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Control of cytomegalovirus viremia after T cell depleted allogeneic stem cell transplantation

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Chapter 4

CMV seronegative donors: effect on clinical severity of CMV infection and reconstitution of CMV-specific immunity

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Abstract

Cytomegalovirus (CMV)-specific T cells are crucial to prevent CMV disease. CMV seropositive recipients transplanted with stem cells from a CMV seronegative allogeneic donor (R⁺D⁻) may be at risk for CMV disease due to absence of donor CMV-specific memory T cells in the graft. We analyzed the duration of CMV reactivations and the incidence of CMV disease in R⁺D⁻ and R⁺D⁺ patients after alemtuzumab-based T cell depleted allogeneic stem cell transplantation (TCD alloSCT). To determine the presence of donor-derived primary CMV-specific T cell responses we analyzed the origin of CMV-specific T cells in R⁺D⁻ patients. The duration of CMV reactivations (54 versus 38 days, respectively, p=0.048) and the incidence of CMV disease (0.14 versus 0.02, p=0.003 at 1 year after alloSCT) were higher in R⁺D⁻ patients compared to R⁺D⁺ patients. In R⁺D⁻ patients, CMV-specific CD4⁺ and CD8⁺ T cells were mainly of recipient origin. However, in 53% of R⁺D⁻ patients donor-derived CMV-specific T cells were detected within the first year. In R⁺D⁻ patients, immunity against CMV was predominantly mediated by recipient T cells. Nevertheless, donor CMV serostatus significantly influenced the clinical severity of CMV reactivations indicating the role of CMV-specific memory T cells transferred with the graft, despite the ultimate formation of primary donor-derived CMV-specific T cell responses in R⁺D⁻ patients.

Introduction

The presence of anti-viral T cell immunity is crucial for effective and sustained protection against cytomegalovirus (CMV) following allogeneic stem cell transplantation (alloSCT)¹. In vitro and in vivo T cell depletion (TCD) via addition of the anti-CD52 monoclonal antibody alemtuzumab to the stem cell graft (alemtuzumab “in the bag”) is used to reduce the incidence of acute Graft versus Host Disease (GVHD) following alloSCT²⁻⁴. Alemtuzumab does not exclusively eliminate alloreactive T cells, but affects presumably all T cells, including donor-derived CMV-specific T cells in the graft and residual CMV-specific T cells of the recipient. Despite the profound TCD, protection against CMV is observed early after TCD alloSCT in CMV seropositive recipients (R⁺) transplanted with a CMV seropositive donor (R⁺D⁺) mediated by CMV-specific T cells that can either originate from the donor via transfer with the graft or from the recipient as residual memory T cells. In CMV seropositive recipients (R⁺) transplanted with a CMV seronegative donor (R⁺D⁻) donor-derived CMV-specific memory T cells are not present in the graft and R⁺D⁻ patients must therefore rely on residual CMV-specific T cells of recipient origin and/or a donor-derived primary CMV-specific T cell response to control CMV reactivations. If despite the in vivo T cell depletion mediated by the free alemtuzumab transferred with the graft, recipient-derived T cell immunity predominates in the protection against CMV, the incidence and severity of CMV reactivation and disease would not differ between R⁺D⁺ and R⁺D⁻ patients. Because the function of the thymus is likely to be impaired after TCD alloSCT⁵, it is not known if or when to expect a donor-derived primary immune response after TCD alloSCT. Demonstrating donor derived CMV-specific T cells after transplantation with a CMV seronegative donor (R⁺D⁻) would be indicative of a newly developed CMV-specific primary T cell response.

In this study we analyzed the effect of donor CMV serostatus on the incidence of CMV reactivation and CMV disease in R⁺D⁻ patients versus R⁺D⁺ patients following TCD alloSCT using alemtuzumab in the bag (20 mg). Furthermore we analyzed the origin of circulating CMV-specific CD4⁺ and CD8⁺ T cell populations in R⁺D⁻ patients by chimerism analysis to detect donor derived CMV-specific T cells indicative of a donor derived primary CMV-specific T cell response.

Objectives

The objectives of this studies were to analyze the effect of donor CMV serostatus on the incidence of CMV reactivation and CMV disease following T cell depleted allogeneic stem cell transplantation and to detect CMV-specific primary T cell responses by demonstrating donor derived CMV-specific CD4⁺ and CD8⁺ T cell populations in seropositive recipients transplanted with stem cells from a CMV seronegative allogeneic donor.

Material and Methods

Patients and CMV monitoring

General institutional policy with respect to patients' informed consent for inclusion into the study, approved by the ethical institutional board, was applied. Consecutive patients transplanted in the period 2004-2010 were included. Patients with haplo-identical or cord blood transplantation were excluded from the analysis. We retrospectively analyzed CMV PCR loads, determined as part of regular post transplantation monitoring. The real-time quantitative PCR for detection of CMV DNA in plasma was performed according to the method described previously⁶. CMV DNA load guided pre-emptive therapy was initiated according to a protocol based on criteria established in a previous study⁷. CMV reactivation was defined as previously described by the detection of two consecutive positive CMV DNA loads ($>\log_{10} 2.7$ (>500)/ml copies plasma) and CMV disease was defined as previously published⁸. Post transplantation sampling for T cell analysis was scheduled every 3 months and continued for 1 year after alloSCT or longer if deemed necessary.

T cell depletion and transplantation

T cell depletion of the graft was performed by *in vitro* incubation of the graft with alemtuzumab (20 mg). The stem cell product was infused on day 0. Pre-transplantation conditioning was performed either according to a myeloablative (MA) conditioning protocol or a nonmyeloablative (NMA) conditioning (RIC) protocol as described previously^{9,10}.

Detection and isolation of CMV-specific CD4⁺ and CD8⁺ T cells based on CD137 expression

CMV-specific CD4⁺ or CD8⁺ T cells were detected by flow cytometric analysis of expression of the activation marker CD137 upon stimulation of PBMC with protein spanning overlapping peptide pools of the CMV-derived proteins pp65 and IE1¹¹⁻¹³. A cluster of ≥ 5 CD137⁺ events on FACS analysis within a total of 10.000 acquired events was considered positive based on the low level of background seen in CMV seronegative individuals. The isolation of CMV-specific CD4⁺ or CD8⁺ CD137⁺ T cells was performed as described previously¹³. In short, after thawing, PBMCs at a concentration of 10^6 /ml were stimulated with 10^{-6} M CMV-derived pp65 and IE1 protein spanning peptide pools in culture medium supplemented with 10 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands) for 24 hours at 37°C and 5% CO₂. Viability after thawing was consistently $>75\%$. After stimulation the cells were stained with CD137-allophycocyanin (APC, BD Pharmingen, Franklin Lakes, USA), fluorescein isothiocyanate-labelled CD16 (BD, Franklin Lakes, USA), CD14 (BD Pharmingen), CD19 (BD) and TCR $\gamma\delta$ (BD) (dump gate), phycoerythrin (PE labelled CD4, BD Pharmingen), Alexa fluor 700 labelled CD8⁺ (Invitrogen, Waltham, MA, USA) and PE Texas Red labelled CD3 (Invitrogen) for 30 minutes at 4°C. Isolation was performed by Fluorescence Activated Cell Sorting using the FACS Aria (BD). CD16, CD14, CD19 and TCR $\gamma\delta$ negative and CD3/CD4/CD137 triple positive and CD3/CD8⁺/CD137 triple positive cells were sorted in bulk for chimerism analysis.

Chimerism analysis

Chimerism analysis on sorted CMV-specific CD4⁺ and CD8⁺ CD137⁺ T cells was performed as described previously¹⁴. In short, we performed PCR analysis with primers specific for patient and donor selected polymorphic short tandem repeats using the AmpFLSTR Profiler Plus ID amplification kit (Applied Biosystems, Waltham, MA, USA) and a GeneAmp 9700 thermocycler (Applied Biosystems) using AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR products were analyzed using the ABI PRISM 3100 Genetic Analyzer and Genemapper V3.5 analysis software (Applied Biosystems). Maximum sensitivity of the markers was set at 2% for all markers.

Statistical analysis

Analysis of CMV reactivation and CMV disease was performed using competing risk analysis as described earlier¹⁵. Factors taken into account as competing risks were death, non-engraftment, rejection, systemic immune suppression, DLI and relapse. Additional analyses were performed using Student T-test IBM SPSS Statistics version 22.

Results

CMV reactivation and disease in CMV seropositive patients following TCD alloSCT

From the cohort of 157 CMV seropositive patients, 51 were transplanted with a CMV seronegative donor (R⁺D⁻) and 106 were transplanted with a CMV seropositive donor (R⁺D⁺). The donor and patient demographics (age, gender, type of conditioning regimen, unrelated/related donor) did not significantly differ between the two patient groups (**table 1**). The cumulative incidences of CMV reactivations and CMV disease were compared by separate competing risks analyses, taking non-engraftment, rejection, immune suppression, DLI, relapse and death of the patient without any of these events into account as competing risks. Non-engraftment did not occur and the cumulative incidence of rejection was very low in both groups (cumulative incidence 0.02 and 0.03 in R⁺D⁻ and R⁺D⁺ respectively). The cumulative incidence of CMV reactivation did not differ between the R⁺D⁻ cohort and the R⁺D⁺ cohort (0.80 versus 0.74 at 1 year after alloSCT, respectively; Gray's test p=0.91), nor did the moment of onset of CMV reactivation after alloSCT (27 days versus 22 days, range 4-129 vs. 4-271, respectively; p=0.7). In the patients who developed at least one CMV reactivation, the mean number of episodes of CMV reactivation was found to be similar in both groups (1.4 versus 1.4 CMV reactivations per patient in the R⁺D⁻ (n=44) and R⁺D⁺ (n=84) group, respectively). However, the median duration of individual CMV reactivations was significantly longer in the R⁺D⁻ cohort compared to the R⁺D⁺ cohort (54 versus 38 days, respectively, p=0.048). The cumulative incidence of CMV disease was significantly higher in the R⁺D⁻ cohort compared to the R⁺D⁺ cohort (0.14 versus 0.02 at 1 year after alloSCT, respectively; Gray's test p=0.003; **table 1**). The cumulative incidences of the competing

events non-engraftment, rejection, immune suppression, DLI, relapse and death did not differ significantly between the two groups.

Table 1. Outcome of CMV reactivation and disease in CMV seropositive recipients transplanted with a CMV seronegative donor (R⁺D⁻) compared to CMV seropositive recipients transplanted with a CMV seropositive donor (R⁺D⁺) patients up to one year after TCD alloSCT.

	R ⁺ D ⁻	R ⁺ D ⁺	P
Total number of patients	51	106	
Male/Female	29/22	62/44	NS
Median age (years)	52	51	NS
Myelo-ablative conditioning	25 (49%)	49 (46%)	NS
Nonmyelo-ablative conditioning	26 (51%)	57 (54%)	NS
Matched related donor	24 (47%)	54 (51%)	NS
Matched unrelated donor	27 (53%)	52 (49%)	NS
CI Relapse	0.33	0.33	NS
CI Non relapse mortality	0.33	0.18	NS
Onset CMV reactivation (days after TX, range)	27 (4-129)	22 (4-271)	NS
Mean number of CMV reactivations	1.4	1.4	NS
Median days of CMV reactivation	54	38	0.048
CI CMV reactivation*	0.80	0.74	NS
CI CMV disease*	0.14	0.02	0.003
CI Systemic immune suppression	0.22	0.23	NS
CI Donor Lymphocyte Infusion	0.24	0.31	NS

* Competing risks analyses taking non-engraftment, rejection, systemic immune suppression, Donor Lymphocyte Infusion, relapse and death of the patient without CMV reactivation or CMV disease, respectively, into account as competing risks.

CMV = cytomegalovirus; TCD = T cell depleted; alloSCT = allogeneic stem cell transplantation; NS = not significant; CI = cumulative incidence; NRM = non-relapse mortality; CMV reactivation = defined by the detection of two consecutive positive CMV DNA loads ($>\log_{10} 2.7$ (>500)/ml copies plasma); Days of CMV reactivation = number of days between first positive CMV DNA load ($\log_{10}>2.7$) and first negative CMV DNA load ($\log_{10}<2.7$). CMV disease = defined as previously published⁸.

Origin of CMV-specific T cells in R⁺D⁻ patients following TCD alloSCT

Chimerism analysis of circulating CMV-specific T cells in R⁺D⁺ patients demonstrated recipient and donor origin, ranging from mixed donor/recipient chimerism to full donor chimerism or full recipient chimerism (n=6, donor origin in 5/6, data not shown). To investigate the presence and origin of anti-viral immunity in R⁺D⁻ patients, we performed in-depth analyses in the cohort of R⁺D⁻ patients, allowing the discrimination of pre-existing patient-derived memory T cells and the possible induction of a primary CMV-directed immune response mediated by donor T cells. CMV-specific CD4⁺ or CD8⁺ T cells were detected by flow

cytometric analysis of expression of the activation marker CD137 upon stimulation of PBMC with protein spanning overlapping peptide pools of the CMV-derived proteins pp65 and IE1¹¹⁻¹³. A representative example of CD137 expression on T cells following stimulation with CMV-derived pp65 and IE1 protein spanning peptide pools and the corresponding negative control without peptide stimulation is shown in **Figure 1**.

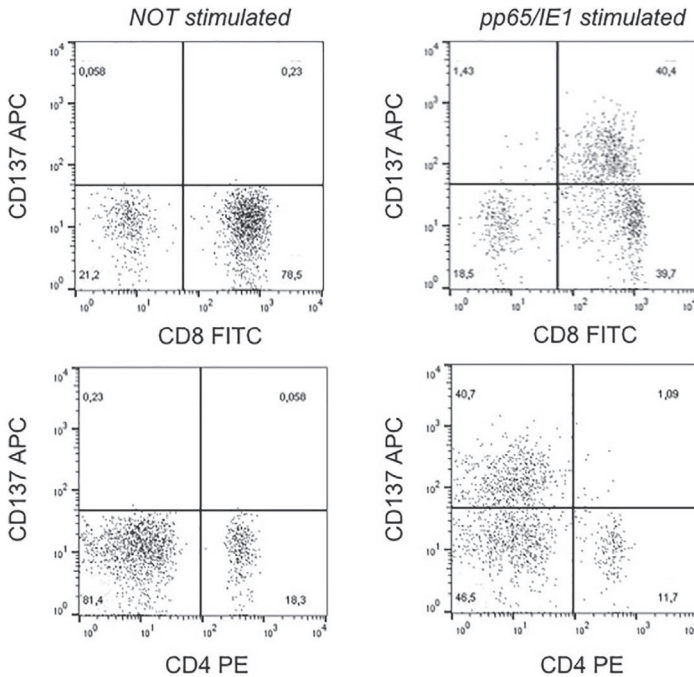


Figure 1.

Representative example for CD137 expression on unstimulated T cells and following stimulation of PBMC from R⁺D⁻ patient with 10⁻⁶ M CMV-derived pp65 and IE1 protein spanning peptide pools for 24 hrs. Left panels show CD137 expression of unstimulated T cells (CD8⁺ T cells on top panels) and CD4⁺ T cells on bottom panels) and right panels demonstrate CD137 expression of stimulated T cells. Additional staining allowed for a gating strategy for bulk sorting of CD16, CD14, CD19 and TCRγδ negative and CD3/CD4/CD137 triple positive and CD3/CD8⁺/CD137 triple positive cells.

From the cohort of 51 R⁺D⁻ patients, 26 patients were excluded from this analysis due to graft failure, early disease relapse, therapeutic use of systemic immune suppression, early death or lack of cryopreserved samples for analysis. Twenty-five patients were eligible for analysis of the presence of CMV-specific CD4⁺ and/or CD8⁺ T cells. Samples were cryopreserved as part of routine follow-up after alloSCT (irrespective of viral load). In 19/25 (76%) of the analyzed patients of the R⁺D⁻ cohort, visible frequencies of CMV-specific CD4⁺ and/or CD8⁺ T cells were detected (median of 198 (range 85-361) days after TCD alloSCT). The median frequency of CD4 CMV-specific T cells in the CD4 compartment was 3.3%, (range 0.4 to 5.1%; n=8) and the median CD8⁺ CMV-specific T cells in the CD8⁺ compartment was 6.2 (range 0.4 to 26.2%; n=18) (**Figure 2A**). To analyze the origin of these CMV-specific CD4⁺ and/or CD8⁺

T cells, chimerism analysis was performed on CMV-specific T cells purified from peripheral blood of the 19 patients with detectable frequencies of circulating CMV-specific T cells. Of these 19 patients, 17 had developed a CMV reactivation within the first year following TCD alloSCT. As expected, in most patients the majority of these CMV-specific T cells were of recipient origin (median 95.5%, range 0-100; n=8) in CMV-specific CD4⁺ T cells versus 100% (range 0-100; n=18) in CMV-specific CD8⁺ T cells. However, although in varying frequencies, in 10/19 (53%) of patients in this R⁺D⁻ cohort CMV-specific CD4 and/or CD8⁺ T cells of donor origin were detected within the first year following TCD alloSCT (**Figure 2B**). In the 2 patients without detectable CMV reactivation within the first year following TCD alloSCT (marked in green in Figure 2), unexpected high numbers of CD4⁺ and CD8⁺ CMV-specific T cells were detected (4.1% and 5.1% in CD4⁺ compartment and 1.3% and 5.9% in CD8⁺ compartment in both patients, respectively, analysis on day 85 and day 99). Part of these CMV-specific T cells was even found to be of donor origin in both patients (4% and 5% within CD4⁺, and 0% and 9% in CD8⁺ CMV-specific T cells, respectively).

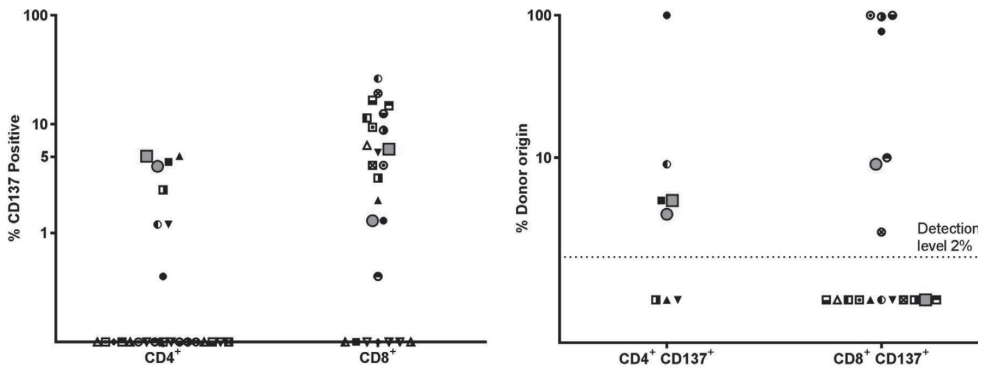


Figure 2.

Frequencies and origin of CMV-specific T cells in CMV seronegative patients after TCD alloSCT with a CMV seronegative donor (R⁺D⁻). (A) Frequencies of CMV-specific CD4⁺ and CD8⁺ T cells following TCD alloSCT were detected by flow cytometric analysis of CD137 expression upon stimulation with CMV-derived pp65 and IE1 protein spanning peptide pools in 19/25 R⁺D⁻ patients. Frequencies of CMV-specific T cells in individual patients are depicted as unique symbols. The symbols in green represent 2 patients without detectable CMV reactivation in the first year following alloSCT. (B) Chimerism analysis of isolated CMV-specific CD4⁺ CD137⁺ and CD8⁺ CD137⁺ T cells. Samples were cryopreserved as part of routine follow-up after alloSCT (irrespective of viral load). The dotted line represents the detection level of 2% in chimerism analysis. Donor origin of CMV-specific T cells in individual patients is depicted as unique symbols. The symbols in green represent 2 patients without detectable CMV reactivation following alloSCT.

Discussion

The observed effect of the donor serostatus on the course of CMV reactivations in CMV seropositive patients suggests that *in vitro* TCD by addition of 20 mg of alemtuzumab to the bag is not 100% effective in fully depleting grafts from T cells. This importance of donor-derived CMV-specific memory T cells for sustained control of CMV reactivation has been demonstrated in previous studies⁵. Our clinical data on CMV reactivation are in agreement with these studies and suggest that donor-derived CMV-specific memory T cells are able to survive profound TCD and provide protective immunity. Indeed, chimerism analysis to assess the origin of CMV-specific T cells circulating in R⁺D⁺ patients demonstrated that CMV-specific immunity in these patients can be mediated by CMV-specific T cells of donor origin, patient origin or a mixture of these. A recent study¹⁶ described loss of expression of the Alemtuzumab target antigen CD52 as a possible escape mechanism allowing survival of T cells (including virus-specific donor T cells) following alemtuzumab based TCD alloSCT.

The data in our manuscript confirm previous data on the origin of CMV-specific T cells following TCD alloSCT in CMV seropositive patients transplanted with a CMV seronegative donor (R⁺D⁻) and demonstrate that also recipient CMV-specific memory T cells are able to survive alemtuzumab based TCD and are the main actors supplying protective immunity to prevent CMV disease in these patients^{5, 17}. However, the demonstration of donor-derived CMV-specific T cells, as indicator of the development of a donor-derived primary immune response after TCD alloSCT in R⁺D⁻ patients, adds an important novel insight to the findings made in previous studies. It may provide a rationale for adoptive cell transfer (ACT) of CMV-specific T cells from healthy third party donors or autologous CMV-specific T cells harvested prior to the transplant for bridging the period of severe T cell deficiency prior to development of the primary T cell response¹⁸⁻²⁰. Although these strategies imply a risk of rapid rejection, a short-term protective effect may be sufficient to prevent CMV disease while allowing the development of donor-derived CMV-specific T cells.

In previous studies, analysis of CMV-specific T cells after alloSCT consistently demonstrated mainly cells of patient origin^{5, 17}. A possible explanation for the better detection of donor-derived CMV-specific T cells in our study may be the utilized methodology for detection and purification of virus-specific T cells. Whereas previous studies were focused only on CMV-specific CD8⁺ T cells, isolated using peptide/MHC multimers, in our study we analyzed both CMV-specific CD4⁺ and CD8⁺ T cells, isolated based on expression of the activation marker CD137 upon stimulation with CMV-derived pp65 and IE1 protein spanning peptides, which allows the analysis of a broader repertoire of CMV-specific T cells¹¹⁻¹³.

In conclusion, we demonstrated a significantly increased duration of CMV reactivation and a significantly increased incidence of CMV disease in CMV seropositive patients transplanted with a CMV seronegative donor (R⁺D⁻) compared to CMV seropositive patients transplanted with a CMV seropositive donor (R⁺D⁺) following TCD alloSCT, illustrating that despite alemtuzumab-based TCD, memory T cells can be transferred from the graft to provide protective anti-viral immunity. Furthermore, we demonstrated that protective immunity

against CMV was predominantly mediated by T cells from recipient origin in patients transplanted with a CMV seronegative donor (R⁺D⁻) within the first year after TCD alloSCT, but that a primary donor-derived CMV-specific T cell response was frequently observed within the first year following TCD alloSCT, even as early as 3 months following TCD alloSCT.

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