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Activity of broad-spectrum T-cells as treatment for AdV, EBV, CMV, BKV and HHV6 infections after HSCT

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Abstract

It remains difficult to treat the multiplicity of distinct viral infections that afflict immunocompromised patients. Adoptive transfer of virus-specific T-cells (VSTs) can be safe and effective, but such cells have been complex to prepare and limited in anti-viral range. We now demonstrate the feasibility and clinical utility of rapidly-generated single-culture VSTs that recognize 12 immunogenic antigens from 5 viruses (Epstein-Barr virus, adenovirus, cytomegalovirus, BK virus, and Human Herpesvirus 6) that frequently cause disease in immunocompromised patients. When administered to 11 recipients of allogeneic transplants, 8 of whom had up to 4 active infections with the targeted viruses, these VSTs proved safe in all subjects and produced an overall 94% virological and clinical response rate that was sustained long-term.

Introduction

Severe or fatal infections with a broad array of viruses remain a common problem for immunocompromised humans(1;2). Although treatment with small molecule anti-viral drugs may benefit some individuals, for many viruses they are of limited efficacy and have substantial toxicities. An alternative strategy for treatment of immunocompromised patients is to adoptively transfer T lymphocytes that are specific to virus-associated antigens.

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Competing interests: The authors declare that they have no competing interests relevant to the subject matter of this article. JFV is a consultant for Wilson Wolf Manufacturing. CMR, MKB and HEH have a licensing agreement with Cell Medica for EBV-specific T cells to treat EBV-associated lymphoma or nasopharyngeal carcinoma. MKB is on the scientific advisory board of CanVAC and has share options in both CanVAc and bluebird bio. The Center for Cell and Gene Therapy has a research collaboration with Celgene. None of these relationships is relevant to the subject matter presented in this manuscript. The trial is registered at www.clinicaltrials.gov as NCT01570283.

Author contributions: This study was developed and designed by UG, AML, J.F.V., HEH and CMR and the principal investigators on the clinical trial were HEH and AML. UG developed the method for generating multivirus-specific T cells. AP, UK, and AML grew the T cell lines in the GMP facility and all clinical grade products were reviewed and released by A.P.G. GK, RAK, CM, AK-N and KL enrolled patients on the study and HL provided statistical support. AP, UK, and IT performed follow-up studies. The data was analyzed by AP and AML. All co-authors critically reviewed the manuscript. AP, AML, HEH and MKB contributed to writing the manuscript.

Following stem cell transplantation, for example, administration of donor-derived T cells with specificity for cytomegalovirus (CMV), adenovirus (AdV) or Epstein Barr virus (EBV) have all produced frequent and sustained anti-viral and clinical benefits, even for patients suffering from advanced and drug resistant infections(3–6;6). More recently, “off the shelf”, or banked, partially HLA-matched virus-specific T lymphocytes (VSTs) have shown promise in treating intractable virus infections in solid organ and stem cell transplant recipients(7–11). This promise notwithstanding, broader application of VSTs is limited by the restricted number of viruses that have been targeted and the lengthy, complex and costly methodology required for production.

Optimally, an immunocompromised patient with viral disease should be treated with a single preparation of VSTs containing a polyclonal mixture of T cells specific for a large number of antigenic epitopes in a multiplicity of pathogenic viruses, thereby broadening the antiviral coverage and reducing the risk of immune escape by viral escape mutants. This preparation should be as simple as possible to manufacture and provide prolonged protection. Unfortunately, few of these characteristics have yet been met by available products. Current approaches for making multi-specific VSTs sustain T cells specific for only a limited number of the viruses that afflict the immunocompromised host, due to antigenic competition between immunodominant components of each viral antigen(12–14). Moreover, manufacture of these VSTs frequently requires preparation of specialized antigen presenting cells (APCs), the use of viruses or viral vectors to provide target antigens, and prolonged *ex vivo* culture and antigen restimulation. These necessities both increase the cost and complexity of preparation and preclude urgent treatment of seriously ill patients, unless T cells have been prepared well in advance and with prophylactic intent.

We now report the development and clinical activity of single preparations of VSTs made by direct stimulation of peripheral blood mononuclear cells (PBMCs) with overlapping peptide libraries that incorporate EBV, CMV, AdV, BK virus (BKV), and human herpesvirus 6 (HHV6) antigens. These multivirus (m)VSTs can meet the desired specifications of multi-viral specificity, rapid production and sustained and broad anti-viral activity in immunocompromised patients.

Results

Rapid generation of polyclonal mVSTs from stem cell donors

Forty eight clinical-grade mVST lines were manufactured from allogeneic stem cell donors, as described in Materials and Methods (Supplementary Materials). From 3×10^7 PBMCs we produced a mean of $40.1 \pm 2.7 \times 10^7$ cells (median: 35.7×10^7 cells, range: $9.9\text{--}82.5 \times 10^7$; $n=48$) representing an average 13 fold total expansion within 9–11 days (Figure 1A). The lines were almost exclusively CD3⁺ T cells ($98 \pm 0.2\%$; mean $\pm$ SEM) containing both helper CD4⁺ ($57 \pm 2\%$) and cytotoxic CD8⁺ ($35 \pm 2\%$) T cell subsets that expressed central CD45RO⁺CD62L⁺ ($62 \pm 3\%$) and effector memory markers CD45RO⁺/CD62L[−] ($10 \pm 1\%$) (Figure 1B).

Anti-viral specificity of mVST lines and donor serostatus

The anti-viral specificity of the patients' mVSTs was assessed by interferon γ (IFN γ) enzyme-linked immunospot (ELISPOT) assay after we re-exposed the T cells to each of the viral antigens used for stimulation. A line was considered specific for a given virus when the sum of IFN γ -producing spot forming cells (SFC) directed against all antigens from the target virus was $30/2 \times 10^5$ input cells. Of the 48 lines generated, 14 had activity against all 5 stimulating viruses (pentavalent), 9 recognized 4 viruses (tetraivalent), 12 were trivalent, 11 were divalent, 1 was monovalent and 1 failed to recognize any of the targeted viruses (Figure 2A). Donor serostatus to CMV was determined prior to transplant, and we examined the proportion of seropositive donors from whom we could subsequently generate CMV reactive lines. Twenty six donors were CMV seropositive, and all 26 lines generated from these donors contained a CMV-reactive component. None of the CMV seronegative donors produced lines containing CMV-directed T cells. These data highlight the robustness of our manufacturing process but also emphasize the requirement for prior viral exposure for the broadest range of anti-viral reactivity in the line derived from each donor. Indeed, consistent with the lower likelihood for virus exposure in younger donors, increasing age correlated with an increased probability of recognizing multiple viruses ($p=0.003$) (Figure 2B). Overall, AdV was the most frequently recognized virus (45 reactive lines - Hexon: 470 ± 71 (mean $\pm$ SEM); Penton: 366 ± 86 SFC/ 2×10^5); 37 had EBV-directed reactivity [latent membrane protein 2 (LMP2): 137 ± 76 ; Epstein-Barr virus nuclear antigen 1 (EBNA1): 123 ± 52 ; BZLF1: 99 ± 75 SFC/ 2×10^5]; 29 lines recognized HHV6 (U90: 109 ± 78 ; U11: 37 ± 17 ; U14: 84 ± 26 SFC/ 2×10^5); 28 had BKV-directed reactivity (Large T: 123 ± 61 ; VP1: 208 ± 89 SFC/ 2×10^5); while 26 lines had CMV-directed activity (pp65: 1048 ± 446 ; IE1: 356 ± 157 SFC/ 2×10^5) (Figure 2C). Supplementary Table 1 shows the frequency of T cells directed to each of the individual antigens in each of the lines generated for clinical use, alongside the age of the donors from whom the lines were generated.

Alloreactivity of mVSTs

The VSTs described above were obtained following a single *in vitro* stimulation. To determine whether this process leaves residual alloreactive cells capable of cross-reacting with normal recipient cells, we measured cytotoxicity against recipient or haploidentical phytohaemagglutinin (PHA) blasts. There was no evidence of alloreactivity in any line generated (Supplementary Figure 1). Specific lysis of uninfected recipient/haploidentical PHA blasts of $<10\%$ at an E:T ratio of 20:1 was a clinical release criterion and was met by all lines (mean $1 \pm 0.4\%$ specific lysis, E:T 20:1, $n=43$).

Safety of mVSTs

Having demonstrated the feasibility of rapidly generating VSTs with specificity for multiple clinically relevant viruses we next tested the safety and activity of these cells by administering them to 11 recipients of matched related ($n=5$), matched unrelated ($n=3$), mismatched unrelated ($n=2$) or haploidentical ($n=1$) hematopoietic stem cell transplants (HSCTs). The mVSTs were infused between days 38 and 139 (Table 1). Each patient received 0.5 to 2×10^7 cells/ m^2 ; 8 subjects received the cells as treatment for established viral infections/disease and 3 as prophylaxis (Table 2). There were no immediate infusion-related

toxicities. One patient developed *de novo* graft versus host disease (GvHD) of the skin (Grade II) approximately 4 weeks after receiving the cells, which improved with the administration of topical steroids. Two patients who received the mVSTs as prophylaxis developed transplant-associated microangiopathy (TAM) 11 and 19 weeks, respectively, after receiving the cells. Since this complication occurs in up to 10% of HSCT recipients, and we saw neither evidence of virus-specific T cell amplification nor significant changes in plasma cytokines, we considered this delayed toxicity unrelated to mVST infusion. Of note, no patient, irrespective of viral infection, had an elevation of plasma cytokines after mVST infusion [representative examples for patients P3975 (patient who developed TAM at week 19), P3940, P4052, P3987, P3925 and P4165 are shown in Supplementary Table 2].

Anti-viral efficacy of mVSTs

Of the 3 patients who received VSTs prophylactically between days 38 and 43 after HSCT, all remained virus-infection free for >3 months following infusion. Eight patients received the cells as treatment for one (n=4), two (n=3) or three (n=1) active viral infections/reactivations between days 40 and 139 days after HSCT (Table 2) and 4 of those subjects subsequently reactivated viruses other than those for which they were initially treated. Thus, 2 patients were treated for one virus (P4052 and P3850), 3 patients were treated for 2 viruses (P3908, P3925, P4165), 2 patients were treated for 3 viruses (P3940, P3987) and 1 subject was treated for 4 viruses (P3022). We saw no correlation between the cell dose infused and either safety or anti-viral activity.

Patients with a single virus who responded to mVSTs—Two patients with a single viral infection (P4052 – AdV and P3850 – BKV) received VSTs. In the 3 weeks prior to mVST infusion the AdV load detected in the peripheral blood of patient P4052 progressively increased from 1,600 DNA copies/ml to a maximum of 434,000 copies/ml and remained elevated despite administration of cidofovir (Figure 3A). Within 2 weeks of receiving VSTs, the AdV load had halved, and by week 4 had fallen below the detection threshold, where it remained. Viral clearance corresponded with an increase in the circulating frequency of AdV-specific T cells from 0 SFC/ 5×10^5 PBMCs on the day of infusion to 165 SFC by week 4 (Figure 3A). Similarly, patient P3850 had a persistent BKV infection with 400 copies/ml detected in peripheral blood on the day of infusion of 5×10^6 mVSTs/m². This patient had pre-existing endogenous T cells directed against BKV (72 SFC/ 5×10^5 PBMCs) but there had been no control of disease. Within four weeks of VST administration, however, the viral load became undetectable and remained so until week 5, at which time the patient was taken off study to receive a second infusion of CD34+ cells due to poor graft function that had antedated VST infusion (Figure 3B).

Patients with two viruses who responded to mVSTs—Three patients had infections with two viruses, and VSTs controlled both pathogens in all of them (Figure 4). Patient P3908 initially presented with a persistent, ganciclovir-resistant, CMV reactivation, with confirmed CMV pneumonitis, colitis, and skin infiltration. On the day of VST infusion her CMV viral load was 13,200 copies/ml. Within one week of receiving 5×10^6 mVSTs/m², the viral load decreased to 3,800 copies/ml and continued to decline thereafter, coincident with an increase in the frequency of CMV-directed T cells (Figure 4A). At week 3 after VST

infusion, the same patient had a BKV reactivation (100 copies/ml), which peaked at week 8 (800 copies/ml) and resolved thereafter, coinciding with an increase in her circulating BKV-specific T cells (Figure 4B). Subject P3925 was initially treated for elevated BKV load in both his peripheral blood (1,000 copies/ml) and urine ($>10^{10}$ DNA copies/ml) accompanied by tissue disease with severe hemorrhagic cystitis. Administration of mVSTs resulted in a resolution of hematuria by week 4 and an associated rapid decrease in his viral load, which became undetectable in peripheral blood by week 6 and in urine by week 10 after infusion. This clinical response correlated with an increase in the frequency of circulating BKV T cells reactive against the antigens targeted in the T cell line (from undetectable levels on day 0 to a peak of 71 SFC/ 5×10^5 by week 3 after infusion), with no activity against the “non-targeted” but highly immunogenic antigen, small T(15) (Figure 4C, D, and Supplementary Figure 2A). Five weeks after mVSTs, the patient experienced worsening bladder pain, a symptom that coincided with a 5 log fall in BKV DNA in the urine. Cystoscopy at that time showed mucosal thickening and inflammation, and a mucosal biopsy confirmed T- and inflammatory-cell infiltrates. T cells obtained from this biopsy were expanded *ex vivo* and showed a high level of BKV-reactivity, supporting the capacity of the infused cells to home to distant sites of infection (Figure 4E). The patient was treated with one dose of methylprednisolone (1 mg/kg) with benefit. At 8 weeks after mVST infusion, this patient then developed an EBV reactivation (EBV DNA rising from 0 to 343 copies/ μ g DNA), but this reactivation was rapidly controlled, with normalization of EBV DNA levels associated with a corresponding increase in his EBV-specific T cells (Figure 4F). He remains well and free of infection at 5 months after transplant. Finally, patient P4165 eliminated both BKV (700 copies/ml) and EBV (666 copies/ μ g DNA) within 2 weeks of receiving VSTs, coincident with a corresponding increase in both T cells directed against the BKV- and EBV antigens targeted in the mVST line infused (Figure 4G and H) and not against non-targeted antigens (BKV – small T; EBV – EBNA3a, 3b, 3c, LMP1 – Supplementary Figure 2B and C).

Patients with three viruses who responded to mVSTs—Two study participants developed reactivation of 3 viruses. Subject P3987 received VSTs to treat reactivation of CMV (1,900 copies/ml) and EBV (2,161 copies/ μ g DNA): both were controlled by the infused mVSTs (Figure 5A and B). This patient also had BKV-associated hemorrhagic cystitis at the time of VST infusion, with elevated viral load detected in urine (900 copies/ml). Within 1 week of VSTs there was a marked improvement in his hematuria and BKV became undetectable (Figure 5C). Subject P3940 was our only “mixed” responder. This patient initially presented with elevated HHV6 (100 copies/ml) and BKV (500 copies/ml) in peripheral blood and at week 6 after infusion also transiently reactivated EBV (566 copies/ μ g DNA). HHV6 was successfully cleared after VST administration, coincident with a temporal increase in the frequency of T cells directed against the antigens targeted in the line infused (Figure 5D) and not against the non-targeted U54 antigen(16–18) (Supplementary Figure 2D). Similarly the EBV reactivation was rapidly controlled in association with a rise in EBV-reactive T cells (Figure 5E). The BKV load, however, continued to increase (Figure 5F). Examination of the specificity of the infused VST line, however, (#5469 – Supplementary Table 1), showed that it lacked specificity for BKV thus explaining its inability to control BKV in this patient (Figure 5F). Ultimately subject P3940

was taken off study at week 12 after infusion to receive a CD34 top-off due to continued poor graft function but remains well almost 7 months after receiving the cells without viral issues.

Patient with four viruses who responded to mVSTs—Subject P3022 presented with an exponentially increasing EBV viral load, reaching 154,089 copies/μg DNA on the day of infusion (Figure 6A) and frank EBV-associated post-transplant lymphoproliferative disease (EBV-PTLD) with extensive disease in multiple lymph nodes (Figure 6B). She also had elevated BKV levels in her blood and urine (200 and 4.1×10^7 copies/ml, respectively), with clinical symptoms of hemorrhagic cystitis. After infusion her EBV load rapidly and progressively declined corresponding with an increase in T cells directed against EBV antigens targeted in the line infused and not against “non-targeted” (EBNA3a, EBNA3b, EBNA3c, LMP1)(19) EBV antigens (Supplementary Figure 2E). Her BKV-associated hematuria also improved within 2–3 weeks of receiving the mVSTs coincident with a decrease in viral load to 0 and 8×10^5 copies/ml in blood and urine, respectively, at week 8 after infusion (Figure 6C and D). Though there was a subsequent increase in her viral load levels to 200 and 1.5×10^7 copies/ml in blood and urine, respectively, long term immune reconstitution studies could not be performed as this patient died of diabetic complications 18 weeks after receiving mVSTs (Table 2). Finally, four weeks after mVSTs she developed HHV6 and CMV reactivations (4,800 HHV6 copies/ml and 5–10 CMV antigen positive cells/50,000), both of which rapidly resolved without additional intervention (Figure 6E and F).

Discussion

We have used synthetic peptides to generate single T cell lines from stem cell donors, which consistently have specificity for up to five viruses (AdV, EBV, CMV, BKV and HHV6) representing the most frequent causes of viral morbidity and mortality after HSCT. Two of these viruses - BKV and HHV6 - have not previously been targeted by VSTs. When administered to patients, the VSTs expanded *in vivo* and produced clinical responses in all patients who had viral reactivations, without adverse events. The range of viruses that can be treated, the accelerated manufacture and the avoidance of biohazardous agents such as live viruses should facilitate broader introduction of this approach for intractable virus infection in the immunocompromised host.

Many factors contribute to the vulnerability of HSCT recipients to endogenous (latent) and exogenous (community) viruses. These include the cytotoxic drugs administered during conditioning, the delay in endogenous immune recovery after transplant, and the immunosuppressive therapies given to prevent GvHD(1). Some viral infections, including CMV, BKV, EBV, AdV and HHV6 are frequent and endemic, while others are seasonal (e.g. influenza, parainfluenza, and respiratory syncytial virus) but all can contribute to substantial transplant-associated disease or death(20–22). This broad spectrum of problematic viruses makes it impracticable to develop products for a single virus in a single patient. Here we show the feasibility of generating T cell lines with simultaneous specificity for multiple viruses and demonstrate that the infused product provides both immediate and sustained broad spectrum antiviral benefits at all dose levels tested. The safety and efficacy

of mVSTs suggest that if made widely available, they could be included as a standard part of any T-cell depleted stem cell therapy.

Although we used synthetic peptides derived from 12 immunogenic antigens within CMV, BKV, EBV, AdV and HHV6 to generate the multivirus donor-derived T cell lines, examination of VST specificities showed that only 14 of the 48 had activity against antigens from all 5 stimulating viruses. We determined whether this deficiency simply reflected lack of donor exposure to the virus (i.e. seronegativity) or whether it indicated a flaw in the approach we used that had led to antigenic competition and the consequent overgrowth of T cells targeting immunodominant antigens. We first examined the CMV serostatus of the donors, since this is the only virus for which serological screening is routinely performed. We expanded a CMV-reactive T cell component from all of the 26 CMV seropositive but none of the 22 seronegative donors. We also found a correlation between the number of viruses recognized by a given line and the donors' age, consistent with increasing likelihood of viral exposure over time. To further exclude a contribution from antigenic competition to this pattern of response, we compared the frequency of AdV-reactive T cells in mono-, bi-, tri-, tetra-, and pentavalent-specific T cell products. We saw no significant differences. For example, in a mono-specific line in which AdV alone was recognized, the frequency of reactive cells (1170 ± 54 SFC/ 2×10^5) was similar to the frequency of AdV-reactive cells in the 14 lines that recognized all 5 viruses (mean 1283 ± 201 SFC/ 2×10^5). Thus, our manufacturing protocol supports the generation of broad-spectrum VSTs, whose range is currently only limited by the prior viral exposure of the VST donor.

While even intractable CMV, EBV and/or AdV infections have proved to be responsive to adoptive T cell transfer(3–9;23–32), neither BKV nor HHV6, both of which may cause severe and intractable disease in HSCT recipients(33–39), had previously been targeted using this approach. BKV is a ubiquitous polyomavirus which establishes a latent, asymptomatic infection in >90% of the general population. In both solid organ and HSCT allograft recipients, however, viral reactivation is frequent and correlates with the absence of circulating BKV-specific T cells(40). Thus, urinary shedding of BKV occurs in 60–80% of HSCT recipients, and develops into BKV-associated hemorrhagic cystitis in 5–15%, resulting in prolonged hospital stays, severe morbidity and increased mortality(1). Although BKV was discovered more than 40 years ago, there are still no approved antiviral agents for treatment. Balduzzi and colleagues, however, reported a 19 year old HSCT patient diagnosed with progressive multifocal leukoencephalopathy (PML) (caused by the highly homologous JC polyomavirus), whose disease responded to infusions of donor-derived JC-specific T cells targeting VP1 and large T viral proteins(41). Their observation prompted us to explore a similar approach targeting the BKV homologs. In our study, of the 7 patients who reactivated BKV we achieved 5 complete and 1 partial response. The single treatment failure had received a donor line lacking activity for this virus, likely reflecting the serostatus of the donor. Three of the treated patients had tissue disease with severe hemorrhagic cystitis that had persisted despite administration of cidofovir and full supportive measures. All 3 had marked symptomatic and virological responses with disappearance of hematuria within 2–4 weeks of receiving cells. One patient subsequently had an episode of transient but severe bladder pain in association with inflammation seen on cystoscopy, which coincided with detection of BKV-specific T cells in his bladder and a 5

log fall in urine BKV viral load. These symptoms resolved after a single infusion of steroids. In contrast to the effects seen in recipients receiving tumor-directed chimeric antigen receptor-modified T cells(42;43), there was no clinical evidence of any systemic inflammatory response, and no rise in plasma cytokines.

HHV6, like CMV, is a member of the β -Herpesvirus family, and primary infection occurs in >90% of individuals before the age of two(44). The virus subsequently persists in a latent form lifelong. Although viral reactivation may occur in healthy individuals, disease is usually observed only in individuals who are immunocompromised. HHV6 reactivation and viremia occur in 40–60% of HSCT recipients, and may become associated with CNS disease (including encephalitis and meningitis), pneumonitis, transplant rejection, or delayed engraftment(35–37;45–47). Although ganciclovir, cidofovir and foscarnet have all been used as treatment, these agents are associated with substantial toxicities and limited benefit(35;36;48;49). The lack of known immunogenic and protective antigens for HHV6 had precluded the use of immune-based therapies. We recently showed, however, that in healthy seropositive donors and HSCT recipients with HHV6 reactivation, the immediate early (U90), early (U14) and late (U11) HHV6 genes are immunodominant targets for effector T cells(18). Our current results support the suitability of these antigens as therapeutic targets since HHV6 VSTs produced clinical benefit in both patients who reactivated this virus, and viral clearance coincided with an increase in the frequency of T cells targeting our chosen antigens.

Intermittent, low level viremia is frequently detected after allogeneic HSCT and may spontaneously resolve without any specific intervention. However, in the current study, of the 8 patients treated with mVSTs for 18 viral episodes, elevated viral load measurements were detected on at least 3 consecutive occasions in 13 cases (median 8, range 3–15). Furthermore, 4 patients were clinically symptomatic with confirmed tissue disease (3 with BK-hemorrhagic cystitis and 1 with EBV-PTLD). Nevertheless, absent a large scale randomized controlled study it is not possible to definitively attribute clinical responses against the targeted viruses to the infused cells. However, the current results from our phase I study support the safety of these mVSTs and provide preliminary evidence to support the in vivo activity of the infused T cells, including against the newly targeted BKV and HHV6.

The ultimate value of multivirus-specific T cell lines will depend both on their anti-viral potency and safety and also on the speed with which they can be made available. Although our accelerated manufacturing process provides cells within 10 days of commencing culture, other approaches such as streptamer isolation(50;51) or IFN γ -capture(52–56) of virus-specific T cells from peripheral blood may provide VSTs even more rapidly. These direct selective approaches, however, have several limitations including a requirement for a large volume of blood (particularly when T cells directed to a given virus are present at low frequency), the difficulty in obtaining a broad spectrum of antigenic specificities across a wide range of HLA polymorphisms when using streptamers, or the selection of only the subset of VSTs that produce IFN γ . Finally, the small numbers of cells isolated limits the crucial purity, identity and potency testing that can be performed prior to infusion. As a consequence, both approaches are restricted to viruses with a high circulating precursor frequency. Thus, our multipathogen-targeted VSTs compare favorably since T cells specific

for a broad range of viruses can readily be generated from a broad range of donor and recipient pairs. Additionally, these cells should be suitable for use in the partially HLA-matched setting as an “off the shelf” product for the treatment of intractable viral illness(7), which will both facilitate the broader implementation of cellular therapies and overcome the difficulty of preparing virus-directed products from non-immune sources, such as cord blood donors.

In summary, multivirus-directed T cell lines VSTs administered as treatment to 8 patients who had a total of 18 viral infections/reactivations with 5 different virus infections produced a 94% response rate (15 complete and 2 partial responses); in all cases viral clearance was associated with an increase in the frequency of T cells directed against the infecting virus(es). Of note, these benefits were obtained without toxicities such as severe GvHD despite our short (10 day) manufacturing process, indicating that this approach adequately depletes alloreactive T cells. Multi-specific VSTs may therefore prove a safe and cost-effective therapeutic alternative for immunocompromised patients with severe viral infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cell expansion and immunophenotype of mVSTs generated for clinical use
Panel A shows the T cell expansion of mVSTs achieved over a 9–11 day period based on cell counting using trypan blue exclusion. **Panel B** shows the phenotype of the mVST cell lines on the day of cryopreservation. Each symbol represents an individual T cell line (n=48), and the black bar (–) represents the mean.

Figure 2. Virus-specific activity of mVST lines

Panel A shows the spectrum of specificity of each mVST cell line vs the age of the donor from whom the T cell line was generated. Each circle represents a T cell line (n=48). The mean is represented as a black line. **Panel B** shows the estimated probability of activity against one or more viruses compared to the donor's age, analyzed by the binomial regression model. Each circle represents one donor. **Panel C** shows the frequency of AdV (Hexon and Penton), CMV (IE1 and pp65), EBV (LMP2, EBNA1, BZLF1), BKV (Large T and VP1), and HHV6 (U11, U14 and U90) reactive T cells in mVST cell lines as measured

by IFN γ ELISpot. Only T cell lines with a total of ≥ 30 SFC/ 2×10^5 for a given virus were considered to be positive. Each symbol represents the mean $\pm$ SEM SFC/ 2×10^5 input cells of an individual T cell line. Control was IFN γ release in response to stimulation with no pepmix.

Figure 3. *In vivo* expansion and clinical benefits of mVSTs in subjects infected with one virus
Panel A shows the AdV viral load in blood (copies/ml) and frequency of AdV-specific T cells before and after infusion in subject P4052 who was treated for an AdV infection. **Panel B** shows the BKV viral load in blood (copies/ml) and frequency of BKV-directed T cells before and after infusion in subject P3850 who was treated for a BKV reactivation. In both cases dotted lines represent the viral loads and solid lines the frequency of VSTs as measured by IFN γ ELISpot and results are presented as average SFC/5 $\times$ 10 5 PBMCs. The arrow indicates the time of mVST infusion.

Figure 4. *In vivo* expansion and clinical benefits of mVSTs in subjects with two viral infections/reactivations

Panels A and B show the CMV and BKV viral load in blood (copies/ml) and frequency of CMV- and BKV-reactive T cells before and after infusion in subject P3908 who was treated for a CMV infection and had a subsequent BKV reactivation. **Panels C and D** show the BKV load (copies/ml) in blood and urine respectively, and frequency of BKV-specific T cells before and after infusion in subject P3925 who was treated for BKV infection. **Panel E** shows the frequency of BKV-reactive T cells expanded from a bladder biopsy sample taken from patient P3925, versus unstimulated T cells, which served as a negative control. **Panel F** shows the EBV viral load in blood (copies/μg DNA), and frequency of EBV-specific T cells before and after infusion in subject P3925 who reactivated EBV. **Panels G and H** show the BKV and EBV loads (copies/ml and copies/μg DNA, respectively) in blood and frequency of BKV- and EBV-directed T cells before and after infusion in subject P4165 who had both BKV and EBV infections. In all cases dotted lines represent the viral loads and solid lines the frequency of VSTs measured by IFN γ ELISpot and results are presented as average SFC/5x10⁵ PBMCs. The arrow indicates when the mVSTs were administered.

Figure 5. *In vivo* expansion and clinical benefits of mVSTs in subjects with three viral infections/reactivations

Panels A, B and C show the CMV, EBV and BKV viral loads detected in the peripheral blood (copies/ml, copies/μg DNA and copies/ml, respectively) and frequency of CMV-, EBV- and BKV- reactive T cells before and after infusion in subject P3987 who was treated for CMV, EBV and BKV. **Panels D, E and F** show HHV6, EBV and BKV viral loads detected in the peripheral blood (copies/ml, copies/μg DNA and copies/ml, respectively) and frequency of HHV6-, EBV- and BKV-reactive T cells before and after infusion in subject P3940 who was treated for active HHV6 and BKV and subsequently reactivated EBV. In all cases dotted lines represent the viral loads and solid lines the frequency of VSTs measured by IFN γ ELISpot and results are presented as average SFC/5x10⁵ PBMCs. The arrow indicates the infusion of mVSTs.

Figure 6. *In vivo* expansion and clinical benefits of mVSTs in one subject with four viral infections/reactivations

Panel A shows the EBV viral load (copies/ μ g DNA) in blood and the frequency of EBV-specific T cells in subject P3022 who was treated for EBV-PTLD. **Panel B** shows the PET scan of the subject P3022 before and after mVSTs. **Panels C and D** show the levels of BKV in blood and urine while **Panels E and F** show the HHV6 and CMV viral loads in the peripheral blood (copies/ml and copies/cell, respectively). The frequency of BKV-, HHV6- and CMV- reactive T cells in blood before and after infusion of mVSTs is also shown. In all cases dotted lines represent the viral loads and solid lines the frequency of VSTs measured by IFN γ ELISpot and results are presented as average SFC/ 5×10^5 PBMCs. The arrow indicates the infusion of mVSTs.

Table 1

Patient characteristics

Patient #	Age/sex	Ethnicity	Disease	Donor	GvHD prophylaxis	Day after HSCt when mVSTs infused	Cell dose	Immune suppression at time of mVST infusion	Changes to immune suppression drug doses
P4052	6 m/M	Hispanic	Leukocyte Adhesion Deficiency	Haploidentical	Alemtuzumab, tacrolimus	74	2 × 10 ⁷ /m ²	Tacrolimus	On tacrolimus - not weaned until wk4 after VSTs when AdV PCR had fallen to 5,200 copies/ml
P3850	12 y/M	Hispanic	ALL	Matched related	Tacrolimus, prednisone	139	5 × 10 ⁶ /m ²	MMF, prednisone, topical triamcinolone	Remained on MMF and triamcinolone cream. On prednisone for previous GvHD - dose from 7.5 mg – 20 mg daily while on study
P3908	7 y/F	Hispanic	Sickle cell disease	Matched related	Alemtuzumab, tacrolimus, MMF	126	5 × 10 ⁶ /m ²	Tacrolimus, MMF	Tacrolimus and MMF with no dose changes
P3925	18 y/M	Black	MDS (GATA2 deficiency)	Mismatched unrelated (HLA-A)	Alemtuzumab, tacrolimus, prednisone	86	2 × 10 ⁷ /m ²	Tacrolimus, MMF, topical triamcinolone	Remained on MMF and triamcinolone cream with no dose changes. Tacrolimus weaned by 25% at wk2 after VSTs
P4165	9 y/M	White	SCID variant	Matched unrelated	Tacrolimus, topical triamcinolone	40	2 × 10 ⁷ /m ²	Tacrolimus, topical triamcinolone	Remained on tacrolimus and triamcinolone cream. No tapering in first 6 wks after infusion
P3987	5 y/M	Asian	Thalassemia	Matched related	Cyclosporine	64	1 × 10 ⁷ /m ²	Cyclosporine, topical triamcinolone	Cyclosporine weaned from 50 mg bid on day of VST infusion to 50 mg daily at wk3 then discontinued at wk6.
P3940	19 y/F	White	HLH	Matched unrelated	Alemtuzumab, MMF, prednisone	59	1 × 10 ⁷ /m ²	MMF, prednisone	Changed from MMF to tacrolimus wk1 as not tolerating MMF. On prednisone for previous GVHD -dose from 50 mg to 15 mg daily while on study
P3022	53 y/F	White	Myelodysplasia	Mismatched unrelated HLA-A (HVG:6/6; GVH: 5/6)	Tacrolimus, topical triamcinolone	107	5 × 10 ⁶ /m ²	Tacrolimus, topical triamcinolone	Tacrolimus with no dose changes
P3975	15 y/M	White	Very high risk ALL	Matched related	Tacrolimus, methotrexate	40	1 × 10 ⁷ /m ²	Tacrolimus, topical triamcinolone	n/a
P3938	9 y/F	White	ALL	Matched related	Tacrolimus, methotrexate	38	1 × 10 ⁷ /m ²	Tacrolimus, topical triamcinolone	n/a
P3914	54 y/M	White	NHL	Matched unrelated	Tacrolimus, methotrexate	41	5 × 10 ⁶ /m ²	Tacrolimus, topical triamcinolone	n/a

Abbreviations: ALL, Acute Lymphoblastic Leukemia; MDS, Myelodysplastic syndrome; SCID, Severe Combined Immunodeficiency; GvHD, Graft-versus-host disease; HLH, Haemophagocytic lymphohistiocytosis; NHL, non-Hodgkin’s lymphoma; HVG, Host-versus-graft; GVH, Graft-versus-host; MMF, Mycophenolate Mofetil; wk, week; BID, bis in die (twice daily)

Table 2

Viral responses and outcomes after mVSTs (up to 12 weeks)

Patient #	# of mVST infused	Reason for mVST infusion (copies/ml; for EBV copies/ μ g DNA)	History of infection	Subsequent reactivations	Anti-viral therapy before mVSTs	Anti-viral therapy after mVSTs	Antiviral response	Long term outcome (>12 weeks)
P4052	5624	AdV (237,000)	AdV became detectable 6 wks before infusion and viral load increased despite cidofovir	None	Cidofovir (started 2 wks before mVSTs)	None - last dose of cidofovir 2 days before mVSTs	AdV - CR	Alive with mixed chimerism - received donor leukocyte infusion
P3850	5393	BKV (blood: 400)	BKV consistently present in blood for 3 months before mVSTs	None	None	None (off study at week 5)	BKV - CR	Off study at wk5 - Received a CD34 top off for poor graft function
P3908	5435	CMV (13,200)	CMV elevated for at least 6 wks with confirmed skin infiltration and presumed colitis and pneumonia BKV: no data before infusion	BKV	iv foscarnet for at least 4 wks, weekly cidofovir, cytogram X 5	Ganciclovir for 6 wks	BKV - CR; CMV - PR	Alive
P3925	5450	BKV [blood: 1,000; urine >1 $\times$ 10 ¹⁰ (upper limit assay)]	BKV in blood: elevated for 7 wks before mVSTs; urine elevated for 9 wks before mVSTs. No EBV reactivation before mVSTs	EBV	Foscarnet for 2 months before mVSTs, valganciclovir for previous CMV infection- stopped before mVSTs	Foscarnet stopped 1 wk after mVSTs	BKV - CR; EBV - CR	Alive
P4165	5787	BKV (700); EBV (666)	BKV: no data before infusion EBV elevated for 3 wks before mVSTs	None	None	None	BKV - CR; EBV - CR	Alive
P3987	5553	CMV (1,900); EBV (2,161); BKV urine (900)	History of CMV reactivation and increasing for 2 wks before mVSTs; EBV reactivation with increasing load for 3 wks before mVSTs; BKV in urine elevated for 6 wks before mVSTs	None	Cidofovir for 5 wks before mVSTs, foscarnet for 6 wks before mVSTs, ganciclovir from wk-8 until wk-4	Cidofovir for 2 wks and ganciclovir for 6 wks after mVSTs	CMV - CR; EBV - CR; BKV - CR	Alive
P3940	5469	HHV6 (100); BKV (500)	HHV6: 1 st reactivation at infusion; BKV: no data before infusion; No EBV reactivation before mVSTs	EBV	None	None	HHV6 - CR; BKV - NR; EBV - CR	Off study at wk12 - Received a CD34 top off for poor graft function

Patient #	# of mVST infused	Reason for mVST infusion/Viral load at infusion (copies/ml; for EBV copies/ μ g DNA)	History of infection	Subsequent reactivations	Anti-viral therapy before mVSTs	Anti-viral therapy after mVSTs	Antiviral response	Long term outcome (>12 weeks)
P3022	5381	EBV-PTLD (blood: 154,089 with disease on imaging); BKV (blood: 200; urine: 4.1×10^7)	EBV: Elevated for at least 2 wks before mVSTs and elevated at time of EBV lymphoma diagnosis; BKV: elevated for at least 3 wks before mVSTs; CMV and HHV6 reactivations previously but negative at infusion	CMV; HHV6	Cidofovir (for one month before mVSTs), foscarnet and ganciclovir (for previous CMV and HHV6 reactivations)	Valganciclovir after 6 wk follow-up; foscarnet from wks 4 to 6 for HHV6 reactivation	EBV-PTLD -CR; BKV - PR; HHV6 - CR; CMV - CR	Died of diabetic complications. Official cause of death AML
P3975	5518	prophylaxis (n/a)	n/a	None	None	None	N/A	Developed TAM, which improved, patient had poor renal function
P3938	5468	prophylaxis (n/a)	n/a	None	None	None	N/A	Died of TAM
P3914	5442	prophylaxis (n/a)	n/a	None	None	None	N/A	Alive

Abbreviations: Adv, adenovirus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; PTLD, post-transplant lymphoproliferative disease; HHV6, Human herpesvirus 6; GvHD, Graft-versus-host disease; CR, complete response; PR, partial response; NR, no response; TAM, transplant-associated microangiopathy; AML, Acute Myeloid Leukemia.