Persistent numbers of tetramer$^+$ CD8$^+$ T cells, but loss of interferon-γ$^+$ HIV-specific T cells during progression to AIDS

Stefan Kostense, Kristin Vandenberghe, Jeanine Jolinge, Debbie Van Baarle, Nening Nanlohy, Erik Manting, and Frank Miedema

Although CD8$^+$ T cells initially suppress human immunodeficiency virus (HIV) replication, cytotoxic T-cell precursor frequencies eventually decline and fail to prevent disease progression. In a longitudinal study including 16 individuals infected with HIV-1, we studied both the number and function of HIV-specific CD8$^+$ T cells by comparing HLA-peptide tetramer staining and peptide-induced interferon-γ (IFN-γ) production. Numbers of IFN-γ$^+$-producing T cells declined during progression to acquired immunodeficiency syndrome (AIDS), whereas the number of tetramer$^+$ T cells in many individuals persisted at high frequencies. Loss of IFN-γ$^+$-producing T cells correlated with declining CD4$^+$ T-cell counts, consistent with the need of CD4$^+$ T-cell help in maintaining adequate CD8$^+$ T-cell function. These data indicate that the loss of HIV-specific CD8$^+$ T-cell activity is not due to physical depletion, but is mainly due to progressively impaired function of HIV-specific CD8$^+$ T cells. (Blood. 2002; 99:2505-2511)

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Patients, materials, and methods

Subjects and samples

Sixteen HIV-1 infected participants of the Amsterdam Cohort Studies were selected for HLA types corresponding to available major histocompatibility complex (MHC)–peptide tetramers (A2, B8, or B57). Furthermore, the subjects were selected on the basis of recorded strong responses to one or more peptide-antigen combinations.

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more of the tested epitopes (see below), and to include a wide range of disease progression rates. During the follow-up, 11 of the 16 subjects developed AIDS (Table 1); these are referred to as progressors. The 5 subjects who remained asymptomatic during the follow-up are referred to as asymptomatics. Peripheral blood mononuclear cells (PBMCs), 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. PBMCs from HIV+ or HLA-mismatched donors served as controls for specificity of tetramers and peptide-specific stimulation.

Tetrameric HLA-peptide complexes

Refolding of HLA heavy chains and tetramer formation was performed as described previously.11 HLA heavy chains and β2-microglobulin genes were constructed in pET plasmids and expressed in BL21
dilution. 25 Peptides derived from p17 Gag and Pol (SL YNTV A TL,
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>131</td>
<td>Kaposi sarcoma</td>
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<td>SLY</td>
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<td>123‡</td>
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<td>33‡</td>
<td>33</td>
<td>Pneumocystis carinii</td>
<td>E1Y</td>
</tr>
</tbody>
</table>

Cryp indicates cryptococcus; CMV, cytomegalovirus.

*Peptides listed in materials section are indicated with the first 3 amino acids.
†Months after seroconversion at which zidovudine (AZT) treatment is initiated.
§Months after seroconversion at which HAART is initiated.
¶Months after seroconversion at which AZT plus didoxysine (DDI) treatment is initiated.

Antigen-specific stimulation

Two million PBMCs per milliliter were stimulated with the peptide used in the corresponding tetramer complexes at 37°C for 4 hours in the presence of 3 μM monensin. Stimulation protocols were tested for peptide concentrations varying from 0.1 to 10 μg 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 peptide from a healthy HIV-EBV donor was used as negative control, and stimulation with phorbol myristate acetate (PMA)/ionomycin was used as a positive control. After incubation, cells were washed and stained with tetramers (PE or APC) and anti-CD8 T cells. Incubation with 1 μg and 10 μg peptide/mL is shown to induce equal amounts of IFN-γ+ T cells, but 10-μg peptide induced a loss of tetramer+ T cells compared to 1 μg peptide. Longer incubation periods (6, 24 hours, not shown) decreased the number of IFN-γ+ T cells.

Results

Validation of the stimulation protocol

Incubation of PBMCs from an HIV+ donor with HIV-peptide or with PMA and ionomycin results in production of IFN-γ by CD8+ T cells as indicated in Figure 1A.12 To establish the optimal stimulation protocol, we stimulated PBMCs from a healthy HIV-EBV+ donor and an HIV+ donor with the EBV peptide "RAKFQL" or the HIV-Nef "FLKEKGGL" peptide, respectively, at varying concentrations and durations. To evaluate IFN-γ production of tetramer+ T cells, we combined intracellular IFN-γ detection and tetramer staining. In Figure 1B, tetramer binding cells and IFN-γ-producing cells are given as percentages of CD8+ T cells. Incubation with 1 μg and 10 μg peptide/mL is shown to induce equal amounts of IFN-γ+ T cells, but 10-μg peptide induced a loss of tetramer+ T cells compared to 1 μg peptide. Longer incubation periods (6, 24 hours, not shown) decreased the number of IFN-γ+ T cells.

In some studies antibodies against CD28 or CD49d were used to provide costimulation during peptide-specific stimulation assays.4,20 When costimulation was performed by adding CD28 and peptide-specific stimulation.
CD49d antibodies, the number of IFN-γ-producing T cells was further increased, but did not alter the peptide-dependent response kinetics (Figure 1C). The percentage of tetramer+ T cells producing IFN-γ reached 74% for EBV-specific T cells, which is in agreement with previous findings for virus-specific CD8+ T cells.23,24 Although costimulation may be relevant for CD4+ T cells to show antigen-induced IFN-γ production, we chose to limit the CD8+ T-cell stimulation protocol to presentation of 1 μg peptide-antigen/mL without costimulation.

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

Frequencies of HIV-specific CTL precursors are reported to decline during the course of infection in most patients, which may result from physical deletion in HIV-specific T cells. By the use of HLA-peptide tetramers we investigated whether the decrease of HIV-specific T-cell numbers preceded progression to AIDS. In Figure 2, data from all individuals analyzed are shown, including CD4+ and CD8+ T-cell counts and viral RNA load. Because 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 microliter blood. Subjects’ PBMCs were stained with all described tetramers appropriate for individual HLA alleles. IFN-γ responses were tested for the same peptides as used in the tetramers. The middle panels in Figure 2 show the number of tetramer+ T cells in time; HLA restriction and dominant peptides recognized are indicated. Absolute numbers of HIV-specific tetramer+ T cells decreased (7 of 16 individuals) or increased (4 of 16) or remained stable (5 of 16) during the follow-up. In general, no significant decrease was observed (Figure 3A). These data indicate that depletion is not the main cause for the loss of HIV-specific CTLs.

**Numbers of HIV-specific IFN-γ-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 IFN-γ production was evaluated in PBMC samples drawn during the follow-up period. The middle panels in Figure 2 and Figure 3 show that, although numbers of tetramer+ T cells could rise or fall, the number of IFN-γ-producing T cells decreased in most individuals before AIDS diagnosis (10 of all 16 subjects, 9 of 11 progressors). We designated the data point closest to AIDS diagnosis or the last sample before start of highly active antiretroviral therapy (HAART) as clinical end point and compared this with the earliest sample tested. Figure 3B shows that IFN-γ+ T cell numbers in all 16 subjects decreased from early to late in infection (median from 3.24 to 1.07/μL blood) and this was mainly due to decreasing IFN-γ+ T-cell numbers of the 11 progressors (median from 2.42 to 0.66/μL blood). In the 5 subjects who remained asymptomatic, IFN-γ+ T cells remained stable or increased; one asymptomatic subject (1140) had severely decreased IFN-γ+ T cells, but therapy may have stopped disease progression.

Tetramer+ T cells not producing IFN-γ after peptide-specific stimulation may produce other cytokines or these cells may be in cell division. However, we and others observed that IFN-γ was the predominant cytokine produced (data not shown and Appay et al23), and TNF-α was mainly produced by T cells that also produced IFN-γ. Furthermore, the number of HIV-specific T cells in cell division was too low to explain the lack of IFN-γ production (mean 3.5% of tetramer+ T cells, n = 4) and no longitudinal correlation or complementation was observed for IFN-γ+ T cells and tetramer+ T cells expressing the cell division marker Ki67 (data not shown).

In most individuals progressing to AIDS, a relatively stable number of tetramer+ T cells was accompanied by an early or late loss of IFN-γ-producing T cells. A representative example is shown in Figure 4, in which one individual’s tetramer+ T cells and IFN-γ+ T cells are shown for an early and a late time point. This discrepancy between tetramer staining and IFN-γ production increased with time. These different kinetics are illustrated by the fraction of IFN-γ+ T cells within the tetramer+ T cells (IFN-γ+/tetramer+ × 100%) in the lower panels of Figure 2. In 13 of 16 individuals a decrease of the percentage of IFN-γ+ T cells within tetramer+ T cells was observed. When comparing the IFN-γ+ fraction early after seroconversion, with the IFN-γ+ fraction around AIDS diagnosis, start of HAART, or latest time point available in the asymptomatic phase, a significant decrease was observed (median from 19.3% to 12.0%, n = 16), and specifically the progressors showed a significant decrease (Figure 5A).
percentages are in agreement with earlier findings in cross-sectional studies\(^\text{18,21}\) and with data obtained by combining Elispot assay and tetramer staining, which showed an average of 18% of tetramer\(^+\) T cells producing IFN-\(\gamma\) in 14 HIV-infected individuals.\(^\text{49}\) Interestingly, individuals who received HAART, but occasionally also dual therapy or monotherapy, generally showed an increase in the IFN-\(\gamma\) fraction after initiation of HAART (Figure 2, dotted vertical lines).

From 5 subjects we repeated tetramer staining and IFN-\(\gamma\) measurement on the early and late samples. When available the same time points were used. These second measurements showed similar IFN-\(\gamma\)/tetramer percentages except for one aberrant measurement of subject 57 (closed triangles in Figure 2, lower panels). Furthermore, 2 subjects (748 and 658) were simultaneously tested in Elispot assays, and Elispot results matched intracellular cytokine staining results (data not shown).

To investigate whether costimulation would result in different changes or IFN-\(\gamma\)\(^+\) T cells in HIV-infected patients over time, we tested early and late samples of 4 individuals in a 6-hour incubation assay including 10 \(\mu\)g peptide/mL, CD28, and CD49d antibodies. This resulted in higher numbers of IFN-\(\gamma\)\(^+\) T cells than obtained without costimulation, but similar trends in time were found (Figure 5B).

To determine whether the T cells in the samples investigated were in principle capable of IFN-\(\gamma\) production, we stimulated T cells with PMA and ionomycin to bypass early receptor signaling, and followed the IFN-\(\gamma\) production of CD8\(^+\) T cells and tetramer\(^+\) T cells in PBMCs collected during the course of HIV infection.
There is good evidence that CTL function is dependent on CD4+ T-cell help and that this help is reduced in HIV infection.27-29 When including all time points of the individuals investigated, numbers of tetramer+ T cells showed no correlation with CD4+ T-cell counts (Figure 6A). IFN-γ+ T cells and tetramer+ T cells showed a strong correlation as reported before24 (Figure 6B). In contrast to numbers of tetramer+ T cells, absolute numbers of IFN-γ–producing T cells did correlate with CD4+ T-cell counts (Figure 6C). The percentages of IFN-γ+ cells in the tetramer+ T cells and CD4+ T-cell numbers were strongly correlated (Figure 6D). CD4+ T cells and IFN-γ+ T cells or IFN-γ/tetramer percentages showed best correlations within the progressors, whereas tetramer staining and IFN-γ production were best correlated within the asymptomatic group (data not shown).

In a multivariate stepwise regression analysis including absolute numbers and percentages of tetramer+ T cells and IFN-γ+ T cells, percentage IFN-γ/tetramer and viral load, only the latter 2 variables were predictive for CD4+ T-cell numbers. The percentage IFN-γ/tetramer was the strongest predictor with β = 0.614, P < .0001 compared to β = 0.42, P = .006 for viral load.

**Discussion**

Despite mounting evidence that HIV-specific CTLs are critical for suppressing HIV viral load, CTLs appear to decline and lose control of virus replication, resulting in progression to AIDS in almost all HIV-infected individuals.10 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 suppressed after in vitro stimulation with peptide, peptide and costimulation, or PMA and ionomycin. Data from progressors (n = 11, ●) include early samples versus samples drawn during AIDS diagnosis. Data from asymptomatics (n = 5, ○) are early samples versus latest sample available before HAART. (A) IFN-γ+ fraction of tetramer+ HIV-specific T cells early versus late in infection, measured by stimulation of PBMCs with 1 μg peptide/mL. Thirteen of all 16 individuals showed a decrease in the fraction of antigen-induced IFN-γ production during disease progression (n = 16; P = .028, Wilcoxon). Progressors and asymptomatics had different dynamics of the IFN-γ+ fraction as shown by different P values. (B) IFN-γ+ fraction of tetramer+ HIV-specific T cells after stimulation with 10 μg peptide in the presence of CD28 and CD49d costimulation. (C) PBMCs of HIV-infected individuals were incubated with PMA and ionomycin to investigate anti-tetramer IFN-γ production during the course of HIV infection. Included are percentages IFN-γ producing CD8+ T cells (left panel) and tetramer+ T cells (right panel).

Figure 5. Percentages of IFN-γ–producing T cells early and late in HIV infection determined after in vitro stimulation with peptide, peptide and costimulation, or PMA and ionomycin. Data from progressors (n = 11, ●) include early samples versus samples drawn during AIDS diagnosis. Data from asymptomatics (n = 5, ○) are early samples versus latest sample available before HAART. (A) IFN-γ+ fraction of tetramer+ HIV-specific T cells early versus late in infection, measured by stimulation of PBMCs with 1 μg peptide/mL. Thirteen of all 16 individuals showed a decrease in the fraction of antigen-induced IFN-γ production during disease progression (n = 16; P = .028, Wilcoxon). Progressors and asymptomatics had different dynamics of the IFN-γ+ fraction as shown by different P values. (B) IFN-γ+ fraction of tetramer+ HIV-specific T cells after stimulation with 10 μg peptide in the presence of CD28 and CD49d costimulation. (C) PBMCs of HIV-infected individuals were incubated with PMA and ionomycin to investigate anti-tetramer IFN-γ production during the course of HIV infection. Included are percentages IFN-γ producing CD8+ T cells (left panel) and tetramer+ T cells (right panel).

IFN-γ/tetramer was the strongest predictor with β = 0.614, P < .0001 compared to β = 0.42, P = .006 for viral load.
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 readout for CD8\(^+\) T-cell functionality, which was found to decrease in time, irrespective of total numbers of HIV-specific CD8\(^+\) T cells.

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 the tested epitopes revealed similar dynamics, other epitopes may be involved. This may explain why the fraction of IFN-\(\gamma\)-producing CD8\(^+\) T cells correlated better with CD4\(^+\) T-cell numbers than absolute numbers of IFN-\(\gamma\)-producing CD8\(^+\) T cells (Figure 5). The difference between IFN-\(\gamma\)-producing CD8\(^+\) T cells and IFN-\(\gamma\)-producing CD8\(^+\) T cells correlated better with absolute numbers of tetramer\(^+\) T cells. (B) IFN-\(\gamma\)-producing CD8\(^+\) T cells correlate with absolute numbers of CD8\(^+\) T cells. (C) IFN-\(\gamma\) percentage of tetramer\(^+\) T cells correlates with CD4\(^+\) T-cell counts.

Figure 6. Correlations between HIV-specific T cells CD4\(^+\) T cells IFN-\(\gamma\)-producing CD8\(^+\) T cells and IFN-\(\gamma\)-producing CD8\(^+\) T cells. All time points of all subjects are included in each plot. Filled symbols indicate progressors (\(n = 11\)), open symbols, asymptomatics (\(n = 5\)). HLA restrictions are indicated. (A) Absolute numbers of tetramer\(^+\) T cells do not correlate with CD4\(^+\) T-cell counts. (B) Absolute numbers of IFN-\(\gamma\)-producing CD8\(^+\) T cells correlate with absolute numbers of tetramer\(^+\) T cells. (C) Absolute numbers of IFN-\(\gamma\)-producing CD8\(^+\) T cells correlate with absolute numbers of CD4\(^+\) T cells. (D) IFN-\(\gamma\) percentage of tetramer\(^+\) T cells correlates with CD4\(^+\) T-cell counts.

Secretion of IFN-\(\gamma\) is an important effector function of viral suppression in HIV infection and other viral infections.\(^{30,32}\) IFN-\(\gamma\) production is typically induced shortly after antigenic stimulation,\(^{31}\) and has been shown to be a correlate for cytotoxic T-cell function.\(^{34}\) In healthy individuals, EBV-specific T cells could also be divided in IFN-\(\gamma\)^- and IFN-\(\gamma\)^+ T cells, paralleled by CD62L-selectin and CCR7 expression.\(^{17}\) These populations may represent functionally different subsets that can be distinguished by CCR7 expression and have different activation requirements.\(^{17,35}\) However, HIV-specific T cells were found to be mainly CCD7\(^-\), but also CD45RA\(^-\), indicating a skewed maturation, different from other virus-specific T cells.\(^{36}\)

The HIV-specific T cells that do not produce IFN-\(\gamma\) in our assay may indeed have impaired activation kinetics whereby antigen stimulation sufficient to trigger T cells in early asymptomatic conditions may fail later in HIV infection. Our results show that costimulation increases the range of IFN-\(\gamma\) production, resulting in higher numbers of IFN-\(\gamma\)-producing T cells. Costimulation may decrease the threshold for T cells to respond and may partially compensate for decreased T-cell reactivity, as has been shown before.\(^{37}\) Interestingly, T-cell function on antigen-independent stimulation (PMA + ionomycin) did not show the same decline as antigen-induced IFN-\(\gamma\) production. Apparently, the activation pathways downstream of PKC activation and Ca\(^{2+}\) mobilization are not affected during progression to AIDS, but the T cell is not able to induce IFN-\(\gamma\) production on cognate TCR triggering. In this respect, the CD3z 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.\(^{38,39}\) These results are consistent with the reported loss of CD3/TCR-mediated T-cell activation during the course of HIV infection.\(^{37}\)

The CD4\(^+\) T cell counts were correlated with the fraction of IFN-\(\gamma\)^+ T cells, which suggests that CD4\(^+\) T-cell help is required for functional CD8\(^+\) T cells. Zajac et al\(^{40}\) have shown that CD4 knockout mice can maintain high numbers of virus specific CD8\(^+\) T cells, but that these cells lack IFN-\(\gamma\) production, whereas wild-type mice had high numbers of IFN-\(\gamma\)^+ T cells and were able to eliminate lymphocytic choriomeningitis virus infection. Several additional studies have pointed to the dependence of specific CD8\(^+\) T-cell function on CD4\(^+\) T-cell help.\(^{27,29,41}\) Shortly after primary infection, HIV-specific CD4\(^+\) T cells are no longer detectable\(^{28}\) and only in long-term asymptomatics are CD4\(^+\) T-cell responses, to some extent, preserved.\(^{42}\) 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 CD4\(^+\) 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 IFN-\(\gamma\) production of HIV-specific CD8\(^+\) T cells gradually deteriorates during disease progression. This provides an explanation for the failure to prevent progression to AIDS despite initially strong HIV-specific CD8\(^+\) T-cell responses. Attempts have been made to restore T-cell immunity to HIV by autologous CTL transfusion; however, no long-term improvement has been observed.\(^{43,44}\) To improve T-cell function, various immunogenic\(^{35,46}\) or immunotherapeutic drugs have been used,\(^{47,48}\) 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.

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References


