

# Cytomegalovirus (CMV)-Reactivation Influences T-Cell Differentiation and CMV-Specific T-Cell Reconstitution after Stem Cell Transplantation

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## Abstract

CMV-specific T cells were shown to be important for protection against CMV-disease in SCT recipients. Here we investigated specific T-cell features like effector cell differentiation and perforin-expression as well as CMV-specific T cells after SCT in relation to CMV-reactivation. To this end, CD4<sup>+</sup> and CD8<sup>+</sup> T-cell characteristics (differentiation, activation and functional CMV-specific immunity) of SCT patients with (n=13) or without (n=8) CMV-reactivation were analysed longitudinally by flow cytometry. CMV-specific IFN $\gamma$ -production as measured by intracellular staining and proliferation as measured by CFSE dye dilution were analysed after stimulation with overlapping peptide pools of the tegument protein pp65 and immediate early antigen 1. A more differentiated phenotype, up-regulation of the activation markers CD38 and HLA-DR on CD4<sup>+</sup> T cells and increased expression of perforin on CD8<sup>+</sup> T cells was more frequently observed in patients with CMV-reactivation compared to patients without reactivation. Interestingly, these T-cell features were often already different early after SCT. In addition, CMV-specific CD8<sup>+</sup> T-cell responses, both based on IFN $\gamma$ -production as well as proliferation, directed against both pp65 and IE1 tended to be present more frequently in patients with CMV-reactivation compared to patients without reactivation. These data suggest that CMV-reactivation influences CMV-specific T-cell reconstitution after SCT and that early T-cell differentiation differences may be helpful in predicting viral reactivations.

**Keywords:** SCT; CMV; T-cells

**Abbreviations:** CMV: Cytomegalovirus; EBV: Epstein-Barr Virus; SCT: Stem Cell Transplantation; SOT: Solid Organ Transplantation; TBI: Total Body Irradiation; ATG: Antithymocyte Globulin; PBMC: Peripheral Blood Mononuclear Cells; EDTA: Ethylenediamine Tetraacetic Acid; CFSE: 5,6-carboxyfluorescein diacetate

## Introduction

CMV is a widespread persistent human  $\beta$ -herpesvirus [1-3]. Serious illness can develop during primary infection or reactivation of the virus in immunocompromised patients, such as after SCT. Timely reconstitution of CMV-specific T-cell responses has been reported to be important for protection against disease in SCT [4-9] and SOT [10-12]. A similar pattern of reconstitution of CMV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells after SCT was observed [13], indicating that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are important for control of CMV-infection. It is known that CMV-infection drives T cells to an effector phenotype in healthy individuals [14]. In renal transplant patients a dramatic change in phenotype has been observed after primary infection, resulting in increased numbers of CMV-specific CD45RA<sup>+</sup>CD27<sup>+</sup>CCR7<sup>+</sup> or CD45RO<sup>+</sup>CD27<sup>+</sup>CCR7<sup>+</sup> CD8<sup>+</sup> T cells [15]. Differences in CD8<sup>+</sup> T-cell function were demonstrated to depend on differentiation status [16].

As it is unclear whether CMV-reactivation is required for CMV-specific T-cell reconstitution or whether absence of CMV-specific T cells is responsible for lack of viral control, we investigated specific T-cell features like effector cell differentiation and perforin expression as well as CMV-specific T cells after SCT in relation to CMV-reactivation.

## Patients and Methods

### Study population and conditioning regimen

Between October 2005 and September 2006, 65 patients received a SCT at the University Medical Center Utrecht. Patients were included when either donor or recipient serostatus was CMV-positive (n=56)

and when blood samples were available from at least two time points (n=53). Only patients without EBV-reactivation were included (n=22), to be able to observe effects of CMV-reactivation only. As a result 14 SCT patients with CMV-reactivation and 8 without reactivation were included. Patient characteristics are shown in Table 1. Nonmyeloablative conditioning consisted of Fludarabine 30 mg/m<sup>2</sup>/day intravenously during 3 days followed by TBI of 200 cGy or TBI alone. Myeloablative conditioning consisted of cyclophosphamide 60 mg/kg/day during 2 days followed by TBI, 600 cGy/day for 2 days. Patients receiving grafts from an unrelated donor or a human leukocyte antigen (HLA) mismatched donor were given ATG in a dose of 2 mg/kg/day during 4 days.

Blood samples were drawn just before conditioning for SCT and 2, 4, 6, 9 and 12 months after SCT. PBMC were isolated by Ficoll-hypaque density centrifugation and cryopreserved. The study was approved by the local ethical committee and all participants gave written informed consent.

### CMV and EBV monitoring

CMV and EBV-monitoring was based on realtime TaqMan<sup>TM</sup> CMV

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or EBV DNA PCR assay in EDTA anticoagulated plasma [17-20] and performed weekly in all patients until day 120 post-transplantation. Pre-emptive antiviral therapy with valganciclovir (twice daily 900 mg) was initiated when CMV-DNA load exceeded 500 copies/ml plasma. Valaciclovir was given to all patients prophylactic (2×500 mg daily). Viral reactivation and/or infection were defined as CMV load exceeding 50 copies/ml plasma.

### Analysis of lymphocyte markers by flow cytometry

Differentiation and activation status of lymphocytes was analysed by six-colour fluorescence flowcytometry. To this end, PBMC were stained with CD3-PerCP, CD4-PE Cy7, CD8-APC Cy7, in combination with either CD45RO-PE, CD27-APC and PD-1 FITC or HLA-DR FITC (Becton Dickinson, San José, California, United States) and CD38 PE (Sanquin, Amsterdam, The Netherlands). Before staining with Perforin-FITC (BD) cells were permeabilized (FACS Permeabilizing Solution and FACS Lysis Solution, BD), washed and stained with specific antibodies. 100,000 cells were acquired by the LSRII flow cytometer (BD). Based on the expression of CD45RO and CD27, T cells were divided into naïve (CD27<sup>+</sup>CD45RO<sup>-</sup>), central-memory (CD27<sup>+</sup>, CD45RO<sup>+</sup>) and effector-memory (CD27<sup>-</sup>CD45RO<sup>+</sup>) or effector (CD27<sup>-</sup>CD45RO<sup>-</sup>) T-cell populations [21].

### T-cell stimulation

PBMC were stimulated with overlapping peptide pools consisting of 15-mer peptides with 11 amino acid overlap. Peptide pools spanned the entire pp65 or IE-1 protein (138 and 120 peptides, respectively) (Mimotopes, Melbourne, Australia) and consisted of a final concentration of 1 mg/ml of each peptide, dissolved in DMSO.

### Intracellular cytokine staining after antigenic stimulation

1-2×10<sup>6</sup> PBMC, were stimulated for 6 hours with pp65 or IE1 peptide pool (2 µg/ml) and anti-CD28 (1 µg/ml) as costimulation at

37°C. After one and half hour 1:1000 Monensin was added (Golgistop, BD Biosciences) to allow accumulation of cytokines in the cytosol. As a negative control PBMC were stimulated with medium and co-stimulation alone. As a positive control PBMC were stimulated with PMA (10 ng/ml) and ionomycin (2 µg/ml). PBMC were washed and stained with CD3-PerCP and CD4-APC Cy7 (BD), permeabilized (FACS Permeabilizing Solution and FACS Lysis Solution, BD), washed again and stained with specific antibodies for IFNγ-APC and IL-2-PE (BD). 200,000 cells were acquired by the LSRII flow cytometer (BD) and data were analysed by BD FACSDiva software. In case of IFNγ- and IL-2-measurements, the number of responding T cells was calculated after subtraction of the negative control values.

### Antigen-specific T-cell proliferation

PBMC were pelleted at a final concentration of 6×10<sup>6</sup> PBMC/ml and labelled with CFSE (Molecular Probes) according to the manufacturer's protocol. 1-2×10<sup>6</sup> PBMC were stimulated with 2 µg/ml pp65 or IE-1 peptide pool or with anti-CD3 and anti-CD28 (positive control) or medium (RPMI with pen/strep and 10% human pooled serum) alone (negative control) for 5 days at 37°C. PBMC were stained with CD3 PerCP, CD8 APC (BD) and CD4 APC Cy7 (BD Pharmingen), and 200,000 cells were acquired by the LSRII flow cytometer (BD) and analysed by BD FACSDiva software. Stimulation indices (SI) were calculated by dividing the percentage proliferating cells after stimulation with antigen by the percentage of proliferating cell after culture with medium.

### Statistical analyses

Statistics were computed by SPSS 15.0 for Windows (SPSS, Inc., Chicago, Illinois, USA). Differences between groups were compared using the Fisher's Exact test in case of discrete variables. In case of continuous variables the Mann-Whitney test was used. *P*-values <0.05 were considered significant.

		Total		CMV reactivation		no reactivation		p-value
		n	%	n	%	n	%	
Total included		22		14		8		
Sex	male	11	50	8	57	3	38	0,659
	female	11	50	6	43	5	63	
Age (years)	median (range)	56	(18-66)	58	(19-66)	52	(18-61)	0,165
Diagnosis	AML	8	36	5	36	3	38	0,423
	MM	5	23	2	14	3	38	
	NHL	6	27	5	36	1	13	
	Hodgkin	1	5	1	7	0	0	
	ALL	1	5	1	7	0	0	
	MDS	1	5	0	0	1	13	
HLA mismatch		2	9	2	14	0	0	0,515
Donor	related	17	77	11	79	6	75	1,000
	unrelated	5	23	3	21	2	25	
Conditioning	nma	19	86	13	93	6	75	0,527
	ma	3	14	1	7	2	25	
ATG		7	32	4	29	3	38	1,000
CMV serostatus	R+/D+	8	36	8	57	0	0	0,010
	R+/D-	9	41	5	36	4	50	
	R-/D+	5	23	1	7	4	50	
	R-/D-	0	0	0	0	0	0	

AML: Acute Myeloid Leukemia; MM: Multiple Myeloma; NHL: Non-Hodgkin Lymphoma; ALL: Acute Lymphatic Leukemia; MDS: Myelodysplastic Syndrome; NMA: Non-myeloablative; MA: Myeloablative; ATG: Anti-thymocyte Globulin; CMV: Cytomegalovirus; SCT: Stem Cell Transplantation  
Differences between groups were compared using the Fisher's Exact test in case of discrete variables. In case of continuous variables the Mann-Whitney test was used.

**Table 1:** Patient characteristics.

## Results

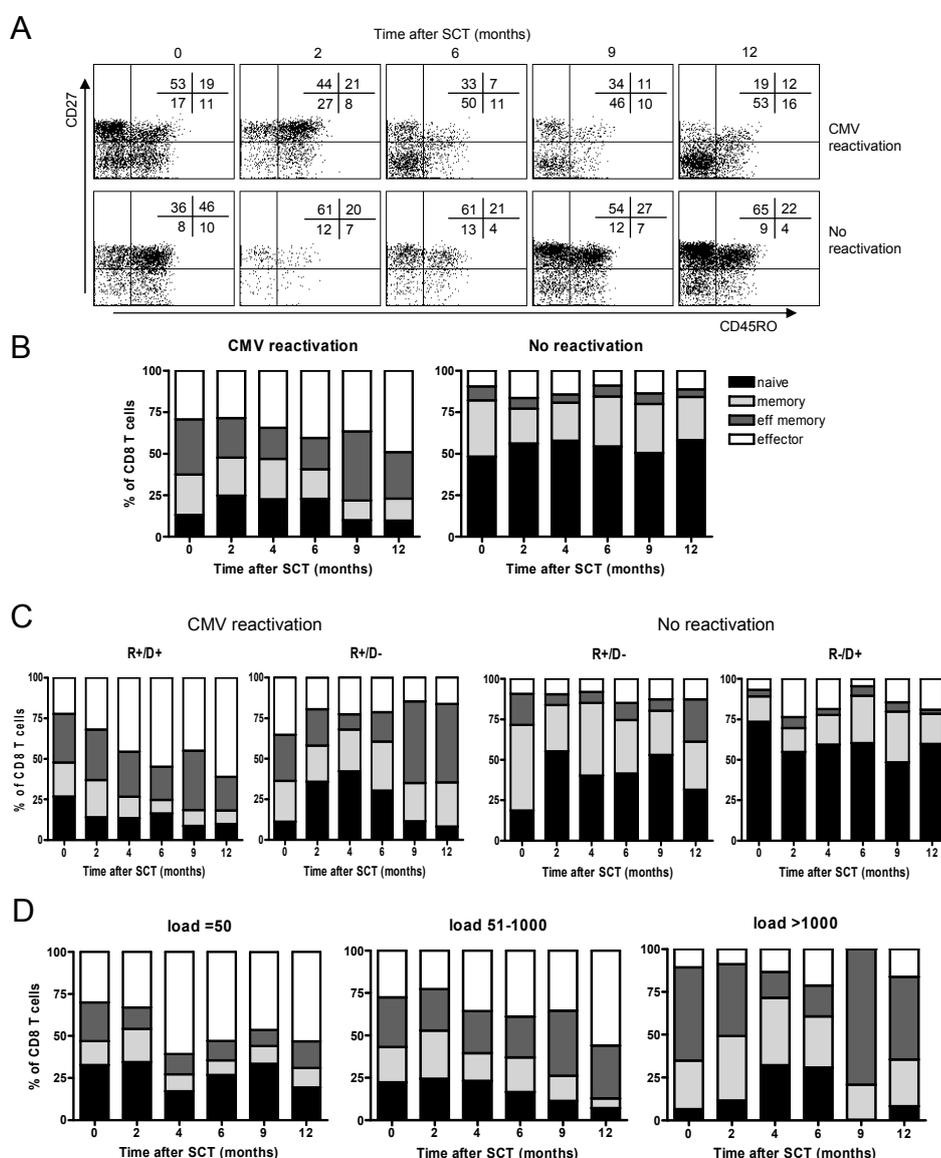
### Characteristics of SCT recipients with and without CMV reactivation

CMV-reactivation occurred in 14 out of 22 SCT patients (64%), mostly within 2 months after SCT (86%) (Table 1). Only donor and recipient CMV-serostatus were associated with CMV-reactivation after SCT ( $p=0.010$ ).

### CMV-reactivation skews to a more differentiated T-cell phenotype

As CMV-infection drives T cells to a differentiated phenotype in healthy subjects [14], we investigated the influence of CMV-reactivation

on phenotype differentiation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells after SCT. Figure 1A shows a representative T-cell phenotype development after SCT of a patient with and without CMV-reactivation. Interestingly, CD8<sup>+</sup> T cells from patients without CMV-reactivation consisted for the major part of naïve (CD45RO<sup>-</sup>/CD27<sup>+</sup>, range 54-65% post-SCT) T cells and showed a slow recovery of naïve and memory T cells (Figure 1A, lower panel). Percentages of naïve T cells after SCT were significantly higher in patients without reactivation compared to those with CMV-reactivation ( $p=0.008$ , Figure 1B). CD8<sup>+</sup> T cells from reactivating patients mainly consisted of effector-memory (CD45RO<sup>+</sup>/CD27<sup>-</sup>) and effector (CD45RO<sup>-</sup>/CD27<sup>-</sup>) T cells which recovered shortly after SCT and increased further after SCT (Figure 1A, upper panel). Percentages of effector-memory T cells after SCT were significantly increased in the



**Figure 1: Longitudinal phenotype differentiation after SCT in patients with and without CMV-reactivation.** A. Representative examples of phenotype differentiation of CD8<sup>+</sup> T cells from a patient with (upper panel) and without CMV-reactivation (lower panel). Percentages in each quadrant are shown in the upper right corner. B. Mean percentage of naïve (CD45RO<sup>-</sup>/CD27<sup>+</sup>, black), central-memory (CD45RO<sup>+</sup>/CD27<sup>+</sup>, light grey), effector-memory (CD45RO<sup>+</sup>/CD27<sup>-</sup>, dark grey) and effector (CD45RO<sup>-</sup>/CD27<sup>-</sup>, white) CD8<sup>+</sup> T cells in patients with and without CMV-reactivation. C. Phenotype differentiation of CD8<sup>+</sup> T cells depending on recipient and donor CMV-serostatus in patients with and without CMV-reactivation. D. Phenotype differentiation of patients with CMV reactivation depending of the height of the peak viral load within one year post-SCT (copies/ml).

patient group with CMV-reactivation ( $p=0.006$ , Figure 1B). Already before SCT, percentages of effector-memory CD8<sup>+</sup> T cells, but not CD4<sup>+</sup> T cells, were increased in patients with CMV-reactivation ( $p=0.002$ , Figure 1B), although there is no knowledge of CMV-reactivation at that time.

Therefore, we investigated the impact of CMV-serostatus of donor and recipient on the differentiation of CD8<sup>+</sup> T cells, which may explain the observed effector-memory T-cell dominance pre-SCT. The group with detectable CMV load post SCT was represented by only 1 R<sup>+</sup>/D<sup>+</sup> patient who developed CMV-disease and was left out of this analysis. We compared the R<sup>+</sup>/D<sup>+</sup> and R<sup>+</sup>/D<sup>-</sup> patients with CMV-reactivation ( $n=8$  and  $n=5$ , respectively) and the R<sup>+</sup>/D<sup>-</sup> and R<sup>-</sup>/D<sup>+</sup> patients without CMV-reactivation ( $n=4$  and  $n=4$ , respectively; Figure 1C). The most differentiated phenotype was observed in the R<sup>+</sup>/D<sup>+</sup> patients with CMV-reactivation. After SCT, percentages of naïve (CD45RO<sup>-</sup>/CD27<sup>+</sup>) and central-memory (CD45RO<sup>+</sup>/CD27<sup>+</sup>) CD8<sup>+</sup> T cells were significantly decreased in the R<sup>+</sup>/D<sup>+</sup> patients compared to the R<sup>+</sup>/D<sup>-</sup> patients with CMV-reactivation ( $p=0.030$  and  $p=0.045$ , respectively).

To determine the effect of the viral load level on differentiation of CD8<sup>+</sup> T cells, patients with CMV-reactivation were divided into three groups based on their peak viral load level within one year post-SCT (Figure 1D). Interestingly, the patient group with a low (detectable,  $\leq 50$  copies/ml,  $n=3$ ) or intermediate (50-1000 copies/ml,  $n=7$ ) peak viral load tended to differentiate towards the so-called terminally-differentiated effector (CD45RO<sup>+</sup>/CD27<sup>-</sup>) CD8<sup>+</sup> T cells, whereas in the group with high viral peak load ( $>1000$  copies/ml,  $n=3$ ) more effector-memory (CD45<sup>+</sup>/CD27<sup>-</sup>) CD8<sup>+</sup> T cells were observed.

### Effect of CMV-reactivation on immune activation and expression of perforin

Next, generalized activation of T lymphocytes by expression of both HLA-DR and CD38 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells was measured. Increased activation was apparent in CD4<sup>+</sup> T cells, but not in CD8<sup>+</sup> T cells, in patients with CMV-reactivation after SCT ( $p=0.037$ ; Figure 2A). This difference in activation was already present before SCT (T0,  $p=0.011$ ). Programmed death (PD)-1 receptor, a negative regulator of T-cell activity which is up-regulated after activation, has been suggested to be a prognostic indicator of CMV-disease in solid-organ transplant patients [17]. Figure 2B shows that PD-1 expression was similar between both groups in CD4<sup>+</sup> as well as CD8<sup>+</sup> T cells. Interestingly, high PD-1 expression (MFI  $>500$ ) was only observed in patients with a viral load  $>1000$  copies/ml on CD4<sup>+</sup> as well as CD8<sup>+</sup> T cells (data not shown).

The expression of perforin was measured in CD8<sup>+</sup> T cells, as a marker for effector CD8<sup>+</sup> T cells with cytolytic activity. Figure 2C shows representative FACS stainings of perforin-expression by CD8<sup>+</sup> T cells from a patient with (left panel) and without CMV-reactivation (right panel), two months post SCT. In patients with CMV-reactivation, perforin-expression was significantly increased ( $p=0.045$ , median perforin-expression by CD8<sup>+</sup> T cells from all time points: 23.9%) compared to patients without CMV-reactivation (median perforin-expression by CD8<sup>+</sup> T cells from all time points: 9.0%; Figure 2C and 2D).

### CMV-specific T-cell responses are dominated by pp65

We measured CMV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses towards pp65 and IE1 overlapping peptide pools in patients with and without CMV-reactivation. The maximum T-cell response against

CMV-antigen within one year post-SCT is shown in Figure 3A. Pp65-specific CD8<sup>+</sup> T-cell responses tended to be more frequently (8 out of 13 patients, 62%) above background (0.2% of CD8<sup>+</sup> T cells, measured in healthy CMV-seronegative controls) in patients with CMV-reactivation compared to patients without reactivation (2 out of 8 patients, 25%). Although less pronounced, also IE1-specific CD8<sup>+</sup> T-cells responses tended to be more regularly detected in patients with CMV-reactivation (5 out of 13 patients, 38%) compared to patients without CMV-reactivation (2 out of 8 patients, 25%). Differences in CD4<sup>+</sup> T-cell responses directed against either pp65 or IE1 were not observed. In addition, IL-2 production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells was very low in both groups (data not shown).

### IE1-specific T-cell proliferation is mainly present in patients with CMV-reactivation

Proliferative capacity in response to either pp65 or IE1 was studied by CFSE dye dilution. As proliferative responses in general were of low magnitude, patients were divided in three groups based upon their proliferative capacity during the whole time period post-SCT; A stimulation index (SI) $<1.5$  indicative of lack of proliferative capacity,  $1.5<SI<2.0$  representing intermediate proliferative capacity and  $SI>2.0$  indicating good proliferative capacity. Proliferative capacity in response to pp65 was observed in CD4<sup>+</sup> and CD8<sup>+</sup> T cells in both patients with and without CMV-reactivation (Figure 3B). However, responses towards IE1 were absent or very low in patients without CMV-reactivation, whereas proliferative capacity was present in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in approximately half of the patients with CMV-reactivation.

### Immunological features of a SCT patient with primary CMV-infection

We analysed features and function of T-cell responses during primary CMV-infection in a patient who was CMV-seronegative before transplantation and was transplanted with stem cells from a CMV-seropositive donor. CD8<sup>+</sup> T cells differentiated to predominantly effector-memory and effector T cells (Figure 4A) and were highly activated, as measured by the expression of both HLA-DR and CD38 (reaching up to 78% of CD8<sup>+</sup> T cells). During acute CMV-infection the percentage perforin<sup>+</sup>CD8<sup>+</sup> T cell strongly increased (from undetectable to 58% of CD8<sup>+</sup> T cells), higher than patients with CMV-reactivation (median 10.7% of CD8<sup>+</sup> T cells), and remained high for at least one year post-SCT, though viral load was undetectable after treatment. Two and four months post SCT no CMV-specific T-cell responses were observed. More than four months after SCT CMV-specific immunity started to reconstitute. Although IL-2 producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells were hardly present (Figure 4D), both CD4<sup>+</sup> and CD8<sup>+</sup> T cells produced IFN $\gamma$  (Figure 4B) and displayed proliferative capacity in response to pp65 and IE1 (Figure 4C). Especially CD8<sup>+</sup> T-cell responses directed towards IE1 were high and remained at least until one year post-SCT (Figure 4D).

### Discussion

To investigate the causal relationship between CMV-reactivation and reconstitution of CMV-specific T-cell responses, immunological features of SCT patients with and without CMV-reactivation were determined.

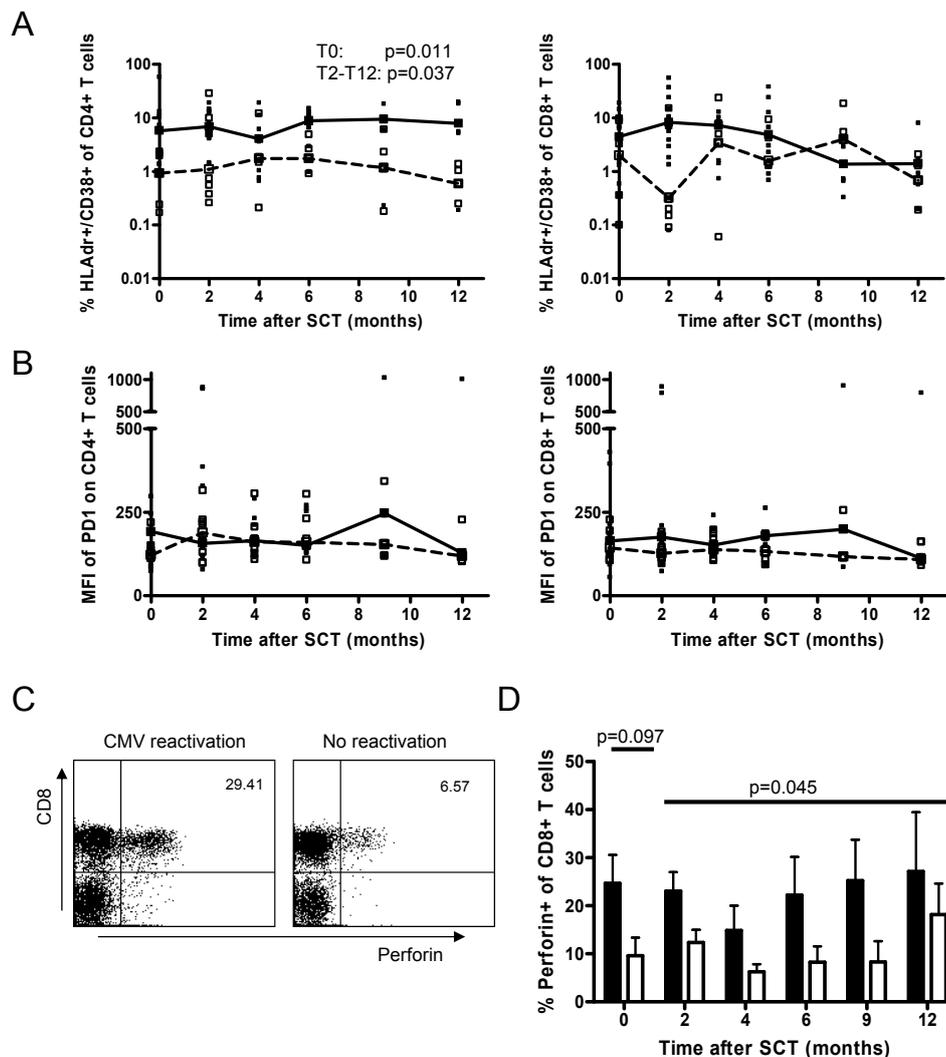
We observed a more differentiated phenotype of CD8<sup>+</sup> T cells, but not of CD4<sup>+</sup> T cells, in patients with CMV-reactivation compared to those without, which fits with the knowledge that CD8<sup>+</sup> T cells

are more pronounced in their differentiation status compared to CD4<sup>+</sup> T cells [14]. Interestingly, this difference in T-cell phenotype is already observed before SCT, suggesting that parameters prior to SCT may influence T-cell phenotype. In line with the observation that CMV drives CD8<sup>+</sup> T-cell differentiation, in the patient group with CMV-reactivation recipients were already CMV-seropositive before transplantation, whereas only half of the patients without CMV-reactivation were CMV-seronegative pre-SCT and displayed therefore a more naïve phenotype. After SCT the antigenic drive for T-cell differentiation is absent in patients without CMV-reactivation and reconstitution mainly results from thymic output. In the R<sup>+</sup>/D<sup>-</sup> pairs from the patient group with CMV-reactivation the development of a more differentiated T-cell phenotype is therefore somewhat delayed. In the R<sup>+</sup>/D<sup>+</sup> pairs CMV-specific T cells from the donor will expand rapidly, whereas in the R<sup>+</sup>/D<sup>-</sup> pairs reconstitution of a new CMV-specific T-cell pool is required.

The CMV load level did not appear to have a major impact on

T-cell differentiation, as a relatively small amount of virus is already sufficient for a differentiated T-cell phenotype. Interestingly, the high viral load group tended to display a more effector-memory phenotype, whereas the patients with low or intermediate viral load level showed a more effector phenotype. As high viral load levels were accompanied by recurrent reactivation, this may have led to differences in CD8<sup>+</sup> T-cell differentiation. A recent study showed lack of CD45RA<sup>+</sup>CD27<sup>-</sup>CD28<sup>-</sup> T cell in patients with uncontrolled CMV-reactivations [22]. Furthermore, Gamadia et al. [11] showed in renal transplant patients loss of CD45RO only when CMV load had dropped, suggesting that CMV drives T cells to an effector-memory phenotype during periods of antigen exposure, whereas T cells differentiate into terminally-differentiated effector type T cells only when antigen levels are reduced.

Prolonged CMV-reactivation may lead to exhaustion of T cells as has been suggested for other antigens [23,24]. Interestingly, high expression of the exhaustion marker PD-1 (MFI>500) was only observed in patients with a viral load above 1000 copies/ml. Previously

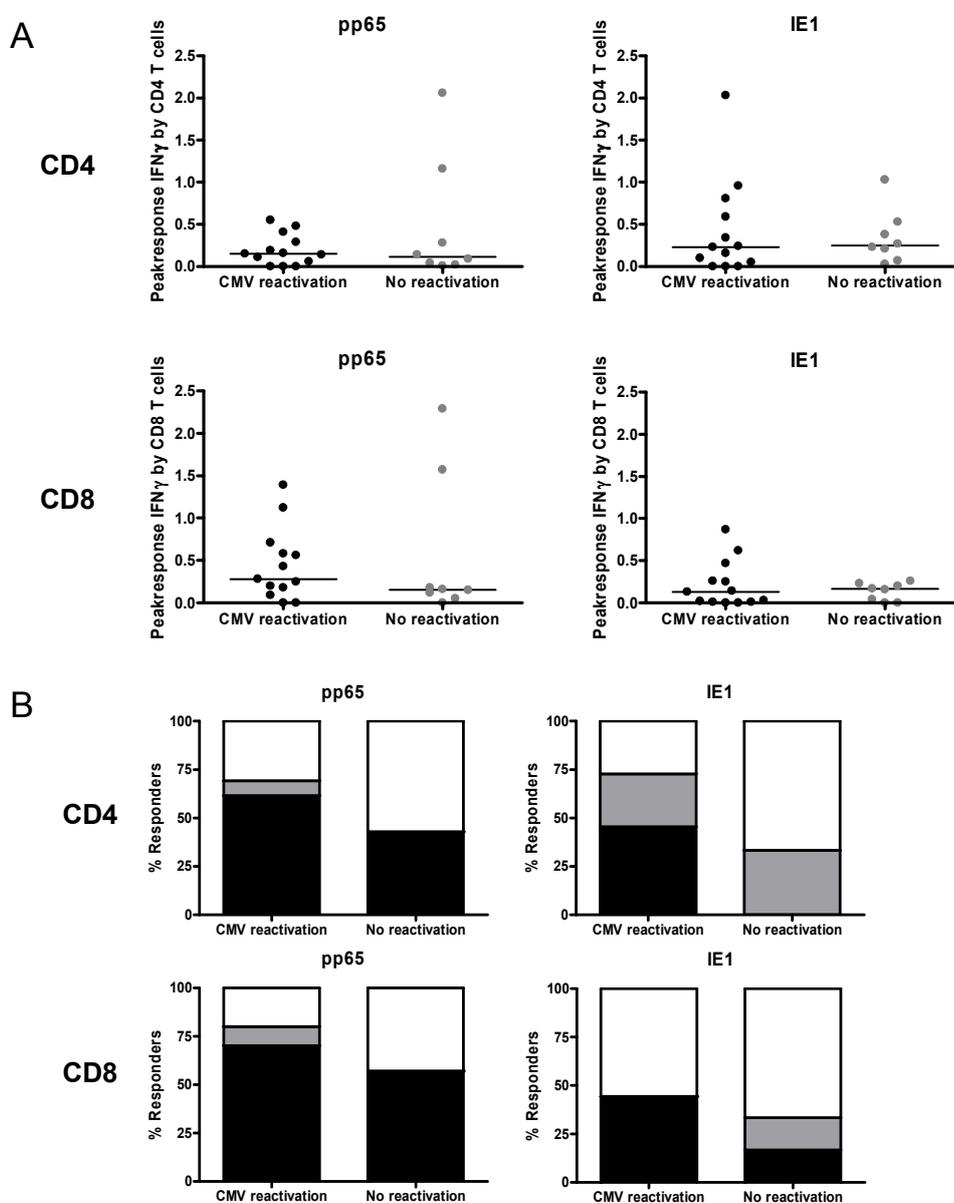


**Figure 2: T-cell activation, PD-1 and perforin-expression in patients with and without CMV-reactivation.** Percentage of HLA-DR<sup>+</sup>/CD38<sup>+</sup> (A) and MFI of PD-1 (B) in CD4<sup>+</sup> and CD8<sup>+</sup> T cells of patients with (black square; median straight line) and without (open square; median dashed line) CMV-reactivation. C. Representative example of perforin-expression by CD8<sup>+</sup> T cells in a patient with and without CMV-reactivation, two months after SCT. Percentages of perforin<sup>+</sup> T cells within the CD8<sup>+</sup> T cells are indicated in the upper right corner. D. Mean (and S.E.M.) percentage of perforin-expression in patients with (black bars) and without (white bars) CMV-reactivation.

Barber et al. [25] showed only upregulation of PD-1 on CD8<sup>+</sup> during the late phase of chronic infection.

Activation of CD4<sup>+</sup> T cells was increased in patients with CMV-reactivation; however this difference was also observed before SCT. No significant differences in activation of CD8<sup>+</sup> T cells were observed, probably due to low numbers of patients in both groups. Especially early after SCT (2 months), CD8<sup>+</sup> T-cell activation is different between the two groups, but this difference is lost during follow-up. Increased CD8<sup>+</sup> T-cell activation was observed clearly during acute infection in a patient subsequently developing CMV colitis, which may represent activation of specific immunity. As a measure for potent CD8<sup>+</sup> T-cell

function, perforin levels were increased in patients with CMV-reactivation compared to those without reactivation. Previous studies described high levels of perforin-expression by CMV-specific CD8<sup>+</sup> T cells [15,25,26], indicating that high levels of perforin<sup>+</sup>CD8<sup>+</sup> T cells measured in this study may be a consequence of high levels of CMV-specific T cells. Remarkably, perforin-expression tended to be increased in patients with CMV-reactivation already before reactivation. Since effector T cells, which are preferentially present in CMV-seropositive individuals, express more perforin compared to T cells with a more early phenotype, the difference in perforin-expression present before SCT might reflect the more differentiated phenotype of T cells in



**Figure 3: CMV-specific IFN $\gamma$ -production and proliferative capacity.** A. IFN $\gamma$ -production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells in response to pp65 or IE1 in patients with and without CMV-reactivation after SCT were measured by ICCS. Presented are the maximum T-cell responses within one year post-SCT. The median is indicated by a straight line. B. Proliferative capacity of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in response to pp65 or IE1 was measured by CFSE-labelling. At each time point SI was determined and categorized into good (S.I.>2.0), intermediate (1.5<S.I.<2.0) or no proliferative capacity (S.I.<1.5). Presented is the percentage of patients with most responses after SCT in the represented category. Good proliferative capacity S.I.>2.0, black; Intermediate proliferative capacity 1.5<S.I.<2.0, grey; No proliferative capacity S.I.<1.5, white.

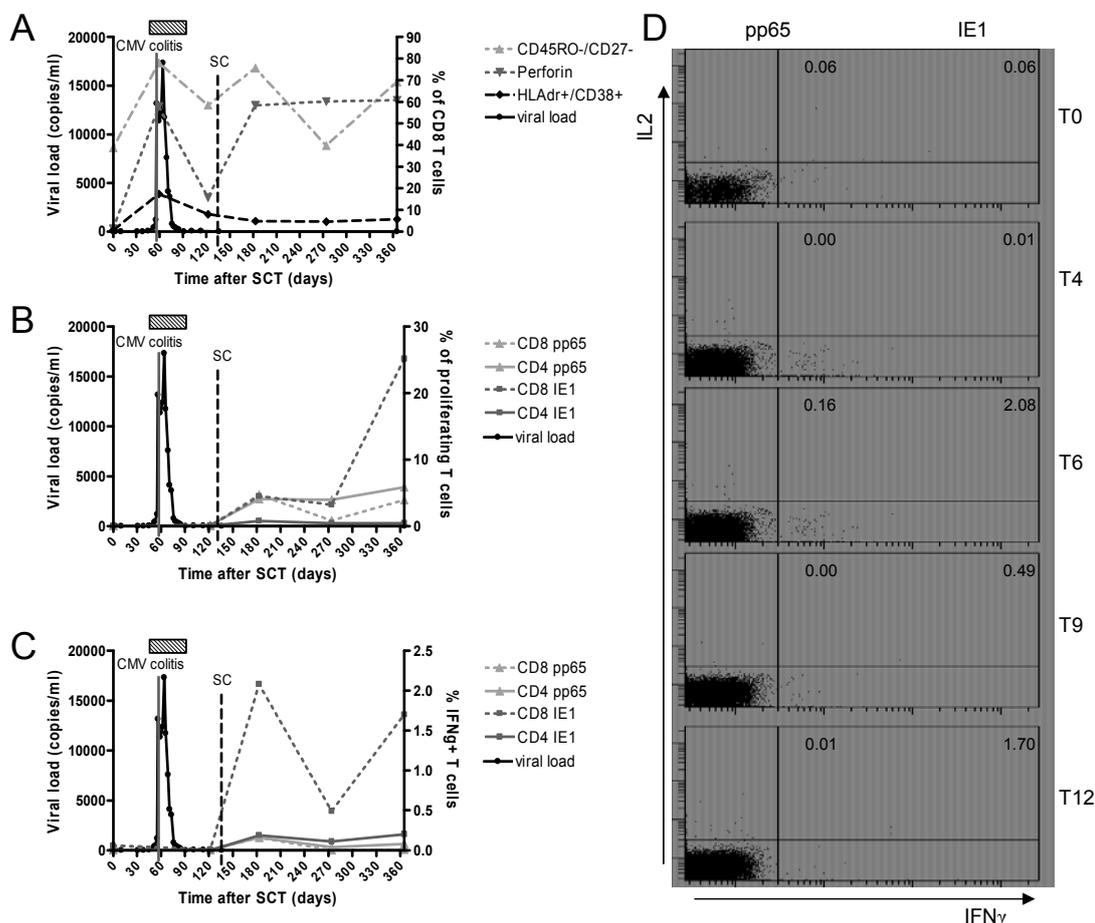
patients who are CMV-seropositive before SCT. Alternatively, perforin-expression may indicate subclinical reactivation or alloreactivity.

CD8<sup>+</sup> T-cell responses against pp65 and IE1 were observed more frequently in patients with CMV-reactivation, compared to patients without reactivation. This suggests that the recent encounter of virus leads to increased IFN $\gamma$ -production by CMV-specific CD8<sup>+</sup> T cells, a characteristic of effector T cells, which were abundantly present [16]. Furthermore, pp65-specific CD8<sup>+</sup> T-cell responses were increased compared to IE1-specific T cells, which is in accordance with previous findings [27-29]. However, these pp65-specific responses were not correlated with protection against reactivation [23,24]. Furthermore, IFN $\gamma$  production after CMV-antigen stimulation by CD3<sup>+</sup> T cells was reported to correlate with lower peak viral loads four weeks after SCT [30]. Sacre et al. [31] reported a correlation between IE1-specific T-cell reconstitution in the first three months post SCT and protection against CMV-replication. Low pp65-specific CD4<sup>+</sup> T-cell responses and low

IE1-specific CD8<sup>+</sup> T-cell responses have been reported to correlate with recurrent CMV-reactivation after SCT, whereas in patients with single or no reactivations better responses were observed [28].

In the patient who developed a primary CMV-infection after SCT, delayed reconstitution of CMV-specific responses, as measured by IFN $\gamma$ -production, may have led to the development of CMV-colitis. In line with our observation, previous studies reported suppressed [32] or lack of IFN $\gamma$ -production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells in patients with CMV-disease [32]. In line with Morita-Hoshi et al. [32] we observed an increase in responses with recovery of disease.

In conclusion, in this study a more differentiated phenotype, up-regulation of the activation markers CD38 and HLA-DR on CD4<sup>+</sup> T cells and increased expression of perforin on CD8<sup>+</sup> T cells was more frequently observed in patients with CMV-reactivation compared to patients without reactivation. This suggests that active CMV-



**Figure 4: Immunological features of a patient with primary CMV-infection and subsequent development of CMV-colitis.** A. Viral load (copies/ml), straight line, black; Percentage of activated CD8<sup>+</sup> T cells (HLA-DR<sup>+</sup>/CD38<sup>+</sup>), dashed line, black; Percentage of perforin-expression by CD8<sup>+</sup> T cells, dashed line, dark grey; Percentage effector cells (CD45RO/CD27<sup>+</sup>) of CD8<sup>+</sup> T cells. Percentages of proliferating (B) and IFN $\gamma$ -producing (C) CD4<sup>+</sup> and CD8<sup>+</sup> T cells in response to pp65 or IE1. Viral load (copies/ml), straight line, black; Percentage responding CD4<sup>+</sup> (straight line) and CD8<sup>+</sup> (dashed line) T cells in response to pp65 (light grey) and IE1 (dark grey). S.C. CMV-seroconversion, striped box; treatment with ganciclovir. D. FACS staining on several time points post-SCT of the production of IFN $\gamma$  and IL-2 by CD8<sup>+</sup> T cells after stimulation with pp65 or IE1. Percentages of IFN $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells are indicated.

This patient was transplanted with stem cells from a matched, related, CMV-seropositive donor after non-myeloablative conditioning. After SCT, CMV load became detectable on day 45 and the patient subsequently developed CMV-colitis after 58 days, as diagnosed by histology of sigmoid biopsies. The patient had not developed GVHD at time of diagnosis of CMV-colitis. CMV load reached a maximum viral load of  $1.7 \times 10^4$  copies/ml on day 64. The patient was treated with intravenous ganciclovir from day 56 till day 92. From day 90 onwards CMV load remained undetectable for at least one year. Serology was performed to confirm primary CMV-infection. IgM and IgG became detectable three months after the first detection of CMV load (day 136 post SCT).

replication plays a role in immune activation, thereby influencing T-cell features as phenotype differentiation and perforin-expression. The early occurrence of these differences, some already detectable before SCT, may indicate these parameters as potential prognostic or diagnostic markers. Interestingly in this light is our recent observation that perforin<sup>+</sup> CD8<sup>+</sup> T cells very early after SCT (first 3 weeks) predict the severity of viral-reactivation in a prospective cohort study [33].

In addition, CMV-specific CD8<sup>+</sup> T-cell responses directed against both pp65 and IE1 tended to be present more frequently in patients with CMV-reactivation compared to those without reactivation. These data fit with earlier studies showing that activation of CMV is a potent stimulator of T-cell function [27,28] and suggest that CMV-antigen may be required for reconstitution of CMV-specific T-cell responses. Future studies should aim to identify the main determinant of CMV-reactivation in the interaction between CMV load and CMV-specific immunity in the immunocompromised host.

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