Shift of CMV-specific CD4⁺ T-cells to the highly differentiated CD45RO⁻CD27⁻ phenotype parallels loss of proliferative capacity and precedes progression to HIV-related CMV end-organ disease

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Abstract To identify factors related to progression to CMV end-organ disease, cytokine production, proliferative capacity and phenotype of CMV-specific CD4⁺ T-cells were analysed longitudinally. Numbers of IFNγ⁺CD4⁺ and IFNγ⁺IL-2⁺CD4⁺ T-cells tended to decrease in individuals progressing to AIDS with CMV end-organ disease (AIDS–CMV), whereas they remained detectable in long-term asymptomatics (LTAs) and progressors to AIDS with opportunistic infections (AIDS–OI). In parallel, CMV-specific proliferative capacity was lost in AIDS–CMV. Initially, the majority of the CMV-specific IFNγ⁺CD4⁺ T-cells were of the CD45RO⁺CD27⁻ subset, but during progression to AIDS–CMV a shift in phenotype to the CD45RO⁻CD27⁻ subset was observed. Our data indicate that a decrease in CMV-specific cytokine production and proliferative capacity precedes progression to AIDS–CMV. Accumulation of CD4⁺ T-cells with a CD45RO⁻CD27⁻ phenotype suggests that persistent antigen exposure drives differentiation of CMV-specific CD4⁺ T-cells towards a poorly proliferating, and highly differentiated "effector" subset, which eventually fails to produce IFNγ in patients developing AIDS–CMV.
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Introduction

CMV-infection normally does not lead to symptomatic disease but can cause serious clinical complications in immunocompromised individuals. CD4⁺ T-cells were shown to play an important role in protection from clinical complications. Primary CMV-infection occurring in CMV-seronegative transplant recipients receiving a CMV-seropositive organ, only leads to symptomatic disease in individuals who

showed a delayed IFNγ+CD4+ T-cell response [1]. Similarly, CMV-reactivation in CMV-seropositive transplant recipients was associated with absence of IFNγ+CD4+ T-cells [2]. In HIV-infected individuals, loss of IFNγ+CD4+ T-cells was observed prior to onset of symptomatic disease only in individuals who progressed to AIDS−CMV [3]. A recent study reported that IFNγ-producing CMV-specific T-cells were indicative for development of CMV-viraemia and progression to CMV end-organ disease as measured by ELISPOT assay [4].

Recently, additional qualitative aspects of virus-specific T-cells have been suggested to be relevant for effective memory T-cell responses. HIV-specific IL-2+CD4+ T-cells with proliferative properties have been associated with low viral load and long-term non-progressing HIV-1-infected individuals [5,6]. Furthermore, the presence of such IL-2+CD4+ T-cells were protective in *Leishmania major* infection in mice [7]. In addition, besides classification into different subsets based on production of cytokines [8], CD4+ T-cells can be classified based on the expression of differentiation markers, such as CD27 and CCR7, in combination with CD45RO. Based on these molecules, the CD4+ T-cell population can be divided in naïve, (central) memory, effector (memory) and highly (/terminally)-differentiated effector T-cells [9,10]. IL-2-production and proliferative capacity are features of (central) memory CD4+ T-cells, whereas IFNγ-production at the site of infection and decreased proliferative capacity are characteristics of effector (memory) CD4+ T-cells [11,12].

In this study, we investigated several qualitative aspects of CMV-specific CD4+ T-cells in relation to their phenotypic characteristics in progression to CMV end-organ disease. To analyse the specificity of the results we also investigated kinetics of CMV-specific CD4+ T-cell proliferation and cytokine production in individuals that did not progress to CMV end-organ disease [3,4,13,14].

Methods

Study population

This study was performed on PBMC samples from participants of the Amsterdam Cohort Studies on HIV-1-infection. Five of the 18 HIV-seropositive individuals were defined as progressors to AIDS with CMV retinitis and/or other end-organ disease (AIDS−CMV), 7 as progressors to AIDS with opportunistic infections (AIDS−OI), and 6 as long-term asymptomatics (LTAs) with more than 7 years of asymptomatic follow-up, CD4 counts>300/μl. All individuals received neither anti-viral therapy against CMV nor highly active anti-retroviral therapy (HAART) during the study period.

Diagnostic criteria for CMV retinitis was a characteristic appearance on ophthalmologic exam (with an adequate response to specific therapy) and for CMV other end-organ disease characteristic inclusion bodies upon histological exam of the affected tissue and/or positive stain for CMV on immunohistochemistry. In addition, all LTA and AIDS−OI showed clear cytokine production upon stimulation with CMV lysate, confirming their CMV+ status. Several subjects included in the present study (3 AIDS−OI and 5 AIDS−CMV) were part of a previous study [3], in which CMV seropositivity was confirmed by serology or by the detection of CMV load (in the AIDS−CMV group).

For all individuals, an early time point (one year after HIV-seroconversion or study entry) was compared with a late time point. For AIDS−CMV, the late time point was between 9 and 12 months before CMV-diagnosis, a time point slightly earlier than used in our previous study [3], which was chosen to be able to measure phenotype of CMV-specific IFNγ+CD4+ T-cells. The late time point studied in AIDS−OI and LTA was around 5 years (time of AIDS-diagnosis), and 10 years after HIV seroconversion, respectively. Characteristics of the study participants are summarised in Table 1.

Intracellular cytokine staining after antigen-specific stimulation

Four-colour fluorescence analysis was performed as described previously [15,16]. Briefly, PBMC were stimulated with 10 μl/ml CMV lysate (Microbix Biosystems, Toronto, Canada) in the presence of 2 μg/ml anti-CD28 (Sanquin Reagents, Amsterdam, The Netherlands) and CD49d (Becton Dickinson (BD), San José, CA, USA) mAb at 37 °C for 6 h. After 1 h, 1 μl/ml Brefeldin A ("GolgiPlug", BD) was added. As a positive control, PMA and ionomycin (Sigma-Aldrich, Zwijndrecht, The Netherlands; 5 ng/ml and 1 μg/ml, respectively) were added. Unstimulated cells were used as a negative control. Subsequently, cells were placed at 4 °C overnight, and stained extracellularly with fluorochrome-conjugated mAb against CD45RO (Pharmingen, San Diego, CA, USA). After fixation and permeabilisation (BD reagents), cells were stained (intracellularly) with fluorochrome-conjugated mAb against IFNγ and/or IL-2, CD4, CD3 (BD), CD27 (Sanquin Reagents). Cells were fixed in Cellfix (BD), and up to 300,000 events were acquired using a FACSCalibur flow cytometer (BD). Lymphocytes were gated by forward−sideward scatter, and data were analysed using the software program CELL Quest (BD). Percentages of IFNγ+and IL-2+ T-cells were calculated after subtracting negative control values (median 0.02%), and considered positive when the percentage of cytokine production exceeded levels observed in CMV-negative HIV-negative individuals (median 0.01% of CD4+ T-cells; data not shown) [17,18], and consisted of more than 50 events in the cytokine gate. Data are expressed in absolute numbers per microliter blood, to take into account that during progression to AIDS, CD4+ T-cell numbers decrease. At the time of PBMC isolation, absolute numbers of CD4+ T-cells were enumerated in parallel in whole blood using a 3-color, single-platform immunostaining technique. Absolute numbers of circulating CMV-specific cytokine-producing CD4+ T-cells were calculated from the proportion of CD4+ T-cells producing cytokines and the simultaneously obtained absolute CD4+ T-cell count [19,20]. CD45RO+CD27+, CD45RO+CD27− and CD45RO−CD27− CD4+ T-cells were expressed as percentages within these three T-cell subsets. In a group of 6 healthy blood bank donors, detection of single IFNγ+CD4+ T-cells ranged from 0.104 to 19.344/μl blood (median 2.808/μl), single IL-2+CD4+ T-cells from 0 to 2.184/μl blood (median 0.208/μl) and IFNγ+IL-2+CD4+ T-cells from 0 to 9.048/μl (median 0.624/μl). Total number of IFNγ+CD4+ T-cells ranged from 0.104 to 28.392/μl blood (median 4.264/μl), total number of IL-2+CD4+ T-cells from 0 to 11.232/μl blood

Table 1 Characteristics of the study population

Subject	Classification [a]	AIDS-defining illness [b]	Months AIDS-free	CD4 count early [c]	CD4 count late [c]	Age at AIDS-diagnosis
8001 [d]	CMV disease	CMV disease, Candidiasis, KS	36	630	70	44
8332 [d]	CMV disease	CMV disease	84	470	60	46
0594	CMV retinitis	KS	72	940	50	31
0026	CMV retinitis	Toxoplasmosis	95	370	50	45
1112	CMV retinitis	CMV retinitis	66	360	30	45
0057	LTA	–	>168	680	470	>53
0126	LTA	–	>170	550	460	>53
0044	LTA	–	>170	470	370	>49
1171	LTA	–	>156	770	470	>40
0658	LTA	–	>146	980	700	>49
1160	LTA	–	>128	575	370	>46
0208	Progressor	PCP	54	790	40	35
0656	Progressor	Cryptosporidiasis	73	240	120	35
1211	Progressor	C. meningitis	70	700	180	31
0159	Progressor	PCP	32	590	210	48
1234	Progressor	PCP	67	460	380	36
0547	Progressor	PCP	65	1155	390	45
0748	Progressor	Microsporidiosis	67	300	150	34

[a] CMV = cytomegalovirus; CMV disease = CMV other end-organ disease than retinitis; LTA = long-term asymptomatic.
[b] PCP = *Pneumocystis carinii* pneumonia; KS = Kaposi sarcoma; C. meningitis = Cryptococcal meningitis.
[c] CD4 counts are in absolute numbers/μl and correspond to the time points measured.
[d] 8332 was specified as CMV-associated colitis; 8001 as other than lymph node, liver or spleen.

(median 0.832/μl). The phenotypes CD45RO$^+$CD27$^+$, CD45RO$^+$ CD27$^-$ and CD45RO$^-$CD27$^-$ CD4$^+$ T-cells in IFNγ-producing CD4$^+$ T-cells were detected with medians of 18.6%, 46.8% and 16.4%, respectively, and in IL-2-producing CD4$^+$ T-cells with medians of 28.5%, 48.3% and 17%, respectively (data not shown).

Antigen-specific proliferation assay

In vitro T-cell proliferation to CMV lysate was measured using CFSE (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's protocol. Briefly, PBMC were labelled with CFSE and incubated with 10 μl/ml CMV lysate at 37 °C for 6 days. As a positive control, 0.4 μg/μl anti-CD3 and 4 μg/μl anti-CD28 (Sanquin Reagents) were added and medium alone was used as a negative control. At day 6, cells were stained extracellularly with fluorochrome-conjugated mAb against CD4 and CD3 (BD). Stimulation indices (SI) were calculated by dividing percentage proliferation of CD3$^+$CD4$^+$ T-cells after stimulation by percentage proliferation of unstimulated cells. In healthy CMV$^+$ donors, SI ranged from 1.6 to 33.8 with a median of 6.73 (data not shown). SI values >1.5 were considered positive, based on SI values observed in HIV-negative individuals (median SI CMV lysate in CMV-seronegative HIV-negative healthy individuals is 1.07, data not shown) [17].

As HIV-infected individuals suffer from large proportions of activated cells, negative control stimulated cells might provide a high background in the CFSE^{low} staining, possibly resulting in an overestimation of the actual proliferation. We focussed on the longitudinal dynamics using the more strict SI as a measure of 'proliferative capacity' instead of the percentage of proliferation [17].

Statistical analysis

Wilcoxon tests were performed to compare values at early and late time points, within patient groups. Differences between patient groups were analysed using Mann–Whitney tests. Correlations were tested using Spearman's non-parametric correlation tests. All statistical analyses were performed using software programme SPPS 11.5 (SPSS Inc., Chicago, IL, USA).

Results

CMV-specific CD4$^+$ T-cells mainly produce IFNγ

After stimulation with CMV lysate, IFNγ-producing CMV-specific CD4$^+$ T-cells could be detected in all individuals (Fig. 1). In LTA, IFNγ^+CD4$^+$ T-cells were readily detected and their number increased in 3 out of 5 individuals (median of 1.76/μl to 5.81/μl; $p = 0.225$, Wilcoxon; Fig. 1A; and median of 0.21% to 0.83%, data not shown). Progressors to AIDS–OI initially had similar numbers of IFNγ^+CD4$^+$ T-cells but they decreased in 5 out of 7 individuals (median of 6.90/μl to 1.54/μl; $p = 0.176$, Wilcoxon; Fig. 1A; and median of 1.5% to 0.79%; data not shown). In progressors towards AIDS–CMV, IFNγ^+CD4$^+$ T-cells were detected in 4 out of 5 individuals initially, but they tended to decrease to low levels in all individuals a year before onset of CMV end-organ disease (median of 6.88/μl to 0.63/μl; $p = 0.080$, Wilcoxon; Fig. 1A; and median of 1.91% to 1.39%; data not shown).

As IL-2$^+$ and especially IL-2$^+$IFNγ^+ T-cells have been implicated in better viral control [21], IL-2-producing T-cells were measured in addition to IFNγ. In all groups, the number of IL-2$^+$CD4$^+$ T-cells was lower than the number of IFNγ^+CD4$^+$
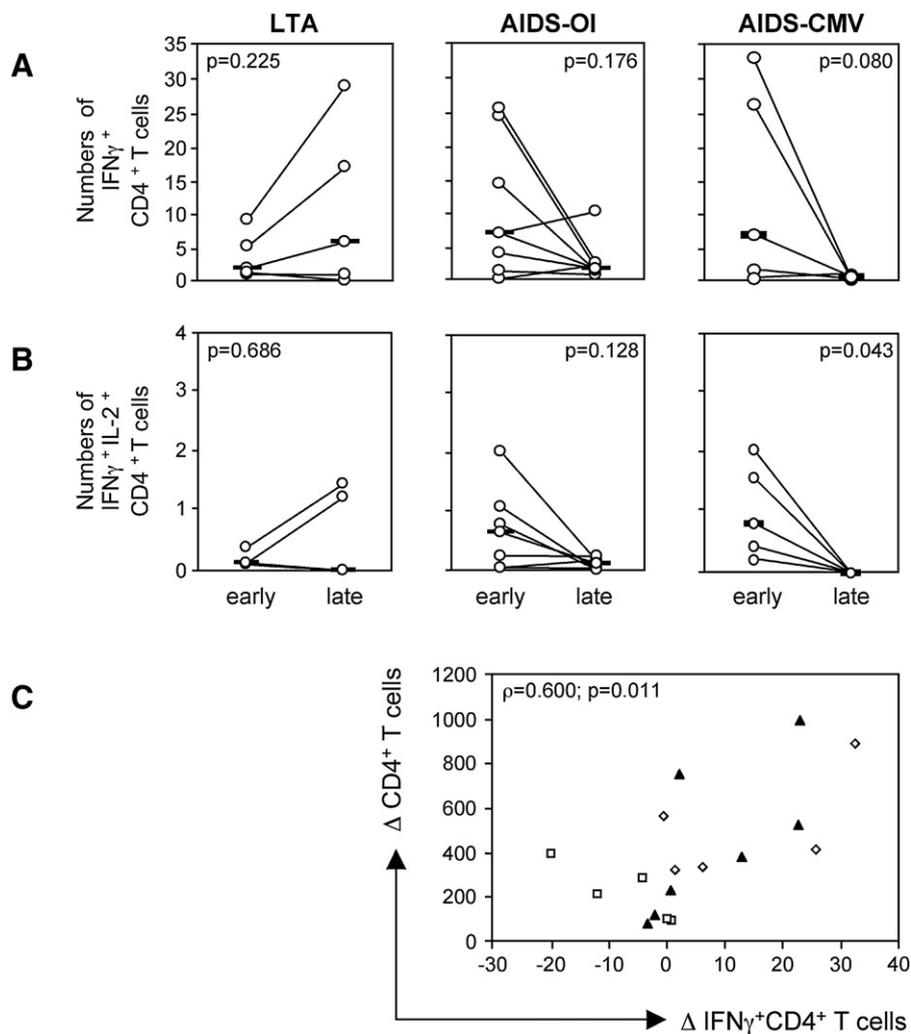
Figure 1 Early and late CMV-specific cytokine-producing CD4$^+$ T-cell responses. Early and late time points from the longitudinal data of LTA ($n=5$; left column), progressors to AIDS−OI ($n=7$; middle column) and progressors to AIDS−CMV ($n=5$; right column) were compared. Depicted are: (A) numbers of CMV-specific IFNγ$^+$CD4$^+$ T-cells/μl blood; (B) numbers of IFNγ$^+$IL-2$^+$CD4$^+$ T-cells/μl blood; and (C) correlation between decrease in CD4$^+$ T-cell numbers and decrease in numbers of IFNγ$^+$CD4$^+$ T-cells. Dots represent individual patients and the median is shown as a bar. Two-tailed p-values from Wilcoxon tests comparing early and late time points are depicted in the upper right corner of the graphs (A and B). White squares represent LTA, grey diamonds represent progressors to AIDS−CMV, and black triangles represent progressors to AIDS−OI; Spearman's ρ is the correlation coefficient; the ρ- and p-values are depicted in the upper left corner of the graph (C).

T-cells (data not shown). In the majority of LTA and progressors to AIDS−OI, IFNγ$^+$IL-2$^+$CD4$^+$ T-cells decreased over time but could still be detected at both time points (median of 0.14/μl to 0/μl; $p=0.686$, Wilcoxon; Fig. 1B; and median of 0.02% to 0%; data not shown; LTA; and median of 0.644/μl to 0.114/μl; $p=0.128$, Wilcoxon; Fig. 1B; and median of 0.06% to 0.03%; data not shown; AIDS−OI), whereas IFNγ$^+$IL-2$^+$CD4$^+$ T-cells significantly decreased to non-detectable levels in all progressors to AIDS−CMV a year before onset of CMV end-organ disease (median of 0.828/μl to 0/μl IFNγ$^+$IL-2$^+$; $p=0.043$, Wilcoxon; Fig. 1B; and median of 0.22% to 0%; data not shown). Interestingly, the percentage of IFNγ$^+$IL-2$^+$CD4$^+$ T-cells also decreased significantly over time in progressors to AIDS−CMV ($p=0.043$, Wilcoxon; data not shown). At the late time point in the AIDS−CMV group, the number of IFNγ$^+$IL-2$^+$ CMV-specific CD4$^+$

T-cells tended to be lower than in AIDS−OI ($p=0.048$, Mann−Whitney).

When analysing the decrease in CD4$^+$ T-cell numbers relative to the decrease in numbers of IFNγ$^+$CD4$^+$ T-cells, there seemed to be a correlation ($\rho=0.6$, $p=0.011$, Spearman's; Fig. 1C). This was particularly true for AIDS−OI ($\rho=0.9$, $p=0.007$, Spearman's). Interestingly, no correlation between these parameters could be detected in AIDS−CMV ($\rho=0.4$, $p=0.5$, Spearman's). In LTA, there seemed to be a negative correlation ($\rho=-0.9$, $p=0.037$, Spearman's).
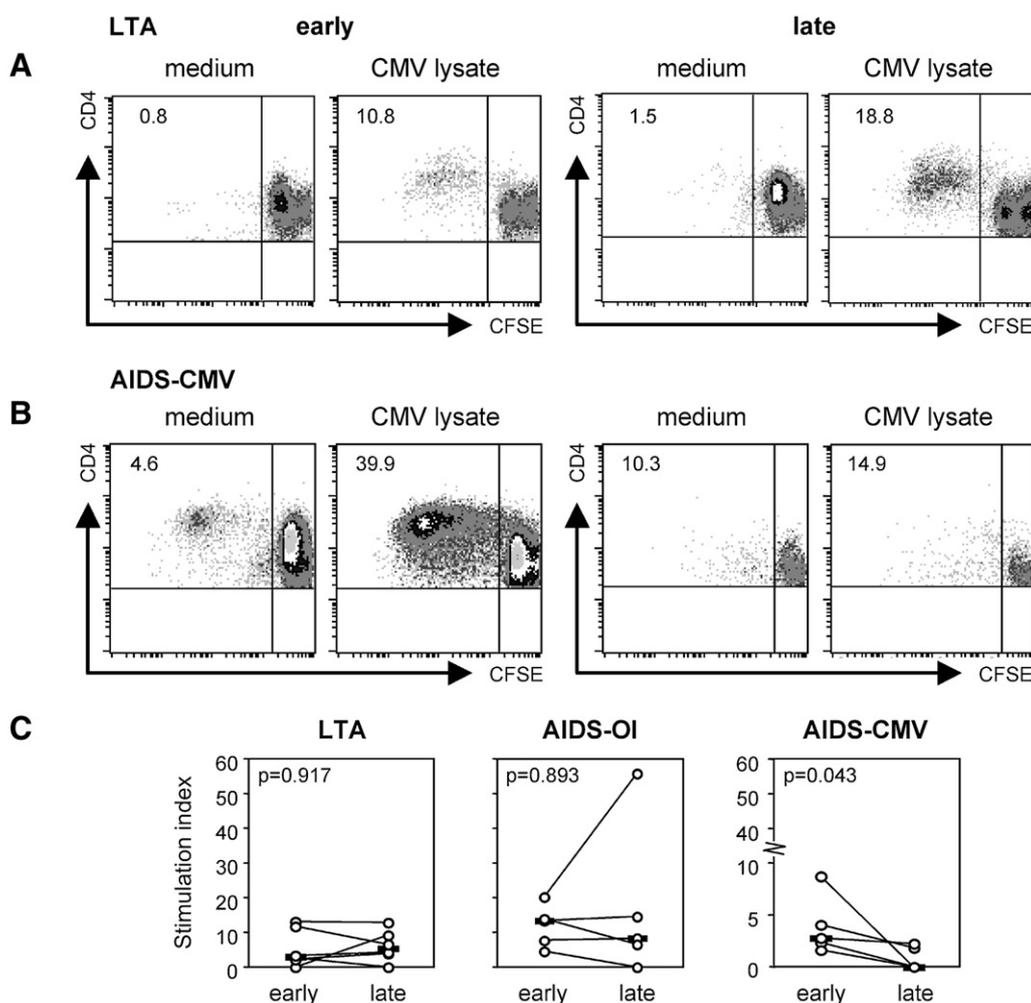
CMV-specific CD4$^+$ T-cells lose proliferative capacity a year before onset of CMV end-organ disease

To investigate proliferative capacity of the CMV-specific CD4$^+$ T-cells, PBMC were labelled with CFSE dye and stimulated for

6 days with CMV lysate. CMV-specific proliferation remained relatively stable in LTA (median SI 3 to 5.4; $p=0.917$, Wilcoxon; Figs. 2A and C). In progressors to AIDS−OI, proliferation was high initially and remained high (median SI 13.4 to 8.4; $p=0.893$, Wilcoxon; Fig. 2C). In progressors to AIDS−CMV, however, CMV-specific CD4+ T-cell proliferation was already initially lower compared to progressors to AIDS−OI ($p=0.028$, Mann−Whitney) and decreased significantly in all individuals towards progression to CMV end-organ disease (median SI 2.8 to 1.4; $p=0.043$, Wilcoxon; Figs. 2B and C). At the late time point in the AIDS−CMV group, the proliferative capacity of CMV-specific CD4+ T-cells was significantly lower than in LTA ($p=0.030$, Mann−Whitney) and borderline significantly lower in AIDS−OI ($p=0.052$, Mann−Whitney).

In 9 out of 16 patients, we were also able to detect CD3+CD4− (i.e. CD8+) T-cell proliferation upon stimulation with CMV lysate. Of these 32 analyses in total (16 patients, two time points each), only 8 could be detected at the early time point and 3 at the late time point. CD8+ T-cell proliferation tended to be lower compared to CD4+ T-cell proliferation (SI-range from 0 to 5.39 for CD8+ versus 0 to 55.89 for CD4+ T-cells; Fig. 3). When a positive response was observed within the CD8+ T-cells, there was also a positive response within the CD4+ T-cells. High responses in CD4+ T-cells were high in CD8+ T-cells as well. However, the height of the response only weakly correlated between CD8+ and CD4+ T-cells ($\rho=0.435$, $p=0.013$, Spearman's; data not shown). Although CD8+ T-cell responses against CMV seemed to follow similar kinetics and were especially low in AIDS−CMV patients, no firm conclusions can be drawn on the proliferative capacity of CD8+ T-cells.

Figure 2 Proliferative capacity of CMV-specific CD4+ T-cells. (A) FACS analysis after 6 days of stimulation with CMV lysate of CFSE-labelled PBMC of a representative LTA at the early (left) and late (right) time point; (B) representative progressor to AIDS−CMV at the early and late time point. Figures were gated on CD3+ T-cells. Numbers indicate percentages of CFSE^lowCD4+ T-cells that were used to calculate stimulation indices. (C) Early and late time points from the longitudinal data of LTA ($n=6$; left column), progressors to AIDS−OI ($n=5$; middle column) and progressors to AIDS−CMV ($n=5$; right column; note the different Y-axis scale) were compared. Proliferative capacity as indicated by stimulation index is shown on the y-axis. Dots represent individual patients and the median is shown as a bar. Two-tailed p-values from Wilcoxon tests comparing early and late time points are depicted in the upper left corner of the graphs.
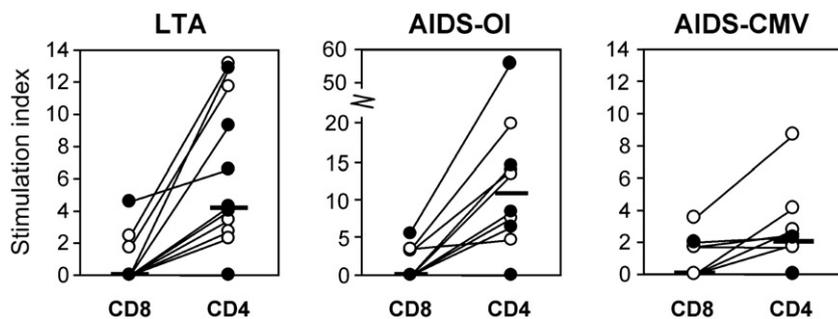
Figure 3 Comparison of proliferative capacity of CMV-specific CD4$^+$ versus CD8$^+$ T-cells. All time points from the data of CFSE^{low}CD4$^+$CD3$^+$ T-cells of LTA ($n=6$; left column), progressors to AIDS–OI ($n=5$; middle column; note the different Y-axis scale) and progressors to AIDS–CMV ($n=5$; right column) as shown in Fig. 2C were compared to the data of CFSE^{low}CD4$^-$CD3$^+$ T-cells (i.e. the proliferating CD8$^+$ T-cells). Proliferative capacity as indicated by stimulation index is shown on the y-axis. Dots represent individual patients and the median is shown as a bar. Early time points are represented by white dots, and late time points by black dots.

CMV-specific IFNγ$^+$CD4$^+$ T-cells shift to the CD45RO$^-$CD27$^-$ phenotype a year before onset of CMV end-organ disease

IFNγ- and IL-2-producing CD4$^+$ T-cells after stimulation with CMV lysate were stained with cell surface markers CD45RO and CD27 (Figs. 4A and B for a representative progressor to AIDS–CMV). The three major subsets (CD45RO$^+$CD27$^+$, CD45RO$^+$CD27$^-$, and CD45RO$^-$CD27$^-$) within IFNγ$^+$CD4$^+$ T-cells are depicted in Fig. 4C.

In both LTA and progressors to AIDS–OI, most IFNγ$^+$CD4$^+$ T-cells resided in the CD45RO$^+$CD27$^-$ effector subset both early and late in HIV-infection (median of 65.2% to 73.4%; $p=0.465$ and 77.2% to 81%; $p=0.600$, respectively, Wilcoxon). In progressors to AIDS–CMV, initially the majority of the IFNγ$^+$CD4$^+$ T-cells were of the CD45RO$^+$CD27$^-$ effector subset, but during progression to AIDS–CMV a shift in phenotype to the CD45RO$^-$CD27$^-$ subset was observed (median of 67.6% to 23% CD45RO$^+$CD27$^-$ versus median of 18.5% to 73% CD45RO$^-$ CD27$^-$; $p=0.068$ for both subsets, Wilcoxon). HIV (gag)- specific cytokine-producing CD4$^+$ T-cells in the same individuals did not progress towards the CD45RO$^-$CD27$^-$ compartment [18].

Unfortunately, in several individuals, IL-2$^+$CD4$^+$ T-cells could not be analysed for phenotypic markers due their low numbers. Only in the AIDS–OI group, 1 progressor to AIDS–CMV and 2 LTA, enough IL-2-producing T-cells were present. Interestingly, the largest subset of IL-2$^+$CD4$^+$ T-cells was of the CD45RO$^+$CD27$^+$memory phenotype (median of 54.2% to 34.4%; $p=0.500$, Wilcoxon), followed by CD45RO$^+$CD27$^-$ effector (median of 42% to 29.3%) and CD45RO$^-$CD27$^-$ (median of 5.6% to 0%) CD4$^+$ T-cells (Fig. 4B and data not shown). Overall, CD45RO$^+$CD27$^+$ memory cells were more abundant in IL-2-producing compared to IFNγ-producing CD4$^+$ T-cells, especially early (median of 51.38% versus 4.3%; $p=0.008$, Wilcoxon) and in most cases late (median of 33.3% versus 5.5 %; $p=0.093$, Wilcoxon) in HIV-infection.

To see whether a decrease in proliferative capacity was related to further differentiation towards the CD45RO$^-$CD27$^-$ highly differentiated phenotype within our study, we analysed whether these parameters correlate. Indeed, when taking all data points together, and analysing the percentage of CD45RO$^-$CD27$^-$ highly differentiated IFNγ-producing CD4$^+$ T-cells and the proliferative capacity of CMV-specific CD4$^+$ T-cells, there seemed to be a negative correlation ($\rho =-0.551$, $p=0.004$; Spearman's; Fig. 4D).

Discussion

In this study we analysed different functional aspects in relation to phenotype of CMV-specific CD4$^+$ T-cells in HIV-infected individuals with different clinical end-points. Longitudinal analysis during the natural course of HIV-infection enabled us to identify factors, which might be related to progression to CMV end-organ disease.

CMV-specific IFNγ-producing T-cells were higher compared to IL-2-producing CD4$^+$ T-cells, compatible with a study in which HIV-1-infected individuals with various patient characteristics were analysed [22]. In primary CMV-infection and during CMV-latency in transplantation patients, CMV-specific CD4$^+$ T-cells have been reported to produce IFNγ and TNFα, but no IL-2 or IL-4 [2]. Even though Harari et al. [6] reported a relatively equal distribution of IFNγ and/or IL-2-producing CMV-specific CD4$^+$ T-cell populations, single IFNγ$^+$ T-cell numbers were still shown to be highest. In progressors to AIDS–CMV, a trend to a decline was observed in IFNγ$^+$CD4$^+$ T-cells and CMV-specific IFNγ$^+$IL-2$^+$CD4$^+$ T-cells significantly decreased. In a previous study we observed a significant decline in IFNγ-producing T-cells. However, because we now aimed to study phenotypic characteristics of the cytokine-producing population, we chose to study a slightly earlier time point, where cytokine-producing T-cells had not yet declined to undetectable levels.

One possibility for the observed decreases in cytokine-producing T-cells may be the lower CD4$^+$ T-cell numbers in our AIDS–CMV group (median of 55/μl) compared to the AIDS–OI group (210/μl). As a proportion (in this study 2/5) of the AIDS–CMV patients first progress to other AIDS-defining illnesses before developing CMV end-organ disease, it is difficult to match subjects according to CD4$^+$ T-cell numbers. We have matched according to follow-up, which was similar between these groups. Inclusion of an extra time point with comparable CD4$^+$ T-cells as the AIDS–OI group (270 vs. 210/ μl) showed that at this time point both IFNγ$^+$and IFNγ$^+$IL-2$^+$ CMV-specific T-cells were still detectable, suggesting similar kinetics of CMV-specific T-cells in progressors to AIDS–OI and

IFNγ
IL-2
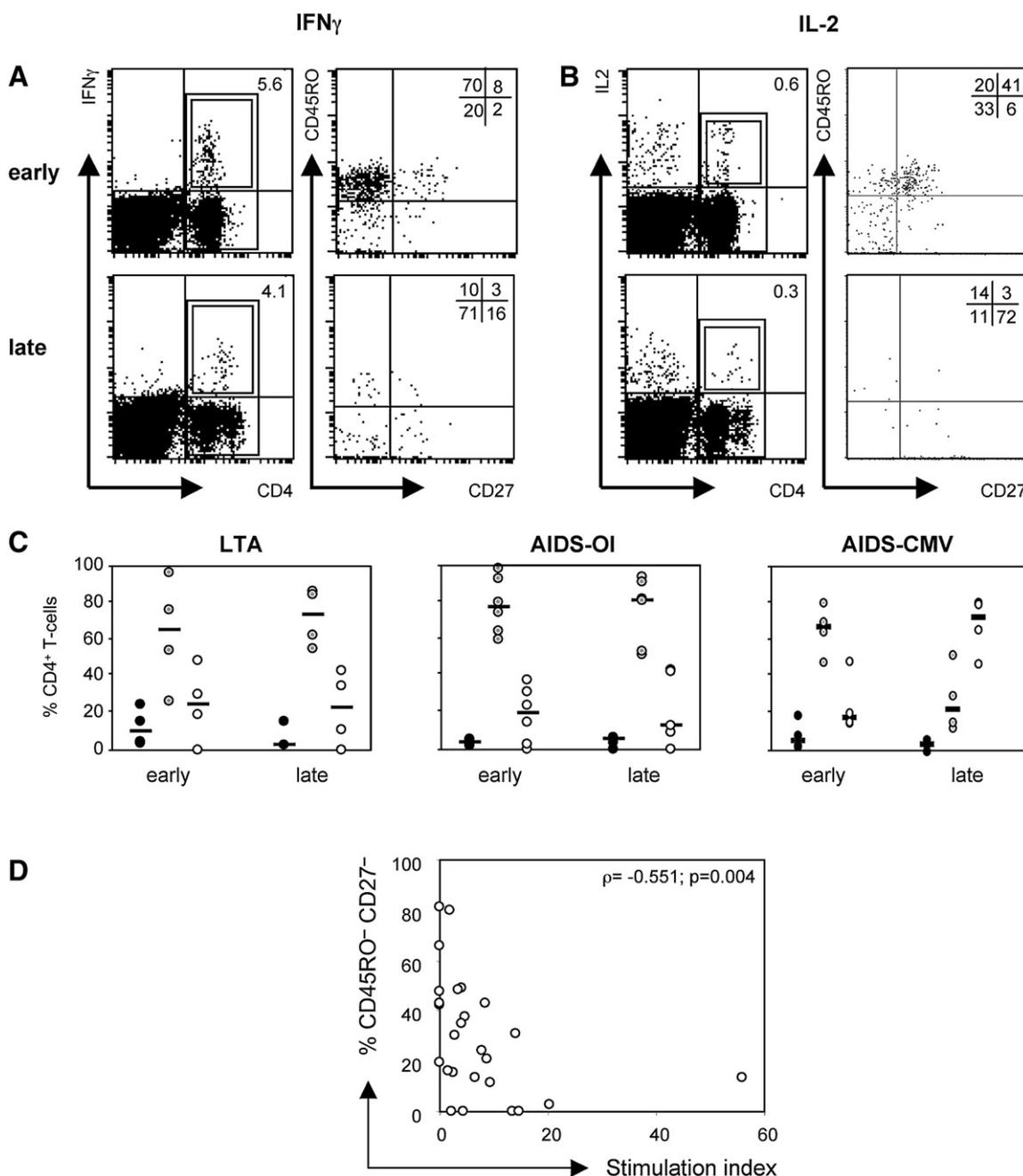
Figure 4 Phenotype of CMV-specific total IFNγ- or IL-2-producing CD4+ T-cells. (A) IFNγ-producing CD4+ T-cells and phenotype of a representative progressor to AIDS–CMV early (upper) and late (lower); (B) IL-2-producing CD4+ T-cells and phenotype of the same individual. Numbers in the upper right-hand corner indicate percentages of IFNγ+(or IL-2+) within CD4+ T-cells and percentages of the 4 quadrants, respectively. (C) Early and late time points from the longitudinal data of IFNγ+CD4+ T-cells of LTA (n=4; left column), progressors to AIDS–OI (n=6; middle column) and progressors to AIDS–CMV (n=4; right column) were compared. Percentages of CD4+ T-cells in a particular subset are shown on the y-axis. Memory CD45RO+CD27+ T-cells are depicted by black circles, effector CD45RO+ CD27− T-cells as grey circles, and highly differentiated CD45RO−CD27− T-cells as white circles. Dots represent individual patients and the median is shown as a bar. (D) Correlation between the percentage of highly differentiated CD45RO−CD27− IFNγ-producing CD4+ T-cells and the proliferation capacity of CMV-specific CD4+ T-cells. Spearman's ρ is the correlation coefficient; the ρ- and p-values are depicted in the upper right corner of the graph.

AIDS–CMV. When analysing the decrease in CD4+ T-cell numbers relative to the decrease in numbers of IFNγ+CD4+ T-cells, there seemed to be a correlation, suggesting that the IFNγ response would be influenced by CD4 counts. This was particularly true for AIDS–OI. As there was no obvious cor-relation between these parameters in AIDS–CMV, this suggests that either IFNγ+CD4+ T-cells decrease because CD4+ T-cells as a whole decrease or due to a specific IFNγ decrease. In AIDS–CMV patients who first develop an opportunistic infection as AIDS-defining illness, it may well be that the

decrease may be caused by loss of CD4+ T-cells, whereas in the other AIDS−CMV patients specific loss of IFNγ+CD4+ T-cells may be more pronounced. A negative correlation in LTA may be explained by the fact that even though CD4 counts decrease in this group, they remained well above critical levels and could respond to reactivation of the virus, which perhaps happens more frequent late in infection. Interestingly, the IFNγ+IL-2+CD4+ T-cells do also show a significant decrease when analysing the percentages. This could suggest that IL-2 production may be lost at an early stage, when CD4+ T-cell numbers are still intact, and that IFNγ responses may be better preserved.

Proliferative capacity was also lost with progression to AIDS−CMV. Initially, proliferative capacity in AIDS−CMV was already lower compared to AIDS−OI. This may be caused by increased levels of CMV reactivation in AIDS−CMV leading to subsequent impairment of proliferation. Impaired proliferation due to high levels of antigen has been suggested previously to occur in HIV-specific CD4+ T-cells during HIV-infection [6], and proliferative capacity has been suggested to be a function of the level of antigen following a bell-shaped curve [17]. Initially, proliferation is driven by antigen until the optimal balance between viral levels and proliferation is reached. If the level of antigen further increases, proliferation becomes impaired. HIV-infection is associated with more frequent CMV reactivation [23], which may be low in LTA, resulting in the observed low proliferative (and cytokine) responses, intermediate in AIDS−OI, resulting in optimal proliferation, and highest in AIDS−CMV, in which proliferation was impaired already at an early stage. Proliferative capacity subsequently became progressively impaired in AIDS−CMV with progression towards disease, at which time point high levels of CMV−DNA could be detected in an earlier study [3].

Remarkably, our phenotypical analysis revealed that within IFNγ+CD4+ T-cells in progressors to AIDS−CMV a striking shift from CD45RO+CD27− effector to the CD45RO−CD27− subset occurred during progression to AIDS−CMV. In LTA and AIDS−OI, IFNγ+CD4+ T-cells were mainly found within the CD45RO+CD27− effector subset during the course of HIV-infection, a subset frequently found also in healthy CMV-seropositive individuals [24]. The shift from CD45RO+CD27− to CD45RO−CD27− in individuals developing CMV end-organ disease is surprising given the fact that in healthy controls only a small percentage of CD4+ T-cells will down-regulate CD45RO and re-express CD45RA+ [10,25]. Within the CMV-specific IFNγ+ and IL-2+CD4+ T-cells in healthy controls we observed a median of 16.4% and 17%, respectively of the CD45RO−CD27− phenotype. However, in our patients with prog ession to CMV end-organ disease, these cells accumulate up to a median of 73% (within the IFNγ producers) at the late time point. Again, antigenic load has been associated with antigen-specific CD4+ (and CD8+) T-cell differentiation [5,26−28] and loss of IL-2+CD45RO+CCR7+ central memory cells. CMV replication may well play a role in the shift in differentiation status of CMV-specific T-cells, not only because of its high antigenic properties but also since high CMV load (indicative of active CMV replication) could be detected in PBMC of the same individuals just before onset of symptomatic disease [3]. CMV-specific CD8+ T-cells with a "late" differentiation phenotype were shown to be predominant in CMV+ individuals and active CMV disease in

immunodeficient individuals is associated with a diminished repertoire and diversity next to a reduction to hardly detectable levels of CMV-specific CD8+ T-cells with an "early" or "intermediate" phenotype [13]. The loss of these "early" and "intermediate" CMV-specific cells is implied in progression to disease, similar to our observed shift to the CD45RO−CD27− subset in CD4+ T-cells. In addition, Casazza et al. [24] recently showed that a more differentiated phenotype is associated with loss of IL-2 production of CMV-specific CD4+ T-cells.

Also other examples of chronic antigen exposure, specifically HIV-infection, have shown that persistent antigen-exposure drives differentiation of antigen-specific T-cells [5,29]. It is postulated that under persistent antigenemia CD4+ T-cells are rapidly induced to differentiate into IFNγ-only-producing cells with an effector (memory) phenotype that are prone to die more rapidly. Furthermore, memory (i.e. the ability to mount an accentuated response to antigen re-encounter) of an immune response can be found in different phenotypic subsets depending on antigen persistence and antigen load [27]. A dominant single IL-2+CD4+ T-cell response was associated with a model in which antigen can be cleared. Polyfunctional (single IL-2+ plus IL-2+/IFNγ+ plus single IFNγ+) CD4+ T-cell responses were associated with antigen persistence and low antigen levels. A dominant single IFNγ+CD4+ T-cell response was associated with a model of antigen persistence and high antigen levels. For HIV-specific CD4+ T-cells, IL-2-production and CD45RO+CCR7+ (central) memory phenotype has been associated with protective immunity, i.e. low viral load and a long-term asymptomatic clinical course, and is considered a better marker for disease progression [5,6]. CMV-specific immunity may be different in that CMV-specific memory CD4+ T-cells may also produce IFNγ and express a slightly more differentiated phenotype, possibly due to repetitive antigen exposure. In addition, CMV-specific CD4+ T-cells with a differentiated phenotype may also harbour cytolytic potential, which may have direct effect on control of viral infection in vivo. CMV-specific CD4+ T-cells have been shown to contain perforin [22]. A recent paper by Casazza et al. [24] showed that next to expression of perforin, granzyme B was expressed and CD107a mobilised, suggestive of cytolytic potential. These cells were shown to have a CD27−CD45RO+CD57+ phenotype.

We observed that proliferative capacity negatively correlated with differentiation towards the CD45RO−CD27− highly differentiated phenotype. In general, due to increased antigenic load, and hence enhanced activation of antigen-specific T-cells, cells will proliferate extensively. Activation and proliferation will lead to down-regulation of markers like CD27 (but also CD28), whereby the cells become more differentiated, produce less IL-2 and more IFNγ and are less able to proliferate.

In conclusion, IFNγ- and IL-2-producing CMV-specific CD4+ T-cells as well as CMV-specific proliferative capacity tended to decline in progressors to AIDS−CMV before onset of CMV end-organ disease. CMV-specific IFNγ+CD4+ T-cells shifted towards highly differentiated CD45RO−CD27− phenotype during progression towards AIDS−CMV, and eventually disappeared. These parameters could possibly be used as surrogate markers to predict disease progression. Antigenic presence could play an important role in driving the

differentiation of the CMV-specific CD4$^+$ T-cells towards the CD45RO$^-$CD27$^-$ phenotype. Probably, decline of these CMV-specific IFNγ^+CD4$^+$ T-cells plays a role in uncontrolled dissemination of the virus. Although neither IFNγ nor IL-2 have been directly implicated in CMV-control, T-cells displaying these features have been associated with viral control in general and may likely also be associated with CMV-control. The parallel decrease in CMV-specific IFNγ^+CD8$^+$ T-cells [3] suggests that exhaustion of CMV-specific T-cells occurs with progression to CMV disease. Whether this is the actual cause of disease progression, or consequence of other factors, remains to be elucidated. Especially in HIV-infection, where chronic immune activation seems to exhaust immune responses in general, this could play an important role.

Acknowledgments

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