# PROTECTION AGAINST HIV-DISEASE PROGRESSION:

## *FROM IMMUNE ACTIVATION TO T-CELL IMMUNITY*

Beschermende elementen in HIV-ziekte progressie:

*Van imuunactivatie tot T-cel immuniteit*

(met een samenvatting in het Nederlands)

**Proefschrift**

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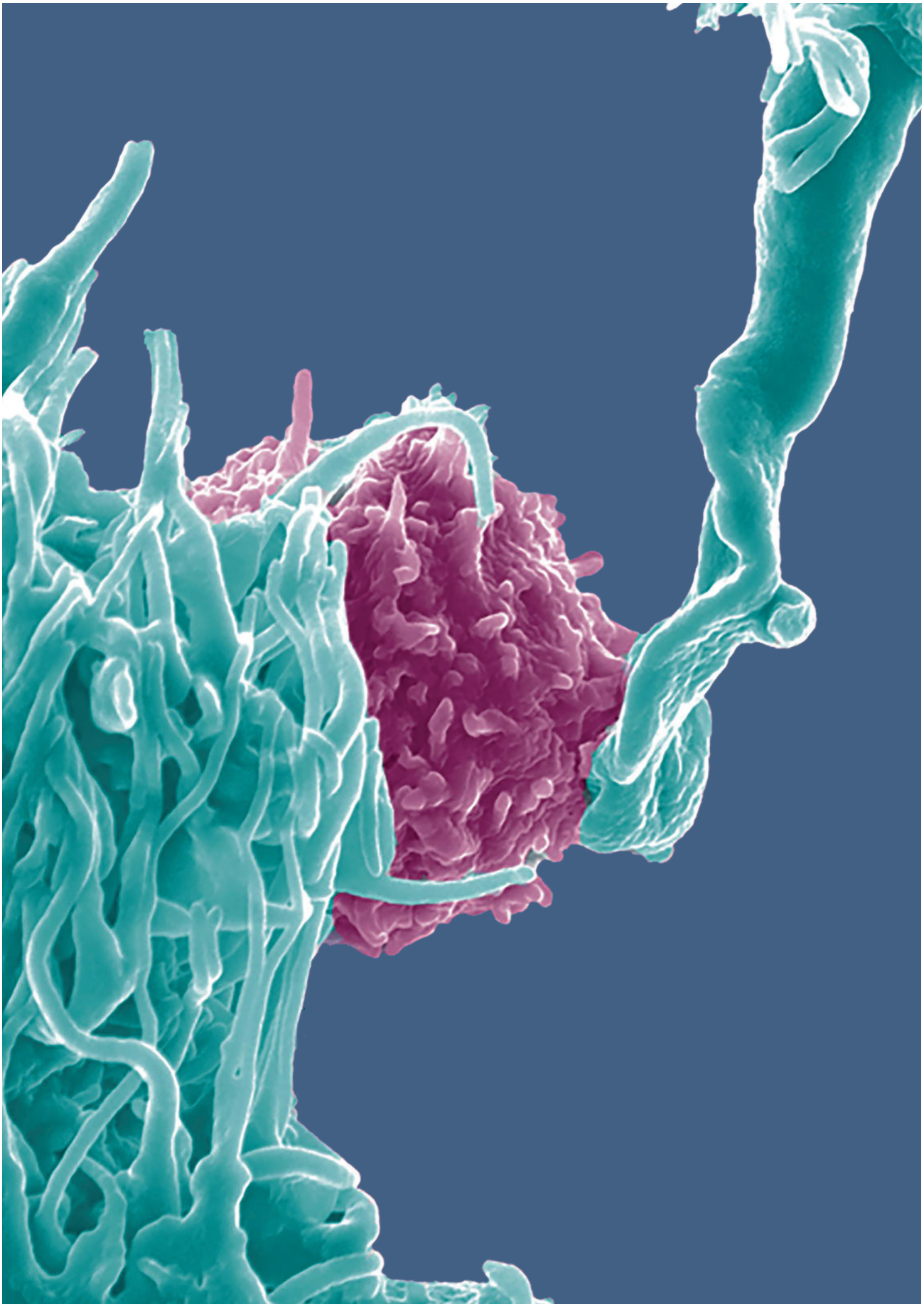

# 1

# GENERAL INTRODUCTION

1

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### *Pathogenesis of HIV infection*

As the name suggests, human immunodeficiency virus (HIV) causes the immune response to fail, most notably by triggering $CD4^+$ T cell loss. Initially after infection there is an enormous peak in virus production followed by a nadir (see Figure 1). This viral nadir is called the viral setpoint and is reached after the immune system has developed HIV-specific $CD8^+$ T cells that target the virus [1, 2]. The viral load at the viral setpoint is predictive of the time it takes before control of the virus is lost, the $CD4^+$ T-cell counts plummet and the HIV-infected individual progresses to acquired immunodeficiency syndrome (AIDS) [3]. $CD8^+$ T cells are very important in controlling the virus [1, 2, 4-7]. Due to continuous exposure to HIV, the immune system will attain a state of chronic systemic inflammation. The overall immune activation has a debilitating effect on the $CD4^+$ and $CD8^+$ T cells, causing increased rates of T-cell turnover and accelerated immune aging (see Figure 1) [8-11].

### *The quest for a HIV-cure*

In 1985, shortly after the isolation of HIV-1, the US Human Services Secretary famously declared "We hope to have a vaccine [against AIDS] ready for testing in about two years" [12]. That turned out to be overly optimistic and now, 30 years of dedicated research later, we still have no 'working' vaccine or cure for HIV infection.

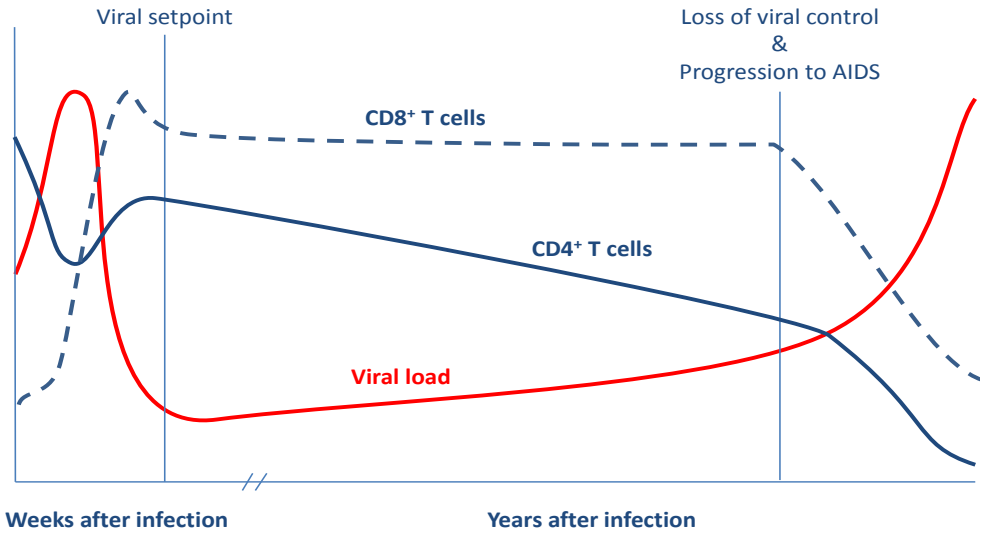


**Figure 1. schematic overview of HIV-pathogenesis.**

The dynamics of $CD4^+$ (blue solid line) and $CD8^+$ (blue dashed line) T-cell counts and viral load (red line) in HIV-disease progression.

*Drug treatment*

The turbulent road to a cure for HIV-infection has directed the HIV-field to focus on a more successful alternative; suppression of HIV and prevention of HIV disease progression. In 1987 the first antiretroviral drug, a nucleoside reverse transcriptase inhibitor (NRTI), was approved for use. However replication of HIV-1 is very error prone and characterized by high mutation rates. Combined with its high replication rate in vivo the virus has a tremendous capacity to adapt [13]. This ability to rapidly diversify allows HIV-1 to evade the host's immune system but also to develop resistance to antiretroviral drugs [14]. Especially as AZT is able to reduce but not fully suppress HIV viral load, drug-resistance mutations rapidly arise, rendering the drug ineffective. It wasn't until 1996 when the so-called triple drug regimen (containing a protease inhibitor and 2 reverse transcriptase inhibitors) was introduced, that it was possible to effectively suppress HIV-1 [15]. Not only did combined antiretroviral therapy (cART) cause reduction of HIV RNA, individuals also showed improved immune function, regression of opportunistic infections, and reduced mortality [15-17]. cART made it possible to lead a life free of HIV-associated complications and has become the primary treatment. In more recent years it has become apparent that starting treatment (very) early –during primary infection– has an additive positive effect. Temporary cART during primary HIV infection transiently lowers the viral setpoint [18]. Moreover, long-term cART initiated during primary infection can enable up to 15% of individuals to maintain viral control 12 months after treatment interruption [19, 20].

Nevertheless, cART is not the holy grail of HIV-therapy. The drugs can have side effects and the daily intake of multiple drugs is taxing. An even larger issue in the quest for effective therapy is that, even on cART, many HIV-infected individuals do not fully recover their immune function and therefore AIDS-related diseases remain a threat [21]. Treatment-mediated $CD4^+$ T-cell reconstitution is often incomplete, especially in mucosal lymphoid tissues, even after many years of viral suppression [22]. The damage to the mucosal tissue leads to gut permeability, subsequent translocation of bacterial products into the blood and finally results in chronic (low-level) immune activation and a diminished capacity of the adaptive immune system to function effectively. As compared to age-matched uninfected adults, treated HIV-infected adults still have higher morbidity and mortality rates [23]. Early treatment seems to be beneficial in terms of effective immune functioning; however as the main body of HIV-infected individuals is not diagnosed with clinical manifestations until after primary infection, this approach is only available for a few exceptional cases.

*Vaccines*

Despite the great advances made in effective antiretroviral treatment, a therapeutic or prophylactic HIV vaccine is therefore still of vital importance. While a truly successful HIV-vaccine has not been produced yet, there have been significant developments. Since the first trial in 1986, more than 250 clinical phase I and II trials have been conducted [24]. Nevertheless only one vaccine –the Thai vaccine trial– has shown a positive (albeit modest) outcome [25]. During the study, there was a 31.2% reduction of HIV infections in the vaccine-group compared to the placebo group. However of the 3 statistical tests used to analyse the trial, only one showed that the lower rate of infection in the vaccine group compared to the placebo group was (borderline) statistically significant. Additionally, once individuals had been infected, no effect could be found on the viral load and $CD4^+$ T-cell counts. Moreover

in 2008 the largest vaccine trial to date –'the STEP trial' which was jointly initiated by Merck and the National Institutes of Health– had to be halted because of a lack of protection, no effect on viral set points, and an increased risk of infection in vaccinated individuals with pre-existing immunity against the viral vector that was used in the vaccine [26].

Though we have come a long way since HIV-1 was first isolated, effective treatment remains elusive. The persistently increased mortality and morbidity during drug therapy and the substandard outcomes of vaccine trials made us realize that we 'must step back to the drawing board' and try to develop a better understanding of the mechanisms that drive HIV-pathogenesis and how to oppose them [27, 28].

What do we know about the cause of AIDS and the control of HIV-1 disease progression?

***Cause of AIDS and HIV-related diseases***

*Activation*

The hallmark of HIV disease is the gradual loss of $CD4^+$ T cells. Initially it was thought that the direct cytopathic effects of HIV were the primary cause of the loss of $CD4^+$ T cells. Strikingly, the majority of the dying $CD4^+$ T cells turned out to be uninfected, the so-called 'bystander' cells. In part the reason for bystander death might be abortive infection, whereby accumulation of defective RNA causes apoptosis [29]. However a strong case has also been made that it is the chronic activation that causes activation of $CD4^+$ T cells, which in turn results in T-cell proliferation and high T-cell loss rates [10, 30].

Simian immunodeficiency virus (SIV) monkey models offer an excellent opportunity to figure out more. SIV –the simian counterpart of HIV– is able to induce pathogenic and non-pathogenic infection based on the infected host [11, 31, 32]. Sooty mangabey's (SM) and African green monkey's (AGM), which are the natural hosts of SIV show very limited disease progression, even though SIV viral load is very high in these animals [33]. In contrast, in SIV-infected 'non-natural' hosts, such as rhesus macaques (RM), disease progression does occur. Strikingly the main difference between these two groups is that the natural hosts show very low immune activation levels [34-36]. Similar to SIV infection in natural hosts, a very small and select group of HIV-infected individuals does not progress to AIDS despite high viral loads. Again, the most distinctive characteristic of these individuals is that they have very low levels of immune activation [37].

Other evidence also points towards a pivotal role for immune activation in HIV-pathogenesis. Not only has immune activation been found to be a good predictor of the rate of $CD4^+$ T-cell loss in HIV infected individuals, it was even better than, and independent of viral load [8, 9]. In the past, many markers of systemic immune activation, such as neopterin, sIL-2R, sCD163 and IP10 as well as cellular immune activation markers, have been used as surrogate markers for HIV-disease progression [38-48]. Especially type 1 interferons (IFNs) are thought to contribute a great deal to ongoing systemic immune activation. Once again, strong evidence came from non-pathogenic SIV-infection. In acute infection, SM were shown to mount strong innate and adaptive immune responses to the virus, which resemble the immune responses during HIV infection in humans. Strikingly, however, within 8 weeks after infection, the innate immune response to the virus in SMs was found to be naturally down-modulated [49, 50].

Mechanisms to contain immune activation include reduction of monocyte, macrophage and plasmacytoid dendritic cells (pDC) function, leaving them resistant to further TLR stimulation, and downregulation of type I IFN response genes. In contrast, in pathogenic SIV-infection in RM, the type I IFN response continues throughout the chronic phase of infection [51]. As touched on above, chronic immune activation and inflammation even partially persist under cART. The effects thereof have not been fully elucidated but some of the increased markers of inflammation are predictive of cardiovascular diseases (CVD) and mortality in HIV disease [52].

*Source of immune activation in HIV-1 infection*
The exact mechanisms by which HIV infection induces chronic immune activation are highly complex and still under debate. Two main sources have been pinpointed. One is that HIV directly triggers both the adaptive and the innate immune system. The prolonged stimulation of T cells during HIV-infection causes excessive activation, proliferation, functional impairment and finally loss of T cells. Innate immune stimulation is elicited through the activation of toll like receptors (TLR) 7 and 8 by single stranded HIV RNA, which initiates a signaling cascade that results in activation of pDCs, monocytes and NK cells, and production of type I IFNs [53-57].

A more indirect effect of HIV-1 infection and additional cause of immune activation is the leakage of bacterial products. This so-called bacterial translocation is caused by massive depletion of central memory $CD4^+$ T cells from the gut associated lymphoid tissue (GALT), which is one of the earliest effects of HIV infection [22]. The early injury to the gut immune system, together with the subsequent damage exerted to the gut epithelial cells, is thought to induce gut permeability and translocation of microbial products such as lipopolysaccharides (LPS) [58]. In turn, LPS and other microbial products trigger for instance TLR4 leading to pro-inflammatory cytokine production and systemic activation.

It is unclear which markers of systemic immune activation in chronic HIV-infection are caused by HIV directly and which are driven by bacterial translocation. To be able to exploit these mechanisms as possible therapeutic targets we should first fully understand their functioning.

***Control of HIV-disease progression***
The most important question regarding a CTL vaccine in HIV-infection is how to control the virus. The idea that $CD8^+$ T cells are paramount in controlling HIV infection is supported by a whole range of observations. Firstly, there is a temporal association between the appearance of HIV-specific CTL responses and the decline of viral load during acute infection [7]. Furthermore it was shown that CTL escape mutations —i.e. mutations that leads to loss of recognition by CTL— arise both in acute and chronic infection [1]. Thirdly, depletion of $CD8^+$ T cells in SIV-infection has been shown to lead to an increase in viral load and disease progression [2]. Fourthly, HIV-disease progression is strongly correlated with the human leukocyte antigen (HLA) molecules expressed by the cells of infected individuals. These molecules, which are highly polymorphic, determine which viral peptides are presented to the adaptive immune system and thereby determine the specific CTL response that is mounted. Among the very select group of individuals who are able to control the virus and delay progression to AIDS

—so-called long term non progressors (LTNP)— there is an enrichment for the expression of the molecules HLA-B*27 and B*57, while among individuals with relatively fast progression there is an enriched presence of HLA-B*3503 [4, 59]. A global genome-wide association study (GWAS) even showed that the only singly nucleotide polymorphisms (SNPs) that are significantly associated with the rate of HIV disease progression lie within the peptide-binding pockets of HLA class I molecules [60].

While only a very small group in humans does not progress to AIDS, in chimpanzees AIDS is the exception [61, 62]. The absence of certain major histocompatibility complex (MHC) molecules in the chimpanzee population suggests that chimpanzees experienced a selective sweep due to a lentiviral infection, which strongly affected their MHC class I repertoire [63]. The MHC molecules that are still present in the chimpanzee population may therefore hold important information as to how HIV can be effectively controlled. Interestingly, it was found that the MHC molecules of chimpanzees present viral peptides that are similar to those presented by the relatively "protective" HLA molecules HLA-B*27 and B*57 [64].

Both the MHC I molecules of chimpanzees and protective HLA alleles in humans preferentially target a structurally very constrained part of HIV, the p24-Gag protein [65]. Viral mutations in these regions tend to lead to severe viral fitness loss [66, 67]. Importantly, even irrespective of their HLA-restriction, the number of Gag-specific CTL responses was found to correlate negatively with HIV viral load and with the rate of HIV-disease progression [6], suggesting a central role for targeting Gag. Other mechanisms by which protective HLA-molecules may provide protection include the possibility that their restricted CTL are more immunodominant and of higher functionality than CTL restricted by non-protective HLA molecules [68-70]. Indeed, $CD8^+$ T cells of LTNPs have been shown to be superior in terms of magnitude, frequency, polyfunctionality and functional avidity [71-73].

**SCOPE OF THIS THESIS**

Following the failure of the STEP vaccine trial, Anthony Fauci responded by cancelling the planned Partnership for AIDS Vaccine Evaluation (PAVE) study, and called on to first "determine which immune responses correlate with protection, the central mystery in the frustrating search for an AIDS vaccine" before spending more money on expensive trials (Science 321, 25 July 2008, page 472). The aim of this thesis was to gain insights that are a prerequisite for successful vaccine and therapeutic developments (see Figure 2). These insights should help us understand:

i) the main source of immune activation in HIV infection, and
ii) what the correlates of a protective CTL response are

*Part 1: Activation*
**Chapter 2** dissects the contribution of two main sources of chronic immune activation in HIV-infection –bacterial translocation and HIV-RNA– to the systemic immune activation driving HIV disease progression.

In **chapter 3** we investigated the immunological status of HIV-infected individuals whose viral load set point had been lowered by early cART. It was investigated whether early treatment protects against progression to AIDS by preventing excessive immune activation. Alternatively we explored whether HIV disease progression is limited because HIV-RNA depletion allows for maintenance of functional CTL responses.

*Part2: T-cell immunity*
**In Chapter 4** we investigated whether protective CTL can shorten the lifespan of HIV infected cells producing virus. Thereby we aimed to solve the controversies that have arisen in literature, when studies in rhesus macaques showed that $CD8^+$ T cell depletion does not influence the lifespan of cells productively infected with SIV-1 [74, 75]. As this was previously assumed to be the mechanism that cytotoxic T cells used to target and control HIV infection, we ascertained if not even the most protective CTL-responses shorten the lifespan of productively infected cells.

**Chapter 5** delves further into the characteristics of the immune response needed to delay HIV-disease progression**.** Especially HLA-B*27 and B*57 are associated with relatively slow progression to AIDS. By comparing the T cell responses restricted by these protective HLA molecules and comparing them with HLA-A restricted T cells responses, *within the same individual*, we studied the mechanisms by which these HLA molecules confer protection against disease progression.

In **chapter 6** we concentrated specifically on one component of a protective $CD8^+$ T cell response: its polyfunctionality. By investigating the association between the T cell receptor (TCR) ζ chain –the functional component of the TCR– and polyfunctionality we aimed to elucidate part of the mechanism behind a protective $CD8^+$ T cell response in HIV infection.

The results described above are jointly discussed in **chapter 7**, and put in a broader perspective.

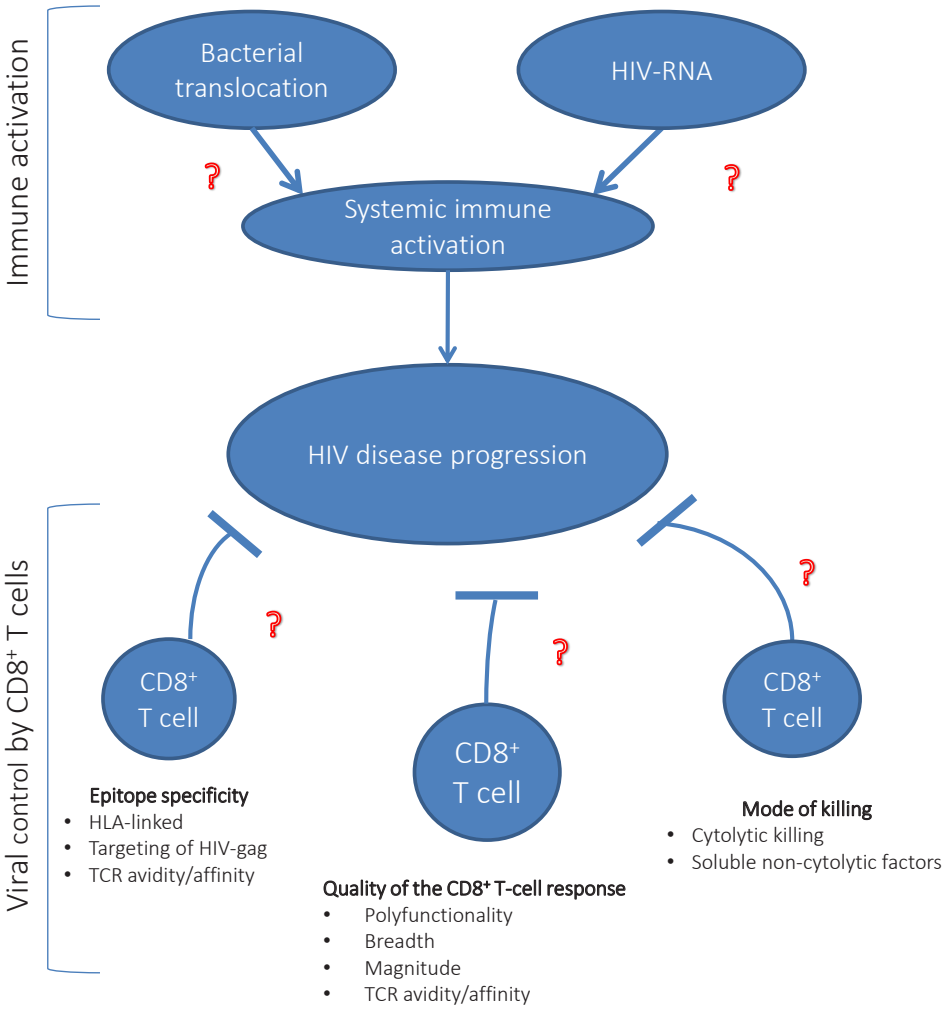


**Figure 2. Schematic outline of subjects explored in this thesis.**

Immune activation is a major determinant of HIV-disease progression. It is poorly understood what the drivers of systemic immune activation are, though two major players are thought to be bacterial translocation and HIV-RNA. In chapter 2 we studied the contribution of HIV-RNA and bacterial translocation to immune activation. While in chapter 3 we investigated whether early treatment can prevent immune activation in HIV-infection.

In contrast $CD8^+$ T cells are known to play an important role in delaying disease progression. In this thesis we explored which characteristics of $CD8^+$ T cells are important in HIV-infection. In chapter 4 we focused on the mode of killing of HIV-infected cells, chapter 5 looks into the quality of the T-cell response and finally, chapter 6 discusses the polyfunctionality of $CD8^+$ T cells.

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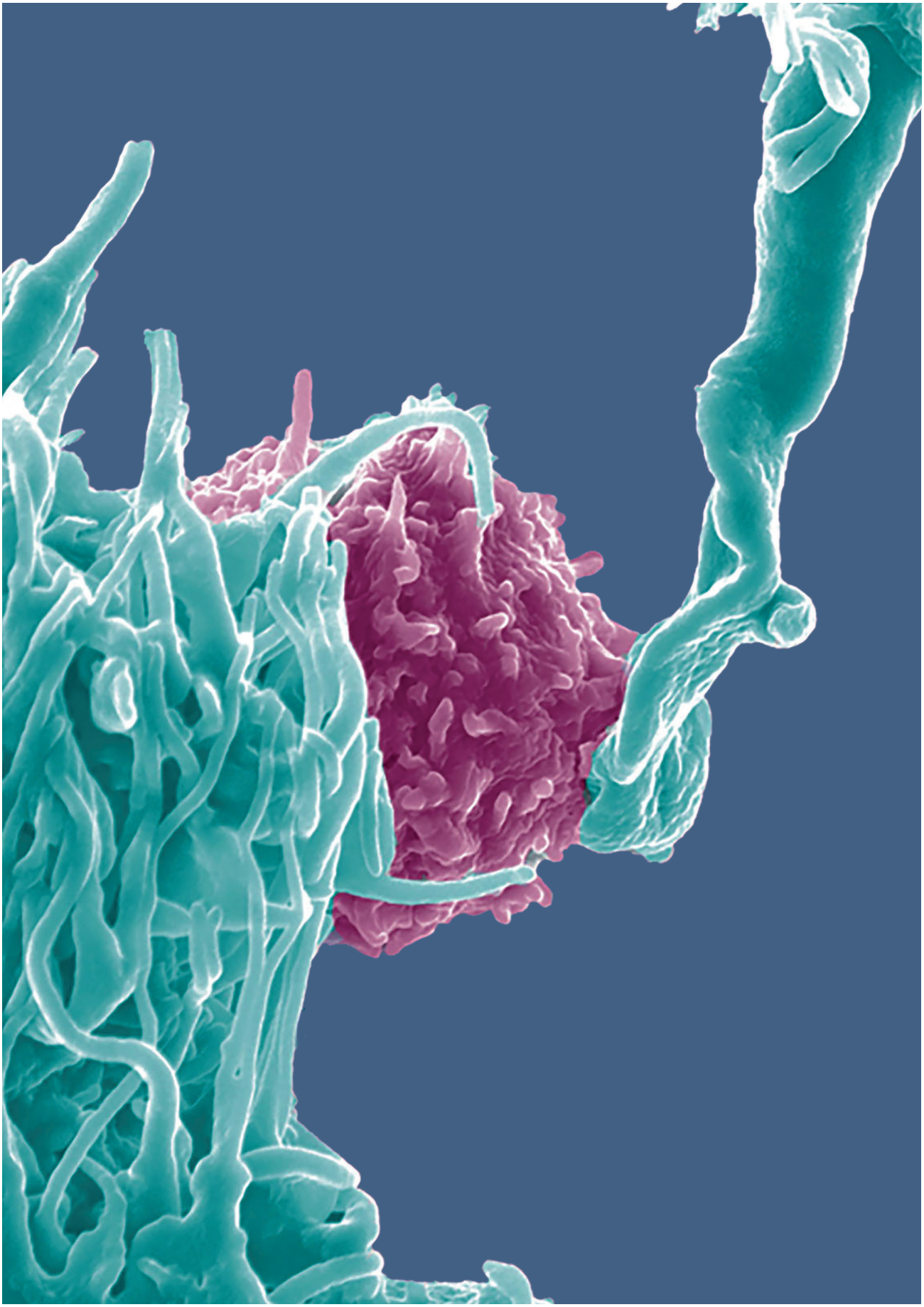

# 2

# CHRONIC IMMUNE ACTIVATION IN HIV-INFECTION: DIFFERENT ROLES FOR HIV-REPLICATION AND BACTERIAL TRANSLOCATION

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*Introduction***:** Chronic immune activation plays a fundamental role in HIV pathogenesis, and is one of the best predictors of the rate of $CD4^+$ T-cell loss in HIV-infected individuals, even better than, and independent of viral load. Both TLR-stimulation by HIV and bacterial translocation as a result of the breach of the gut barrier are thought to be main drivers of immune activation in HIV infection. It remains unclear, however, which features of chronic immune activation are caused by HIV directly and which are driven by bacterial translocation.

*Methods:* Here, we followed the decay dynamics of both sources of immune activation, HIV-RNA and sCD14 –as a marker for bacterial translocation–, longitudinally for a year after cART initiation. Subsequently we correlated these to the decay dynamics of a large group of immune activation markers, expressed by T cells, NK cells and monocytes, as well as soluble factors in plasma.

*Results:* Of the markers that were significantly elevated in HIV-infected individuals, most $CD8^+$ T-cell activation markers and the plasma markers IP-10, sIL-2R and sTNF-R2 tended to follow the decay of HIV-RNA, and rapidly declined in the first 28 days. In contrast, the macrophage activation marker sCD163 and the percentage of $CD16^{++}$ monocytes remained high during therapy, similar to sCD14. $CD4^+$ T-cell activation markers, $CD38^+$ $HLA\text{-}DR^+$ expression on $CD8^+$ T cells and sCD163 decreased on cART but lagged behind the decay of HIV-RNA.

*Conclusion:* The results suggest that immune activation markers of $CD8^+$ T cells are mostly driven by HIV-RNA. While, in contrast, immune activation markers of $CD4^+$ T cells and innate cells tend to remain elevated on cART, despite the significant decline in HIV-RNA.

## INTRODUCTION

It has become evident that immune activation plays a fundamental role in HIV-pathogenesis [1]. Immune activation has been found to be a good predictor of the rate of $CD4^+$ T-cell loss in HIV infected individuals, even better than, and independent of viral load [2-4]. Further evidence supporting the role of immune activation in HIV pathogenesis comes from observations made in sooty mangabey's (SM) and African green monkey's (AGM), which are the natural hosts of simian immunodeficiency virus (SIV), the simian counterpart of HIV [5, 6]. Although SIV viral load is very high in these animals, immune activation levels and disease progression are both limited [7]. In contrast, when SIV is introduced to a 'non-natural' host, such as rhesus macaques (RM), both immune activation and disease progression occur [8]. Similar to SIV infection in natural hosts, a select group of HIV-infected individuals does not progress to AIDS despite high viral loads. The most distinctive characteristic of these individuals is that they have very low levels of immune activation [9].

HIV infection triggers full-blown systemic inflammation as reflected by increased expression of soluble activation markers which are independently linked to HIV-disease progression, including IFNα, IP-10, sIL-2R, IL-6, sCD163 and sTNF-R2 [10-16] and (innate) cellular immune activation markers, such as $HLA\text{-}DR^+CD38^+$ and Ki67 on T cells, CD163 on $CD16^{++}$monocytes [2, 17, 18]. The exact mechanisms by which HIV infection induces immune activation are probably multi-faceted and still under debate.

One important source of immune activation is that HIV directly triggers both the adaptive immune and the innate immune system. The prolonged antigenic stimulation of T cells by HIV-1 causes excessive activation, proliferation, functional impairment and finally loss of T cells [19, 20]. The innate immune stimulation is elicited through the activation of toll like receptors (TLR) 7 and 8 by single stranded HIV RNA, which initiates a signaling cascade that results in activation of plasmacytoid dendritic cells (pDCs), monocytes and NK cells and production of type 1 interferons (IFNs) [21-23]. Especially type I IFNs are thought to contribute a great deal to ongoing systemic immune activation [24], which is illustrated by observations in sooty mangabeys [25]. During primary infection, these animals mount strong innate and adaptive immune responses to the virus, which resemble the immune responses during HIV infection in humans, in whom elevated amounts of type I IFNs and pro-inflammatory cytokines are produced. Strikingly, however, within 8 weeks after infection, the innate immune response to the virus in SMs is naturally down-modulated, despite continuous high viral loads [26, 27]. Mechanisms to contain immune activation are reduction of monocyte, macrophage and pDC function, leaving them resistant to further TLR stimulation, and the downregulation of type I IFN response genes. In contrast, in pathogenic HIV infection, the type I IFN response continues throughout the chronic phase of infection [24].

A more indirect effect of HIV-1 infection and additional cause of immune activation is the leakage of bacterial products through the gut epithelial. This so-called bacterial translocation is caused by massive depletion of central memory $CD4^+$ T cells from the gut associated lymphoid tissue (GALT), which is one of the earliest effects of HIV infection [28, 29]. The instant and massive early injury to the gut immune system, together with the subsequent damage exerted to the gut epithelial cells, is thought to induce gut permeability and the

translocation of microbial products such as lipopolysaccharides (LPS) [30]. In turn, LPS and other microbial products elicit secretion of soluble CD14 (sCD14) by activated monocytes and macrophages. sCD14 then enables the transfer of LPS to TLR4 leading to pro-inflammatory cytokine production and systemic activation [31]. To date it remains unclear how much of the immune activation in chronic HIV-1 infection is caused by HIV directly and how much is driven by bacterial translocation.

Here we investigated the decay dynamics of both sources of immune activation, HIV load and sCD14 –as a marker for bacterial translocation–, frequently and longitudinally after cART initiation. These changes were related to the normalization of a range of immune activation markers, to unravel the contributions of both sources to chronic immune activation in HIV infection.

## MATERIALS AND METHODS

### *Patients*

Blood samples were obtained from 20 treatment naive adult HIV-1 subtype B infected participants who initiated cART (see table 1). During the first year of therapy, blood was drawn at day 0, 4, 7, 14, 28, 42, 56, 60, 180, 270 and 360 to accurately determine the changes of HIV viral load and the various activation markers (see Figure 1A for sampling schematic). Study subjects received cART in the form of 600 mg of efavirenz, 200 mg of emtricitabine, and 245 mg of tenofovir disoproxil. All individuals were antiretroviral-therapy naive before enrolment in this study. In addition, a single blood sample was collected from 30 healthy individuals. The study was approved by the medical ethics committees of the UMC Utrecht and written informed consent was obtained from all individuals.

### *Blood separation*

Chelated blood samples were centrifuged, and cell-free plasma samples were obtained and stored at –80°C until they were analyzed. Subsequently, for the samples collected during the first three months after start of therapy, PBMC were isolated from the chelated blood using Ficoll (GE Healthcare Lifesciences) density separation, as described previously [32]. PBMC were isolated, cryopreserved and stored in liquid nitrogen within 24 hours of collection. All cellular experiments were performed on previously frozen PBMC.

### *Cell phenotyping and Flow cytometry analysis*

Surface staining was performed with monoclonal antibodies described in Table S1. All incubations were performed at 4°C (20 minutes). After fixation and permeabilisation (BD) for 10 min, cells were stained intracellularly (see Table S1). Cells were fixed in cellfix (BD) and flowcytometry was performed. At least 300.000 events were acquired, using the Fortessa flow cytometer (BD). Data were analysed using the DIVA software (BD).

### *Plasma markers*

Levels of TNF-R2, IP-10, sCD14 and sIL-2R in plasma samples were measured using an in-house developed and validated multiplex immunoassay based on Luminex technology (xMAP, Luminex Austin TX USA), as described previously [33, 34]. Measurements were performed

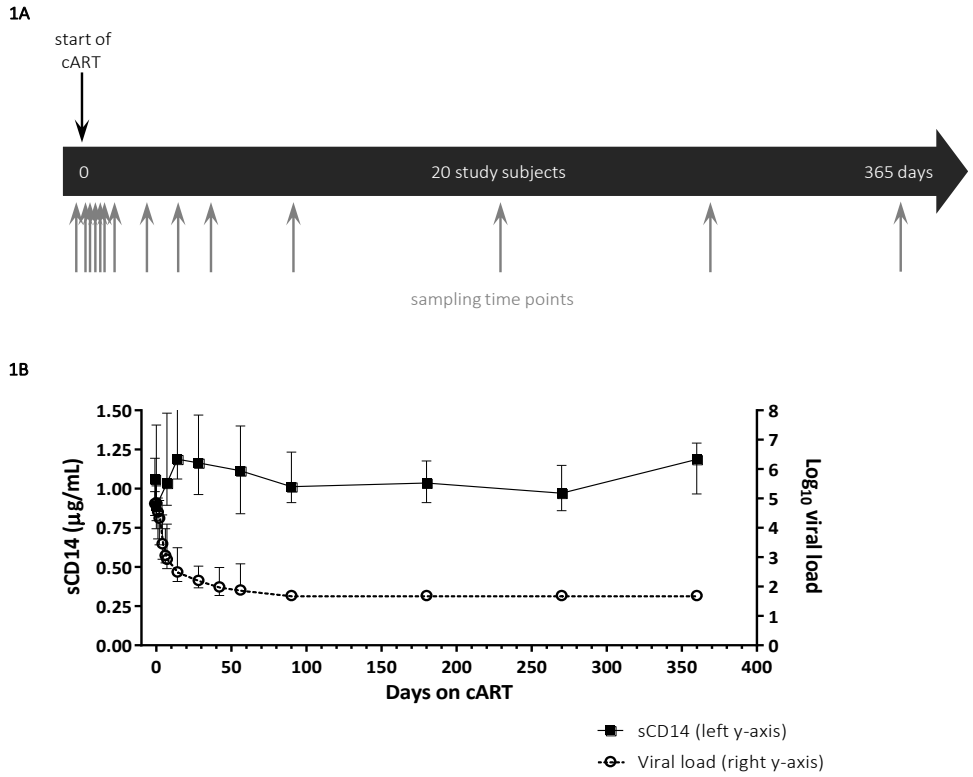



**Figure 1. Suppression of HIV-load, but not of sCD14 –the marker for bacterial translocation- on cART.**
In Figure 1A a schematic overview of the sampling regimen can be found. In Figure 1B, the effect of cART on the sources of immune activation, log10 of HIV-RNA load (right Y-axis, open dots) and sCD14 (left Y-axis, closed squares) was shown longitudinally for 365 days in 20 HIV-infected individuals. Dots/squares represent the median, with interquartile range.

using A-specific heterophilic immunoglobulins that were preabsorbed from all samples with heteroblock (Omega Biologicals, Bozeman MT, USA). Acquisition was performed with the Biorad FlexMAP3D (Biorad laboratories, Hercules USA) in combination with xPONENT software version 4.2 (Luminex). Data was analyzed by 5-parametric curve fitting using Bio-Plex Manager software, version 6.1.1 (Biorad). sCD163 levels were determined by a commercially available ELISA kit (eBioscience).

***Statistical analysis***

Differences between HIV-infected and healthy groups were analysed using a Mann-Whitney test or Kruskal-Wallis test if the analysis pertained more than 2 parameters, while temporal differences of biological markers were analysed with a Wilcoxon matched pair signed rank test. Changes in biological markers were studied using linear mixed effects models with one or several slopes depending on the best fit (defined by Akaike Information Criteria, the lower the

**Table 1. Baseline characteristics.**

| | |
|---|---|
| ***Study population*** | |
| N | 20 |
| Age (years)[1] | 40 (25 – 60) |
| Male[2] | 19 (95) |
| $CD4^+$ T-cell count (x $10^6$/L)[1] | 418 (143-836) |
| HIV viral load (copies/mL)[1] | 70300 (3340-5220000) |
| Time since first positive HIV test (years)[1] | 3.1 (0.1 – 7.9) |
| ***HLA background*** | |
| Protective HLA alleles (HLA B2705/B5701)[2] | 4 (20) |
| Detrimental HLA alleles (HLA B3503)[2] | 1 (5) |

[1] Median with range is given for total study population
[2] Number and percentage is given for total study population

better). The time taken for the slope to change was determined for all patients by likelihood profile. To achieve normality and homoscedasticity of measurement error distributions, a logarithm-10 transformation was used instead of the natural markers when necessary. All statistical analyses were performed with SAS software (version 9.2, SAS institute Inc.) and the software program SPSS 19.0 (SPSS Inc, Chicago, Illinois). Differences were considered statistically significant for p-value < 0.05.

## RESULTS

### *Study population*

We investigated how the elimination of HIV-RNA on successful cART related to normalisation of the immune activation profile of HIV-infected patients. To this end we longitudinally studied 20 HIV-1 subtype B infected individuals at short time intervals up to 56 days after start of treatment, after which they were sampled every 3 months up to 1 year after start of cART (see Figure 1A. All but one of the study subjects are male and the age of the subjects varied from 25 to 60 years. The majority of individuals (at least 70%) initiated cART during chronic infection. At baseline, viral loads varied widely between individuals from 3,340 to 5,220,000 HIV-1 RNA copies/mL, with a median of 70,200 HIV-1 RNA copies/mL, while $CD4^+$ T-cell counts ranged from 143 to 836 cells/µl, with a median of 418 cells/µl (see Table 1).

### *No decrease of sCD14 –a marker for bacterial translocation– on cART*

As expected [35], cART was effective in decreasing the viral load and allowing restoration of $CD4^+$ T-cell counts during the first year of treatment (overall median increase of 50 cells/µL, Figure S1). Nineteen of the 20 individuals attained an undetectable viral load within 1 year, while 1 individual attained an undetectable viral load after 390 days of treatment. In fact, the HIV-RNA load plummeted in the first 7 days on cART, then steadily declined till day 56, where after it became undetectable (Figure 1B). In contrast, sCD14 levels –which we used as a marker for bacterial translocation– remained elevated despite effective suppression of viral

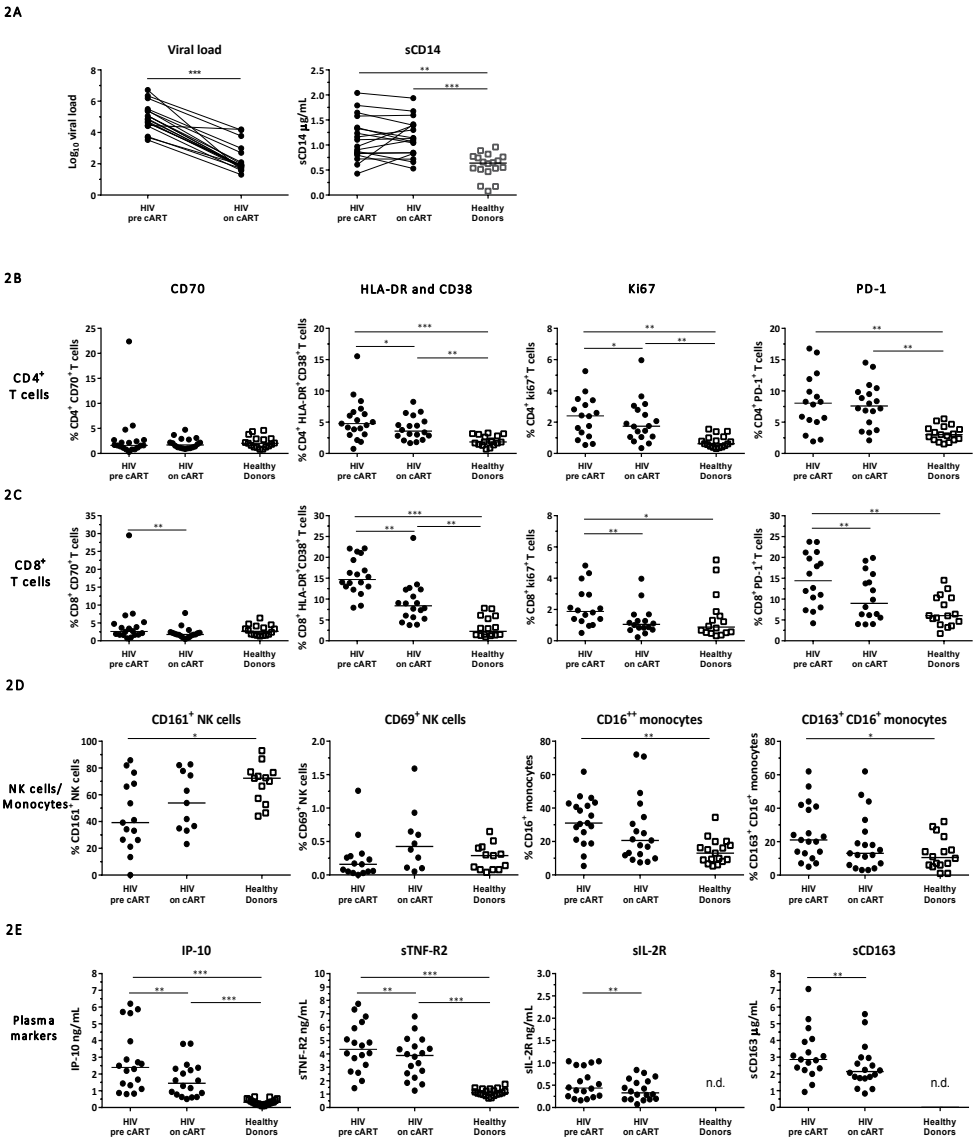



**Figure 2. Overall decrease, but no normalization of elevated immune activation markers in HIV-infected individuals on cART.**

To assess the effect of cART on the sources of immune activation (Figure 2A), log10 HIV-RNA load and plasma sCD14 levels (Y-axis) were measured 'before' and '56 days' on cART in HIV-infected individuals (n=20, black dots) and in healthy donors (n=17, black squares). The effect of HIV-RNA suppression on the expression levels of activation markers (Y-axis) was assessed on $CD4^+$ T cells (Figure 2B), $CD8^+$ T cells (Figure 2C), monocytes and NK-cells (Figure 2D) and in the plasma (Figure 2E). The markers were compared between; HIV-infected individuals before start of cART and 56 days on cART and healthy donors.

Dots/squares represent individuals, horizontal bars represent the median. * p-value =0.05- 0.01 ** p-value =0.01- 0.0001, *** p-value < 0.0001

load (Figure 1). This is in line with previous literature showing continued elevation of markers of bacterial translocation on cART [30, 36, 37]. The clear-cut divergence in decay dynamics of these two sources of immune activation enabled us to link the different markers of immune activation in HIV infection to their probable cause.



***Activation markers lower but not normalized in HIV-infected individuals on cART***

We investigated change of immune activation markers early after start of cART in in comparison to HIV-1 RNA and sCD14 (Figure 2A). To this end we compared the different immune activation markers between healthy and HIV-infected individuals both before and after 56 days on cART

CD4$^+$ T cells

A number of markers for systemic activation on CD4$^+$ T cells that have previously been associated with HIV-disease progression, including HLA-DR$^+$CD38$^+$, the proliferation marker Ki67 and exhaustion marker PD-1, were significantly higher in HIV-infected individuals before start of cART compared to healthy individuals (Figure 2B, $p<0.0001$, $p=0.0004$ and $p=0.0007$, respectively). In contrast, the expression of the immune activation marker CD69 (data not shown) and the co-stimulatory molecule CD70 on CD4$^+$ T cells (Figure 2B) did not differ between HIV-infected and healthy individuals. After 56 days of cART the percentages of the immune activation markers HLA-DR$^+$ CD38$^+$ and Ki67 on CD4$^+$ T cells were significantly lower, but they did not return to healthy donor levels. The frequency of PD-1$^+$ expressing cells in the CD4$^+$ T-cell pool were not significantly different 'before' compared to 'on' cART. Together these data show that despite the strong viral load decrease during the first 56 days on cART, CD4$^+$ T-cell activation markers were lower on cART, but remained markedly elevated.

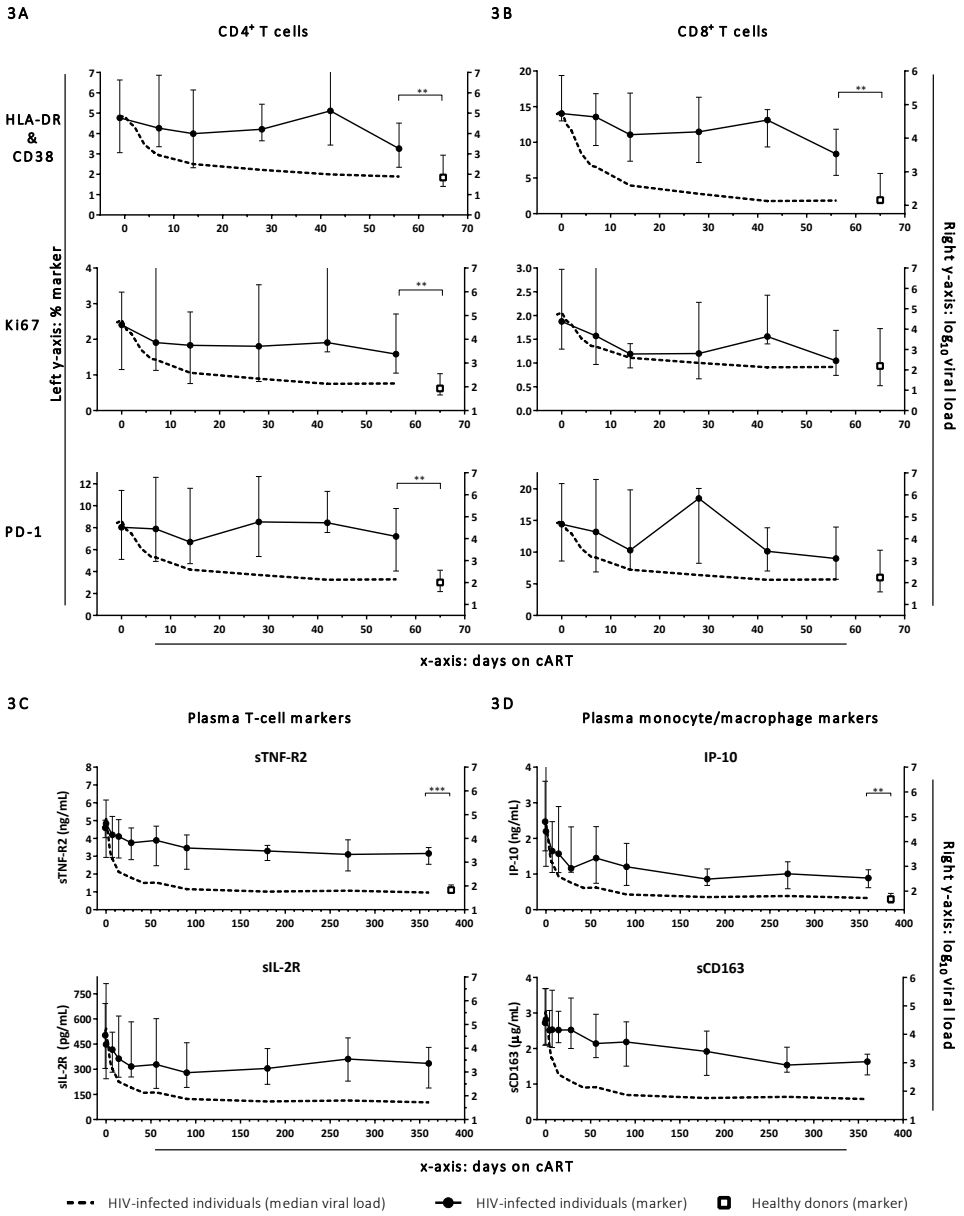
CD8$^+$ T cells

The expression levels of HLA-DR$^+$CD38$^+$, Ki67 and PD-1 expression were also significantly elevated on CD8$^+$ T cells in the HIV-infected pre-cART individuals compared to healthy individuals (Figure 2C $p<0.0001$, $p=0.04$ and $p=0.0009$, respectively), while CD69 (data not shown) and CD70 (Figure 2C) expression by CD8$^+$ T cells did not differ between HIV-infected and healthy individuals. However, in contrast to CD4$^+$ T cells, the percentage of PD1 and Ki67 expressing CD8$^+$ T cells were comparable to healthy donor levels at 56 days on cART. Moreover the HLA-DR$^+$CD38$^+$ fraction of the CD8$^+$ T-cell pool was significantly lower (though not entirely at healthy levels) after the first 56 days on cART ($p=0.001$), suggesting an association of these CD8+ T-cell immune activation markers with viral load.

Innate cells

In the innate arm, the percentage of CD14$^+$CD16$^{++}$ monocytes –a subset of proinflammatory monocytes– was significantly increased ($p=0.008$) in HIV-infection. Moreover, on the CD16$^{++}$ monocytes the scavenger receptor (and activation marker) CD163 was significantly elevated in HIV-infected individuals (Figure 2D, $p=0.04$). Meanwhile, lower percentages of CD161$^+$ NK

**Figure 3. Decay dynamics of immune activation markers during HIV-RNA suppression by cART.** Changes of the activation markers HLA-DR/CD38, Ki67 and PD-1 (left Y-axis) levels were longitudinally assessed in CD4$^+$ (Figure 3A) and CD8$^+$ T cells (Figure 3B) for HIV-infected individuals (n=20, black circles), during the first 56 days on cART. Expression levels measured pre cART, 56 days on cART in HIV-infected individuals and healthy




donors (n=17, black squares) were statistically compared. Similarly, in Figure 3C and 3D, the longitudinal changes of the plasma T-cell activation markers and monocyte activation markers (left y-axis), are depicted. Comparisons were made between pre-cART HIV-infected individuals (black dots), 365 days on cART, and healthy donors (black squares). In all graphs the median log10 HIV-RNA load of the HIV-infected individuals is depicted (n=20, grey line, right Y-axis) and is scaled to the pre-baseline levels and healthy donor levels of the appropriate activation markers. Dots/squares represent the median, with interquartile range. * p-value =0.05 - 0.01 ** p-value =0.01- 0.0001, *** p-value < 0.0001

cells (p= 0.02) and no differences in percentages of activated CD69$^+$ NK cells were found in untreated HIV-infected individuals as compared to healthy controls (Figure 2D). In conclusion, the innate immune markers did not seem to respond rapidly to a lower viral load as none had significantly changed after 56 days on cART.



Plasma activation markers

Immune activation in HIV infection is also characterized by an increased concentration of soluble (pro-inflammatory) mediators in plasma [13]. Accordingly, we found chemokine IP-10 and sTNF-R2 to be significantly higher in HIV-infected individuals prior to therapy (Figure 2E). 56 days on cART the soluble mediators IP-10 and sTNF-R2 were lower, but did not fully normalize to healthy levels. No data from healthy individuals were available for the plasma activation markers sCD163 and sIL-2R. However compared to other studies [11, 38] both were significantly elevated in untreated HIV-infected patients (Figure 2E), but did not normalize to the levels of healthy individuals during the first 56 days of cART.

***Dynamics of immune activation markers after HIV-RNA suppression by cART***

The immune activation markers that normalized when HIV-viral load became undetectable were most likely driven by HIV-RNA. However, for the immune activation markers that remained relatively high after 56 days on cART it could mean these markers simply were not driven by HIV-RNA directly. On the other hand, their initial decay dynamics may have been less pronounced or merely lagging behind. Therefore we specifically examined whether decay dynamics of the immune activation markers followed the initial HIV-RNA decrease. Confirming our previous results, the shape of the curves of CD4$^+$T-cell activation markers in no way resembled the HIV-RNA decay (Figure 3A). The CD4$^+$ T-cell activation markers decayed gradually over 56 days, in comparison to the steep decline of HIV-RNA in the first weeks. In contrast, the markers PD-1 and Ki67 expressed by CD8$^+$ T cells strongly associated with HIV-RNA decline and showed an immediate drop in the first 14 days on therapy, after which they leveled off (Figure 3B).

The plasma activation markers were followed longitudinally for 365 days in parallel with the viral load (Figure 3C,D). We observed that sTNF-R2, sIL-2R and IP-10 showed a tri-phasic decline following the HIV-RNA one, indicating that HIV-RNA might directly trigger a higher plasma concentration of these soluble mediators. Indeed, the slope of IP-10 concentration seemed to very closely mimic that of HIV-RNA. In contrast, it took longer for sCD163 to drop, suggesting that its high concentration during chronic infection was less directly correlated with viral load.

## DISCUSSION

By following the decay dynamics of both HIV RNA and sCD14, two major sources of immune activation in HIV-1 infection, and a set of immune activation markers on cells and in plasma during cART, we studied which features of systemic immune activation in chronic HIV infection are caused by HIV directly and which are driven by bacterial translocation. We observed a clear-cut difference between the decline in viral load and sCD14 upon start of cART: while HIV-RNA declined dramatically on cART, sCD14 levels remained elevated. Of the

immune activation markers that were significantly elevated pre-cART, most cellular $CD8^+$ T-cell activation markers as well as the plasma markers IP-10, sIL-2R and sTNF-R2 tended to follow the decay of HIV-RNA during the first year of therapy, though their expression levels did not return to those of healthy donors. In contrast, $CD4^+$ T-cell activation markers, $CD38^+$ $HLA\text{-}DR^+$ expression by $CD8^+$ T cells and sCD163 showed a slower decay than HIV-RNA. Finally, the macrophage activation marker CD163 and the number of $CD16^{++}$ monocytes remained high during therapy, just like sCD14.

In literature, most observations concerning immune activation parameters on cART were made at least six months after initiation of cART [16, 38]. These studies have suggested that monocyte activation markers remain elevated on cART [39] while T cell activation markers such as sIL-2R, PD-1 and ki67 on $CD8^+$ T cells strongly decline, which is in line with our data. Also it was found –again in line with our data– that most plasma immune activation markers strongly decrease after a year on cART but do not normalize, with the exception of sCD14 which was shown to remain high or to decrease only slightly on cART [16, 38]. However previous studies primarily looked at immune activation markers only at time points when the HIV-RNA had long been undetectable. As a result it was unclear if the immune activation markers that declined on cART followed the dynamics of viral load, which would be a strong suggestion that they were directly driven by HIV-RNA. Therefore we longitudinally followed immune activation markers in the initial phase of cART and HIV-RNA suppression. We were able to complement earlier data with the decay dynamics of the individual immune activation markers and to study how these related to HIV-RNA decline.

From the continuous high levels of bacterial translocation we concluded that the immune activation markers that (almost) normalized during the first year of cART could not have been driven by bacterial translocation, but by HIV-RNA. The frequent sampling enabled us to pinpoint that the percentage of PD-1 and Ki67 on $CD8^+$ T cells and IP-10, sIL-2R and sTNF-R2 in the plasma followed the HIV-RNA decline on cART. We showed that these immune markers, even though some of the markers had not yet normalized to healthy levels at 56, were likely- in part- caused by HIV directly.

Inversely, markers that had decreased but did not follow the HIV-RNA decay, such as CD163 and the activation markers of $CD4^+$ T cells, may be driven by bacterial translocation or have other origins. A number of additional options could have caused specific immune activation markers to demonstrate a delayed/ or lack of response to HIV-1 depletion. First, the prolonged exposure to an inflammatory environment -before cART- could have caused irreversible alterations to the immune system, which prevented the immune activation markers from returning to normal levels. Secondly, the immune activation markers on $CD4^+$ T cells could have displayed aberrant kinetics compared to HIV-RNA decay because they are recovering from depletion. As could be seen in Figure S1 and S2a, there was a steady increase in $CD4^+$ T cell numbers particularly early after cART which could have spurred higher levels of $CD4^+$ T-cell activation/proliferation markers. Thirdly, even when the HIV load is effectively repressed below detection level (50 copies/mL), low level viral replication still occurs and could induce immune action [40]. Both very low levels of HIV replication and microbial translocation could be the reason for the continued immune activation under cART. Several studies have shown that the initial damage to the gut is irreversible and though the microbial translocation found

will slightly decrease over 10 years on cART it does not normalize by far [41, 42].

Our interpretation that the immune activation markers that follow the immediate steep decay of HIV-RNA are driven directly by the virus and not by bacterial translocation, hinges on the assumption that sCD14 –which remains high on cART – is a solid marker for bacterial translocation. In literature the most widely used marker to measure bacterial translocation is lipopolysaccharide (LPS) [43, 44]. Recently, increasing numbers of studies have started to substitute LPS for surrogate markers of bacterial translocation, in particular by sCD14, because measurement of LPS is very sensitive to contamination. sCD14 indeed seems a solid substitute marker for bacterial translocation, as previous studies have shown a strong correlation between LPS and sCD14 levels [45], and sCD14 can readily be measured. Our interpretation that the observed high levels of sCD14 after one year of therapy reflect continuously elevated levels of bacterial translocation is also in line with previous studies, which showed that both LPS and sCD14 stayed constantly high under cART [16, 30].

There are risks connected to the fact that even after 360 days on effective cART IP10, sCD163, sTNF-R2 and sIL-2R were still significantly elevated. While shorter follow-up only allowed us to examine the cellular $CD4^+$ T cell activation markers empirically till 56 days after start of cART, the slopes computed with the mixed effect models indicated these markers would also still be increased at one year on cART compared to healthy donors. The chronic inflammation during long-term cART, gives rise to increasing clinical problems in HIV-infected patients [39, 46], such as premature immunological aging and cardiovascular disease (CVD), which contribute to increased morbidity and mortality.

As it has become clear that therapy to suppress HIV-RNA is no longer sufficient and additional immunomodulatory interventions are necessary, it is fundamental to fully comprehend what the prime driver for HIV-induced immune activation is. Moreover if we better understand the mechanisms causing the maintenance of HIV-associated chronic immune activation, it may be possible to directly target the causal agents, both on and off cART, thus reducing its negative impact on overall immune function. Only if we fully understand the precise mechanisms causing chronic (T-cell) immune activation both on and off cART, will we be able to optimize strategies directed at reducing inflammation-associated disease**.**

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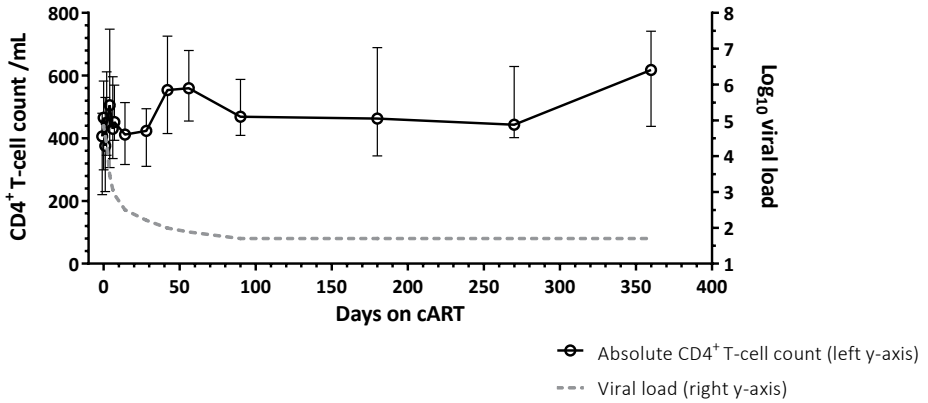
## SUPPLEMENTAL DATA

**Table S1. Monoclonals.**

| Marker | Fluorochrome | Manufacturer |
|---|---|---|
| CCR5 | FITC | Biolegend |
| CD1c | APC | eBioscience |
| CD3 | AF700 | Biolegend |
| CD4 | PB | Biolegend |
| CD4 | PerCP | Biolegend |
| CD8 | V500 | BD |
| CD11c | BV711 | BD |
| CD14 | APC eFluor780 | eBioscience |
| CD16 | BV605 | BD |
| CD16 | V500 | BD Horizon |
| CD19 | BV786 | BD |
| CD19 | eFluor 450 | eBioscience |
| CD27 | BV786 | BD |
| CD38 | PE | eBioscience |
| CD45RO | APC-Cy7 | Biolegend |
| CD56 | PE-Cy7 | BD |
| CD56 | PerCP(Cy5.5) | Biolegend |
| CD62L | AF700 | Biolegend |
| CD69 | APC | Biolegend |
| CD70 | FITC | Biolegend |
| CD141 | PE-Cy7 | Biolegend |
| CD161 | eFluor 450 | eBioscience |
| CD163 | PE | eBioscience |
| CD196 | PE-Cy7 | eBioscience |
| CD303 | FITC | Miltenyi Biotec |
| HLA-DR | APC-Cy7 | Biolegend |
| HLA-DR | BV605 | BD |
| ki67 | FITC | Zebra bioscience |
| M-DC8 | PE | Miltenyi Biotec |
| PD-1 | PerCP(Cy5.5) | Biolegend |

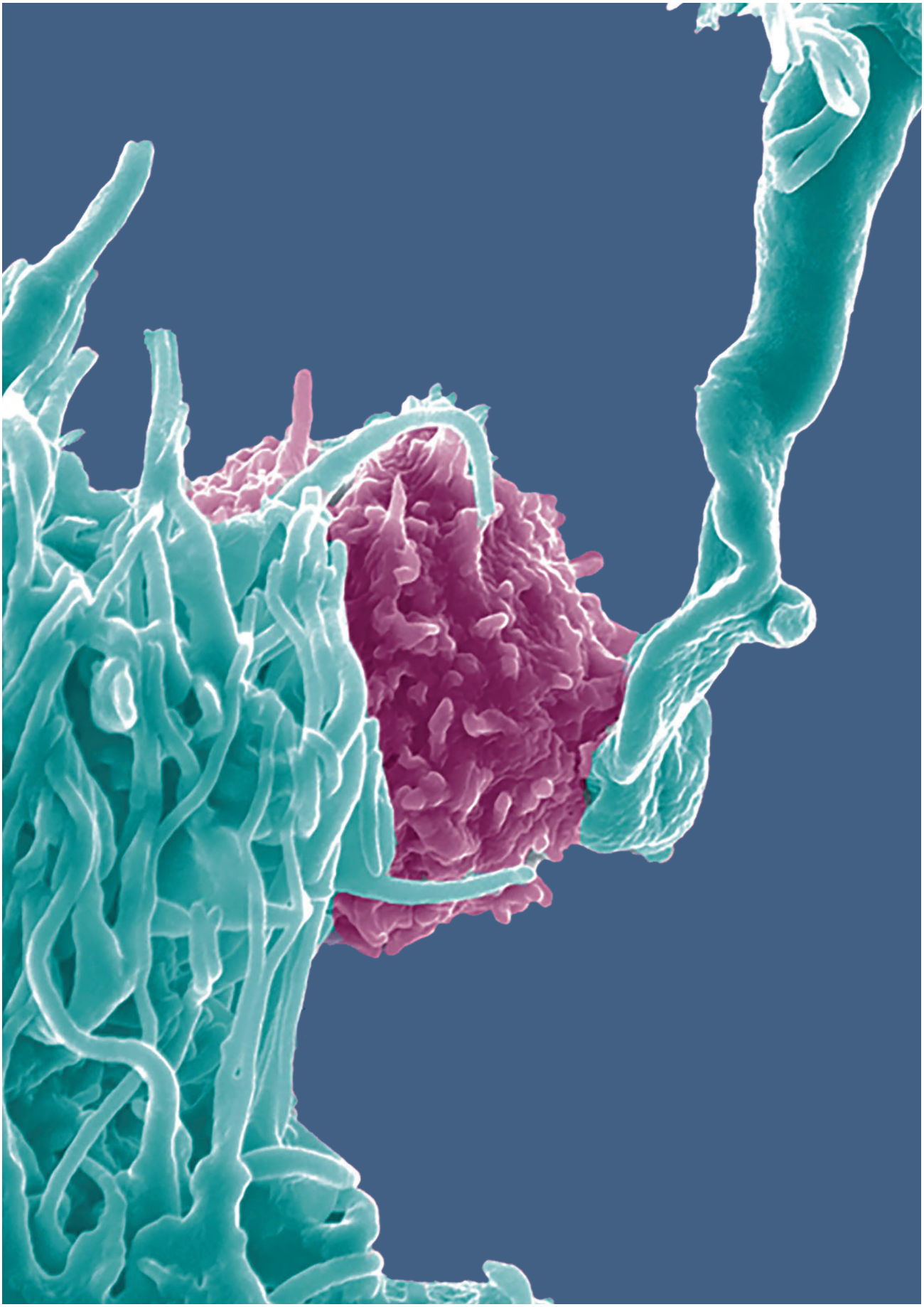
S1




**Supplementary Figure 1. Longitudinal analysis of CD4⁺ T-cell recovery and decline of HIV RNA load on cART.**

The changes upon cART of the log10 HIV-RNA load (right Y-axis) and absolute CD4⁺ T-cell count (left Y-axis, open dots) were measured longitudinally for 365 days in 20 HIV-infected individuals.

The median log10 HIV-RNA load of the HIV-infected individuals is depicted (grey line). Dots represent the median, with interquartile range.



# 3

# A LOWER VIRAL SETPOINT, BUT LITTLE IMMUNOLOGICAL IMPACT AFTER EARLY TREATMENT DURING PRIMARY HIV INFECTION

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3

*Introduction***:** The Primo-SHM trial, a multicenter randomized trial comparing no treatment with 24- or 60-weeks of combination antiretroviral therapy (cART) during primary HIV infection(PHI), recently demonstrated that temporary early cART lowered the viral setpoint and deferred the need for reinitiation of cART during chronic HIV infection. We studied whether the beneficial effect of early treatment was caused by preservation of immunological responses.

*Methods***:** Twenty-seven treated and twenty untreated PHI-individuals participating in the Primo-SHM trial were compared at viral setpoint, i.e. 36 weeks after baseline or after treatment interruption (TI), respectively, for a diverse set of immunological parameters.

*Results:* Our results show no differences between treated and untreated individuals at the level of effector T-cell formation or replication capacity of the T cells, regulation of various T, B, NK or dendritic cells, polyfunctionality of the $CD8^+$ T cells, preservation of $CD4^+$ T cells in the GALT, and immune activation. There were subtle differences in the quality of the cytolytic $CD4^+$ T-cell response: 11% (median) of $CD4^+$ T cells of the early treated individuals produced the cytolytic molecule perforin, compared to 5% in the untreated individuals (p=0.046), and treatment caused a modest increase of $CD4^+$ T cells expressing both perforin and granzyme B (median 9% versus 4% of $CD4^+$ T cells, p=0.045).

*Conclusions***:** Early treatment had a modest positive effect on the quality of the $CD4^+$ T-cell response. It remains unclear, however, whether these subtle immunological differences were the cause or a result of the lower viral setpoint in patients who received early treatment.

## INTRODUCTION

In HIV infection, a low viral setpoint and low levels of immune activation correlate with slower disease progression [1, 2]. The Primo-SHM trial, a multicenter randomized study comparing no treatment with 24- or 60-weeks of combination antiretroviral therapy (cART) during primary HIV infection (PHI), recently demonstrated that temporary early cART transiently lowered the viral setpoint and deferred the need for reinitiation of cART during chronic HIV infection [3]. Factors such as baseline $CD4^+$ T-cell count, stage of PHI, and HLA-background could not explain the differences observed in viral setpoint [3, 4].

There are many immunological parameters that play a role in controlling HIV disease progression. Foremost, there is compelling evidence that (gag-specific) CTL responses correlate with HIV viral load and the rate of HIV-disease progression[5-13]. Current consensus in the field is that such a 'protective' T-cell response in HIV infection is comprised of two elements, namely effective cytolytic killing and production of multiple cytokines. Recent work from Soghoian et al. highlights the importance of vigorous cytolytic activity by HIV specific $CD4^+$ T cells in controlling disease progression [12]. The presence during PHI of HIV-specific $CD4^+$ T cells which are able to express both perforin and high levels of granzyme A, was found to be highly predictive of slower disease progression and good clinical outcome. Likewise, HIV-nonprogressors preferentially maintain more polyfunctional HIV specific T cells, i.e. T cells with the capacity to secrete multiple cytokines and chemokines upon antigenic stimulation [6]. Almeida et al. demonstrated that superior control of HIV infection by $CD8^+$ T cells was reflected by a polyfunctional and high avidity phenotype [5].

The effectivity of HIV-specific immune responses can be severely limited during disease progression**,** as persistent viraemia has deleterious effects on HIV-specific $CD4^+$ and $CD8^+$ T-cell immunity [9]. For instance, chronic antigen exposure leads to a terminally differentiated phenotype of the $CD4^+$ and $CD8^+$ T cells, with upregulated CD57 expression and a diminished functional proliferative capacity [14]. Also, HIV viraemia prevents the establishment of highly functional memory $CD4^+$ T cells that retain the capacity to proliferate upon antigen stimulation [15, 16]. Finally, chronic antigenic stimulation induces upregulation of inhibitory receptors, most notably PD-1 and CTLA-4, which may interfere with HIV-specific T-cell responses and ultimately leads to T-cell anergy and loss of HIV-specific T cells [17, 18].

One of the earliest effects of HIV infection is a massive depletion of central memory $CD4^+$ T cells from the gut associated lymphoid tissue (GALT) [19]. The instant and massive early injury to the gut immune system, together with the subsequent damage exacted to the gut epithelial cells, is thought to induce gut permeability and the translocation of microbial products such as lipopolysaccharides (LPS). This is thought to contribute to systemic immune activation, which characterizes HIV-infection [20]. It is widely accepted that chronic immune activation drives progression to AIDS [1, 21-23]. In fact, immune activation is more strongly associated with the rate of $CD4^+$ T-cell loss in HIV infected individuals than viral load [1].

We and others hypothesized that viral suppression in PHI might prevent the excessive depletion of the GALT, thereby reducing the level of immune activation and delaying the $CD4^+$ T-cell loss. Depletion of $CD4^+$ T cells in the GALT can indirectly be assessed in blood

by measuring the level of α4β7 expression on T cells. Indeed, in primary SIV infection the reduction of $\alpha4\beta7^{high}$ $CD4^+$ T cells in the peripheral blood was shown to parallel the reduction of $CD4^+$ T cells in intestinal tract biopsies [24, 25]. Likewise, the finding that $\alpha4\beta7^{high}$ $CD4^+$ T cells are preferentially depleted in the blood in untreated PHI [26] likely reflects the depletion of $CD4^+$ T cells from the GALT.



To determine the cause of the lower viral setpoint in individuals treated during PHI, we evaluated whether i) effector T-cell formation, ii) effectivity of the T-cell response (in terms of cytolytic function and polyfunctionality) and, iii) inhibitory receptor expression on immune cells were altered in individuals treated during PHI compared with untreated PHI-individuals. To investigate whether viral suppression in PHI caused preservation of $CD4^+$ T cells in the GALT, we evaluated whether expression of the gut homing receptor α4β7 on $CD4^+$ T cells from the blood was altered in individuals treated during PHI. Finally, we assessed whether early treatment contributed to delayed disease progression by preventing excessive systemic T cell activation.

## MATERIALS AND METHODS

### *Study Population*

Blood samples were obtained from participants of the Primo-SHM trial. The Primo-SHM trial was a multicentre, open-label randomized controlled trial comparing temporary early cART (for 24 or 60 weeks) with no treatment. Detailed procedures have been described elsewhere [3]. Briefly, inclusion criteria were age over 18 years and laboratory evidence of PHI, defined as a negative or indeterminate Western blot in combination with detectable plasma HIV-1 RNA (Fiebig stage I–IV) or, in case of a positive Western blot, a documented negative HIV screening test in the previous 180 days (Fiebig stage V–VI).

20 untreated and 27 treated PHI-individuals were selected based on sample availability. Both patients treated for 24 weeks and patients treated for 60 weeks were included. Immunological parameters were measured at inclusion (baseline) and 'viral setpoint', defined as the plasma viral load 36 weeks after baseline or TI, respectively (see figure 1). In addition, blood samples of 18 healthy donors were obtained through the UMC Utrecht. For the studies in both patients and healthy donors approval was obtained from the Medical Ethics Committee of each participating site, and written informed consent was obtained from all participants.

### *T cell activation and phenotype*

Expression of activation markers on $CD4^+$ and $CD8^+$ T cells was measured after staining of the cells with antiCD4-PB, antiCD8-Amcyan, antiCD38-PE and antiHLA-DR-APC monoclonal antibodies (BD). The phenotype of the cells was determined using antiCD27- APC-Cy7 (Biolegend) and antiCD45RO-PECy7 (BD). In the same sample, cells were also stained with antiPD-1-PerCPcy5.5. All incubations were performed at 4°C (20 minutes) after which cells were fixed in cellfix (BD) and analysed with the LSRII flow cytometer.

### *Ex vivo T cell function*

Ex vivo surface staining was performed with antiCD3-eFluor450 (eBioscience), antiCD8-

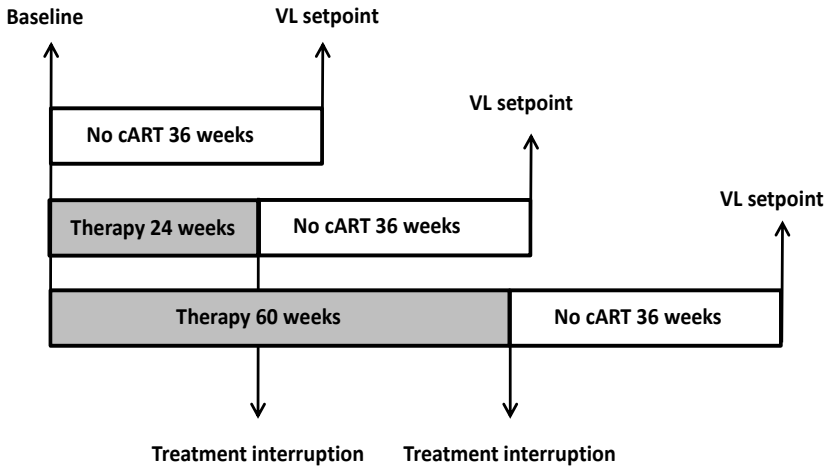


**Figure 1. Schematic overview of timeline.**
All immunological parameters were measured at the time of patient inclusion and randomization (baseline) and/or 36 weeks after inclusion or treatment interruption (viral setpoint).

V500, antiα4β7-APC, antiCD57-FITC, antiCD45RO-PE-Cy7 (all BD) and antiCD27-APC-Cy7 (Biolegend) monoclonal antibodies for 20 min at 4°C. After fixation and permeabilisation (permeabilisation reagents, BD) for 10 min, cells were stained for cytotoxic molecules with antigranzyme A-Pe or antigranzyme B-Pe (Sanquin) and antiperforin-PerCP-Cy5.5. Hereafter cells were fixed in cellfix (BD) and flowcytometry was performed.

***CD8⁺ T-cell stimulation and intracellular cytokine staining***
Cryopreserved PBMC were thawed and aliquotted at $2x10^6$ cells per ml in round bottom tubes (Becton Dickinson (BD), San Jose, California). $CD8^+$ T-lymphocytes were stimulated for 6 hours with a gag-peptide pool (15mers with 11 overlap, final concentration of the individual peptides was 2 µg/ml, Consensus B 2007, NIH AIDS Research and Reagent program, Bethesda, Maryland, United States). As a positive control, PMA and ionomycin (Sigma-Aldrich, Zwijndrecht, The Netherlands; 5 ng/ml and 1 µg/ml respectively) were used. After 1.5 hours, Brefeldin A (3 µM, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added. Surface staining was performed with antiCD3-PerCP, antiCD8-V500, antiα4β7-APC (all BD) and antiCD27-APC-Cy7 (Biolegend) monoclonal antibodies for 20 min at 4°C. After fixation and permeabilisation (permeabilisation reagents, BD) for 10 min, cells were stained with antiIFNγ-Pe-Cy7 (eBioscience), antiTNFα-FITC, antiMIP1β-PE and antiIL-2-PB (BD) for 20 min at 4°C. Cells were fixed in cellfix (BD) and flowcytometry was performed.

***Characterisation of inhibitory markers***
Expression of inhibitory markers was assessed on $CD4^+$ and $CD8^+$ T cells, B cells, NK cells and dendritic cells. A surface staining was performed for $CD4^+$ and $CD8^+$ T cells (antiCD3-eFluor450 (eBioscience), antiCD8-V500 (BD)), B cells (antiCD19-PerCP (BD)), NK cells (antiCD56-APC (BD)) and dendritic cells (antiHLA-DR-APC-Cy7 (BD), antiCD11c-PE-Cy7 (BD)). These sets were completed with either antiCD31–PE (BD)/3D3 antisirl-FITC or antiLAIR-PE/antiILT4-FITC or antiIREM-1-PE/antiKLRG-1-FITC or isotype controls. After staining for 20 minutes at 4 °C, cells

were fixed in cellfix (BD) and flowcytometry was performed.

***Flow cytometry analysis***

At least 100.000 events were acquired after phenotypical staining and at least 300.000 events were acquired after intracellular cytokine stainings, using the LSRII flow cytometer (BD). Data were analysed using the DIVA software (BD). The events were gated for either lymphocytes or monocytes in a FSC-A versus SSC plot. Following this, events were gated using the markers described above. T-cell polyfunctionality was analysed by Flowjo software (version 9.2). After determining the lymphocyte gate in a FSC-A versus SSC plot, cells were sequentially gated for CD3 and CD8. Subsequently, within the $CD8^+$ T-cell population a gate was created for the 4 respective functions; IFNγ, TNFα, MIP1β, and IL-2. Hereon a Boolean gating was performed resulting in 20 different combinations. All data were background-subtracted using the unstimulated samples.



***Statistical analysis***

Differences between treated and untreated individuals and between healthy donors and patients were analysed using the Mann-Whitney test, because we could not assume a normal distribution of the data. Whenever more than 2 groups were compared, a Kruskal–Wallis test was used. All statistical analyses were performed using the software program SPSS 19.0 (SPSS Inc, Chicago, Illinois).

**Table 1. Patient characteristics.**

| | *Treated[n] (n=27)* | *Not Treated (n=20)* | *p-Value* |
|---|---|---|---|
| *A. Patient characteristics at baseline* | | | |
| Age (years), median (range) | 41 (30-59) | 41 (25-55) | 0.39 |
| Male | 25 (92%) | 20 (100%) | 0.22 |
| MSM | 21 (78%) | 17 (85%) | 0.76 |
| Caucasian | 23 (85%) | 18 (90%) | 0.63 |
| Stage of PHI | | | |
| Fiebig I–IV | 21(78%) | 17 (85 %) | 0.54 |
| Fiebig V–VI | 6 (22%) | 3 (15%) | |
| $CD4^+$ T-cell count (cells/$mm^3$), median (range) | 570 (150-1050) | 520 (200-1110) | 0.26 |
| $CD8^+$ T-cell count (cells/$mm^3$), median (range) | 1129 (260-2570) | 1057 (160-2740) | 0.98 |
| Plasma HIV-1 RNA ($log^{10}$ copies/ml), median (range) | 4.8 (1.7-6.7) | 5.4 (1.8-6.5) | 0.42 |
| *B. Patient characteristics at viral setpoint* | | | |
| $CD4^+$ cell count (cells/$mm^3$), median (range) | 630 (110-1280) | 430 (180-940) | 0.03 |
| $CD8^+$ cell count (cells/$mm^3$), median (range) | 860 (380-2220) | 860 (310-2540) | 0.29 |
| Plasma HIV-1 RNA ($log^{10}$ copies/ml), median (range) | 4.2 (1.8-5.5) | 5.0 (3.0-5.4) | 0.002 |

Numbers are n(%), unless otherwise indicated.

[n] Treatment consists of either 24 or 60 weeks of cART; MSM, men who have sex with men; PHI, primary HIV infection

## RESULTS

### *Patient characteristics*

In the patients selected for the present study, the median viral loads of treated and untreated patients were not significantly different at baseline (for all patient characteristics see Table 1). Comparable with the complete study group [3], at setpoint the pVL was significantly lower in patients who were treated during PHI: median 4.2 versus 5.0 $^{10}$log copies/ml for treated and untreated individuals, respectively (p=0.002). $CD4^+$ and $CD8^+$ T-cell counts at viral setpoint also resembled those of the patients in the complete Primo-SHM trial [3]. $CD8^+$ T-cell counts were not significantly different between treated and untreated individuals, both at baseline and at viral setpoint. $CD4^+$ numbers were comparable at baseline (p=0.26), but at viral setpoint median $CD4^+$ T-cell counts were significantly higher in treated than in untreated patients (630 [range 110-1280] versus 430 [180-940] $CD4^+$ T cells/mm$^3$, p=0.03).

### *No change in T cell subsets by early treatment*

To investigate a possible immunological cause of the lower viral setpoints in individuals treated during PHI, we studied whether T cells of treated individuals had a different maturation state

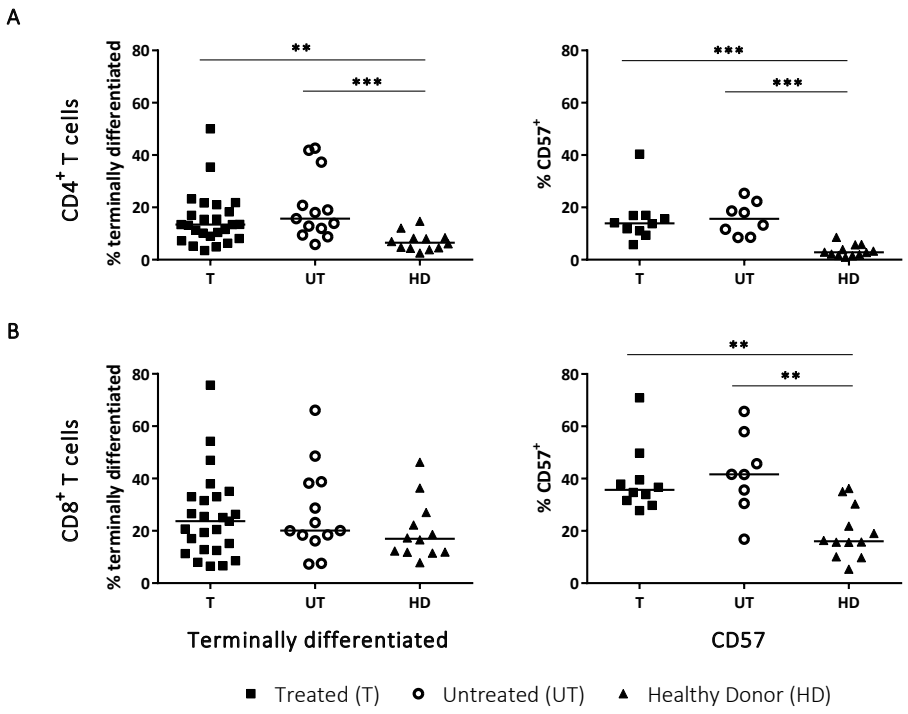


**Figure 2. No change in T cell subsets after early treatment.**

Markers of maturation (CD45RO-CD27-) and senescence (CD57+) on CD4+ (Figure 2A) and CD8+ T cells (Figure 2B) are shown for treated (n=25, square), untreated (n=13 for terminally differentiated and n=8 for senescent T cells respectively, open circle) and healthy (n=12, triangle) individuals. Dots represent individuals, with a bar at the median. ** p-value =0.01- 0.001, *** p-value < 0.001

and/or a less exhausted phenotype, with a resulting more effective immune response at viral setpoint. We analyzed the level of terminal differentiation (based on the expression of CD45RO and CD27) and replicative senescence (based on the marker CD57) of the $CD4^+$ and $CD8^+$ T cells of treated and untreated individuals at viral setpoint (Figure 2). We found that irrespective of early treatment, $CD4^+$ T cells of HIV-infected individuals had a more terminally differentiated phenotype ($CD45RO^-CD27^-$) and increased replicative senescence ($CD57^+$) than healthy volunteers (p=0.0002 and p<0.0001, respectively). The level of senescence of $CD8^+$ T cells was also higher in HIV-infected individuals compared to healthy volunteers (p=0.008). However, no differences could be observed between treated and untreated PHI individuals.
To reveal whether HIV-specific cells displayed differences in replicative capacity between treated and untreated patients, an in vitro proliferation assay was performed. Cells were stimulated with an overlapping gag-peptide pool and after 6 days the stimulation index was determined. Early treatment had no effect on the gag-specific proliferative capacity of either $CD4^+$ or $CD8^+$ T cells (data not shown).

***Ex vivo $CD4^+$ cytolytic T-cell activity is enhanced by early treatment***

To assess the direct ex vivo cytolytic functionality of T cells from treated and untreated patients, we measured the levels of granzyme A, granzyme B and perforin expression of total

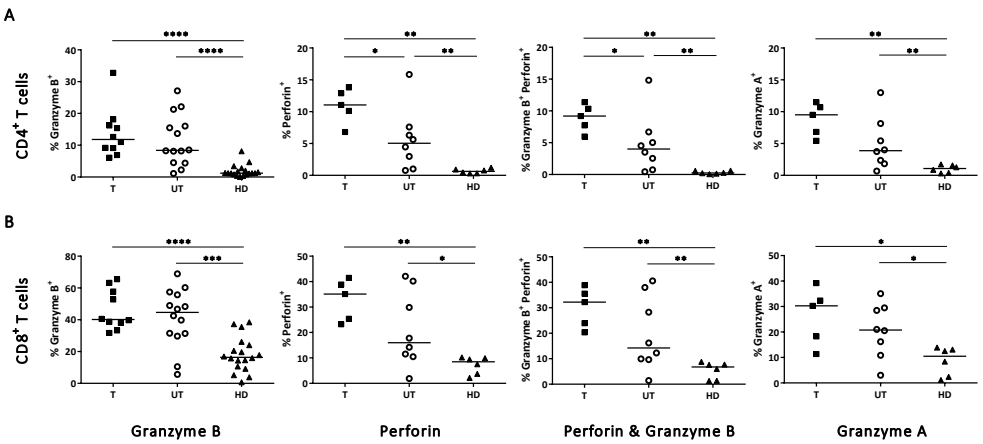


**Figure 3. Ex vivo cytolytic T-cell activity is not enhanced at viral setpoint after treatment.**

The ex-vivo effector functions of $CD4^+$ (Figure 3A) and $CD8^+$ T cells (Figure 3B) in terms of granzyme A, granzyme B and perforin production in treated (n= 5, square), untreated (n= 8, open circle) and healthy (n= 6, triangle) individuals was assessed. Individual production Granzyme B by $CD4^+$ and $CD8^+$ T cells was assessed in a larger group of individuals (n=10, 14 and 18 for treated, untreated and healthy individuals, respectively). Dots represent individuals, with a bar at the median. *p-value =0.05 - 0.01 ** p-value =0.01 - 0.001, *** p-value 0.001- 0.0001, **** p-value < 0.0001

$CD4^+$ and $CD8^+$ T cells at viral setpoint. The percentage of $CD4^+$ and $CD8^+$ T cells expressing granzyme A, perforin and/or granzyme B was elevated in both treated and untreated HIV-infected individuals compared to healthy individuals (Figure 3). At viral setpoint early treated individuals showed an increased level of $CD4^+$ T cells expressing perforin compared to untreated individuals (median of 11% vs. 5% of $CD4^+$ T cells, p=0.045). In addition, treatment

caused a modest increase of CD4⁺ T cells expressing both perforin and granzyme B (median of 9% versus 4% of CD4⁺ T cells, p=0.045).

***Treatment does not preserve a more polyfunctional T-cell response***

Another measure for an effective T-cell response is its poly-functionality. We performed a stimulation assay with an overlapping gag-peptide pool (Figure 4) and measured which proportion of CD4⁺ and CD8⁺ T cells produced one or more of the cytokines TNFα, MIP1β, IFNγ and IL-2. No differences were observed between treated and untreated PHI individuals at viral setpoint in the total amount of CD8⁺ T cells that produced these cytokines/chemokines in response to HIV gag (median 1.0 % versus 0.7% of CD8⁺ T cells, respectively; p=0.74, data not shown). The CD4⁺ T-cell response to stimulation was either absent or too low to interpret. When we compared the functional profiles of the CD8⁺ T-cell responses by expressing each functional component as a proportion of the total response [6], we found that treated individuals did not exhibit a more polyfunctional response than untreated PHI-individuals

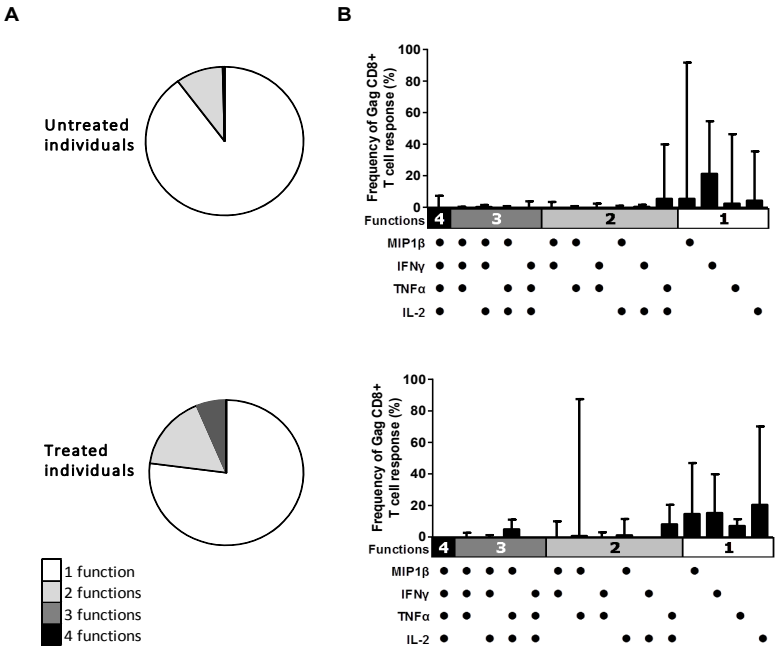


**Figure 4. Preservation of a more polyfunctional T-cell response due to treatment.**

PHI- individuals with treatment (n=10) and no treatment (n=8) were compared. The piecharts depict the relative contribution of the number of functions, i.e. cytokines/chemokines secreted, which are produced by CD8⁺ T cells in response to HIV-gag (Figure 4A). The graphs zoom in on the relative contribution of the cytokine (Mip1β, INFγ, TNFα and IL-2) combinations to the total CD8⁺ T-cell response (Figure 4B). The bars represent the frequency of the CD8⁺ T-cell response expressing the particular combination of functions shown. Each dot indicates Mip1β, INFγ, TNFα and/or IL-2 positivity. In all graphs medians (with range) are shown and the number of functions (1 function = white; 2 functions = grey; 3 functions = dark grey; 4 functions = black) are depicted.

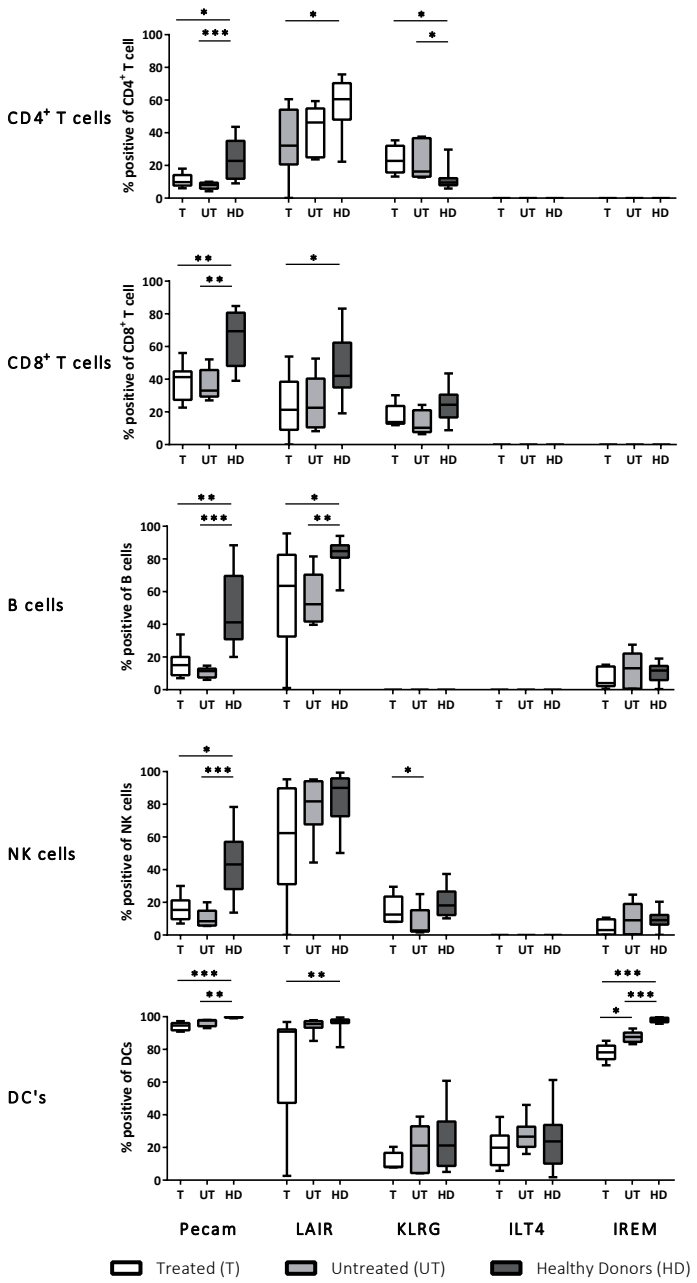


**Figure 5. No changes in regulatory and inhibitory molecules after early treatment.**

To assess the level of inhibitory receptor expression, the % (y-axis) of inhibitory receptors on $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells and DC's was determined. The parameters were compared between; healthy individuals (n=13, black), treated individuals (n=8, dark grey) and untreated individuals (n=10, white). Bars represent the median with range.* p-value = 0.01- 0.05, ** p-value =0.01- 0.001, *** p-value < 0.001

(Figure 4A). In treated individuals, a median of 24% of the gag-specific $CD8^+$ T cells displayed more than one function compared to 13 % of $CD8^+$ T cells in the untreated individuals (p=0.59). Likewise no significant differences could be found in the type of cytokine that was produced by the $CD8^+$ T cells in response to gag-antigen. For instance, in untreated individuals IFNγ production by HIV-specific $CD8^+$ T cells made up approximately 21% of the total response, while its median contribution did not exceed 17% in treated individuals (p=0.72) (Figure 4B).

***No changes in expression of regulatory and inhibitory molecules after early treatment***

We analysed markers of regulation of the immune response, and selected a panel of 'well recognized' but also recently characterized and largely unexplored inhibitory receptors [27] and analysed their level of expression on $CD4^+$ and $CD8^+$ T cells, B cells, NK cells and dendritic cells at viral setpoint (see Figure S1 for gating strategy).

Pecam-1 (CD31), which is known to inhibit apoptosis [28], was expressed less in HIV infected individuals compared to healthy controls in all measured cell types (Figure 5 & S2 first panel). Similarly, in (most) HIV infected individuals a distinct down-regulation of LAIR-1 (known to inhibit cytolytic function) was seen on T-, B- and dendritic cells (Figure 5 & S2 second panel). KLRG-1, an NK cell inhibitor, was increased on $CD4^+$ T cells in HIV-infected individuals, reflecting an exhausted phenotype (Figure 5 & S2 third panel). On dendritic cells no difference was seen in the expression of ILT4, a molecule that inhibits CTL function (Figure 5 & S2 forth panel). Finally, IREM-1, which inhibits TLR signalling in dendritic cells (Figure 5 & S2 fifth panel), was the only receptor that showed a significant effect of early treatment. However, instead of mirroring the healthy controls, the expression of this receptor was decreased even more in treated compared to untreated individuals (median 78 versus 88% of dendritic cells positive for IREM-1, respectively). The significance of this finding was lost after correcting for multiple testing. Also the level of PD-1 expression did not differ between patients who did and those who did not receive treatment during PHI (p=0.30, data not shown).

***No effect on GALT T cell depletion and T cell activation after early treatment***

To study whether treatment in PHI could overcome the rapid depletion of T cells from the GALT that is typically observed in untreated HIV infection, we evaluated the level of expression of the gut homing receptor α4β7 on $CD4^+$ and $CD8^+$ T cells of treated and untreated individuals at viral setpoint (Figure 6A & B). Both treated and untreated HIV-infected individuals exhibited a marked decrease in the levels of $\alpha4\beta7^{high}$ cells in the naive $CD4^+$ T-cell compartment compared to healthy controls. Also, in the effector $CD4^+$ T-cell compartment the percentage of α4β7 expression was lower in the treated HIV-infected individuals. Despite the lower viral setpoint in patients treated during PHI, there were no significant differences between treated and untreated individuals in the levels of $\alpha4\beta7^{high}$ cells, suggesting that treatment did not lead to the preservation of $\alpha4\beta7^{high}$ $CD4^+$ T cells. In contrast, no significant differences were found between HIV-infected individuals and healthy donors in α4β7 expression on $CD8^+$ T cells.

Because immune activation levels are known to be even more predictive of the rate of disease progression than plasma viral load, we analyzed whether early treatment affected the overall level of immune activation, by measuring CD38 and HLA-DR expression on T cells. No differences were found between treated and untreated individuals in the expression of CD38 and HLA-DR on $CD4^+$ or $CD8^+$ T cells at viral setpoint (data not shown).

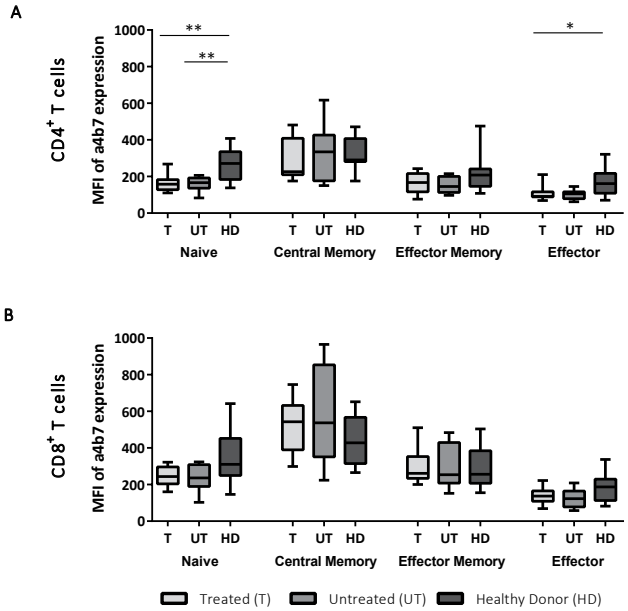


**Figure 6. No differences in gut homing marker expression between treated and untreated patients.**
The expression of gut homing marker a4b7 within the T cell compartments (naïve, central memory, effector memory and effector) on $CD4^+$ T cells (Figure 6A) and $CD8^+$ T cells (Figure 6B), is depicted. The mean fluorescent intensity of a4b7 was compared for treated (n=8, light gray), untreated (n=10, dark grey) and healthy (n=12, black) individuals. Bars represent the median with range.* p-value = 0.05- 0.01, ** p-value =0.01- 0.001

## DISCUSSION

We investigated whether the reported beneficial effect of treatment during PHI [3] was caused by preservation of immunological responses, and whether early treatment could overcome the rapid depletion of $CD4^+$ T cells from the GALT, and its immune activating effect. Our data suggest that treatment during PHI does not induce differences at the level of i) effector T cell formation or replication capacity of the T cells, ii) polyfunctionality of HIV-specific $CD8^+$ T cells, iii) expression of inhibitory receptors (Pecam-1, LAIR, ILT4, IREM-1, KLRG-1) on $CD4^+$ and $CD8^+$ T cells, B cells, NK cells and dendritic cells, iv) preservation of $CD4^+$ T cells in the GALT, and v) immune activation. We did see a modestly higher cytolytic capacity of $CD4^+$ T cells in patients at viral setpoint receiving early treatment.

The observation that early treatment did not decrease the level of senescence of the effector T cells at viral setpoint is quite surprising, as previously chronic antigen exposure was shown to lead to a terminally differentiated phenotype of $CD4^+$ and $CD8^+$ T cells, with a diminished functional proliferative capacity [14]. In addition, after correction for multiple testing, also inhibitory receptor expression levels on immune cells were not significantly altered in treated compared to untreated individuals. Formally, we cannot exclude the possibility that the immune parameters that we measured were initially preserved by early treatment, but

regained the features of a disrupted immune response rapidly after treatment interruption.

Along the same line, our data suggest that treatment in PHI-individuals did not preserve the quality of HIV-specific $CD8^+$ T-cell responses, in terms of their cytokine polyfunctionality. So far, not *one single* function of HIV-specific T cells has been proven to correlate with control of HIV infection [29]. The current consensus is that the more HIV-specific functions a $CD8^+$ T cell performs, the more protective it is. Indeed, HIV-specific $CD8^+$ T cells of long term non-progressors have been shown to be more prone to perform five functions (IL-2, IFNγ, TNFα, MIP1β, and CD107a) simultaneously than HIV-progressors [6]. However, neither the separate functions nor the combined functions of $CD8^+$ T cells were better preserved in individuals treated during PHI.

Finally, early treatment had no effect on preservation of $CD4^+$ T cells in the GALT. As there is an acute and severe depletion of $CD4^+$ T cells in the GALT in PHI, we hypothesized that treatment during PHI would prevent this. All individuals in the Primo-SHM trial were recruited early after infection, at least within 100 days of HIV-infection and 73% of the individuals even within 30 days. However, previous work in SIV infection showed that severe depletion of $CD4^+$ T cells in the GALT was seen within days and not weeks [30]. Therefore, we cannot exclude that in our study population depletion may already have occurred before start of treatment, which could explain why we saw no differences between treated and untreated PHI.

Jointly, gut permeability with subsequent translocation of microbial products such as LPS and HIV itself are thought to be responsible for systemic immune activation in untreated HIV infection [22]. Indeed, immune activation has been shown to be an even stronger predictor of disease progression than viral load. Thus, we hypothesized that the delayed reinitiation of cART, as was seen in the Primo-SHM trial [3], might be caused by diminished level of immune activation. However neither the $CD4^+$ nor $CD8^+$ T-cell activation was diminished 36 weeks after treatment interruption in our study population.

The only significant effect of early treatment that we identified was a slightly higher cytolytic functionality of $CD4^+$ T cells in early treated individuals. In the existing literature, increased granzyme A production after HIV-specific stimulation was shown to be predictive of better clinical outcome [12]. Moreover, the cytolytic response is thought to be more antiviral when producing a combination of various granzymes and perforin against HIV-gag [12]. In addition, proliferated HIV-specific $CD8^+$ T cells, but not $CD4^+$ T cells of long term non-progressors have previously been shown to be more prone to produce perforin in comparison with $CD8^+$ T cells of progressors [9]. In contrast, we found elevated levels of perforin and granzyme B expression in the $CD4^+$, but not in the $CD8^+$ T-cell pool. Though not completely in line with existing data, similarly our findings hint at the importance of highly functional cytotoxic $CD4^+$ T cells.

While we found a modest impact of treatment in PHI, recently other groups found far more profound differences in immunological results after early treatment [31, 32].
There are two possible explanations. First it might be the timing of the treatment during PHI. Paci et al have shown that the progression of HIV-markers corresponded to the timing –i.e. Fiebig's stage- whereat the treatment was initiated [33, 34]. Secondly early treatment

which persists over a longer period of time has been shown to be more effective [31, 32]. In the VISCONTI trial around 15% of individuals treated during PHI maintained viral control 12 months after treatment interruption. These individuals had been treated for a median of 36 months. It has been shown that immunological characteristics of predominantly $CD4^+$ and $CD8^+$ T cells are markedly improved after prolonged periods of treatment initiated during PHI [35, 36]. Therefore even though a 24-60 weeks' treatment period during PHI caused a one log decrease in viral load at viral load setpoint in the PRIMO-SHM trial, the effect was too modest to find more than moderate effects on the immune functions.

To broaden the scope of innate and adaptive immune mechanism we have explored even further, it would be fascinating to elucidate the effect of early treatment on cellular factors such as APOBEC3G, TRIM5α, and tetherin which also play an important role in restriction of HIV-1. Another question that should be addressed in further research is whether the observed preservation of the cytotoxic capabilities of the $CD4^+$ T cells after early treatment is the cause of a lower viral load setpoint in these individuals, or that a lower viral load allows for the preservation of the cytotoxic capabilities of the $CD4^+$ T cells.

In conclusion, our results show that early treatment during PHI has a limited impact on the immunological parameters measured. The observation that early treated individuals had $CD4^+$ T cells with modestly higher cytolytic capabilities may be a result of the reduced viral load rather than the cause of the lower viral setpoint.

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## AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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## SUPPLEMENTAL DATA

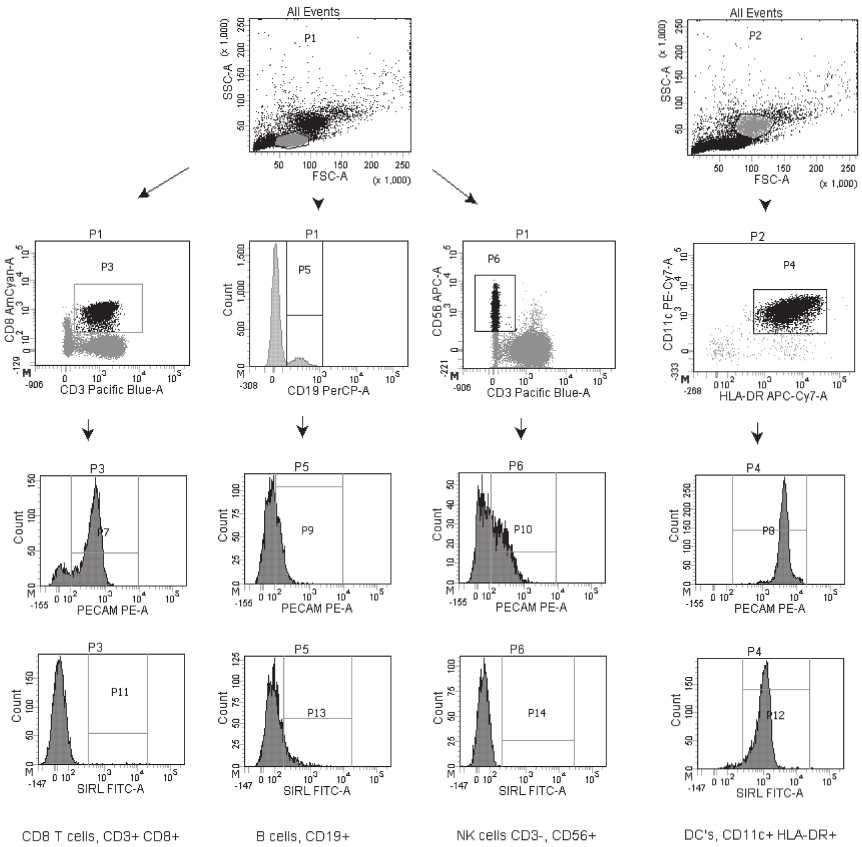



**Figure S1. Gating strategy for flow cytometry analysis of inhibitory receptor expression.**

In this sample gating, cells were first gated for lymphocytes or monocytes (FSC-A vs. SSC-A). Within the lymphocyte gate surface expression of $CD3^{+}CD8^{+}$ ($CD8^{+}$ T cells), $CD19^{+}$ (B cells) and $CD3^{-}CD56^{+}$ (NK cells) is used to determine the various cell subsets. Within the monocyte gate (P2) HLA-DR and CD11c are used to determine the DC's. The expression of various inhibitory receptors are then determined in these gated populations, SIRL and PECAM are shown in the example. The gate is determined by isotype controls.

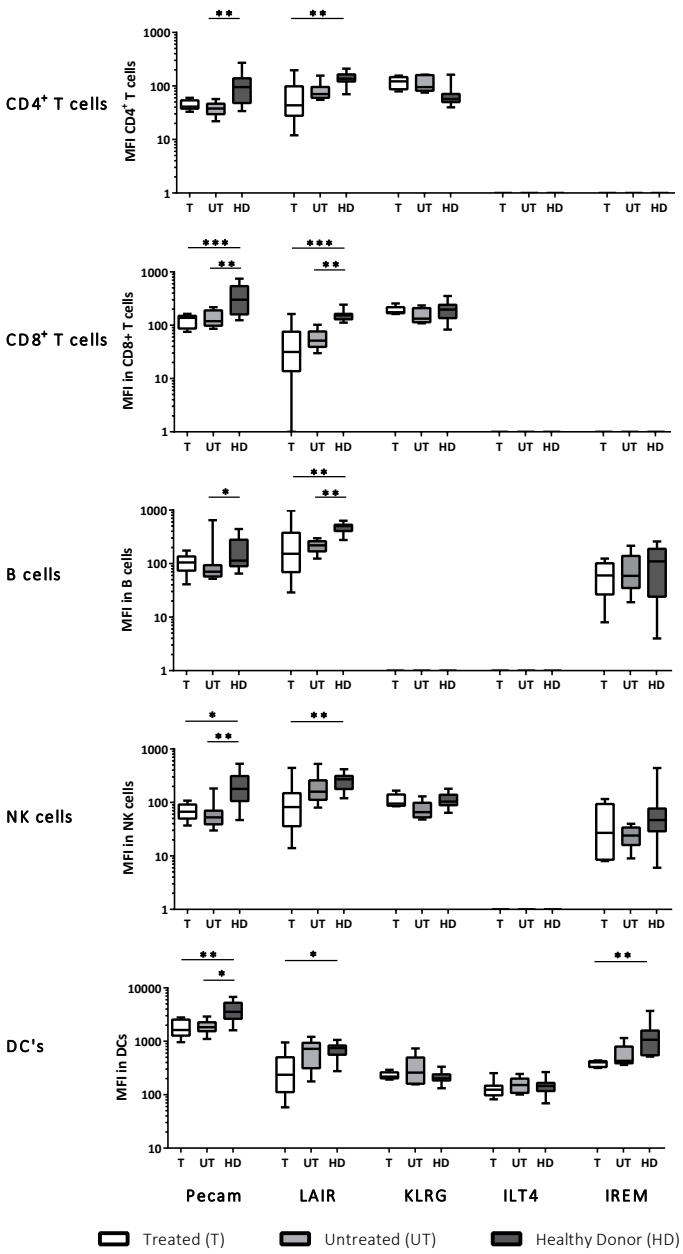
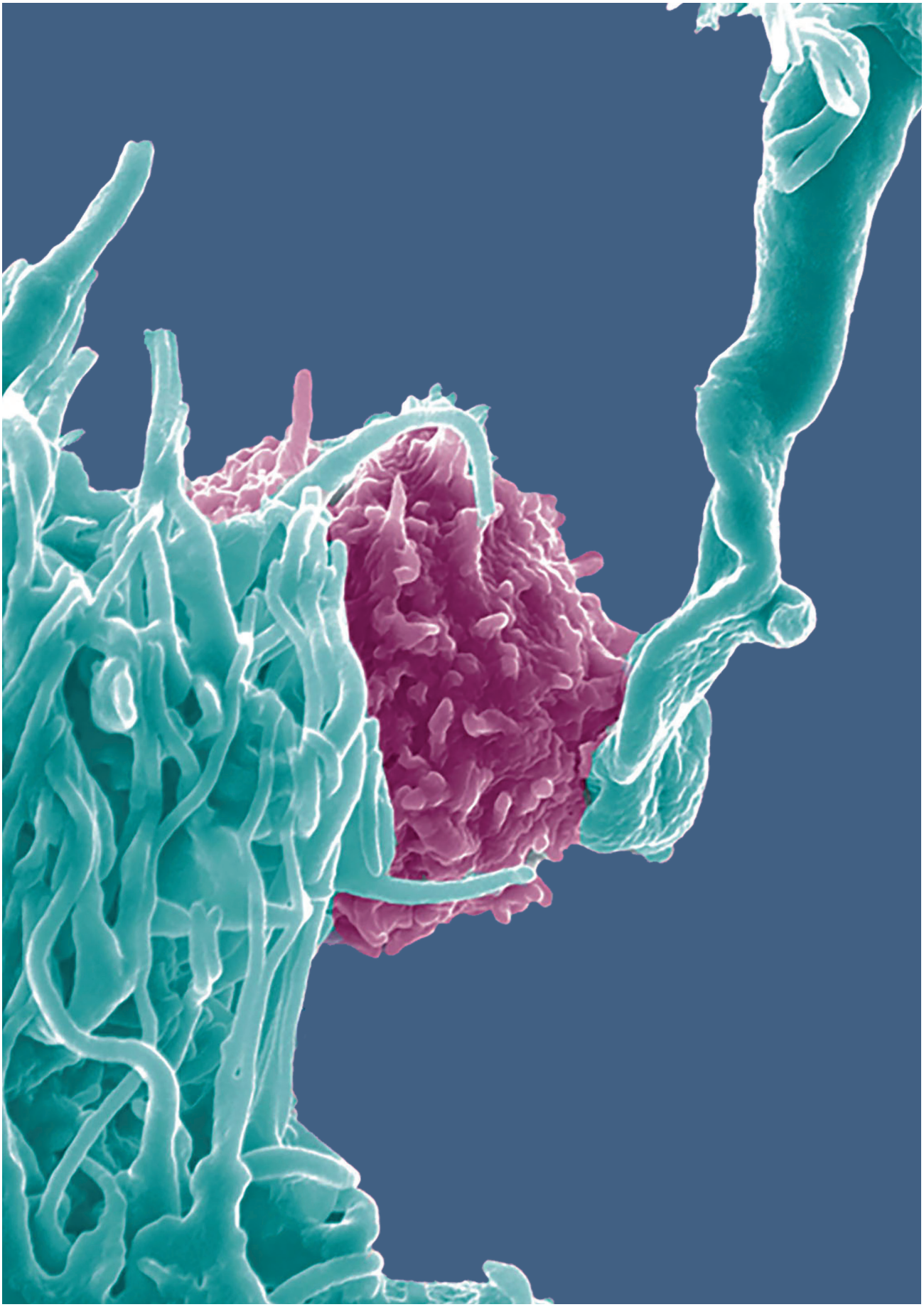



**Figure S2. No changes in regulatory and inhibitory molecules after early treatment.**

To assess the level of inhibitory receptor expression, the mean fluorescence intensity (y-axis) of inhibitory receptors on $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells and DC's was determined. The parameters were compared between; healthy individuals (n=13, black), treated individuals (n=8, dark grey) and untreated individuals (n=10, white). Bars represent the median with range.* p-value = 0.01- 0.05, ** p-value =0.01-0.001, *** p-value < 0.001



# 4

# PROTECTIVE CTL DO NOT SHORTEN THE LIFESPAN OF PRODUCTIVELY-INFECTED CELLS IN HIV-1 INFECTED INDIVIDUALS

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*Introduction***:** Cytotoxic T lymphocytes (CTL) are important in the control of HIV-infection. Although CTL are thought to reduce the lifespan of productively-infected cells, CTL depletion in SIV-infected rhesus macaques showed no effect on the lifespan of productively-infected cells. Since CTL-responses that successfully delay HIV-disease progression occur only in a minority of HIV-infected individuals, we studied the hypothesis that the ability of CTL to reduce the lifespan of productively-infected cells is limited to protective CTL responses only.
*Methods***:** We correlated features of CTL that are associated with control of HIV-infection, i.e. restriction by protective HLA alleles, and/or a broad, high or polyfunctional Gag-specific CTL-response, to the lifespan of productively-infected cells in 36 HIV-1 infected individuals, by measuring their plasma viral load declines immediately after start of cART.
*Results***:** The average lifespan of productively HIV-infected cells varied greatly between individuals, from 1.01 to 3.68 days (median 1.82 days) but was not different between individuals with or without the protective HLA molecules B27 or B57 (p=0.76, median 1.94 and 1.79 days, respectively). While the CTL-response against HIV-Gag was the dominant HIV-specific T-cell response, its magnitude ($r^2$=0.02, p=0.5), breadth ($r^2$=0.03, p=0.4), and poly-functionality ($r^2$=0.01, p=0.8), did not correlate with the lifespan of productively HIV-infected cells.
*Conclusions:* The features of CTL-responses that have clearly been associated with control of HIV infection do not correlate with a reduced lifespan of productively-infected cells in vivo. This suggests that protective CTL exert their effect on target cells before onset of productive infection, or via non-cytolytic mechanisms.

## INTRODUCTION

$CD8^+$ cytotoxic T lymphocytes (CTL) are thought to play an important role in the control of HIV infection, and current vaccines are developed based on that premise. The importance of CTL in HIV-control is suggested by a temporal association between the appearance of HIV-specific CTL responses and the decline of viral load during acute infection [1]. More directly, depletion of CTLs in SIV-infected rhesus macaques (RM) during chronic infection has been shown to cause a dramatic increase in SIV viral load [2]. In HIV-infected humans, the presence of certain HLA molecules is associated with the rate of progression to AIDS and viral load setpoint, suggestive of an important role for CTL [3, 4]. The latter finding was further corroborated by a world-wide GWAS in long-term non-progressors (LTNP), where the only significant SNPs were found within the peptide-binding pockets of HLA class I molecules [5].

Not all CTLs have the same impact on HIV-disease progression. CTL restricted through HLA-B27 and B57 (henceforth referred to as protective CTL), for example, are clearly associated with relatively slow disease progression while the presence of HLA-B*35:03 is associated with rapid progression [6-9]. Multiple studies independently found that individuals with slow HIV-1 disease progression tend to make broad and strong CTL responses against HIV-1 Gag, while CTL responses of individuals with rapid disease progression and high HIV-1 viral loads are more focused towards epitopes from Env and accessory/regulatory proteins [10-16]. Importantly, even irrespective of their HLA-restriction, the number of Gag-specific CTL responses was found to correlate negatively with HIV viral load and with the rate of HIV-disease progression [13].

It is important to understand what makes CTL associated with slower HIV-1 disease progression more protective than other CTL. Part of the answer probably lies in the preferential targeting of the p24-Gag protein[17], which is structurally very constrained. As a consequence, viral mutations in these regions tend to lead to severe viral fitness loss. Immune escape mutations in the immuno-dominant HLA-B27 and B57 restricted epitopes KK10 and TW10 – both part of the p24-Gag protein – for example, have been shown to lead to a dramatic loss in viral fitness [18-20]. Such detrimental mutations will only be selected for, if they outweigh the selective pressure exerted by the specific CTL response. Differences in the quality of CTL responses may therefore also contribute to the different levels of protection conveyed by CTL. Indeed, CTLs of LTNPs have been shown to be highly poly-functional, i.e. they have the capacity to produce a broad spectrum of cytokines [6].

It is generally thought that CTL recognize and kill productively-infected cells, and thereby effectively shorten the time during which infected cells produce new viral particles [21]. However, two studies have challenged this belief. In both studies, the $CD8^+$ T-cell pool of SIV-infected RM was completely depleted, and its effect on the lifespan of productively SIV-infected cells was measured by estimating the slope of viral decay in the first weeks after start of successful anti-viral treatment [22, 23]. Although an increase in viral load could be observed after depletion of the CTLs, most likely due to the role of CTLs in viral control, both studies found no difference between the lifespans of productively-infected cells in RM with or without CTLs. The authors concluded that the CTLs did not exert their antiviral effect through the 'classical' cytotoxic killing of productively-infected cells, but instead may suppress viral

replication by non-cytolytic mechanisms or via cytolytic killing of infected cells *before* their productive infection.

An alternative explanation for these findings may be that only the most protective CTL responses are capable of shortening the lifespan of productively HIV-infected cells. If only few individuals carry CTL responses that are capable of reducing the lifespan of productively-infected cells, their effect may go undetected in studies in which such protective CTL responses are rare or even absent. Here, we investigated whether CTL with features that are known to be associated with slow HIV-1 disease progression – including restriction by one of the protective HLA molecules or a strong, broad and poly-functional CTL response to HIV-Gag – are associated with a reduced in vivo lifespan of productively-infected cells in HIV-1 infected humans.



## MATERIALS AND METHODS

***Patient characteristics***

Blood samples were obtained from 36 HIV-1 subtype B infected patients, 20 of whom participated in the THILIHT-study, which was designed specifically to correlate immunological parameters with the lifespan of productively-infected cells. Before start of cART, a large amount of blood was drawn to perform immunological experiments. During the first week of therapy, blood was sampled at day 0, 1, 2, 4, 6 and 7 to accurately determine the slope of the plasma viral load decline. Study subjects received 600 mg efavirenz, 200 mg emtricitabine, and 245 mg tenofovir disoproxil.

The other 16 individuals were part of the ERA-study, of which the primary objective was to study the effect of highly suppressive combination antiretroviral treatment on the plasma viral load decay. Detailed procedures have been described elsewhere[24]. All subjects were antiretroviral-therapy naive before enrolment in this study, except for ERA-005, who received 1 year of zidovudine and lamivudine up to 1 year prior to enrolment. Both studies were approved by the medical ethics committees of the participating centres and written informed consent was obtained from all donors.

***The lifespan of productively-infected cells***

EDTA plasma HIV-1 RNA decay after the initiation of antiretroviral therapy was used to estimate the lifespan of productively-infected cells. In the 20 THILIHT-subjects HIV-1 levels were quantitated by COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test(v2.0), with a sensitivity of >20copies/mL, and in the ERA-subjects as described previously[24] with a final sensitivity of >50copies/mL. Plasma viral loads were measured for 12 weeks after start of therapy. The slope *s* of the HIV-1 RNA decline during the first 7 days was used to calculate the average lifespan of the productively-infected cells as 1/*s*, as published previously [25, 26].

***HLA-typing***

High-resolution HLA-typing was performed for all included subjects using sequence-based typing for 2 loci (HLA-A, and HLA-B) according to the manufacturers' guidelines (GenDx). All allele and genotype ambiguities at the 2-field level were resolved.

***PBMC's and blood separation***
PBMC's were isolated from heparinized blood before start of treatment using Ficoll (GE Healthcare Lifesciences) density separation, as described previously[27]. PBMC's were isolated, cryopreserved and stored in liquid nitrogen within 24 hours of collection. All experiments were performed on previously frozen PBMC's.

***Interferon (IFN)γ enzyme-linked immunospot (ELISpot) assay***
IFNγ-producing antigen-specific CTLs were measured using the IFNγ Elispot assay, as described previously [28]. The IFNγ CTL response was measured after stimulation with total overlapping peptide pools (15mers with 11 overlap, Consensus B 2007, NIH AIDS Research and Reagent program) of Env, Nef, Gag or Pol at a concentration of 2µg/ml. PHA stimulation served as a positive control. Results were analysed with an AELVIS ELIScan (ELIAnalyse Software v4). The number of IFNγ-producing cells was calculated by subtracting the unstimulated control value. Samples with at least twice the number of spot forming units (SFU) of the negative control were considered positive.

***Matrix-based Elispot assay***
Initial screening for T-cell responses was performed using the IFNγ-Elispot assay (as described above) with the peptides in a matrix format[29]. Subtype B Gag-peptide sets (15mers with 11 overlap, Consensus B 2007) were pooled in an 11-by-11 matrix format, with a final concentration of the individual peptides of 2µg/ml. The resulting potential epitopes were matched to the epitopes predicted from the Gag Consensus B 2007 reference strain (IEBD analysis resource, http://tools.immuneepitope.org/ processing/, date:1-Apr-2014) and all Gag-epitopes recorded in the Los Alamos database (http://www.hiv.lanl.gov/content/immunology/maps/ctl/Gag.html date:1-Apr-2014). In vitro confirmation of the positive matrix responses was performed for the 20 subjects included in the THILIHT study using the corresponding individual peptides at a concentration of 10µg/ml, as depicted in Figure S1. PHA stimulation served as a positive control, and medium served as a negative control.

***T-cell phenotype***
Phenotype and expression of senescence markers on $CD4^+$ and $CD8^+$ T cells was measured after extracellular staining with the monoclonal antibodies described in Table S1. All incubations were performed at 4°C (20 minutes) after which cells were fixed (cellfix,BD) and analysed by flow cytometry.

***$CD8^+$ T-cell stimulation and intracellular cytokine staining***
$CD8^+$ T lymphocytes (at $2x10^6$ cells/ml) were incubated with 2mg/mL Gag-peptide pool (see above) and antiCD107a-FITC (BD) for 6 hours. As a positive control, PMA and ionomycin (Sigma-Aldrich, The Netherlands; 5ng/ml and 1µg/ml respectively) were used. After 1.5 hours, Brefeldin A (3µM, BD) and Monensin (2µM, BD) were added. Surface staining was performed with monoclonal antibodies described in Table S1. After fixation and permeabilisation (BD) for 10 min, cells were stained intracellularly (see Table S1) and fixed in cellfix (BD) for flowcytometry.

***Flow cytometry analysis***
100.000 events were acquired after phenotypical staining and 300.000 events were acquired

after intracellular cytokine staining, using the LSRII flow cytometer (BD). Data were analysed using the DIVA software (BD). The events were gated for either lymphocytes or monocytes in a FSC-A versus SSC plot. T-cell poly-functionality was analysed by Flowjo software (v9.2). Within the $CD8^+$ T-cell population a boolean gating was created for the 5 respective functions; CD107a, IFNγ, TNFα, MIP1β, and IL-2, resulting in 31 different combinations. All data were background-subtracted using the unstimulated samples. To convert the 5-dimensional poly-functional profile to a one-dimensional value taking into account all cells performing 0,1,2 until 5 functions we used the poly-functionality index, as developed and described previously[30].



***Statistical analysis***

Differences between groups were analysed using a Mann-Whitney test. All statistical analyses were performed using the software program SPSS 19.0 (SPSS Inc, Chicago, Illinois). For further details, we refer to the legends of the figures.

## RESULTS

***Study population***

We included 36 HIV-1 infected individuals in our analyses. The viral declines of 16 of these individuals were published previously [24]. All patients were treated with at least a triple drug combination, and 35 of the individuals attained an undetectable viral load within 1 year. At baseline, viral loads ranged widely between individuals from 3,340 to 5,220,000 HIV-1 RNA copies/mL, with a median of 72,200 HIV-1 RNA copies/mL, while $CD4^+$ T-cell counts ranged from 30 to 851 cells/μl, with a median of 360 cells/μl (see Table 1). All, but one, of the study subjects were male and the age of the subjects varied from 25 to 60 years. The majority of individuals (24[67%]) initiated cART during chronic infection. Twenty-eight percent of the study population carried at least one of the protective HLA-alleles HLA-B*27:05 or B*57*01,

**Table 1. Baseline characteristics**

| | **Study population** |
|---|---|
| Number | 36 |
| Age (years)[1] | 40.1 (25.9- 60.5) |
| Male[2] | 35 (97%) |
| $CD4^+$ T-cell count (x $10^6$/L)[1] | 360 (30-851) |
| HIV viral load (copies/mL)[1] | 72200 (3340-5220000) |
| Time since first positive HIV test (years)[1] | 2.7 (0.1- 10.4) |
| | |
| **HLA background** | |
| Protective HLA alleles (HLA B*27:05/B*57:01)[2] | 10 (28%) |
| Detrimental HLA alleles (HLA B*35:03)[2] | 2 (6%) |
| HLA homozygosity[3] | 9 (27%) |

[1] Median with range is given for total study population

[2] Number and percentage is given for total study population

[3] Homozygosity at 1 or more of the HLA-A or B loci

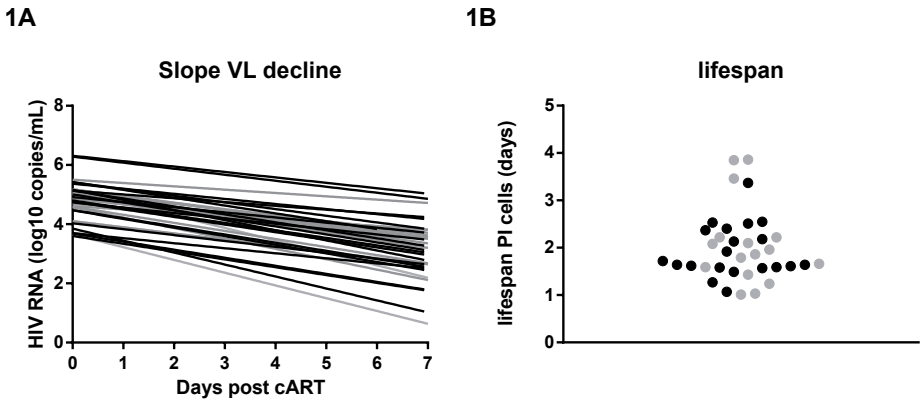


**Figure 1. Spread in lifespans of productively-infected cells between individuals.**
An overview of the 36 individual HIV-1 RNA declines in the first week after start of antiretroviral therapy (panel A); the linear regression lines are depicted. The average lifespan of the productively-infected cells of these individuals (panel B) was calculated as the inverse of the slopes of the regression lines of panel A (see Supplemental Figure 2 and Weverling et al.[24] for the individual viral RNA measurements). The results of the 16 individuals of the ERA-study are depicted in grey and of the 20 individuals of the THILIHT study in black.

and 6% carried the detrimental HLA-allele, i.e. HLA-B*35:03. The complete 2-field HLA-A and B typing of the study population is shown in Table S2.

***Large spread in the lifespans of productively-infected cells***

We investigated whether the lifespan of productively HIV-1 infected cells is shorter in individuals with CTL responses with features that are known to be associated with relatively slow HIV-1 disease progression. For each individual we estimated the lifespan of productively HIV-1 infected cells based on the slope of the viral decay after start of therapy. For an accurate calculation of the first phase decline, at least 4 time points were measured in the first week after therapy initiation, as previously described [25, 31, 32]. Figure S2 depicts the individual HIV-1 RNA declines of the newly included individuals. The viral decay of the other 16 study subjects was published previously [24]. The expected lifespan of productively-infected cells varied between individuals from 1.01 to 3.68 days (median 1.82 days, see Figure 1 and Table S3). Despite their known association with the rate of progression to AIDS, neither baseline $CD4^+$ T-cell count (Figure S3a) nor baseline plasma HIV-1 viral load (Figure S3b) correlated with the lifespan of productively-infected cells[31].

***The presence of protective HLA alleles does not affect the lifespan of productively-infected cells***

Because of the clear association between CTL restricted through HLA-B27 and B57 and slow disease progression [3, 6-9, 33, 34], we studied whether HLA-genotypes associated with slow progression correlated with a reduced lifespan of productively-infected cells. When the expected lifespans of productively-infected cells in individuals with at least one of the protective HLA-alleles HLA-B*27:05 or B*57:01 were compared to those who did not possess any protective HLA allele, we found no evidence that productively-infected cells are shorter lived in people with a protective HLA molecule (figure 2A, p=0.76). Likewise, the relative

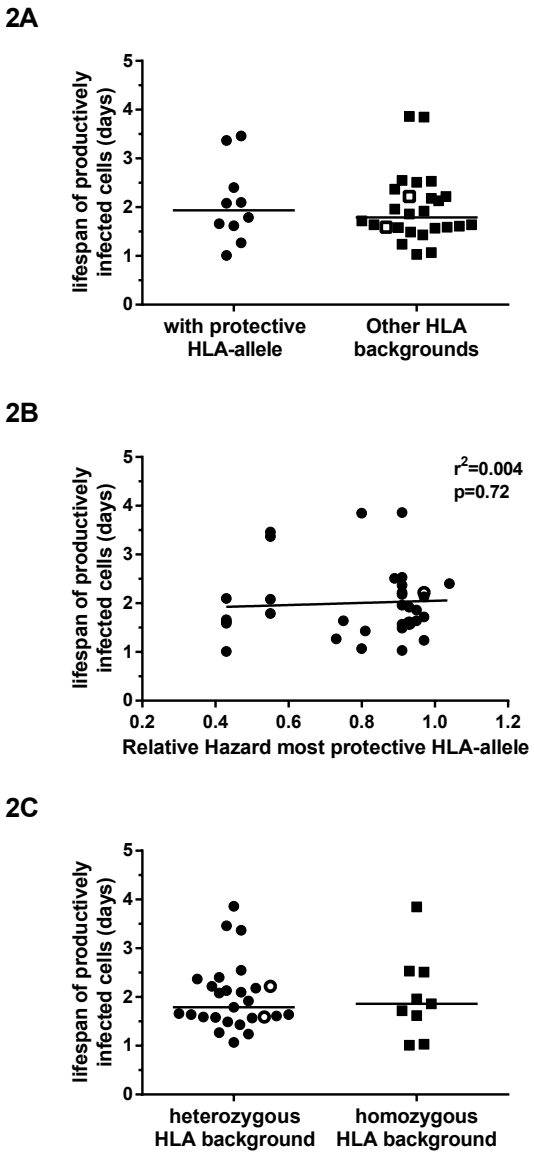


**Figure 2. No effect of the HLA background on the lifespan of productively-infected cells.**

Panel A shows the lifespan of productively-infected cells in individuals expressing one of the protective HLA alleles B*27:05 or B*57:01 (n=10) as compared to individuals without these HLA alleles (n=26). Panel B depicts the (lack of) correlation between the relative hazard of the most protective HLA A and HLA B allele of the 36 study participants and the lifespan of their productively-infected cells. Panel C gives the lifespan of productively-infected cells in individuals with a fully heterozygous (n=27) HLA background and in individuals with a partially homozygous (n=9) HLA background. Median lifespans in the different groups are depicted by horizontal lines. A linear regression is depicted in panel B, with corresponding $r^2$ and p-values. The open dots/squares represent individuals with the detrimental HLA allele B*35:03.

hazard of the most protective HLA allele per individual did not correlate with the expected lifespan of productively-infected cells (Figure 2B, $r^2$=0.004, p=0.72). Productively-infected cells were also not shorter-lived in individuals heterozygous for all HLA-A,-B and-C loci (n=17), another HLA-phenotype related to delayed disease progression in HIV-1 infection [35] (Figure 2C, p=0.948). Thus, the HLA-background of an HIV-infected individual does not seem to affect the lifespan of productively-infected cells.

***No impact of Gag-specific CTLs on the lifespan of productively-infected cells***

As CTLs targeting Gag are associated with a relatively low viral load and slow HIV-disease progression, even irrespective of their HLA-restriction [6, 8, 13], we also investigated whether the magnitude, breadth and/or poly-functionality of Gag-specific CTL responses had an impact on the lifespan of productively-infected cells. These experiments were performed in a subgroup of 20 subjects in whom extra material was collected before start of cART.

The magnitude of the HIV-specific T-cell response against the overlapping peptide pool of Gag was measured using an IFNγ Elispot. As a control we also measured the responses elicited by the overlapping peptide pools of HIV Pol, Env and/or Nef. We found that the Gag-restricted T-cell response – when present – was dominant in all individuals. Despite its dominance, the magnitude of the CTL response against the Gag-peptide pool (Figure 3A) was not associated with the lifespan of productively-infected cells. Even when we compared individuals with the highest IFNγ-response against HIV-Gag (over 1000 SFU/$10^6$ cells) with individuals without such a response, we observed no significant difference in the lifespan of their productively-infected cells (p=0.74, data not shown).

In addition, we studied the breadth of the Gag-specific T-cell response. We first made a 'quick scan' of the total Gag-protein using an 11 by 11 matrixpool Elispot (see M&M), and combined with the use of a prediction programme this suggested that the total CTL-response against Gag was driven by up to 15 potential epitopes per individual. For each individual, we subsequently confirmed which of these potential epitopes truly contributed to the immune response to Gag, by measuring whether the individual epitopes also elicited a T-cell response in vitro. This revealed a median breadth of 1.5 [range 0-7] Gag-specific $CD8^+$T-cell responses per individual. We observed no significant association between the number of CTL responses against HIV-Gag and the lifespan of productively-infected cells (Figure 3B). Even in individuals with more than 3 responses against HIV-Gag, which has previously been shown to be associated with a relatively low viral load [13], the productively-infected cells did not live longer than in individuals with fewer than three responses against HIV-Gag (p= 0.65, data not shown).

Finally, we determined the functional profile of the Gag-restricted CTL response in terms of the capacity to produce multiple cytokines/chemokines, including TNFα, MIP1β, IFNγ, IL-2 and CD107a. A median of 1.5% of CTLs (range 0.2–4.5%) responded to HIV-Gag (data not shown). We observed no significant differences between the specific functions exhibited by the CTLs in response to HIV-Gag of the 5 individuals with the shortest-lived and the 5 individuals with the longest-lived productively-infected cells (Figure 3C). In both groups, CD107a and MIP1β production by HIV-specific CTLs made up approximately 80% of the total functional response while the contribution of TNFα and IFNγ was much lower, and IL-2 was hardly produced. When expressing the average number of functions of the Gag-specific T cells as a poly-functionality index [30], we did not find any association between T-cell poly-functionality and the lifespan of the productively-infected cells (Figure 3D). Even

the four individuals with the most poly-functional T cells did not have significantly shorter-lived productively-infected cells (p=0.42, data not shown). Also CTL exhaustion (as measured by the markers PD-1 and CD57) was not associated with the estimated lifespan of the productively cells (data not shown).

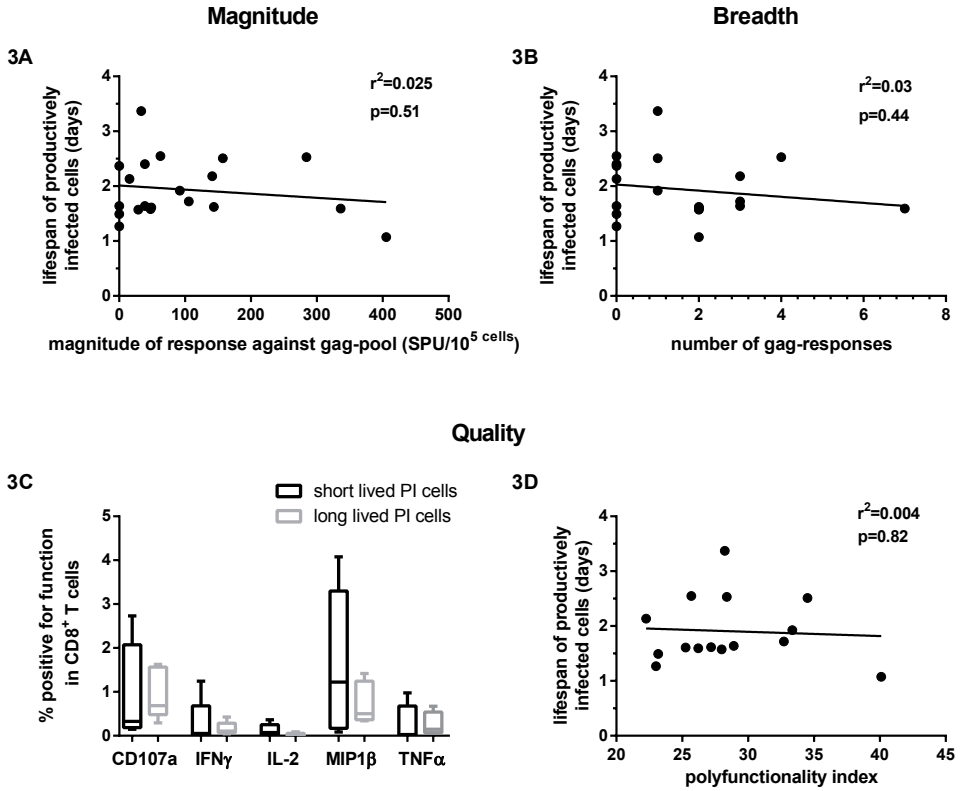


**Figure 3. Characteristics of the HIV-specific CTL response are not associated with the lifespan of productively-infected cells.**

In 20 HIV-infected individuals neither the magnitude (Panel A), nor the breadth (Panel B) or the functionality (Panels C and D) of the Gag-specific CTL response was correlated with the lifespan of the productively-infected cells. Linear regressions are depicted for each graph, with corresponding $r^2$ and p-values. Panel C shows that there were no significant differences in the expression of CD107a, IFNγ, IL-2, MIP1β and TNFα by Gag-specific CTLs between the 5 individuals with the longest-lived productively-infected cells (grey boxplots) and the 5 individuals with the shortest-lived productively-infected cells (black boxplots). Median and range are depicted for each group.

## DISCUSSION

Following the $CD8^+$ depletion studies in rhesus macaques [22, 23], which showed no influence of $CD8^+$ T-cell depletion on the lifespan of productively SIV-infected cells, we studied whether shortening of the lifespan of productively-infected cells may be limited to the most protective

CTL responses only. Although the average lifespan of the productively-infected human cells varied considerably between our study subjects, it did not correlate with any of the factors known to be associated with slow disease progression, including the presence of protective HLA-alleles, or a high, broad or poly-functional CTL response against HIV-Gag. Thus, even the most protective HIV-specific CTL responses do not seem to exert their effect by shortening the lifespan of productively HIV-infected cells.

Although measuring the decay rate of plasma HIV-RNA following effective cART provides an elegant way to deduce the lifespan of productively-infected cells before treatment, this approach may introduce a bias when applied to patients. In the past, cART was typically only initiated in asymptomatic persons because of certain clinical end-points, such as a decrease of the $CD4^+$ T-cell count under a predefined value, or an increase in viral load. Under such circumstances, the HIV-specific CTLs may already have an exhausted phenotype, and therefore may not be representative for the typical HIV-specific T-cell response during chronic infection. Our study group is quite unique in this respect, because as many as 75% of our study subjects started therapy *without* clinical progression. It is thus highly unlikely that the lack of effect of even the most protective HIV-specific CTL responses on the lifespan of productively-infected cells that we report is due to such a bias. Indeed, we found no difference in the lifespan of productively-infected cells between individuals with acute or chronic HIV-infection, and omitting the data from individuals who started therapy because of clinical progression did not change the conclusions of our study.

In light of the plethora of prior evidence for a pivotal role of CTLs in HIV control [1-3, 5, 13, 36], our findings suggest that CTL control of HIV infection is based on other mechanisms than cytolytic killing of productively-infected cells. An alternative mechanism via which CTL may reduce the viral load is through secretion of non-lytic soluble factors that prevent or reduce infection of uninfected cells. The best-known soluble non-cytolytic factors secreted by CTLs are the CCR5-binding molecules RANTES, MIP1α and MIP1β. These chemokines function as competitive binders and down-modulators of the CCR5 receptor and thereby reduce HIV entry and infection [37]. Likewise, the cytokines IFNγ and TNFα are thought to have anti-viral properties that can inhibit HIV-1 infection [38, 39]. Indeed, depletion of CTLs in RM has been shown to lead to a marked reduction in the plasma levels of RANTES, MIP1α, MIP1β, IFNγ and TNFα [22]. An in silico modeling study also demonstrated that non-lytic T cells can drive (slow) HIV-immune escape, while previously it was presumed that HIV immune escape mutations were exclusively caused by lytic T cells [40].

A second possibility is that CTLs perform their cytolytic effects *before* the productive stage of infection of the target cells, i.e. within the first ~24 hours after infection of the cell. Importantly, if target cells can be recognized by CTL very early after they have been infected, they have not had the chance to produce new viral particles, and the expression of HLA molecules on the cell surface has not yet been down-regulated. Both our study and the two studies in RM would not detect such effects of CTL since the read-out was the death of productively-infected cells. Indeed, it was shown that Gag-specific CTL clones are indeed able to recognize SIV-infected cells as early as 2 hours after infection of the target cell [41]. The presented Gag-epitopes are derived from the infecting virions, which can contain up to 5000 copies each [42]. Moreover, in HIV-1 infected humans a T-cell clone specific for the immuno-dominant HLA-B*27:05 Gag-

restricted KK10 epitope recognized infected target cells within 6 hours post-infection, while sub-dominant HLA-B*27:05-restricted Vpr VL9 epitopes were not recognized until 18 hours after infection[43]. Previous mathematical modeling has shown that such cytolytic effects of CTL on infected cells before their productive stage of infection could also reconcile the large inter-individual differences in set-point viral load, despite the relatively small inter-individual differences in the rate of viral load decline during treatment [44]. It remains to be investigated which factors underlie these latter, albeit smaller, differences in viral dynamics on treatment.

It is fundamental to fully comprehend the mechanisms via which CTLs contribute to control of HIV infection. Puzzling outcomes of some vaccine trials have clearly shown how incomplete our understanding is of how CTL exert control. Despite a significant (although modest) positive outcome of the Thai vaccine trial, the absence of an effect on viral load and $CD4^+$ T-cell counts, and extremely low CTL reactivity in the vaccinated, question the role of CTL to the induced protection [45]. Likewise, the STEP-trial had to be halted because of a lack of protection, no effect on viral set points, and an increased risk of infection in vaccinated individuals with pre-existing immunity against the viral vector [46]. Only if we fully understand the $CD8^+$ T-cell restricted mechanisms that control HIV-1 infection, will we be able to effectively apply this knowledge in the design of a functional HIV-vaccine**.**



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Author contributions: H.B.S., I.M.M.S., M.N., T.M, D.v.B., and J.A.M.B. designed the study; H.B.S., T.F. and T.M., organized patient inclusion and sample collection; J.P., A.M.J.W., and E.S. provided data; H.B.S performed the experiments; H.B.S., I.M.M.S., D.v.B., and J.A.M.B. analyzed data; H.B.S., I.M.M.S., D.v.B., and J.A.M.B. wrote the manuscript. All authors were involved in critically appraising the manuscript and approve the final version.
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## SUPPLEMENTAL DATA

### S1 Gag Epitope Map

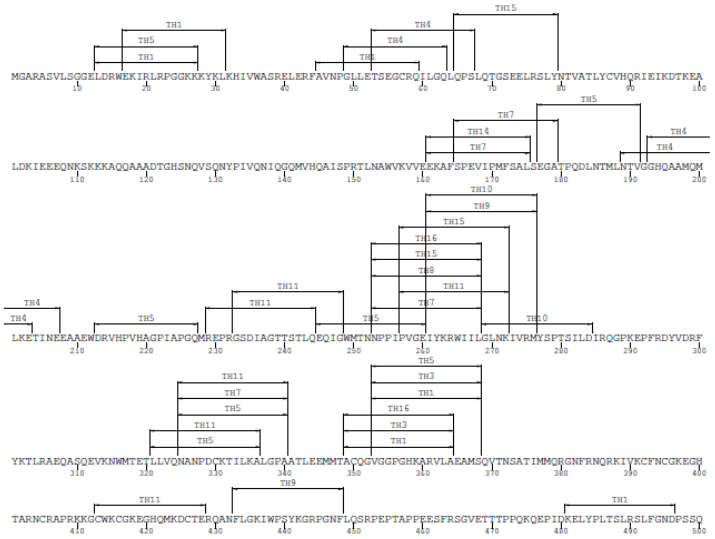



**Figure S1. The location of the Gag-epitopes on HIV-Gag.**
The location of the Gag-epitopes, which were confirmed by IFNγ Elispot for the 20 individuals, are depicted here on the Gag Consensus B 2007 reference strain.

**Figure S2. Estimating the lifespan of productively-infected cells (next page).**
The HIV-1 RNA declines in the first week after start of antiretroviral therapy of the 20 THILIHT individuals; linear regressions with the corresponding 95% confidence intervals are depicted. The average lifespan of the productively-infected cells of these individuals was calculated as the inverse of the slopes of these regression lines, and are depicted in Figure 1.

S2
TH 1 Lifespan = 2.53 days
TH 2 Lifespan = 2.55 days
TH 3 Lifespan = 1.92 days
TH 4 Lifespan = 1.07 days
TH 5 Lifespan = 1.59 days
TH 6 Lifespan = 2.40 days
TH 7 Lifespan = 1.72 days
TH 8 Lifespan = 2.51 days
TH 9 Lifespan = 1.58 days
TH 10 Lifespan = 1.62 days
TH 11 Lifespan = 2.18 days
TH 12 Lifespan = 1.27 days
TH 13 Lifespan = 2.13 days
TH 14 Lifespan = 3.37 days
TH 15 Lifespan = 1.61 days
TH 16 Lifespan = 1.57 days
TH 17 Lifespan = 1.64 days
TH18 Lifespan = 1.49 days
TH19 Lifespan = 2.37 days
TH20 Lifespan = 1.64 days
HIV RNA (log10 copies/mL)
Days after start therapy

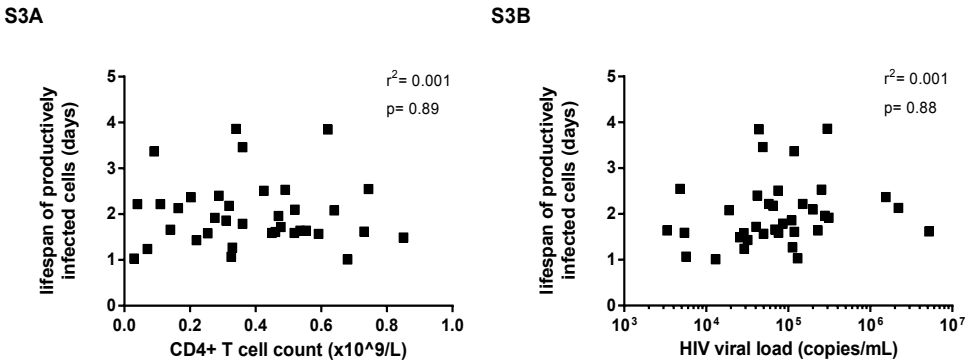



**Figure S3. No associations between the lifespan of productively-infected cells and parameters of HIV-disease progression.**

In 36 HIV$^-$infected individuals the estimated lifespans of the productively-infected cells was not correlated with their CD4$^+$ T-cell count (Panel A) or viral load (Panel B). P-values were analyzed with a Spearman rank test.

**Table S1. monoclonals.**

| *Staining* | *Marker* | *Fluorochrome* | *Manufacturer* |
|---|---|---|---|
| **Senescence** | | | |
| | CD95 | APC | BD |
| | CD28 | PE | BD |
| | CD57 | FITC | BD |
| | PD-1 | PerCP-c5.5 | Biolegend |
| | CD3 | AF700 | Biolegend |
| | CD4 | PB | Biolegend |
| | CD8 | V500 | BD |
| | CD27 | APC-cy7 | eBioscience |
| | CD45RO | Pe-cy7 | BD |
| **Poly-functionality** | | | |
| Extra-cellular | CD3 | PerCP | eBioscience |
| | CD8 | V500 | BD |
| Intra-cellular | IFNγ | Pe-Cy7 | eBioscience |
| | TNFα | APC | BD |
| | MIP1β | PE | BD |
| | IL-2 | PB | BD |

**Table S2. HLA characteristics.**

| Patient | HLA-A Allele 1 | HLA-A Allele 2 | HLA-B Allele 1 | HLA-B Allele 2 |
|---|---|---|---|---|
| Era 2 | A*03:01 | - | B*07:02 | **B*27:05** |
| Era3 | A*03:01 | A*68:01 | **B*51:01** | **B*57:01** |
| Era4 | A*02:01 | A*68:02 | **B*27:02** | B*53:03 |
| Era5 | A*01:01 | A*24:02 | B*08:01 | ***B*35:03*** |
| Era6 | A*02:01 | A*31:01 | B*07:02 | **B*27:05** |
| Era8 | A*01:01 | A*03:01 | **B*27:05** | B*37:01 |
| Era9 | A*03:01 | A*68:01 | B*44:02 | **B*57:01** |
| Era10 | A*02:01 | - | B*14:01 | **B*57:01** |
| Era13 | A*24:02 | A*68:01 | B*18:01 | B*44:03 |
| Era14 | A*02:01 | A*03:01 | B*07:02 | - |
| Era15 | A*01:01 | A*03:01 | B*08:01 | B*35:01 |
| Era16 | A*02:01 | - | B*07:02 | B*40:01 |
| Era17 | A*02:01 | ***A*66:01*** | B*07:02 | B*41:02 |
| Era18 | A*02:01 | A*03:01 | B*07:02 | B*15:01 |
| Era19 | A*01:01 | - | B*15:01 | B*40:01 |
| Era21 | A*24:02 | - | B*07:02 | B*38:01 |
| TH1 | A*02:01 | A*03:01 | B*07:02 | - |
| TH2 | A*02:01 | A*31:01 | B*07:05 | **B*51:01** |
| TH3 | A*03:01 | A*31:01 | B*07:02 | B*49:01 |
| TH4 | ***A*66:01*** | A*68:01 | B*38:01 | B*41:02 |
| TH5 | A*03:01 | A*31:01 | B*07:02 | ***B*35:03*** |
| TH6 | A*01:01 | A*24:02 | B*15:01 | **B*57:01** |
| TH7 | A*01:01 | A*26:01 | B*08:01 | - |
| TH8 | A*25:01 | A*32:01 | B*08:01 | - |
| TH9 | A*02:01 | A*11:01 | **B*27:02** | B*35:01 |
| TH10 | A*02:01 | - | B*15:01 | **B*27:05** |
| TH11 | A*02:01 | A*03:01 | B*08:01 | B*40:01 |
| TH12 | A*02:01 | A*11:01 | B*35:01 | **B*57:01** |
| TH13 | A*03:01 | A*23:01 | B*07:02 | B*44:03 |
| TH14 | A*24:02 | A*25:01 | B*15:01 | **B*57:01** |
| TH15 | A*01:01 | A*31:01 | B*08:01 | B*44:02 |
| TH16 | A*02:05 | A*34:02 | B*08:01 | B*50:01 |
| TH17 | A*01:01 | A*03:01 | B*07:02 | B*40:01 |
| TH18 | A*01:01 | A*02:01 | B*08:01 | **B*27:02** |
| TH19 | A*02:01 | A*68:02 | B*40:01 | B*53:01 |
| TH20 | A*30:01 | A*30:04 | B*08:01 | B*13:02 |

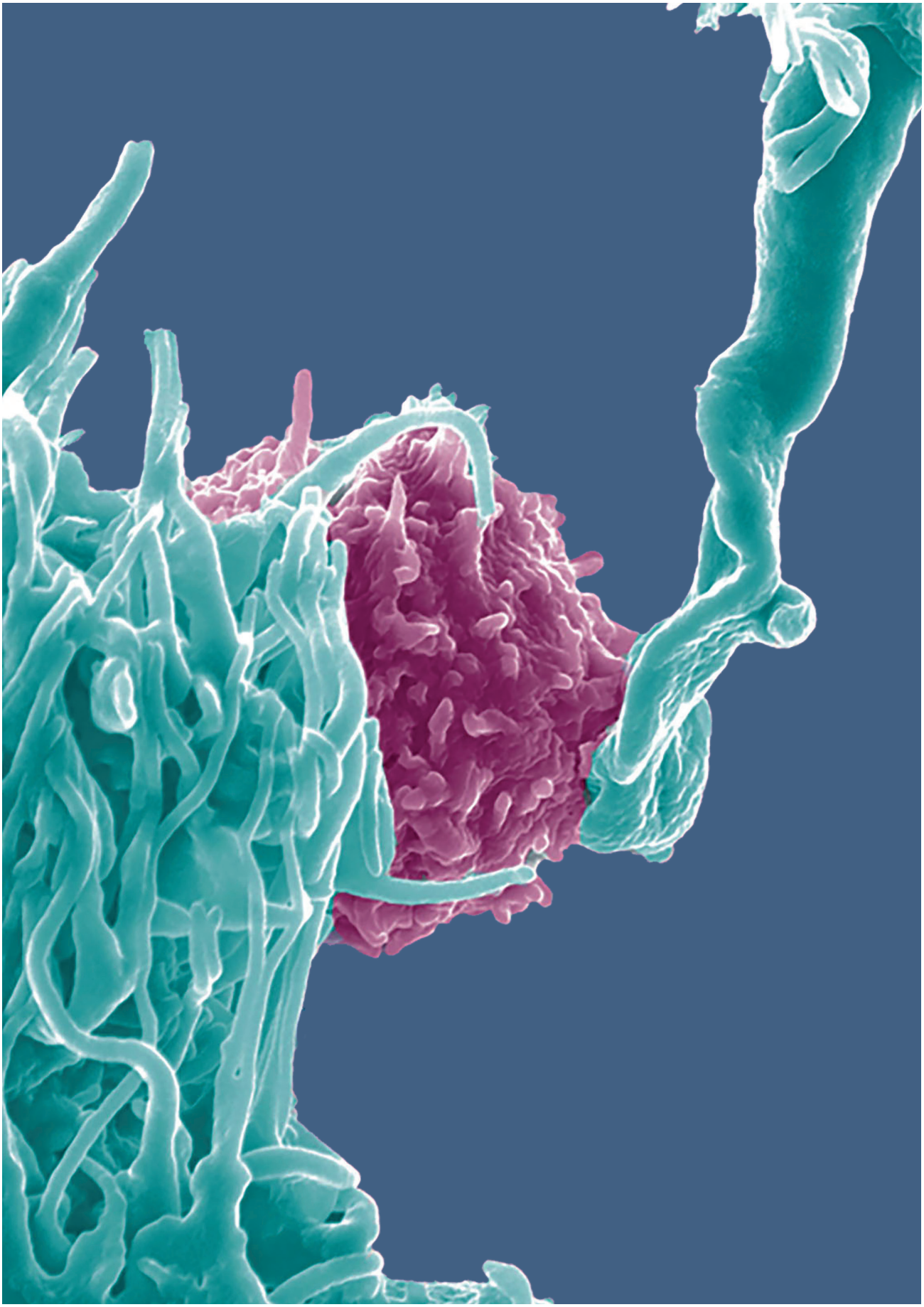
**Table S3. Baseline characteristics, comparison THILIHT and ERA-study.**

| | THILIHT | ERA | p-Value[3] |
|---|---|---|---|
| Number | 20 | 16 | |
| Age (years)[1] | 40 (25-60) | 40 (27 – 57) | 0.81 |
| Male[2] | 19 (95%) | 16 (100%) | 1.00 |
| $CD4^+$ T-cell count (x $10^6$/L)[1] | 443 (91- 851) | 350 (30-680) | 0.25 |
| HIV viral load (copies/mL)[1] | 70300 (3340- 5220000) | 72500 ( 13000- 300000) | 0.96 |
| Time since first positive HIV test (years)[1] | 3.17 (0.11- 7.94) | 1.3 (0.01- 10.45) | 0.21 |
| Lifespan of productively-infected cells (days)[1] | 1.17 (0.74-2.34) | 1.32 (0.70-2.70) | 0.91 |

[1] Median with range are given for total study population

[2] Number and percentage are given for total study population

[3] P-values were determined using a Mann-Whitney U test



# 5

# DIFFERENTIAL CHARACTERISTICS OF CYTOTOXIC T-LYMPHOCYTES RESTRICTED BY THE PROTECTIVE HLA ALLELES B*27 AND B*57 IN HIV-1 INFECTION

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*Introduction:* HLA-B*27 and B*57 are associated with relatively slow progression to AIDS. Mechanisms held responsible for this protective effect include the immunodominance and high magnitude, breadth and affinity of the CTL response restricted by these HLA molecules, as well as superior maintenance of CTL responses during HIV-1 disease progression.
*Methods:* We examined CTL responses from HIV-1 infected individuals restricted through protective and non-protective HLA-alleles within the same host, thereby excluding any effects of slow or rapid progression on the CTL response.
*Results***:** We found that neither immunodominance, nor high magnitude and breadth nor affinity of the CTL response are general mechanisms of protection against disease progression. HLA-B*57 restricted CTL responses were of exceptionally high affinity and dominated the HLA-A*02 restricted CTL response in individuals co-expressing these HLA alleles. In contrast, HLA-B*27 restricted CTL responses were not of particularly high affinity and did not dominate the response in individuals co-expressing HLA-B*27 and HLA-A*02. Instead, in individuals expressing HLA-B*27, the CTL response restricted by non-protective HLA alleles was significantly higher and broader, and of higher affinity than in individuals expressing these alleles without HLA-B*27. Even though HLA-B*27 and B*57 are thought to target the most conserved parts of HIV, during disease progression, CTL responses restricted by HLA-B*27 and B*57 were lost at least as fast as CTL responses restricted by HLA-A*02.
*Conclusion***:** Our data show that many of the mechanisms of CTL that are generally held responsible for slowing down HIV-1 disease progression hold for HLA-B*57, but do not hold for HLA-B*27.

## INTRODUCTION

It has consistently been shown that certain HLA class I alleles (e.g. HLA-B*27 and HLA-B*57) are associated with relatively slow progression to AIDS (reviewed in Carrington et al. [1]). The mechanism behind these associations is not fully understood but most likely involves both virological and immunological mechanisms. Several studies have shown that the cytotoxic T lymphocytes (CTL) restricted by these HLA molecules play an important role. A recent study suggested that the actual presence of certain HLA-B*57 or B*27 HIV-specific $CD8^+$ T-cell responses during primary HIV infection better defines HIV-disease progression than the HLA genotype alone [2]. Moreover, conformational differences in peptide presentation due to polymorphisms in amino acids located in the HLA-peptide binding groove have been implicated in control of HIV infection [3].

Viral escape from CTL responses targeting immunodominant CTL epitopes restricted by HLA-B*27 and B*57 often requires the accumulation of several mutations, including mutations that can compensate for the loss of viral fitness caused by the actual CTL escape mutation(s) [4-6]. The immunological mechanisms involved in the protective effect of HLA-B*27 and B*57 are less well understood. It is even unknown if the mechanism of protection is similar for both HLA molecules. HLA-B*57-restricted CTL are known to be immunodominant and of high functionality, features frequently proposed to be correlates of protection against disease progression [7-9]. It has therefore been suggested that $CD8^+$ T cells restricted by protective HLA alleles in general may confer protection because they are superior in terms of e.g. magnitude, frequency, or functional avidity [10-12].

HLA-alleles associated with slow disease progression are known to have an intrinsic preference to present Gag p24-derived epitopes [13-16]. P24 is one of the most conserved regions of the HIV genome [17], and higher order constraints on viral evolution are present [18]. Persons who durably control HIV spontaneously often target multidimensionally constrained regions of p24 [18]. Therefore the protective HLA alleles B*27 and B*57 might be associated with slow disease progression because the CTL responses against epitopes restricted via these alleles may be better preserved throughout the course of infection than CTL responses restricted via other HLA alleles.

Here we present a comprehensive study comparing CTL responses restricted via protective and non-protective HLA molecules within the same host. By analysing HLA-B*27 and B*57 separately, we reveal important insights in differences and similarities in their potential mechanism of protection.

## MATERIALS AND METHODS

### *Patient selection*

Twenty-four HIV-1 infected individuals from the Amsterdam Cohort Studies on HIV-1 infection and AIDS (ACS) with a known date of seroconversion were included based on HLA expression. Eighteen individuals expressed HLA-A*02, of which three co-expressed HLA-B*57 and eight co-expressed HLA-B*27. Six individuals expressed HLA-B*57 or B*27

**Table 1. Patient characteristics per HLA expression group.**

| HLA group | n¤ | CD4 counts¥ | CD8 counts§ | Viral load† |
|---|---|---|---|---|
| **HLA-A*02** | 7 | 350 | 800 | 76.000 |
| **HLA-B*08** | 5 | 330 | 780 | 52.000 |
| **HLA-B*27** | 1 | 600 | nd‡ | <1.000 |
| **HLA-B*57** | 5 | 580 | 960 | <1.000 |
| **HLA-A*02+B*27** | 13 | 430 | 1.000 | 4.800 |
| **HLA-B*08+B*27** | 5 | 470 | 920 | 4.800 |
| **HLA-A*02+B*57** | 6 | 690 | 920 | 2.550 |

¤ number of included individuals who express the specific HLA class I allele(s)

¥. $CD4^+$ T cell counts ($x10^6$ cells/ul)

§. $CD8^+$ T cell counts ($x10^6$ cells/ul)

†. HIV-1 RNA load (copies/ml), detection limit 1.000 copies/ml

‡. Not determined



without co-expressing HLA-A*02 (see Table 1 for more details). Additionally, 8 HIV-1 seroprevalent individuals (5 co-expressing HLA-A*02 and B*27 and 3 co-expressing HLA-A*02 and B*57) from the ACS were included during asympotomatic chronic infection. All individuals were treatment naive at the time of analysis. 2-Digit genotyping of the HLA class I loci was performed as described elsewhere [19]. Informed written consent was obtained from all participants and the study was approved by the Medical Ethical Committee of the Academic Medical Center Amsterdam. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

***Interferon (IFN)-γ enzyme-linked immunospot assay***

IFN-γ-producing antigen-specific $CD8^+$ T cells were measured using the IFN-γ ELISpot assay as described previously [20]. To avoid a bias in peptide selection we included known HIV-1 peptides published in the Los Alamos Database, and epitopes predicted to be presented via these HLA alleles [21], see Supplementary Table 1 and [20]. Phytohemagglutinin (PHA) stimulation served as a positive control, and medium without peptide or PHA served as a negative control. IFN-γ-producing cells were detected as dark spots and counted using an AELVIS EliScan (EliAnalyse Software version 4, Hanover, Germany). The number of IFN-γ-producing cells was calculated by subtracting the negative control value and was reported as number of spot-forming units (SFU) per $10^6$ PBMC. Samples with >100 spots per million PBMC, after subtraction of the negative control values were considered positive.

***Tetramer dissociation assay***

PBMC were thawed and washed twice. Subsequently $3*10^6$ cells were stained for 60 min at 4°C with mAb for CD8 (PerCP) and one of the following PE labelled tetramers: A*02-tetramers SLYNTVATL (SL9) or ILKEPVHGV (IV9), B*27-tetramers KRWIILGLNK (KK10) or KRKGGIGGY (KY9), B*57-tetramers KAFSPEVIPMF (KF11) or IATESIVIW (IW9). Cells were washed and resuspended in PBA. After removing 500.000 cells for $T_0$, the remaining cells were incubated with a five times excess APC labelled tetramer for 90 min. After 5, 10, 15, 30, 60 and 90 min, 500.000 cells were removed, washed and fixed. Per sample 200.000 events were acquired

using a FACS LSRII (BD). Data were analysed using BD FACSDiva software. The natural log (LN2) of the geometric mean fluorescent intensity of the PE-labelled tetramer was plotted against time. The half-life of the TCR-tetramer interaction was derived from the slope of this curve ($T_{1/2}$=LN2/slope).

***PCR amplification and sequencing***

Clonal HIV-1 variants were obtained by cocultivation of increasing numbers of patient PBMC with 2-3 day PHA stimulated PBMC from a healthy donor (PHA-PBMC) as described [22, 23]. Total DNA was isolated using the L6 isolation method [24] and DNA was amplified as described [25]. PCR products were purified using High pure PCR product purification kits (Roche Diagnostics) and sequenced using ABI Prism Big Dye Terminator v1.1/3.1 Cyclesequencing Kits (Applied Biosystems) with nested PCR primers. Sequences were analyzed on an Applied Biosystems/Hitachi 3130 xl Genetic Analyzer.

***Prediction of CTL epitopes***

Epitopes were predicted using the proteasomal cleavage/TAP transport/MHC class I combined predictor available at http://tools.immuneepitope.org, using the most abundant four-digit HLA type of each HLA serotype. Cut-off values used were 1.135 for proteasomal cleavage, -0.56 for TAP transport and-2.7 for MHC binding [26] .

***Statistical analysis***

Data was analysed using SPSS 15.0 software (SPSS, Chicago, Illinois, USA). using Wilcoxon Signed Ranks, Chi Square or Mann-Whitney tests. Correlations were tested using Spearman's correlation test. A p-value ≤ 0.05 was considered statistically significant.

## RESULTS

***Magnitude and breadth of CTL responses restricted by protective and non-protective HLA alleles***

It is well known that HLA-B*57 dominates the total HIV-specific CTL response during both acute and chronic infection [7, 8]. For HLA-B*27 most studies focused only on the dominant p24 Gag-derived KK10 epitope (e.g. [5, 6, 27, 28]). To investigate whether a high magnitude and/or breadth of the CTL response is a general mechanism for protection against disease progression, we here performed a detailed analysis of the CTL responses restricted by HLA-B*27 and B*57. To this end we measured CTL responses against 78 peptides derived from the entire HIV-1 subtype B genome (30 HLA-A*02, 29 HLA-B*57, and 19 HLA-B*27 restricted peptides, respectively, Supp. Table 1) using the IFN-γ enzyme linked immunospot (Elispot) assay. HIV-1 infected individuals were selected from the Amsterdam Cohort Studies on HIV-1 infection and AIDS (Table 1A) on the basis of expression of one of the protective HLA alleles HLA-B*27 (RH = 0.43, p=0.001) or HLA-B*57 (RH = 0.55, p=0.04) and/or a non-protective HLA allele (either HLA-A*02 (RH = 0.91, p=0.41) or HLA-B*08 (RH=0.97, p=0.82)) [29].

In individuals co-expressing HLA-A*02 and HLA-B*57, the CTL response indeed tended to be dominated by HLA-B*57 restricted CTL, although this difference was not statistically significant (Figure 1A,C). In contrast, in individuals expressing both HLA-A*02 and HLA-B*27,

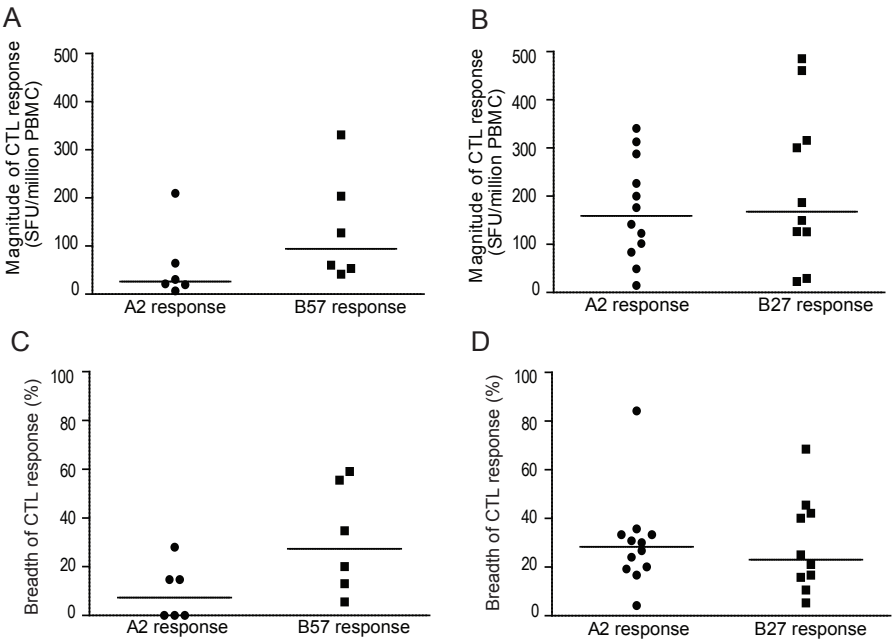


**Figure 1. Comparison of the magnitude and breadth of CTL responses restricted by protective and non-protective HLA molecules.**

The magnitude and breadth of the HLA-A*02, B*27, and B*57 restricted CTL response was analysed based on IFNγ Elispot assays during chronic HIV-1 infection. The breadth was determined as the fraction of tested peptides that elicited a positive CTL response. The left panels show the magnitude (A) and breadth (C) of the HLA-A*02 and B*57 restricted CTL response in individuals co-expressing these HLA alleles in Spot Forming Units (SFU) per million PBMC. The right panels show the magnitude (B) and breadth (D) of the HLA-A*02 and B*27 restricted CTL response in individuals co-expressing these HLA alleles. Each dot represents one individual, the black line represents the median value for the whole group. No differences were observed in individuals with a known date of seroconversion and seroprevalent individuals.

the magnitude (Figure 1B) and breadth (Figure 1D) of the CTL response restricted by HLA-A*02 and HLA-B*27 were similar. Because none of our patients expressed both HLA-B*27 and B*57, responses restricted by these HLA molecules could not be compared within the same individual. Since viral loads in individuals expressing B*27 or B*57 were very similar, however, we did compare these CTL responses and found that the magnitude and breadth of HLA-B*27 and B*57 restricted responses were not significantly different (p=0.113 and p=0.979, respectively). Thus, although both protective HLA alleles induce CTL responses of comparable magnitude (Figure 1A vs. B) and breadth (Figure 1C vs. D), HLA-B*57 restricted responses are, but B*27-restricted responses are not, immuno-dominant in individuals co-expressing HLA-A*02 and one of the protective HLA class I alleles.

***Differences in affinity of the TCR for different peptide-MHC complexes***

We next investigated whether the affinity of the T cell receptors (TCRs) for their cognate peptide-HLA (pHLA) complexes is associated with HIV-1 disease progression rates by performing tetramer decay assays (see Figure 2A for an example). In line with our previous

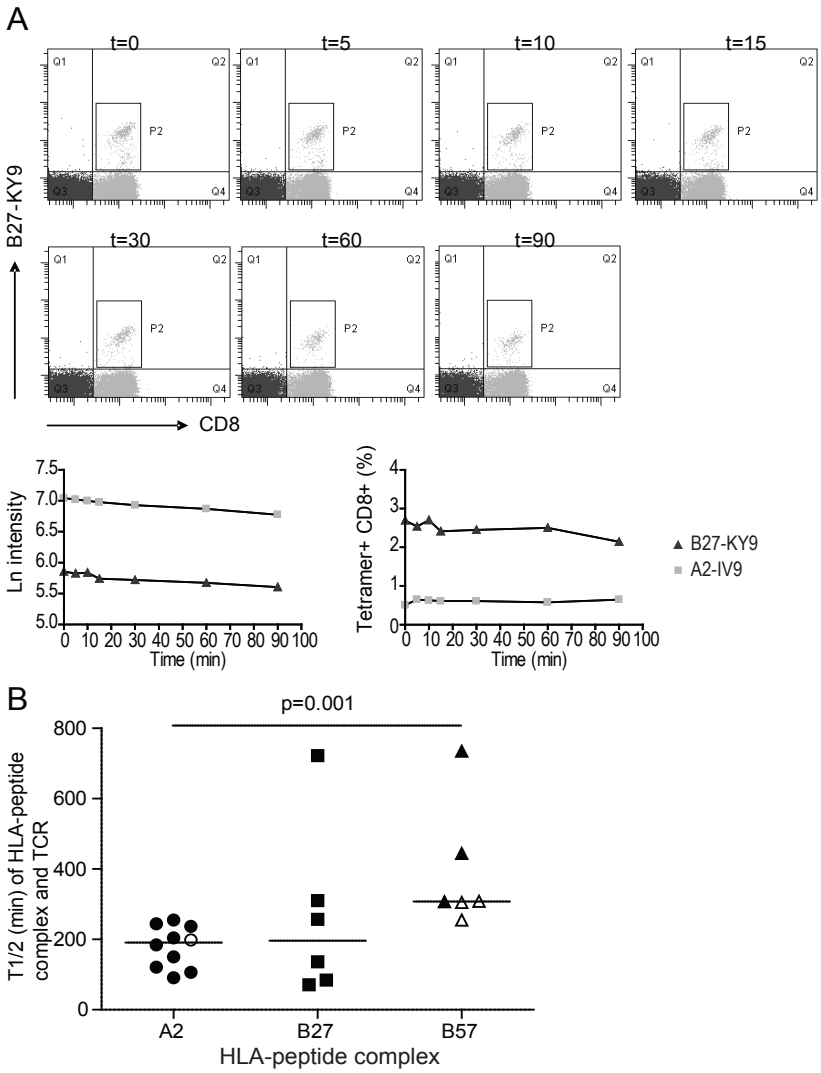


**Figure 2. Comparison of the half-life of HLA-peptide complexes and the TCRs for protective and non-protective HLA molecules.**

In (A) an example of the tetramer decay assay is shown for the B*27-KY9 tetramer. The mean fluorescent intensity of the tetramer$^+$ CD8$^+$ T cells (P2) decreases over time. An example of a dissociation graph of the B*27-KY9 (black triangles) and A*02-IV9 (grey squares) tetramers is shown (left panel, patient 8, $t_{1/2}$ KY9 257 minutes, $t_{1/2}$ IV9 244 minutes), as well as the percentage of tetramer positive cells (right panel), which remains relatively constant throughout the experiment. (B) The half-lives of the interaction between the HLA-peptide complexes and the TCR for peptides presented via HLA-A*02 (n=9, black dots; the white dot depicts data from our previous study [30]), B*27 (n=6, black squares) and B*57 (n=3, black triangles; the white triangles depict data from our previous study [30]). Some individuals did not have high enough frequencies of the specific CTL to perform our analyses and were therefore not included. Significant differences between the groups ($p \leq 0.05$, Mann-Whiney) are depicted when applicable.

**Table 2. Patient characteristics per HLA expression group.**

| | | CD4 counts¥ | | CD8 counts§ | | Viral load† | |
|---|---|---|---|---|---|---|---|
| **Subject** | **HLA type** | **early** | **late** | **early** | **late** | **early** | **late** |
| 1 | **A2**,A32,**B27**,B40 | 0,43 | 0,28 | 0,7 | 0,8 | <1.000 | 5.800 |
| 2 | **A2**,A30,**B27**,B40 | nd‡ | 0,3 | nd‡ | 1 | 32.000 | <1.000 |
| 3 | **A2**,A1,**B27**,B8 | 0,67 | 0,57 | 0,81 | 1,25 | 13.000 | nd‡ |
| 4 | **A2**,A68,**B27**,B7 | 0,54 | 0,38 | 0,6 | 0,6 | 8.600 | 27.000 |
| 5 | **A2**,**B27**,B15 | 1,06 | 0,58 | 1,3 | 1,75 | 12.000 | 1.700 |
| 6 | **A2**,**B27**,B8 | 0,84 | 0,45 | 0,5 | 0,9 | 3.100.000 | 4.800 |
| 7 | **A2**,**B27**,B8 | nd‡ | 0,43 | nd‡ | 1,3 | 12.000 | 4.800 |
| 8 | **A2**,A31,**B27**,B35 | 1,39 | 0,82 | 2,1 | 3,03 | 11.000 | <1.000 |
| | **median** | 0,76 | 0,44 | 0,76 | 1,13 | 12.000 | 4.800 |
| 9 | **A2**,A1**,B57**,B41 | 0,74 | 1,56 | 0,5 | 1 | <1.000 | <1.000 |
| 10 | **A2**,B37**,B57** | 1,13 | 1,19 | 0,5 | 0,64 | <1.000 | 3.100 |
| 11 | **A2,**A32,B14**,B57** | 0,53 | 0,45 | 0,7 | 0,71 | 33.000 | 16.937 |
| | **median** | 0,72 | 0,87 | 0,55 | 0,81 | <1.000 | 9.050 |
| 12 | **A2**,A3,B7,B40 | 0,58 | 0,37 | 0,8 | 0,8 | 37.000 | 74.000 |
| 13 | **A2**,A1,B8,B35 | 0,6 | 0,24 | 0,6 | 0,3 | 31.000 | 110.000 |
| 14 | **A2**,A1,B8,B40 | 0,81 | 0,69 | 0,4 | 1,1 | 64.000 | <1.000 |
| 15 | **A2**,A1,B7,B15 | 0,58 | 0,35 | 0,5 | 0,7 | 220.000 | 90.000 |
| 16 | **A2**,A11,B40 | 0,72 | 0,6 | 1 | 0,9 | <1.000 | 60.000 |
| 17 | **A2**,B18,B44 | 0,47 | 0,15 | 1,6 | 1,1 | 770.000 | 59.000 |
| 18 | **A2,**A1,B8,B38 | 1,38 | 0,3 | 0,9 | 0,6 | 15.000 | nd‡ |
| | **median** | 0,6 | 0,35 | 0,8 | 0,8 | 37.000 | 67.000 |
| 19 | A24,A26,B8,**B27** | nd‡ | 0,6 | nd‡ | nd‡ | 33.000 | <1.000 |
| 20 | A1,A11,B8,**B57** | 0,56 | 0,58 | 0,5 | 1,17 | 8.800 | 5.450 |
| 21 | A1,A68,B14,**B57** | 1,11 | 0,74 | 0,7 | 0,4 | <1.000 | <1.000 |
| 22 | A1,A32,B15,**B57** | 1,08 | 0,81 | 0,8 | 1,02 | <1.000 | <1.000 |
| 23 | A1,A24,B35,**B57** | 0,36 | 0,29 | 1 | 0,96 | 19.000 | <1.000 |
| 24 | A3,A68,B35,**B57** | 0,42 | 0,46 | 0,5 | 0,8 | 15.000 | 72.000 |
| | **median** | 0,56 | 0,58 | 0,7 | 0,96 | 8.800 | <1.000 |

¥. $CD4^+$ T cell counts ($x10^6$ cells/ul)

§. $CD8^+$ T cell counts ($x10^6$ cells/ul)

†. HIV-1 RNA load (copies/ml), detection limit 1.000 copies/ml

‡ not determined

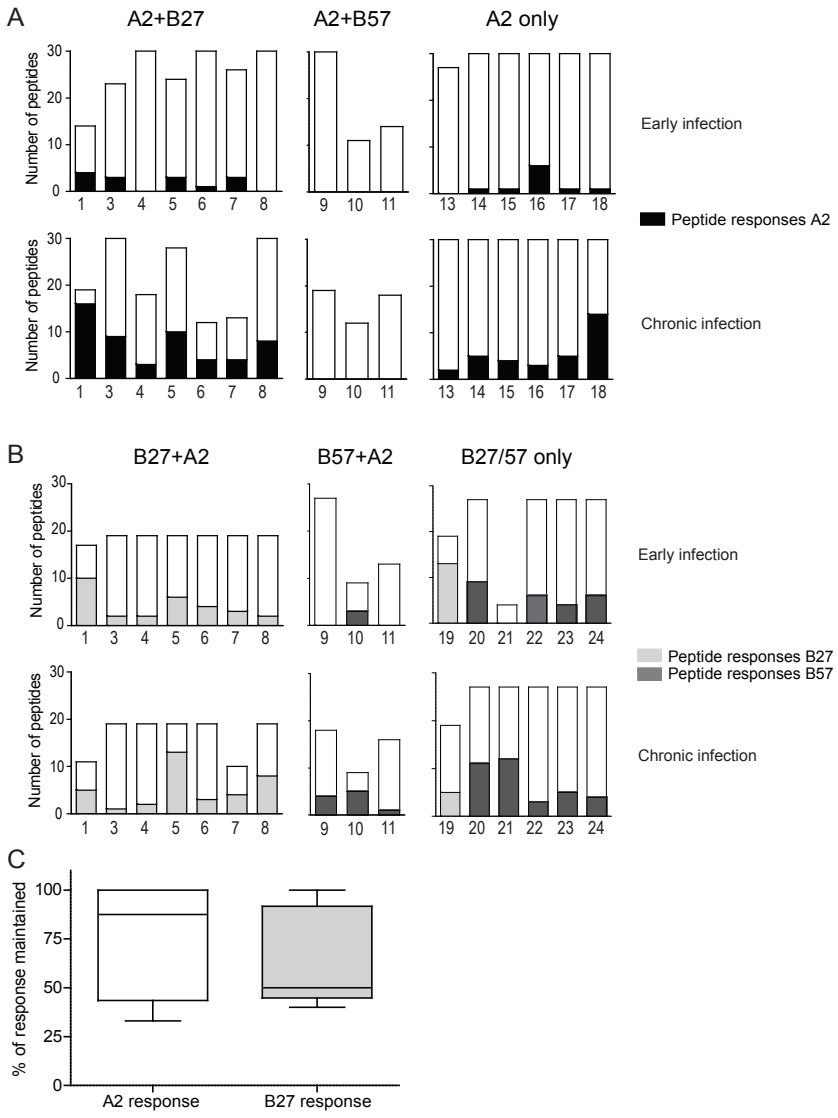


**Figure 3. Changes in CTL response between early and chronic HIV-1 infection.**

CTL responses towards a maximum of 30 HLA-A*02, 19 HLA-B*27 and 29 HLA-B*57 restricted peptides were measured within 6 months after seroconversion (Early infection) and 5 years later (Chronic infection). (A) Shows the HLA-A*02 restricted CTL response per patient for patients co-expressing HLA-A*02 and B*27 (A2+B27), patients co-expressing HLA-A*02 and B*57 (A2+B57), and patients expressing HLA-A*02 without any of the protective HLA alleles (A2 only). Likewise, (B) shows the HLA-B*27 or B*57 restricted CTL response per patient for patients co-expressing HLA-B*27 and A*02 (B27+A2), patients co-expressing HLA-B*57 and A*02 (B57+A2), and patients expressing HLA-B*27 or B*57 without HLA-A*02 (B27/B57 only). The Y axis represents the number of tested peptides; the whole bar shows the total number of tested peptides, filled bars show the number of peptides to which a CTL response was observed (black: HLA-A*02 binding peptides,

*(continued on the next page)*

findings [30], we observed a stronger interaction for HLA-B*57-restricted peptides (median half-life 444 minutes, range 307-735 minutes) compared to HLA-A*02-peptide complexes (median 184, range 91-255; p=0.001, Figure 2B). In contrast, the half-life of the interaction between HLA-B*27-peptide complexes and the TCRs was not significantly higher than that observed for HLA-A*02-peptide complexes (Figure 2B). Even when comparing protective and non-protective HLA alleles *within* the same individual, no difference was observed in the affinity of the TCR for the HLA-A*02-peptide complexes and the HLA-B*27-peptide complexes (data not shown).
These data indicate that a low relative hazard of disease progression of an HLA molecule can (HLA-B*57) but is not always (HLA-B*27) associated with a stronger interaction between the peptide-HLA complex and the TCRs.

***CTL responses restricted by protective HLA alleles are not better preserved***



Another mechanism that is thought to play a role in the protective effect of HLA-B*27 and B*57 is maintenance of the CTL response throughout the course of infection. To investigate this, we analysed HIV-1 specific CTL responses at two sequential time points, within 6 months after seroconversion (early infection) and approximately 5 years later (during asymptomatic chronic infection). Table 1B shows detailed characteristics of the individuals included for this analysis. In line with previous studies [31-34], the CTL response in chronic infection was broader than early after seroconversion (Figure 3A and B). Remarkably, this broadening of the CTL response was observed for HLA-A*02 restricted responses (Figure 3A, p=0.002) but not for HLA-B*27 and HLA-B*57 restricted responses (Figure 3B, p=0.155, Wilcoxon Signed Ranks test), which were already relatively broad during early HIV-1 infection, in line with previous results [8].

Because the maintenance of CTL responses may be affected by disease progression itself [35, 36], we next confined our analysis to patients who co-expressed HLA-A*02 *and* a protective HLA allele (either HLA-B*27 (n=7) or B*57 (n=3)), allowing us to compare the evolution of CTL responses restricted by both types of HLA within the same host. We included only CTL responses that were present during early HIV-1 infection and analysed whether these responses were still present five years later. Figure 3C shows that within an individual, CTL responses towards peptides presented via the protective HLA allele B*27 were not better (if not even worse) preserved during disease progression (median 50% of CTL responses is preserved, range 40-100%) than CTL responses restricted by the non-protective HLA-A*02 (median 87.5%, range 33-100%). Individuals co-expressing HLA-A*02 and HLA-B*57 hardly

**Figure 3. Continued.**

light grey: HLA-B*27 binding peptides, dark grey: HLA-B*57 binding peptides). Two patients (#2 and #12) were not included due to a lack of PBMC from the early infection timepoint. Samples with >100 spots per million PBMC, after subtraction of the negative control values were considered positive. In (C) we analysed whether the responses that were present during early HIV-1 infection were still present during chronic infection in individuals co-expressing HLA-A*02 and one of the protective HLA alleles. Within each box, the median is indicated by a horizontal line, the bar represents the 25-75% interval, and whiskers represent minimum and maximum values. (D) The pie charts depict the fraction of MHC-binding epitopes that were predicted to be present during early infection and maintained (grey) or lost (white) during chronic infection based on HIV sequences derived from individuals carrying the respective HLA molecule.

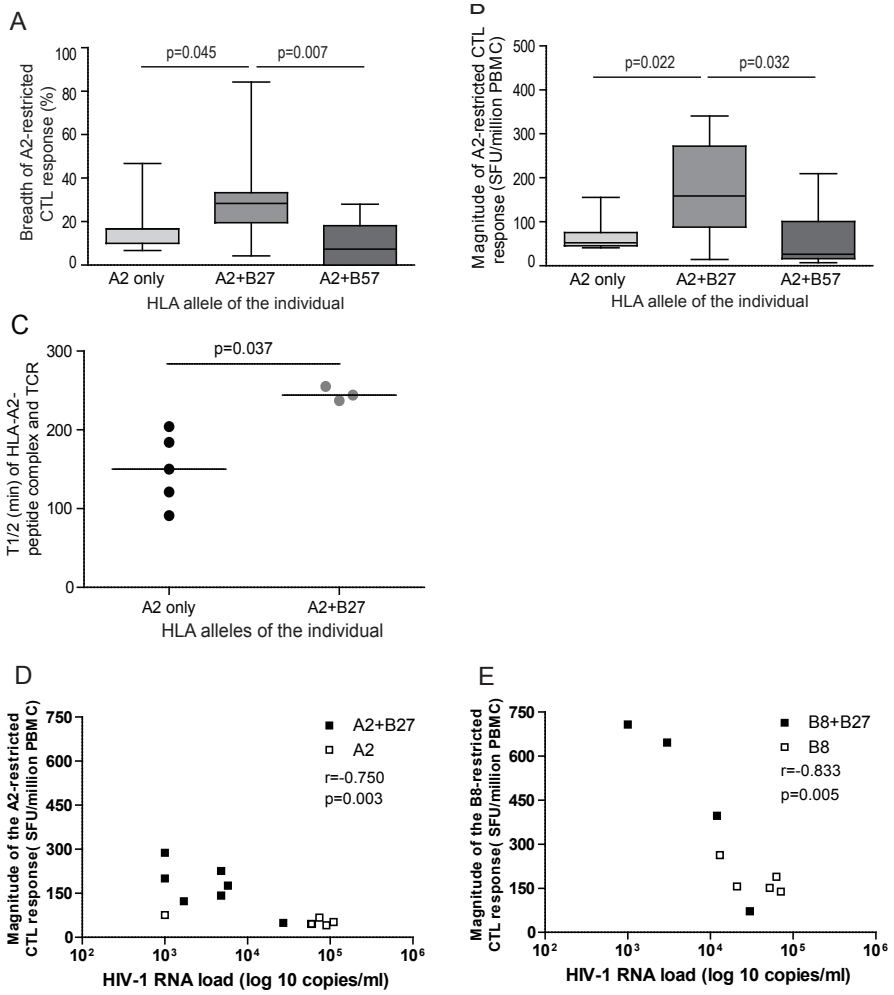


**Figure 4. The influence of co-expression of protective HLA alleles on the CTL response restricted by non-protective HLA alleles.**

The breadth (A) or magnitude (B) of the HLA-A*02 restricted CTL response in individuals expressing HLA-A*02 without any of the protective HLA alleles (A2 only) or in combination with HLA-B*27 (A2+B27) or HLA-B*57 (A2+B57) is shown. An additional 8 HIV-1 seroprevalent individuals (3 individuals co-expressing HLA-A*02 and B*57 and 5 individuals co-expressing HLA-A*02 and B*27) were included to increase the sample size. Within each box, the median is indicated by a horizontal line, the bar represents the 25-75% interval, and whiskers represent minimum and maximum values. (C) The half-lives of the interaction between the HLA-A*02-peptide complexes and the TCRs of the responding CTL for individuals expressing HLA-A*02 alone (black dots) and individuals co-expressing HLA-A*02 and B*27 (grey dots). Significant differences between the groups ($p \leq 0.05$, Mann-Whiney) are depicted when applicable.

HIV-1 RNA load was plotted against the magnitude of the HLA-A*02 (D) or HLA-B*08 (E) restricted CTL response in individuals expressing HLA-A*02 or B*08 (open squares) and individuals co-expressing HLA-B*27 and HLA-A*02 or B*08 (black squares). Each square represents the mean HLA-A*02 (D) or B*08 (E) restricted CTL response of one individual. Correlations were tested using Spearman's correlation test.

showed responses towards peptides presented via HLA-A*02 (Figure 3A), making it impossible to compare the maintenance of both responses. Therefore, we additionally used peptide prediction programs (http://immuneepitope.org [21]) to reveal the number of potential CTL epitopes present in HIV sequences obtained during early and chronic infection, which resulted in similar findings. On average, 91% (range 80-100%) of the HLA-A*02 restricted epitopes predicted to be present early are still present during chronic infection which is again comparable with (or even higher than) the 85% (range 73-100%) and 74% (range 70-78%) for HLA-B*27 and B*57-restricted CTL epitopes, respectively (data not shown).

***Co-expression of HLA-B*27, but not B*57, results in enhanced responsiveness of HIV-specific T-cells restricted through HLA-A*02***

It has previously been shown that HLA alleles might have an impact on CTL responses restricted by other HLA alleles expressed by individuals [37, 38]. Indeed, when we compared the HLA-A*02 restricted CTL response in individuals expressing either HLA-A*02 alone or both HLA-A*02 and HLA-B*27 or B*57, we found that individuals co-expressing HLA-A*02 and B*27 responded to more HLA-A*02 restricted peptides (Figure 4A, p=0.045) and with higher magnitude (Figure 4B, p=0.022) compared to individuals without HLA-B*27. Individuals co-expressing HLA-A*02 and B*57 on the other hand responded to fewer HLA-A*02 restricted peptides (Figure 4A, p=0.007) and with lower magnitude (Figure 4B, p=0.032). Also the half-life of the HLA-A*02-peptide-TCR interaction was significantly higher in individuals co-expressing HLA-B*27 compared to individuals expressing HLA-A*02 without B*27 (p=0.037, Figure 4C). These data indicate that the HLA-A*02 restricted CTL response is differentially influenced by the presence of the different protective HLA alleles.

When we plotted the magnitude of the HLA-A*02 restricted CTL response against HIV-1 viral load, including individuals who did and did not co-express HLA-B*27, a negative correlation was observed (r=-0.750, p=0.003, Figure 4D). Since the presence of HLA-B*27 results in a low viral load, we hypothesized that proper viral suppression, due to the presence of HLA-B*27, preserves CTL function including CTL restricted by non-protective HLA alleles. To substantiate this finding, we repeated our analysis for another non-protective HLA molecule. We selected 10 patients expressing HLA-B*08, which is not associated with delayed disease progression (RH=0.97, p=0.82) [29], 5 of which co-expressed HLA-B*27. Again, the magnitude of the CTL response restricted by the non-protective HLA allele B*08 was negatively correlated with HIV-1 RNA load (r=-0.833, p=0.005, Figure 4E), with individuals co-expressing HLA-B*27 showing the highest magnitude. This shows that expression of HLA-B*27 also leads to preservation of HLA-B*08 restricted CTL responses.

Taken together, our data suggest that while HLA-B*57 restricted responses are of exceptionally high affinity and down-regulate CTL responses restricted through other HLA molecules, HLA-B*27 restricted responses are not exceptionally high, broad, or of high affinity, but have a beneficial effect on CTL responses restricted by other HLA molecules of the host. Moreover, even though protective HLA alleles are thought to target the most conserved parts of HIV, we found that CTL responses restricted by HLA-B*27 and B*57 were lost at least as fast during the course of infection as CTL responses restricted by a non-protective HLA allele.

## DISCUSSION

Functional studies focusing on those relatively rare patients able to spontaneously control HIV-disease progression are key to obtaining insights into the characteristics of CTL responses needed to delay HIV-disease progression. Such studies have previously pinpointed differences in CTL functions between HIV controllers and patients with progressive disease (see for example [10, 39-46]). Our study uniquely compared CTL responses restricted by non-protective and protective HLA alleles within the same host, such that the effects of slow or rapid progression are not interfering with our read-out. Moreover, we analysed the two HLA-alleles most convincingly associated with slow disease progression separately, to reveal potential different and/or shared mechanisms of protection. In concordance with previous studies [7, 8], we found that the CTL response in individuals co-expressing HLA-B*57 was dominated by CTL against HLA-B*57 binding peptides. In contrast, the CTL response in B*27+ patients was not dominated by CTL against HLA-B*27 binding peptides. In fact, CTL responses restricted by HLA-B*27 and HLA-A*02 were indistinguishable in height and breadth. Moreover, although it has convincingly been shown that CTL specific for the dominant HLA-B*27-restricted epitope KK10 are highly polyfunctional and have a superior functional capacity compared to other HIV-1 specific CTL [10], our data suggest that this is not due to an increased half-life of the interaction between the TCRs and the HLA-B*27-KK10 complex, or the pHLA-B*27 complex in general, indicating that a strong interaction between pHLA complexes and the TCR is not a prerequisite for a protective T-cell response. Thus, even though immunodominance, breadth, magnitude, and affinity of the T cell response might be associated with protection against progression to AIDS in HLA-B*57 expressing individuals, our data show that this is not the case for HLA-B*27.

Additionally, we found that CTL responses restricted by the protective HLA alleles HLA-B*27 and B*57 were lost at least as fast as CTL responses restricted via the non-protective HLA allele HLA-A*02. Even at the HIV-sequence level we found no evidence that HLA-B*27 or B*57-binding epitopes are more conserved during disease progression than HLA-A*02 binding epitopes. Maintenance of CTL responses per se is therefore also not a main determinant of protection against progression to AIDS. The finding that protective HLA alleles contribute strongly to the total CTL response during primary infection, as was shown before [8], might however certainly add to their protection.

Our data clearly show that T-cell responses restricted by different HLA molecules influence each other differently, which has also been described for other viruses [37,38]. Even though the mechanism behind these observed associations are not well understood, this phenomenon has implications for the design of epitope-specific vaccines. In our case, it seems that the effect of HLA-B*27 on the magnitude and breadth of responses restricted through other HLA alleles is due to the beneficial effect of HLA-B*27 on HIV viral load. The low HIV viral load and activation level in patients co-expressing HLA-B*27 might result in a decreased level of exhaustion of all HIV specific CD8+ T cells, hence also the ones restricted by HLA-A*02 or HLA-B*08. Our findings thereby also illustrate the difficulty in interpreting the quality of CTL responses, as a high and broad (HLA-A*02 or B*08 restricted) CTL response apparently not necessarily means that the HLA in question is driving the favourable clinical outcome.

**Box 1. Similarities and differences between HLA-B*27 and B*57 revealed in this study**

Similarities:

- CTL responses restricted through both HLA molecules are already broad during early HIV infection.
- Both HLA molecules induce CTL responses that are not better maintained during disease progression than those restricted by non-protective HLA alleles.
- During chronic HIV infection, CTL responses restricted through both HLA molecules are comparable in breadth and magnitude.

Differences:

- HLA-B*57 restricted responses are of exceptionally high affinity, which is not observed for HLA-B*27 restricted responses.
- While HLA-B*57 restricted responses seem to down-regulate CTL responses restricted through other HLA molecules, HLA-B*27 restricted responses have a clear beneficial effect on CTL responses restricted by other HLA molecules of the host.



HLA-B*27 is also associated with beneficial outcome of hepatitis C virus (HCV) infection. A recent study showed that functional avidity, the functional profile, antiviral efficacy or naïve precursor frequency of the immunodominant HLA-B*27-restricted HCV-specific CD8+ T-cell epitope was not superior to T cells targeting epitopes restricted by HLA-A*02 [47], in line with our current observations. However, epitope generation was much more efficient for this B*27-restricted peptide compared to the A*02-restricted peptides, indicating that kinetics of antigen processing might be associated with HLA-B*27-mediated protection in HCV infection [47]. Such a mechanism might also play a role in HIV infection.

Our data indicate that there are at least two different strategies via which HLA class I alleles can be protective, which include i) inducing a very dominant CTL response (e.g. HLA-B*57), and ii) preservation of total T-cell responses (as observed for HLA-B*27). The marked differences between the two protective HLA alleles that we observed are an important new insight, as previous studies often did not distinguish between HLA-B*27 and B*57 when investigating HIV control (e.g. [41, 44-46]). Box 1 depicts the observed similarities and discrepancies between HLA-B*27 and B*57, which may contribute to their protective effect. The observation that HLA-B*27 and B*57 exert their protective effect at distinct moments after HIV infection [48] suggests the existence of a different mechanism of protection. The effect of HLA-B*57 already occurs early after infection, before the $CD4^+$ T-cell count drops below 200 cells/µl, whereas HLA-B*27 delays progression to AIDS-defining illnesses when the $CD4^+$ T cell counts have already dropped below 200 cells/µl [48]. This fits with our observation that the beneficial effect of HLA-B*27 is evident during chronic infection, but not early during infection (within 6 months after seroconversion, data not shown).

In conclusion, the actual mechanism(s) of protection offered by an HLA molecule involve both virological as well as immunological features. Several virological features are known, [4-6], but the immunological mechanisms are less well understood. We here show that certain mechanisms at least are not required for protection against disease progression. Our

data indicate that while HLA-B*57 restricted responses are more likely to be of exceptionally high affinity and down-regulate CTL responses restricted through other HLA molecules, HLA-B*27 restricted responses are of moderate affinity, but have a clear beneficial effect on CTL responses restricted by other HLA molecules of the host.


## ACKNOWLEDGEMENTS

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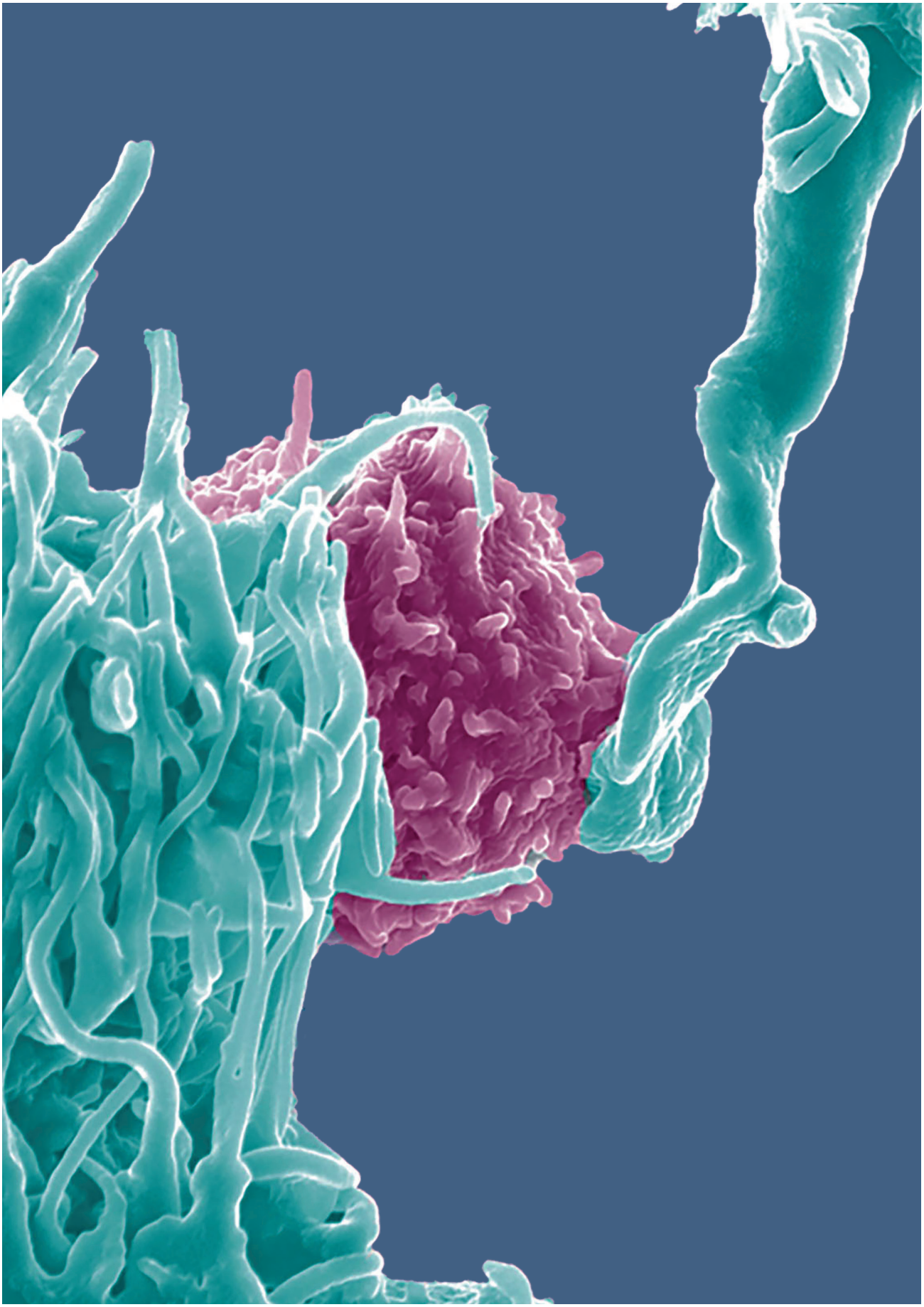

# 6

# A DIRECT LINK BETWEEN LOSS OF T-CELL FUNCTION AND DOWNREGULATION OF TCRZ EXPRESSION IN CHRONIC VIRAL INFECTIONS?

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6

*Introduction:* In chronic infection both T-cell dysfunction and downregulation of the T-cell receptor ζ chain (TCRζ) are found. Here we studied the direct relationship between the level of TCRζ expression and $CD8^+$ T-cell function in terms polyfunctionality based on production of particular cytokines and chemokines.

*Methods:* PBMC from healthy and HIV-infected individuals were stimulated with either TCR-bypassing or TCR dependent mitogens. Both magnitude and polyfunctionality of the responding T cells and their TCRζ expression levels were determined by intracellular measurement of IFNγ, IL-2, TNFα and MIP1β and TCRζ.

*Results:* The magnitude and polyfunctionality of $CD8^+$ T cells positively correlated with TCRζ expression levels, in both healthy and HIV-infected individuals. $CD8^+$ T cells producing IL-2 alone or a combination of IL-2 and MIP1β consistently exhibited low TCRζ expression levels, in all individuals. However, in HIV infected individuals, low TCRζ levels extended to all $CD8^+$ T cells producing IL-2.

*Conclusion:* Here we demonstrated, on a cellular basis, the direct correlation between polyfunctionality and magnitude of cytokine production in $CD8^+$ T cells and TCRζ expression level, both in healthy and HIV-infected individuals.

## INTRODUCTION

$CD8^+$ T cells play a critical role in resolving various acute viral infections and controlling chronic infections, such as HIV infection. Both in HIV infection and in other diseases wherein persistent viraemia and chronic antigen exposure are involved, $CD8^+$ T-cell defects have been observed [1-5]. One of such effects is the downregulation of the T-cell receptor ζ-chain (TCRζ) [1-5]. The ζ-chain disulphide-linked homodimer is part of the T-cell receptor (TCR) multisubunit complex, where it is indispensable for linking TCR engagement to various signal-transduction pathways. These pathways ultimately lead to transcription of cytokine genes, secretion of cytokines and proliferation of T cells [1]. It has been suggested that the ζ-chain dictates the amount of TCR expression on the cell surface, as it is a limiting factor in both the assembly and transfer of the TCR multi-subunit complex to the cell surface [6]. Once the TCR is expressed on the cell surface, TCRζ stabilizes the TCR complex and preserves cell-surface expression [7]. This contributes to sustained signaling, which needs to occur for full $CD8^+$ T-cell activation, as this requires interaction between the TCR and its ligands for several hours [8]. After activation TCRζ is temporarily down regulated [9].

Another $CD8^+$ T-cell effect that takes place during chronic viral infection is the loss of cytokine production. According to current consensus, T cells capable of secreting multiple cytokines, so-called polyfunctional $CD8^+$ T cells, are key players in an effective immune response [10]. These cells are thought to produce specific combinations of cytokines, and by production of a greater absolute quantity of cytokines mediate key functions in the control of viral infections [11]. In HIV infection, long-term nonprogressors-who maintain low viral loads and high $CD4^+$ T-cell counts in chronic infection- have T-cell responses of higher polyfunctionality including concomitant production of IL-2, TNFα, IFNγ, and MIP1β compared to HIV-progressors [12, 13]. The presence of polyfunctional HIV-specific $CD8^+$ T cells has been shown to correlate inversely with viral load [13]. In the majority of HIV-infected individuals, however, $CD8^+$ T cells have an "exhausted" phenotype, characterized by expression of inhibitory receptors -most notably PD1 and CTLA4-, diminished functional proliferative capacity (reviewed in [14]), and reduced production of cytokines. In particular, loss of $CD8^+$ T-cell derived IL-2, a cytokine that promotes T-cell proliferation, is a correlate of poor viral control and disease outcome in HIV infection [15]. Remarkably, the loss of each individual $CD8^+$ T-cell function during chronic infection occurs in a time-dependent fashion [16]. As was also found in chronic LCMV infection, IL-2 production is one of the first functions to be lost during early HIV infection. Subsequently, secretion of TNFα gets impaired, which is followed by impairment of IFNγ and MIP1β production [16]. In progressive chronic HIV-infection, $CD8^+$ T cells were shown to have the propensity to produce IFNγ but not IL-2 and to have a low level of TCRζ expression [17]. However in literature a direct link between these various functional $CD8^+$ T-cell defects has not been demonstrated to date. Therefore we studied the relationship between the level of TCRζ expression and $CD8^+$ T-cell function, in terms of magnitude of particular cytokines and chemokines, and polyfunctionality both in healthy and HIV-infected individuals.

## MATERIALS AND METHODS

### *Study Population*

Blood samples were collected from 10 healthy donors after having provided written informed consent. In addition, blood samples of eight HIV-infected participants were obtained. Four untreated and four 24-week treated primary HIV-infected individuals were selected based on sample availability and immunological parameters were measured at 36 weeks after inclusion or stop of treatment [18]. Approval was obtained from the Medical Ethics Committee of each participating site, and written informed consent was obtained from all participants.

### *PBMC's / blood separation*

PBMC's were isolated from heparinized blood before start of treatment using Ficoll (GE Healthcare Lifesciences) density separation, as described previously [19]. PBMC's were isolated, cryopreserved and stored in liquid nitrogen within 24 hours of collection. All experiments were performed on previously frozen PBMC's.

### *$CD8^+$ T-cell stimulation*



Cryopreserved PBMC were thawed and aliquotted at $2x10^6$ cells per ml in round bottom tubes (Becton Dickinson (BD), San Jose, California) in complete medium (RPMI supplemented with 10% FCS and 1% pen/strep). PBMC were stimulated for 1, 2, 3, 4, 5, and/or 6 hours with the respective stimulant, see table S1A at 37° C. After 1 hour, Brefeldin A (3 µM, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added and at the end of the allotted period all cells were stained.

### *Intracellular cytokine staining*

Surface staining was performed with monoclonal antibodies (see table S1B) for 20 min at 4°C. Cells were fixed and permeabilized using the FOXp3/transcription factor staining buffer set (eBioscience), that has been optimized for staining with antibodies to transcription factors and nuclear proteins, as well as cytokines and chemokines, according to the manufacturer's instructions. Thereafter cells were stained with monoclonal antibodies for the intracellular markers (see table S1B) for 20 min at 4°C. Cells were fixed in cellfix (BD) and flow cytometry was performed.

### *Flow cytometry analysis*

At least 300.000 events were acquired after intracellular cytokine staining, using the LSRII flow cytometer (T-cell polyfunctionality was analysed by Flowjo software (version 9.2). After determining the lymphocyte gate in a FSC-A versus SSC plot, cells were sequentially gated for CD3 and CD8. Subsequently, within the $CD8^+$ T-cell population a gate was created for the 4 respective functions; IFNγ, TNFα, MIP1β, and IL-2. Hereon a Boolean gating was performed resulting in 16 different combinations (see Figure S1 for a gating strategy).

### *Statistical analysis*

Differences between healthy donors and HIV-infected individuals were analysed using the Mann-Whitney test. Whenever more than 2 groups were compared, a Friedman test was used. All statistical analyses were performed using the software program SPSS 21.0 (SPSS Inc, Chicago, Illinois).

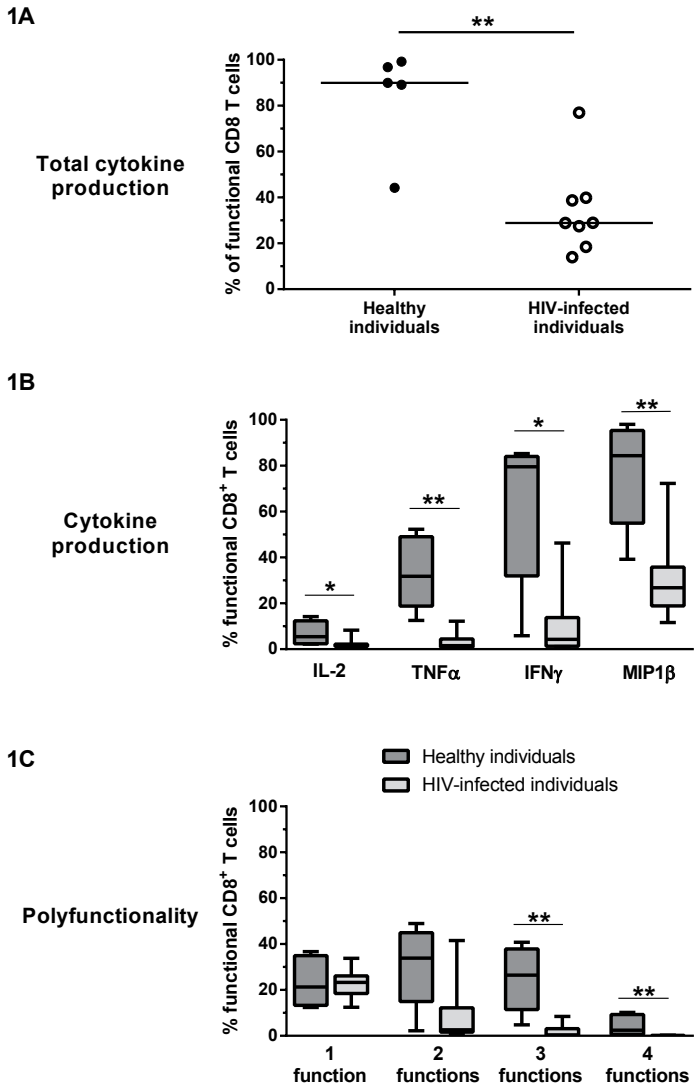


**Figure 1. Decreased production of individual cytokines and polyfunctionality in CD8⁺ T cells during early HIV-1 infection.**

PBMC from 5 healthy individuals and 8 early HIV infected individuals were stimulated with PMA/ionomycin and the production of IFNγ, IL-2, TNFα and MiP1β was measured in CD8⁺ T cells by flow cytometry. In Figure 1A the total percentage of functional CD8⁺ T cells (left y-axis) is compared between healthy donors (●) and HIV-infected individuals (o). Each symbol represents an individual, with a bar at the median. The total CD8⁺ T-cell response was broken down to compare the individual production of cytokines/chemokines (Figure 1B) and the production of multiple functions (Figure 1C) by the CD8⁺ T cells. The boxplots depict median and range, and Mann Whitney U tests were used to compare healthy donors (dark grey) with HIV-1 infected individuals (light grey). *p-value =0.05- 0.01 ** p-value =0.01- 0.001.

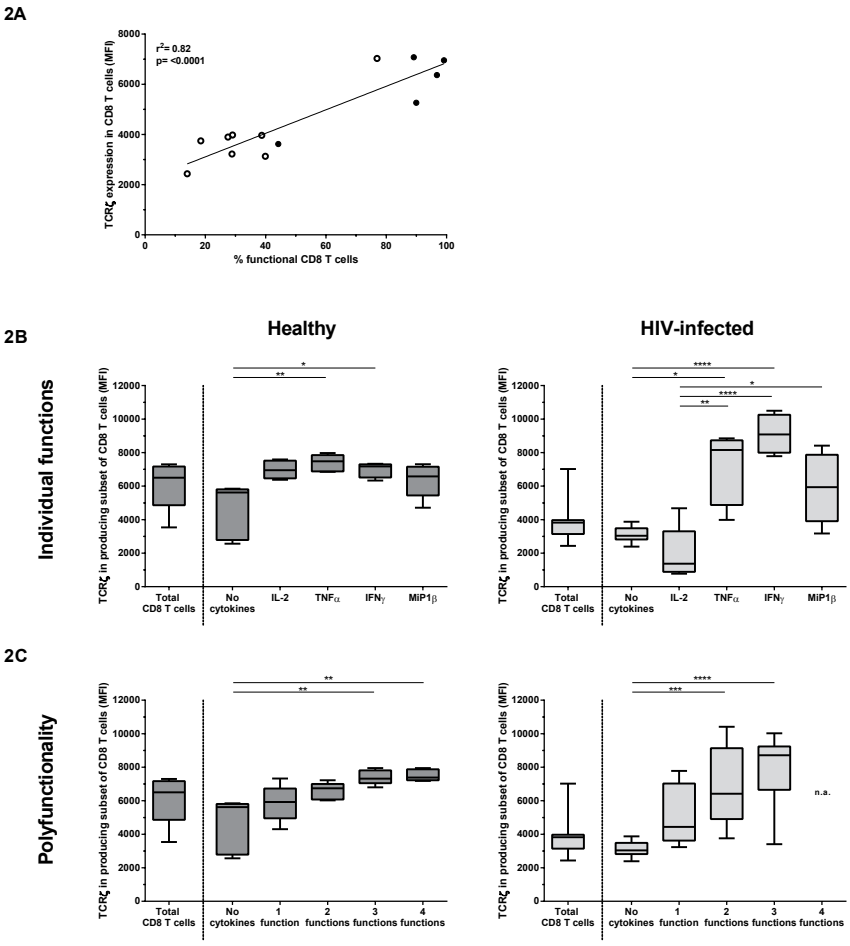


**Figure 2. Cytokine expression and polyfunctionality are correlated with TCRζ .**

PBMC's of 5 healthy donors and 8 early HIV infected individuals were stimulated with PMA/ionomycin and the TCRζ-expression (MFI) and polyfunctionality in terms of IFNγ, IL-2, TNFα and MiP1β production was measured by flow cytometry. In Figure 2A the total percentage of functional $CD8^+$ T cells (x-axis) and the TCRζ expression (right Y-axis) was correlated in healthy donors (●) and HIV-infected individuals (**o**). The graphs in Figure 2b zoom in on the expression of TCRζ in the $CD8^+$ T-cell subsets performing the functions MiP1β, IFNγ, TNFα and IL-2 (x-axis). The y-axis denotes the TCRζ expression as median fluorescent intensity (MFI). The boxplots depict median and range. *p-value=0.05- 0.01 ** p-value=0.01- 0.001, *** p-value=0.001- 0.0001, *** p-value<0.0001.

## RESULTS

### *Decreased production of individual cytokines and polyfunctionality by $CD8^+$ T cells during early HIV-1 infection*

We investigated the individual cytokine/chemokine production, magnitude and

polyfunctionality of CD8⁺ T cells in HIV-infected individuals-within one year after seroconversion- compared to healthy donors. In line with previous publications [20, 21], a significantly lower magnitude of CD8⁺ T cells produced cytokines upon in vitro stimulation in HIV-infected compared to healthy individuals (Figure 1A; p=0.003). The low overall cytokine production in HIV-infected individuals was reflected in a severely reduced production of all individual cytokines/chemokines, in particular TNFα and IL-2 (Figure 1B). The near loss of IL-2 and TNFα production by CD8⁺ T cells in HIV-infected individuals also affected the polyfunctionality of

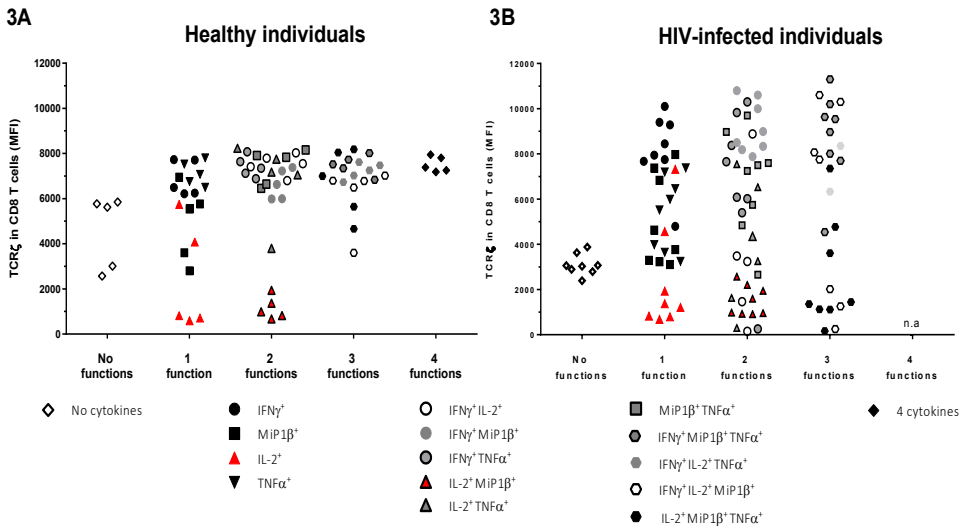


**Figure 3. The association between cytokine combinations and the level of TCRζ expression.**
PBMC's of 5 healthy donors (Figure 3A) and 8 early HIV infected individuals (Figure 3B) were stimulated with PMA/ionomycin and polyfunctionality was measured for IFNγ, IL-2, TNFα and MiP1β by flow cytometry. TCRζ expression (MFI) was determined across 16 different functional subsets and the responses were grouped per number of functions. TCRζ expression is low/downregulated in PBMC's producing only IL-2 or IL-2/MiP1β both in healthy donors and HIV-1 infected individuals.

the CD8⁺ T cells. The proportion of CD8⁺ T cells that simultaneously produced 3 or 4 of the cytokines/chemokines was significantly lower in HIV infected individuals compared to healthy donors (Figure 1C, p=0.007 and p=0.002 for 3 and 4 functions, respectively).

***Total cytokine expression and polyfunctionality are correlated with TCRζ expression level***
In line with previous studies we found that TCRζ expression levels tended to be lower in HIV-infected individuals than healthy donors (data not shown). Therefore, we explored whether an association exists between the level of cytokine production and TCRζ expression of T cells in healthy and HIV-infected individuals. The total cytokine response by CD8⁺ T cells significantly correlated with their TCRζ expression level (Figure 2A; p=<0.0001, $r^2$=0.82). Interestingly, the outliers of both the healthy and HIV-infected groups –wherein the total cytokine production was determined- supported this result, as they clustered convincingly in the linear regression. Hereafter we zoomed in on the TCRζ expression levels of specific CD8⁺ T-cell populations. Both in healthy and HIV-infected donors, CD8⁺ T cells producing either

TNFα or IFNγ had a significantly higher median TCRζ expression compared to CD8⁺ T cells that did not produce cytokines (Figure 2B). In healthy donors median TCRζ expression levels did not differ between the CD8⁺ T cells producing a specific cytokine/chemokine. In contrast, in HIV-infected individuals, the CD8⁺ T cells producing IL-2 had a significantly lower TCRζ expression level – similar to or lower than CD8⁺ T cells not producing cytokines- than CD8⁺ T cells producing TNFα, MIP1β and/or IFNγ.
Next, we examined whether the polyfunctionality of CD8⁺ T cells correlated with higher TCRζ expression levels on a cellular level. We found that the number of functions exerted by CD8⁺ T cells after stimulation showed a trend towards a higher TCRζ expression level, both in HIV-infected individuals and healthy donors (Figure 2C). In particular, CD8⁺ T cells producing the most functions simultaneously had significantly higher TCRζ levels than CD8⁺ T cells not producing cytokines, both in HIV-infected and healthy donors.

***IL-2 and IL-2/MIP1ɓ producing cells have low TCRζ expression***

The major discrepancy we observed between healthy donors and HIV-infected individuals was a significantly lower TCRζ expression level of IL-2 producing CD8⁺ T cells in HIV-infected individuals. This observation might be an intrinsic characteristic of the IL-2 producing CD8⁺ T cells as a result of HIV infection. However, an alternative explanation for the lower TCRζ expression levels in the IL-2 producing CD8⁺ T cells during HIV-infection could be that the IL-2 producers are less polyfunctional, which in itself correlates with TCRζ expression. Therefore, we investigated TCRζ expression levels of the various IL-2 producing CD8⁺ T-cell subsets. In HIV-infected individuals, *all* functional combinations that contained IL-2 showed



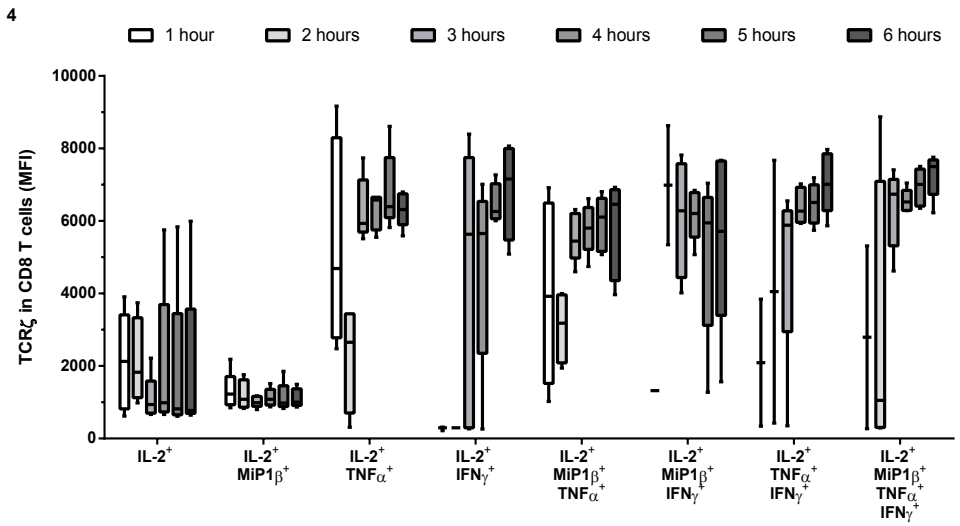


**Figure 4. The IL-2 and IL-2/Mip1β producing CD8⁺ T cells have a continuously lower TCRζ expression after stimulation.**

PBMC from 5 healthy individuals were stimulated with PMA/ionomycin and polyfunctionality was measured for IFNγ, IL-2, TNFα and MiP1β by flow cytometry after 1, 2, 3, 4, 5 and 6 hours. TCRζ expression (MFI) was determined across 16 different functional subsets and the responses are grouped per number of functions. TCRζ expression is continuously low/downregulated in PBMC's producing only IL-2 or IL-2/MiP1β

low TCRζ expression levels (Figure 3B), whereas CD8⁺ T cells not producing IL-2 had higher TCRζ expression levels. In contrast almost all functional CD8⁺ T-cell subsets in healthy donors

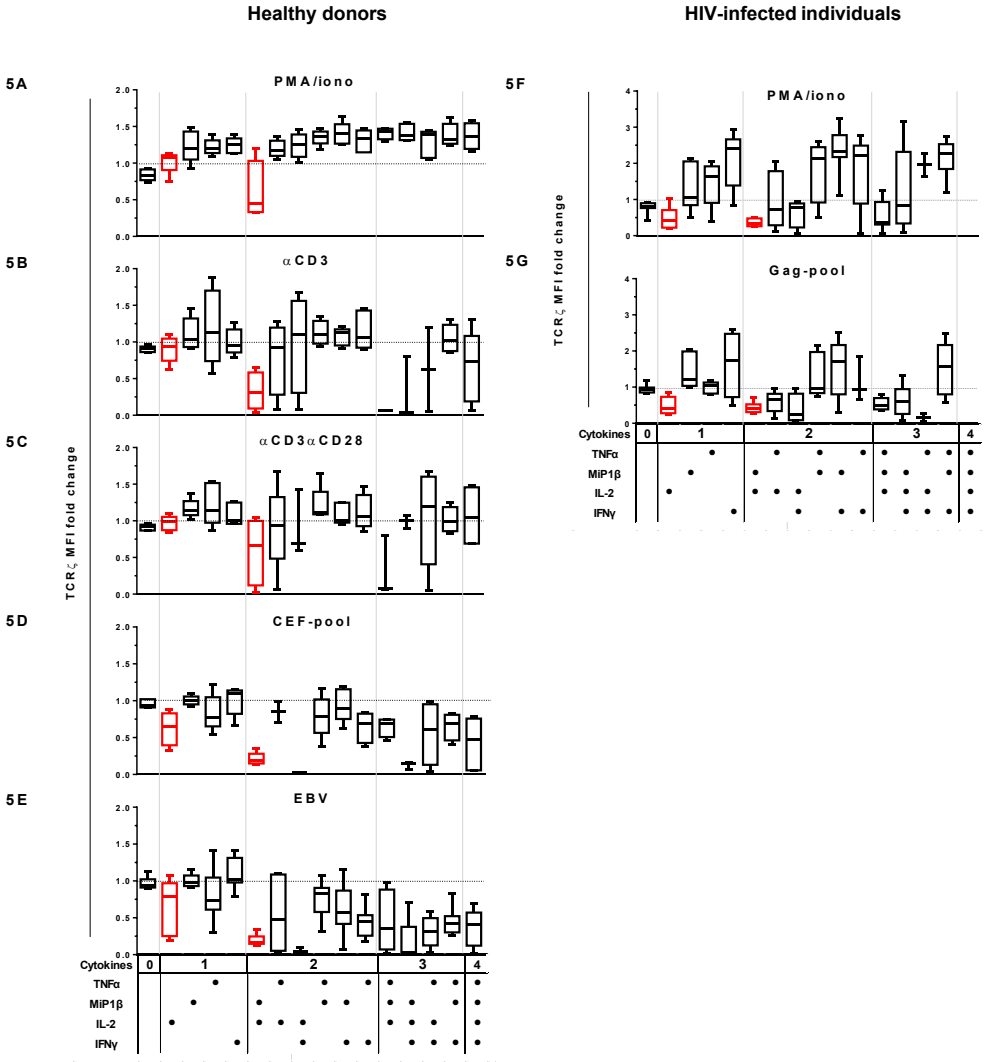


**Figure 5. TCRζ expression is low in IL-2/MiP1β producing cells independent of their stimulus.**
Distribution of responding CD8⁺ T-cells across 16 different functional subsets are shown when PBMC from 5 healthy donors are stimulated with PMA/iono (A), aCD3 with or without aCD28 (B/C), CEF-pool (D) or EBV-peptides (E). In the 8 HIV-infected individuals the PBMC were stimulated with PMA/iono (F) or Gag-peptidepool (G). The y-axis denotes the TCRζ expression as median fluorescent intensity (MFI) fold change compared to the total MFI of unstimulated PBMC's. The graphs zoom in the on the expression of TCRζ in the CD8⁺ T-cell subsets performing various combinations of functions (Mip1β, IFNγ, TNFα and IL-2).The boxplots represent the relative change of the TCRζ MFI compared to unstimulated cells (medium). On the X-axis, each dot indicates IL-2, Mip1β, TNFα, and/or IFNγ positivity. In all graphs medians (with range) are depicted.

displayed high TCRζ expression levels (Figure 3A), with two exceptions: $CD8^+$ T cells producing IL-2 or IL-2/ MIP1β, which exhibited low TCRζ expression levels. The low TCRζ expression levels of the IL-2 and the IL-2/MIP1β producing $CD8^+$ T cells in healthy donors only became apparent after separating TCRζ expression of the various $CD8^+$ T cell subsets, because these populations are relatively minor and thus had a negligible impact on the mean TCRζ expression level of all IL-2 producing $CD8^+$ T cells.

To explore whether the low TCRζ expression within the IL-2 and/or MIP1β producing $CD8^+$ T-cell subsets were a result of greater downregulation of the TCRζ-chain after stimulation or a diminished recovery of TCRζ after stimulation, we performed a time course experiment. As can be seen in Figure 4, all functional subsets exhibited high TCRζ expression levels from the start or gradually regained TCRζ expression over time (Figure 4 and S2), except for the IL-2 and IL-2/ MIP1β producing T cells which showed persistently low TCRζ expression levels over time. Therefore, it seems that these 2 functional subsets show a diminished recovery of TCRζ expression levels compared all other subsets measured.

***TCRζ expression is low in IL-2/MIP1β producing cells independent of their stimulus***

To investigate whether the observed TCRζ expression patterns are a general phenomenon and independent of the type of T-cell stimulus, we stimulated $CD8^+$ T cells of healthy donors and HIV-infected individuals with a wide range of TCR dependent and independent mitogens (Figure 5). Strikingly, there was more variation in TCRζ expression levels in healthy donors after TCR dependent stimulation (Figure 5B to 5E) compared to stimulation with the TCR-bypassing mitogens PMA and ionomycin (Figure 5A).
After stimulation with the TCR-dependent superstimuli αCD3 with or without CD28 all T-cell subsets (co)producing IL-2 exhibited a low TCRζ expression (Figure 5B and 5C). Likewise, after stimulation with peptide pools containing epitopes for chronic viruses, $CD8^+$ T cells of healthy donors –who were latently infected with the viruses CMV and EBV-, exhibited low TCRζ expression levels in almost all functional subsets (Figure 5D and 5E).

IL-2 and IL-2/ MIP1β producing $CD8^+$ T cells had lower TCRζ expression levels under all conditions (figure 5A to 5E). This was mirrored by HIV-infected individuals where TCRζ expression was also lower in the IL-2 and IL-2/MIP1β subsets after both TCR independent (Figure 5F) as well as TCR dependent stimulation (Figure 5G). Furthermore, in HIV-infected individuals all $CD8^+$ T-cell subsets producing IL-2 had lower TCRζ expression levels.

Taken together, these data demonstrate that cytokine production in $CD8^+$ T cells correlates with TCRζ expression levels, both in healthy and HIV-infected individuals. In HIV infection, a marked decrease of TCRζ expression levels takes place that is associated with a loss of cytokine production.

## DISCUSSION

Previously it has been shown that both TCRζ expression levels and polyfunctionality decline in chronic infection. However, so far, only circumstantial evidence linked these separate $CD8^+$ T-cell characteristics. Here we demonstrated the existence of a direct correlation

between polyfunctionality and magnitude of cytokine production in $CD8^+$ T cells and the TCRζ expression level, both in healthy and HIV-infected individuals. Furthermore we found that TCRζ expression levels were associated with the production of particular cytokines in $CD8^+$ T cells. Both in healthy and HIV-infected individuals TCRζ expression levels of $CD8^+$ T cells producing IL-2 and IL-2/ MIP1β were consistently low. In HIV infected, but not healthy individuals, strikingly low TCRζ expression levels were observed in all $CD8^+$ T cells producing IL-2.

We focused on $CD8^+$ T-cell polyfunctionality in terms of IFNγ, IL-2, MIP1β and TNFα production. Recently, Makedonas et al. demonstrated that perforin production is an important additional function of the $CD8^+$ T cells in battling viral infections [22]. Additionally, they demonstrated that $CD8^+$ T cells that swiftly upregulate perforin show a strong effector phenotype and accommodate high levels of T-bet but low expression levels of CD28. In line with those results, we found that the level of TCRζ expression positively correlated with the level of T-bet expression, and negatively correlated with CD28 expression (Figure S3A and S3B). The fact that TCRζ expression levels also correlate with other functional markers of $CD8^+$ T cells, which are described in literature, further strengthens our observation that there is a direct association between TCRζ expression levels and $CD8^+$ T cell function.

The loss of cytokine production by $CD8^+$ T cells in chronic HIV-infected infection has previously been shown to follow a hierarchical pattern; first the loss of IL-2 production takes place, followed by TNFα and IFNγ production [16]. In this study, we specifically investigated HIV-infected individuals who had been infected for less than a year [18]. Even despite the early stage of HIV-infection, IL-2 and TNFα production by $CD8^+$ T cells was already severely diminished compared to healthy controls. We found low levels of TCRζ expression in the IL-2 producing $CD8^+$ T cells, seemingly reflecting the loss of IL-2 production. In contrast, however, we did not find low TCRζ expression levels in the remaining TNFα producers. It seems therefore, also taking into account the divergent TCRζ expression levels of certain IL-2 producing $CD8^+$ T cell subsets in healthy donors, that it is more probable that low TCRζ expression is an intrinsic characteristic of IL-2 producing cells due to repeated antigen stimulation. This is supported by the fact that PD-1, which is associated with reduced cytokine production, has a greater impact on IL-2 production than on IFNγ and MIP1β production [23]. As PD-1 functions by inhibition of immediate downstream signaling molecules of TCRζ, the combined effect of low TCRζ expression levels and the additional inhibitory signal of PD-1 ensures that $CD8^+$ T cells producing IL-2 are virtually resistant to antigen stimulation very early during HIV-infection.

In both healthy donors and HIV-infected individuals specifically the IL-2 and the IL-2/ MIP1β producing $CD8^+$ T-cell subsets exhibited relatively low TCRζ expression levels after stimulation, even lower or comparable with the $CD8^+$ T-cell subset that did not produce any cytokines/ chemokines. However, mechanistically it is unclear why these subsets have different TCRζ expression levels after stimulation. After activation of the $CD8^+$ T cell the entire TCR complex is internalized and degraded, however, it will immediately start being resynthesized intracellularly. Our data showed a far greater (variation in) downregulation of TCRζ expression levels within various functional $CD8^+$ T-cell subsets induced by TCR dependent than by TCR independent mitogens (see Figure 5). The reason for the discrepancy in TCRζ expression levels after the two different stimuli are the two independent mechanisms modulating TCR

downregulation after CD8+ T-cell activation[24]. TCR dependent mitogens activate so-called ligand-induced downregulation of the TCR, which is dependent on the protein tyrosine kinases p56lck and p59fyn. In contrast, the TCR independent mitogen PMA is an upstream regulator of the protein kinase C (PKC) which causes TCR internalization and ζ degradation via ZAP-70 and CD3γ [25]. TCR dependent mitogens also trigger the negative PKC feedback loop. Our data suggest that the TCRζ expression levels of the IL-2 and IL-2 and IL-2/MIP1β producing CD8+ subsets are specifically linked to the PKC mediated TCR downregulation. From an evolutionary viewpoint the fact that there is a separate feedback loop especially for the IL-2 and IL-2/MIP1β producing cells supports that it is crucial to control these subsets. The reason for the tight regulation of the IL-2 and IL-2/Mip1β producing CD8+ T cells may lie in their function. IL-2 production is known to be an important signal for proliferation of T cells whereas MIP1β sensitizes cells to IL-2 stimulation by upregulation of the IL-2receptor. We hypothesize that subsets with low TCRζ expression levels *after* stimulation, -the CD8+ T-cell subsets that previously produced IL-2 or IL-2/MIP1β- would be resistant to restimulation. This would make it impossible for the CD8+ T cells to be reactivated and produce IL-2 de novo. It is possible that the specific downregulation of TCRζ expression levels in the T cells producing these cytokines is an immune mechanism that prevents excessive proliferation of immune cells and protects the immune system against unwanted activation.



Though we showed the existence of a direct relationship between TCRζ expression levels and cytokine production in CD8+ T cells, it remains speculative whether the observed association between them is one of cause or of effect. There are two probable options, 1) the TCRζ expression levels that are measured after T-cell stimulation could be a result of a feedback loop that causes downregulation/upregulation of TCRζ expression levels after cytokine production and, 2) the level of TCRζ expression *after* stimulation might be similar to the level of TCRζ expression *before* stimulation, which dictates the potential strength of the activating signal and therefore the amount and combinations of cytokines that are produced by CD8+ T cells. In practice it is most likely a combination of both options and the TCRζ dictates the amount of cytokines produced, however in certain CD8+ T cells cytokine producing subsets TCRζ expression levels will remain 'downregulated' after stimulation. Nevertheless we cannot exclude the possibility that TCRζ expression is not caused by or does not cause certain cytokine expression levels but both are a reflection of the intrinsic capabilities of the cells.

Taken together, the direct correlation between the amount of cytokine production and polyfunctionality of CD8+ T cells with TCRζ expression levels suggests that a qualitatively better T cell has high TCRζ expression levels. These conclusions are supported by the results found in the HIV-infected individuals where a marked shift of TCRζ expression levels in the IL-2 producing T-cell subsets is associated with low IL-2 cytokine production. In the future this interesting association between T-cell function and TCRζ expression levels could possibly be exploited to modulate and maybe even augment CD8+ T-cell responses.

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## SUPPLEMENTAL DATA

Table S1

| a. Stimulants | Concentration | Characteristics | Manufacturer |
|---|---|---|---|
| Medium | - | - | - |
| Gag-pool | 2 µg peptide/mL | 15mers with 11 overlap spanning the HIV-Gag protein, Consensus B 2007 | NIH AIDS Research and Reagent program, Bethesda, Maryland, United States |
| CEF-pool | 2 µg peptide/mL | 32 peptides, 8-12 amino acids in length, with sequences derived from human CMV, EBV and Influenza | NIH AIDS Research and Reagent program, Bethesda, Maryland, United States |
| EBV-peptides | 2 µg peptide/mL | GLC, RAK, YVL, FLR, RPP, AVF | |
| CMV-NLV | 0.1, 1, 10 µg/mL | | |
| HIV-KAF | 0.1, 1, 10 µg/mL | | |
| PMA | 5 ng/mL | | Sigma-Aldrich, The Netherlands |
| Ionomycin | 1 µg/mL | | Sigma-Aldrich, The Netherlands |
| αCD3 | 1 µg/ml | | Biolegend |
| αCD28 | 1 µg/ml | | eBioscience |
| **b. Monoclonals** | **Marker** | **Fluorochrome** | **Manufacturer** |
| *Extracellular* | CD3 | PerCP | eBioscience |
| | CD8 | V500 | BD |
| | CD28 | PE Cy7 | eBioscience |
| *Intracellular* | TCRζ | FITC | Biolegend |
| | MIP1β | PE | BD |
| | IFNγ | PE Cy7 | eBioscience |
| | IL-2 | PB | BD |
| | TNFα | APC | Biolegend |
| | Perforin | BV421 | Biolegend |
| | T-bet | PE Cy7 | Biolegend |

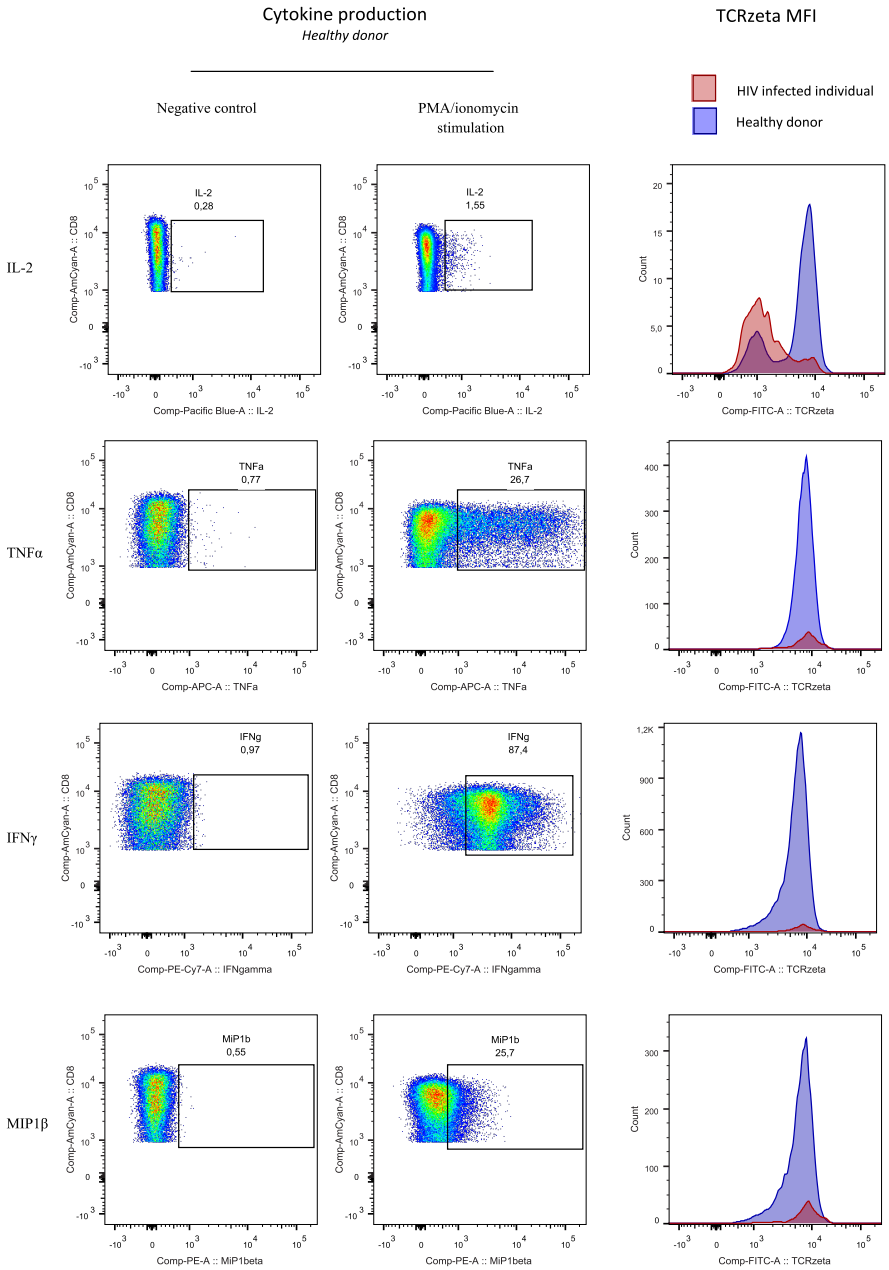



**Figure S1. Gating strategy for flow cytometry analysis of TCRζ-expression.**

In this sample gating, cells were first gated for lymphocytes or monocytes (FSC-A vs. SSC-A). Within the lymphocyte gate surface expression of $CD3^{+}CD8^{+}$ was used to define the $CD8^{+}$ T cell subset. The fraction of cells producing cytokines (IL-2, TNFα, IFNγ or MIP1β) was measured in response to PMA/ionomycin. The gates were set to unstimulated controls. Within the cytokine producing subsets the TCRζ expression levels were determined, as shown in the right column.

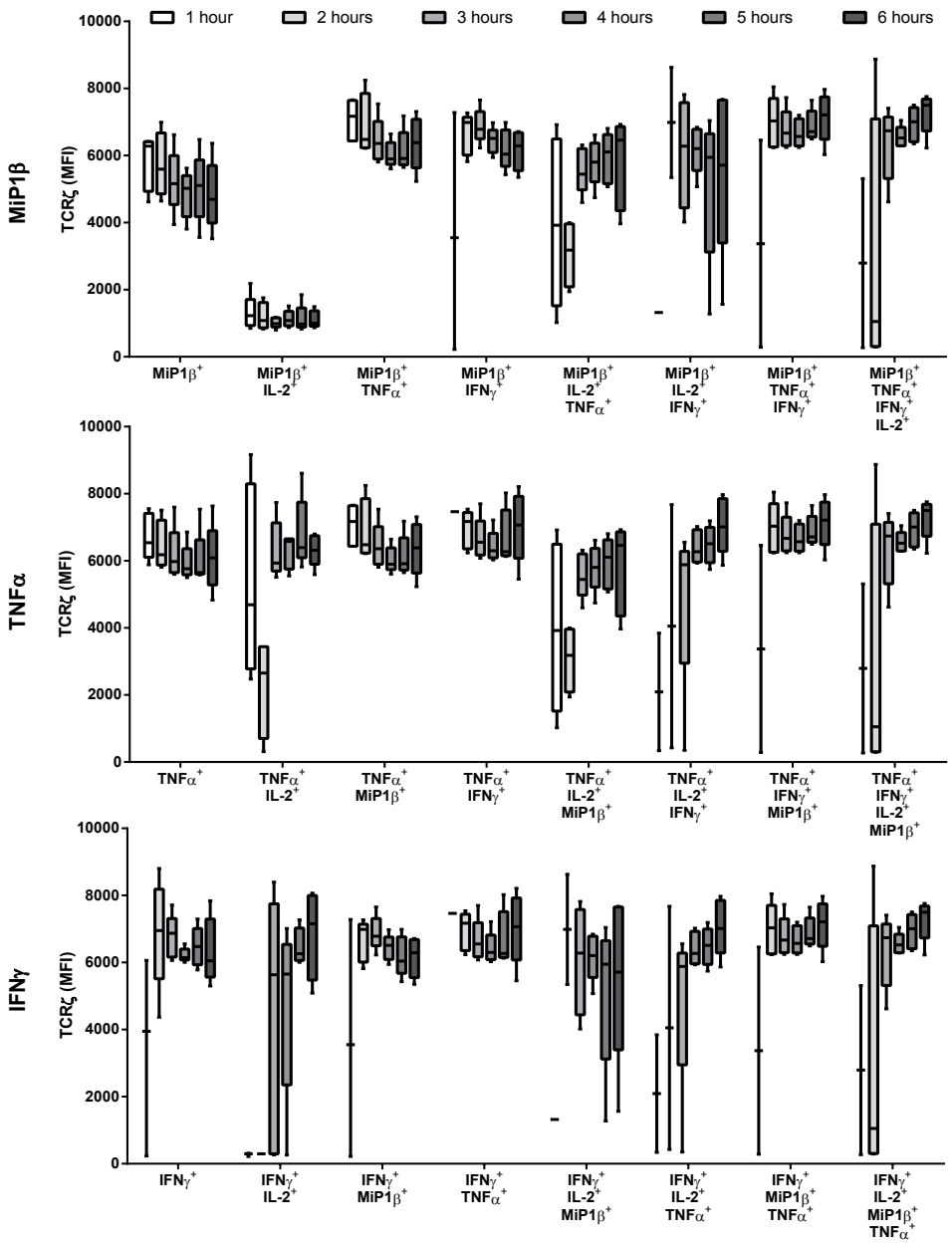



**Figure S2. The IL-2 and IL-2/Mip1β producing CD8⁺ T cells have a continuously lower TCRζ expression after stimulation.**

PBMC from 5 healthy individuals were stimulated with PMA/ionomycin and polyfunctionality was measured for IFNγ, IL-2, TNFα and MiP1β by flow cytometry after 1, 2, 3, 4, 5 and 6 hours. TCRζ expression (MFI) was determined across 16 different functional subsets and the responses are grouped per number of functions. TCRζ expression is continuously low/downregulated in PBMC's producing only IL-2 or IL-2/MiP1β

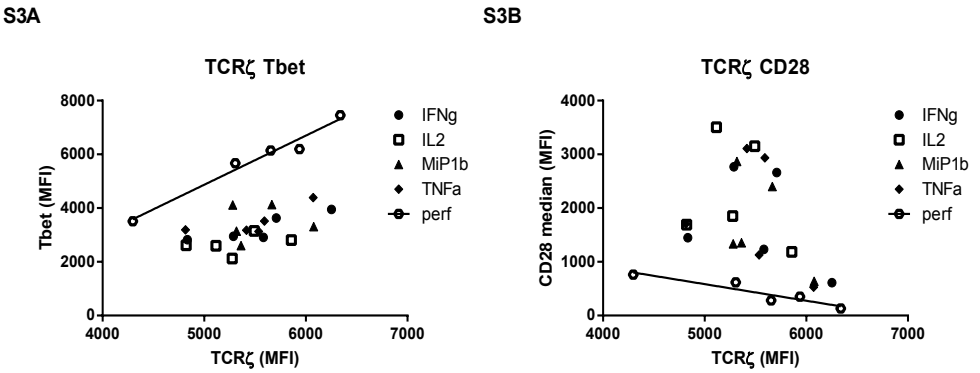
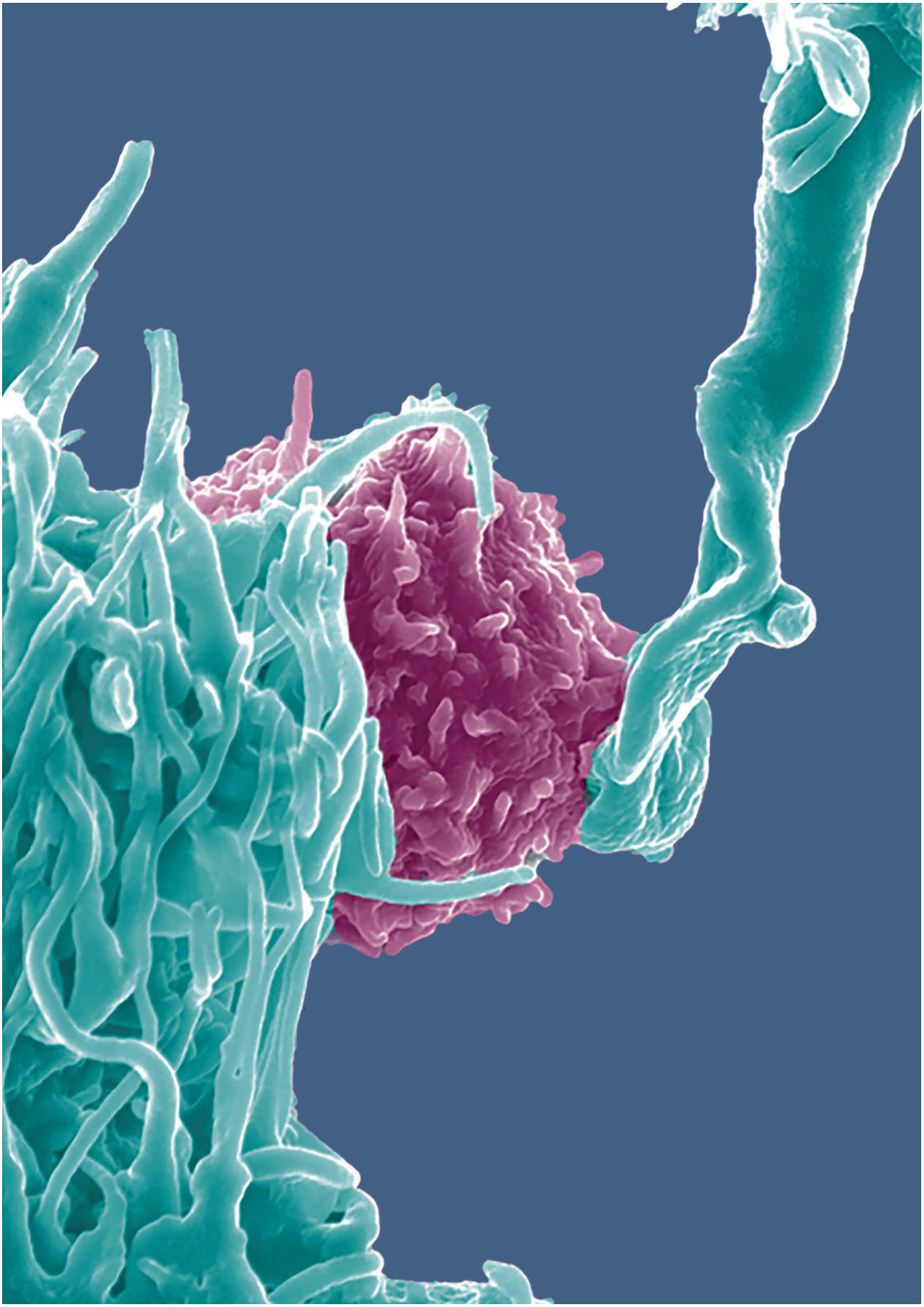



**Figure S3. TCRζ expression correlated with T-bet and CD28 expression.**

PBMC from 5 healthy individuals were stimulated with PMA/ionomycin and polyfunctionality was measured for perforin, IFNγ, IL-2, TNFα and MiP1β by flow cytometry. Tbet (y-axis, Figure S3A), CD28 (y-axis, Figure S3B) and TCRζ expression (x-axis) were determined within the 5 different functional subsets. We found that the level of TCRζ expression positively correlated with the level of T-bet expression, and negatively correlated with CD28 expression, especially in the perforin producing cells.



# 7

# GENERAL DISCUSSION

## DISCUSSION

Despite 30 years of dedicated research there is still a gap in what we know of the mechanisms controlling HIV-disease progression. This thesis focuses on two main players that are known for modulating disease progression: chronic systemic immune activation and cytotoxic T lymphocytes (CTL). If we want to target these mechanisms for therapeutic purposes we have to know exactly how they influence HIV-disease progression. Therefore we set out to investigate 1) what drives the systemic immune activation that predicts disease progression in chronic HIV-infection and, 2) how CTL responses contribute to control of the virus.

### *Chronic HIV infection - The importance of activation*

From the early 90's onwards it has become clear that systemic immune activation is an important determinant in HIV-disease progression. Multiple immune activation markers were found to be predictive of disease progression [1-3], stronger than, and even independent of, viral load [4, 5]. Therefore, a lot of research has been focused on understanding how immune activation causes disease progression, and on uncovering the sources of systemic immune activation in chronic HIV-infection.

*How does immune activation cause $CD4^+$ T-cell loss?*

At least two hypotheses have been put forward to explain how systemic immune activation could contribute to the gradual loss of $CD4^+$ T cells, the hallmark of HIV-disease progression. In labeling studies, in which turnover of T cells was measured by deuterated glucose or BrdU, a high turnover rate was found of $CD4^+$ and $CD8^+$ T cells in HIV-1 and SIV-infection [6-8]. It was initially thought that the high turnover rate of $CD4^+$ T cells was a homeostatic response to low naive $CD4^+$ T cell numbers. Hazenberg et al disproved this theory by showing that immediately after removal of HIV-1 (by cART) the percentage of proliferating $CD4^+$ T cells rapidly declined, even though individuals still had persistent low $CD4^+$ T-cell counts. This suggested that high Ki67 expression was driven by HIV rather than a homeostatic response to low $CD4^+$ T-cell numbers [9]. In support of this, it was shown that, at one and five years after seroconversion, increased proportions of dividing $CD4^+$ T cells and high $CD4^+$ T cell activation marker expression levels were associated with progression to AIDS [10]. Even *pre-seroconversion* $CD4^+$ T cell activation levels were predictive of increased rates of HIV-disease progression once the individuals got infected [10], suggesting that immune activation is causing the loss of $CD4^+$ T cells and disease progression. It was hypothesized that increased turnover of memory and naive $CD4^+$ T cells leads to loss of the cells necessary to maintain the $CD4^+$ T cell compartment, and causes accelerated depletion of the T cell pool [11]. Another mechanism via which activation could contribute to HIV-pathogenesis is by providing easy targets for HIV infection, as activated $CD4^+$ T cells are highly susceptible to infection due to upregulation of the co-receptor CCR5 [12, 13].

*Immune activation markers predictive of HIV-disease progression*

Overall a broad array of immune activation markers are increased in HIV infection and are predictive of disease progression, including both activation markers of the adaptive and of the innate immune system (see table 1[2, 14, 20-22]). It is unclear however, what major activation pathways play the central role in $CD4^+$ T-cell loss, as many of these inflammatory

markers are intricately linked. In fact, the observed increased immune activation markers per se may not even have a direct link to $CD4^+$ T-cell decline; instead, it may be the source(s) that drive the increases in these immune activation markers that are responsible for $CD4^+$ T cell loss and HIV-disease progression.

*Drivers of immune activation in HIV-infection*
It is likely that both bacterial translocation and HIV-RNA contribute to excessive chronic immune activation in HIV infection (as described in chapter 1). The massive $CD4^+$ T cell depletion in the gut associated lymphoid tissue (GALT) during primary HIV infection causes gut-permeability in both HIV and pathogenic SIV-infection which in turn allows bacterial products to cross the gut barrier, to enter the blood and cause activation of the immune system. HIV-RNA in turn can stimulate both the innate (via the TLR-pathway) and the adaptive (by cognate antigen and via TLR-signaling) part of the immune system. A lot of controversy still exists on the relative roles of these two sources of systemic activation in HIV infection. In literature, most observations concerning immune activation parameters on cART were made at least six months after initiation of cART [22, 23]. These studies have shown that monocyte activation markers tend to remain elevated on cART while many T cell activation markers strongly decline[24]. In chapter 2 we aimed to unravel which features of systemic immune activation are caused by HIV directly and which are driven by bacterial translocation by following the decay of these immune activation markers immediately after start of cART.

We followed the decay dynamics of both HIV RNA and sCD14 – as a marker for bacterial translocation – on cART and compared them with the decay dynamics of a range of immune activation markers that have previously been described to correlate with HIV-disease progression (see Table 1). The expression of HLA-DR and CD38 on $CD4^+$ and $CD8^+$ T cells, Ki67 expression of $CD4^+$ T cells and the concentration of sCD163 in plasma showed dynamics that resembled sCD14 after start of cART. Although it is tempting to conclude that the elevated levels of these immune markers were driven by bacterial translocation, we cannot exclude the possibility that these immune markers remained elevated because they were affected irreversibly before cART was started. However an intriguing study in pig tailed macaques (PTM) (a non-natural host which typically progresses to AIDS) showed that administration of Sevelamer – a drug that captures LPS – to acutely SIV-infected PTM strongly decreased the levels of immune activation and coagulation in these animals [25]. Additionally it was shown that percentages of HLA-DR and CD38 expressing $CD4^+$ and $CD8^+$ T cells and Ki67-expressing $CD4^+$ T cells were lowered by Sevelamer administration, which were exactly the cellular immune markers that we found to follow the decay dynamics of sCD14 (Chapter 2). Taken together, these data suggest that the increased expression of these immune activation markers in HIV infected individuals is indeed driven by bacterial translocation. Regretfully, the plasma markers measured in our study did not overlap with those of the Sevelamer study in SIV-infected PTM. It would have been very interesting to see whether sCD163, the other marker that we found to follow the dynamics of sCD14, would also be suppressed by Sevelamer treatment.

In contrast, all markers that followed the HIV-1 decay on cART are most likely directly driven by HIV-RNA. We found that a broad range of immune activation markers decreased to some extent after HIV-RNA depletion by cART. However, only the fractions of Ki67 expressing and

**Table 1 Immune markers predictive of HIV-disease progression in chronic infection**

| Marker | characteristics |
|---|---|
| Ki67 ($CD4^+$ T cells) [10] | $CD4^+$ T-cell proliferation |
| HLA-$DR^+$ $CD38^+$ ($CD4^+$ T cells) [10] | $CD4^+$ T-cell activation |
| Ki67 ($CD8^+$ T cells) [10] | $CD8^+$ T-cell proliferation |
| HLA-$DR^+$ $CD38^+$ ($CD8^+$ T cells) [10] | $CD8^+$ T-cell activation |
| β2M (plasma)[14] | Measured in plasma. Component of MHC class I molecules. |
| sCD163 (plasma) [15] | A scavenger receptor present on macrophages, shed when activated |
| Neopterin (plasma)[16] | A catabolic product of guanosine triphosphate, synthesized by monocytes and macrophages upon stimulation with IFNγ, associated with increased production of reactive oxygen species |
| LPS (plasma) [17] | Bacterial product, activates immune system through binding of TLR's |
| sCD14 (plasma) [18] | A monocyte activation marker, also correlated with LPS |
| sIL-2R (plasma)[16] | Soluble IL-2R is part of a membrane receptor for interleukin-2, which is predominantly shed by activated T cells |
| IP10 (plasma)[19] | Chemo-attractant for T cells (among others), produced predominantly by monocytes in response to type 1 IFN stimulation |
| sTNF-R2 (plasma)[14] | Receptor for TNFα on T cells, monocytes and macrophages where it is involved in the apoptotic cascade. The extracellular domain is shed when the cell is activated |
| IL-6 (plasma)* | Pro-inflammatory cytokine secreted by T cells and macrophages to stimulate the immune response |
| CRP (plasma)* | Acute-phase protein of hepatic origin that increases following IL-6 secretion from macrophages and T cells |
| D-dimer (plasma)* | Fibrin degradation product, present when coagulation cascade is activated |

* Associated with morbity and mortality on cART

PD-1-expressing $CD8^+$ T cells and the plasma markers IP-10, sIL-2R and sTNF-R2 followed the decay dynamics of HIV-RNA, suggesting that the increased expression of these immune activation parameters in HIV-infected individuals was driven directly by HIV. Interestingly, the Sevelamer administration study in PTM reported that Ki67 expression by $CD8^+$ T cells was unaffected after Sevelamer administration [25], which is completely in line with this interpretation. Although many immune activation markers decreased in the footsteps of HIV-RNA, and were thus most likely caused by HIV directly, bacterial translocation seemed to have a bigger influence on immune activation of $CD4^+$ T cells, and may thereby be responsible for excessive proliferation and finally exhaustion of $CD4^+$ T cells that drives HIV-disease progression.

Indeed, Brenchley and colleagues showed that LPS levels in HIV infected individuals are clearly associated with the rate of HIV disease progression [26]. Furthermore, microbial translocation takes place during pathogenic SIV-infection of non-natural hosts, while in contrast, the integrity of mucosal barrier is either not undermined, or restored in chronic non-pathogenic SIV infection, despite a severe depletion of mucosal T cells [27, 28]. Consequently, there is no increase in microbial translocation in non-pathogenic SIV infection [27, 28]. Interestingly, when microbial translocation was mimicked by administering LPS to chronically SIV-infected natural hosts (AGMs), LPS increased the levels of systemic immune activation and inflammation and

boosted viral replication [29]. Controversially, however it was also shown that even without any signs of microbial translocation, an attenuated variant of pathogenic SIVmac239 was able to cause T-cell activation, $CD4^+$ T-cell loss, and progression to AIDS [30].

*Can we prevent immune activation?*
We observed that after one year of cART many of the immune activation markers had not normalized (chapter 2). Even markers such as IP10, sTNF-R2 and sIL-2R that followed the early dynamics of HIV-decline were still higher compared to healthy volunteers. An obvious explanation for this residual activation on cART may be the persistence of microbial translocation. Another factor contributing to continued immune activation on cART could be low level viral replication. It has previously been shown that even under 'effective' therapy low amounts of virus (<50 copies/mL) are still present in the blood [31, 32]. This residual virus production is likely released from previously infected cells, the so-called viral reservoir. Another factor possibly playing a role in the continued high levels of activation under cART is that irreversible alterations to the immune system may have occurred before start of cART.

It has been investigated whether the residual immune activation seen in patients on cART could be prevented by initiating cART very early, during acute HIV-infection. Indeed, early treatment has shown far more promising results than treatment in chronic infection [33-35]. In chapter 3 we investigated whether early treatment in the Primo-SHM trial prevented residual activation. The Primo-SHM trial is a multicenter randomized trial comparing no treatment with 24- or 60-weeks of cART during primary HIV infection. It was found that temporary early cART successfully (although transiently) lowered the viral setpoint and deferred the need for reinitiation of cART during chronic HIV infection [36]. Within a representative subgroup of the study subjects we investigated whether early cART had i) prevented depletion of immune cells in the gut (and therefore gut integrity), ii) limited immune activation or, iii) preserved T-cell immunity. These parameters were assessed 36 weeks after inclusion or treatment interruption.

We found that early treatment did not prevent depletion of $CD4^+$ T cells in the GALT, using (the absence of) the gut homing marker α4β7 on lymphocytes in the blood as an indirect measure for GALT depletion. This was in line with other studies studying the effect of cART during acute infection on reconstitution of mucosal $CD4^+$ T cells, where biopsies of the gut were taken [37, 38]. Mehandru et al examined a group of 18 individuals longitudinally by taking yearly recto-sigmoid biopsies up to 3 years on cART [37]. At 1 year on cART (corresponding to our time frame) they found higher percentages of $CD4^+$T cells in the GALT than at baseline (15.7% versus 34.6%), but they were and remained significantly lower than in HIV-uninfected individuals. It must be noted that these findings were done while still on cART. In a study that started cART in primary HIV-infection, treated for 6 months and then took biopsies 6 months later, it was shown that if cART was started more than 20 days after seroconversion there was no beneficial effect on gut preservation. On the other hand, it was also shown that if individuals started treatment in Fiebig stage 1 – within 15 days after transmission – as opposed to later, less depletion of $CD4^+$ $CCR5^+$ cells took place in the gut mucosa [38]. These results are perhaps not surprising, given that previous work in SIV infection showed that severe depletion of $CD4^+$ T cells in the GALT occurs within days and not weeks after seroconversion [39]. Since the individuals in the Primo-SHM trial were recruited within 100

days of HIV-infection, the reason for lack of effect might be that overall treatment initiation was too late to prevent the depletion of the cells from the GALT. In general, most newly infected individuals are not diagnosed within the first 2 weeks after infection; we believe that early treatment may therefore not be a feasible way to prevent microbial translocation in HIV-infected humans. This conclusion is slightly worrying as the bacterial translocation induced immune activation seems to be the driver for $CD4^+$ T cell activation (chapter 2). Several strategies are being investigated to prevent or control bacterial translocation, including modification of the intestinal microbiome by probiotics, reduction of inflammation in the gut, and capture of certain translocated microbial products such as LPS (reviewed in [40].

While we primarily looked into the effect of early cART after treatment interruption on *cellular* markers of activation, literature teaches us that the effects of early cART on plasma activation markers are heterogeneous. Liovat et al showed that like initiation of cART in chronic infection, cART initiated during acute HIV infection caused decreased levels of IFNα, D-dimer, and IP-10 [21]. Interestingly, sCD14 levels increased under early cART, suggesting continuous bacterial translocation [21]. In contrast cART initiated within 1 year of infection normalized levels of sCD163 [41], which we (and others) observed to remain elevated even after a year of cART in chronic HIV-infection (chapter 2). This suggests that sCD163 might not be driven by bacterial translocation as we previously assumed. It also hints that early cART may not affect all inflammatory pathways similarly [42].
Finally, in the Primo-SHM trial (chapter 3) we found no differences between treated and untreated individuals in the expression of CD38 and HLA-DR on $CD4^+$ or $CD8^+$ T cells at viral setpoint (chapter 3). This was rather surprising as one of the main results of the VISCONTI trial- in which individuals were treated early in HIV infection with cART for 36 months- was that the immune activation of $CD4^+$ and $CD8^+$ T cells was markedly reduced in individuals who controlled the virus [35]. One factor that may explain the different findings in our Primo-SHM trial and the VISCONTI trial could be the difference in treatment length. Because we only had a 24-60 weeks' treatment period during primary HIV-infection we may have missed possibly longer-term effects on these immune activation parameters.

It is quite remarkable that we did not see any effect of early cART on $CD8^+$T cell activation, as $CD8^+$ T cell activation specifically is strongly associated with HIV-disease progression [4, 5]. Persistent activation of $CD8^+$ T cells may lead to their exhaustion, as was shown (among others) by Kostense et al [43-46]. In a longitudinal study with 16 patients it was shown that the progressive loss of $CD8^+$ T-cell function was not due to the loss of the cells per se (as they were still demonstrable by tetramer staining) but by the loss of their capacity to produce IFNγ. Loss of IFNγ producing $CD8^+$ T cells correlated with declining $CD4^+$ T-cell counts [46]. Overall, during the course of HIV infection, there is a gradual loss of several T cell functions, including proliferation, cytokine secretion and cytotoxic potential. Quite possibly this leads to loss of $CD8^+$ viral control, and subsequently disease progression [47].

***The importance of CTL in the control of HIV-1 disease progression***

There is an abundance of evidence for a pivotal role of $CD8^+$ T cells in HIV control [48-52]. A strong case was made, for instance, by a world-wide genome wide association study (GWAS) in HIV-controllers, who were characterized as individuals whose HIV RNA remained below

2000 copies/mL for at least a year in absence of cART. It was found that the only single nucleotide polymorphisms (SNPs) that significantly correlate with HIV control lie within the peptide-binding pockets of HLA class I molecules [51]. Each individual has a diverse set of HLA-molecules–also called major histocompatibility complex (MHC) I molecules, which are known for their high population diversity– and each of these molecules has distinctive properties for presenting viral peptides. Because of these differences between MHC molecules, individuals tend to mount different T cell responses to the same virus. The fact that the SNPs, that significantly correlate with HIV control, lie within the peptide-binding sites of these molecules therefore suggests that $CD8^+$ T cell responses play an important role in HIV control and that different $CD8^+$ T cell responses affect HIV disease progression differently. HLA-B*27 and B*57 are especially well-known for their strong association with slower HIV-disease progression [49, 53].

*Why are HLA-B*27 and B*57 associated with slow disease progression?*
Part of the answer to why HLA-B*27 and B*57 are associated with slow disease progression probably lies in the specific viral epitopes that are targeted by these HLA molecules. Chimpanzees are resistant to progression to AIDS [54, 55], and have lost the expression of certain MHC-molecules during evolution, whose presence would be expected based on the trans-species evolution of the MHC. It has been suggested that this loss of MHC molecules in the chimpanzee population was caused by a selective sweep in the past, in response to a lentiviral infection [56, 57]. Interestingly, the MHC molecules that are still present in the current chimpanzee population turn out to present the same viral peptides of the HIV genome as the protective HLA molecules HLA-B*27 and B*57 [58]. These protective MHC molecules seem to preferentially target a structurally very constrained part of HIV, the p24-Gag protein [59]. Viral mutations in these regions tend to lead to severe viral fitness loss [60, 61]. Therefore CTL targeting of these epitopes is highly effective as, without viral mutations there will be permanent CTL pressure and if viral escape mutations do occur the virus will be crippled. Numerous studies have shown that individuals with strong $CD8^+$ T-cell responses against the Gag-protein tend to progress slowly to AIDS, while $CD8^+$ T-cell responses of individuals with rapid disease progression are more focused towards epitopes from other HIV-proteins [62-68]. Intriguingly, the number of Gag-specific CTL responses was found to correlate inversely with the rate of HIV-disease progression, even irrespective of HLA-restriction [65].

Even though HLA-B*27 and B*57 both preferentially present epitopes from the same constrained area of the Gag-protein, they are associated with alterations to HIV-disease pathogenesis at very distinct time intervals after HIV-1 infection [69]. This suggests that different mechanisms of protection exist. The effect of HLA-B*57 already occurs early after infection, before the CD4+ T-cell count has dropped below 200 cells/µl, whereas HLA-B*27 delays progression to AIDS-defining illnesses when CD4+ T cell counts have already dropped below 200 cells/µl [69]. It is possible that the quality of the $CD8^+$ T cell responses to the epitopes presented by HLA-B*27 and B*57 may also play a role. The current consensus is that the more HIV-specific functions a $CD8^+$ T cell performs, the more protective it is. HIV-specific $CD8^+$ T cells of LTNPs have indeed been shown to be more prone to perform five functions (IL-2, IFN-γ, TNF-α, MIP-1β, and CD107a) simultaneously than HIV-specific $CD8^+$ T cells of HIV-progressors [48]. Although this seems to suggest that poly-functional $CD8^+$ T cells are more protective, one can in fact not discriminate what is cause and effect, because CTL function

is known to be affected by HIV-disease progression [46]. We therefore studied qualitative differences between CTL responses restricted by different HLA molecules within the same individual, and thereby ruled out viral load as a confounding factor (chapter 5). We found that divergent protective CTL mechanisms were responsible for control by HLA-B*27 and B57 restricted T cell responses. HLA-B*57-restricted CTL responses were of exceptionally high affinity and dominated the HLA-A*02-restricted CTL response in individuals co-expressing these HLA alleles. Instead, in individuals expressing HLA-B*27, the CTL response restricted by 'non protective' HLA alleles was significantly higher and broader, and of higher affinity than in individuals expressing these alleles without HLA-B*27.

*$CD8^+$ T cell targeting of HIV-infected cells*

Another player important in determining what a qualitatively high CTL response must be comprised of is the mode of targeting the HIV-infected cells. Until recently it was widely assumed that $CD8^+$ T cells kill HIV-infected cells during productive infection, i.e. ~24 hours after HIV first enters a cell. In productive infection an infected cell starts producing new viral particles and therefore an abundance of viral peptides is available for presentation on MHC molecules, where they can be targeted by $CD8^+$ T cells. Two studies in 2008 shattered the belief that infected cells are targeted during productive infection, by showing that depletion of $CD8^+$ T cells did not change the lifespan of productively infected cells in RM [70, 71]. Since $CD8^+$ T cell-responses that successfully delay HIV-disease progression occur only in a minority of HIV-infected individuals, we studied the hypothesis that the ability of CTL to reduce the lifespan of productively-infected cells might be limited to protective $CD8^+$ T-cell responses only.



In a group of 36 HIV-infected individuals we showed that not even the correlates thought to be most protective in HIV-infection – i.e. being carrier of a protective HLA-genotype, a broad $CD8^+$ T cell response against HIV-Gag or high levels of $CD8^+$ T cell poly-functionality – correlated with a reduced lifespan of productively infected cells. With respect to the earlier RM studies [70, 71], this suggested that $CD8^+$ T cell mediated control of HIV-infection functions via other mechanisms than cytolytic killing of productively-infected cells. Two alternative mechanisms have been suggested via which $CD8^+$ T cells may control HIV-replication.

*CTL function through secretion of non-lytic soluble factors*

The best-known soluble non-cytolytic factors secreted by CTLs are the CCR5-binding molecules RANTES, MIP1α and MIP1β. These chemokines function as competitive binders and down-modulators of the CCR5 receptor and thereby reduce HIV entry and infection [72]. Likewise, the cytokines IFNγ and TNFα are thought to have anti-viral properties that can inhibit HIV-1 infection [73, 74]. Indeed, depletion of $CD8^+$ T cells in RM has been shown to lead to a marked reduction in the plasma levels of RANTES, MIP1α, MIP1β, IFNγ and TNFα [70]. The increase in virus could therefore be caused by depletion of soluble non-cytolytic factors more than the direct depletion of CTL. This is seemingly in conflict with the observation that there is a selection of HIV-variants that escape recognition of $CD8^+$ T cells. Though it is apparent that a viral variant that has escaped a CTL response could be more fit than wild-type, it is less clear that this also happens when faced with non-lytic control, as the soluble factors would target both variant and wild-type infected cells. Using a 3D spatio-temporal model it was shown, however, that non-cytolytic T cells can nevertheless drive immune escape, while previously it

was assumed that CTL were exclusively the cause of immune escape mutations [75].

CTL function through secretion of non-lytic factors may also explain another paradigm in the HIV-field: the more polyfunctional the T cell is the more effective its response [48]. Betts et al. showed that HIV non-progressors have significantly more polyfunctional $CD8^+$ T cells than do HIV progressors [76], but as already pointed out above, such correlations fail to unveil whether the increased frequency of polyfunctional $CD8^+$ T cells is the cause for or the consequence of better viral control. An in silico model that aimed to resolve whether the strength, the functional diversity, or a specific function of CTL mainly determines disease progression, rather controversially demonstrated that the functional diversity of CTL has only a minor influence while the overall strength of the response is a good predictor of disease progression [77]. The model predicted, however, that when nonlytic effector functions dominate the immune response they have a positive effect on disease outcome [77]. Interestingly, we found that after treatment interruption of early cART (chapter 3), the only moderately positive effects in the treatment group compared to the untreated group were increased cytolytic functions (in terms of perforin and granzyme B expression) and only a trend in increased non-cytolytic T cell function.

*CTL target cells before productive infection*

A second possibility is that CTLs perform their cytolytic effects on HIV-infected cells before the productive stage of infection of the target cells, i.e. within the first ~24 hours after infection of the cell. Importantly, if target cells can be recognized by CTL very early after they have been infected, they have not had the chance to produce new viral particles, and the expression of HLA molecules on the cell surface has not yet been down-regulated. At 12 hours after infection HIV-nef starts to downregulate the expression of MHC class I molecules and as a consequence epitope presentation is mostly abrogated. Indeed, it was shown that Gag-specific CTL clones are able to recognize SIV-infected cells as early as 2 hours after infection of the target cell [78]. Even though some other HIV-proteins may also be presented early, Gag seems to have a higher immunogenicity. The reason is probably the high level of Gag present early after infection of a cell, as the infecting virions can contain up to 5000 copies of Gag each [79]. In HIV-1 infected humans a T-cell clone specific for the immuno-dominant HLA-B*27:05 Gag-restricted KK10 epitope recognized infected target cells within 6 hours post-infection, while sub-dominant HLA-B*27:05-restricted Vpr VL9 epitopes were not recognized until 18 hours after infection [80]. It was also demonstrated that primary $CD8^+$ T cells from HLA-B*57/5801 LTNP were able to efficiently eradicate resting and activated primary HIV-infected $CD4^+$ T cells before productive infection. Moreover it was found that $CD8^+$ T cells from LNTP were superior at eliminating these cells compared to $CD8^+$ T cells from HIV-progressors [81]. Although differences in the targeted HIV proteins probably play an important role in determining whether a response is effective during the first hours of the infected cell cycle, it stands to reason that also characteristics of the CTL response (e.g. affinity and poly-functionality) determine the chance of achieving recognition and killing before productive infection.

The hypothesis that target cell killing takes place before productive infection fits very well with what we know of correlates of protection in HIV-infection. First of all, broad responses against HIV-Gag are associated with viral control, independent of the HLA-restriction [65]. As

an effect these individuals are able to recognize and target the epitopes that are presented before productive infection. Moreover HLA-B*27 and B*57 even preferentially target HIV-Gag. If protection against disease progression is indeed conferred by targeting infected cells before their virus producing stage, this would imply that only a small timeframe exists for recognition by effective CTL.

We found that HLA-B*57 restricted CTL responses were of exceptionally high affinity and dominated the HLA-A*02 restricted CTL response in individuals co-expressing these HLA alleles. In order to achieve targeting before productive infection, fast and effective presentation of epitopes is paramount. If the CTL-MHC peptide complex is of high affinity, the triggering of the CTL, and subsequent killing of the target cell is expected to be more effective. In contrast, HLA-B*27 restricted CTL responses were not of particularly high affinity and did not dominate the response in individuals co-expressing HLA-B*27 and HLA-A*02. Instead, in individuals expressing HLA-B*27, the CTL responses restricted by the non-protective HLA alleles A*02 and B*08 were significantly higher and broader, and of higher affinity than in individuals expressing these alleles without HLA-B*27. This means that overall a broader and higher affinity CTL response could be generated which would allow for a better chance to recognize a viral epitope in the short time frame during which infected cells are not yet productively infected. Overall a mechanism that may also account for the protective features of HLA-B*27 and B*57 compared to ''non-protective" HLA-alleles such as HLA*A2, is that both are highly expressed on the cell surface, which will enhance the chance of recognition by a CTL [82]. Similarly T cells with high levels of the signaling molecule (ζ-chain) of the T cell receptor (TCR) might be more sensitive to activation after antigen engagement. In chapter six we showed a direct positive association between the expression levels of TCRζ on the T-cell and its poly-functionality in terms of non-cytolytic mechanisms. It would be interesting whether these cells were also able to perform more cytolytic functions.



***In conclusion***

Simian models suggest that two different pathways can be modulated to control disease progression. SM can retain low levels of immune activation, despite high viral loads and therefore hardly progress to AIDS. Chimpanzees on the other hand, express a select group of MHC class I molecules, which regulate a superior CTL response that is able to control the virus [56]. Based on the work presented in this thesis, what have we learned with respect to these two pathways: Immune modulation and CTL-control? (See Figure 1)

*Immune activation* HIV-RNA directly drives a broad range of immune activation markers, including $CD8^+$ T cell proliferation and activation ($PD\text{-}1^+$) and plasma markers that are associated with HIV-disease progression. However, our data suggest that microbial translocation plays an important role in the proliferation and activation of $CD4^+$ T cells specifically. One may wonder if bacterial translocation thereby has a stronger impact on disease progression, as increased turnover of $CD4^+$ T cells is thought to lead to $CD4^+$ T cell loss. Future experiments should focus on unraveling the precise mechanisms whereby immune activation causes loss of $CD4^+$ T cells.

*CTL control* In humans, the most protective CTL likely target HIV-infected cells before they are producing new virus (chapter 4). As this suggests that only a small timeframe is open for efficient (HIV-Gag) epitope targeting, CTL able to control the virus should be of superior quality and target the epitopes that are presented early in the infected cell cycle.

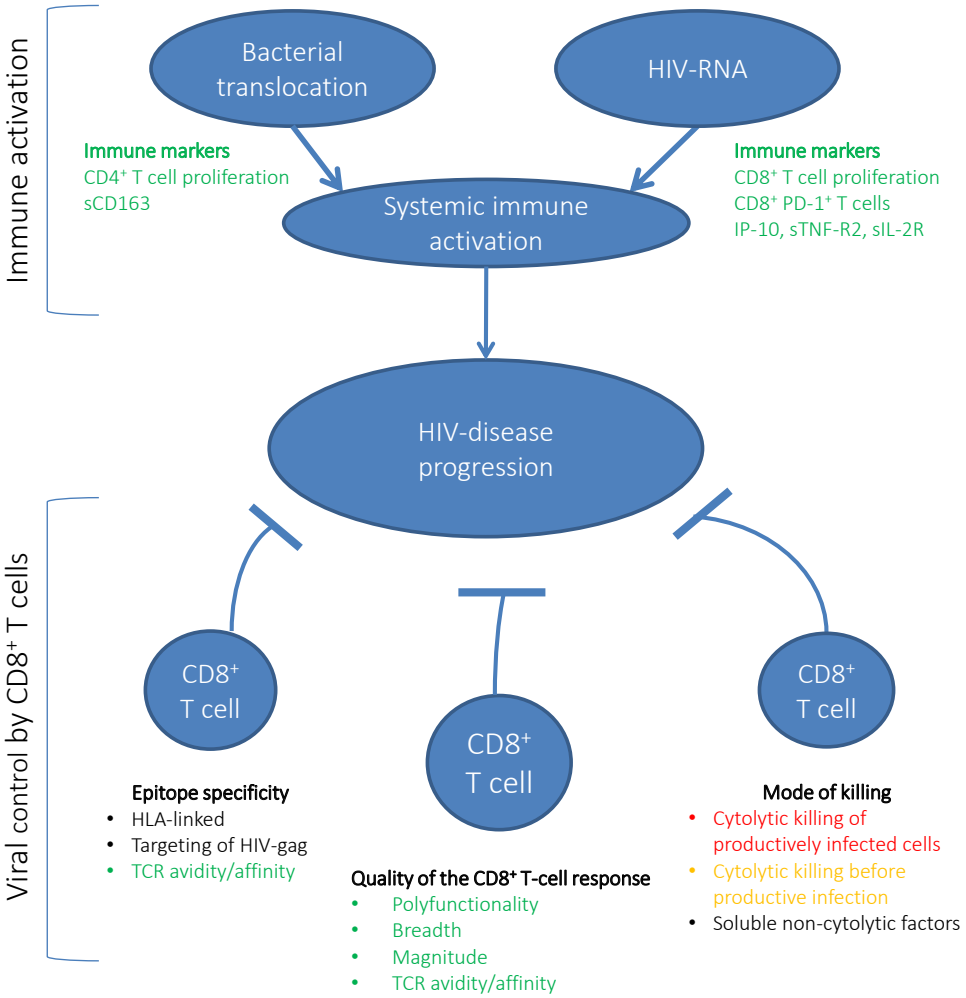



**Figure 1. Schematic outline of thesis results.**

Many different factors contribute to the modulation of HIV-disease progression. In this thesis we researched a broad number of these factors (though of course not all). All factors that were indeed associated with disease outcome are given in green. Results that disproved a hypothesis are given in red and finally in orange are the alternatively hypothesized pathways which we did not prove but consider most likely given our own findings as well as those of others. We found that the levels of $CD8^+$ T cell proliferation, $CD8^+$ $PD\text{-}1^+$ T cells, IP-10, sTNF-R2 and sIL-2R were driven by HIV RNA while $CD4^+$ T cell proliferation and sCD163 were likely driven by bacterial translocation. Also, we found that TCR affinity, and the polyfunctionality, breadth and magnitude of the $CD8^+$ T cell response were important correlates of protection in HIV-infection. In contrast we rejected the hypothesis that cytolytic killing of productively infected cells contributes to CTL control (in red). Instead we think it is most likely that cytolytic killing of infected cells takes place before productive infection, which fits nicely with our other results

In line with this, HIV-responses restricted by the protective HLA-alleles B27 and B57 were found to preferentially target HIV-Gag, and to be relatively high, broad and of high affinity (chapter 5). High affinity T cells are usually more polyfunctional and therefore may contribute to viral control (chapter 2,6). Further studies should show i) if CTL definitely target cells before productive infection and ii) if a protective phenotype of CTL can be induced or if it's intrinsically limited to the CTL responses restricted by certain HLA-alleles.

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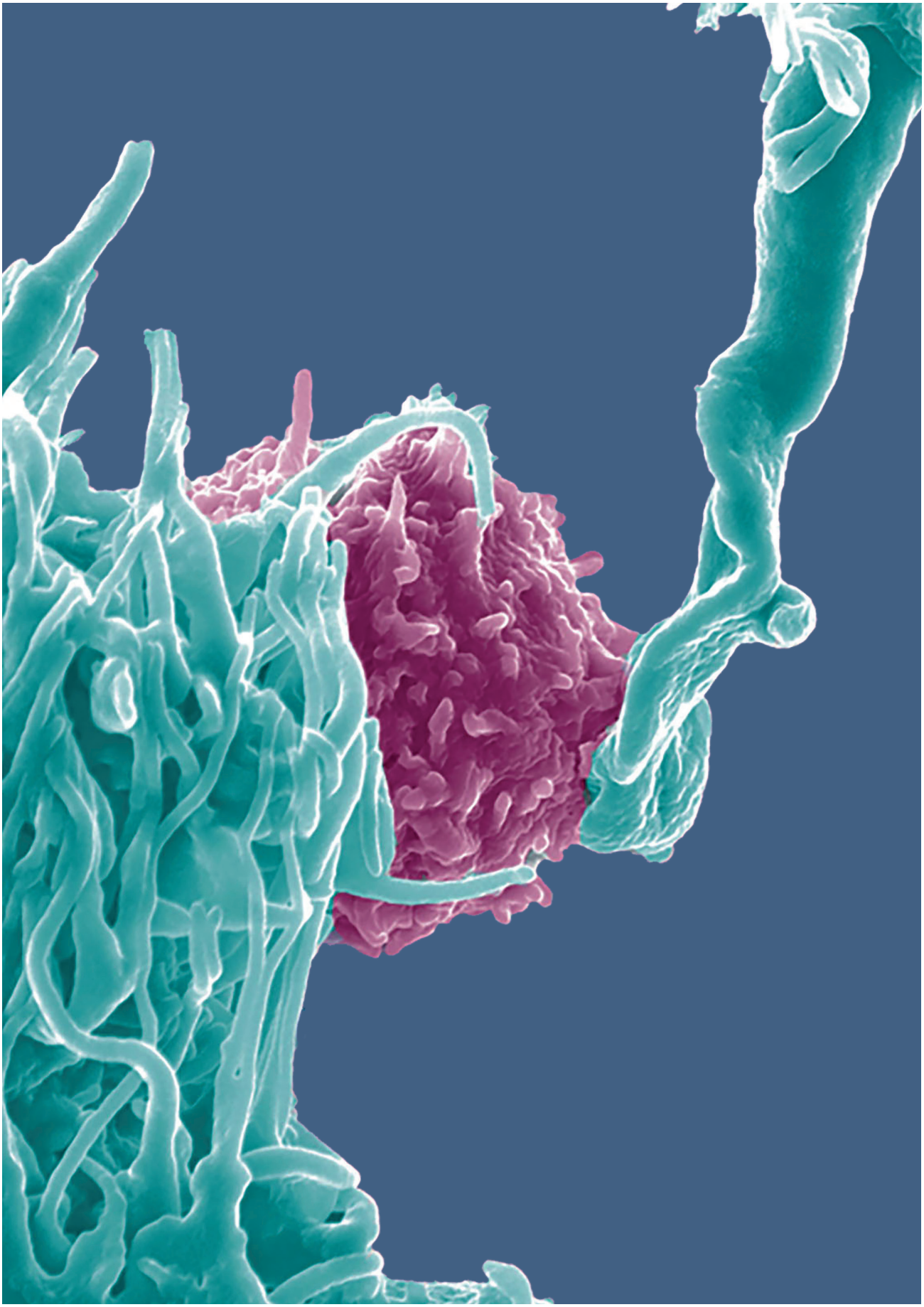

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NEDERLANDSE SAMENVATTING

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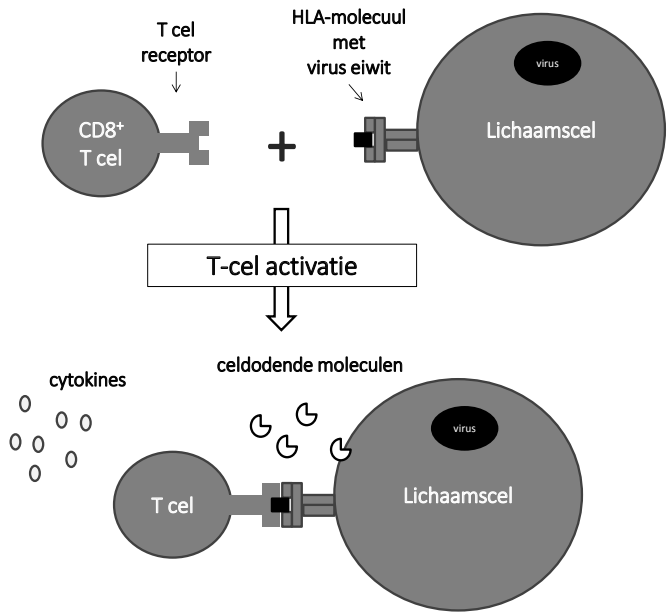
## SAMENVATTING

***Het immuunsysteem*** beschermt ons tegen ziekteverwekkers van buitenaf, zoals virussen en bacteriën. Op het moment dat een ziekteverwekker het lichaam binnendringt, wordt het immuunsysteem geactiveerd. In eerste instantie vindt een aspecifieke afweer plaats, waarbij verschillende cellen betrokken zijn die slechts herkennen dat de ziekteverwekkers niet lichaamseigen zijn. Mocht dat niet voldoende zijn om de ziekteverwekker te doden, dan komt de specifieke afweer op gang, die een 'op maat gemaakte' afweerreactie voor iedere ziekteverwekker kan ontwikkelen. Een zeer belangrijk onderdeel van de specifieke afweer zijn de T-cellen. De T-cellen herkennen om welke ziekteverwekker het gaat doordat er op alle lichaamscellen (behalve op rode bloedcellen) HLA-moleculen zitten (zie Figuur 1). Deze HLA-moleculen laten stukjes eiwit uit de cel zien aan de T-cellen. Op het moment dat een virus of een bacterie in een cel zit, zullen ook daarvan specifieke stukjes eiwit getoond worden aan de T-cellen. Deze herkennen de viruseiwitten en ondernemen actie. De twee meest voorkomende soorten T-lymphocyten (of T-cellen) zijn $CD4^+$ T-cellen en $CD8^+$ T-cellen. $CD4^+$ T-cellen worden ook wel T-helper cellen genoemd en houden zich vooral bezig met de afweer tegen bacteriën. $CD8^+$ T-cellen worden ook wel T-killer cellen genoemd en houden zich voornamelijk bezig met virussen die in de cel zitten.



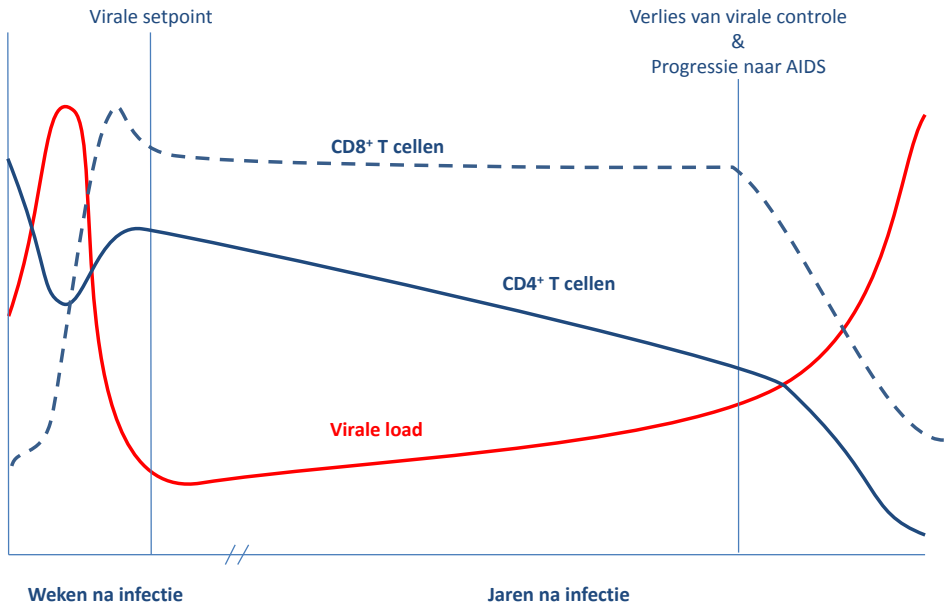

**Figuur 1. Schematische weergave van $CD8^+$ T-cel activatie.** Alle lichaamseigen cellen - behalve rode bloedcellen - hebben HLA- moleculen waarmee ze verschillende stukjes eiwit uit hun cel aan $CD8^+$ T-cellen laten zien. Wanneer een $CD8^+$ T-cel een specifiek stukje virus eiwit herkent, gaat de T-cel 'aan'. De $CD8^+$ T-cel gaat dan celdodende moleculen uitscheiden om de geïnfecteerde lichaamscel stuk te maken en/of scheidt signaleringsmoleculen uit (cytokines) die de rest van het immuunsysteem waarschuwen dat er een infectie is.

***Human immunodeficiency virus (HIV)*** is een RNA-virus. Het virus dringt cellen binnen waar het zijn eigen genetische informatie verbergt tussen de genetische informatie van de humane cel. Op deze manier zit het virus goed verstopt als de humane cel in rust is. Als de cel geactiveerd wordt, kan het virus meeliften en zichzelf vermenigvuldigen.

HIV infecteert niet zomaar alle cellen, maar voornamelijk $CD4^+$ T-cellen. Met het infecteren van de $CD4^+$ T-cellen wordt de functie van het immuunsysteem ernstig aangetast. Nadat een individu is geïnfecteerd, is er een enorme piek in de virusproductie, gevolgd door een dip (zie Figuur 2). Deze virale dip (ook wel het virale *setpoint* genoemd) ontstaat nadat het immuunsysteem HIV-specifieke $CD8^+$ T-cellen heeft ontwikkeld die tegen het virus gericht zijn [1, 2]. $CD8^+$ T-cellen zijn zeer belangrijk voor het beheersen van het virus [1-6]. De hoeveelheid virus op het virale setpoint voorspelt hoe lang het zal duren tot het immuunsysteem de controle over het virus verliest. Uiteindelijk zullen de $CD4^+$ T-cel aantallen dalen en ontwikkelt het HIV-geïnfecteerde individu verschillende opportunistische infecties, waar het verzwakte immuunsysteem niets tegen kan verrichten. Bij zulke lage $CD4^+$ T-cel aantallen en het voorkomen van opportunistische infecties spreekt men van *acquired immune deficiency syndrome* (**AIDS**) [7] (zie Figuur 2).

***Wat weten we over de oorzaak van AIDS?***

Zoals hierboven genoemd is hét kenmerk van HIV-infectie het geleidelijke verlies van $CD4^+$ T-cellen. Aanvankelijk werd gedacht dat de celdodende-effecten van HIV de belangrijkste oorzaak waren van het verlies van $CD4^+$ T-cellen. Opvallend was echter dat de meerderheid van




**Figuur 2. Schematische weergave van HIV-pathogenese.**

De relatieve dynamiek van $CD4^+$ (blauwe doorgetrokken lijn) en $CD8^+$ (blauwe onderbroken lijn) T-cel aantallen en virale load (rode lijn) in HIV-ziekteprogressie.

de stervende $CD4^+$ T-cellen bleek te bestaan uit niet-geïnfecteerde cellen [8]. Wat bleek: door continue blootstelling aan HIV, wordt bij het immuunsysteem een toestand van chronische lichaamsbrede inmuunactivatie bereikt. De chronische imuunactivatie veroorzaakt door de HIV-infectie, zorgt ervoor dat $CD4^+$ T-cellen ook geactiveerd worden. Door de continue activatie zullen de $CD4^+$ T-cellen sneller delen, maar ook sterven en uiteindelijk leidt dit tot een verlies van $CD4^+$ T-cellen [9-11]. De algehele immuunactivatie heeft een vergelijkbaar slopend effect op $CD8^+$ T-cellen, want hoewel de $CD8^+$ T-cellen beter bestand zijn tegen continue activatie, zullen zij uiteindelijk versneld verouderen en uitgeput raken [11-14].

Het doel van dit proefschrift is om inzichten te vergaren die kunnen bijdragen aan een succesvol HIV-vaccin en nieuwe therapeutische ontwikkelingen. We hebben ons met name gericht op de volgende vragen:

i) Wat is de voornaamste bron van immuunactivatie in HIV-infectie?
ii) Wat houdt een beschermende T-celrespons in HIV-infectie precies in?

**Deel 1: activatie**

***De bron van immuunactivatie in HIV-infectie***

De exacte mechanismen waarmee HIV-infectie chronische immuunactivatie veroorzaakt, zijn zeer complex en staan nog steeds ter discussie. De twee belangrijkste oorzaken zijn gevonden. Eén daarvan is dat HIV zowel het adaptieve als het aspecifieke immuunsysteem direct activeert. De onophoudelijke stimulatie van T-cellen bij HIV-infectie veroorzaakt verhoogde activatie, proliferatie, functionele beperkingen en uiteindelijk verlies van T-cellen [15-21]. De tweede oorzaak van immuunactivatie (en een indirect effect van HIV-infectie) is het lekken van bacteriële producten vanuit de darm. Deze zogenaamde 'bacteriële translocatie' wordt veroorzaakt door een massaal verlies van $CD4^+$ T-cellen in het darmgeassocieerde lymfoïde weefsel (**GALT**), wat één van de eerste effecten van HIV-infectie is [22]. Men neemt aan dat de vroege schade aan het darmimmuunsysteem, permeabiliteit en translocatie van microbiële producten induceert. Microbiële producten zoals lipopolysacchariden (**LPS**) [23] activeren op hun beurt weer het immuunsysteem.



***De oorsprong van HIV-geassocieerde immuunactivatie***

Het is echter nog niet duidelijk hoeveel van de lichaamsbrede activatie tijdens HIV-infectie direct wordt veroorzaakt door HIV en hoeveel door bacteriële translocatie. In **hoofdstuk 2** is geprobeerd dit vraagstuk te ontrafelen. Daarvoor zijn, gedurende een jaar na aanvang van therapie, de dynamiek van beide bronnen van immuunactivatie gevolgd: HIV-RNA en een marker voor bacteriële translocatie. Tijdens therapie werd het HIV-RNA in het bloed effectief onderdrukt, terwijl bacteriële translocatie zeker het eerste jaar nog aanwezig bleef. Vervolgens is de dynamiek van deze twee activatiebronnen gecorreleerd met de dynamiek van een grote groep immuunactivatiemarkers op T-cellen, NK-cellen en monocyten, en met oplosbare factoren in het bloedplasma. Het idee was dat de immuunactivatiemarkers die verhoogd waren vanwege het virus binnen twee weken sterk verminderd zouden zijn, zoals ook het geval was bij HIV-RNA.

Er is gevonden dat de immuunactivatie markers op $CD8^+$ T-cellen voor het grootste deel veroorzaakt werden door de aanwezigheid van het virus. Daarentegen hadden de immuunactivatiemarkers op $CD4^+$ T-cellen en de cellen van de aspecifieke immuunrespons de neiging verhoogd te blijven tijdens therapie, ondanks de aanzienlijke daling van HIV-

RNA. Dit zou erop kunnen wijzen dat $CD4^+$ T-cel activatie veroorzaakt werd door bacteriële translocatie, maar het kan ook betekenen dat de activatie van $CD4^+$ T-cellen onomkeerbaar is en daarom niet verdwijnt. De vraag die mede onbeantwoord blijft, is wat de progressie naar AIDS veroorzaakt: het HIV-RNA of de bacteriële translocatie.

***Behandeling van HIV met medicatie***

Sinds 1996 is het mogelijk om HIV goed te behandelen. Met gecombineerde antiretrovirale therapie (**cART**)- een combinatie van tenminste drie verschillende antiretrovirale drugs- is het mogelijk om het virus onder controle te houden. Toch is cART niet de ultieme HIV-therapie. De drugs kunnen bijwerkingen veroorzaken en de dagelijkse inname van geneesmiddelen is erg belastend. Een nog groter probleem is dat, zelfs op therapie, het immuunsysteem niet volledig herstelt. Daardoor kunnen AIDS-gerelateerde ziekten een bedreiging blijven vormen [24]. Eén van de redenen dat het immuuunsysteem zich niet herstelt, is waarschijnlijk dat de meeste therapieën pas tijdens chronische infectie gegeven worden. In de laatste jaren is het echter duidelijk geworden dat het (zeer) vroeg starten van therapie- tijdens primaire infectie - een positief effect heeft. Tijdelijke therapie tijdens primaire HIV-infectie verlaagt het virale setpoint [25]. Bovendien kan langdurige therapie die tijdens primaire infectie geïnitieerd wordt erin resulteren dat tot 15% van de behandelde individuen het virus tot 12 maanden na stopzetting van de behandeling onderdrukt kan houden [26, 27].

In **hoofdstuk 3** is bekeken wat de reden is dat (tijdelijke) vroege therapie ervoor zorgt dat het virale setpoint verlaagd is. Een aantal mogelijke redenen dat is onderzocht is: minder activatie van $CD8^+$ T-cellen, preventie van GALT-depletie en dus bacteriele translocatie, en/ of behoud van T-cel immuniteit. Hiervoor is de PRIMO-SHM studie gebruikt- een multicenter gerandomiseerde trial waarin groepen individuen vergeleken werden die ofwel onbehandeld waren ofwel korte tijd therapie kregen tijdens primaire HIV-infectie. Op het virale setpoint vonden we tot onze verrassing geen verschillen tussen behandelde en onbehandelde individuen, als we keken naar de preventie van bacteriële translocatie en de immuunactivatie van de $CD8^+$ T-cellen. Wel waren er subtiele verschillen in de kwaliteit van de cytolytische $CD4^+$ T-celrespons: iets meer $CD4^+$ T-cellen in de behandelde groep produceerden de celdodende moleculen perforine en Granzyme B (welke de geïnfecteerde cel kapot maken). Het blijft echter onduidelijk of deze subtiele immunologische verschillen de oorzaak of het gevolg waren van het lagere virale setpoint bij patiënten die vroeg behandeld werden.

**Deel 2: T-cel immuniteit**

***Vaccins***

Ondanks dat medicatie HIV kan onderdrukken is er geen totale genezing. Daarom is een therapeutisch of preventief HIV-vaccin van groot belang. Door de belangrijke rol van $CD8^+$ T-cellen in het controleren van HIV-infectie is er in het verleden al veel onderzoek gedaan naar een T-cel vaccin. Sinds de eerste trial in 1986, zijn er meer dan 250 klinische fase I- en II studies uitgevoerd. Desondanks is een echt succesvol HIV-vaccin nog niet geproduceerd. Slechts één vaccin – onderzocht in The Thai vaccine Trial- heeft een (matig) positief effect laten zien [28]. Tijdens het onderzoek was er 31,2% vermindering van de HIV-infecties in de vaccin-groep vergeleken met de placebogroep, maar het het effect was niet significant bij twee van de drie gebruikte statistische methodes.

In 2008 werd de grootste vaccin studie tot nu toe- de 'STEP trial'- stopgezet als gevolg van een

gebrek aan bescherming door het vaccin, geen effect op de virale setpoints en een verhoogd risico van infectie bij gevaccineerde individuen met reeds bestaande immuniteit tegen de virale vector die werd gebruikt in het vaccin [29]. Dit liet op een zeer pijnlijke manier zien hoe ver we verwijderd zijn van het begrijpen van anti-HIV immuniteit.

***Wat weten we wel over beschermende T-cellen in HIV-infectie?***

Er zijn maar weinig individuen die HIV-geïnfecteerd zijn en het virus zelf over een lange periode onder controle kunnen houden. Dit gebeurt in minder dan 5% van alle geïnfecteerden, die *longterm non progressors* (**LTNP's**) worden genoemd. Het is niet helemaal duidelijk waarom deze mensen langer dan 10 jaar AIDS-vrij kunnen blijven maar zoals eerder genoemd zijn er sterke aanwijzingen dat $CD8^+$ T-cellen daar een grote rol in spelen. Onder de LTNP's is er een groter percentage individuen met de HLA-moleculen HLA-B*27 en B*57, terwijl er bij personen met relatief snelle progressie naar AIDS een verhoogde aanwezigheid is van HLA-B*3503 [3, 30]. Zoals hierboven beschreven zijn HLA-moleculen bepalend voor welke specifieke stukjes eiwit aan de T-cellen getoond worden en bepalen ze daarmee de T-celrespons die wordt gevormd.

Een probleem voor T-celresponsen tegen HIV is dat HIV heel snel muteert. Omdat T-cellen specifiek zijn voor bepaalde stukjes eiwit, kunnen virus mutaties ervoor zorgen dat de gemaakte T-celrespons de geïnfecteerde cellen niet meer herkent, of zelfs dat de stukjes eiwit niet meer door de HLA-moleculen gepresenteerd worden. Beschermende HLA-moleculen presenteren bij voorkeur een deel van het HIV-gag-eiwit [31]. Relatief weinig mutaties treden op in het gag-eiwit omdat virale mutaties in die regio ervoor zorgen dat HIV niet meer zo goed kan functioneren [32, 33].

Het is duidelijk dat het herkennen van stukjes gag-eiwit door T-cellen bijdraagt om HIV onder controle te houden. Het aantal gemaakte gag-specifieke T-celresponsen correleert namelijk negatief met de HIV virale load en met HIV-ziekteprogressie [5], ongeacht welke HLA-moleculen erbij betrokken zijn. Andere kenmerken die een beschermende T-celrespons van LTNP's heeft ten opzichte van mensen die wel HIV-ziekteprogressie vertonen, zijn [34-36]:

1) Sterkere T-celresponsen (meer T-cellen die geactiveerd worden door een specifiek stukje viruseiwit).
2) Bredere T-celrespons (een T-celrespons tegen meer verschillende stukjes HIV-eiwit).
3) Meer T-cel polyfunctionaliteit (de T--cellen die geactiveerd worden door een geïnfecteerde cel kunnen op verschillende manieren tegelijkertijd de infecteerde cel doden en extra hulp inroepen).

***Wanneer doden T-cellen HIV-geïnfecteerde cellen?***

Om een goed werkend HIV-vaccin te kunnen ontwikkelen is het belangrijk om te begrijpen hóe $CD8^+$ T-cellen HIV-geïnfecteerde cellen doden, omdat ze op verschillende manieren een infectie tegengaan. Allereerst zijn er $CD8^+$ T-cellen die stofjes uitscheiden die de geïnfecteerde cellen zelfmoord laten plegen de zogenaamde cytotoxische T-cellen (**CTL**). Daarnaast zijn er $CD8^+$ T-cellen die zogenaamde chemokines en cytokines uitscheiden, waarmee ze verschillende functies kunnen uitoefenen, zoals het aansturen van andere immuuncellen of het tegengaan van nieuwe HIV-infecties. Tot voor kort werd aangenomen dat $CD8^+$ T-cellen HIV-infectie voornamelijk tegengaan door op een cytotoxische manier HIV-geïnfecteerde cellen te doden. Er werd van uitgegaan dat het doelwit van de CTL met name de HIV-geïnfecteerde cellen zijn

die alweer nieuwe virusdeeltjes produceren (productief geïnfecteerde cellen). In de eerste 12 uur na infectie van een cel zijn er namelijk slechts enkele HIV-virusdeeltjes in de cel aanwezig, wat het erg lastig maakt om stukjes hiervan te detecteren.
Het HIV-veld werd verrast toen in een aapmodel met *simian immunodeficiency virus* (SIV), de aapvariant van HIV, aangetoond werd dat de afwezigheid van CTL geen invloed heeft op de levensduur van productief geïnfecteerde cellen [37, 38]. Omdat CTL-responsen die HIV-ziekteprogressie tegengaan slechts in een klein aantal HIV-geïnfecteerde individuen voorkomen (de LTNP's), hebben we in **hoofdstuk 4** onderzocht of wellicht alleen beschermende CTL-responsen in staat zijn de levensduur van productief geïnfecteerde cellen te verminderen. Bij 36 HIV-geïnfecteerden hebben we onderzocht of er een correlatie bestaat tussen de levensduur van productief geïnfecteerde cellen en het hebben van CTL-responsen die geassocieerd zijn met controle van HIV-infectie. Dat wil zeggen CTL die beschermende HLA-moleculen herkennen of CTL die sterk reageren op HIV-gag. Tot onze verbazing vonden we dat zelfs de aanwezigheid van de meest beschermende CTL- responsen niet correleerde met een verkorte levensduur van productief geïnfecteerde cellen *in vivo*. Dit suggereert dat beschermende $CD8^+$ T-cellen op een andere manier bescherming bieden, bijvoorbeeld doordat ze HIV-geïnfecteerde cellen al herkennen nog voordat ze in staat zijn nieuwe virusdeeltjes te produceren. Een andere mogelijkheid is dat de $CD8^+$ T-cellen niet op een cytotoxische manier maar door het het uitscheiden van cytokines bescherming bieden. Het belang van vroege herkenning van de infectie van een cel zou een extra verklaring kunnen vormen voor het belang van gag-specifieke T-celresponsen in controle van HIV-ziekteprogressie. Gag is één van de weinige HIV-eiwitten die het infecterende virusdeeltje in grote hoeveelheden mee de humane cel inneemt en zal dus met grotere kans gepresenteerd worden door een HLA-molecuul in de korte tijd dat er nog geen virus geproduceerd wordt [39].

***Wat is er zo speciaal aan HLA-B*27 en B*57?***

Iedere persoon heeft tot 6 verschillende HLA-moleculen die specifieke stukjes eiwit aan $CD8^+$ T-cellen laten zien. Van de honderden verschillende HLA-moleculen zijn met name HLA-B*27 en B*57 sterk geassocieerd met een relatief langzame progressie tot AIDS. In het verleden is laten zien dat dit gedeeltelijk komt doordat deze HLA-moleculen een bredere en effectievere $CD8^+$ T-celrespons aansturen. Voor het ontwikkelen van vaccins is het echter belangrijk om verder uit te diepen waarom deze T-celresponsen effectiever zijn. Daarnaast zijn deze observaties gedaan in LTNP's waarin de virale load vaak erg laag was. Daardoor was onduidelijk of de goede T-celrespons een oorzaak of gevolg was van een lage virale load. Om dit onafhankelijk te bekijken hebben we, in **hoofdstuk 5**, CTL-responsen vergeleken die aangestuurd werden door beschermende en niet beschermende HLA-moleculen, binnen *één en hetzelfde individu.* We bestudeerden daarvoor individuen die zowel één van de beschermende HLA-moleculen B*27 of B*57 hadden als het niet beschermende HLA-molecuul A*02. Interessant genoeg vonden we dat de beschermende T-celresponsen aangestuurd door HLA-B*27 en B*57 niet dezelfde kenmerken vertoonden. Er was een zeer sterke binding tussen het HLA-B*57 molecuul en de $CD8^+$ T-cel (waardoor deze waarschijnlijk makkelijker geactiveerd kan worden). Daarnaast waren de door HLA-B*57 geïnduceerde T-celresponsen hoger dan de (niet-beschermende) HLA-A*02 T-celresponsen. Daarentegen bonden de HLA-B*27-moleculen niet bijzonder sterk met de T-cellen en overheersten deze responsen niet in individuen die zowel HLA-B*27 als HLA-A*02 moleculen hadden. In plaats daarvan waren in individuen met HLA-B*27 ook de T-celresponsen aangestuurd door niet-

beschermende HLA-moleculen significant hoger en breder dan in individuen zonder HLA-B*27 moleculen. Dit onderzoek suggereert dat het mogelijk is om op meerdere manieren een effectieve T-celrespons op te wekken.

***Waarom worden T-cellen minder functioneel in chronische HIV-infectie?***

Een groot probleem in chronische HIV-infectie is dat de $CD8^+$ T-celresponsen minder effectief worden omdat ze uitgeput raken door constante activatie. HIV-geïnfecteerden die lange tijd het virus kunnen controleren hebben minder last van uitgeputte T-cellen. Zo hebben ze bijvoorbeeld meer T-cellen die meerdere functies tegelijkertijd kunnen uitvoeren – zogenaamde polyfunctionele T-cellen – ten opzichte van mensen die wel snel AIDS krijgen. Een ander verschijnsel dat optreedt in chronische infectie is een afnemende expressie van de T-celreceptor ζ keten (**TCRζ**). Dat zorgt ervoor dat cellen minder makkelijk geactiveerd kunnen worden. In **hoofdstuk 6** hebben we onderzocht of er ook een directe relatie is tussen de mate van TCRζ expressie en $CD8^+$ T-cel functie, wat betreft polyfunctionaliteit en productie van bepaalde cytokines. We vonden dat zowel de omvang van de T-celrespons als de polyfunctionaliteit van $CD8^+$ T-cellen positief correleerden met TCRζ expressieniveaus, zowel in gezonde als in HIV-geïnfecteerde individuen. Dit suggereert dat de verlaagde expressie van TCRζ die we zien in chronische infecties ook een direct effect zou kunnen hebben op de functionaliteit van de cel. Als we dit breder trekken suggereert dit dat de effectieve T-cellen die je zou willen induceren met een vaccin hoge TCRζ expressie moeten hebben.

***Conclusies***

*Activatie* - In dit proefschrift hebben we laten zien dat de lichaamsbrede immuunactivatie die HIV-ziekte progressie veroorzaakt meerdere oorzaken heeft. De activatie van $CD8^+$ T-cellen wordt voornamelijk direct veroorzaakt door HIV-RNA. Daarentegen wordt $CD4^+$ T-cel activatie naar alle waarschijnlijkheid voor een groot deel veroorzaakt door bacteriële translocatie, al kunnen we niet uitsluiten dat het een irreversibel effect is van HIV-RNA. In de toekomst is het belangrijk te achterhalen welke bron van activatie het meest belangrijk is voor HIV-ziekteprogressie. Ondanks het feit dat HIV-geïnfecteerden die vroeg behandeld werden daarna een betere prognose hadden, voorkwam tijdelijke vroege therapie noch bacteriële translocatie, noch $CD8^+$ T-cel activatie.

*T-cel immuniteit* – We hebben laten zien dat zelfs beschermende T-celresponsen geen invloed hebben op de levensduur van geïnfecteerde cellen die nieuwe HIV-deeltjes produceren. Voor de ontwikkeling van een vaccin moeten we ons dus waarschijnlijk richten op T-cellen die HIV-geïnfecteerde cellen herkennen vóór de productieve infectie, of die non-cytolytisch zijn. Dit strookt met onze bevindingen over de beschermende moleculen HLA-B*27 en B*57. Naast het feit dat deze HLA-moleculen een sterke en brede T-celrespons mogelijk maken, zorgen ze voor een sterke binding met de T-cel. Doordat deze HLA-moleculen zo een effectieve, vroege T-celrespons kunnen stimuleren, kunnen ze mogelijk betere bescherming bieden. Als laatste hebben we laten zien dat sterke en polyfunctionele T-celresponsen geassocieerd zijn met een hoge TCRζ expressie.

Samenvattend, zijn in dit proefschrift fundamentele stappen gezet in het identificeren van de parameters van een beschermende T-celrespons in HIV-infectie. De uitdaging is nu om deze kennis om te zetten tot de uiteindelijke ontwikkeling van een werkend T-celvaccin.

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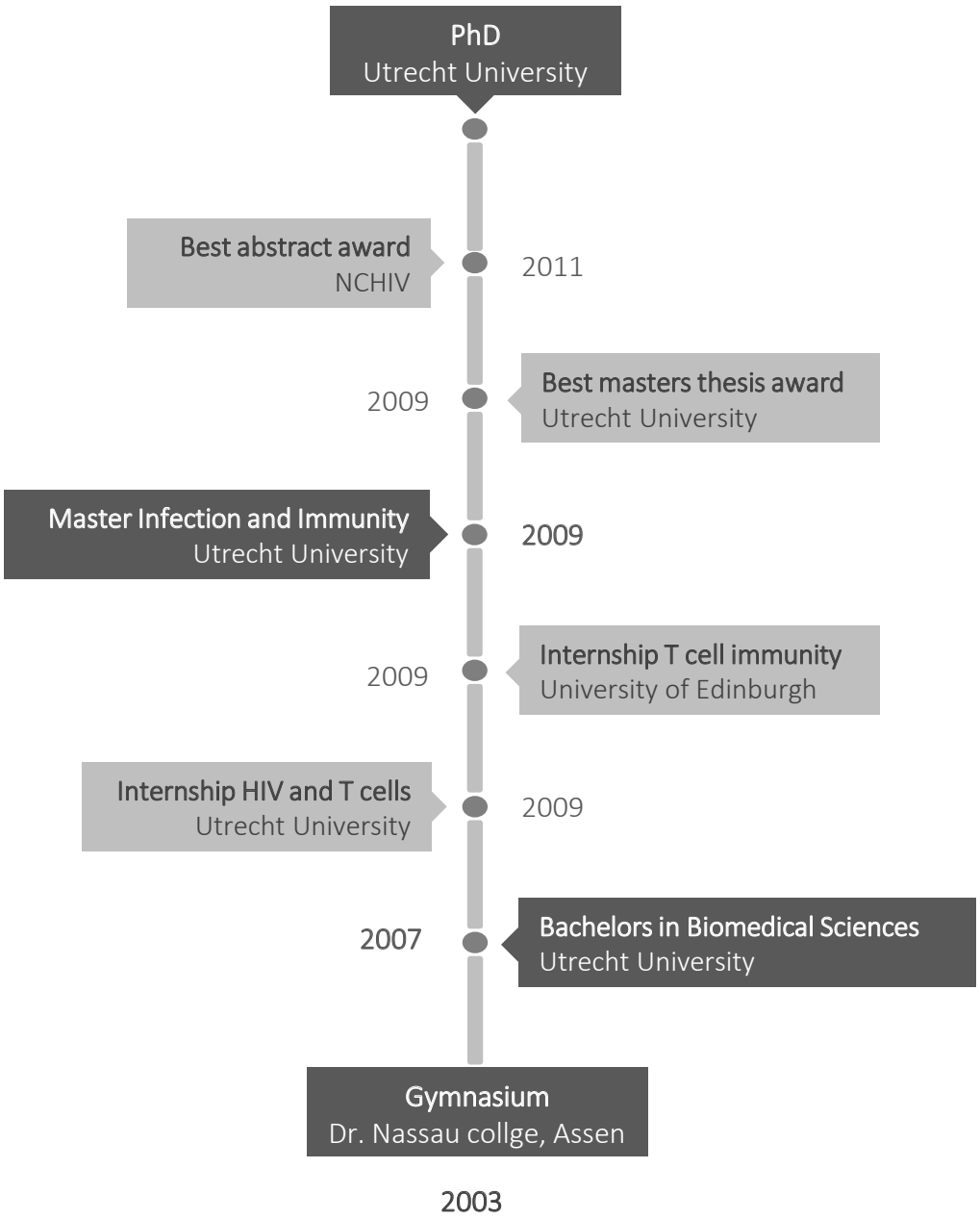
# CURRICULUM VITAE

EDUCATION



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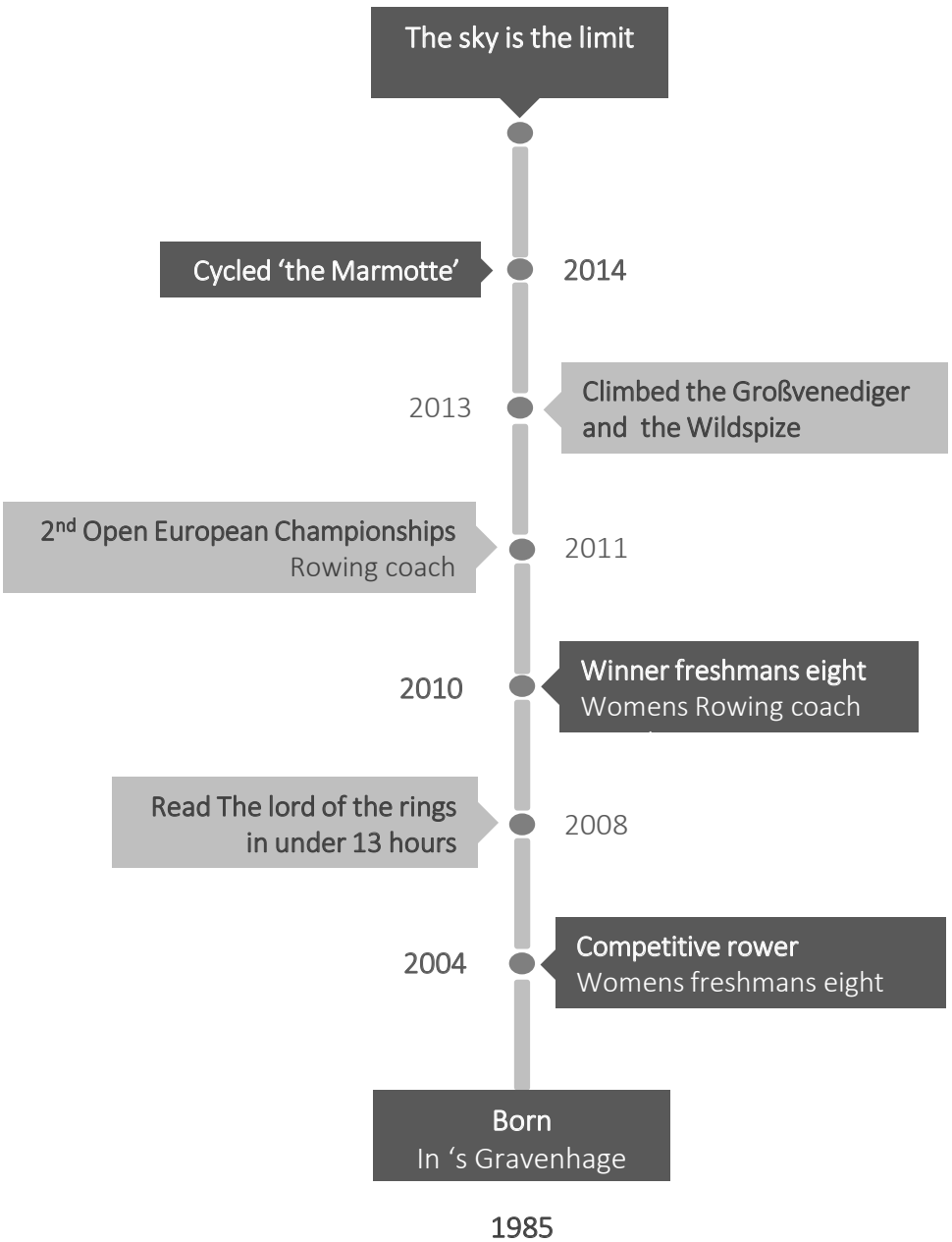

## LIST OF PUBLICATIONS

Schellens IM, Spits HB, Navis M, Westerlaken GH, Nanlohy NM, Coffeng LE, Kootstra N, Miedema F, Schuitemaker H, Borghans JAM, van Baarle D. Differential characteristics of cytotoxic T lymphocytes restricted by the protective HLA alleles B*27 and B*57 in HIV-1 infection. *J Acquir Immune Defic Syndr* 2014; **67(3)**:236-245.

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Spits HB, Mudrikova T, Schellens IM, Wensing AMJ, Prins JM, Feuth T, Spierings E, Nijhuis M, van Baarle D, Borghans JAM. Protective CTL do not shorten the lifespan of productively-infected cells in HIV-1 infected individuals. *Accepted for publication in AIDS.*

de Roock S, Wienke J, Janssen WJM, Scholman RC, Spits HB, van Gijn ME, Boes ML, van Montfrans JM, Moes ND. A novel human STAT3 mutation presents with autoimmunity involving Th17 hyperactivation. *Accepted for publication in Oncotarget.*

Spits HB, Drylewicz J, Mudrikova T, Wensing AMJ, de Jager W, Schellens IM, van Baarle D, Borghans JAM. Chronic immune activation in HIV-infection: different roles for HIV-replication and bacterial translocation. *Submitted for publication.*

Spits HB, Nanlohy NM,Koning D, Prins JM, Borghans JAM, van Baarle D, Schellens IM. A direct link between loss of T-cell function and downregulation of TCRζ expression in chronic viral infections? *Submitted for publication.*

## DANKWOORD

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&

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