T and NK cell immunity after hematopoietic stem cell transplantation

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Chapter 2

Early CMV reactivation leaves a specific and dynamic imprint on the reconstituting T cell compartment long term post hematopoietic stem cell transplantation

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Abstract

Human Cytomegalovirus (CMV) reactivation frequently occurs during the early phase of immune recovery after allogeneic hematopoietic stem cell transplantation (HSCT). Whereas the recovery of virus-specific immunity in the early phase after HSCT is extensively studied, the impact of CMV on the reconstitution and composition of the T cell compartment long term post HSCT is unknown. We analyzed T cell reconstitution one to two year after HSCT in 131 pediatric patients. One year post HSCT, patients with early CMV reactivation (n=46) had three-fold higher $CD8^+$ T cell numbers (median 1323 vs. 424 cells/µl, $p<0.0001$) compared to patients without CMV reactivation (n=85). This effect, caused by a major expansion of $CD8^+$ effector memory (EM) and end-stage effector (EMRA) T cells, was independent of pre-transplant donor and recipient CMV serostatus and not seen after Epstein-Barr virus or Adenovirus reactivations. At one and two year post HSCT, the absolute numbers of $CD8^+$ naive and central memory (CM) T cells as well as $CD4^+$ naïve, CM, EM and EMRA T cells did not differ between patients with and without CMV reactivation. In the second year post HSCT, a significant contraction of the initially expanded $CD8^+$ EM and EMRA T cell compartments was observed in patients with early CMV reactivation. In conclusion, CMV reactivation early after pediatric HSCT leaves a specific and dynamic imprint on the size and composition of $CD8^+$ T cell compartment without compromising the reconstitution of $CD8^+$ and $CD4^+$ naive and central memory T cells pivotal in the response to neo- and recall antigens.

Introduction

In immunocompetent individuals, CMV infection generally causes mild symptoms after which latency is established and asymptomatic reactivations can occur sporadically throughout life.[139] In contrast, CMV reactivation occurs frequently during the transient period of immune deficiency after allogeneic hematopoietic stem cell transplantation (HSCT) in case of a CMV seropositive donor and / or recipient. This donor- or recipient derived CMV infection or reactivation, hereafter called reactivation, can lead to disseminated infections causing interstitial pneumonitis, colitis and hepatitis which are, despite the pre-emptive use of antiviral medication, a major cause of morbidity and mortality.[46;140] Reconstitution of $CD4^+$ and $CD8^+$ T cell immunity is pivotal to provide protection against and achieve sustained control of CMV reactivations.[37;141;142] In the first months after HSCT, the repopulation of the T cell compartment is facilitated by cytokine- and antigen-driven homeostatic peripheral expansion of T cells that were transplanted with the graft.[143;144] The thymic-dependent T cell reconstitution of naive and central memory cells, essential for a balanced immune system with a broad specificity against neo- and recall antigens is delayed for many months to years.[143]

The differentiation stages of human T cells can be discerned based on the expression of cell surface markers CD45RA and CCR7. Naive ($CD45RA^+$ $CCR7^+$) cells differentiate upon antigen exposure into central memory (CM, $CD45RA^-$ $CCR7^+$) and effector memory (EM, $CD45RA^-$ $CCR7^-$) cells and eventually regain CD45RA when differentiating into end-stage effector (EMRA, $CCR7^-$ $CD45RA^+$) cells.[70] Whereas the expression of co-stimulatory molecules and chemokine receptors varies between the differentiation stages, ex-vivo cytolytic capacity increases during differentiation and telomere length shortens.[70;145] With increasing age, a continuous accumulation of CMV-specific $CD8^+$ T cells, also called "memory inflation", has been described in healthy CMV seropositive individuals. These cells mainly represent EM and EMRA T cells.[146-148] A durable expansion of these late differentiated T cells has also been observed in infants with congenital and post-natal CMV infections [149-152] and after primary CMV infection or reactivation in patients continuously receiving immunosuppressive medication after solid organ transplantation (SOT).[147;153;154]

In the recent years, the interaction between viral reactivations and early immune reconstitution after HSCT has been the studied extensively.[37;141;142;155;156] However, apart from an early report in 1985, the influence of viral reactivations occurring during the early phase of immune reconstitution on the composition of the T cell compartment in steady state conditions after HSCT is largely unknown.[157] In this study, we report the impact of early CMV reactivations on the reconstitution and composition of the T cell compartment one and two year after HSCT in a large cohort of pediatric HSCT recipients.

Methods

Ethics Statement

All transplantations were performed according to European society for Blood and Marrow Transplantation guidelines. Blood samples were routinely obtained and analyzed after approval by the institutional review board (protocol P01.028). Informed consent was provided by the patient and/or a parent or guardian.

Patients and blood sampling

Between 1-1-2002 and 31-12-2011, 227 children received a Bone Marrow or Peripheral Blood Stem Cell transplantation following myeloablative conditioning for malignant and non-malignant hematological disorders in the Leiden University Medical Center (LUMC). A total of 59 patients died in the first year and 11 were lost to follow up (Figure 2.S1). Of the 157 remaining patients, 26 were excluded, resulting in a cohort of 131 patients. Exclusion criteria were relapse of malignant disease (n=8) or autologous reinfusion (n=1) in the first year after HSCT, systemic immunosuppressive drugs at one year post HSCT (n=15), or clinically relevant CMV infection after day +250 (n=2). Patients who received two transplantations (n=14) were analyzed only after their second transplantation. Peripheral blood samples were obtained for routine analysis at different time points after HSCT and the sample drawn closest to one year (median 363 days, range 302-478 days) post HSCT was analyzed in this study. In 76 patients, this sample could be compared with a sample obtained two year (median 729, range 498-849 days) post HSCT and at least 180 days after the first sample.

Monitoring and treatment of viral reactivations

After HSCT, plasma was routinely screened for Epstein Barr Virus (EBV), CMV and Human Adenovirus (HAdV) DNA by real time quantitative (RQ) PCR.[158-160] The limit of detection in these assays was 50 (1.7 log) viral DNA copies/ml. Reactivation of CMV, EBV and HAdV was defined as detection of viral DNA in plasma at least once, whereas pre-emptive treatment with respectively Ganciclovir, Rituximab or Cidofovir was initiated upon detection of 1000 (3 log) or more viral DNA copies/ml at two or more consecutive time points.[161-163]

Flow cytometric analysis

To determine the size of individual lymphocyte populations and subsets, peripheral blood mononuclear cells (PBMC) were analyzed by flow cytometry. PBMC were separated using ficoll-isopaque density gradient centrifugation (LUMC Pharmacy, Leiden, NL). PBMC were stained with CD45, CD14, CD33, CD235a, CD3, CD19, CD56, CD4, CD8 and TCR-γδ antibodies, listed in Table 2.S1. Four-color flow cytometry was performed on a BD FACS Calibur II flow cytometer (Becton Dickinson Biosciences (BD), Franklin Lakes, NJ, US) and data were analyzed using BD Cellquest software. Lymphocytes were defined as $CD45^+$ CD33/CD235a/$CD14^-$ cells within the forward / sideward scatter lymphocyte gate and absolute cell numbers per µl of peripheral blood were calculated. In a representative subcohort of 53 consecutive patients who

		no CMV reactivation		CMV reactivation		p - value	p - value
All patients		**85**		**46**		*Univariate*	*Multivariate*
Age of recipient	*Year*	7.9	(0.4 - 18)	13	(0.5 - 19)	*0.001 (c)*	*0.016 (d)*
Sex of recipient	*Male*	56	(66 %)	32	(70 %)	*0.70 (a)*	
CMV serostatus of recipient	*Positive (anti-CMV IgG)*	26	(31 %)	38	(83 %)	*<0.0001 (a)*	*<0.0001 (d)*
Age of donor	*Year*	24	(2.0 - 52)	28	(1.0 - 57)	*0.58 (c)*	
Sex of donor	*Male*	43	(51 %)	23	(50 %)	*1.0 (a)*	
CMV serostatus of donor	*Positive (anti-CMV IgG)*	26	(31 %)	36	(78 %)	*<0.0001 (a)*	*<0.0001 (d)*
Indication for HSCT	*Non-malign hematology*	27	(32 %)	21	(46 %)	*0.13 (a)*	
	Malign hematology	58	(68 %)	25	(54 %)		
Donor type	*Identical Related*	35	(41 %)	17	(37 %)	*0.72 (b)*	
	Haplo-identical	9	(11 %)	7	(15 %)		
	Matched Unrelated	41	(48 %)	22	(48 %)		
Graft source	*Bone Marrow*	71	(84 %)	36	(78 %)	*0.48 (a)*	
	Peripheral Blood Stem Cells	14	(17 %)	10	(22 %)		
Conditioning regimen	*Total Body Irradiation based*	37	(44 %)	18	(39 %)	*0.71 (a)*	
	Chemotherapy based	48	(57 %)	28	(61 %)		
Serotherapy	*None*	23	(27 %)	10	(22 %)	*0.49 (b)*	
	Anti-Thymocyte Globulin	53	(63 %)	28	(61 %)		
	Alemtuzumab	9	(11 %)	8	(17 %)		
T cell depletion	*Yes*	9	(11 %)	8	(17 %)	*0.29 (a)*	
Donor Lymphocyte Infusion	*Yes*	5	(6 %)	2	(4 %)	*1.0 (a)*	
Stem Cell Boost	*Yes*	2	(2 %)	1	(2 %)	*1.0 (a)*	
Acute GVHD ≥ grade 2	*Yes*	13	(15 %)	5	(11 %)	*0.60 (a)*	
Peak CMV load	*Log copies / mL*	N.A.		3.8	(2.3 - 6.0)	N.A.	
Duration CMV reactivation	*Day*	N.A.		36	(1 - 185)	N.A.	
Start CMV reactivation	*First day PCR ≥ 1.7 log*	N.A.		26	(5 - 62)	N.A.	
End CMV reactivation	*Last day PCR ≥ 1.7 log*	N.A.		80	(13 - 211)	N.A.	
Ganciclovir therapy for CMV	*DNA load ≥ twice ≥ 3 log*	N.A.		33	(72 %)	N.A.	
EBV reactivation	*EBV DNA load ≥ 1.7 log*	50	(59 %)	22	(48 %)	*0.20 (a)*	
Rituximab therapy for EBV	*DNA load ≥ twice ≥ 3 log*	11	(13 %)	7	(15 %)	*0.79 (a)*	
HAdV reactivation	*HAdV DNA load ≥ 1.7 log*	20	(24 %)	15	(33 %)	*0.30 (a)*	
Cidofovir therapy for HAdV	*DNA load ≥ twice ≥ 3 log*	7	(8 %)	2	(4 %)	*0.49 (a)*	

Table 2.1. Characteristics of patients with and without early cytomegalovirus reactivation.

Categorical data are displayed as: number (percentage). Numerical data are displayed as: median (range). p-values: (a) Fisher's Exact test, (b) Pearson Chi Square test, (c) Mann-Whitney U test, (d) logistic regression analysis. N.A.: Not Applicable, HSCT: Hematopoietic Stem Cell Transplantation, CMV: Cytomegalovirus, EBV: Epstein-Barr-virus, HAdV: Human Adenovirus, GVHD: Graft-versus-Host-Disease. Applied T cell depletion methods: erythrocyte rosetting (n=2), alemtuzumab in the bag (n=4) and CliniMACS CD34 enrichment (n=11).
transplantation between 2008 and 2011, T cell differentiation was also analyzed based on CD45RA and CCR7 expression.

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Statistical analysis

Statistical analysis was performed using SPSS Statistics 20 (IBM SPSS Inc, Chicago, IL, US). GraphPad Prism software (version 6.00; GraphPad Software, San Diego, CA, US) was used to construct figures. Because cell numbers did not follow Gaussian distribution, Mann-Whitney U test (2 groups), Kruskall-Wallis test (> 2 groups) and Wilcoxon Matched-pairs signed ranks test (paired analysis) were used for the univariate analysis of numerical parameters. Pearson's correlation test was performed on log-transformed data for analysis of univariate correlations. Fisher exact test (2 groups) and Pearson's Chi-square test (> 2 groups) were used for univariate analysis of categorical parameters. Logistic regression analysis and linear regression analysis on log-transformed data were performed for multivariate analysis of parameters with p-values ≤ 0.10 in univariate analysis.

Results

Incidence of CMV reactivations and description of patients

In the study cohort of 131 pediatric stem cell transplantation recipients who were available for follow up one year post HSCT, 46 patients (35%) experienced a CMV reactivation in the first 100 days post HSCT. The median duration of CMV viremia was 36 (range 1 – 185) days, starting 5 – 62 (median 26) days after HSCT. The criteria for pre-emptive Ganciclovir treatment were met in 33 of 46 patients (Table 2.1). At the time of transplantation, patients with CMV reactivation after HSCT were more often CMV seropositive (83 vs. 31 %, *p<0.0001*) and were older (median 13.0 vs. 7.9 year, *p=0.001*) than patients without CMV reactivation. Also, patients receiving a graft from a CMV seropositive donor more often experienced a CMV reactivation (78 vs. 31 %, *p<0.0001*). As shown in Table 2.1, no significant differences were observed in other HSCT related parameters. In a logistic regression analysis of parameters with p-values < 0.10 in univariate analysis, pre-transplant CMV serostatus of recipient and donor as well as patient age differed significantly between children with and without CMV reactivation post HSCT (*p<0.0001, p<0.0001* and *p=0.017,* respectively*).*

Patients with early CMV reactivation had higher $CD8^+$ T cell numbers one year post HSCT

Compared with 85 patients without CMV reactivation, 46 patients with early CMV reactivation had significantly higher lymphocyte numbers one year after HSCT. This could be attributed to an increase of CD3+ T cells (median 2083 vs. 1257 cells/µl, p<0.0001) whereas NK- and B cell numbers were three-fold higher (median 1323 vs. 424 cells/µl, p<0.0001) in patients with CMV

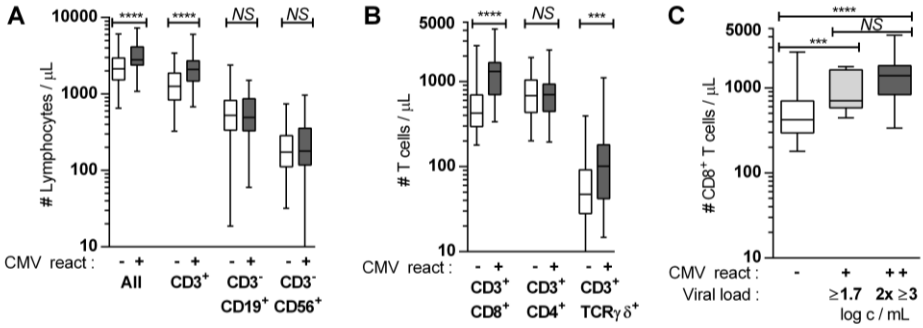

Figure 2.1. Impact of early CMV reactivation on lymphocyte numbers and -subsets one year post HSCT. **(A)** Absolute numbers of lymphocytes, CD3+ T cells, CD3-CD19+ B-cells and CD3-CD56+ NK cells and **(B)** CD8+ T cells, CD4+ T cells and T cell receptor γδ+ T cells from 85 patients without CMV reactivation (white bars, CMV DNA load always < 1.7 log copies/ml (c / ml)) and 46 patients with CMV reactivation (gray bars, CMV DNA load ≥ 1x >1.7 log). **(C)** Absolute numbers of CD8+ T cells of 85 patients without detectable CMV reactivation (white bars), 13 patients with detectable CMV DNA in plasma but not ≥ 3 log c / ml at two consecutive time points (light gray bars) and 33 patients with a CMV load ≥ 3 log c / ml at two or more consecutive time points (dark gray bars). Shown are cells/µl of peripheral blood one year post HSCT. Bars: median, interquartile range and overall range. p-values: Mann-Whitney test, NS: p > 0.05, ***: p<0.001, ****: p<0.0001

reactivation (Figure 2.1B). Patients with a low level CMV reactivation (n=13), i.e. CMV plasma DNA load above the limit of detection, but not at two consecutive time points above 3.0 log copies/ml, already had significantly higher $CD8^+$ T cell numbers one year post HSCT than patients without CMV reactivation (median 709 vs. 424 cells/µl, *p=0.0001*, Figure 2.1C). The highest measured plasma CMV DNA load and the time between CMV clearance and analysis at one year post HSCT did not influence the number of $CD8^+$ T cells at one year post HSCT (Figure 2.S2). Also, the impact of early CMV reactivation on the $CD8^+$ T cell compartment did not differ between patients who received a stem cell graft from an HLA-identical related donor, matched unrelated donor or haplo-identical donor (data not shown).

$CD4^+$ T cell numbers were comparable in patients with and without CMV reactivation (median 702 vs. 684 cells/µl, *p=0.79*, Figure 2.1B). Although γδ T cells only represented a small proportion (median 4 %) of all T cells, γδ T cell levels were twofold higher in patients with an early CMV reactivation (median 104 vs. 47 cells/µl, *p=0.0005*, Figure 2.1B).

The expansion of the $CD8^+$ T cell subset was independent of pre-transplant CMV serostatus and not seen after EBV and HAdV reactivation

In univariate analysis, $CD8^+$ T cell numbers - but not $CD4^+$ T cell numbers (data not shown) - were significantly influenced by CMV reactivation after HSCT (median 1323 vs. 424 cells/µl, *p<0.0001*) as well as by pre-transplant CMV serostatus of donor (median 780 vs. 460 cells/µl, *p<0.0001*) and recipient (median 880 vs. 440 cells/µl, *p<0.0001*, Figure 2.S3). However, in multivariate regression analysis, pre-transplant serostatus of the donor (*p=0.22)* and recipient (*p=0.14*) did not influence $CD8^+$ T cell numbers independently. This is illustrated in Figure 2.2A,

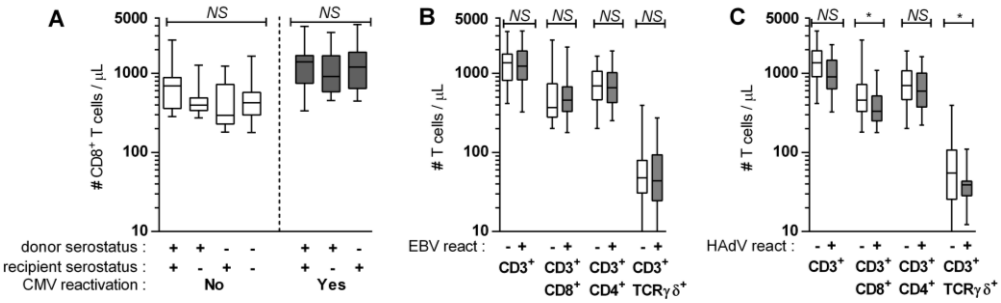

Figure 2.2. Influence of pre-transplant CMV serostatus and early EBV and HAdV reactivation.
(A) Influence of donor and recipient pre-transplant CMV serostatus on $CD8^+$ T cell numbers in patients without CMV reactivation (white bars, + / +: n=14, + / -: n=12, - / +: n=13 and - / -: n=46) and patients with CMV reactivation (gray bars, + / +: n=28, + / -: n=8 and - / +, n=10). **(B & C)** Absolute numbers of $CD3^+$, $CD4^+$, $CD8^+$ and T cell receptor $\gamma\delta^+$ T cell in 85 patients without CMV reactivation. (B) 35 patients without vs. 50 patients with early Epstein-Barr virus (EBV) reactivation and (C) 65 patients without vs. 20 patients with early Human Adenovirus (HAdV) reactivation. White bars (-): plasma DNA load always < 1.7 log. Gray bars (+): plasma DNA load at least once > 1.7 log. Shown are cells/µl of peripheral blood one year post HSCT. Bars: median, interquartile range and overall range, *p*-values: Kruskall-Wallis test (A) or Mann-Whitney test (B & C). *NS: p > 0.05, *: p<0.05*.

showing the size of the $CD8^+$ T cell compartment for patients with- and without early CMV reactivation distributed over the different pre-transplant donor-recipient serostatus combinations.
Epstein-Barr Virus (EBV) or Human Adenovirus (HAdV) reactivations as well as other HSCT related variables including T cell depletion of the graft, serotherapy and age of donor and recipient did not significantly influence $CD8^+$ T cell numbers one year post HSCT (Figure 2.S3). Considering the high impact of CMV reactivation on the $CD8^+$ T cell compartment, we separately analyzed the effect of early EBV and HAdV reactivations in the 85 patients without CMV reactivation. One year post HSCT, T-, B- and NK cell numbers did not differ between patients with and without EBV or HAdV reactivations (Figure 2.S4). Also, the size of CD8, CD4 and γδ T cell subsets did not differ between patients with (n=50) and without (n=35) EBV reactivation (Figure 2.2B). Patients with HAdV reactivations (n=20) had lower numbers of $CD8^+$ T cells and γδ T cells than patients (n=65) without HAdV reactivation (Figure 2.2C). However, patients with and without HAdV reactivations were not fully comparable (Table 2.S2). After multivariate correction for alemtuzumab serotherapy and T cell depletion of the graft, the impact of early HAdV reactivation on $CD8^+$ T cell numbers one year post HSCT was not statistically significant (*p=0.11*).

The increase of $CD8^+$ T cells after CMV reactivation was caused by an expansion of EM and EMRA cells

In a sub-cohort of 53 consecutive patients transplanted between 2008 and 2011, we further determined the differentiation stages of $CD4^+$ and $CD8^+$ T cells. Patients in this sub-cohort did not differ significantly from the whole cohort with respect to the parameters listed in Table 2.1 (data not shown). In both T cell subsets, but most pronounced in the $CD8^+$ T cell subset, the proportion

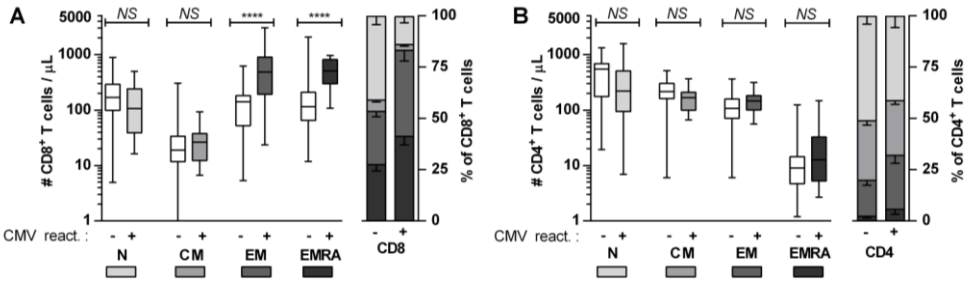

Figure 2.3. Early CMV reactivation and T cell differentiation one year post HSCT.
Absolute numbers (left panels) and relative contribution (right panels) of T cell differentiation stages to the **(A)** $CD8^+$ and **(B)** $CD4^+$ T cell subsets in a subcohort of 35 patients without (-) and 18 patients with (+) CMV reactivation. N: naive (very light gray, $CD45RA^+$ $CCR7^+$), CM: central memory (light gray, $CD45RA^-$ $CCR7^+$), EM: effector memory (gray, $CD45RA^-$ $CCR7^-$), EMRA (dark gray, $CD45RA^+$ $CCR7^-$). Absolute numbers: shown are cells/µl of peripheral blood one year post HSCT. Bars: median, interquartile range and overall range. *p*-values: Mann-Whitney test. *NS: p > 0.05, ****: p<0.0001.* Relative contribution: bars show mean percentage of the $CD8^+$ or $CD4^+$ T cell subset with Standard Error of the Mean.

of effector memory and end-stage effector T cells was enlarged in patients with (n=18) compared to patients without (n=35) early CMV reactivation. Together, the cells of the EM and EMRA phenotype constituted a median of 88% of the $CD8^+$ T cell subset in patients with early CMV reactivation compared to 55% in patients without CMV reactivation (*p<0.0001,* Figure 2.3A).
Next, we calculated absolute cell numbers for the T cell differentiation stages. The altered memory differentiation in the $CD8^+$ T cell subset in patients with early CMV reactivation was caused by a major expansion of $CD8^+$ EM and EMRA T cells (median 485 vs. 141, *p<0.0001* and 509 vs. 114 cells/µl, *p<0.0001*, Figure 2.3 A). The size of the $CD8^+$ naïve and CM T cell compartment were not significantly affected by early CMV reactivation. The increased contribution of $CD4^+$ memory T cells in patients with early CMV reactivation was caused by a non-significant increase of $CD4^+$ and EMRA T cells and a non-significant reduction of $CD4^+$ naive and CM T cell numbers (Figure 2.3B).

Dynamics of $CD8^+$ T cell expansion in patients with early CMV reactivation after HSCT

Within the 46 patients with an early CMV reactivation, we categorized patients based on the size of the primary $CD8^+$ T cell recovery at the moment of CMV clearance. In patients with $CD8^+$ T cell numbers < 500 cells/µl at the moment of CMV clearance (n=21), the $CD8^+$ T cell number gradually increased after the clearance of CMV (Figure 2.4A). In contrast, in patients with $CD8^+$ T cell numbers > 500 cells/µl at the moment of CMV clearance (n=25), this subset did not further expand (Figure 2.4A). Whereas at one year post HSCT, the $CD8^+$ T cell number was not influenced by T cell depletion of the graft (Figure 2.S3), all patients (n=8) who received a T cell depleted graft had $CD8^+$ T cell numbers < 500 cells/µl at the moment of CMV clearance (p=0.001,

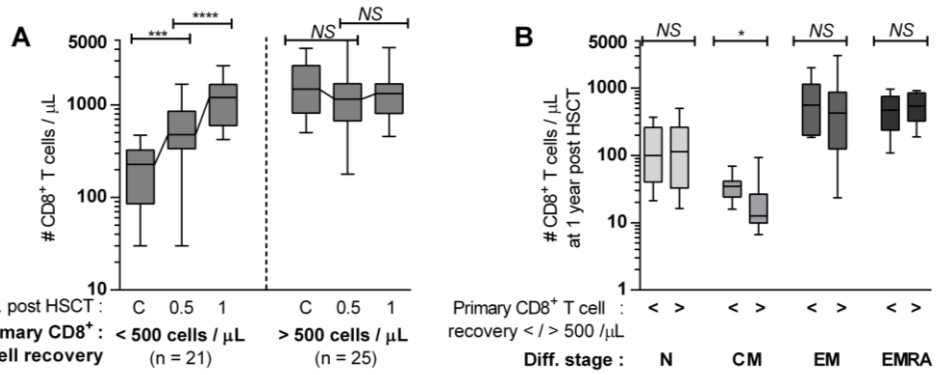

Figure 2.4. Dynamics of CD8+ T cell expansion in patients with early CMV reactivation.

(A) Dynamics of CD8+ T cell reconstitution after CMV clearance in 21 patients with a primary CD8+ T cell recovery < 500 cells/µl and 25 patients with a primary recovery > 500 cells/µl. Shown are cells/µl of peripheral blood at the moment of CMV clearance (C), 0.5 year and 1 year after HSCT. Bars: median, interquartile range and overall range. Lines connect median values. p-values: Wilcoxon Matched-pairs signed ranks test. NS: p > 0.05, ***: p<0.001, ****: p<0.0001.

(B) Absolute numbers of T cell differentiation stages one year after HSCT in 9 patients with a primary $CD8^+$ T cell recovery < 500 cells/µl and 9 patients with a primary recovery > 500 cells/µl. N: naive (very light gray, $CD45RA^+$ $CCR7^+$), CM: central memory (light gray, $CD45RA^-$ $CCR7^+$), EM: effector memory (gray, $CD45RA^-$ $CCR7^-$), EMRA (dark gray, $CD45RA^+$ $CCR7^-$). Shown are cells/µl of peripheral blood one year post HSCT. Bars: median, interquartile range and overall range. *p*-values: Mann-Whitney test. *NS: p > 0.05, *: p<0.05.*

Table 2.S3). Although the dynamics of the $CD8^+$ T cell expansion differed, the size and composition of the $CD8^+$ T cell compartment at one year post HSCT were comparable between patients with high or low $CD8^+$ T cell numbers at the time of CMV clearance (Figure 2.4B).

Ongoing reconstitution of naive and CM cells in parallel with contraction of EM and EMRA $CD8^+$ T cells in the second year post HSCT

Finally, we investigated the influence of early CMV reactivations on the T cell reconstitution in the second year after HSCT. The number of $CD8^+$ T cells decreased in patients with early CMV reactivation (n=34, median 1340 to 1141 cells/µl, *p=0.010*) whereas an increase in $CD8^+$ T cell numbers was seen in patients without CMV reactivation (n=42, median 473 to 619 cells/µl, *p=0.043,* Figure 2.5A). Still, the $CD8^+$ T cell number at two years post HSCT remained significantly higher in patients with early CMV reactivation (*p=0.0002*). In both groups, $CD4^+$ T cell numbers increased in the second year after HSCT.

More profound changes were observed in the separate T cell differentiation compartments in a sub-cohort of 31 HSCT recipients transplanted between 2008 and 2010. In patients with early CMV reactivation (n=12), a contraction of late differentiated $CD8^+$ EM (median 825 to 618, *p=0.0068*) and EMRA T cells (555 to 378 cells/µl, *p=0.0342*) was observed. This was accompanied by a further reconstitution of $CD8^+$ naive and CM T cells (Figure 2.5B). In patients without CMV reactivation (n=19), $CD8^+$ naive and CM cell numbers increased, while the size of

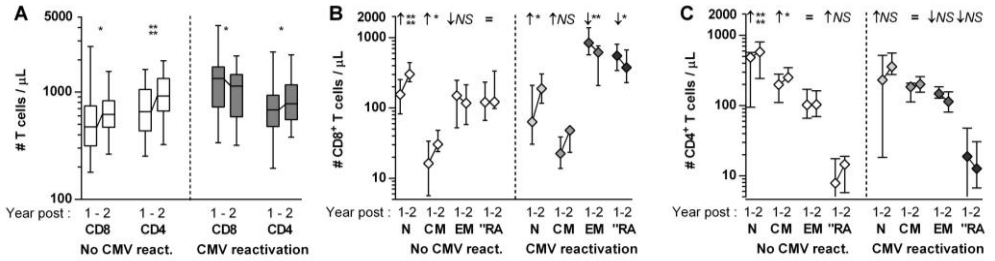

Figure 2.5. Dynamics of T cell reconstitution one and two year after HSCT.

(A) Paired analysis of absolute numbers of $CD8^+$ and $CD4^+$ T cells in 42 patients without and 34 patients with early CMV reactivation at one and two year post HSCT. Shown are cells/µl of peripheral blood one and two year post HSCT.

(B & C) Paired analysis of absolute numbers of (B) $CD8^+$ and (C) $CD4^+$ N, CM, EM and EMRA T cells in 19 patients without (-) and 12 patients with (+) CMV reactivation at one and two year post HSCT. N: naive (very light gray, $CD45RA^+$ $CCR7^+$), CM: central memory (light gray, $CD45RA^-$ $CCR7^+$), EM: effector memory (gray, $CD45RA^-$ $CCR7^-$), EMRA (dark gray, $CD45RA^+$ $CCR7^-$). Shown are cells/µl of peripheral blood one and two year post HSCT. Bars (A): median, interquartile range and overall range. Diamonds (B & C): Median and interquartile range. Lines connect median values one and two year post HSCT. Arrows (↑ / ↓): increase / reduction. *p*-values: Wilcoxon Matched-pairs signed ranks test. *= : p > 0.10, NS: 0.05 < p<0.10, *: p<0.05, **: p<0.01, ****: p<0.001.*

the $CD8^+$ EM and EMRA compartments remained stable over time. The increase of $CD4^+$ T cell numbers was caused by an expansion of naive and CM cells both in patients with and without early CMV reactivation (Figure 2.5C).

Discussion

Here, we provide evidence that early and transient CMV reactivation leaves a long-lasting, dynamic and specific signature on the composition of the T cell compartment in pediatric HSCT recipients. One year post transplant, patients that had encountered (and cleared) early CMV reactivation showed a marked relative as well as absolute expansion of the $CD8^+$ EM and EMRA T cell populations. This typical pattern was not seen in patients with early EBV or HAdV reactivation and early CMV reactivation did not compromise the reconstitution of the naive and CM compartments. Furthermore, the CMV signature on T cell reconstitution was subjected to dynamic changes in the year thereafter. This throws a new light on the early findings by Würsch *et al.* in 1985, describing the impact of early CMV infection on the developing T cell immunity in adult HSCT recipients.[157]

One year post HSCT, none of the patients included in this study had active CMV infection. Also, pre-transplant CMV seropositivity of donor or recipient without detectable CMV viremia post HSCT did not trigger the expansion of $CD8^+$ T cells. Therefore, we hypothesize that CMV reactivation and -viremia in the immunocompromized period early post HSCT leads to infection

of large numbers of cells, which remain infected latently and can lead to subclinical reactivations, providing antigenic stimulus for the ongoing expansion and differentiation of CMV-specific T cells in a pro-inflammatory environment.[143;144;164;165] This proceeds after discontinuation of immunosuppressive medication post HSCT and is most evidently observed in patients with a relatively small number of $CD8^+$ T cells at the moment of CMV clearance. However, the relative contribution of peripherally expanded memory cells which have been transplanted with the graft and differentiated, newly educated naive T cells to the pool of $CD8^+$ EM and EMRA T cells is not known.[143;144] Although our observations do not allow for a definitive conclusion about the specificity of the expanded $CD8^+$ T cells, the differentiation of virus-specific T cells has been studied extensively. CMV specific $CD8^+$ T cells are mainly of the late differentiated EM and EMRA phenotypes and especially the EMRA phenotype is more often seen in the context of CMV compared to other viruses.[146;147;166] Of note, T cell exhaustion, characterized by the upregulation of inhibitory receptors like PD-1 and the progressive loss of cytokine production, proliferative capacity and cytolytic function,[167] has not been found applicable for CMV-specific T cells.[168-170]

$CD4^+$ T cells are essential for the maintenance of CMV-specific $CD8^+$ T cell responses and can also acquire direct cytolytic capacity.[171-173] Indeed, one and two year post HSCT, a minor but stable expansion of $CD4^+$ T cells of the late EMRA phenotype was found in patients with early CMV reactivation. More profound changes were present in the γδ-T cell subset. Their role in CMV clearance has been well established and these cells can compose up to 30% of T cells at the time of CMV clearance. [174;175]

We show that, although their relative contribution to the respective T cell subsets was decreased, the numerical reconstitution of naive and central memory cells in both the $CD8^+$ and the $CD4^+$ T cell subset was not negatively affected by early CMV reactivation. This indicates that thymic output and the generation of a central memory compartment are not disturbed. The reconstitution of early differentiated T cells is essential for a healthy and balanced adaptive immune system with the capacity to produce cellular immune responses against neo- and recall antigens.[143] However, in contrast to for example recall vaccination responses, the evaluation of T cell responses against neo antigens it is very difficult. Still, this hypothesis is supported by the observation that the memory compartment is flexible and expansion of CMV-specific memory cells does not result in lower numbers of EBV- and influenza specific T cells after solid organ transplantation.[166] Furthermore, the accumulation of late differentiated CMV-specific memory cells has not been observed in lymph nodes, leaving immunologic space for the generation of new immune responses.[176]

CMV reactivation in adult solid organ transplant (SOT) recipients receiving life-long immunosuppressive medication has been correlated to an accelerated and ongoing accumulation of late differentiated T cells with a stable (relative) contribution of CMV specific T cells.[154;165;177] Although the number of $CD8^+$ T cells expanded after CMV clearance in pediatric HSCT recipients with a small primary $CD8^+$ T cell response, a stable condition was reached early in children with high $CD8^+$ T cell numbers at the moment of CMV clearance. Also, we did not observe a further expansion of memory cells in the second year after HSCT but even noticed a reduction of the $CD8^+$ EM and EMRA compartment in children with early CMV reactivations.

The SOT setting differs fundamentally from HSCT as in HSCT recipients, CMV reactivations occur in a newly reconstituting immune system. Furthermore, SOT recipients receive life-long immunosuppressive medication, while in our cohort immunosuppression was tapered 3 to 6 months after transplantation.

We compared our results to reported findings in a more physiological setting of CMV infection in healthy Gambian infants as well. One year post infection, the proportion of late differentiated $CD8^+$ T cells was much smaller (mean 17%) in CMV infected infants than in our cohort but, similarly to our data after HSCT, the relative contribution of these cells to the $CD8^+$ T cell compartment decreased slightly in the second year after infection.[151;152] Although no absolute cell numbers were assessed in these studies, the difference might be explained by a large contribution of naive T cells in infants compared to pediatric HSCT recipients as well as the severity of the CMV infection after HSCT as discussed above.

Although we analyzed T cell reconstitution in a large cohort of HSCT recipients, the size and diversity of the subcohort of patients with an hematological malignancy did not allow for a reliable evaluation of the impact of early CMV reactivation on the relapse risk.[178] Also, extended follow up is required to evaluate the impact of early CMV reactivation on long term clinical outcome. One year after HSCT, the phenotype of the $CD8^+$ T cell subset of pediatric HSCT recipients with early CMV reactivation closely resembled that of the $CD8^+$ T cells of elderly CMV seropositive individuals.[148] However, the expansion of naive and CM cells together with a dynamic contraction of the $CD8^+$ late differentiated memory T cell compartment in the second year after HSCT imply that an ongoing process of immune-regulation and further reconstitution is operative in modeling the cellular immune system after discontinuation of immunosuppressive medication. Altogether, CMV reactivation early after HSCT leads to a dynamic expansion of late differentiated $CD8^+$ T cells on top of a normal reconstitution of the naive and central memory compartment.

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All authors declare that no potential financial conflicts of interest exist.

Supplemental data

Type	Antibody	Fluorochrome	Clone	Supplier
A) Lymphocyte subsets				
Lymphocyte gate	**CD45**	FITC	2D1	BD
	CD14	PE	MOP9	BD
	CD33	PE	P67.6	BD
	CD235a	PE	11E4B-7-6	BC
Lymphocyte subset	**CD3**	PerCP-Cy5.5	SK7	BD
	CD19	APC	J4.119	BC
	CD56	APC	N901	BC
T-cell subset	**CD4**	PE	SK3	BD
	CD8	APC	B9.11	BC
	TCR-γδ	FITC	11F2	BD
B) T-cell differentiation				
T-cell subset	**CD3**	PerCP-Cy5.5	SK7	BD
	CD8	APC	B9.11	BC
	CD4	APC	13B8.2	BC
T-cell differentiation	**CD45RA**	PE	2H4	BC
	CCR7	FITC	150503	R&D

Table 2.S1. Antibodies used for flow cytometry.

Staining was performed for 30 minutes at 4°C in FACS buffer: Phosphate Buffered Saline (Braun, Melsungen, GE) + 10 mg / ml Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO, US) + 3 mM Ethylenediamine-tetraacetic Acid (Merck Darmstadt, GE). FITC: Fluorescein isothiocyanate, PE: phycoerythrin, PERCP: Peridinin chlorophyll, Cy: Cyanine, APC: Allophycocyanin. BD: Becton Dickinson Biosciences, Franklin Lakes, NJ, US, BC: Beckman Coulter, Brea, CA, US, R&D: R&D systems, Minneapolis, MN, US.

		no HAdV reactivation		HAdV reactivation		*p* - value
All patients		**65**		**20**		
Age of recipient	*Year*	7.9	(0.4 - 17)	7.9	(2.1 - 18)	*0.79 (c)*
Sex of recipient	*Male*	45	(69 %)	11	(55 %)	*0.28 (a)*
CMV serostatus of recipient	*Positive (anti-CMV IgG)*	23	(35 %)	3	(15 %)	*0.10 (a)*
Age of donor	*Year*	24	(2.0 - 52)	29	(3.4 - 52)	*0.095 (c)*
Sex of donor	*Male*	32	(49 %)	11	(55 %)	*0.80 (a)*
CMV serostatus of donor	*Positive (anti-CMV IgG)*	22	(34 %)	4	(20 %)	*0.28 (a)*
Indication for HSCT	*Non-malign hematology*	18	(28 %)	9	(45 %)	*0.17 (a)*
	Malign hematology	47	(72 %)	11	(55 %)	
Donor type	*Identical Related*	30	(46 %)	5	(25 %)	
	Haplo-identical	4	(6 %)	5	(25 %)	*0.033 (b)*
	Matched Unrelated	31	(48 %)	10	(50 %)	
Graft source	*Bone Marrow*	59	(86 %)	15	(75 %)	*0.30 (a)*
	Peripheral Blood Stem Cells	9	(14 %)	5	(25 %)	
Conditioning regimen	*Total Body Irradiation based*	29	(45 %)	8	(40 %)	*0.80 (a)*
	Chemotherapy based	36	(55 %)	12	(60 %)	
Serotherapy	*None*	22	(34 %)	1	(5 %)	
	Anti-Thymocyte Globulin	39	(60 %)	14	(70 %)	*0.006 (b)*
	Alemtuzumab	4	(6 %)	5	(25 %)	
T-cell depletion	*Yes*	4	(6 %)	5	(25 %)	*0.03 (a)*
Donor Lymphocyte Infusion	*Yes*	3	(5 %)	2	(10 %)	*0.59 (a)*
Stem Cell Boost	*Yes*	0	(0 %)	2	(10 %)	*0.053 (a)*
Acute GVHD ≥ grade 2	*Yes*	10	(15 %)	3	(15 %)	*1.0 (a)*
EBV reactivation	*EBV DNA load ≥ 2.3 log*	37	(57 %)	14	(70 %)	*0.43 (a)*
Rituximab therapy for EBV	*DNA load ≥ twice ≥ 3.0 log*	5	(8 %)	6	(30 %)	*0.018 (a)*
Cidofovir therapy for HAdV	*DNA load ≥ twice ≥ 3.0 log*			7	(35 %)	*NA*

Table 2.S2. Characteristics of patients with and without early Adenovirus (HAdV) reactivation (CMV reactivation excluded)

Categorical data are displayed as: number (percentage). Numerical data are displayed as: median (range). *p* - values: (a) Fisher's Exact test, (b) Pearson Chi Square test, (c) Mann-Whitney U test. N.A.: Not Applicable, HSCT: Hematopoietic Stem Cell Transplantation, CMV: Cytomegalovirus, EBV: Epstein-Barr virus, HAdV: Adenovirus, GVHD: Graft-versus-Host-Disease

		<500 CD8⁺ T cells / µL		>500 CD8⁺ T cells / µL		*p* - value		*p* - value
All patients		**21**		**25**		*Univariate*		*Multivariate*
Age of recipient	*Year*	11	(2.7 - 16)	13	(0.5 - 19)	*0.12*	*(c)*	
Sex of recipient	*Male*	13	(61 %)	19	(76 %)	*0.30*	*(a)*	
CMV serostatus of recipient	*Positive (anti-CMV IgG)*	17	(81 %)	21	(84 %)	*0.78*	*(a)*	
Age of donor	*Year*	34	(2.0 - 57)	23	(1.0 - 43)	*0.027*	*(c)*	*0.22 (d)*
Sex of donor	*Male*	9	(43 %)	14	(56 %)	*0.38*	*(a)*	
CMV serostatus of donor	*Positive (anti-CMV IgG)*	19	(91 %)	17	(68 %)	*0.07*	*(a)*	*0.12 (d)*
Indication for HSCT	*Non-malign hematology*	10	(47 %)	11	(44 %)	*0.81*	*(a)*	
	Malign hematology	11	(53 %)	14	(56 %)			
Donor type	*Identical Related*	6	(52 %)	11	(44 %)	*0.07*	*(b)*	
	Haplo-identical	6	(29 %)	1	(4 %)			*0.84 (d)*
	Matched Unrelated	9	(29 %)	13	(52 %)			
Graft source	*Bone Marrow*	15	(71 %)	24	(84 %)	*0.30*	*(a)*	
	Peripheral Blood Stem Cells	6	(29 %)	4	(16 %)			
Conditioning regimen	*Total Body Irradiation based*	10	(48 %)	8	(32 %)	*0.28*	*(a)*	
	Chemotherapy based	11	(52 %)	17	(68 %)			
Serotherapy	*None*	2	(10 %)	8	(32 %)	*0.15*	*(b)*	
	Anti-Thymocyte Globulin	14	(67 %)	14	(56 %)			
	Alemtuzumab	5	(24 %)	3	(12 %)			
T cell depletion	*Yes*	8	(38 %)	0	(0 %)	*0.001*	*(a)*	*0.99 (d)*
Donor Lymphocyte Infusion	*Yes*	0	(0 %)	2	(8 %)	*0.19*	*(a)*	
Stem Cell Boost	*Yes*	1	(5 %)	0	(0 %)	*0.27*	*(a)*	
Acute GVHD ≥ grade 2	*Yes*	1	(5 %)	4	(16 %)	*0.23*	*(a)*	
Peak CMV load	*Log copies / mL*	3.9	(2.3 - 6.0)	3.7	(2.3 - 5.9)	*0.88*	*(c)*	
Duration CMV reactivation	*Day*	35	(1 - 134)	36	(2 - 185)	*0.37*	*(c)*	
Start CMV reactivation	*First day PCR ≥ 1.7 log*	19	(6 - 46)	26	(5 - 62)	*0.88*	*(c)*	
End CMV reactivation	*Last day PCR ≥ 1.7 log*	73	(13 - 146)	83	(27 - 211)	*0.15*	*(c)*	
Ganciclovir therapy for CMV	*DNA load ≥ twice ≥ 3 log*	6	(29 %)	7	(28 %)	*0.97*	*(a)*	
EBV reactivation	*EBV DNA load ≥ 1.7 log*	10	(48 %)	12	(48 %)	*0.98*	*(a)*	
Rituximab therapy for EBV	*DNA load ≥ twice ≥ 3 log*	3	(14 %)	4	(16 %)	*0.87*	*(a)*	
HAdV reactivation	*HAdV DNA load ≥ 1.7 log*	6	(29 %)	9	(36 %)	*0.60*	*(a)*	
Cidofovir therapy for HAdV	*DNA load ≥ twice ≥ 3 log*	1	(5 %)	1	(4 %)	*0.90*	*(a)*	

Table 2.S3. Characteristics of patients with a primary CD8+ T cell recovery < 500 or > 500 cells / µl at the time of CMV clearance

Categorical data are displayed as: number (percentage). Numerical data are displayed as: median (range). p - values: (a) Fisher's Exact test, (b) Pearson Chi Square test, (c) Mann-Whitney U test, (d) logistic regression analysis. N.A.: Not Applicable, HSCT: Hematopoietic Stem Cell Transplantation, CMV: Cytomegalovirus, EBV: Epstein-Barr virus, HAdV: Adenovirus, GVHD: Graft-versus-Host-Disease. Type of T cell depletion: alemtuzumab in the bag (n=3) and CliniMACS CD34 enrichment (n=5).

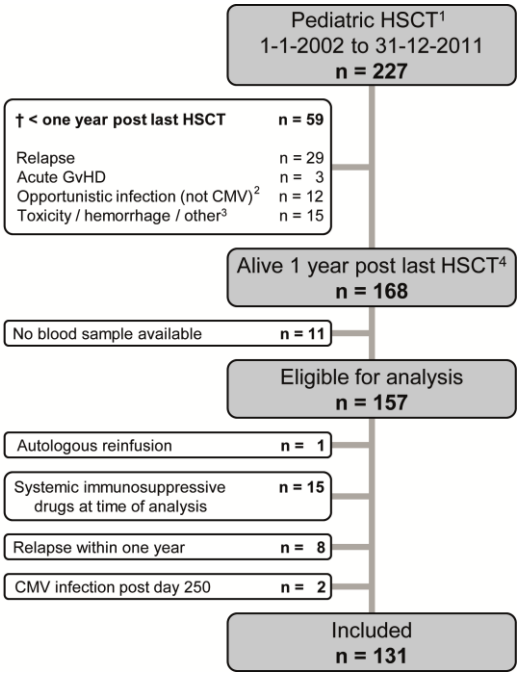

Figure 2.S1. Flow chart of patient inclusion.

[1]Bone Marrow or Peripheral Blood Stem Cell transplantation following myeloablative conditioning for malignant and non-malignant hematological disorders.

[2]Death from human adenovirus, respiratory syncytial virus, aspergillus or candida infection or bacterial sepsis.

[3]Death from transplant toxicity, veno occlusive disease, respiratory failure, hemorrhage, hyperhemolysis syndrome or post-transplant lymphoproliferative disease.

[4]Patients who received >1 HSCT were only included after the last HSCT. Abbreviations: GvHD: graft-versus-host disease, CMV: cytomegalovirus.

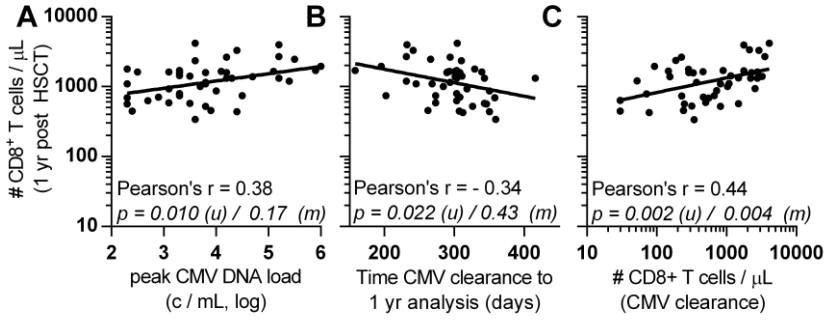

Figure 2.S2. Factors influencing CD8$^+$ T cell numbers one year post HSCT in 46 patients with early CMV reactivation.

Correlation between CD8$^+$ T cell numbers one year post HSCT and (A) highest measured plasma CMV DNA load, (B) time between CMV clearance and analysis 1 year post HSCT and (C) CD8$^+$ T cell numbers at time of CMV clearance. Shown are cells/µl of peripheral blood. Pearson's r: Pearson's correlation coefficient, univariate *p*-values (u): Pearson's correlation test, multivariate *p*-values (m): linear regression analysis.

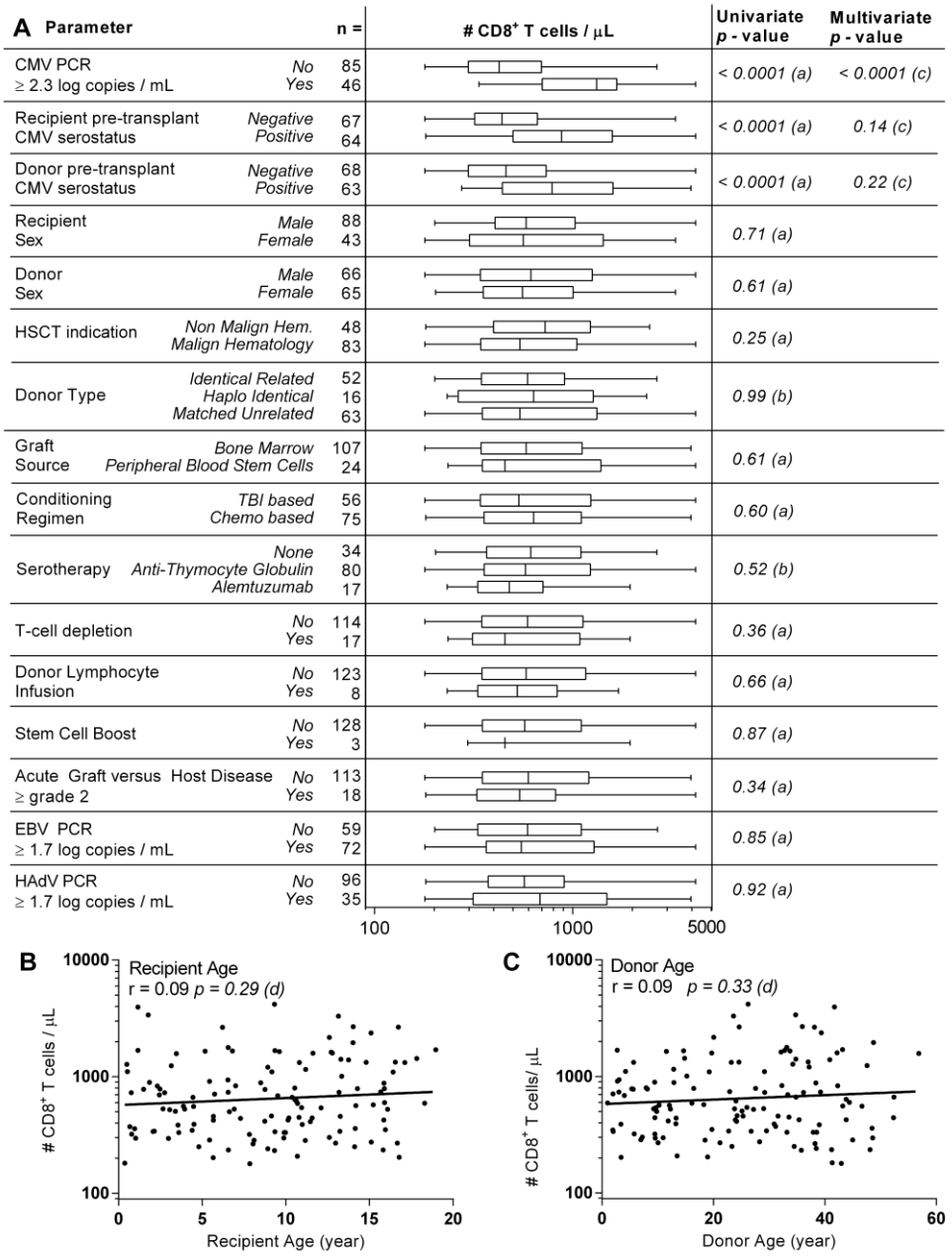

Figure 2.S3. Influence of HSCT related parameters on CD8+ T cell numbers one year post HSCT in all 131 patients.

Influence of **(A)** HSCT parameters, **(B)** recipient age and **(C)** donor age on CD8+ T cell numbers one year post HSCT in all 131 patients. CMV: Cytomegalovirus, EBV: Epstein-Barr virus, HAdV: Human Adenovirus.

Shown are cells/µl of peripheral blood one year post HSCT. Bars: median, interquartile range and overall range. p-values: a: Mann-Whitney test, b: Kruskall-Wallis test, c: linear regression analysis, d: Pearson's correlation test. r: Pearson's correlation coefficient.

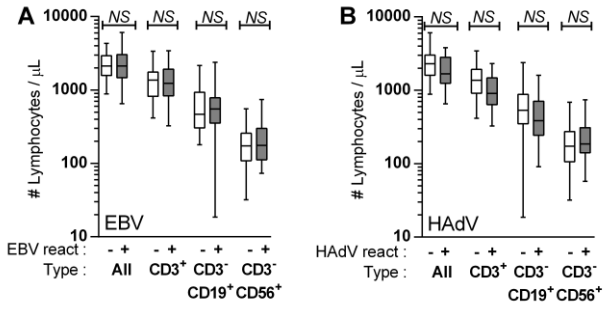

Figure 2.S4. Influence of EBV and HAdV reactivations.

Absolute numbers of lymphocytes, $CD3^+$ T cells, $CD3^-CD19^+$ B-cells and $CD3^-CD56^+$ NK cells in 85 patients without CMV reactivation. **(A)** 35 patients without vs. 50 patients with early Epstein-Barr virus (EBV) reactivation and **(B)** 65 patients without vs. 20 patients with early Human Adenovirus (HAdV) reactivation. White bars (-): plasma DNA load always < 1.7 log. Gray bars (+): plasma DNA load at least once > 1.7 log. Shown are cells/µl of peripheral blood one year post HSCT. Bars: median, interquartile range and overall range, *p*-values: Mann-Whitney test. *NS: p > 0.05.*