Activity of Broad-Spectrum T Cells as Treatment for AdV, EBV, CMV, BKV, and HHV6 Infections After HSCT

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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 antiviral range. We now demonstrate the feasibility and clinical utility of rapidly generated single-culture VSTs that recognize 12 immunogenic antigens from five 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 four 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 antiviral 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. After stem cell transplantation, for example, administration of donor-derived T cells with specificity for cytomegalovirus (CMV), adenovirus (AdV), or Epstein-Barr virus (EBV) has all produced frequent and sustained antiviral and clinical benefits, even for patients suffering from advanced and drug-resistant infections (3–6). More recently, “off the shelf,” or banked, partially human leukocyte antigen (HLA)–matched virus-specific T cells (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 multivirus-specific T cells (mVSTs) sustain T cells specific for only a limited number of the viruses that afflict the immunocompromised host, because of antigenic competition between the immunodominant components of each viral antigen (12–14). Moreover, manufacture of these mVSTs frequently requires preparation of specialized antigen-presenting cells, 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 mVSTs 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 mVSTs can meet the desired specifications of multiviral specificity, rapid production, and sustained and broad antiviral 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 Supplementary Materials and Methods. From 3 × 10⁷ PBMCs, we produced a mean of 40.1 ± 2.7 × 10⁷ cells (median, 35.7 × 10⁷ cells; range, 9.9 × 10⁷ to 82.5 × 10⁷; n = 48) representing an average 13-fold total expansion within 9 to 11 days (Fig. 1A). The lines were almost exclusively CD3⁺ T cells (98 ± 0.2%; mean ± SEM) containing both helper CD4⁺ (57 ± 2%) and cytotoxic CD8⁺ (35 ± 2%) T cell subsets that expressed central CD45RO⁺CD62L⁺ (62 ± 3%) and effector memory markers CD45RO⁺/CD62L⁻ (10 ± 1%) (Fig. 1B).

Antiviral specificity of mVST lines and donor serostatus

The antiviral specificity of the patients’ mVSTs was assessed by interferon-γ (IFN-γ) enzyme-linked immunospot (ELISpot) assay after we reexposed 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 (SFCs) directed against all antigens from the target virus was ≥30/2 × 10⁵ input cells. Of the 48 lines generated, 14 had activity against all five stimulating viruses (pentavalent), 9 recognized four viruses (tetravalent), 12 were trivalent, 11 were divalent, 1 was monovalent, and 1 failed to recognize any of the targeted viruses (Fig. 2A). Donor serostatus to CMV was determined before 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 not only highlight the robustness of our manufacturing process but also emphasize the requirement for previous viral exposure for the broadest range of antiviral 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 ($P = 0.003$) (Fig. 2B). Overall, AdV was the most frequently recognized virus [45 reactive lines: hexon, 470 ± 71 (mean ± SEM); penton, 366 ± 86 SFCs/2 × 10⁵ input cells]; 37 had EBV-directed reactivity [latent membrane protein 2 (LMP2): 137 ± 76; Epstein-Barr virus nuclear antigen 1 (EBNA1): 123 ± 52; BZLF1: 99 ± 75 SFCs/2 × 10⁵ input cells]; 29 lines recognized HHV6 (U90: 109 ± 78; U11: 37 ± 17; U14: 84 ± 26 SFCs/2 × 10⁵ input cells); 28 had BKV-directed reactivity (large T: 123 ± 61; VP1: 208 ± 89 SFCs/2 × 10⁵ input cells); whereas 26 lines had CMV-directed activity (pp65: 1048 ± 446; IE1: 356 ± 157 SFCs/2 × 10⁵ input cells) (Fig. 2C). Table S1 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 mVSTs described above were obtained after 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 phytohemagglutinin (PHA) blasts. There was no evidence of alloreactivity in any line generated (fig. S1). Specific lysis of uninfected recipient/haploidentical PHA blasts of <10% at an effector/target (E/T) ratio of 20:1 was a clinical release criterion and was met by all lines (mean ± SEM, 1 ± 0.4% specific lysis; E/T ratio, 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).

**Table 1. Patient characteristics.** ALL, acute lymphoblastic leukemia; BID, twice daily; GVH, graft versus host; HLH, hemophagocytic lymphohistiocytosis; HVG, host versus graft; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; n/a, not applicable; NHL, non-Hodgkin's lymphoma; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>Ethnicity</th>
<th>Disease</th>
<th>Donor</th>
<th>GVHD prophylaxis</th>
<th>Days after HSCT when mVSTs were infused</th>
<th>Cell dose</th>
<th>Immunosuppression at time of mVST infusion</th>
<th>Changes to immunosuppression drug doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4052</td>
<td>6 months/M Hispanic</td>
<td>Leukocyte adhesion deficiency</td>
<td>Haploidentical</td>
<td>Alemtuzumab, tacrolimus</td>
<td>74</td>
<td>2 x 10^7/m^2</td>
<td>Tacrolimus</td>
<td>On tacrolimus—not weaned until week 4 after VSTs when AdV PCR had fallen to 5200 copies/ml</td>
<td></td>
</tr>
<tr>
<td>P3850</td>
<td>12 years/M Hispanic</td>
<td>ALL</td>
<td>Matched related</td>
<td>Tacrolimus, prednisone</td>
<td>139</td>
<td>5 x 10^6/m^2</td>
<td>MMF, prednisone, topical triamcinolone</td>
<td>Remained on MMF and triamcinolone cream. On prednisone for previous GVHD—dose from 7.5 to 20 mg daily while on study</td>
<td></td>
</tr>
<tr>
<td>P3908</td>
<td>7 years/F Hispanic</td>
<td>Sickle cell disease</td>
<td>Matched related</td>
<td>Alemtuzumab, tacrolimus, MMF</td>
<td>126</td>
<td>5 x 10^6/m^2</td>
<td>Tacrolimus, MMF</td>
<td>Tacrolimus and MMF with no dose changes</td>
<td></td>
</tr>
<tr>
<td>P3925</td>
<td>18 years/M Black</td>
<td>MDS (GATA2 deficiency)</td>
<td>Mismatched unrelated (HLA-A)</td>
<td>Alemtuzumab, tacrolimus, prednisone</td>
<td>86</td>
<td>2 x 10^7/m^2</td>
<td>Tacrolimus, MMF, topical triamcinolone</td>
<td>Remained on MMF and triamcinolone cream with no dose changes. Tacrolimus weaned by 25% at week 2 after VSTs</td>
<td></td>
</tr>
<tr>
<td>P4165</td>
<td>9 years/M White</td>
<td>SCID variant</td>
<td>Matched unrelated</td>
<td>Tacrolimus, topical triamcinolone</td>
<td>40</td>
<td>2 x 10^7/m^2</td>
<td>Tacrolimus, topical triamcinolone</td>
<td>Remained on tacrolimus and triamcinolone cream. No tapering in first 6 weeks after infusion</td>
<td></td>
</tr>
<tr>
<td>P3987</td>
<td>5 years/M Asian</td>
<td>Thalassemia</td>
<td>Matched related</td>
<td>Cyclosporine</td>
<td>64</td>
<td>1 x 10^7/m^2</td>
<td>Cyclosporine, topical triamcinolone</td>
<td>Cyclosporine weaned from 50 mg BID on day of VST infusion to 50 mg daily at week 3 and then discontinued at week 6</td>
<td></td>
</tr>
<tr>
<td>P3940</td>
<td>19 years/F White</td>
<td>HLH</td>
<td>Matched unrelated</td>
<td>Alemtuzumab, MMF, prednisone</td>
<td>59</td>
<td>1 x 10^7/m^2</td>
<td>MMF, prednisone</td>
<td>Changed from MMF to tacrolimus at week 1 as not tolerating MMF. On prednisone for previous GVHD—dose from 50 to 15 mg daily while on study</td>
<td></td>
</tr>
<tr>
<td>P3022</td>
<td>53 years/F White</td>
<td>Myelodysplasia</td>
<td>Mismatched unrelated HLA-A (HVG: 6/6; GVH: 5/6)</td>
<td>Tacrolimus, topical triamcinolone</td>
<td>107</td>
<td>5 x 10^6/m^2</td>
<td>Tacrolimus, topical triamcinolone</td>
<td>Tacrolimus with no dose changes</td>
<td></td>
</tr>
<tr>
<td>P3975</td>
<td>15 years/M White</td>
<td>Very high risk ALL</td>
<td>Matched related</td>
<td>Tacrolimus, methotrexate</td>
<td>40</td>
<td>1 x 10^7/m^2</td>
<td>Tacrolimus, topical triamcinolone</td>
<td>n/a</td>
<td></td>
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<tr>
<td>P3938</td>
<td>9 years/F White</td>
<td>ALL</td>
<td>Matched related</td>
<td>Tacrolimus, methotrexate</td>
<td>38</td>
<td>1 x 10^7/m^2</td>
<td>Tacrolimus, topical triamcinolone</td>
<td>n/a</td>
<td></td>
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<tr>
<td>P3914</td>
<td>54 years/M White</td>
<td>NHL</td>
<td>Matched unrelated</td>
<td>Tacrolimus, methotrexate</td>
<td>41</td>
<td>5 x 10^6/m^2</td>
<td>Tacrolimus, topical triamcinolone</td>
<td>n/a</td>
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</table>
Table 2. Viral responses and outcomes after mVSTs (up to 12 weeks). AML, acute myeloid leukemia; CR, complete response; NR, no response; PR, partial response.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>No. of mVST infused</th>
<th>History of infection</th>
<th>Subsequent reactivations</th>
<th>Antiviral therapy before mVSTs</th>
<th>Antiviral therapy after mVSTs</th>
<th>Antiviral response</th>
<th>Long-term outcome (&gt;12 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4052</td>
<td>5624</td>
<td>AdV (237,000)</td>
<td>AdV became detectable 6 weeks before infusion, and viral load increased despite cidofovir</td>
<td>None</td>
<td>Cidofovir (started 2 weeks before mVSTs)</td>
<td>None—last dose of cidofovir 2 days before mVSTs</td>
<td>AdV—CR</td>
</tr>
<tr>
<td>P3850</td>
<td>5393</td>
<td>BKV (blood: 400)</td>
<td>BKV consistently present in blood for 3 months before mVSTs</td>
<td>None</td>
<td>None</td>
<td>None (off study at week 5)</td>
<td>BKV—CR</td>
</tr>
<tr>
<td>P3908</td>
<td>5435</td>
<td>CMV (13,200)</td>
<td>CMV elevated for at least 6 weeks with confirmed skin infiltration and presumed colitis and pneumonia. BKV: no data before infusion</td>
<td>None</td>
<td>Intravenous foscarnet for at least 4 weeks, weekly cidofovir, CytoGam × 5</td>
<td>Ganciclovir for 6 weeks</td>
<td>BKV—CR; CMV—PR</td>
</tr>
<tr>
<td>P3925</td>
<td>5450</td>
<td>BKV (blood: 1000; urine: &gt;1 × 10^10 (upper limit assay))</td>
<td>BKV in blood: elevated for 7 weeks before mVSTs; urine elevated for 9 weeks before mVSTs. No EBV reactivation before mVSTs</td>
<td>None</td>
<td>Foscarnet (for 2 months before mVSTs), valganciclovir for previous CMV infection—stopped before mVSTs</td>
<td>Foscarnet stopped 1 week after mVSTs</td>
<td>BKV—CR; EBV—CR</td>
</tr>
<tr>
<td>P4165</td>
<td>5787</td>
<td>BKV (700); EBV (666)</td>
<td>BKV: no data before infusion. EBV elevated for 3 weeks before mVSTs</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>BKV—CR; EBV—CR</td>
</tr>
<tr>
<td>P3987</td>
<td>553</td>
<td>CMV (1900); EBV (2161); BKV urine (900)</td>
<td>History of CMV reactivation and increasing for 2 weeks before mVSTs. EBV reactivation with increasing load for 3 weeks before mVSTs. BKV in urine elevated for 6 weeks before mVSTs</td>
<td>None</td>
<td>Cidofovir for 5 weeks before mVSTs, foscarnet for 6 weeks before mVSTs, and ganciclovir from week 8 until week 4</td>
<td>Cidofovir for 2 weeks and ganciclovir for 6 weeks after mVSTs</td>
<td>CMV—CR; EBV—CR; BKV—CR</td>
</tr>
<tr>
<td>P3940</td>
<td>5469</td>
<td>HHV6 (100); BKV (500)</td>
<td>HHV6: first reactivation at infusion. BKV: no data before infusion. No EBV reactivation before mVSTs</td>
<td>None</td>
<td>EBV</td>
<td>None</td>
<td>HHV6—CR; BKV—NR; EBV—CR</td>
</tr>
<tr>
<td>P3975</td>
<td>5518</td>
<td>Prophylaxis (n/a)</td>
<td>n/a</td>
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<td>None</td>
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<tr>
<td>P3938</td>
<td>5468</td>
<td>Prophylaxis (n/a)</td>
<td>n/a</td>
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<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>P3914</td>
<td>5442</td>
<td>Prophylaxis (n/a)</td>
<td>n/a</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>
Patients with a single virus who responded to mVSTs. Two patients with a single viral infection [P4052 (AdV) and P3850 (BKV)] received mVSTs. In the 3 weeks before mVST infusion, the AdV load detected in the peripheral blood of patient P4052 progressively increased from 1600 DNA copies/ml to a maximum of 434,000 copies/ml and remained elevated despite administration of cidofovir (Fig. 3A). Within 2 weeks of receiving mVSTs, 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/5 × 10^5 PBMCs by week 4 (Fig. 3A). Similarly, patient P3850 had a persistent BKV infection with 400 copies/ml detected in peripheral blood on the day of infusion of 5 × 10^5 mVSTs/m^2. This patient had preexisting endogenous T cells directed against BKV (72 SFC/5 × 10^5 PBMCs), but there had been no control of disease. Within 4 weeks of mVST 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 because of poor graft function that had antedated mVST infusion (Fig. 3B).

Patients with two viruses who responded to mVSTs. Three patients had infections/reactivations with two viruses, and mVSTs controlled both pathogens in all of them (Fig. 4). Patient P3908 initially presented with a persistent, ganciclovir-resistant CMV reactivation, with confirmed CMV pneumonitis, colitis, and skin infiltration. On the day of mVST infusion, her CMV viral load was 13,200 copies/ml. Within 1 week of receiving 5 × 10^6 mVSTs/m^2, the viral load decreased to 3800 copies/ml and continued to decline thereafter, coinciding with an increase in the frequency of CMV-directed T cells (Fig. 4A). At week 3 after mVST 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 (Fig. 4B). Subject P3925 was initially treated for elevated BKV load in both his peripheral blood (1000 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 PBMCs by week 3 after infusion), with no activity against the “nontargeted” but highly immunogenic antigen small T (15) (Fig. 4, C and D, and fig. S2A). 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 (Fig. 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/μg DNA), but this reactivation was rapidly controlled, with normalization of EBV DNA levels associated with a corresponding increase in his EBV-specific T cells (Fig. 4F). He remained well and free of infection at 5 months after transplant. Finally, patient P4165 eliminated viruses (P3940 and P3987), and one subject was treated for four viruses (P3022). We saw no correlation between the cell dose infused and either safety or antiviral activity.

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

Each patient received 0.5 × 10^7 to 2 × 10^7 cells/m^2; eight subjects received the cells as treatment for established viral infections/disease, and three as prophylaxis (Table 2). There were no immediate infusion-related toxicities. One patient developed de novo graft-versus-host disease (GVHD) of the skin (stage II) about 4 weeks after receiving the cells, which improved with the administration of topical steroids. Two patients (GVHD) of the skin (stage II) about 4 weeks after receiving the cells, which improved with the administration of topical steroids. Two patients 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 (Fig. 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/μg DNA), but this reactivation was rapidly controlled, with normalization of EBV DNA levels associated with a corresponding increase in his EBV-specific T cells (Fig. 4F). He remained well and free of infection at 5 months after transplant. Finally, patient P4165 eliminated viruses (P3940 and P3987), and one subject was treated for four viruses (P3022). We saw no correlation between the cell dose infused and either safety or antiviral activity.
both BKV (700 copies/ml) and EBV (666 copies/µg DNA) within 2 weeks of receiving mVSTs, coincident with a corresponding increase in both T cells directed against the BKV and EBV antigens targeted in the mVST line infused (Fig. 4, G and H) and not against nontargeted antigens (BKV—small T; EBV—EBNA3a, EBNA3b, EBNA3c, and LMP1; fig. S2, B and C).

Patients with three viruses who responded to mVSTs. Two study participants developed reactivation of three viruses. Subject P3987 received VSTs to treat reactivation of CMV (1900 copies/ml) and EBV (2161 copies/µg DNA); both were controlled by the infused mVSTs (Fig. 5, A and B). This patient also had BKV-associated hemorrhagic cystitis at the time of mVST infusion, with elevated viral load detected in urine (900 copies/ml). Within 1 week of mVSTs, there was a marked improvement in his hematuria, and BKV became undetectable (Fig. 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/µg DNA). HHV6 was successfully cleared after mVST administration, coincident with a temporal increase in the frequency of T cells directed against the antigens targeted in the line infused (Fig. 5D) and not against the nontargeted U54 antigen (16–18) (fig. S2D). Similarly, the EBV reactivation was rapidly controlled in association with a rise in EBV-reactive T cells (Fig. 5E). The BKV load, however, continued to increase (Fig. 5F).

Examination of the specificity of the infused mVST line (#5469; table S1), served as a negative control. (F) 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. (G and H) 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, solid lines represent the frequency of VSTs measured by IFN-γ ELISPOT, and results are presented as average SFC/5 × 10^5 input cells. The arrow indicates when the mVSTs were administered.

Fig. 4. In vivo expansion and clinical benefits of mVSTs in subjects with two viral infections/reactivations. (A and B) 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. (C and D) 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. (E) Frequency of BKV-reactive T cells expanded from a bladder biopsy sample taken from patient P3925 versus unstimulated T cells, which

however, showed that it lacked specificity for BKV, thus explaining its inability to control BKV in this patient (Fig. 5F). Ultimately, subject P3940 was taken off study at week 12 after infusion to receive a CD34 top-off because of continued poor graft function but remained 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 (Fig. 6A) and frank EBV-associated posttransplant lymphoproliferative disease (EBV-PTLD) with extensive disease in multiple lymph nodes (Fig. 6B). She also had elevated BKV levels in her blood and urine (200 and 4.1 × 10⁷ 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 infused line and not against nontargeted (EBNA3a, EBNA3b, EBNA3c, and LMP1) (19) EBV antigens (fig. S2E). Her BKV-associated hematuria also improved within 2 to 3 weeks of receiving the mVSTs, coincident with a decrease in viral load to 0 and 8 × 10⁵ copies/ml in blood and urine, respectively, at week 8 after infusion (Fig. 6, C and D). Although there was a subsequent increase in her viral load levels to 200 and 1.5 × 10⁷ copies/ml in blood and urine, respectively, long-term immune reconstitution studies could not be performed because this patient died of diabetic complications 18 weeks after receiving mVSTs (Table 2). Four weeks after receiving mVSTs, she developed HHV6 and CMV activations (4800 HHV6 copies/ml and 5 to 10 CMV antigen-positive cells/5 × 10⁴ PBMCs), both of which rapidly resolved without additional intervention (Fig. 6, E 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 mVSTs 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, whereas others are seasonal (such as 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 mVST specificities showed that only 14 of the 48 had activity against antigens from all five stimulating viruses. We determined whether this deficiency simply reflected lack of donor exposure to the virus (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 because 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 monovalent-, bivalent-, trivalent-, tetravalent-, and pentavalent-specific T cell products. We saw no significant differences. For example, in a monospecific line in which AdV alone was recognized, the frequency of AdV-reactive cells (1170 ± 54 SFCs/2 × 10⁵ input cells) was similar to the frequency of AdV-reactive cells in the 14 lines that recognized all five viruses (mean, 1283 ± 201 SFCs/2 × 10⁵ input cells). Thus, our manufacturing protocol supports the generation

**Fig. 6. In vivo expansion and clinical benefits of mVSTs in one subject with four viral infections/reactivations.** (A) EBV viral load (copies/µg DNA) in blood and the frequency of EBV-specific T cells in subject P3022, who was treated for EBV-PTLD. (B) Positron emission tomography scan of subject P3022 before and after mVSTs. (C to F) Levels of BKV in blood and urine (C and D) and HHV6 and CMV viral loads in the peripheral blood (E and F) (copies/ml and copies per 5 × 10⁴ cells, 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, solid lines represent the frequency of VSTs measured by IFN-γ ELISpot, and results are presented as average SFC/5 × 10⁵ PBMCs. The arrow indicates the infusion of mVSTs.
of broad-spectrum VSTs, whose range is currently only limited by the previous viral exposure of the VST donor.

Whereas even intractable CMV, EBV, and 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 that 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 to 80% of HSCT recipients and develops into BKV–associated hemorrhagic cystitis in 5 to 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 (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, among the seven patients who reactivated BKV, we achieved five complete and one partial responses. 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 three had marked symptomatic and virologic responses with disappearance of hematuria within 2 to 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 2 years (44). The virus subsequently persists lifelong in a latent form. 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 to 60% of HSCT recipients and may become associated with central nervous system disease (including encephalitis and meningitis), pneumonitis, transplant rejection, or multifocal leukoencephalopathy. They may become associated with central nervous system disease (including encephalitis and meningitis), pneumonitis, transplant rejection, or multifocal leukoencephalopathy.

Our current results support the suitability of these antigens as therapeutic targets because 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 HHV6 VSTs may therefore prove a safe and cost-effective therapeutic alternative for immunocompromised patients with severe viral infections.

**SUPPLEMENTARY MATERIALS**

www.sciencetranslationalmedicine.org/cgi/content/full/6/242/242ra83/DC1

**Materials and Methods**

Fig. S1. Lack of alloreactivity of mVSTs generated for clinical use.

Fig. S2. In vivo expansion of T cells directed against targeted and nontargeted viral antigens.

Table S1. Specificity of mVSTs.

Table S2. Plasma cytokine concentrations before and after infusion of mVSTs (pg/ml).

**REFERENCES AND NOTES**


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Abstracts

One-sentence summary: Rapidly generated broad-spectrum T cells can simultaneously treat multiple viral infections after hematopoietic stem cell transplant.

Editor’s Summary:
Killing Multiple Viruses with One Stone

Bone marrow or stem cell transplantation is becoming increasingly common for cancer as well as for other blood disorders and genetic diseases. Although patient outcomes are often good and are continuing to improve as technology evolves, the patients are still at risk for a variety of complications. One of the deadliest complications for newly transplanted patients is infection due to their severely compromised immune function. Viral infections are especially problematic, because many viruses have no specific treatments. In a small clinical trial, Papadopoulou et al. demonstrated a way to quickly generate antiviral T cells and give them to transplant patients, to help them safely clear up to four (and potentially five) simultaneous viral infections.