Sequential Anti-Cytomegalovirus Response Monitoring May Allow Prediction of Cytomegalovirus Reactivation after Allogeneic Stem Cell Transplantation

Sylvia Borchers1,*, Melanie Bremm2,*, Thomas Lehrnbecher2, Elke Dammann1, Brigitte Pabst3, Benno Wölk4, Ruth Esser2, Meral Yildiz2,5, Matthias Eder1, Michael Stadler1, Peter Bader2, Hans Martin5, Andrea Jarisch2, Gisbert Schneider6, Thomas Klingebiel1, Arnold Ganser1, Eva M. Weissinger1,*, Ulrike Koehl2,7

1 Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School, Hannover, Germany, 2 Pediatric Hematology and Oncology, Johann Wolfgang Goethe-University, Frankfurt, Germany, 3 Institute of Human Genetics, Hannover Medical School, Hannover, Germany, 4 Institute of Virology, Hannover Medical School, Hannover, Germany, 5 Internal Medicine II, Johann Wolfgang Goethe-University, Frankfurt, Germany, 6 Institute of Pharmaceutical Science and Biostatistics, ETH Zürich, Switzerland, 7 Institute of Cellular Therapeutics, IFB-Tx, Hannover Medical School, Hannover, Germany

Abstract

Background: Reconstitution of cytomegalovirus-specific CD3+CD8+ T cells (CMV-CTLs) after allogeneic hematopoietic stem cell transplantation (HSCT) is necessary to bring cytomegalovirus (CMV) reactivation under control. However, the parameters determining protective CMV-CTL reconstitution remain unclear to date.

Design and Methods: In a prospective tri-center study, CMV-CTL reconstitution was analyzed in the peripheral blood from 278 patients during the year following HSCT using 7 commercially available tetrameric HLA-CMV epitope complexes. All patients included could be monitored with at least CMV-specific tetramer.

Results: CMV-CTL reconstitution was detected in 198 patients (71%) after allogeneic HSCT. Most importantly, reconstitution with 1 CMV-CTL per μl blood between day +50 and day +75 post-HSCT discriminated between patients with and without CMV reactivation in the R+/D+ patient group, independent of the CMV-epitope recognized. In addition, CMV-CTLs expanded more daramatically in patients experiencing only one CMV-reactivation than those without or those with multiple CMV reactivations. Monitoring using at least 2 tetramers was possible in 63% (n = 176) of the patients. The combinations of particular HLA molecules influenced the numbers of CMV-CTLs detected. The highest CMV-CTL count obtained for an individual tetramer also changed over time in 11% of these patients (n = 19) resulting in higher levels of HLA-B*0801 (IE-1) recognizing CMV-CTLs in 14 patients.

Conclusions: Our results indicate that 1 CMV-CTL per μl blood between day +50 to +75 marks the beginning of an immune response against CMV in the R+/D+ group. Detection of CMV-CTL expansion thereafter indicates successful resolution of the CMV reactivation. Thus, sequential monitoring of CMV-CTL reconstitution can be used to predict patients at risk for recurrent CMV reactivation.

Introduction

Reactivation of cytomegalovirus (CMV) remains one of the major complications after allogeneic hematopoietic stem cell transplantation (HSCT) [1,2,3]. The latent virus is controlled mainly by CMV-specific T cells (CMV-CTLs) in healthy persons, but in immune-compromised patients CMV reactivation occurs frequently due to impaired T cell reconstitution and post-HSCT immunosuppressive therapy. CMV reactivation, if not controlled, can lead to severe and multiple manifestations of CMV disease, such as CMV retinitis, gastroenteritis or pneumonia [4]. CMV disease is also associated with a high risk for bacterial or fungal infections. Thus, monitoring of CMV-CTL reconstitution is a key goal in HSCT care.
Design and Methods

Ethics statement
Sample collection and analyses were part of an extended monitoring program conducted in the course of routine sampling for clinical follow-up. The study was approved by the University Hospital Ethics Committees (Hannover and Frankfurt, Germany), and is registered as #2906 with the Ethics Committee at the Hannover Medical School and as #50/07 with the Ethics Committee at the University Hospital Frankfurt. Written informed consent was obtained from all patients or legal guardians.

Patient characteristics

Patients were transplanted between January 2006 and December 2010 in the Departments of Pediatric Hematology and Oncology and Internal Medicine in Frankfurt and the Department for Hematology, Hemostasis, Oncology and Stem cell transplantation at Hannover Medical School. This prospective study included all patients (n = 278) who had at least one HLA molecule corresponding to a set of 7 commercially available HLA-CMV tetramers (Coulter; USA). Patients were transplanted for leukemia, lymphoma, myelodysplastic or benign hematopoietic dysfunction syndromes or solid tumors in pediatric patients after the first relapse of the underlying disease. HLA-matched donors were available for 78% (n = 218/278) of patients, while 22% (n = 60/278) received grafts from mismatched donors. Bone marrow (BM; n = 35), cord blood (CB; n = 2), peripheral blood stem cells (PBSC; n = 220) or T cell-depleted (TCD) PBSC (n = 21) were used as grafts. T cell depletion was done ex vivo as described previously [20,21] using good manufacturing practice (GMP), and reduced T cells in the graft by 13,000-fold compared to unselected PBSC and 800-fold compared to BM. Sixty-eight percent (n = 190/278) of the patients were CMV-seropositive (R+), and 73% (n = 139/190) of this group were transplanted from CMV-seropositive donors (D+), with the remaining 27% (n = 51/190) receiving grafts from CMV-seronegative donors (D−). Prior to HSCT, 88 recipients (32%) were CMV-seronegative (R−), of whom 56% (n = 49/88) received grafts from CMV-seronegative donors, while 44% (n = 39/88) were transplanted from CMV-seropositive donors. Patient and graft characteristics are summarized in Table 1.

Multimers, such as the tetrameric HLA epitope complexes (tetramer), are commonly used to monitor CMV-specific CTL reconstitution [13,14,15,16,17,18], and can be used as a tool to analyze the reconstitution process. Although several studies have investigated CMV-CTL levels as a possible predictor for CMV reactivation, no clear protective threshold has yet been defined. While differences between patient cohorts and transplantation protocols may make the definition of a reliable threshold value for therapy initiation or withdrawal difficult, the HLA molecules and tetramer combinations used to detect CMV-CTLs may contribute to the variation observed to date in meaningful CMV-CTL levels. Monitoring time-points or time-frames of patients also differed in these studies, further complicating the interpretation of the results [14,15,16,17]. To address these questions, we prospectively monitored the reconstitution of CMV-specific immune responses in 278 patients using 7 commercially available tetramers [15,19] representing various CMV epitopes.

We hypothesized that monitoring using tetramers could be a valuable tool to individualize antiviral therapy, especially for patients at increased risk for developing multiple CMV reactivations. We analyzed factors that influence the CMV-CTL level, to assess the possibility of identifying the minimal number of CMV-CTLs required to provide protection against reactivation and with the intent of defining optimal monitoring time-point(s) for CMV-CTL reconstitution in HSCT patients.

Detection of CMV infection/reactivation after allogeneic HSCT

Blood/serum samples from all patients were routinely monitored for CMV DNA load using PCR [18] or for pp65 expressing cells per 400,000 leukocytes in peripheral blood mononuclear cells (PBMCs) using immunohistochemistry [13]. Pre-emptive antiviral therapy was initiated when a) the CMV DNA load increased by more than 0.5 log levels above the baseline, b) more than 2 pp65-expressing cells were present per 400,000 leukocytes in 2 consecutive tests or c) more than 5 pp65-expressing cells were present per 400,000 leukocytes in a single test. The initial therapy was ganciclovir (GCV; 5 mg/kg twice a day), which was adjusted according to the presence of pp65-expressing PBMC or CMV DNA load [13].

Flow cytometric quantification of CMV-CTLs

We monitored patients on days +30, +60, +90, +120 and +200 (all time-points +/−15 days) after HSCT. Patients who a) experienced CMV reactivation or b) at increased risk for reactivation due to increased immunosuppression were monitored weekly. Weekly monitoring was stopped when a) CMV reactivation was resolved or b) immunosuppression reduced. CMV-CTLs were quantified using HLA-CMV epitope tetramers as previously described [13,18]. HLA-typing of patients and donors was conducted during preparation for HSCT via high-resolution multiplexed PCR [22].

CD3+CD8+ T cells were quantified using 100 μl of EDTA-anticoagulated blood stained with 10 μl CD3-PECy5/7-labelled anti-CD3 antibody (Beckman Coulter, Germany), 10 μl FITC-labelled anti-CD8 clone T8 antibody (Beckman Coulter, Germany), 20 μl PE-labelled anti-CD4 antibody (Beckman Coulter, Germany) and fluorescent beads (FlowCount™ beads, Beckman Coulter, Germany) added according to the manufacturer’s instructions. CMV-CTLs were quantified using the following commercially available set of PE-labelled tetramers: HLA-A*0101-VTEHDTLLY, pp50 amino acids (aa) 243–255; HLA-A*0201-NLVPMVATV, pp65 aa 495–503; HLA-A*1101-ATVQGQNLK, pp65 aa 501–509; HLA-A*2402-QYDPVAALF, pp65 aa 341–349; HLA-B*0702-TPRVTGGGAM, pp65 aa 417–426; HLA-B*0801-ELRKKMYM, IE-1 aa 199–207; HLA-B*3501-IPSNVIIHY, pp65 aa 123–131 (all: Beckmann-Coulter, Germany). Each tetramer corresponding to a HLA-
Table 1. Patient characteristics.

| Patient cohort          | n = 278          |
|-------------------------|------------------|
| Age mean (range)        | 43.5 (2–72)      |
| Gender                  |                  |
| Female                  | 119              |
| Male                    | 159              |
| Diagnosis               |                  |
| ALL                     | 41               |
| AML                     | 104              |
| sAML                    | 29               |
| CLL                     | 5                |
| CML                     | 9                |
| MDS                     | 35               |
| solid tumors/lymphoma   | 31               |
| other diseases with indication for HSCT | 24 |
| CMV reactivations/CMV DeNovo Infection in the various groups | n/n |

| CMV-serostatus recipient (R)/donor (D) group | n/n |
|---------------------------------------------|-----|
| All patients                                | 117/278 |
| R+/D+                                       | 77/139 |
| R+/D−                                       | 30/51  |
| R−/D+                                       | 7/39   |
| R−/D−                                       | 3/49   |

| Donor |                  |
|-------|------------------|
| MRD   | 87               |
| MUD   | 131              |
| MMUD  | 40               |
| MRD   | 20               |

| Transplant |                  |
|------------|------------------|
| BM         | 35               |
| PBSC without TCD | 220 |
| CD34 *10^6/kgBW (mean): | 3.1 |
| CD3 *10^6/kgBW (mean):  | 25.8 |
| PBSC with TCD | 21 |
| CD34 *10^6/kgBW (mean): | 13.4 |
| CD3 *10^6/kgBW (mean):  | 391.6 |
| other*    | 2                |

| Matched tetramers per patient | n  |
|-------------------------------|----|
| 1 tetramer                    | 102|
| 2 tetramers                   | 126|
| 3 tetramers                   | 49 |
| 4 tetramers                   | 1  |

| CMV-epitopes |                  |
|--------------|------------------|
| HLA-A*0101 (pp50) | 95  |
| HLA-A*0201 (pp65) | 156 |
| HLA-A*1101 (pp65) | 1   |
| HLA-A*2402 (pp66) | 53  |
| HLA-B*0702 (pp65) | 73  |
| HLA-B*0801 (E-1) | 77  |
| HLA-B*3501 (pp65) | 41  |

Table 1. Cont.

| Diagnosis |                  |
|-----------|------------------|
| ALL       | acute lymphatic leukemia |
| AML       | acute myeloid leukemia |
| sAML      | secondary AML |
| CLL       | chronic lymphoid leukemia |
| CML       | chronic myeloid leukemia |
| MDS       | myelodysplastic syndrome |
| MMUD      | mismatched unrelated donor |
| MRD       | matched related donor |
| TCD       | T cell depletion |
| BM        | bone marrow |
| PBSC      | peripheral blood stem cell |

molecule expressed in the patient was measured separately and required 200 µl EDTA-anticoagulated blood. Aliquots were stained with 10 µl αCD3, 10 µl αCD8 and 5 µl tetramer. One negative control was performed per patient using 200 µl of EDTA-anticoagulated blood and 5 µl negative control tetramer, to which none of the cells should specifically bind to, provided by the manufacturer (Beckman Coulter, Germany). All samples were labeled for 30 min at room temperature (RT), followed by erythrocyte lysis (15 min, RT) and fixation. The gating strategy and staining results are shown in Figure 1. At least 10,000 lymphocytes, 1000 CD3^+CD8^+ cells and 10 tetramer-positive cells were counted for each tetramer-stained sample. At least 10,000 lymphocytes and 1000 CD3^+CD8^+ cells were counted for each negative control tetramer-stained sample and in addition 1100 fluorospheres were counted for each CD3^+CD8^+ T cell quantification.

Absolute numbers of CMV-CTLs were calculated using fluorescent beads (FlowCount™ beads, Beckman Coulter, Germany) in a single-platform, no-wash analysis according to the manufacturer’s directions. Briefly, samples were washed and analyzed, after erythrocyte lysis (VersaLyse, Lysing Solution; Ion 3, Fixative Solution 10×; Beckman Coulter, Germany), on a FC500 flow cytometer (Beckman Coulter, Germany) [13][18]. The absolute number of CMV-CTLs in a sample was calculated by subtracting negative-control-tetramer-binding cells from CMV-CTLs binding only to CMV-tetramers. Table 1 summarizes the tetramers corresponding to HLA-molecules present in patients. Quality constraints were determined in our previous studies [13][18] and we determined that only whole blood samples containing at least 50 CD3^+CD8^+ T-cells per µl blood gave reliable and between centers reproducible results to cytometrically detect multipositive cells at a reliable event rate as detailed above. The detection limit is 0.05 multipositive cells per µl blood using these quality constraints. Application of these quality constraints allowed to include 92% (n = 1712) of 1861 samples in blood using these quality constraints. Application of these quality constraints was performed per patient using 200 lymphocytes and 1000 CD3 were counted for each tetramer-stained sample. At least 10,000 lymphocytes and 1000 CD3^+CD8^+ cells were counted for each negative control tetramer-stained sample and in addition 1100 fluorospheres were counted for each CD3^+CD8^+ T cell quantification.

Data management and statistical analysis

CMV-CTL data were collected and stored in a mySQL database. The general purpose PHP5 (Personal Home Page tools 5; open source license) scripting language was used for queries in the database. The mean number of CMV-CTLs from all tests using one tetramer was calculated for each patient to evaluate whether monitoring using single tetramers resulted in similar CMV-CTL counts in patients with detectable immune responses. For patients monitored with more than 1 tetramer, the CMV-CTL counts obtained for each tetramer were calculated individually after HSCT. Clinical data were correlated with CMV-CTL reconstitution. The influence of the presence or absence of CMV-CTL on the occurrence of CMV reactivation and vice versa was evaluated. CMV-CTL reconstitution was analyzed at different intervals after HSCT.
thresholds: namely >0, ≥1, 3, 5, 7 or 10 CMV-CTL per µl blood). CMV-CTL levels before and after 1st CMV reactivation were counted, and the resulting slope between this 2 time-points was calculated. For the determination of CMV-CTL expansion after CMV reactivation, the following patient/donor pairs were excluded: a) CMV-seronegative patients who were transplanted from seronegative donors and did not develop de novo CMV infections during follow-up; b) patients who died before day +100, c) patients with early relapse of the underlying disease by day +100 and d) patients for whom sampling could not be achieved prior to and after CMV reactivation. Statistical and Kaplan-Meier analyses were performed with GraphPad Prism 4 and 5 (GraphPad Software, San Diego, USA). Graphs were plotted using GraphPad Prism 4 and 5). P-values ≤0.05 were considered significant, and the significance test applied is indicated for all p-values in the figure legends.

Results

CMV-CTL levels vary depending on the tetramer used for detection, on HLA-molecules expressed and on occurrence of CMV reactivation

CMV-CTL reconstitution was observed in 71% (n = 198/278) of the patients. The median level of CMV-CTLs varied considerably for each HLA allele investigated, ranging from 2–30 CMV-CTLs per µl blood (Figure 2A). CMV-CTLs recognizing the HLA-A*2402 tetramer were detected at significantly lower levels in all patients with this HLA molecule compared to CMV-CTLs corresponding to other CMV epitope and tetramer combinations. In addition, 73% of all FACS analyses with the HLA-A*2402 tetramer detected no CMV-CTLs per µl blood, and HLA-A*2402-specific CMV-CTLs did not correlate with the total number of CD3⁺CD8⁺ T cells observed. CMV reactivations did not occur more frequently in patients with HLA-A*2402. In contrast, CMV-CTL levels detected by HLA-A*0101, HLA-A*0201, HLA-B*0702, HLA-B*0801 and HLA-B*3501 tetramers correlated with overall CD3⁺CD8⁺ immune reconstitution (0.5 ≤ r ≤ 0.8; p < 0.0001). The HLA-A*1101-pp65 tetramer was used to monitor only one patient, thus data obtained with this tetramer are not shown in Figure 2.

CMV-reactivation occurred in 42% (n = 117/278) of the patients and had a negative impact on overall survival (p < 0.04; Log-rank Test; Figure S1) as expected. After CMV reactivation the median CMV-CTL levels were always higher independent of CMV-epitope tetramer combination used (Figure 2B). The difference in CMV-CTL numbers prior to and after CMV reactivation was statistically significant for HLA-A*0101 (p < 0.001) and for HLA-A*2402 (p < 0.01).
Sixty-three percent of the patients (n = 176/278) could be monitored with more than one CMV tetramer (Table 1) and CMV-CTL levels were influenced by the presence of other HLA-tetramer combinations. For example, the level of T cells detected by HLA-A*0201-pp65 was significantly lower, if the HLA molecules expressed by the patients corresponded to both HLA-A*0201 and HLA-B*0702 rather than only the HLA-A*0201 molecule (Figure 3A; median: 1.6 versus 18.5 CMV-CTLs per μl blood; p<0.05; t-Test with Welch’s correction). The number of CMV-CTLs detected by HLA-B*0702 did not differ whether the patients also expressed the HLA-A*0201 molecule or not (median: 21.6 and 27.8 CMV-CTLs per μl blood; Figure 3A). A change in the most abundant CMV-CTLs detected with particular tetramers occurred in 11% (n = 19/176) of the patients monitored with at least 2 tetramers. We detected 30 alterations of the most abundant CMV-CTL lines after HSCT. Figure 3B shows a typical example of such a change. This patient experienced CMV reactivation on day +41. A shift from HLA-A*0201 to HLA-B*0702 was detected by day +100, when the level of HLA-B0801 CMV-CTLs rose above the level of HLA-A0201 CMV-CTL. In 14 patients, 17 changes (57%; 17/30) led to higher levels of HLA-B*0801 (IE-1) recognizing CMV-CTLs (Table S2) after day +100 post-HSCT. Interestingly, these increases/decreases of most abundant CMV-CTLs did not correlate with the time of CMV reactivations after HSCT (Table S2).

Early CMV-CTL reconstitution correlates well with protection against CMV reactivation in the R+/D+ group

To identify common features for CMV-CTL reconstitution in our patient cohort, we analyzed the kinetics of CMV-CTL reconstitution. CMV-seropositive recipients (R+, n = 190/278; 68%) were grouped into patients receiving a transplant from a CMV-seropositive donor (R+/D+, n = 139/190) or a seronegative donor (R+/D−, n = 51/190). Monitoring of reconstitution of CMV-CTLs was initiated on day 30 (+/-15 days) post-HSCT in the R+/D+ group. In the R+/D+ group sixty two patients had no CMV reactivation after HSCT. By day +50 38 patients (62%, p<0.02) had 1 CMV-CTL/μl blood, by day +75 46 patients (74%, p<0.04) had achieved 1 CMV-CTL/μl blood. These were significantly more patients than those reactivating CMV at least once (37% by day+50; n = 47/77) (Figure 4A). Interestingly, increasing the threshold level to between 5 and 10 CMV-CTLs per μl blood did not improve discrimination between patients with and without CMV reactivations (Figure S2C-F). A typical example for the R+/D+ patient group illustrates early reconstitution of CMV immunity by day +60 following CMV reactivation (Figure 4B). Patients from the R+/D+ group without detectable CMV-CTLs or with detectable CMV-CTLs that did not expand during or after the first CMV reactivation (12.5% of patients, n = 20), Those were at risk for multiple CMV reactivations (n = 13) similar to patients transplanted from seronegative donors. (R+/D−) showed a delayed reconstitution of CMV immunity, which occurred on or after day +120 (Figure 4C). There is no significant difference in number of patients in the R+/D− group achieving
more, low-level preexisting CMV-CTLs did not proliferate upon CMV reactivation in 27% (n = 14/51) of R+D+ patients. As expected, reconstitution of CMV-CTLs was delayed in patients who received TCD grafts, and the course of reconstitution is similar to R+ patients transplanted from CMV-seronegative donors (Figure S3). Patients in the groups R−/D− or R−/D+ were not included in statistical analyses, since patient numbers were small and CMV reactivations (R−/D+ 7/39) or de novo infections (R−/D− 3/49) were rare events (Table 1) and did not contribute to the elucidation of CMV reactivation in the context of CMV-CTL reconstitution.

To investigate whether incidence, severity and treatment of aGvHD influenced the numbers of CMV-CTLs detected, we analyzed the associated data for the R+D+ group. The number of CMV-CTL did not differ significantly in patients without or with aGvHD grade I or II (Figure S4).

Expansion of CMV-CTLs is associated with single but not multiple CMV reactivations

CMV-CTL levels detected by the different tetramers (Figure 2) increased after CMV reactivation, thus, defining a time-dependent threshold of CMV-CTL that protects the patient against CMV reactivation is difficult. In our patient cohort, the first CMV reactivation occurred by day +46 (mean; range: +11 to +170) for patients of the R+D+ group. Thus, we reasoned that protective CMV-CTL levels could probably be predicted by monitoring on days +30 (+/−15) and +60 (+/−15) (Figure 5). Sixty-five of 139 patients of the R+D+ group were included in these analyses. Figure 5A compares the number of CMV-CTLs on days +30 and +60 in patients who experienced no or only 1 CMV reactivation. CMV-CTLs hardly expanded in patients without CMV reactivation. In contrast, CMV-CTL numbers increased significantly (p<0.004, t-Test with Welch’s correction) between days +30 (range: +15 to +45) and +60 (range: +46 to +99) in patients with a single reactivation of CMV. These data illustrate the impact of the first CMV reactivation on CMV-CTL expansion and demonstrate that CMV-CTL levels cannot serve as predictors for the first CMV reactivation. Thus, we assessed whether the proliferation of CMV-CTLs could act as a predictor for a successful restoration of the CMV immune response. We calculated the slope of the CMV-CTL expansion between time-points prior to and after reactivation, and compared CMV-CTL levels in patients without, or with a single or recurrent CMV reactivations (Figure 5B). The expansion slope was significantly higher in R+D+ patients with only 1 CMV reactivation. In contrast, no significant increase of the slope was seen between days +30 and +60 in patients experiencing recurrent CMV reactivation, indicating that CMV-CTL expansion may be a prerequisite to resolve CMV reactivation.

Discussion

CMV reactivation following HSCT is a consequence of the massive immunosuppression and insufficient lymphocyte reconstitution, and occurs more frequently after T cell depletion of the graft or after transplantation of CMV-seropositive patients with grafts from seronegative donors. CMV reactivations still constitute significantly to post-HSCT morbidity, despite advancements made to reduce CMV disease by monitoring for viral reactivation and pre-emptive therapy.

Monitoring CMV-CTL reconstitution can be achieved in about 85% of all transplanted patients [13,18], using commercially available CMV-tetramers. A large cohort of 278 patients could be monitored with the tetramers, 198 (71%) actually developed detectable CMV-CTLs following HSCT. The CMV-CTL levels detected varied greatly (1–1235 per μl blood), and correlated only...
weakly with reconstitution of the CD3⁺CD8⁺ T cells, as described previously [23]. Restoration of CMV-specific immunity is frequently analyzed using tetramer staining [14,15,24]. However, absolute values for the CMV-CTL levels required to protect the patient from reactivation are still actively debated to date. Our data indicate that CMV-CTL numbers vary considerably for individual combinations of HLA molecules and CMV epitopes. Many authors focus on the HLA allele that is most common in the Caucasian population, HLA-A*0201. Using the HLA-A*0201-NLVP tetramer, 10 to 20 CMV-CTLs per μl blood by day +60 were described as being protective [25,26]. Our data for CMV-CTL levels detected by HLA-A*0201-NLVP are similar, with a mean level of 10 CMV-CTL per μl blood (Figure 2). However, analyzing additional HLA molecules in our large patient cohort showed that different HLA types yield quite different median values of detectable CMV-CTLs (Figure 2). We and others observed that CMV-CTL levels detected by HLA-A*0101 (pp50243–255) or HLA-B*3501 (pp65123–131) were considerably higher or much lower than CMV-CTL levels detected by HLA-A*2402 (pp65341–349), which yielded a mean of 4 per μl blood (Figure 2) [15,18,19,27]. We detected no CMV-CTLs using the HLA-A*2402 tetramer in 73% of the tests. This is in agreement with data published by others, who detected low levels of CMV-CTLs using the HLA-A*2402 tetramer when monitoring CMV-CTL reconstitution [19,27,28,29,30,31,32]. We also identified no correlation between the immune reconstitution of CD3⁺CD8⁺ T cells and CMV-CTL levels detected using the HLA-
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Figure 5. Kinetics of CMV-CTL expansion after CMV reactivation. (A) CMV-CTL levels around day +30 (the interval from day +15 to +45 was assessed, filled symbols) and day +60 (the interval from day +46 to +99 was assessed, filled symbols) in patients who experienced (circles) or did not experience (triangles) a CMV reactivation prior to day +50 in the R+/D+ group. Significant differences between groups were assessed by t-test with Welch’s correction, and included the 34 data points not depicted because they lie outside axis limits. (B) Patients in the R+/D+ group who experienced no (triangles), a single (circles) or multiple (diamonds) CMV reactivations before day +100 were compared (t-test with Welch’s correction) using the difference between the slopes of the lines created by the CMV-CTL levels during the interval from day +15 to +45 and the interval from day +46 to +99. The dotted line indicates no change in CMV-CTL level between the measurements in both intervals. ** p<0.01.

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days), and measured the expansion of CMV-CTLs within that time period. Patients with a protective response after the first CMV reactivation showed a significantly increased expansion of CMV-CTLs within this time period compared to patients with recurrent CMV reactivations. Patients without CMV reactivation also expanded CMV-CTLs during this time interval, but to a lesser extent than those with 1 CMV reactivation. The inability of CMV-CTLs to expand after CMV reactivation may be due to even minor HLA incompatibilities between donor and recipient. Our results indicate that analyses using a single tetramer at only one time point, for instance on day +60, do not allow prediction of pending or, more importantly, recurrent CMV reactivations. Despite the fact that patients without CMV reactivation showed an earlier reconstitution of at least 1 CMV-CTL per µl blood, the wide range of CMV-CTL levels does not allow definition of definitive protective value that is broadly applicable for all HSCT patients. However, monitoring the level of CMV-CTL expansion between days +30 and +60 (+/- 15 days) after CMV reactivation can indicate successful restoration of CMV immunity. In summary, our results show that sequential tetramer monitoring rather single time point cut offs of the post-transplant CMV-CTL immune reconstitution allows a more accurate interpretation of an individual patient’s response to CMV. In addition, CMV-CTL expansion after the first CMV reactivation indicates recurrence of CMV reactivation even in R+/D+ patients after allogeneic HSCT. Analysis of the CMV-CTL expansion rate may facilitate implementation of patient-specific antiviral strategies, including adoptive transfer of CMV-CTLs to recipients unable to respond to CMV reactivations.

Supporting Information

Figure S1 Impact of CMV reactivation on survival. Patients in whom CMV reactivation occurred (n = 117) had a significantly lower probability for survival (p<0.04) than patients not experiencing CMV reactivation (n = 161). (TIF)

Figure S2 CMV-CTL analysis applying different threshold levels. [A–F] Reconstitution of CMV-CTLs with respect to reactivation in the R+/D+ patient group using different thresholds of CMV-CTLs per µl blood. Percentage of patients reaching the threshold level is plotted against time after HSCT. [A–C] Using thresholds of >0 to ≥3 CMV-CTLs per µl blood showed significant differences between patients with and without CMV reactivation. [D–F] Using thresholds of ≥5 or higher (per µl blood) detected no significant differences in the reconstitution of CMV-CTLs between patients with and without CMV reactivation. The asterisk (*) indicates significant differences at day +50; the plus symbol (+) indicates significant differences at day +75.

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