

Immunologic special forces: anti-pathogen cytotoxic T-lymphocyte immunotherapy following hematopoietic stem cell transplantation

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Abstract: Anti-pathogen adoptive T-cell immunotherapy has been proven to be highly effective in preventing or controlling viral infections following hematopoietic stem cell transplantation. Recent advances in manufacturing protocols allow an increased number of targeted pathogens, eliminate the need for viral transduction, broaden the potential donor pool to include pathogen-naïve sources, and reduce the time requirement for production. Early studies suggest that anti-fungal immunotherapy may also have clinical benefit. Future advances include further broadening of the pathogens that can be targeted and development of T-cells with resistance to pharmacologic immunosuppression.

Keywords: immunotherapy, stem cell transplantation, T-cell, virus, fungus

Introduction

Since the advent of hematopoietic stem cell transplantation (HSCT), infections have remained a leading cause of morbidity and mortality in patients.¹⁻⁴ Although advances in prophylactic therapy have reduced the early burden of viral and fungal infections, therapeutic options for breakthrough infections are complicated by toxicities, and for many viral infections there are no effective treatments.⁵⁻⁹ It has been well established that T-cell reconstitution is the most important factor in preventing viral infection following HSCT, and factors that influence the speed of T-cell recovery also impact the risk of viral infection in this period.^{2,3} As transplantation protocols have progressed to allow an increasing number of donor sources for transplantation, clinicians have had to balance the risks of graft versus host disease (GVHD) when using a T-cell replete graft versus delayed T-cell engraftment when using T-cell depletion or a naïve donor source such as cord blood.^{10,11}

Given the importance of T-cells to antiviral immunity, use of donor lymphocyte infusions from the stem cell donor was discovered to be an effective salvage therapy for viral infections in HSCT recipients prior to T-cell recovery.¹² However, the high rate of potentially fatal GVHD has relegated this treatment to a course of last resort. However, subsequent advances in immunobiology and culturing techniques have permitted great progress in improving the safety and efficacy of cytotoxic T-lymphocyte (CTL) immunotherapy following HSCT. These include: an improved knowledge of conserved T-cell epitopes for various pathogens,¹³⁻¹⁵ improvements in ex vivo culture of T-cells and antigen-presenting cells,¹⁶⁻¹⁸ and rapid tests to evaluate the effector function and major histocompatibility complex (MHC) restriction of T-cells.^{19,20} In essence, CTL therapy allows clinicians to bypass the months required for T-cell engraftment and a subsequent primary immune response to a pathogen.

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Although trials utilizing antiviral CTLs represent the bulk of the studies to date, preclinical studies and early clinical trials of antifungal CTLs have also shown promise. Adoptive immunotherapy targeting tumor targets is also a burgeoning field, and has recently been reviewed.²¹ In this review, we summarize the methodologies and results of recent and current trials of anti-pathogen CTL therapy, and recap recent preclinical advances that provide the framework for future CTL clinical studies.

Methodologies of CTL production

In CTL production protocols to date, two concepts are essential, ie, harnessing pathogen-specific T-cells, and the exclusion of alloreactive T-cells. This has been accomplished previously by either direct selection of donor cells, or stimulation and ex vivo culture of donor T-cells from peripheral blood mononuclear cells (PBMCs).

Direct selection relies on cell sorting of donor PBMCs, usually after a short stimulation with the antigen of interest.²² Selection can be achieved via multimer selection (selecting for T-cells with a T-cell receptor of known antigen specificity), or by column selection of interferon-gamma-producing T-cells following a brief stimulation with an antigen of interest (Figure 1). It has the advantage of a minimal time requirement for product manufacturing, and uses existing Good Manufacturing Practice-compliant sorting technologies. However, this technique requires leukapheresis of donors in order to collect sufficient cells for clinical use. Additionally, it requires that there be detectable pathogen-specific T-cells in the periphery, and thus it would not be a viable option for manufacturing of CTLs from pathogen-naïve donors nor for pathogens that induce a poor memory response. Multimer selection has the disadvantage of selecting only CD8+ T-cells of limited specificity and MHC restriction, which could allow pathogen evasion and possibly impair CTL persistence.²³ Additionally, previous studies have suggested that residual binding of multimers may impact T-cell function in vitro,²⁴ although the clinical impact of this effect is unclear. The recent development of reversible streptamer technology for selection bypasses this potential risk.²⁵ Interferon-gamma selection allows inclusion of polyclonal antigen-specific CD4+ and CD8+ T-cells, and allows selection of a wider range of antigen-specific cells in the final product.

Alternatively, stimulation and ex vivo culture permits expansion of single or multiple pathogen-specific CTLs. Culture has several advantages over cell selection, including generation of polyclonal CTLs, and expansion of cells to clinically useful volumes from a small volume of blood.²⁶

These advantages come at the expense of the culture and processing time required for CTL stimulation and expansion, which can vary from 10 days to more than 3–4 weeks, depending on the donor source. Loss of the ability of cells to self-renew and impaired persistence in vivo has been a long-standing concern with the use of prolonged ex vivo culture and expansion.²⁷ However, clinical trials to date have demonstrated prolonged persistence in spite of ex vivo culture.²⁸ Additionally, studies have demonstrated that ex vivo culturing with pathogen-specific stimuli eliminates alloreactivity,¹⁵ likely due to cell death or inability to compete with pathogen-specific T-cells, and residual alloreactivity in manufactured CTLs has been shown to be clinically insignificant.²⁹ Early trials of CTL therapy depended on the use of virus-infected antigen-presenting cells, such as cytomegalovirus (CMV) lysates, CMV-infected fibroblasts, or Epstein Barr virus (EBV)-lymphoblastoid cell lines as a stimulant for expansion of donor-derived memory T-cells.^{30–32} Subsequent knowledge of dominant and highly conserved antigens such as CMV-pp65 and Adenovirus (Adv) hexon and penton have permitted the replacement of live virus with antigen stimulation using either 15-mer peptide pools spanning viral proteins, or with transduction of DNA plasmids encoding viral antigens into antigen-presenting cells.^{33,34} New methods to rapidly grow and manipulate antigen-presenting cells have also enabled the use of a wider population of donors and targeting of a greater number of pathogens in a single CTL culture.^{16,35} Optimization of cytokine cocktails for CTL culture has also allowed improved yields and targeted cellular phenotypes. In the recent rapid CTL protocol, interleukin (IL)-4 and IL-7 were shown to produce CD4+ T-cells with a predominantly Th1 phenotype, whereas IL-2 and IL-15 seem to favor proliferation of natural killer cells at the expense of T-cells.³⁴ Finally, studies have shown that central memory T-cells (characterized by expression of chemokine receptors CCR7, CD62L, and CD45RA) have superior persistence in vivo following adoptive transfer, and may be the ideal cell population for adoptive immunotherapy.^{36,37} Consequently, studies using both selection and culture methods have demonstrated the development of central memory T-cells in the resulting CTL products.^{25,34}

Clinical studies of anti-viral CTLs

Clinical studies utilizing cell selection

Cell selection has been used in several prior studies to treat patients following HSCT (Table 1). Cobbold et al published the first clinical report in which CD8+ CMV-specific CTLs were isolated via tetramer selection.³⁸ Complete or

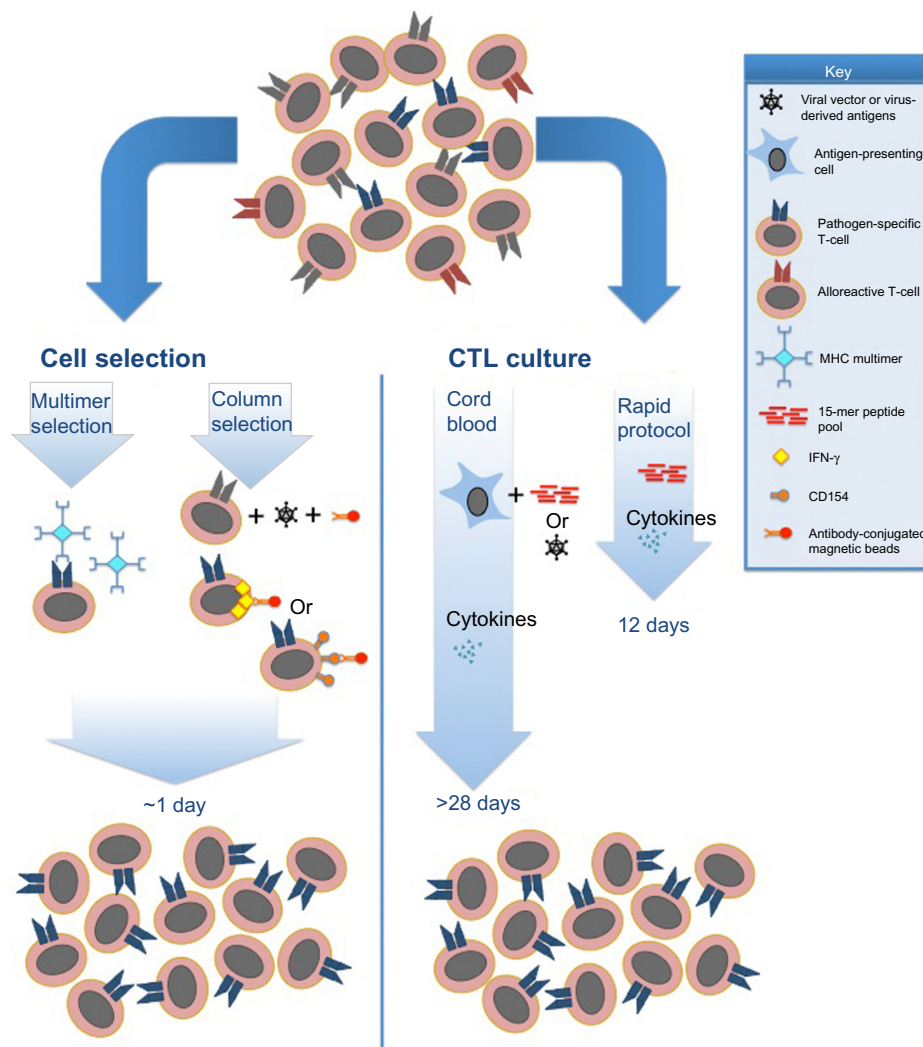


Figure 1 Current Good Manufacturing Practice-compliant approaches for generation of antipathogen CTL products.

Notes: Cell selection utilizes either multimers displaying a pathogen-derived peptide in the setting of a type I human leukocyte antigen molecule, or column selection utilizing ex vivo stimulation of T-cells with antigens followed by selection of interferon-gamma or CD154-expressing T-cells via antibody-coated immunomagnetic beads. Ex vivo cell culture utilizes stimulation of T-cells by antigen-displaying antigen-presenting cells, which can be produced via antigenic peptide pools, viral transduction, or nucleofection. A rapid protocol can produce cytotoxic T-lymphocytes in 10–12 days from virus-seropositive donors after a single stimulation, whereas production of cytotoxic T-lymphocytes derived from cord blood requires three stimulations over a minimum of 28 days.

Abbreviations: CTL, cytotoxic T-lymphocyte; IFN, interferon.

partial clinical responses were achieved in nine patients who received infusions, although there were limited data on long-term persistence of infused CTLs. Feuchtinger et al utilized interferon-gamma column selection (Gamma capture assay; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to produce CMV-CTL, resulting in partial to complete responses in 15 of 18 patients who were given a single dose.³⁹ Peggs et al also used interferon-gamma selection to produce CMV-CTL, using either recombinant pp65 or an overlapping peptide pool of 15-mers covering the pp65 protein as stimulants.⁴⁰ They were successful in protecting seven patients who were prophylactically treated, while in vivo expansion of CMV-CTLs was detected in 11 patients

infused who had detectable CMV.⁴⁰ Schmitt et al produced CMV-CTL from HSCT donors utilizing reversible streptamers with MHC-restricted pp65 peptides.²⁵ These products were used to successfully treat two patients who developed CMV reactivation during treatment of GVHD after HSCT.

Fewer clinical studies have been performed using these techniques to produce Adv-specific or EBV-specific CTL. Feuchtinger et al successfully produced Adv CTL by interferon-gamma selection for treatment of nine patients with treatment-refractory Adv infections.⁴¹ In vivo CTL expansion was demonstrated in five of six patients tested, and four patients had clearance of disease. Uhlin et al used

Table 1 Previous clinical trials of pathogen-specific T-cell therapy

Strategy	Study	Pathogen specificity	Donor	Methodology	Patient accrual	Centers (n)	Advantages/disadvantages
Cell selection	Cobbold et al ³⁸	CMV	HSCT donor or third-party	Tetramer selection	9	1	Advantages Rapid development Uses existing GMP compliant technology
	Feuchtinger et al ³⁹	CMV	HSCT donor or third-party	Interferon-gamma column selection	18	1	
	Peggs et al ⁴⁰	CMV	HSCT donor	Interferon-gamma column selection	18	1	
	Schmitt et al ²⁵	CMV	HSCT donor	Reversible Streptamer selection	2	1	Disadvantages Limited repertoire with multimer use (low cell yield) Requires presence of pathogen-specific memory T-cells
	Feuchtinger et al ⁴¹	Adv	HSCT donor	Interferon-gamma column selection	9	1	
	Uhlin et al ⁴²	EBV	Related haploidentical donor	Multimer selection	1	1	
	Moosman et al ⁴³	EBV	HSCT donor	Interferon-gamma column selection	6	1	
	Qasim et al ⁵⁴	Adv	Third party	Interferon-gamma column selection	1	1	
	Uhlin et al ⁵²	CMV/EBV/Adv	HSCT donor or third-party	Pentamer selection	8	1	
Cell culture	Perruccio et al ⁶⁹	CMV or Aspergillus	HSCT donor	Stimulation of PBMC with CMV antigen or inactivated conidia	10	1	Advantages Yields large number of polyclonal CTL Allows CTL development from pathogen-naïve donors
	Leen et al ¹⁷	CMV/EBV/Adv	HSCT donor	Ad5f35pp65 transduced LCL	26	3	
	Micklethwaite et al ⁴⁵	CMV/Adv	HSCT donor	Ad5f35pp65 transduced DC	12	1	
	Leen et al ⁴⁴	EBV/Adv	HSCT donor	Ad5f35 null transduced LCL	13	3	
	Barker et al ⁵⁰	EBV	Third-party donor	EBV-LCL stimulation	2	1	Disadvantages Time-intensive (2–5 weeks) Regulatory requirements for GMP culturing
	Rooney et al 2010 ³¹	EBV	HSCT donor	Irradiated EBV-LCL	114	3	
	Balduzzi et al ⁵⁵	JCV	HSCT donor	Pemix-pulsed PBMC	1	1	
	Leen et al ⁵¹	CMV/EBV/Adv	Third-party donor	Ad5f35pp65 transduced LCL	47	8	
	Gerdemann et al 2013 ⁷²	CMV/EBV/Adv	HSCT donor	Nucleofection of DCs	12	2	
	Blythe et al 2013 ⁴⁶	CMV or CMV/Adv	HSCT donor	NLV-peptide pulsing or Ad5f35pp65 transduction of DCs	50	2	

Abbreviations: Adv, adenovirus; CMV, cytomegalovirus; CTL, cytotoxic T-lymphocyte; DC, dendritic cells; EBV, Epstein Barr virus; GMP, Good Manufacturing Practice; LCL, lymphoblastoid cell lines; HSCT, hematopoietic stem cell transplantation; PBMCs, peripheral blood mononuclear cells; JCV, John Cunningham virus.

human leukocyte antigen (HLA)-A2-specific pentamers to produce EBV-CTL from the haploidentical mother of a patient who underwent cord blood transplantation and subsequently developed EBV-induced post-transplant lymphoproliferative disease.⁴² A complete clinical response was obtained following two doses of CTLs. Moosman et al treated six patients with EBV-induced post-transplant lymphoproliferative disease with EBV-CTL developed by interferon-gamma selection, and achieved complete responses in three patients with early disease, but no response in three patients with advanced, multiorgan disease.⁴³ Of note, in all studies utilizing cell selection, no significant GVHD

occurred, and clinical impacts were seen in spite of very low cell doses ($<5 \times 10^4$ cells/kg in most studies).

Clinical studies utilizing cell culture

CTL production utilizing ex vivo cell culture has been the most common methodology to date, and accounts for the majority of patients treated in clinical trials of antipathogen adoptive immunotherapy (Table 1) over the past decade. Walter et al were among the first to show that stimulation of donor PBMC by CMV extracts resulted in expansion of CMV-specific CTLs, which lost alloreactivity after several weeks of ex vivo culture but retained antiviral activity.³² There

have been many advancements in ex vivo CTL culture since then, which have decreased both the manufacturing time and cost. Early notable strides involved the culture and manipulation of antigen-presenting cells for CTL culture. Rooney et al successfully used irradiated EBV-lymphoblastoid cell lines (EBV-LCL) to generate EBV-specific CTL, which were effective as prophylaxis or treatment for EBV-induced post-transplant lymphoproliferative disease in 114 patients.^{28,31} Of note, the first 26 patients received gene-marked CTLs, and follow-up studies showed persistence of the gene-marked cells as long as 105 months following infusion.

The development of clinical grade Adv vector Ad5f35pp65, which contains immunodominant CMV antigen pp65, permitted transduction of either donor-derived dendritic cells or EBV-LCL for use as antigen-presenting cells for CTL culture. Leen et al used this strategy to produce triviral (CMV, EBV, Adv-specific) CTLs, which were utilized in a dose-escalation trial to treat 26 patients.¹⁷ No adverse effects were seen at doses ranging from 5×10^6 to 1×10^8 cells/m², and all patients were effectively protected against CMV, EBV, and Adv disease. However, although EBV-specific and CMV-specific CTLs showed persistence by interferon-gamma ELISPOT, Adv-specific CTLs were not detectable except in the setting of infection. A follow-up trial utilized Ad5f35-transduced EBV-LCL to produce EBV-specific and Adv-specific CTL, which were infused into 13 patients as prophylaxis or treatment of EBV and Adv following HSCT.⁴⁴ Although the products provided effective protection against EBV and Adv in vivo, Adv-specific CTLs were again not detectable except in the setting of Adv infection, suggesting that even at levels below the limits of detection by interferon-gamma ELISPOT, the Adv-specific CTL provided protection and was able to undergo expansion in the setting of viral infection. Ad5f35pp65-transduced dendritic cells were similarly used by Mickelthwaite et al to produce CMV-specific and Adv-specific CTLs, which were clinically effective in 12 patients who received infusions following HSCT.⁴⁵ Only two subsequent episodes of CMV reactivation occurred in the setting of administration of prednisone at levels as low as 0.5 mg/kg/day. Blyth et al similarly treated 50 patients following HSCT with triviral (CMV, EBV, Adv-specific) CTLs which were derived by a mix of methods: ten were produced by pulsing donor dendritic cells with the HLA-A2-restricted CMV peptide NLVPMVATV and 40 were produced using Ad5f35pp65-transduced donor dendritic cells.⁴⁶ Only five of the 50 patients developed CMV reactivations following CTL infusions, and one of these five required antiviral pharmacotherapy after being treated with steroids for acute GVHD.

Further protocol advances have validated the use of 15-mer peptide pools encompassing immunodominant viral antigens in place of viral transduction of antigen-presenting cells, thus removing the potential safety and regulatory barriers associated with use of viral vectors.³³ The use of gas-permeable rapid-expansion (G-Rex) bioreactors has further simplified CTL culture.⁴⁷ Gerdemann et al combined these two advances to develop a rapid protocol that yields CTL at clinical volumes in 10–12 days, and provided effective antiviral protection in ten patients who were infused following HSCT.³⁴ The ongoing ARMS (Administration of Rapidly Generated Multivirus-Specific Cytotoxic T-Lymphocytes for the Prophylaxis and Treatment of EBV, CMV, Adv, human herpesvirus 6 [HHV6], and BK virus infections post Allogeneic Stem Cell Transplant; NCT01570283) study has further modified this rapid protocol to produce five virus-specific CTL from a monoculture.

Gerdemann et al have further modified the rapid CTL protocol by utilizing nucleofection of DNA plasmids containing viral epitopes into donor-derived dendritic cells.⁴⁸ The resulting CTL cultures showed antiviral activity in vitro by interferon-gamma ELISPOT and Cr⁵¹ cytotoxicity assays comparable with that of similar products derived via stimulation with 15-mer peptide pools for the same viral epitopes.

Adverse events following administration of ex vivo cultured CTL products in 381 infusions for 180 patients on 18 protocols were recently reviewed by the groups at Baylor College of Medicine.⁴⁹ Twenty-four mild adverse events were reported within 6 hours of infusion, with nausea and vomiting being most common, and 22 nonserious adverse events (fever, chills, nausea) occurring within 24 hours. No significant GVHD was attributable to CTL infusion. The only significant complications of CTL therapy have been rare reports of systemic inflammatory responses following EBV-CTL therapy in patients with bulky EBV+ lymphoma. Blyth et al reported that seven cases of acute GVHD occurred following CTL infusion, although some were attributable to corticosteroid weaning prior to CTL infusion, and additionally the authors noted that the degree of HLA mismatch was greater in patients who received CTL therapy versus controls.⁴⁶

Recent developments

Third-party CTL use, expanded viral targets, T-cell receptor gene transfer, and CTL manufacture from pathogen-naïve donors

Until recently, the selection or culture of antipathogen CTLs was dependent on the presence of pathogen-specific

memory T-cells in donor blood. These protocols failed to help recipients of pathogen-naïve stem cell products, a population that has been well described to be at increased risk of viral infection following HSCT.

One answer to this problem is the use of “off-the-shelf” CTLs derived from third-party donors. This approach has been successfully used in several prior studies.^{50,51} Barker et al successfully treated two patients with refractory EBV-induced post-transplant lymphoproliferative disease following cord blood transplantation with third-party EBV-specific CTLs.⁵⁰ Leen et al utilized a bank of 32 CTL lines with characterized activity against EBV, CMV, and Adv to identify matched lines for 50 patients with refractory viral infections.⁵¹ These infusions resulted in antiviral responses in 74%, 78%, and 67% of those with CMV, Adv, and EBV, respectively. This represents a dramatic improvement from the standard therapy response rate in eight patients for whom a matched line could not be found, who had a response rate of 13% and a mortality rate of 75%. In spite of only partial HLA matching (1–4 loci), only two patients developed grade I GVHD. The lower rate of response against EBV relative to CMV and Adv may be reflective of a greater breadth of immunodominant epitopes that differ by MHC types, which complicates the task of selecting the ideal third-party line with both antiviral activity and proper MHC restriction.

Third-party CTL treatment has also been successful using selection methodology. Uhlin et al used pentamer selection to produce anti-viral CTL specific for CMV, EBV, or Adv from related third-party donors for six patients with refractory viral infections (four with CMV, and one each with EBV and Adv).⁵² Five of six patients had partial or complete responses. Notably, an infant with severe combined immunodeficiency was treated prior to cord blood transplantation with CMV-CTL derived from her mother, with a ten-fold reduction in her CMV DNA level. Wy and Qasim used interferon-gamma selection to manufacture Adv-CTL from related third-party donors to treat two patients who underwent HSCT and subsequently developed Adv viremia.⁵³ Although treatment successfully cleared the Adv infection in one patient, she developed grade III skin and liver GVHD.⁵⁴ Curiously, cytogenetic studies of liver tissue showed infiltration with T-cells from the original HSCT donor but not the CTL donor. The authors postulated that this was due to a “bystander” effect of CTLs on the HSCT donor cells; however, such an effect has not been seen in larger trials utilizing third-party CTL therapy.

A small number of other viruses have been targeted via adoptive immunotherapy. John Cunningham virus (JCV) is an ubiquitous polyoma virus which can cause progressive

multifocal leukoencephalopathy, a devastating neurologic disease, in patients who are profoundly immunocompromised, including recipients of HSCT or solid organ transplants and patients with advanced human immunodeficiency virus or primary immunodeficiency disorders. Balduzzi et al described the use of donor-derived JCV-specific CTL in a 14-year-old patient who developed progressive multifocal leukoencephalopathy in the setting of prolonged steroid treatment for GVHD following HSCT.⁵⁵ These CTL were manufactured using 15-mer peptide pools encompassing the JCV antigens VP1 and LT, and were cultured for 26 days. The patient received two doses of JCV-specific CTLs, and had a remarkable and sustained improvement, including clearance of JCV-DNA from the cerebrospinal fluid and substantial improvements in his neurologic status.

Although not a frequent problem following HSCT, human papillomavirus (HPV) is not an uncommon late complication of HSCT, particularly in patients treated for primary immunodeficiency disorders. HPV has also been evaluated in preclinical studies as a potential target for CTL therapy. Ramos et al have described the use of peptide pools spanning the HPV E6 and E7 proteins to generate HPV-specific CTLs from patients with oropharyngeal or cervical cancer, many of which arise due to HPV16 infection.⁵⁶ The resulting CTLs showed specific activity against HPV E6 and E7, and also showed antitumor activity against CaSki, an HPV16 cervical cancer cell line.

Several studies have explored the possibility of transducing CTL with a T-cell antigen receptor of known viral specificity.^{57–59} This offers a novel strategy to develop CTL from pathogen-naïve donors, but imposes the additional regulatory requirements of transgenic technology. Additionally, the use of a single antiviral T-cell antigen receptor may risk antigenic escape by the pathogen. Nonetheless, a current trial of transgenic CTL utilizing a retroviral vector with a CMV-specific T-cell antigen receptor is being conducted in the UK by Emma Morris (principal investigator).⁶⁰

An important landmark in the field of adoptive immunotherapy has been the successful development of virus-specific CTLs from virus-naïve donors. Hanley et al first demonstrated that CTL could be produced in a 20% fraction from cord blood using donor-derived dendritic cells and an EBV-lymphoblastoid cell line as antigen-presenting cells, and Ad5f35pp65 transduction as a source of CMV and Adv antigens.¹⁶ The resulting cell lines had specific antiviral activity against CMV, EBV, and Adv in interferon-gamma ELISPOT analysis as well as Cr⁵¹ cytotoxicity assays, with no evidence of alloreactivity. Curiously, epitope mapping showed that the immunodominant

epitopes recognized by cord blood-derived CTLs differed from CTLs manufactured from CMV-seropositive and EBV-seropositive adult donors, with the HLA-A2 restricted epitope NLVPMVATV notably absent in the cord blood-derived lines. Despite this finding, CTLs manufactured from cord blood have been used successfully in 12 cord blood transplant recipients to date in the ongoing ACTCAT (Safety, Toxicity and MTD of One Intravenous IV Injection of Donor CTLs Specific for CMV and Adenovirus; NCT00880789) trial.

Most recently, Hanley et al have successfully manufactured multiviral CTLs from CMV-naïve adult donors.³⁵ To do so, CMV-CTLs were produced from CD45RA+ naïve T-cells isolated via column selection, and stimulated by donor dendritic cells pulsed with CMV 15-mer peptide pools. Preclinical data suggest that they have similar antiviral activity, and the current MUSTAT (Multivirus-Specific Cytotoxic T-Lymphocytes for the Prophylaxis and Treatment of EBV, CMV, and Adenovirus Infections post Allogeneic Stem Cell Transplant; NCT01945814) trial will seek to compare the clinical efficacy of CTLs derived from CMV-seropositive versus CMV-naïve donors.

Anti-fungal CTLs

Fungal infections are a well described risk after HSCT. The importance of Th17 immunity in controlling *Candida* infections has been well demonstrated by forms of primary immunodeficiency such as Hyperimmunoglobulin E syndrome and chronic mucocutaneous candidiasis, as well as human immunodeficiency virus infection.^{61,62} The importance of T-cell immunity in defense against invasive aspergillosis and mucormycosis is less clear, while their ties to innate defense (most notably neutrophil function) are well established. Interestingly, a recent study of patients with chronic granulomatous disease showed that they have abundant *Aspergillus*-specific T-cells with increased interferon-gamma production compared with healthy controls.⁶³ Despite these uncertainties, fungal infections may be a valid target for treatment via adoptive immunotherapy after HSCT.

Several preclinical studies have been successful in developing CTLs with activity against *Candida*, *Aspergillus*, and *Rhizopus* species (Table 2). Beck et al successfully produced *Aspergillus*-specific CTLs by stimulation of PBMCs with antigens from *Aspergillus* extracts, followed by interferon-gamma selection and culture.⁶⁴ The resulting population was predominantly CD4+ memory (CD45RO+) cells, but demonstrated interferon-gamma production in response to several species of *Aspergillus* as well as *Penicillium*. The authors also showed that these T-cells enhanced hyphal

Table 2 Preclinical studies of novel antipathogen T-cell therapies

Study	Pathogen specificity	Donor	Methodology
Beck et al ⁶⁴	<i>Aspergillus</i>	Healthy donors	Interferon-gamma selection after stimulation of PBMCs with <i>Aspergillus</i> extracts
Khanna et al ⁶⁶	<i>Aspergillus</i> , <i>Candida</i> , <i>Rhizopus</i>	Healthy donors	CD154 selection after stimulation of PBMCs with fungal extracts
Gerdemann et al ³⁴	CMV/EBV/Adv/ HHV6/RSV/ BK/influenza	Healthy donors	Nucleofection or Pempix stimulation of DCs
Ramos et al ⁵⁶	HPV13	Cervical or nasopharyngeal cancer patients	Pempix stimulation of DCs
Tramsen et al ⁶⁵	<i>Aspergillus</i> , <i>Candida</i> , <i>Rhizopus</i>	Healthy donors	Interferon-gamma selection after stimulation of PBMCs with fungal extracts

Abbreviations: Adv, adenovirus; CMV, cytomegalovirus; DCs, dendritic cells; EBV, Epstein Barr virus; PBMCs, peripheral blood mononuclear cells; HHV, human herpesvirus 6; HPV, human papillomavirus; RSV, respiratory syncytial virus.

damage by neutrophils and antigen-presenting cells in vitro. Tramsen et al similarly used interferon-gamma selection following stimulation with cellular extracts from *Candida albicans*, *Aspergillus fumigatus*, and *Rhizopus oryzae* to produce multifungal-specific CTL lines, which were also almost exclusively CD4+ CD45RO+ HLA-DR+.⁶⁵ These lines displayed pathogen-specific activation markers (interferon-gamma CD154, tumor necrosis factor-alpha) and also enhanced oxidative activity of neutrophils when coincubated with antigen and antigen-presenting cells and tested via the 123-dihydrorhodamine assay. Khanna et al described a novel selection method based on upregulation of CD154 to produce multipathogen-specific T-cells against CMV, EBV, Adv, *Candida*, and *Aspergillus*.⁶⁶ Donor PBMCs were incubated with peptide libraries from CMV-pp65, EBV-LMP2, Adv-Hexon, *Candida* MP65, and a 15-mer peptide from *Aspergillus* CRF1. Following 14 days of culture, the authors showed pathogen-specific interferon-gamma production, proliferation, and cytotoxicity in vitro. Although these results are intriguing, there are very limited data regarding the relative importance of MP65 and CRF1 in antifungal immunity.^{67,68}

As of the time of this review, only one clinical trial of anti-fungal CTLs has been published. Perruccio et al developed CTLs via stimulation of donor PBMCs with inactivated conidia from *A. fumigatus*, followed by several weeks of culture, resulting in clonal CD4+ CTLs with anti-*Aspergillus* activity by interferon-gamma ELISPOT.⁶⁹ Clinical use of these lines in patients with pulmonary aspergillosis resulted

in survival of eight of nine patients treated, compared with a survival rate of 7/13 in patients with aspergillosis who did not receive infusions. There was no difference in the length of antifungal therapy required for survivors in the two groups.

Although these studies are intriguing, several key issues require attention before anti-fungal CTL trials begin to catch up with their antiviral brethren. First, a better understanding of the immunodominant T-cell targets for various fungal species is needed. Second, standardized Good Manufacturing Practice-compliant fungal antigen sources are necessary to allow consistency and valid comparisons between future clinical trials.

Future of CTL therapy Expanding the breadth of monoculture CTL lines: is there an antigen limit?

As manufacture of CTLs expands to include more pathogens in a single culture, the possibility of antigenic competition between the different pathogen-specific T-cells has caused many to question the limits of CTL monocultures. This concern has certainly been validated in attempts to produce multivirus-specific CTLs from donors who are CMV-naïve, in which the resulting culture is dominated by memory-derived EBV-specific and Adv-specific T-cells. Although the relative proportions of individual virus-specific CTLs decrease as the number of antigens increases, this has not seemed to impact the efficacy of these products in clinical trials. Recent studies have challenged the upper antigen limit of CTL monoculture, as Gerdemann et al successfully produced CTLs specific for seven viruses (CMV, EBV, Adv, BK, HHV6, respiratory syncytial virus (RSV), and influenza) utilizing peptide pools for 15 antigens, and demonstrated specific activity against all targeted viruses via interferon-gamma ELISPOT.³⁴ As additional preclinical studies attempt to add further pathogens to monoculture, it remains to be seen whether an increased number of targets will compromise specific CTL function or persistence in vivo.

Engineering resistance to immunosuppression

The need for immunosuppressive medications is common in recipients of HSCT, and unfortunately the use of these drugs also suppresses CTL products. Most existing protocols require recipients to be receiving less than 0.5 mg/kg/day prednisone and at least 30 days out from any anti-T-cell serotherapy in order to receive a CTL infusion. Calcineurin inhibitors such as cyclosporin A, tacrolimus, or sirolimus would similarly impact the clinical benefits of CTL at therapeutic doses.

One answer to this problem is to produce genetically modified CTLs that have resistance to immunosuppressive

medications. Several recent studies have successfully demonstrated the viability of this concept. De Angelis et al produced EBV-specific CTLs with resistance to tacrolimus by knockdown of FKBP12 via a retrovirally-transduced specific siRNA.⁷⁰ Transduction of CTLs did not impact antiviral activity, and the cells showed activity in a mouse EBV-lymphoma model in the presence of tacrolimus. Brewin et al similarly produced EBV-specific CTLs with resistance to both cyclosporin A and tacrolimus by direct mutation of calcineurin.⁷¹ The mutation had no impact on the phenotype or antiviral activity of the CTL in vitro, and mutated cells showed a growth advantage in the presence of calcineurin inhibitors.

Although similarly modified cells have not been used clinically to date, they have great potential in treating both HSCT and solid organ transplant recipients. Future extension of these studies could potentially allow production of CTLs with resistance to monoclonal biologic agents such as alemtuzumab.

Conclusion

With several hundred patients having been treated successfully, antipathogen CTLs have been established as a safe and highly effective therapy following HSCT. Further studies to identify preserved viral T-cell epitopes, probe the antigen limits in CTL monoculture, and test the clinical efficacy of immunosuppressive-resistant CTLs will further broaden the usefulness of this therapy. As rapid advances in protocols and multiple available methods of manufacture broaden the availability of this therapy, in time CTL therapy may become the standard of care following HSCT.

Acknowledgment

This work was supported by a National Institute of Child Health and Human Development award to MDK (K12-HD-001399) and Cancer Prevention and Research Institute of Texas (R01 RP100469) and National Cancer Institute (P01 CA148600-02) awards to CMB.

Disclosure

The authors report no conflicts of interest.

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