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REVIEW

Accelerating immune reconstitution after hematopoietic stem cell transplantation

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Viral infections remain a significant cause of morbidity and mortality after hematopoietic stem cell transplantation. Pharmacologic agents are effective against some pathogens, but they are costly and can be associated with significant toxicities. Thus, many groups have investigated adoptive T-cell transfer as a means of hastening immune reconstitution and preventing and treating viral infections. This review discusses the immunotherapeutic strategies that have been explored.
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Allogeneic hematopoietic stem cell transplantation (HSCT) is increasingly applied as a curative treatment for a variety of both malignant and nonmalignant hematological diseases.[1–5] However, the majority of patients requiring an allogeneic transplant do not have an available human leukocyte antigen (HLA)-identical sibling donor, necessitating the use of alternative stem cell sources including HLA-matched or -mismatched unrelated donors, haploidentical donors or cord blood units. The extension of HSCT to these higher-risk patients who either receive more extensively manipulated products (for example, T cell-depleted grafts) or require more intensive and prolonged post-transplant immunosuppression has resulted in a coincident rise in infection-related morbidity and mortality associated with a range of viruses including cytomegalovirus (CMV), Epstein–Barr virus (EBV), BK virus, human herpesvirus 6, influenza, parainfluenza, human metapneumovirus, adenovirus (AdV) and respiratory syncitial virus.[6–10] Pharmacologic agents are effective against only some of these pathogens, but they are costly and can be associated with significant toxicities. In addition, as the use of antivirals does not accelerate immune reconstitution, infections frequently recur after treatment termination.[11–13] This has led a number of groups to investigate whether adoptive T-cell transfer approaches might provide a safe and effective means of providing both immediate and long-term antiviral protection. Our review discusses some of the immunotherapeutic strategies that have been explored.

DONOR LYMPHOCYTE INFUSION (DLI)

The first adoptive transfer studies involved the infusion of unmanipulated donor lymphocytes containing a spectrum of T cells specific for all pathogens encountered by that donor over their lifetime. The therapeutic potential of this approach for viral infections was first demonstrated in the context of EBV-related post-transplant lymphoproliferative disease (EBV-PTLD),[14] and subsequently for AdV[15] and CMV.[16] However, despite promising results, the adoptive transfer of unmanipulated T cells also carries with it the risk of causing graft-versus-host disease (GvHD), because of the high frequency of alloreactive cells present in the infused product.[17] Furthermore, because of the low circulating frequency of T cells reactive against many common viruses including AdV, respiratory syncitial virus, parainfluenza and human metapneumovirus, higher DLI doses may be required to confer clinical benefit, further increasing the risk of GvHD.

ALLOREACTIVE T-CELL DEPLETION

In order to retain the activity but enhance the safety of DLIs, a number of groups have investigated strategies to selectively deplete or inactivate alloreactive T cells.

Selective allodepletion *ex vivo*

One approach to safely accelerate immune reconstitution is to remove alloreactive T cells from the donor graft before infusion. To identify the alloreactive sub-population within the DLI product, donor lymphocytes are first co-cultured with recipient-derived antigen presenting cells (APCs), including peripheral blood mononuclear cells, keratinocytes, EBV-transformed B lymphoblastoid cell lines (EBV-LCL), dendritic cells (DCs) or fibroblasts.[18–23] Activated T cells that proliferate and upregulate activation markers, including CD25, CD69, CD71, CD134, CD137 and HLA-DR, can subsequently be eliminated using immunomagnetic depletion, immunotoxin-conjugated antibody-based depletion, photodepletion or induction of apoptosis.[21–29]

Van Dijk *et al.*[19] were the first to demonstrate that the depletion of alloantigen-activated T cells was feasible. In their preclinical study, the investigators cultured donor T cells with patient keratinocytes in the presence of an anti-CD28 antibody, followed by incubation with

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microbeads conjugated to monoclonal antibodies directed to CD25, CD69, CD71 and HLA-DR, used individually or in combination. After selective depletion by immunomagnetic separation, they reported a 10-fold decrease in the frequency of T-cell precursors reactive against the same patient-derived APCs, whereas activity against third-party cells remained unchanged.[19] Similar studies have been performed using a CD25 antibody coupled to the deglycosylated ricin A chain (dgA) (called RFT5-SMPT-dgA). Indeed, Montagna *et al.*[18] demonstrated that after activation with haploidentical peripheral blood mononuclear cells, incubation with this antibody-conjugated immunotoxin efficiently depleted alloreactive T cells, whereas those with antiviral activity were retained. This preclinical study was translated to a clinical phase I/II trial in which 15 pediatric transplant patients, 13 of whom received haploidentical grafts, were administered allodepleted donor T cells at doses ranging from 1 to 8×10^5 cells per kg between days 15 and 47 post transplant. Even at the highest dose, the infusions proved safe, with only four patients developing acute GvHD (aGvHD; grade I/II) and one patient presenting with limited chronic GvHD (cGvHD). In addition, the allodepleted cells showed evidence of antiviral activity *in vivo*. Specifically, in three patients with active CMV and/or EBV reactivations unresponsive to antiviral drugs, infection-associated symptoms resolved post infusion.[23] Solomon *et al.*[29] applied this strategy in the adult setting for recipients of matched sibling donor HSCTs. They incubated donor peripheral blood mononuclear cells with *ex vivo* expanded recipient-derived T cells followed by allodepletion with RFT5-SMPT-dgA that produced a mean five fold decrease in host-reactive cells. Subsequently, on the day of transplant, a substantially larger dose of cells (median 1×10^8 allodepleted T cells per kg) was administered to 16 elderly patients with advanced hematologic malignancies who were considered high risk for developing GvHD. Post infusion, 8 patients developed acute GvHD that was severe in 4 (2 grade III and 2 grade IV), and the severity of GvHD appeared to correlate with the efficiency of allodepletion.[29] Finally, Amrolia *et al.*[30] performed a phase I dose-finding clinical study using EBV-LCL-depleted donor T cells in the haploidentical setting for pediatric and adult patients with high-risk hematologic malignancies. Sixteen patients received 10^4 or 10^5 cells per kg that were administered on days 30, 60 and 90 post transplant. Two patients developed significant acute GvHD (1 patient at each dose tested) that evolved to extensive chronic GvHD in both cases and was lethal in one individual. Importantly, only the top dose level of 10^5 cells per kg supported immune reconstitution, and in this group rapid recovery of CMV- and EBV-specific immunity was observed as early as 2–4 months post HSCT.[30]

Photodynamic purging has also been used to deplete alloreactive T cells. This platform was developed to overcome fluctuations in activation-based surface marker expression and to achieve a more consistent and effective allodepletion. The technique is based on the impaired ability of activated T cells to excrete a phototoxic rhodamide-like dye (TH9402) because of activation-induced changes in a multidrug-resistance P-glycoprotein. Consequently, exposure of TH9402-exposed, alloactivated cells to visible light results in apoptosis.[31]

In a phase I clinical trial, Roy *et al.*[32,33] prepared allodepleted cells using photodynamic purging that were administered on day 34 ± 6 at doses ranging from 1×10^4 to 5×10^6 cells per kg to 19 adults, all of whom had undergone T cell-depleted haploidentical HSCTs. The allodepleted cells caused acute GvHD (grade I/II) in 4 patients, but chronic GvHD developed in 9 patients, the majority of whom received the higher doses. Reconstitution of $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells to 'normal' levels occurred more rapidly in patients administered the top cell dose and 67% of patients in this group remained infection free during the first 6 months post infusion in comparison with only 14% of patients who received the lowest cell dose.[32,33] Given the apparent safety and antiviral protection conferred by the cell infusions, Mielke *et al.*[34] performed a follow-up study in which 24 adults with hematological malignancies (16 high risk) received 5×10^6 per kg selectively photodepleted donor T cells (from an HLA-matched sibling) on the day of transplant. The frequency of GvHD was relatively high, with a probability of acute GvHD grade II–IV of $38 \pm 10\%$ and $13 \pm 7\%$ for grade III–IV, whereas the probability of developing chronic GvHD was $65 \pm 11\%$. However, serious viral, bacterial and fungal infections were common and persistent. Of the patients infused, 20 developed CMV reactivations that required treatment, 5 patients experienced viral-associated hemorrhagic cystitis and 1 patient died of respiratory syncitial virus pneumonitis. Recurrent bacterial infections were detected in five patients, four of whom died, and four patients developed invasive fungal infections. Ultimately, the trial was prematurely terminated because of both unexpected duration and the severity of these infectious complications.[34]

In vivo T-cell depletion

Alloactivated T cells can also be depleted *in vivo* by genetically engineering the infused cells to incorporate a 'safety switch' that can be triggered should adverse effects occur.[35,36] Various suicide gene systems have been evaluated preclinically, including CD20 and rituximab,[37] varicella zoster virus-derived thymidine kinase and the prodrug 6-Methoxypurine arabinoside,[38] cytosine deaminase and 5-Fluorocytosine,[39] purine nucleoside phosphorylase and 6-methylpurine-2-deoxyriboside,[40] Carboxypeptidase A and Methotrexate-a-peptides.[41] Two platforms have been tested clinically—herpes simplex viral thymidine kinase (HSV-tk) and inducible Caspase 9 (iC9). The HSV-tk gene functions by converting the prodrug ganciclovir to its active triphosphate form, leading to inhibition of DNA synthesis and death of dividing cells. In a phase I/II multicenter study, 28 haploidentical HSCT recipients received $0.9–40 \times 10^6$ HSV-tk-modified donor lymphocytes per kg from day 28 post transplant. These infusions supported rapid (median 23 days post T cells) immune reconstitution, defined as circulating $CD3^+$ T-cell numbers of at least 100 cells per μl detected on two consecutive occasions in 22 patients, all of whom received $\geqslant 0.9 \times 10^6$ cells per kg. In addition, these individuals had fewer and less severe infectious complications than those who did not reconstitute (infection-related mortality of 9% versus 60%). In this study, 10 patients developed acute (grade I–IV) GvHD between days 8 and 86 post infusion and 1 patient developed extensive chronic GvHD on day 146. In 1 patient with cutaneous grade I GvHD, the rash resolved spontaneously, but in the other 10 patients ganciclovir was administered, resulting in a significant reduction (40–93%) in the circulating frequency of HSV-tk-modified T cells and consequent complete clinical responses in all cases.[42] However, there are several shortcomings associated with the use of HSV-tk as a suicide system that must also be taken into consideration, including the inherent immunogenicity of the virus-derived transgene that may lead to the premature elimination of the infused cells. In addition, its mechanism of action requires interference with DNA synthesis, and hence cell killing may be prolonged. Finally, the requirement for ganciclovir to activate the suicide gene removes the possibility of administering this agent as a treatment of viral infections post transplant.[42–44] Nevertheless, the

activity of this approach is currently being tested in late-phase clinical studies.

An alternative suicide strategy is iC9, which is nonimmunogenic and triggered upon administration of a small-molecule dimerizer that produces apoptosis within 24 h, even in nondividing cells.[45] The safety and activity of iC9-modified T cells was assessed in a phase I dose-escalation study where donor cell numbers ranging from 0.1 to 1×10^7 cells per kg were infused to 5 haploidentical pediatric HSCT recipients at 30–90 days after transplantation. Four patients developed grade I/II GvHD and, following a single dose of the dimerizing drug AP1903, >90% of cells underwent apoptosis within 30 min, resulting in rapid resolution of GvHD (24–48 h) without recurrence. Importantly, virus-specific T cells (VSTs) were spared.[46]

Induction of anergy

Functional inactivation rather than physical depletion of T cells has also been used as a means of protecting against GvHD. In order to become activated, T cells require 'signal 1' provided by T-cell receptor engagement with peptide presented in the context of HLA, as well as 'signal 2' provided by costimulatory molecules on T cells engaging their ligands on APCs. Of the latter, the interaction between CD28 and its ligands, B7-1 (CD80) and B7-2 (CD86), is one of the major positive costimulatory signals. This interaction can be specifically blocked using a fusion protein, for example CTLA4-Ig, that binds to B7 with higher affinity than CD28, or monoclonal antibodies to CD80 or CD86. In the event of stimulation with signal 1 in the absence of signal 2, cells enter a state of allospecific anergy.

This approach was tested clinically by Guinan *et al.*[47] who reported the outcome of 12 patients aged 0.5–26 years who received alloanergized haploidentical bone marrow grafts for high-risk hematologic malignancies. To induce alloantigen-specific anergy, bone marrow from the donor was cocultured with recipient lymphocytes in the presence of CTLA4-Ig, resulting in a decrease in alloreactive precursors by a median of 4 logs. A median of 16×10^6 $CD3^+$ T cells per kg was infused on the day of HSCT and just 3 patients developed aGVHD (grade II/III), but all resolved.[47] In order to better assess the safety and effectiveness of this technique with respect to its ability to promote immune reconstitution and control infections, the same group performed two follow-up phase I trials. A total of 24 patients (pediatric and adult) with high-risk hematologic malignancies or bone marrow failure received haploidentical stem cell grafts containing a median of 29×10^6 CD3 per kg that had been alloanergized using CTLA4-Ig ($n = 19$) or anti-B7.1 and anti-B7.2 antibodies ($n = 5$). Despite the high T-cell dose infused, GvHD of ⩾grade III was seen in only five patients. Moreover, both absolute lymphocyte and $CD4^+$ T-cell recovery occurred within 3 months of infusion and there was evidence of virus-specific T cell expansion *in vivo*. Indeed, in three evaluable patients with CMV reactivation, a rapid expansion of $CD8^+$ T cells to levels exceeding those required for protection was observed.[48]

An alternative approach to reduce GvHD *in vivo* is to infuse regulatory T cells (Tregs) that have the capacity to inhibit activated T cells. In a phase I clinical trial of this approach, 23 adults with advanced-stage hematologic malignancies received two cord blood units followed by 1 or 2 infusions of Tregs on days +1 and +15 ($1–30 \times 10^5$ and 30×10^5 per kg, respectively). The incidence of aGVHD (grade III/IV) was 17% overall and 11% for those who received a Treg dose of $\geqslant 30 \times 10^5$ per kg.[49] Similarly, Di Ianni *et al.*[50] administered Tregs (2×10^6 or 4×10^6 per kg) to 28 haploidentical HSCT patients 4 days before transplant, at which point they also received a DLI (0.5×10^6, 1×10^6 or 2×10^6 per kg). Only 2 of 26 evaluable patients developed ⩾grade II aGvHD, both of whom also received the highest doses of both Tregs and DLI. The Tregs did not inhibit immune reconstitution, with CD4 and CD8 counts reaching 50 per μl within 1 month after transplantation that reflected an expansion of T-cell populations directed against a range of pathogens including *Aspergillus fumigatus*, *Candida albicans*, CMV and AdV. Overall, fewer episodes of CMV reactivation occurred compared with those reported after haploidentical HSCT, and no patient developed CMV disease.

Adoptive transfer of VSTs

The adoptive transfer of selected or *ex vivo* expanded VST populations has also been utilized to provide *in vivo* antiviral protection.

RAPID ISOLATION STRATEGIES

Several groups have focused on selection approaches that facilitate the isolation of VST populations directly from peripheral blood for immediate transfer to patients with acute infections. These include the isolation of HLA-peptide multimer-binding T cells or the selection of cytokine-producing T cells following a short (12–16 h) *in vitro* stimulation.

HLA-peptide multimers consist of individual peptide epitopes refolded with appropriate HLA class I molecules that can bind to epitope-specific $CD8^+$ T cells and are subsequently selected for infusion using magnetic beads. Cobbold *et al.*[51] used a panel of CMV IE1 and pp65 tetramers (a complex of 4 peptide-loaded HLA molecules) to select specific T cells from nine HSCT donors that were administered to 6 recipients of matched related and 3 recipients of matched unrelated transplants with CMV reactivations. Although only small doses (1.2×10^3–3.3×10^4 cells per kg) of exclusively $CD8^+$ T cells were infused, post transfer the cells were able to expand and persist *in vivo*, as indicated by their detection 100 days post infusion. In addition, clearance of CMV DNA was documented in eight of nine infused patients, with no case of CMV disease reported.[51] Uhlin *et al.*[52] used pentamers to select $CD8^+$ T cells reactive against not only CMV (HLA B7, A2 and B35) but also EBV (HLA A2) and AdV (HLA A1) either from frozen DLI products or from the peripheral blood of haploidentical relatives. These cells ($0.8–24.6 \times 10^4$ cells per kg) were infused to 6 patients with refractory CMV, 1 with EBV-PTLD and 1 with an AdV infection, and produced clinical responses with a reduction in viral titer documented in 6 of the 8 treated patients.[52]

Despite the apparent clinical benefit associated with the adoptive transfer of multimer-selected cells, concerns have recently been raised regarding the impact that multimer binding may have on the activity of the selected T-cell populations. Indeed, Maile *et al.*[53] demonstrated that prolonged HLA-peptide/T-cell receptor interaction can induce epitope-specific tolerance in a dose-dependent manner, resulting in clonal exhaustion/anergy of the multimer-bound T cells.[53] To address this problem, Neudorfer *et al.*[54,55] designed streptamers that have the binding properties of traditional multimers but can be rapidly dissociated from T cells by the addition of a competitor molecule, subsequently making the isolated population phenotypically and functionally indistinguishable from untreated T cells. In a recent clinical trial of this approach, Schmitt *et al.*[56] administered a single infusion of selected CMV-directed $CD8^+$ T cells to two allogeneic HSCT patients with CMV infections who were unresponsive to antiviral drugs. Patient 1 received a single infusion of 2.2×10^5 HLA A24/pp65 cells per kg that resulted in an increase in the circulating frequency of CMV-specific T cells from 0 to 27.1% of all

peripheral blood T cells. Similarly, in patient 2, who received only 0.35×10^5 HLA B7/pp65 cells per kg, there was an increase in the frequency of reactive cells from 0.03 to 0.48%. In both cases, the T-cell infusions resulted in CMV clearance without GvHD.[56]

The second method (γ-capture) for rapid selection of virus-specific cells relies on the capacity of memory T cells to secrete interferon-γ (IFNγ) in an antigen-dependent manner. Feuchtinger *et al.*[57] first used this method to select AdV-directed T cells for the treatment of systemic AdV infections in nine pediatric allograft recipients. Donor-derived AdV-reactive cells were activated by short-term (16 h) *in vitro* stimulation using species C adenoviral antigen as a stimulus, followed by magnetic selection of the IFNγ-secreting population that contained both $CD4^+$ (mean 63.2%) and $CD8^+$ (mean 28.7%) T cells. The infusions were safe and despite the small numbers of cells infused (range $1.2–50 \times 10^3$ per kg) 5 of 6 evaluable patients exhibited a substantial reduction in viral load in both peripheral blood and stool with a corresponding increase in the circulating frequency of AdV-specific T cells. This group later applied this strategy to treat chemorefractory CMV disease or reactivation ($n = 18$ children and adults who had received a T cell-depleted allo-HSCT), and in 83% of cases CMV was either eliminated or patients had a >1 log viral load reduction, including 2 patients with CMV encephalitis.[58] Peggs *et al.*[59] also used IFNγ-captured CMV pp65-specific T cells as prophylaxis and preemptive treatment. In a phase I/II study, 18 patients received a median of 2840 $CD4^+$ and 630 $CD8^+$ CMV-specific T cells per kg early (median day 35) post transplant, and expansion of both cell subset populations was observed. However, aGvHD (grade I–III) occurred in eight patients and extensive cGvHD in three patients.[59]

Moosmann *et al.*[60] used IFNγ-captured cells to treat refractory EBV-PTLD in 6 allo-HSCT patients. In this study, antigen-specific cells were isolated following stimulation with 23 EBV epitope peptides (19 HLA class I and 4 HLA class II) derived from 11 lytic and latent antigens. Following a single infusion of $0.4–9.7 \times 10^4$ polyclonal T cells per kg, 3 patients had complete and durable responses that corresponded with a rapid increase in EBV-specific $CD8^+$ T cells in 2 patients. However, the other 3 patients, all with end-stage disease, failed to respond.[60] Feuchtinger and coworkers[61] also targeted EBV, but focused solely on EBNA1 as a stimulus to activate both $CD4^+$ and $CD8^+$ T cells. Ten pediatric and adult patients with EBV viremia and/or PTLD after HSCT were infused with IFNγ-captured EBNA1-directed T cells that were activated either with whole EBNA1 protein or EBNA1 overlapping peptide pools. After administration of a mean T-cell dose of 5794 $CD3^+$ cells per kg, no acute toxicities were observed and only 1 patient developed aGvHD grade I/II. *In vivo* expansion of EBNA1-specific T cells was seen in 8 of 10 patients, 7 of whom demonstrated a clinical and virologic response, defined as a decrease in viral load of >1 log and resolution of PTLD.[61]

Thus, to date these direct selection approaches have proven safe and allow for the rapid preparation and infusion of virus-specific precursors. However, multimer selection requires the generation of HLA-specific reagents for each viral epitope and thus far has been restricted to $CD8^+$ T cells. Instead, IFNγ capture provides an HLA-unrestricted means of selecting both $CD4^+$ and $CD8^+$ memory T cells. However, both methods require large volumes of donor blood, and even then only small numbers of captured cells are obtained, so that only viruses with a high frequency of circulating T-cell precursors can be targeted.

Table 1 Clinical trials using *in vitro* expanded VSTs

Stimulation	*Target*	*Patients*	*Prophylaxis or treatment (number of patients)*	*Viral outcomes*	*References*
CMV-infected fibroblasts	CMV	14	Prophylaxis	No CMV infections	Walter *et al.*[62]
CMV lysate-stimulated PBMCs	CMV	8	Treatment	6 CR	Einsele *et al.*[63]
				1 PR	
				1 NR	
CMV antigen-pulsed DCs	CMV	28	Prophylaxis	23 Responded to VSTs with antivirals	Peggs *et al.*[66]
			Treatment		
pp65-pulsed or Ad5f35pp65 vector-transduced DCs	CMV	50	Prophylaxis	26 Patients developed CMV infections	Blyth *et al.*[69]
				9 Required antivirals	
				1 CMV-related death	
EBV-LCLs	EBV	118	Prophylaxis (105) Treatment (13)	No new EBV infections	Rooney *et al.*[70]
				11 CR	Heslop *et al.*[71]
				2 Deaths	Rooney *et al.*[72]
					Heslop *et al.*[20]
EBV-LCLs	EBV	6	Treatment	5 Displayed a decrease in viral load	Gustafsson *et al.*[74]
				1 EBV-related death	
EBV-LCLs	EBV	3	Treatment	3 CR	Comoli *et al.*[73]
EBV-LCLs	EBV	19	Treatment	13 CR	Dubrovina *et al.*[75]
				1 EBV-related death	
Ad5f35pp65 vector-transduced	EBV	11	Prophylaxis (10)	3/3 CR of EBV infection/PTLD	Leen *et al.*[77]
EBV-LCLs and PBMCs	AdV		AdV treatment (1)	3/3 CR of CMV infection	
	CMV			6/6 CR of AdV infection/disease	
Plasmid-nucleofected DCs	EBV	10	EBV treatment (4)	3 CR	Gerdemann *et al.*[82]
	AdV		AdV treatment (5)	5 CR	
	CMV		CMV treatment (5)	4 CR, 1 patient with persistent colitis proceeded with colectomy	

Abbreviations: AdV, adenovirus; CMV, cytomegalovirus; CR, complete response; DC, dendritic cell; EBV, Epstein–Barr virus; EBV-LCL, EBV-transformed B lymphoblastoid cell line; NR, non-responder; PBMC, peripheral blood mononuclear cell; PR, partial response; PTLD, post-transplant lymphoproliferative disease; VST, virus-specific T cell.

ADOPTIVE TRANSFER OF *EX VIVO* EXPANDED VSTs

An alternative strategy to enhance immune recovery after HSCT is to infuse VSTs that have been selectively expanded *ex vivo* to enrich for pathogen-specific populations with a corresponding dilution in alloreactive cells. This approach has been implemented by a number of groups for the prevention and treatment of infections associated with CMV, EBV and AdV, which will be summarized in the following section (Table 1).

CMV

CMV is a latent β-herpesvirus that frequently reactivates in immunocompromised allogeneic HSCT recipients in the absence of CMV-specific $CD4^+$ and $CD8^+$ T cells. Thus, a number of groups have investigated whether *in vitro* expanded VSTs generated using CMV-infected fibroblasts, peptide/antigen-loaded DCs or adenovector-transduced APCs could provide clinical benefit. Walter *et al.*[62] were the first to test this approach clinically using $CD8^+$ T-cell clones that had been activated and expanded using autologous fibroblasts infected with the AD169 strain of CMV. These 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 per kg from day 30 post transplant. The infusions were safe, with only three patients developing *de novo* grade I/II GvHD, but all responded to steroid treatment. Before adoptive transfer, all 14 patients lacked CMV-specific T cells, but these became detectable by days 12 to 19 post infusion and persisted for at least 8 weeks after cessation of therapy, as indicated by T-cell receptor clonotyping studies. Consequently, none of the infused patients developed CMV reactivation or disease. However, the magnitude of the CMV-specific T-cell response progressively declined over time in patients who did not develop a concomitant endogenous CMV-specific $CD4^+$ response, highlighting the importance of $CD4^+$ T-cell help in sustaining antiviral activity.[62]

In an effort to promote *in vivo* persistence, Einsele *et al.*[63] prepared polyclonal CMV-specific T cells by incubating donor peripheral blood mononuclear cells with CMV lysate followed by restimulation with autologous irradiated feeder cells loaded with CMV antigen. After 4 stimulations, the product for infusion contained a mixture of $CD4^+$ (mean $77 \pm 10\%$) and $CD8^+$ (mean $6 \pm 3\%$) T cells that were CMV specific as measured by intracellular IFNγ staining, but lacked alloreactive cells as tested in a mixed lymphocyte culture. Eight recipients of matched or mismatched sibling or unrelated donor grafts, all of whom had drug-resistant CMV reactivations, received a single dose of 10^7 CMV-specific T cells per m^2 a median of 120 days post HSCT. The infusions were safe and resulted in a significant viral load reduction in all seven evaluable patients that was durable in 5 patients and corresponded with an increase in CMV-reactive T cells.[63] Similar positive results were reported by Peggs *et al.*[64,65] who generated reactive cells using DCs loaded with inactivated CMV as a stimulus. Following administration to 28 adult allogeneic HSCT recipients at high risk for both CMV and GvHD, the cells proved safe (4 cases of aGvHD (grade II/III) and 2 cases of cGvHD) and promoted *in vivo* immune reconstitution in all 28 patients.[66] Finally, Gottlieb and colleagues[69] recently published their phase II results where 50 recipients (4–68 years old) of HLA-matched or -mismatched allografts were prophylactically infused with 2×10^7 donor-derived HLA-A2-peptide or pp65-reactive CMV-specific T cells per m^2. The infusions were safe, with only 2 patients developing *de novo* aGvHD $\geqslant$III, whereas the incidence of cGvHD was 42% overall; similar to the rate detected in a contemporaneously treated control cohort ($n = 128$), and overall and progression-free survival was similar in both groups. However, both the percentage of patients requiring CMV antiviral therapy and the total number of treatment days was decreased in those who received CMV-specific T cells (17 vs 36% and 3.4 days vs 8.9 days, respectively). Thus, VSTs can directly prevent and treat CMV reactivations but also result in a decreased requirement for antiviral therapies with a corresponding reduction in pharmacotherapy costs and drug and disease-associated morbidity.[67–69]

Epstein–Barr virus

EBV has also proven amenable to VST therapy. Our group first targeted EBV reactivations post transplant with *in vitro* expanded donor-derived EBV-specific T cells generated using EBV-LCL as a stimulus. In our initial study, 3 patients with EBV-PTLD and 7 high-risk patients received $0.4–1.8 \times 10^8$ cells per m^2 with no adverse effects and complete viral elimination was achieved in all patients with active disease.[70] We subsequently extended this study and infused a total of 39 high-risk allograft recipients with 2–4 infusions of EBV-specific T cells that established a dose of 2×10^7 cells per m^2 as safe and effective. In 12 patients where immune reconstitution was measured, there was a median 32-fold increase in EBV-specific T-cell precursors 1 month post treatment, and 6 patients with elevated EBV-DNA levels at the time of infusion had a 2–4 log decrease in their viral load within 2 to 3 weeks of receiving cells.[72] More recently, we have reviewed the long-term outcome of 114 patients, aged 0.5–38 years, who were infused with 2×10^7 per m^2 EBV-specific T cells as prophylaxis ($n = 101$) or treatment ($n = 13$) for EBV-PTLD. The cells were effectively able to control PTLD in 80% of patients who received the cells therapeutically and were 100% effective in those who were prophylactically infused, in comparison with a PTLD incidence of 11.5% in controls. In the first 26 patients, the T-cell lines were genetically marked with a retroviral vector encoding the neomycin resistance gene that facilitated tracking studies and demonstrated that functional T cells persisted for up to 9 years.[20]

These results have been reproduced by numerous centers,[73,74] including the group from Memorial Sloan Kettering Cancer Center who infused 19 patients with biopsy-proven EBV-PTLD with EBV-LCL-activated T cells. The patients received 3 weekly doses of 10^6 cells per kg that proved safe and induced a complete response in 13 (68%) patients. Interestingly, in 3 patients who failed to respond to T-cell therapy, the investigators demonstrated that the infused T-cell lines, expanded using EBV B95-8-transformed B cells as APCs, failed to recognize patient-derived infected B cells, implying antigenic differences between the EBV B95-8 laboratory strain and the endogenous virus responsible for the PTLD. Furthermore, in two recipients of haploidentical or HLA-mismatched grafts, the infused donor-derived T-cell lines were found to selectively recognize EBV peptides presented in the context of non-shared HLA alleles, facilitating tumor immune escape.[75] Our group reported a similar outcome associated with the infusion of a line that failed to recognize an endogenous virus deletion mutant.[76] These reports highlight the importance of manufacturing and infusing polyclonal T-cell lines that ideally recognize multiple $CD8^+$ and $CD4^+$ T-cell epitopes from multiple antigens and have been confirmed to recognize recipient-derived targets via a shared HLA allele.

Multipathogen-directed VSTs

In order to broaden the specificity of viruses that could be targeted by a single T-cell line, we subsequently developed a strategy to simultaneously activate and expand T cells reactive against CMV, AdV and EBV using, as APCs, monocytes and EBV-LCLs transduced

with an adenoviral vector encoding the immunodominant CMV pp65 antigen. Although it was feasible to generate these trivirus VSTs, in general the lines were dominated by the CMV-reactive component. Nevertheless, when small doses (ranging from 5×10^6 to 1.5×10^8 cells per m^2) were infused to 11 recipients of haploidentical, HLA-matched related or unrelated donor transplants, the cells proved safe and effective against all 3 viruses *in vivo*.[77]

SIMPLIFYING VST PRODUCTION

Although the administration of *ex vivo* expanded donor-derived VSTs has proven to be safe and effective in the post-HSCT setting, broader implementation is limited by the logistics of manufacture and the requirement for individualized products. For example, for the production of trivirus-directed T-cell lines, the generation of the EBV-LCLs alone requires 4–6 weeks. Subsequently, VSTs are activated and expanded using monocytes and EBV-LCLs transduced with the adenopp65 vector, resulting in an additional 4–6 weeks of culture. The infectious virus (EBV B95-8) and clinical grade adenoviral vector required for VST generation are expensive to make and test, whereas the prolonged period of culture (8–12 weeks for VST manufacture and 1–2 weeks for product release testing) is lengthy and complex, necessitating the speculative manufacture of VSTs for all at-risk subjects without guarantee of their eventual need. Fortunately, it has now become possible to address many of the manufacturing issues enabling cost-effective and rapid VST production using simplified technologies that should facilitate the introduction of the approach into broader clinical practice (Figure 1).

Reducing manufacturing time

To eliminate the requirement for live virus/viral vectors, we have investigated alternative antigen sources including DNA plasmids encoding a range of immunodominant and subdominant viral antigens from EBV, CMV and AdV as T-cell stimulators. These plasmids were introduced into DCs using the AMAXA nucleofection system (Lonza, Inc., Allendale, NJ, USA) and resulted in efficient VST generation in just 17 days.[78] More recently, we have also evaluated the direct stimulation of donor peripheral blood mononuclear cells with commercially available peptide mixtures (pepmixes) as an approach to rapidly expand antigen-specific populations. These pepmixes consist of 15-mer peptides overlapping by 11 amino acids spanning immunogenic target antigens. As each peptide is 15 amino acids in length, pepmixes covered the majority of both $CD8^+$ and $CD4^+$ epitopes[79] and produced VSTs that were phenotypically and functionally equivalent to conventionally generated T-cell lines, thus eliminating the requirement for DCs or other specialized APCs. Thus, by incorporating pepmixes as a stimulus, the manufacturing time is reduced to just 10 days.[80]

Reducing complexity

Traditionally, VSTs have been generated using standard tissue-culture treated 24-well plates, with weekly T-cell stimulation and frequent manipulation (counting, splitting and feeding). Thus, the process is laborious and time consuming, limiting scalability. To address this issue, our group, in collaboration with Wilson Wolf Manufacturing, has developed and tested a gas-permeable culture device (G-Rex) with a silicone membrane at the base that allows for efficient O_2 and CO_2 exchange. Incorporation of this silicone membrane thus allows for the initial input media volume to be increased which in turn, increases the available nutrients and dilutes waste products with minimal cell handling required. These optimized growth conditions increase output by up to 20-fold (from $2–3 \times 10^6$ per cm^2 in traditional plastic ware to $\sim 10^7$ cells per cm^2 in the G-Rex).[81]

Reducing antigenic competition

Until recently, the maximum number of viruses that have been targeted by a single line has been limited to three because repetitive *in vitro* stimulation of T-cell cultures leads to antigenic competition within our cultures. For example, our trivirus (EBV + CMV + AdV)-specific products were dominated by CMV-specific T cells at the expense of the AdV- and EBV-specific components. This antigenic competition and the resultant production of lines with restricted viral specificities limits their clinical value. To overcome this limitation and retain both high- and low-frequency T cells, we supplemented our cultures with different Th1 (T helper type 1) polarizing and prosurvival cytokines (including interleukin (IL)-15, IL2 and a combination of IL4 and IL7) that have been reported to prevent activation-induced cell death, and found that the combination of IL4 and IL7 supported the expansion and survival of both $CD4^+$ and $CD8^+$ virus-specific T cells that recognized multiple viral epitopes and killed virus-infected targets. The induced cells were Th1 polarized and lacked alloreactive T cells, even when the VSTs had been produced by only a single *in vitro* stimulation with pepmixes. By implementing this change, we were able to mitigate the impact of antigenic competition, allowing us to incorporate additional specificities within our VSTs.[80]

Clinical trials using rapidly generated VSTs

To test the safety and clinical activity of rapidly generated VSTs, we first performed a phase I clinical trial using trivirus (AdV, EBV, CMV)-directed VSTs that had been activated using plasmid-nucleofected DCs as APCs and expanded for 9–11 days in a G-Rex device in media supplemented with IL4 and IL7. Ten patients with reactivations of one or more viruses received cell infusions ranging from 0.5 to 2×10^7 VSTs per m^2 between day 27 and month 52 post HSCT. The infusions were safe, with only one patient developing a mild and localized skin rash post infusion. In addition, the cells produced clinical benefit and complete virological responses in 8 of 10 patients treated for active infections associated with one or more of the targeted viruses.[82] We are currently testing the activity of pepmix-stimulated VSTs directed against EBV, AdV, CMV, human herpesvirus 6 and BK virus (clinicaltrials.gov)[83] (Figure 1).

EXTENDING THE APPLICABILITY OF VSTs

The range of viruses that can be treated, the avoidance of biohazardous agents such as live viruses and the accelerated manufacture should facilitate broader introduction of VST-based approaches for intractable virus infection in the immunocompromised host. However, the individualized nature of the products for infusion as well as the requirement for seropositive donors preclude extending the strategy to recipients of grafts from seronegative donors. Two approaches may overcome these barriers.

VST generation from seronegative donors

The activation and expansion of VSTs from seronegative donors and cord blood (CB) remains challenging because of both the naive phenotype of the T cell and their low circulating precursor frequency. Hence, the generation of VSTs *in vitro* requires the use of professional APCs (for example, DCs) as well as potent activating/Th1-polarizing cytokines including IL12, IL15 and IL7. In preclinical studies, Park *et al.*[84] were the first to demonstrate the feasibility of activating and expanding CMV-directed VSTs from CB, whereas more recently

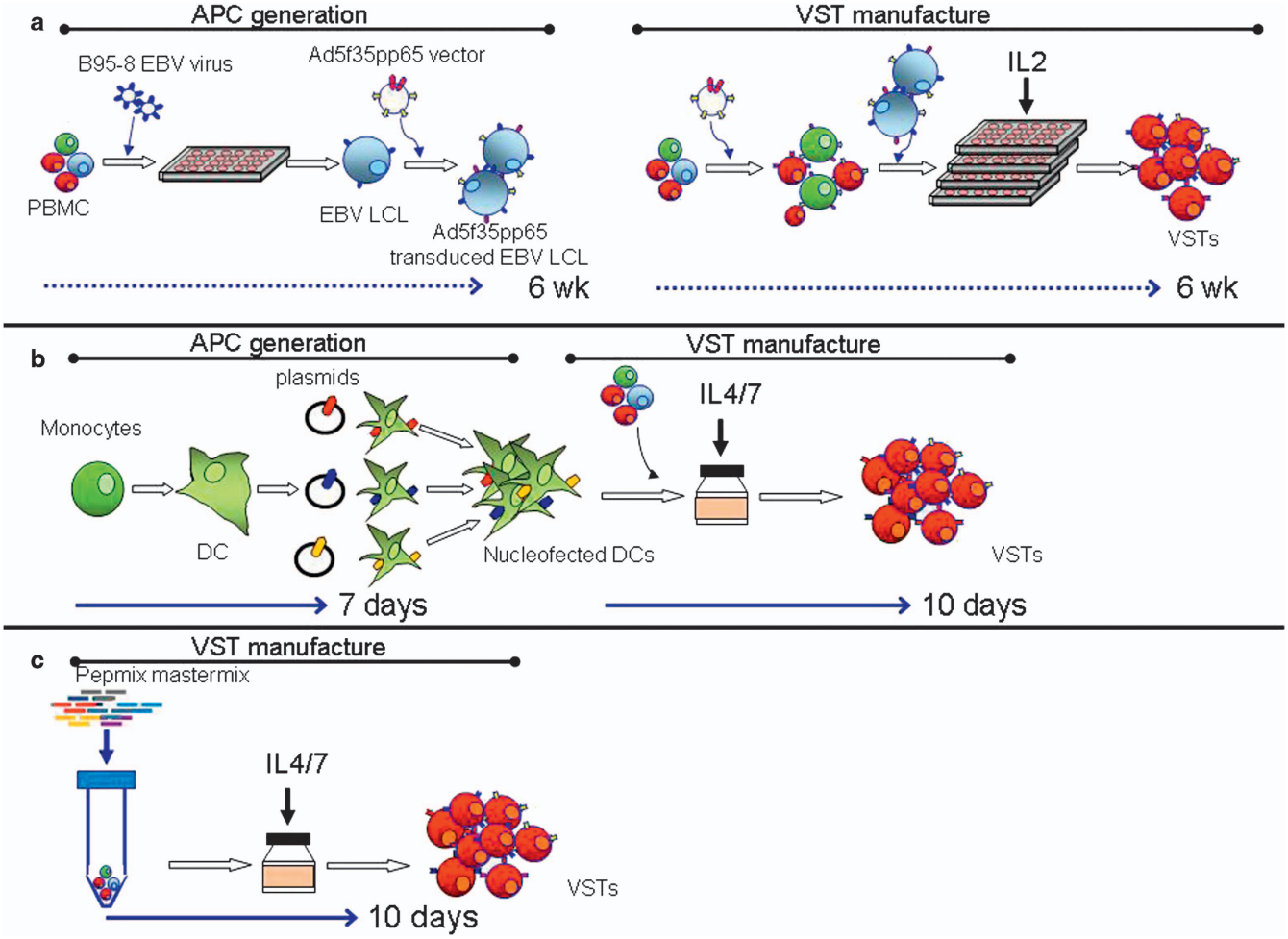

Figure 1 Improvement of VST manufacture. (**a**) Shown is our original protocol for generating VSTs directed against AdV, EBV and CMV using Ad5f35pp65-transduced monocytes and EBV-LCLs as APCs in a process that took up to 12 weeks. (**b**, **c**) Shown are our more recent streamlined manufacturing approaches to generate multivirus-directed VSTs using either DCs nucleofected with viral antigen-encoding plasmids as APCs (**b**) or direct peripheral blood mononuclear cell (PBMC) exposure to overlapping peptide libraries (**c**) to generate VSTs in 17 and 10 days, respectively.

Hanley *et al.*[85] have generated trivirus-directed T cells targeting AdV, EBV and CMV using the 20% fraction of the CB graft. They have clinically translated their approach and, to date, 7 transplant recipients have been infused with these CB-derived cells at doses ranging from 0.5 to 2.5×10^7 cells per m^2 on days 63–146 post transplant, with no infusion-related toxicities or subsequent GvHD. Two of these patients had evidence of viral reactivation; the first with both AdV and CMV, and the second patient had an EBV reactivation. In both cases, the viruses were controlled, although for CMV two infusions were required.[86] Hence, it appears that adoptively transferred CB VSTs can support immune reconstitution, although whether such cells will have the same *in vivo* persistence and antiviral activity as VSTs generated from seropositive donors remains to be seen.

Third-party VST banks

An alternate approach to more broadly implement VST-based therapies involves the use of banked T-cell lines that have been prepared in advance from seropositive individuals with common HLA polymorphisms. These cells can be administered in the partially HLA-matched third-party setting as an 'off the shelf' product available for immediate use. However, one must consider the potential for inducing GvHD with these often substantially HLA-mismatched products. Nevertheless, a number of studies have demonstrated the feasibility, safety and clinical benefit associated with this therapy. For example, Haque *et al.*[87,88] prepared a bank of EBV-specific T cells that were used to treat patients with EBV-PTLD after solid organ ($n = 31$) or stem cell ($n = 2$) transplant. In their initial study, lines for infusion were selected based on the best HLA- A, B and DR match and were administered in 4 weekly doses of 2×10^6 VSTs per kg. Overall, the infusions proved safe with no acute toxicity, GvHD or adverse effects on the transplanted organ. At 5 weeks and 6 months after the first infusion, the complete or partial response rates were 65 and 52%, respectively. Responses were associated with the number, but not the loci of HLA matches, that ranged from 2/6 to 5/6 HLA, and the level of overall HLA match correlated with a significantly better response to T-cell therapy at 6 months.[87,88] Similarly, the Memorial Sloan Kettering group reported that third-party EBV-specific T cells produced complete responses in four of five patients with EBV PTLD after HSCT (including two cord blood transplant recipients).[75,89] Finally, in a recent multicenter study, our group administered partially HLA-matched trivirus-directed VSTs to 50

allogeneic HSCT recipients with refractory EBV, CMV and AdV infections. From a bank of just 32 lines, we were able to identify a suitable product for 90% of patients screened for study participation, and selection of lines for infusion was based on the specificity of the line for the target virus through a shared HLA allele as well as the overall level of HLA match. Eighteen VST lines were administered as treatment for persistent CMV ($n = 23$), AdV ($n = 18$) and refractory EBV-PTLD ($n = 9$). The infusions were well tolerated, and even though the lines infused matched at 1 to 4 of the recipients' HLA antigens, *de novo* GVHD occurred in only 2 patients, grade I in both cases. Overall, the infusions were associated with clinical benefit as 74% of treated patients had a complete or partial response—74%, 79% and 67% for CMV, AdV and EBV, respectively, that was durable in the majority of cases. ELIspot assays performed on pre- and post-infusion follow-up samples demonstrated an increase in the frequency of virus-reactive cells in $\sim$50% of responders. In addition, *in vivo* T-cell persistence, monitored using deep sequencing analysis, indicated that donor-derived T-cell receptor sequences persisted for 4–12 weeks post infusion, though our expectation is that as endogenous host immunity recovered the infused cells were eliminated.[90] As the data from three centers support both the safety and efficacy of banked VSTs, it should be possible to use this approach as a means of more broadly implementing VST therapy.

FUTURE PERSPECTIVES

Evidence from over 20 years of adoptive VST and selected T-cell transfer studies overwhelmingly supports the safety and clinical benefit associated with this therapeutic modality as a means to prevent and treat viral infections and support endogenous immune reconstitution. However, there is still limited access to this therapy for most HSCT patients. The ability to simultaneously protect against multiple viruses using a broad-spectrum VST product is more cost effective and less toxic that the alternative of administering multiple conventional antivirals—features that are attractive from the perspective of both the patient and their insurance company, whereas the ease of manufacturing makes this approach appealing for commercial entities to explore for commercialization. Thus, these advances should serve to move T-cell immunotherapies to a standard of care.

CONFLICT OF INTEREST

AML has patents in the area of cellular therapy, and the Center for Cell and Gene Therapy has a collaborative research agreement with Celgene for genetically modified T cells.

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