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Challenges of T Cell Therapies for Virus-associated Diseases after Hematopoietic Stem Cell Transplantation

Ann M. Leen, **Tamara Tripic**, and **Cliona M. Rooney**
Center for Cell and Gene Therapy, Baylor College of Medicine, The Methodist Hospital, and Texas Children's Hospital, Houston, Texas

Abstract

Importance of the field—Hematopoietic stem cell transplantation (HSCT) is the treatment of choice for many hematological malignancies and genetic disorders. A majority of patients do not have a human leukocyte antigen (HLA) identical sibling donor, and alternative stem cell sources include HLA-matched or mismatched unrelated donors and haploidentical related donors. However, alternative donor HSCT are associated with three major complications (i) graft rejection, (ii) graft-versus-host disease (GvHD) and (iii) delayed immune reconstitution leading to viral infections and relapse.

Areas covered in this review—Graft rejection and the risk of GvHD can be significantly reduced by using intensive conditioning regimens, including *in vivo* T cell depletion as well as *ex vivo* T cell depletion of the graft. However, the benefits of removing alloreactive T cells from the graft are offset by the concomitant removal of T cells with anti-viral or anti-tumor activity as well as the profound delay in endogenous T cell recovery post-transplant. Thus, opportunistic infections, many of which are not amenable to conventional small-molecule therapeutics, are frequent in these patients and are associated with significant morbidity and high mortality rates. This review discusses current cell therapies to prevent or treat viral infections/reactivations post-transplant.

What the reader will gain—The reader will gain an understanding of the current state of cell therapy to prevent and treat viral infections post-HSCT, and will be introduced to preclinical studies designed to develop and validate new manufacturing procedures intended to improve therapeutic efficacy and reduce associated toxicities.

Take home message—Reconstitution of HSCT recipients with antigen-specific T cells, produced either by allodepletion or in vitro reactivation, can offer an effective strategy to provide both immediate and long-term protection without harmful alloreactivity.

Viral Infections After HSCT

Increasing numbers of viral pathogens have been implicated in infectious complications after HSCT, due to a combination of more intensive screening using improved detection methods and the extension of HSCT to higher risk patients who either receive more extensively manipulated products or who require more intensive and prolonged post-transplant immunosuppression[1–6]. Infections caused by endogenous herpesviruses like Epstein-Barr virus (EBV) and cytomegalovirus (CMV) are well documented, while more recently infections caused by human herpesvirus (HHV)-6, BK virus and the respiratory viruses respiratory syncytial virus (RSV), parainfluenza, metapneumovirus, adenovirus, and bocavirus are increasingly reported[1–3;5;7–19] (Table 1). Pharmacologic agents are standard therapy for some

Correspondence: Ann M. Leen, Center for Cell and Gene Therapy, Baylor College of Medicine, 1102 Bates Street, 760.01, Houston, TX 77030, Phone: 832-824-4690, Fax: 832-825-4732, amleen@txccc.org.

infections, but most have substantial toxicities, drive the outgrowth of resistant viral variants, and are not effective against all viruses. Since the use of anti-virals does not improve virus-specific immunity, infections frequently recur after termination of treatment. In contrast, reconstitution of HSCT recipients with antigen-specific T cells can offer an effective non-toxic strategy to provide both immediate and long-term protection. Such immunotherapeutic strategies have been explored by a number of groups.

Donor lymphocyte infusions

The first adoptive T cell transfer protocols in the allogeneic HSCT setting were based on the premise that donor peripheral blood contained T cells able to mediate antitumor and/or antiviral activity in the HSCT recipient. Accordingly, donor lymphocyte infusions (DLI) have been extensively used to provide anti-tumor immunity[20–23], and to a lesser extent, antiviral immunity. DLIs should contain memory T cells specific for a broad range of viruses, however, while successful for the treatment of a proportion of infections with adenovirus and EBV[24; 25], the efficacy of this therapy is limited by the low frequency of T cells specific for many common “acute” viruses (such as RSV and parainfluenza) and the relatively high frequency of alloreactive T cells. The high ratio of alloreactive to virus-specific T cells is especially problematic in recipients of haploidentical transplants in whom a higher incidence of GvHD limits the tolerable DLI dose, severely limiting the dose of virus-specific T cells received [26; 27].

Depletion of alloreactive T cells

To preserve the benefits and enhance the safety of DLI, strategies for the selective removal or inactivation of recipient-specific alloreactive T cells have been evaluated.

Induction of anergy—Antigen specific T-cell anergy can be induced ex vivo by T cell receptor (TCR) signaling in the absence of costimulation. T cells require at least two signals to become activated; signal 1 involves TCR engagement with peptide-loaded MHC molecules, while signal 2 is mediated by co-stimulatory molecules on T cells engaging their ligands on APCs. The interaction between the CD28 receptor on T cells and its ligands, B7-1 (CD80) and B7-2 (CD86) on APCs is one of the major positive co-stimulatory signals, and this can be blocked by fusion proteins, such as CTLA4-Ig, or monoclonal antibodies to CD80 or CD86. Guinan and colleagues investigated whether blockade of this interaction could be used clinically to render alloreactive donor T cells anergic. In their initial study they showed that co-culture of whole bone marrow with irradiated recipient cells in the presence of CTLA4-Ig for 36hrs reduced the frequency of alloreactive T cells, while reactivity to 3rd party cells was unaffected[28]. In 11 evaluable patients the alloanergized marrow could reliably engraft, and infusion of relatively large cell doses (median 28x10^6 CD3+ T cells/kg) was not associated with excessive GvHD [28].

In two follow-up phase I clinical trials the same group analyzed immune reconstitution, infection, and development of acute and chronic GvHD in a larger patient cohort who received haploidentical HSCT after ex vivo induction of alloantigen-specific anergy in donor T cells, again achieved using CTLA4-Ig. Again they found that alloanergization did not appear to impair immune reconstitution. The median absolute lymphocyte count (ALC) on day +30 was 0.42×10^9/L, which is similar to that of patients receiving unmanipulated BMT from HLA-matched sibling donors[29;30]. Further, despite relatively high numbers of anergized T cells (median CD3+ T cell dose was 29×10^6/kg) was not associated with an increased incidence of steroid-refractory acute or chronic GvHD. Of 11 high-risk patients (donor and/or recipient CMV seropositive), 5 reactivated CMV, but all were able to clear the infection with a short course (3 days) of antiviral therapy, and none developed either CMV disease or EBV PTLD. The authors are currently conducting a follow-up study using escalating doses of alloanergized

T cells to define the optimal cell dose to improve immune reconstitution without causing severe GvHD.

Comoli and colleagues investigated a similar approach to induce alloantigen-specific T cell anergy by adding a combination of CTLA4-Ig and cyclosporine A (CsA) to *in vitro* primary mixed lymphocyte reactions. This induced a state of unresponsiveness to recipient alloantigens in donor PBMC, leaving anti-viral activity intact [31]. However, the efficacy of this strategy remains to be tested clinically.

An alternative route to alloantigen-specific immune tolerance is to use the inhibitory/suppressive characteristics associated with regulatory T cells (Treg)32;33. To establish a role for Tregs in preventing GvHD after allogeneic SCT Rezvani and colleagues quantified the number of CD4+FOXP3+ Tregs in 32 donor grafts infused into HLA-matched siblings and found that a high frequency of Tregs in the donor was associated with a reduced risk of GvHD, while in 21 SCT recipients they found that a low CD4+FOXP3+ cell count early (day 30) after transplant was associated with an increased risk of GvHD34. The authors suggest that assessmentof Treg content can be used as a predictor of risk for acute GvHD and that *ex vivo* expanded Treg infusions could prevent or treat GvHD[34]. To this end, Hoffmann and colleagues validated the *ex vivo* expansion of large numbers of functional Tregs using cross-linked anti-CD3 and anti-CD28 antibodies together with high dose IL2[35]. Recently this has been translated to the clinic and Trzonkowski et al adoptively transferred *ex vivo* expanded Tregs to two patients, one with chronic and one with acute GvHD. The infusions were associated with clinical benefit. In the case of the patient with chronic GvHD complete resolution of symptoms was achieved, while the patient with grade IV acute GvHD showed a transient clinical improvement[36].

Selective allodepletion—A potentially more permanent approach to GVHD is to remove alloreactive T cells from the donor graft prior to infusion. Recipient-specific T cells activated by *in vitro* exposure to recipient cells, such as EBV-transformed lymphoblastoid cell lines (EBV-LCL) [37;38], activated lymphocytes39 or fibroblasts40, upregulate activation markers such as CD25[37;41–45], CD69[43;45;46], and CD137[40;47], and proliferate, allowing their removal or elimination by immunomagnetic depletion[47;49], apoptosis induction[50;51], photodepletion[39;48;52;53], or immunotoxin-conjugated antibodies[37;38;42;54;55].

Three clinical trials using allodepleted T cells have been reported, all prepared using the CD25-immunotoxin[42;54;55]. Two were performed in pediatric recipients of haploidentical stem cell transplants and one in adults receiving an HLA-matched related donor transplant. Compared to earlier studies reporting GvHD of grade II or greater in 40% of patients after infusion of 1×10^5 unmanipulated donor T lymphocytes/kg[56;57], Andre-Schmutz and colleagues found that infusion of doses as high as 8×10^5 allodepleted T cells/kg were safe and retained a virus-specific immune component since three of the infused patients with active CMV had a rapid increase in antigen-specific T cells post-infusion and subsequent resolution of their infections55. Solomon et al infused 16 elderly patients at high risk of severe GvHD with allodepleted T cells[42]. In eight patients who developed acute GvHD ranging from grade I/II (6 patients) to grade III/IV (2 patients), and the severity of disease correlated with the efficiency of depletion[42]. Finally, Amrolia and colleagues showed that the infusion of allodepleted cells to haploidentical SCT recipients was safe but a minimum dose of 1×10^5 cells/kg was required to produce accelerated anti-viral T cell recovery[54].

Taken together these studies demonstrated the feasibility of add-back T cell therapy for clinical use but they also highlighted a number of limitations with current strategies. First, the availability of the clinical grade IT may be an issue for larger phase II/III studies. Second, in the study from Amrolia and colleagues, recipient-derived EBV-LCLs, which require 4–6 weeks

to establish, were used as the T cell allo-stimulus. While EBV-LCLs provide an unlimited source of tumor-free professional antigen-presenting cells (APCs), LCL production increased the time required for T cell preparation[54]. This study also demonstrated the small window between the minimum cell dose for immune reconstitution and the maximum tolerate dose54. Third, achieving sufficient T cells for infusion can be challenging since the recovery of donor cells after allodepletion is approximately 10%. Thus a donor leukapheresis may be required and this is not feasible for unrelated stem cell donors. Finally, T cells specific for a most of pathogens circulate with lower frequency than those specific for persistent viruses like EBV and CMV58–60, therefore, even higher doses of allodepleted T cell may be required to provide full spectrum protection.

To allow the safe administration of larger T cell doses, suicide transgenes have been evaluated to mediate self destruction in case of adverse effects *in vivo*. The thymidine kinase gene from herpes simplex virus I (HSV-tk) has been used and validated clinically. TK phosphorylates the nontoxic prodrug ganciclovir, which then becomes phosphorylated by endogenous kinases to GCV-triphosphate, causing chain termination and single-strand breaks upon incorporation into DNA, which kills dividing cells. Several phase I-II studies, and a more recent Phase III study have shown that Ganciclovir administration can be used to reduce transferred TK-modified cells *in vivo*61–64. However, the TK gene product may be immunogenic and specific immune responses directed to this transgenic protein have been detected in vivo which may lead to the premature and unintentional elimination of infused cells65. Our group has investigated an alternative minimally-immunogenic approach in which allodepleted T cells were transduced with a retroviral vector encoding an inducible human caspase 9 (iCasp9) suicide gene66 and a selectable marker (truncated human CD19)67. Even after allodepletion, donor T cells could be efficiently transduced, expanded, and subsequently enriched by CD19 immunomagnetic selection, and that the engineered cells retained anti-viral specificity. Following, following iCasp activation with a small-molecule dimerizer over 90% of cells underwent apoptosis67. Thus, the scale-up of allodepletion doses should be feasible, and this is currently being tested at our Center in the Haploidentical transplant setting.

Infusion of *ex vivo* expanded CTL

An alternative strategy to prevent and treat specific viral infections after HSCT is the adoptive transfer of *ex vivo*-expanded T cells with antiviral activity. The specific expansion of virus-reactive T cells has the advantage of increasing the numbers of virus-specific T cells that can be infused without increasing alloreactive T cells.

Cytomegalovirus (CMV)—CMV is a persistent beta-herpesvirus that is frequently reactivated from recipient or host tissues after allogeneic SCT. Fatal pneumonitis may follow new infection or reactivation, and available therapies may fail or prove toxic[10]. Riddell and colleagues infused *in vitro* expanded cytomegalovirus (CMV) reactive CD8+ T cell clones into 14 allogeneic HSCT patients to prevent CMV reactivation, and found that the cells were safe and able to restore anti-viral immunity *in vivo*. The transferred cells persisted for at least 8 weeks based on T cell Receptor (TCR) clonotyping studies, but progressively declined in patients who did not develop a concomitant endogenous CMV-specific $CD4^+$ T helper response[68]. Subsequently, Einsele and colleagues generated polyclonal CMV-specific CTL lines containing both CD4+ and CD8+ T cells and infused them in patients with antiviral chemotherapy-resistant CMV viremia. The clinical results were impressive, and infusion of small numbers of cells (10^7cells/m^2) significantly reduced the viral load in 7 evaluable patients, an effect that was sustained long term in 5 subjects but was transient in the two who had the highest virus load (>10^5 CMV-DNA copies/mL). A second T cell infusion controlled infection completely in one patient, but the other eventually succumbed to fatal CMV encephalitis after refusing a second dose of CTL.

Similarly encouraging results using polyclonal CMV-specific CTL lines were published by Peggs and colleagues and more recently by Micklethwaite et al, although the CTL lines were generated using different antigen sources[69;70]. Peggs et al used DCs loaded with inactivated CMV antigen produced from human lung fibroblast cell cultures infected with human CMV (Towne strain) to stimulate PBMCs from allogeneic HSCT donors [69;71]. Sixteen patients were treated with 1×10^5 cultured CMV-specific T cells/kg at a median of 36 days post-transplant, after the first episode of CMV viremia. The infused cells were safe, did not cause GvHD, and expanded *in vivo* as confirmed by tetramer analysis in donors with informative HLA types. Furthermore, the cells appeared to be effective, resulting in reconstitution of viral immunity and in eight of the ten cases additional antiviral drugs were not required[69]. Micklethwaite and colleagues generated donor-derived, CMV-specific T cells for prophylactic use in 12 adult HSCT patients by stimulating polyclonal CD4+ and CD8+ T cells with DCs transduced with a chimeric adenoviral vector encoding the immunodominant CMV antigen pp65. There was no infusion-related toxicity and although four patients reactivated CMV the titer was low and antiviral therapy was not needed to achieve viral control[70].

Epstein Barr Virus (EBV)—T cell therapy has also been successfully used to prevent and treat viral EBV associated lymphoproliferative disorders (post transplant lymphoproliferative disease; PTLD) after HSCT or solid organ grafting. Although most patients respond to withdrawal of immunosuppression and/or the anti-B cell antibody rituximab[72], the disease may progress, with a fatal outcome. Rooney and colleagues generated EBV-specific CTL using EBV-LCLs as stimulators and transferred them to immunocompromised patients at risk of developing EBV-associated PTLD. Since 1993 this group has infused over 100 SCT recipients with donor-derived polyclonal T cell lines and established that a dose of 2×10^7 CTL/m^2 is safe and effective for both prophylaxis and treatment[73–75]. A similar approach has been used by other groups to achieve similar results[76;77]. The first 26 patients enrolled in the Rooney study received CTLs which were genetically marked with a retroviral vector containing the neomycin resistance gene (*neo*). Long-term follow-up showed that the marked cells could be detected for as long as 9 years post infusion.

Although effective in a majority of patients treated for active disease, CTL therapy for EBV has failed in exceptional cases. Although the EBV-LCLs used as APCs express a range of viral latent and early lytic antigens, their immunogenicity is hierarchical and HLA dependent and some CTL lines display specificity for a limited number of epitopes from 1 or 2 viral proteins[78–80]. Therefore, efforts to treat EBV-LPD may fail if the tumor mutates an immunodominantviral target antigen which is the major specificity contained within the CTL line. This complication was discovered in a patient with EBV-LPD whose CTL line was largely HLA-A11-restricted with specificity for 2 epitopes in EBNA3B, both of which were deleted in the tumor virus[81] This highlights the importance of infusing a CTL product which is polyclonal (CD4+ and CD8+) with broad antigen and epitope specificity in order to minimize the potential for tumor immune evasion.

Multivirus CTL—More recently the safety and efficacy of CTL lines simultaneously targeting EBV, CMV, and adenovirus (Adv) has been demonstrated in HSCT recipients. APCs were produced by expressing the immunodominant CMV-pp65 antigen in activated monocytes[82] and EBV-LCLs using a chimeric adenoviral vector83. These APCs consistently reactivated CTLs specific for all three viruses in a single culture, although their specificity was dominated by CMV-reactive T cells, with a smaller fraction of EBV- and adenovirus-reactive T cells[84;85]. Infusion of donor-derived, trivirus-specific CTLs was safe in recipients of HLA-matched related or unrelated donors and the infused cells demonstrated apparent activity against all three viruses *in vivo*. Strikingly, however, only the CTLs directed to EBV and CMV showed evidence of *in vivo* expansion and persistence. By contrast, adenovirus-specific CTLs were detected in the peripheral blood after infusion only in patients who also had positive

adenoviral cultures, demonstrating the importance of antigen *in vivo* as a stimulus for the infused cells[84]. However, none of the trivirus-specific CTL recipients developed adenovirus infections, by contrast to 68% of similar patients who did not receive CTLs[12], suggesting that the adenovirus-specific CTLs may survive and enter memory, likely residing in the spleen and circulating only during periods of infection. This supposition was supported by the observation that adenovirus-specific T cells could be detected if first expanded by antigenic restimulation *in vitro*[86]. Thus it appears that 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 anti-viral protection.

Limitations of current CTL generation protocols

Although the administration of *ex vivo* activated and expanded antigen-specific T cells with single or multivirus specificity appears to be a safe and effective means of preventing and/or treating viral infections that arise in the immunocompromised host, there are a number of limitations to the broader implementation of T cell immunotherapy. These include; (i) time taken to produce clinical grade CTL, (ii) costs associated with CTL production, (iii) complexity of production, (iv) competition between multiple viral antigens for HLA molecules on APCs and (v) the wide range of viruses that require coverage.

Time to manufacture CTL lines—In the case of EBV and trivirus CTL, the generation of the EBV-LCL used as APCs requires 4 to 6 weeks followed by an additional 4 to 6 weeks for CTL activation and expansion, followed by a 1–2 weeks to perform identity, sterility and potency testing. This precludes urgent treatment of seriously ill patients, and CTL must be prepared speculatively and in advance for patients judged to be high risk so that they are available if needed.

Cost—Besides the infrastructure cost of building and maintaining a GMP facility and maintaining regulatory components (quality assurance, quality control, data management), there are a number of production costs which must also be taken into consideration including the technician time to produce APCs and CTL for clinical use, the cost of manufacturing and testing clinical grade viral vectors that are used for genetic modification of T cells and APCs, the reagents and media for CTL production, and the release testing that must be performed on CTL lines prior to infusion to ensure identity, purity and potency. In 2009 the cost for manufacturing, testing and infusing of an EBV CTL line was $6,095[87], while the generation of a trivirus line was $10,559, excluding professional time. Although each line is a patient-specific product, it should be noted that this therapeutic modality nevertheless compares favorably with others; for example, CD20 monoclonal antibody therapy for treatment of EBV-LPD is $9,000 per dose.

Complexity—The production process itself is relatively complex, necessitating the generation and genetic modification of APCs for weekly CTL stimulation, repeat feeding of open culture systems, and multiple skilled “judgment calls”, which also serves to limit scalability.

Antigenic competition—While in our study trivirus-specific CTL could be generated consistently in a single culture[84] the lines were heavily dominated by CMV-reactive T cells[85]. This competition is likely due to a combination of factors including the lower frequency of circulating adenovirus-specific T cells relative to EBV and CMV-specific T cells in healthy donors[60;80;88], and to competition from the high affinity or more stable CMV and EBV epitopes for presentation by HLA molecules in the APCs[89;90]. This may limit the number of organisms to which a single CTL line can be reactive.

Spectrum of viruses—The range of viruses detected post-transplant is continually increasing as more reagents become available for screening and detection (Table 1). Some of these such as EBV, CMV, Adv, BK virus and HHV6 are clearly associated with graft failure and/or morbidity and mortality post-transplant, while more recently identified viruses such as metapneumovirus and bocavirus, though detected in the post-transplant setting, have not been definitively connected with severe disease[1;6]. Nevertheless, these emerging viruses must be considered in the development of future CTL protocols, should prospective studies identify them as causative factors in post-transplant morbidity and mortality.

Overcoming limitations of CTL therapies

Rapid CTL production—The direct isolation of HLA-multimer-binding T cells, or the selection of IFN-γ expressing T cells following stimulation with either recombinant protein or peptide stimulation allow rapid selection of virus-reactive T cells for direct infusion into patients. Several groups have demonstrated that small numbers of *ex vivo* selected, antigen-specific T cells can expand substantially after infusion into HSCT recipients and protect against the targeted pathogen[91–93]; a median of 8.6×10^3 per kg of tetramer selected and $1.2–50\times10^3$/kg per kg of T cells selected by their secretion of γ–IFN in response to antigen stimulation in the Miltenyi gamma catch system proved clinically effective. However, there are also limitations to these approaches; tetramer selection is restricted to CD8+ T cells with known epitope specificities and to viruses, such as CMV, with a high frequency of circulating reactive T cells[91;94]. The IFN-γ-capture assay provides an HLA unrestricted means to select specific T cells with both effector and central memory characteristics[95–98] that should persist *in vivo* and mediate long-term protection against viral challenge[99]. However a low frequency of circulating cells specific for certain viruses may limit T-cell recovery[100]. One potential approach to enhance T-cell recovery is to stimulate T cells with combinations of whole antigens from different viruses[96]. Increased numbers of activated and selected cells may support the survival of low frequency antigen-reactive cells, however a minimum effective dose for infusion is yet to be established.

Third party banks—To bypass the need to grow CTLs for individual patients, banks of CTL lines that are available as an “off the shelf” product for immediate use have been evaluated. Since it is unlikely that a completely HLA-matched line will be available, the most closely HLA-matched line is administered. This raises two potential concerns; (i) the risk of inducing GvHD by administering a 3rd party CTL product101–[103] and (ii) limited *in vivo* persistence, due to recipient alloreactivity to non-shared HLA antigens. However, a number of small studies have shown the feasibility of this approach and reported clinical responses in the patients with EBV lymphoma arising after HSCT or solid organ transplant[104;105]. Haque and colleagues used 3rd party EBV-specific CTLs to treat PTLD after solid organ transplant or SCT and showed an encouraging response rate of 64% and 52% at 5 weeks and 6 months, respectively[106]. In this study patients received 4 doses of 2×10^6 CTL/kg at weekly intervals. 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 PHA blasts. The degree of HLA matching ranged from 2/6 to 5/6 antigens and there was a statistically significant trend towards a better outcome with closer matching at 6 months. Importantly no patient developed GVHD post CTL administration[106]. In another report two solid organ recipients with CNS lymphoma received closely matched EBV-specific T cells resulting in complete resolution of their brain lesions[107].

Given the promising results using “allogeneic” EBV-specific CTL we are currently evaluating the safety and feasibility of using “off-the-shelf” trivirus CTL for treating HSCT recipients with CMV, adenovirus or EBV infections that persist despite standard therapy. In this multicenter phase I clinical trial, CTL lines for infusion are chosen based first on the presence

of activity against the problem virus through the shared HLA allele(s), and second on the overall degree of HLA matching. For example, we would favor a CTL line matched at a single allele through which there was documented antiviral activity over a line matched at three alleles through which antiviral reactivity was not detected. Preliminary results in ten recipients, most of whom had received alternative donor transplants were encouraging. None experienced acute GvHD and complete or partial responses were achieved in 5 of 8 evaluable recipients. If this trend continues we will generate a larger CTL bank to cover as many racial groups as possible and progress to a Phase II clinical trial where we can ask more specific questions regarding the persistence and function of the CTL *in vivo*.

Reducing the production time and increasing the efficacy of APCs—Trivirus-specific T cells are produced by reactivating peripheral blood T cells with autologous monocytes transduced with an Ad5f35 vector expressing CMV-pp65, followed on days 9, 16 and 23 by restimulation with autologous EBV-LCL transduced with the same vector[84]. The infectious viruses, EBV for EBV-LCL production and clinical grade adenoviral vector required for CTL stimulation are expensive to make and test. To reduce costs and avoid the use of viral vectors, we have investigated alternative sources of antigen, and have evaluated DNA plasmids that encode antigens from all three viruses and can be introduced into APCs, such as monocytes or DCs, using the clinically applicable AMAXA nucleofection system. After transfer, high level transgene expression is achieved with good APC viability during the period of T cell activation. Plasmids are non-infectious, non-replicative, and integrate poorly into the transfected cell genome, and clinical grade DNA can be rapidly and cost-effectively produced in scalable quantities with excellent long term stability. We estimate that the substitution of plasmids reduces the cost of manufacture by more than 50%, by eliminating LCL manufacture and viral vector testing testing, since the cost of plasmid testing is about one tenth that of adenovirus vector and EBV testing. Plasmids also reduce antigenic competition for HLA molecules since APCs can be nucleofected separately with each plasmid. Further, we can add to our clinical grade plasmid library as new protective antigens from other viruses are identified, which will allow us the flexibility to increase the spectrum of antigens targeted by our CTL as and when needed.

Simplifying CTL production—Most current protocols for the activation and expansion of antigen-specific CTL *ex vivo* are complicated and labor intensive, limiting the broad application of this therapy. Many groups expand antigen-specific CTL for clinical use in the $2cm^2$ wells of 24-well plates, which are not suitable for routine production of large cell numbers. In standard static culture vessels, the depth of medium is limited by oxygen diffusion to about $1mL/cm^2$, a volume that limits the supply of nutrients and concentrates waste products including lactic acid and CO_2. As a result, the maximum cell density that can be achieved is about 2×10^6 cells/cm^2/mL. Consequently, to produce large T cell numbers, skilled GMP technologists must frequently divide the cultures and replenish media and growth factors to sustain expansion.

To improve cell output with minimal cell handling, a number of closed-system bioreactors have been explored. Mechanical rocking or stirring can be used to increase the availability of O_2 in the culture, while media and nutrients can be exchanged by perfusion[109–113]. Examples of such bioreactors include stirred tank bioreactors as well as static hollow fiber bioreactors. Stirred bioreactors allow high density cell growth and can readily be scaled up, but shear stress associated with the stirring rate reduces cell viability, and cultures require frequent medium sampling to evaluate growth-limiting factors like glucose and waste metabolites. In contrast, constant medium perfusion in the hollow fiber bioreactors results in the dilution of metabolites without shear stress, but cell sampling to assess T cell status during the culture is difficult. High cell densities can also be achieved in culture bags on rocking platforms, and the Wave Bioreactor has been used by Jensen and colleagues for therapeutic T cell production[114].

Although all are GMP applicable and can produce large numbers of cells, their disadvantages are the cost of purchase and the space required for specialized equipment, as well as the complexity of running and maintaining the equipment. Moreover, although genetically-engineered, mitogenically-activated T cells can be cultured in bioreactors, they have proven inefficient for antigen-specific CTL production, since CTL have strict requirements for prolonged interaction with APCs and feeder cells that are disrupted by mechanical agitation.

Vera and co-workers have described an improved manufacturing system for virus-specific CTL using optimized cell seeding densities in a novel cell gas permeable rapid expansion device (G-Rex) that supports medium to large-scale production of cells for clinical use (Vera *et al*, J. of Immunotherapy *In press*). By using cultureware that promotes optimal O_2 and CO_2 exchange, the initial input volume of medium can 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. This allows higher antigen-specific T-cell densities per unit surface area to be achieved ($8–10 \times 10^6$ per cm^2 compared to 2 to 3×10^6 per cm^2 in wells), and simplifies production by minimizing the number and complexity of manipulations. The G-Rex supports and promotes more rapid cell expansion than our current systems that can be further increased by the addition of enhancing cytokines. Thus cells can be made and are available for infusion sooner that would otherwise be possible. It is important to note that this rapid expansion and increased cell numbers are due to reduced cell death rather than increased cell division, thus the CTL are not functionally “exhausted” prior to adoptive transfer. Rapid expansion produces over two logs expansion of virus-specific T cells, with a concomitant but difficult to quantify loss of alloreactive T cells, that favors the ratio of virus-specific to alloreactive T cells. It should also be noted that although the G-Rex device probably cannot readily support the production of massive T cell numbers (up to 10^9 CTLs per G-Rex500) a number of studies have shown that massive quantities of cells are not required for reconstitution of virus-specific immunity after HSCT[74;84;91;92].

Extending CTL therapy to recipients of grafts from virus-naive donors

Despite the promising results of CTL therapy in both the HLA matched and mismatched allogeneic HSCT setting, it may be more challenging to translate this therapeutic modality to recipients of grafts from seronegative donors or to the cord blood transplant setting - an increasingly important alternative source of HLA mismatched stem cells. Generation of a virus-specific T cell product for infusion is complicated by the naive phenotype of virus-reactive T cells and their vanishingly low frequency. Hence, the generation of CTL requires the priming and extensive expansion of naive T cells rather than the more simple direct expansion of a pre-existing virus-specific memory T cell population. Modifications to traditional CTL generation schema using optimized APCs and enhancing cytokines have allowed functional CTLs to be generated even from this starting population[115;116]. Whether CTL derived from naive T cells will have the same *in vivo* persistence and antiviral activity as CTLs from peripheral blood remains to be evaluated.

Expert opinion section

Ideally, viral antigen-specific CTL preparation from allogeneic donors should ***rapidly*** and ***selectively*** produce CTL in numbers sufficient to reproducibly provide therapeutic benefit without harmful alloreactivity. This should require small amounts of donor blood, which could be obtained and cryopreserved at the time of transplant, even from unrelated donors. The CTL product should protect against a wide range of infectious agents, and not only the commonly detected CMV, Adv, EBV, BK virus, and HHV6 but also less common viruses including RSV and parainfluenza (Table 1). While these viruses are detected less frequently or may have a seasonal detection pattern, taken together they contribute significantly to patient care costs and

virus-related mortality rates after allogeneic HSCT, and thus rapidly-generated broad-spectrum CTL may offer a cost-effective and safe therapy.

In the previous sections we have outlined various improvements to CTL generation protocols and we are currently combining these strategies to develop and validate new manufacturing procedures which **simplify** and **shorten** CTL production and extend the number of viruses targeted. Immunogenic antigens from a range of viruses will be expressed from plasmids after nucleofection into DCs or monocytes[117]. The CTL will be expanded in the G-Rex device to support optimal expansion, and after 9–12 days in culture the CTL will be tested for identify, sterility and function and then can be infused either prophylactically or therapeutically (Figure 1). Implementation of these modifications in our CTL production processes will enable the extension of T cell therapy to a broad spectrum of clinically relevant viruses using a single CTL product which will be more cost-effective (we predict a reduction in the cost of CTL manufacture from $10,559 to $3,505) and less toxic that administering multiple antiviral agents, even if these were able to deliver the same breadth of protection.

Article highlights box

- Viral infections are frequent after hematopoietic stem cell transplant
- T cell therapy can offer an effective non-toxic strategy to provide both immediate and long-term protection
- Alloreactive T cells must be removed or inactivated to enhance the safety and improve the efficacy of donor leukocyte infusions
- Adoptive transfer of cytotoxic T lymphocyte (CTL) lines targeting single or multiple viruses simultaneously can prevent and treat infections in immunocompromised individuals
- Current preclinical work aims to overcome manufacturing limitations to allow the broad implementation of T cell therapy

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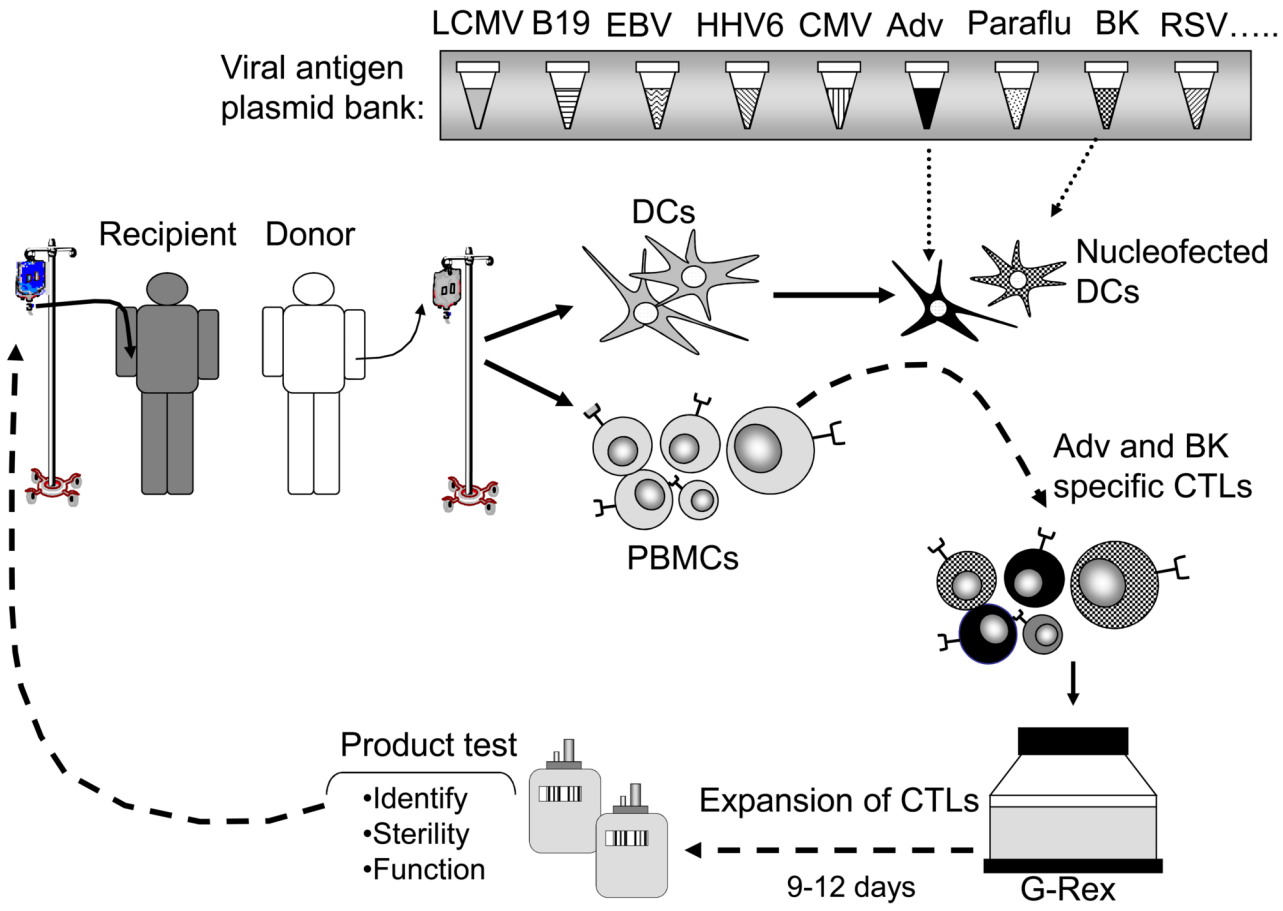

Figure 1. Rapid generation of multivirus-specific CTL

Our CTL manufacturing process will be shortened from >10 to <2 weeks by using plasmid nucleofected DCs to activate T cells, which will then be efficiently and rapidly expanded in the G-Rex.

Table 1

Emerging viral pathogens implicated in complications after HSCT and SOT.

Virus	Incidence in bone marrow and solid organ transplant
Adenovirus	SOT and HSCT[5;6;11;12;118–121]
Bocavirus	HSCT[122]
Coronavirus	SOT and HSCT[123–128]
HHV6	SOT and HSCT[18;19;129–138]
LCMV	SOT[139–142]
Mumps and Measles	SOT and HSCT[143–146]
Metapneumovirus	SOT and HSCT[147–156]
Parainfluenza	SOT and HSCT[1;128;157–162]
Parvovirus B19	SOT and HSCT[163–174]
RSV	SOT and HSCT[13;128;175–178]
Rotavirus	SOT[179–181]
West Nile virus	SOT and HSCT[182–190]
BK Virus	SOT and HSCT[17;191–195]