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Technology Insight: applications of emerging immunotherapeutic strategies for Epstein–Barr virus-associated malignancies

Rajiv Khanna*, Denis Moss and Maher Gandhi

SUMMARY

The Epstein–Barr virus (EBV) is uniquely associated with a broad range of human malignancies. In spite of their diverse cellular origin, most of these malignancies share common features, including the expression of either some or all of the EBV latent proteins, which can be potentially exploited for immune-based therapies. Here we discuss new and emerging strategies to manipulate the immune response to specifically boost T-cell immunity towards viral proteins that are expressed in EBV-associated malignancies. These strategies are used either alone or as an adjuvant therapy in combination with chemotherapy and/or monoclonal antibodies. Overall, this strategy may serve as a new paradigm for the successful multi-modality treatment of malignancies.

KEYWORDS cancer, Epstein–Barr virus, immunotherapy, T cells, transplant, vaccine

REVIEW CRITERIA

Data for this review were identified by searching the PubMed database and references from relevant articles. Numerous articles were identified through searches of the authors' files. Search terms were "Epstein Barr virus", "renal transplant", "lung transplant", "heart transplant", "liver transplant", "bone marrow transplant", "immunotherapy", "CTL" and "immunity". Only English language papers were reviewed. No time limits for these searches were applied.

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INTRODUCTION

Epstein–Barr virus (EBV)-associated malignancies arise in both immunosuppressed and immunocompetent individuals. These malignancies are of variable cellular origin. Some involve specific genetic lesions but all involve the expression of either some or all of the EBV latent proteins, EBV-associated nuclear antigens (EBNAs) 1, 2, 3A, 3B and 3C, and latent membrane proteins (LMPs) 1, 2 (Figure 1).[1] In the immunosuppressed patient, these malignancies are of B-cell lymphocyte origin. These malignancies emerge as a result of a depressed immune response mediated by either graft-directed immunosuppressive therapy (post-transplant lymphoma [PTL], defined as early and late PTL) or HIV infection (Figure 1). The Asian population seems to be particularly susceptible to EBV-associated malignancies in immunocompetent individuals, suggesting a unique interaction of environmental and genetic factors that are not fully understood.[2–4] These malignancies are of both epithelial and lymphoid origin and include nasopharyngeal carcinoma (NPC) and gastric carcinoma together with the lymphoid malignancies, Hodgkin's lymphoma (HL), and T-cell and natural killer cell lymphomas (Figure 1).

Our understanding of the immune control of EBV infection in healthy carriers[5] has now reached the stage where this response can be considered for targeting towards viral proteins expressed in the EBV-associated malignancies. In healthy individuals, CYTOTOXIC T LYMPHOCYTES (CTLS) have a major role in the immune recovery phase stimulated in response to primary EBV infection, and in controlling the latent life-long EBV infection present in previously exposed individuals.[6] Much of the immune recovery response is directed towards EBNA 3A, 3B and 3C proteins, whereas the response to LMPs is known to be relatively weak. This review considers new and emerging strategies to manipulate this immune response, by specifically boosting CTL numbers to determinants known to be present in EBV-associated malignancies, either alone or in conjunction with adjuvant therapies.

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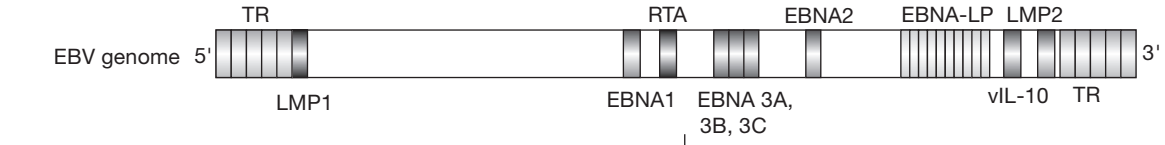

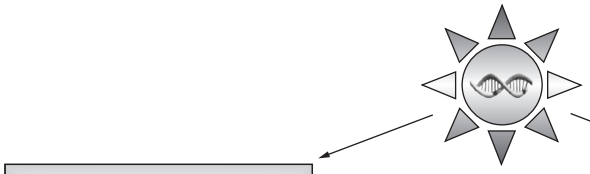

Malignancies in immunosuppressed host

Malignancies in immunocompetent host

EBV malignancy	Viral gene expression	Cellular origin	Genetic lesion
Post-transplant lymphomas (early and late onset)	**Early:** *EBNA1*, *2*, *3A*, *3B*, *3C*, *LP*, *LMP1* and *LMP2* **Late:** *EBNA1*, *2*(+/–), *3A*, *3B*, *3C*(+/–), *LP*, *LMP1*(+/–) and *LMP2*	**Early:** B-cell lymphocyte (polyclonal or monoclonal) **Late:** B-cell lymphocyte (monoclonal)	**Early:** none **Late:** *BCL-6* mutations
HIV-associated lymphomas	**Primary CNS lymphoma** (*EBNA1*, *2*, *3A*, *3B*, *3C*, *LP*, *LMP1* and *LMP2*)	Germinal center or post-germinal center B cells	*cMyc*, *BCL-2* and *p53* as well as *BCL-6*
	Burkitt's lymphoma (*EBNA1*)	Germinal center B cells	
	Primary effusion lymphoma (*EBNA1*) **Diffuse large B-cell lymphoma** (*EBNA1*) **Immunoblastic lymphoma** (*EBNA1*, *2*, *3A*, *3B*, *3C*, *LP*, *LMP1* and *LMP2*)	Germinal center or post-germinal center B cells	

EBV malignancy	Viral gene expression	Cellular origin	Genetic lesion
Burkitt's lymphoma	*EBNA1*	B cell (germinal centroblast; monoclonal)	*Ig/cMYC* translocation *p53* mutations
Hodgkin's lymphoma	*EBNA1*, *LMP1* and *LMP2*	Post-germinal center B cells (monoclonal)	*Ig* gene transcription
T cell and NK cell lymphoma	*EBNA1*, *LMP1*(+/–), *LMP2* and *BARF1*	$CD3^-$, CD56* NK cell	Deletion of 6q
Nasopharyngeal carcinoma	*EBNA1*, *LMP1*(+/–), *LMP2* and *BARF1*	Undifferentiated epithelial cell	Deletion in 3p, 9p, 11q, 13q and 14q
Gastric carcinoma	*EBNA1*, *LMP2*, *BART*s and *BARF1*	Gastric epithelial cell	Hyper-methylation of *INK4A*

Figure 1 Epstein–Barr virus-associated malignancies in immunocompetent and immunocompromised individuals. Each malignancy is characterized by a unique pattern of latent viral gene expression, cellular origin and genetic lesions.[1] The viral gene expression is a crucial determinant for designing novel immunotherapeutic strategies for these malignancies. CNS, central nervous system; EBNA 1, 2, 3A, 3B, 3C, EBV-associated nuclear antigens 1, 2, 3A, 3B, 3C; EBV, Epstein–Barr virus; Ig, immunoglobin; LMP1 and 2, latent membrane proteins 1 and 2; LP, leader protein; NK, natural killer; RTA, viral immediate-early protein, also called BRLF1 or R;TR, terminal repeat; vIL-10, viral interleukin-10.

CURRENT STATUS OF THE TREATMENT OF EBV-ASSOCIATED MALIGNANCIES

At present, treatment of EBV-associated diseases predominately exploits the malignancies' cellular properties, including cellular origin/level of differentiation, viral gene expression and chemo/radiosensitivity. EBV-associated malignancies are a heterogenous group of tumors that vary in their response to conventional therapies. They are in general relatively radiosensitive and chemosensitive in their early stages, but are less amenable to conventional modality therapy in late stage or relapse.[7,8] This is the case particularly in early-stage disease, with 5-year survival rates as high as 90%.[7] By contrast, in the case of late-stage/relapse disease, survival outcomes as low as 50–70% are more typical;[8] but these might drop to below 20% in

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CYTOTOXIC T LYMPHOCYTES (CTLs) T lymphocytes that have a cytolytic function on target-cell binding; they generally recognize antigenic peptides presented by HLA class I molecules

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Table 1 Selected completed/ongoing trials of cellular immunotherapy in Epstein–Barr virus-associated malignancies.

Reference and disease	Patient population	Study type	Results/comments
PTL			
Rooney *et al.* 1998[11]	39, after TCD alloSCT	Prophylactic	Gene-marked donor LCL-stimulated CTLs. No PTL occurred in infused patients, against 11% in historical controls.
Comoli *et al.* 2002[12]	7, after SOT	Pre-emptive (high viral load)	Autologous LCL-stimulated CTLs. Viral load reduced/ stabilized, and no patients developed PTL.
Papadopoulos *et al.* 1994[13]	5, after TCD alloSCT	Therapeutic	Donor leukocyte infusion for established PTL. 5 CR (2 with GvHD), sustained in 3.
Khanna *et al.* 1999[14]	4, after SOT	Therapeutic	Autologous LCL-stimulated CTLs for established PTL. 2 sustained CR, 1 PR, 1 NR.
Haque *et al.* 2002[15]	8, after SOT	Therapeutic	LCL-stimulated haploidentical unrelated-donor CTLs. 3 CR, 1 PR, 4 NR. No GvHD.
Hodgkin's lymphoma			
Lucas *et al.* 2004[17]	6	Therapeutic	LCL-stimulated haploidentical related/unrelated-donor CTLs. 1 CR, 4 PR, 1 stable disease. 1% of CTLs were LMP2A-specific. No GvHD observed.
Roskrow *et al.* 1998[18] and Bollard *et al.* 2004[16]	14	Therapeutic (11/14 had measurable disease, and 3/14 in CR)	Prior to infusion with autologous LCL-stimulated CTLs. 'Super expansion' cocktail containing irradiated allogeneic PBMC used to assist in vitro expansion. 1% of CTLs LMP2A-specific. Gene marking demonstrated CTL localisation to sites of disease. 2/11 CR, 1/11 PR, 5/11 had stable disease, 3 NR, and 3/3 remain in CR.
Ongoing studies	Patients with prior multiply relapsed disease	Therapeutic	NCI/Baylor sponsored study (NCT00070226/62868/82 225) using recombinant AdLMP2A transduced dendritic cells (gene-marking optional) as stimulators to expand autologous LMP2A-specific CTLs. CTLs infused following CD45 antibody administration.
	Patients with progressive, relapsed, or refractory disease	Therapeutic	NCI/Hershey sponsored study (http://clinicaltrials.gov/ct/gui NCT00006100) using HLA matched/haploidentical donor EBV-specific CTLs administered following fludarabine chemotherapy. IL-2 given after CTL infusion.
NPC			
Chua *et al.* 2001[21]	4	Therapeutic (advanced disease)	Autologous LCL-stimulated CTLs, with restoration of EBV-specific T-cell immunity and reduction of EBV plasma levels. No discernable anti-tumor efficacy observed.
Lin *et al.* 2002[22]	16	Therapeutic (local recurrence)	Patients received LMP2A-specific peptide-pulsed dendritic cell immunisations. T-cell responses elicited in 9/16, with PR in 2/16.
Comoli *et al.* 2004[20]	1	Therapeutic (refractory disease)	LCL-stimulated, HLA-identical related-donor allogeneic CTLs, resulted in temporary stabilization of disease. 3% of CTLs were LMP2A-specific. TCR spectra typing demonstrated localisation of CTLs to sites of disease.
Straathof *et al.* 2004[19]	10	Therapeutic	Autologous LCL-stimulated CTLs. Up to 5.5% of CD8 T cells LMP2A peptide-specific. Modest increase in T-cell activity and reduction in EBV viral load observed. 6/10 had measurable tumor, 2/6 CR, 1 PR, 1 had stable disease and 2 NR.
Ongoing studies	NA	Prophylactic	NIH sponsored study (http://clinicaltrials.gov/ct/gui NCT00078494) using LMP2A peptides emulsified in Montanide ISA-51 as a prophylactic vaccine to prevent recurrence in patients with treated NPC. 99 patients to be enrolled.

AdLMP2A, adenoviral vector containing LMP2A; alloSCT, allogeneic stem-cell transplant; CR, complete remission; CTLs, cytotoxic T lymphocytes; EBV, Epstein–Barr virus; GvHD, graft versus host disease; IL-2, interleukin 2; LCL, EBV-transformed lymphoblastic cell lines; LMP2A, latent membrane protein 2A; NA, not applicable; NCI, National Cancer Institute; NIH, National Institutes of Health; NPC, nasopharyngeal carcinoma; NR, no response; PBMC, peripheral blood mononuclear cells; PR, partial response; PTL, post-transplant lymphoma; SOT, solid organ transplantation; TCD, T-cell depletion, TCR, T-cell receptor.

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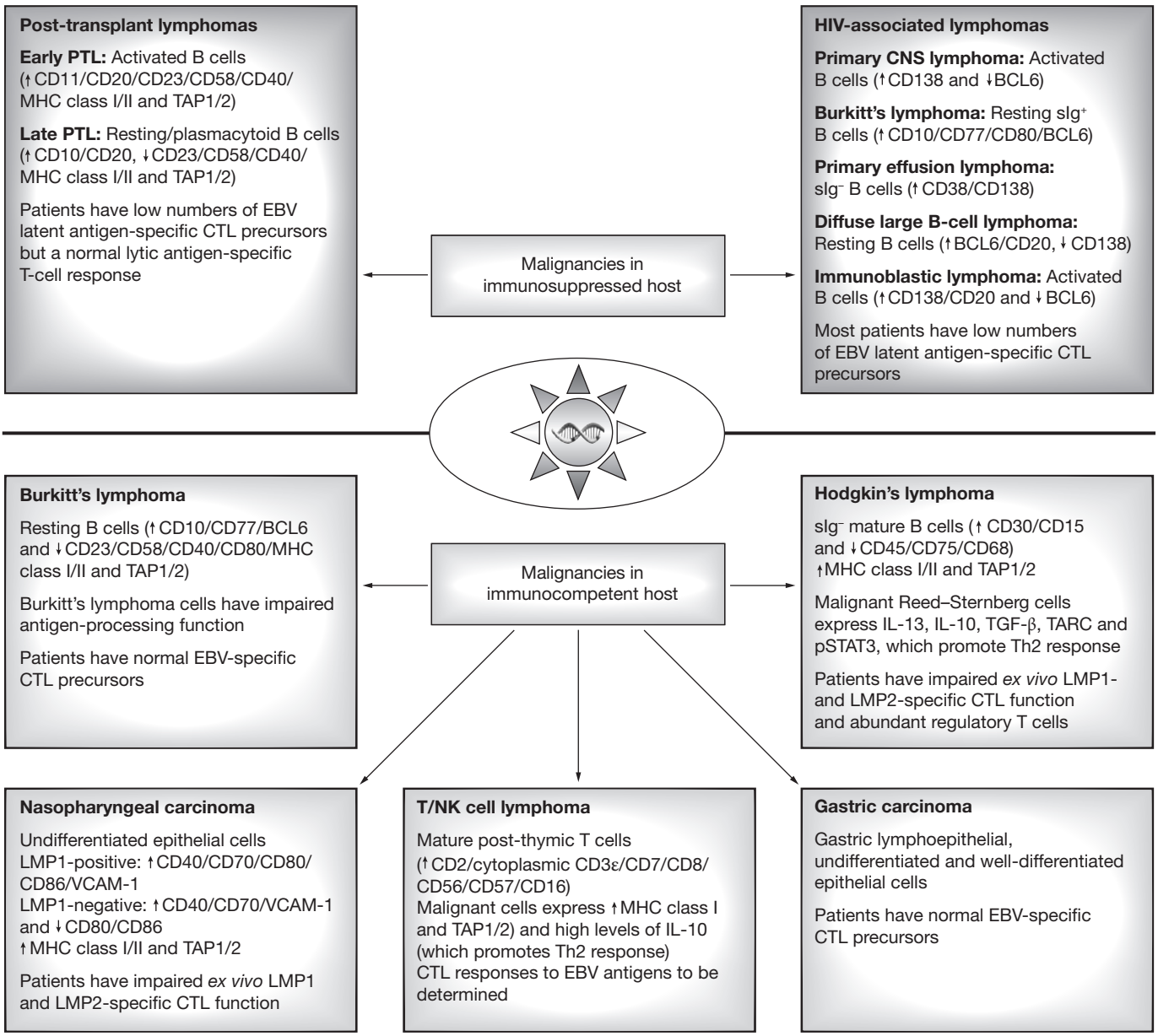

Figure 2 Cellular and immunologic phenotype of Epstein–Barr virus-associated malignancies in immunocompetent and immunocompromised individuals.[21] BCL, B-cell lymphoma; CNS, central nervous system; CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; IL-10 and IL-13, interleukins 10 and 13; LMP, latent membrane protein; pSTAT3, phosphorylated signal transducer and activator of transcription 3; PTL, post-transplant lymphoma; sIg, surface immunoglobulins;TAP1 and 2, transporters 1 and 2; TARC, thymus and activation-regulated chemokine; TGF-β, transforming growth factor-beta; Th2, T-helper cell 2; T/NK lymphomas,T-cell and natural killer lymphomas; VCAM, vascular cell adhesion molecule.

developing countries. These data have raised the possibility of combining immunotherapy with chemo/radiotherapy, particularly in advanced disease, to improve survival. The potential tolerability of this form of therapy in terms of its modest treatment-related toxicity compared with more conventional treatments is appealing. Histologic examination of most of these malignancies shows a heavy lymphoid infiltrate, which suggests that these malignant cells might be immunoactive. The commonly held view is, however, that this infiltrate might exert an immunosuppressive effect.[9,10] To circumvent this, attempts have been made to expand EBV-specific T-cell immunity *in vitro* in the absence of an immunosuppressive environment, and

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Table 2 Limitations in the treatment of Epstein–Barr virus-associated malignancies and potential strategies to overcome these limitations.

Limitations	Potential strategies to reverse limitations
Viral gene expression restricted to poorly immunogenic proteins	Targeting poorly immunogenic proteins to professional APC to enhance quality and quantity of antigen-specific T cells *In vivo* stimulation of antigen-specific T-cell responses using recombinant viral vectors encoding either full-length antigens or immuno-reactive determinants as a 'string-of-beads' (prophylactic or therapeutic) Drug-induced demethylation of EBV genome to induce expression of highly immunogenic latent and lytic proteins Specific targeting of EBNA1 for proteasomal degradation
Tumor cell phenotype, which limits T-cell control	Treatment with cytokines/chemokines, which induces differentiation of malignant cells
High tumor load	Surgical or chemo/radiotherapy de-bulking
Loss of antigen-processing gene expression	Treatment with CD40L to induce expression of antigen-processing genes Drug-induced demethylation of EBV genome to induce expression of LMP1, which reverses the down-regulated expression of antigen-processing genes
EBV-specific T cells: (i) Insufficient precursors (ii) Lag time between diagnosis and T-cell expansion (iii) Lack of function (anergy/tolerance) and specificity	*In vitro* stimulation, expansion and adoptive transfer of autologous latent antigen-specific T cells (prophylactic or therapeutic) Adoptive transfer of haplo-identical EBV-specific T cells from EBV CTL bank Active immunization with recombinant viral vectors encoding either full-length antigens or immuno-reactive determinants as a 'string-of-beads' (prophylactic or therapeutic) *In vitro* and *in vivo* targeted stimulation of T cells using autologous dendritic cells to enhance T-cell precursors specific for EBV antigens expressed in malignant cells (prophylactic or therapeutic) Directing T cell to the tumor site Adoptive immunotherapy with EBV-specific T cells following anti-CD45 mediated cytoreduction Redirecting T cells with recombinant antigen-specific T-cell receptors using retroviral transduction Reverse tumor-induced tolerance through the expression of dominant negative form of STAT3 and/or A20
Compromised immune status of the patient	Reduce immunosuppressive drug therapy Reconstitute immunity by anti-retroviral therapy
Presence of immunosuppressive microenvironment: (i) Cytokine mediated (ii) Regulatory T-cell mediated	Co-delivery of recombinant viral vectors encoding either full-length antigens or immuno-reactive determinants as a 'string-of-beads' with Th1 promoting cytokines (e.g. IL-15) (prophylactic or therapeutic) Transduction of EBV-specific CTLs with dominant negative TGF-β receptor to block immunosuppressive effects of this cytokine Depletion of regulatory T cells by treating patients with anti-CD25

APC, Adenomatous polyposis coli, CTL, cytotoxic T lymphocyte; EBNA1, EBV-associated nuclear antigen 1; EBV, Epstein–Barr virus; IL-15, interleukin-15; LMP1, latent membrane protein 1; STAT3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor-beta; Th1, T-helper cell 1.

to transfer the resulting expanded T cells for the treatment of high-risk patients[11–21] (Table 1). Indeed, despite potential immunosuppression generated by the tumor, *in vivo* generation of EBV-specific CTLs has been achieved successfully in patients with advanced NPC, following immunization with autologous peptide-pulsed dendritic cells.[22] This form of therapy has been particularly successful in the treatment of early PTL, which is characterized by the expression of a full complement of EBV latent genes, an activated B-cell phenotype with high levels of expression of antigen-processing genes, and a proven susceptibility to EBV-specific CTLs (Figures 1 and 2). The direct application of this technology to other EBV-associated malignancies will need modifications to overcome the significant barriers—as outlined below (Table 2).

Each EBV-associated malignancy is characterized by a unique cellular phenotype, which is to some extent influenced by the pattern of EBV gene expression within the malignant cell.[23] The full complement of latent EBV gene expression in early PTL is coincident with an activated cell phenotype and renders this malignancy an ideal target for T-cell-based immunotherapy. Reduced EBV gene expression (i.e. *EBNA1* only), and/or a consistent loss of antigen-processing function through the MHC class I pathway, severely restricts the potential use of CTL-based

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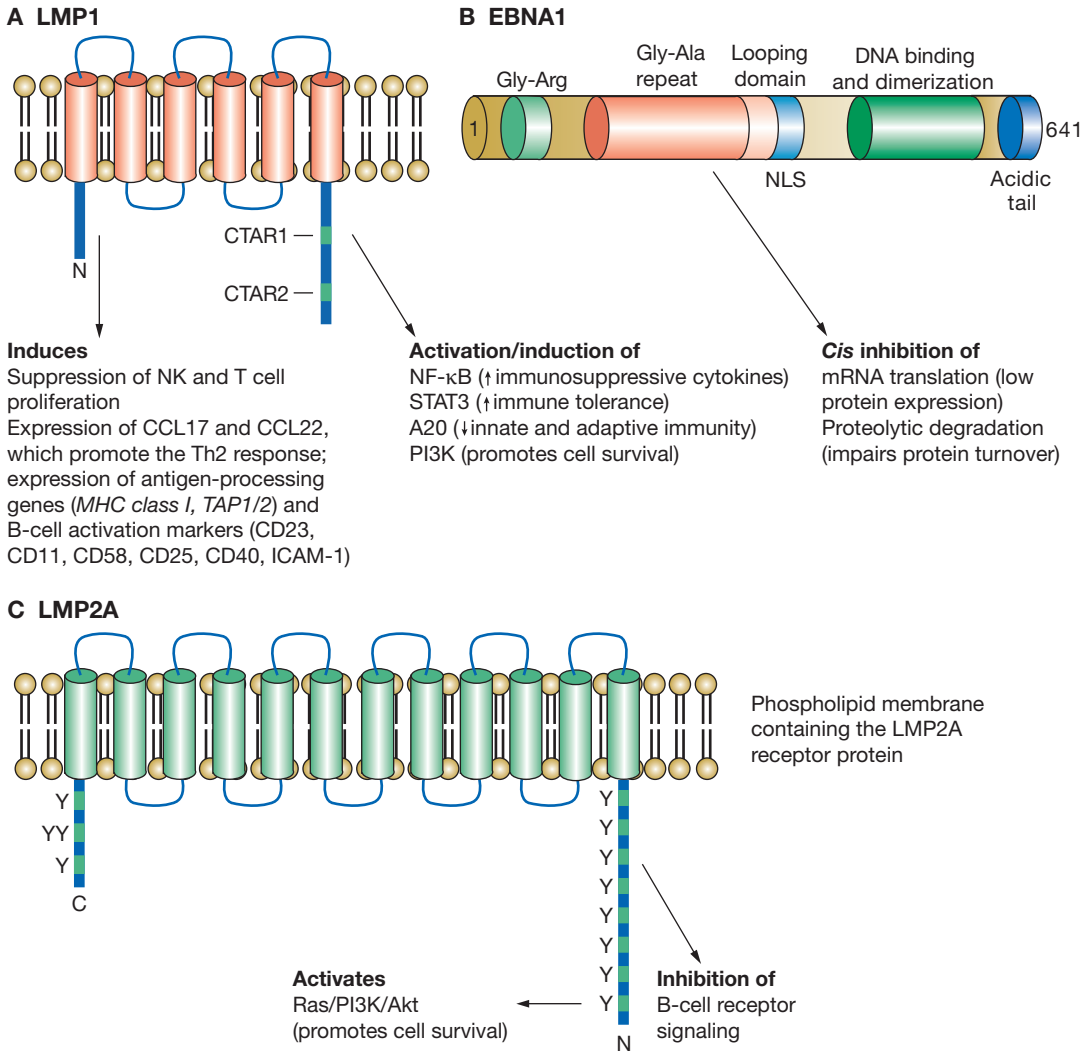

Figure 3 Immunomodulatory effects of Epstein–Barr virus-encoded latent proteins expressed in Epstein–Barr virus-associated malignancies. **(A)** The LMP1 transmembrane oncogenic protein contains three major domains: first, an amino-terminal cytoplasmic tail, which binds LMP1 to the plasma membrane; second, six hydrophobic transmembrane domains, which are involved in self-aggregation and oligomerization; third, a long carboxy-terminal cytoplasmic region, which contains the signaling activity of the protein. Two distinct functional domains referred to as C-terminal activation regions 1 and 2 (CTAR1 and CTAR2) have been identified on the basis of their ability to activate NF-κB, STAT3, PI3K and induce expression of the A20 protein. The activation of these transcription factors and other mediators has direct impact on the innate and adaptive immunity. **(B)** The Gly-Arg-rich domain has been shown to mediate homotypic interactions at a distance between DNA-bound EBNA1 molecules, as well as heterotypic interactions with cellular proteins. The Gly-Ala repeat domain is known to block mRNA transcription and proteasomal degradation of EBNA1. The core domain contains an eight-stranded antiparallel β-barrel, which forms the DNA binding and dimerization interface, and two α-helices per monomer. The extreme carboxyl terminus of EBNA1 is highly acidic. This acidic tail has been reported to have roles both in transactivation and in oriP plasmid maintenance. **(C)** The LMP2A protein includes 12 transmembrane domains, a 27-amino-acid cytoplasmic carboxyl terminus and a 119-amino-acid cytoplasmic amino-terminal domain that contains 8 tyrosine residues, 2 of which (Tyr74 and Tyr85) form an immunoreceptor tyrosine-based activation motif (ITAM). The LMP2A ITAM blocks signaling from the B-cell receptor. LMP2A also induces activation of various kinases including Ras, Akt and PI3K, which promote cell survival. CCL 17 and 22, chemokines 17 and 22; CTAR1 and CTAR2, C-terminal activation regions 1 and 2; EBNA 1, EBV-associated nuclear antigens 1; EBV, Epstein–Barr virus; Gly-Ala, glycine-alanine; Gly-Arg, glycine-arginine; LMP1 and 2A, latent membrane proteins 1 and 2A; mRNA, messenger RNA; NF-κB, nuclear factor-kappa B; NK, natural killer; NLS, nuclear localization signal; PI3K, phosphatidylinositol 3-kinase; STAT3, signal transducer and activator of transcription 3; TAP1 and 2, transporters 1 and 2; Th2, T-helper cell 2.

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GLOSSARY

EBNA1
A protein that regulates the replication and segregation of the viral episomes in latently-infected cells and transactivates the expression of other EBV latent proteins

ADOPTIVE IMMUNOTHERAPY
Ex vivo activation of antigen-specific T cells that circumvent host immune-suppressive mechanisms, allowing expansion of antigen-specific cytotoxic T lymphocytes

immunotherapeutic strategies. Examples of such malignancies include Burkitt's lymphoma, HIV-associated lymphomas and late PTLs (Figure 2). Although loss of expression of immunodominant viral antigens (the EBNA3 proteins) in HL, NPC, T-cell and natural-killer-cell lymphoma and gastric carcinoma limits potential viral targets, recent studies have suggested that LMP1 and LMP2 can be efficiently targeted for immune-based therapies.[16,22] *Ex vivo* profiling of EBV-specific T-cell responses, however, revealed that patients with HL and NPC might have impaired LMP1 and LMP2-specific T-cell function at the time of diagnosis, which rebounds to normal levels following tumor regression (M Gandhi and R Khanna, unpublished data). Although the precise mechanism for the loss of this T-cell function is unknown, there is some indication that the expansion of tumor cells is coincident with the emergence of immunoregulatory T cells that express high levels of CTLA4 (M Gandhi and R Khanna, unpublished data). Furthermore, *in vivo* analysis of tumor biopsies shows that malignant cells in HL and NPC express immunosuppressive cytokines, for example interleukins IL-10 and IL-13, transforming growth factor-beta (TGF-β) and thymus and activation-regulated chemokine, which might be induced by the activation of certain transcriptional factors, such as the signal transducer and activator of transcription STAT3.[24]

Apart from using restricted viral gene expression, immune-based therapies are also constrained by the immunomodulatory effects of certain EBV proteins expressed in malignant cells. These EBV proteins act at the level of antigen processing and presentation, cytokine regulation, regulation of adhesion molecule expression, and T-cell recognition[25–27] (Figure 3). A classic example is the modulation of innate and adaptive immunity by LMP1. Previous studies have shown that this protein can activate various transcription factors including nuclear factor-kappa B and STAT3, which can induce expression of immunosuppressive cytokines or cause immune tolerance.[28,29] Furthermore, LMP1 induces the expression of the DNA-binding zinc finger protein A20, which has recently been shown to inhibit inflammatory responses.[30] Similarly, LMP2 is known to inhibit B-cell receptor signaling and to promote tumor cell survival.[31,32] The glycine-alanine amino acid repeat within EBNA1 stabilizes the mature protein by preventing its degradation by the proteasome; this is in contrast to its previously assumed role as an immune evasion domain.[33,34] This repeat also acts as a *cis* inhibitor of mRNA translation and thus limits the availability of EBNA1 for immune recognition.[35]

MODIFYING THE TUMOR MICROENVIRONMENT TO ENHANCE IMMUNE-BASED THERAPIES

It is now well established that tumor growth is critically dependent on a microenvironment that is resistant to immune control.[36] There have been a number of attempts to modify the tumor microenvironment to promote immune recognition. These attempts include the use of pharmacological and/or gene therapy constructs designed to express either cytotoxic or inhibitory proteins selectively in tumor cells. Examples to date include treatment with the CD40 ligand to induce the expression of antigen-processing genes,[37] drug-induced demethylation of the EBV genome in order to reverse expression of viral latent proteins,[38] transduction of dominant negative forms of STAT3 and/or A20,[28,29] and treatment with cytokines/chemokines, which induce differentiation of malignant cells (Table 2). Another approach is based on specific targeting of LMP1 and EBNA1 using N-end rule targeting (i.e. the *in vivo* half-life of a protein that relates to its N-terminal residue) or E3 ubiquitin-protein ligases to direct degradation of these proteins.[39–41]

EMERGING CELL-BASED THERAPIES FOR TREATMENT OF EBV-ASSOCIATED MALIGNANCIES

The effectiveness of T-cell-based immunotherapy for the prevention and treatment of a range of EBV-associated malignancies necessitates its wider application, perhaps in combination with chemotherapy and monoclonal antibody therapies. ADOPTIVE IMMUNOTHERAPY involves the *ex vivo* activation of EBV-specific T cells, thus circumventing *in vivo* host immune-suppressive mechanisms, to permit the expansion of large numbers of virus-specific CTLs for administration to the autologous host. A major constraint of the wider application of autologous CTL-based therapy is the delay between diagnosis and the preparation of a therapeutic dose of T cells (Table 2). To overcome this limitation, banks

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of cryopreserved, haploidentical EBV-specific CTLs derived from healthy seropositive subjects can be established.[42] This strategy has proven clinical efficacy[13] (Figure 4) and offers a distinct logistic advantage of speed of access and ease of generation that might permit its wide-scale use in treating both PTL and other malignancies, such as HL and NPC. This may be particularly relevant for developing countries where T cells could be transported to remote locations in which access to standard chemo/radiotherapy is restricted. Most importantly, infusion of haploidentical allogeneic CTL[15,17] is associated with low rates of graft-versus-host disease, suggesting that prolonged *in vitro* culture diminishes alloreactivity.

Another constraint for the widespread use of autologous or allogeneic CTLs, especially where latent EBV gene expression is limited, is the antigen specificity. Recent studies indicate the potential use of recombinant viral vectors expressing individual latent antigens,[43,44] or T-cell epitopes linked as a POLYEPITOPE[45] in combination with autologous dendritic cells, to expand T cells of the relevant specificity (Figure 5). Indeed, a clinical study aimed at expanding LMP2A-specific CTLs using autologous dendritic cells infected with a recombinant adenovirus encoding this antigen has been initiated for the treatment of relapsed HL (Table 1).

Another significant challenge is presented by the *in vivo* efficacy of adoptively transferred CTLs in the remit of tumor-induced immune evasion strategies. This barrier might be reversed by the transduction of EBV-specific CTLs with a dominant negative TGF-β receptor[46] or depletion of regulatory T cells by treating patients with anti-CD25 (Table 2 and Figure 4). Alternatively, the blockade of tumor and microenvironment-secreted cytokines such as IL-10, IL-13 and TGF-β might be an effective approach to the enhancement of anti-tumor immunity.[47] It has also been proposed that anti-CD45-mediated cytoreduction prior to adoptive immunotherapy can facilitate rapid expansion of EBV-specific CTLs[48] (Table 2 and Figure 5).

Much of the optimism about T-cell-based therapies is because of the measurement of surrogate markers, which are regulated in response to a reduction of viral load and/or reconstitution of EBV-specific T-cell immunity. An objective analysis of disease outcome is, however, less impressive, highlighting the need to explore alternative strategies for circumventing tumor-evasion mechanisms that preclude the development of a robust anti-tumor response (Table 2). Recent data from large patient cohort studies of EBV-associated malignancies provide ample evidence that nontargeted intensive chemotherapy is associated with short-term and long-term adverse effects[7], and that responses to monoclonal antibody therapy are frequently not sustained,[49] most likely owing to the selection of tumor escape mutants. Triple combination therapy comprising chemo-radiotherapy, monoclonal antibodies and T-cell adoptive immunotherapy, aims to augment efficacy through the synergistic actions of multi-modality agents, while reducing treatment-related toxicities and the risk of tumor escape. Low-intensity chemotherapy not only debulks the tumor but potentially improves the host's-immune environment by eliminating regulatory T cells[50] and providing space within the lymphocyte compartment to facilitate the reconstitution of virus-specific T cells.[51] Triple combination therapy for EBV-associated malignancies could serve as a new paradigm for the successful multi-modality treatment of malignancies.

GLOSSARY

POLYEPITOPE
Multiple DNA-encoded epitopes linked without their flanking regions in a synthetic construct, which retain broad-spectrum immunogenicity

EMERGING VACCINE STRATEGIES FOR TREATMENT OF EBV-ASSOCIATED MALIGNANCIES

One of the most important outcomes of adoptive immunotherapy has been the demonstration that $CD8^+$ EBV-specific CTLs alone can provide therapeutic benefit by reversing the growth of EBV-associated malignancies (Table 1). As the success of these therapies becomes established there will be an increasing demand for their application in general clinical practice; however, the requirement for specialized facilities, as well as regulatory constraints for these therapies, is likely to preclude their widespread use, particularly in developing countries, unless new co-operative approaches be adopted. One option is national or international tissue banks of cryopreserved GMP grade T-cell lines, perhaps administered along the lines of current international registries such as those used in the field of bone marrow transplantation. Reconstitution of $CD8^+$ T-cell immunity using low-cost prophylactic/therapeutic vaccine formulations incorporating immunogenic determinants are likely to overcome these limitations. Indeed, preclinical studies based on recombinant viral vectors or plasmid DNA

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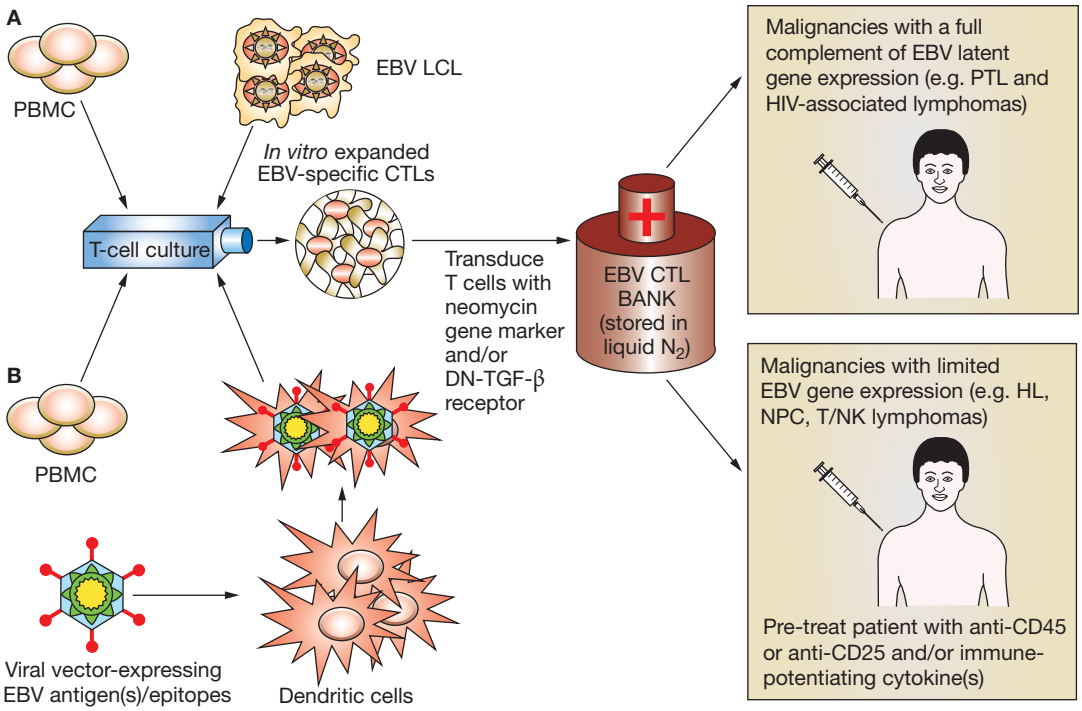

Figure 4 T-cell-based adoptive immunotherapeutics for Epstein–Barr virus-associated malignancies. Two broad strategies are currently being explored for the expansion of EBV-specific CTLs. **(A)** PBMC from the patient or from a healthy seropositive haploidentical donor are stimulated with autologous EBV-transformed LCLs for 3–4 weeks in the presence of recombinant interleukin-2 (IL-2). **(B)** Alternatively, PBMC from the patient or from a healthy seropositive haploidentical donor are stimulated with dendritic cells transduced with recombinant viral vectors encoding either full-length EBV protein or CTL epitopes linked together as a polyepitope. Following stimulation, these expanded T cells are assessed for antigen specificity and allospecific reactivity. CTLs generated from approach A or B can be either immediately transferred into the patient or stored as part of an EBV CTL bank for future adoptive immunotherapy. The establishment of T-cell banks from haploidentical healthy donors allows rapid access to the T cells for the treatment of EBV-associated malignancies. These CTLs can be used to treat malignancies with a full complement of EBV latent proteins (e.g. PTL and HIV-associated lymphomas). In the case of HL, NPC and T/NK lymphomas, however, the *in vivo* efficacy of these effectors cells can be enhanced significantly by transduction with dominant negative TGF-β receptor or depletion of regulatory T cells by treating patients with anti-CD25 and anti-CD45 mediated cytoreduction prior to adoptive immunotherapy. For *in vivo* tracking, the effector cells can be transduced with a neomycin gene marker. CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; HL, Hodgkin's lymphoma; LCL, lymphoblastoid cell lines; NPC, nasopharyngeal carcinoma; PBMC, peripheral blood mononuclear cells; PTL, post-transplant lymphoma; T/NK lymphomas, T-cell and natural killer lymphomas.

expressing either full-length EBV antigens or CTL epitopes linked together as a poly-epitope, have shown encouraging results[44,45,52] (Figure 5). Another technology currently under development is scrambled antigen vaccines (SAVINE). This technology is designed to incorporate each of the relevant EBV proteins encoded as overlapping peptides, rearranged and rejoined in such a way that potential CTL epitopes are retained[53] for safer vaccine delivery. As in the case of adoptive immunotherapy, this vaccination strategy will need to counter tumor-induced immune evasion strategies, such as the expression of immunosuppressive cytokines, the presence of regulatory T cells and/or loss of antigen-processing function. Co-delivery of immune-potentiating cytokines and specific targeting of recombinant antigens through the MHC class I/II pathways is likely to enhance the efficacy of these vaccines.[54] These formulations could be delivered either directly or following *in vitro* transduction of autologous dendritic cells (Figure 5), alone or as a component of combination therapy.

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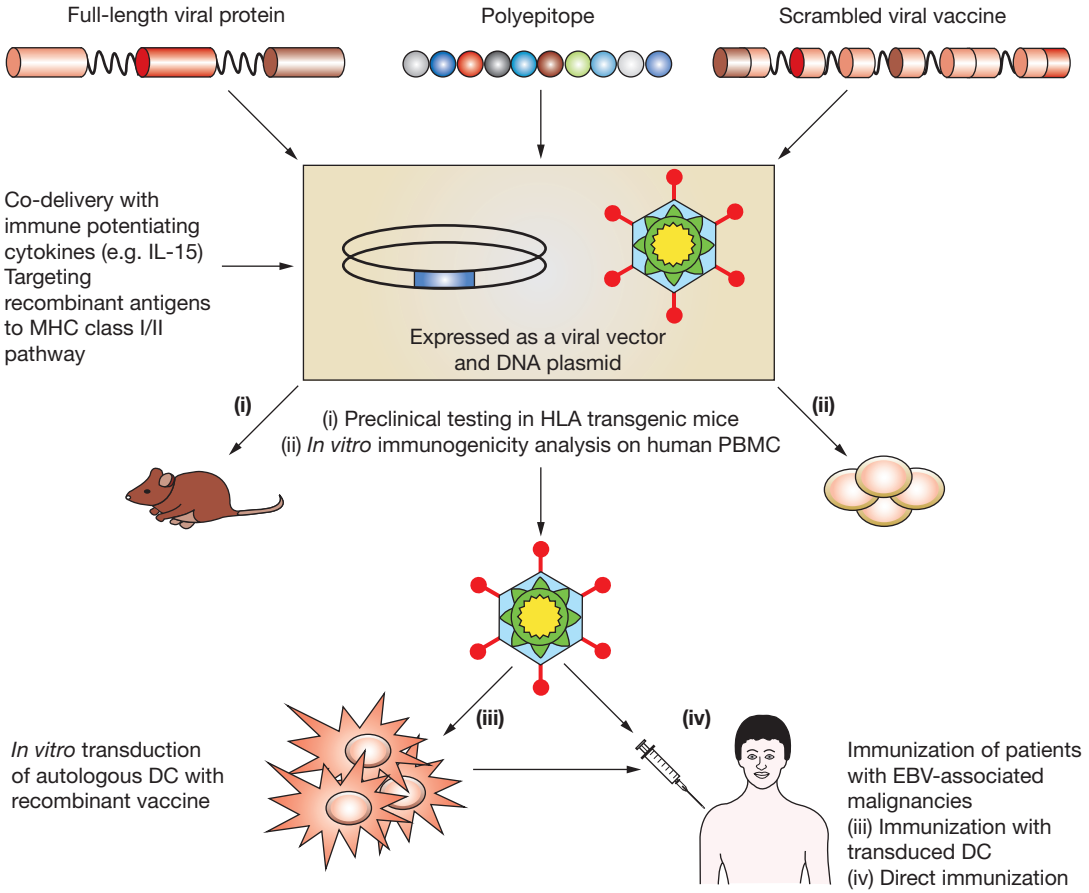

Figure 5 Emerging prophylactic/therapeutic vaccine strategies for EBV-associated malignancies. Three different vaccine formulation strategies based on recombinant viral vectors or plasmid DNA expressing full-length EBV antigens, CTL epitopes linked together as a polyepitope, or scrambled antigen vaccines, are currently being tested for the treatment of EBV-associated malignancies. Each of these vaccine strategies is designed to incorporate relevant EBV proteins in such a way that they induce a strong antigen-specific CTL response. **(i)** Preclinical testing and **(ii)** *in vitro* immunogenicity analysis has demonstrated the potential of these approaches to induce EBV-specific CTL responses. To counter tumor-induced immune evasion strategies such as the expression of immunosuppressive cytokines, the presence of regulatory T cells and/or loss of antigen processing function, co-delivery of immune-potentiating cytokines and specific targeting of recombinant antigens through the MHC class I/II pathways may be required to enhance the efficacy of these vaccines. These formulations could be delivered either indirectly **(iii)** following *in vitro* transduction of autologous DCs, either alone or as a component of combination therapy as discussed in the text, or directly **(iv)** into the patient. CTL, cytotoxic T lymphocyte; DC, dendritic cells; EBV, Epstein–Barr virus; IL-15, interleukin-15; PBMC, peripheral blood mononuclear cells.

CONCLUSION

Despite major clinical and scientific advances within the discipline, the aim of routinely supplementing pharmacologic strategies with immune cell-based therapies remains an elusive goal. As scientific advances are made, so new challenges for laboratories developing novel therapies emerge. EBV-associated malignancies could serve as a new prototype for the successful multi-modality treatment of malignancies (T-cell immunotherapy in combination with monoclonal antibodies and chemotherapy). Among the key challenges facing researchers attempting to pioneer such strategies are the regulatory issues involved in immunotherapy. Too often, regulatory authorities apply restrictive criteria based on a model of product development that is better suited to the drug industry than to the academic research institute (i.e. a patented commercial product to be manufactured by a company). For the future benefit of patients with EBV-associated malignancies, it is vital that there is regulatory understanding so that there can be continued academic-led interventional research into this evolving field.

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Competing interests
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