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Antiviral T-cell therapy

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Acknowledgements

This work was supported in parts by NIH grants P50CA126752, PO1CA94237, U54HL081007, N01-HB-10-03, and the Production Assistance for Cellular Therapies (PACT) program (NHLBI contract #HHSN268201000007C). We also appreciate the support of shared resources by the Dan L. Duncan Cancer Center support grant P30CA125123. H. E. H. is supported by a Dan L. Duncan Chair and M. K. B. by a Fayez Sarofim Chair. The authors all have patents in the area of cellular therapy and the authors' Center has a collaborative research agreement with Celgene for genetically modified T cells. The authors declare no conflict of interest.

This article is part of a series of reviews covering Transplantation appearing in Volume 258 of *Immunological Reviews*.

Immunological Reviews 2014

Vol. 258: 12–29

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Immunological Reviews

0105-2896

Summary: Serious viral infections are a common cause of morbidity and mortality after allogeneic stem cell transplantation. They occur in the majority of allograft recipients and are fatal in 17–20%. These severe infections may be prolonged or recurrent and add substantially to the cost, both human and financial, of the procedure. Many features of allogeneic stem cell transplantation contribute to this high rate of viral disease. The cytotoxic and immunosuppressive drugs administered pretransplant to eliminate the host hematopoietic/immune system and any associated malignancy, the delay in recapitulating immune ontogeny post-transplant, the immunosuppressive drugs given to prevent graft versus host disease (GvHD), and the effects of GvHD itself, all serve to make stem cell transplant recipients vulnerable to disease from endogenous (latent) and exogenous (community) viruses, and to be incapable of controlling them as quickly and effectively as most normal individuals.

Keywords: T cells, immunotherapies, transplantation

Introduction: viral disease after hematopoietic stem cell transplantation

Serious viral infections are a common cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). They occur in the majority of allograft recipients and are fatal in 17–20% (1, 2). These severe infections may be prolonged or recurrent and add substantially to the cost, both human and financial, of the procedure (3–5). Many features of allogeneic HSCT contribute to this high rate of viral disease. The cytotoxic and immunosuppressive drugs administered pretransplant to eliminate the host hematopoietic/immune system and any associated malignancy, the delay in recapitulating immune ontogeny post-transplant, the potential for induction of immune tolerance/T-cell anergy upon exposure on an immature system to an active viral infection, the immunosuppressive drugs given to prevent graft versus host disease (GvHD), and the effects of GvHD itself, all serve to make HSCT recipients vulnerable to disease from endogenous (latent) and exogenous (community) viruses, and to be incapable of controlling them as quickly and effectively as most normal individuals.

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Immunological Reviews 258/2014

Over the past 20 years, great strides have been made in identifying and implementing effective therapeutic agents for the prevention and treatment of many of the serious virus infections that afflict HSCT recipients. By combining effective agents with rapid molecular analyses that can detect the early reactivation of latent infections, it has been possible to transform the outcome of patients with post-transplant cytomegalovirus (CMV) and Epstein Barr virus (EBV). Agents such as ganciclovir and rituximab (6–8), respectively, can now be used to control CMV and EBV before they cause serious or fatal illness. Despite these and other advances in early detection and prompt treatment, post-transplant viral illness remains a major threat. As might be expected, improvements in viral prophylaxis and treatment have encouraged clinicians to extend the range of patients to whom HSCT can be offered as a curative option for their disease. A high proportion of patients who are now transplanted lack siblings who are identical for human leukocyte antigens (HLAs) (major histocompatibility antigens), necessitating the use of alternative stem cell sources including HLA-partially matched unrelated donors, haploidentical related donors or cord blood units. These patients may require donor T cells to be removed from the infused stem cell preparation or may need to receive more intensive or prolonged post transplant immunosuppression, increasing their susceptibility to viral disease and prolonging the duration of this enhanced susceptibility. Moreover, the considerable success of ganciclovir and rituximab against CMV and EBV disease has yet to be followed by the availability of additional agents that can as effectively and safely treat the many other virus infections to which transplant patients are susceptible (Table 1). Even when pharmacological therapies are available, these agents are costly, associated with significant toxicities, and may lead to the outgrowth of drug-resistant mutants. Since none of these agents improve

Table 1. Emerging viral pathogens implicated in complications after hematopoietic stem cell transplantation (HSCT)

Virus	Incidence after allogeneic HSCT
HHV6	33–48% (120–125)
HHV7	10–57% (126, 127)
Parvovirus	Up to 30% (128–131)
BK virus	10–25% (132–136)
Coronavirus	8.8% (137–139)
Parainfluenza	4–7% (3, 140–143)
Influenza	1.3–2.6% (143, 144)
RSV	1.8–6% (140, 143, 145)
Metapneumovirus	5% (146, 147)
Bocavirus	5% (148, 149)
KI virus/WU virus	1–8% (148–151)
JC virus	Approximately 1% (152, 153)

virus-specific T-cell immunity, the root cause of post-transplant viral infection, these diseases frequently recur after termination of treatment. In this review, we describe the immunotherapeutic strategies that have been explored by our own and other groups to achieve safe, effective, and sustained antiviral prophylaxis and treatment following HSCT and discuss the success and limitations of this approach.

Donor lymphocyte infusions (DLI)

The first adoptive T-cell transfer studies after allogeneic HSCT were based on the premise that peripheral blood from the stem cell donor would contain T cells able to mediate anti-tumor and antiviral activity in the HSCT recipient (9–13). The expectation was that these cells would expand in the recipient after adoptive transfer and provide protective immunity. In terms of antiviral activity, providing that the donor has had prior virus exposure, donor peripheral blood does indeed contain memory T cells specific for a broad range of viruses, which reflect the life-time exposure of the host to an array of pathogens, and DLI has proved to be an effective treatment for both adenovirus (AdV) infections and EBV-associated post-transplant lymphoproliferative disease (PTLD) (14–16). Unfortunately, broader application is limited by the low frequency of virus-specific T cells compared with the much higher frequency of alloreactive T cells that can cause GvHD, a particular concern in HLA mismatched recipients who are most at risk of viral illness. Up to 10% of circulating T cells may be alloreactive (16, 17), while the frequency of T cells with a given antiviral specific-

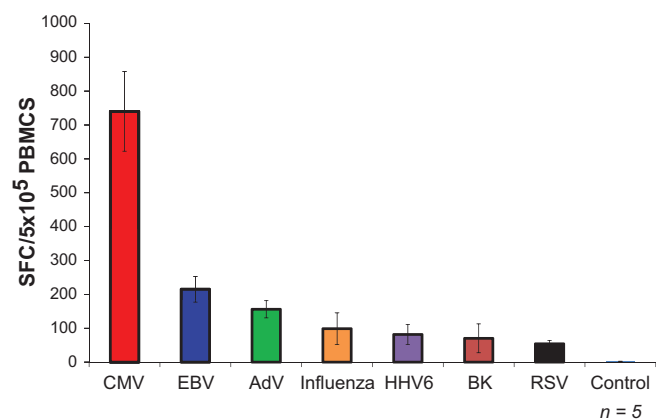


Fig. 1. Frequency of virus-specific T cells in peripheral blood. The frequency of circulating virus-specific T cells directed against CMV (IE1 and pp65), EBV (LMP1, LMP2, EBNA1, EBNA3a, EBNA3b, EBNA3c, BZLF1), AdV (Hexon and Penton), Influenza (NP1 and MP1), HHV6 (U11, U14, and U90), BK virus (Large T and VP1), and RSV (N and F) was evaluated by IFN γ ELISpot. Results are presented as spot forming cells (SFC) per 5×10^5 PBMCs \pm SEM ($n = 5$).

ity may be many logs lower (Fig. 1). Even when DLI is used to successfully treat EBV infections, a high rate of GvHD is observed, and T cells reactive to many common community viruses such as parainfluenza, respiratory syncytial virus (RSV), and human metapneumovirus (hMPV) are frequently all but undetectable in the peripheral blood of the stem cell donor, making this approach too high risk to be widely used to prevent or treat associated viral disease post-transplant.

Reducing alloreactive T cells in DLI

To preserve the benefits and enhance the safety of DLI, techniques to selectively inactivate or deplete recipient-specific alloreactive T cells have been evaluated.

Inactivation of alloreactive T cells

Alloantigen-specific T cells can be selectively anergized by exploiting the requirement of T cells to receive both an HLA-restricted, antigen-specific signal, and secondary co-stimulatory signals if they are to become successfully activated and proliferate. One such costimulatory signal is delivered by the B7.1 and B7.2 molecules on antigen-presenting cells (APCs) to the CD28 receptor on T cells, and blockade of this interaction using fusion proteins (such as CTLA4-Ig) or monoclonal antibodies (such as anti-B7.1 and -B7.2) during T-cell receptor (TCR)-APC engagement has been demonstrated to induce antigen-specific hyporesponsiveness in T cells. Guinan et al. (18, 19) have successfully used both blockade strategies clinically. Two days prior to transplant they harvested haploidentical donor marrow, which was incubated with recipient-derived mononuclear cells in the presence of either CTLA-4-Ig or anti-B7 monoclonal antibodies. These anergized T cells were then administered at the same time as the HSCT. In two pilot studies, they found that even when products contained large numbers of alloanergized donor T cells (range $7\text{--}129 \times 10^6$ CD3⁺ cells/kg) there was acceptable engraftment with less severe acute GvHD than historical control recipients of haploidentical stem cells replete with unmanipulated T cells. In addition, they reported that the treated subjects had more rapid immune reconstitution than historic controls, with detectable *in vivo* expansion of virus-specific T cells (20).

Selective allodepletion *ex vivo*

Alternatively, alloreactive T cells may be physically removed from the donor graft prior to infusion. Several groups have co-cultured donor T cells with recipient-derived APCs of

various origin including peripheral blood mononuclear cells (PBMCs), activated T cells (21), EBV-transformed lymphoblastoid cell lines (EBV-LCL) (22, 23), dendritic cells (DCs) (24), and fibroblasts (25). Any recipient-reactive T cell within the donor cell population should be stimulated by the recipient APC, and subsequently upregulate activation markers such as CD25 (23, 26–29), CD69 (30), CD71 (24, 31), CD137 (25, 32), and HLA-DR and proliferate. These reactive cells can then be selectively eliminated by magnetic-bead coupled monoclonal antibodies targeted to activation antigens or by immunotoxins, induction of apoptosis, or photodynamic purging (21, 23, 25–27, 32–39).

CD25-immunotoxin and photodynamic depletion-based approaches have both been used clinically. Andre-Schmutz et al. (40) used CD25 conjugated to the ricin α chain to allodeplete haploidentical ($n = 13$) or matched unrelated ($n = 3$) donor T cells that were then infused early (days 15–47) post-transplant to a cohort of pediatric patients. Each patient received multiple infusions of $1\text{--}8 \times 10^5$ allodepleted cells/kg, with no GvHD prophylaxis. Even at the highest dose level, the allodepleted T cells were found to be safe, in contrast with earlier studies reporting GvHD \geq Grade II in 40% of patients after infusion of 1×10^5 unmanipulated donor T cells/kg. In addition, the investigators found evidence that the allodepleted cells retained a virus-specific component. Two patients with EBV or CMV developed a high frequency of circulating virus-specific T-cell precursors postinfusion and both cleared their respective infections. Solomon et al. (27) applied this approach to adults, infusing 16 patients (median 65 years) with haploidentical T cells that were also allodepleted with an anti-CD25 immunotoxin. This group of patients would have been considered to be at high risk of developing GvHD but in fact only eight patients developed this complication, which was mild to moderate (GvHD Grades I–II) and steroid responsive in six and severe only in two. The development of GvHD appeared to correlate with the efficiency of allodepletion. Our group also demonstrated that the infusion of CD25-allodepleted cells to haploidentical HSCT recipients was safe and that a threshold of at least 1×10^5 allodepleted T cells/kg was required to accelerate antiviral T-cell recovery (23, 41, 42).

More recently, Mielke et al. (43) used photodynamic purging to prepare allodepleted T cells for infusion. They used a photoactive rhodamine derivative, 4,5-dibromorhodamine 123 (TH9402), which efficiently diffuses into both resting and activated T cells. While resting T cells can actively extrude the dye, it is selectively retained in the mitochondria of activated cells due to the inactivation of the multidrug transporter. These

activated cells are then sensitive to visible light (514 nm), which results in mitochondrial oxidation and cell death. Taking advantage of this property, Mielke *et al.* (43) co-cultured donor cells with activated recipient-derived T cells followed by addition of TH9402 and exposure to visible light. Twenty-four HLA-identical sibling HSCT recipients received a dose of 5×10^6 photodepleted cells/kg on day 0. Unfortunately, the results were disappointing. Both acute and chronic GvHD were frequent, and infectious complications associated with viral (CMV, AdV, BK virus, RSV), bacterial and fungal pathogens were common and unexpectedly prolonged and severe so that the trial was halted. In this study, it was unclear whether severe GvHD was associated with variability in the infused photodepleted products.

Overall, the above studies demonstrated the feasibility of add-back allodepleted T-cell therapy. They also emphasized the importance of choosing the optimal recipient-derived stimulator cell to achieve robust alloactivation, the impact that variability in the efficiency of allodepletion can have on *in vivo* safety, and the narrow and unpredictable therapeutic window between the desired antiviral activity and unwanted GvHD. Moreover, since T cells specific for the majority of viral pathogens circulate at frequencies that are lower than EBV and CMV (Fig. 1), it is likely that extremely high doses of allodepleted T cells would be needed to provide full spectrum anti-viral protection and that, even after allodepletion, the cell doses required would likely exceed the GvHD threshold. Finally, the allodepletion approach, at present, can only reliably be used when donor and recipient are haploidentical. Where there are fewer HLA disparities or differences only at minor histocompatibility antigens (e.g. in an HLA matched sibling transplant), the degree of alloactivation produced in the mixed lymphocyte cultures is insufficient for consistent removal of all alloreactive T cells. Consequently, the ratio of viral reactive to alloreactive T cells is not sufficiently improved to obtain beneficial antiviral effects in the absence of increased GvHD.

Selective allodepletion *in vivo*

An alternative to *ex vivo* allodepletion is to administer donor T cells that incorporate a suicide or safety switch that can be activated only in the event of GvHD, allowing recipients to take full advantage of the antiviral benefits associated with donor T-cell infusions. Moreover, if the suicide switch is functional only in activated cells, and the patient has GvHD but no viral infection, induction of suicide may deplete the alloreactive component while sparing virus-reactive cells

capable of responding to future virus reactivation or infection.

The most widely tested *in vivo* allodepletion approach uses the thymidine kinase gene from herpes simplex virus I (HSV-tk) (44). TK expression in transgenic T cells catalyzes the phosphorylation of the non-toxic prodrug ganciclovir into the active agent. After transformation into the final triphosphate form by cellular kinases, the drug acts as a GTP analog, thus inhibiting DNA chain elongation and killing dividing cells. Several phase I–II studies have shown that ganciclovir administration can be used to deplete transferred TK-modified cells *in vivo* and no adverse events related to gene transfer have been reported (45–50). However, induction of transgenic cell death may require many days and is usually incomplete, potentially delaying clinical benefit. In addition, since ganciclovir is required for cell elimination this precludes its use as an antiviral agent (e.g. for the treatment of CMV) in this highly susceptible patient population. Finally, the TK gene product can be immunogenic (51, 52). For example, the relatively immune competent patients post HLA-identical HSCT can mount a TK-directed CD8⁺ T-cell response leading to the premature and unintentional elimination of infused cells (53, 54). Despite these potential limitations, phase I and II clinical studies have shown TK-T cells can consistently benefit immune reconstitution and that GvHD can be controlled by ganciclovir administration so that the approach is now being evaluated in a multicenter, multi-national phase III study that it is hoped will allow licensure of this important approach.

We have investigated an alternative safety-switch in which we transduced allodepleted T cells with a retroviral vector encoding an inducible human caspase 9 (iC9) suicide gene and a selectable marker (truncated human CD19) to enable enrichment of the transduced cells (55–57). The iC9 gene product is activated by exposure to a small molecule chemical inducer of dimerization (CID) leading to rapid T-cell death by triggering the intrinsic (mitochondrial) apoptosis pathway. We gave iC9-expressing T cells to haploidentical pediatric HSCT recipients, and if the patients developed GvHD, we gave a single dose of the dimerizing drug AP1903. We found that CID treatment eliminated 90% of the infused transgenic cells within 30 min, with a further log depletion during the next 24 h (55). The patients' GvHD responded fully and did not recur even when the residual transgenic T cells re-expanded. The recovering iC9 T cells, however, did retain antiviral activity, suggesting selective sparing of these cells over the more activated

alloreactive iC9 T cells that had caused GvHD. We found no evidence of an immune response against the transgenic cells. The use of an otherwise bioinert small molecule to dimerize and activate iC9 allows the retention of important antiviral agents, including ganciclovir, for therapeutic use.

Direct enrichment of virus-specific T cells

An alternative means of safely providing antiviral protection after HSCT relies on the direct isolation of virus-specific T cells from donor peripheral blood for subsequent adoptive transfer. Peptide-HLA multimers and cytokine-secretion capture columns have both been adapted to serve this purpose. Multimer selection isolates T cells based on the ability of their antigen-specific receptor (TCR) to bind to a complex of synthetic peptide-loaded recombinant HLA molecules. While the approach is therefore independent of a defined phenotypic or functional characteristic, it requires prior knowledge of immunodominant epitopes and is restricted by HLA type. At present, multimers are most readily made with class I HLA antigens, which can select only CD8⁺ T cells and not the class II HLA-restricted CD4⁺ T-cell subset. This may limit the breadth and duration of any immune response following adoptive transfer. Even when class I HLA antigens are used, individual multimer complexes vary unpredictably in their stability and affinity for a given TCR, so that it is not possible at present to make effective multimers for every immunodominant epitope for each HLA class I polymorphism. In contrast, the cytokine capture approach selects T cells (both CD4⁺ and CD8⁺) based on their ability to secrete effector cytokines in response to viral antigen stimulation and is thus neither limited to specific peptides nor particular HLA types.

Selection of virus-specific T cells by multimers

T cells detect antigens via TCR interaction with peptides bound to self-HLA molecules presented on the surface of APCs. The TCR/peptide-HLA interaction is very weak and typically lasts for no longer than a few seconds at physiological temperatures. Advances over the past 15 years, however, have produced multimeric forms of soluble peptide-HLA molecules with enhanced avidity that can be utilized *ex vivo* to both visualize and select T cells that bear cognate TCRs. To date, two distinct multimer formats, tetramers, and pentamers, have been used to select T cells for adoptive transfer. Tetramers consist of four peptide-loaded HLA molecules held in a tetrahedral configuration that typically allows only 3 HLA-peptide complexes to bind to cognate TCRs (Fig. 2).

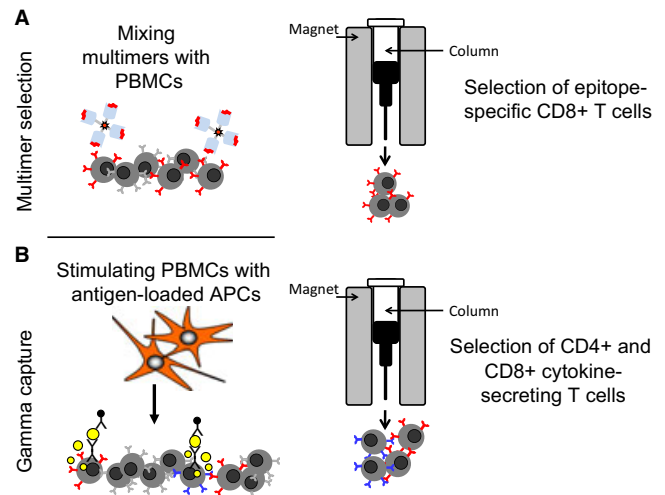


Fig. 2. Rapid selection of virus-specific T cells. Peptide-HLA multimers (A) and cytokine-secretion capture (B) have both been used clinically to select virus-specific T cells for adoptive transfer. Multimer selection isolates T cells based on the ability of their TCR to bind to a complex of synthetic peptide-loaded recombinant HLA molecules, while the cytokine capture approach selects T cells (both CD4⁺ and CD8⁺) based on their ability to secrete effector cytokines in response to viral antigen stimulation.

The five HLA-peptide groups of HLA-pentamers, however, are arranged in a planar configuration so that all five complexes are available for TCR binding thereby increasing both the binding sensitivity and avidity.

Cobbold et al. (58) were the first to use multimer-selected T cells clinically. They selected CMV-specific T cells, since CD8⁺ T cells specific for this clinically important virus are found at high frequency in healthy seropositive individuals (typically 0.5–4% of the CD8⁺ T-cell pool) which facilitates their direct isolation *ex vivo* (58). The investigators used a panel of CMV IE1 and pp65 tetramers to select specific T cells from nine SCT donors that were administered to six recipients of a matched related donor transplant and three recipients of matched unrelated transplants who had CMV reactivation. Although the infused product was composed exclusively of CD8⁺ T cells, the cells expanded by several logs *in vivo*, and in eight of nine cases CMV viral load became undetectable post transfer. No case of CMV disease was seen. Uhlin et al. (59) used pentamers to isolate T cells from the mother of a cord blood transplant recipient with rituximab-resistant EBV-PTLD. Based on shared haplotypes, available pentamers, and detectable specific populations in peripheral blood, the investigators used two HLA-A2-restricted epitopes from EBV BMLF1 (lytic) and LMP2 (latent) to select specific T cells for adoptive transfer. These directly selected cells expanded postinfusion and produced a

complete clinical response. Although antiviral protection was non-sustained, since EBV-PTLD recurred at 12 months, the disease was eradicated by a second infusion of the same cells (59). The same group subsequently used multimer selection to isolate virus-specific T cells either from cryopreserved donor material or from third party donor peripheral blood to treat eight patients with CMV ($n = 6$; HLA-A2, B7 and B35 pp65 peptide-loaded pentamers), AdV ($n = 1$; HLA-A1 hexon-derived peptide), or EBV ($n = 1$; HLA-A2 LMP2- and BMLF1-derived peptides) infections after allogeneic HSCT (60). Six of these eight patients showed a decrease in viral titers within 2 weeks of receiving the selected cells (60).

More recently, researchers have prepared streptamers for clinical application, which are multimers in which the binding between the complex of peptide-loaded HLA molecules and the TCR of antigen-specific T cells is reversible by addition of a competitor molecule that causes the streptamer to monomerize (61). The rapid release of the streptamer from the T cells means that the final infused product is indistinguishable from untreated T cells. As a consequence, the European Medicines Agency considers streptamer-selected cells as 'non-advanced therapy medicinal products' (non-ATMP), which require substantially less testing prior to clinical use than agents classified as ATMPs. Recently Schmitt et al. (62) reported the first clinical streptamer experience in two patients with refractory CMV. After a single infusion of 2.2×10^5 HLA-B7⁺/CMV pp65-specific CD8⁺ T cells/kg (purity of 97%), the frequency of CMV-specific T cells in patient 1 increased from 0% to a maximum of 27.1% of all peripheral blood T cells. Patient 2 received a lower cell dose (0.37×10^5 HLA-A24⁺/CMVpp65-specific CD8⁺ T cells/kg) but nevertheless had a detectable increase of CMV-specific CD8⁺ T cells, from 0.03% to 0.48%. These expanded cells were confirmed to be donor in origin by analysis of donor chimerism through single-tandem repeats analysis, T-cell receptor excision circles, and V β -chain typing. Clinically, the T-cell infusion resulted in CMV clearance without GvHD.

While these clinical successes hold promise, multimer selection of cells currently remains limited to patients who are infected with immunologically well-characterized viruses (e.g. CMV or EBV) with defined epitopes, known HLA-restricting elements, and who have donors with a relatively high frequency of circulating reactive T cells in their peripheral blood. Otherwise sufficiently large starting numbers of donor PBMCs may not be readily available (e.g. if the donor is unrelated to the recipient).

IFN γ -capture

An alternative means by which virus-specific T cells can be rapidly selected for adoptive transfer is based on their ability to produce and secrete effector cytokines. To date, only cells that secrete IFN γ in an antigen-dependent manner have been used clinically. The IFN γ capture technique is based on the premise that antigen-specific T cells are capable of secreting IFN γ following short-term (12–16 h) antigen exposure, and these populations can be specifically captured by labeling cells with an anti-IFN γ monoclonal antibody conjugated to a leukocyte-specific (CD45) antibody followed by magnetic selection with anti-IFN γ microbeads. Thus, unlike multimer selection this approach can be used to select both CD4⁺ and CD8⁺ antigen-specific T cells in an HLA unrestricted manner.

Feuchtinger et al. (63) were the first to use this selection platform as a means of treating AdV infections in the post-HSCT setting. AdV has a particularly high incidence after pediatric HSCT and remains a significant cause of morbidity and mortality in immunocompromised individuals, in whom it may produce pneumonia, hemorrhagic cystitis, nephritis, colitis, hepatitis, and encephalitis (64, 65). Several reports have shown that clearance of infection is associated with detection of AdV-specific T cells (66, 67). Thus, in this study Feuchtinger et al. (63) stimulated donor PBMCs (matched related – $n = 1$, matched unrelated – $n = 3$, mismatched unrelated – $n = 4$, haploidentical – $n = 1$) with AdV lysate overnight, followed by magnetic selection of the IFN γ -secreting population and then infused the cells into nine pediatric patients with systemic AdV infections (defined as persistent or recurrent AdV DNA in peripheral blood and/or stool after 2 weeks of antiviral chemotherapy and lacking AdV-specific T cells). The infusions were found to be safe and despite the small numbers of infused polyclonal T cells (range 1.2×10^3 – 5×10^4 CD3⁺ T cells/kg: CD4⁺, mean 63%, CD8⁺, mean 29%), were associated with clinical benefit, since five of six evaluable patients showed a significant decrease in viral load in peripheral blood and stool with a corresponding increase in the frequency of AdV-specific T cells in the blood. In a follow-up study, the group applied this approach to treat chemorefractory CMV disease or reactivation (68). After short-term (16 h) stimulation with the immunodominant CMV-pp65 protein and capture of IFN γ -secreting cells, a mean of 21.3×10^3 CD3⁺ T cells/kg were infused into 18 patients (matched related $n = 1$, matched unrelated $n = 3$, mismatched unrelated $n = 3$, haploidentical $n = 11$). The infusions were

well-tolerated with only a single case of GvHD reported. Fifteen of the 18 infused patients cleared their CMV viremia or had significant reduction (>1 log) of viral load. Peggs et al. (69) also reported the adoptive transfer of IFN γ -captured CMV-specific T cells for disease prophylaxis and pre-emptive treatment. In their study, pp65 protein or peptide pools were used to stimulate T cells. Post-selection a median of 2840 CD4⁺ and 630 CD8⁺ CMV-specific T cells/kg were infused early (median day 35) post-transplant and expansion of both populations was detected *in vivo*. However, acute GvHD was observed in eight of 18 patients (Grades I–III), two of whom required systemic steroid therapy; three patients developed extensive chronic GvHD.

IFN γ capture can also be used to obtain therapeutically effective EBV-specific T cells. Moosmann et al. (70) selected IFN γ -secreting cells following 12 h of exposure to 23 HLA class I and II-associated peptides derived from 11 EBV antigens [five latent antigens (LMP2, EBNA1, EBNA3A, EBNA3B, EBNA3C), four immediate early/early antigens (BZLF1, BRLF1, BMLF1, BHRF1), and two late/structural antigens (BLRF1, BNRF1)] (70). The full set of 23 peptides was used as a generic stimulating pool for each donor, irrespective of the individual's HLA haplotype. The captured cells were given to six allogeneic HSCT recipients who had developed biopsy-proven EBV-PTLD that was progressing following 3–14 days conventional therapy (including reduction in immunosuppression and administration of rituximab or cidofovir). Three of the patients responded, but three, with multiorgan dysfunction at the time of T-cell transfer, showed no/low EBV-specific T-cell activity postinfusion and all failed to respond. Icheva et al. (71) took a similar approach but focused on targeting the EBV EBNA1 antigen, based on its central role in maintaining viral persistence, its universal expression in EBV-PTLD, and the fact that it has been shown to contain immunodominant CD4⁺ and CD8⁺ T-cell epitopes. The investigators stimulated donor PBMCs either with whole EBNA1 protein or EBNA1 overlapping peptide pools, and gave IFN γ captured cells to 10 patients with EBV-PTLD. No toxicities were observed, and eight of 10 patients showed *in vivo* expansion of EBNA1-specific T cells, which was associated with a clinical and virologic response in seven, defined as a decrease of viral load >1 log and resolution of PTLD. In the two patients in whom *in vivo* T-cell expansion was absent, no clinical improvement was observed.

Clinical trials of the IFN γ capture-based approach have shown to be safe and to produce clinical activity for CMV, AdV, and EBV. The availability of 'universal' selection agents

that can be used irrespective of HLA haplotypes, rather than individualized multimers made for each viral epitope and HLA polymorphism and the ability to select both CD4⁺ and CD8⁺ T cells rather than just class I HLA-restricted CD8⁺ T cells are both potential advantages over multimer-based selection techniques. Nevertheless, both γ -capture and multimer selection techniques require large volumes of starting donor blood, and even then only small numbers of captured cells are obtained, limiting the crucial purity and potency testing that is required prior to patient transfer. As a consequence, both approaches are restricted to viruses with a high circulating precursor frequency.

Infusion of *ex vivo* expanded virus-specific T cells

Immune recovery after HSCT can also be safely enhanced by infusing virus-specific T cells (VSTs) that have been selectively amplified *ex vivo* using repetitive rounds of stimulation with APCs expressing target viral antigens. The *in vitro* stimulation with viral antigens selectively enriches virus-reactive T cells and correspondingly dilutes alloreactive T cells. By this means even low frequency virus-directed T cells can be obtained in substantial numbers from small quantities of peripheral blood, so that the strategy can produce VSTs directed to viruses for which the circulating T-cell frequency is below the practicable threshold required for either multimer or IFN γ capture assays.

Ex vivo expansion of virus-specific T cells requires the identification of immunodominant antigens in target viruses that induce protective VSTs as well as a delivery system to transfer the antigen to effective APCs. To avoid expanding alloreactive T cells, the APCs used to generate the VSTs must be autologous and express HLA molecules that present virus-derived peptides. The APC must also express the costimulatory molecules required to support robust T-cell activation and expansion. All reagents must also be compliant with current good manufacturing practices (cGMP) for cell therapies, which limits the use of some cell types, antigen sources, and other reagents. Finally, the time required to produce, test, and release the expanded product is significant. These many obstacles have limited broader application of the approach, but, as we describe below, all are being progressively surmounted.

Epstein Barr virus

EBV is a ubiquitous human herpesvirus, and over 95% of the adult population is seropositive, indicating a previous infection (72, 73). After primary infection, the virus persists

in latent form in B lymphocytes and epithelial cells in the nasopharynx, and periodic replicative reactivations in B cells are tightly controlled by a very strong viral antigen-specific T-cell response so that up to 1–2% of circulating T cells in a normal EBV seropositive individual may be specific for EBV (74–76). After transplant when T-cell immunity is impaired, EBV-infected B cells that would normally be controlled by an effective EBV-specific cytotoxic T-cell response can outgrow, resulting in EBV-PTLD. The risk of this complication after HSCT ranges from 1% to 20% with risk factors including the use of a T-cell-depleted stem cell product, a higher degree of HLA-mismatch between donor and recipient, and the use of anti-thymocyte globulin (ATG) in reduced intensity transplant-conditioning regimens. PTLD developing after HSCT is usually derived from donor B cells but can be of recipient origin, particularly in patients who receive reduced-intensity conditioning regimens that included ATG or alemtuzimab (Campath), or who receive cord blood transplants.

The outgrowing tumor cells in EBV-PTLD are highly immunogenic and express all nine latent EBV antigens. Since almost all transplant donors are EBV seropositive with a high frequency of circulating EBV specific precursors, EBV-PTLD is a good target disease to test the activity of *ex vivo* expanded virus antigen specific T cells. In the early 1990s, we were using selective T-cell depletion with monoclonal antibodies to reduce the risk of GVHD in patients receiving transplants from matched unrelated donors or mismatched family members and about 10% of these patients developed EBV PTLD (77). We therefore devised a strategy to prevent and treat this complication by infusing EBV-specific T cells

generated by stimulating donor mononuclear cells with an irradiated EBV transformed donor LCL (78). LCLs could be easily generated from any donor by incubation with a laboratory strain of EBV and were excellent APCs that had the same pattern of viral gene expression as the outgrowing tumor cells. EBV-specific T-cell lines grown after stimulation with LCLs were polyclonal, containing both CD4⁺ and CD8⁺ T-cells that recognized multiple latent and early lytic cycle EBV antigens.

We have reviewed our experience administering EBV-specific T cells generated with this methodology as prophylaxis to over 100 high risk patients and as treatment for 13 patients with established PTLD (Tables 2 and 3). Of the patients who received the VSTs as prophylaxis, none developed PTLD compared with an 11.5% incidence in controls (22, 79–81). Thirteen patients received EBV-specific T cells for active disease, and 11 achieved sustained complete remissions. In the first 26 patients, the T-cell lines were genetically marked with a retroviral vector encoding the neomycin resistance gene, which enabled us to track the cells and show they could expand by up to 3–4 logs and persist for up to 9 years (22). This long-term persistence may have been bolstered by infusion into lymphopenic recipients, in whom homeostatic mechanisms promoted lymphoid expansion and by continued T-cell stimulation by periodic EBV reactivations. Animal studies also suggest that the presence of CD4⁺ T cells in the infused line is important for persistence and access to the memory pool. The infusions were well-tolerated, although in patients with bulky disease, we observed reversible inflammatory reactions at disease sites due to infiltration with activated T cells. Of

Table 2. Outcome of prophylaxis with donor virus-specific T cells (VSTs) in our studies

Specificity	Virus	Patients	No viral infection	Failure	References
Monovirus VSTs with prolonged culture LCL-induced EBV VSTs	EBV	105	105	0	Rooney <i>et al.</i> (80) Heslop <i>et al.</i> (79) Rooney <i>et al.</i> (81) Heslop <i>et al.</i> (22)
Bivirus VSTs with prolonged culture AD5/35 induced EBV/ADV CTLs	EBV	13	13	0	Leen <i>et al.</i> (101)
	AdV	12	12	0	
Trivirus VSTs with prolonged culture AD5/35 pp65 induced CMV/ADV/EBV VSTs	EBV	20	20	0	Leen <i>et al.</i> (102)
	AdV	20	20	0	
	CMV	14	14	0	
Trivirus VSTs with rapid culture Plasmid-induced CMV/ADV/EBV VSTs	EBV	9	9	0	Gerdemann <i>et al.</i> (112)
	AdV	7	7	0	
	CMV	6	6	0	
Total	EBV	147	147	0	
	AdV	39	39	0	
	CMV	20	20	0	

Table 3. Outcome of treatment with donor virus-specific T cells (VSTs) in our studies

Specificity	Virus	Patients	Partial or complete response	Failure	References
Monovirus VSTs with prolonged culture LCL-induced EBV VSTs	EBV	13	11	2	Rooney <i>et al.</i> (80) Heslop <i>et al.</i> (79) Rooney <i>et al.</i> (81) Heslop <i>et al.</i> (22)
Bivirus VSTs with prolonged culture AD5/35 induced EBV/ADV VSTs	EBV	1	1	0	Leen <i>et al.</i> (101)
	AdV	2	1	1	
Trivirus VSTs with prolonged culture AD5/35 pp65 induced CMV/ADV/EBV VSTs	EBV	6	6	0	Leen <i>et al.</i> (102)
	AdV	6	5	1	
	CMV	11	10	1	
Trivirus VSTs with rapid culture Plasmid induced CMV/ADV/EBV VSTs	EBV	2	2	0	Gerdemann <i>et al.</i> (112)
	AdV	4	4	0	
	CMV	5	4	1	
Total	EBV	22	20	2	
	AdV	12	10	2	
	CMV	16	14	2	

note, the infused cells were not alloreactive and there was no *de novo* GVHD after infusion (82).

Similar response rates were seen in another study from Memorial Sloan Kettering Cancer Center (MSKCC), where Doubrovina *et al.* (83) treated 14 patients with active PTLD with donor-derived EBV-specific T cells and showed complete responses in 10. The T cells given to the three patients who failed to respond recognized the LCLs transformed with the laboratory strain B95-8 used to stimulate them but not the strain of EBV expressed by the patients' tumors. Similarly, in one of the patients in our study who had an initial response then progressed, we found expression of an EBV variant with a deletion in the immunodominant epitopes recognized by the infused line (84). The infused line was able to target the parental tumor cells but the tumor cells with the deletion subsequently grew out. In another patient in the MSKCC series the donor VST line was skewed in its response to EBV antigens by an HLA antigen A1101, which was present only in the donor and the tumor was derived from recipient cells. The patient subsequently responded to a third party line that was matched at fewer antigens but had strong EBV activity through a shared antigen (83). This experience emphasizes the importance of ascertaining whether PTLD is of donor or recipient origin and of infusing a line with antiviral activity through a shared antigen.

The results of these and other studies confirmed that donor-derived EBV-specific T-cell therapy using T-cell lines generated using LCLs as stimulator cells is safe and effective as prophylaxis and treatment for PTLD after HSCT (85–88). While the strategy has been implemented at multiple

institutions, a limitation is the time taken to generate the LCL line (around 4 weeks) and then the VST line (3–6 weeks). Furthermore, as illustrated above, the use of a laboratory strain of EBV to generate the LCL lines may stimulate a line that does not recognize all EBV strains and makes moving the approach to licensing studies more difficult.

Cytomegalovirus

CMV is a latent β -herpesvirus that in immunocompetent individuals usually causes asymptomatic infection. However, reactivation of CMV in immunocompromised individuals can result in significant morbidity and mortality, with clinical manifestations including interstitial pneumonitis, gastroenteritis, fevers, hepatitis, encephalitis, and retinitis (8, 89). In the immunocompetent host, CMV-specific CD4⁺ and CD8⁺ T cells play an important role in immune protection from both primary infection and subsequent reactivations. Therefore, a number of groups have investigated whether the adoptive transfer of *in vitro* expanded donor-derived VSTs can provide protection during the immunodeficient phase that follows HSCT.

In pioneering work, Walter *et al.* (90) used fibroblasts infected with the AD169 strain of CMV to stimulate donor T cells followed by limiting-dilution cloning to isolate cytolytic CD8⁺ CMV-directed T cells for adoptive transfer. Cells were administered prophylactically to 14 recipients of HLA-matched transplants at weekly intervals in doses escalating from 3.3×10^6 to 1×10^9 /kg from 30 to 40 days post-transplant. While the majority of patients lacked any

CMV-specific T-cell activity prior to infusion, such activity was subsequently detected in all recipients. TCR clonotyping studies showed that the transferred cells persisted for at least 8 weeks but progressively declined in patients who did not develop a concomitant endogenous CMV-specific CD4⁺ T-cell response. This observation highlights the importance of CD4⁺ helper T cells in sustaining antiviral activity *in vivo*. Neither CMV viremia nor disease developed in any of the treated patients. More recently, the same group performed a phase II study in which donor-derived CD8⁺ and CD4⁺ T-cell clones were combined and infused prophylactically to 35 CMV seropositive HSCT recipients. The infusions were safe and immediately augmented CMV-specific T-cell immunity. Perruccio *et al.* (91) also prepared T-cell clones using donor PBMCs loaded with CMV lysate as a stimulus. In this study, the investigators exclusively generated CD4⁺ CMV-specific T-cell clones, which were infused to 25 haploidentical HSCT recipients at doses ranging from 10⁵ to 3 × 10⁶ cells/kg. Again the cells proved to be safe *in vivo* and infusion accelerated the recovery of endogenous CD8⁺ CMV-directed T cells and controlled CMV antigenemia (91).

As an alternative to infusing clones with a limited diversity of antiviral specificity, other groups have used polyclonal T cells directed to a broader array of CMV antigens and epitopes to prevent and treat CMV infections post-transplant. For example, Einsele *et al.* (92) generated CMV-directed lines using donor PBMCs loaded with CMV lysate as a stimulus. The resultant VSTs contained both CD4⁺ and CD8⁺ T cells, which were infused to eight matched or mismatched related HSCT recipients who lacked endogenous CMV-directed immunity and who were suffering from drug-resistant CMV viremia (defined as the presence of CMV DNA in the peripheral blood after a minimum of 4 weeks of antiviral chemotherapy). Infusions of 1 × 10⁷ cells/m² were safe and resulted in a significant viral load reduction in seven evaluable patients, which was durable in five (92). In the two patients with the highest viral loads, however, the antiviral effect was only transient. One of these patients responded to a second VST infusion, whereas the other succumbed to fatal CMV encephalitis after refusing a second dose of cells. Similar successful outcomes were reported by Peggs *et al.* (93), who used DCs loaded with inactivated CMV antigen to stimulate PBMCs from allogeneic HSCT donors. Sixteen patients were treated with 1 × 10⁵ VSTs/kg at a median of 36 days post-transplant. The infusions were safe and reconstituted sufficient antiviral immunity to enable additional antiviral drugs to be avoided for eight patients. Finally, Gottlieb *et al.* reported a phase II study in which

CMV-VSTs were generated using either peptide-loaded DCs or DCs transduced with an adenoviral vector encoding the full length pp65 antigen. A total of 50 patients received a single dose of 2 × 10⁷ VSTs/m² from day 28 post-transplant, and their outcomes were compared with a group of contemporaneous controls. There was no difference in acute or chronic GvHD between the groups and overall and progression-free survival was similar. However, there was a reduction in the percentage of patients who required CMV-directed antiviral therapy (17% versus 36%) and in the total number of treatment days in the VST cohort (3.4 days versus 8.9 days) (94–96). Thus, VSTs can be of direct benefit in preventing/treating CMV reactivations, reducing the requirement for antiviral therapy with a corresponding reduction in drug costs and drug and disease-associated morbidity.

To increase the practicality of using adoptively transferred T cells and to extend the application to additional viruses, we have developed protocols for the generation of VSTs with simultaneous activity against multiple agents.

Multivirus VSTs

To generate VSTs directed to the most common post-transplant serious viral infections (CMV, AdV, and EBV), we prepared APCs consisting of activated monocytes and EBV-LCLs transduced with a clinical grade adenoviral vector, either encoding the immunodominant CMV-pp65 antigen for the generation of trivirus VSTs from CMV seropositive donors (97, 98), or modified with a null vector for the generation of bivirus-directed VSTs from CMV seronegative donors (99–101). Thus, the EBV-LCL presents viral antigens derived from the transforming EBV, the transducing adenoviral vector, and the encoded CMV pp65 transgene. When used to stimulate donor PBMCs, these 'double' or 'triple-positive' LCLs consistently reactivated polyclonal VSTs with activity against the target viruses in a single culture. We administered small numbers (5 × 10⁶–1.5 × 10⁸ cells/m²) of these *in vitro* expanded donor-derived bivirus or trivirus VSTs to recipients (age range 1–63 years) of haploidentical, HLA-matched related or unrelated donor transplants, none of whom developed GVHD. Postinfusion, we observed a detectable increase in the precursor frequency of T cells directed against both EBV and CMV in peripheral blood, but circulating AdV-specific T cells increased only in those recipients who also had evidence of an AdV infection, demonstrating the importance of *in vivo* restimulation by viral antigens to support the expansion of the infused cells. We also saw evidence of activity against all three viruses *in vivo* (101, 102).

Ten of 11 patients with CMV reactivation (defined as increased CMV DNA or CMV antigen positive) had a complete or partial response (defined as >50% reduction in the viral load) to VSTs, with a coincident increase in CMV-specific T cells. Similarly, seven patients with evidence of EBV reactivation (including 1 with EBV-PTLD) also responded to VSTs, with a marked expansion of EBV-reactive T cells. Finally, six or eight recipients with AdV infections (defined as culture positive in their respiratory tract, blood, or stool) or disease at the time of VST infusion had a marked reduction in their adenoviral load concomitant with a rise in the frequency of their circulating AdV-specific T cells. This included one patient with progressive adenoviral pneumonia requiring maximal ventilatory support who had a complete response to VSTs with clearance of AdV from his respiratory tract. All results are summarized in Tables 2 and 3. It is also noteworthy that neither bivirus nor trivirus VST recipients developed *de novo* AdV infections post-infusion, in contrast with an incidence of 68% in similar patients who did not receive VSTs (67), suggesting that the infused cells may survive at levels below the threshold for detection and protect patients long term, perhaps by entering the memory pool. This possibility is supported by our ability to detect AdV-reactive cells after a single *ex vivo* restimulation of blood T cells with adenoviral antigens (99, 100, 103). Hence, broad spectrum antiviral protection and treatment can be provided from a single infusion of cells and small numbers of T cells can provide long-term antiviral protection.

Manufacturing limitations for VSTs

As described above, the administration of *ex vivo* activated and expanded antigen-specific VSTs with single or multivirus specificity has a proven clinical track record of safety and efficacy in the immunocompromised host that spans almost 20 years. Unfortunately, however, until recently the approach has proved difficult to scale for broad application. Manufacturing of VSTs is technically demanding and prolonged; for trivirus VSTs, for example, the generation of the EBV-LCL requires 4–6 weeks followed by an additional 4–6 weeks for VST activation and expansion, and a further 1–2 weeks for identity, sterility, and potency testing. In addition, manufacturing must be performed in a facility approved to make cells that meet cGMP standards, and such facilities are expensive to build and maintain. In addition, the costs of manufacturing and release testing live EBV virus (B95-8 strain) and clinical grade viral vectors are also high. Finally, all these processes demand high skill and substantial time commitment from manufacturing technologists. Fortu-

nately, it has now become possible to address many of these issues of cost, complexity, and scalability.

Reducing the VST manufacturing cost

To reduce costs and alleviate concerns associated with the use of live virus/viral vectors to manufacture VSTs, we have investigated alternative sources of antigen with which to stimulate donor PBMCs. We replaced our adenovector and EBV-LCLs by generating DNA plasmids encoding immunogenic antigens from EBV (EBNA1, LMP2, BZLF1), CMV (IE1, pp65), and AdV (Hexon, Penton), which could be introduced into APCs, such as DCs, using the clinically acceptable AMAXA nucleofection system. In preclinical studies, we achieved high level transgene expression following nucleofection of DCs with good viability during the period of T-cell activation and the reproducible generation of VSTs that were phenotypically similar to our conventionally generated VSTs (mix of CD4⁺ and CD8⁺ T cells, a proportion of which retained expression of the central memory marker CD62L), and that contained virus-specific T cells at frequencies that were comparable or superior to conventional products (104). Clinical grade plasmid-DNA can be rapidly and cost-effectively produced in scalable quantities with excellent long-term stability. Since plasmids are non-infectious, non-replicative, and integrate poorly into the transfected cell genome, this approach also alleviates safety concerns. Thus, transition to such an antigen source would substantially lower the production costs of VSTs.

More recently we evaluated direct stimulation of donor PBMCs with commercially available GMP-grade peptide mixtures (pepmixes). These consist of 15mer peptide libraries derived from viral antigens that overlap by 11 amino acids. Since each peptide is 15 amino acids in length, the pepmixes cover all possible CD8⁺ and the majority of CD4⁺ epitopes (105). These 15mer-produced VSTs were phenotypically and functionally equivalent to conventionally generated VSTs. This refinement eliminates the need for DCs or other specialized APCs and for virus/viral vectors for stimulation, resulting in a >50% reduction in manufacturing costs.

Reducing VST manufacturing complexity

Conventional VSTs are made by sequential expansion steps in traditional 24-well tissue-culture plates, with weekly restimulation and regular splitting of the cultured cells. This system is extremely labor intensive, highly skilled, and

essentially unscalable. The frequent media changes required to optimize nutrient and cytokine levels and remove waste products make contamination a constant risk and still allow T cells to undergo significant cell death. Efforts to substitute the closed and scalable bioreactor systems that are widely used in other types of clinical cell culture systems failed to produce VSTs with the same anti-viral specificity and effector function. To address this logistic issue, we collaborated with Wilson Wolf Manufacturing to develop a new disposable gas permeable rapid expansion device (G-Rex), which dramatically reduces T-cell apoptosis during culture, resulting in more efficient *in vitro* expansion. In this static device, gas exchange (O_2 in and CO_2 out) occurs across a gas permeable silicon membrane at the base, allowing for the initial input media volume to be increased, which in turn increases the available nutrients and dilutes waste products without the need for culture agitation, frequent culture feeding, or continuous medium perfusion (106–108). These optimized growth conditions allow for higher antigen-specific T-cell densities per unit surface area to be achieved ($8\text{--}10 \times 10^6$ per cm^2 in the G-Rex compared with approximately 1.5×10^6 per cm^2 in wells), and incorporation of this platform substantially simplifies VST production by minimizing the number and complexity of cell manipulations without affecting the phenotype or function of the generated T-cell lines (109).

Reducing antigenic competition

Until recently, a maximum of three viruses could be successfully targeted in a single VST. Efforts to make VSTs that were consistently specific for additional viruses had foundered on the problem of antigenic competition. This phenomenon occurs due to competition between virus-derived peptides to become bound to HLA molecules expressed on shared APCs and the physical constraints on the capacity of APCs to simultaneously engage T cells with different specificities if these are present at substantially different frequencies. Indeed, even the conventional trivirus (EBV+CMV+AdV)-directed VSTs described above are dominated by CMV-specific T cells at the expense of the EBV and AdV-specific components, so that both the breadth of AdV epitopes recognized and the magnitude of the response to them is less in the trivirus products than in bivirus products generated using EBV-LCLs transduced with an adenoviral vector lacking the CMV-pp65 transgene (100). This antigenic competition and the resultant production of lines with restricted viral specificities precludes the generation of potent, broad-spectrum effector T cells that

would be effective against the complete array of pathogens present in HSCT recipients.

Our conventional VST manufacturing process did not incorporate exogenous cytokines, which we discovered adversely affected VST proliferation and survival *in vitro*, increasing their susceptibility to activation-induced cell death (AICD), and contributing to a restricted repertoire of epitope recognition. Thus, we increased the range of viral antigens that could be recognized by a single VST line and thereby mitigated the impact of antigenic competition. We supplemented cultures of peptide-pulsed PBMCs with cytokines shown to support T-cell proliferation *in vitro* and *in vivo* (IL-2, IL-15), as well as cytokine combinations (IL-4+IL-7) that support retention of a central memory phenotype, and promote activated T-cell survival by upregulating anti-apoptotic molecules (110, 111). When lines were supplemented with IL-4+IL-7, we observed expansion and survival of both $CD4^+$ and $CD8^+$ virus-specific T cells within the PBMCs, which recognized multiple viral epitopes and killed virus-infected targets (105). The induced cells were Th1-polarized despite exposure to IL-4, a prototypic Th2 cytokine. Importantly, these VST cultures lacked alloreactive T cells, even when the VSTs had been produced by only a single *in vitro* stimulation with viral peptides. By this approach, we were able to alleviate antigenic competition both within the APC and between T cells.

Clinical results using rapidly generated VSTs

To learn whether our manufacturing modifications produced clinically active VSTs that performed as well as or better than conventionally generated VSTs, we initiated two clinical studies. In the first clinical trial, we assessed the safety and potency of trivirus VSTs, produced using plasmid-nucleofected DCs as APCs followed by expansion for 10 days in the G-Rex device in media supplemented with IL-4+IL-7. These rapidly generated trivirus VSTs were infused to 10 allogeneic HSCT recipients (five haploidentical, four matched unrelated donor, one mismatched unrelated donor, and one matched related donor), all of whom had active infections with one or more of our target viruses (3 CMV, 2 AdV, 2 EBV, 2 EBV+AdV, and 2 CMV+AdV). Each patient received $0.5\text{--}2 \times 10^7$ cells/ m^2 from day 27 post-HSCT. One patient developed a mild and localized skin rash postinfusion, but this patient also had an intercurrent BK infection and had presented with a similar rash during an earlier episode of BK reactivation. No other infusion-related toxicities were noted. Overall, our clinical response rate was equivalent to that achieved in our previous trials of trivirus-directed T cells generated using EBV and AdV

vectors for manufacturing, with 90% of subjects with active AdV, CMV, and/or EBV infections having a measurable increase in circulating T cells directed against the infecting virus(es), and clearance of both single and dual viral reactivation/disease. Importantly, the lines in this study were generated without exposure to biohazards (live virus/viral vectors) and were manufactured in only 17 days (versus 8–12 weeks for 'conventional' VSTs) and from 60 ml of peripheral blood without evident loss of efficacy (112).

More recently, we have initiated a clinical trial based around a further manufacturing simplification, in which donor PBMCs are stimulated by pepmix combinations spanning 12 immunogenic antigens from five clinically relevant viruses (EBV, CMV, AdV, BK, HHV6) followed by expansion for 10 days in the G-Rex device using media supplemented with IL-4+IL-7 (112, 113). To date, we have infused these pentavalent (p)VSTs to 10 allogeneic HSCT recipients at doses ranging from 5×10^6 to $2 \times 10^7/m^2$ with no immediate infusional toxicities, and no *de novo* acute GvHD. This study is still ongoing, but to date the infused pVSTs have successfully controlled active infections associated with all five of the target viruses. Thus, it appears that rapidly produced (10 days) VSTs targeting five clinically relevant pathogens that are generated from 20 ml of peripheral blood can produce clinical benefit, and we are currently exploring the extension of this platform to include additional clinically relevant viruses.

The manufacturing modifications described above should help VST therapies become a standard of care for transplant recipients by facilitating adoption of the approach in sponsored late phase clinical studies.

Extending VST therapy to recipients irrespective of donor availability/immunity

Despite the promising results of VST therapy in the allogeneic HSCT setting, it may be more challenging to translate this therapeutic modality to recipients of grafts from seronegative donors such as those derived from cord blood, an increasingly important alternative source for stem cells. Two approaches may be used to serve such individuals.

VSTs from cord blood donors

Generation of a virus-specific T-cell product from cord blood for infusion is complicated by the low numbers of available cells, their naive phenotype, and the associated low frequency of virus-reactive T cells. Hence, the generation of VSTs *in vitro* requires the use of donor DCs as professional

APCs and the addition of enhancing cytokines IL-12, IL-15, and IL-7. Whether such cells will have the same *in vivo* persistence and antiviral activity as VSTs generated from seropositive donor peripheral blood remains to be seen (114). At last report (Hanley et al., American Society of Hematology annual meeting, 2012), seven patients received between 5×10^6 and 2.5×10^7 cells/ m^2 on days 63–146 after cord blood transplant, with no infusion-related toxicities or subsequent GvHD. Five patients had no initial infection or reactivation episodes. Two patients had evidence of CMV or EBV reactivation, both of which were controlled, although for CMV two VST infusions were required. Thus, early reports indicate that the infusion of these cord blood derived antigen-specific T cells can support immune reconstitution *in vivo*.

Third party VST banks

Despite the clinical benefit associated with VST therapy and the ease of manufacturing afforded by our production refinements, the need to generate specific VSTs for each individual patient renders this approach impractical for widespread or urgent use, or when the donor lacks viral immunity (as for cord blood transplant described above). To circumvent this requirement, investigators have prepared and administered banks of closely HLA-matched VSTs that are 'off the shelf' products and so are available for immediate use. Administration of such products is, however, associated with some concerns. Most lines administered will be mismatched at one or more HLA-loci, which may either increase GvHD postinfusion due to alloreactivity of the line or reduce *in vivo* persistence and antiviral benefit, due to the alloreactivity of the recipient. Despite these concerns, a number of studies have shown that the approach is feasible, safe, and effective, with reports of a high level of clinical responses. For example, Haque et al. (115, 116) used third party EBV-specific VSTs to treat PTLD after solid organ or HSCT transplantation. In this study, patients received four doses of 2×10^6 VSTs/kg at weekly intervals and the lines were selected for matching by low resolution typing and screened for high level killing of donor EBV-LCLs and low level killing of patient-derived PHA blasts. No patient developed organ rejection or GVHD, and the study showed response rates of 64% and 52% at 5 weeks and 6 months respectively. The degree of HLA matching between the line and the recipient ranged from 2/6 to 5/6 HLA antigens, and at 6 months, there was a statistically significant trend toward a better outcome with closer matching (115, 116). In a second report, two solid organ transplant recipients

with CNS lymphoma received closely matched EBV-specific T cells leading to complete resolution of brain lesions due to EBV-LPD (117). Similarly, the Memorial Sloan Kettering group reported that third party EBV-specific VSTs produced complete responses in four of five patients with EBV PTLD after HSCT (including two cord blood transplant recipients) (83, 118). More recently, our group applied this approach to treat patients with refractory CMV, AdV, or EBV infections (119). In this multicenter study, we established a bank of 32 trivirus-directed VSTs using conventional manufacturing approaches, 18 of which were administered to 50 recipients of allogeneic HSCTs [marrow ($n = 24$), peripheral blood ($n = 33$), or cord blood ($n = 12$ single and 13 double units)]. Our bank was of sufficient size for us to identify a suitable line for 90% of the patients screened for participation. Lines for infusion were chosen based on the presence of activity against the infecting virus through a shared HLA allele(s), which requires a comprehensive analysis of viral epitopes in the VSTs and the identification of the HLA restricting elements associated with each. Once activity against the infecting virus through a shared HLA allele(s) was confirmed, further selection of the best line was based on the overall degree of HLA match. Of the 50 patients who were treated, 74% had a complete or partial response (74% for CMV, 78% for AdV and 67% for EBV), the majority of which were durable (Table 4). *In vivo* T-cell persistence, monitored using deep sequencing analysis, indicated that donor VST-derived TCR sequences became apparent concomitant with a reduction in viral titers and persisted 4–12 weeks postinfusion. Although long-term (beyond 3 months post-infusion) analysis was not performed the expectation is that the third party cells were eliminated concomitant with endogenous immune recovery. Indeed, of the 50 patients treated, 14 required between 2 and 6 VSTs to sustain clinical benefit. However, we saw no significant *de novo* GvHD,

Table 4. Outcome of treatment with third party virus-specific T cells (VSTs) in our studies

Specificity	Virus	Patients	Complete or partial response	Failure	References
Trivirus VSTs with prolonged culture					
AD5/35 pp65 induced CMV/	EBV	9	6	3	Leen et al. (119)
AdV/EBV VSTs	AdV	18	14	4	
	CMV	23	17	6	
Total		50	37	13	

and only one episode of secondary stem-cell graft failure (in a patient with relapse). Thus, this study, like the other applications of banked VSTs, observed no associated increase in toxicity.

Since the available clinical data support both the safety and clinical activity of third party VSTs, it should be possible to use such banks on a larger scale, particularly given the availability of more rapid VST manufacturing technology (see section ‘Manufacturing limitations for VSTs’ above), thereby making the adoptive transfer of VSTs a standard of care for transplant recipients in which these third party cells may be used as an immediate measure while the need for specific donor-derived VSTs can be assessed and, if necessary, this additional product made.

Future perspectives

It is likely that VSTs will have an increasing role to play in the prevention and management of post-transplant viral infections. Our ability to rapidly select VSTs for viruses such as CMV and EBV and to relatively rapidly culture cell lines specific for a broad range of common viral antigens, coupled with the feasibility of developing third-party off the shelf VSTs will ensure that the advantages of this approach compared with available small molecule therapies will become increasingly evident.

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