Pediatric HIV-1-infection: perspectives on vaccination strategies and immune reconstitution during long-term antiretroviral therapy
Bekker, V.

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

Vincent Bekker *
Corine Bronke *
Henriëtte J. Scherpbier
Jan F. Weel
Suzanne Jurriaans
Pauline M. E. Wertheim-van Dillen
Frank van Leth
Joep M.A. Lange
Kiki Tesselaar
Debbie van Baarle
Taco W. Kuijpers

* Both authors contributed equally to the work presented.

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Abstract

Objective: To analyze the effect of viral co-infections on immune reconstitution in HIV-1 infected children (< 18 years) taking highly active antiretroviral therapy.

Results: Prior cytomegalovirus (CMV) infection correlated with an increased number of CD8+ effector T cells (i.e. CD45RA+CD27-) at baseline (CMV-seropositive vs. CMV-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 interferon-γ (IFN-γ)-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: In HIV-1-infected children, CMV infection correlated 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-γ-producing CD8+ T cell response was diminished, which could explain the inability to suppress CMV completely in 41% of HIV-1-infected children.
Introduction

Absolute CD4⁺ T cell numbers recover more rapidly following suppression of HIV replication by highly active antiretroviral therapy (HAART) in children compared with adults. In contrast, CD8⁺ T cell numbers do not change over time during treatment [1].

CD8⁺ cytotoxic T lymphocytes are recognized as important in combating intracellular infections, such as with viruses. Using a combination of phenotypic markers CD8⁺ T cells can be divided into different subsets. In this study, the phenotypic marker CD27, which is downregulated upon interaction with its ligand CD70, is used with CD45RA to divide CD8⁺ T cells into four different subsets: CD8⁺CD45RA⁺CD27⁺ (naïve), CD8⁺CD45RA⁻CD27⁺ (memory), CD8⁺CD45RA⁻CD27⁻ (memory/effector) 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 naïve to effector and then to memory [3], during chronic viral infections in humans, a sequential maturation pattern has been suggested to follow the path from naïve to memory and via memory/effector to effector [4].

Herpesviruses, 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. 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 seropositive for HIV-1 only [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 pediatric population, 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 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.

The present study analyses the effect of CMV infection and replication on CD8⁺ T cell differentiation in HIV-1-infected children taking HAART. To our knowledge, this is the first study to describe CMV-related immune reactivity in HIV-1-infected children associated with persistent CMV replication.

Methods

Patients
The Amsterdam pediatric HIV-1 cohort consists of children and young adolescents under the age of 18. The present study comprised all children who started HAART
between 1997 and 2002. The Medical Ethical Committee approved this study and 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 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**
CMV antibodies were defined by Axsym assays (Abbott Diagnostics, Amstelveen, the Netherlands) and 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-specific IgG remaining positive during follow-up in order to exclude the confounding contribution of maternal antibodies in the very young.

**Culture of cytomegalovirus**
Patient urine was cocultivated 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 procedures using FACScan (Becton Dickinson, San Jose, California, USA). Activation and maturation of the CD4+ and CD8+ T cells were determined using monoclonal antibodies against HLA-DR (Becton Dickinson), CD38, CD45RA (Coulter Immunology, Paris, France) and CD27 (Sanquin Reagents, Amsterdam, the Netherlands).

**Flow cytometry and intracellular IFN-γ staining after antigen-specific stimulation**
Peripheral blood mononuclear cells were stimulated either with 2 µg/ml 15-mer 11 amino acid residue overlap CMV-derived pp65 peptide pool, or with CMV lysate 10 µl/ml (Microbix Biosystems, Toronto, Canada) in the presence of anti-CD28 (Sanquin Reagents) and CD49d (Becton Dickinson) for 6 h. After 1 h, brefeldin A was added. Subsequently, cells were stained with anti-CD27, CD45RO and either CD8 or CD4; after fixation and permeabilization, the cells were stained intracellularly with anti-IFN-γ (Becton Dickinson). A FACSCalibur flowcytometer (Becton Dickinson) was used to acquire 200,000-400,000 events. For clarity of interpretation, CD45RO+ and CD45RO− were considered as CD45RA− and CD45RA+, respectively. For intracellular
IFN-γ 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. HIV-1-RNA below 400 copies/mL was considered undetectable.

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

**Results**

**Population characteristics and response to antiretroviral therapy**
The study comprised 52 HIV-1-infected children who 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 was initially suppressed below detection levels in 49 of 52 children in a median of 9.1 weeks (interquartile range (IQR) 3.4-18.9). After 48 weeks, 44 children still had undetectable plasma HIV RNA. Median CD4+ T cell numbers increased from 480 to 1185 cells/µL during 48 weeks of follow-up (p<0.001).

**Cytomegalovirus infection and CD8+ T-cell differentiation**
Since CMV-infection was associated with faster progression to AIDS and with the outgrowth of CD8+ effector T cells, CMV-seropositive children in our cohort were compared with CMV-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 CMV-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 at baseline did not differ between CMV-seropositive and CMV-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 group (5/37 vs. 1/12, p=0.54). Table 2 shows immunophenotypic comparisons of CMV-seropositive and CMV-seronegative children.
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</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 (white vs. non-white)</td>
<td>5/47</td>
</tr>
<tr>
<td>Sex (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>Median CD4+ T cell number (cells/µL) (IQR)</td>
<td>480 (175-835)</td>
</tr>
<tr>
<td>Median CD8+ T cell number (cells/µL) (IQR)</td>
<td>1230 (763-2203)</td>
</tr>
<tr>
<td>Median CD4+ T cell % (IQR)</td>
<td>17.0 (11.0-24.0)</td>
</tr>
<tr>
<td>Median CD8+ T cell % (IQR)</td>
<td>49.0 (33.5-60.0)</td>
</tr>
<tr>
<td>Median Plasma HIV-1 RNA (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>Number with IgG positive for CMV</td>
<td>37</td>
</tr>
<tr>
<td>Epstein-Barr virus</td>
<td>45</td>
</tr>
<tr>
<td>Varicella zoster virus</td>
<td>27</td>
</tr>
</tbody>
</table>

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

The absolute CD8+ effector T cell number was higher in CMV-seropositive compared with the CMV-seronegative children at baseline (p=0.009); this 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 showed that in CMV-seropositive children at baseline only CD8+CD45RA+CD27- effector T cells were higher (Table 2). After 48 weeks on HAART the CD8+CD45RA-CD27- memory/effector T cells were also higher. In contrast, the naive CD8+CD45RA+CD27+ T cells were lower in CMV-seropositive children.

**Cytomegalovirus-associated outgrowth of effector T cells and chronic immune activation**

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

At baseline, absolute numbers of CD8+ effector T cells correlated with CD4+ T cells (r=0.45, p=0.005) and activated (HLA-DR+CD38+) CD4+ and CD8+ T cells (r=0.71 and r=0.82, p<0.001), which persisted in the activated CD8+ T cells after 48 weeks (r=0.61, p<0.001). In contrast, age and plasma HIV-1 RNA did not correlate with CD8+ effector T cell numbers.
Table 2A. T cell phenotype (median cells/μL) CMV-seropositive (n=30) vs. CMV-seronegative patients (n=12).

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>p-value</th>
<th>48 weeks</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMV+ vs. CMV-</td>
<td></td>
<td>CMV+ vs. CMV-</td>
<td></td>
</tr>
<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>0.69</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+ (CD45RA+ &amp; CD45RA-)</td>
<td>605 vs. 158</td>
<td>0.02</td>
<td>423 vs. 59</td>
<td>&lt;0.001</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) vs. CMV-seronegative (n=12) patients.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>p-value</th>
<th>48 weeks</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMV+ vs. CMV-</td>
<td></td>
<td>CMV+ vs. CMV-</td>
<td></td>
</tr>
<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>

Primary cytomegalovirus infection during HAART

Three girls seroconverted for CMV during treatment with HAART. In patient A and B, the number of CD8+CD45RA+CD27- T cells increased and stabilized at a level above baseline (Figure 1A and B). Patient C developed acute CMV infection, when she only had a CD4+ T cell count of 6 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, multidrug-induced liver failure.

Cytomegalovirus serology and 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: 13 (41%) 11 boys and two girls, had persistent CMV shedding and 19 (59%), three boys and 16 girls, had negative urine cultures (p<0.001). CMV secretors were younger than non-secretors (median 5.3 vs. 1.0 years, p=0.001) (Figure 2A). This difference in age and gender was also present when only the 27 vertically HIV-1-infected children were analyzed (p=0.007 and p=0.002, respectively). Furthermore, CMV-specific IgG increased in CMV secretors and stabilized
in non-secretors (median 73.3 vs. 0.00 arbitrary units/mL; p=0.02) (Figure 2B). Among patients with prolonged viral secretion, five 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 and illustrated in Figure 1C, none of the patients developed clinical CMV-related disease that needed treatment under HAART.

**Cytomegalovirus shedding in the urine and T cells**

At baseline, 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 vs. 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 at any time (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 (421 cells/μL) and naive CD8\(^+\)CD45RA\(^+\)CD27\(^+\) T cells (710 cells/μL) at week 36 for all CMV-seropositive children was used to define binary variables, above or under the median. In a univariable
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+HILA-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) were found in CMV secretors vs. non-secretors at 36 weeks after the start of HAART.

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, HIV RNA at 36 weeks, CDC classification at presentation, and prior varicella zoster virus or EBV infection gave no higher chance of CD8+CD45RA+CD27- numbers > 421 cells/μL. A multivariable 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). There was no two-way interaction found between gender and CMV secretion.
In a univariable 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. In a multivariable 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). Therefore, 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 multivariable analysis for the total (p=0.03), naive (p=0.04) and memory (p=0.04) CD4\(^+\) T cells at 36 weeks.

**Figure 3.** CMV-specific IFN-\(\gamma\) production in CD4\(^+\) and CD8\(^+\) T cells. In A, absolute numbers of IFN-\(\gamma\)-producing CD4\(^+\) and CD8\(^+\) T cells in CMV non-secretors (-) (n=8) and secretors (+) (n=8). Shown are median cells/\(\mu\)L (IQR and range). In B, absolute numbers of CD8\(^+\)CD45RA\(^-\)CD27\(^-\) T cells within the IFN-\(\gamma\)-producing CD8\(^+\) T cells in CMV non-secretors and secretors. Shown are median, IQR and range. In C, proportions of CD27\(^+\) and CD27\(^-\) cells within IFN-\(\gamma\)-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\(^{high}\) T cells are depicted in the upper left quadrant, memory CD45RO\(^+\) CD27\(^+\) T cells in the upper right quadrant, memory/effector 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.
Cytomegalovirus-specific T cell responses

The number of IFN-γ-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, IFN-γ 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: eight secretors and eight non-secretors. Neither absolute numbers nor percentages of CMV-specific IFN-γ-producing CD4+ T cells were different in secretors vs. 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 IFN-γ-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 IFN-γ-producing CD8+CD45RA+CD27- effector T cells were found in CMV secretors compared with non-secretors (median 1.0 vs. 3.8 cells/μL, p=0.04) (Figure 3B), as was true for the CD8+CD27- T cells in children with prolonged CMV shedding (median 2.0 vs. 6.0 cells/μL, p=0.01). Whereas IFN-γ-producing T cells were 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 favor of the CD27+ (median 62%) over the CD27- subset (median 36.7%) (p=0.04) (Figure 3C). Age was not associated with the number of IFN-γ-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, the EBV-specific CD8+ T cell responses in the same patients upon stimulation with an EBV-lytic antigen derived BZLF-1 peptide pool were compared between the two groups; there were no differences in the numbers of IFN-γ-producing T cells (data not shown).

Discussion

In the present study, the kinetics of CD8+CD45RA+CD27- effector T cells were analyzed 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-1-infected children, we now demonstrate, that the outgrowth of these CD8+ effector T cells is similarly related to CMV, as is further exemplified by the kinetics of these cells in patients with acute CMV infection under HAART.

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 two 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. In contrast, such correlation was not found with plasma HIV RNA at any time. 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.

CMV-seropositive but otherwise healthy children have been found to have a median of 67 cells/μL CD8+ effector T (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 cells/μL CD8+ effector. In contrast, children who had primary CMV infection during treatment with immunosuppressive therapy had a median of 413 cells/μL CD8+ effector T. 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 had higher numbers of CD8+ effector T cells and part of them showed continuing replication of CMV and mucosal shedding that was 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 plasma HIV RNA at baseline or after 48 weeks. CMV replication was reflected by persistent secretion of CMV in the urine, and periodic CMV DNA in the peripheral blood. Furthermore, CMV secretors showed increasing titers of CMV-specific IgG and increased numbers of CD8+ effector T cells while CMV-specific IFN-γ-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. [15] found that after CMV infection in very young children, 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. In contrast, our results in HIV-1-infected children showed that CMV secretion was associated with a decreased number of functional CMV-specific IFN-γ-producing CD8+ T cells in the presence of equal numbers of CMV-specific CD4+ T cells.

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 did not seem to affect (CMV-specific) CD4+ effector T cells in our study. Moreover, CMV-specific IFN-γ-producing CD8+ effector T cells were not able to suppress CMV replication completely.

An alternative explanation would relate to CMV-specificity and responsiveness. The number of CMV-specific IFN-γ producing T cells 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 fewer CMV-specific IFN-γ producing CD8+ T cells [18].

Since HLA-typing precluded the use of the standard tetramer staining (i.e. HLA-A2, B7) 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. Virus-specific peptide-induced IFN-γ 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 that suppressed CMV replication.

Recent experimental studies [20-23] have revealed that ability of “unhelped” memory CD8+ T cells to produce IFN-γ 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 are defective before HAART, CD8+ T cell responses start to fail and viral replication returns. In such a scenario, the increased antibody response in secretors may act to contain replication [24].

In addition, the naive CD8+ T cells and the total, memory and memory-effector CD4+ T cells seemed to expand more strongly in CMV-secretors than in non-secretors, but multivariable analysis demonstrated that this association was, in contrast to CD8+ effector T cells, confounded by age and not related to CMV. The expansion of naive T cells upon HAART is assumed to be largely antigen-independent. Cytokines, such as IL-7 and —to a lesser extent— IL-15, may play a role [26-29]. Whether CMV infection and prolonged shedding results in increased cytokine levels or a different cellular sensitivity affecting selective outgrowth of certain T cell subsets, 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, which is not seen with other herpesviruses tested nor with 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 IFN-γ-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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