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REVIEW

Immunotherapy against cancer-related viruses

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Approximately 12% of all cancers worldwide are associated with viral infections. To date, eight viruses have been shown to contribute to the development of human cancers, including Epstein-Barr virus (EBV), Hepatitis B and C viruses, and Human papilloma virus, among others. These DNA and RNA viruses produce oncogenic effects through distinct mechanisms. First, viruses may induce sustained disorders of host cell growth and survival through the genes they express, or may induce DNA damage response in host cells, which in turn increases host genome instability. Second, they may induce chronic inflammation and secondary tissue damage favoring the development of oncogenic processes in host cells. Viruses like HIV can create a more permissive environment for cancer development through immune inhibition, but we will focus on the previous two mechanisms in this review. Unlike traditional cancer therapies that cannot distinguish infected cells from non-infected cells, immunotherapies are uniquely equipped to target virus-associated malignancies. The targeting and functioning mechanisms associated with the immune response can be exploited to prevent viral infections by vaccination, and can also be used to treat infection before cancer establishment. Successes in using the immune system to eradicate established malignancy by selective recognition of virus-associated tumor cells are currently being reported. For example, numerous clinical trials of adoptive transfer of *ex vivo* generated virus-specific T cells have shown benefit even for established tumors in patients with EBV-associated malignancies. Additional studies in other virus-associated tumors have also been initiated and in this review we describe the current status of immunotherapy for virus-associated malignancies and discuss future prospects.

Keywords**:** virus-associated malignancies; immunotherapy; Epstein-Barr virus; HPV; HBV; HCV

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Introduction

An estimated 15%-20% of cancers are associated with infection [1]. Bacteria and multicellular parasites are responsible for a fraction of these infection-related cancers, but the majority is associated with viral infection. At least 1.3 million new cases of cancer worldwide every year, or 10%-12% of the total number of new diagnoses, are likely related to viral infection [1, 2]. If these virus-associated tumors were prevented, there would be 19% fewer cancers in developing countries and 3.8% fewer in developed countries [1]. Although infection-associated cancers are less frequent in developed countries, their incidence is increasing due to the rising number of patients who are immunosuppressed either iatrogenically (e.g., transplantation recipients on immunosuppressive drugs) or due to infection with the human immunodeficiency virus (HIV). In addition, the incidence of Human papilloma virus (HPV)-associated head and neck cancers has increased in developed countries due to changes in sexual practices [3].

To date, at least 5 DNA viruses, Epstein-Barr virus (EBV), HPV, Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8), Merkel cell polyomavirus (MCV), and Hepatitis B virus (HBV), and 3 RNA viruses, Human T lymphotropic virus type-1 (HTLV-1), Hepatitis C virus (HCV), and HIV have been associated with human cancers, though this number is likely to increase over time. These viruses produce oncogenic effects by three distinct mechanisms. First, viruses can directly induce transformation of the infected cells. The genes that viruses express following integration or after establishing a stable episome can regulate host cell growth and survival. Alternatively, recognition of viral genes by host cells can initiate the DNA damage response (DDR) which many viruses need for their replication. DDR increases

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genetic instability, which in turn raises the mutation rate and accelerates the acquisition of oncogenic chromosomal alterations in host cells. Second, viral infection can lead to cancer by inducing chronic inflammation [4]. For example, HBV and HCV induce chronic hepatic inflammation associated with oxidative DNA damage followed by macronodular cirrhosis, contributing to the subsequent development of hepatocellular carcinoma (HCC) [5-6]. Finally, some viruses such as HIV are not themselves oncogenic but inhibit the patient's immune system, disrupting immunosurveillance and allowing for the emergence of hyper-mutated malignant cells [7]. In this review we will not discuss cancers caused by the third mechanism. Immunotherapy against HIV has been developed and reviewed elsewhere [8-9]. Immunotherapy has potential beneficial effects for many virus-associated cancers, as it can work at three different phases: preventing viral infections, treating infection before it causes a malignancy, or eradicating an established malignancy by selective recognition of virus-infected cells. The development of effective prophylactic vaccines reduces the risk of virus-associated cancer irrespective of the mechanism of cancer induction. Such vaccines are already available for HPV and HBV, and extensive research to develop EBV, HCV and other vaccines continues [10]. However, as described in each section below, these prophylactic vaccines cannot induce the sterilizing immunity required to prevent or eliminate tumors in previously infected patients. Once viral infection is established, immunotherapy can be used to target the viral gene products in infected cells that cause inflammation or unregulated cell proliferation. Unlike traditional cancer treatments such as chemotherapy, radiotherapy and surgery, cell-based immunotherapies can distinguish virus-infected cells from non-infected cells. As we describe below, the feasibility of developing and using virus-specific T cells (VSTs) as cellular immunotherapeutics has greatly increased due to simplified manufacturing [11] and the success of "off the shelf" partially HLA-matched VSTs [12-13].

In this review, we will focus on EBV and HPV as examples of viruses that directly cause cancers, and on HCV and HBV as illustrations of viruses that cause cancer mainly by indirect mechanisms. Other cancer-associated viruses, HTLV-1 [14], KSHV [15], and MCV [16-17], have been reviewed elsewhere [18-20].

More recently chimeric antigen receptor (CAR) T cell therapy has shown significant success in the treatment of B-cell malignancies. Their application to the treatment of virus-associated malignancies is less advanced, however, since viral antigens are generally presented as processed peptides in association with MHC molecules, and are recognized by conventional T cell receptors (TCRs) rather than by CARs. This article therefore focuses on TCR-mediated immunotherapies in virus-associated cancers.

EBV

EBV life cycle

EBV is an enveloped DNA virus whose linear, double-stranded DNA genome encodes approximately 90 genes [21]. EBV is the causal agent of infectious mononucleosis (IM), a common benign disorder, and is associated with several human malignancies [22-23]. Worldwide, more than 95% of adults are infected with EBV [24], with the majority of infections occurring during childhood when they produce minimal symptoms. Post adolescence, infection is associated with IM, a self-limiting lymphoproliferative disease [25-26].

Primary infection with EBV occurs in the oropharyngeal cavity, where EBV can infect both B lymphocytes and epithelial cells. The initial phase of EBV infection of B lymphocytes requires EBV attachment to the host cell mediated by a viral envelope glycoprotein gp350/220 which binds to the complement receptor type 2 (CR2), also known as CD21 [27]. In contrast, attachment to epithelial cells, which lack or express very low levels of CR2, is mediated by viral glycoprotein gH and is much less efficient. Following attachment, entry into B lymphocytes requires a complex of three envelope glycoproteins, gH, gL, and gp42, whereas entry into epithelial cells requires a complex without gp42 [28-29].

The EBV life cycle can be divided into the lytic phase, in which EBV replicates, and the latent phase, in which EBV shuts down most of its protein-encoding genes. EBV latency occurs in both B cells and epithelial cells with different latency patterns. Latently infected B cells have at least four patterns of gene expression, termed Latency 0, 1, 2 and 3. Each pattern expresses up to 10 EBV-derived proteins, including EBV nuclear antigens (EBNAs) 1, 2, 3a, 3b, 3c and LP, BARF1 and latent membrane proteins (LMPs) 1, 2a, and 2b as well as regulatory RNAs, including the BamHI A rightward transcripts (BARTs) and EBV-encoded RNAs (EBERs).

Latency 3 drives B-cell transformation and proliferation, and the expression of all 10 EBV latency-associated proteins makes these cells highly immunogenic. In an immunocompetent host, therefore, Latency 3 B cells are controlled and eliminated by T cells specific for EBV-associated proteins, of which EBNA3 proteins are the dominant targets. As a result, there is a selection for infected B cells that only express the less immunogenic EBNA1, LMP1, and LMP2 antigens — termed Latency 2 B cells. These cells enter lymphoid follicles where they prolifer-

ate [30]. After exiting the lymph node, they shut down all viral proteins that can be detected by the immune system (Latency 0), or only express EBNA1 (Latency 1), which is essential for viral genome replication during B cell divisions [31] (Figure 1).

Mechanisms of oncogenesis

All of the above phases of the EBV life cycle and latency patterns are reflected in the formation of different types of EBV-associated tumors with the exception of Latency 0 (Figure 1). Although we do not yet have a comprehensive understanding of the underlying pathogenic sequence that causes EBV-associated tumors, several potential mechanisms have been identified. For example, LMP1 is essential for the ability of EBV to immortalize B cells [24], and the underlying mechanism

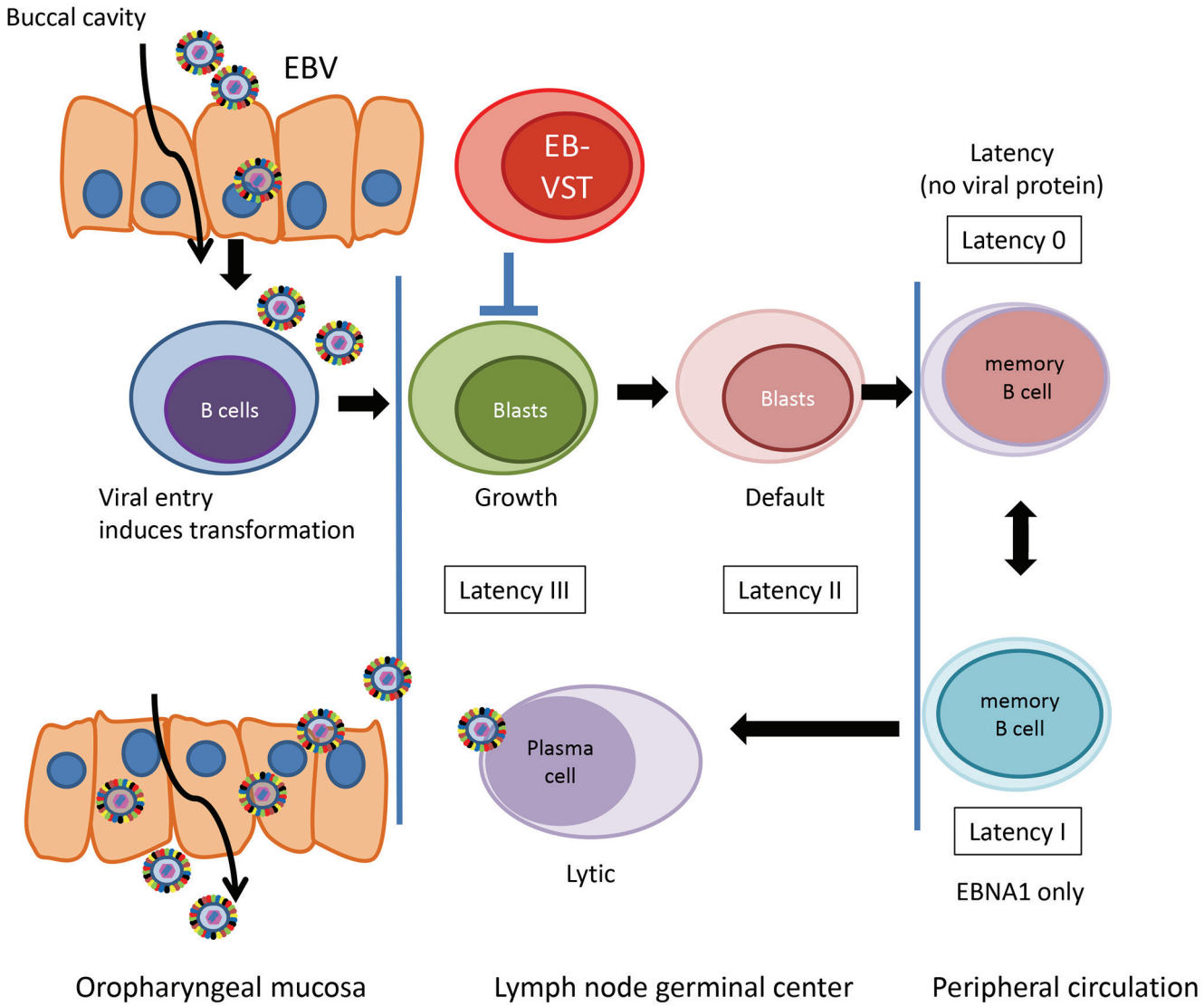

Figure 1 The model of EBV life cycle and latency states. EBV primary infection occurs in the oropharyngeal cavity. EBV infects naive B cells and expresses its entire latency genes (Latency 3, growth program). Although Latency 3 drives B cell transformation and proliferation, these cells are highly immunogenic, and thus EB-VSTs eliminate these Latency 3 cells in immunocompetent hosts. The infected B cells downregulate the expression of its immunogenic proteins, allowing viral persistence (Latency 2, default program). Then these cells migrate to the peripheral blood where they express EBNA1 (Latency 1, EBNA1 only program) or no viral proteins at all (Latency 0, Latency program). These memory compartments are not detected by the immune system and are likely the sites of long-term persistence. The latently infected memory B cells undergo terminal differentiation into plasma cells which can produce viruses (Lytic program) [148]. Viruses released from plasma cells can infect epithelial cells where they are amplified before shedding [149].

may involve the effects of LMP1 in regulating the mitogen-activated protein kinase (MAPK), NF-κB, and PI3K pathways [32]. Similarly, LMP2a, forms tyrosine-phosphorylated aggregates in the plasma membrane, associates with Lyn and Syk and stimulates B-cell receptor (BCR) signaling, thereby also inducing activation of the PI3K/AKT survival pathway [24, 33].

EBV also plays an unequivocal, albeit incompletely understood, role in the pathogenesis of undifferentiated nasopharyngeal carcinoma (NPC), more than 90% of which are EBV-positive [34], and express the type 2 latency pattern of antigens. These type 2 latency genes likely contribute to the establishment of multiple hallmarks associated with these epithelial malignancies, such as resistance to cell death and evasion of growth suppression [35].

Immunotherapy against EBV-associated tumors

EBV vaccines As with any virus-associated malignancy, in principle EBV-associated tumors could be prevented by effective vaccination strategies before infection and latency are established. To date, most preventative vaccines have focused on the EBV glycoprotein gp350, which is the most abundant protein on the virus and in virus-infected cells and is the major target of neutralizing antibodies. In a phase 2 clinical study, the incidence of IM was reduced by 78% in 181 EBV-seronegative (i.e., presumptively uninfected) young adults who received the recombinant EBV gp350 vaccine compared to the placebo control group. However, the rate of seroconversion, defined by the subsequent production of antibodies to nonvaccine EBV antigens in the absence of IM, was unchanged, indicating that the vaccine cannot protect against asymptomatic EBV infection [36-37]. The same gp350 vaccine (albeit in a different adjuvant) was also used at lower doses to immunize seronegative patients awaiting kidney transplantation to treat chronic renal failure [38]. Unfortunately, in this patient subset the vaccine was poorly immunogenic, with the transient appearance of neutralizing antibody to EBV detected in only 4 of 13 patients [38]. Elliot *et al*. tested an EBV peptide subunit vaccine in ten seronegative HLA B*08:01 subjects using an EBNA-3A peptide in combination with tetanus toxoid in an oil-in-water emulsion in an attempt to generate a cell-mediated rather than an antibody-based immune response to the virus. By using gamma interferon enzyme-linked immunospot assay, the investigators detected an increase in peptide-reactive T-cells in 8/9 evaluated vaccine recipients and 0/4 placebo controls. After 2 to 12 years of follow up, 4 out of 4 vaccinated patients studied had seroconverted asymptomatically [39]. Barriers currently preventing the further development and evaluation of a truly effective prophylactic vaccine for EBV include lack of knowledge as to whether gp350 is the optimal protein to induce a protective antibody response and the delay between primary EBV infection and the development of associated tumors. Thus, it will be important to identify and validate suitable markers that can predict future tumor development and thus allow more rapid assessment of the potential capacity of a new vaccine to prevent the onset of EBV-associated malignancy [40].

Notwithstanding the difficulties of developing an antibody-inducing EBV vaccine, investigators have tested the ability of therapeutic immunization to eradicate established infection/EBV-associated tumors by inducing EB virus-specific T cells (EB-VSTs), CD4+ and CD8+ effector T cells capable of recognizing EBV-infected target cells through their native TCRs. A phase I clinical study for patients with EBV-positive nasopharyngeal cancer (NPC) used a modified vaccinia virus expressing the CD4+ T cell epitope-rich C-terminal fragment of EBNA1 and the full-length sequence of LMP2. The vaccine induced CD4+ and CD8+ EB-VSTs in a dose-dependent manner; however, only 2/16 vaccinated patients showed clinical benefit. Determining a correlation between detectable immune reactivity and clinical benefit for this therapy will require an expanded cohort of patients [41-42].

Treatment of EBV-associated tumors All EBV-related cancers are associated with the viral latency cycle and the expression of viral proteins. These viral proteins contribute to the malignant transformation process and are true neo-antigens, making them excellent targets for immunotherapy. Because most EBV antigens are intracellular proteins (e.g., EBNA2) or are tightly embedded within cell membrane (e.g., LMP1 and LMP2), they cannot be effectively targeted by antibody-mediated therapy. They are, however, processed and presented on the surface of the infected cells in association with Class I and Class II MHC molecules, making them excellent targets for viral antigen-specific T cells.

Treatment of type 3 latency tumors

Type 3 latency tumors express the full panoply of EBV latency antigens, making them highly immunogenic. These tumors therefore flourish only in immunocompromised hosts, for example in patients who have received hematopoietic stem cell transplant (HSCT) or solid organ transplant (SOT). More than 25 years ago, Papadopoulos *et al*. [43] reported that lymphocyte infusions from EBV-seropositive donors contained sufficient numbers of EB-VSTs to induce complete responses in 5/5 patients who developed donor-derived EBV-associ-

ated immunoblastic lymphoma after allogeneic stem cell transplantation. However, these unselected lymphocytes also contained large numbers of alloreactive T cells that caused severe graft-versus-host disease (GvHD). Subsequently, many different studies in multiple centers have confirmed that infusing non-alloreactive EB-VSTs generated from HSCT donor blood was able to prevent severe EBV reactivation (prophylaxis) as well as treat established and bulky immunoblastic lymphoma. These cells were even effective against lymphomas that were resistant to conventional therapies such as CD20 antibody (Rituximab). For example, our own center reported that none of more than 100 HSCT recipients who received prophylactic EB-VSTs developed EBV-associated post transplant lymphoproliferative disease (PTLD), compared to 12.5% of patients who did not receive EB-VSTs. When used as a treatment against PTLD, EB-VST infusion led to sustained complete response in 11 out of 13 patients [44]. Although several hundred patients have now been treated, these VSTs have not induced significant acute or chronic GvHD [44-45]. Other investigators have observed similar results with these cells [46-49].

Patients who receive SOT are also iatrogenically immunodeficient and may develop PTLD. However, there are several differences between the PTLDs that develop after SOT and HSCT. After SOT, > 90% of PTLDs are derived from the recipients' own B cells, whereas > 90% of PTLDs are of donor origin after HSCT. While T cells for preparing EB-VSTs to treat PTLD can readily be obtained from HSCT donors, availability is less common after SOT, since many organs are from cadaveric donors. Moreover, since most PTLDs after SOT are derived from the recipients' B cells, donor-derived VSTs will not work unless they share the MHC alleles through which EBV antigens can be recognized. Another obstacle is that SOT patients continue long-term immunosuppressive therapy, and these drugs may suppress the infused cells. Despite these limitations, several groups including our own have reported successful clinical trials using autologous EB-VSTs after SOT [50-52]. In these studies, adoptive transfer of the patients' own VSTs did not induce organ rejection or produce other adverse events, although *in vivo* T cell expansion was lower than that observed in HSCT patients who received similar doses of EB-VSTs. Despite the difference in T cell expansion *in vivo*, which is attributed to more prolonged treatment with immunosuppressive drugs in SOT versus HSCT recipients, response rates in SOT patients were promisingly high [51].

Over the past 20 years, major improvements have been made to the manufacture and processing of these EB-VSTs, many of which have also facilitated the manufacture of T cells directed to other oncogenic viruses (see HPV below) [11, 53-54]. In early studies, EBV-transformed B-lymphoblastoid cell lines (LCLs), which express the same viral antigens as Latency 3 tumors and high levels of HLA class I/class II and co-stimulatory molecules, were used to generate EB-VSTs [44-46]. LCLs could be generated easily by incubating cells from healthy EBV seropositive donors with a laboratory strain of EBV and were excellent antigen-presenting cells (APCs), having the same pattern of viral gene expression as the outgrowing tumor cells [55]. However, this EB-VST manufacturing process takes at least 10 weeks, including 6 weeks for LCL production, limiting the extension of EB-VST therapy to a broader range of patients.

To decrease manufacturing time, investigators used an approach first developed for CMV-specific T cells [56]. First, they isolated EB-VSTs by using HLA-peptide multimers or streptamers [57] or by selecting T cells that secrete IFN-γ in response to EBV antigen stimulation (γ-capture) without any *ex vivo* expansion [53] (Figure 2). A small number of T cells responding *in vitro* to two HLA A2-restricted EBV-associated peptides (GLC and CLG) showed substantial *in vivo* expansion and dramatic clinical effects [53]. Similarly, IFN-γ capture has been used clinically by Moosmann and colleagues [54], who isolated EB-VSTs by stimulating donor leukapheresis products overnight with EBV peptides, followed by IFN-γ capture and immunomagnetic separation (Figure 2). They treated six post-HSCT PTLD patients, and although 3 patients with late-stage disease showed no response, 3 patients at earlier stage of the disease had complete remissions (CRs), 2 of which were sustained for more than 2 years [54].

However, *ex vivo* selection strategies may require leukapheresis of donors or patients, which may not always be feasible, especially for unrelated HSCT donors. Even when leukapheresis is possible, the number of VSTs obtained by these selection approaches may still be too low [58].

For the above reasons, investigators have since developed rapid *ex vivo* expansion strategies. Initially, researchers substituted EBV-LCLs with dendritic cells (DCs) transfected with EBV plasmids, but subsequent studies have focused on pulsing DCs with virus-derived peptides [11]. These studies have shown that even peripheral blood mononuclear cells (PBMCs) could be pulsed with peptides in the presence of cytokines to rapidly expand EB-VSTs, eliminating the need to make DCs. The cells manufactured using these accelerated and simplified strategies appear to be clinically effective [59], and the substantial reduction in time, complexity and cost has enabled the study of this approach in multi-national trials in lymphoma and NPC [55].

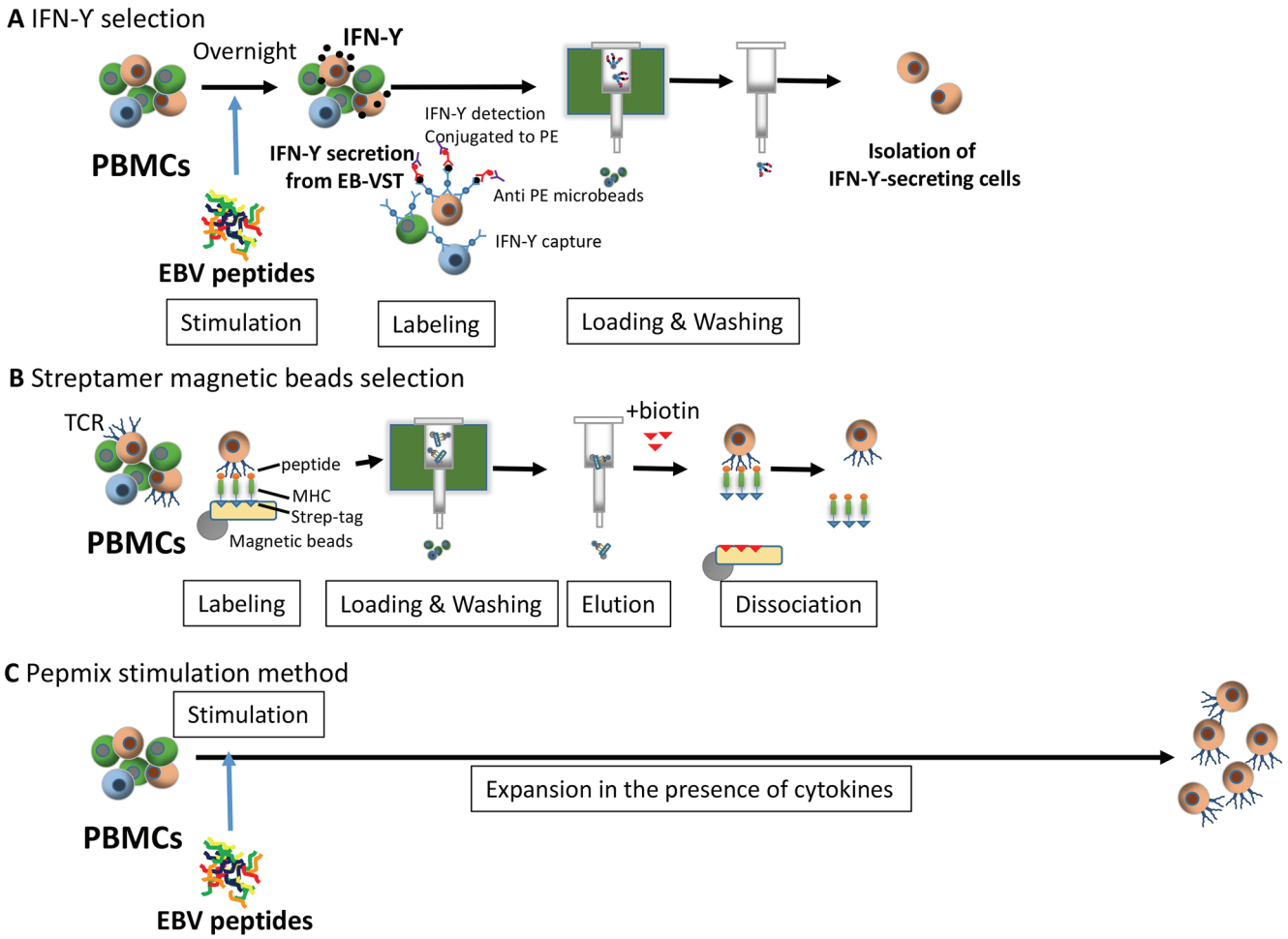

Figure 2 Manufacturing of EB-VSTs. **(A)** IFN-γ selection: stimulate PBMCs with EBV peptides and capture IFN-γ-secreting cells with magnetic beads. **(B)** Streptamer magnetic beads selection: select EB-VSTs using HLA-peptide streptamer and isolate them with magnetic beads. **(C)** Pepmix stimulation method: stimulate PBMCs with EBV peptides and expand them in the presence of cytokines.

Notwithstanding the improvements described above, individualized cell therapies will always be more complex than standardized "off the shelf" approaches, and some patients need treatment more urgently than the cells can be manufactured. Moreover, for post-HSCT treatments the T cells must be obtained from the HSCT donor, and if the donor is unavailable or EBV seronegative (e.g., cord blood) then individualized treatment is not feasible. For these reasons, investigators have begun developing banks of characterized, HLA-typed EB-VSTs from third-party donors that are immediately available for most patients.

Haque and colleagues first reported the use of EB-VSTs from partially HLA-matched third-party donors to treat EBV-PTLD in an SOT recipient [60]. The patient achieved CR without infusion-related toxicity or GvHD. Many groups have now shown the feasibility, safety and efficacy of this approach in patients after SOT and HSCT, reporting that the infused cells can target the viral antigen presented by a shared MHC allele on the tumor cells [12-13, 47, 60-61]. However, the complete mechanism of action of third-party T cells remains uncertain because these cells undergo little expansion in peripheral blood of recipients (unlike the donor-specific EB-VSTs given to HSCT recipients). Functional T cells may still exist at tumor sites or the cell infusion may create an inflammatory response that induces the proliferation of endogenous tumor-specific T cells against non-viral antigens [58].

This "off the shelf" approach has not yet been used successfully to treat lymphoma and NPC occurring in immunocompetent hosts (see below), in whom host

alloreactivity is more potent and the tumor is less immunogenic than in immunocompromized SOT or HSCT patients.

Treatment of type 2 latency tumors

The success of VST therapy against type 3 latency tumors encouraged investigators to extend the strategy to the treatment of EBV-related Latency 2 tumors, including Hodgkin's lymphoma (HL), non-Hodgkin's lymphoma (NHL), NPC, and some gastric carcinomas in immunocompetent hosts. Type 2 latency cells only express EBNA1, which is required for the maintenance and replication of the viral episome in EBV-infected cells, and LMP1 and LMP2, which are essential for B cell immortalization and transformation of post-germinal center B cells as described above (Mechanisms of oncogenesis). EBNA1 is poorly presented by HLA class I molecules because of its glycine-alanine repeats [62]. LMP2 has been identified as a source of epitopes for several HLA class I alleles, but the number of reactive T cells in EBV-infected individuals is generally low, and immune reactivity to LMP1 is even lower. The basis of the differences in immunogenicity between Latency 3- and Latency 2-associated EBV antigens is still not understood [63]. In practical terms, however, since EBNA1, LMP1 and LMP2 are less immunogenic and T cells specific for EBV type 2 latency tumor antigens may be suppressed or anergized by the tumor microenvironment [64], treating type 2 latency tumors is more challenging. 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 to stimulate cytotoxic T lymphocytes (CTLs) [65-66]. Manufacturing these APCs *ex vivo* takes at least 12 weeks and may be impossible for patients with lymphoma who have received the CD20-directed monoclonal antibody Rituximab, a drug that is the standard of care for many lymphoma patients and markedly depletes the normal CD20+ B cells that are required to form EBV-LCLs [58]. Subsequent studies have therefore used PBMCs or DCs pulsed with peptides derived from type 2 latency antigens to activate antigen-specific effector T cells, and then expanded these effector T cells using autologous activated T cells pulsed with the same antigens as artificial APCs combined with an HLA-negative K562 cell line transduced with CD80, CD83, CD86 and 4-1BBL as cells providing co-stimulatory signals. Using this method, we can avoid the requirement for EBV-LCL to present these tumor-associated EBV antigens [67]. Although, thus far, the EBV-LCLs method is more widely used, this new method may allow the generation of T cell products for patients whose EBV-LCLs are not available. The clinical efficacy of this method remains to be determined.

HL and NHL In immunocompetent patients, Latency 2 EBV infection is associated with both HL and NHL. HL is a unique malignancy, as the bulk of the tumor is composed of normal cells with malignant Hodgkin-Reed-Sternberg (HRS) cells interspersed throughout. HRS cells likely originate from germinal center B cells. HL is one of the most frequent lymphomas in the Western world and EBV-encoded RNA or protein is detected in HRS cells in up to 40% of cases [68-69]. In North America, 20%-50% of HLs are EBV+ [70-72], but in developing countries the percentages of EBV positivity in HL are much higher. In Kenya, for example, LMP1 has been detected in lymph node biopsies from 66% of adults and 100% of children with HL [73], while intermediate values of approximately 57% were reported from China [74].

NHL is commoner than HL, accounting for about 4% of all malignancies. NHL arises from lymphoid tissue, and has heterogeneous clinical and biological features [75]. The majority of NHLs originate from B cells, but T cells, NK-T cells and NK cells may also form these NHLs. Overall, up to 40% of NHLs are EBV-positive, but the association with EBV is subtype-dependent and may be as high as 90% in some NHL subgroups including EBV-positive diffuse large B cell lymphoma of elderly [76-78].

While chemotherapy and radiation remain the initial treatment of HL and NHL, immunotherapy is an attractive alternative for patients with relapsed disease or those who fail to enter remission [79-80]. One immunotherapeutic approach is vaccination to enhance the proliferation of endogenous VSTs using peptides or DNA as the source of EBV antigen, and LCLs or DCs as APCs, but this therapy's success has been hampered by the anergy of VSTs in patients with EBV-associated malignancy [81].

An alternative is to use adoptive transfer of T cells specific for type 2 latency EBV antigens. We have reported beneficial effects from autologous LMP-specific T cell therapy against EBV-related lymphoma [82]. In our study, 28 of 29 patients with high-risk/multiply relapsed disease who received LMP-specific T cells as adjuvant therapy remained in remission for a median of 3.1 years after infusion. Of 21 patients with active disease at the time of VST infusion, 13 had clinical responses, including 11 CRs. Patients with EBV+ NK-T cell lymphoma were also included in this study. All 5 treated patients who were in first or second remission at the time of infusion had a sustained remission and 3/5 patients with active disease achieved sustained CRs [82], results that

are particularly encouraging given the poor prognosis of NK-T cell lymphomas treated by conventional therapy. Consistent with these data, Cho and colleagues reported that NK-T cell lymphoma patients who received autologous LMP1/2a-specific T cell therapy combined with chemotherapy, radiotherapy and/or high-dose chemotherapy followed by stem cell transplantation had 4-year overall survival and progression-free survival of 100% and 90%, respectively, with a median follow-up of 55.5 months [83].

NPC NPC is a squamous cell carcinoma arising from the nasopharyngeal epithelium. The disease is most frequent in South-East Asia and Southern China and is strongly associated with EBV infection. Over 90% of undifferentiated NPCs are EBV-positive, and patients have high levels of EBV antibodies directed to lytic cycle antigens. Whether the infection is an initiating event or acts as a sustained driver of the malignancy remains controversial [34, 84].

Although the precise contribution of EBV to NPC pathogenesis remains elusive, its strong association with EBV infection provides a rationale for treating NPC patients with EB-VSTs. We applied autologous EB-VST infusion to treat 23 patients with locoregional or metastatic recurrent/refractory NPC [85-86]. Out of 15 patients who had active disease at the time of treatment, three of 4 patients with locoregional disease had CRs, but only 1/11 with metastatic disease had a CR [85]. Comoli *et al*. reported control of disease in 6 of 10 patients with stage-4 NPC and observed similar response rates in a later study of 11 patients that added lymphodepleting chemotherapy prior to EB-VST infusion [87-88]. In a larger study in Singapore, 35 patients received up to six doses of EB-VSTs after four cycles of gemcitabine and carboplatin, producing a response rate of 71.4% with 3 CRs and 22 partial responses [89]. The 2- and 3-year overall survival rates were 62.9% and 37.1%, respectively, and anti-tumor responses correlated with the presence of LMP2-specific T cells in the infused line products [89]. A phase III randomized trial is now comparing the efficacy of this strategy with chemotherapy alone [58].

Treatment of type 1 latency tumors

EBV-positive Burkitt's lymphoma (BL) is a high-grade, malignant small non-cleaved cell lymphoma that occurs with high frequency in its endemic form in equatorial Africa, with intermediate frequency in Central America and low frequency (sporadic) elsewhere. Over 95% of endemic BLs are associated with type 1 latency EBV infection, but the association of EBV with sporadic cases is lower and in the Unites States only 20% of BLs are EBV-positive. More recently, about 10% of gastric carcinomas (GCs) have been shown to be associated with EBV [90], and these patients may have superior outcomes than patients with EBV-negative forms of the disease [91]. The precise role of EBV in the pathogenesis of GCs remains to be determined, but the absence of EBV infection in pre-malignant gastric lesions supports the suggestion that viral infection is a relatively late event in this tumor [92].

BL and GC express only EBNA1 (Latency 1). As described above, EBNA1 is thought to be poorly presented to the immune system by HLA class I molecules and thus to be incapable of mediating an effective cytotoxic immune response by CD8+ effector T cells. More recently, however, several groups have shown that EBNA1 can in fact be presented by certain HLA class I alleles (e.g., HLA B35) in infected cells, likely due to the translation of defective EBNA1 RNA [93-95]. Further, EBNA1 contains numerous HLA class II-restricted epitopes [63, 95] and the reactive CD4+ T cells can produce substantial cytotoxic effects towards type I latency tumor targets [96-98]. Hence, EBNA1 may in fact be an excellent target antigen for immunotherapy of EBV-associated malignancies since it is expressed in all EBV-positive malignancies and induces both CD4+ helper/killer T cells and in some cases (depending on HLA polymorphisms) CD8+ VSTs as well [95]. As yet, however, no clinical trials have evaluated T-cell therapy for EBV-positive BLs or GCs.

Genetic modifications to improve EB-VST functions

While the adoptive transfer of EB-VSTs is an effective therapy for PTLD post HSCT with sustained complete response rates of > 90%, EB-VST infusion is less effective for PTLD after SOT, and only half as effective for type 2 latency malignancies. Investigators therefore have modified EB-VST effector T cells in order to enhance their functionality.

In SOT recipients, immunosuppressive drugs are normally administered long-term to prevent graft rejection and these agents inhibit T cell expansion and function. To render EB-VSTs resistant to immunosuppressive drugs, such as calcineurin inhibitors, rapamycin, or mycophenolate mofetil (MMF), investigators have exploited several different gene modifications. Ricciardelli *et al*. showed that overexpression of a calcineurin A mutant in EB-VSTs provided resistance to the calcineurin inhibitor FK506 and restored the cells' ability to eliminate established LCLs in NOD/SCID/IL2Rγ-null mice engrafted with human EBV-LCLs in the presence of FK506 [99]. Silencing FK-binding protein 12 by siRNA in EB-VSTs also showed similar results [100]. Huye *et al*. [101]

expressed a rapamycin-resistant mTOR in CD19-specific CAR T cells that synergized with rapamycin in the elimination of B-cell lymphoma, a strategy that could be adapted to EB-VSTs for SOT recipients receiving this drug. Finally, investigators have rendered T cells resistant to MMF by expressing a mutant inosine monophosphate dehydrogenase II in T cells [102].

For type 2 latency malignancies, the problems are more complex and include the limited array of EBV antigens expressed, the lack of lymphoid "space" to expand (no lymphodepletion), and the immunosuppressive tumor microenvironment. Several genetic modifications have been evaluated as potential countermeasures to these roadblocks [58]. Systemic cytokine administration may in principle overcome the limitation of *in vivo* expansion of EB-VSTs due to insufficient growth cytokines or immunosuppressive cytokines derived from the tumor or tumor-infiltrating cells. However, this approach is not specific to VSTs and carries the risk of serious adverse effects. Investigators therefore have also tried to "arm" T cells by engineering them to express their own growth-promoting cytokines/receptors, such as IL-7Rα [103], and others [104]; or by expressing receptors that block inhibitory signals, such as the dominant-negative TGFβ receptor type II (DNR) [105], or that convert an immunoinhibitory signal to an activation signal [106]. DNR has been tested in the clinic and an abstract disclosed that DNR-modified EB-VSTs benefited patients who failed therapy with unmodified EB-VSTs [58]. These approaches may also be applied to T cells used to treat other virus-associated malignancies.

HPV

Epidemiology and pathogenesis

HPV is a small non-enveloped DNA virus that is associated with benign papillomas or warts and human cancers of the cervix, anus, penis, and head and neck. To date, approximately 200 HPV serotypes have been characterized [107]. These viruses are classified into high-risk and low-risk groups according to the propensity for malignant progression of the lesions that they cause. Persistent infection of high-risk subtypes, such as HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, or 66, has long been known to predispose to the development of cervical cancer [108] and is also associated with carcinomas of the oropharynx and anogenital region. HPV infects basal epithelial cells where viral genomes are persistently maintained as low-copy number episomes [4]. As these cells differentiate and move toward the surface of the epithelium, the virus is induced to replicate and releases infectious particles into the mucosa. In most people, HPV infection is asymptomatic and in more than 90% of cases HPV can be cleared within 1 to 2 years [18]. Lesions that are not cleared by the immune system can persist for several decades, and during that time partial viral genomes become integrated into the host cell genome. Lesions caused by a high-risk HPV that integrate the E6 and E7 oncogenes have a high chance of inducing cancer since E6 and E7 inhibit several natural "tumor suppressor' pathways. For example, E6 and E6-associated protein induce the proteasomal degradation of p53 following ubiquitination [18, 109]. Similarly, E7 promotes cell proliferation by competing with the E2F transcription factor for binding to the retinoblastoma tumor suppressor protein (pRB) [107], releasing E2F and thus enabling DNA synthesis by facilitating cell entry into the S phase. As a consequence, these viral proteins induce the proliferation of cervical carcinoma cells [110-111] and are also likely involved in the induction and maintenance of other HPV-associated malignancies. Their sustained involvement in the development and growth of HPV-associated tumors makes E6 and E7 strong candidates for targeting in order to elicit an antiviral and hence anti-tumor immune responses.

Immunotherapy against HPV-associated tumors

HPV vaccines There are three approved effective prophylactic vaccines for high-risk HPV genotypes. In 2006, the first of these was approved in the United States for prevention of both cervical cancer and genital warts. This quadrivalent vaccine (Gardasil, Merck & Co., Inc.) targets HPV genotypes 6, 11, 16, and 18, which are responsible for approximately 66% of cervical cancers and 90% of genital warts [112]. A bivalent vaccine targeting oncogenic HPV genotypes 16 and 18 (Cervarix, GalaxoSmith-Kline) has been marketed for female vaccine programs. A nona-valent vaccine (Gardasil 9, Merck & Co., Inc.) that covers five additional HPV genotypes (31, 33, 45, 52, and 58) responsible for an additional 15%-20% of cervical cancer cases has more recently been approved by the FDA [113]. In general, HPV vaccines are safe and effective as prophylaxis against infection [114-115], but have no activity against established disease since they lack specificity for E6/E7, the only viral proteins expressed in HPV-associated tumors. Therapeutic vaccines are currently under investigation [116].

Adoptive cell therapy Stevanovic and colleagues reported a clinical study using tumor-infiltrating lymphocytes (TILs) from cervical cancer biopsies. These TILs had reactivity against E6 and E7 and were expanded and infused into patients after lymphodepletion; 3/9 patients who received TILs had clinical responses, including 2

CRs [117]. The same group has begun a clinical study using genetically engineered T cells expressing a high-affinity T cell receptor (TCR) against E6 (NCT02280811) [118]. Unlike TILs, these engineered T cells can target only a single epitope of E6, increasing the risk of tumor "editing" and immune escape, and are also restricted to patients with one specific HLA polymorphism (HLA-A02:01).

More recently, a study has opened in which polyclonal HPV-specific T cells (HP-VSTs) are generated from patient PBMCs *ex vivo* [119]. Briefly, peripheral blood T cells were stimulated with DCs loaded with pepmixes (peptide libraries) derived from E6/E7 and the resulting HP-VSTs were administered to patients with advanced HPV-associated malignancies of the head and neck or anogenital region (NCT02379520) [119]. The efficacy of administration of these HP-VSTs remains to be determined.

HCV and HBV

Epidemiology and pathogenesis

HCV is an enveloped single-stranded, positive-sense RNA virus of the Hepacivirus genus in the Flaviviridae family. Its genome of ~9.6 kb contains a single open reading frame encoding a 3 000 amino acid residue polyprotein precursor that is cleaved into 10 smaller proteins by cellular and viral proteases [120]. More than 270 million people are infected by HCV worldwide. In the majority of infected individuals, HCV establishes a persistent and life-long infection [24]. Of those infected, 20% eventually develop liver complications due to chronic inflammation and the development of macronodular cirrhosis. These patients then have a 4%-7% annual risk of progression to HCC [121]. Although *in vitro* studies have shown that both the core protein and the NS3, NS4B and NS5A proteins of HCV have oncogenic potential [122], the precise viral oncogene causing HCC is unclear because, unlike EBV- or HPV-associated malignancies, virus-derived proteins are not always present in the malignant cells themselves.

HBV is a DNA virus consisting of a genome that is mostly double stranded [19] in the Hepadnaviridae family and blood-borne HBV infection may result in acute and chronic hepatitis, macronodular cirrhosis and HCC. HBV infection persists in 1%-2% of immunocompetent adult individuals after acute hepatitis [123]. Up to 50% of patients with chronic HBV infection and cirrhosis will develop HCC. The progression to cancer is mediated in part by dysregulated repair/regeneration responses, and in part by the oncogenic potential of the viral proteins HBX and HBS and microdeletions in the host DNA due to partial integration of the viral genome [122].

Immunotherapy against HCV- and HBV-associated tumors

Therapy against HBV and HCV infections used to rely on (pegylated) IFN-α, an unpleasant drug to take with substantial side effects; however, nucleoside analogues against HBV and several new protease inhibitors against HCV have now been introduced with very good response rates [124]. Drug toxicities and viral resistance remain concerns, and these agents do not work against established tumors. This is an unfortunate limitation, since the overall prognosis for HCC patients is poor, with a reported 5-year survival of around 50% even in patients with early, small HCC (< 3 cm) who undergo surgical resection. Most patients present with unresectable advanced disease [125].

Prophylactic vaccination for HBV and HCV Although an effective HBV prophylactic vaccine has been widely available for three decades, it has been estimated that nearly 400 million individuals have chronic HBV infections [126]. HCV is poorly suited to vaccine strategies because of its high mutation rate [127]. As a result, HCC is increasing in incidence.

Adoptive cell therapy Given the limited efficacy of current therapies, there has been great interest in developing an immunotherapeutic strategy for HCC. There are, however, a number of obstacles. Since the tumor cells usually lack viral antigens, the targets for the immune response cannot be derived directly from the infecting virus, as they are in HPV- and EBV-associated malignancies, but must come instead from host antigens present on the tumor cells. Many of these host-derived antigens are shared with normal hepatocytes and other organs in the body. Hence, the risks of "on target, but off cancer" tissue damage are considerable. Moreover, the liver is characterized by a highly immunosuppressive microenvironment [128]. Hepatocytes themselves can induce an anergic phenotype in CD8+ T cells and there are in addition several subsets of phagocytic cells present that may act as inhibitory or tolerogenic APCs, namely liver sinusoidal endothelial cells, Kupffer cells and liver DCs [129].

To date, clinical trials have evaluated transfer of several different immune effector cells for the treatment of HCC, including IL-2- and anti-CD3-activated autologous PBMCs [130], cytokine-induced killer (CIK) cells [131-132], NK and NKT cells (NCT02008929 and NCT01801852), and TILs (NCT01462903). Takayama *et al*. reported 76/150 patients who had undergone curative

resection of HCC received adjuvant autologous lymphocyte infusions. There was prolongation of recurrence-free survival but no improvement of overall survival (OS) [130]. Immunotherapy using CIK cells significantly improved both OS and progression-free survival of HCC patients, although further study through a large-scale, multi-center, randomized clinical trial should be conducted [131-132]. A preliminary report about an alternative TIL approach also showed encouraging outcomes. After a median follow-up of 14 months, 12 out of 15 patients treated with autologous TILs following tumor resection showed no evidence of disease [133]. In addition to adoptive transfer of lymphoid cells, investigators have used DCs pulsed with autologous tumor lysates [134] or peptide-based therapeutic cancer vaccines targeting tumor-associated antigens, such as telomerase [135], alpha-fetoprotein (NCT00022334) [136], or NY-ESO-1(NCT01522820). That these antigens are not restricted to malignant cells is a continuing concern for their specificity [128-129, 137-138]. More recently, efforts have been made to induce immune responses to HCC-associated neoantigens, which are unique to the tumor itself, initially by using checkpoint blockade combined with TIL infusion. The feasibility and benefits of targeting such neo-antigens remain uncertain [139]. Several review articles are available for further information on HCC immunotherapy [128-129, 137-138].

Conclusions

Foreign viral antigens are ideal targets for immunotherapy using T cells carrying the native TCR. Based on the success of donor-derived EB-VST transfer against PTLD after HSCT, the field of immunotherapy against cancer-related viruses has been expanding to cover more patients and additional viruses with promising results. However, to improve clinical outcomes several obstacles must be overcome. Infused T cells must expand and persist long-term. To this end, they must evade tumor-derived inhibition as well as suppressive elements in the host environment. They must also receive sufficient positive signals to ensure their expansion upon antigen encounter and yet they also must be safe. These improvements will require modifications both to the T cells themselves and to the tumor environment. For example, upregulation of PD-1 expression on T cells during chronic infections has been extensively reported and the PD-1 expression is associated with T cell exhaustion [140-142]; thus, in the future genetically modified T cells can be tested clinically in conjunction with checkpoint-blockade antibodies which have shown promising anti-tumor activity as a single agent against several cancers including HL [143-146]. Also a preclinical study has shown that epigenetic modifiers can enhance VST function [147]. Combinations of these novel approaches have the potential to induce improved outcomes. We therefore think that in the future T cell immunotherapy will likely be more widely used against cancer-related viruses and with greater success.

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