Mechanisms of decreasing HIV-1 specific CD8+ T cell activity during progression to AIDS
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Persistent numbers of tetramer\(^+\) CD\(^8\)\(^+\) T cells, but loss of IFN\(_\gamma^+\) HIV-specific T-cells precedes progression to AIDS.

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Although initially CD\(^8\)\(^+\) T cells suppress HIV replication, cytotoxic T-cell precursor (CTLp) frequencies eventually decline and fail to prevent disease progression. In a longitudinal study including 14 HIV-1 infected individuals, we studied both number and function of HIV-specific CD\(^8\)\(^+\) T cells by comparing HLA-peptide tetramer staining and peptide-induced IFN\(_\gamma\) production. Numbers of IFN\(_\gamma\)-producing T cells declined during disease progression, whereas numbers of tetramer\(^+\) T cells in many individuals persisted at high frequencies. Loss of IFN\(_\gamma\)-producing T cells correlated with declining CD\(^4\)\(^+\) T cell counts, consistent with the need of CD\(^4\)\(^+\) T cell help for adequate CD\(^8\)\(^+\) T cell function. These data indicate that the loss of HIV-specific CD\(^8\)\(^+\) T cell activity is not lost due to physical depletion, but is mainly due to progressively impaired function of HIV-specific CD\(^8\)\(^+\) T cells.

**Introduction**

CD\(^8\)\(^+\) cytotoxic T lymphocytes (CTL) are generally regarded to have a major contribution to elimination or control of viral infections (3,190). In HIV-1 infected individuals, HIV-specific CD\(^8\)\(^+\) T cells are frequently reported to suppress viral replication and to delay disease progression (13,15,16,23,26,32), but eventually CTL precursor frequencies decline and fail to protect the infected individuals against progression to AIDS (30,34). This CTL decline may be due to physical depletion of HIV-specific CD\(^8\)\(^+\) T cells, or due to T cell dysfunction.

In order to distinguish between these two mechanisms, two different techniques are available. Tetrameric HLA-peptide complexes allow the detection of CD\(^8\)\(^+\) T cells that express a T cell receptor (TCR) specific for a given peptide restricted to a given HLA molecule (47). In addition, peptide-specific T cells can be detected by intracellular cytokine staining, where antigen-responsive T cells are detected by virtue of their cytokine production upon stimulation with the peptide of interest (49). Studies using tetrameric peptide-HLA complexes have shown high frequencies of HIV-specific T cells at various stages of the natural course of infection (26,88,162). This would argue against HIV-specific CD\(^8\)\(^+\) T cells being depleted. Alternatively, an increasing number of studies have reported a dissociation between numbers of HCV\(^+\), EBV\(^+\), HIV\(^-\), or SIV-specific CD\(^8\)\(^+\)tetramer\(^+\) T cells and number of T cells that responded to functional assays with antigen specific IFN\(_\gamma\) production (88,161,162,183,185,189,191-194). In other studies including many long-term asymptomatic HIV-carriers (LTA) or treated individuals, cytokine production by a relatively high percentage of HIV-specific T cells was observed (177,195). These data possibly point to clinical conditions where virus specific CTL may be relatively non-functional.

In these studies no universal stimulation protocol was employed. Some studies used extensive co-stimulation to induce cytokine production. Although costimulation may be relevant for CD\(^4\)\(^+\) T cells to show antigen induced IFN\(_\gamma\) production in vitro, we chose to limit the CD\(^8\)\(^+\) T cell stimulation protocol to presentation of peptide-antigen without costimulation. First, whereas costimulation is provided to CD\(^4\)\(^+\) T cells by their natural antigen presenting cells, effector CD\(^8\)\(^+\) T cells should be able to respond to an infected target cell which does not provide costimulatory signals. Second, in HIV infection, most CD\(^8\)\(^+\) T cells lack CD28. Therefore the effect of CD28 monoclonal antibody is likely to be an indirect stimulus. Third, subtle defects in antigen responsiveness may be masked when too strong stimuli are provided.

Here we studied in detail the dynamics of HIV-specific CD\(^8\)\(^+\)T cells in 3 HIV-infected long-term asymptomatic and 11 individuals progressing to AIDS. Presence and function of HIV-specific T cells was measured by simultaneous tetramer staining and peptide induced IFN\(_\gamma\) production, using several well characterized HLA-A2 or HLA-B8 restricted HIV peptides. The kinetics of antigen-specific and antigen-responsive CD\(^8\)\(^+\) T cells were analyzed in relation with disease progression and CD\(^4\)\(^+\) T cell numbers to elucidate the mechanism of the CTL dysfunction in HIV-infection.
Table 1: Clinical data of the individuals investigated.

<table>
<thead>
<tr>
<th>Subject</th>
<th>HLA</th>
<th>Start of therapy</th>
<th>Time to AIDS</th>
<th>AIDS diagnosis</th>
<th>Dominant response</th>
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<td>ELY</td>
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<sup>1</sup> Months after seroconversion at which HAART is initiated.
<sup>2</sup> Months after seroconversion at which AZT+DDI treatment is initiated.
<sup>3</sup> Months after seroconversion at which AZT treatment is initiated.
<sup>4</sup> Subjects 1140 and 434 did not develop AIDS but started HAART because of high viral load and decreasing CD4 T cell numbers.
<sup>5</sup> Peptides listed in materials section are indicated with the first 3 amino acids.

Materials and methods

Subjects and samples
A total of 14 HIV-1 infected participants of the Amsterdam Cohort Studies were selected for HLA types corresponding to available MHC-peptide tetramers (A2, B8 or B57). Furthermore, the subjects were selected on recorded (189) strong responses to one or more of the tested epitopes (see below), and to include a wide range of disease progression rates. During the follow up, 11 of the 14 subjects developed AIDS (Table 1); these are referred to as progressors. The 3 subjects who remained asymptomatic during the follow-up are referred to as asymptomatics. Peripheral blood mononuclear cells (PBMC), cryopreserved according to a standard computerized freezing protocol, were selected to establish a longitudinal range from seroconversion to the latest sample available or to AIDS diagnosis. PBMC from HIV negative, or HLA mismatched donors served as controls for specificity of tetramers and peptide specific stimulation.
**Figure 1.** Validation of the stimulation protocol. A. CD69 and IFNy expression of CD8+ T cells after incubation with 2µl dimethylsulfoxide (DMSO), 1µg HIV-peptide dissolved in 2µl DMSO or PMA and ionomycin. Upper panels show total PBMC, lower panels are gated on CD8+ T cells. B. Response of EBV- and HIV-specific T cells to in vitro peptide stimulation. The FACS dot-plots are gated on CD8+ T cells and demonstrate tetramer+ cells (Y-axis), and IFNy production (X-axis). Percentages in the right upper corner indicate number of IFNy+ peptide specific T cells (right from the dotted line) of the CD8+ T cell population. C. IFNy-producing antigen-specific T cells after incubation with various stimuli. IFNy+ T cells are shown as percentage of the total number of tetramer+ T cells in the non-stimulated sample.

**Tetrameric HLA-peptide complexes**

Refolding of HLA heavy chains and tetramer formation was performed as described previously (47). HLA heavy chains and B2 microglobulin genes were constructed in pET plasmids and expressed in BL21 E.coli strains. Heavy chain, B2m and peptides were refolded by dilution (167). Peptides derived from p17 Gag and Pol (SLYNTVATL, ILKEPVHGV respectively) were complexed with HLA-A2; p24Gag and Nef peptides (EYKRWII, FLKEKGGGL) were combined with HLA B8 proteins; the peptide KAFSPEVIPMF(p24 Gag) was combined with HLA-B57. Monomeric complexes were concentrated, biotinylated, FPLC purified on a Superdex 200 HR16/60 column (Amersham Pharmacia, Little Chalfont, UK) and bound to streptavidin-PE or streptavidin-APC (Sigma, St Louis, MO). Tetrameric product was FPLC purified.
Loss of HIV-specific IFN\(\gamma^+\)CD8\(^+\) T cells

**Figure 2** Panel I. Longitudinal data of HIV infected individuals during the course of infection. Months are given from time of seroconversion, AIDS diagnosis is indicated with arrows and therapy is indicated with dotted lines. Upper panels show CD4\(^+\) (-■-) and CD8\(^+\) (-●-) T cell numbers and viral RNA copies / ml serum (thick lines). Middle panels indicate absolute numbers of tetramer* T cells (-■-) and IFN\(\gamma\) producing T cells (-○-). HLA restrictions and dominant peptide responses are indicated by the first 3 amino acids of the peptides described in the methods section. The IFN\(\gamma^+\) fraction of tetramer* T cells is plotted in the lower panels (-Δ-).
Figure 2. panel II.

**Antigen-specific stimulation**

Two million PBMC/ml were stimulated with the peptide used in the corresponding tetramer complexes at 37°C for 4 hours in the presence of 3µM monensin. Stimulation protocols were tested for peptide concentrations varying from 0.1 to 10µg peptide/ml, for 4 or 6 hours incubation (for some experiments in the presence of CD28 and CD49d antibodies). HLA mismatched peptide, matched irrelevant peptide, or medium alone were used as negative controls, and stimulation with PMA/ionomycin was used as positive control. After incubation, cells were washed, stained with tetramers (PE or APC) and anti-CD8 (PerCP, Becton Dickinson, San Jose, CA). Additional phenotyping of tetramer+ cells was performed by co-staining for Ki67 FITC (Immunotech), or CD69 APC (BD). Cells were fixed with 4% paraformaldehyde, permeabilised (Permeabilisation kit, BD) and stained intracellularly with IFNγ FITC or PE (BD) and TNFα (FITC, BD). Cells were analyzed using Cellquest software (BD) and gated on live lymphocytes. The IFNγ gate was determined by the negative and positive controls of each individual’s CD8 T cells. The percentages of tetramer+ CD8 T cells and IFNγ+ CD8 T cells were back calculated to absolute numbers per
volume blood by multiplication with absolute CD8+ T cell counts/µl blood. IFNγ+ fractions were calculated by taking the sum of IFNγ+ T cells specific for all tested peptides divided by the sum of tetramer+ T cells for all peptides, as determined in the non-stimulated control sample.

**Statistical analyses**

Early and late timepoints were compared using Wilcoxon signed rank tests and correlations were analysed using Spearman correlation tests.

**Results**

**Validation of the stimulation protocol**

Incubation of PBMC from an HIV+ donor with HIV-peptide or with PMA and ionomycin results in production of IFNγ by CD8+ T cells(49) as indicated in Figure 1A. To establish the optimal stimulation protocol, we stimulated PBMC from a healthy HIV+ EBV+ donor and an HIV+ donor with the EBV peptide "RAFKQKLL" or the HIV-NeF "FLKEKGGL" peptide, respectively, at varying concentrations and durations.

To evaluate IFNγ production of tetramer+ T cells, we combined intracellular IFNγ detection and tetramer staining. In figure 1B, tetramer binding cells and IFNγ producing cells are given as percentages of CD8+ T cells. Incubation with 1µg and 10µg peptide /ml is shown to induce equal amounts of IFNγ+ T cells, but 10µg peptide induced a loss of tetramer+ T cells compared to 1µg peptide. Longer incubation periods (6, 24 hours, not shown) decreased the number of IFNγ+ T cells.

In some studies antibodies against CD28 or CD49d were used to provide costimulation during peptide specific stimulation assays (172,195). When co-stimulation was performed by adding CD28 and CD49d antibodies, the number of IFNγ producing T cells was further increased, but did not alter the peptide dependent response kinetics (Figure 1C). The percentage of tetramer+ T cells producing IFNγ reached 74%, which is in agreement with previous findings for virus specific CD8+ T cells (177,195). Although costimulation may be relevant for CD4+ T cells to show antigen-induced IFNγ production, we chose to limit the CD8+ T cell stimulation protocol to presentation of peptide-antigen without costimulation. We selected the 4-hour stimulation with 1µg peptide / ml for further studies, as IFNγ production reached the maximum under the selected conditions.

**HIV-specific T cells are not physically depleted during progression to AIDS.**

Frequencies of HIV-specific CTLp are reported to decline during the course of infection in most patients, which may result from physical deletion of HIV-specific T cells. By the use of HLA-peptide-tetramers we investigated whether decrease in numbers of HIV specific T cells preceded increase of viral load and progression to AIDS. In figure 2, data from all individuals analyzed are shown, including CD4+ and CD8+ T cell counts and viral RNA load. Since an overall decrease in CD8+ T cell numbers could mask loss of HIV-specific T cells when these are expressed as percentages of CD8+ T cells, we evaluated absolute numbers of tetramer+ T cells per µl blood. Depending on their HLA haplotype, subjects' PBMC were stained with all described tetramers appropriate for individual HLA alleles. Figure 2 middle panels show the number of tetramer+ T cells in time; indicated is HLA restriction and dominant peptides recognized. Absolute numbers of HIV-specific tetramer+ T cells decreased (6 of 14 individuals) or increased (3 of 14) or remained stable (5 of 14) during the follow up. For the whole group, no significant decrease was observed (p=0.64 wilcoxon, Fig.3A). These data indicate that depletion is not the main cause for the loss of HIV-specific CTL.

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**Figure 3.** Absolute numbers of tetramer+ T cells and IFNγ+ T cells early in infection compared to late in infection A. Numbers of HIV-specific tetramer+ T cells/µl blood early after seroconversion compared to late in the asymptomatic state but before start of HAART (asymptomatics, open circles) or around AIDS diagnosis (filled circles). B. Numbers of antigen specific IFNγ producing T cells are significantly decreased from early to late in HIV-infection.
HIV-specific IFNγ producing CD8+ T cells decrease during progression to AIDS.

T cells were stimulated with the same peptide as complexed in the tetramers and peptide-induced IFNγ production was evaluated in PBMC samples drawn during the follow up period. Figure 2, middle panels, and figure 3 show that, although numbers of tetramer+ T cells could rise or fall, the number of IFNγ-producing T cells decreased in most individuals before AIDS diagnosis (12 of 14). We designated the datapoint closest to AIDS diagnosis or the last sample before start of highly active antiretroviral therapy (HAART) as clinical endpoint and compared this with the earliest sample tested. Figure 3B shows that IFNγ+ T cell numbers significantly decreased from early to late in infection (median from 2.4 to 1.1 / μl blood, Wilcoxon, p=0.026). In 2 of 3 subjects who remained asymptomatic, IFNγ+ T cells remained stable (658) or increased (434); the third asymptomatic subject had decreasing IFNγ+ T cells (1140), but therapy may have stopped disease progression.

For 6 individuals, 2 samples were repeated and 2 of these were simultaneously tested in both intracellular cytokine staining and Elispot assay, which showed similar numbers and kinetics of IFNγ+ T cells. Tetramer+ T cells not producing IFNγ after peptide specific stimulation may produce other cytokines or these cells may be in cell division. However, we and others observed that IFNγ was the predominant cytokine produced (data not shown)[177], and TNFα was mainly produced by T cells that also produced IFNγ. Furthermore, the number of HIV-specific T cells in cell division was too low to explain the lack of IFNγ production (mean 3.5% of tetramer+ T cells) and no longitudinal correlation or complementation was observed for IFNγ+ T cells and tetramer+ T cells expressing the cell division marker Ki67 (data not shown).

In most individuals a relatively stable number of tetramer+ T cells was accompanied by an early or late loss of IFNγ producing T cells. A representative example is shown in Figure 4, in which one individuals tetramer+ T cells and IFNγ+ T cells are shown for an early and a late time-point. This discrepancy between tetramer staining and IFNγ production increased with time. These different kinetics are illustrated by the fraction of IFNγ+ T cells within the tetramer+ T cells (IFNγ+/ tetramer+ *100%) in the lower panels of figure 2. In 11 of 14 individuals a decrease of the percentage of IFNγ+ T cells within tetramer+ T cells was observed. In subject 658 no decrease in the percentage or number of IFNγ producing T cells was observed and this individual remained asymptomatic throughout the follow up period.

When comparing the IFNγ+ fraction early after seroconversion, with the IFNγ+ fraction around AIDS diagnosis or to start of HAART, also a significant decrease was observed (median from 20.3% to 12.0%, Wilcoxon, p=0.041, Figure 5A). These percentages are in agreement with earlier findings in a cross-sectional study (189) and with data obtained by combining Elispot assay and tetramer staining, which showed an average of 18% of tetramer+ T cells producing IFNγ+ in 14 HIV infected individuals (Kostense et al. submitted). Interestingly, individuals who received HAART, but also monotherapy, generally showed an increase in the IFNγ+ fraction after initiation of HAART (Figure 2, dotted vertical lines).

To investigate whether costimulation would result in different changes for IFNγ+ T cells, we tested early and late samples of four individuals in a 6 hour incubation assay including 10μg peptide /ml, CD28- and CD49d- antibodies. This resulted in higher numbers of IFNγ+ T cells than obtained without costimulation, but similar trends in time were found (data not shown).
Loss of HIV-specific IFNγ+CD8+ T cells

IFNγ producing HIV-specific T cells correlate with CD4+ T cell counts
There is good evidence that CTL function is dependent on CD4+ T cell help and that this help is reduced in HIV infection (163,184,196). When including all time points of the individuals investigated, numbers of tetramer+ T cells showed no correlation with CD4+ T cell counts (Figure 6A). IFNγ+ T cells and tetramer+ T cells showed a strong correlation as reported before (195) (Figure 6B). In contrast to numbers of tetramer+ T cells, absolute numbers of IFNγ-producing T cells did correlate with CD4+ T cell counts (Figure 6C; p=0.007, R=0.34, Spearman). The percentages of IFNγ+ cells in the tetramer+ T cells and CD4+ T cell numbers were strongly correlated (Figure 6D, p<0.001, R=0.54, Spearman). In a multivariate stepwise regression analysis including absolute numbers and percentages of tetramer+ T cells and IFNγ+ T cells, percentage IFNγ/tetramer and viral load, only the latter two variables were predictive for CD4+ T cell numbers. The percentage IFNγ/tetramer was the strongest predictor with Β=0.614, p<0.001 compared to Β=0.42, p=0.006 for viral load.

Discussion
Despite mounting evidence that HIV-specific CTL are critical for suppressing HIV-viral load, CTL apparently decline and lose control of virus replication, resulting in progression to AIDS in almost all HIV-infected individuals (34). In this study we compared physical presence with functional responsiveness of HIV-specific CD8+ T cells, to investigate whether CTL control is lost due to physical depletion or due to impairment of function. HIV-specific T cells, as measured by tetrameric HLA-peptide complexes, were not depleted during progression to AIDS. In contrast, numbers of in vitro antigen-inducible IFNγ producing T cells decreased in most individuals progressing to AIDS. Similar results have been found for EBV-specific T cells in individuals progressing to AIDS related non-Hodgkin lymphoma (185). Previously, it has been observed that CTLp frequencies decline during progression to AIDS (30), indicating that cytolytic activity of HIV-specific T cells is impaired in the late stages of HIV-infection. Here we used antigen-induced cytokine production as a read out for CD8+ T cell functionality, which was found to decrease in time, irrespective of total numbers of HIV-specific CD8+ T cells.

To determine whether the T cells in the samples investigated were in principle capable of IFNγ production, we stimulated T cells with PMA and ionomycin to bypass early receptor signaling, and followed the IFNγ production of CD8+ and tetramer+ T cells in PBMC collected during the course of HIV-infection. Figure 5B shows that IFNγ production in total CD8+ and tetramer+ T cells under these stimulation conditions did not significantly change during disease progression. This indicates that downstream from protein kinase C (PKC) activation and calcium mobilization, the signaling pathways remained intact during HIV-infection.

Figure 5. Percentages of IFNγ producing T cells early and late in HIV-infection determined after in vitro stimulation with peptide or PMA and ionomycin. A. IFNγ+ fraction of tetramer+ HIV-specific T cells early in infection versus AIDS diagnosis. Twelve of 14 individuals decrease in the fraction of antigen induced IFNγ production during disease progression (p=0.041, Wilcoxon). B. PMA+ ionomycin induced IFNγ production of CD8+ and tetramer+ T cells early and late in HIV infection. PBMC of HIV infected individuals were incubated with PMA and ionomycin to investigate the antigen independent IFNγ production. Filled circles: progressors; open circles: asymptomatics.
In order to compare presence and function of HIV-specific CD8+ T cells, we used the combination of HLA-peptide tetramers and peptide stimulation. Therefore we were restricted to a limited number of epitopes. Although we selected the subjects based on recorded strong responses to the epitopes included, and tested epitopes revealed similar dynamics, other epitopes may be involved. This may explain why the fraction of IFNy+ tetramer+ T cells correlated better with CD4+ T cells than absolute numbers of IFNy+ T cells (Figure 5). The difference between IFNy and tetramer+ T cells may be a good indicator of the general immune function, more or less independent on the peptides investigated (189).

Figure 6. Correlations between HIV specific T cells CD4+ T cells IFNy+ T cells and IFNy+ fractions. All time-points of all subjects are included in each plot. A. Absolute number of tetramer+ T cells do not correlate with CD4+ T cell counts. B. Absolute numbers of IFNy producing CD8+ T cells correlate with absolute numbers of tetramer+ T cells. C. Absolute numbers of IFNy producing CD8+ T cells correlate with absolute numbers of CD4+ T cells. D. IFNy+ percentage of tetramer+ T cells correlates with CD4+ T cell counts.
Secretion of IFNγ is an important effector function of viral suppression in HIV infection and other viral infections (6,7,178). IFNγ production is typically induced shortly after antigenic stimulation (179), and has been shown to be a correlate for cytotoxic T cell function (144). It has been suggested that IFNγ- and IFNγ+ T cells represent central and effector memory T cells, analogous to CD62L-selectin and CCR7 expression (191). In that respect, HIV-specific CD8+ T cells acquire preferentially a central memory phenotype with progressive disease and increasing viral load. The central memory T cells require additional activation to become effector cells (197), which may not be provided for in HIV-infection. HIV-specific T cells that do not produce IFNγ in our assay may indeed have impaired activation kinetics by which antigen stimulation sufficient in early asymptomatic conditions may fail to trigger T cells later in HIV-infection. Costimulation may decrease the threshold for T cells to respond, and may partially compensate for the decreased reactivity, as has been shown before (44).

Interestingly, antigen-independent stimulation (PMA + ionomycin) did not show the same decline as antigen-induced IFNγ production. Apparently, the activation pathways downstream of PKC activation and Ca2+ mobilisation are not affected during progression to AIDS, but the T cell is not capable to induce IFNγ production upon cognate TCR triggering. In this respect, the CD3ζ chain may be involved in this defect, as this component of the TCR complex is down-regulated in CD8+ T cells of HIV-infected individuals (198,199). These results are consistent with the reported loss of CD3/TCR mediated T cell activation during the course of HIV-infection (42,44).

CD4+ T cell counts were correlated with the fraction of IFNγ+ T cells, which suggests that CD4+ T cell help is required for functional CD8+ T cells. Zajac et al. have shown that CD4 knock out mice can maintain high numbers of virus specific CD8+ T cells, but that these cells lack IFNγ production, whereas wild type mice had high numbers of IFNγ+ T cells and were able to eliminate LCMV infection (146). Several additional studies have pointed to the dependence of specific CD8+ T cell function on CD4+ T cell help (163,196,200). Early after primary infection, HIV-specific CD4+ T cells are no longer detectable (184) and only in long-term asymptomatics CD4+ T cell responses may to some extend be preserved (173). Our results are in agreement with the need of CD4+ T cell help, but additional studies are required to show that depletion of HIV-specific CD8+ T cells precedes the CD8+ T cell dysfunction observed here.

In conclusion, our results show that the decrease in CTL activity is not invariably caused by a physical depletion of the number of HIV-specific T cells but that antigen induced IFNγ production of HIV-specific CD8+ T cells is gradually deteriorated during disease progression. This provides an explanation for the failure to prevent progression to AIDS despite initially strong HIV-specific CD8+ T cell responses. It has been attempted to restore T cell immunity to HIV by autologous CTL transfusion, which showed no long-term improvement until now (27,145). To improve T cell function, various immunogenic (201,202) or immuno-therapeutic drugs have been used (81,203), with varying success. Our results show that immune-based therapies should focus primarily on improving T cell quality above quantity, to enhance efficient HIV-specific CD8+ T cell responses.

Acknowledgements
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