Abundant cytomegalovirus (CMV) reactive clonotypes in the CD₈⁺ T cell receptor alpha repertoire following allogeneic transplantation

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Accepted for publication 20 January 2016
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Summary

Allogeneic stem cell transplantation (SCT) has greatly improved the prognosis for patients with malignant hematological diseases. However, the required immunosuppression following SCT as well as the use of in-vivo T cell depletion with anti-thymocyte globulin (ATG) increases patients’ susceptibility to infections. This risk is increased further in the case of intensified immunosuppression in patients with graft-versus-host disease (GVHD). Cytomegalovirus (CMV) infections are one of the most frequent complications following SCT as well as the use of anti-thymocyte globulin (ATG) increases patients’ susceptibility to infections. More than half of stem cell recipients develop a CMV infection, and untreated infections can lead to severe CMV organ-disease, such as enterocolitis, pneumonia, hepatitis and/or retinitis [2,3]. Currently, pre-emptive treatment is the most common therapeutic approach when increasing virus load or antigenaemia is observed. The standard treatments of CMV infections are ganciclovir, foscarnet in conditions of neutropenia or cidofovir [3,4].

T cells are a major line of defence against CMV. CD₈⁺ T cells directed against CMV epitopes expand quickly upon active infection and individuals exposed previously to CMV infection can have large numbers of circulating CMV-targeting CD₈⁺ T cells in the peripheral blood. Analyzing the T cell receptor (TCR) repertoire provides new insights into virus specific immunity and immunoreconstitution in transplanted patients. New technologies, such as next-generation sequencing (NGS), offer the opportunity to assess the TCR repertoire on an unprecedented level. So far, most TCR sequencing in the setting of allogeneic transplantation has focused on assessment of the TCR repertoire and its clinical relevance in patients following stem cell transplantation. Using next-generation sequencing we examined the TCR repertoire of CD₈⁺ T cells and CMV-specific CD₈⁺ T cells in four patients. Additionally, we performed single-cell TCR-αβ sequencing of CMV-specific CD₈⁺ T cells. The TCR-α composition of human leucocyte antigen (HLA)-A*0201 CMVpp65- and CMVIE-specific T cells was oligoclonal and defined by few dominant clonotypes. Frequencies of single clonotypes reached up to 11% of all CD₈⁺ T cells and half of the total CD₈⁺ T cell repertoire was dominated by few CMV-reactive clonotypes. Some TCR-α clonotypes were shared between patients. Gene expression of the circulating CMV-specific CD₈⁺ T cells was consistent with chronically activated effector memory T cells. The CD₈⁺ T cell response to CMV reactivation resulted in an expansion of a few TCR-α clonotypes to dominate the CD₈⁺ repertoires. These results warrant further larger studies to define the ability of oligoclonally expanded T cell clones to achieve an effective anti-viral T cell response in this setting.

Keywords: allogeneic transplantation, CMV, next-generation sequencing, T cell receptor alpha, T cell receptor repertoire

Introduction

Allogeneic stem cell transplantation (SCT) has greatly improved the prognosis for patients with malignant hematological diseases. However, the required immunosuppression following SCT as well as the use of in-vivo T cell depletion with anti-thymocyte globulin (ATG) increases patients’ susceptibility to infections. This risk is increased further in the case of intensified immunosuppression in patients with graft-versus-host disease (GVHD). Cytomegalovirus (CMV) infections are one of the most frequent complications after SCT [1,2]. More than half of stem cell recipients develop a CMV infection, and untreated infections can lead to severe CMV organ-disease, such as enterocolitis, pneumonia, hepatitis and/or retinitis [2,3]. Currently, pre-emptive treatment is the most common therapeutic approach when increasing virus load or antigenaemia is observed. The standard treatments of CMV infections are ganciclovir, foscarnet in conditions of neutropenia or cidofovir [3,4].

T cells are a major line of defence against CMV. CD₈⁺ T cells directed against CMV epitopes expand quickly upon active infection and individuals exposed previously to CMV infection can have large numbers of circulating CMV-targeting CD₈⁺ T cells in the peripheral blood. Analyzing the T cell receptor (TCR) repertoire provides new insights into virus specific immunity and immunoreconstitution in transplanted patients. New technologies, such as next-generation sequencing (NGS), offer the opportunity to assess the TCR repertoire on an unprecedented level. So far, most TCR sequencing in the setting of allogeneic transplantation has focused on assessment of the TCR-β chain [5–7]. A recent report assessed the TCR-β repertoire of CMV-specific T cells by NGS and demonstrated a high prevalence of dominant clonotypes in kidney allograft
recipients [8]. Similarly, single TCR-β chain analyses of CMV-specific cells following allogeneic transplantation showed the presence and persistence of dominant clonotypes in the antigen-specific repertoire [9,10]. However, the role and composition of the TCR-α chain in the context of CMV infections in patients receiving allogeneic stem cells remains largely unknown. Here, we report the TCR repertoire of CMV_{pp65} and CMV_{IE}-specific CD8^+ T cells, restricted to human leucocyte antigen (HLA)-A*0201, in transplanted patients positive for CMV using single-cell TCR-α and TCR-β sequencing and NGS for TCR-α. We report findings from four patients with acute myeloid leukaemia (AML) or chronic lymphocytic leukaemia (CLL).

**Materials and methods**

**Patients**

Four human leucocyte antigen (HLA)-A*0201-positive patients transplanted for AML or CLL were analysed for their peripheral blood CD8^+ T cell and CMV-specific CD8^+ T cell TCR repertoires (Table 1). Patients were enrolled after written informed consent and ethical review board approval (EK-187052011 and EK-279072013).

**Chimerism analysis**

Donor chimerism analyses were provided by AgenDix GmbH (Dresden, Germany) using polymerase chain reaction (PCR)-based amplification of short tandem repeats (STRs) [11]. As part of routine procedures for transplanted patients, donor chimerism was analysed every 2 weeks from day 14 onwards. Chimerism >98% was considered as a full donor chimerism.

**Immunophenotyping and sorting**

CMV-specific CD8^+ T cells were detected using reversible phycoerythrin (PE)-labelled streptamers (IBA GmbH, Göttingen, Germany). Streptamers were composed of HLA A*0201 bound to CMV-phosphoprotein-65 (pp65495–503, peptide sequence: NLVPMVATV) and CMV-immediate early (IE316–324, peptide sequence: VLEETSVML) epitopes. The staining panel included the following antibodies: CMV-streptamer-PE (IBA GmbH), CD45-V500, CD3-peridinin chlorophyll (PerCP)-cyanin (Cy5-5), CD8-allophycocyanin (APC)-H7, CCR7-fluorescein isothiocyanate (FITC), CD45RA-Pacific Blue (all from BD Biosciences, San Jose, CA, USA). For TCR repertoire analyses, 250 000 CD3^+CD8^+ T cells were isolated (purity: 99%) and between 8 × 10^4 and 1 × 10^5 CD3^+CD8^+ CMV streptamer^+ T cells were sorted according to their CMV_{pp65} (purity: 99%) and CMV_{IE} (purity: 83%) specificity. Cells were sorted using BD fluorescence activated cell sorter (FACS)Aria II and directly frozen in RLT buffer + 10 μl/ml β-mercaptoethanol. CD3^+CD8^+ and

### Table 1. Patients’ characteristics.

| Patient | Disease status at Tx | Remission | Age/sex | Conditioning regimen | Immunosuppression prophylaxis | Donor | HLA-matching | CD3^+ cells in graft | Viraemia (pg/ml) | Prophylaxis |
|---------|----------------------|-----------|---------|----------------------|--------------------------------|-------|--------------|----------------------|----------------|-------------|
| 1       | AML                  | IF        | 60/m    | MUD                   | CSA + MTX                      | IF    | MUD 10/10    | 2.3 × 10^6/kg        | 0.5             | Aciclovir   |
| 2       | AML                  | PR        | 71/m    | MUD                   | CSA + MMF                      | PR    | MUD 10/10    | 3.5 × 10^6/kg        | 0.5             | Aciclovir   |
| 3       | CLL                  | CR        | 48/f    | MUD                   | OFatumumab                     | CR    | MUD 09/10    | 3.4 × 10^6/kg        | 0.5             | Aciclovir   |
| 4       | AML                  | CR        | 76/f    | MUD                   | CSA + MTX                      | CR    | MUD 10/10    | 5.5 × 10^6/kg        | 0.5             | Aciclovir   |

All patients received peripheral blood stem cells. No T cell depletion or further graft manipulation was performed. AML = acute myeloid leukaemia; CLL = chronic lymphocytic leukaemia; CMV = cytomegalovirus; CSA = cyclosporine; IF = induction failure with 8% residual bone marrow blasts prior to conditioning; MUD = matched unrelated donor; RIC = reduced intensity conditioning; MAC = myeloablative conditioning. Tx = transplant.
CD3⁺CD8⁺ CMV-specific cells were single-cell sorted directly into 96-well PCR plates containing 5 µl phosphate-buffered saline (PBS).

**Chromium-51 release cytotoxicity assay**

See methods in the Supporting information.

**RNA isolation and cDNA synthesis by template switch**

For RNA isolation, cells were thawed and processed using the RNAeasy Mini and Micro Kit (Qiagen, Valencia, CA, USA). Quantification and quality control was performed with the RNA 6000 Pico Kit on the Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). First-strand cDNA was synthesized by a 5’ RACE (template-switching) reverse transcriptase using 200 U SuperScript® II (Invitrogen, Life Technologies, Carlsbad, CA, USA), as described previously [13,14]. For samples with <250 000 cells for RNA isolation, 50 000 Jurkat cells (human T cell leukaemia, DSMZ no: ACC 282) with a monoclonal T cell receptor (amino acid sequence CAVSDELPSNBIKKF) were added prior to cDNA amplification. Further steps were performed as described previously [14]. Purification of cDNA was performed using the MinElute PCR Purification Kit (Qiagen).

**Library preparation**

The total purified cDNA was PCR amplified to add Illumina adaptors and barcodes using PrimeSTAR HS DNA polymerase (Takara Bio Inc., Shigata, Japan). Whole cDNA was amplified in three rounds of PCR, as described previously [14]. The final PCR product was purified using the QIAquick PCR purification Kit (Qiagen).

**NGS**

The final TCR-α construct obtained from the library preparation contains the nucleotide sequence for the variable region of TCR-α (V and J segments) with the entire complementary determining region (CDR3); 150 base pair (bp) reads were generated using the Illumina HiSeq 2500. TCR CDR3 region sequence extraction and PCR error correction was carried out as described with MiTCR [15]. The libraries were sequenced with 20 reads/cell, resulting in a mean of 69% of usable reads (range 49–81%). Non-productive TCR sequences were removed. TCR-α chains were considered as one clonotype if they had identical CDR3 amino acid sequences (AA).

**TCR-αβ sequencing of single cells**

Paired TCR-α and TCR-β chain repertoires were analysed at the single-cell level. Reverse transcription–polymerase chain reaction (RT–PCR) amplification and sequencing of TCR-α and TCR-β chains from single T cells, as well as subsequent cloning of PCR fragments, was performed as described previously [16]. Analysis of TCR-α and TCR-β sequences and junction peptide amino acid sequence extraction was conducted with reference to the IMGT database [17]. Junction peptides were analysed using KNIME version 2.5.2 [18].

**Isolation of influenza-specific T cells**

Influenza-specific CD8⁺ T cell clones used in this study were propagated from FluMP58–66 multimer-positive single cells of a HLA-A*0201-positive donor according to the protocol described by Skowera and colleagues [19]. Specificity of the approach was verified by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) and cytotoxicity assays using propagated clones together with peptide-loaded K562/A*0201 cells for antigen presentation, as described previously [20]. For multi-parameter gene expression analysis, single cells of expanding clones cultured in X-VIVO 15 supplemented with 2.5% human AB serum, interleukin (IL)-7 (10 ng/ml), IL-15 (10 ng/ml) and 5% Cellkine (ZeptoMetrix, New York, NY, USA) were sorted into 96-well plates and processed as described above.

**Gene expression profiles**

Gene expression profiles on single cells were examined as described previously [16], with some modifications. cDNA was synthesized with Quanta qScript TM cDNA Supermix directly on cells. Total cDNA was pre-amplified for 16 cycles [1 × 95°C 8’, 16 × (95°C 45°, 49°C 1’, 72°C 1.5’)] 1 × 72°C 7’] (* with 0.3°C increment/cycle) with the TATAA GrandMaster Mix (TATAA Biocenter, Göteborg, Sweden) in a final volume of 35 µl in the presence of 31 primer pairs designed for the following genes at 25 nM final each: CD52, macrophage inflammatory protein-1B (MIP-1B), tumour necrosis factor (TNF)-SF6, CD127, nuclear factor of activated T cells (NFAT)-C2, IFN-γ, CD3ε, E2F transcription factor 1 (E2F-1), PD1, TGF-α, perforin 1 [pore-forming protein-1] (PRF-1), CCRI7, GATA binding protein 3 (GATA-3), regulated on activation, normal T cell expressed and secreted (RANTES), IL-10, IL-21, granzyme A (GZMA), V-Rel avian reticuloendotheliosis viral oncogene homologue (rel), Eomesodermin (EOMES), IL-17f, granzyme B (GZMB), regulator of G-protein signalling-16 (RGS-16), T-box gene expressed in Th3 cells (Tbet), granzyme H (GZMH), TNF-SF10 and lymphocyte-activation gene-3 (LAG-3) (Supporting information, Table S1). Pre-amplified DNA (10 µl) was treated with 1.2 U Exonuclease I and expression quantified by real-time PCR on the BioMark™ HD System (Fluidigm Corporation, San Francisco, CA, USA) using the 96-96 Dynamic Array IFC and the GE 96 × 96 fast PCR + melt protocol and SsoFast EvaGreen Supermix with low ROX (BioRad, Hercules, CA, USA) with 5 µM primers for each assay. Raw data were analysed and pre-processed using the
Fluidigm real-time PCR analysis software and KNIME version 2.5.2.

Statistical analysis

Data analysis was conducted using KNIME version 2.5.2 and R programming software (version 2.15.2 and Studio version 0.98.945; Boston, MA, USA). In order to identify biologically meaningful multivariate gene expression patterns, we performed a t-distributed stochastic neighbour embedding (t-SNE) [21] on the Ct values that were corrected for confounding effects via a linear model, as described previously [22]. Comparison of expression profiles on single cells was performed using Wilcoxon’s rank sum test. Two-tailed P-values of <0.05 were considered statistically significant.

Results

Course of CMV-specific T cells during CMV infection

Patient 1 was transplanted for AML from a matched unrelated donor; cyclosporin A (CSA) and methotrexate (MTX) were used for immunosuppression (Table 1). Within the first month following transplantation, patient 1 developed CMV-specific immunity with high frequencies of CMVpp65-specific CD8+ T cells between days 27 and 86 post-transplantation. Up to 43% of all CD3+CD8+ T cells were measured as CMVpp65-specific and appeared sufficient to control the CMV infection (Fig. 1). Consistent with this, CMVpp65-specific CD8+ T cells isolated at day 389 were able to specifically kill CMV pp65-pulsed cells (Supporting information, Fig. S1). On day 74 after transplantation, the patient was diagnosed with acute GVHD requiring prednisolone (2 mg/kg). Following steroid exposure whole lymphocytes (not shown) and CMVpp65-specific CD8+ T cells decreased and the patient developed a CMV reactivation with CMV enteritis requiring anti-viral treatment. Samples for next-generation sequencing (NGS) of CMVpp65-specific CD8+ T cells were taken at days 74, 173, 404, 525 and 547 following transplantation. The patients showed a donor chimerism >98% from day 27 and >99% from day 62 following transplantation throughout the observed period. Total CMVpp65-specific CD8+ T cells (solid line) were calculated based on total lymphocyte count in peripheral blood (10⁹/l).

Next-generation sequencing of TCR-α in CMV-specific T cells

To characterize the TCR composition of the CMVpp65-specific CD8+ T cells, NGS of the TCR-α was performed at
different time-points (days 74, 173, 404 and 525) following transplantation in patient 1. At all time-points donor chimerism was >99%. The first sample was taken at day 74 following allogeneic transplantation before the CMV infection became clinically apparent and when 26/C11% of CD3\(^+\)CD8\(^+\) T cells were positive for the CMVpp65 streptamer. NGS of the CMVpp65-specific CD8\(^+\) T cells revealed an oligoclonal TCR-\(\alpha\) composition (Fig. 2a). Eight clonotypes had read frequencies >1%, with one clonotype [TCR-\(\alpha\)-pp65-1; CAVNYGMLTF; T cell receptor alpha variable gene (TRAV)-3, T cell receptor alpha joining gene (TRAJ)-39] having a read frequency of 75/C10–90% of reads. Following CMV-enteritis, the TCR-\(\alpha\)-pp65-1 clonotype remained dominant within the CMVpp65-specific CD8\(^+\) T cells at each time-point up to day 525. The read frequencies of the other seven clonotypes varied during follow-up, but all remained detectable at day 525. One additional clonotype was found with a read frequency >1% only in the sample from day 525 (Supporting information, Table S2). Previously described common motifs within the CDR3 amino acid sequence of CMV-specific T cells [23–25] were identified in four of the nine clonotypes with >1% read frequencies (NNNDM, GNQF and YGQNF, Fig. 2a).

NGS of the TCR-\(\alpha\) was also performed on CMVIE-specific CD8\(^+\) T cells at day 547 following allogeneic transplantation. At this point donor chimerism was 100%. Two clonotypes dominated the repertoire (TCR-\(\alpha\)-IE-1; CAELNAGNNRKLIW; TRAV-5, TRAJ-38 and TCR-\(\alpha\)-IE-2; CAEGGNQFYF, TRAV-12-2, TRAJ-49) with read frequencies of 55/C11 and 41/C44%, respectively (Fig. 2b). The CDR3 sequence of TCR-\(\alpha\)-IE-2 contained a known common motif for CMV-specific T cells (GNQF [23–26]).

The overall frequency of the CMV-specific CD8\(^+\) T cells was estimated by NGS of TCR-\(\alpha\) in the whole CD3\(^+\)CD8\(^+\) fraction isolated at days 525 and 547 following transplantation (Fig. 2c, Table 2). Nearly half the reads were composed of the two dominant CMVIE-reactive clonotypes (TCR-\(\alpha\)-IE-1, TCR-\(\alpha\)-IE-2) and the one dominant CMVpp65-reactive clonotype (TCR-\(\alpha\)-pp65-1). Five other clones that were not frequent in the CMVpp65-specific CD8\(^+\) T cells or CMVIE-specific CD8\(^+\) T cells had read frequencies >1% in the CD8\(^+\) repertoire.
Table 2. Frequent clonotypes in T cell receptor alpha (TCR-α) repertoire of CD8+ T cells in patient 1.

| Specificity | CDR3α          | TRAV      | TRAJ      | Read frequency (%) |
|-------------|----------------|-----------|-----------|--------------------|
|             |                |           |           | d525               | d547               |
| pp65-specific† | CAVNYGNMLTF *  | TRAV-3    | TRAJ-39   | 10.95              | 10.31              |
|             | CAGVPFTGRRALTF | TRAV-12-2 | TRAJ-5    | 0.34               | 0.33               |
|             | CILDNNDMRF [24] | TRAV-26-2 | TRAJ-43   | 0.29               | 0.31               |
|             | CAVFNGVFLHC *   | TRAV-3    | TRAJ-35   | 0.30               | 0.24               |
|             | CILREDNQGQNVF   | TRAV-26-2 | TRAJ-26   | 0.10               | 0.10               |
|             | CILRDGGGGSEKLVF | TRAV-26-2 | TRAJ-57   | 0.05               | 0.08               |
|             | CAMRFYNNAGNMLTF | TRAV-14/DV4 | TRAJ-39  | 0.06               | 0.05               |
|             | CAVYGNNQYFF *   | TRAV-3    | TRAJ-49   | 0.07               | 0.05               |
|             | CIRDNNDMRF      | TRAV-26-2 | TRAJ-43   | 0.04               | 0.06               |
| IE-specific‡ | CAILNAGNNRKLIW  | TRAV-5    | TRAJ-38   | 20.29              | 23.92              |
|             | CAEGQNNQYF      | TRAV-12-2 | TRAJ-49   | 14.09              | 15.53              |
| Unknown     | CAVNGGTIGRLYF   | TRAV-12-2 | TRAJ-18   | 8.30               | 9.06               |
|             | CGADPSGTYKYYF   | TRAV-34   | TRAJ-40   | 7.64               | 8.01               |
|             | CGADQGTKYFYIF   | TRAV-34   | TRAJ-40   | 2.89               | 2.70               |
|             | CAARKGTGGFKTFIF | TRAV-13-1 | TRAJ-9    | 0.98               | 1.19               |
|             | CVVATDNLIF      | TRAV-8-2  | TRAJ-34   | 1.12               | 0.91               |

Frequent clonotypes (>1% of reads) identified within cytomegalovirus (CMV)pp65-specific and CMVIE-specific T cells and their read frequency (% reads) within the CD8+ repertoire are listed. Five more clonotypes of unknown specificity found in >1% of reads were identified within the CD8+ repertoire. Previously published public CMV motifs are underlined. Sequences that were also identified by single-cell sequencing are marked by *. Numbers in parentheses refer to reference number in which the sequence was also found. †Representing 11-4% of CD3+CD8+ T cells as measured by flow cytometry on day 525 following transplantation (see Fig. 1). ‡Representing 31-88% of CD3+CD8+ T cells as measured by flow cytometry on day 547 following transplantation. TRAV = T cell receptor alpha variable gene; TRAJ = T cell receptor alpha joining gene.

Single-cell sequencing and gene expression profiling of CMVpp65-specific cells

Analysis of the CMVpp65-specific CD8+ T cells at day 525 identified a discrete population of high streptamer binding [population (pop) I; 10-3% of CD8+ T cells] and a second population with lower fluorescence intensity (pop II; 0.7% of CD8+ T cells, Fig. 3a). Pop I cells were predominantly CCR7-CD45RA+ effector memory cells (84-3%) and fewer CCR7-CD45RA+ terminal differentiated effector memory cells (15-3%). In contrast, pop II cells were predominantly CCR7-CD45RA+ terminally differentiated effector memory cells (77.2%, Fig. 3b).

Paired TCR-α and TCR-β sequence analysis was performed on single sorted cells from pop I and pop II of the CMVpp65-specific CD8+ T cells. All 70 pop I cells represented the dominant clonotype identified by NGS (TCR-α-pp65-1) paired with TCR-β CASSKTSGSPYNEQFF [T cell receptor beta variable gene (TRBV)-27, T cell receptor beta joining gene (TRBJ)-2-1, T cell receptor beta diversity gene (TRBD)-2, Fig. 3a]. Pop II cells (n = 35) contained five different clonotypes, including the TCR-α-pp65-1. Within the amino acid sequence of the identified clonotypes, public CMV motifs were identified in two of these clonotypes (GNQF; NFGNEK [23–25]).

To assess these two populations further we analysed single-cell gene expression profiles for 26 T cell-relevant genes in pop I cells, pop II cells, in expanded influenza-specific T cells (FluMP58-66 CD8+) as an example of activated and expanded memory CD8+ T cells, and CD8+ T cells from a healthy control (Fig. 4). Compared to CD8+ T cells from the healthy control, the influenza-specific CD8+ T cells had increased expression of granzyme A, granzyme B, Tbet, PRF-1, TNF-α and IFN-γ (all P < 0.001) and decreased expression of CD127 (P = 0.01), CCR7 (P = 0.003) and RGS-16 (P = 0.01), consistent with the expected profile of activated memory CD8+ T cells. The pop I and pop II CMVpp65-specific CD8+ T cells were similar in their gene expression and resembled the influenza-specific CD8+ T cells. Notable differences to the influenza-specific CD8+ T cells were a marked expression of granzyme H (P < 0.001) and decreased cytokine expression (IFN-γ, TNF-α, TNSF-10, all P < 0.001) and TNSF-6 (P = 0.01) in the CMVpp65-specific CD8+ T cells.

Validation of results in additional patients

The TCR repertoire of two additional patients with a high frequency of CMVpp65-specific T cells was analysed. One of these patients was transplanted for CLL from a matched donor. ATG was additionally used for GVHD prophylaxis (patient 2, Fig. 5, Table 1). At day 71 following transplantation, samples were taken for NGS of TCR-α of CMVpp65-specific T cells and CD8+ T cells. Additionally, paired TCR-αβ sequencing of single CMVpp65-specific T cells was
Fig. 3. Sorting, phenotype and T cell receptor alpha (TCR-α) sequences of cytomegalovirus (CMV) pp65-specific CD8$^+$ T cells (day 547 following transplantation). (a) CD3$^+$CD8$^+$ T cells (52.0% of lymphocytes) and CMV$_{pp65}$-specific cells (11.4% of CD3$^+$CD8$^+$ T cells) were sorted for next-generation sequencing (NGS) analyses of the TCR-α. Additionally, single-cell sequencing with the amplification of TCR-α and TCR-β was performed on sorted single cells of the two CMV$_{pp65}$-specific populations (pop I: 10.3% of CD3$^+$CD8$^+$ T cells and pop II: 0.7% of CD3$^+$CD8$^+$ T cells). One single clonotype was identified in pop I cells, whereas clonotypes of pop II cells were heterogeneous. (b) Phenotyping of pop I and pop II cells of CD3$^+$CD8$^+$ CMV$_{pp65}$-specific T cells. Pop I cells were mainly CCR7–CD45RA– effector memory (EM) cells (84.3% of CD3$^+$CD8$^+$ CMV$_{pp65}$-specific T cells) and for pop II, 77.2% were CCR7$^+$CD45RA$^+$ terminally differentiated effector memory (TEMRA) cells.

Fig. 4. Gene expression analyses of single cytomegalovirus (CMV)$_{pp65}$-specific CD8$^+$ T cells (day 547 following transplantation). Heatmap for selected genes. Pop I of CMV$_{pp65}$-specific cells (blue, $n = 84$) and pop II of CMV$_{pp65}$-specific cells (green, $n = 84$) showed comparable expression patterns. Gene expression profiles of influenza-specific CD8$^+$ T cell clones (FluMP, red, $n = 36$) and CD8$^+$ T cells from a healthy donor (purple, $n = 80$) are shown for comparison. Whole gene names are provided in Supporting information, Table S3.
performed on the same day. The patient had a CMV reactivation requiring valganciclovir 1 month prior to sample acquisition, donor chimerism was 99-7%. Patient 2 did not develop a GVHD and did not receive steroids during the time of observation, unlike patient 1. At the time of sampling no CMV virus load was detectable and the patient had a high frequency of CMVpp65-specific CD8+ T cells (23-3% of CD3+CD8+ cells), the majority of which had an effector memory phenotype (57-9%). The TCR-α repertoire of the CMVpp65-specific CD8+ T cells demonstrated an oligoclonal composition. Six clonotypes with read frequencies >1% were identified. Of these, three dominant clonotypes constituted 87-9% of all CMVpp65-specific CD8+ T cell clonotypes (Fig. 6, Table 3). One of the most frequent clonotypes contained a public CMV motif (NNNDM [23–25]). The clonotypes identified in the CMVpp65-specific CD8+ T cells represented 22-5% of all detectable reads in the total CD8+ T cells. Assessing the rest of the CD8+ compartment for public CMV motifs [23–26] revealed three additional highly represented clonotypes that contained the CMV motifs GNQF and NTGNQF within their CDR3 sequence. Paired TCR-αβ sequencing of single CMVpp65-specific T cells identified four different clonotypes in 33 analysed cells. All four clonotypes were also found within the CD8+ sample. These four clonotypes accounted for 16-5% of all detectable TCR-α NGS reads. Public motifs for TCR-α and TCR-β [23–25] were found in this patient (Table 4). A third patient was transplanted for AML (patient 3, Fig. 5, Table 1). This patient was transplanted from a donor with a HLA-B mismatch and received ATG. On the day of sampling, donor chimerism was 99-3%. Although the patient did not develop CMV reactivation, a small peak of CMV virus load below the cut-off level was observed prior to sampling. CMVpp65-specific CD8+ T cells were frequent in the peripheral blood (10-9% of CD3+CD8+ T cells) on day 48 following transplantation and were predominantly (95-5%) of the effector memory phenotype. Three clonotypes (in 13 cells) were identified by single-cell paired TCR-αβ sequencing in the CMVpp65-specific CD8+ T cells. Two of these clones were detected by NGS of the whole CD8+ repertoire and represented 8-1% of the TCR-α reads and one of these clonotypes contained a TCR-αβ public motif [23–26] (Table 4).

Finally, we analysed the TCR-α repertoire of a patient with low frequency of CMVpp65-specific CD8+ T cells (1-3% of CD3+CD8+ T cells). This patient (patient 4, Fig. 5, Table 1) was transplanted for AML from a CMV seronegative donor and developed CMV reactivation requiring a 3-week therapy with oral valganciclovir 10 months before sampling for TCR sequencing. On the day of sampling (day 369 following transplantation), this patient was diagnosed with a slight GVHD of the skin not requiring steroids. No virus load was detectable at the day of sampling and overall chimerism was 99-7%. Immunophenotyping of CMVpp65-specific cells from patient 4 revealed them as terminally differentiated effector memory (TEMRA) cells expressing
CD45RA (60-3%). By single-cell sequencing, seven TCR-α clonotypes were identified in CMVpp65-specific CD8\(^+\) T cells. One of the TCR-α clonotypes (CAFPYNNNDMRF) was also found in patient 2. The paired TCR-β was different to patient 2, but also contained a public CMV motif [23,25] (Table 4). All clonotypes were also identified within the total CD8\(^+\) repertoire. However, the frequency of these clonotypes was very low, accounting for only 0.9% of all reads in the CD8\(^+\) cells.

**CMV-specific TCR sharing**

Comparing all TCR-α read frequencies of the CMVpp65-specific CD8\(^+\) T cells for patients 1 and 2, we identified 34 clonotypes (>1% in CMVpp65\(^+\)) with CMV motif

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CAASATGNQFYYF
CAMREGSNTNQFYYF
CALSEANTGNQFYYF
other clonotypes
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**Further clonotypes (>1% in CD8\(^+\)) with CMV motif**

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CAVFKGGRAGNNRKLIW*
CAFPYNNDMRF*
CAVTGNNDKLIF
CALGWANNLFF*
CARNTGNQFYYF*
CAGPKKTSYDKVIF
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**Table 3. T cell receptor alpha (TCR-α) clonotypes in patient 2.**

| Specificity | CDR3α | TRAV | TRAJ | Read frequency (%) in CD8\(^+\) T cells |
|-------------|-------|------|------|---------------------------------------|
| pp65-specific\(^†\) | CAVFKGGRAGNNRKLIW | TRAV-21 | TRAJ-38 | 7.49 |
| | CAFPYNNNDMRF * [24] | TRAV-24 | TRAJ-43 | 7.10 |
| | CAVTGNNDKLIF | TRAV-12-2 | TRAJ-37 | 5.76 |
| | CALGWANNLFF * | TRAV-16 | TRAJ-36 | 1.15 |
| | CARNTGNQFYYF * [24] | TRAV-24 | TRAJ-49 | 0.72 |
| | CAGPKKTSYDKVIF | TRAV-35 | TRAJ-50 | 0.31 |
| Unknown but with CMV motif | CAASATGNQFYYF | TRAV-29/D5 | TRAJ-49 | 17.60 |
| | CAMREGSNTNQFYYF | TRAV-14/DV4 | TRAJ-49 | 8.49 |
| | CALSEANTGNQFYYF | TRAV-19 | TRAJ-49 | 3.34 |
| Unknown | CAVKGNNGGFTIF | TRAV-12-2 | TRAJ-9 | 1.68 |
| | CAVEAPDDKIF | TRAV-36/DV7 | TRAJ-30 | 1.10 |
| | CVLTSGGYNKLIF | TRAV-12-1 | TRAJ-4 | 1.07 |

**Table 3. T cell receptor alpha (TCR-α) clonotypes in patient 2.**

**Fig. 6.** Distribution of T cell receptor alpha (TCR-α) clonotypes in patient 2. Cytomegalovirus (CMV)pp65-specific CD8\(^+\) T cells and CD8\(^+\) T cells from day 71 following transplantation were sequenced by next-generation sequencing (NGS) for their TCR-α. Clonotypes with a read frequency >1% in the CMVpp65-specific cells are shown in colour and mapped in the CD8\(^+\) repertoire. Additionally, frequent clonotypes containing a public CMV motif are shown in yellow. Previously published public CMV motifs are underlined. Sequences that were also identified by single-cell sequencing are marked by *.

Frequent clonotypes (>1% of reads) identified within cytomegalovirus (CMV)pp65-specific T cells or clonotypes seen in >1% of reads within the CD8\(^+\) T cell repertoire that contained a public CMV motif are listed. Three more frequent (>1% of reads) clonotypes of unknown specificity were identified within the CD8\(^+\) repertoire. Previously published public CMV motifs are underlined. Sequences that were also identified by single cell sequencing are indexed*. Numbers in parentheses refer to reference number in which the sequence was also found. *Representing 23-3% of CD3\(^+\)CD8\(^+\) T cells as measured by flow cytometry on day 71 following transplantation (see Fig. 5). TRAV = T cell receptor alpha variable gene; TRAJ = T cell receptor alpha joining gene.
Table 4. Single-cell T cell receptor alpha (TCR-αβ) sequencing of cytomegalovirus (CMV) pp65-specific CD8\(^+\) T cells.

| Patient | TRBV | TRBJ | TRBD | CDR3\(β\) | TRAV | TRAJ | CDR3\(ζ\) | Counts | % of counts | Frequency of total CD8 reads (%) |
|---------|------|------|------|-----------|------|------|-----------|--------|-------------|----------------------------------|
| 1-Pop I | 27*01 | 2-1*01 | 2*02 | CASSKTSGSYPXEQFF | 3*01 | 39*01 | CAVNYGNMLTF | 70 | 100 | 10.95 |
| 1-Pop II | 27*01 | 1-4*01 | 1*01 | CASSRTGGAPEKLF | 3*01 | 35*01 | CAVFFGNVLHC | 17 | 48-57 | 0.30 |
|         | 27*01 | 2-5*01 | 1*01 | CASKTTGAPETQYF | 3*01 | 49*01 | CAVGYGNQYF | 13 | 37-14 | 0.07 |
|         | 27*01 | 2-1*01 | 2*02 | CASKRTGSPXEQNF | 3*01 | 39*01 | CAVNYGNMLTF | 3 | 8-57 | 10.95 |
|         | 12-3*01 | 2-2*01 | 2*01 | CATAGTGGGLFF | 3*01 | 31*01 | CAVRDISARLMF | 1 | 2.86 | 0.06 |
|         | n.a. | n.a. | n.a. | n.a. | 41*01 | 48*01 | CAVRSNEGNELTF | 1 | 2.86 | 0.02 |
| 2       | 27*01 | 1-1*01 | 1*01 | CASSLEGLYTEAFF [24] | 24*01 | 43*01 | CAFPYNNNDMRF [24] | 27 | 81-82 | 7-10 |
|         | 27*01 | 2-2*01 | 1*01 | CASSPGTGGTELFF | 16*01 | 36*01 | CALGWANNLFF | 3 | 9-09 | 1.15 |
|         | 6-5*01 | 1-2*01 | 1*01 | CASSXTGGTYGXF | 24*01 | 49*01 | CARNTGNUYF [24] | 2 | 6-06 | 0.72 |
|         | n.a. | n.a. | n.a. | n.a. | 21*01 | 38*01 | CAVFKGGRAGNNKLIW | 1 | 3-03 | 7-49 |
| 3       | 6-5*01 | 1-2*01 | 1*01 | CASSPVTGGTYGXF | 24*01 | 49*01 | CAFVTGNQYF | 9 | 69-23 | 2.78 |
|         | 24-1*01 | 2-5*01 | 2*02 | CATSPGLAGYEQYF | 26-2*01 | 32*02 | CILRGDDATNKLIF | 3 | 23-08 | 5-36 |
|         | 24-1*01 | 2-5*01 | 2*02 | CATSPGLAGYEQYF | 16*01 | 30*01 | CALRDDKIIF | 1 | 7-69 | 0.00 |
| 4       | 27*01 | 1-1*01 | 1*01 | CASQIQGYTEAFF | 24*01 | 43*01 | CAFPYNNNDMRF [24] | 3 | 21-43 | 0.05 |
|         | 25-1*01 | 2-1*01 | 2*01 | CASSLVEQQQF | 8-2*01 | 48*01 | CVPPYELTLF | 3 | 21-43 | 0.07 |
|         | 3-1*01 | 2-3*01 | 2*01 | CASSQDVSRALHLSTDQQN | 22*01 | 43*01 | CAVHVNDMRF | 2 | 14-29 | 0.07 |
|         | 25-1*01 | 2-1*01 | 1*01 | CVPPYELTLF | 8-2*01 | 48*01 | CVPPYELTLF | 1 | 7-14 | 0.07 |
|         | 20-1*01 | 2-7*01 | 2*01 | CASSPGALGGWQQYF | 17*01 | 20*01 | CATGDDDYKLSF | 1 | 7-14 | 0.48 |
|         | 3-1*01 | 1-2*01 | 2*01 | CSARDNLNANYGF | 17*01 | 20*01 | CATGDDDYKLSF | 1 | 7-14 | 0.48 |
|         | 27*01 | 2-7*01 | 2*01 | CASNPFAGLPQEQYF | 3*01 | 39*01 | CAVSYGNMFTF | 1 | 7-14 | 0.04 |
|         | n.a. | n.a. | n.a. | n.a. | 26-1*01 | 26*01 | CIASDNYGQNFVF | 1 | 7-14 | <0.01 |
|         | n.a. | n.a. | n.a. | n.a. | 8-2*01 | 28*01 | CVVSDPGQASSQYLF | 1 | 7-14 | 0.22 |

Single CMV pp65-specific CD8\(^+\) T cells were sequenced for their TCR-αβ. Previously published public CMV motifs are underlined. Numbers in parentheses refer to reference number in which the sequence was also found. TRAV = T cell receptor alpha variable gene; TRAJ = T cell receptor alpha joining gene; TRBV = TCR-β variable gene; TRBJ = TCR-β joining gene; TRBD = TCR-β diversity gene; n.a. = not available.
potentially overlapping clonotypes including two of the frequent clonotypes (read frequency >1% in CMVpp65-specific CD8⁺ T cells) of patient 2 (CARNTGNQFYF, 3.33% in patient 2 and 0.03% in patient 1; CAFFYNNDMRF, 29.76% in patient 2 and 0.0018% in patient 1). None of the overlapping clonotypes had read frequencies >1% in both patients (Fig. 7). The frequent clonotypes from patient 1 (read frequency >1% in CMVpp65-specific CD8⁺ T cells) were not found in patient 2. We screened the CD8⁺ repertoire of all four patients for the frequent CMV TCR-α clonotypes (read frequency >1% in CMV⁺CD8⁺ T cells from patients 1 and 2). Four of the frequent CMV clonotypes were also identified in CD8⁺ T cell samples from patients 3 and 4 with frequencies up to 0.05% (Supporting information, Table S3a).

Discussion

Delayed and insufficient immune reconstitution following haematopoietic SCT has been associated with poor outcome [27]. Previous studies evaluating the CMV CD8 response in both normal and patients following SCT using conventional methods have revealed oligoclonal T cell composition [28,29]. We used the CD8⁺ T cell response to CMV following SCT to assess diversity of T cell clones in patients following SCT. The findings show a limited diversity, with CMV-specific clonotypes persistently dominating the CD8⁺ T cell repertoire in some patients. While we cannot draw general conclusions from these results due to the heterogeneity and small number of patients in this study, the observed response with a limited repertoire appeared sufficient to control infection in two of our patients.

Novel in our study was the analysis of the TCR-α chain repertoire with the simultaneous typing of TCR-α and TCR-β by single-cell sequencing, and a semi-functional characterization of cells by their gene expression. Previous studies have focused on TCR-β and few studies have included whole TCR-β repertoire analysis using NGS [5–7,30]. A recent study found marked expansion of CMV-specific TCR-β clones together with a contraction of effector memory CD8⁺ T cell diversity, suggesting a compromised CD8⁺ T cell immune status [31]. Previous studies using spectratyping and conventional sequence analyses showed that the CD8⁺ TCR repertoire becomes restrictive after stimulation with CMVpp65 peptide [32]. Our findings are consistent with this, as we find that the whole repertoire is skewed toward a few clonotypes, thereby suggesting that the diversity of the remaining repertoire might be restricted. Such expansion of a few clones at the expense of clones directed against other antigens may limit the ability to survey virus and tumour antigens and therefore contribute to the increased risk of infection and cancer post-SCT. Thus, a measure of overall TCR diversity following SCT may provide prognostic value.

Despite advances in methodology, our study has some limitations. First, the number of included patients was small, and they were heterogeneous with respect to underlying disease and treatment, limiting the generalizability of the findings. Secondly, CMV-specific CD8⁺ T cells were defined by binding to a very restricted number of epitopes (CMVpp65 and CMVIE) and only for HLA A*0201.
Therefore, we have little knowledge of how much of the remaining \( \text{CD}^8^+ \) T cell repertoire is \( \text{CMV} \)-specific or whether it is similarly restricted in diversity. We were able to identify highly frequent clonotypes that contained motifs that have been described previously as \( \text{CMV} \)-specific [23–26], suggesting that the \( \text{CMV} \)-specific repertoire may be more extensive and more dominant in these patients.

The determination of the T cell repertoires in our described setting is affected by the low T cell counts in the patients as well as by therapeutic intervention. This has to be considered when interpreting the data, as it is likely to impact the composition of the T cell population as well as their diversity. Our and previous findings, including data from single-cell analyses on T cells specific for another \( \text{CMVpp65} \) peptide (HLA A*0201, QYDPVAALF), indicate that \( \text{CMV} \)-specific \( \text{CD}^8^+ \) T cells are composed of dominant clonotypes post-allogeneic SCT [9,10]. Estimation of the TCR-\( \beta \) repertoire reconstitution by NGS following SCT in patients with a CMV or Epstein–Barr virus (EBV) infection also shows a restricted TCR-\( \beta \) diversity [6]. Similar results are found in patients after solid organ transplantation showing that CMV infection leads to a narrowing of the TCR repertoire assessed by NGS of the TCR-\( \beta \) and TCR-\( \alpha \beta \) single-cell sequencing [8,23]. In this context, whether more diverse or rather the presence of specific clonotypes is required to control CMV infection is controversially discussed. While there are data suggesting that TCR diversity has no functional advantage to control virus infections [9,33], it has been shown that the CMV-specific \( \text{CD}^8^+ \) diversity was more important than the magnitude of CD8\(^+\) T cell response for the control of persistent CMV infection in immunocompetent adults [25]. In two of our patients, it appeared that a large response with relatively restricted clonotype diversity was sufficient to control CMV infection, and it was noted that the CMV-specific \( \text{CD}^8^+ \) T cells from patient 1 were able to kill peptide-loaded target cells in vitro. Additionally in our study, we were able to examine the phenotype of the CMV-specific \( \text{CD}^8^+ \) T cells. CMV-specific T cells of patients transplanted from a seropositive donor were mainly effector memory phenotype. In contrast, the cells from patient 4 with a seronegative donor were characterized as terminally differentiated. This may be linked to the clearance of CMV in this patient. It is reported that re-expression of CD45RA on memory cells is associated with a controlled CMV infection and absence of virus antigen [34,35]. This remains to be elucidated in further studies. Single-cell surface marker and gene expression in cells from patient 1 provided evidence for chronically stimulated, granzyme-replete, \( \text{CCR7} \) CD45RA\(^-\) effector memory \( \text{CD}^8^+ \) T cells, suggesting that there was low-level persistence of virus and that, despite activation, a large proportion of cells did not assume a terminally differentiated effector memory phenotype. From a detection and characterization viewpoint, it was impressive to see how different clonotypes of \( \text{CD}^8^+ \) T cells specific to one peptide could be distinguished by their binding characteristics, as shown by the pop I and pop II \( \text{CMVpp65} \)-specific \( \text{CD}^8^+ \) T cells. However, although the high binding pop I \( \text{CMVpp65} \)-specific cells were dominated by a single TCR, the same TCR was also found in the lower binding pop II cells, consistent with variation in the ability of \( \text{CD}^8^+ \) T cells to interact with target, presumably because of TCR expression differences between cells.

Comparing NGS data between patients remains problematic with respect to the high sensitivity of this method. This is especially the case for studies with few patients. Moreover, it is difficult to rule out false positive results. Overlapping TCRs should be considered with caution. When comparing NGS data from our patients, we focused on comparing clonotypes with ample frequencies. The assessment of larger cohorts is required to identify virus-specific T cell receptors of frequent or dominant clonotypes. We were unable to find fully paired TCR-\( \alpha \) and -\( \beta \) chain CMV-specific clonotypes that were shared between patients. Prior to NGS analyses, it was already assumed that virus-specific T cells show similar amino acid motifs in their TCR CDR3 regions [32]. Nevertheless, there was evidence for overlap in the use of single chains between patients and with reports in the literature, and between CMV epitopes. One clonotype (CDR3\( \alpha \): CAF-PYNNNDMRF) was detected in patients 2 and 4 in single-cell sequencing analyses. This amino acid sequence and the corresponding CDR3\( \beta \) sequence from patient 2 (CDR3\( \beta \): CASSLEGYTEAFF) were also identified by another group that examined \( \text{CMVpp65} \)-reactive T cell clonotypes [24]. Another single-cell \( \text{CMVpp65} \) clonotype of patient 2 (CDR3\( \alpha \): CARNTGQNYYF) was also described previously in \( \text{CMVpp65} \)-specific cells [24]. Of interest is the finding that the reported CMV motif that was present in this clonotype (NTGQF) was present in clonotypes observed in \( \text{CD}^8^+ \) T cells binding the \( \text{CMVpp65} \) peptide and in a substantial proportion of other \( \text{CD}^8^+ \) T cells from this patient. Potentially more intriguing is the observation that the reported CMV motif GQNF was identified in patient 1 \( \text{CD}^8^+ \) T cells that bound the \( \text{CMVpp65} \) peptide and cells that bound the \( \text{CMVIE} \) peptide. If confirmed, these observations could suggest that there may be general motifs for multiple peptides of a single virus.

Cross-reactive \( \text{CMVpp65} \)-specific T cells have been described previously. Following solid organ transplantation, cross-reactivity was demonstrated between HLA A\(^*\)02\(^+\) and HLA B\(^*\)27\(^+\) \( \text{CMVpp65} \)-specific T cells [23]. HLA A\(^*\)02\(^+\) and HLA B\(^*\)27\(^+\) T cells contained the same amino acid residues within the CDR3 sequences (CDR3\( \alpha \): RXNNARL, TRAV-3, TRAJ-31; CDR3\( \beta \): SXVNEA, TRBV-12-4, TRBJ-1-1). While this specific signature was not observed in our patients, we found a similar CDR3 sequence containing the amino acid combination ARL (TRAV-3, TRAJ-31) in our single-cell analyses from
patient 1. Further studies will be required to identify virus- or peptide-specific T cells.

We identified a further nine CDR3 sequences in our CMV NGS analyses that were described previously as CMV-associated [24,26]. These included three of our dominant clonotypes (CAFPYNNNDMRF and CARNTGNNQYFF from patient 2 and CILDNNNDMRF from patient 1), all of which had CMV motifs (Supporting information, Table S3b).

There is evidence to suggest that CMV reactivations after allogeneic SCT may protect against relapse in patients with AML [36,37]. Also, the onset of acute GVHD has been associated with CMV reactivations [38]. NGS data on the TCR repertoire may provide new insights if applied as longitudinal measurements in larger cohorts of patients.

In conclusion, our findings show that the TCR repertoire narrows and becomes skewed following CMV infections in transplanted patients, and that these changes persist for long periods of time. These results warrant further larger studies to define the ability of oligoclonal expansion of TCR-α clonotypes to achieve an effective anti-viral T cell response in this setting. Furthermore, it remains unclear if a reduced overall diversity may compromise the immunocompetence of patients with regard to other infections.

To our knowledge, this is the first time that the TCR-α chain repertoire was assessed by NGS in this context. With an increasing accessibility to NGS, TCR repertoire analyses may turn out to be a promising tool for a patient-specific monitoring of allogeneic transplanted patients.

Acknowledgements

This work was supported by the DFG Research Center and Cluster of Excellence – Center for Regenerative Therapies Dresden (FZ 111) and the BMBF (STRATOS consortium, FKZ 01GIN1108A). The authors acknowledge Christiane Gläser and Ines Partzsch for their technical assistance.

Disclosure

The authors declare no disclosures.

Author contributions

C. S. L. designed and performed the research, analysed data and wrote the manuscript. A. E. analysed data and contributed to the writing of the manuscript. F. H. performed research and contributed to the writing of the manuscript, E. R. B., M. Schmie, U. O, D.K., S. D. and Y. F. performed research. C. K. analysed data. A. D. and A. M. J. D. performed research and analysed data. M. Schmitz, G. E. and M. B. contributed to the writing of the manuscript. J. S. and E. B. analysed data and contributed to the writing of the manuscript. All authors critically reviewed and edited the manuscript.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher’s web-site:

Table S1. Gene expression primers.
Table S2. Frequent clonotypes of cytomegalovirus (CMV)pp65-specific T cells, patient 1.
Table S3. Genes used in Fig. 5.
Table S4. Read frequencies (read frequency %) of (a) T cell receptor alpha (TCR-a) clonotypes from CMV-specific CD8(+) T cells in CD8 next-generation sequencing (NGS) reads from patients 1–4 and (b) of previously reported CMV-reactive TCR-a clonotypes identified in NGS reads from patients 1–4.
Fig. S1. Chromium-51 release assay.