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Adoptive T-cell Immunotherapy

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Abstract

Epstein-Barr virus (EBV) is associated with a range of malignancies involving B-cells, T-cells, natural killer (NK)-cells, epithelial cells and smooth muscle. All of these are associated with the latent life cycles of EBV, but the pattern of latency-associated viral antigens expressed in tumor cells depends on the type of tumor. EBV-specific T cells (EBVSTs) have been explored as prophylaxis and therapy for EBV-associated malignancies for more than two decades. EBVSTs have been most successful as prophylaxis and therapy for post-transplant lymphoproliferative disease (PTLD), which expresses the full array of latent EBV antigens (type 3 latency), in hematopoietic stem cell transplant recipients. While less effective, clinical studies have also demonstrated their therapeutic potential for PTLD post solid organ transplant, and for EBV-associated malignancies such as Hodgkin's Lymphoma, Non-Hodgkin's Lymphoma, and nasopharyngeal carcinoma that express a limited array of latent EBV antigens (type 2 latency),. Several approaches are actively being pursued to improve the antitumor activity of EBVSTs including activation and expansion of T cells specific for the EBV antigens expressed in type 2 latency, genetic approaches to render EBVSTs resistant to the immunosuppressive tumor environment and combination approaches with other immune-modulating modalities. Given the recent advances and renewed interest in cell therapy, we hope that EBVSTs will become an integral part of our treatment armamentarium against EBV-positive malignancies in the near future.

1. INTRODUCTION

Epstein-Barr virus (EBV) is associated with a range of malignancies involving B-cells, T-cells, natural killer (NK)-cells, epithelial cells and smooth muscle. All of these are associated with the latent life cycles of EBV, but the pattern of latency-associated viral antigens expressed in tumor cells depends on the type of tumor. True latency (no expression of viral antigens) is found only in normal memory B-cells and never in EBV-associated malignancies.

The viral antigens expressed in EBV-positive tumors provide target antigens for immune based therapies and T-cells specific for each of the latency-associated antigens have been

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detected in patients with malignancies, as well as in healthy individuals (Figure 1). Therefore even tumors, such as Burkitt's lymphoma (BL) and gastric carcinoma (GC) that express only EBNA1 and BARF1 (type 1 latency) can, in principal, be targeted by T-cells. Malignancies such as B-, T- and NK-cell lymphomas and nasopharyngeal carcinoma (NPC) express additional, more immunogenic target antigens, LMP1 and LMP2, a pattern termed type 2 latency. Type 3 latency involves the expression of all latency-associated antigens and adds EBNA's −2, −3a, −3b, −3c and -LP to the range of viral antigens that can be targeted. This highly immunogenic form of latency is observed only in patients who are severely immunosuppressed for example by stem cell or solid organ transplantation, congenital immunodeficiency or HIV infection. All healthy seropositive individuals and most patients carry a broad repertoire of T-cells specific for a range of EBV latency antigens that can be reactivated and expanded ex vivo for therapeutic use. The frequency of T-cells specific for EBV early lytic cycle antigens is usually higher than for the latency antigens,[1;2] and while these T-cells likely control virus spread by killing lytically infected cells before they can release infectious, their role, if any, in the control of malignancies is unknown.

EBV-specific T cells (EBVSTs) have had outstanding success for the treatment of immunogenic type 3 latency, and infusion of donor-derived EBVSTs in hematopoietic stem cell transplant (HSCT) recipients rapidly restores EBV-specific immunity. EBVSTs are less effective in type 2 malignancies that develop in immune competent hosts because these have developed sophisticated immune evasion strategies. However, EBVSTs have produced CRs in patients with locoregional NPC[3] and prolonged overall survival in a larger group of patients with more extensive disease.[4] Responses in type 2 latency lymphoma were achieved by only focusing T-cells on the type 2 latency antigens, but such T-cells produce tumor responses in over 70% of patients and complete responses (CRs) in over 50%.[5–7] However, to ensure clinical efficacy in all patients, additional strategies will be required to overcome tumor immune evasion strategies and enable T-cell expansion and continued anti-tumor function after infusion.[8] Gene-modifications of EBVSTs may be used to provide intrinsic resistance to inhibitory molecules, to express growth-promoting genes or to provide additional specificity for stromal cells. Alternatively EBVSTs may be combined with other immunomodulatory agents, such as checkpoint inhibitors or vaccines.

There are many advantages to the use of EBVSTs for the treatment of EBV-associated malignancies, not least of which their lack of short or long-term toxicities demonstrated in hundreds of patients who continue with their normal lives during and after therapy. Further, a single infusion of a small dose of T-cells can proliferate exponentially in the patient, eliminate tumors, enter the memory compartment and provide life-long anti-tumor immunity. While previously thought to be a boutique therapy available only in Institutions with specialized cell culture facilities, recent successes of gene-modified T-cells in more common malignancies[9–12] have captured the interest of the pharmaceutical industry which is now bringing T-cell therapies to a wider patient population.

Other chapters have discussed the different malignancies associated with EBV (Chapters in book section '**Viral Associated Diseases**'), their complex patterns of gene expression and the functions of the latency-associated genes (Chapters in book section '**EBV Latency**'). For the purpose of this chapter, we will discuss the protein products of latency genes

predominantly as targets for adoptive immunotherapy with EBVSTs in malignancies with different patterns of latent gene expression.

2. T-CELLS FOR TYPE 3 MALIGNANCIES

2.1. Adoptive transfer of EBVSTs for the prevention and treatment of EBV-associated lymphoproliferative disease (PTLD) after HSCT

Prior to the advent of the B-cell-depleting monoclonal antibody (MAb) to CD20, Rituxan®, and FDA approval of the CD52-specific MAb Campath® in the US in 2001 allowing *in vitro* or *in vivo* depletion of T- and B-cells, EBV-associated post transplant lymphoproliferative disease (PTLD) was a significant problem in recipients of T-cell depleted stem cells from HLA-mismatched or unrelated donors. While T-cell depletion reduces the incidence of graft versus host disease (GVHD) it leaves patients more vulnerable to infections with viruses including EBV, allowing investigators to evaluate the safety and efficacy of T-cells for the treatment of malignancy. The first studies used unmanipulated donor lymphocyte infusions (DLIs) that had proved effective for the treatment of leukemic relapse after allogeneic HSCT (Figure 2; Table 1).[13] DLIs were also effective for PTLD, but were associated with significant toxicity due to GVHD.[4]

Our group therefore specifically activated EBVSTs from stem cell donors using autologous EBV-transformed B-lymphoblastoid cells lines (LCLs) as antigen presenting cells (APCs) in the hope that the incidence of GVHD would be reduced.[14;15] T-cells reactivated using LCLs are ideal for the treatment of type 3 malignancies since they express the same range of viral antigens and stimulate CD4- and CD8-positive T-cells with specificity for a broad range of viral and non-viral tumor antigens.[16] LCLs are outstanding antigen presenting cells (APCs), since they can be prepared from most donors, provide an unlimited source of autologous professional APCs, and present a full gamut of EBV antigens. 101 patients received EBVSTs as prophylaxis and 13 as treatment for active PTLD.[17] After infusion, T-cells proliferated extensively, restored immunity to EBV and persisted for up to 10 years as demonstrated by gene-marking in the first 26 patients.[15;18;19] No patient who received EBVSTs as prophylaxis developed PTLD, by comparison with 12% of historical controls and high virus loads were reduced. Of the 13 patients who received EBVSTs as treatment for active disease, 11 patients had complete and permanent tumor responses. Importantly there were few short- and no long-term toxicities associated with treatment. There was no incidence of de novo GVHD and no patients suffered cytokine storm, even during the elimination of bulky tumors. This contrasts with responses of B-cell malignancies to adoptively transferred CD3- and CD28-activated T-cells modified with chimeric antigen receptors (CARs) for CD19.[11;20]

EBVSTs produce similar clinical efficacy when infused as a component of multivirus-specific T-cells.[21;22] Peripheral blood mononuclear cells (PBMCs) stimulated with monocytes and LCLs transduced with a recombinant adenovirus vector expressing pp65 of cytomegalovirus (CMV) demonstrated robust specificity for EBV and CMV pp65 as well as to hexon and penton of adenovirus that are processed and presented from the virion. As few as 2×10^7 total T-cells were effective at clearing all three viruses, and since EBVSTs

comprised only a minor fraction of the total, these studies showed that very small numbers of EBVSTs could reconstitute EBV-specific immunity in the HSCT setting.[21]

2.2. Adoptive transfer of EBVSTs for the prevention and treatment of PTLD after solid organ transplant (SOT)

PTLDs occurring after SOT are usually of recipient origin and therefore EBVSTs are ideally autologous. EBVSTs are readily generated from SOT recipients, even those with active PTLD, despite their *in vivo* suppression with drugs such as steroids, calcineurin inhibitors or mTor inhibitors, suggesting that EBVSTs are present, but are unable to respond adequately to virus reactivation.[23] SOT recipients receiving EBVSTs must remain on immunosuppressants to prevent graft rejection and unlike HSCT recipients are not lymphodepleted. Nevertheless Haque et al showed that autologous EBVSTs infused in 3 escalating doses one month apart, expanded after infusion into 3 SOT recipients and reduced virus load for 3 months without causing graft rejection.[24] Comoli et al[25;26] generated EBVSTs from 23 SOT recipients deemed at high risk for PTLD based on a high virus load. Seven of these were safely infused with up to 5 doses of EBVSTs with a decrease of virus load in 5 patients and increases in the EBV-specific T-cell precursor frequency in those tested (Table 2). The demonstration of safety in this patient population is important, but the ability of the adoptively transferred cells to persist and function long term remains unclear.[27–29] To enable adoptively-transferred EBVSTs to expand and function in patients on immunosuppressant drugs to prevent graft rejection, two groups rendered T-cells resistant to specific immunosuppressive drugs.[30–32] These will be discussed in the section '**Rendering T-cells resistant to immunosuppressive medications**'.

2.3. Rapid selection of EBVSTs from donor blood for adoptive transfer

The activation and expansion of EBVSTs using LCLs is a lengthy process, requiring six weeks to establish LCLs, then at least 4 weeks to expand EBVSTs followed by two weeks for quality control testing (Figure 3). Since PTLD is rapidly progressive, EBVSTs must be made in advance to be of clinical benefit and since the incidence of PTLD is low, many lines would never be infused. Therefore unless all patients are treated prophylactically, this type of manufacturing is not practical for wider use. Hence investigators evaluated strategies to isolate virus-specific T-cells (VSTs) directly from donor peripheral blood. This strategy was first evaluated for the control of CMV reactivation. CMV-specific T-cells (CMVSTs) were selected either using HLA-peptide multimers or streptamers, or by magnetic isolation of T-cells that secrete interferon (IFN)-γ in response to antigen stimulation (gamma capture).[33–35] Even using large starting blood volumes, the VST numbers recovered are generally small allowing only small doses of cells to be infused.[33;34;36] The silver lining to this drawback was finding that a very few VSTs could expand exponentially in HSCT recipients and control disease. Less than 10^4 tetramer-selected CMVSTs per kg of patient body weight were able to expand in patients and eliminate CMV viremia.[33] Similarly, a mean of 21 × 10^3/kg CMV pp65-specific gamma captured T-cells eliminated CMV viremia in 83% of patients.[37]

One problem with tetramer/streptamers selection is that these are limited to single epitopes presented by single HLA alleles, so that multiple clinical grade reagents would be required

for the treatment of all patients and a lack of reagents for the selection of CD4-positive T-cells. This may be overcome as more reagents including class II multimers become available. By contrast, the 'gamma capture strategy' is not HLA-dependent and can use whole antigen to activate polyclonal CD4+ and CD8+ T-cells. This is ideally suited to CMV and adenovirus, since all seropositive individuals recognize pp65 and immediate early (IE) of CMV and hexon and penton of adenoviruses and T-cells with these specificities have proved protective. However, the antigen specificity of EBVSTs is broad and highly HLA dependent and there are no universally dominant EBV antigens, so that an even wider range of clinical grade tetramers/streptamers would be required to cover all individuals. Further it is not clear which antigen or antigens are required to induce protective T-cells, T-cells elicited by LCLs recognize a range of latency antigens, early lytic cycle antigens and phosphoproteins and studies using LCL-activated T-cells did not identify critical target antigens for tumor recognition.[16;38] However, two recent studies used donor-derived IFN-γ-captured EBVSTs in HSCT recipients with PTLD or viremia (Table 1).[39;40] In one of these studies patients received EBNA1-specific T cells, which expanded in 8 of 10 patients between 3 and 45 days after transfer and produced clinical and anti-viral responses in seven.[40] This was an important finding, since EBNA1 is poorly presented by MHC class I and hence has not been considered an ideal target antigen.[41;42]

2.4. Rapid expansion of EBVSTs from donor blood for adoptive transfer

Rapid selection strategies require large amounts of donor blood that cannot always be obtained from unrelated donors and in some donors the frequency of VSTs is limiting. Gerdemann et al[43] developed a rapid expansion strategy in which small numbers of donor PBMCs were stimulated for 10 days with autologous dendritic cells transfected with DNA plasmids expressing EBNA1, LMP2 and the immediate early lytic cycle antigen, BZLF1 as well as pp65 and IE of CMV and hexon and penton of adenovirus in the presence of interleukin (IL)-4 and IL-7. The total manufacturing time including 7 days for dendritic cell (DC) manufacture was 17 days, plus 7 days for quality control testing.[44;45] PBMCs expanded by about 1.5 logs in 9 to 11 days, so that starting with 15×10^6 PBMCs, a median of 212.5×10^6 VSTs was obtained, more than sufficient for the infusion of 5 to 20×10^6 VSTs per m^2. 10 patients were treated for 12 viral infections, including four patients with EBV reactivations. 80% of patients had CRs and a single patient whose EBV load did not respond to T-cell infusion did not develop PTLD.

This rapid expansion strategy was shortened by using overlapping peptide libraries (pepmixes) as a source of antigen instead of plasmids. These could be pulsed directly onto PBMCs eliminating the requirement for DCs and 7 days of culture. Pepmixes comprising 15 mers overlapping by 11 amino acids that span the entire protein sequence of the antigen of interest, contain all possible class I restricted epitopes and many class II-restricted epitopes.[46] Papadopolou infused pentavirus-specific T-cells targeting EBV, CMV, adenoviruses, BKV and human herpes virus (HHV)6 into 8 patients with 18 viral infections including 5 with PTLD or EBV viremia. CRs were observed in 80% of patients, including all 5 with EBV.[47] The EBV pepmixes used spanned EBNA1, LMP2 and BZLF1. Although EBNA1 and LMP2 are not the strongest antigens, they are recognized by most individuals, are expressed in most EBV-associated malignancies and EBNA1 strongly induces CD4-

positive T-cells. BZLF1 was included as a strong antigen recognized by most donors that may mediate elimination of productively infected cells before they can release infectious virus and therefore should help control virus spread after HSCT. Together these antigens induced T-cells able to eliminate bulky PTLD.[47]

2.5. Off the shelf, third party T-cells

Even 10 days of culture may be too long to wait to treat a patient with rapidly progressive PTLD, and in the case of HSCT recipients, the stem cell donor is not always available or willing to provide additional blood and the donor may be seronegative or cord blood. Further patients developing PTLD after solid organ transplant do not have a healthy donor. Haque et al therefore established a bank of 60 LCL-activated EBVST lines and used them to treat 33 transplant recipients with PTLD occurring after SOT (31) or HSCT (2).[48;49] All patients had failed standard therapies and EBVST lines were selected based on best HLA match and ability to kill patient LCLs if available. 64% of patients showed tumor responses at 5 weeks post infusion and 14 (42%) had CRs. Responses correlated with the number of HLA matches and the presence of CD4+ T-cells. The major anticipated toxicity was graft rejection or GVHD, but no adverse events were observed. TCR spectratyping in 5 patients revealed the presence of donor T-cells for up to 7 days, but major T-cell expansion and persistence, as observed with donor T-cells in the post HSCT setting was not observed. Subsequently other third party banks have been established at Memorial Sloan-Kettering Cancer Center (MSKCC)[50;51] and Baylor College of Medicine (BCM),[52] and a new bank consisting of EBVSTs from New Zealanders has been established and used by the Edinburgh group to avoid potential transmission of Creutzfeld-Jacob disease.[53] Our (BCM) bank comprised 32 lines with specificity not only for EBV, but CMV and adenovirus. Eighteen lines, selected for their ability to recognize the culprit virus through the shared HLA allele(s), were administered to 50 patients (23 for CMV, 18 for adenovirus and 9 for EBV) in a multicenter trial. Responses to all viruses were observed in around 75% of recipients at 6 weeks post infusion and of the responders, only 4 patients recurred or progressed. Despite the HLA disparities between donor and recipient de novo GVHD occurred in only two patients. Together these results support the development of third party banks in the transplant setting, since the cells are rapidly available, safe and have high efficacy. The mechanism of action of third party T-cells is however mysterious. The type of EBVST expansion seen in the peripheral blood of HSCT recipients receiving - EBVTs stem cell donor is not recapitulated. It is possible T-cells may remain active at tumor sites or may create an inflammatory response that induces endogenous tumor-specific T-cells specific for non-viral antigens.

3. T-CELLS FOR TYPE 2 MALIGNANCIES

3.1. LCL-activated EBVSTs for the treatment of type 2 latency malignancies

NPC and the T- and B-cell lymphomas occurring outside the transplant setting express only EBNA1, LMP1, LMP2 and the BARF1 gene products at the protein level, while EBERs and miRNAs are also expressed but not translated (type 2 malignancy).[54–59] LCL-activated EBVSTs are usually dominated by T-cells specific for early lytic cycle antigens and EBNA's 3A, 3B and 3C, with unpredictable activity towards type 2 latency antigens.[1;2] This

problem is exacerbated in patients with malignancies, since T-cells specific for tumor antigens may be suppressed or anergized by the tumor microenvironment.[60] Nevertheless LCL-activated EBVSTs were evaluated in patients with Hodgkin's lymphoma (HL) and NPC.[3–5;61–65] In 14 patients with multiple-relapsed HL, EBVSTs were able to control B-symptoms and reduce peripheral blood EBV load.[5] Two patients with minimal disease had CRs, one had a partial response and five had stable disease (Table 3).

Our group also infused EBVSTs into twenty-three patients with recurrent/refractory NPC.[3;65] Three of 4 patients with locoregional disease had CRs. By contrast only 1 CR was observed in 11 patients with metastatic disease.[3] Comoli et al reported control of disease in 6 of 10 patients with stage 4 NPC and similar results in a later study that used lymphodepleting chemotherapy prior to EBVST infusion in 11 patients.[63;64] In a larger study in Singapore, 35 patients received up to six doses of EBVSTs after four cycles of gemcitabine and carboplatin producing a response rate of 71.4% with 3 CRs and 22 partial responses.[4] The 2-year and 3-year overall survival (OS) rate was 62.9% and 37.1%, respectively. Tumor responses correlated with the presence of LMP2-specific T-cells in the infused cell line. FF CanVac is now planning a phase III trial to compare the efficacy of this strategy with chemotherapy alone.

3.2. Targeting T-cells to type 2 latency antigens

To increase the frequency of T-cells specific for type 2 latency antigens, our group used APCs overexpressing LMP1 and/or LMP2 from recombinant adenovirus (Ad) vectors encoding either LMP2 alone (Ad5f35.LMP2) or LMP2 and a non-toxic, truncated from of LMP1 (Ad5f35.ΔLMP1-I-LMP2) to stimulate PBMCs.[66;67] DCs in the first stimulation proved essential to ensure the activation of anergic LMP1- and LMP2-specific T-cells from patient PBMCs, differing from healthy donors in this respect. Subsequent stimulations used LCLs transduced with the same Ad vectors to produce sufficient antigen-specific T-cells for infusion. In our hands this protocol has evolved over time and our ability to reactivate and expand LMP-specific T-cells from patients has been improved by the incorporation of cytokines and superior media formulations. Nevertheless LMP-specific T-cells could not always be detected, although LCL killing was almost always observed.[7]

We performed two clinical trials using these LMP-directed T-cells in patients with EBV-associated HL and Non-Hodgkin Lymphoma (NHL), including NKT lymphoma; the first trial targeted LMP2 alone and the second targeted LMP1 and LMP2.[6;7] When the two trials are considered together, 50 patients received LMP-targeted T-cell lines. Twenty-nine patients received LMP-targeted EBVSTs as adjuvant therapy after autologous HSCT or chemotherapy and 28 remained in remission for a median of 3.1 years and although 8 patients died during this time, deaths resulted from complications of prior chemotherapy. Twenty-one patients received T-cell therapy for active disease and of 13 patients with objective responses, 11 were complete. EBVSTs could be generated in 91% of patients and LMP1- and or LMP2-specificifity was detected in about 66%. After infusion, increases in LMP-specific activity could be detected in the majority of responders and epitope spreading to non-viral tumor antigens was detected only in responders.[7]

In an Australian study, EBVSTs enriched in LMP- and EBNA1-specific T cells generated by stimulation of PBMCs with monocytes transduced with an Ad vector encoding HLA-class I-restricted LMP1 and LMP2 epitopes and the n-terminus of EBNA1 (Ad5f35.E1ΔGA-LMPpoly) were given as an adjuvant to sixteen patients with NPC, and these patients had a prolonged median OS in comparison to patients (n=8), who did not receive cells (523 vs. 220 days).[68]

3.3. Overcoming problems with manufacturing using LCLs as APCs

The generation of LCLs for use as APCs adds at least 6 weeks to the EBVST manufacturing time with an additional four weeks to expand T-cells and 2 weeks for quality control (QC) testing. During this time, patients may progress and become ineligible for infusion. Further, EBV-LCLs cannot be generated from patients who have received the B-cell depleting monoclonal antibody Rituxan®, and while this was not a problem in early studies, this drug has become standard of care for patients with B-cell malignancies. Finally, the presence of live EBV, is a regulatory hurdle, particularly in European and some Asian countries even though it has not presented a problem in hundreds of patients who have received LCL-activated T-cells.

Pepmixes can also be used to generate EBVSTs for the adoptive immunotherapy of type 2 latency malignancies. Again, in our hands, DCs were required for the first stimulation of PBMCs with pepmixes from most patients,[69] and since DCs are limiting, especially in patients, for the second stimulation we developed an antigen-presenting complex comprising pepmix-pulsed, autologous activated T-cells (ATCs) and HLA-negative K562 costimulatory cells (K562cs) from a master cell bank to replace autologous LCLs.[69] ATCs upregulate HLA class II molecules and therefore can present peptide epitopes in association with HLA class I and II, while HLA class I and II-negative K562cs cells have been gene-modified to express the costimulatory molecules CD80, CD86, CD83 and 4-1BB ligand.[70] Using this antigen-presenting complex, we have generated T-cells lines with specificity for LMP1, LMP2, EBNA1 and BARF1 from patients with lymphoma and NPC, and are currently evaluating them clinically in patients with lymphoma. Of note, the resultant T-cell lines, even from healthy donors, rarely recognize all four antigens, but T-cells specific for at least one and up to 4 antigens can be generated from most patients.

Improving media formulations, the addition of cytokines and the use of gas-permeable GRex culture vessels has further reduced the manufacturing time by increasing the rate of T-cell expansion, so that pepmix-activated EBVST for the treatment of patients with type 2 malignancies can now be manufactured and released in about one month; seven days for DC differentiation and maturation, 16 days for T-cell expansion and 7 to 14 days for QC (Table 4).

While the pepmix strategy provides a more specific T-cell product, caveats may be that T-cells specific for non-viral phosphoproteins that are reactivated by LCLs,[16] may be important clinical target antigens.[38] Thus EBV-LCL-activated EBVSTs have been shown to recognize uninfected B-cell blasts, but not other autologous cells.[38] Further, T-cells specific for early lytic antigens are present in LCL-activated T-cell lines.[2] These T-cells may play an important role in the control of malignancies that support even low levels of virus

replication, since lytic cycleantigens are abundant relative to latent cycle antigens and may be cross-presented by other tumor cells after death of even a minority of lytically infected cells.

4. T-CELLS FOR TYPE 1 MALIGNANCIES

BL and GC express type 1 latency, thus provide only EBNA1 and BARF1 as target antigens for T-cells.[71–74] EBNA1 contains a glycine alanine repeat (GAr) that was shown to prevent EBNA1 transfer to the endoplasmic reticulum for processing and presentation on HLA class I molecules; transfer of the GAr domain to other immunogenic proteins conferred resistance to processing and presentation, and deletion of the GAr from EBNA1 increased its immune recognition in LCLs.[75;76] Later it was suggested that the purine-rich mRNA sequence encoding the GAR reduced the rate of EBNA1 translation initiation and the availability of protein for processing and that it was the nucleotide sequence, not the amino acid sequence that reduced EBNA1 presentation.[77–79] Regardless, it was assumed for a long time that EBNA1 would not be a good target for CD8-positive cytotoxic VSTs. More recently several groups showed that EBNA1 could be presented on specific HLA class I alleles for example HLA B35, perhaps due to processing from defective ribosomal products (DRiPs).[76;80;81] Further, EBNA1 contains numerous HLA class II-restricted epitopes[81–83] and CD4-positive T cells were shown to kill BL cell lines *in vitro*.[82;84;84] EBNA1-specific T-cell clones were also able to inhibit the outgrowth of EBV-infected B-cells *in vitro*,[85] so that EBNA1 may in fact be an ideal target antigen, being expressed in all EBV-positive malignancies and inducing CD4-positive helper/killer T-cells and some CD8-positive VSTs.[81] However, as yet, no clinical trials have evaluated T-cell therapy for either EBV-positive BL or GC.

5. GENETIC MODIFICATIONS TO IMPROVE ANTITUMOR ACTIVITY OF T CELLS

While the adoptive transfer of EBVSTs is an effective therapy for PTLD post HSCT, EBVSTs have been less effective for PTLD in SOT recipients and for type 2 latency malignancies for a number of reasons. Most SOT recipients receive immunosuppressive medications that inhibit T-cell function, and are not lymphodepleted; a prerequisite for significant *in vivo* expansion of adoptively transferred T cells. For type 2 malignancies, the limited array of EBV antigens expressed, lack of lymphoid space to expand and the immunosuppressive tumor microenvironment are key factors that may limit EBVST efficacy. Several genetic modifications have been evaluated as potential countermeasures to these roadblocks (Table 5) and are discussed in this section.

5.1. Rendering T-cells resistant to immunosuppressive medications

Most SOT recipients receive immunosuppressive medications such as FK506, rapamycin or mycophenolate mofetil (MMF) to prevent graft rejection. Ricciardelli et al showed that overexpression of a calcineurin A mutant in EBVSTs provided resistance to the calcineurin inhibitor FK506 and restored their ability to eliminate established LCLs in NOD/SCID/ IL2rγ^{null} mice in the presence of FK506.[86] This strategy is under evaluation clinically. De Angelis et al produced similar results by silencing the expression of FK binding protein 12

in EBVSTs.[87] Huye et al expressed a rapamycin-resistant mTOR in CD19-specific chimeric antigen receptor (CAR)-modified T-cells that synergized with rapamycin in the elimination of B-cell lymphoma, a strategy that could be adapted to EBVSTs for SOT recipients receiving this drug.[32] Lastly, investigators have rendered T-cells resistant to MMF by expressing a mutant inosine monophosphate dehydrogenase II in T-cells.[88] Any of these strategies should improve the ability of T-cells to function in patients receiving immunosuppressive drugs to prevent graft rejection and are at elevated risk for viral infections.

5.2. Enhancing T-cell expansion *in vivo*

While administration of lymphodepleting chemotherapy such as cyclophosphamide and/or fludarabine prior to T-cell infusion enhances the *in vivo* expansion of adoptively transferred T-cells,[89] this approach lacks specificity and carries the risk of serious adverse events. Vaccination post T-cell transfer enhances the expansion of adoptively transferred T cells in preclinical models,[90] however there is no commercially available EBV vaccine to adapt this approach to cell therapy with EBVSTs. To test this concept in humans we are currently evaluating the potency of commercially available varicella zoster virus vaccines to boost adoptively transferred varicella-specific T cells *in vivo*. Systemic administration of IL-2 has shown promise in patients with melanoma to promote T-cell expansion *in vivo*. However systemic administration of IL-2 is associated with serious toxicities such as capillary leak.[91] In addition, IL-2 potentially expands inhibitory, regulatory T cells (Tregs) that could potentially inhibit the antitumor activity of infused EBVSTs. To overcome this limitation, investigators have explored the use IL-15. While IL-15 shares the growth-promoting effects of IL-2 on effector T cells and does not preferentially promote Treg expansion, its systemic administration is also toxic, preventing its wider use.[92],Therefore investigators have genetically modified EBVSTs to express IL-15. Genetically-modified EBVSTs expressing IL-15 at low levels, produced autocrine expansion in an antigen-dependent fashion[93] and were resistant to Treg-mediated immunosuppression.[93] Transgenic expression of cytokine receptors that sensitize EBVSTs to cytokines with low systemic toxicity is another potential strategy to enhance T-cell expansion. For example, expressing IL-7Rα on EBVSTs restores their sensitivity to IL-7, a cytokine that has an encouraging safety profile in humans.[94–96]

5.3. Rendering T-cells resistant to the immunosuppressive tumor microenvironment

Hodgkin Reed Sternberg (HRS) cells, NHL and NPC cells have developed - like other malignant cells - an intricate system to suppress the immune system.[97–102] For example, they 1) secrete immunosuppressive cytokines such as transforming growth factor (TGFβ) or IL-10, 2) recruit immuonsuppressive cells such Tregs or myeloid derived suppressor cells, 3) express molecules on the cell surface that suppress immune cells including FAS ligand (FAS-L) and PD-L1, and 4) create a metabolic environment (e.g. high lactate, low tryptophan) that is immunosuppressive.

TGFβ expression is employed by many tumors as an immune evasion strategy since it promotes tumor growth, limits T-cell effector function, and activates Tregs.[103] These detrimental effects of TGFβ can be overcome by modifying T cells to express a dominant-negative TGFβ receptor type II (DNR), which lacks its intracellular signaling domain.[104;105]

DNR expression blocks TGFβ-signaling and restores T-cell effector function in the presence of TGFβ. A clinical study evaluating this strategy is in progress for patients with EBV-positive NHL and preliminary results indicate that DNR-modified EBVSTs benefit patients who failed therapy with unmodified EBVSTs.[106] Preclinical studies have further shown that not only is it possible to render T cells resistant to the detrimental effects of TGFβ, but also to convert the 'negative' TGFβ signal into a 'positive' signal by expressing a chimeric cytokine receptor in T-cells, which consists of the extracellular domain of the TGFβ receptor type II and the endodomain of toll-like receptor (TLR) 4. Transgenic expression of this chimeric cytokine induced T-cell activation and expansion in the presence of TGFβ and in the absence of growth promoting cytokines.[107] Similar approaches have been developed to 'convert' the inhibitory effects of IL-4 on T cells.[108;109]

Silencing negative regulators or transgenic expression of cytokines are other strategies to render EBVSTs resistant to the immunosuppressive microenvironment. For example HRS cells as well as NPC cells express FAS-L, and preclinical studies have shown that silencing FAS-expression in EBVSTs renders T-cells resistant to the FAS-mediated apoptosis.[110] As mentioned in the section '**Enhancing T-cell expansion *in vivo***', transgenic expression of IL-15 renders EBVSTs resistant to Tregs.[93] Transgenic expression of another cytokine, IL-12, is also actively being explored to render T cells resistant to the immunosuppressive tumor microenvironment (or to convert the environment to one more conducive to T-cell growth).[111–113]

5.4. Enhancing T-cell homing to tumor sites

T-cell homing to tumor sites depends on the secretion of chemokines by the tumor and expression of the corresponding chemokine receptors on T cells. Often, there is a 'chemokine/chemokine receptor mismatch' limiting T-cell homing to tumor sites. HL cells secrete thymus- and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), which both preferentially attract inhibitory T cells such as Tregs and T helper (TH)2 cells that express the corresponding chemokine receptor CCR4, but not CCR4 negative, CD8-positive cytotoxic T cells. In a preclinical HL xenograft model, transgenic expression of CCR4 on effector T-cells that were specific for the HL antigen CD30 showed improved migration towards tumor sites and antitumor activity, indicating that this approach could potentially improve the antitumor activity of EBVSTs.[114] Of note however, EBV-positive lymphomas often express RANTES that is induced by LMP1 and recruits CCR5-expressing effector T-cells.[115]

5.5. Redirecting T-cells to non-EBV antigens

Redirecting EBVSTs to non-EBV antigens is actively being explored not only to enhance their anti-tumor activity against EBV-positive malignancies, but also to broaden their utility for malignancies that are not virus-associated and lack strong viral antigens. CARs, which consist of an antigen-binding domain, most commonly derived from a MAb, a transmembrane domain, and intracellular domain signaling domains[116] can redirect EBVSTs to tumor antigens, since CARs do not interfere with the native T-cell receptor expressed on EBVSTs. In addition, CARs recognize cell surface antigens in an MHC-independent fashion, rendering the CAR/antigen recognition immune to commonly used immune evasion

strategies such as down regulation of MHC class I expression or defects in the antigen processing machinery of tumor cells. Antigens that have been explored for type 2 malignancies include CD30 and CD70, which are expressed on HRS or NPC cells respectively.[117;118] A clinical study with CD30-specific CAR T cells is in progress.

Since latently infected memory B-cells maintain infused EBVSTs in EBV-seropositive patients,[17] our group expressed a CAR directed to GD2 (a disialoganglioside) on EBVSTs and gave them to 11 children with advanced neuroblastoma.[119;120] Three of them had complete responses while an additional two with bulky tumors showed substantial tumor necrosis. This approach has been adapted to prevent and treat recurrent CD19-positive B-cell malignancies with CD19-specific CAR modified VSTs post HSCT.[121]

In summary, gene transfer is an attractive approach to enhance the anti-tumor activity and broaden the scope of EBVSTs. While the majority of approaches have, as yet, only been evaluated in preclinical studies, the encouraging results obtained so far warrant further active exploration of this modified approach to immunotherapy with EBVSTs. While gene transfer has resulted in malignant transformation of hematopoietic stem cells,[122;123] no such event has been observed in more than 500 patients, who received T-cells that were genetically modified with retroviral or lentiviral vectors.[124] Nevertheless, some of the discussed genetic modifications such as transgenic expression of cytokines might increase the risk of autonomous T-cell growth and/or transformation. Thus, depending on the inserted transgene, inclusion of an inducible suicide gene is advisable so that selective T-cell death can be induced in the event of unwanted toxicities. In this regard, several inducible suicide systems have been developed and evaluated in preclinical or clinical studies. For example, T-cells that express an inducible, modified caspase 9 gene can be effectively ablated in preclinical models and in patients by administration of a ‘chemical inducer of dimerization’.[125;126] Other strategies include the expression of a cell surface antigen on T cells such as truncated CD20 or EGFR, which allows the elimination of T-cells with FDA-approved MAbs.[127;128]

6. Combinatorial T-cell therapy

Combining EBVSTs with other therapies is an attractive approach to increase their anti-tumor activity. For example, type 2 and 1 malignancies can potentially be rendered more sensitive to EBVST-mediated killing by inducing the expression of immunodominant, lytic EBV antigens. In preclinical studies several agents including chemotherapy, histone deacetylase inhibitors or proteasome inhibitors have shown promise in inducing the expression of lytic cycle antigens.[129–131] However, clinical experience indicate so far, that the induction of EBV lytic antigens with currently available agents in type 2 malignancies is either limited in humans[132;133] or requires their continued infusion to be effective.[134]

Combining EBVSTs with MAbs that block immune-cell-intrinsic checkpoints is another strategy to enhance their antitumor activity. In this regard, MAbs that block the inhibitory receptor CTLA-4 on T cells or the interaction between the inhibitory receptor PD-1 and its ligand (PD-L1) have shown promising antitumor activity as single agents in early Phase clinical studies for patients with solid tumors and HL.[135–138] While CTLA-4 blockade or

PD-1/PD-L1 blockade has not been combined with the adoptive transfer of T-cells including EBVSTs in humans, these MAbs enhance the antitumor activity of adoptive T-cell therapies in preclinical models.[139]

7. CONCLUSIONS

EBVSTs have been explored as prophylaxis and therapy for EBV-associated malignancies for more than two decades. While most successful for PTLD post HSCT, clinical studies have also demonstrated their therapeutic potential for PTLD post SOT and type 2 malignancies including HL, NHL, and NPC. Advances in the production technology of EBVSTs and renewed interest of biotech companies should facilitate later phase clinical studies. Lastly, gene transfer and approaches to combine EBVSTs with other targeted therapies hold the promise to further improve their anti-tumor activity. Giving the recent advances in the field, we hope that EBVSTs will become an integral part of our treatment armamentarium against EBV-positive malignancies in the near future.

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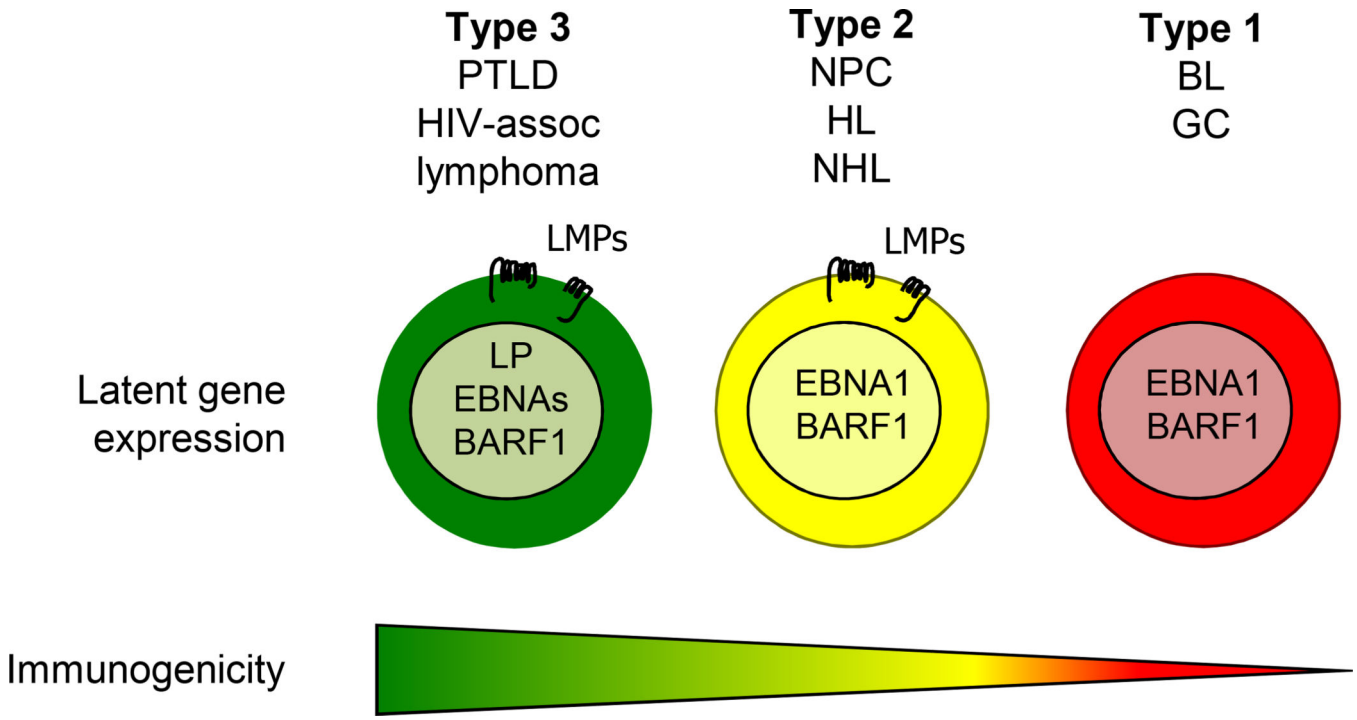

Figure 1. Immunogenicity of EBV-positive tumors according to latency

For details see text. EBNAs: EBNAs −2, −3a, −3b, and −3c; LMPs: LMP1 and LMP2.

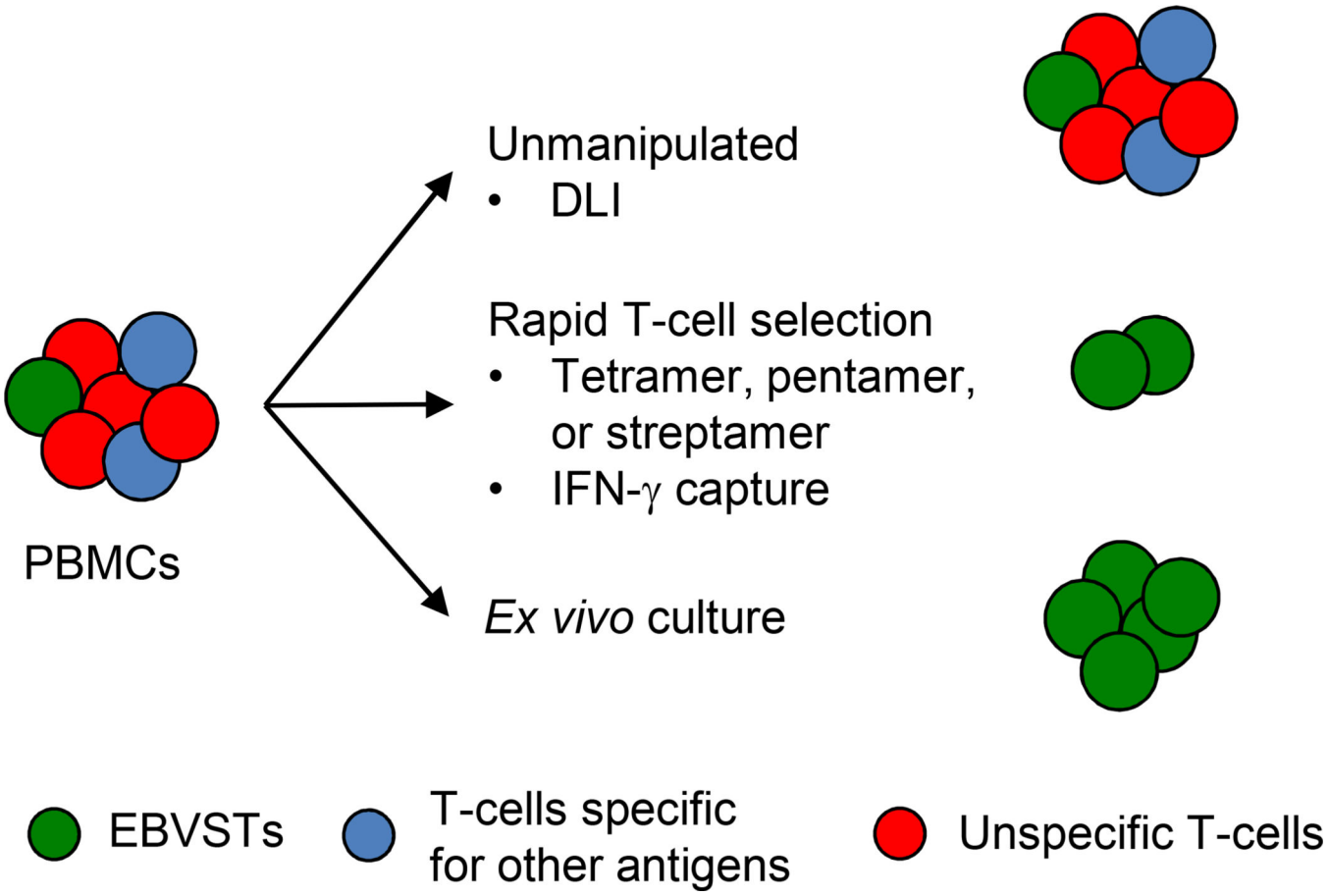

Figure 2. T-cell products for EBV-positive malignancies
For details see text. DLI: Donor lymphocyte infusion.

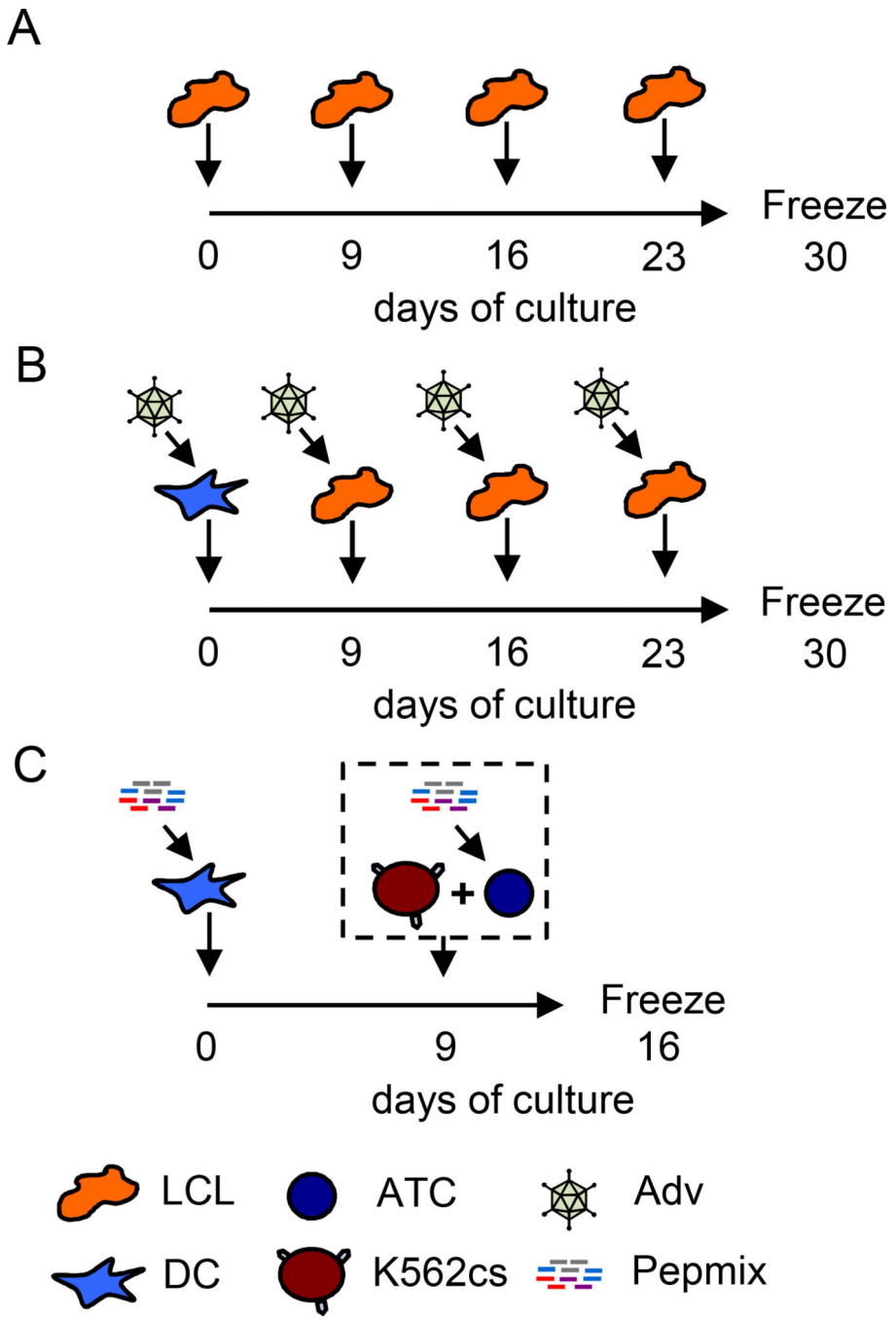

Figure 3. ***Ex vivo*** **generation of EBVSTs for clinical studies**

(**A**) For most clinical studies EBVSTs have been generated using LCLs as antigen presenting cells (APCs). (**B**) In an effort to increase the frequency of T cells for type 2 latency antigens, DCs and LCLs have been used as APCs that are modified with recombinant adenoviruses expressing LMP2 or LMP1 and LMP2. (**C**) EBVST generation not requiring LCLs or recombinant adenoviruses. For additional details see text.

Table 1

Selected T-cell Therapy Clinical Studies for Type 3 Malignancy – PTLD post HSCT

T-cell product	Production method	Comment
Allogeneic – donor derived		
DLI	n/a	*MSKCC experience*: Thr (n=30): 22 CR or PR; Incidence of GVHD: 17% [51] Smaller case series have reported similar response rates, but a higher incidence of GHVD (up to 40%)
EBV-specific Ts	LCL IFN-γ capture post stim with peptides derived from 11 EBV antigens IFN-γ capture post stim with EBNA1 antigen Pentamer selection	*SJCRH and BCM experience*: Pro (n=101): no PTLD development; Thr (n=13): 11 CR17 *MSKCC experience*: Thr (n=19): 13 CR or PR[51] Smaller case series have reported similar response rates Thr (n=6): 3 CR[39] Thr (n=10): 7 CR or PR[40] Thr (n=1): 1 CR[36]
EBV-, Adv-specific Ts	LCL modified with Ad5f35	Pro (n=13): no PTLD development Thr (n=1): 1 CR22
EBV-,CMV-, Adv-specific Ts	LCL modified with Ad5f35.CMVpp65 DC/plasmidE1AGA-LMP2-I-BZLF1	Pro (n=20): no PTLD development Thr (n=6): 6 CR[21] Pro (n=9): no PTLD development Thr (n=2): 2 CR[43]
EBV-,CMV-, Adv-, BKV HHV6-specific Ts	Monocytes loaded with pepmixes forEUGA, LMP2, BZLF1	Thr (n=5): 5 CR[47]
Allogeneic – 3rd party		
EBV-specific Ts	LCL	*Edinburgh experience:* Thr (n=3): 2 CR[48] *MSKCC experience*: Thr (n=2): 2 CR[50]
EBV-,CMV-, Adv-specific Ts	LCL modified with Ad5f35.CMVpp65	Thr (n=9): 6 CR or PR[49]

DCs: Dendritic cells; CR: Complete response; PR: Partial Response; Pro: Prophylaxis; Thr: Therapy

Table 2

Selected T-cell Therapy Clinical Studies for Type 3 Malignancy – PTLD post SOT

T-cell product	Production method	Comment
Autologous		
EBV-specific Ts	LCL	*Edinburgh experience:* Pro (n=3): decrease in viral load; no PTLD development[24] *Pavia experience:* Pro (n=7): decrease in viral load in 5/7 patients; no PTLD development[26] *BCM experience:* Pro (n=12): variable effects on viral load; no PTLD development[27] *Two case reports:* Thr (n=2): 1 CR or 1 PR[28;29]
Allogeneic – 3rd party		
EBV-specific Ts	LCL	Thr (n=38): 23 CR or PR[48;49]

CR: Complete response; PR: Partial Response; Pro: Prophylaxis; Thr: Therapy

Table 3

Selected T-cell Therapy Clinical Studies for Type 2 Malignancy

T-cell product	Production method	Comment
HL and NHL		
auto EBV-specific Ts	LCL	Thr (n=14): 2 CR, 1 PR, 5 SD5
	LCL/Ad5f35.LMP2 or Ad5f35.ΔLMP1-I-LMP2	Thr (n=21): 11 CR, 2 PR[6;7]
	DCs and ATCs/K562cs loaded with pepmixes for E1AGA, LMP1,LMP2,BARF1	Clinical study in progress
allo EBV-specific Ts	LCL	T cells (n=3): 3 PR; Chemo + T cells (n=3): 1 SD, 2 PR[62]
NPC		
auto EBV-specific Ts	LCL	*Brisbane experience* (n=4): 4 NR[61] *Pavia experience:* T-cells (n=10): 2 PR, 4 SD; Chemo + T-cells (n=11): 6 SD[63;64] *BCM experience* (n=15): 3 CR, 2 CRu, 2 PR, 3 SD3;65 *Singapore experience* (n=35): Chemo + T-cells; 3 CR, 22 PR; 3-year OS: 37.1%[4]
	Monocytes modified with Ad5f35.EUGA-LMPpoly	As adjuvant (n=16); prolonged OS in comparison to patients (n=8) who did not receive T-cells[68]

Allo: Allogeneic, Auto: Autologous; ATCs: Activated T cells; Chemo: chemotherapy; CR: Complete response; CRu: Complete response undefined; DCs: Dendritic cells; NR: no response; OS: Overall survival; PR: Partial Response; Pro: Prophylaxis; Thr: Therapy;

Table 4

Release criteria for pepmix-activated EBVSTs*

Test	Method	Release criterion	Specification
Antigen-specific function	Elispot	No	Recognition of type 2 latency antigens
Antigen-specific function	Chromium release	No	Killing of LCLs and pepmix-pulsed targets
Phenotype subset and memory markers	Flow cytometry	No	None
Killing of autologous targets	Chromium release	Yes	< 10% killing at E:T ratio of 20:1 in a 4 hour assay
Phenotype	Flow cytometry	Yes	< 0.1% K562 cells
Identity	HLA PCR	Yes	HLA identical with blood
Mycoplasma	MycoAlert PCR	Yes	Negative
Endotoxin	Endosafe PCR	Yes	<5.0 EU/ml
Fungus and bacteria	Bactec and CFR sterility	Yes	Negative at 7 to 21 days

* T-cells are fully characterized for research purposes and to develop potency and purity criteria for later phase studies.

Table 5

Genetic modifications to improve anti-tumor activity of T-cells

Goal	Genetic Modification
Rendering T-cells resistant to immunosuppressive Thr	*Molecules conferring resistance:* Calcineurin A mutant[30;86]; rapa-resistant mTOR[32]; mutant IMDH II[88]*Gene silencing:* FKBP12[87]
Enhancing T-cell expansion	*Cytokines:* IL-15[93] *Cytokinereceptors:* IL-7Rα[94;95]
Rendering T-cells resistant to immunosuppressive tumor environment	*Cytokines:* IL-12[111–113], IL-15[93] *Dominant negative receptors (DNR):* TGFβ RII DNR[104;105] *Chimeric cytokine receptors:* TGFβ RII/TLR4[107]; IL-4/IL-7[108]; IL-4/IL-2[109] *Silencing negative regulators:* FAS[110]
Enhancing T-cell homing to tumor sites	*Chemokine receptors:* CCR4[114]
Redirecting T-cells to non-EBV antigens	*CARs:* CD30[117], CD70[118], GD2[119;120]

CAR: Chimeric antigen receptor; FKBP: FK binding protein; IMDH: inosine monophosphate dehydrogenase; mTOR: mammalian target of rapamycin; Rapa: rapamycin