Cytomegalovirus-specific T-cell dynamics in HIV infection
Bronke, C.

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Characterisation of virus-specific CD8$^+$ effector T cells in the course of HIV-1 infection: longitudinal analyses in slow and rapid progressors

Christine A. Jansen$^1$, Erwan Piriou$^1$, Corine Bronke$^1$, José Vingerhoed$^2$, Stefan Kostense$^{1,*}$, Debbie van Baarle$^{1,#}$ and Frank Miedema$^{1,2,#}$

$^1$Department of Clinical Viro-Immunology, Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands
$^2$Department of Human Retrovirology, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands
$^*_{Current address:}$ Department of Immunology, University Medical Centre Utrecht, Lundlaan 6, 3584 EA Utrecht, the Netherlands
$^#_{Current address:}$ Crucell Holland BV, Archimedesweg 4, 2301 CS Leiden, the Netherlands

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ABSTRACT

Studies in humans have provided evidence that CD8$^+$ T cells exhibit distinct phenotypical and functional properties dependent on virus specificity. It is not known how these T-cell phenotypes develop over the course of infection. Dynamics and properties of T cells specific for human immunodeficiency virus (HIV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) in HIV infection were investigated in relation to viral load. In rapid progressors, HIV-specific CD8$^+$ T cells were less differentiated early in infection and did not develop a more differentiated phenotype. In slow progressors, perforin expression of HIV-specific CD8$^+$ T cells slightly increased over time. HIV and EBV load was detectable in all individuals, while CMV load could not be detected. Thus, in individuals with progressive HIV infection, HIV-specific T cells are less differentiated already early in infection. This apparent block in differentiation may be partly caused by chronic viremia or lack of CD4$^+$ T-cell help.
virus-specific CD8+ T cells in HIV infection

INTRODUCTION

CD8+ cytotoxic T lymphocytes (CTL) may be able to control human immunodeficiency virus (HIV) for some time, but eventually, this control fails leading to progression to AIDS [1]. In contrast, infection with other persistent viruses like cytomegalovirus (CMV) and Epstein-Barr virus (EBV) is controlled for life in most people. After exposure to antigen, CD8+ CTL are activated and can induce death of infected targets via several mechanisms [2]. They can produce cytokines like TNFα and IFNγ, capable of inhibiting viral replication [3]. Another mechanism to induce cell death is the Fas-FasL pathway, via activation of the caspase cascade [4,5], but the main mechanism by which CD8+ CTL are able to induce death of their targets is the granule exocytosis pathway that is mediated by the pore-forming protein perforin and granzyme B [6,7]. Granzyme B, the principle granzyme in CTL mediated apoptosis [8,9], belongs to the family of the neutral serine proteases and induces apoptosis by triggering activation of caspases within target cells [10]. Granzymes and perforin induce target-cell apoptosis cooperatively [11].

Upon antigen recognition, naive CD8+ T cells differentiate into memory and effector T cells. In humans, this is most likely mediated by a linear differentiation process that eventually results in the formation of fully differentiated effector T cells [12,13]. Different stages of CD8+ T-cell differentiation [14] can be characterised based on the expression of TNF-receptor family members like CD27 [15,16]. Expression of this molecule is irreversibly lost upon differentiation to CD8+ effector T cells.

Although HIV-, EBV- and CMV-specific T cells are present in high numbers during chronic infection [17-20], they are not always capable of controlling viral infection. For example, in HIV infection, immune control is lost in individuals progressing to AIDS, despite high numbers of HIV-specific T cells during the course of infection [21]. We have shown that EBV-specific CD8+ T-cell numbers are maintained in HIV-infected individuals who develop AIDS-related non-Hodgkin lymphoma (AIDS-NHL), whereas the number of EBV-specific IFNγ-producing cells decreases [22]. Several groups have studied the effector phenotype of virus-specific T cells and showed that functional impairment may be a consequence of either impaired maturation [23-25] or anergy [2,26]. However, until now, results have been inconclusive and contradictory. Recently, we showed that in the course of HIV infection, in transsectional studies in general, a lack of differentiation of HIV-specific but not of EBV-specific CTL can be observed. However, in HIV-infected individuals who develop AIDS-NHL, EBV-specific CTL remain of the CD27+ phenotype. This indicates that the block in differentiation is not specific for HIV-specific CTL but may be related to disease progression. Furthermore, a correlation between the percentage CD27+ T cells and high IFNγ production by virus-specific T cells was found [27].

In cross-sectional analyses, it is difficult to establish a relation between numbers of HIV-specific CD8+ T cells, their differentiation status, high viremia and progressive disease [28,29]. Longitudinal studies in which expression of CD27, perforin and granzyme B by different virus-specific T cells was analysed during HIV infection have not been reported so far. Here, a detailed longitudinal analysis of HIV-, EBV- and
CMV-specific CD8\(^+\) T cells was performed in the course of HIV infection to investigate the role of T-cell differentiation and expression of effector molecules for the different viruses in control of viral infection. Both differentiation and effector phenotypes of virus-specific CD8\(^+\) T cells against HIV, EBV and CMV were investigated during HIV infection in rapid and slow progressors to AIDS.

**Materials and Methods**

**Study population**

This study was performed on samples from participants of the Amsterdam Cohort on HIV-1 infection and AIDS. HIV-seropositive male individuals were selected for HLA types corresponding to available MHC-tetramers. Only HLA-A2- or B8-positive individuals were included, one individual was positive for HLA-A2 and B8. Peripheral blood mononuclear cells (PBMC) were cryopreserved according to a standard protocol in a computerised freezing device. Longitudinal PBMC samples from 13 individuals were analysed. Six of these individuals were defined as rapid progressors based on progression to AIDS within six years of infection and CD4 counts of < 300/\(\mu l\) during the fourth year of follow-up. One individual progressed to AIDS within six years despite CD4 counts of > 300 cells per \(\mu l\). This individual is also included as rapid progressor.

**Table I. Characteristics of the study participants**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Classification</th>
<th>HLA type</th>
<th>Months AIDS</th>
<th>CD4 T-cells (cells/(\mu l))</th>
<th>CD8 T-cells (cells/(\mu l))</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>early</td>
<td>late*</td>
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<tr>
<td>57</td>
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<td>A2</td>
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<tr>
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<td>177</td>
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<td>B8</td>
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<td>A2</td>
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<td>A2</td>
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<td>A2</td>
<td>60</td>
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<td>220</td>
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<tr>
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<td>A2, B8</td>
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<td>rapid</td>
<td>B8</td>
<td>73</td>
<td>300</td>
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</table>

\(^a\) CD4\(^+\) and CD8\(^+\) T cell counts at time points analysed in this study. Early: 1 year after seroconversion; late: around AIDS diagnosis or in the case of slow progressors on average 10 years after seroconversion. For definitions of slow and rapid progressors, see Material and Methods.
The group of slow progressors consists of seven individuals, characterised by a period of more than 7 years of asymptomatic follow-up and CD4 counts of > 400/μl during the seventh year of follow-up. Individuals were analysed both early (1 to 2 years after HIV seroconversion) and late (around AIDS diagnosis or in the case of slow progressors on average 10 years after seroconversion). None of these individuals received anti-retroviral therapy during the study period. From all participants of the Amsterdam Cohort studies on HIV infection and AIDS informed written consent was obtained, and this study has been approved by the Medical Ethical Committee. Characteristics of these HIV-infected individuals are summarised in Table 1.

**Flow cytometry and tetramer staining**

MHC class I tetramers complexed with virus-specific peptides were produced as previously described [21,30]. Immunodominant peptides from HIV [31,32], EBV [33,34] and CMV [35] were selected (Table 2) and synthesised (Netherlands Cancer Institute, Amsterdam, the Netherlands). Refolded HLA-peptide complexes were biotinylated and subsequently tetramerised by the addition of allophycocyanin streptavidin. Four-colour flow cytometry analysis was performed. Briefly, PBMC were thawed and 1.5 x 10^6 cells were stained in PBS supplemented with 0.5% (v/v) bovine serum albumin (PBA) with MHC class I tetramers and fluorochrome-conjugated monoclonal antibodies CD8 and CD27 (Becton Dickinson (BD), San José, California, USA; Sanquin Reagents, Amsterdam, the Netherlands). After fixation and permeabilisation (BD reagents), cells were stained intracellularly with fluorochrome-labelled monoclonal antibodies against the effector molecules perforin (BD) and granzyme B (Sanquin Reagents). Next, cells were fixed in Cellfix (BD) and at least 250,000 events were acquired using a FACSCalibur flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter. Data were analysed using the software program CELLQuest (BD).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Epitope</th>
<th>Protein</th>
<th>HLA</th>
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</thead>
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<tr>
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<td>SLYNTVATL</td>
<td>Gag</td>
<td>A2</td>
</tr>
<tr>
<td></td>
<td>FLKEKKGL</td>
<td>Nef</td>
<td>B8</td>
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<td>RAKFKQQL</td>
<td>BZLF-1</td>
<td>B8</td>
</tr>
<tr>
<td>CMV</td>
<td>NLVPMVATV</td>
<td>pp65</td>
<td>A2</td>
</tr>
</tbody>
</table>

**Viral load determination**

HIV RNA load was quantitated in serum using NASBA (Organon Teknika, Boxtel, the Netherlands), with a detection limit of 1,000 copies/ml. EBV load was determined by real-time quantitative PCR amplification as described previously [36,37], using 1 x 10^5 PBMC in duplicate. The detection limit of this assay is 50 copies. CMV viral load was determined in 1 x 10^6 PBMC with the COBAS AMPLICOR™ CMV MONITOR.
KIT (Roche Diagnostics, Almere, the Netherlands) [38,39]. The detection limit is 100 copies. Copy numbers between 1 and 100 are considered to be positive but cannot be quantitated.

**Statistical analyses**

Nonparametric statistical tests were used to avoid assumption of normally distributed data sets. Differences between the virus-specific CD8⁺ T cells were initially analysed by Kruskal-Wallis tests. If significant differences were observed, Mann-Whitney tests between two groups were performed. Differences between slow and rapid progressors were analysed using Mann-Whitney tests. Wilcoxon tests were performed to determine differences within virus-specific T cells in time. Correlations were tested using the Spearman’s non-parametric correlation test. All statistical analyses were performed using the software program SPSS 11.5 (SPSS Inc., Chicago, Illinois, USA).

**RESULTS**

**HIV-, EBV- and CMV-specific CD8⁺ T-cell numbers in the course of HIV infection in slow and rapid progressors to AIDS**

To enumerate virus-specific CD8⁺ T cells in the course of HIV infection, PBMC from HIV-infected individuals were stained early and late in infection using HLA-A2 tetrameric complexes loaded with immunodominant peptides derived from HIV, EBV or CMV (Table 2). Two groups of HIV-infected individuals with a known date of seroconversion were selected based on disease progression. A representative staining using the different tetramers in a slow and rapid progressor at a time point late in infection (Figure 1) shows that virus-specific T cells for all three viruses were readily detectable during HIV infection. In the course of HIV infection, in slow progressors, the median percentage of CMV-specific T cells within CD8⁺ T cells increased from 2.27% (range 0.3-2.8) to 4.2% (range 0.7-6.6), p=0.08, Wilcoxon. The median percentage of EBV-specific T cells remained constant during HIV infection (early 0.2% (range 0.12-0.25); late 0.2% (range 0.03-0.38), p=1.0). The median percentage of HIV-specific CD8⁺ T cells decreased from 0.46% (range 0.1-1.2) to 0.20% (range 0.1-2.85), p=0.04. In individuals who progressed rapidly to AIDS no changes in median percentages of HLA-A2-restricted virus-specific CD8⁺ T cells were observed during HIV infection. Late in infection frequencies of HIV- and EBV-specific HLA-A2-restricted CD8⁺ T cells in rapid progressors were similar to frequencies in slow progressors. However, frequencies of HLA-A2-restricted CMV-specific CD8⁺ T cells were increased (median 4.2% (range 0.7-6.6), p=0.02, Mann-Whitney).

**Effector phenotypes, as determined by the expression of perforin, granzyme B and CD27 in HIV-, EBV- and CMV-specific CD8⁺ T cells in the course of HIV infection**

To investigate possible differences in expression of CD27 and the effector molecules perforin and granzyme B by CD8⁺ T cells specific for HIV, EBV or CMV the
expression of the different molecules was analysed within the tetramer-positive CD8⁺ T-cell populations in 5 slow and 5 rapid progressors. Figure 2 shows a representative staining of a slow progressor (A) and a rapid progressor to AIDS (B) at a time point late in infection. Results of analyses of effector molecules in all HLA-A2⁺ individuals (n=10) in this study are shown in Figure 3. In rapid progressors, early in infection, significantly more CD27⁺ T cells were found within the CMV-specific CD8⁺ T cells (median 52.9%) compared to both HIV- (median 23.7%, p=0.03, Mann-Whitney) and EBV-specific CD8⁺ T cells (median 29.9%, p=0.03). In the course of HIV infection, the fraction of CD27⁺ CMV- and EBV-specific CD8⁺ T cells tended to increase to, respectively, 61.5% (p=0.35, Wilcoxon) and 43.2% (p=0.07), while expression of CD27 by HIV-specific CD8⁺ T cells remained stable (23.5%, p=0.89). This resulted in significant difference in CD27 expression between CMV- and HIV-specific CD8⁺ T cells late in infection (p=0.01, Mann-Whitney), which has been reported in cross-sectional studies before (Figure 3F).

The median percentage of CMV-specific CD27⁺ CD8⁺ T cells in slow progressors increased from 68.5 to 85.6% (p=0.08, Wilcoxon), the percentage of EBV-specific CD27⁺ CD8⁺ T cells slightly increased, although not significant, from 32.1 to 48.1% (p=0.50). Similar results were found for the percentage of HIV-specific CD27⁺ CD8⁺ T cells (median early 18.2%, late 31.6%, p=0.35). No differences in CD27 expression were found between HIV-, EBV- and CMV-specific CD8⁺ T cells, although the latter showed a trend towards less CD27 expression compared to EBV-specific CD8⁺ T cells (early p=0.06; late p=0.10, Mann-Whitney) (Figure 3C).
Figure 2. Representative staining of virus-specific CD8\(^+\) T cells for the differentiation marker CD27 and the effector molecules perforin and granzyme B. PBMC of a slow progressor (upper panel) and an individual progressing to AIDS (lower panel) were stained late in infection for CD27, perforin and granzyme B in combination with different HLA-A2 tetramers. Numbers indicate the percentage positive cells within the tetramer\(^+\) CD8\(^+\) population.

Perforin showed a similar expression pattern as CD27. In rapid progressors, the perforin expression of CMV- and EBV-specific CD8\(^+\) T cells slightly increased in the course of HIV infection from, respectively, 15.6 and 14.8\% (early) to 22.5 and 20.4\% (late) (p=0.14 and p=0.14, respectively, Mann-Whitney) while the expression of HIV-specific CD8\(^+\) T cells remained constant (median early 6.5\%, late 5.7\%, p= 0.69). This resulted in a slightly higher perforin expression of CMV-specific CD8\(^+\) T cells compared
to HIV-specific CD8+ T cells (p=0.10, Mann-Whitney) (Figure 3D). Interestingly, in slow progressors, the expression of perforin tended to increase in all virus-specific CD8+ T cells during HIV infection from, respectively, 27.6 to 65.8% (CMV, p=0.14, Wilcoxon), 16.3 to 56.8% (EBV, p=0.14) and 17.8 to 41.9% (HIV, p=0.14) (Figure 3A).

Figure 3. Differences in expression of CD27 (A, D), perforin (B, E) and granzyme B (C, F) in HIV-infected individuals. A longitudinal analysis was performed on PBMC of slow progressors (left panel) and individuals more rapidly progressing to AIDS (right panel). Cells were stained for CD27, perforin and granzyme B in combination with different HLA-A2 tetramers. Within each box, the median is indicated by a bar. Whiskers represent minimum and maximum values. Significant differences (p<0.05, Mann-Whitney) are indicated by asterisks.
In both rapid and slow progressors, granzyme B expression within HIV-specific CD8+ T cells increased from, respectively, 47.2 to 66.3% (p=0.04, Wilcoxon) and 45.1 to 59.8% (p=0.04), resulting in similar levels of granzyme B expression in individuals with different disease progression (Figures 3 and E).

B8-restricted T cells specific for HIV and EBV showed a similar expression of granzyme B and CD27 as A2-restricted CD8+ T cells in both slow and rapid progressors. However, in individuals with a slow disease progression, perforin expression in B8-restricted virus-specific T cells was much lower (median early 2.1% (0.9-3.2) (HIV) and 0.94% (0.6-1.3) (EBV); median late 1.0% (0.6-1.5) (HIV) and 3.1% (1.9-4.3) (EBV), data not shown).

Thus, early in infection HIV-specific CD8+ T cells in rapid progressors express more CD27 and less perforin compared to EBV- and CMV-specific CD8+ T cells. Furthermore, in the course of HIV infection, no decrease in CD27 and increase in perforin expression within HIV-specific CD8+ T cells are observed in rapid progressors. This is in contrast with the decreased expression of CD27 and increased expression of perforin within EBV- and CMV- specific CD8+ T cells over time and the increase in perforin expression of HIV-specific CD8+ T cells in slow progressors. Interestingly, an increase in granzyme B expression within HIV-specific CD8+ T cells during follow-up was observed both in rapid and slow progressors.

CD8+ T-cell phenotype and viral load
To investigate possible influences of viral load on the differentiation of virus-specific CD8+ T-cell populations, viral load of HIV (in serum), EBV (on PBMC) and CMV (on PBMC) was determined (Table 3). Late in infection, HIV load in rapid progressors tended to be higher compared to slow progressors (median 64,000 copies/ml vs. 1,000 copies/ml, p=0.06, Mann-Whitney). No differences in EBV load were found between slow and rapid progressors (median 1,724 copies/10^6 PBMC vs. 170 copies/10^6 PBMC, p=1.00, Mann-Whitney). Interestingly, CMV load could only be detected in one rapid progressor, although the amount of virus was too low to quantify. Thus, differentiation status of virus-specific CD8+ T cells could not only be explained by differences in viral load.

Higher expression of perforin and granzyme B in more differentiated CD27- virus-specific CD8+ T cells
We then investigated the effect of differentiation on the expression of the effector molecules perforin and granzyme B in more detail, regardless patient groups or time points. In some individuals, not enough tetramer-positive events were gated to distinguish between CD27+ and CD27- perforin or granzyme B expressing CD8+ T cells, and these individuals were excluded from these analyses. As shown in Figure 4A, the CD27+ population contained significantly more perforin-positive cells than the CD27- population in both HIV- (median CD27+ 8.4% vs. CD27- 15%, p=0.05), EBV- (median CD27+ 14.1% vs. CD27- 44.35%, p=0.02) and CMV- (median CD27+ 16.5% vs. CD27- 34.55%, p=0.002, Mann-Whitney) specific T cells. In both HIV- and EBV-
specific T cells significantly higher granzyme B expression was observed in CD27- versus CD27+ T cells (HIV median 66.5% vs. 47.5%, p=0.001, Mann-Whitney; EBV median 54.25% vs. 30.3%, p=0.004). Granzyme B expression in CD27- CMV-specific CD8+ T cells also tended to be higher (median CD27- 74.4% vs. median CD27+ 56.7%, p=0.10, Mann-Whitney) (Figure 4B).

Furthermore, a strong negative correlation was found between the expression of CD27 and perforin (R= - 0.71, p<0.001, Spearman's test). Correlations were also found between the expression of granzyme B and the percentage of CD27- cells (R=0.56, p=0.002, Spearman's test), and between the expression of perforin and granzyme B (R=0.61, p=0.001, Spearman's test) (results not shown).

### Table 3. Viral load data of slow and rapid progressors

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<thead>
<tr>
<th></th>
<th>EARLY</th>
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<tbody>
<tr>
<td></td>
<td>HIVaEBVB CMVc HIVEBVCMV</td>
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<tr>
<td>Slow progressors</td>
<td>17000 (1000-41500)</td>
<td>1000 (400-74000)</td>
</tr>
<tr>
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<td>311 (17-847)</td>
<td>1724 (21-2531)</td>
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<tr>
<td>Rapid progressors</td>
<td>12000 (5700-220000)</td>
<td>64000 (21000-250000)</td>
</tr>
<tr>
<td></td>
<td>974 (84-1554)</td>
<td>170 (119-2586)</td>
</tr>
</tbody>
</table>

a HIV load in copies/ml; median (range). Load was determined in 6 slow progressors and 5 rapid progressors.

b EBV load in copies/million PBMC; median (range), samples of 3 slow progressors and 5 rapid progressors.

c CMV load was measured in 4 slow progressors and 5 rapid progressors.

d Load was detectable in one rapid progressor, but too low to quantify. nd: not detectable.

### Co-expression of perforin and granzyme B in virus-specific CD8+ T cells

Co-expression of perforin and granzyme B of HLA-A2-restricted virus-specific CD8+ T cells was analysed in 5 slow and 5 rapid progressors to AIDS. In slow progressors, as shown in Figure 5A, the majority of the virus-specific T cells was either granzyme B+ (HIV range 17.9-40.7%; EBV range 27-54.7%; CMV range 32.7-47.6%), perforin-granzyme B+ (HIV range 11.2-24.9%; EBV range 10.4-33.5%; CMV range 19.7-44.8%), or perforin-granzyme B- (HIV range 42.4-50.5%; EBV range 33.9-49%; CMV range 5.2-14.5%). Only a small percentage of the cells (up to 4%) was single perforin+. In individuals rapidly progressing to AIDS (Figure 5B) a similar pattern of expression could be observed as in slow progressors, and no significant differences between the different virus-specific T cells were detectable.

In the course of HIV infection, EBV- and CMV-specific T cells in slow progressors showed an increase in co-expression (early median EBV 10.4%, CMV 28.2%; late median EBV 33.5%; CMV 44.8%) compared to HIV-specific CD8+ T cells (early median 29.1%, late 24.9%). However, this was not significant.
Figure 4. Differences in perforin and granzyme B expression in CD27+ CD8+ T cells compared to CD27− CD8+ T cells. To investigate the effect of differentiation on the expression of effector molecules, the expression of perforin and granzyme B was determined in CD27+ versus CD27− HLA-A2-restricted CD8+ T cells specific for HIV, EBV and CMV. As we were interested in the overall characteristics of CD27+ versus CD27− CD8+ T cells, early and late samples of both slow and rapid progressors were combined. In some individuals, not enough tetramer-positive events were gated to distinguish between CD27+ and CD27− perforin or granzyme B expressing CD8+ T cells. These individuals were excluded from the analyses.

Figure 5. Co-expression of perforin and granzyme B. Co-expression of perforin and granzyme B was analysed in HLA-A2-restricted virus-specific CD8+ T cells during HIV infection in 5 slow progressors (A) and in 5 individuals who rapidly progress to AIDS (B). H indicates HIV; E, EBV; C, CMV.
DISCUSSION

In this study, differences in differentiation and expression of effector molecules were investigated longitudinally for CD8\(^+\) T cells specific for HIV, EBV and CMV in relation to control of viral infection. For this purpose, CD8\(^+\) T cells specific for immunodominant epitopes of HIV, EBV and CMV were investigated in slow progressors and in individuals progressing more rapidly to AIDS.

During HIV infection, no increase in the number of HLA-A2-restricted HIV- and EBV-specific T cells was observed, in line with our earlier finding that functional properties but not the number of virus-specific T cells may be a determinant of HIV- and EBV-associated disease progression [21]. In contrast, the number of HLA-A2-restricted CMV-specific T cells increased in time despite a lack of detectable viremia in the blood, which may be related to CMV replication at different anatomical sites or by periodical reactivation of the virus. Recently, this increase in time of CMV-specific T cells has also been described in mice infected with murine CMV, despite undetectable viral replication in the blood [40].

Early in infection, HIV-specific CD8\(^+\) T cells in rapid progressors were already less differentiated, as reflected by less expression of perforin and a lower fraction of CD27\(^-\) CD8\(^+\) T cells. In the course of HIV infection, the fraction of HIV-specific CD27\(^-\) CD8\(^+\) T cells did not increase, nor did their perforin expression. In contrast, EBV- and CMV-specific CD8\(^+\) T cells developed a more differentiated phenotype. This suggests that the impaired maturation as observed in HIV-specific CD8\(^+\) T cells [23-25] is established very early in infection. Interestingly, in slow progressors, perforin expression of HIV-specific CD8\(^+\) T cells tends to increase during HIV infection, while the expression of CD27 remains constant. This may imply that partial differentiation of HIV-specific CD8\(^+\) T cells (increase in expression of effector molecules) is possible in some individuals during HIV infection. In both rapid and slow progressors, granzyme B expression of HIV-specific CD8\(^+\) T cells also increased during follow-up. Although this increase was not paralleled by a decrease in HIV load, it may be an indication that, even in individuals with progressive disease, HIV-specific CD8\(^+\) T cells are not completely anergic, as was suggested previously [2,26]. The lack of perforin expression of HIV-specific CD8\(^+\) T cells has been reported before [24,41]. Our data suggest that, in individuals who slowly progress to AIDS, a slight increase in perforin expression of HIV-specific CD8\(^+\) T cells may occur, although to a lesser extend than the increase and expression level within EBV and CMV-specific CD8\(^+\) T cells. Interestingly, late in infection, the expression of granzyme B did not differ between the virus-specific CD8\(^+\) T cells and was much higher than the expression of perforin. Similar results have been reported for granzyme A [41]. A possible explanation for the higher levels of granzyme B may be that the expression of this molecule is less tightly regulated. As perforin plays an important role in both killing and immune regulation [42], it may be required that its expression is more tightly controlled to avoid immunopathology.
With regards to co-expression of perforin and granzyme B, few single perforin-positive cells were observed. This was in contrast with the high number of cells that express only granzyme B. These results are in agreement with findings in healthy individuals where only $6 \pm 2\%$ is perforin-positive compared to $27 \pm 8\%$ of the cells that is granzyme A-positive [41,43]. All virus-specific T cells contained a fraction of cells that co-expressed perforin and granzyme B. However, during HIV infection, EBV- and CMV-specific T cells in slow progressors showed a small increase in the percentage of cells that co-expressed both effector molecules, while the percentage in HIV-specific T cells remained fairly constant. Considering the fact that the granule exocytosis pathway acts mainly by the dual action of perforin and granzyme [6,44], it may be that, because of higher co-expression, CMV- and EBV-specific CTL are more capable of killing, resulting in more efficient anti-viral immunity. Although perforin by itself may be an important mediator in the clearance of viral infections [24,45,46], this appears to be dependent on the type of virus. Granzyme B seems to be less required for clearing viral infections [10], however, the exact function of this molecule is not clear. Smyth et al [47] have demonstrated in five different tumour models that granzyme A and B clusters are not essential for CTL-mediated rejection of spontaneous and experimental tumours. However, mice lacking both the granzyme A and granzyme B clusters have been demonstrated to be as susceptible to ectromelia virus infection as perforin-deficient mice [48]. Furthermore, an indispensable role for granzymes was observed in controlling acute murine cytomegalovirus infection [49]. These results suggest that the role of granzyme B in the absence of perforin may depend on the nature of the pathogen-host interaction.

When CD$^{27^-}$ and CD$^{27^+}$ cells are compared with regard to their expression of perforin and granzyme B, it was shown that, during differentiation, the expression of the effector molecules perforin and granzyme B increased. This effect was strongest in HIV-specific T cells, suggesting that in principle HIV-specific CD$^{8^-}$ T cells are not completely impaired in maturation to a more differentiated effector phenotype. A possible explanation for the largest increase in expression of effector molecules with differentiation of HIV-specific T cells may be that perforin and granzyme B expression in these cells is the lowest compared to CD$^{27^+}$CD$^{8^-}$ T cells specific for the other viruses. Furthermore, we found a negative correlation between the expression of CD27 and the expression of both perforin and granzyme B. It has already been shown that down-modulation of CD27 correlates with perforin expression on CD$^{8^-}$ T cells [50], and here we showed that this is true for CD$^{8^-}$ T cells specific for three different viruses. Furthermore, this correlation could also be demonstrated for the expression of granzyme B and CD27. Moreover, a correlation between perforin and granzyme B was observed.

Our data suggest a possible influence of viral load on the differentiation state of EBV- and HIV-specific CD$^{8^+}$ T cells, in that chronic high load in the blood compartment may be associated with an enrichment of CD$^{8^+}$ T cells with a less differentiated phenotype. Viremia may result in a decrease in proliferative capacity and IL-2 production of virus-specific T cells as has been reported by Migueles et al [51]. This may subsequently lead to a decreasing number of virus-specific CD$^{8^+}$ T cells and a
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diminished effector function. Interestingly, CMV-specific CD8+ T cells express less CD27, have more perforin and granzyme B, while no CMV load could be detected in the blood. It has been reported that CMV-specific CD4+ T-cell responses are stronger than HIV-1-specific CD4+ T-cell responses [29,52] compatible with the higher differentiation state of CMV-specific compared to HIV-specific CD8+ T cells. No differences in EBV load were observed between rapid and slow progressors. This may be due to the small sample size, however, in a previous study where we investigated EBV load in a large group of non-progressors and progressors, no differences in EBV load between the two groups of HIV-infected individuals were found [53].

In conclusion, in individuals who rapidly progress to AIDS, HIV-specific CD8+ T cells are already early in infection less differentiated compared to EBV- and CMV-specific CD8+ T cells. This block in differentiation may be partly caused by the prolonged presence of high amounts of virus. However, other factors like CD4+ T-cell help probably play an important role the induction and maintenance of an efficient CD8+ T-cell response.

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