Specific Adoptive T-Cell Therapy for Viral and Fungal Infections

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Introduction

Infections remain the leading cause of mortality and morbidity during the first 3 months after hematopoietic stem cell transplantation (HSCT) [1–4]. Despite advances in prophylactic viral and fungal therapy to minimize the viral and fungal burden early after HSCT, breakthrough viral and fungal infections remain life-threatening, and for some viral and fungal infections, there are no effective therapies [5–9]. Vaccine strategies to induce immunity to CMV began in the 1970s but have been limited in their success [10–12]. The conditioning regimens for HSCT that vary from non-myeloablative to myeloablative create an immunodeficiency that leaves the allogeneic HSCT recipient susceptible to viral and fungal infections while immune reconstitution occurs during the first 6–9 months after HSCT. Immune reconstitution is further abrogated by intensive immunosuppression used to prevent and/or control GVHD. It is clearly established that the kinetics and rate of T-cell reconstitution are critical to controlling viral infections. Factors that speed T-cell recovery will decrease the risk of viral infection during the first 3 months after HSCT [2, 3, 13]. Early studies showed that donor lymphocyte infusions (DLI) given before T-cell reconstitution from the stem cell donor were effective for treating viral infections in HSCT recipients but were associated with a high risk of GVHD [14]. Since the early 1990s, investigators began to develop virus-specific cytotoxic T lymphocyte (vCTL) for adoptive immunotherapy against specific targets early during immune reconstitution after HSCT [15, 16].

Advances in vCTL therapy have benefited from (1) advances in understanding of immune responses to conserved T-cell epitopes for various pathogens [17–19], (2) technological advances in ex vivo expansion of T cells and advances in the preparation of antigen-presenting cells [20–22], and (3) assays that evaluate vCTL activity and the MHC restriction of vCTL [23, 24].

In this chapter, we review the following areas of how: (1) T cells have been expanded to target multiple pathogens; (2) vCTL production no longer requires viral infection or viral vector transduction of antigen-presenting cells (APCs); (3) The source of lymphocytes is no longer restricted to donors who are immune to the pathogens; (4) Naive T cells have been redirected with chimeric antigen receptor T cells (CARTs) to target pathogen-infected cells; (5) Bispecific antibody

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B. H. Segal (ed.), *Management of Infections in the Immunocompromised Host*,
https://doi.org/10.1007/978-3-319-77674-3_20

(BiAb)-armed T cells (BATs) can mediate vCTL activity; and (6) Pathogen-specific T-cell products can be manufactured by third parties and banked for "off-the-shelf" use post-HSCT.

We summarized the methodological approaches, clinical trials using vCTL, promising preclinical studies, and early clinical trials of anti-pathogen CTLs that have promise. These advances provide the rationale and impetus for future vCTL adoptive immunotherapy.

Production of vCTL As a guiding principle to decrease the risk of GVHD in allogeneic HSCT recipients, strategies excluded alloreactive T cells by selecting virus-specific T cells. Four major approaches were used: (1) stimulation with viral antigen(s) during ex vivo culture of donor T cells from peripheral blood mononuclear cells (PBMC), (2) direct selection of donor cells, (3) genetic modification of T cells to confer specific recognition of pathogen or pathogen-infected cells, or (4) arming of ex vivo expanded T cells with bispecific antibody to target the viral antigen (Fig. 20.1).

Antigen Stimulated Expansion Numerous ex vivo culture approaches have been used to produce cytomegalovirus (CMV)-specific CTL or Epstein-Barr virus (EBV)-specific CTL [15, 16, 25–30]. CMV viral- or peptide-specific stimulation in vitro expands single or multiple pathogen-specific vCTL. The advantages of culture over cell selection are the generation and expansion of polyclonal vCTL to clinically useful quantities of vCTL from small amounts of blood [31]. However, the major disadvantages of this strategy is the daunting task of culturing and processing after stimulation to expand the vCTL (up to more than 1 month) and the HLA-histocompatibility requirement of finding a closely matched donor. During these longer-term cultures, the vCTL may lose their capacity to self-renew and to persist in vivo, particularly after prolonged ex vivo culture [32]. It should be noted that clinical trials infusing ex vivo expanded vCTL post-HSCT showed prolonged persistence [33] and that ex vivo expansion using pathogen-specific stimuli decreased alloreactivity [19]. This may be due to selection of virus-specific clones and deselection of alloreactive clones. One study showed that residual alloreactivity seen in vCTL is clinically insignificant [34]. The initial trials of vCTL therapy required CMV lysates on APC, CMV-infected fibroblasts, or EBV-lymphoblastoid cells lines as a stimulant for expansion of donor-derived memory T cells [25, 27, 35]. The discovery of dominant and highly conserved antigens such as CMV-pp65 and adenovirus hexon and penton led to replacement of live viral stimulation with either 15-mer peptide pools spanning viral proteins or DNA plasmid-transduced antigen-presenting cells [36, 37]. The newer approaches to rapidly expand and manipulate APCs enabled use of a less restricted population of donors and the targeting of an increased number of pathogens in a single culture [20, 38]. In a recent rapid vCTL protocol, the addition of IL-4 and IL-7 leads to production of CD4+ T cells with a Th_1 phenotype, whereas IL-2 and IL-15 tended to favor in vitro natural killer (NK) cell expansion [37]. The ideal population to adoptively transfer may be ex vivo expanded central memory T cells with a CD62L and CD45RA phenotype as these cells have a superior ability to persist in vivo after adoptive transfer [39, 40].

Direct Selection via Cell Capture Sorting Direct selection relies on cell sorting of immune donor PBMCs, usually after pulsing them with the antigen(s) of interest, to drive expansion of virus-specific T-cell clones [41]. This approach would not be viable for obtaining immune CTLs from pathogen-naive donors. Multimer selection is achieved by binding of HLA-peptide complexes to T-cell receptors (TCRs) of known antigen specificity, followed by purification of bound cells, e.g., by magnetic column separation. Alternatively, antiviral T cells expressing interferon-γ (IFN-γ) can be isolated using the gamma capture assay. Direct selection methods have the advantage of rapid manufacturing time. Unfortunately, these approaches require apheresis of donors in order to collect sufficient cells for sorting and processing for clinical appli-

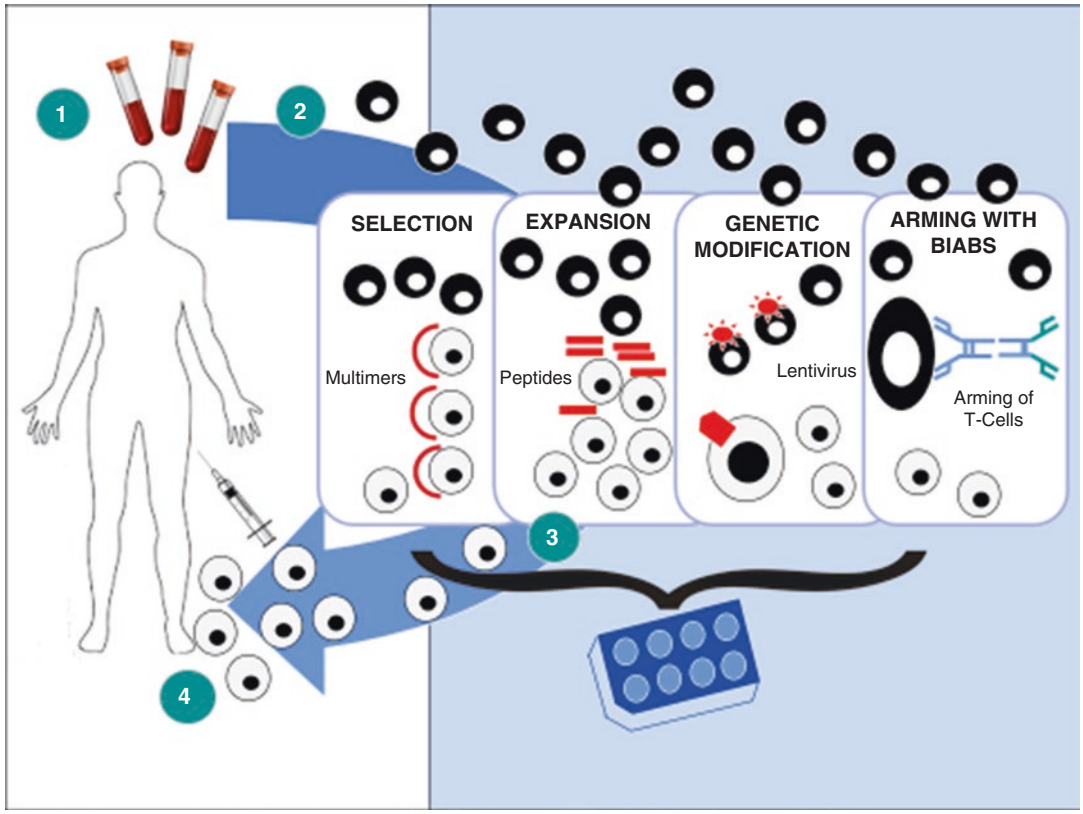

Fig. 20.1 Approaches to produce pathogen-specific CTL. (1) Blood is obtained from donors (autologous, allogeneic, or umbilical cord blood) or is drawn or apheresis is performed to obtain a larger quantity of blood; (2) PBMCs are processed via: (a) cell *selection* panel using multimers with a pathogen-derived peptide associated with a type-I HLA molecule *or* column selection after in vitro stimulation of T cells with antigens followed by binding of IFNɣ or CD154-expressing T cells with antibody-coated immunomagnetic beads; (b) cell *expansion* by stimulating the PBMC with APCs produced by antigenic peptide pools, viral transduction, or nucleofection; (c) *genetic modification* that involves the transfer of high-affinity pathogen-specific TCRs or CARs to redirect the specificity of the T cells; and (d) polyclonal expansion of T cells for 8–14 days and arming with BiAbs directed at the pathogen of interest on one hand and the TCR on the other hand; (3) quality control and release testing; and (4) infusion into patients

cations and pre-existing and detectable pathogen-specific T cells in the blood. Multimer selection is major histocompatibility (MHC)-restricted and selects only CD8+ T cells of a limited specificity. This could possibly allow pathogen evasion and impair persistence of vCTL in vivo [42]. Earlier studies suggested that persistent binding of multimers to the TCR may impair T-cell function [43]. Recent reversible Streptamer technology for direct selection may overcome the problem of impaired function [44]. IFN-γ positive selection captures polyclonal antigen-specific CD4+ and CD8+ T cells and selects for a wider range of antigen-specific cells. Combining direct selection, culture expansion methods, and cytokine cocktails can optimize the selection of central memory T cells in vCTL products and improve yields on targeted cellular phenotypes [37, 44].

TCR or CAR Gene Modifications T cells can be modified to redirect their specificity with retroviral and lentiviral vectors to introduce the transgenes for high-affinity TCRs or chimeric antigen receptors (CARs) consisting of a single-chain variable fragments (scFvs). High-affinity TCR genes can be cloned and transduced into polyclonal T cells to generate a large population of

TCR pathogen-specific CTLs [45]. A similar strategy was used to produce tumor-specific T cells after TCR gene transfer [18]. In contrast, CARs have an extracellular region that consists of a scFv that binds to antigen, with an intracellular signaling complex composed of TCR zeta chain for first-generation CARs, the TCR zeta chain and the CD28 for second-generation CARs, and TCR zeta and CD28 or 41BB for third-generation CARs [46–48]. The high-affinity TCR-transduced CTLs have been used to target CMV-infected cells [49], HPV-infected cells [50], hepatitis B-infected cells [51], hepatitis C-infected cells [52], tuberculosis-infected cells [53], SARS-infected cells [54], chlamydia-infected cells [55], and HIV-infected cells [56]. CAR T cells were used to target CD4 in HIV-infected cells [57–60] and for recognition of β-glucans in fungi [61].

Clinical and Preclinical Studies of Antiviral CTLs

Cytomegalovirus

Ex vivo CTL expansion is the most common method for producing clinical CTLs for most clinical trials (Table 20.1). Walter et al. were first to demonstrate that CMV stimulation of donor PBMC expanded CMV-specific CTLs and the expanded T cells lost alloreactivity after several weeks of ex vivo culture while retaining antiviral cytotoxicity [25].

CMV has been the primary focus of the first virus targeted therapy trials and remains a primary focus in subsequent studies (Table 20.1). The first clinical report in which $CD8^+$ CMV-specific CTLs were isolated via tetramer selection [62] generated complete or partial clinical responses in nine patients, but there was limited data on long-term persistence of the infused CMV-specific CTLs.

IFN-γ column selection (Gamma capture, Miltenyi) to produce CMV-CTLs was associated with partial and complete responses in 15 of 18 patients who were given one dose of CMV-CTLs [63]. IFN-γ selection after stimulation with recombinant pp65 or an overlapping peptide pool of 15-mers covering the pp65 protein was used to produce CMV-CTL [64]. Infusions of CMV-CTLs administered prophylactically after stem cell transplantation successfully protected seven patients from the development of viral reactivation and disease. Further, in vivo expansion of CMV-CTLs was detected in 11 patients [64]. CMV-CTLs from HSCT donors using reversible Streptamers with MHC-restricted pp65 peptides were used to successfully treat two patients with CMV reactivation after HCT [44].

Bispecific Antibody-Armed T Cells Targeting CMV

The strategy for using bispecific antibodies (BiAbs) to target cancer was nearly abandoned due to cytokine storm reactions. However, the last 10 years has seen a resurrection of interest particularly for targeting T cells to various cancer antigens. Studies using retargeted T cells have been reported for HER2 in breast and prostate cancer using anti-CD3 x anti-Her2 BiAb ATC [89, 90]; EGFR in colorectal, pancreatic, and lung cancer using anti-CD3 x anti-EGFR BiAb ATC [91]; and CD20 in non-Hodgkin's lymphoma using anti-CD3 x anti-CD20 BiAb ATC [92–94]. Since chemical or molecularly engineered constructs could be used to target the TCR on one hand and tumor-associated antigen (TAA) on the other hand, we reasoned that CMV could be targeted by chemically heteroconjugating OKT3 (anti-CD3, anti-TCR) with Cytogam® (polyclonal donor-derived anti-CMV IgG, designated CMVBi) to kill CMV-infected fibroblasts [95]. In this strategy shown in Fig. 20.1, anti-CD3 monoclonal antibody-activated T cells (ATC) which expanded in low-dose IL-2 were the T effector cells. ATC alone do not kill CMV-infected targets. Arming doses of CMVBi ranging from as low as 0.01 ng/10^6 ATC to 50 ng/10^6ATC exhibited high levels of specific anti-CMV cytotoxicity in targets infected with CMV at multiplicities of CMV infection (MOI) ranging from 0.01 to 1. The polyclonal nature of the Cytogam may provide multiple antibody clones directed at multiple CMV epitopes on the

Table 20.1 Previous clinical trials of virus-specific T-cell therapy

Methodology	Pathogen specificity	Setting	Donor	Patient accrual	Number of centers	Methodology
Tetramer selection	CMV	HSCT	HCT donor or third-party	9	1	Cobbold [62]
IFN-γ column selection	CMV	HSCT	HCT donor or third-party	18	1	Feuchtinger [63]
IFN-γ column selection	CMV	HSCT	HCT donor	18	1	Peggs [64]
EBV-LCL stimulation	EBV	SOT	Autologous	3	1	Haque [65]
Irradiated EBV-LCL	EBV	SOT	Third-party	1	1	Haque [66]
Irradiated EBV-LCL	EBV	SOT	Third party	8	1	Haque [67]
Irradiated EBV-LCL	EBV	SOT	Third party	33	1	Haque [68]
Multimer selection	EBV	HSCT	Related haploidentical donor	1	1	Uhlin [69]
IFN-γ column selection	EBV	HSCT	HCT donor	6	1	Moosman [70]
EBV-LCL stimulation	EBV	HSCT	Third-party donor	2	1	Barker [71]
Irradiated EBV-LCL stimulation	EBV	HSCT	HCT donor	114	3	Heslop [33]
EBV-LCL stimulation	EBV	HSCT	Autologous	1	1	Basso [72]
IFN-γ column selection	Adv	HSCT	Third party	1	1	Qasim [73]
IFN-γ column selection	Adv	HSCT	HCT donor	9	1	Feuchtinger [74]
CD8+ HIV-specific ex vivo expanded	HIV	N/A	Autologous	6	1	Lieberman [75]
CD4-ɣ CAR transduction	HIV	N/A	Autologous	24	1	Mitsuyasu [76]
CD4-ɣ CAR transduction	HIV	N/A	Autologous	40	5	Deeks [77]
Transduction with antisense gene to HIV env	HIV	N/A	Autologous CD4-T cells	17	1	Tebas [78]
CCR5 gene editing via ZFN	HIV	N/A	Autologous CD4-enriched	12	1	Tebas [79]
PepMix-pulsed PBMC	JCV	HSCT	HSCT donor	1	1	Balduzzi [80]
Pentamer selection	CMV/EBV/Adv	HSCT	HSCT donor or third-party	8	1	Uhlin [81]
Stimulation of PBMC with CMV antigen or inactivated conidia	CMV or *Aspergillus*	HSCT	HSCT donor	10	1	Perruccio [82]
Ad5f35pp65 transduced LCL	CMV/EBV/Adv	HSCT	HSCT donor	26	3	Leen [83]
Ad5f35pp65 transduced DC	CMV/Adv	HSCT	HSCT donor	12	1	Mickelwaite [84]
Ad5f35null transduced LCL	EBV/Adv	HSCT	HCT donor	13	3	Leen [21]
Ad5f35pp65 transduced LCL	CMV/EBV/Adv	HSCT	Third-party donor	47	8	Leen [85]
Nucleofection of DCs	CMV/EBV/Adv	HSCT	HSCT donor	12	3	Gerdemann [86]
NLV-peptide pulsing or Ad5f35pp65 transduction of DCs	CMV or CMV/Adv	HSCT	HSCT donor	50	2	Blyth [87]
Rapidly generated EBV, adenovirus, CMV, BK virus, HHV6-specific T cells following stimulation with peptide mixes	Adv, EBV, CMV, BKV, HHV6	HSCT	HSCT donor	11	1	Papadopoulou [88]

CMV-infected targets leading to the increased potency at a low arming dose of CMVBi. Cytotoxicity was evident at effector-to-target ratios (E:T) of 25:1, 13:1, 6:1, and 3:1 compared to unarmed ATC alone. At an MOI of 1.0, the mean % specific anti-CMV-specific cytotoxicities at E:T of 3, 6, and 13 were 79%, 81%, and 82%, respectively, whereas unarmed ATC at the same E:Ts killed <20%. Unarmed ATC, Cytogam®, or CMVBi alone did not exhibit significant killing of uninfected or CMV-infected fibroblasts. Furthermore, cultures of CMVBi-armed ATC with CMV-infected targets induced cytokine and chemokine release from CMVBi-armed ATC. This simple targeting strategy bypasses MHC-restricted cytotoxicity for treating viral disease in organ transplant and HSCT recipients. It was shown that CMVBi ATC do not react to alloantigens in vitro in a mixed lymphocyte culture, and they can be frozen and reinfused at different time points as an "off-the-shelf" drug. Although promising, it is not clear from these data whether targeting CMV or other disease agents using this approach will be clinically effective.

Epstein-Barr Virus

EBV-CTLs have been used for prevention and treatment of post-HSCT lymphoproliferative disease (PTLD) as well as EBV^+ lymphoma. Irradiated EBV-lymphoblastoid cells (EBV-LCL) were used to generate EBV-specific CTLs in vitro for prophylaxis or treatment for EBV-PTLD in 114 patients [27, 33]. Remarkably, the first 26 patients received gene-marked CTLs, and follow-up studies showed the gene-marked cells persisted up to 105 months after HSCT (Table 20.1).

HLA-A2-specific pentamers and IFN-γ selection procedures were used to produce EBV-CTLs. HLA-A2 specific pentamers were used to produce EBV-CTLs from the haploidentical mother of a patient with EBV-PTLD who had received a cord blood transplantation [69]. A complete clinical response was obtained following two infusions of EBV-CTLs. Three of six patients with early EBV-induced PTLD treated with EBV-CTLs produced by IFN-γ selection achieved complete responses whereas three patients with advanced, multiorgan disease did not respond [70]. The latest strategy is to target EBV with multiviral CTL products (below) or third-party-derived EBV-CTLs.

Adenovirus

Most studies targeting adenovirus (Adv) use multiviral CTLs [21, 81, 83, 84]. A few exclusively target Adv by selection technology. Adv-CTLs produced by IFN-γ selection was used for treatment of nine patients with drug-refractory Adv infections [74]. There was in vivo CTL expansion in five of six patients and four patients cleared their disease. In all studies using cell selection, clinical benefit was observed in spite of very low doses of vCTLs infused ($<5 \times 10^4$ cells/kg in most studies) [73, 74].

Multiviral CTL Trials

Recent antiviral CTL therapy trials target multiple viruses (CMV, EBV, and Adv as primary targets). CMV, EBV, and Adv are the three leading causes of viral-associated mortality after allogeneic HSCT. Clinical-grade Adv vector Ad5f35pp65 contains the immunodominant CMV antigen pp65, providing a unique opportunity to transduce donor-derived dendritic cells or EBV-LCL to serve as APCs for the CTL cultures. Triviral (CMV, EBV, and Adv-specific) CTLs were tested in a dose-escalation trial involving 26 patients [83]. There were no adverse effects at doses ranging from 5×10^6 to 1×10^8 cells/m^2, and all patients were effectively protected against CMV, EBV, and Adv. Interestingly, although EBV- and CMV-specific CTLs were detected by IFN-γ ELISpots, Adv-CTLs were not detectable except during infection. In a follow-up trial using Ad5f35-transduced EBV-LCL to produce EBV- and Adv-CTLs, 13 patients received prophylaxis or treatment for EBV and Adv infections after HSCT [21]. Although the CTLs provided protection in vivo, the Adv-CTLs could not be

detected except in the setting of Adv infection; these data suggest that levels of specific vCTLs below the limits of detection by IFN-γ ELISpots provide protection and infection induces clonal expansion. Similarly, Ad5f35pp65 transduced dendritic cells (DC) used to produce CMV- and Adv-CTLs were clinically effective in 12 patients after allogeneic HSCT [84]. There were a few cases of CMV reactivation in the setting of low-dose prednisone. This approach was applied to 50 patients after allogeneic HSCT with triviral (CMV, EBV, Adv-specific) CTLs using two methods: 10 were produced by pulsing donor DCs with the HLA-A2 restricted CMV peptide NLVPMVATV and 40 were produced using Ad5f35pp65-transduced donor DCs [87]. Only 5 of 50 patients had CMV reactivation after CTL infusions and only 1 of 5 patients required antiviral drug therapy after steroid treatment for acute GVHD.

Advances in processing protocols have validated 5-mer peptide pools that include immunodominant viral antigens that replace viral transduction of APC thereby removing safety and regulatory barriers associated use of viral vectors [36]. The use of gas-permeable rapid-expansion (G-Rex) bioreactors has simplified CTL culture [96]. These advances in technology led to the development of a rapid manufacturing protocol for expanding virus-specific T-cell products (VSTs) that yield clinically relevant numbers of VSTs in 10–12 days. Further, VST products targeting multiple viral antigens have been shown to provide effective antiviral protection (against CNV, EBV and Ad) in ten patients after HSCT [37]. This rapid manufacturing protocol was subsequently adapted to produce five virus-specific CTLs targeting EBV, CMV, Adv, HHV6, and BK virus infections in a single T-cell product for patients following allogeneic HSCT [88]. Fourteen of 48 VST products manufactured from HSCT donors recognized all 5 viral components while 35 (73%) recognized 3 or more by IFN-γ ELISpots. Unexpectedly 22 of the donors were CMV seronegative and VSTs produced predictably lacked CMV specificity. These VSTs were used to treat 11 patients after HSCT. The 3 patients treated prophylactically remained free of viral infections and 8 patients with 18 viral reactivations received VSTs, with all experiencing partial or complete responses in their CMV, EBV, Adv, or HHV6 infections.

CTL Therapy for Human Immunodeficiency Virus

Although there was intense interest in the use of CTL therapy for HIV, there was only limited success to date [97]. Attempts to expand and reinfuse autologous HIV-specific CTLs resulted in only transient improvements in viral load [75]. A larger number of clinical trials focused on genetically modified CTL to target HIV using transduction of a modified TCR or CARs. These trials established safety, but exhibited limited antiviral efficacy [76, 77]. A major challenge for this approach is the outgrowth of escape mutants expressing alterations of the target epitope so the infected cell can no longer be targeted by the effector cells. A more successful approach has been inserting genes that would provide HIV resistance. This approach was clinically tested when antisense gene complementary to HIV *env* was transduced into T cells from 17 patients using lentiviral vectors [78]. The CTLs persisted for 5 weeks, homed to gut-associated lymphoid tissue, and were well-tolerated with clinical toxicities. Infusions of CTLs in two of eight patients who underwent antiviral treatment interruption keep the viral load u

ndetectable for 4 and 14 weeks. When CCR5-delta32 mutations were introduced to CD4-enriched T cells through the use of a zinc-finger nuclease [79], the CCR5-edited T cells were subsequently infused in 12 patients, and engineered T cells were detectable in the peripheral blood for up to 42 months post infusion. In six patients who underwent antiviral treatment interruption, the absolute number of gene modified CD4+ T cells decreased at a lower rate than non-modified T cells. Recent studies showed that dual gene editing of CXCR4 and CCR5 via zinc-finger nucleases was successful in a T-cell line, and preclinical studies show that the T cells were highly resistant to HIV infection [98]. It is not clear whether this

approach could prevent primary infection or have a clinical impact as an HIV cure strategy.

CTL Therapy for Other Viruses

There are a few studies that target other viruses with adoptive immunotherapy. The John Cunningham virus (JCV) is a ubiquitous polyoma virus which can cause progressive multifocal leukoencephalopathy (PML), which occurs in immunocompromised individuals such as acquired immunodeficiency syndrome (AIDS), recipients of HSCT or solid organ transplants, or primary immunodeficiency disorders. Donor-derived JCV-specific CTLs were used in a 14-year-old patient with PML after prolonged steroid treatment for GVHD following HSCT. Cells were manufactured using 15-mer peptide pools that included JC antigens VP1 and LT and infused twice leading to clearance of JV-DNA from the cerebrospinal fluid with improvements in neurologic status [80].

Human papillomavirus (HPV) disease can be a late complication of HSCT. Peptide pools spanning the HPV E6 and E7 proteins were used to generate HPV-specific CTLs from patients with oropharyngeal or cervical cancer that arise after HPV16 infection [99]. The CTLs exhibited specific activity directed at HPV E6 and E7 and antitumor activity against the HPV16 cervical cancer cell line CaSki.

Adverse Events in Antigen-Specific CTL Therapy

Adverse events after 381 infusions for 180 patients on 18 protocols by the groups at Baylor College of Medicine were reported [100]. Side effects were limited to 24 mild adverse events observed within 6 h of infusion; nausea and vomiting were most common with 22 nonserious adverse events (fever, chills, nausea) that occurred within 24 h. No significant GVHD was attributed to CTL infusions. The only significant complications were rare reports of systemic inflammatory responses in patients with bulky EBV+ lymphomas following EBV-CTL therapy. Seven cases of acute GVHD occurred in patients who had a greater degree of HLA mismatch than controls after infusions of EBV-CTL. Some of the cases of GVHD were attributable to reducing the corticosteroid dose prior to the CMV-CTL infusions [87].

Third-Party CTL

For years, the selection or culture of anti-pathogen CTLs was dependent on the presence of pathogen-specific memory T cells in the blood of donors, and, therefore, the approach could not help allograft recipients of pathogen-naive hematopoietic cell products after HSCT. One strategy to address this problem is to provide "off-the-shelf" pathogen-specific CTLs derived from third-party donors. This strategy was first validated in a phase I trial involving 8 patients who received partially matched EBV-CTLs for PTLD that developed after solid organ transplantation [66, 67] and confirmed in a cohort of 33 patients in a phase II trial [68]. The latter trial showed a response rate of 64% at 5 weeks and 52% at 6 months; the outcomes correlated with the degree of HLA matching between the CTL donor and recipient. In the HSCT patients, two patients with refractory EBV-PTLD after cord blood transplantation (CBT) with third-party EBV-specific CTLs [71]. A bank of 32 CTL lines with characterized activity against EBV, CMV, and Adv were used to match for 50 patients with refractory viral infections. This strategy resulted in partial or complete antiviral responses in 74%, 78%, and 67% of those with CMV, Adv, and EBV, respectively [85]. This is a marked improvement from standard therapy response rate of 13% in eight patients for whom a matched line could not be identified. Despite partial HLA matching at one to four loci, there were only two patients who developed grade I GVHD. Clones that are responsible for GVHD have been selected against in the expansion culture and may exist at such low precursor frequencies after culture that they do not expand enough to cause clinically significant GVHD. The lower

rate of response against EBV relative to CMV and Adv may reflect selective expansion of T cells against immunodominant epitopes of the latter two viruses, thereby complicating the selection of an ideal third-party pathogen-specific line that fulfills the requirements of antiviral activity and MHC-restriction against multiple pathogens. The methods for producing third-party-virus-specific CTL include pentamer selection for Ad, CMV, EBV [81], and IFN-γ selection for Adv-CTL [73].

TCR Gene Transfer

A few studies reported transducing CTLs with a virus-specific TCR [49, 101, 102]. A trial of transgenic CTLs using a retroviral vector that expresses a CMV-specific TCR is ongoing in the United Kingdom (Morris E. et al. MRC# G0701703). Alternatively, Kumaresan et al. transduced T cells with the β-glucan receptor dectin [61]. Since the carbohydrate β-glucan is found in the cell wall of most fungi [103], investigators used its natural receptor, dectin-1, as a recognition receptor coupled to a CD28 (a key co-stimulatory molecule) and CD3-zeta transgene to initiate signaling and killing in T cells. The same group showed that the antifungal CARTs could mediate damage to hyphae in vitro and in vivo [61]. These novel approaches would allow creation of specific CTLs from pathogen-naive donors; however, they are subject to the regulatory challenges in gene transfer technology. Furthermore, use of a single antifungal TCR allows for antigenic escape.

Production of CTL from Pathogen-Naive Donors

A major advance in adoptive viral CTL therapy was development of virus-specific CTLs from virus-naive donors. CTL could be produced from a 20% fraction from cord blood using donor-derived DCs and EBV-lymphoblastoid cell line (LCL) as APC and Ad5f35pp65 transduction as a source of CMV and Adv antigens [20]. The resulting viral CTLs exhibited specific anti-CMV, EBV, and Adv IFN-γ ELISpots responses as well as specific ^{51}Cr cytotoxicity with no alloreactivity. Epitope mapping showed that the immunodominant epitopes recognized by cord blood-derived CTLs were different from the immunodominant epitopes recognized by the CMV and EBV seropositive adult donors. The HLA-A2-restricted epitope NLVPMVATV was notably absent in the cord blood-derived lines. CTLs derived from cord blood were successfully infused in 12 CBT recipients in the ongoing ACT-CAT trial (Safety, Toxicity and MTD of One Intravenous IV Injection of Donor CTLs Specific for CMV and Adenovirus, # NCT00880789).

Recently, multiviral CTLs were produced from CMV-naive adult donors using column-selected CD45RA+ naive T cells stimulated by donor DCs pulsed with CMV 15-mer peptide pools [38]. Preclinical studies suggest that multiviral CTLs will exhibit similar anti-CMV activity to DCs pulsed with CMV 15-mer peptide pools. The current MUSTAT trial (Multivirus-Specific Cytotoxic T-Lymphocytes for the Prophylaxis and Treatment of EBV, CMV, and Adenovirus Infections Post Allogeneic Stem Cell Transplant, # NCT01945814) compares the clinical efficacy of CTLs derived from CMV-seropositive vs. CMV-naive donors.

CTL for Solid Organ Transplant Recipients

EBV-PTLD is a significant long-term risk in solid organ transplant recipients. Rituximab can be effective, but treatment often requires reduction of immunosuppression which can lead to graft rejection. Autologous EBV-CTLs have been used in this setting [72]. Several prophylactic infusions of autologous EBV-CTLs reduced the EBV viral load without adverse reactions despite ongoing treatment with calcineurin inhibitors [65]. A heart transplant recipient who developed Hodgkin's lymphoma-type PTLD 8 years after transplant had remission after being treated with autologous EBV-CTLs in combination with chemotherapy without alterations in

his immunosuppression [72]. This observation supports the prior observations that calcineurin inhibitors block proliferation, but do not impair CTL activity.

Fungal-Specific CTLs

Fungal infections are a major cause of morbidity and mortality in allogeneic HSCT recipients, with GVHD being the major risk factor. Candidal infections can range from mucocutaneous colonization of the skin and mouth to life-threatening systemic infections. *Aspergillus* species are ubiquitous molds that cause invasive pulmonary infections as well as widespread infection including central nervous system dissemination in highly immunocompromised patients [104]. Patients with inherited immunodeficiencies (e.g., chronic granulomatous disease), patients with prolonged neutropenia after repeated rounds of chemotherapy (e.g., for acute leukemia), and those receiving immunosuppression after lung transplant or allogeneic HSCT are at the highest risk for mycoses [105]. The importance of T-cell immunity in defense against invasive aspergillosis and other filamentous fungi is not clear, since patients with these invasive fungal diseases usually have severe deficiencies in multiple components of the immune system. In patients with advanced AIDS, invasive aspergillosis is an uncommon complication and generally occurs when other forms of immune impairment (e.g., neutropenia and use of corticosteroids) are present. Despite these unknowns, it may be clinically useful to target fungal infections with fungus-specific T cells after HSCT.

The adaptive immune response against invasive aspergillosis is believed to be orchestrated by CD4+ T cells. Table 20.2 summarizes preclinical studies that developed fungal-specific CTLs against *Candida*, *Aspergillus*, and *Rhizopus* (a member of the *Mucorales* group) species. *Aspergillus*-specific CTLs were produced by stimulation of PBMC with antigens from aspergillus extracts, selection with IFN-γ secretion, and culture [106]. The CTLs were predominantly CD4+, CD45R0+ memory cells that secrete IFN-ɣ in response to *Aspergillus* and *Penicillium*. The fungal-specific CTL enhanced hyphal damage by neutrophils and APCs. IFN-ɣ selection and stimulation with *Candida albicans*, *Aspergillus fumigatus*, and *Rhizopus oryzae* extracts were used to produce multifungal-specific CTL lines, which were also nearly all CD4+ CD45RO+ HLA-DR+ that exhibited activation markers of IFN-ɣ, CD154, and TNFα and enhanced oxidative activity of neutrophils when co-incubated with antigen and APCs [108]. Several studies target the *Candida* MP65 and *Aspergillus* CRF1 antigens. To produce multipathogen-specific T cells that secrete IFN-ɣ, proliferate, and kill CMV, EBV, Adv, *Candida*, and *Aspergillus*, donor PBMCs were incubated with peptide libraries from CMV-pp65, EBV-LMP2, Adv-Hexon, *Candida* MP65, and a 15-mer peptide from aspergillus CRF1 [107]. However, it remains unclear what the significance of MP65 and CRF1 is in antifungal immunity [117] [113]. Expanded memory/effector Th1 cells following stimulation with *Rhizopus* extracts were used to generate memory/effector Th1 cells for mucormycosis, and the product exhibited specificity to the original *Rhizopus oryzae* extract as well as other *Mucorales* species [118]. *Candida*-specific T cells generated with cellular extracts of *Candida albicans* released cytokines that caused hyphal damage and increased neutrophil activity against hyphae [111].

CTLs produced by stimulation with inactivated conidia (spores) from *Aspergillus fumigatus* resulted in clonal CD4+ CTLs with anti-*Aspergillus* activity by IFN-ɣ ELISpots [82]. These donor T-cell clones specific for *Aspergillus* antigens were then infused in patients following haploidentical HSCT. Of 23 patients who developed invasive aspergillosis, 10 patients received anti-aspergillus CTLs, while 13 patients did not. Nine of 10 treated patients cleared their infections whereas only 7 of 13 untreated patients cleared their infections. *Aspergillus*-specific CTLs were detected in high frequencies in patients who received immunotherapy while they were barely detectable in untreated patients [82].

Table 20.2 Preclinical studies of T-cell therapy against nonviral infections

Methodology	Pathogen specificity	Donor	Investigator
IFN-γ selection after stimulation of PBMCs with aspergillus extracts	*Aspergillus*	Healthy donors	Beck [106]
CD154 selection after stimulation of PBMCs with fungal extracts	*Aspergillus, Candida, Rhinopus*	Healthy donors	Khanna [107]
IFN-γ selection after stimulation of PBMCs with fungal extracts	*Aspergillus, Candida, Rhinopus*	Healthy donors	Tramsen [108]
Transduction of CTL with chimeric antigen receptor directed against B-glucans	*Aspergillus* (potentially other pathogens	Healthy donors	Kumaresan [61]
Expansion of anti-*Aspergillus* T cell using *Aspergillus* extracts	*Aspergillus*	Healthy donors	Tramsen [109]
Expansion of fungus-specific T cells using a lysate from *A. fumigatus*	*Aspergillus, Candida, Penicillium*	Healthy donors	Gaundar [110]
IFN-γ capture of T cells following stimulation with C. albicans cellular extract	*Candida*	Healthy donors	Tramsen [111]
Crf1-stimulated T cells	*Candida* and *Aspergillus*	Murine cells	Stuehler [112]
CD137 selection following stimulation with Crf1 and catalase 1	*Aspergillus*	Healthy donors	Jolink [113]
Transgenic TCR-transduced cells	Tuberculosis	Murine cells	Feng [114]
MHC-Streptamer-enriched antigen-specific T cells	*Listeria*	Murine cells	Stemberger [115]
(Proof of principle) – targeting of OVA-expressing parasites	*Leishmania*	Murine cells	Polley [116]
Transgenic TRC-transduced cells	*Chlamydia*	Murine cells	Roan [55]

Despite notable advances in antifungal CTLs, a better understanding of the immunodominant T-cell targets that should be selected for various fungal species is needed, and standardized clinical-grade cGMP fungal antigen sources are needed to provide consistency between trials.

Controversies and Challenges

Although there have been major advances in producing pathogen-specific CTLs, important questions remain regarding methods that affect potency and efficacy of the T-cell products. It is unclear whether manufacturing CTLs to include more pathogens in a single culture will affect potency and specificity in the CTL cultures. Although the proportions of virus-specific CTLs for each virus decrease as the number of antigens increases, these effects have not seemed to impact clinical trials. CTLs specific for 7 viruses (CMV, EBV, Adv, BK, HHV6, RSV, and influenza) produced using peptide pools for 15 antigens exhibited specific activity against all targeted viruses [37]. The question remains as to whether adding additional viral targets will skew specific cytotoxicity, alter potency for each target, induce alloreactive T cells, or compromise in vivo responses.

A major challenge is achieving consistent and optimal culture conditions for generating the most effective CTL product. Although multiple rounds of stimulation with antigen select and expand the specific antiviral clones, prolonged culture may lead to T-cell exhaustion. Some groups have decreased production time using newer bioreactors [96]. Identification of the "correct" subset of T cells for clinical use (however selected) will require well-designed randomized phase II trials using a specific CTL product made by the same group or a common standard operating procedure (SOP) in a homogeneous group of HSCT patients. Assays for measuring IFN-ɣ ELISpots and cytotoxicity need to be standardized and the timing of the studies needs to be the same. Recently, a new population of

"stem cell memory T cells" has been putatively identified – which possess characteristics ideal for use in adoptive immunotherapy. Unfortunately, there are no randomized phase II trials to date to support continued development and commercialization of clinically effective CTLs.

The presence of immunosuppression remains a barrier for optimal immunotherapy after allogeneic HSCT and solid organ transplantation since most agents also suppress CTL functions. Nearly all protocols require recipients to be receiving less than 0.5 mg/kg/day of prednisone and wait at least 30 days after anti-T-cell serotherapy to be eligible to receive CTL therapy. Virtually all of the calcineurin inhibitors (cyclosporin A, tacrolimus, or sirolimus) at therapeutic doses impair CTL activity. EBV-specific CTLs can be made resistant to tacrolimus by knockdown of FKBP12 via a retrovirally transduced specific siRNA and exhibit anti-EBV lymphoma activity in the presence of tacrolimus [119]. Similarly, EBV-specific CTLs can be made resistant to both cyclosporine A and tacrolimus by mutating calcineurin [120]. The mutation does not alter the phenotype or antiviral activity of the CTLs and mutated cells have a growth advantage in calcineurin inhibitors. Although they have not been applied clinically, they have great potential for treating HSCT and solid organ transplant recipients.

There is one preclinical report of T cells used to target bacterial and parasitic infections [116], but there are no clinical trials evaluating T-cell immunotherapy for bacterial and parasitic infections. Despite numerous studies evaluating in vitro T-cell responses, there is no consensus on the role of T cells in defense against aspergillosis.

Conclusion

Infusions of anti-pathogen CTLs in several hundred patients over the past several decades have been established as a safe and highly effective therapy following allogeneic HCT. Identifying preserved viral T-cell epitopes, probing the antigen limits in CTL monoculture, testing the clinical efficacy of immunosuppressive-resistant CTLs, and improving conditions for rapid and specific expansion will further broaden the usefulness of this treatment strategy. As advances in protocols and methods for manufacture achieve acceptable clinical standards that can be supported commercially, CTL therapy may become an integral component of care offered to allogeneic HSCT or immunodeficiency patients.

Acknowledgments Special thanks to the clinical coordinators for dedicating their efforts to serve the immunotherapy patients. We thank Manley Huang for his thoughtful reading of the chapter. The studies were supported in part by R01 CA140314 (LGL) and R01 CA182526 (LGL), Translational Grants #6092-09 (LGL) and #6066-06 (LGL) from the Leukemia & Lymphoma Society, and UVA Cancer Center Support Grant NCI 5P30CA044579-24. LGL is a founder of Transtarget, Inc. CMB is supported in part by the NICHD K12-HD-001399 award to MDK and CPRIT R01 RP100469 and NCI P01 CA148600-02 awards to CMB.

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