cells (each absolute Pearson's coefficient < 0.24, each p value >0.45). These findings indicate that the daily increase of the CD4 count plays a limited role in the decay rate of T-cell activation.

**Correlation between activation and proliferation status of $CD4^+$ and $CD8^+$ cells**

We confirm observations by Sachsenberg et al. (14) that $Ki67^+$ expression on $CD4^+$ and $CD8^+$ T cells is positively correlated (Table 3, Figure 2d). Similarly, the percentage of HLA-$DR^+$ $CD4^+$ T cells was positively correlated with the percentage of HLA-$DR^+$ or $CD38^+$ $CD8^+$ T cells (Figure 2c). This suggests that $CD4^+$ and $CD8^+$ T-cell activation is driven by a common mechanism.

The fact that the positive correlation between $CD4^+$ and $CD8^+$ T-cell activation persists after controlling for the indirect effect of CD4 count (Table 3), suggests that other factors than CD4 homeostasis drive $CD4^+$ and $CD8^+$ T-cell activation. This positive correlation however also persists when we control for plasma HIV RNA level, and when we control for both CD4 count and plasma HIV RNA levels. These findings indicate that additional factors may play a role in T-cell activation, such as immune activation by other infections or HIV antigens that are not correlated with the plasma HIV RNA load.

## Discussion

The aim of this study was to determine the mechanisms involved in increased activation and division of T lymphocytes in HIV infected patients. We found a negative correlation between the CD4 count and the percentage of activated $CD4^+$ T cells, which remains after controlling for plasma HIV RNA load. Observations like this suggest a homeostatic response of the $CD4^+$ T-cell population to compensate for the $CD4^+$ T-cell depletion in HIV infection (23,24). Several other observations however argue against a general role for homeostasis in the increased activation of $CD4^+$ and $CD8^+$ T cells in HIV infection. First, the activation and proliferation is also in the expanded $CD8^+$ T-cell population. Second, because the expression of activation markers on $CD4^+$ and $CD8^+$ T cells remains positively correlated after controlling for the CD4 count (Table 3), factors other than $CD4^+$ T-cell depletion appear to play a role in driving the activation of both $CD4^+$ and $CD8^+$ T cells. Third, the percentage of $CD4^+$ T cells expressing HLA-DR and Ki67 decreased rapidly after the start of HAART eventhough $CD4^+$ T cells were still depleted (15). Fourth, at no time point during therapy, the decrease in the expression of HLA-DR and $Ki67^+$ on $CD4^+$ T cells was correlated to the increase in CD4 count (data not shown).

The plasma viral load correlated positively with the expression of HLA-DR, CD38 and Ki67 on T cells. This positive correlation suggests that plasma HIV RNA load and HIV replication drive T-cell activation.

However, two of our observations suggest that additional factors play a role. First, even though at week 48 of HAART all patients had plasma HIV RNA loads below 50 copies/ml for a mean interval of 32 weeks, the level of T-cell activation and proliferation remained significantly higher than in healthy controls. Second, the decay rate of the percentage of activated and proliferating T cells was not correlated with the elimination rate of HIV RNA from plasma. Third, the expression of activation markers on $CD4^+$ and $CD8^+$ T cells remains positively correlated after controlling for plasma HIV RNA, suggesting that other factors than plasma HIV RNA contributes to T-cell activation.

What additional factors, apart from CD4 homeostasis and plasma HIV RNA levels, could contribute to the T-cell activation? The positive correlation between fractions of activated cells in the $CD4^+$ and $CD8^+$ T-cell population, which persist after controlling for the CD4 count and the plasma HIV viral load, suggests that $CD4^+$ and $CD8^+$ T-cell activation are governed by similar factors. Thus, we believe that the most parsimonious explanation for the hyperactivation of both $CD4^+$ and $CD8^+$ T-cell populations is antigenic stimulation. This may involve (long-lived) antigens from HIV and/or other (opportunistic) pathogens.

The negative correlation between the CD4 count and T-cell activation, which could be taken as evidence for a homeostatic response of the $CD4^+$ T-cell population, may also be explained by antigenic stimulation. A low CD4 count increases the risk of developing opportunistic infections with Pneumocystis Carinii, Cytomegalovirus (CMV) or Mycobacterium Avium Complex (33,34). Moreover, in the blood of patients with low CD4 counts signs of active CMV and Epstein-Barr virus replication have been observed (35-38). These (Opportunistic) infections are associated with increased T-cell activation (11,39-41). Antigenic stimulation also explains the positive correlation between the plasma viral load and T-cell activation. A high plasma viral load is associated with an increases the risk of developing opportunistic infections, and replication of HIV itself will also increase the antigenic load.

The slow decay of T-cell activation during HAART may be explained in two ways. First, the clearance of antigens from other anatomic compartments than the blood, e.g. lymphoid tissue, is expected to be slow

(42-44). In line with this, we observed persistence of HIV p24 antigen in lymphoid tissue, after 18 months of HAART with plasma viral loads below 50 copies / ml (data not shown). Secondly, low level ongoing HIV replication during HAART may play a role. Based on theoretical considerations (45) and the detection of HIV mRNA in lymphoid tissue of patients on HAART with plasma viral load < 50 copies / ml (46-47), it has been hypothesized that a low level of HIV replication may occur during HAART. In addition, it has been demonstrated that the presence of episomal HIV-1 infection intermediates perisist in patients with undectable plasma HIV RNA levels during HAART (48).

There is a strong interaction between HIV replication and T-cell activation because productive HIV infection is largely restricted to $CD4^+$ T cells that are activated (22,25). Several predator-prey type mathematical models of HIV infection describe this interaction, assuming that activated $CD4^+$ T cells are the primary target cells of HIV (49). In contrast to our observations (Figure 1) however, the number of activated $CD4^+$ T cells increases during HAART in these models. Thus, the number of target cells increases if HIV is suppressed. Our results therefore suggest that current mathematical models should be extended with mechanisms for $CD4^+$ T-cell activation by HIV and/or other antigens. One such mechanism, obviously, would be to allow for the immune response to HIV (and/or other antigens).

In conclusion, our results suggest that antigenic stimulation is the dominant mechanism of T-cell activation in HIV infection, rather than $CD4^+$ T-cell homeostasis. Persistence of HIV antigens, or low level ongoing HIV replication during HAART may explain why T-cell activation fails to revert to levels of healthy individuals after 48 weeks of therapy.

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# 9

# Transient relapses ("blips") of plasma HIV RNA levels during HAART are associated with drug resistance

James Cohen Stuart[1]
Colin Kovacs[2]
Maike Rigthart[1]
Dorien de Jong[1]
Steve Kaye[3]
Rob Schuurman[1]
Corjan J. T. Visser[1]
Charles A. B. Boucher[1]

1 Department of Virology, Eijkman-Winkler Institute, University Medical Center Utrecht
2 Division of Internal Medicine, Department of Emergency Medicine, University of Toronto, Canada
3 Department of Virology, Royal Free and University College Medical School, University College London, England

*(Submitted for publication)*

## Abstract

*Introduction*: In a large number of patients on HAART who achieved plasma HIV RNA levels below the limit of detection (50 copies/ml), transient relapses of HIV RNA levels ("blips") are observed.
*Objective*: To determine whether relapses of plasma HIV RNA during HAART are associated with development of drug resistance.
*Methods*: Plasma samples from 15 patients with a transient viral load relapse during HAART were studied. All regimens contained lamivudine (3TC). We used an ultrasensitive sequence approach to analyze the presence of protease and reverse transcriptase drug resistance mutations during the relapse.
*Results*: The median plasma HIV RNA load of the relapse was 72 copies/ml (range 50-1253). In 11 of 15 cases, a genotype of HIV could be obtained. Mutations in the RT and protease gene conferring resistance to one or more drugs were observed in 8 of 11 patients, 6 of whom had the M184V substitution. During a median follow up of 12 months after the relapse, plasma HIV RNA levels remained undetectable in 14 of 15 patients.
*Conclusion*: Plasma HIV RNA blips during HAART are associated with selection of drug resistant HIV in the majority of cases. This indicates that viral replication occurs during HAART, probably due to a temporary decrease in active drug concentrations. In some instances, blips are due to the production of wild type viruses, which can be due to activation of the latent virus reservoir. Independent of the cause, blips did not preclude successful inhibition of viral replication below 50 copies/ml during a year follow-up.

**Keywords:** antiretroviral therapy, transient viral load relapse, resistance, genotype.

## Introduction

In a majority of patients, highly active antiretroviral therapy (HAART) results in a sustained suppression of plasma HIV RNA levels to below 50 copies/ml. However, during prolonged follow up of patients on HAART, transient relapses of the plasma viral load to levels above 50 copies/ml ("blips") can be observed in up to 40% percent of the patients (1-3). The clinical implications of these blips are unclear.

Two mechanisms may account for blips of the plasma viral load during HAART. First, activation of latently infected memory pool could result in the production of HIV particles, analogous to increased plasma HIV RNA levels during opportunistic infections (4,5), after interleukin-2 administration (6) and after influenza pneumococcal vaccination (7-9). Under such circumstances, second rounds of replication of HIV will be prevented by HAART and consequently, no drug resistant virus will be generated. Second, blips of the plasma viral load during HAART could be caused by diminished inhibition of HIV replication as a result of temporary reductions of the concentrations of antiretroviral drugs. This could be due to of interactions with other drugs, intermittent adherence to the regimen or temporary alterations in absorption. As consequence of continuous replication during suboptimal suppression the replicating viruses will acquire drug resistance mutations.

The goal of this study was to distinguish between the two mechanisms underlying transient relapses of the plasma viral load during HAART. We therefore determined the genotype of the plasma HIV population using a ultrasensitive approach during a transient viral load relapse in a well defined cohort of 15 intensively monitored patients on HAART. The regimens of all patients contained lamivudine (3TC). Because continuing HIV replication in the presence of 3TC results in rapid selection of HIV variants harbouring the M184V mutation in the reverse transcriptase (RT) gene of HIV (11-14), emergence of M184V variants could be used as a marker for HIV replication during the relapse.

**Table 1.** *Characteristics of patients.*

| patient number | HAART regimen | Pre-HAART viral load (copies/ml) | Pre-HAART treatment | Duration of HAART at relapse (months) | Duration of follow up after relapse (months) |
|---|---|---|---|---|---|
| 1 | azt/3tc/idv | 50294 | azt | 22 | 10 |
| 2 | d4t/3tc/nfv | 2808 | azt | 22 | 14 |
| 2 | d4t/3tc/nfv | 2808 | azt | 23 | 13 |
| 2 | d4t/3tc/nfv | 2808 | azt | 26 | 10 |
| 3 | azt/3tc/sqv | unk¶ | naïve | 22 | 9 |
| 4 | d4t/3tc/nvp | 60000 | naïve | 18 | 10 |
| 5 | 3tc/ddi/rtv/sqv | 6313 | azt/ddi | 28 | 14 |
| 5 | 3tc/ddi/rtv/sqv | 6313 | azt/ddi | 29 | 13 |
| 6 | d4t/3tc/nfv/sqv | 202900 | naïve | 17 | 12 |
| 7 | d4t/3tc/idv | 2559 | azt | 18 | 13 |
| 8 | d4t/3tc/rtv/sqv | 689600 | azt/3tc | 21 | 15 |
| 9 | azt/3tc/idv | 2137 | naïve | 20 | 5 |
| 10 | d4t/3tc/idv | 50550 | naïve | 17 | 12 |
| 11 | d4t/3tc/idv | 9756 | azt/3tc | 19 | 12 |
| 12§ | d4t/3tc/idv | 9039 | naive | 29 | 15 |
| 13§ | d4t/3tc/idv | 38560 | naive | 26 | 13 |
| 14§ | d4t/3tc/idv | 205042 | azt/ddc | 11 | 12 |
| 15§ | d4t/3tc/nfv/sqv | unk* | naive | 36 | 15 |

¶ No baseline plasma HIV RNA level available.

§ No HIV RNA amplification at blip

## Materials & Methods

### Patients

A cohort of 15 patients with transient plasma HIV RNA relapses during HAART was selected from the outpatient clinic of the division of internal medicine of the university of Toronto, Canada. Patients were selected if

they were on antiretroviral therapy containing at least three antiretroviral drugs, one of which was lamivudine. A transient relapse was defined as having a detectable viral load in an ultrasensitive assay (cut-off 50 copies/ml) during HAART, whereas a plasma viral load < 50 copies/ml was achieved during HAART prior to the blip, and at least one sample obtained subsequent to the blip had a plasma viral load < 50 copies/ml.

**Antiretroviral Regimens**

In 10 of the 15 patients, the antiretroviral regimen at the time of the plasma viral load relapse consisted of lamivudine plus one other nucleoside analogue RT inhibitor (NRTI) and one or two protease inhibitors (Table 1). Four patients were treated with lamivudine, stavudine, plus two protease inhibitors (patients 5, 6, 8 and 15) and one patient was treated with lamivudine, stavudine plus nevirapine (Patient 4). Seven patients were pretreated with mono or dual therapy prior to initiation of HAART (Table 1).

**Plasma HIV RNA load**

The ultrasensitive HIV RNA plasma levels were measured using a bDNA assay with a lower limit of detection of 20 copies/ml (Quantiplex HIV RNA assay version 3.0; Chiron Corp., Emeryville, Calif.). In some cases (as indicated in Figure 1) HIV RNA plasma levels were measured using a bDNA assay with a lower limit of detection of 500 copies/ml (Quantiplex HIV RNA assay version 2.0; Chiron Corp., Emeryville, Calif.).

**Ultrasensitive HIV RNA isolation and amplification**

To be able to isolate and amplify low copy numbers of HIV RNA from the plasma at the moment of relapse, RNA was extracted from large volumes (1.7 to 3.7 ml) of EDTA plasma using the method by Boom et al. (15). An equivalent of 340µl-740µl serum was used for reverse transcription and amplification of the RT gene. The RNA input in the RT-PCR assay varied between 26 and 446 copies of HIV RNA.

A previously described one-tube RT-PCR procedure (16) was performed using oligonucleotides RT18 (sense) 5'-GGAAACCAAAAATGATAGG GGGAATTGGAGG-3' (nucleotides 2376-2406) and RT21 (antisense) 5'-CTGTATTTCTGCTATTAAGTCTTTTGA

TGG-3' ( nucleotides 3511-3538) for the RT gene and oligonucleotides 5'prot1 (sense) 5'-AGGCTAATTTTTTAGGGAAGATCTGGCCTTCC-3' (nucleotides 2076-2108) and 3'prot1 (antisense) 5'-GCAAATACTGGAG TATTGTATGGATTTTCAGG-3' (nucleotides 2733-2702) for the protease gene. A nested PCR was used to amplify the amount of DNA from the first PCR, using oligonucleotides RT19 (sense) 5'-GGACATAAAGCTATAGGTACAG-3' (nucleotides 2453-2474) and RT20 (antisense) 5'-CTGCCAGTTCTAGCTCTGCTTC-3' (nucleotides 3461-3440) for direct sequencing of the RT gene, and oligonucleotides 5'prot2 (sense) 5'-TCAGAGCAGACCAGAGCCAACAGCCCCA-3' (nucleotides 2135-2166) and 3'prot2 (antisense) 5'-AATGCTTTTATTT TTTCTTCTGTCAATGGC-3' (nucleotides 2620-2649) for the protease gene. A nested PCR using oligonucleotides SPP2A (sense) 5'-TGTTGAC TCAGATTGGTTGCACTTTA-3' (nucleotides 2518-2543) and SPP6A (antisense) 5'-TTCTGTATGTCTTAGACAGTCCAGCT-3' (nucleotides 3325-3300) was used for the point mutation assay.

**Population Sequencing**

After nested PCR amplification, fragments were ethanol precipitated and used for direct sequencing according to the Big-dye-terminator protocol (Perkin-Elmer, Foster City, CA, USA). Sequence analysis was performed using the ABI automated sequencer 377 (ABI, Foster City, CA, USA). The relative amount of wildtype and mutant variants was estimated from the sequence electropherograms (cut-off approximately 10%).

**Point Mutation Assay**

To be able to detect minority HIV populations with higher sensitivity than with population sequencing, the wildtype / mutant ratio of amino acid position 184 of the HIV-RT gene was determined using a primer guided nucleotide incorporation assay or point mutation assay (detection level 3%), as previously described (17).

**Drug resistance mutations**

For the protease gene, we distinguished primary mutations that confer drug resistance by themselves from secondary or compensatory mutations, that improve the fitness of HIV containing one or more primary mutations

without increasing resistance (18). Primary resistance mutations are M46I/L and/or V82A/F/T for indinavir, V82A for ritonavir, G48V and/or L90M for saquinavir and D30N and N88D for nelfinavir (18,19).
In the RT gene, mutations considered to confer drug resistance were are M41L, T69N, K70R, T215Y and K219Q for zidovudine, L74V for didanosine, M184V for lamivudine, Y181C/I and/or G190A for nevirapine and V75T for stavudine. In addition, the presence of AZT mutations were also considered to confer resistance to d4T (20,21).

## Results

**Plasma HIV RNA levels during HAART**

The median plasma HIV RNA load at the start of HAART was 44427 copies/ml (range 2137-689600 copies/ml)(Table 1). All patients achieved plasma HIV RNA levels below <50 copies / ml after initiation of HAART. The median viral load at the time of relapse was 72 copies/ml (range 50-1239 copies/ml)(Figure 1). Figure 1 shows the plasma HIV RNA levels patterns during therapy of each patient.
Two patients had two transient relapses throughout the course of HAART (Patients 2 and 7). One patient (Patient 1) had failure of antiretroviral therapy after the transient relapse, i.e. had a viral load more than 400 copies/ml at two or more consecutive time points without achieving a plasma viral load < 50 copies/ml at a later time point while continuing at the same HAART regimen (Figure 1).

**CD4 counts**

The median CD4 count at the moment of the plasma viral load blip was 547 cells/$mm^3$ (range 300-1084 cells/$mm^3$). The CD4 counts at the time of the relapse were not significantly different from the CD4 count measurements directly preceding or following the relapse (Wilcoxon Signed Rank Test for two related samples: p=0.3 and p=0.2, respectively, data not shown).

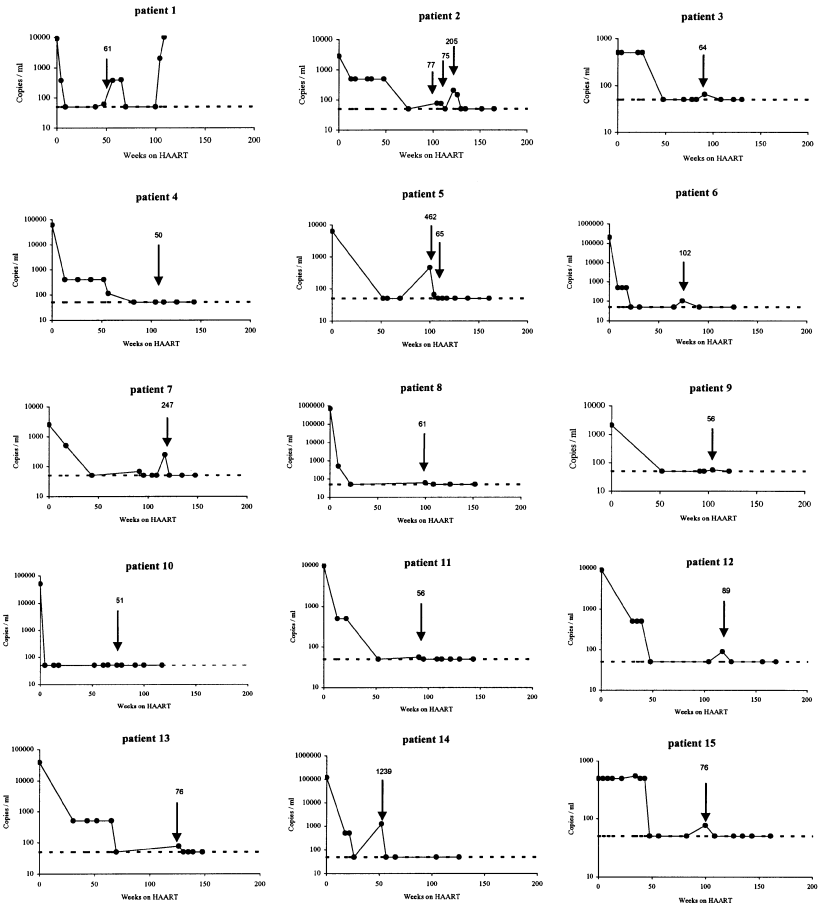


**Figure 1.** *Plasma HIV RNA levels during HAART. Triangles refer to plasma HIV RNA levels that were undetectable in an assay with a lower limit of detection of 500 copies/ml. Arrows refer to the moment and plasma HIV RNA level at the time of relapse.*

**Time characteristics of the relapses**

The median duration that patients were on HAART at the time of relapse was 21.5 months (range 5-36 months) (Table 1). The median duration of follow up after the relapse was 12 months (5-15 months) (Table 1). The median duration that plasma HIV RNA levels were < 50 copies/ml prior to the blip was 11 months (range 2-13 months). The median duration that

**Table 2.** *Characteristics of Blips.*

| Patient | HAART regimen | Plasma HIV RNA Load at blip | M184V mutation in RT % 184V (Sequencing) | %184V (PMA) | Mutations in RT | Primary Mutations in Protease | Secondary Mutations in Protease |
|---|---|---|---|---|---|---|---|
| 1 | azt/3tc/idv | 61 | 10% | 6% | M184V | - | M36I, L63P,V77V/I |
| 2 | d4t/3tc/nfv | 77 | 100% | >97% | D67N,T69T/N,K70R, M184V, K219Q | D30N,N88D | L10I, L63T |
| 3 | azt/3tc/sqv | 64 | 30% | 40% | M184V | - | L63P |
| 4 | d4t/3tc/nvp | 50 | 100% | >97% | Y181C, M184V, G190A | - | L63V |
| 5 | 3tc/ddi/rtv/sqv | 462 | 100% | >97% | L74V, M184V | G48V, V82T | L10V, I54V,V77I |
| 6 | d4t/3tc/nfv/sqv | 102 | 40% | 11% | M41L, M184V, T215Y | - | L63S |
| 7 | d4t/3tc/idv | 247 | 0% | <3% | K70R,T215Y/C | - | - |
| 8 | d4t/3tc/rtv/sqv | 61 | 0% | <3% | M41L,L210W,T215D | - | - |
| 9 | azt/3tc/idv | 56 | 0% | <3% | - | - | - |
| 10 | d4t/3tc/idv | 51 | 0% | <3% | - | - | - |
| 11 | d4t/3tc/idv | 56 | 0% | N.D. | - | - | - |

plasma HIV RNA levels were < 50 copies/ml after the blip was 12 months (range 1-16 months). The median duration of a blip (from the last moment that the plasma viral load was < 50 copies/ml preceding the blip to the first moment after the blip) was 27 weeks (range 13-87 weeks).

**Genotype of HIV plasma variants at the time of the relapse**

From 11 of the 15 patients, HIV RNA could be successfully extracted and amplified for sequencing and the point mutation assay. The drug resistance mutations at the time of relapse are shown in Table 2. Eight patients had mutations conferring resistance to one or more of the antiretroviral agents given at the time of relapse (Table 2: patients 1-8). In 3 patients, wild type viruses were observed at the time of relapse (Table 2: patients 9, 10 and 11).

From patients 2 and 5, the genotype of the HIV plasma population was determined at two and three moments respectively (Figure 1, Table 1). In patient 5, the same drug resistance mutations were observed at the two time points, which were part of the same relapse (Figure 1). In patient 2, the same mutations were observed at the two time points during the first relapse. During the second relapse, an additional mutation at amino acid position 71 in protease was observed (A71V).

**The M184V mutation in RT**

Sequencing of the RT gene revealed that HIV variants with the M184V mutation in were present in 6 of 11 patients at the time of the plasma viral load relapse (Table 2). Using the point mutation assay, M184V variants were detected in the same 6 patients (Table 2). In general, the observed wild type/mutant ratio was similar in both assays.

The M184V mutation was the most common resistance mutation. Patients with the M184V mutation had more drug resistance mutations in protease than patients who had HIV that was wild type at codon 184. No primary drug resistance mutations in protease were observed in the absence of the M184V mutation.

## Discussion

In this study we investigated in 15 patients on HAART whether resistant HIV variants emerge during a transient relapse of the plasma viral load from below 50 copies/ml to a median load of 72 copies/ml. Using an ultrasensitive genotyping procedure, the genotype from the HIV plasma population could be determined in 11 of 15 patients. Mutations in the RT and protease gene conferring resistance to one or more of the given drugs were observed in 8 of 11 evaluable patients. Thus, blips of the plasma HIV RNA during HAART are associated with drug resistance in the majority of cases.

The emergence of drug resistant HIV variants during a blip of the viral load indicates that HIV replication occurred during HAART, probably due to a transient reduction in bioavailability of antiretroviral drugs. Suboptimal drug levels that allow HIV replication while still exerting a selective pressure will cause a rapid selection of drug resistant HIV variants.

We propose that a significant amount of replication must have occurred in the patients in which the M184V mutation emerged, because the M184I was not observed in these patients. Emergence of M184I variants usually precedes the emergence of M184V variants when HIV is allowed to replicate in the presence of 3TC (11).

In three patients (9, 10 and 11), no drug resistant HIV variants were observed. Assuming that the plasma virus population during a blip may contain a mixture of wildtype and resistant virus variants, it could be argued that the observed absence of drug resistant HIV variants in these three patients is due to selective amplification of wildtype virus during the RT-PCR reaction. Selective amplification effects have been observed with ultra low genotyping, when plasma HIV RNA levels were below 60 copies/ml. (24). Because patients 9, 10 and 11 had low plasma HIV RNA levels during the blip ($\leq$ 54 copies/ml), it can not be excluded that besides wild type HIV variants, drug resistant variants were present in these patients.

The transient outgrowth of drug sensitive, wild type viruses during HAART in patients 9, 10 and 11 could be explained by several

mechanisms. It could result from production of wildtype HIV virions by activated memory T cells that were infected prior to the start of HAART. RT inhibitors and protease inhibitors cannot inhibit the production of HIV virions in latently infected cells, although the produced virions will not be infectious for new cells due to the activity of protease inhibitors (22,23).

Immunologic stimulation of latently infected cells could be due to concomitant infections, although this may not always be clinically evident. A clinically evident concomitant infection (influenza) was observed in one of the patients at the moment of the relapse (patient 11). The HIV plasma population of this patient was indeed sensitive to all agents in the regimen (Table 2).

Alternatively, transient outgrowth of drug sensitive viruses during HAART could be due to temporary discontinuation of the antiretroviral therapy. It seems an unlikely explanation since the patients in this study indicated that they were fully compliant as assessed by patient interviews. In the absence of plasma drug levels however it can not be excluded that therapy non-compliance may have played a role.

Our observations may have clinical implications. We show that, independent of the presence of drug resistant HIV variants, transient relapses of the plasma viral load are not predictive of treatment failure during a 12 months follow up. Only one of 15 patients experienced a treatment failure after the relapse, as defined by a plasma viral load >400 copies/ml at two consecutive time points (patient 1). More research is required to determine the long term consequences of transient relapses of plasma HIV RNA levels.

It is surprising that in three patients (2,4,and 5) with two or more resistance mutations conferring resistance to two or more of the agents, the plasma HIV RNA levels could be re-suppressed to <50 copies/ml after the relapse. It is unlikely that this is due to the natural course of HIV infection since the plasma HIV RNA loads in these patients prior to start with HAART were 2808, 60.000 and 6313 copies/ml, respectively. Suppression by HAART of genotypically multidrug resistant HIV can be explained in several ways. First, the viruses may still be phenotypically sensitive to the combination administered drugs, despite the presence of multiple mutations that are known to confer resistance to each individual drug. Second, multidrug resistant variants could have a compromised capacity to

replicate, i.e. have a reduced fitness (25-29). Third, because the three patients with multidrug resistant HIV during the relapse were on HAART for a relatively long period time (~100 weeks), reconstitution of the immune system may play a role in re-suppression of HIV after the relapse. HAART has been shown to diminish the activation of $CD4^+$ T cells (30,31), thus reducing the number of primary target cells for HIV, which are the activated (HLA-DR+) $CD4^+$ T cells (32).

Because 6 of the 8 patients with drug resistance mutations to the given drugs had the M184V mutation, this mutation could also play a role in the re-suppression of HIV after the relapse. It has been suggested that selection of M184V HIV variants during antiretroviral therapy may be beneficial for therapy outcome due to several reasons. First, M184V variants have reduced replication capacity in vitro due to a decreased processivity of the M184V RT as compared to the wildtype enzyme (13,25). Second, resistance to AZT is delayed by the presence of M184V variants in patients who were treated with AZT/3TC dual therapy (12-14). In vitro it was shown that AZT resistance is suppressed by the M184V mutation. Third, it has been demonstrated that the fidelity of the M184V enzyme is higher as compared to the wildtype enzyme (33,34). It was hypothesized that this might reduce the mutation rate of the mutant HIV and delay the generation of mutations conferring resistance to the other compounds of the regimen. However, it was shown that the increased fidelity of the M184V enzyme does not reduce the overall error rate of the M184V virus (35). Fourth, cytotoxic T lymphocytes that specifically target M184V HIV variants have been observed. It has been suggested that the M184V specific CTL´s may play a role in the suppression of HIV after selection of the M184V mutation (36).

In conclusion, our data show that HIV RNA blips during HAART are associated with the development of drug resistance in the majority of the cases. This indicates that viral replication may occur during HAART, probably caused by a temporary decrease in the active drug concentrations. In the remaining cases, blips are due to the production of drug sensitive wildtype viruses, which may be caused by activation of pre-therapy infected memory cells. Independent of the cause, transient relapses of the plasma viral load did not preclude successful inhibition of viral replication below 50 copies/ml during a year follow-up after the relapse.

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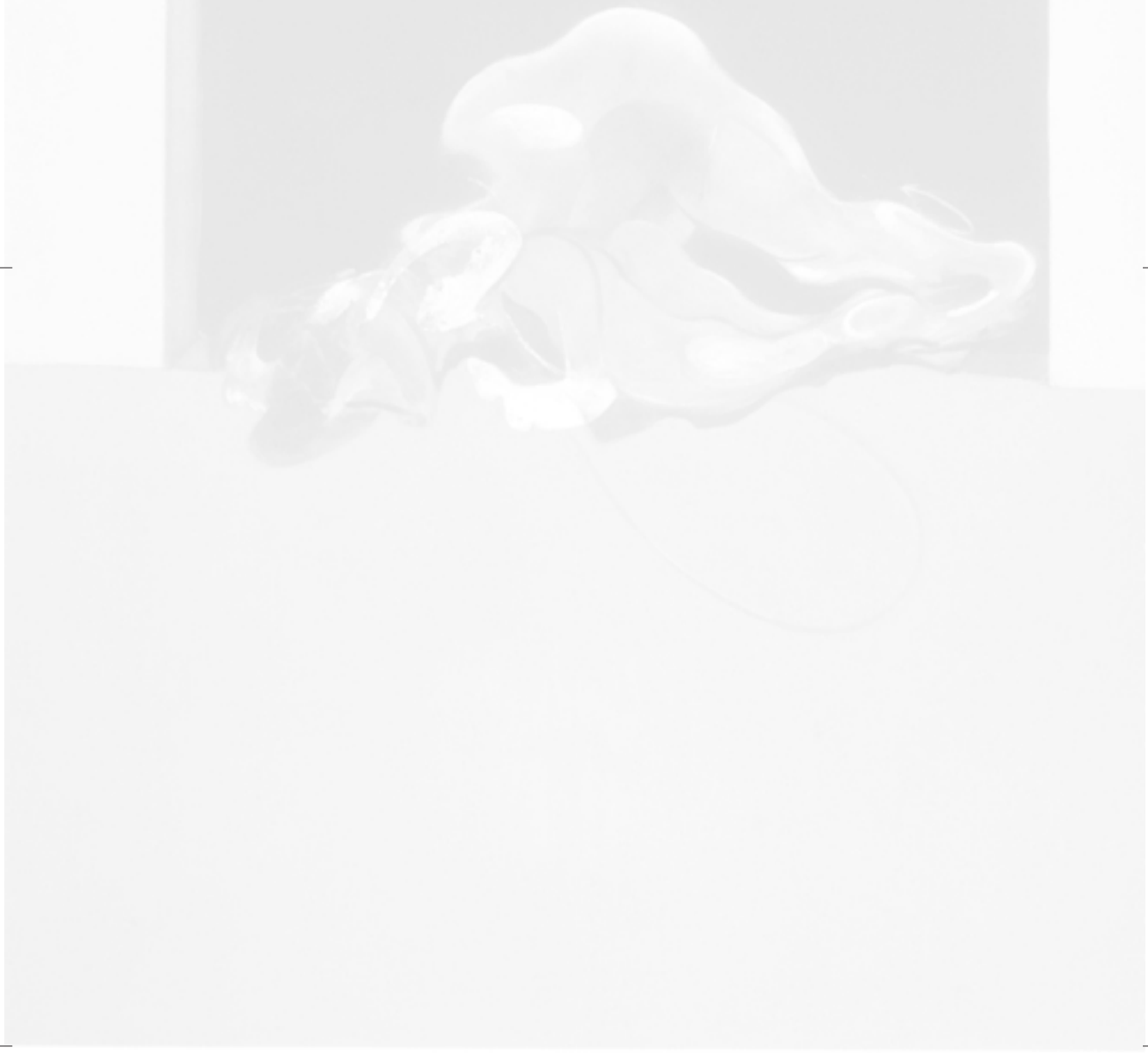


## Inleiding

Het humaan immuundeficiëntie virus (HIV) is de veroorzaker van het verworven immuundeficiëntie syndroom (Acquired Immunodeficiency Syndrome, AIDS). Een infectie met HIV leidt tot een afname van het aantal $CD4^+$ T cellen. Deze cellen spelen een centrale rol bij de afweer van het lichaam tegen ziekmakende micro-organismen, zoals gisten, schimmels, bacteriën en virussen. De $CD4^+$ T cellen worden wel de helper cellen van het afweer systeem genoemd. Afname van het aantal helper cellen door een HIV infectie maakt het afweersysteem minder effectief en uiteindelijk ontwikkelt zich bij de meeste HIV geïnfecteerde patiënten AIDS.
De schadelijke werking die HIV uitoefent op het afweersysteem kan afgeremd worden door behandeling met een combinatie van HIV remmers. Deze geneesmiddelen worden ook wel antiretrovirale geneesmiddelen genoemd. HIV is immers een zogenaamd retrovirus. In de kliniek worden op het ogenblik twee typen antiretrovirale middelen gebruikt, die elk een specifiek enzym van HIV remmen: protease remmers en reverse transcriptase remmers. In dit proefschrift worden onderzoeken beschreven naar het effect van deze combinaties ("cocktails") van antiretrovirale geneesmiddelen op de replicatie (vermenigvuldiging) van HIV en het naar het herstel van het afweersysteem tijdens zo'n combinatie therapie.

## De hoofdstukken

Als inleiding op dit proefschrift wordt in **hoofdstuk** 1 een overzicht gegeven van de huidige kennis over de pathogenese van HIV infectie en de effecten van antiretrovirale therapie.

**Hoofdstuk** 2 beschrijft de CHEESE studie. CHEESE staat voor **C**omparative trial in **H**IV infected patients **E**valuating **E**fficacy and **S**afety of saquinavir **E**nhanced oral formulation and indinavir given as part of a

triple therapy. In deze studie werd de antiretrovirale effectiviteit en de bijwerkingen van twee combinatie therapieën met elkaar vergeleken, te weten een combinatie van saquinavir soft-gelatin-capsules (SGC) + zidovudine (AZT) + lamivudine (3TC) versus een combinatie van indinavir + AZT + 3TC (de standaard therapie toen de studie werd gestart). Het was de eerste studie waarin twee protease remmers (saquinavir en indinavir) met elkaar vergeleken werden, toegediend in combinatie met dezelfde reverse transcriptase remmers. Het was een gerandomiseerde, "open label" studie (dus iedereen wist welke medicatie er na loting aan de patiënten toegediend werd), uitgevoerd in 8 ziekenhuizen in Nederland. In totaal namen er 70 HIV geïnfecteerde (volwassen) patiënten deel aan de studie, 35 in elke arm. De patiënten waren voor het begin van deze studie nooit behandeld met HIV remmers.
De antiretrovirale effectiviteit van beide therapie armen was niet verschillend na 24 weken en na 48 weken therapie. In beide behandelarmen van de studie was een snelle afname van het aantal virus deeltjes in het plasma waarneembaar. Na 24 weken therapie was het percentage patiënten met minder dan 50 kopieën HIV RNA (het genetisch materiaal van HIV) per milliliter plasma 74.3% in de saquinavir groep en 71.4% in de indinavir groep. Dit verschil is niet significant. De toename van het aantal $CD4^+$ T cellen in de eerste 24 weken was hoger in de saquinavir-SGC groep dan in de indinavir groep (162±20 VS. 89±21 cellen per mm3). Echter, na 32 weken therapie was geen verschil in het CD4 getal meer aantoonbaar was tussen beide therapie armen.

De soft-gel-capsules van saquinavir zijn ontwikkeld omdat de oorspronkelijke toedieningsvorm van saquinavir, de hard-gel-capsules, een lage biologische beschikbaarheid heeft van slechts 4% (het wordt niet goed opgenomen in het lichaam). **In hoofdstuk 3** is onderzocht of gebruik van de saquinavir soft-gel-capsules leidt tot hogere saquinavir spiegels in plasma bij patiënten dan bij gebruik van de hard-gel-capsules. Een hogere biologische beschikbaarheid van de soft-gel-capsules ten opzichte van de hard gel capsules kon niet aangetoond worden. Tevens kon geen relatie aangetoond worden tussen de saquinavir spiegels in het plasma van de patiënten en de antiretrovirale repons gedurende 48 weken therapie.

In **hoofdstuk** 4 en **hoofdstuk 5** is de relatie onderzocht tussen de leeftijd van HIV geïnfecteerde patiënten en de regeneratie (de heraanmaak) van nieuwe T cellen gedurende antiretrovirale combinatie therapie. Nieuwe T cellen worden aangemaakt in de thymus (zwezerik). Dit is een orgaan dat vlak bij het hart ligt. Nieuwe T cellen worden ook wel naïeve T cellen genoemd omdat ze nooit in aanraking zijn geweest met lichaamsvreemd materiaal (antigeen). Een infectie met HIV leidt tot een afname van het aantal naïeve T cellen waardoor de afweer tegen nieuwe infecties gecompromitteerd kan worden. Onze hypothese was dat de snelheid waarmee het aantal naïeve T cellen toeneemt tijdens antiretrovirale combinatie therapie afneemt met de leeftijd, omdat de thymus kleiner wordt als iemand ouder wordt. De snelheid van naïeve T-cel aanmaak bleek inderdaad omgekeerd evenredig te zijn met de leeftijd van de patiënten. Dit geldt zowel voor kinderen in de leeftijdscategorie van 0-16 jaar (**hoofdstuk** 4) als voor volwassenen van 25 tot 56 jaar (**hoofdstuk 5**).

In **hoofdstuk 6** is onderzocht of de productie van naïeve T cellen door de thymus is verminderd in HIV geïnfecteerde patiënten. Door sommige onderzoekers is gesteld dat De T-cel productie van de thymus bepaald kan worden door het aantal T cellen te meten die recent de thymus verlaten hebben. Deze nieuwe T cellen kan men herkennen aan de aanwezigheid van een T-cel receptor excisie cirkel (TREC). Deze TRECs zijn cirkelvormige stukjes DNA die ontstaan bij het T-cel rijpingsproces in de thymus, tijdens de vorming van de antigeen receptor van de T cel. Een infectie met HIV leidt tot een afname van het aantal T cellen dat TRECs bevat. Gebruik makend van een wiskundig model hebben wij aangetoond dat de verlaging van het aantal TREC positieve T cellen tijdens HIV infectie beter verklaard kan worden door de toename van deling van de naïeve T cellen dan door een vermindering van de thymus functie.

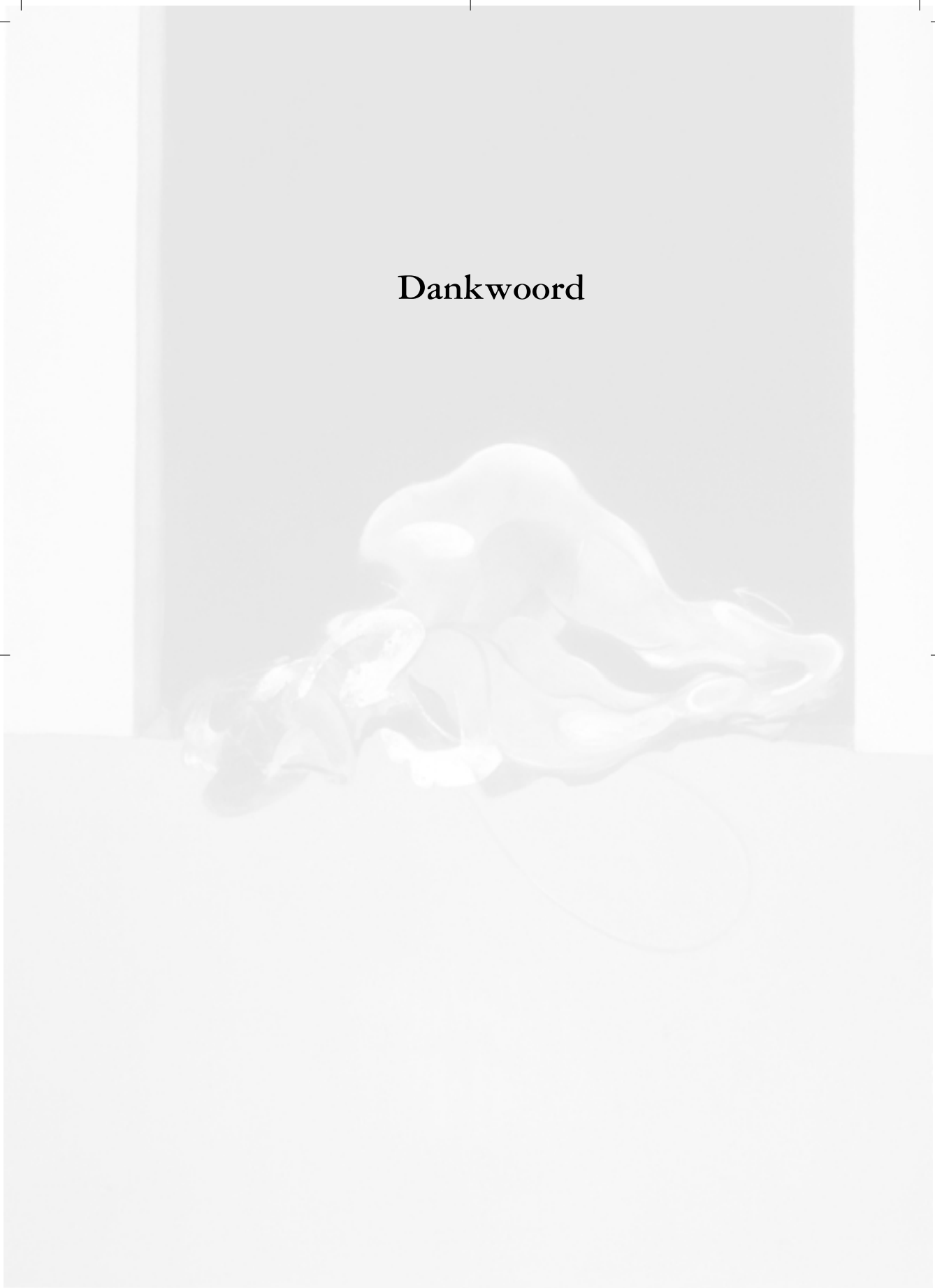
In **hoofdstuk** 7 en **hoofdstuk 8** is de activatie en deling van T cellen onderzocht in onbehandelde HIV geïnfecteerde patiënten en in patiënten tijdens combinatie therapie. Een infectie met HIV leidt tot een toename in de activatie en deling van T cellen. Deze T-cel hyperactivatie kan verklaard worden met twee modellen. Volgens het eerste model is de hyperactivatie het gevolg van een gegeneraliseerde immuunactivatie, veroorzaakt door

antigenen van HIV zelf, en van andere pathogenen (bijvoorbeeld van opportunistische pathogenen). Volgens het andere model is de toegenomen activatie en deling van T cellen tijdens HIV infectie het gevolg van een regelmechanisme van het afweersysteem, dat probeert de afname van het aantal $CD4^+$ T cellen door HIV te compenseren (een homeostatische respons). In **hoofdstuk 7** en **hoofdstuk 8** is geprobeerd uit te maken welk van deze twee modellen het beste de T cel hyperactivatie verklaart. Stimulatie door antigenen lijkt de beste verklaring voor de T-cel hyperactivatie in HIV infecties.

Bij een meerderheid van de patiënten leidt krachtige antiretrovirale combinatie therapie tot een zodanige onderdrukking van HIV dat geen HIV RNA kopieën in het plasma meer aantoonbaar is, althans minder dan 50 kopieeen per milliliter. Echter, nadat het virus eenmaal ondetecteerbaar is geworden in het plasma tijdens combinatie therapie, vinden bij een aanzienlijk deel van de patiënten (tot 40%) kortdurende perioden van HIV viremie plaats. Deze perioden van viremie heten hebben wij "blips" genoemd. Het doel van de studie in **hoofdstuk 9** was om te bepalen of deze blips geassocieerd zijn met de ontwikkeling van resistentie van HIV tegen combinatie therapie. Bij een meerderheid van de patiënten met een blip was HIV resistentie aantoonbaar op genetisch niveau.

**Hoofdstuk 10** is de algemene discussie.

# Dankwoord



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Mijn promotor, professor Hoepelman. Andy, je vermogen om de grote lijnen te zien en die te integreren met details is uitzonderlijk. Ten prooi aan veel verbazing enerzijds en bewondering anderzijds heb ik met je samengewerkt. Je bent uniek en inspirerend. Dank voor je steun tijdens mijn queeste naar een professionele toekomst.

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De theoretisch biologen: Rob de Boer en André Noest. Wiskunde is goed voor de mens.

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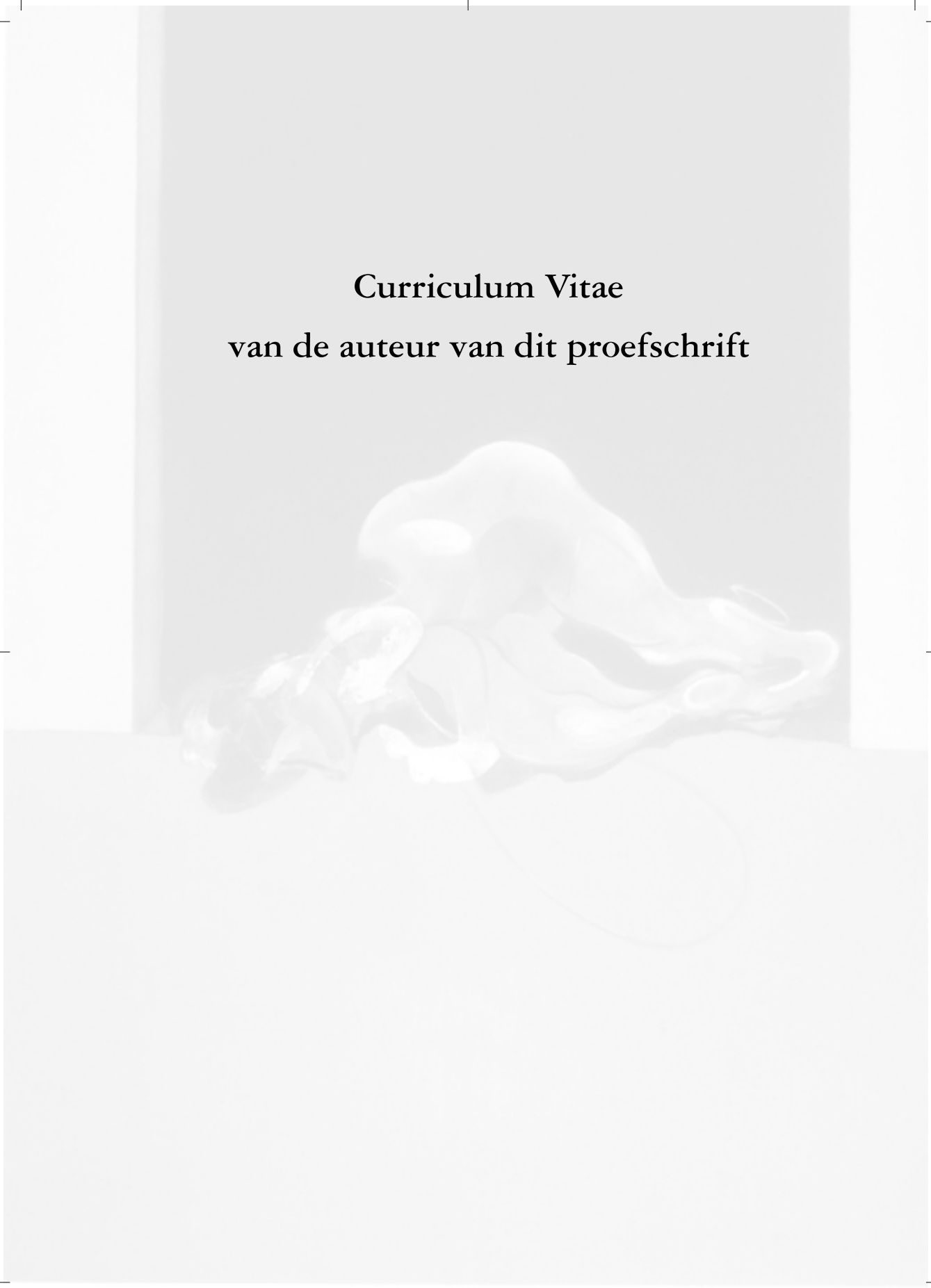
Lieve Ingrid, weet dat de vogels niet dood zijn, ze zijn gevlogen.

Mijn vrienden, ouders en andere dierbaren vormen het fundament van wat ik ben.

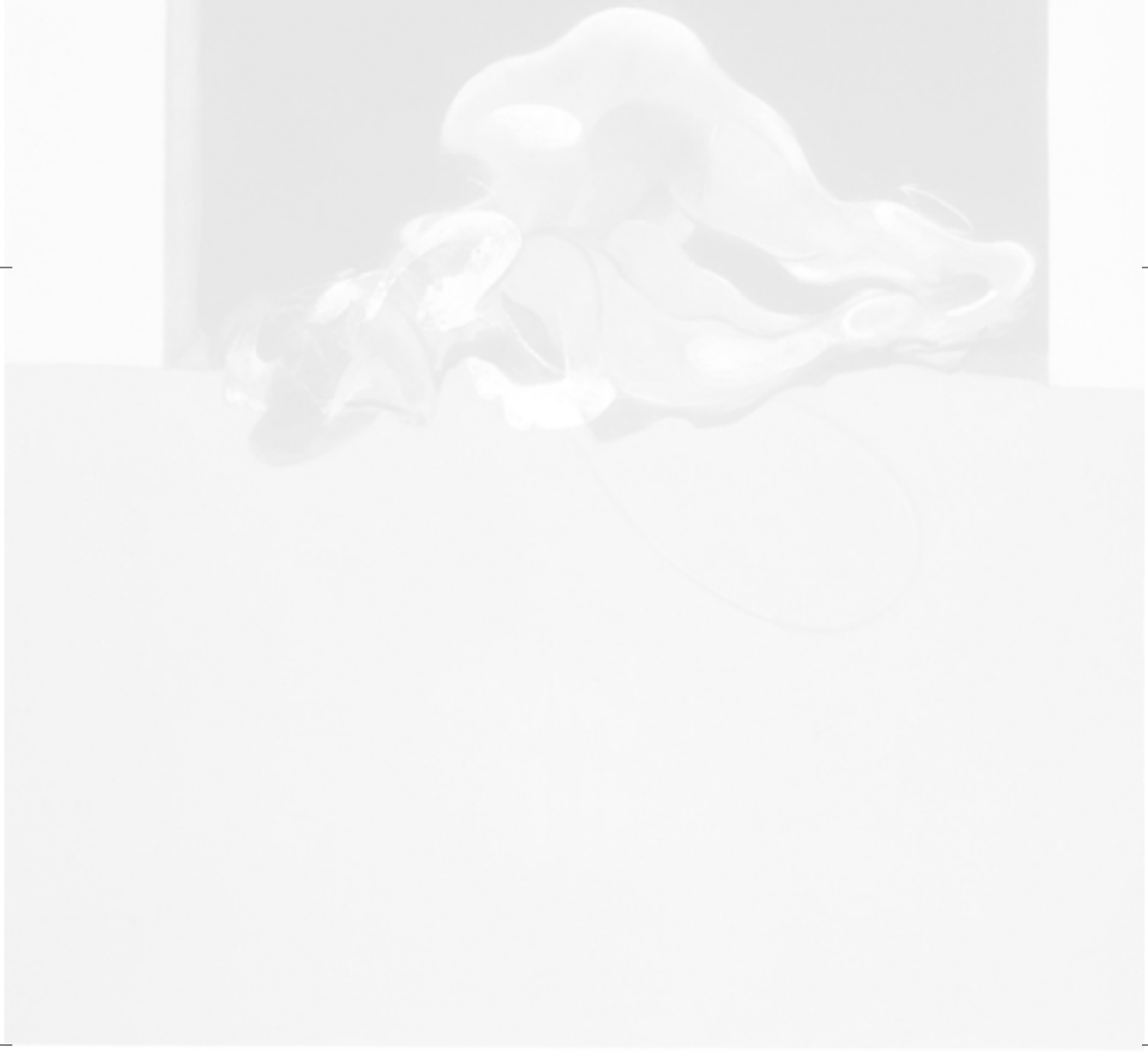
*Jij, die het eind van ieder streven,*
*begrijpt in het geleidend licht.*
*Kun jij mij sneeuw en storm vergeven,*
*mijn waanzin, duister en gedicht.*
Jean Pierre Rawie

# Curriculum Vitae
# van de auteur van dit proefschrift



| | |
|---|---|
| 1981-1987 | Gymnasium Haganum te 's Gravenhage. |
| 1987-1988 | Propedeuse Geneeskunde, Universiteit van Amsterdam. |
| 1988-1992 | Doctoraal Geneeskunde, Universiteit van Amsterdam. |
| 1992-1994 | Doctoraal Medische Biologie, Universiteit van Amsterdam, cum laude. |
| 1994-1996 | Co-assistentschappen. |
| 1996 | Examen tot Arts, cum laude. |
| 1997-2000 | Promotie onderzoek bij het Laboratorium voor Medische Microbiologie, Eijkman-Winkler Instituut, en de afdeling Interne Geneeskunde, onderafdeling Infectieziekten & AIDS, Universitair Medisch Centrum Utrecht |
| 2000 | Virology Networks, Utrecht |
| 2001- | McKinsey & Company, Amsterdam |

# Publications



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