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Therapeutic Approaches to Epstein-Barr Virus Cancers

Samantha S. Soldan,
Troy E. Messick,
Paul M. Lieberman

The Wistar Institute, Philadelphia, PA 19104

Summary

Epstein–Barr virus (EBV) establishes a lifelong latent infection that can be a causal agent for a diverse spectrum of cancers and autoimmune disease. A complex and dynamic viral lifecycle evades eradication by the host immune system and confounds antiviral therapeutic strategies. To date, there are no clinically approved vaccines or therapies that selectively target EBV as the underlying cause of EBV-associated disease. Here, we review the challenges and recent advances in the development of EBV-specific therapeutics for treatment of EBV-associated cancers.

Epstein–Barr virus prevalence, lifecycle, and disease burden

Epstein–Barr virus (EBV) is a human gamma-herpesvirus that establishes lifelong latency in over 95% of the adult population [1]. EBV is also an etiological agent for ~2% of all human cancers, most notably endemic Burkitt's lymphoma, 50% of Hodgkin's lymphoma (HL), ~10% of gastric carcinomas (GC), and undifferentiated nasopharyngeal carcinomas (NPC) [2,3]. In addition, EBV is a triggering factor in autoimmune disorders, especially multiple sclerosis (MS) [4,5]. EBV has a complex lifecycle that enables high-transmissibility and lifelong persistence [1]. These attributes, combined with geographical, socioeconomic, and genetic variations in virus and host, contribute to the challenges of developing successful and comprehensive EBV-selective therapeutics.

The complex EBV lifecycle presents many challenges to therapeutic strategies. After primary infection in the oropharynx, the virus enters and replicates in oropharyngeal epithelial cells and infects local infiltrating B-lymphocytes. EBV infection of resting B-cells through CD21 receptor leads to a germinal center-like hyperproliferation followed by the emergence of slower cycling and long-lived memory B-cells [1,6]. During latency, EBV can adopt different gene-expression programs that are associated with the different infected cell and tumor types [7]. EBV latent genes drive B-cell proliferation and persistence in reservoirs of long-lived memory B-cells that evade host immune elimination. Viral reactivation can occur through several pathways, including the terminal differentiation of latently infected memory B-cells. Numerous viral genes have been implicated in EBV-disease pathogenesis, including those associated with latent and lytic cycle, and it remains unclear which viral

Conflict of interest statement

The authors PML and TEM hold patents on EBNA1 inhibitors that are in clinical trial with potential for royalty payments. PML is a founder and advisor of Vironika, LLC.

genes provide the best targets for therapeutic intervention [8,9]. Here, we review recent efforts in vaccines, cellular–immune-based therapies, and small molecules to treat EBV-associated cancers.

Epstein–Barr virus therapeutics

Vaccines

Several vaccine strategies to prevent EBV infection or treat EBV-associated disease are under investigation (Table 1). While vaccines to human herpesvirus 3 (Varicella-Zoster virus (VZV)) have demonstrated remarkable efficacy in preventing disease from primary childhood infection and late-life reactivation, this success has not yet been repeated for other members of the herpesvirus family [10–12]. Attenuated live virus has been used successfully as the basis for other herpes-viruses vaccines, including Marek's disease virus and VZV, but this strategy is technically and clinically challenging for EBV because of the relatively low yield and potential residual oncogenic activity of an attenuated virus. Consequently, better-defined viral-like particles (VLPs) and non-EBV viral vectors have received more attention for EBV vaccines. EBV-derived VLPs, including EBV mutants with deletions in oncogenic genes or DNA-packaging genes [13], have been produced by inducing cell lines to enter the lytic phase and subsequently purifying VLPs from cell supernatants. Several of these EBV VLPs also contain deletions in key lytic genes (BFLF1/BFRF1, BBRF1, BFLF2, and terminal repeats) to prevent virus replication and packaging to address safety concerns [13]. Recently, more immunogenic EBV VLPs were generated by fusing latent antigens (e.g. Epstein-Barr Nuclear Antigen 1 (EBNA1) and EBNA3C) to the tegument protein BNRF1 to stimulate a greater CD4+ T-cell response [14]. As an alternative to EBV-based VLPs, a New Castle disease virus VLP platform to generate an EBV-specific immune response was developed. NDP–VLP-based vaccines, including the EBV membrane antigen BLLF1 (gp350) ectodomain in combination with additional envelope proteins and latent antigens (e.g. gH/gL-EBNA1 and gB-LMP2), generate high titers of neutralizing antibodies and EBV-specific T-cell responses in mouse models, representing a safe and rapid method of producing VLPs for EBV vaccines [15].

Recombinant protein vaccination studies have focused mostly on gp350 to prevent EBV infection. Early studies showed that purified gp350 elicited sufficient immunity to protect against EBV-induced malignant lymphomas [16]. Further work demonstrated that a multivalent, tetrameric gp350 created by fusing two gp350 proteins to a C-terminal leucine zipper with or without specific T-cell epitopes, enhanced neutralizing antibody responses in rabbits [17]. In addition, gp350 nanoparticles that induce high neutralizing antibody titers and provide protection against EBV infection in cynomolgus macaques have been produced [18]. Other studies have used other EBV glycoproteins and latent proteins, including gH/gL and gp42, as immunogens to elicit high neutralizing antibody titers. Ferritin-based nanoparticles containing fusion-apparatus component gH/gL or gH/ gL/gp42 were found to provide protection against EBV infection of B-cells and epithelial cells in culture [19]; a clinical trial with ferritin–gp350 is underway (Table 1) [10].

Most recently, nucleic-acid-based vaccines have come to the fore with the rapid development, success, and widespread use of mRNA vaccines for SARS-CoV-2 [20,21].

DNA vaccines using three EBV-latency genes (EBNA1, Latent membrane protein 1 (LMP1), and LMP2A) or a combined EBV–LMP2A–CD40L plasmid have been tested [22,23]. While LMP1 vaccination was antigenically weak and did not provide robust T-cell immunity, EBNA1 and LMP2A DNA-based vaccines were highly immunogenic, induced dominant CD8+ T-cell responses, and prevented tumor growth [22,24]. Based on the recent success of mRNA vaccines for SARS-Cov2, an EBV-targeted mRNA vaccine encoding the major EBV glycoproteins (gp350, gB, gH/gL, and gp42) is being tested for safety and reactogenicity among healthy participants ages of 18–30 (Table 1).

Cellular–immune-therapy approaches

Cellular immunotherapies are in development for the treatment of EBV-associated cancers and autoimmune disorders. Cellular–immune therapy may be achieved by delivering antigen-presenting cells loaded with EBV tumor antigens or by vaccination. Alternatively, cytotoxic cells (cytotoxic T-cell lines (CTLs) or natural killer (NK) cells), from adoptive transfer of the patient's cells or from an Human Leukocyte Antigens (HLA)-matched donor that are primed and expanded to target and kill cancer cells or, in the case of multiple sclerosis, latently infected EBV-positive B-cells, are also under investigation [25,26]. Finally, EBV-specific allogeneic T-cells/ chimeric antigen-receptor T-cells (CAR T-cells) genetically engineered to produce an artificial TCR for use in immunotherapy are under development with EBV antigen (EBNA3C, LMP1, and gp350)-specific chimeric antigen receptors and showed good efficacy in preclinical models [27–30] (Table 2).

Donor-derived EBV-specific CTLs have proven successful in the treatment of post-transplantation lymphoproliferative disorders, with low rates of graft-versus-host disease [31–33]. In addition, the adoptive transfer of CTLs has been used for various EBV-associated cancers and, especially lymphomas [34]. In these studies, a more restricted group of EBV antigens is expressed and CTLs specific for EBNA1, LMP1, and LMP2 have shown clinical efficacy in EBV-associated lymphomas and NPC [35–37]. For patients with advanced EBV-positive NPC, EBV-specific CTLs were administered in combination with chemotherapy (gemcitabine and carboplatin) with promising response and survival rates and some patients experiencing stable disease and reduced tumor growth [38]. In another Phase-I/II clinical trial, EBV-specific CTL therapy, generated using the patients' own EBV (B95–8)-transformed LCLs to generate and expand CTLs, was used alone in 21 patients with recurrent, metastatic NPC [39]. Although the overall response rate was low, a subset of patients demonstrated robust responses to chemotherapy regimens that they had previously failed, suggesting that the enhancement of the immune response by EBV-specific CTLs had a broad effect that restored the patients' response to conventional chemotherapy [39].

Checkpoint inhibitors

Most cancer types, including EBV cancers, are known to upregulate immune-checkpoint ligands, especially PD-L1, making them susceptible to immune-checkpoint blockade, either alone or in combination with other antiviral treatment modalities [40–43]. Immune-checkpoint therapy is under investigation for the treatment of EBVaGC [44]. PD-L1 expression is frequently observed in EBVaGC by immunohistochemistry [45,46] and

EBVaGC is responsive to the antiPD-L1 antibody avelumab [47]. In addition, a Phase-II trial of pembrolizumab (antiPD-1 antibody) monotherapy for metastatic gastric carcinoma demonstrated that a EBVaGC was more responsive (100%) than EBV-negative gastric cancers (50–85.7%).

Based on the prognostic value of PD-L1 in NPC, the PD-1/PD-L1 checkpoint has been extensively investigated in NPC. More than 70% of NPC patients present with advanced disease at diagnosis and chemoradiotherapy has limited efficacy for advanced metastatic disease [48]. Higher levels of tumor-infiltrating lymphocytes have been described in EBV-positive NPC compared with EBV-negative NPCs. Accordingly, CD8+ cells within EBV-positive NPC tumors are associated with higher expression of PD-L1 (found in 50–80% of NPC tissues and preclinical models), CD68+ tumor-associated macrophages and Forkhead box P-3+ Tregs, T-cell immunoglobulin mucin-3, and lymphocyte-activating 3 [49] and CD4+ cells with CTLA4 [50]. Combinatorial approaches using antibodies targeting the PD-1/PD-L1 checkpoint with chemotherapeutic agents, including cisplatin, may improve outcomes by enhancing tumor recognition and further decreasing the immunosuppressive milieu of the tumor microenvironment [51,52]. Clinical trials determining the combined inhibition of PD-1 with CTLA4, DKY709 (an immunomodulatory agent that targets Tregs), and Tabelecleucel (an EBV-specific CTL therapy generated from healthy donors) are underway [41].

Immune-checkpoint inhibitors are also of interest for their therapeutic potential in EBV-associated lymphomas. EBV LMP1 and LMP2 upregulate PD-L1 expression in a subset of classic HL patients, making these lymphomas sensitive to PD-1 blockade [53]. HL patients have been demonstrated to be responsive to nivolumab (PD-1 inhibitor) and pembrolizumab (PD-L1 inhibitor) [54,55] and, consequently, both drugs were given accelerated FDA approval for the treatment of relapsed/refractory HL. However, PD-L1 is upregulated in both EBV-negative and EBV-positive tumors, as well in the surrounding lymphocytes [56]. Therefore, it has yet to be determined whether the high sensitivity of EBV-positive HL to nivolumab and pembrolizumab is specifically counteracting PD-L1 induction by EBV LMP1/2 in these tumors. High RNA expression of PD-1 and PD-L2 has been demonstrated in Primary Central Nervous System Lymphoma (PCNSL) brain specimens and this high expression is associated with a poor prognosis [57]. AntiPD-1 antibodies were tested in a pre-clinical model of CNS lymphoma using murine lymphoma cells that express PD-L1 [58]. Although immune-checkpoint inhibitors have not been rigorously investigated in EBV+ PCNSL, anecdotal reports have shown promise and the available data support the further development of PD-1/PD-L1 inhibitors against EBV-positive PCNSL in the CNS [59].

Small-molecule approaches (viral targets)

Small molecules have been highly successful for targeted treatment of a few viruses, notably Human Immunodeficiency Virus (HIV), Hepatitis C virus (HCV), Herpes Simplex Virus (HSV), and most recently SARS-CoV-2. To date, there are no FDA-approved small molecules that selectively target EBV and those used to treat EBV-associated cancers have no known selectivity for the virus. Most EBV-associated disease is treated agnostically

with regard to EBV status. However, EBV-positive tumors provide several unique viral targets and there are several EBV-specific treatment strategies under investigation with some innovative therapeutic approaches entering clinical trials (Table 3).

BXLF1 and BGLF4 (viral-encoded kinases)

EBV-positive tumor cells express a limited number of EBV-encoded latent genes with relatively few tumor cells expressing lytic genes. One approach is to induce the lytic cycle and viral proteins that are susceptible to small-molecule inhibition [60]. This ‘kick and kill’ strategy pushes the virus into lytic replication, rendering EBV vulnerable to inhibitors of lytic enzymes, especially viral kinases and DNA polymerases [61–64]. For EBV cancers, incorporation of nucleoside-chain terminators has the added benefit of inhibiting cellular DNA replication, providing viral-specific antitumor activity.

Histone deacetylase (HDAC) inhibitors are known to be potent activators of the lytic cycle [65]. Lytic replication is dependent on the expression of two immediate–early genes — BZLF1 and BRLF1. HDAC inhibitors derepress the promoters of these two genes, allowing the expression of Zta and Rta to activate a cascade of lytic genes, resulting in the production of viral-encoded replication enzymes [66–68]. During lytic induction, EBV expresses BamHI X Left Frame 1 (BXLF1) and BamHI G Left Frame 4 (BGLF4) that encode for thymidine kinase and protein kinase (PK). EBV-PK phosphorylates and converts the nucleoside analogs into their active, cytotoxic form in EBV-infected cells [69]. Aciclovir and ganciclovir and their prodrug forms valciclovir and valganciclovir are analogs of 2′-deoxyguanosine. After phosphorylation to the monophosphate form by EBV-PK, cellular kinases convert the analogs to the active triphosphate forms, which are incorporated by viral and cellular DNA polymerases into the replicating viral and cellular DNA, resulting in chain termination that preferentially blocks viral DNA replication, but can also cause host cell-cycle arrest and apoptosis [70]. Viral resistance to various PK inhibitors has been identified and represents a challenge for long-term treatments [70].

Several noncyclic nucleoside analogs, such as tenofovir alafenamide (TAF), that were developed as specific inhibitors of reverse transcriptases to treat HIV and HBV infection, were identified as potent inhibitors of EBV DNA polymerase [71]. TAF was found to be twice as potent as ganciclovir in direct inhibition of EBV DNA polymerase activity in vitro and viral DNA replication in cell culture [72]. There is some evidence suggesting that tenofovir may provide some benefit for treatment of MS and may be related to its antiviral activity directed toward lytic EBV [73].

An early clinical trial on the use of arginine butyrate in combination with ganciclovir showed modest therapeutic benefit for EBV lymphoma [63]. Further optimization of HDAC and nucleoside analog improved efficacy, especially in EBV-positive NK/T-cell lymphoma. Presently, a Phase-2 clinical trial is in progress for the HDAC inhibitor nanatinostat in combination with valganciclovir to determine its efficacy in various EBV-positive hematological malignancies, including PTLD, Diffuse Large B-Cell Lymphoma (DLBCL), and HL. Another HDAC inhibitor, HQK-1004, has also been tested in combination with valganciclovir in patients with relapsed or refractory EBV-positive lymphoid malignancies

or other lymphoproliferative disorders. HDAC inhibitors have been found to induce cellular differentiation of EBV-positive NPC, suggesting they may provide direct antineoplastic activity independent of viral reactivation [74].

In addition to HDACs, numerous other agents have been found to induce the EBV lytic cycle with some dependence on host-cell type. A class of thiosemicarbazone that chelate intracellular iron was found to be potent activator of EBV and has been explored therapeutically [75,76]. A family of tetrahydro-β-carbolines, with a lead termed C60, were found to induce EBV lytic reactivation through protein stabilization of BZLF1 (ZTA) and a mechanism involving perturbation of Cullin-associated and neddylation-dissociated 1 protein (CAND1)-dependent regulation of CRL F-box ubiquitin-ligation complex [77–79].

There are some limitations to this approach. Response to lytic induction is heterogeneous with on average 2–60% of tumor cells expressing lytic genes, depending on which lytic activator drug is used. Lytic activation is also variable in terms of the cellular context with some cell lines (many derived from solid tumors) only weakly induced [67,80,81]. Common side effects from the HDAC inhibitor, vorinostat Suberoylanilidine hydroxamic acid (SAHA), for example, include anemia, thrombocytopenia, and intestinal issues [82]. It is also not yet known if the systemic reactivation of EBV may increase risk for subsequent malignancies or immune disorders.

EBNA1 (DNA-binding episome maintenance)

EBNA1, a viral-encoded regulatory protein critical for the replication, maintenance, and survival of EBV during latency. EBNA1 is the only protein that is expressed in all oncogenic forms of latency and in all EBV-positive tumors [83]. Thus, EBNA1 may be considered an attractive target for therapeutic intervention. The structure of the C-terminal domain of EBNA1 has been solved alone and bound to cognate DNA [84–86]. The C-terminal DNA-binding domain of EBNA1 is an obligate homodimer. Small molecules or peptides that perturb the dimer–dimer interface have activity in vitro and in vivo [87–89]. Several pockets in the dimeric C-terminal domain were found to be susceptible to small-molecule binding and inhibition of DNA binding [90,91]. Using a combination of fragment-based lead discovery and structure-based design, a unique series of 2-,3-substituted benzoic acids was found to inhibit EBNA1–DNA binding [92]. These inhibitors blocked EBNA1–DNA interaction in vitro and EBNA1-dependent replication and oriP binding in cellular assays. Moreover, EBNA1 inhibitors specifically blocked the proliferation of EBV-positive cells in cellular assays and preclinical models [92,93]. Treatment with EBNA1 inhibitors also resulted in significant loss of EBV genomes and viral gene expression. One EBNA1 inhibitor (VK-2019) has progressed to clinical studies for treatment of patients with advanced NPC (Table 3).

BILF1 (viral G-protein-coupled receptor)

EBV encodes a G-protein-coupled receptor (GPCR), BamHI I Left Frame 1 (BILF1), that can be expressed at variable levels in different infection scenarios and tumor types. BILF1 is a lytic protein that downregulates the expression of a broad range of surface HLA

class-I molecules and impedes presentation of viral antigens, allowing the virus to evade cytotoxic T-cells [94,95]. GPCRs are 7-transmembrane proteins that are frequently targeted by small-molecule inhibitors. Small molecules that bind and inhibit BILF1 signaling are under development to treat EBV-driven disease [96].

Latent membrane protein1/LMP2A (viral membrane oncogenes)

Latent membrane protein 1 (LMP1) is a functional homolog and acts as a constitutively active receptor of CD40, recruiting cellular signaling molecules associated with tumor necrosis factor receptors. Together with LMP2A, LMP1 activates numerous pathways, including Nuclear Factor-kappa B (NF-κB), phosphatidylinositol-3-kinase, mitogen-activated protein kinase, Interferon Regulated Factor 7 (IRF7), and Signal Transducers and Activators of Transcription (STAT), and drives cellular survival and proliferation. Recently, affibody molecules that interact with LMP2A N-terminal or C-terminal domains were shown to inhibit proliferation of NPC cells [97,98].

Other Epstein–Barr virus-encoded targets

EBV encodes many additional proteins and noncoding RNAs that are implicated in cancer pathogenesis that represent attractive targets for small-molecule inhibition. These include BamHI A Right Frame 1 (BARF1), a Cytokine Stimulatory Factor 1 (CSF1)-interacting protein expressed in many EBV-epithelial tumors [99], latency-associated nuclear regulatory protein Epstein-Barr Nuclear Antigen 2 (EBNA2) [100], the viral-encoded ubiquitin ligase BamHI P Left Frame 1 (BPLF1) [101], and the viral-encoded ribonucleotide reductase BamHI O Right Frame 2 (BORF2) that also inhibits APO-BEC3B [102], to name just a few. It is not yet clear which of these early-stage targets are most likely to produce an efficacious inhibitor to treat EBV-driven cancers.

Conclusions

Selective therapies to treat EBV-associated disease have been challenged by the complexity of the EBV lifecycle, host immunity, the heterogeneity of viral gene expression, and the diversity of diseases caused by EBV infection. Various immune strategies are likely to be effective in reducing transmission and disease burden and provide new and safe methods to treat EBV malignancies. Small molecules targeting EBV may also provide selective modalities that, in combination with existing cancer therapies, or with newly developed immune approaches, may provide precision approaches for EBV-driven cancers and autoimmune disorders.

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Table 1.

Selection of completed and ongoing EBV vaccine trials

NIH Clinical Trials Identifier	Vaccine platform	EBV antigen	Dose and route	application	Outcome measures	Results	Status as of January, 2022
NCT04645147	Ferritin nanoparticle	gp350	3 doses, i.m.	IM	Ab and CD4+ to gp350	pending	Recruiting
NCT05164094	mRNA	gH, gL, gp220	3 doses, i.m.	IM	Immune response	pending	Recruiting
NCT00430534	recombinant	gp350	3 doses, i.m.	IM	Immune response, development of IM, seroconversion	prevents IM, does not prevent asymptomatic infection	Completed (Phase II; Sokal, 2007)
NCT01094405	MVA	EBNA1/LMP1	3 doses, i.d.	NPC	Stable disease, immune response, survival	Increased CD4+, CD8+, and Ab	Completed (Phase I; Hui 2013))

Table 2.

Selection of cell-based immunotherapy trials for EBV-associated diseases

NIH Clinical Trials Identifier	Intervention	Platform	Dose and route	application	Outcome measures	Results	Status as of January, 2022
NCT03648697	Adoptive T-cell immunotherapy	LMP1, LMP2, and EBNA1-TCR-T cells	1 dose, i.v.	NPC	Adverse events, clinical	pending	Recruiting
NCT03925896	Adoptive T-cell immunotherapy	LMP2 specific TCR-T cells	1 dose, i.v.	NPC	Maximum Tolerated Dose	pending	Recruiting
NCT00430534	Adoptive T-cell immunotherapy	gemcitabine-carboplatin followed by EBV-specific CTL	Multiple doses gemcitabine-carboplatin, Followed by 4 doses of EBV-specific CTL	NPC	Survival, disease progression	pending	Active (Phase III)
NCT01498484	Adoptive T-cell immunotherapy	EBV specific CTLs from HLA matched donor (ATA129)	3 doses, i.v.	lymphoma	Clinical response rate	Complete or partial remission in over 58%	Completed (Phase II; Prockop, 2020)
NCT03283826	Adoptive T-cell immunotherapy	EBV specific CTLs from HLA matched donor (ATA129)	3 doses, i.v.	MS	Adverse events, clinical progression (EDSS), MRI, IgG Index	pending	Recruiting

Table 3.

Selection of clinical trials for small molecule approaches to EBV-associated malignancies

NIH Clinical Trials Identifier	Compound	Class	application	Status as of January, 2022
NCT05011058	Nanatinostat	Class I HDAC inhibitor	In combination with Valganciclovir in patients with relapsed/refractory EBV-positive lymphomas, including: EBV- associated lymphoproliferative disease; PTLD, Non-Hodgkin lymphoma, extranodal NK/T, DLBC, Hodgkin lymphoma, PTCL	Recruiting (Phase II)
NCT04925544	VK-2019	EBNA1 inhibitor	Advanced NPC	Recruiting (Phase II)
NCT02670616	Ibrutinib	BTK inhibitor	In combination with Rituximab-CHOP for diffuse large B-cell lymphoma	Completed (Phase II)
NCT01094405	HQK-1004	HDAC inhibitor	In combination with Valganciclovir for relapsed/refractory EBV lymphoid malignancies and lymphoproliferative disorders	Completed (Phase II)

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