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ORIGINAL ARTICLE

Early reconstitution of effector memory CD4+ CMV-specific T cells protects against CMV reactivation following allogeneic SCT

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Reactivation of CMV is a common complication following allogeneic haematopoietic SCT and is associated with significant morbidity and mortality. The relative importance of the CD4+ and CD8+ components of the CMV-specific immune response in protection from reactivation is unclear. The CMV-specific CD4+ and CD8+ immune response was measured at serial time points in 32 patients following allogeneic HSCT. Intracellular cytokine staining following CMV lysate stimulation and HLA-peptide tetramers were used to determine CMV-specific CD4+ and CD8+ responses, respectively. A deficient CMV-specific CD4+ T-cell immune response within the first 30–50 days post transplant was associated with high risk of viral reactivation. Patients with combined impairment of the CD4+ and CD8+ immune response within the first 100 days were susceptible to late viral reactivation. The frequency of CMV-specific CD4+ T cells correlated with CMV-specific CD8+ T cells, comprising 10% of the whole T-cell repertoire. Early CMV-specific CD4+ T-cell reconstitution was dominated by effector memory cells with normal levels of IL-2 resuming 6 months following transplantation. In summary, both CD4 and CD8 CMV-specific immune reconstitution is required for protection from recurrent activation. Measurement of the magnitude of the CMV-specific CD4+ immune response is useful in managing viral reactivation following HSCT.
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Introduction

Allogeneic haematopoietic SCT is a powerful strategy for the treatment of many haematological disorders. The cellular immune response is impaired for many months post-HSCT and contributes to serious morbidity and mortality from infectious complications.[1] In particular, CMV seropositivity remains an important risk factor for transplant-related mortality after sibling donor and matched unrelated donor haematopoietic stem cell recipients,[2,3] particularly in the setting of T-cell-depleted conditioning regimens. The development of effective T-cell immunity is important for the control of CMV infection and disease. A strong association has been found between the lack of CMV-specific CD8+ T cells and the development of CMV disease after transplantation.[4,5] In another study the recovery of CMV-specific CD8+ T cells to levels of >10 cells/μl was associated with protection from CMV disease.[6] Although CD8+ CTL have been considered as the predominant antiviral effector cells, there is an increasing interest in the role of CD4+ T cells in controlling and maintaining the cellular immune response. It has been shown that infusion of CMV-specific CD8+ T-cell clones in allogeneic transplants leads to detectable CD8 responses *in vivo* but the magnitude of the responses declines in those who lack a detectable CMV-specific CD4 response.[7] A correlation between CD4 deficiency and CMV reactivation has been seen in patients with HIV infection and following solid organ transplantation.[8] In renal transplant recipients the development of CMV-specific CD4+ T-cell immunity before virus-specific CD8+ expansion was associated with asymptomatic control of primary CMV viraemia. In contrast, symptomatic disease was observed only in patients who exhibited delayed CMV-specific CD4 immunity.[9] In HSCT patients a low lymphocyte count and CD4+ T-cell count of less than 40 per μl at 3 months post transplant have been demonstrated as risk factors for development of late CMV disease.[10] Hakki *et al.*[11] also showed that low CD4 (<100 per μl) and CD8 (<50 per μl) T-cell counts at this time point are associated with poor CMV-specific immunity. The functional potential of the cellular immune response is also critical and dysfunctional Ag-specific CD8+ T cells have been found as risk factors for CMV reactivation.[12] These data suggest

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that both a low absolute T-cell count and poor Ag-specific immune reconstitution are predictive of protection from viral reactivation but were measured at 3 months post transplant and not at earlier time points. Avetysian *et al*. have recently reported that the total T-cell response to CMV Ag at 4 weeks post-HSCT is inversely correlated with viral load and Lilleri have suggested an absolute CD4 and CD8 T-cell response at day 60 which may confer protection against viraemia in young patients.[13,14] Here we have looked at the CD4+ and CD8+ T-cell response to CMV at several time points following SCT, including an early 30 day analysis, to see if earlier detection of poor Ag-specific T-cell immunity can predict for CMV reactivation.

Patients and methods

Patients

The study group consisted of 32 allogeneic HSCT recipients at the QE hospital in Birmingham in which either the donor or the recipient, or both were CMV seropositive and were recruited from January 2002 to December 2003 (Table 1). Patients gave informed consent for the study approved by the South Birmingham Ethics Committee. The patients had an age range between 21 and 60 years with a median of 42 at the time of transplantation. Of the patients, 23 had sibling donors whereas 9 had volunteer matched unrelated donors (MUD). Seventeen patients (53.2%) underwent myeloablative conditioning consisting of CY and TBI except for one patient who received a melphalan and TBI regimen. The non-myeloablative conditioning regimens consisted of BEAM (BCNU 300 mg/m^2 for 1 day, Etoposide 200 mg/m^2/day for 4 days, ara C 200 mg/m^2/day for 4 days. Melphelan 140 mg/m^2/day for 1 day)/Campath ($n=6$; 18.7%), fludarabine/melphalan/Campath ($n=7$; 28.1%) or BU/CY ($n=2$; 6.3%)). CMV reactivation was routinely monitored by quantitative PCR twice weekly up to 100 days, weekly up to 6 months and then as clinically indicated. The sensitivity of this assay was 400 copies/ml. Nineteen patients had CMV reactivation in the first 3 months post transplantation and were termed as early reactivation. Three patients had late reactivation in addition to early reactivation and this occurred between days 100 and 180. Sixteen patients (50%) suffered from GVHD (acute, chronic or both).

Table 1 Clinical characteristics of HSCT patients

Patients age (years): median (range)	41.8 (21–60)
Donors age (years): median (range)	44.1 (37–67)
Gender, number (%)	
Male	18 (56.3)
Female	14 (43.7)
Underlying disease, number (%)	
AML	11 (34.4)
ALL	2 (6.3)
CML	7 (21.8)
CLL	1 (3.1)
NHL	6 (18.7)
HD	2 (6.3)
Myeloma	2 (6.3)
MDS	1 (3.1)
Median follow up time (day)	201
Transplant type, number (%)	
Sibling	23 (71.9)
Unrelated donor	9 (28.1)
Conditioning regimen, number (%)	
CY/TBI	16 (50)
Mel/TBI	1 (3.1)
BEAM/CAM	6 (18.7)
Flu/Mel/CAM	7 (28.1)
BU/CY	2 (6.3)
Source of stem cell, number (%)	
BM	3 (9.4)
PB	29 (90.6)
GVHD, number (%)	
Yes	16 (50)
No	16 (50)
CMV reactivation (PCR) (%)	
Early reactivation only	15 (46.8)
Early and late reactivation	4 (12.6)
None	13 (40.6)
Patients/donor CMV status, number (%)	
P/P	17 (53.1)
P/N	11 (34.4)
N/P	4 (12.5)

MoAb

The following MoAb were used in this study: anti-IFN-γ (FITC, PE), anti-IL-2 (FITC, PE), mouse IgG_{2a} (FITC, PE), mouse IgG1 (FITC, PE), anti-CD49d and anti-CD28 (BD Biosciences, Oxford, UK); anti-CD4 (ECD, PC5), anti-CD8 (FITC), anti-CD3 (PE), anti-CD69 (PC5), mouse IgG_1 (ECD, TC; Beckman Coulter, High Wycombe, UK); anti-CD8 (TC; Caltag Medsystems, Buckingham, UK).

Assessment of CMV-specific CD4+ T-cell responses

CMV-specific CD4+ T-cell frequency was determined according to the previously described method.[15] Briefly, sodium-heparinized whole blood was aliquoted into 15 ml propylene tubes. Costimulatory mAbs, anti-CD28 and anti-CD49d, were added to the samples at a 1 μg/ml final concentration. Patient samples were divided into no treatment (unstimulated), SEB (Sigma-Aldrich, Poole, UK) (positive control) and CMV lysate (Microbix Biosystem Inc., Toronto, Canada). The culture tubes were incubated at 37 °C in a humidified 5% CO_2 atmosphere for a total of 6 h in the presence of 10 μg/ml of the cytokine secretion inhibitor, brefeldin A (Sigma-Aldrich), for the last 4 h of culture. EDTA (Sigma-Aldrich) was then added to a final concentration of 2 mM for 15 min at room temperature. In some experiments, the cultures were stored overnight at 4 °C before EDTA treatment. After EDTA treatment, the RBC were lysed and leucocytes fixed with 1 × FACS lysing solution (BD Biosciences) for a 10 min incubation at room temperature. The cells were washed in PBS containing 0.5% BSA (Sigma-Aldrich), 0.1% sodium azide (Fisher, Loughborough, UK) (wash buffer) and

permeabilized by incubation with 1 × FACS permeabilization buffer (BD Biosciences) for 10 min at room temperature. The cells then were washed in wash buffer and subsequently stained with directly conjugated mAbs for 30 min at room temperature in the dark. After staining, the cells were washed, fixed in 1% paraformaldehyde (Fisher) in PBS and stored at 4 °C in the dark until flow cytometric analysis. Four-colour flow cytometric analysis was performed on a Beckman Coulter XL-MCL machine (Beckman Coulter). Data was analysed using WinMDI software (Scripps Institute, La Jolla, CA, USA) whereby lymphocytes were gated on FSC/SSC and CD4/CD3 and then displayed as dot plots of cytokine (IL-2/IFN-γ) vs CD69 fluorescence to determine frequency of cytokine-positive and CD69-positive cells. Background intracellular isotype staining was subtracted from IFN-γ and IL-2 responses for both unstimulated and stimulated PBMC. Spontaneous cytokine production from unstimulated PBMC was then subtracted from CMV-lysate stimulation to yield actual frequency of CMV-specific T cells. Enumeration was examined using IFN-γ production primarily but all IL-2 producing cells produced IFN-γ. The absolute number of CMV-specific CD4+ T cells was calculated using total lymphocyte count, frequency of CD4+ T cells in lymphocyte and CD3+ gate and the frequency of cytokine-positive cells.

Cell staining with tetramers

CMV tetramers derived from pp65, immediate early Ag (IE-1) and pp50 proteins were produced using a previously described method.[16] Tetramers used in this study were HLA-A2 restricted ($pp65_{495–503}$, NLV, $IE\text{-}1_{316–324}$, VLE), -B7 restricted ($pp65_{417–426}$, TPR, $IE\text{-}1_{88–96}$, QIK), -A1 restricted ($pp50_{245–253}$, VTE, $pp65_{363–373}$, YSE) and -B8 restricted (2 natural variants of $IE\text{-}1_{199–207}$, ELK, ELR). PBMC (5×10^5) were incubated with 0.1–0.5 μg of PE-conjugated tetramer at 37 °C for 15 min. After washing with wash buffer, the cells were resuspended and incubated with CD8 mAb for 30 min at 4 °C. The cells were washed and analysed on the FACS immediately or fixed in 2% paraformaldehyde (Fischer) for later analysis. The absolute number of tetramer-positive cells was quantitated using total lymphocyte count, frequency of CD8+ T cells in the lymphocyte gate and frequency of tetramer-positive cells in the CD8+ T-cell subset. In subjects with more than one CMV-specific response, the sum of the tetramer-positive cells was made.

Statistical methods

All statistical analyses were performed using Prism (Graphpad, San Diego, CA, USA). To identify associations in the data, Pearson's χ^2-test was used for categorical variables (Continuity adjustment and Fisher's exact test were used as appropriate). The non-parametric Mann–Whitney *U*-test for comparison of two groups, and the Kruskal–Wallis test followed by the Dunn's post-test for more than two samples were used to compare groups of continuous measurements. For correlation analyses the Spearman rank test was used. A *P*-value of less than 0.05 was considered statistically significant.

Results

A higher frequency of CMV-specific CD4+ T cells between days 30 and 50 post-HSCT affords protection from CMV reactivation

CMV reactivation occurred only when the patient was CMV seropositive (Patient (P)−/Donor (D)+: 0/4; P+/D−: 7/11; P+/D+: 12/17; $P<0.05$) but was not influenced by the type of donor, the development of GVHD or the conditioning regimen. CMV-specific CD4 responses were measured by CMV lysate stimulation followed by IL-2 and IFN-γ intracellular cytokine staining on all 32 HSCT patients (MFI average 72.3 ± 11.9 s.d.). Where sufficient cells were available, 25 patients were also analysed for CMV-specific CD8 responses by HLA-class I CMV tetramers. Assays were performed on serial samples collected from 30 to 390 days post-HSCT.

Of the 32 patients, 19 had at least one episode of CMV reactivation following HSCT; their serostatus, conditioning, donor type, day of reactivation and immune reconstitution are presented in Table 2. The majority of reactivation episodes occurred between days 30 and 50 post-HSCT, and the CMV-specific CD4+ and CD8+ T-cell response was measured during this time. Immune function was also determined at days 80–100 and at days 180–200 in all patients (Figure 1). Between days 30 and 50, the patients who experienced at least one episode of CMV reactivation had a markedly reduced CMV-specific CD4 T-cell count (median 0.4 (0.01–7.8) cells/μl) in comparison to patients who did not reactivate (6.5 (0.01–34.9) cells/μl; $P=0.03$; Figure 1a). The CMV-specific CD8+ T-cell count was threefold lower, although NS in patients with an episode of viral reactivation (10.2 (0.01–169) vs 32 (0.03–250) cells/μl; Figure 1b). The CMV-specific CD4+ and CD8+ T-cell responses in patients with or without viral reactivation did not differ between days 80 and 100 nor between days 180 and 200 (Figures 1a and b).

A lower frequency of CD4+ and CD8+ CMV-specific T cells within the first 100 days post-SCT is associated with recurrent CMV reactivation

Late CMV reactivation is an increasing concern with SCT and has been reported in up to 30% of patients in some cohorts.[17] Five patients experienced more than one episode of CMV reactivation in this study (Table 2). Patients with recurrent viral reactivation had a significantly lower number of CMV-specific CD4+ T cells within the first 100 days post transplant compared to patients with only one reactivation; however between days 30 and 50 this was NS because samples were not collected from all the five patients. Within this group the median peak level of CMV-specific CD4+ T cells during the first 100 days was 0.4 (0.01–10) cells/μl, which is the same value that was recorded for reactivation during the first 50 days (Figure 1a). Patients who did not suffer more than one episode of viral reactivation exhibited an increase in the CD4+ T-cell response to a median of 10 (0.2–103) cells/μl within 100 days ($P=0.01$; Figure 2a). A similar profile was seen for CMV-specific CD8+ T cells with a median of only 0.26 (0.02–17) CMV-specific cells/μl being seen in patients with

Table 2 Clinical features and CMV-specific immunity in CMV-reactivated HSCT patients

Patient ID	*Patient/Donor CMV serostatus*	*Conditioning*	*Sibling (Sib)/MUD*	*Day (d) or period of reactivation*[a]	*Peak CMV-specific CD4 30–50 days*	*Peak CMV-specific CD8 30–50 days*
PT1	+/+	Non-mye.	Sib	<29	ND	ND
PT2	+/+	Non-mye.	Sib	<100	ND	ND
PT3	+/+	Non-mye.	Sib	<100	3	ND
PT4	+/+	Non-mye.	Sib	<20	11.6	ND
PT10	+/−	Mye.	Sib	42	7.6	58.3
PT11	+/+	Mye.	Sib	80	<0.01	ND
PT12	+/+	Non-mye.	Sib	30–44	1.6	169
PT16	+/+	Non-mye.	Sib	40	9.1	8.1
PT18	+/+	Mye.	MUD	14–24	0.6	ND
PT20	+/+	Mye.	MUD	40	0.2	ND
PT25	+/+	Non-mye.	Sib	21	<0.01	27.7
PT26	+/−	Mye.	Sib	53–66	ND	126
PT30	+/+	Non-mye.	Sib	42–59	0.5	0.03
PT31	+/−	Non-mye.	Sib	35–52	ND	ND
PT8	+/−	Mye.	Sib	43–72 143–167	0.17	<0.01
PT27	+/+	Non-mye.	Sib	51 83	3.4	ND
PT28	+/−	Mye.	MUD	29–75, 135–162	ND	ND
PT29	+/−	Non-mye.	Sib	36 51–61	0.1	ND
PT19	+/−	Mye.	MUD	30 73 162	ND	0.16

Abbreviations: MUD = matched unrelated donor; Mye. = myeloablative conditioning; ND = not done.
[a] < Exact date of reactivation not known but occurred less than specified day post-SCT.

multiple reactivation episodes compared to a value over 180-fold higher (48 (0.03–262) cells/μl) in patients with only one episode ($P = 0.02$; Figure 2b).

The CMV-specific T-cell response comprises 10% of the T-cell pool and is correlated with the total lymphoid cell count

To determine if the total CD4 and CD8 T-cell count could act as a surrogate marker for prediction of CMV reactivation, the number of CMV-specific CD4+ and CD8+ T cells was then correlated with these subsets (Figures 3a and b, respectively). A strong positive correlation was observed between the magnitude of the CMV-specific CD4+ T-cell count and the total CD4 count ($P = 0.001$; Figure 3a). Interestingly the peak total CD4 count was 60% lower in patients who exhibited CMV reactivation compared to those without reactivation (median 68 (4.4–385) vs 165 (3.3–607); $P = 0.05$). A similar correlation was observed for the CMV-specific CD8+ and total CD8 T-cell counts ($P < 0.0001$; Figure 3b). The CMV-specific immune response comprised approximately 10% of the total CD4 and CD8 lymphoid response.

The number of CMV-specific CD4+ and CMV-specific CD8+ T cells are highly correlated during immune reconstitution

The number of CMV-specific CD4+ T cells was then compared with the number of CMV-specific CD8+ T cells as determined by HLA-peptide tetramer staining (Figure 3c). A significant positive correlation was observed between the CMV-specific CD4+ and CMV-specific CD8+ T-cell count ($P = 0.001$), which were also broadly comparable in magnitude.

The CMV serostatus of the recipient and donor influence the frequency and magnitude of CMV-specific T-cell reconstitution

Within the first 100 days post-HSCT, all CMV-positive recipients (+/+) who received a transplant from a CMV-seropositive donor developed a CMV-specific CD4+ T-cell response, whereas 7/11 CMV-positive recipients (+/−) with a seronegative donor and only 1/4 CMV-seronegative recipients (−/+) with a seropositive donor had a response. In addition the magnitude of the CMV-specific CD4+ T-cell response was greatest for +/+ recipients, median 10.3 (0.6–103) cells/μl, followed by +/− recipients, median 0.4 (0–42) cells/μl and then −/+ recipients, median 0 (0–0.7) (Kruskal–Wallis test, $P = 0.01$). The link between serostatus and the CMV-specific CD4+ T-cell immunity was observed throughout the period of study ($P = 0.02$). This trend was also true for the CMV-specific CD8+ T-cell response, although not statistically different ($P = 0.08$). However, it should be noted that despite poor CMV-specific T-cell recovery in the seronegative patients there were no episodes of CMV reactivation within this group (data not shown).

Poor CMV-specific T-cell recovery was observed in recipients of MUD grafts in comparison to sibling recipients (median value $\leqslant 0.1$ vs $\geqslant 11.1$; $P < 0.01$). Only two of the nine MUD recipients developed a CMV-specific T-cell response in the first 100 days post transplant but this

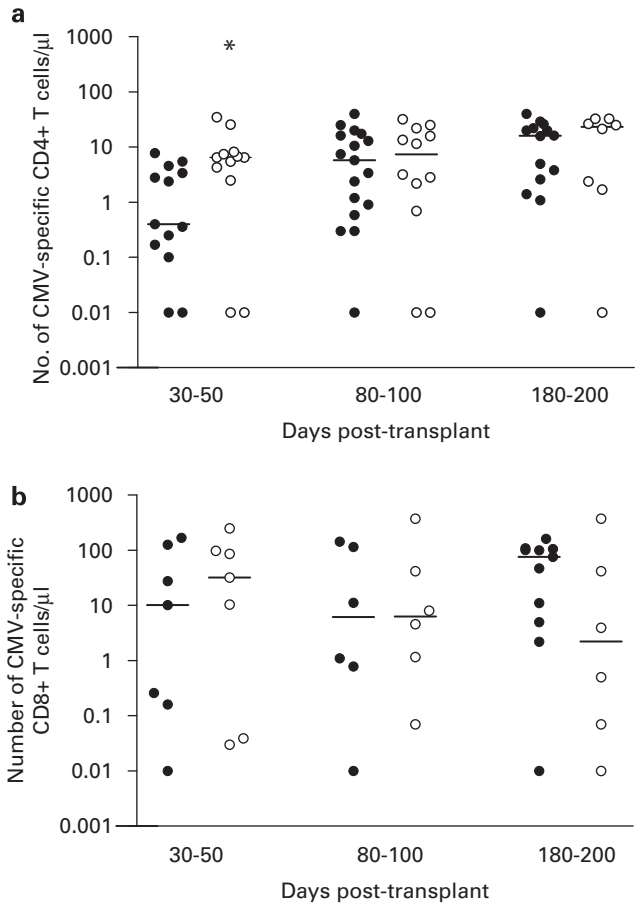

Figure 1 CMV-specific T-cell response in patients with and without CMV reactivation at different time periods. (**a**) Higher frequency of CMV-specific CD4+ T cells between days 30 and 50 is associated with protection from CMV reactivation. The number of CD4+ T cells responding to CMV lysate within peripheral blood was assessed by intracellular cytokine staining (ICS; *y* axis). These measurements were made on samples collected between days 30 and 50, 80 and 100 and 180 and 200 (*x* axis) post transplantation. *Indicates $P<0.05$ (Mann–Whitney *U*-test) between the two groups, CMV reactivated (solid circles) vs non-reactivated (open circles). (**b**) No difference in number of CMV-specific CD8+ T cells and CMV reactivation status. HLA-class I tetramers spanning a wide number of HLA types and the CMV proteins pp65, immediate early Ag (IE-1) and pp50 proteins were used to determine the number of CMV-specific CD8+ T cells (*y* axis) at different time periods post transplant (*x* axis). CMV reactivated (solid circles) vs non-reactivated (open circles).

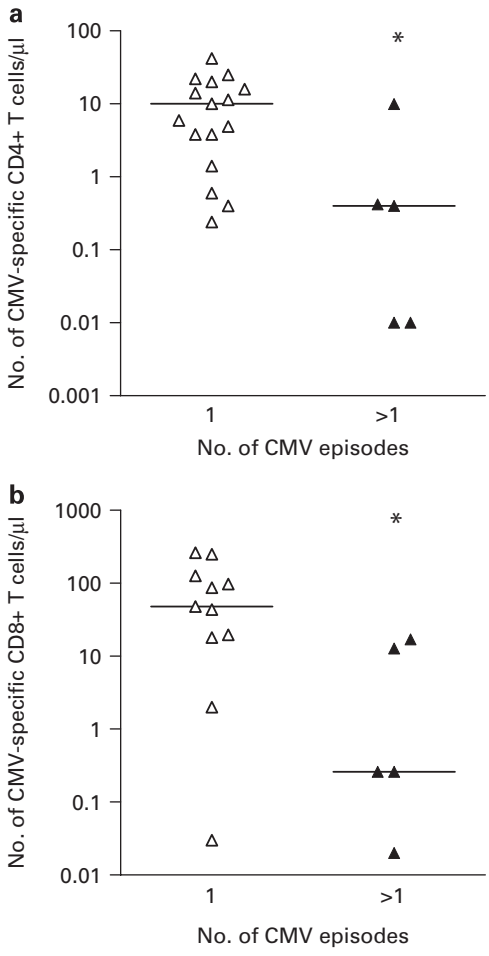

Figure 2 A lower number of CMV-specific T cells is associated with more than one CMV episode. The peak value of CMV-specific T cells (*y* axis) in first 100 days post transplantation was compared between patients with one CMV episode and those with more than one episode (*x* axis) for CD4-specific (**a**) and CD8-specific (**b**) T cells and analysed using the Mann–Whitney *U*-test for significance. *Indicates a significant difference ($P<0.05$) between 1 vs more than 1 CMV episode.

was extremely weak (0.2 and 0.6 cell/µl; Table 2) and both patients experienced viral reactivation. Of the remaining seven transplant pairs with no CMV-specific immunity, three patients were seronegative and did not reactivate, possibly relating to low viral load; the other four patients were CMV seropositive with CMV seronegative donors and two of these did reactivate CMV without a detectable immune response. Myeloablative or non-myeloablative transplant conditioning did not have a significant effect on CMV-specific CD4+ and CD8+ T-cell reconstitution and a non-significant increase in the median value was observed in the non-myeloablative group (2.3 (0–169) vs 10.3 (0–103) and 13.9 (0.02–143) vs 17.5 (0–262) for CMV-specific CD4+ and CD8+ T-cell response, respectively).

CMV-specific CD4+ T cells show impaired production of IL-2 for the first 6 months following SCT

IL-2 production is important for sustaining T-cell expansion and is produced following initial T-cell priming and also by central memory T cells. In contrast an IFN-γ+ IL2-cytokine profile is typical of cells with an effector memory phenotype.[18] Cytokine production by CMV-specific CD4+ T cells was determined by flow cytometry after stimulation with CMV lysate. No detectable production of IL-2 by CMV-specific T cells was observed in the first 4 weeks following transplantation. IL-2 production did increase thereafter and the ratio of IL-2/IFN-γ production by CMV-specific T cells at different time points is shown in Figure 4. This ratio increased after 50 days but was still less than 50% before day 180. After 180 days over half of all CMV-specific CD4+ T cells showed IL-2 production, which is typical of the profile observed in healthy non-SCT donors. Unlike IFN-γ production by CMV-specific CD4+ T cells, IL-2 was not associated with reduced CMV reactivation in the first 100 days post transplant (data not shown).

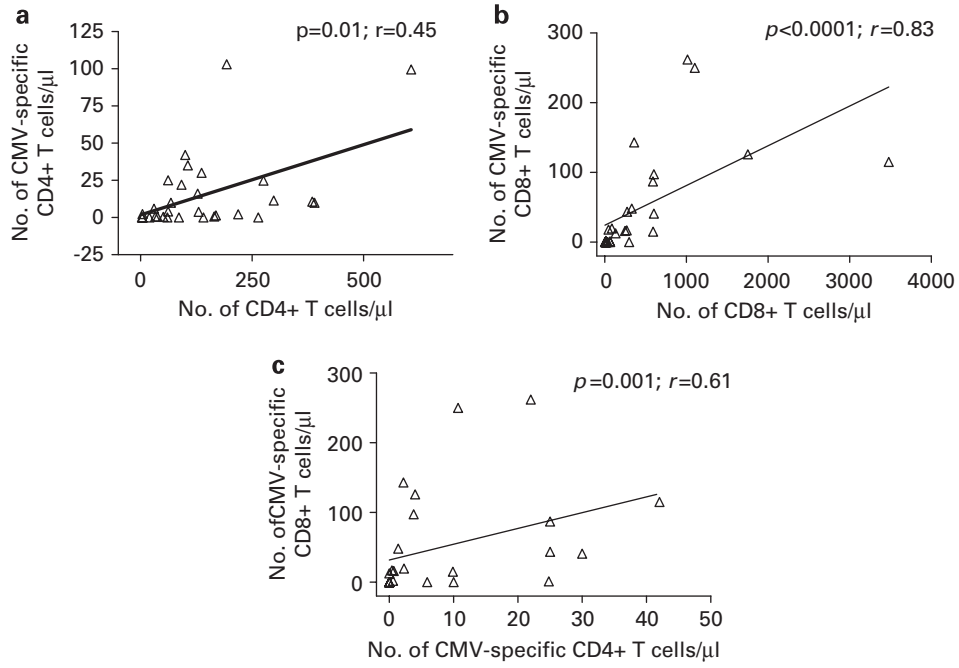

Figure 3 Influence of the overall T-cell recovery on CMV-specific T-cell reconstitution. Within the first 100-day period post transplant the peak T-cell count (*x* axis) vs the CMV-specific T-cell count (*y* axis) was compared using the Spearman rank correlation test for CD4 (**a**) and CD8 (**b**) T cells. (**c**) Correlation between CMV-specific CD4+ and CD8+ T-cell response. The number of CMV-specific CD4+ T cells (*x* axis) was compared to the number of CMV-specific CD8+ T-cell response (*y* axis) using the Spearman rank correlation test. The *P*-value indicates level of significance and *r*, the correlation coefficient.

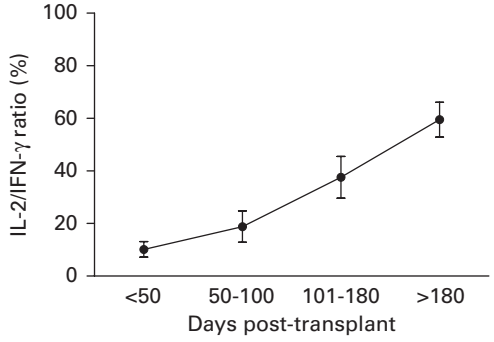

Figure 4 Conversion from effector memory to central memory CD4+ T cells occurs during immune reconstitution post transplant. The ratio of IL-2 to IFN-γ production within CMV-specific CD4+ T cells (*y* axis) was analysed in relation to the time post transplant (*x* axis). The mean with s.e. bar is for five patients measured at the four different time periods.

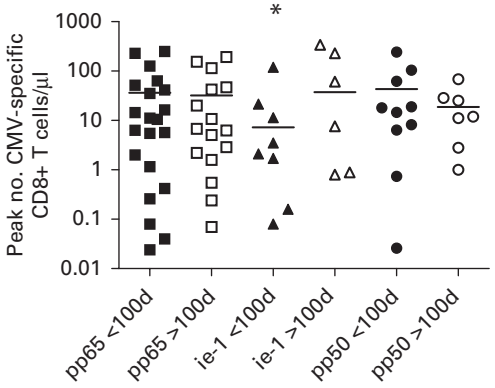

Figure 5 Analysis of the antigenic specificity of the CD8+ T-cell response to CMV. CMV-specific class I tetramers were generated using peptides from three different CMV proteins (pp65, pp50 and immediate early Ag (IE-1)). The peak CMV-specific response for each protein is represented for each patient at time points <100 days and >100 days post transplant. A significant difference in CMV-specific CD8+ T cells is indicated * between pp65 and IE-1 (<100 days) using the Kruskal–Wallis test and Dunn's post-test. Bars represent the mean.

CD8+ T-cell immunity to IE-1 is suppressed in the first 100 days after transplantation

The magnitude and specificity of the CMV-specific CD8 T-cell immune response was assessed by staining of PBMC with HLA-peptide tetramers containing a range of individual peptides from the immunodominant CMV proteins pp65, pp50 and IE-1. To determine the hierarchy of immunodominance against these proteins the number of CD8+ T cells staining with each tetramer was determined at <100 days and >100 days post transplant (Figure 5). The magnitude of the CD8+ T-cell responses to pp65 and pp50 were comparable (mean 36 vs 43 cells/µl) but responses to IE-1 were five times lower (mean 7.2 cells/µl) within the first 100 days post transplantation. After 100 days the IE-1-specific CD8+ T-cell count increased and comparable immune levels were seen against all three proteins.

Discussion

Reactivation of CMV remains a significant clinical problem following allogeneic SCT despite improvements in antiviral

therapy.[19] The nature of the conditioning regimen plays a major role in determining the risk of viral reactivation and T-cell depletion of the donor stem cell infusion is associated with a particularly high risk of viraemia. There remains debate as to the relative risk of CMV reactivation following non-myeloablative transplantations in comparison to myeloablative programmes[20] and CMV-specific T-cell immune reconstitution can occur promptly even after alemtuzumab-containing conditioning regimens.[21] In our cohort reactivation occurred only in CMV seropositive patients and confirmed similar studies showing the primacy of patient serostatus over donor graft origin in development of viraemia.[22]

Relatively few studies have studied the importance of both CMV-specific CD4+ and CD8+ T-cell immune reconstitution in relation to reactivation risk. Most reports have focussed on the CD8+ CMV-specific T-cell response and demonstrate that robust CD8+ immune reconstitution is associated with a reduction in both CMV reactivation and disease.[6,23,24] These studies have incorporated a variety of techniques including *in vitro* expansion of effector populations and staining with HLA-peptide tetramers. We have used CMV lysate as an antigenic stimulus to identify CMV-specific CD4+ T cells in the peripheral blood of patients following SCT. This technique is being used widely in the identification of these cells in healthy immunocompetent donors and has demonstrated that the CMV-specific CD4+ T-cell response is dominated by large oligoclonal expansions of cells with cytotoxic activity and predominant production of IFN-γ.[25,26] These cells have been shown to expand with age and display many phenotypic and functional characteristics in common with CD8+ T-cell populations. In HSCT, several studies have demonstrated the importance of both the CD4+ and CD8+ CMV-specific T cells in protection from CMV reactivation.[27,28] More recently, Lilleri proposed that an absolute CD4 and CD8 count of 1 and 3 cells/μl, respectively, may be valuable in protection from recurrent CMV reactivation in young HSCT patients.[14]

Our data shows a clear association between reconstitution of the CD4+ CMV-specific T-cell immune response within the first 50 days and protection against CMV reactivation. Patients who experienced CMV reactivation displayed a mean CMV-specific CD4 response of 0.4 cells/μl, which was 16 times lower than that seen in patients without reactivation. It was not possible to discern an absolute specific CD4+ T-cell response that was associated with protection from reactivation and no CMV-specific CD4+ T-cell response was observed in two patients despite absence of viral reactivation. In this regard, it must be appreciated that CMV lysate contains only a proportion of the total CMV proteome and it is likely that a component of the CMV-specific CD4+ T-cell response is therefore not detected by this methodology. Avetisyan *et al*.[13] have reported that the total CMV-specific T-cell response at 4 weeks correlates inversely with subsequent CMV viral load but did not demonstrate a specific protective role for the CD4+ population. In this study, the CD8+ CMV-specific T-cell response was not predictive of protection from a single episode of viral reactivation and suggests that the efficient reconstitution of CD4+ CMV-specific T cells plays a more important role in the early period post transplantation. These findings are comparable to studies in renal transplantation and HIV infection where the magnitude of the CMV-specific CD4+ immune response is highly correlated with protection from viral disease.[26,29]

Late CMV disease is becoming an increasing problem with current transplant conditioning regimens. In our study we found that patients with a combination of poor CD4+ and CD8+ CMV-specific T-cell responses within the first 100 days post transplantation were at very high risk of recurrent viral reactivation and this is likely to explain the observation that T-cell lymphopenia during this time period is also associated with recurrent activation.[17] These data potentially allow for the identification of patients in whom recurrent viral reactivation will be likely and can thus direct appropriate antiviral therapy.

The absolute lymphocyte count has been shown to be a powerful determinant of patient outcome in a variety of settings in haematological malignancy. The magnitude of the CMV-specific immune response is such that it has been shown to have a positive influence on the total lymphocyte count within peripheral blood, and this may be at least partly reflected in our study.[30] Lymphopenia is known to be a predictive factor for CMV reactivation after allogeneic transplantation[11,31] and may potentially be used as a surrogate marker for poor virus-specific immunity in situations where technology for ascertaining the CMV-specific immune response is not available.

The data showed a strong correlation between the magnitude of the CMV-specific CD4+ and CD8+ T-cell immune responses. Indeed the CMV-specific CD4+ and CD8+ T-cell responses both represented 10% of their respective T-cell repertoire. Such extreme immunodominance of CMV in the post transplant period has been observed in other studies[28] and may reflect the observation of CMV latency and reactivation in myeloid DCs.[32]

The CMV-specific CD4+ T-cell immune response is unusual in that the great majority of the population is comprised of cytotoxic CD4 cells, a minority population within the CD4+ T-cell repertoire. Cytotoxic CD4+ T cells show very comparable phenotypic and functional features with CD8+ cytotoxic T cells and this is reflected in their cytokine production. Almost all CMV-specific cytotoxic CD4+ T cells secrete IFN-γ following antigenic stimulation, with subpopulations also showing production of tumour necrosis factor-α and IL-2. In young healthy donors approximately half of the CMV-specific CD4+ T-cell pool is capable of IL-2 production but this proportion falls within the elderly population.[33] The mechanisms that determine the pattern of cytokine profile within cytotoxic CD4+ populations have not yet been ascertained but at least a component of this subset is likely to comprise central memory cells which are characterized by IL-2 production. A notable feature of our findings was the clear increase in the proportion of cells expressing IL-2 in the first year following transplantation. The most likely explanation for this finding is viral reactivation in the early post transplant period could lead to the expansion of effector memory CMV-specific CD4+ T-cell populations even in the absence of clinically detectable viraemia.[34] As

viral reactivation becomes controlled, reversion of Ag-specific populations into central memory and resting memory phenotype could then occur. Alterations in general immune homeostasis in the early post transplant period may also impact on the cytokine profile of effector populations.

The study also allowed us to investigate the antigenic specificity of the CD8+ T-cell response to CMV following allogeneic transplantation. The majority of studies within this area have focussed on the magnitude and function of the CD8+ T-cell response to peptides derived from pp65, a component of the viral matrix and an immunodominant Ag for CD8+ T-cell immunity.[35] In this study we were able to use HLA-peptide tetramers which contain peptides derived from an additional two immunodominant proteins from within the CMV proteome. These included the IE-1, and the immune response against this protein has been shown to be an important determinant for control of CMV reactivation following solid organ transplantation.[36] Functional differences between pp65 and IE-1-specific CD8+ T cells have been reported in the SCT setting which may influence their relative efficacy in control of viral reactivation,[37] however T-cell function could not be addressed here. We also studied the CD8+ immune response to a peptide from pp50 from which a number of potent immunodominant peptides have now been characterized. Using the panel of tetramers we observed a decreased frequency to IE-1 at less than 100 days in comparison to pp65 and pp50. Interestingly Lacey *et al*.[37] have also observed comparable functional responses to pp50 and pp65 post-SCT, but impaired responses to IE-1. The CD8+ immune response to IE-1 peptides was not predictive of increased protection from viral reactivation in our study supporting two other HSCT studies[38,39] but in contrast to other studies in solid organ transplantation. However it should be noted that not all patients had both <100 days and >100 days samples hence variation in CMV immune reconstitution in differing HLA contexts could potentially explain our findings in the small patient cohort.

In conclusion, our findings revealed that the magnitude of the CMV-specific CD4+ T-cell immune response to CMV in the first 50 days following transplantation is predictive of the risk of viral reactivation. Protection from viral reactivation appears to be mediated through populations of effector memory CD4+ cells, as defined according to their cytokine profile, producing high levels of IFN-γ. CD8+ immune reconstitution was not predictive of reactivation at this early stage but poor recovery was predictive of recurrent episodes of viral reactivation. These findings may help to direct future forms of immunotherapy to assist reconstitution of the CMV-specific immune response and imply that techniques, which enhance both CD4+ and CD8+ immune reconstitution may exhibit enhanced level of clinical efficacy.

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