Automated production of specific T cells for treatment of refractory viral infections after allogeneic stem cell transplantation

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Abstract

Therapy-resistant viral reactivations contribute significantly to mortality after hematopoietic stem cell transplantation. Adoptive cellular therapy with virus-specific T cells (VST) has shown efficacy in various single-center trials. However, the scalability of this therapy is hampered by laborious production methods. In this study we describe the in-house production of VST in a closed system (CliniMACS Prodigy® system, Miltenyi Biotec). In addition, we report the efficacy in 26 patients with viral disease following hematopoietic stem cell transplantation in a retrospective analysis (adenovirus, n=7; cytomegalovirus, n=8; Epstein-Barr virus, n=4; multi-viral, n=7). The production of VST was successful in 100% of cases. The safety profile of VST therapy was favorable (n=2 grade 3 and n=1 grade 4 adverse events; all three were reversible). A response was seen in 20 of 26 patients (77%). Responding patients had a significantly better overall survival than patients who did not respond ($P<0.001$). Virus-specific symptoms were reduced or resolved in 47% of patients. The overall survival of the whole cohort was 28% after 6 months. This study shows the feasibility of automated VST production and safety of application. The scalability of the CliniMACS Prodigy® device increases the accessibility of VST treatment.

Introduction

Hematopoietic stem cell transplantation has shown great efficacy in various malignant and non-malignant diseases.[1] A significant drawback of this therapy is opportunistic infections due to immunosuppression and prolonged T-cell aplasia, which impose relevant transplant-related morbidity and mortality.[2-4] Delayed immune reconstitution can occur when using matched unrelated donors and especially in haplo-identical stem cell transplants because of extensive *in vivo* or *ex vivo* T-cell depletion, performed to prevent graft-*versus*-host disease (GvHD).[5]
Patients should therefore be routinely monitored after hematopoietic stem cell transplantation for reactivation of a latent infection with Herpesviridae (human cytomegalovirus [CMV] and Epstein-Barr virus [EBV]) or infection with human adenovirus (ADV), because of the high prevalence and morbidity of these viral infections.[4,6-10] Antiviral therapy, either therapeutic or preemptive, against ADV and CMV is mainly based on antiviral agents such as cidofovir, foscarnet, ganciclovir[11,12] and letermovir,[13] but therapy failure due to viral resistance or toxic side effects is repeatedly observed.[14-17] Recovery of cellular immunity is essential for eradication of the viral infections.
A current extension to virostatic agents is to transplant antiviral immunity via adoptive transfer of virus-specific T cells (VST) against ADV, CMV or EBV.[18-21] Even multi-specific T cells have been evaluated.[22] VST have shown promising antiviral efficacy as well as establishment of long-term immunity. Unfortunately, the extensive clean-room procedures for *ex vivo* culturing of VST are restricting the use of cellular therapies to specialized centers and it takes several weeks until the cells are ready to be used.[3]
A possible approach to these limitation is an automated closed system of VST production using the interferon (IFN) cytokine capture system (CCS), as described by Kim *et al.* and Kállay *et al.*[3,23] This method of production is based on the presentation of viral antigens to donor lymphocytes and subsequent magnetic separation of VST reacting to antigen stimulation with IFN-γ expression in a fully automated way, using the CliniMACS Prodigy® system from Miltenyi Biotec (Bergisch-Gladbach, Germany).
Following a retrospective analysis of real-world data, we report the results of 31 VST preparations and the experience of 12 treating centers after application of our VST with regard to safety and response to treatment in 26 patients.

Methods

Virus-specific T cells

Unstimulated apheresis products containing at least 1×10^9 T cells from the stem cell donor or, in the case of ineligibility, a third-party donor (n=5), were collected. Third-party donors were haploidentical family donors except one unrelated third-party donor who was tested on two HLA alleles with 50% matching. VST were isolated using the IFN-γ CCS (Miltenyi Biotec) on a CliniMACS Prodigy® (Figure 1). Within this device, cells were stimulated with viral peptides (ADV: MACS GMP PepTivator AdV5 Hexon; CMV: MACS GMP PepTivator HCMV pp65 or EBV: MACS GMP PepTivator Select, all from Miltenyi Biotec) for 4 h. For multi-specific VST, the appropriate antigens were combined. Apheresis products were labeled with the CliniMACS CCS Catchmatrix Reagent, capturing the secreted IFN-γ on the surface of activated T cells. Labeled cells were separated using CliniMACS IFN-γ Enrichment Reagent, consisting of IFN-γ-specific antibody-conjugated superparamagnetic particles. The final product

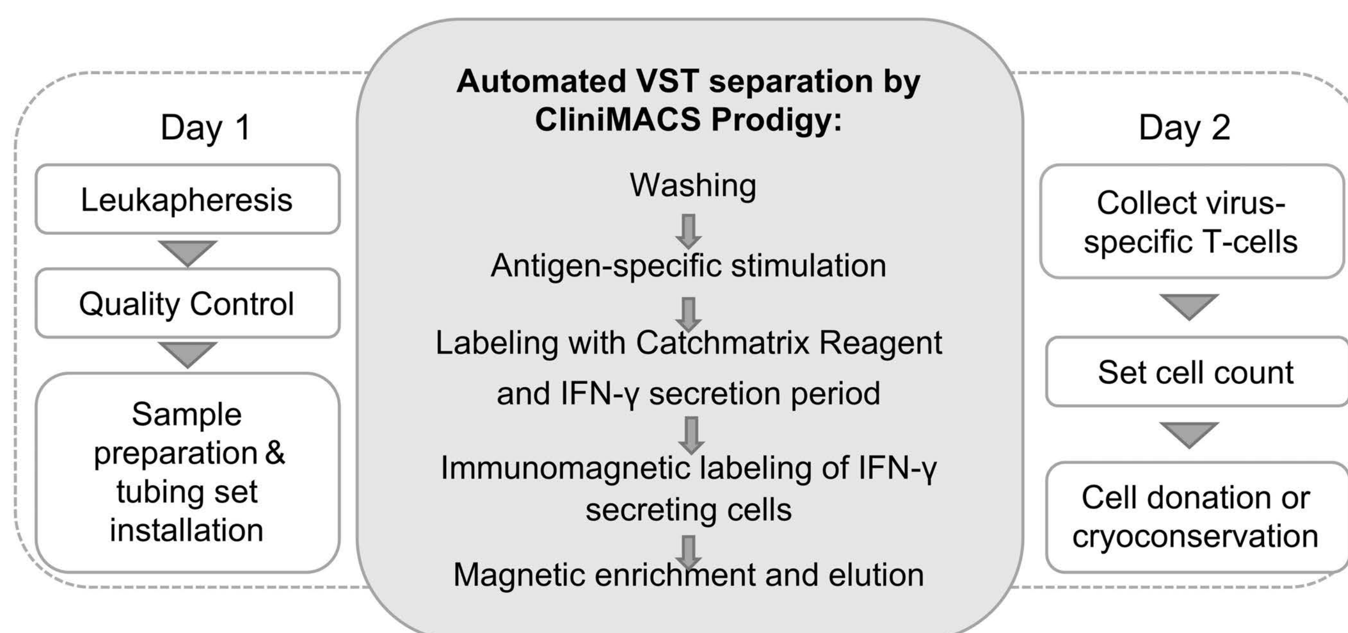


Figure 1. Schematic of the production process for virus-specific T cells using the CliniMACS Prodigy® system. VST: virus-specific T cells; IFN: interferon.

was ready for infusion within 2 days. Release criteria for infusion were: (i) sterility; (ii) absence of endotoxins; (iii) purity (>20% $CD3^+$ T cells); and (iv) cell viability (>60% of $CD3^+$ cells). A maximum of $25x10^3$ and $50x10^3$ T cells/kg were infused per donation from haploidentical donors and matched donors, respectively.

Patients

Between January 2015 and December 2017, VST preparations for 42 patients were produced at the University Children's Hospital in Tübingen. Patients were referred from Tübingen and the University Clinics in Berlin, Regensburg, Giessen, Marburg, München, Düsseldorf, Aachen, Ulm, Jena, Freiburg and Erlangen.

There were no fixed inclusion criteria, as this publication reports real-world data outside clinical trials. Usually, VST were given at increasing viral load despite anti-viral pharmacotherapy after allogeneic stem cell transplantation. Patients were excluded if they had active acute GvHD grade ≥2 or were receiving strong immunosuppressive drugs (prednisolone ≥1.0 mg/kg/day or equivalent).

Data collection and evaluation

Blood samples for viral polymerase chain reaction analysis were collected according to standard-of-care protocols in the different centers. Side effects, response and survival data as well as concomitant, prior or subsequent antiviral treatment were reported by the participating centers using standardized case report forms. Safety was assessed using Common Terminology Criteria for Adverse Events, version 3.

The data analysis was conducted with the formal approval of the institutional review board (University Tübingen, approval number 044/2017BO2) and in accordance with the Declaration of Helsinki, as revised in 2008. The requirement for informed consent was waived by the ethics committee due to the retrospective character of this analysis.

Definition of terms and statistics

Response was defined as reduction in viral load by ≥1 log. In the case of multi-viral infection, reduction of the viral load by ≥1 log in at least one infection was defined as response. Response kinetics were differentiated into straight response (reduction of viral load by ≥1 log within 8 weeks with no subsequent increase of viral load), transient response (reduction of viral load by ≥1 log within 8 weeks with subsequent re-increase of viral load by ≥1 log), and no response, as previously described.[24] Viral control was defined as a viral load below 1,000 copies per microliter, while final clearance was defined as no evidence of viral load. Relapse was defined as an increase of viral load ≥1 log after former viral control.

Statistics were calculated using IBM SPSS 26 (Armonk, NY, USA) and GraphPad 8 (San Diego, CA, USA). For overall survival, time from infusion of VST to death due to any cause or last follow-up was calculated. For comparison of overall survival levels, the log-rank test was used. Results were considered statistically significant at P values <0.05.

Results

Patients

Out of 42 patients for whom VST were produced, 32 received the T cells (7 products were conserved for later use in patients with a stable viral load and/or absence of urgent clinical symptoms at the time the VST product had been manufactured, 2 patients succumbed to disease prior to administration of the product, and in 1 patient the disease was cured prior to administration of the product). Six patients had to be excluded from the retrospective analysis of this cohort because of missing data (n=5) or death due to the patient's underlying disease 1 day after administration of the product (n=1). In total, 26 patients were eligible for analysis; 31 preparations were manufactured, as VST were produced repetitively for two patients. The detailed characteristics of the eligible patients are presented in Table 1 and *Online Supplementary Figure S1*. Their median age was 13 years, with six adults also analyzed in this study. Leukemia was the most common indication for transplantation (n=11), followed by hematologic diseases plus immunodeficiency (n=10). Haploidentical donors were used more frequently than HLA-identical donors (14 *vs*. 12 patients). One patient received a reduced intensity conditioning regimen due to Fanconi anemia. All HLA-identical donors (11 matched unrelated donors, 1 matched sibling donor) were matched for 10/10 HLA alleles with the patients.

CMV infection was the most common reason for VST treatment (n=15), followed by ADV (n=11) and EBV (n=7), adding up to 33 viral reactivations in 26 patients (7 patients had multiple reactivations). Most patients received intensive antiviral treatment regimens with ≥2 antiviral drugs before therapy with VST was initiated.

Virus-specific T-cell production

In all 31 eligible procedures the product characteristics fulfilled the predefined criteria for infusion of at least $1x10^3$ VST (range, $59.4x10^3$–$2,948x10^3$). A minimum of $1x10^9$ cells were harvested during apheresis. The proportion of VST was determined by flow cytometry analysis as exemplified in Figure 2. The $CD3^+$ cells in the apheresis product contained 0.02-0.94% IFN-γ^+ cells after stimulation. After isolation of the IFN-γ^+ fraction, the mean number of VST was $773x10^3$ with a mean recovery rate over 81%, defined as the proportion of IFN-γ^+ cells in the

final cell product related to the count in the apheresis product. These products contained 76% VST on average. CMV- and EBV-specific T cells were more abundant in the apheresis product than were ADV-specific T cells (mean: 0.25% CMV; 0.33% EBV; 0.13% ADV), resulting in a higher VST yield after separation (mean: 560x10^3 CMV; 420x10^3 EBV; 150x10^3 ADV). The purity was also higher for those products (75% CMV; 84% EBV; 68% ADV) but with a recovery of 93%, cell loss was lowest for ADV VST products (85% CMV; 52% EBV). Enriched T cells were more likely to be CD4$^+$ than CD8$^+$ for ADV (78% CD4$^+$; 18% CD8$^+$), whereas EBV-VST were mainly CD8$^+$ T cells (12% CD4$^+$; 80% CD8$^+$). For CMV the two subgroups were equally represented (41% CD4$^+$; 52% CD8$^+$). The highest yield of VST was observed when generating multi-specific T cells using a combination of peptides (880x10^3 ADV/CMV; 2,611x10^3 CMV/EBV). Per donation, 1x10^3-50x10^3 T cells/kg were used. The summarized production data are shown in Table 2.

Safety

With regard to the safety assessment of VST infusion, infusion-related symptoms were reported for 23 patients (no data for 3 patients). In total, nine adverse events attributed to VST infusion were reported in a total of three patients (1 with a HLA-identical donor, 2 with haploidentical donors, none in patients receiving third party-derived VST). Six of nine of the adverse events were grade 1 or 2, as presented in *Online Supplementary Table S1*. All infusion-related severe adverse events abated.

Grade 4 acute GvHD occurred in two out of 23 evaluable patients after transplantation, with complete control of GvHD symptoms prior to the administration of VST. These patients did not experience a relapse of GvHD after VST infusion.

One patient developed grade 2 acute GvHD of the skin after transplantation, also with control of the symptoms prior to VST infusion. After infusion of ADV-specific T cells in this patient, grade 3 diarrhea and grade 1 nausea occurred after infusion of VST. Rectoscopic biopsy showed diffuse T-cell infiltration. This patient was considered to have grade 3 GvHD and received immunosuppression, with complete relief of gastrointestinal symptoms. The ADV infection was cured, and the patient is alive with chronic GvHD of the skin.

In one patient, the occurrence of grade 4 diarrhea and grade 3 nausea after infusion of ADV-specific T cells was reported. This patient had no history of GvHD prior to VST infusion. T-cell expansion was measured in this patient, showing simultaneous expansion of VST at the onset of symptoms. Thus, these gastrointestinal symptoms were considered as direct infiltration of ADV-specific T cells into the gastrointestinal tract, not GvHD, and the patient was not treated with immunosuppression. No rectoscopic biopsy was taken. The gastrointestinal symptoms resolved spontaneously, and the ADV infection was cleared.

Response

Response to VST treatment was achieved by 18 of the 26 patients within 4 weeks (73%). After 8 weeks, two more patients responded, making a total of 20/26 responding patients (77%) and six non-responders.

Table 1. Characteristics of the 26 patients who received virus-specific T cells.

Characteristic	
Male/female, N	15/11
Age in years, median (range)	13 (1-74)
Age ≤18 years, N	20
Age >18 years, N	6
Diagnosis, N	
Acute lymphoblastic leukemia	6
Acute myeloid leukemia	5
MDS/BMF	5
Solid tumor	3
Immune disorders	3
Sickle cell disease	2
EBV PTLD	1
Not specified	1
Donor, N	
Matched sibling	1
Matched unrelated	11
Haploidentical	14
Conditioning regimen, N	
Busulfan-based	3
Melphalan-based	8
TBI-based	6
Treosulfan-based	5
Reduced intensity conditioning	1
Unknown	3
T-cell depletion, N	
Antithymocyte globulin	19
Campath	2
None	1
Unknown	4
Ex vivo immunomagnetic T-/B-cell depletion	15
Acute GvHD prophylaxis, N of reported	
Methotrexate	12/23
Cyclosporine A	13/24
Mycophenolate mofetil	9/23
Tacrolimus	1/22
No data	2
Single viral infection, N	
Adenovirus	7
Cytomegalovirus	8
Epstein-Barr virus	4
Multiple viral infections, N	
Adenovirus, cytomegalovirus	4
Epstein-Barr virus, cytomegalovirus	3
Antiviral drug treatments before VST, N	
1	11
2	5
3	10

MDS: myelodysplastic syndrome; BMF: bone marrow failure; EBV: Epstein-Barr virus; PTLD: post-transplant lymphoproliferative syndrome; TBI: total body irradiation; GvHD: acute graft-*versus*-host disease; VST: virus-specific T cells.

The median time for a 1 log reduction in plasma viral load was 17.5 days (range, 6-41 days). Figure 3 shows the response rates within 8 weeks with regard to ADV-, CMV-, EBV- and multi-specific VST. The response rate to VST against CMV was 100%, whereas response rates were lower for VST against ADV (71%), multi-specific targets (71%) and EBV (50%).

A detailed investigation of response kinetics (Figure 4A) revealed that 13 of the 20 responders had a straight response (Figure 4B), leading directly to sustained viral control until the end of follow-up. Seven of the 13 straight responders had final clearance of the viral load after 8 weeks, whereas the viral load was cleared in 9/13 at the end of the follow-up. Four of the straight responders died

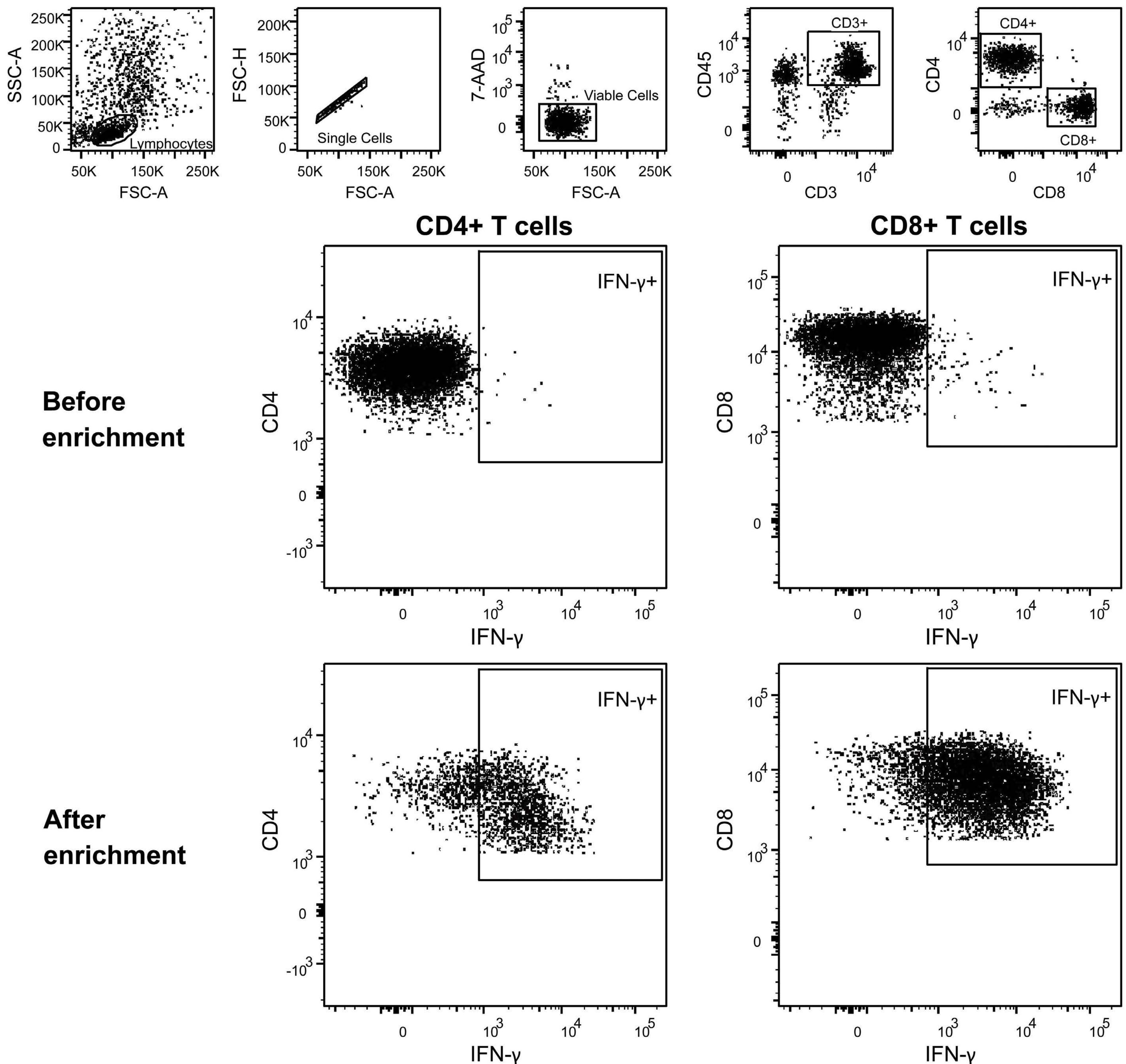


Figure 2. General gating strategy for determining the T-cell frequency and the proportion of virus-specific T cells in the cell product by flow cytometry before and after interferon-γ enrichment. Cells were gated on lymphocytes and single cells according to their morphological characteristics in forward scatter and sideward scatter. Cell viability was assessed by exclusion of cells stained by 7-aminoactinomycin D. $CD45^+CD3^+$ T-cell frequency was determined by antibody staining and further classified into $CD4^+$ or $CD8^+$ T cells. Cells that bound interferon-γ on their surface during the interferon-γ secretion phase were considered to be virus-specific T cells. SSC: side scatter; FSC: forward scatter; 7-aminoactinomycin D; IFN: interferon.

from non-virus-related causes shortly after responding to treatment, before achieving viral clearance.

Four of 19 responders showed a transient response (Figure 4C) with a secondary increase of viral load after the initial response, leading to delayed viral control. Two of these patients relapsed after achieving viral control, which diminished afterwards without requiring further antiviral treatment. Both patients were alive at the end of follow-up. Viral clearance was achieved in three of these four patients at the end of follow-up; the fourth patient did not completely clear the virus, but maintained a state of viral control (<1000 copies/μL).

The remaining three of 20 responders showed transient responses not leading to viral control (Figure 4D). One died due to the viral disease (n=1, ADV), while the deaths of the other two were potentially correlated with viral disease (n=1 ADV, n=1 EBV).

In total, final clearance of viral load was achieved after a median of 55 days (range, 13-350 days) and was observed in 12 of the 20 responding patients at the end of follow-up. Viral clearance was observed after a median of 42 days in patients with a straight response and 86 days after transient response with viral control (Figure 5).

Sixty-five percent of patients (15 out of 23 reported; 3 without data) had virus-specific symptoms at the onset of treatment, with nausea (n=7), pneumonia (n=7), diar-

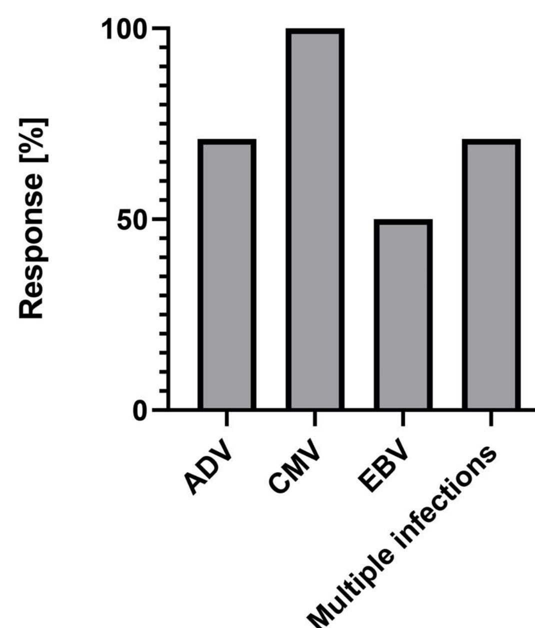


Figure 3. Proportions of patients responding to treatment with virus-specific T cells within 8 weeks, distinguishing between T cells against adenovirus, cytomegalovirus, Epstein-Barr virus or multi-specific targets. ADV: adenovirus; CMV: cytomegalovirus; EBV: Epstein-Barr virus.

Table 2. Product characteristics of 23 preparations of virus-specific T cells (data missing for 8 cases).

Characteristic, mean (range)	ADV (N=3)	CMV (N=12)	EBV (N=2)	ADV+CMV (N=3)	CMV+EBV (N=3)	Total (N=23)
$CD3^+$ IFN-γ^+ cells before enrichment, %	0.13 (0.02-0.3)	0.25 (0.07-0.9)	0.33 (0.3-0.35)	0.37 (0.07-0.94)	0.66 (0.4-0.84)	0.3 (0.02-0.94)
$CD3^+$ IFN-γ^+ cell yield, $x10^3$	149.80 (80.7-208)	560.42 (59.4-1,955)	419.90 (214-625)	879.50 (66.8-1,686)	2,611.13 (2,227-2,948)	772.95 (59.4-2,948)
Purity[a], %	68.4 (60.0-75.5)	74.6 (56.1-94)	84.4 (79-89.7)	85.0 (74-90.7)	74.6 (49.8-89.2)	76.0 (49.8-94)
$CD4^+CD3^+$ IFN-γ^+, %	77.7 (62.4-95.8)	41.1 (6.2-72.5)	11.6 (7.1-16.1)	46.3 (19.2-86.8)	23.7 (10-48.6)	40.6 (6.2-95.8)
$CD8^+CD3^+$ IFN-γ^+,%	17.7 (3.2-35.3)	52.3 (24.8-86.7)	80.0 (74.9-85.1)	49.4 (11.3-70.3)	65.4 (34.1-89.9)	52.6 (3.2-89.9)
Recovery[b], %	92.6 (78-99.9)	84.9 (22-99.9)	52.0 (4-99.9)	68.6 (24-99.9)	87.3 (69-99.9)	82.0 (4-99.9)
T cells given, $x10^3$/kg	7.82 (1.02-13)	14.9 (3.96-38.76)	4.5 (2.27-6.73)	11.6 (1.85-24.71)	35.65 (22.6-50)	14.74 (1.02-50)
IFN-γ^+ cells given, $x10^3$/kg	5.05 (0.77-656)	11.33 (4.50-29.88)	3.91 (1.79-6.04)	10.41 (1.37-22.41)	24.74 (2,016-2,917)	11.50 (0.77-29.88)
IFN-γ^- cells given, $x10^3$/kg	2.77 (0.25-5.19)	3.57 (0.60-9.64)	0.58 (0.48-0.69)	1.19 (0.48-2.30)	10.91 (2.43-25.1)	3.85 (0.25-25.1)

[a]Purity: proportion of $CD3^+$ IFN-γ^+ T cells to total cell count in the final cell product. [b]Recovery: proportion of IFN-γ^+ cells in the final cell product related to the count in the apheresis product. ADV: adenovirus; CMV: cytomegalovirus; EBV: Epstein-Barr virus; IFN-γ: interferon gamma.

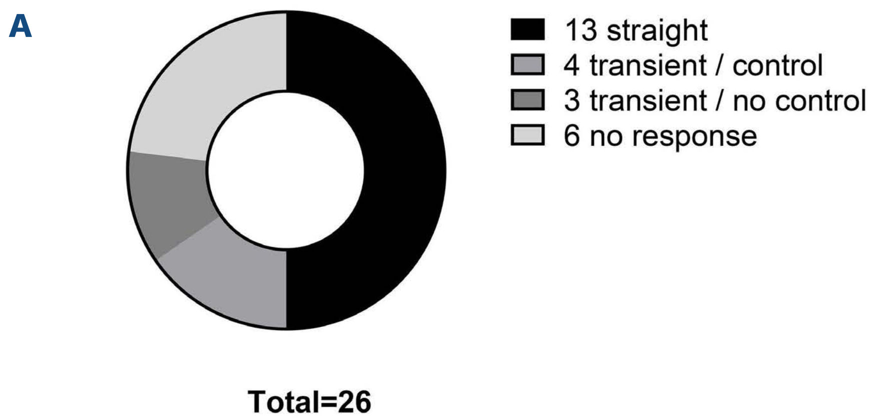
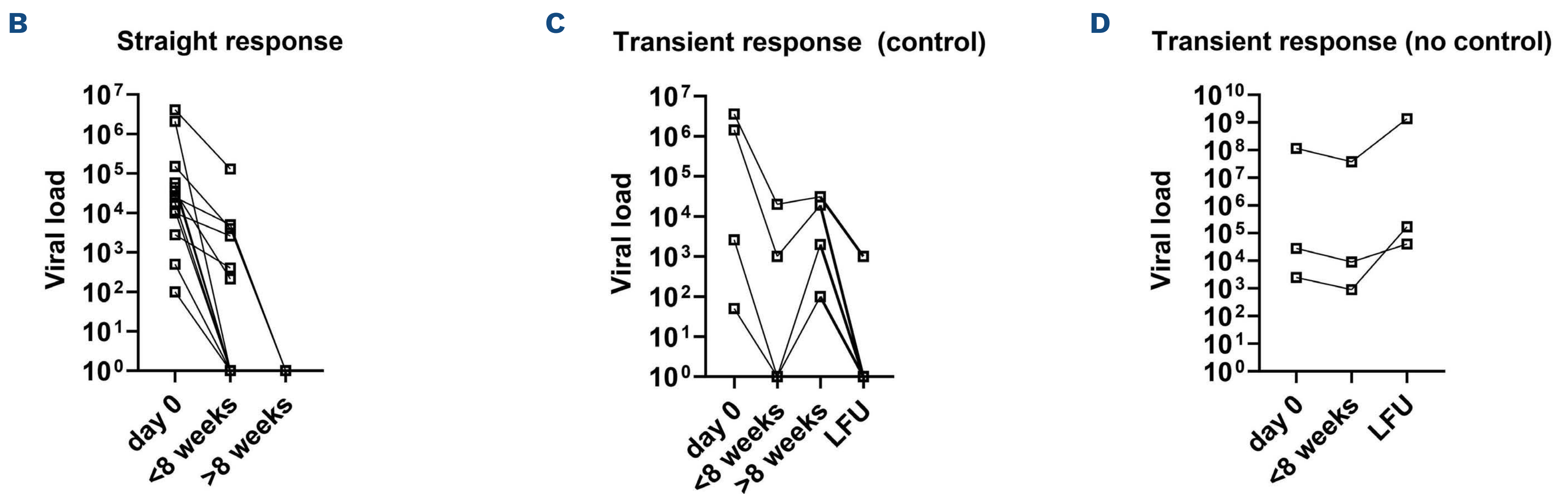


Figure 4. Response kinetics, as previously defined, after infusion of virus-specific T cells. (A) Proportion of patients showing straight and transient responses with or without viral control or no response. (B-D) Changes in viral load in the 20 responding patients at different time points, with viral load measured by copies per μL at the time of infusion (day 0), lowest value within the first 8 weeks, highest value after 8 weeks, and value at the last follow-up in patients who had a straight response (B), a transient response with viral control (C) and a transient response without viral control. The definitions of the different responses are those described by Feucht *et al.*[24] LFU: last follow-up.



rhea (n=6) and hepatitis (n=3) being the most prevalent, as listed in *Online Supplementary Table S2*. Resolution of all symptoms occurred in 47% of patients (7 out of 15 patients with reported symptoms) within 13 weeks. For the remaining patients symptoms persisted (n=3) or worsened (n=5) until the end of follow-up.

A correlation analysis between VST dose and response revealed a correlation coefficient of r=0.16 and Pearson *P*=0.4, so no dose-dependent effect of VST on response was observed.

The response rate to third-party VST (80%, 4/5 patients) was effectively the same as that to VST acquired from the stem cell donor (76%, 16/21 patients) with no statistically significant difference in survival (log-rank test: *P*=0.63). Patients transplanted from an HLA-identical donor had a higher response rate (91%, 11/12 patients) than patients with a haploidentical stem cell donor (64%, 9/14 patients), but this difference was not statistically significant (χ^2-test, *P*=0.1). There was no difference in survival (log-rank, *P*=0.4).

Concomitant antiviral treatment and immunosuppression

At the time point of VST infusion, six of the seven patients suffering from ADV infection were being treated with cidofovir (n=4), brincidofovir (n=1) or an unknown drug (n=1); the seventh patient started treatment with brincidofovir 4 weeks after VST infusion despite a prior response to the VST. All eight patients with CMV infection received concomitant treatment with valaciclovir/ganciclovir (n=5) or foscavir (n=3). All patients with EBV infection received rituximab during VST treatment, and one patient was also given foscavir. Patients suffering from multi-virus infection (n=7) were treated against one (n=3) or both (n=4) infections with concomitant antiviral medications.

Immunosuppression at the time of VST infusion was applied in 2/13 evaluable patients after haploidentical transplantation because of GvHD: in one case the immunosuppression was achieved with a calcineurin inhibitor (CNI) plus imatinib, in

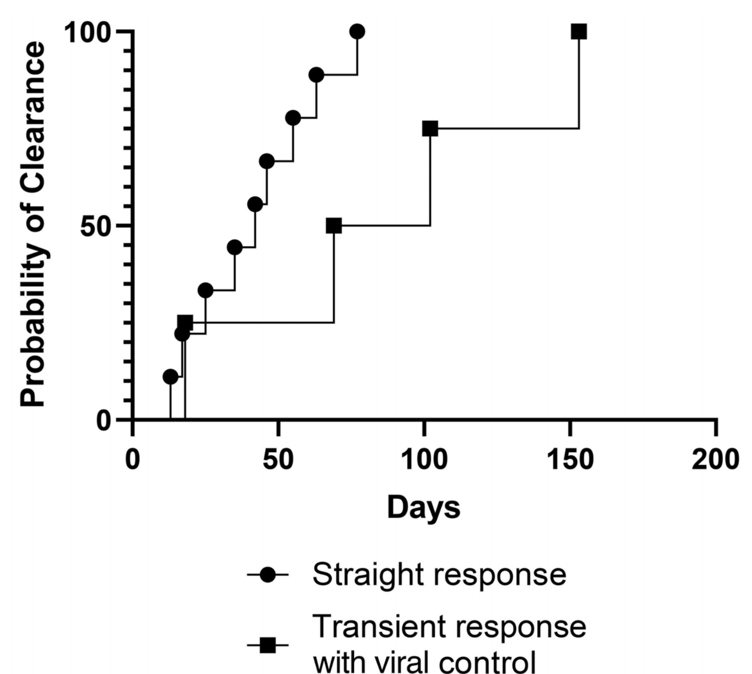


Figure 5. Days until achievement of viral clearance depending on response kinetics (straight response *vs.* transient response with viral control).

the other case a CNI + prednisolone <1 mg/kg/day was used. After HLA-identical transplantation, 6/11 evaluable patients had ongoing immunosuppression with a CNI (n=4) or a CNI plus mycophenolate mofetil (n=1) due to early VST treatment within the first 100 days after transplantation or with CNI plus prednisolone <1 mg/kg/day (n=1) due to GvHD. There was no statistically significant difference in terms of response to VST treatment between patients receiving concomitant immunosuppression or no immunosuppression (88% *vs.* 69% response rate; χ^2, *P*=0.3); likewise there was no statistically significant difference in survival (log-rank, *P*=0.2).

Outcome

The median follow-up time was 70 days (range, 4-440 days). Eight patients died within the first 30 days, with the deaths being potentially related to the viral infection in half of these patients. The 6-month overall survival (Figure 6A) of the entire cohort was 28% (95% confidence interval [95% CI]: ±19%). Patients showing a response (Figure 6B) had a 6-month overall survival of 37% (95% CI: ±12%) with 3/12 patients dying due to or with their viral infection, as shown in *Online Supplementary Table S3*, whereas all six non-responders died within 100 days (*P*<0.001). Patients with a straight or transient response with viral control showed a 6-month overall survival of 45% (95% CI: ±27%), whereas all patients with a transient response without viral control died after a median of 75 days and non-responders after a median of 26 days (Figure 6C). There was no difference in outcome between patients with ADV, CMV, EBV or multi-viral infection (*P*=0.63) (Figure 6D). Of the seven patients in the multi-viral group, four had short follow-up because of early deaths (2 virus-related, 2 not virus-related) within <30 days after VST administration.

Discussion

The aim of this retrospective analysis was to evaluate the feasibility of VST production with the CliniMACS Prodigy® system as well as the safety and efficacy of VST against different viral targets, based on real-world data.

Concerning feasibility, VST production using the automated IFN-γ CCS CliniMACS Prodigy® was reliable. VST against CMV, ADV, and EBV, as well as multi-specific VST, could be produced with sufficient cell numbers for 100% of the patients. The final cell product was ready for infusion within 2 days,

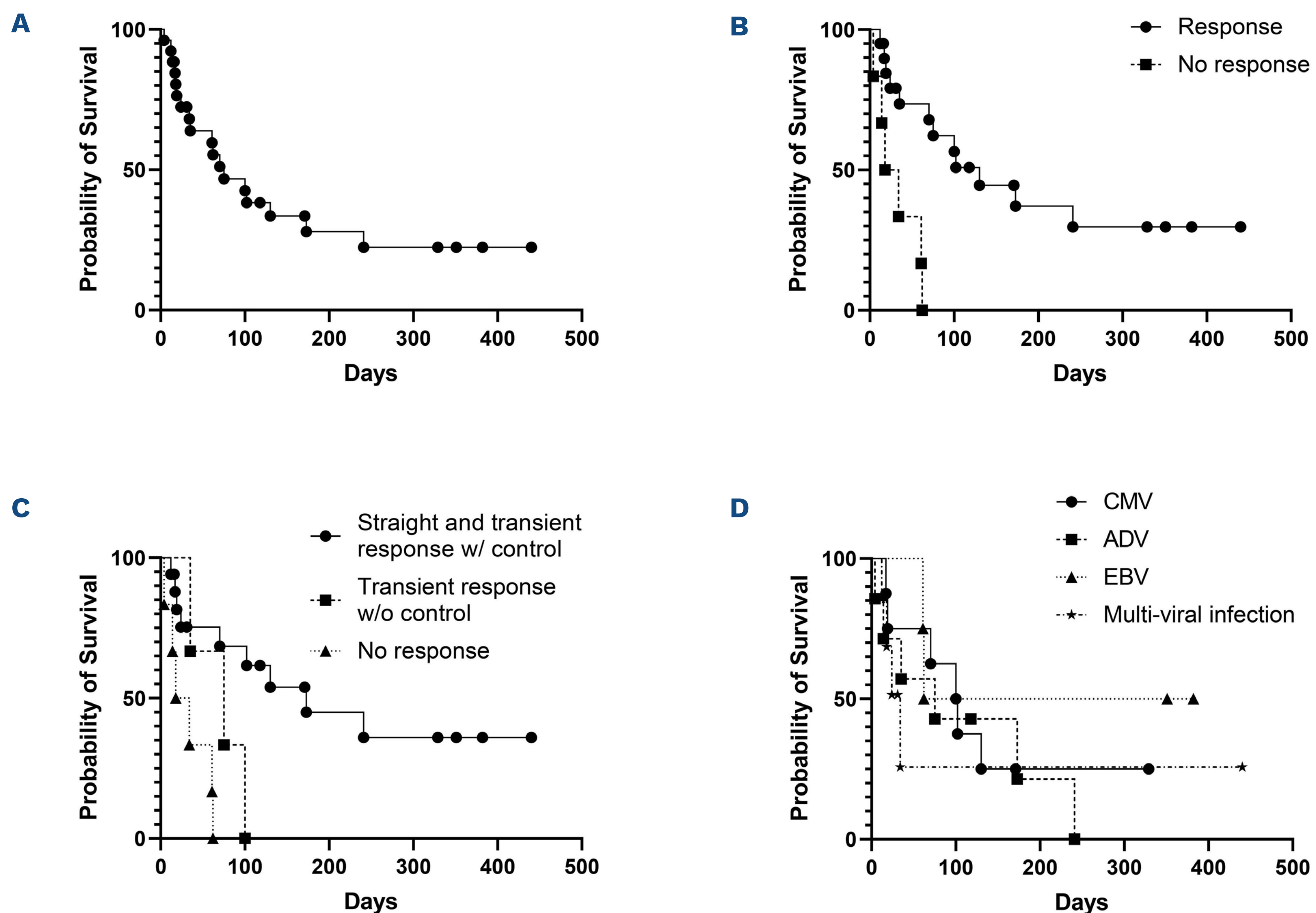


Figure 6. Overall survival of the investigated cohort. (A) Overall survival (OS) of the entire cohort. (B) OS depending on response to treatment with virus-specific T cells. (C) OS depending on response kinetics. (D) OS depending on type of infection. CMV: cytomegalovirus; ADV: adenovirus; EBV: Epstein-Barr virus; w/: with; w/o: without.

which is a significant reduction of production time compared with former *ex vivo* culture methods taking 2-12 weeks to be completed.[25,26] In our experience, the fully automated CliniMACS Prodigy® system shortened hands-on time from 14 hours to 2-4 hours during regular working time, which reduces infrastructure requirements and the burden on the Good Manufacturing Practice team. These manufacturing times are in line with those reported by Priesner *et al*.,[27] who compared CliniMACS Prodigy®-based production with manufacturing with the CliniMACS Plus®. In that study three healthy donors were used to produce CMV-specific T cells by both methods. The recovery rate was comparable, but the purity was higher using the CliniMACS Prodigy® (purity range on Prodigy®: 79.2-96.4% *vs*. 19.2-81.1% on the Plus®). The comparable pre-clinical study by Kim *et al*.[3] extensively investigated the product characteristics of five production runs of CMV-specific T cells from healthy donor leukapheresis products. The final VST yield was lower than in our cohort, ranging from 2.7-470x10^3 IFN-γ^+ T cells, despite a comparable percentage of CMV-specific T cells in the leukapheresis products. Kallay *et al*.[23] reported on the clinical use of CMV-specific T cells produced with the CliniMACS Prodigy® in a pediatric cohort and described a purity range of 26.5-94.4% for CD4$^+$IFN-γ^+ and 29.9-98.7% CD8$^+$IFN-γ^+ cells or 39.0-94.4% and 53.8-98.7%, respectively, when two outliers were excluded. These results are in line with our data for CMV-specific T cells as well as with those reported by Priesner *et al*.[27]

Regarding the safety profile of VST treatment, no major safety concerns arise from our data, in accordance with earlier studies evaluating the use of VST.[20,23,26,28,29] One patient with GvHD grade 2 of the skin before VST treatment had a worsening to GvHD grade 3 with nausea and diarrhea after treatment with ADV-specific T cells, with histological proof of lymphocytic invasion into the gastrointestinal tract. Another patient treated in a different center, received ADV-specific T cells and developed the same symptoms. However, this patient was not considered to have developed GvHD, and showed spontaneous abatement of symptoms without the use of immunosuppression. So, it remains ambiguous whether these symptoms were a sign of GvHD, an invasion of ADV-specific T cells clearing the infection, or a combination of both. A comparable case of cystitis with lymphocytic invasion of BKV-reactive T cells after clearance of BKV using VST has been described.[30]

The very low risk of inducing *de novo* GvHD after T-cell infusion in our cohort might be correlated with the high purity of VST administered. In our opinion, it is of utmost importance to allow only minimal numbers of contaminating, unspecific T cells. Such cells can be potentially alloreactive and therefore cause GvHD, so we respected the limits of 25x10^3 IFN-γ-negative T cells for haploidentical transplants and 100x10^3/kg for HLA-identical transplants in the manufactured products, which were defined as risk thresholds for GvHD according to our in-house experience.

Looking at the efficacy of VST treatment, we can report responses in 77% (50-100%) of all patients, which is consistent with the reported response rate of 74% (62-85% depending on the virus targeted) in the pooled meta-analysis by Käuferle *et al*.[31] Furthermore, we can confirm the highly predictive value of response to VST treatment for survival and the observation of different response kinetics, as already shown:[24] a large group of straight responders with fast, direct achievement of viral control had a relatively favorable outcome compared to transient responders. Transient response was correlated with delayed viral control in some and uncontrolled viral replication and death in other cases. With transient response not achieving viral control, at least median survival could be prolonged when compared with that of patients with no response. It does, however, remain an interesting question whether the response in these patients was achieved due to initial clonal expansion of VST, followed by secondary T-cell exhaustion, or whether these patients never had sufficient VST expansion, and the response was only caused by concomitant virostatic treatment. Unfortunately, we cannot answer this question through this retrospective dataset.

Two patients showed a relapse of their viral disease, but both achieved slow viral clearance without further antiviral treatment. This suggests a long-term efficacy of VST treatment.

Of course, the validity of our data is impaired by the co-administration of antiviral drugs. This prevented the effects of VST and virostatic treatment from being separated clearly, especially as no parallel measurement of clonal expansion of VST was performed during the study.

Unfortunately, overall survival was still poor with a 28% survival rate after 6 months in the whole cohort and 9/18 deaths potentially correlated with the viral infection, including the deaths of patients with unspecific multi-organ failure. Mortality rates of 82% for disseminated ADV viremia[32] and almost 100% for CMV pneumonia[33] were reported for historical cohorts. Pre-emptive virostatic treatment greatly improved survival rates for both post-transplant ADV and CMV reactivation,[10,34] but a direct comparison of our results to these datasets is not feasible, as our cohort mainly suffered from refractory viral infections.

A main issue of VST treatment are early deaths <30 days which contributed to four of the virus-related fatalities. This underlies the importance of beginning treatment early, as the prognosis deteriorates quickly through uncontrolled viral replication.[24] An automated IFN-γ CCS might be an important step in the direction of speeding up treatment, but regulatory barriers still limit this production method to specialized centers. Therefore, additional measures such as the implementation of donor databases[35,36] may be needed to further broaden availability of cellular-based antiviral therapy. Another promising approach might be the pre-emptive use

of third-party VST at the first signs of CMV/EBV reactivation, as performed by Wei Jiang *et al*.,[37] which led to an impressive response rate of 94% and in comparison with historical controls, a lower percentage of patients receiving third-line antiviral therapy. Although these results cannot be directly compared to our data, as refractory patients were included in our cohort, we can confirm that third-party VST seem to produce equal response rates compared to those of donor-derived VST. Xu-Ying Pei *et al*. made the same observation.[38] CMV-directed VST as prophylactic therapy has also been reported to be safe in matched unrelated donor recipients.[39] The efficacy of this approach will be examined in a subsequent study; however, this method has previously been reported to be effective in preventing EBV-driven lymphoproliferative disease.[40]
Lastly, experimental approaches such as vaccination of the donor with viral peptides to boost the cell count of VST before apheresis, or PD-L1 inhibition in the patient to inhibit T-cell exhaustion[41] may be attractive steps for further investigation, and controlled clinical trials, such as the TRACE study (NCT04832607), which is currently evaluating the feasibility and efficacy of decentralized VST production in different European countries, will be of great importance to prove the effectiveness of VST treatment in a randomized setting.

Disclosures
No conflicts of interest to disclose.

Contributions
AD, FGJC and LM acquired the data, which ATH, FGJC, AD and LM analyzed. CB, DA, MS, FH and AMA produced the virus-specific T cells. CS, MD, AS, FRS, RM, BS, JFi, BH, SS, GS, DS, BG, KMD, JFo, JHS, WW, CMK, JT, and TF reported data on the treatment and clinical outcome of treated patients. ATH and FGJC wrote the manuscript. RH and PL supervised the study. All authors contributed substantially to the manuscript.

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Data-sharing statement
The data that support the findings of this study are available from the corresponding author, ATH, upon reasonable request.

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