Cytomegalovirus-specific T-cell dynamics in HIV infection
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Chapter 6

In HIV-1-infected children CMV rather than HIV triggers the outgrowth of effector CD8^+CD45RA^+CD27^+ T cells

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Submitted for publication
**ABSTRACT**

**Objective:** We analysed the absolute lymphocyte numbers of various subsets of CD8$^+$ T cells, to study the effect of viral co-infections on immune reconstitution under HAART in HIV-1-infected children under the age of 18.

**Results:** Prior CMV infection correlated with an increased number of CD8$^+$ effector T cells (i.e. CD45RA$^-$CD27$^-$) at baseline (CMV-seropositive versus seronegative patients; p=0.009), as well as an increased state of T-cell activation as defined by HLA-DR and CD38 expression. The expansion of effector CD8$^+$ T cells persisted over time, independent of the HIV response to HAART. Numbers of CD8$^+$ effector T cells were significantly higher in patients with CMV replication, as reflected by persistent urinary CMV shedding and periodic CMV DNAemia (p=0.02). These patients also showed an increase in CMV-specific antibodies, compared to those without CMV shedding (p=0.007). The number of CMV-specific IFN$\gamma$-producing CD8$^+$ T cells was lower in children who persistently shed CMV compared to those who did not (p=0.02). In contrast, CMV-specific CD4$^+$ T-cell responses were detected at similar levels in both groups.

**Conclusions:** Our findings demonstrate that, in HIV-1-infected children, CMV infection correlates with the outgrowth of CD8$^+$CD45RA$^-$CD27$^-$ effector T cells. Activation of the immune system by persistent CMV secretion resulted in increasing CMV-specific IgG and higher numbers of CD8$^+$ effector T cells. Despite these increases, the CMV-specific IFN$\gamma$-producing CD8$^+$ T-cell response is diminished, which could explain the inability to suppress CMV completely in 41% of HIV-1-infected children.
INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infection causes a progressive and severe immunodeficiency. Since 1997 HIV-1-infected children in the Netherlands are treated with highly active antiretroviral therapy (HAART). HAART suppresses HIV replication, followed by immune reconstitution. Compared to adults absolute CD4\(^+\) T-cell numbers in children recover more rapidly, whereas CD8\(^+\) T-cell numbers do not change over time during treatment with HAART [1].

CD8\(^+\) cytotoxic T lymphocytes (CTL) are recognised as important cells to fight intracellular infections, such as viruses. Using a combination of phenotypic markers CD8\(^+\) T cells can be divided into different subsets. For this study we have chosen CD27, which is downregulated upon interaction with its ligand CD70. Using this functional molecule combined with CD45RA, CD8\(^+\) T cells can be divided into 4 different subsets: CD8\(^+\)CD45RA\(^-\)CD27\(^+\) naive, CD8\(^+\)CD45RA\(^-\)CD27\(^+\) memory, CD8\(^+\)CD45RA\(^-\)CD27\(^-\) memory/effecto-r and CD8\(^+\)CD45RA\(^-\)CD27\(^-\) effector T cells [2]. Although the sequential development of CD8\(^+\) T cells during primary infection in mouse was shown to go from naive to effector and then to memory [3] during chronic viral infections in humans, a sequential maturation pattern has been suggested to go from naive to memory and via memory/effecto-r to effector [4].

Herpes viruses, such as cytomegalovirus (CMV), Epstein-Barr virus (EBV) and varicella zoster virus (VZV), establish latency after initial infection. These latent infections have the potential to reactivate, which is accompanied by low levels of transient viral replication. CMV is a frequent infection in HIV-1-infected children [5,6]. In the pre-HAART era, children infected with both CMV and HIV-1 were more likely to have disease progression, than children who were HIV-1-seropositive, but CMV-seronegative [7]. Even in the era of HAART, CMV is associated with an increased risk of disease progression to AIDS and decreased survival [8,9].

In a healthy paediatric population in the Netherlands, CMV-seroprevalence at the age of 18 years was found to reach 75% [10]. In this control-population it has been shown that CMV infection is associated with the outgrowth of CD8\(^+\)CD45RA\(^-\)CD27\(^-\) effector T cells. Since we and others have found that CMV-specific cytotoxic CD8\(^+\) T cells are preferentially of the CD45RA\(^-\)CD27\(^-\) phenotype [4,10-12], it seems likely that CMV relates to these CD8\(^+\) effector T-cell expansions. In the present study, we have analysed the effect of CMV infection and replication on CD8 T-cell differentiation in HIV-1-infected children under HAART. To our knowledge this is the first study to describe CMV-related immune reactivity in HIV-infected children associated with persistent CMV replication.
Chapter 6

METHODS

Patients
The Amsterdam paediatric HIV-1 cohort consists of children and young adolescents under the age of 18. Since 1997, patients received HAART consisting of at least 1 protease inhibitor and 2 nucleoside-analogue reverse-transcriptase inhibitors. For the present study we selected all children who started therapy between 1997 and 2002. The Medical Ethical Committee approved this study. All caregivers gave written informed consent.

Blood samples
Blood and urine samples were obtained at each visit at intervals of 2-4 months. Serology for CMV, EBV and VZV was performed at start of HAART. If positive, the test was repeated at least 48 weeks thereafter. If negative, serological analysis was performed upon each visit until seroconversion.

Serology
Specific IgM and IgG against EBV viral capsid antigen (EBV-VCA) and specific IgG to Epstein-Barr nuclear antigen (EBNA) were determined by enzyme immunoassay (EIA) (Biotest, Dreieich, Germany). Specific antibodies to VZV were determined by Vidas tests (Biomerieux, Lyon, France). CMV antibodies were defined by Axsym assays (Abbott Diagnostics, Amstelveen, the Netherlands), expressed as Arbitrary Units. All tests were performed following the instructions of the manufacturers. Seropositivity was defined by the presence of a positive specific IgG after the age of 12-18 months or by CMV IgG remaining positive during follow-up in order to exclude the confounding contribution of maternal antibodies in the very young.

CMV culture
Patient urine was co-cultivated with human diploid fibroblasts for culture of CMV according to standard procedures. Repeated cultures were done prospectively in 32 CMV-seropositive children. Prolonged CMV shedding was defined as the presence of at least two positive CMV cultures after more than 36 weeks on HAART. A patient was defined as definitively negative if two urine cultures were negative at an interval of 2 months after more than 36 weeks on HAART, while secreting CMV at the start of HAART.

Lymphocyte subsets and enumeration of cytotoxic effector cells
Numbers of B cells (CD19+), T cells (CD3+) and subsets (CD3+CD4+, CD3+CD8+) were determined by Standard FACScan procedures. Activation and maturation of the CD4+ and CD8+ T cells were determined using monoclonal antibodies against CD4, CD8, HLA-DR (Becton Dickinson (BD), San José, CA, USA), CD38, CD45RA (Coulter Immunology, Haleah, FL, USA) and CD27 (Sanquin Reagents). Similar to
our study in healthy children [10], the cut-off for a positive result in CD8\(^+\)CD45RA\(^-\)CD27\(^-\) effector T-cells was set at 20 cells/μL.

**Flow cytometry and intracellular IFN\(\gamma\) staining after antigen-specific stimulation**

PBMCs were stimulated either with 2 μg/ml of a 15-mer 11 amino acids overlap CMV-derived pp65 peptide pool, a similar overlapping EBV-derived BZLF-1 peptide pool (Jerini AG, Berlin, Germany), or with CMV lysate (Microbix Biosystems, Toronto, Canada; 10 μl/ml) in the presence of anti-CD28 (Sanquin Reagents) and CD49d (BD) for 6 hours at 37°C. Phorbol myristate acetate and ionomycin were used as a positive control, and unstimulated cells as a negative control. After 1 hour, Brefeldin A was added. Cells were placed at 4°C overnight, washed and stained with anti-CD27 (BD) and CD45RO (DAKO, Carpinteria, California, USA), and either CD8 in case of peptide pools or CD4 in case of CMV lysate. After fixation and permeabilisation (BD reagents), cells were stained intracellularly with anti-IFN\(\gamma\) (BD). Cells were fixed in Cellfix (BD), and 200,000 to 400,000 events were acquired using a FACSCalibur flow cytometer (BD). For clarity of interpretation we considered CD45RO\(^+\) and CD45RO\(^-\) as CD45RA\(^-\) and CD45RA\(^+\), respectively. In case of intracellular IFN\(\gamma\) measurements, the number of responding T cells was calculated after subtracting negative control values.

**Plasma HIV-1 RNA determination**

Plasma HIV-1 RNA concentration was determined either using Nuclisens HIV-1 RNA QT (Biomérieux, Boxtel, the Netherlands) or Versant HIV-1 RNA 3.0 (Bayer, Tarrytown, NY, USA). All tests were performed according to the instructions of the manufacturers. All HIV-1-RNA below 400 copies/mL were considered as undetectable.

**Statistical analyses**

Statistical analyses were performed using SPSS for Windows version 11.5 (SPSS, Chicago, USA). All p-values were two-tailed. P-values smaller than 0.05 were considered statistically significant. Continuous data were analysed using a Mann-Whitney-U test. Categorical data were compared with a Fisher’s exact test. A paired sample t-test was used to analyse differences between baseline and 48 weeks of follow-up. Correlation was tested using the Spearman’s correlation test. Univariate and multivariate logistic regression models were used to identify independent factors associated with T-cell numbers above or under the median of the group, using a stepwise backward model.
RESULTS

Population characteristics and virologic and immunologic response to HAART

Fifty-two HIV-1-infected children started HAART between 1997 and 2002. All children had completed a follow-up of 48 weeks on treatment at the time of analysis. Table 1 shows baseline characteristics of the cohort. Plasma HIV-1 RNA concentration was initially suppressed below detection levels in 49 of 52 children in a median period of 9.1 weeks (inter-quartile range (IQR) 3.4-18.9). After 48 weeks, 44 children still had undetectable HIV RNA. Median CD4⁺ T-cell numbers subsequently increased from 480 to 1185 cells/μL during 48 weeks of follow-up (p<0.001).

Table 1. Baseline characteristics of study patients at start HAART

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients, n</td>
<td>52</td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>5.0 (0.8-17.9)</td>
</tr>
<tr>
<td>Race/ethnicity, n (white vs. non-white)</td>
<td>5/47</td>
</tr>
<tr>
<td>Sex, n (female/male)</td>
<td>29/23</td>
</tr>
<tr>
<td>Mode of transmission (vertically vs. sexually)</td>
<td>43/9</td>
</tr>
<tr>
<td>CDC-classification, n*</td>
<td></td>
</tr>
<tr>
<td>Non C</td>
<td>29</td>
</tr>
<tr>
<td>C</td>
<td>23</td>
</tr>
<tr>
<td>Prior treatment</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>37</td>
</tr>
<tr>
<td>Mono/Duotherapy</td>
<td>15</td>
</tr>
<tr>
<td>CD4 cell number (cells/μL), median (IQR)</td>
<td>480 (175-835)</td>
</tr>
<tr>
<td>CD8 cell number (cells/μL), median (IQR)</td>
<td>1230 (763-2203)</td>
</tr>
<tr>
<td>CD4 percentage, median (IQR)</td>
<td>17.0 (11.0-24.0)</td>
</tr>
<tr>
<td>CD8 percentage, median (IQR)</td>
<td>49.0 (33.5-60.0)</td>
</tr>
<tr>
<td>Plasma HIV-1 RNA, median log copies /mL (IQR)</td>
<td>4.95 (4.25-5.78)</td>
</tr>
<tr>
<td>Median follow-up, weeks (IQR)</td>
<td>192 (103-271)</td>
</tr>
<tr>
<td>CMV IgG (Number positive)</td>
<td>37</td>
</tr>
<tr>
<td>EBV IgG (Number positive)</td>
<td>45</td>
</tr>
<tr>
<td>VZV IgG (Number positive)</td>
<td>27</td>
</tr>
</tbody>
</table>

*Clinical categories as defined by the US Centres for Disease Control and Prevention (CDC).[30]

CMV infection and CD8⁺ T-cell differentiation

Since CMV infection seems to be associated with faster progression to AIDS and was found to be associated with the outgrowth of CD8⁺ effector T cells, CMV-seropositive children in our cohort were compared with seronegative children.

Thirty-seven children had contracted CMV before initiation of HAART. Three patients seroconverted during treatment and all others remained seronegative during follow-up. Median age at baseline did not differ between CMV-seropositive and seronegative children (4.7 vs. 4.8 years, p=0.5), as was the case for sex (male patients 43% vs. 47%, p=1.0). Plasma HIV-1 RNA concentration at initiation of HAART did
not differ between CMV-seropositive and seronegative children (5.0 vs. 4.6 log copies/mL, p=0.12). Furthermore, children not able to suppress HIV replication during 48 weeks on HAART were equally present in the CMV-seropositive and seronegative (5/37 vs. 1/12, p=0.54). In Table 2A, immunophenotypic comparisons of CMV-seropositive versus seronegative children are listed. The absolute number of CD8\(^+\) effector T cells (i.e. CD45RA\(^+\)CD27\(^-\)) was higher in CMV-seropositive compared to seronegative children at initiation of HAART (p=0.009), which persisted after 48 weeks (p<0.001), and was still present after 96 weeks on HAART (median 406 vs. 53 cells/μL, p=0.001; data not shown). Comparing the fraction of each of the subsets shows that in CMV-seropositive children at baseline only CD8\(^+\)CD45RA\(^+\)CD27\(^-\) effector T cells were higher (Table 2B). After 48 weeks on HAART also the CD8\(^+\)CD45RA\(^+\)CD27\(^-\) memory/effecto r T cells were higher. In contrast, the naive CD8\(^+\)CD45RA\(^+\)CD27\(^+\) T cells were lower in CMV-seropositive children. There was no difference in CD8\(^+\)CD45RA\(^-\)CD27\(^-\) memory T cells.

**Table 2A. T-cell phenotype (median cells/μL) CMV-seropositive (n=30) versus CMV-seronegative patients (n=12)**

<table>
<thead>
<tr>
<th></th>
<th>At baseline</th>
<th>p-value</th>
<th>48 weeks</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4(^+), total</td>
<td>585 vs. 400</td>
<td>0.29</td>
<td>1125 vs. 1150</td>
<td>0.66</td>
</tr>
<tr>
<td>CD8(^+), total</td>
<td>1280 vs. 1230</td>
<td>0.22</td>
<td>1530 vs. 1130</td>
<td>0.09</td>
</tr>
<tr>
<td>CD45RA(^+)CD27(^+)</td>
<td>472 vs. 369</td>
<td>0.41</td>
<td>724 vs. 773</td>
<td>0.98</td>
</tr>
<tr>
<td>CD45RA(^-)CD27(^+)</td>
<td>452 vs. 186</td>
<td>0.20</td>
<td>348 vs. 328</td>
<td>0.62</td>
</tr>
<tr>
<td>CD45RA(^-)CD27(^-)</td>
<td>154 vs. 69</td>
<td>0.03</td>
<td>83 vs. 18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD45RA(^+)CD27(^-)</td>
<td>369 vs. 101</td>
<td>0.009</td>
<td>323 vs. 41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD27(^+)</td>
<td>605 vs. 158</td>
<td>0.02</td>
<td>423 vs. 59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(CD45RA(^+) &amp; CD45RA(^-))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4(^+) HLA-DR(^+)CD38(^+)</td>
<td>82 vs. 35</td>
<td>0.02</td>
<td>112 vs. 99</td>
<td>0.59</td>
</tr>
<tr>
<td>CD8(^+) HLA-DR(^+)CD38(^+)</td>
<td>640 vs. 308</td>
<td>0.07</td>
<td>347 vs. 192</td>
<td>0.20</td>
</tr>
</tbody>
</table>

**Table 2B. T-cell phenotype (median %) CMV-seropositive (n=30) versus CMV-seronegative (n=12) patients**

<table>
<thead>
<tr>
<th></th>
<th>At baseline</th>
<th>p-value</th>
<th>48 weeks</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8(^+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45RA(^+)CD27(^+)</td>
<td>28 vs. 48</td>
<td>0.2</td>
<td>47 vs. 65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD45RA(^-)CD27(^+)</td>
<td>31 vs. 38</td>
<td>0.6</td>
<td>20 vs. 26</td>
<td>0.2</td>
</tr>
<tr>
<td>CD45RA(^-)CD27(^-)</td>
<td>10 vs. 7</td>
<td>0.2</td>
<td>6 vs. 2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD45RA(^+)CD27(^-)</td>
<td>22 vs. 5</td>
<td>0.006</td>
<td>23 vs. 3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
**CMV-associated outgrowth of effector T cells and chronic immune activation**

To test whether CMV infection is associated with chronic activation of the immune system, we analysed the activation markers HLA-DR and CD38 on CD4$^+$ and CD8$^+$ T cells. CD4$^+$HLA-DR$^+$CD38$^+$ T cells at start of HAART were higher in CMV-seropositive than in seronegative children ($p=0.02$). CD8$^+$HLA-DR$^+$CD38$^+$ T cells were nearly significantly increased in CMV-seropositive versus seronegative individuals at start of HAART ($p=0.07$) (Table 2A). Differences in both CD4$^+$ and CD8$^+$ compartments disappeared during follow-up.

At baseline, absolute numbers of CD4$^+$ T cells were correlated with absolute numbers of CD8$^+$ effector T cells ($r=0.45$, $p=0.005$). Interestingly, there was also a correlation between these CD8$^+$CD45RA$^-$CD27$^-$ effector T cells and activated (i.e. HLA-DR$^+$CD38$^+$) CD8$^+$ and CD4$^+$ T cells at baseline ($r=0.82$, $p<0.001$ and $r=0.71$, $p<0.001$, respectively) and with activated CD8$^+$ T cells after 48 weeks ($r=0.61$, $p<0.001$). This suggests that the expansion of CD8$^+$ effector T cells is associated with chronic immune activation. However, age, absolute CD4$^+$ T-cell counts and HIV load did not correlate with the number of CD8$^+$ effector T cells ($p=0.51$, $p=0.90$ and $p=0.79$, respectively).

**Primary CMV infection during HAART**

In our cohort, 3 girls seroconverted for CMV during treatment with HAART. In patient A and B, the number of CD8$^+$CD45RA$^+$CD27$^-$ T cells increased and stabilised at a level above baseline (Figures 1A and B). Patient C developed acute CMV infection, when she only had 6 CD4$^+$ T cells/μL. The number of CD8$^+$CD45RA$^+$CD27$^-$ T cells did not increase above the cut-off of 20 cells/μL (Figure 1C). She was treated with gancyclovir. Hepatic dysfunction and cachexia progressed, and she developed a fatal, multi-drug-induced liver failure.

![Figure 1. Primary CMV infection during treatment with HAART.](image-url) Absolute CD4$^+$ (white squares), CD8$^+$ (white circles) and CD8$^+$CD45RA$^+$CD27$^-$ (black diamonds or triangles) T-cell numbers in 3 patients seroconverting for CMV. Arrows indicate time of seroconversion.
CD8+ T-cell subset outgrowth in viral infections

CMV serology and CMV shedding in urine during latency
After infection, CMV remains dormant in the kidneys. Urinary shedding is a marker of CMV replication. After congenital infection it can be found for up to 10 years [13]. In healthy asymptomatic children and adolescents, urine samples are intermittently positive for CMV up to 30 weeks after primary infection [14].

Based on this report an extended period of 36 weeks as arbitrary cut-off for persistent CMV shedding was chosen. Regular urine tests for CMV were prospectively performed in 32 of 37 CMV-seropositive children. The patients with persistent CMV shedding (n=13, 41%) consisted of 11 boys and 2 girls, in contrast to the group with negative urine cultures (n=19, 59%) with 3 boys and 16 girls (p<0.001). Median age of the secretors was 1.0 year at start of HAART, that of non-secretors 5.3 years (p=0.001) (Figure 2A). This difference in age and gender was also present when only vertically HIV-1-infected children were analysed (n=27, p=0.007 and p=0.002, respectively). Furthermore, CMV IgG increased in CMV secretors and stabilised in non-secretors (median 73.3 vs. 0.00 Arbitrary Units/mL; p=0.02) (Figure 2B). Among patients with prolonged viral secretion, 5 patients were positive for CMV DNA as measured by quantitative PCR on whole blood, compared to none in non-secretors. Apart from the single patient mentioned earlier in Figure 1C, none of the patients developed clinical CMV-related disease that needed treatment under HAART, irrespective of persistent secretion or periodic detection of low concentrations of CMV DNA in blood.

CMV shedding in the urine and T cells
At start of HAART, the total numbers of CD4+, CD8+ T cells, and CD8+ effector T cells were not different between secretors and non-secretors (Figure 2C, left panel). In contrast, 36 weeks after the initiation of HAART, CMV-secreting patients had a higher number of total CD4+ T cells (p=0.01). This was also true for total CD8+ T cells (p=0.003), CD8+CD45RA+CD27- effector T cells (p=0.01) (Figure 2C, right panel) and CD8+CD45RA+CD27- naive T cells (median 1067 vs. 484 cells/μL, p=0.006) in CMV secretors versus non-secretors. These differences persisted until 96 weeks of follow-up (data not shown).

The relative fractions of each of the subsets within the CD8+ T cells were not different between secretors and non-secretors, both at baseline and 36 weeks on HAART (data not shown). This finding underscores the idea that continuous replication is associated with absolute numbers of effector T cells instead of relative changes in subset distribution.

The median number of CD8+CD45RA+CD27- T cells at week 36 for all CMV-seropositive children (421 cells/μL) was used to define a binary variable, denoting a high or low number of CD8+CD45RA+CD27- T cells. In a univariate analysis, prolonged CMV shedding (Odds Ratio [OR] 7.9 [95% CI 1.1-56.1]; p=0.04) and male gender (OR 7.5 [95% CI 1.3-43.0], p=0.02) were associated with a higher chance of increased numbers of CD8+CD45RA+CD27- T cells. In contrast, age at start of HAART, HIV RNA concentration at 36 weeks, CDC classification at presentation,
and prior VZV or EBV infection gave no higher chance of CD8⁺CD45RA⁺CD27⁻ numbers above 421 cells/μL. A multivariate regression model showed that CMV secretion was the only predictor of having a high number of CD8⁺ effector T cells at week 36 (OR 7.9; p=0.04). No two-way interaction was found between gender and CMV secretion.

Figure 2. Age, differences in CMV IgG, and T-cell subsets in relation to CMV shedding.
In A, age at start of therapy and CMV secretion (years), horizontal lines represent median of the groups (non-secretors vs. secretors; median 5.3 vs. 1.0; p=0.001). In B, differences in CMV IgG between time before start with HAART and minimal 48 weeks on HAART (median 73.3 vs. 0.00; p=0.02), horizontal lines represent medians of the groups. In C, absolute numbers of T-cell subsets in CMV non-secretors (-) (n=14) and secretors (+) (n=11) at week 0 (left panel) and week 36 (right panel). Shown are median cells/μL (IQR and range). Higher numbers of total CD4⁺ T cells (median 1535 vs. 630 cells/μL, p=0.01), activated CD4⁺HLA-DR⁺CD38⁺ T cells (median 168 vs. 64 cells/μL, p=0.049), total CD8⁺ T cells (median 2365 vs. 1310 cells/μL, p=0.003), CD8⁺CD45RA⁻CD27⁻ effector T cells (median 650 vs. 270 cells/μL, p=0.01) and CD8⁺CD45RA⁻CD27⁻ naive T cells (median 1067 vs. 484 cells/μL, p=0.006) was found in the CMV secretors versus non-secretors at 36 weeks after the start of HAART.
Naive CD8+ T-cell numbers were also higher in CMV secretors. Again the median number of CD8+CD45RA+CD27+ T cells at week 36 for all CMV-seropositive children (710 cells/µL) was used to define a binary variable for high or low numbers of naive CD8+ T cells. In a univariate analysis, male gender (OR 5.1 [95% CI 1.0-26.8]; p=0.06) and age (OR 0.8 [95% CI 0.6-0.9]; p= 0.007) were associated with a higher number of naive CD8+ T cells and not CMV shedding, which is in direct contrast to the aforementioned multivariate regression analysis for CD8+ effector T cells. In a multivariate analysis, age was the only independent predictor of the number of naive CD8+ T cells (OR 0.6 [95% CI 0.3-0.9]; p=0.03). Thus, in contrast to CD8+ effector T cells, high naive CD8+ T cells were independently associated with younger age. The same association was found in a multivariate analysis for the total (p=0.03), naive (p=0.04) and memory (p=0.04) CD4+ T cells at 36 weeks.

**CMV-specific T-cell responses**

The number of IFNγ-producing T cells can be used as a measure of the number of virus-specific T cells present in the blood. To study CMV-specific T-cell immunity, IFNγ production by CD4+ and CD8+ T cells was measured after stimulation with either CMV lysate or a peptide pool derived from the immunodominant pp65 antigen in 16 CMV-seropositive children, 8 secretors and 8 non-secretors. Neither absolute numbers nor percentages of CMV-specific IFNγ-producing CD4+ T cells were different in secretors versus non-secretors (Figure 3A, and data not shown). In contrast to the increased total CD8+ T-cell numbers and its CD45RA+CD27+ effector subset in CMV secretors, the numbers of CMV-specific IFNγ-producing CD8+ T cells were lower in CMV secretors compared to non-secretors (median 6.1 vs. 13.6 cells/µL, p=0.02) (Figure 3A). Also, lower numbers of IFNγ-producing CD8+CD45RA+CD27+ effector T cells were found in CMV secretors compared to non-secretors (median 1.0 vs. 3.8 cells/µL, p=0.04) (Figure 3B). This held also true for the CD8+CD27+ T cells within the IFNγ-responsive T cells in children with prolonged CMV shedding (median 2.0 vs. 6.0 cells/µL, p=0.01). Whereas equally detected in the CD27+ (median 53.5%) as in the CD27- subset (median 46.8%) in patients with complete CMV suppression, patients with persistent CMV shedding showed a difference in favour of the CD27+ over the CD27- subset (median 62% and 36.7%, respectively; both p=0.04) (Figure 3C). Age was not associated with the number of IFNγ-producing CD8+CD45RA+CD27+ T cells. Together, these findings may suggest an incomplete functional differentiation of CMV-specific CD8+ T cells despite a higher frequency of total CD8+ T cells with an effector phenotype.

As a control, we compared the EBV-specific CD8+ T-cell responses in the same patients upon stimulation with an EBV-lytic antigen derived BZLF-1 peptide pool. The EBV-specific IFNγ-producing CD8+ T-cell numbers were not different between the 2 groups. The distribution of the cells over the different subsets characterised for CD45RA and CD27 (data not shown) was not different either.
**Fig. 3. CMV-specific IFNγ production in CD4⁺ and CD8⁺ T cells.** In A, absolute numbers of IFNγ-producing CD4⁺ and CD8⁺ T cells in CMV non-secretors (−) (n=8) and secretors (+) (n=8). Shown are median cells/µL (IQR and range). In B, absolute numbers of CD8⁺CD45RA⁺CD27⁻ T-cells within the IFNγ-producing CD8⁺ T cells in CMV non-secretors and secretors. Shown are median cells/µL (IQR and range). In C, proportions of CD27⁺ and CD27⁻ cells within IFNγ-producing CD8⁺ T cells in CMV non-secretors and secretors. Shown are median, IQR and range. In D, representative FACS analyses of CD27 and CD45RO staining of CD8⁺ T cells of a secretor (right panel) and a non-secretor (left panel) are shown. Total CD8⁺ T cells are depicted in the upper graphs, CMV-specific T cells as defined by tetramer staining in the lower graphs. Naive CD45RO⁻CD27⁺ T cells are depicted in the upper left quadrant, memory CD45RO⁺CD27⁺ T cells in the upper right quadrant, memory/effectort CD45RO⁻CD27⁻ T cells in the lower right quadrant and effector CD45RO⁺CD27⁻ T cells in the lower left quadrant. Numbers indicate percentages of CMV-specific CD8⁺ T cells in the four quadrants.

**DISCUSSION**

In the present study the kinetics of CD8⁺CD45RA⁺CD27⁻ effector T cells were analysed in HIV-1-infected children treated with HAART. In healthy children, a significant association between the number of circulating CD8⁺CD45RA⁺CD27⁻ T cells and CMV seropositivity was found [10]. In HIV-infected children we now demonstrate that the outgrowth of these CD8⁺ effector T cells is similarly related to
CD8+ T-cell subset outgrowth in viral infections

CMV, as is further exemplified by the kinetics of these cells in patients with acute CMV infection under HAART. The expansion of CD8+ effector T cells was triggered by acute CMV infection, reaching a plateau with stable numbers in two months.

In our cohort there was no difference in CD4+ or CD8+ T-cell numbers at baseline and during follow-up between the CMV-seropositive and seronegative group, although CD4+ and CD8+ T cells were more activated in children with prior CMV infection as indicated by CD38 and HLA-DR expression. This difference in activation-state between these 2 groups disappeared after initiation of HAART. A correlation between activated CD8+ T cells and CD8+ effector T cells was found both at baseline and after 48 weeks of follow-up. These data demonstrate that in HIV-1-infected children, apart from the effects of HIV itself, ongoing CMV replication may contribute to chronic alteration of the immune system. The HIV load at baseline and during follow-up was not different between the groups, suggesting that the differences found in the T-cell compartment are not solely a reflection of HIV disease, but may be influenced by additional factors such as prior CMV infection.

In this respect, it is interesting that CMV-seropositive otherwise healthy children were found to have a median of 67 CD8+ effector T cells/µL (mean 85 cells/µL [10]), which is much lower than the median of 369 cells/µL at baseline and 323 cells/µL after 48 weeks HAART found in our cohort of HIV-1-infected children. In the same study it was found that children who had primary CMV infection prior to organ transplant had a median of 74 CD8+ effector T cells/µL. In contrast children who had primary CMV infection during treatment with immunosuppressive therapy had a median of 413 CD8+ effector T cells/µL. This suggests that the ability of the immune system to suppress CMV is inversely correlated with the number of CD8+ effector T cells. This is in line with our finding that HIV-1-infected CMV-seropositive children have higher numbers of CD8+ effector T cells and part of them shows continuing replication of CMV and mucosal shedding, associated with the outgrowth of this subset.

Of the prospectively tested HIV-1-infected children, 41% showed persistent CMV secretion in the urine for more than 36 weeks after start of HAART, irrespective of their HIV load at baseline or after 48 weeks. CMV replication is reflected by prolonged and persistent secretion of CMV in the urine, as well as by the finding of periodic CMV DNA in the peripheral blood of patients. Furthermore, CMV secretors showed increasing titers of CMV-specific IgG and increased numbers of CD8+ effector T cells while CMV-specific IFNγ-producing CD8+ T cells were reduced, when compared with non-secreting patients in our cohort. These data suggest inadequate cellular immunity to CMV in children with prolonged secretion. Tu et al found that in very young children after CMV infection CD4+ T-cell responses were diminished in a selected group that secreted CMV after 1-2 years, while CD8+ T-cell responses were comparable to adults [15]. In contrast, our results in HIV-1-infected children show that, in both absolute and relative terms, CMV secretion is associated with a decreased number of functional CMV-specific IFNγ-producing CD8+ T cells in the presence of equal numbers of CMV-specific IFNγ-producing CD4+ T cells.

103
There are several explanations possible for our findings. First, reduced numbers of CMV-specific CD8\(^+\) T cells can be explained by differences in distribution of these cells over the various anatomical compartments. CMV-specific cells may have become trapped in the target organs and draining lymph nodes, whereas the increase in CD8\(^+\) effector T cells could represent an epiphenomenon. However, CMV-specific T cells are preferentially found in the peripheral blood instead of extravascular tissues [16], and redistribution does not seem to affect (CMV-specific) CD4\(^+\) effector T cells in our study. Moreover, CMV-specific IFN\(\gamma\)-producing CD8\(^+\) effector T cells are not able to suppress CMV replication completely. An alternative explanation would relate to CMV specificity and responsiveness per se. The number of responsive CMV-specific T cells, as measured by the number of IFN\(\gamma\)-producing T cells upon stimulation with CMV-derived pp65 peptide pool, was significantly lower in children with prolonged CMV shedding. Despite a higher frequency of CD8\(^+\) effector T cells, incomplete functional differentiation of CMV-specific CD8\(^+\) T cells may be present [17]. This is in line with the finding that in HIV-1-infected male adults, progressors to AIDS with CMV end-organ disease showed increased CMV-specific tetramer\(^+\) CD8\(^+\) T cells, but dysfunction of CMV-specific CD8\(^+\) T cells in terms of IFN\(\gamma\). In addition, loss of CMV-specific IFN\(\gamma\)-producing CD4\(^+\) T cells occurred in the year before onset of CMV end-organ disease. This was paralleled by detection of CMV DNA in PBMC [18].

Since HLA-typing precluded the use of the standard tetramer (i.e. HLA-A2, B7) staining to enumerate CMV-specific CD8\(^+\) T cells in our cohort, a functional read-out for CMV-specific activity was used instead. Therefore, we cannot discriminate between the possibilities of an increase in CMV-related CD8\(^+\) T cells with a virus-specificity different from CMV-pp65, an increased number of dysfunctional CMV-pp65-directed T cells, or an indirect bystander phenomenon, being CMV-related yet with little or no CMV specificity. Other cellular functions remain to be studied. On the other hand, in functional assays it was found that after stimulation with virus-specific peptides \textit{in vitro} the IFN\(\gamma\) production correlated with cytotoxicity against target cells loaded with the same peptides [19]. We describe for the first time that numbers of functional CMV-specific CD8\(^+\) T cells are reduced in CMV-shedding children compared to children suppressing CMV replication successfully.

Recent experimental studies [20-23] revealed that ability of "unhelped" memory CD8\(^+\) T cells to produce IFN\(\gamma\) when restimulated was strongly reduced compared with "helped" memory CD8\(^+\) T cells. These experimental studies also demonstrated that restored CD4\(^+\) help (as seen under HAART) of previously "unhelped" memory CD8\(^+\) T cells did not remedy the defective CD8\(^+\) T-cell response [20-25]. If CMV-specific CD4\(^+\) T cells were defective before HAART, the CD8\(^+\) T-cell response starts to fail and viral replication returns. In such a scenario, the increased antibody response in secretors may act to contain replication [24].

Apart from CD8\(^+\) effector T cells, also the naive CD8\(^+\) T cells (i.e. CD45RA\(^+\)CD27\(^+\)) total, memory and memory-effector CD4\(^+\) T cells seemed to
expand more strongly in CMV secretors than in non-secretors. Multivariate analysis demonstrated that this association was, in contrast to CD8⁺ effector T cells, confounded by age and not related to CMV shedding. The expansion of naive T cells upon HAART is assumed to be largely antigen-independent in which cytokines may play a role, such as IL-7 and, to a lesser extent, IL-15 [26-29]. Whether CMV infection and prolonged shedding results in an increase of cytokines or different cellular sensitivity to these cytokines, hence affecting selectively the outgrowth of certain T-cell subsets such as the CD8⁺ effector T cells, remains to be determined.

In conclusion, our findings demonstrate that, similar to healthy age-matched controls [10], in HIV-1-infected children CMV infection is associated with the outgrowth of CD8⁺CD45RA⁺CD27⁻ effector T cells, and neither one of the other herpes viruses tested, nor to HIV-1 itself. Endogenous stimulation of the immune system by persistent CMV secretion results in progressively increasing CMV-specific IgG and higher numbers of CD8⁺ effector T cells. Despite these increases, the CMV-specific IFNγ-producing CD8⁺ T-cell response is diminished, which could explain the inability to suppress CMV completely in 41% of HIV-infected children, irrespective of HIV RNA and immune reconstitution under HAART.

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CD8+ T-cell subset outgrowth in viral infections


