Chapter 3

Progression to CMV end-organ disease in HIV-1-infected individuals despite abundance of highly differentiated CMV-specific CD8+ T cells

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CMV co-infection. Comparing T-cell phenotypes in this unique group of patients to long-term asymptomatic HIV-infected individuals and progressors to AIDS without CMV end-organ disease as well as CMV-seropositive HIV-negative controls enabled us to characterise CMV-specific T cells in the natural course of HIV infection and progression to CMV-related disease.

**METHODS**

**Study population**
This study was performed on longitudinal peripheral blood mononuclear cell (PBMC) samples from participants of the Amsterdam Cohort Studies on HIV-1 infection and AIDS among homosexual men. In comparison, 10 HIV-seronegative, CMV IgG-seropositive individuals were analysed cross-sectionally. All individuals were selected according to their HLA-type (HLA-A2). Six of the 16 HIV-seropositive individuals were defined as progressors to AIDS with CMV retinitis and/or other end-organ disease (AIDS-CMV), 5 as progressors to AIDS, but without CMV end-organ disease, and 5 as long-term asymptomatics (LTA) with more than 7 years of asymptomatic follow-up and CD4 counts > 400/μl (classification of the Centres for Disease Control 1993), and without CMV end-organ disease. None of the individuals received anti-(retro)viral therapy during the study period. For the AIDS-CMV group, an early time point just after HIV seroconversion or study entry was compared with a late time point between 5 to 8.5 months before CMV diagnosis. The early time point studied in the progressor and LTA groups was between 1 and 2 years after HIV seroconversion. The late time point studied was on average 5 years (around the time of AIDS diagnosis) in progressors, and 10 years after HIV seroconversion for LTA. Group characteristics of the study participants are summarised in Table 1.

**HLA-class I tetramer staining in combination with phenotypic markers**
It has been well characterised that the immune response to CMV in HLA-A2 individuals is dominated by the NLVPMVATV epitope of the pp65 lower matrix protein [29-31]. HLA-A2 NLVPMVATV peptide-tetrameric complexes were produced, and four-colour fluorescence analysis was performed as described previously [32,33]. Briefly, PBMC were stained with HLA-A2-NLV tetramers. Subsequently, cells were stained extracellularly with fluorochrome-conjugated monoclonal antibodies (mAb) against CD8 (Becton Dickinson, hereafter BD, San José, California, USA) and combinations of CD45RO (DAKO cytometry Inc, Carpinteria, California, USA), CD27, CD57 (BD) (followed by fluorochrome-conjugated anti-mouse IgM; Southern Biotech), CCR7 (BD), CD27, CD45RA (Sanquin Reagents, Amsterdam, the Netherlands), or, after fixation and permeabilisation (BD reagents), cells were stained intracellularly with fluorochrome-conjugated monoclonal antibodies (mAb) against Ki67 (DAKO). In parallel, PBMC were stained with CD4, CD28 (BD), CD45RO (DAKO) and CD27-biotin (Sanquin
Table 1. Characteristics of the study population

<table>
<thead>
<tr>
<th>Subject (n)</th>
<th>Classification</th>
<th>HIV status</th>
<th>Months AIDS free</th>
<th>CD4 count</th>
<th>Months from AIDS to CMV</th>
<th>Age at AIDs diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>CMV disease (3)/ SC (5)/ CMV retinitis (3)</td>
<td></td>
<td>78</td>
<td>30</td>
<td>1.25</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>20</td>
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<td></td>
<td></td>
<td></td>
<td>8</td>
<td>44.5</td>
</tr>
<tr>
<td>5</td>
<td>LTA</td>
<td></td>
<td>(98 to &gt;200)</td>
<td>n.a. ±</td>
<td>-</td>
<td>n.a. ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(42 to &gt;62)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Progressor</td>
<td></td>
<td>65</td>
<td>140</td>
<td>-</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(54 to 71)</td>
<td>(40 to 390)</td>
<td>-</td>
<td>(2 to 39)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(28 to 45)</td>
<td></td>
</tr>
</tbody>
</table>

1 n = number of individuals.

2 CMV = cytomegalovirus; CMV disease = CMV other end-organ disease than retinitis; LTA = long-term asymptomatic.

3 SC = seroconversion; SP = seroprevalence: these individuals have seroconverted approximately between 0.5 to 1.5 years before study entry.

* median and/or range.

4 CD4 counts at time of AIDS diagnosis.

5 CD4 counts at time of CMV diagnosis.

‡ n.a. = non applicable: most individuals are still asymptomatic, so there is no median possible for this parameter.
Statistical analysis
Wilcoxon tests were performed to compare values at early and late time points, within patient groups. Differences between the patient groups were analysed using the Mann-Whitney test. Correlations were tested using the Spearman's non-parametric correlation test. All statistical analyses were performed using the software programme SPSS 11.5 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Ki67 expression within CMV-specific CD8\(^{+}\) T cells increases over time prior to onset of CMV end-organ disease
Staining with HLA-A2-NLV tetramers (Figure 1A) confirmed our previous findings [34] that CMV-specific CD8\(^{+}\) T cells increase over time prior to onset of CMV end-organ disease in progressors to AIDS-CMV (median 1.07 to 4.80%; Figure 1A), and in LTA (median 1.98 to 3.17%). Progressors to AIDS without CMV end-organ disease were generally low (median 0.19 to 0.17%).

To study whether increases in CMV-specific CD8\(^{+}\) T cells are associated with increased proliferation, Ki67 expression was measured intracellularly. Within the tetramer\(^{CD8^{+}}\) T cells, the highest increase in Ki67 expression (Figure 1B; see also Figure 2 and Table 2) was found in the progressors to AIDS-CMV (median 3.42 to 8.86%; p=0.028*, Wilcoxon), which fits with the highest increase of CMV-specific T cells found in the progressors to AIDS-CMV in Figure 1A. In LTA, the percentage of Ki67 remained constant (median 2.56 to 3.07%; p=0.686, Wilcoxon), despite an increase in tetramer\(^{CD8^{+}}\) T cells. Progressors to AIDS without CMV end-organ disease also showed an increase in Ki67 expression (median 3.11 to 6.58%; p=0.043*, Wilcoxon), but this was not reflected in significantly higher percentages of CMV-specific CD8\(^{+}\) T cells. In comparison, Ki67-expressing CMV-specific CD8\(^{+}\) T cells could also be detected in the HIV-negative control group with a median of 5.44%. Ki67 expression in the total CD8\(^{+}\) T-cell population could also be clearly detected (Figure 1C) and might well be related to the chronic immune activation observed in HIV-infected individuals [35]. Overall, it is clear that in the AIDS-CMV group, the large expansion of CMV-specific CD8\(^{+}\) T cells was accompanied by significantly increased Ki67 expression.

CMV-specific CD8\(^{+}\) T cells are CCR7\(^{-}\) and downregulate CD27 over time prior to onset of CMV end-organ disease
To study differentiation and maturation of CMV-specific CD8\(^{+}\) T cells, we investigated CMV-specific CD8\(^{+}\) T cells for the expression of CCR7 as well as CD27 and CD45RO (Figures 2A and B and Table 2). Percentages of CCR7-expressing CMV-specific CD8\(^{+}\) T cells were very low in all individuals, including progressors to AIDS-CMV (median 1.64 to 0.54% CCR7\(^{-}\)). This indicates that CMV-specific CD8\(^{+}\)
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Figure 1. Early and late CMV-specific CD8⁺ T-cell responses. T-cell responses were analysed cross-sectionally in healthy HIV-negative donors (left column). Early and late time points from the longitudinal data of LTA (middle left column), progressors to AIDS without CMV end-organ disease (middle right column) and progressors to AIDS-CMV (right column) were compared. Depicted are a) percentages tetramer⁺ within CD8⁺ T cells; b) percentages Ki67⁺ within tetramer⁺CD8⁺ T cells; c) percentages Ki67⁺ within total CD8⁺ T cells; d) percentages CD27⁻ within tetramer⁺CD8⁺ T cells. Dots represent individual patients and the median is shown as a bar. The p-values from Wilcoxon tests are depicted in the upper left corner of the graphs.

T cells in HIV infection are of the effector memory phenotype, capable of homing to tissues to exert their effector functions.

In LTA, CD27 was downregulated already early in HIV infection (median 68.78 to 72.18% CD27⁻; p=0.080, Wilcoxon; Figure 1D). In progressors to AIDS without CMV end-organ disease, the CD27 expression overall remained higher (54.49 to 59.65% CD27⁻; p=0.138, Wilcoxon). In progressors to AIDS-CMV, CD27 was downregulated significantly to levels similar to the LTA group (median 44.24 to 75.19% CD27⁻; p=0.028*, Wilcoxon).
Figure 2. FACS analysis of CMV-specific CD8$^+$ and total CD4$^+$ T-cells. PBMC of a progressor to AIDS with CMV end-organ disease were stained with HLA-A2 tetrameric complexes loaded with CMV immunodominant NLV peptide, mAb to CD8; and either: mAbs to CD45RO and CD27; CD27 and CD57; CD45RO and CCR7; CD45RO and CD45RA; and
Ki67 and CD57 respectively in order to analyse the percentages of subsets within tetramer*CD8+ T cells at an early (A) and late (B) time point; Simultaneously, these same PBMCs were stained for total CD4+ T-cell subsets, with mAbs to CD27, CD45RO and CD28, again at an early (C) and late (D) timepoint. Numbers indicate percentages of CMV-specific CD8+ T cells or CD4+ T cells respectively in the four quadrants. Note: in CMV-specific CD8+ T-cell subsets based on CD45RO and CD27, naive CD45RO-CD27+ bright T cells are depicted in the lower right quadrant, memory CD45RO-CD27+ T cells in the upper right quadrant, memory/effecter CD45RO-CD27- T cells in the upper left quadrant and effecter CD45RO-CD27- T cells in the lower left quadrant.

CMV-specific CD8+ T cells in the progressors to AIDS-CMV progressed from mainly a CD45RO-CD27+ memory phenotype towards a CD45RO-CD27- memory/effecter and CD45RO-CD27- effector phenotype (Figures 2A and B), whereas the progressors to AIDS without CMV end-organ disease progressed no further than the CD45RO-CD27+ memory/effecter phenotype (data not shown). This was reflected in the percentages of the CD45RO-CD27- effector CD8+ T-cell subset, which decreased in both LTA and progressors to AIDS without CMV end-organ disease (median 52.80 to 48.70% in LTA; median 32.12 to 20.23% in progressors), whereas these showed a significant increase in progressors to AIDS-CMV (median 28.78 to 38.43%; p=0.028*, Wilcoxon; data not shown). Thus, CMV-specific CD8+ T cells do seem to be able to differentiate to a fully mature effector phenotype in progressors to AIDS-CMV.

**High CD57 expression on CMV-specific CD8+ T cells**

Since CD57 has been implied as a marker for replicative senescence, but also as a marker for antigen-induced apoptosis of CD8+ T cells in chronic persistent viral infections, we aimed to investigate CD57 expression on CMV-specific CD8+ T cells. Interestingly, in progressors to AIDS without CMV end-organ disease, the highest percentages of CD57-expressing cells were seen in HIV infection, both early and late (median 77.94 to 84.87% CD57+; Table 2). CMV-specific CD8+ T cells from the LTA and AIDS-CMV group generally showed lower CD57+ percentages (median 55.55 to 60.63% CD57+ in LTA; median 61.33 to 65.70% CD57+ in AIDS-CMV), as compared to the HIV-negative controls (around 60.26% CD57-expressing cells).

Within the CD27- CMV-specific CD8+ T-cell subset, median percentages of CD57-expressing cells seemed slightly higher in HIV-negative controls (median 68.91%), progressors (median 82.61 to 84%), and LTA (median 57.14 to 62.16%). Even though percentages of CD57 expression within CD27- CMV-specific CD8+ T cells in the AIDS-CMV group seemed to start of slightly higher, like the other groups, at the late time point percentages of CD57 expression did remain stable or decreased (median 72.74 to 66.55% CD57+; data not shown). These data thus suggest that in progressors to AIDS-CMV, CMV-specific CD8+ T cells had not become replicative senescent to a greater extent as in the LTA and other progressors.
As expected, CD57 expression was significantly higher in the CD27" subset compared to the CD27" subset, (early: median 68.92 vs. 49.98%; p=0.000, Wilcoxon; late: median 65.54 vs. 61.71%; p=0.004, Wilcoxon; Figure 3A). Analysis of Ki67
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expression within CD57$^+$ or CD57$^-$ CMV-specific CD8$^+$ T-cell subsets, indicated that both subsets expressed Ki67, thus suggestive of cells in division. At the late time point Ki67 expression was significantly higher in the CD57$^-$ versus the CD57$^+$ subset (early: 4.87 vs. 2.02%; $p=0.115$, Wilcoxon; late: 13.25 vs. 3.84%; $p=0.000$; Figure 3B). Surprisingly, in HIV-negative healthy controls, Ki67 expression was enhanced in the CD57$^-$ versus the CD57$^+$ subset in 8 out of 10 individuals.

![Figure 3. CD57 and Ki67 expression within subsets of CMV-specific CD8$^+$ T cells. A) Percentages CD57$^+$ cells within either CD27$^+$ or CD27$^-$ tetramer$^+$CD8$^+$ T cells, either the early or late timepoint, or only in HIV-negative healthy controls. B) Percentages Ki67$^+$ cells within either CD57$^+$ or CD57$^-$ tetramer$^+$CD8$^+$ T cells, either the early or late timepoint, or only in HIV-negative healthy controls. Dots represent individual patients: black circles represent LTA, grey circles represent progressors to AIDS without CMV end-organ disease, white circles represent progressors to AIDS-CMV, and triangles represent HIV-negative healthy controls. The p-values from Wilcoxon tests are depicted above the graphs.](image)

Expansions of CD27$^-$CD45RO$^-$ and CD27$^-$CD28$^-$ CD4$^+$ T cells prior to onset of CMV end-organ disease

Since CMV infection has been implicated in shaping of the CD4$^+$ T-cell pool, we investigated CD4 subsets within the setting of HIV and CMV co-infection (Figures 2C and D). In HIV-negative CMV-seropositive healthy controls, percentages of CD27$^-$CD28$^-$ CD4$^+$ T cells were around 2.90% (Figure 4A). In LTA, the percentages of CD27$^-$CD28$^-$ within the total CD4 population increased over time in 4 out of 5 patients (median 9.07 to 30.10% CD27$^-$CD28$^-$; $p=0.080$, Wilcoxon). In progressors, the percentages increased as well but to a much lesser extent (median 3.83 to 9.96%; $p=0.080$, Wilcoxon). Remarkably, percentages of CD27$^-$CD28$^-$ increased the most in progressors to AIDS-CMV (median 5.44 to 43.99%; $p=0.028^*$, Wilcoxon).
In addition, we found relatively high percentages of CD45RO−CD27− CD4+ T cells in all HIV+ individuals, which are rare within the CD4+ T-cell population in healthy individuals. In LTA, the percentages tended to increase over time (median 3.22 to 9.87%; p=0.080, Wilcoxon; Figure 4B) whereas in progressors they remained stable (median 2.96 to 2.52%; p=0.225, Wilcoxon). In line with the CD27−CD28− data, progressors to AIDS-CMV significantly increased in percentages of CD45RO CD27− "effector" CD4+ T cells (median 2.86 to 20.58%; p=0.028*, Wilcoxon). The increase in this normally rare CD4+ subset was paralleled by a decrease in naive CD4+ T cells in almost all HIV+ individuals. In LTA, the percentages dropped from a median of 39.55 to 18.62% (p=0.043*, Wilcoxon; Figure 4C), while these remained relatively high at 33.74% in progressors without CMV end-organ disease. In progressors to AIDS-CMV however, the percentages decreased sharply from a median of 37.98 to only 6.72% (p=0.028*, Wilcoxon).

![Figure 4. Early and late total CD4+ T-cell responses.](image)

T-cell responses were analysed cross-sectionally in healthy HIV-negative donors (left column). Early and late time points from the longitudinal data of LTA (middle left column), progressors to AIDS without CMV end-organ disease (middle right column) and progressors to AIDS-CMV (right column) were compared. Depicted are a) percentages CD27−CD28− within CD4+ T cells; b) percentages CD45RO−CD27− within CD4+ T cells; c) percentages CD45RO−CD27+ within CD4+ T cells. Dots represent individual patients and the median is shown as a bar. The p-values from Wilcoxon tests are depicted in the upper left corner of the graphs.
As CMV seropositivity was suggested to leave a fingerprint in both the CD8$^+$ and CD4$^+$ T-cell population, we investigated whether there was a correlation between the phenotype of total CD8$^+$ and CD4$^+$ T-cell populations. When plotting percentages CD27$^-$ within the total CD8$^+$ versus percentages CD27$^-$ within the total CD4$^+$ T-cell population, there was a positive correlation within the HIV$^+$ individuals, which was most pronounced late in infection ($\rho=0.700$ and $p=0.003$; Spearman’s; Figure 5).

![Figure 5](image)

**Figure 5.** Correlation between CD27$^-$ in total CD8$^+$ T cells and CD27$^-$ in total CD4$^+$ T cells early and late. Percentages CD27$^-$ within total CD8$^+$ T cells on the y-axis and percentages CD27$^-$ within total CD4$^+$ T cells on the x-axis. Black circles represent LTA, grey circles represent progressors to AIDS without CMV end-organ disease, white circles represent progressors to AIDS-CMV, and triangles represent HIV-negative healthy controls. Spearman's $\rho$ is the correlation coefficient. The $\rho$ and $p$-values are depicted in the upper left corner of each graph.

**DISCUSSION**

In this study, we aimed to define whether the characteristic advanced stages of differentiation typically observed for CMV-specific T cells could be reached even in patients who do progress to AIDS with CMV end-organ disease. Therefore, HIV$^+$ individuals with different clinical end-points were analysed longitudinally in terms of phenotype of CMV-specific CD8$^+$ T cells.

Confirming our previous data [34], CMV-specific CD8$^+$ T cells increased over time in progressors to AIDS-CMV. Just before onset of CMV end-organ disease, higher percentages of CMV-specific CD8$^+$ T cells were present, compared to other virus-specific CD8$^+$ T cells such as HIV and EBV [17,19]. Analysis of Ki67 expression within CMV-specific T cells revealed that CMV-specific CD8$^+$ T cells had significantly increased Ki67 expression during the course of HIV infection. This increase in dividing T cells may be associated with expansions of CMV-specific T cells. In addition, the Ki67 expression excludes the possibility that individuals
progress to AIDS-CMV because of a proliferation defect in their CMV-specific CD8$^+$
T cells as has been suggested for HIV-specific T cells [36,37]. In progressors to AIDS
without CMV end-organ disease, Ki67 expression was also increased. However, this
did not correlate with increased numbers of CMV-specific T cells. Since CD57
expression, implied in replicative senescence but also in antigen-induced apoptosis,
was highest in this group, it may suggest that these CMV-specific T cells are further
differentiated and more prone to apoptosis.

Our observations that a substantial fraction of CD27$^+$ cells could express
CD57, and on the other hand that CD57$^+$ cells could still express Ki67, are not
compatible with the idea that CD57 is an absolute marker of replicative senescence
[8]. However, since Ki67 is found in all stages of cell cycle [10], its expression does
not equate to cell division. Although we cannot conclude from our data that CD57 is a
marker for replicative senescence, it may relate to diminished expansion through
antigen-induced apoptosis compatible with the low numbers of CMV-specific T cells
in progressors to AIDS without CMV end-organ disease.

CD27 downregulation has been generally accepted to be associated with
differentiation to an effector T-cell phenotype in terms of cytokine expression and/or
cytotoxic capacity [2]. We observed a strong downregulation of the CD27 molecule
on CMV-specific CD8$^+$ T cells in progressors to AIDS-CMV, and an increase in the
CD45RO$^-$CD27$^+$ subset, whereas the CD45RO$^-$CD27$^+$ subset was decreased in both
LTA and progressors to AIDS without CMV end-organ disease. So, in contrast to
what has been observed for HIV- and EBV-specific T cells in individuals progressing
to AIDS and AIDS with Non-Hodgkin Lymphoma respectively [12,17,19], there is no
block in CMV-specific T-cell differentiation in progressors to AIDS-CMV in the
course of HIV infection. This is in line with our earlier findings that CMV-specific
CD8$^+$ T cells from progressors to AIDS-CMV express high percentages of both
perforin and granzyme B [34], which is associated with a CD45RO$^-$CD27$^+$ effector
phenotype.

The observed high percentages of cells lacking CCR7 expression are
agreement with other studies who describe CCR7$^-$ cells as an important subset in the
CMV-specific CD8$^+$ T cells [12,38,39]. It seems that for CMV-specific T cells, the
lymph nodes are not an important location for the effector immune response to take
place. Indeed, CMV end-organ disease is not typically located in the lymph nodes, but
in several other organs. In addition, this confirms that CCR7 expression is not a useful
marker for T-cell differentiation status, but provides insights in migration and homing
of the virus-specific T cells [13].

As it was suggested that CMV seropositivity shapes the T-cell subset
distribution in both healthy donors and renal transplant patients with CMV
seropositivity, we investigated the phenotype of the CD4$^+$ T-cell population. In
healthy CMV-seropositive individuals, a median percentage of 2.90% CD28$^-$CD27$^-$
CD4$^+$ T cells was found, which is in the range of percentages found by Van Leeuwen
et al [27]. In HIV-infected individuals, percentages early in infection were in the
range of the percentages observed in healthy individuals, and interestingly, they
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Increased in the course of HIV infection. Progressors to AIDS-CMV showed the highest increase up to a median of 44%. Also marked increases in the percentages of CD45RO-CD27+ "effector" CD4+ T cells were observed, which was accompanied by decreases in the naive CD45RO-CD27- CD4+ T cells. Although very common in CD8+ T cells, this "effector" subset is rare in CD4+ T cells. We observed a median percentage as high as 20.58% just before onset of CMV end-organ disease. Together these data imply that there is a clear CMV-driven maturation in the polyclonal CD4+ T-cell population over time in the context of HIV infection, which is strongest in progressors to AIDS-CMV. This is compatible with our previous findings where high CMV load, indicative of active CMV replication, was only observed in patients who progressed to AIDS-CMV just before onset of symptomatic disease [34].

Our data show clearly that in progressors to AIDS with CMV end-organ disease CMV-specific CD8+ T cells show a phenotypic expression pattern typical of fully differentiated T cells, being CCR7−, CD27− CD45RO+/−, related to functional effector capabilities. Also the characteristic CD27−CD28− phenotype within the total CD4+ T-cell population induced by CMV infection specifically, was found in very high numbers in the HIV+ individuals, interestingly highest in the progressors to AIDS-CMV just before onset of disease. Therefore it seems that despite a strong immune response, these individuals progressed to AIDS-CMV. It may be that this expanded CMV-specific T-cell population is associated with pathology instead of protection from disease.

Acknowledgements

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