Review Article

Adoptive cellular immunotherapy for Epstein-Barr virus-associated lymphoproliferative disease

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Contributions: (I) Conception and design: All authors; (II) Administrative support: None; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: B Wistinghausen, H Dave; (V) Data analysis and interpretation: B Wistinghausen, H Dave; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: This review focuses on the current state of adoptive cellular immunotherapy for Epstein-Barr virus-associated lymphoproliferative disease (EBV-LD). EBV-positive post-transplant lymphoproliferative disease (PTLD) is a major complication of hematopoietic stem cell and solid organ transplantation. First successes with adoptive immunotherapy were described using donor lymphocyte infusion for the treatment of EBV+ PTLD in hematopoietic stem cell transplantation (HSCT) recipients but carried an appreciable risk of graft-versus-host disease (GVHD). Subsequently, EBV-specific T-lymphocytes (EBVSTs) were developed and have been evaluated clinically for the treatment of EBV-LD over the last two decades predominantly in specialized centers. With impending commercialization, current state and knowledge is critically reviewed. The objectives of this review are: (I) to describe different manufacturing strategies of EBV specific T-lymphocytes (EBVSTs); (II) to review different EBVST donor options and their advantages and limitations; (III) to summarize current clinical experience with adoptive cellular therapy for EBV-LD and finally; (IV) to explore the potential for combination therapies with other immunotherapeutic strategies. This review is based on a review of published literature spanning 20 years from 2000 to 2020 in addition to personal experience and data from the authors. EBVSTs can be readily produced from seropositive donors using good-manufacturing-practice (GMP)-compliant protocols and cryopreserved for future use. In clinical trials, adoptive cellular therapy using EBVSTs has shown impressive results in immunocompromised patients with EBV-LD and there is the potential for wider use. Current research is focused on strategies to enhance EBVST potency *in vivo* through genetic engineering as well as combination therapies.

Keywords: Epstein-Barr virus (EBV); lymphoproliferation; adoptive cell therapy; immunosuppression

Received: 15 November 2021; Accepted: 11 April 2022; Published: 30 June 2022.
doi: 10.21037/aol-21-43
View this article at: https://dx.doi.org/10.21037/aol-21-43

Introduction

Epstein-Barr virus (EBV) is a ubiquitous member of the gamma herpes virus family that is associated with a variety of lymphomas and lymphoproliferative disorders (LPD). It infects more than 90% of the adult population worldwide (1). Primary infection occurs in childhood as an asymptomatic or mild infection and/or may result in a more florid infectious mononucleosis syndrome in teenagers and young adults. In healthy seropositive individuals, virus neutralizing antibodies control the spread of infectious virus particles and EBV-specific, human leukocyte antigen (HLA) class I restricted, $CD8^+$ cytotoxic T lymphocytes (CTL)

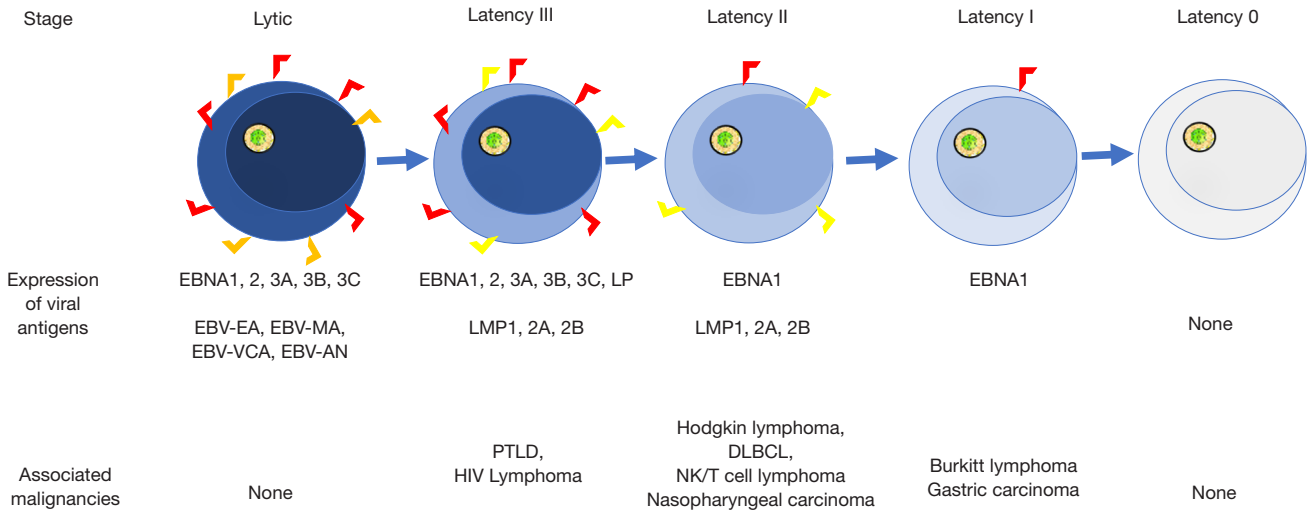

Figure 1 EBV latencies and associated malignancies. After primary EBV infection, most viral antigens are expressed during the lytic stage while the virus is replicating. In EBV-infected B-cells, there is subsequent downregulation of viral antigen expression from latency type III to latency type 0 which allows escape from immune surveillance. Subsequent replication requires return to the lytic stage. EBV, Epstein-Barr virus; EA, early antigen; MA, membrane antigen; VCA, viral capsid antigen; AN, alkaline nuclease; DLBCL, diffuse large B-cell lymphoma; NK, natural killer; PTLD, post-transplant lymphoproliferative disease.

specific to the early lytic cycle proteins kill cells entering the lytic cycle before they are able to release infectious virus particles (2). Regardless of the initial infection, EBV maintains a lifelong latency in B cells and oral epithelial cells. During primary infection, EBV enters the oropharynx replicating within the epithelial cells and infect transiting B-lymphocytes (primarily due to their expression of CD21 which is the major receptor for the virus). EBV can also infect epithelial cells via transfer from infected B cells and other processes (3). EBV-infected naïve B-lymphocytes express proteins comprising the entire EBV genome including the EBV nuclear antigens EBNA1, EBNA2, EBNA3, and EBNALP, membrane proteins LMP1, and LMP2 as well as BARF1 and two small non-translated ribonucleic acids (RNA) (Type III latency) (*Figure 1*). EBV-infected B-lymphocytes then enter the lymphoid follicles and downregulate the immunogenic proteins to express less immunogenic type II latency proteins (EBNA1, LMP1 and LMP2) and thus rescue them into the memory compartment where the virus persists in latently infected B-lymphocytes by further downregulating expression of viral proteins so as to become invisible to EBV-specific T-lymphocytes (EBVSTs) (4). The frequency of EBV-infected B cells in a healthy person remains stable [approximately 0.1–50 EBV-infected B lymphocytes per 1,000,000 peripheral blood mononuclear cells (PBMCs)] over their lifetime, controlled at these levels by a potent EBVST response (5,6). Periodic expansion of EBV-infected B-cells requires re-expression of viral antigens which restimulates the EBVST response.

In individuals with weakened immune systems, such as patients with primary immunodeficiency (PID) or infection with human immunodeficiency virus (HIV), recipients of hematopoietic stem cell transplantation (HSCT) or solid organ transplant (SOT), the lack of a robust EBVST response can lead to uncontrolled proliferation of type 3 latency EBV-infected B cells resulting in EBV-associated LPD and malignancies (7). The transformed B-cells in EBV-associated lymphoproliferative disease (EBV-LD) associated with latency type III present several antigenic viral proteins including EBNA 1-3, LMP1 and LMP2 (*Figure 1*) that induce potent EBVST responses. Such potent T cell immunity maintains the infected B cell pool at <2% of total B cells in immunocompetent individuals but is lacking in immunosuppressed individuals leading to uncontrolled lymphoproliferation of the infected B cells. The scientific rationale for adoptive transfer of EBVSTs is based on harnessing the immunogenicity of type III latency malignancies to control the proliferation of the latently

infected B cells. EBV-associated malignancies associated with type II latency [e.g., Hodgkin's lymphoma (HL), natural killer (NK)/T-cell lymphoma, nasopharyngeal carcinoma (NPC)] or type I latency tumors (e.g., Burkitt lymphoma or gastric carcinoma) can develop in immunocompetent and immune deficient individuals and are much less immunogenic due to downregulation of the immunodominant (e.g., EBNA3 and EBNA2) antigens.

EBV-associated post-transplant lymphoproliferative disease (PTLD) occurs in less than 1% to 25% of HSCT recipients depending on the serostatus of the donor and patient, the degree to which the graft is T-cell depleted, and the post-HSCT immunosuppression (8). In solid organ recipients, incidence of PTLD ranges from 2% to 25% depending on the organ transplanted, passenger lymphocytes in the transplanted organ and type of immunosuppression. However, the biggest risk factor for developing EBV-associated PTLD post-SOT is EBV seronegativity at the time of transplant. Since most children are transplanted at a young age while still being EBV seronegative and convert to EBV seropositivity within 2 years of transplant, EBV driven PTLD is much more common in pediatric SOT recipients (9). More than 90% of EBV-associated PTLD is of mature B-cell origin with cell surface expression of CD20 (10). Therefore, T-cell PTLD is rare and is beyond the scope of this review.

Rituximab, monoclonal antibody targeting CD20 present on the B cells has been an effective monotherapy with response rates of 55% to 100% in HSCT recipients (11-13). Yet, it is limited by increased risk of infection and recurrences. Response rates to rituximab monotherapy in SOT recipients are generally lower and around 50% (9). In pediatric SOT recipients, a combination regimen using low dose cyclophosphamide with prednisone and rituximab has shown event-free survival (EFS) rates of 72% (14). However, these patients with chronic immunosuppression and often impaired organ function have poor chemotherapy tolerance thus there is a need for therapies that address the underlying immune defect, are effective and do not add significant toxicities.

With the expression of multiple immunodominant EBV antigens and the occurrence in the context of immunodeficiency, PTLD is highly immunogenic and amenable to immunotherapy with EBVSTs. Adoptively transferred virus-specific T cells (VSTs) have been evaluated for more than two decades, ranging from the use of donor lymphocyte infusions (DLI) to donor derived multi-antigen specific VSTs in the HSCT setting to autologous as well as allogeneic VSTs in the SOT setting (15-17). However, until relatively recently, this therapy has been available only at specialized centers in the context of single center clinical trials. The objectives of this review are to define current best manufacturing strategies for EBV-specific VSTs, summarize the clinical experience of their use in EBV-related LPD, discuss opportunities to broaden the applicability of this approach and to explore future strategies to enhance their efficacy.

Manufacturing of EBVSTs

EBVSTs can be readily produced from EBV-positive donors using good-manufacturing-grade (GMP) compliant methodologies (*Figure 2*). Donor types include autologous and allogeneic (including third party) sources which are reviewed in more detail below.

Over the years, several strategies have been developed to manufacture EBVST products with minimal alloreactivity and broad specificity against EBV latency proteins or as a multi-virus specific product with activity against multiple viruses (18-22). The most commonly utilized methods consist of *ex vivo* expansion of VSTs versus antigen-specific T cell selection [e.g., interferon γ (IFN-γ) capture].

Ex vivo expansion of EBVSTs

Ex vivo expansion of T cells targeting viral antigens via native T cell receptors was established initially by Smith *et al.* where EBVSTs were selectively expanded utilizing irradiated EBV transformed lymphoblastoid cell lines (LCL) (that express a type III latency pattern of EBV antigen expression) as antigen presenting cells (APCs) to selectively expand EBVSTs (23). LCLs also express high levels of class I and class II HLA and co-stimulatory molecules making them ideal APC for this application (24). LCL-activated EBVSTs consist of a product with activity against early lytic antigens and EBNA 3A, 3B and 3C but unreliable activity towards LMP1 and LMP2 (25,26).

To enhance the specificities to less immunogenic EBV antigens LMP1 and LMP2, several groups have made further modifications to this approach by transducing DC and LCLs with adenovirus vectors to overexpress LMP1 or LMP2 (27). Although this approach helped improve the specificity, the use of LCL and gene engineered APC is complex and time consuming especially when

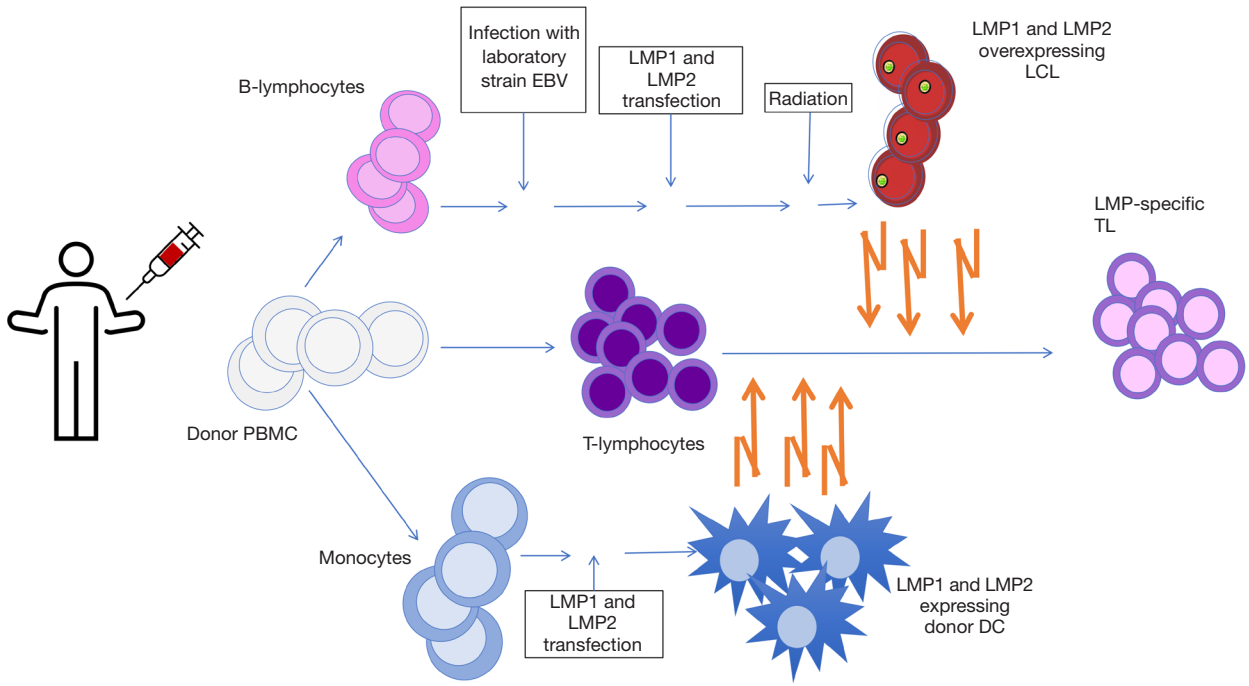

Figure 2 GMP-grade EBV-specific T-cell manufacturing. Mononuclear cells are harvested from the peripheral blood of a donor. B-lymphocytes are infected with laboratory strain EBV and transduced with an adenoviral vector expressing LMP1 and LMP2 and irradiated. Monocytes are separately transduced with the same adenoviral vector and cocultured with T cells followed by a second stimulation by the transduced LCL to create and further expand LMP-specific T-cell product. EBV, Epstein-Barr virus; GMP, good-manufacturing-practice; LCL, lymphoblastoid cell lines; LMP, latent membrane protein.

manufacturing from patients (19,28). Subsequently the use of APC such as dendritic cell (DC) pulsed with overlapping peptide libraries spanning whole antigen coupled with the use of artificial APCs [e.g., the novel antigen-presenting complex (KATpx)] can facilitate the rapid (10–21 days as opposed to 2–3 months) expansion of T cells targeting EBNA1, LMP1 and LMP2 from healthy donors and from patients with type 2 latency EBV-associated malignancies (27,29). A further benefit of *ex vivo* expansion approaches that utilize whole antigen, is that EBVSTs can be manufactured from individuals irrespective of their HLA type. Further, the successful manufacture of EBVSTs derived from EBV seronegative donors has been achieved, including for clinical use, using similar *ex vivo* expansion methodologies (30,31).

Antigen-specific T cell selection

Other rapid methods include: (I) major histocompatibility complex (MHC) multimer selection where oligomeric forms of MHC molecules are designed and conjugated to magnetic beads to isolate typically $CD8^+$ T cells with high affinity to a specific viral epitope/peptide in an HLA restricted manner (32); (II) IFN-γ capture approaches where mononuclear cells are pulsed with antigen [e.g., single peptides, overlapping peptides (pepmixes), etc.] to isolate $CD8^+$ and $CD4^+$ T cells which secrete IFN-γ in response to the viral antigens (33-35). Although these methodologies can manufacture a product in 48 hours, they require seropositive donors with high frequency of circulating antigen/epitope specific T cells which may be technically challenging in the autologous setting. Moreover, the HLA-restriction requirement coupled with the selection of a purely $CD8^+$ T cell product that lacks $CD4^+$ T cell help (necessary for a sustained immune response) limits the overall applicability of the multimer-selection approach (36). Nevertheless, utilizing both of these strategies, good manufacturing practice (GMP)-grade

Table 1 Summary of clinical trial data using allogeneic VSTs in EBV$^+$ PTLD in HSCT recipients

Institution	Indication	Number of patients	Serious adverse events	Outcome
BCM, Houston, TX, USA (17,42,43,45)	Prophylaxis; treatment	101; 13	Recurrence of aGVHD in 8 patients	Prophylaxis: no PTLD; treatment: CR 84.6%
MSKCC, New York, NY, USA (22)	Treatment	19	None	CR 68%, median follow-up 80 months
Karolinska Institute, Stockholm, Sweden (46)	Prophylaxis	6	None	Decrease in viral load in 5 patients, 1 death from PTLD
Children's Research Hospital, Kyoto, Japan (47)	Treatment	1	None	No response

aGVHD, acute graft-versus-host disease; BCM, Baylor College of Medicine; CR, complete response; EBV, Epstein-Barr virus; HSCT, hematopoietic stem cell transplantation; MSKCC, Memorial Sloan Kettering Cancer Center; PTLD, post-transplant lymphoproliferative disease; VST, virus-specific T cell.

EBVST products can be rapidly produced from EBV seropositive autologous and allogeneic donors for clinical use.

Post-HSCT donor-derived T cell therapy

Donor lymphocyte infusions

The earliest reported experience of cellular therapy for the treatment of PTLD utilized unmodified DLI derived from the patient's EBV seropositive HSCT donor which contained effector cells with activity against EBV (37). Although effective in inducing remissions, this therapy carries a significant risk of graft-versus-host disease (GVHD) (19). Strategies such as selective depletion of T-regulatory cells (Tregs) prior to infusion enhances the graft versus lymphoma (GVL) effect while depletion of naïve T cells has been employed to lessen the risk of GVHD (38-40).

Donor-derived EBVSTs

There is extensive reported experience using donor derived EBVSTs for the prevention and treatment of EBV$^+$ PTLD in the post-HSCT setting when the donor is available (17,22,41-47) (*Table 1*).

In the original report using *ex vivo* expanded EBVSTs from healthy seropositive donors, the team at St. Jude's Research Hospital treated ten allogeneic HSCT recipients, three with evidence of EBV reactivation and seven at high risk of reactivation (17). This therapy was well tolerated without significant complications with remarkable reduction in EBV viral copy numbers within 4 weeks, including in a patient with immunoblastic lymphoma. None of the 7 patients who received the EBVSTs as prophylaxis had any EBV reactivation or GVHD and there was evidence of persistence of EBVSTs by tracking of genetic markers on the T cells for a median of 10 weeks. This pivotal study established the safety and early evidence of efficacy of donor-derived EBVSTs for the treatment and prophylaxis of PTLD in transplant recipients (17). These clinical outcomes were replicated in a larger study that reported combined data obtained from St. Jude's (Memphis, TN, USA), Baylor College of Medicine (BCM) (Houston, TX, USA) and Great Ormond Street (London, England). In this report, 114 patients received donor derived EBVSTs after allogeneic HSCT for the treatment and prevention of EBV-related PTLD (43). None of the 101 patients who received this therapy as prophylaxis developed PTLD. Of the 13 patients with active PTLD, 11 patients achieved a complete remission with evidence of VST persistence up to 9 years post-infusion (43). GVHD rates in this study were low with no development of *de novo* acute GVHD and only 8 of 51 patients developed a recurrence of their acute GVHD all of whom responded to GVHD therapy. Of 108 evaluable patients, there were 13 patients with chronic GVHD but only 2 patients had extensive chronic GVHD. The Memorial Sloan Kettering Cancer Center (MSKCC) group also published their experience using adoptively transferred unselected T cells (i.e., donor lymphocyte infusions) or EBVSTs (22). Overall response rates (ORR) were 72% and 68% with DLI and EBVSTs, respectively. GVHD was occurred in 17% of patients with DLI infusions but was not

Table 2 Summary of clinical trial data using autologous EBVSTs for EBV$^+$ PTLD in SOT recipients

Institution	Indication	Number of patients	Serious adverse events	Outcome
BCM, Houston, TX, USA (48)	Prophylaxis; treatment	10; 2	None	No PTLD; 1 CR, 1 PR, follow-up 1 year
IRCCS Policlinico S. Matteo, Pavia, Italy (49)	Prophylaxis	7	None	Reduction in EBV viral load in 5/7

BCM, Baylor College of Medicine; CR, complete response; EBV, Epstein-Barr virus; EBVSTs, EBV-specific T-lymphocytes; PR, partial response; PTLD, post-transplant lymphoproliferative disease; SOT, solid organ transplant.

seen in any of patients who received EBVSTs (22).

Autologous EBVSTs

Administration of autologous EBVSTs has been used for patients with EBV-associated malignancies outside the context of allogeneic HSCT and in SOT recipients with PTLD where donors usually are not available because of the use of cadaveric grafts. In the SOT setting, the production of autologous EBVSTs is technically more challenging because of the ongoing immunosuppression but this can be overcome with modern manufacturing approaches (48). In theory, autologous EBVSTs are preferrable to donor EBVSTs even if available in the post-SOT setting because PTLD is usually of recipient origin and solid organ grafts are not routinely HLA matched to the recipient. However, the challenges in production and the production time leading to delays in initiation of therapy in patients with a rapidly progressive disease impede the routine use of these products in this setting. Comoli *et al.* from Pavia in Italy reported the treatment of seven SOT recipients treated prophylactically for high EBV viral load with autologous EBVSTs (*Table 2*) (49). None of the patients developed PTLD. The group at BCM reported 12 SOT recipients treated with autologous EBVSTs (48). While EBVST infusion did not consistently decrease EBV viremia, none of the patients treated prophylactically progressed to PTLD and the two patients with active PTLD achieved a clinical response [one complete response (CR) and one partial response (PR)].

Autologous EBVSTs have also been used to treat type II latency EBV-associated malignancies including HL, T/NK lymphoma and NPC as adjunctive therapy to chemotherapy and/or in relapsed patients. Such tumors are more challenging targets because of the reduced expression of immunogenic viral antigens in these type II latency tumors which express a more restricted array of antigens (e.g., LMP1, LMP2, EBNA1 and BARF1) compared to type III latency tumors.

EBVST products produced by LCL stimulation alone consist of T cells with specificity predominantly towards early lytic antigens and the immunodominant EBNA3 antigens but less activity towards viral antigens expressed in latency type II (25,26). Several groups have however used these products for the treatment of type II latency EBV-associated malignancies (50,51). A pilot study of 14 patients with relapsed EBV$^+$ HL reported 5 patients who maintained a CR for up to 40 months, two of whom had measurable disease at the time of EBVST infusion. One additional patient achieved a PR and five patients had stable disease (SD) (52). For NPC, locoregional disease control was reported in three out of 4 patients with no activity in metastatic disease (51). Other groups reported response rates of 60–70%. Better responses were observed with EBVST products that included T cells with activity against LMP1 and LMP2 (44,53).

As described above, the BCM group subsequently developed a manufacturing process for a LMP2- and LMP1/2-specific T-cell product (18). In two clinical trials using LMP2- and LMP1/2-specific T cells, respectively, production of EBVSTs was successful in 91% of patients with LMP1 and/or LMP2-specificity detected in 66% of products (27,54). A total of 50 patients with EBV-associated HL or Non-Hodgkin lymphoma (NHL) were treated (27). Of 29 patients receiving latent membrane protein (LMP)-specific T cells as adjuvant therapy after HSCT or chemotherapy, 28 patients remained in a complete remission. Thirteen objective responses, notably 11 CRs were observed in 28 patients with active disease at the time of T cell infusion. These are impressive results in a group of patients with mostly type II latency malignancies which are less immunogenic tumors. LMP-specific T-cell infusions were associated with antigen spreading in responders versus non-responders (27). Specifically, there was a significant increase of T cells specific to lymphoma associated (non-viral) antigens melanoma-associated antigen

A4 (MAGE-A4), survivin and preferentially expressed antigen of melanoma (PRAME). In one of the larger studies that evaluated LMP-specific T cell therapy for NPC, the group from the Queensland Institute of Medical Research (QIMR) (Brisbane, Australia) reported 16 patients with metastatic NPC receiving adjuvant therapy with LMP1/2-specific T cells and experiencing longer median survivals compared to a control of 8 patients without adoptive cell therapy (523 versus 220 days) (55).

Third-party T cells

Even with "rapid" technologies, patient-specific EBVST product (autologous or allogeneic) manufacture can still be prolonged when procurement times of donor or patient are considered. Further, donor cells may not be available (e.g., recipients of umbilical cord blood transplants or cadaveric organ transplants). Hence, manufacture of patient-specific products may not be possible or may be so delayed that patients with rapidly progressive disease are not able to access these therapies. For these reasons, a readily available "off the shelf" T cell therapy product is desirable.

The first third-party EBVST bank of 60 EBVST products was established by Haque *et al.* in the United Kingdom (56). Thirty-three transplant recipients (stem cell, 2; heart, 2; kidney, 13; liver, 10; liver and small bowel, 3; lung, 2; heart and lung, 1) with refractory PTLD between the age of 1–76 years received partially HLA matched EBVSTs. HLA matches ranged from 2 to 5/6 HLA alleles and there was a statistically significant association of better outcome with higher HLA matches. Overall, the response rate (CR and PR) was 64% at 5 weeks and 52% at 6 months. Of note, no significant toxicities were observed, alleviating concerns of graft rejection and/or GVHD with this therapy which fueled the broadened applicability of this approach (20,57-62).

The UK group established a cell bank of 25 donors with HLA alleles prevalent at high frequencies in individuals of European descent (58). Of ten patients treated with products from this bank, 8 achieved a CR. There was one report of grade I skin GVHD but otherwise infusions were well tolerated.

Chiou *et al.* from Birmingham, United Kingdom published their experience in 10 pediatric SOT recipients with PTLD and reported an ORR of 80% (8 out of 10) (61). This favorable response in a pediatric population may indicate differences in the biology of EBV-driven PTLD in this population who is often EBV naïve at the time of transplant and develops PTLD in the earlier post-transplant period compared to the adult population.

The BCM group published extensively on their third-party EBVST products including the use of off-the-shelf multi-VSTs [EBV, cytomegalovirus (CMV), adenovirus, +/– BK virus (BKV) and human herpes virus 6 (HHV6)] products (59,62,63). In a multicenter study led by BCM investigators, utilizing a bank of 33 VST products, 15 products were given to 50 HSCT recipients with severe refractory viral disease after BMT targeting EBV, CMV and/or adenovirus (62). In total, 9 patients receive these third-party multi-VSTs for EBV-associated PTLD. Overall, the response rate to EBV was 66.7% at 6 weeks and only 1 of the responders had a recurrence but achieved a CR with infusion of donor derived EBVSTs. Subsequently, the group established a third-party bank with 59 multi-VST products covering EBV, CMV, adenovirus, BKV and HHV6. In a phase II single center trial, 2 out of 38 patients were treated for EBV reactivation/PTLD and both achieved a CR (59). In both studies, no significant adverse events attributable to the product were observed.

The Memorial Sloan Kettering experience utilizing a third-party cell bank comprising 330 GMP-grade EBVST products was reported by Prockop *et al.* (20). A total of 46 patients post-HSCT (33) or SOT (13) and PTLD were treated with three weekly infusions of third-party EBVSTs. The ORR was 68% in HSCT recipients and 54% in SOT recipients with a 1-year overall survival of 88.9% in patients with a CR or PR and 81.9% OS in patients who achieved SD. Eleven patients had evidence of central nervous system (CNS) involvement. Of those, 5 achieved a CR and 4 a durable PR suggesting that EBVSTs have activity in the brain. Given the dismal prognosis of CNS PTLD with 3-year progression-free survival rates in the 30% range, EBVSTs represent a promising therapeutic option for this patient population. Based on the HLA typing of 400 patients from the ethnically diverse New York area population, the investigators estimated that an EBVST bank with products restricted by 40 HLA alleles would be sufficient to cover 95% of that population.

Third-party EBVSTs have been mostly used in the post-transplant setting. There has been no published experience in patients with HIV-associated lymphomas because in the modern era of highly active anti-retroviral therapy (HAART) the incidence in the Western word has decreased (64) and the logistical support and specialized experience needed does not make EBVSTs an easily accessible option for the treatment of patients in the developing world.

Table 3 Summary of third-party EBVSTs and multi-VSTs in PTLD

Institution	Specificity	Indication	Number of patients	Serious adverse events	Outcome
University of Edinburgh, Edinburgh, UK (56)	EBV	Treatment	2 (HSCT); 31 (SOT)	None	ORR 51.5% (14 CR + 3 PR) at 6 months with 2 subsequent relapses, follow-up 1–7.5 years
MSKCC, New York, NY, USA (20)	EBV	Treatment	33 (HSCT); 13 (SOT)	1 grade I skin GVHD	ORR 68% (19 CR and 3 PR) in HSCT and 54% (2 CR and 5 PR) in SOT, follow-up 6–115 months
Birmingham Woman's and Children's Hospital Foundation NHS Trust, Birmingham, UK (58)	EBV	Treatment	10 (SOT)	None	ORR 80% (7 CR and 1 PR), 5-year OS 85.7%
BCM, Houston, TX, USA (62)	Multi-VST	Treatment	9 (HSCT)	2 TMA, 1 GI hemorrhage, all deemed unrelated	ORR 66.6% (2 CR and 4 PR)

CR, complete response; EBV, Epstein-Barr virus; EBVSTs, EBV-specific T-lymphocytes; GI, gastrointestinal; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; MSKCC, Memorial Sloan Kettering Cancer Center; ORR, overall response rate; OS, overall survival; PR, partial response; PTLD, post-transplant lymphoproliferative disease; SOT, solid organ transplant; TMA, thrombotic microangiopathy; VST, virus-specific T cell.

Surrogate markers of response have been investigated by several groups (52,56,62,65). Prockop *et al.* reported in their series that a 2 $\log_{10}$ reduction in EBV viral load was indicative of response, however, not all patients treated with third-party EBVST's had detectable viral loads at start of therapy (20). Leen *et al.* also showed reduction of EBV viral load correlated with response in HSCT recipients with EBV-associated disease treated with third-party multi virus specific VSTs (62). Moreover, clinical response and reduction of EBV viremia correlated with an increase of EBVSTs (62). However, there is no standardization of EBV viral load measurements by polymerase chain reaction (PCR) making comparisons between different laboratories impossible. EBV viral loads can be measured in plasma or whole blood. Kanakry *et al.* compared EBV viral load in plasma versus PBMCs and found that cell-free (plasma) EBV copy number quantification was superior to PBMC to predict response in both immunocompetent as well as immunosuppressed individuals with EBV-associated lymphomas or lymphoproliferative disease (65).

Overall, these reports are highly encouraging demonstrating the feasibility of third-party cell banks able to cover a majority of the referred patient population while also achieving impressive response rates (*Table 3*). These promising results led to the first cell therapy trial run through a cooperative group with the Children's Oncology Group piloting third-party EBVSTs for the treatment of newly diagnosed PTLD in pediatric SOT recipients (NCT02900976). This study was recently closed and analysis is ongoing. The same group at Children's National Hospital supplied another multi-center phase I/II consortium study (PBMTC SUP1701, NCT 03475212) with third-party EBVSTs through the Pediatric Bone Marrow Transplant Consortium (66). This trial had two arms, one for pediatric patients with refractory CMV, EBV and or adenoviral infections post-HSCT and another for pediatric patients with PID disorders suffering from refractory viral infections prior to HSCT.

Modification of EBVSTs to enhance activity

Overcoming the immune suppressive effects of TGF-β to enhance EBVST activity in vivo

As previously discussed, the efficacy of EBVSTs is dependent on the expression of viral antigens and limited by the paucity of EBV antigen expression in malignancies of latency type I and II. Furthermore, the immune evasion strategies (e.g., TGF-β secretion) employed by the tumor microenvironment in the immunocompetent host suppresses functional antitumor T cell responses *in vivo* (67,68). TGF-β can be released into the tumor microenvironment by tumor cells, fibroblasts and immune cells and creates an immunosuppressive environment by impeding T-cell activation, proliferation and migration. In addition, it affects DC and macrophage antigen presentation and chemotaxis.

The potential to overcome the immunosuppressive properties of TGF-β has been studied in EBV-positive HL. The Baylor group developed an EBV/LMP-specific T-cell (LST) product expressing a dominant negative TGF-β receptor type II (DNRII) (69). The DNRII-LSTs were resistant to otherwise inhibitory concentrations of TGF-β. In a phase I dose escalating study, 8 patients with relapsed EBV-positive HL were treated with 2 to 12 doses of TGF-β resistant LSTs with 4 of the 7 evaluable patients with active disease achieving clinical responses that were complete and sustained in two patients greater than 4 years post-infusion (70). Moreover, DNRII-LSTs expanded *in vivo* and could be detected in the peripheral blood from 2 to 51+ months post-infusion.

Calcineurin resistance to enhance EBVSTs

Given the concerns regarding EBVST persistence in patients who require ongoing immune suppression (e.g., patient post-SOT), several groups have explored gene engineering of virus specific T cells to render them resistant to immune suppressive agents including steroids and calcineurin inhibitors (71-74). In one such example of a proof of principle preclinical study, EBVSTs were genetically engineered to express a mutant form of calcineurin thus rendering them calcineurin inhibitor (CNI) resistant (75). In mouse xenograft models bearing human B-cell lymphoma, treatment with CNI-resistant EBVSTs persisted with enhanced activity in the presence of CNI compared to control EBVSTs.

Chimeric antigen receptor T cells

CD19-chimeric antigen T cells (CD19-CART) have shown impressive efficacy in acute B-lymphoblastic leukemia and in B-cell lymphomas (76-80). In addition to viral antigens, EBV-LD expresses a variety of B-cell antigens targetable by CARTs including CD19, CD20 and CD30. However, their use in EBV-lymphoproliferation is limited by the patient's immunosuppressive state impeding T cell manufacture and the length of production time.

CD19-CART have been administered in three adult SOT recipients with refractory PTLD (81). All patients developed complications to CART therapy including cytokine release syndrome (CRS), neurotoxicity and acute kidney injury. None responded and all ultimately succumbed to their disease. Therefore, while some anecdotal case reports have been published, the wider use of the CART platform for PTLD will likely require an off-the-shelf product (81,82).

Potential for combination strategies administering EBVSTs with other therapeutic modalities to enhance EBVST activity in vivo

Demethylating agents

Newly EBV-infected B-cells express up to 90 viral genes; however rapid CpG-methylation of viral antigens leads to downregulation of viral protein expression and the latency (83-85). Azacytidine and decitabine are potent CpG demethylating agents. In a mouse xenograft of latency type I Burkitt lymphoma, pretreatment with decitabine induced expression of LMP1 and ENBA3 associated with latency type 3 which sensitized tumor cells to subsequent therapy with EBVSTs (86). In contrast, azacytidine did not increase expression of those proteins.

Checkpoint inhibitors

LMP1 has been shown to induce expression of the checkpoint protein PD-L1 in classic HL (CHL) without 9p24.1 alteration (87). When comparing EBV-positive with EBV-negative CHL, EBV-positive CHL had significant higher PD-L1 expression (88). PD-L1 expression scores were inversely correlated with outcome. Similarly, 76–100% of EBV-positive diffuse large B-cell lymphoma (DLBCL) were found to express PD-L1 (89,90). PD-L1 is expressed in 73% of EBV-positive PTLD (87). There have been several trials using checkpoint blockade in DLBCL hinting at single agent activity (91). A phase I study at BCM is exploring combination therapy of checkpoint inhibitors with EBV directed T cells for EBV^+ HL and NHL (NCT02973113). Even though there is a rationale for also combining them with EBVSTs for EBV^+ PTLD, the risk of graft rejection and autoimmunity limits their use in that setting.

BCL-2 inhibitors

Latently EBV-infected cells inhibit proapoptotic signals thus ensuring immortality (92). LMP1 upregulates the expression of the anti-apoptotic protein BCL-2 (93). The INSERM group showed that the BCL-2 inhibitor ABT-737 induce remission in approximately 70% of mice PTLD xenografts (94). In preclinical, *in vitro* studies, pretreatment of malignant B-cell lines with the BCL-2 inhibitor venetoclax increased proapoptotic proteins and sensitivity to CD19-CART; however, co-culture and post-treatment

adversely affected the number of CART (95). Further studies are needed to elucidate whether there is a role in EBV lymphoproliferation.

Conclusions

The use of adoptive immunotherapy, particularly using third-party "off the shelf" EBVSTs for the treatment of EBV-LD has shown promise in several studies conducted at specialized centers (20,56,62). More recently, EBVSTs have become more widely available including in industry led multi-center studies and consortium and cooperative group studies (20,66). Further work is however needed to create widely available and commercialized third-party cell banks to broaden the applicability of this approach beyond boutique centers. In addition, strategies need to be explored to enhance the anti-tumor activity of EBVST therapies especially for the less immunogenic type I and II latency tumors. Preclinical work and early clinical trials are in process exploring various gene engineering and combination therapy approaches to improve the potency of EBVSTs *in vivo*.

Acknowledgments

Funding: None.

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editors (Christopher P. Fox and Claire Shannon-Lowe) for the series "Lymphoma and Viruses" published in *Annals of Lymphoma*. The article has undergone external peer review.

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://aol.amegroups.com/article/view/10.21037/aol-21-43/coif). The series "Lymphoma and Viruses" was commissioned by the editorial office without any funding or sponsorship. CMB is a co-founder and scientific advisory board member for Catamaran Bio and Mana Therapeutics and is on the Board of Directors of Cabaletta Bio which are companies with no specific interest in EBV specific T cell therapies or EBV$^+$ lymphomas. CMB has intellectual property related to developing virus-specific T therapies. CMB has performed ad hoc consultancy for BMS (BCMA-CAR-T for Myeloma), Roche (B-cell NHL) and Pfizer (ALCL). She served on an *ad hoc* advisory board for CDR-Life AG on PTLD. She has received royalties from Cellmedica who licensed the LMP-specific T cell technology. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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doi: 10.21037/aol-21-43
Cite this article as: Wistinghausen B, Dave H, Bollard CM. Adoptive cellular immunotherapy for Epstein-Barr virus-associated lymphoproliferative disease. Ann Lymphoma 2022;6:5.