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# EBV-associated diseases: Current therapeutics and emerging technologies

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EBV is a prevalent virus, infecting >90% of the world's population. This is an oncogenic virus that causes ~200,000 cancer-related deaths annually. It is, in addition, a significant contributor to the burden of autoimmune diseases. Thus, EBV represents a significant public health burden. Upon infection, EBV remains dormant in host cells for long periods of time. However, the presence or episodic reactivation of the virus increases the risk of transforming healthy cells to malignant cells that routinely escape host immune surveillance or of producing pathogenic autoantibodies. Cancers caused by EBV display distinct molecular behaviors compared to those of the same tissue type that are not caused by EBV, presenting opportunities for targeted treatments. Despite some encouraging results from exploration of vaccines, antiviral agents and immune- and cell-based treatments, the efficacy and safety of most therapeutics remain unclear. Here, we provide an up-to-date review focusing on underlying immune and environmental mechanisms, current therapeutics and vaccines, animal models and emerging technologies to study EBV-associated diseases that may help provide insights for the development of novel effective treatments.

## KEYWORDS

high-throughput sequencing technologies, EBV therapeutics, EBV animal models, molecular mechanisms of EBV-host interactions, EBV-associated diseases and cancers, EBV vaccines

## Introduction

Oncogenic viruses cause approximately 15-20% of all human cancers (1, 2). According to the International Agency for Research on Cancer (IARC), there are seven major human oncogenic viruses (3). These include DNA viruses, such as Epstein-Barr virus (EBV; also known as HHV4), Kaposi sarcoma-associated

herpesvirus (KSHV; also known as HHV8), Hepatitis B virus (HBV), human papillomaviruses (HPV) and Merkel cell polyomavirus (MCPyV) and RNA viruses, such as human T-lymphotropic virus 1 (HTLV-1) and Hepatitis C virus (HCV). Despite many differences, these viruses have evolved common mechanisms to persist and replicate within host cells and facilitate escape of infected cells from the host's immune surveillance. EBV and KSHV are two oncogenic viral agents of the  $\gamma$ -Herpesviridae subfamily that are known to modulate a plethora of biological processes in viral-associated cancers. The  $\gamma$ -Herpesviridae family is divided into two genera: *Lymphocryptoviridae* which includes EBV and *Rhadinoviridae* which includes KSHV.  $\gamma$ -herpesviruses encompass a broad range of pathogens in lower mammals ranging from murine herpesvirus-68, bovine herpesvirus 4 and equine herpesvirus 2 that closely resemble the rhadinovirus. Interestingly, to date, lymphocryptoviruses have been found only in primates and humans (4).

EBV was first discovered by Epstein, Achong, and Barr in 1964 who isolated this virus from the cells of a Burkitt lymphoma (BL) patient in Africa (5, 6). Since then, it has become evident that EBV infects ~95% of the world's adult population. The typical transmission route is through bodily fluids, such as saliva, where the orally transmitted virions infect resting B and epithelial cells of the oral cavity. Primary infection is typically asymptomatic, although 35-50% of the human adolescent population develop infectious mononucleosis (IM) approximately 1 month after infection, and the virus persists throughout an individual's life (7-10). After acute infection, a dormant state is established due to a strong, virus-specific T cell response (7). However, when the balance between the virus and host immune system is disrupted, EBV can drive malignant transformation of both lymphoid and epithelial origins, causing ~200,000 deaths annually (11-13).

As a herpesvirus, EBV can cause either latent or lytic infection. In epithelial cells, EBV typically undergoes lytic replication. In B cells, EBV usually establishes lifelong latency with rare sporadic reactivations. During latency only a few essential viral genes are expressed and production of virions are stalled (14-17). The switch from latent to lytic phase is governed by several factors (18, 19). While EBV-encoded products in both phases can play a role in transformation and tumorigenesis, the literature is more extensive on the oncogenic role of latent genes compared to lytic genes. However, it is challenging to target latent EBV using current immunotherapeutic strategies, specifically due to reduced antigen expression. As a result, patients with EBV<sup>+</sup> or EBV<sup>-</sup> tumors are typically subjected to similar treatment regimen. This underscores the need to investigate the complexity of EBV-host interactions to help the development of EBV-specific cancer therapies. In this review, we will first discuss the EBV lifecycle and different types of EBV-associated malignancies. We will then summarize the major underlying molecular mechanisms

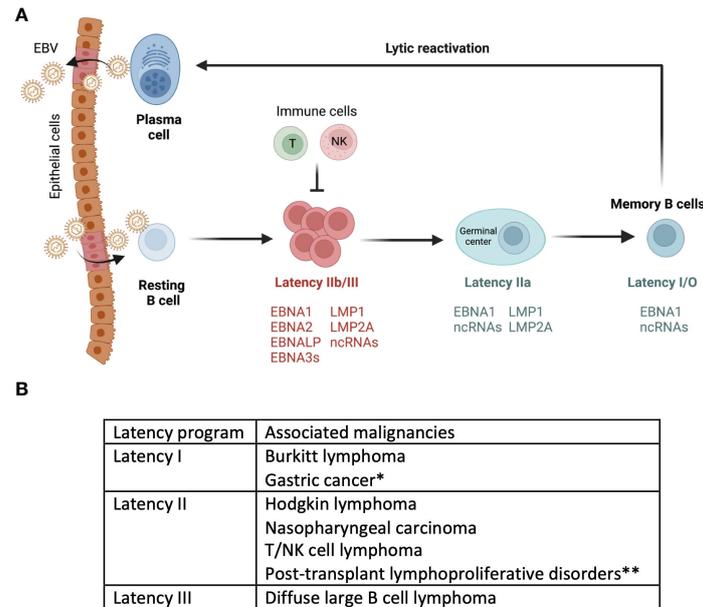
and therapeutic strategies for EBV<sup>+</sup> cancers. Lastly, we will discuss some of the preclinical animal models and emerging technologies for investigating different aspects of host-pathogen interactions in EBV-associated malignancies.

## Epstein-Barr virus infection

### Lifecycle

Epstein-Barr Virus (EBV) exhibits a biphasic lifecycle that includes latent and lytic (replicative) phases (20). Upon infection the virus typically establishes latency within the host cell (21). During latency, only a handful of latent genes that are necessary for the maintenance and persistence of the viral genome are expressed. EBV encodes eight latency genes whose expression in host cells and/or malignancies defines EBV latency programs (20). Based on which of the eight latent viral genes are expressed, viral infection is categorized into three main latency programs, latency III, II and I/0 (22). EBV-infected naive B cells exhibit a latency III program, which allows for the proliferation and expansion of infected cells (23). Latency III genes include 6 EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, LP), 2 latent membrane protein (LMP1 and LMP2), EBV-encoded small RNAs (EBERs), and EBV-encoded microRNAs (miRNAs) (24, 25). The cells in this latency program are highly immunogenic and can be rapidly eliminated by the host immune response, specifically by EBV-specific T cells (26). Latency II has a more restricted expression of EBV genes, namely EBNA1, LMP1, and LMP2A/B making them less immunogenic. Eventually, EBV sequentially shuts down the expression of all the latent genes except EBNA1 and a few EBV-encoded RNAs in latency I. Latency II can also be divided to IIa and IIb based on the expression of LMPs and EBNA2-3 (IIb is EBNA2-3<sup>+</sup>LMP<sup>-</sup>; IIa is EBNA2-3<sup>-</sup>LMP<sup>+</sup>). In most individuals, EBV persists quiescently within a subset of memory B cells (<0.005% B cells in the peripheral blood) without expressing any viral genes in latency 0 state, also referred to as a 'true latency' (24, 27, 28). Latent EBV genes are reported to promote tumorigenesis, inhibit apoptosis, and suppress recognition of infected cells by host immune cells (29). EBV-related malignancies are linked with different EBV latency programs. Lymphoproliferative disorders that are commonly associated with immunosuppression such as post-transplant lymphoproliferative diseases (PTLDs) and acquired immunodeficiency syndrome (AIDS) associated lymphomas exhibit latency III (29). Hodgkin lymphoma, T/NK cell lymphomas and nasopharyngeal carcinoma (NPC) exhibit latency II (30). Gastric carcinoma and Burkitt lymphoma exhibit latency I program (27, 31). So far, EBV in latency 0 has not been associated with any malignancies, presumably due to dormancy during this program (Figure 1).

The lytic phase is necessary for EBV progeny production and horizontal transmission of virus from host to host, so represents an



**FIGURE 1** Model of EBV infection cycle. **(A)** Upon primary infection through saliva, EBV infects B cells. The figure depicts a model of EBV infection, where in EBV drives naive B cells into latency III program. This activation leads to their differentiation into latency I/O memory B cells. This is often followed by spontaneous or induced reactivation of EBV within circulating memory B cells. This figure is adapted/modified from Guo et al (44). **(B)** Depending on the type of latency or lytic program, EBV infected cells are associated with different malignancies.\*Gastric cancer cells also express genes that are associated with latency II programs. \*\*Post-transplant lymphoproliferative disorders express some of the genes in latency III as well.

integral aspect of viral pathogenesis (32). The switch from latent to lytic cycle can be either spontaneous or chemically induced. Some of the commonly used agents to induce lytic cycle include phorbol esters (PMA), sodium butyrate, calcium ionophores, DNA methyltransferase inhibitors (DNMTi), transforming growth factor-beta (TGF-β), doxorubicin and gemcitabine (because these are stress inducing chemotherapeutic drugs) and anti-IgG or anti-IgM as B-cell receptor stimulants (33–35). During lytic reactivation, the full repertoire of >80 viral genes is temporally regulated and expressed during three phases - immediate early (IE), early (E), and late (L). The first phase is primarily initiated by BZLF1 (ZEBRA) and BRLF1, the two key EBV immediate-early (IE) lytic transcription factors. Both genes function to promote their own and each other’s expression, as well as the expression of viral E genes, that code for proteins needed for viral replication (e.g., viral DNA polymerase). BZLF1 forms a homodimer via its basic leucine zipper motif and binds to BZLF1-responsive elements (ZRE) on DNA (36). The binding of BZLF1 to CpG methylated DNA leads to activation of several lytic viral genes that are silenced in latent cells by CpG methylation (37, 38). In addition, binding of BZLF1 to the origin of lytic replication (oriLyt) ZRE promotes lytic viral DNA synthesis (39). Similarly, BRLF1 binds to the BRLF1-responsive elements (RRE) on DNA and is reported to induce lytic replication via the PI3K and ERK signaling pathways (40, 41). Both BZLF1 and

BRLF1 are quintessential for EBV lytic replication since knocking out these genes blocks the latent to lytic switch. In addition, overexpression of BZLF1 and BRLF1 in latently infected cells can induce EBV lytic reactivation (42). This lytic induction leads to a cascade of viral gene expression, which promote viral DNA replication and virion production. Following viral replication, late viral genes code for structural proteins, such as gp350/220 encoded by the *BLLF1* gene are expressed (32). Interestingly, during lytic DNA replication in γ-herpesviruses, continuous DNA synthesis is needed for the transcription of late lytic viral genes but not for early lytic genes (20, 43). The virions can disseminate viral particles within host cells and among hosts. EBV replicates in latency I, II and III via proliferation of activated B cells. Interestingly, lytic replication can only be efficiently induced from latency I/O, and after extensive methylation of the viral genome. This is because BZLF1 prefers binding to methylated CpG sequences to initiate infectious particle production (38, 42).

### Genome organization and DNA methylation

The EBV genome is packaged similarly to that of host cells, that is to say into nucleosomes, except loci that harbor the origin

of plasmid replication (OriP) (44, 45). Nucleosome folding along with several histone modifications at the promoter of lytic genes keep them transcriptionally silent during latent infection. For example, the recruitment of histone deacetylases (HDACs) at BZLF1 and EBNA2 Cp promoters maintain EBV latency (46) (47, 48) and, as expected, HDAC inhibitors, such as sodium butyrate, can activate the EBV lytic cycle (49, 50). Of note, EBV DNA within virions or soon after lytic replication is nucleosome free, potentially to allow its encapsulation into the nucleocapsid (44, 51).

DNA methylation is typically associated with transcriptional silencing (52, 53). The EBV genome is known to be hypermethylated in the latent form and in virions (54). DNA methylation plays a critical role in transcriptional regulation of LMP1 and EBNA2 and thus contributes to the transition among latency programs (55). Consistently, inhibitors of DNA methylation, such as 5-azacytidine can reactivate latently infected lymphoblastoid cells (LCLs) (56). However, since the OriP region is required for EBV transcriptional regulation, it is typically depleted of DNA methylation.

## Entry into host cells

EBV typically exhibits dual tropism with the capacity to actively infect and replicate both in epithelial and B-cells. Sometimes EBV can also infect other targets such as T lymphocytes and natural killer (NK) cells (7, 57). The entry of EBV into target cells is facilitated by its envelope glycoproteins (gp). B-cell entry requires glycoproteins gp350, gH, gL, gB and gp42, whereas epithelial cell entry needs BMFR2, gH, gL and gB (58–61). In epithelial cells, EBV is more likely to be transferred from EBV-positive B lymphocytes that cause lytic infection (62). T-cell and NK-cell entry seem to also require gp350 and gp42, respectively (63, 64).

The EBV virion has a diameter of 150–170 nm, consists of a linear, ~172 kbp double stranded DNA that codes for more than 85 protein coding genes (65, 66). However, the exact function of 30–40% of these genes remains unknown (67). The EBV genome also has several tandem repeat regions that serve various functions (20, 68). The entire genome is enclosed within an icosahedral capsid surrounded by a layer of tegument proteins and a lipid envelope that is made up of several unique glycoproteins. EBV can enter human B cells *via* a high-affinity interaction between viral gp350 and host complement receptor type 2 (CR2) protein. HLA class II can act as a co-receptor (15, 69). These protein-protein interactions stimulate endocytosis of the virus into non-clathrin coated vesicles of B cells. The virus also infects epithelial cells as well as T- or NK-lineage cells albeit at a lower frequency. Ephrin Receptor A2 (EphA2) was recently identified as the entry receptor for EBV in epithelial cells. This

protein interacts with EBV glycoproteins gH/gL and gB (70). Although less is known about the mechanisms of EBV entry into other cells, CR2 has been identified as the entry receptor for T lymphocytes (64) but is apparently not essential for entry into NK cells (71). HLA class II also plays a role for entry into NK cells but its role for T cells remains less clear (63).

Upon entering B cells, the viral genome typically persists in the nucleus as a circular episome, expressing a subset of genes that promote survival of the infected host cell (58, 59). Typically, after initial infection, the EBV genome rapidly circularizes either before or at the same time as the initial phase of viral mRNA synthesis (21, 72–74). After B cell infection, EBV initiates an often asymptomatic, lifelong latency program in a few cells with extremely low viral activity. During this stage, the EBV episome is replicated by the host cell DNA polymerase primed on the EBV origin of plasmid replication (OriP) (75, 76).

## Variants and risk factors

EBV was originally divided into two major sub-variants, type 1 and type 2, based on the sequence of two EBV-encoded genes - EBNA2 and EBNA3 (77, 78). Type 1 is prevalent globally (e.g., B95-8, GD1, and Akata strains) while type 2 (e.g., AG876 and P3HR-1 strains) is endemic to sub-Saharan Africa (79). Currently, more than 71 distinct EBV strains have been identified. EBV variants have different replicative properties and individuals may become infected with two or more strains. With the advent of high-throughput sequencing technologies, it is now possible to sequence EBV genomes from clinical specimens of diverse populations with different malignancies. The first sequenced genome was of the prototypical EBV B95-8 strain. This strain harbored a 12-kb deletion in its genome. It was not until 2014 that this defect was noted and EBV from Raji strains was recovered to get the final complete sequence of wild-type EBV (EBV-wt, 26 GenBank accession no. NC\_007605.1) (65). This is now the gold standard reference sequence for many research groups in the field. It has been reported that certain EBV strains have more oncogenic potential than others. For instance, the NPC derived EBV strain, M81, spontaneously replicates at an unusually high rate in B cells and has an extremely high propensity to infect epithelial cells (80). This 'super infectious' property is attributed to a single nucleotide polymorphism (SNP) in the *BZLF1* promoter region that confers binding by host cellular transcription factors, notably NFATc1 (81). Increasing studies are now investigating the heterogeneity of EBV latent and lytic genes among the different EBV strains in order to identify high-risk EBV strains (79). Doing so will potentially help identify high-risk infected individuals and facilitate development of effective EBV vaccines and anti-EBV T-cell therapies.

## EBV encoded latent gene products and oncogenesis

EBV latent proteins are generally considered key drivers of tumorigenesis in EBV-associated cancers and thus it is important to understand their functions in establishment of persistent infection and cellular transformation. In this section we will briefly describe the function of EBV-encoded latent gene products and their role in transformation and oncogenesis in EBV-associated malignancies.

### Epstein–Barr virus nuclear antigen 1

EBNA1 is a transcription factor that is essential for EBV episomal maintenance and replication (82, 83). Consistently, EBV variants that harbor EBNA1 deletion do not have the capacity to establish episomal latent infection (84). The DNA binding domain of EBNA1 is necessary but not sufficient for EBV replication and requires the N-terminal region (85). Since EBNA1 lacks enzymatic activity, it primarily recruits host cellular factors to replicate EBV episomes and to govern mitotic segregation (86). Of note, EBNA1 can also function as a transcriptional repressor and can downregulate its own transcription in an autoregulatory loop (87–89). In terms of oncogenic potential, EBNA1 is involved in progression of carcinogenesis. Specifically, EBNA1 deletion significantly decreases immortalization efficiency, while its overexpression inhibits apoptosis (90, 91). EBNA1 modulates several cellular signaling pathways that provide survival advantage to infected cells (92). While EBNA1 is reported to enhance phosphorylation of STAT1 in one gastric cancer cell line and two nasopharyngeal cancer cell lines, it inhibits anti-tumor TGF- $\beta$ 1 and NF- $\kappa$ B pathways, promoting tumorigenesis (93, 94). EBNA1 also upregulates several proteins involved in metastasis and oxidative stress in EBV<sup>+</sup> NPC cells (95). In addition, EBNA1 induces loss of promyelocytic leukemia (PML) nuclear bodies and subsequently abrogates PML functions, such as p53 activation and apoptosis, resulting in increased survival of gastric cancer cells (96). The fact that EBNA1 is the only EBV protein that is consistently expressed in all latency types, and therefore in all EBV-associated tumors, makes it a key target for EBV specific therapies. Consistently, pharmacological inhibition of EBNA1 using a small-molecule inhibitor VK-1727 has been tested in various *in vivo* xenograft mouse models for specific EBV<sup>+</sup> cancers. These studies have demonstrated that inhibition of EBNA1 can selectively suppress EBV<sup>+</sup> tumor cell proliferation (97).

### Epstein–Barr virus nuclear antigen 2

EBNA2 is a transcriptional activator of both cellular (e.g., *CD21*, *CD23* and *c-MYC*) and viral genes (e.g., *LMP1* and *LMP2*) (98, 99). EBNA2 plays a crucial role in transcriptional reprogramming of B cells to facilitate growth and survival (100, 101). However, unlike EBNA1, it cannot bind to DNA directly and requires host transcription factors, such as the Notch pathway DNA-binding factor RBP-J $\kappa$  and PU.1 to regulate gene transcription (102). In terms of oncogenic potential, EBNA2 plays a crucial role in the transformation process and functionally mimics Notch (103, 104). Consistently, P3HR-1, a variant EBV strain in which EBNA2 and the last two exons of EBNA-LP are deleted, does not transform B cells *in vitro*. EBNA-LP, a transcriptional co-activator of EBNA2, is also an important EBV oncoprotein that drives B cell transformation and functions by up-regulating the expression of EBNA2 targets (105). In addition, EBNA2 activates *MYC* enhancers *via* long-range interactions. *MYC* can both increase proliferation and sensitize cells to apoptosis. However, it is also a known proto-oncogene, so unsurprisingly, EBNA2-mediated *MYC* activation seems to promote lymphomagenesis in Burkitt lymphoma (106).

### Epstein–Barr virus nuclear antigen 3 family proteins

The EBNA3 protein family members are stable, tightly regulated and consist of EBNA3A, EBNA3B and EBNA3C (107). EBNA3 proteins are well studied classes of transcriptional regulators known to regulate both EBV (e.g., *LMP1*) and host gene (e.g., *CD21*) expression and, depending on context, can function as activators or repressors of gene expression (108–110). All EBNA3 proteins play a role in transformation and prolonged persistence of EBV in infected B cells. EBNA3 transcripts are generated from the Cp latency promoter and are reported to be only expressed in B cells as a part of the latency III program (111). Like EBNA2, EBNA3 proteins also do not directly bind to DNA but are, rather, recruited by cellular DNA binding factors, such as RBP-J $\kappa$  (112, 113). RBP-J $\kappa$  tethers EBNA3s to chromatin, but binding of EBNA2 and EBNA3 to RBP-J $\kappa$  are mutually exclusive (113, 114). In terms of their oncogenic potential, the EBNA3 family of proteins have antagonistic functions but cooperate in a complex to facilitate EBV persistence, as well as to promote oncogenic transformation. EBNA3A and EBNA3C are considered oncogenes since they are also involved in B cell transformation. However, in the absence of EBNA3A and EBNA3C, EBV can latently persist in humanized mice (115). Despite the sequence and structural similarity and reports about their functional cooperativity, EBNA3 proteins are often have

opposing functions. For example, EBNA3B is dispensable for B cell transformation but inhibits EBNA3A- and EBNA3C-mediated oncogenic functions *in vivo*. In addition, like EBNA2, EBNA3 has long-range interactions with enhancers and super-enhancer elements that drive oncogenesis (116).

## Latent membrane protein 1

EBV encoded LMP1 is an essential membrane protein that has an N-terminal cytoplasmic tail, six transmembrane domains and a C-terminal cytoplasmic region that is divided into two C-terminal activation regions 1 and 2 (CTAR1 and CTAR2). These regions are required for tethering LMP1 to the plasma membrane and its signaling activity (117). LMP1 mimics cellular CD40 receptor, a member of the (TNFR) superfamily and can drive growth and differentiation of B cells by substituting CD40 functions *in vivo* (118). LMP1 signaling is primarily mediated by the ability of host TNFR-associated factors (TRAFs) or death domain protein TRADD to interact with CTAR1 or CTAR2 to facilitate activation of upstream regulators of several signaling pathways (119, 120). In terms of oncogenic potential, LMP1 is a well-documented EBV oncogene and is essential for transformation of B cells *in vitro*. LMP1 acts as a constitutively active CD40 receptor and thus can activate target signaling pathways (e.g. the NF- $\kappa$ B pathway) independent of ligand engagement (121–123). These includes pro-tumorigenic functions, such as increase in cell proliferation, cytokine production (IL-6, IL-8), apoptotic resistance (by upregulating the expression of anti-apoptotic protein (Bcl-2, A20)), immune modulation, anchorage-independent growth, metabolism, angiogenesis, metastasis and invasion, all of which are known to contribute to EBV-mediated pathogenesis (124–126).

## Latent membrane protein 2

The LMP2 gene encodes two dominant isoforms, LMP2A and LMP2B. LMP2B is the smaller isoform that structurally lacks a short cytoplasmic N-terminal domain that harbors an essential survival signal known as immunoreceptor tyrosine-based activation motif (ITAM) (75, 127). Interestingly, B cell receptor (BCR) also has an ITAM motif which, upon phosphorylation, recruits and activates the *Src* family and *Syk* protein tyrosine kinases (PTKs) and promotes B cell proliferation and differentiation. However, the association of these PTKs with the phosphorylated ITAM of LMP2A negatively regulates PTK activity (127), thereby inhibiting BCR-driven calcium flux, tyrosine phosphorylation and BZLF1 induction in LCLs (128). In terms of oncogenic potential, LMP2A and LMP2B seems to be dispensable for *in vitro* B-cell

transformation (129). Indeed, LMP2 has anti-oncogenic potential (130, 131).

## Other EBV-encoded latent gene products

In addition to the latent proteins mentioned above, there are a few other EBV-encoded latent gene products including EBERs, BARTs and EBV microRNAs. Two EBERs (EBER 1 and 2) are small non-coding RNAs that are expressed during all latency programs. In terms of oncogenic potential, EBERs do not seem to be essential because EBER-deleted EBV strain can similarly transform primary B-lymphocytes (132). However, they can affect cellular processes by enhancing anti-inflammatory cytokine IL-10 production *via* RIG-I/IRF3 activation (133). In certain B cell lymphomas with restricted type 1 latency, EBER2 and EBNA1 can induce expression of cytokines (e.g., IL6) or cytokine receptors (e.g., CD25) to promote B cell survival (134). The BARTs encode BARF0, RK-BARF0, A73 and RPMS1. The function of BART proteins encoded by corresponding ORFs needs further examinations. The 44 EBV microRNAs are arranged either adjacent to the BHRF1 gene or within the BART introns. These microRNAs are associated with different EBV latency programs (135, 136) and are differently induced during the lytic cycle (137). However, the significance and function of most remain unclear.

## EBV-associated diseases

EBV is associated with a wide variety of diseases and malignancies. Infectious mononucleosis (IM) is an extremely common, self-limiting, and acute disease associated with primary EBV infection. It is characterized by lymphadenopathy, transient fever and hepatosplenomegaly that usually resolves in time. Chronic active EBV infection (CAEBV), although rare, is a severe and fatal condition characterized by unusually high EBV DNA load ( $10^3$ – $10^7$  copies/mL) (138), which is now considered to be one of the EBV<sup>+</sup> T or NK cell lymphoproliferative diseases and can lead to two lethal conditions: hemophagocytic lymphohistiocytosis and chemotherapy-resistant lymphoma (139). Historically CAEBV was partially managed using immunomodulatory agents such as interferon- $\alpha$  (IFN- $\alpha$ ) and IL-2 (140), but JAK/STAT inhibitors are now a standard component of treatment (141).

EBV is also a major risk factor for immunocompromised patients. In HIV patients the lack of efficient and EBV-specific T cell responses significantly increases the risk of developing EBV-associated lymphoma (142, 143). Oral hairy leukoplakia (OHL) is a hyperproliferative disorder observed in immunocompromised patients that is triggered by EBV lytic state (144–147). Post-transplant lymphoproliferative disorder (PTLD) represents severe,

life-threatening uncontrolled B cell proliferation post-organ or bone-marrow transplantation, which in majority cases is associated with EBV reactivation and replication (148). EBV infection is typically associated with cases of early onset (within one year of transplantation) compared to late-onset PTLDs (149) and higher EBV viral loads increase the risk of PTLD (150). One of the main reasons for EBV reactivation during transplantation is the use of immunosuppressants to prevent transplant rejection. However, these immunosuppressants inhibit all T cells, including EBV-specific ones, providing an opportunistic means for EBV to escape from immune surveillance (151). Consistently, pre-emptive treatment with inhibitors of EBV DNA replication can reduce the incidence of PTLD (152).

EBV infection has also been implicated in the development of autoimmune diseases, such as multiple sclerosis (MS) (153). MS is characterized by autoreactive B cells in the cerebrospinal fluid (CSF) that attack the myelin sheath of the central nervous system (CNS). Recently, a large-cohort study on 62 million serum samples taken from over 10 million US military personnel provided compelling evidence suggesting a necessary but not sufficient role of EBV infection towards the development and progression of MS (154). Pathologically, another recent study showed that EBNA1 mimics the CNS protein glial cell adhesion molecule (GlialCAM), which is expressed by myelin sheath-forming cells. Antibodies against a particular region of EBNA1 highly cross-react with GlialCAM in MS patients, potentially resulting in “off-target” autoimmune attack against the myelin sheath in CNS of patients with MS (155).

The ability of EBV to immortalize B cells is testament to its tumorigenic potential (156, 157). Indeed ~1-2% of all human tumors are attributed to EBV, equating to ~300,000 new cases worldwide in 2020 (158–160). EBV infects both genders, however, EBV-associated malignancies are slightly more prevalent in males compared to females (161). EBV infection is associated with various lymphomas, including Burkitt’s lymphoma (BL), Hodgkin lymphoma (HL), diffuse large B cell lymphoma (DLBCL), NK/T cell lymphoma and primary effusion lymphoma (6, 162), as well as epithelial malignancies, such as NPC and gastric carcinoma (GC). Below, we will discuss some EBV-associated malignancies in greater detail.

## Burkitt’s lymphoma

BL is a highly aggressive B cell non-Hodgkin neoplasm first reported in Africa by Denis Burkitt (163). The WHO classification describes three clinical variants of BL: endemic (eBL), sporadic (sBL), and immunodeficiency-related (usually HIV-related) (164). While around 95% of eBL are EBV<sup>+</sup>, only 15% of sBL and 40% of immunodeficiency-related BLs are associated with EBV (67). Despite primarily having the type I latency programs, some other EBV genes (e.g., EBNA2) are sporadically detected (165). Although the role of most EBV

genes in BL pathogenesis remains unclear, it has been shown that EBNA1 can inhibit apoptosis in BL cell lines by interacting with host proteins, such as p53-regulator USP7 and an anti-apoptotic protein survivin (166).

The eBL variant is common in malaria endemic regions and is commonly characterized by the presence of large tumors in the head and abdominal cavity. EBV is detected in nearly all the cells of eBL tumors, however the exact underlying mechanism has not yet been fully elucidated (167). Fortunately, BL tumors typically respond to intensive chemotherapy and are often curable when diagnosed early but it still remains a fatal disease in much of the affected sub-Saharan African population (168). This is attributed to several factors, such as diagnostic delay, inadequate healthcare, poverty, and malnutrition (169, 170). Outside of malaria-endemic regions, the occurrence of BL is about 10-fold lower mostly constitutes the sBL variant, which is concurrent with a lower EBV prevalence (10-30%) (171). Clinically, the majority of sBL cases present as tumors in the abdominal and thoracic cavities. Despite a poor prognosis, current ongoing clinical trials using a modified chemotherapeutic approach are showing some promise (NCI 9177 trial). Nonetheless, more specific and less toxic treatment options are needed.

The best known molecular feature of BL is the translocation of the proto-oncogene MYC to an enhancer locus next to the immunoglobulin heavy chain gene, causing constitutive expression of MYC (172, 173). However, in addition to enhanced MYC activity, the development of BL requires additional genetic or epigenetic aberrations (174). Over the years, extensive genomic and transcriptomic characterization of BL cases have identified genes that are recurrently mutated (e.g., *BCR*, *TCF3*, *ID3*, *CCND3*, *ARID1A* and *SMARCA4*) (175, 176). For instance, it has been experimentally demonstrated that mutations in *ID3* promote proliferation and cell cycle progression (177). Genes in the *ID3*-*TCF3*-*CCND3* pathway are frequently mutated in MYC-rearranged eBLs and may represent one of the major underlying causes of BL (178). Nevertheless, the mutational and transcriptional landscape of EBV<sup>+</sup> BLs is quite distinct from EBV<sup>-</sup> BLs and is primarily attributed to the presence of EBV. Recent studies have explored the spectrum of aberrations in EBV<sup>+</sup> BLs and the complex interplay between specific viral-host transcriptional programs (179, 180). For example, the frequency of *MYC*, *ID3*, *TCF3* and *p53* somatic mutations is lower in sBLs, while the frequency of mutation in *ARID1A*, *RHOA* and *CCNF* are higher in eBLs (179). Interestingly, it has been previously reported that LMP2A enhances MYC driven lymphomagenesis through activation of the PI3K-pathway (181–183), suggesting that activation of PI3K by LMP2A might be an alternative and/or convergent mechanism to the one driven by *TCF3*/*ID3* mutations. In addition to LMP2A, a recent study described the role of LMP1 in MYC-induced lymphomagenesis in a subset of BL cases (184). Further, comparative transcriptome analysis of eBL and sBL tumors have highlighted key mutational differences between the

two types of BLs, with sBL having a significantly higher mutational burden, which correlates strongly with the EBV status rather than geographical distribution (185, 186). A recent study has found a genome-wide increase in aberrant somatic hypermutation (SHM) in EBV<sup>+</sup> BLs, attributed to the higher expression of host activation-induced cytidine deaminase gene AICDA, which is also a target of EBNA3C (187). Overexpression of AICDA increases the likelihood of DNA breaks and MYC translocations as well as pathogenic mutations (188). Other factors, such as co-infection, seem to also contribute to BL pathogenesis. For example, *Plasmodium falciparum* induces DNA damage, which can turn EBV-infected B cells into eBL. Likewise, impaired immune surveillance in HIV-infected patients can induce EBV-associated BLs (179, 189, 190).

## Hodgkin lymphoma

HL is a lymphoid neoplasm that originates from B cell. The two major forms of HL are the classical type (cHL) and the nodular lymphocyte predominant type (NLPHL), the latter being considered as an EBV<sup>-</sup> malignancy. One of the main features of cHL is the presence of large multinucleated cells known as Hodgkin and Reed-Sternberg (HRS) cells (191). Although derived from B cells, HRS cells lack the normal B cell phenotype which is attributed to functional aberrations in key B-cell associated TFs, such as PAX5, EBF1, TCF3/E2A and NF-κB (192, 193). In addition, the HRS cells co-express various hematopoietic cell markers and have anomalous activation of several signaling pathways (e.g., NF-κB and JAK/STAT), attributed to the frequent mutations of key TFs and/or cellular interactions within the tumor microenvironment (TME) (194). Globally, nearly 50% of cHL cases are EBV<sup>+</sup> but the EBV prevalence varies with geography. For instance, about 30-40% of cHL cases in North America and Europe are EBV<sup>+</sup>, while in Africa, Asia, and Latin America, all cases are EBV<sup>+</sup> (192). EBV<sup>+</sup> cHLs are typically characterized by a massive immune cell infiltration (195). Although the exact mechanistic role of EBV in cHL pathogenesis is unclear, the presence of EBV throughout disease progression underscores its role in maintaining the tumor phenotype (196). EBV<sup>+</sup> cHLs exhibit type II latency program, maintaining high levels of LMP1 and LMP2A proteins in all HRS cells (191, 197). LMP1 and LMP2A can both contribute to the pathogenesis of cHLs by mimicking cellular receptors, namely CD40R and BCR, that are essential for cell survival and expansion (99, 198–204).

## Nasopharyngeal cancer

NPC is a unique and complex form of a head and neck epithelial cancer. While the disease prevalence is extremely low

in the Western world, it is endemic in Asia, Southeast Asia, North Africa, Greenland, Alaska, and the Middle East, affecting around 30 per 100,000 individuals. The distinct geographical distribution pattern of NPC cases worldwide suggests both environmental (e.g., consumption of preserved, salt-cured foods) and genetic factors (e.g., mutations in *HLA*, *TNFRSF19*, *CDKN2A/B*, and *TERT*) as its etiology (205–208). Nonetheless, EBV infection is reported to be a critical risk factor and plays an essential role in NPC progression (209–211). About 90% of malignant cells in NPC are either undifferentiated or poorly differentiated squamous epithelial cells that typically express several EBV latency type II gene products (212). These include EBV1/2, EBNA1, LMP1, LMP2, BARF1, and several other EBV-encoded non-coding transcripts. LMP1 is one of the key oncogenic drivers of NPC that is expressed in 20%–60% of NPCs and all pre-malignant or pre-invasive lesions, making it a prime therapeutic target (213).

NPCs harbor a high somatic mutation burden. A recent study identified more than 50 mutations per tumor in a panel of 111 micro-dissected EBV<sup>+</sup> tumor samples. A whole exome sequencing study of NPCs identified a range of somatic mutations in key cellular genes and pathways including p53, HLA, NF-κB, MAPK, and P13K (214). Given that somatic mutations in NF-κB pathway were mutually exclusive to LMP1-overexpressing NPCs, the NF-κB pathway activation either by EBV or mutation seems to be vital for NPC pathogenesis (215). This is corroborated by another genome-wide analysis that reported that ~90% of the EBV<sup>+</sup> NPCs have constitutive activation of NF-κB inflammatory pathways either due to somatic mutations or expression of EBV encoded LMP1 oncogene (216). Additionally, chromosome instability (CIN) is hallmark of NPCs. Early studies have linked EBV infection with genomic instability. Detailed differences in the genomic and epigenomic landscapes of EBV driven epithelial malignancies have been reviewed elsewhere (207).

## EBV-associated gastric cancer

Gastric cancer (GC) is one of the leading causes of cancer-related mortality (165). Nearly 10% of the 950,000 yearly new cases of GC cases are attributed to EBV infection. EBV<sup>+</sup> GC usually mimics the histological features of lymphoepithelioma-like carcinoma, in which dense lymphocytic infiltrates (mainly CD8<sup>+</sup> T cells) are present. EBV<sup>+</sup> GC is, in fact, of the four molecular subtypes of GC, namely EBV<sup>+</sup> GC, GC with microsatellite instability, genomically stable GC, and GC with chromosomal instability (217). EBV<sup>+</sup> GC and GC with microsatellite instability are mutually exclusive. There are two common theories regarding the mechanism of occurrence of EBV<sup>+</sup> GC. First, that EBV enters the digestive tract *via* the saliva and directly infects gastric epithelial cells. Second, EBV within B cells of the stomach is reactivated (through unknown

mechanisms) and infects surrounding gastric epithelial cells (218). EBV<sup>+</sup> GC cells exhibit latency I or intermediate latency I/II programs (219, 220). Consistently, EBNA1 and LMP2A are expressed in 100% and ~50% of EBV<sup>+</sup> GC cases, respectively, but LMP1 is not expressed (221). EBV<sup>+</sup> GCs have distinct genomic aberration, clinicopathological features, cellular gene methylation, and comparatively favorable prognosis compared to EBV<sup>-</sup> GCs (217, 222–224). Unlike NPCs and EBV<sup>-</sup> GCs, p53 mutations are rare in EBV<sup>+</sup> GCs (225). This might also partially explain a comparatively favorable prognosis for EBV<sup>+</sup> patients since it is known that mutations in p53 reduces sensitivity to chemotherapy and radiation (226). EBV can also extensively induce cellular DNA methylation, which could inhibit tumor suppressor genes (e.g. *p16* and *E cadherin*) and thus increase the risk of cancer formation (227). Recent studies report the increased expression of certain immune checkpoint proteins in EBV<sup>+</sup> GC, such as PD-L1 and IDO-1 and their upstream regulators (180), which could explain their favorable response to immune checkpoint therapy (228). The pathogenic role of EBV, underlying molecular mechanisms and current treatment options for EBV<sup>+</sup> GC have been further discussed elsewhere (218).

## Mechanisms underlying EBV induced diseases

To establish infection and persistence, EBV employs different strategies to evade the host immune response and to

compromise innate and adaptive immunity during its life cycle (229). Some of these mechanisms includes genetic and epigenetic alterations, inhibition of apoptosis, enhanced cell proliferation and inhibition of immune recognition of EBV-infected cells (Figure 2). Broadly, tumorigenesis can occur by i) enhancing antiapoptotic or reducing proapoptotic gene expression; ii) promoting cell growth and survival signaling pathways; and iii) shaping the tumor microenvironment for malignant cells to escape immune surveillance. In this section, we will briefly discuss some of these mechanisms, focusing on the role of the tumor microenvironment and immune escape mechanisms in EBV-induced malignancies.

## Tumor microenvironment

EBV is known to alter the cellularity and the properties of the tumor microenvironment (TME) thereby shaping an immunosuppressive environment. This involves inhibition of anti-tumor effector immune cells, such as NK cells and CD8<sup>+</sup> T cells and recruitment and differentiation of immune-suppressive and/or anti-inflammatory cells, such as regulatory T cells (Tregs), dendritic cells (DCs), Th17 cells, M2-polarized tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) (230). In addition to the immune compartment, TME also includes stromal cells, soluble mediators such as chemokines and cytokines that can be modified by EBV. Together, these changes facilitate tumor growth by several mechanisms including promoting immune

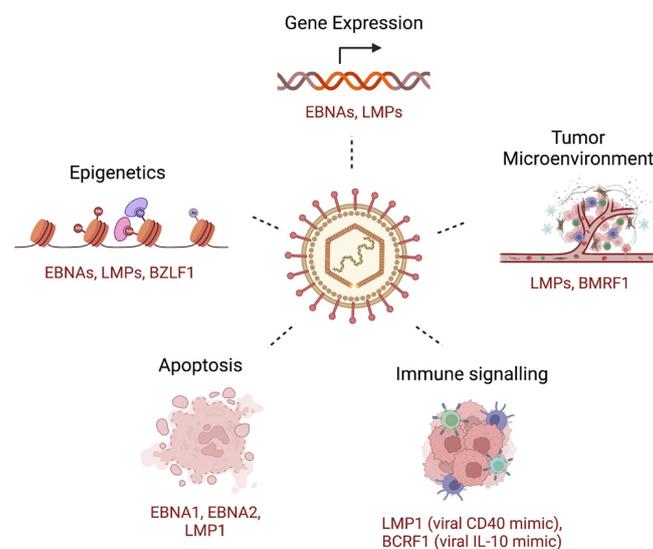


FIGURE 2

A few examples of key host cellular processes perturbed by EBV. Examples of EBV-encoded gene products that are related to the indicated mechanism are shown.

evasion. Extensive research has been done to understand the TME of both EBV<sup>-</sup> and EBV<sup>+</sup> malignancies (67). Studies on the TME are limited for certain EBV<sup>+</sup> malignancies due to low incidence rates (e.g. NK/T cell lymphoma) or high heterogeneity among the sites of the disease and immune compartment (e.g. PTLD).

It is known that tumor cells can affect immune cell infiltration as well as drive the infiltrated immune cells towards a tolerogenic or exhausted state rendering them non-functional. Although highly variable, EBV<sup>+</sup> carcinomas are generally characterized by high immune cell infiltration. This includes CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells (Th1, Th2, Treg cells, etc.) and CD163<sup>+</sup> M2-TAMs (TAMs) (231). T cells are prevalent in EBV<sup>+</sup> epithelial cancers. For example, EBV<sup>+</sup> GCs attract high numbers of CD8<sup>+</sup> T cells better known as cytotoxic T lymphocytes (CTLs) (232) and the CTL infiltration is positively correlated with EBV viral load (233). Despite a significant increase of CD8<sup>+</sup> T cells within the TME of EBV<sup>+</sup> NPCs, they exhibit an exhaustion signature and reduced cytotoxic activity (234, 235). In EBV<sup>+</sup> NPC, LMP1-mediated glycolysis promotes MDSC expansion within TME leading to tumor-induced immunosuppression (236). Tregs and the CD8<sup>+</sup> T cells are dominant in cHL. However, CD8<sup>+</sup> T cells are primarily exhausted due to the high levels of PD-L1 expression (237). In contrast, higher numbers of M2-TAMs seems to be the most prominent in the TME of Burkitt lymphomas (238), which are also known to affect tumor progression *via* upregulation of immune checkpoints and expression of specific cytokines. Similarly, an increased frequency of CD57<sup>+</sup> NK cells is reported in EBV<sup>+</sup> GC, NPCs and cHL compared to their EBV<sup>-</sup> counterparts (231). Unlike T cells, the role and the presence of B cells within the TME of EBV<sup>+</sup> malignancies are mixed and warrant further investigation.

Altered expression profile of certain soluble mediators including cytokines within the TME have an important role in EBV-associated malignancies and often these alterations precede immune cell infiltration. For instance, EBV induces host CXCL9, CXCL10, and CCL20 in some EBV<sup>+</sup> tumors, which in turn attract regulatory T cells into TME (239, 240). The cytokines often have pleiotropic effects. For instance, IL-10 is known to downregulate the expression of HLA class I and II antigens, induce Tregs (which in turn inhibit T-cell proliferation and IFN- $\gamma$  secretion) and inhibit CD8<sup>+</sup> T cell cytotoxic function resulting in an overall immune-suppressive microenvironment within the tumors (241–243). Other soluble mediators such as IL-1 $\beta$ , IL-4, IL-6, IL-8, and IL-13, IFN- $\gamma$ , CXCL10 and CXCL12 are also frequently upregulated in EBV<sup>+</sup> malignancies and implicated in disease progression (231). It is also known that EBV proteins such as LMP1 and EBNA1 can significantly promote an immunosuppressive microenvironment by promoting expression of chemokines and cytokines. Compared to their EBV<sup>-</sup> counterparts, EBV<sup>+</sup> GC cells have an overall higher involvement of Th1 and CD8<sup>+</sup> T cells and produce more

cytokines/chemokines including CCL20, CCL22, and CCL17 (244, 245). Other non-immune cells within the TME (e.g., cancer-associated fibroblasts) are also known to produce pro-inflammatory cytokines and have been reported to surround tumor cells in EBV<sup>+</sup> solid tumors (246).

## Immune evasion

The human immune system has developed several strategies to combat invading pathogens. Central components of such strategies are the innate and adaptive immune responses. While innate immune responses are the first line of defense, they are often non-specific. In contrast, the adaptive immunity is more specific and long lasting and maintains specific memory of invading pathogens. Despite these host immune defense tools, EBV can establish latency within infected cells suggesting that the virus has developed mechanisms to escape, inhibit, or subvert host immune responses to ensure its own persistence. A typical mechanism of innate immune evasion in EBV infection is downregulation of pattern recognition receptors, such as toll like receptors (TLRs). Similarly a general mechanism of adaptive immune-evasion in EBV-associated malignancies is the overexpression of immune checkpoint proteins (e.g., PD-L1, IDO-1, CTLA-4, LAG-3, TIM-3, and VISTA), thus making them susceptible to treatment with immune checkpoint blocking immunotherapy (247). Below, we will briefly discuss the strategies used by EBV to evade the innate and adaptive immune responses.

## Innate immune response and EBV evasion

The innate immune response against EBV originates from both the EBV-infected cells themselves (B and epithelial cells) as well as from bystander cells like myeloid and NK cells. One of the major elements of innate immunity are the pattern recognition receptors (PRRs) that can recognize a diverse array of pathogen associated molecular patterns (PAMPs) and recruit downstream effector mechanisms, such as secretion of type I interferons in response. To date, 10 TLRs have been identified in humans. TLR9 is the key receptor for sensing EBV and is abundantly expressed in B cells and certain myeloid cells. TLR9 specifically senses unmethylated CpGs of EBV DNA motifs present in viral particles immediately after primary infection in B cells. Upon stimulation, TLR9 can activate the NF- $\kappa$ B pathway, which in turn promotes production of pro-inflammatory cytokines and B cell proliferation.

Dendritic cells (DCs) can sense, phagocytose, process and present antigens to cells of the adaptive immune system. DCs are generally classified into two types, conventional DCs (cDCs) and plasmacytoid DCs (pDCs), which express TLR3 and TLR9,

respectively. Unlike TLR9, TLR3 is endosomally located and recognizes dsRNAs, including EBV-RNAs (EBERs). Nevertheless, both TLR3 and TLR9 stimulate type I IFN production when triggered. Even monocytes and macrophages sense EBV *via* TLR3 and TLR9 leading to cytokine and chemokine production. Another interesting cellular player are the NK cells. NK cells are critical cytotoxic innate lymphocytes that target infected cells. Like DCs, NK cells have two broad subsets, CD56<sup>bright</sup> and CD56<sup>dim</sup>, the latter being more relevant in B cells restricted EBV infections (248). This is supported by studies which suggest that deficiencies in NK cells can increase the occurrence of EBV-driven pathologies. Consistent with this notion, NK cells recognize and preferentially target infected cells with lytic EBV infection (249, 250). Despite the intricate network of innate immune players, EBV has developed strategies to counteract innate immunity. For example, EBV can reduce expression of several TLRs. For example, EBV lytic protein BGLF5 reduces TLR9 expression and LMP1 suppresses TLR9 function in EBV<sup>+</sup> PTLDs and cHLs. A detailed review of interplay between EBV and host innate immune responses can be found elsewhere (251).

## Adaptive immune response and EBV evasion

Adaptive immunity can be broadly classified into humoral and cell-mediated processes, which are mediated primarily by B and T cells, respectively. Adaptive humoral responses are the direct product of interaction between antigens and immunoglobulin (Ig) on the surface of naïve B cells, which leads to secretion of antigen-specific antibodies and antigen presentation to T cells. T cells in turn help B cells with Ig-class switching and affinity maturation either specifically *via* CD40L/CD40 binding or non-specifically *via* interleukin/cytokine release (252). Primary EBV infection triggers an immediate IgM response to viral capsid antigen (VCA) and *BMRFI* encoded early antigen diffuse (EaD) complexes. The importance of humoral immune responses and molecular details of antibody response against EBV has been previously reviewed (252). EBV-specific T cells are key players in determining the fate of EBV infected cells. Both types of T cells, namely, CD8<sup>+</sup> cytotoxic and CD4<sup>+</sup> helper T cells can recognize EBV antigens presented on the surface of infected cells by HLA molecules (253, 254). While HLA class I antigens are expressed on almost all nucleated cells, HLA class II antigens are expressed on the surface of antigen presenting cells (APCs) (253). Despite increased infiltration of CD8<sup>+</sup> T cells in EBV<sup>+</sup> tumors compared to EBV<sup>-</sup> tumors (255), EBV has developed several strategies to evade T cell responses, for example by downregulating HLA expression, blocking antigen presentation pathways or creating an immunosuppressive TME. The latter is mediated by increased production of immunosuppressive

cytokines and/or increased expression of immune checkpoint molecules that are known to induce T cell exhaustion (229).

Glycoprotein programmed death ligand 1 (PD-L1) represents one of the several immune checkpoint molecules that is modulated by EBV and is used as a mechanism of immune evasion by many tumors. This occurs as a function of PD-L1 engagement with cell surface receptor programmed death 1 (PD-1 or CD279) expressed on T cells (256). PD-1 interacts typically with its ligand PD-L1 (CD274 or B7-H1) and less frequently with PD-L2 (CD273 or B7-DC) (257). While PD-L1 is expressed by a wide-range of cell types, the latter is expressed only by specific cell types, including DCs, mast cells and macrophages (258). PD-1 is an inhibitory receptor that is rapidly upregulated upon antigen-mediated T cell receptor (TCR) stimulation (259). Recent studies have identified PD-1 expression in a plethora of other immune cell subsets such as B cells, DCs, NK cells, and monocytes (260, 261). The PD-1/PD-L1 pathway plays a crucial role in immune tolerance by fine-tuning the quality and duration of T cell response thereby serving as a 'rheostat' of immune response (262, 263). This is partly achieved by counterbalancing the T cell activation signal triggered by binding of CD28 on T cells with CD80/CD86 on APCs. The binding of PD-1 receptor with its cognate ligands attenuates TCR signaling and leads to T cell exhaustion (264). This inhibitory interaction serves to protect target tissues from hyper-activated immune mediated damage. Tumor cells take advantage of this mechanism by frequently upregulating PD-L1 expression to escape the host anti-tumor immune response (265). Consequently, the PD-1/PD-L1 axis serves as one of the promising targets for immunotherapy in such malignancies. Infectious viruses such as Epstein-Barr virus (EBV), hepatitis C virus (HCV) and hepatitis B virus (HBV) leverage the PD1/PD-L1 pathway to facilitate escape of infected cells from the antiviral immune response (266). Consistently, PD-L1 expression is higher in EBV<sup>+</sup> relative to EBV<sup>-</sup> tumors in NPC, GC and DLBCL (180).

Several mechanisms have been reported for increased PD-L1 expression. These include alterations at the genetic level, specifically the amplification of the chromosomal region 9p24.1, which includes the genes PD-L1, PD-L2, and JAK2 (217, 267). Such genetic alterations have been associated with certain B-cell lymphomas and gastric cancer (268–271). Interestingly, in ~40% of cHLs, increased PD-L1 expression is not due to this amplification but attributed to upregulation by certain EBV-encoded gene products (272). The dysregulated expression of PD-L1 in cancer has been attributed to the oncogenic activation of multiple signaling pathways, including JAK/STAT, PI3K/Akt/mTOR, MEK/ERK, and Jun/AP-1 which can either act independently or synergistically to regulate PD-L1 expression (273–275). In addition, another important mechanism of PD-L1 upregulation is 3'-UTR disruption of PD-L1 by EBV insertion at this locus (276). EBV encoded genes are also known to modulate host immune responses. BZLF1 (Zta) can induce expression of host immunosuppressive

genes, such as *TGFB1*, which further downregulate expression of immune responsive genes, such as *TLR9*, *IFI6*, and *IL23A* (277, 278). LMP1 promotes AP-1, JAK-STAT and NF- $\kappa$ B signaling mediated activation of PD-L1 (279–281), suggesting that LMP1-mediated signaling might also be a key player in the immune escape strategy in cancers that express LMP1 (e.g. NPC, cHLs and DLBCLs). LMP1 also promotes proliferation and survival and LMP1-driven PD-L1 upregulation correlates with poor prognosis in certain lymphomas (282). Lack of LMP1 expression in EBV<sup>+</sup> eBLs, consequently, is associated with absence of PD-L1 expression observed in these tumors (283). Likewise, EBNA1 also modestly promotes IFN- $\gamma$ -induced PD-L1 overexpression in GC cell lines (284). The role of other EBV genes in inducing PD-L1 expression in EBV-associated cancers is less clear and further investigation is needed to determine how different viral gene products affect immune responses and PD-L1 expression in different EBV-associated cancers.

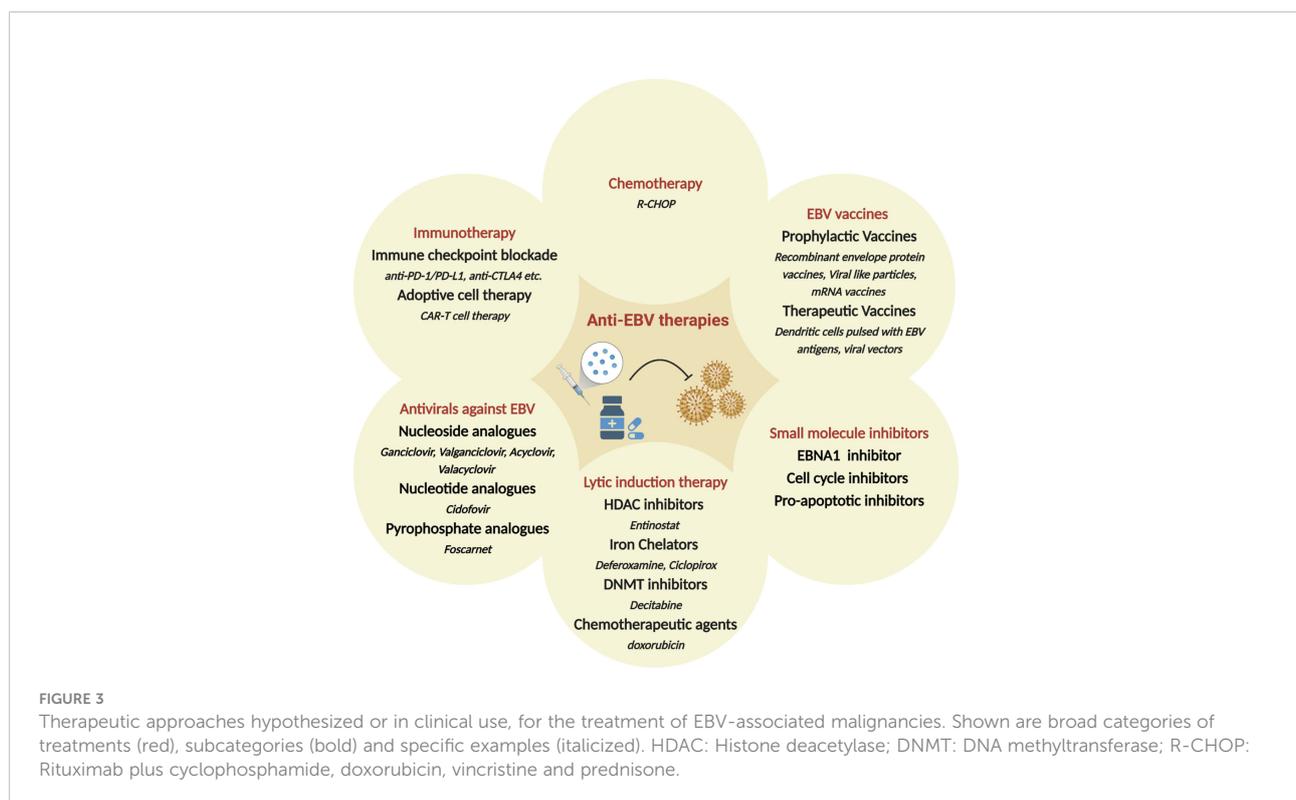
## Therapeutic strategies for targeting EBV-associated malignancies

Since EBV contributes to malignant cell transformation and is found in almost every cell of EBV<sup>+</sup> tumors, it has been considered a potential target for precision medicine and individualized cancer treatment. While non-specific chemotherapy is typically the first line of therapy, several additional strategies have been proposed

specifically targeted towards EBV<sup>+</sup> malignancies (Figure 3). These include i) antivirals against EBV; ii) small molecule inhibitors for EBV-encoded gene products, such as LMP1 and EBNA5; iii) induction of the lytic form of EBV replication in tumor cells in combination with prodrugs that are cytotoxic in lytically infected cancer cells; iv) enhancing the host immune response to viral antigens expressed by EBV-infected tumor cells; v) use of EBV vaccines. Additional strategies that are under consideration include induction of EBV episome loss by treating tumors cells with low-dose hydroxyurea and expressing toxic genes using EBV-dependent approaches (285, 286). There is also considerable enthusiasm for immune checkpoint therapies for the management of EBV associated cancers. Some are approved or under investigation in clinical trials for the treatment of NPC, GC, and HL. Based on initial trial reports, PD-1 targeting treatments, such as pembrolizumab and nivolumab, seem to improve longevity and/or partial response, especially in patients with PD-L1<sup>+</sup> tumors (287–293). Here, we will discuss some of the major therapeutic strategies for EBV<sup>+</sup> cancers, focusing on recent developments and highlighting current gaps and/or challenges.

## Chemotherapy

For lymphomas, such as BLs, the conventional chemotherapeutic regimen include R-CHOP (Rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone), CALGB (cancer and



leukemia group B), Hyper-CVAD +/- R (cyclophosphamide, vincristine, doxorubicin, and dexamethasone, with or without Rituximab), CODOX-M/IVAC +/- R (cyclophosphamide, vincristine, doxorubicin, methotrexate, ifosfamide, etoposide, and cytarabine, with or without Rituximab), and dose-adjusted (DA) R-EPOCH (rituximab, etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin) (294). Although BLs and HLs are generally curable, these regimens for relapsed aggressive B cell lymphomas are typically ineffective. Currently a novel chemotherapeutic approach is under investigation in a phase II clinical trial (NCT01964755) for relapsed EBV-associated lymphomas that utilizes a combination of drugs to potentiate the function of zidovudine (ZDV) to suppress NF- $\kappa$ B and viral latency.

Unlike in lymphomas, there is limited evidence on the clinical gains of chemotherapy alone in EBV<sup>+</sup> epithelial cancers. Surgery (typically gastrectomy) and chemotherapy remain the first line of treatment for patients with EBV<sup>+</sup> gastric cancer. Nevertheless, the efficacy of chemotherapy remains speculative. Corallo et al. reported that 6 EBV<sup>+</sup> GC patients who received fluorouracil and platinum as first-line of chemotherapy had a 3-year survival rate of 80% compared to 26.5% in EBV<sup>-</sup> GC patients (295). However, another observational cohort study with 31 patients reported an overall response rate of only 29% in metastatic EBV-GC patients who received taxane/trastuzumab, fluoropyrimidine and platinum as the first-line therapy (296). Owing to the single center nature of these studies and small sample sizes, it is necessary to confirm these observations in larger cohorts and clinical trials (297). From the limited evidence it seems that patients with EBV<sup>+</sup> GC have few metastases, longer survival, and high disease control rates. Although, chemotherapy helps some patients by increasing the frequency of event free and overall survival, it is still insufficient to treat EBV<sup>+</sup> cancers completely and eradicate infected cells. Combining chemotherapy with immunotherapy has provided encouraging preliminary results but further exploration and development of more effective combinatorial strategies are required.

## Adoptive cell therapy

Adoptive cell therapy (ACT) is a form of immunotherapy where specialized *in vitro* expanded or modified immune (usually T) cells are transferred to patients to enhance or repress immunity. These T cells are either specific for an antigen (e.g. viral protein or tumor-associated antigen) or are genetically engineered to express chimeric antigen receptor (CAR) or modified T cell receptor (TCR). The first ACT was performed in 1994, where donor leukocytes which included EBV-specific cytotoxic T cells (CTLs) were infused into 5 patients who had developed EBV-associated PTLN. Complete remission was observed in 5/5 patients, however all of them developed graft-versus-host disease (GVHD) due to alloreactive

T cells (298). Since then, significant progress has been made in the field of ACT for patients with EBV-associated PTLNs and the concept is expanding to include patients with NPC and HL, as summarized elsewhere (299). One of the strategies to minimize alloreactivity (i.e. to reduce the risk of GVHD) includes infusion of *in vitro* stimulated and expanded EBV-specific CTLs that are donor-derived (300, 301). Interestingly, adoptively transferred CTLs not only restore the anti-EBV immune response but can also establish long-term persistence (302, 303). Similar strategies for ACT have been developed for patients with NPC and HL, where CTLs specific to EBV latent antigens (e.g. EBNA1, LMP1, LMP2) are expanded *ex vivo* and infused into patients (304). Phase I/II clinical trials with such immunotherapy approaches have increased the overall survival of patient with recurrent or refractory NPC (305–307) and HL (308).

A modified version of ACT for the treatment of EBV<sup>+</sup> cancers is to engineer T cell receptors before transferring T cells to the patients. Such EBV-specific TCR-engineered T cell therapy is based on the rationale that TCRs on CD8<sup>+</sup> T cells can be re-engineered to specifically recognize EBV latent and lytic proteins. The stability and anti-tumor effect of these chimeric TCRs have been evaluated in murine models and provide encouraging results (309). For instance, T cells expressing LMP1-specific TCR inhibited tumor growth and prolonged survival in xenograft mice (310). Another type of ACT being widely investigated is EBV-specific CAR T cell therapy. CAR-T cell therapies targeting specific antigens, for instance CD19, CD20, CD22 and CD30, have provided encouraging results for treatment of lymphoma in clinical trials (311–313). One drawback of CAR T cell therapy is that CD8<sup>+</sup> T cells engineered with a CAR will also express their own native TCR, so the potential for auto-reactivity remains. Another the major limitation of CAR-T cell therapy is specificity for tumor-associated antigens, as some of these might be expressed by normal cells. This leads to adverse off-tumor toxicity, cytokine release syndrome and deficiencies in B-cell mediated humoral responses. Some of these limitations have been addressed by developing CAR-T cells that are specific to EBV antigens such as LMP1, since they would be specifically expressed in EBV infected malignant cells (314). In fact, LMP1-specific CAR-T cells exhibit enhanced tumor inhibition in LMP1-positive NPC xenograft mouse models (315). The translation of these therapies for the treatment of EBV-associated cancers warrants further evaluations before it can be prescribed as personalized immunotherapy for EBV<sup>+</sup> cancers in humans.

## Antiviral therapy

Several antiviral drugs have been identified and are being currently evaluated for clinical use. These can be broadly divided into three classes: 1) nucleoside analogs such as acyclovir (ACV),

ganciclovir (GCV), penciclovir (PCV), and their oral prodrugs valacyclovir (VACV), valganciclovir (VGCV) and famciclovir (FAM), respectively; 2) nucleotide analogs such as cidofovir (CDV); and 3) pyrophosphate analogs, including foscarnet. Although these antiviral agents have been clinically evaluated for different viruses, their clinical utility in the context of EBV-associated malignancies is lacking. To date, there is no effective Food and Drug Administration (FDA) or European Medicines Agency (EMA) approved antiviral therapy available for EBV infections. Nevertheless, we will briefly discuss some of these antiviral agents in the context of EBV-associated diseases. A more in-depth review of these drugs can be found published elsewhere (316).

Nucleoside analogs such as ACV and GCV inhibit EBV *in vitro*. The antiviral effect of ACV is attributed to the preferential incorporation of its triphosphate into the viral DNA due to high-affinity interaction with EBV polymerase compared to the cellular polymerase. This process irreversibly and specifically terminates viral DNA elongation and replication (317). The effective dose of ACV against EBV is orders of magnitude lower (0.3 μM) than host cells (250 μM) (318), resulting in a highly favorable therapeutic index and toxicity profile. The antiviral effect of GCV is greater than ACV but it is more toxic. Antiviral (e.g. ACV, GCV or VACV) prophylaxis has significantly reduced development of PTLN in high-risk EBV-seronegative lung transplant patients (319) and reduces EBV viremia in pediatric renal transplant patients (320). Clinical trials administering ACV along with prednisolone have shown the inhibition of EBV replication in the oral cavity, however they do not alleviate the duration or intensity of clinical symptoms (321). Importantly, none of the nucleoside analogs have any effect on latent infections. This is because the viral enzymes that are needed for the prodrug activity are not expressed during latent phase (322). Other nucleoside analogs with efficacy against varicella zoster virus, such as omaciclovir, have not yet been evaluated against EBV (323).

Cidofovir is a nucleotide analog that possesses both antiviral and antiproliferative properties and is metabolized into its active form by cellular kinases (324). The antiproliferative effect of Cidofovir on EBV-infected NPCs has been previously reported (325). Consistently, intra-tumoral injection of cidofovir suppresses tumor growth in EBV<sup>+</sup> NPC xenografts in nude mice (326). Another study showed that treatment of NPC (C15) and BL (Raji) cell lines with cidofovir decreases expression of LMP1 and EBNA2 oncoproteins and increases apoptosis and enhances ionizing radiation (IR)-induced regression of EBV<sup>+</sup> NPC and BL tumor bearing nude mice (327). Tenofovir (TFV) is an acyclic nucleoside/nucleotide analog that has already been approved for the treatment of HIV and HBV infection, where it acts as an inhibitor of the viral reverse transcriptase (328). The prodrugs of tenofovir, disoproxil fumarate (TDF) and tenofovir alafenamide (TAF), are both orally bioavailable and are more potent than ACV, PCV and GCV (329). All of these antiviral

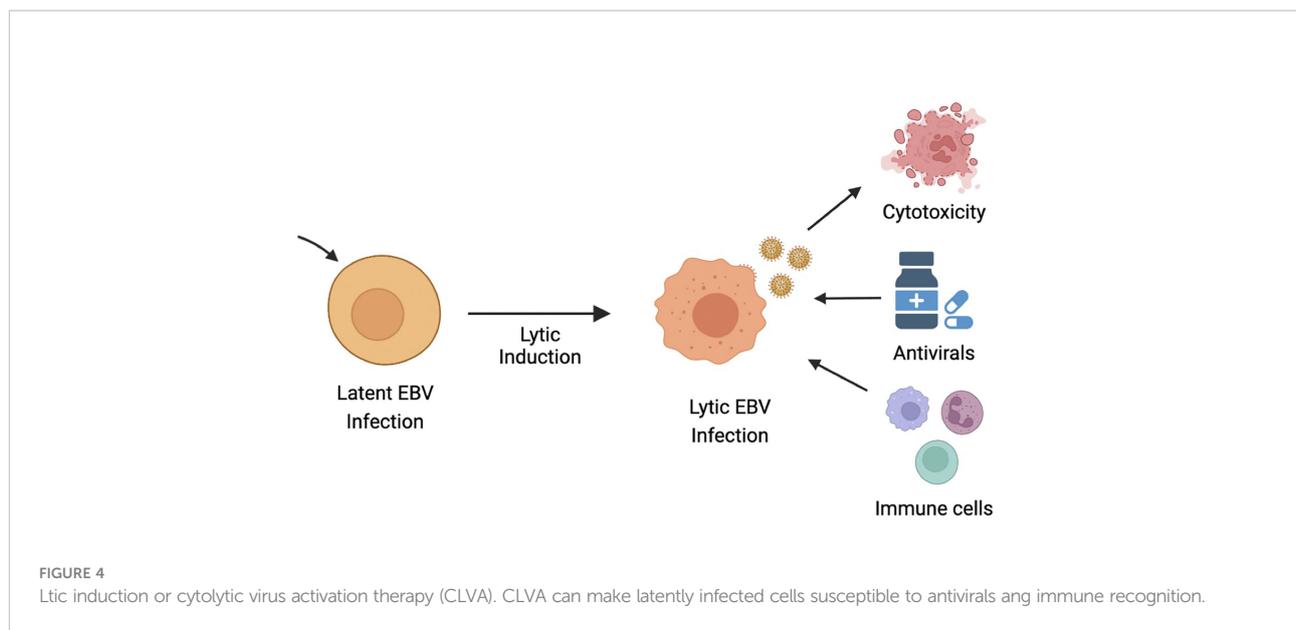
agents target EBV DNA polymerase, however, unlike others, the tenofovir prodrugs are metabolized independently of viral enzymes to their active forms and depend on host enzymes, thus, permitting their usage for latently infected cells (330). Despite the availability of a plethora of antiviral agents, there aren't any effective antivirals for EBV-associated cancers. However, studies on compounds like tenofovir holds some promise and warrants further study (331).

Foscarnet is a pyrophosphate analogue with a broad antiviral activity against the *Herpesviridae* family (332). As a pyrophosphate analogue, it disrupts viral DNA polymerase activity by inhibiting cleavage of pyrophosphate from the nucleoside triphosphate. Unlike ACV and GCV, foscarnet does not depend on viral protein kinases for its activity, making it useful in cases of ACV/GCV acquired resistance. However, it might be less tolerated in patients due to increased toxicity. Several case reports have shown benefits of foscarnet in treatment of EBV<sup>+</sup> PTLNs (333, 334). However, the systematic efficacy of foscarnet in treatments of EBV-associated needs to be further evaluated.

## Lytic induction therapy

As discussed, latent EBV infection is associated with human malignancies, such as BL, PTLN, NPC, GC, HL and non-HLs. GCV and ACV are commonly used antiviral drugs that require the EBV lytic encoded protein kinase (EBV-PK) and thymidine kinase (EBV-TK) for the conversion of pro-drugs into active viral drugs. As a result, these drugs are inefficient in eliminating EBV-infected cells that are in the latent state. One therapeutic approach is therefore the induction of EBV lytic replication, also known as cytolytic virus activation (CLVA), in combination with antiviral drugs to enable specific targeting of tumor cells that harbor EBV in a lytic state (18, 335). CLVA within infected tumor cells can induce i) a cytotoxic or cytostatic effect from the lytic viral proteins; ii) expression of viral enzymes that metabolize and activate antiviral pro-drugs, such as ACV and GCV; and iii) a range of antigenic viral proteins that can now be recognized by host-immune cells (336) (Figure 4).

Although, several classes of lytic inducers have been identified and their mechanism of action has been elucidated in different cell types, only one clinical study has reported a promising outcome in a small fraction of patients with EBV<sup>+</sup> tumors (337, 338). This is primarily because these compounds have three major drawbacks limiting their use in clinical settings. First, most of these compounds have low efficiency and can induce the lytic cycle in only a small percentage of cells, therefore a considerable proportion of cells are refractory (339). Second, the efficiency of these lytic inducers is heavily dependent on the cell type, thus cannot be broadly utilized for all EBV-associated malignancies. Many lytic inducers also have serious side effects, therefore their translation into clinical use is challenging. Lastly,



there is a concern that chemical induction of EBV could promote viral dissemination (340, 341).

Over the years, scientists have identified several classes of organic and chemical compounds that are able to induce the EBV lytic cycle in latently infected cells. Protein kinase C (PKC) activators (PMA), HDAC and DNA methyl transferase (DNMT) inhibitors, chemotherapeutic agents and anti-IgG are among the known classes of lytic inducers. Interestingly, evidence suggests that several distinct mechanisms of lytic induction may exist because synergistic effects have been observed when different lytic inducers are combined. For instance, treatment of BL and GC cell lines with combinations of PMA and sodium butyrate or valproic acid and cisplatin leads to significantly higher EBV lytic reactivation compared to individual treatments. Large-scale chemical library screens in GC and NPC cells have identified two additional distinct compounds that could induce EBV reactivation, one that resembles iron chelators and one that activates the MAPK pathway (342). Different classes of lytic inducers has been previously reviewed here (339). In this section, we will briefly discuss HDAC inhibitors and iron chelators that have been reported to induce the EBV lytic cycle *via* PKC- $\delta$  and HIF-1 $\alpha$  pathways, respectively (343).

### Histone deacetylase inhibitors

Of all the different inducers of the EBV lytic cycle, inhibitors of histone deacetylase (HDACi) have been well studied. These include sodium butyrate (NaB), valproic acid (VPA), suberoyl anilide hydroxamic acid (SAHA or Vorinostat), and romidepsin. Use of HDACi alone or in combination with GCV are currently being tested for use in patients. For example, butyrate in combination with GCV has shown promising results in patients with refractory EBV<sup>+</sup> lymphoid malignancies (344).

However, the efficacy of this combination therapy has received limited success *in vivo* due to the poor oral bioavailability and short half-life of butyrate (345). A systematic study of HDACi have ranked panobinostat, belinostat, butyrate, entinostat, oxamflatin, apicidin, and largazole from highest to lowest in their ability to induce EBV-TK and EBV-PK kinases, suggesting that despite the structural diversity, most HDACi can function as inducers of EBV lytic replication (346). Interestingly, despite belonging to the same class, these HDACi might invoke different mechanisms of action to induce EBV reactivation. For instance, VPA antagonizes the ability of other HDACi to induce EBV lytic reactivation (347).

### Iron chelators

Iron is a nutrient that plays an important role in various aspects of cell biology including growth and differentiation. It is used by heme-containing proteins and serves as a cofactor for many enzymatic activities. Desferioxamine is a widely used drug to treat iron-overload. Desferrithiocin, an orally available iron chelator, is a more potent substitute of Desferioxamine due to higher bioavailability. Both these compounds adversely inhibit proliferation of T cells, which can be rescued by the addition of iron (in the form of ferrous chloride, FeCl<sub>2</sub>) (348). Iron chelators induce the EBV lytic cycle in certain cancer cells by inhibiting enzymatic activity of protein hydroxylases (349). A novel compound, named C7, has been recently described and reported to induce early, but not late, lytic proteins *via* intracellular iron chelation, alleviating the concern about viral dissemination (342, 350). However, C7 seems to direct only a small proportion of cells into the lytic cycle. Moreover, due to the abundance of iron *in vivo* and within the TME, delivery of iron chelators into tumors remains challenging, suggesting that

further studies are needed to identify novel compounds and/or overcome these limitations.

## Small molecule inhibitors

Due to highly penetrant effects, small molecule inhibitors have been of great interest in treating various cancers. In viral-associated cancers, essential viral proteins are great targets because inhibitors against them might have lower toxicity against host cells. In EBV-associated cancers, since EBNA1 has served as a promising target for virus-targeted therapies as it has no cellular homologue and is constitutively expressed in all EBV<sup>+</sup> cancer cells. There are several ways by which EBNA1 can be functionally perturbed, and significant advances are currently being made in the field of EBNA1 targeting therapies. These include inhibiting DNA binding activity of EBNA1 (351, 352), disrupting homodimerization (353), blocking its interaction with key host cellular proteins, such as USP7, CK2 or targeting its oncogenic partners, such as MDM2 (354). VK-2019 is a small molecule inhibitor that binds to EBNA1 and disrupts its DNA binding activity. It is administered orally and is currently undergoing phase 2 clinical trial (NCT03682055) for patients with advanced NPC. Further studies are needed to identify specific inhibitors of other latent EBV proteins, including LMP1, or viral genes that are essential for EBV transformation.

## Prophylactic EBV vaccines

To our knowledge, there are currently no FDA-approved vaccines for EBV. Since EBV is a causative agent for a range of diseases a prophylactic or preventative vaccine would be the most beneficial and cost-effective therapeutic approach to manage EBV-associated IM, malignancies and autoimmune diseases. The rationale for prophylactic vaccination is to prevent EBV infecting its target cells by inducing an antibody response. Below we will briefly discuss strategies for developing prophylactic vaccines against EBV.

Considering that EBV requires multiple envelope proteins to enter target cells, these serve as excellent candidates for developing recombinant envelope protein vaccines. EBV glycoprotein gp350 (BLLF1), is the most abundant envelope protein, and initial studies on EBV vaccine development primarily focused on gp350 (355, 356). However, a large-scale clinical trial using soluble gp350 failed in preventing infection, albeit reducing the development of IM after EBV infection (357). In addition, gp350 vaccine candidates only protect B cells but no other EBV target cells (e.g. epithelial cells) from infection. EBV glycoproteins gH/gL and gp42 bind to HLA-DR on B cells and integrins and ephrin receptor A2 on epithelial cells, respectively, and facilitate EBV fusion to the cell membrane (70, 358–360).

Since, these viral glycoproteins are integral components of the core viral fusion machinery, they also serve as excellent candidates for prophylactic vaccine development (299). To this end, in 2019, Bu et al. developed an EBV gH/gL/gp42 based nanoparticle vaccine. This vaccine inhibits EBV infection of both epithelial and B cells by eliciting an antibody response that targets the virus membrane-fusion proteins in mice and non-human primates-macaques (356). This year, a novel EBV gp350-ferritin nanoparticle vaccine was developed by researchers in NIAID/NIH that has entered the Phase I clinical trial to determine its safety and immunogenicity in humans (NCT04645147). Taken together, these studies suggest that targeting multiple EBV glycoproteins - gH/gL, gB and gp350 - together could synergistically induce highly effective EBV neutralizing activity. Additionally, evidence suggests that these viral glycoproteins can also induce T cell immune responses to further enhance vaccine efficacy by recruiting T cells to either kill or inhibit transformation of recently infected cells if neutralizing antibodies are ineffective, for example due to variations in EBV protein sequences (361, 362).

Recombinant viral vectors are also commonly used to develop therapeutic vaccines. Essentially, these are live viruses that are engineered to express specific proteins that help elicit an immune response. Such vaccines can infect target cells and induce a CD8<sup>+</sup> T cell response, enhance the anti-inflammatory response by serving as adjuvants themselves and have high gene transduction efficiency (363). The first EBV vaccine was developed in 1995 and tested in humans. This was a live recombinant vaccinia-based virus, expressing EBV envelope protein BLLF1/gp350 (364). However, this vaccine was discontinued due to adverse effects. In 2004, Taylor et al. developed a chimeric antigen construct using a modified vaccinia virus “Ankara” (MVA) vector that encoded the C-terminal portion of EBNA1 and entire LMP2 (MVA-EL). Upon transduction, the EL protein can be processed by HLA I and II, resulting in CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses (365). Since these two EBV latent proteins are expressed in NPC, MVA-EL was tested for safety and immunogenicity as a therapeutic vaccine for patients with EBV<sup>+</sup> NPCs in phase I clinical trials (NCT01147991). Indeed, this vaccine was well tolerated and induced EBV-antigen specific T cell responses in 8/14 patients in UK and 15/18 patients in Hong Kong (366). Further studies are needed to determine its translation to the clinical setting for treatment, either alone or in combination with other modalities including T cell therapies. In 2012, another group developed and evaluated the ability of a recombinant adenoviral vector-based vaccine (Ade1-LMPpoly) to induce EBV-specific T cell responses in recurrent or metastatic NPC in a phase I clinical trial (ACTRN12609000675224). Encouragingly, EBV-specific T cells were expanded in 16/24 NPC patients and infusion of Ade1-LMPpoly-generated T cells was tolerated and prolonged survival by 2.3-fold. A phase II randomized clinical trial is necessary to confirm these observations (367). A

comprehensive list of current vaccines is available here (368, 369).

In addition to the EBV envelope protein-based vaccine and recombinant viral vectors, development of viral like particle (VLP) vaccines is another area of active research. The design of such viral particles is based on the rationale that a non-infectious version of EBV will elicit an EBV-specific innate and adaptive immune response in a safe and effective manner (370). Mechanistically, VLPs are phagocytosed and processed by DCs. DCs then activate CD8<sup>+</sup> and CD4<sup>+</sup> T cells by presenting viral antigens on HLA class I and II, respectively (371). While CD8<sup>+</sup> T cells mount a cytotoxic response, CD4<sup>+</sup> T cells elicit an anti-tumor response *via* Th1 and Th2 type responses that produce pro-inflammatory cytokines (e.g. IFN- $\gamma$ , TNF- $\alpha$ , and IL-4, IL-10, respectively) (371). The status of VLP developments against oncoviruses and their biological and chemical characterization has been recently reviewed (372). In 2015, a novel EBV vaccine based on the Newcastle disease virus (NDV) VLP platform was developed, consisting of EBVgp350/220 ectodomain fusion protein that structurally mimicked EBV. This VLP elicited a long-lasting neutralizing antibody response in mice, but the responses were comparable to soluble gp350/220 (373). Thus, a more immunogenic VLP was developed that incorporated additional EBV glycoproteins and latent antigens – EBNA1 and LMP2. Immunization with gH/gL-EBNA1 and gB/LMP2 VLPs produced high neutralizing antibody titers *in vitro* and EBV-specific T cell responses in vaccinated BALB/c mice (374). DNA-free VLPs/LPs typically consists of EBV structural proteins that are weakly immunogenic towards CD8<sup>+</sup> T cells. As a result, humoral and cell-mediated immune responses that recognize these structural proteins offer limited to no protection against latently infected cells. Another strategy is to use EBV particles themselves for VLP vaccines. The first EBV VLP was created by removing the terminal repeats that result in production of large amounts of defective viral particles without the viral DNA that could bind to both B and epithelial cells (375, 376). In subsequent studies, more viral packaging proteins (BFLF1, BFRF1, BBRF1) and viral oncogenes (EBNA2, 3A, 3B and 3C, LMP1 and BZLF1) were deleted to improve the safety profile of these VLPs, while maintaining immunogenic potential (377, 378). In 2018, a more immunogenic EBV VLP was created by fusing EBNA1 and EBNA3C to the EBV tegument protein BNRF1. As a result, only 14% of mice vaccinated with modified VLPs had detectable viral load in the peripheral blood compared to 100% of the control PBS-vaccinated mice (379). While, VLP-based therapeutics are being developed and evaluated in early clinical trials, none have yet reached the phase III efficacy clinical trial stage. This is attributed to two major limitations. First, they suffer from production efficiency and scalability, which is partly because of the use of mammalian cells that lead to low viral titers and the presence of contaminants from human producer cell lines. Second, they offer low immunogenicity as epitope-based vaccines. This necessitates

the need to co-administer an adjuvant or design better antigen delivery systems. Nonetheless, there are some promising ongoing studies on EBV-derived VLP vaccines to prevent EBV<sup>+</sup> cancers.

With the success of mRNA vaccines against SARS-CoV2, researchers have developed an EBV mRNA vaccine based on the same platform. Moderna has recently launched phase I clinical trial of its EBV mRNA vaccine (mRNA-1189) that have shown high EBV neutralizing antibody titers in mice (NCT05164094). This investigational vaccine targets EBV glycoproteins - gp350, gB, gH/gL and gp42 - and is hypothesized to prevent IM and EBV infection. Despite the potential challenges with mRNA-based approach for an asymptomatic virus like EBV (299), results from these trials will help in developing effective preventative treatment approaches for EBV infections and related disorders.

## Preclinical models to study EBV biology

There are several malignancies that associated with EBV infection. Despite decades of research in this field, the precise role of this virus in the tumorigenic process and immunoevasion is not fully understood. While EBV<sup>+</sup> cell lines, including LCLs, serve as a good model system to study EBV-host biology *in vitro*, mouse models help investigate and understand EBV-specific biology *in vivo*. Since the  $\gamma$ -herpesvirus have co-evolved along with their hosts, i.e. humans and monkeys, there is a lack of suitable counterparts in rodents (380). Unfortunately, the murine  $\gamma$ -herpesvirus 68 (MHV-68) lacks EBV's transforming ability and thus fails to recapitulate EBV-induced tumorigenesis (380). Consistently, major differences in molecular mechanisms, tumorigenesis, cellular tropism, and immune responses between MHV-68 and EBV infections have been observed (381). As such, murine models used to study EBV-associated malignancies are typically immunodeficient. Transferring human peripheral blood mononuclear cells (PBMCs) from EBV-seropositive human donors into immunodeficient mice can generate PBMC-derived EBV<sup>+</sup> B cell tumors (382). Zhang et al. further developed a genetically engineered mouse model to study EBV-driven lymphomas found in immunosuppressed patients (383), underscoring the relevance of immunodeficient murine models for the study of EBV disease (382). However, since these mice are immunocompromised, they are unable to induce a host immune response. Transferring human PBMCs to overcome this barrier often leads to severe xeno-graft versus host disease (GVHD) in these mice, limiting the duration of the study (384). Recognizing these limitations, the field has shifted to developing and using lymphocyte-deficient mice which have the potential to be reconstituted with human immune components (385). These mice are valuable resources to investigate human specific viral infections, as well as develop and test therapeutic vaccines (386).

A more comprehensive review of murine models for EBV-associated tumorigenesis can be found elsewhere (386, 387). Despite being an excellent model system for EBV-associated lymphomas, these mice are not suitable to study EBV<sup>+</sup> epithelial cancers since the mice lack human epithelial cells. In these cases, nonhuman primate animal models might be of justified use. These have been reviewed by others (388).

## High-throughput approaches to study EBV-associated cancers

With the advent of massively parallel sequencing technologies, researchers have been able to better characterize the complexity of interactions between host and viral genes and identify novel genomic and epigenomic alterations within EBV-infected cancer cells that are druggable. Specifically, these technologies enable the capture of all the genetic material inside host cells, which can then be used to simultaneously study the biology of both host cells and infecting viruses (180, 389–392). These studies specifically overcome a limitation of laborious traditional EBV genetic studies where only individual viral or host genes are studied in isolation outside of the tumor context. In this section, we will discuss some of the sequencing approaches that are used to explore the genetic, transcriptomic and epigenomic landscapes in the context of EBV-associated malignancies.

### Whole genome sequencing

The investigation of EBV genome sequences is important due to their association with several human malignancies (165). Prior to 2013, GenBank had whole genome sequences from less than 10 strains of EBV (393). Conventional sequencing techniques were inefficient, expensive and could help investigate only a few viral genes at a time. They typically involved digestion of genomic DNA *via* restriction enzymes, cloning and Sanger sequencing. Moreover, the large size of the EBV genome (~172kbp) added to the cost and time. Whole genome sequencing (WGS) technologies marked a new era of EBV genome sequencing and could be performed with or without EBV enrichment (180, 394). The WGS approach is quite sensitive to detecting viral derived sequences as well as integrated viral regions within the host genomes. As such, WGS has not only helped identify distinct EBV variants, mutations and oncogenes but has also allowed for a comprehensive survey of EBV integration in a wide variety of human malignancies as reviewed here (395, 396). Additionally, WGS of EBV-associated

tumors have been extremely informative in identifying a range of distinct host variations that promote cancer (65, 186, 214, 217).

### RNA sequencing

The use of high-throughput sequencing technologies to study the transcriptomic landscapes of disease have become a common practice. The general workflow for such techniques begins with bulk RNA extraction from the biospecimens under investigation, followed by RNA selection (mRNA or ribosomal-free RNA), cDNA synthesis, library preparation and sequencing. In the case of viral-associated malignancies, such techniques have provided key insights into cellular genes and pathways that are affected by virus (397, 398). Additionally, since these sequencing technologies are agnostic of the origin of the RNA within the cells, they capture genetic materials of both host and infecting viruses (391, 392, 399). As such, transcriptomics studies of EBV-associated malignancies have revealed important aspects of EBV biology including expression program and its interactions with the host to promote disease (180, 390, 400, 401). For example, a large-scale transcriptomic study of EBV-associated cancers classified EBV<sup>+</sup> cancer types into molecular sub-types according to activation or repression of interferon signatures which was correlated with expression of several immune checkpoint genes such as PD-L1 and IDO1 (180).

Of note, the heterogeneity of cells within the biospecimens, specifically tumors and their microenvironments, present a challenge for interpreting bulk transcriptomics data. For example, a change in expression of a gene or activation of a pathway could reflect either a change in tumor cells and/or a change in the cellularity of the TME, for example by immune cell infiltration. Recent computational tools that can deconvolute the composition of cells from bulk data have provided some remedy for this issue (402–404). Nevertheless, the accuracy of these tools remains limited, and they have not yet widely applied to study TME in EBV-associated malignancies. The advent of single-cell RNA-sequencing (scRNA-seq) has overcome this challenge and has revolutionized the field of transcriptomics and helped scientists map and generate individual cell atlases (405). Specifically, scRNA-seq has enabled the study of host-pathogen interaction in viral-associated diseases as well as cellular heterogeneity (406, 407). scRNA-seq is rapidly being adopted to study EBV-associated malignancies. For example, recent studies have revealed the landscape of both tumor and infiltrating immune cells in NPCs that are associated with prognosis (234, 235, 408). Additionally, scRNA-seq are now used to delineate rare cell subpopulations such as cancer stem cells, cell-cell interactions *via* receptor-ligand analyses, cell differentiation *via* trajectory and time-resolution analyses

(409). Additional steps also allow TCR, BCR and cell-surface protein expression (CITE-seq) sequencings at the single cell level to supplement scRNA-seq for the study of the diversity of immune cells. Given these technologies, it is exciting to get detailed understanding of how EBV affects these aspects of biology across various diseases and conditions, such as response to therapeutic treatments. It is important to point out that the detection of lowly expressed genes in scRNA-seq is challenging and faces a frequent “drop-out” where it might be only sporadically detected across different cells. This could be an issue for detecting EBV genes in malignancies associated with latency where most EBV genes are expressed at low levels.

## Other sequencing technologies

Transcriptional regulation, epigenetic changes and physical interactions are paramount to EBV biology and understanding EBV-associated diseases. Appending high-throughput sequencing to traditional lab techniques such as chromatin immunoprecipitation (ChIP) has enabled genome-wide detection of transcription factor (TF) bindings and epigenetic changes, such as histone modifications and DNA methylations. For instance, ChIP-seq of EBV TFs has revealed many binding sites across the host genome, which has implications for pathogenic mechanisms (410). Conversely, many cellular TFs can also bind the EBV genome (390). ChIP-seq analysis of EBV infected GC and NPC cell lines reveal a redistribution of characteristic histone marks such as H3K4me1/3 and H3K27ac (411). Of note, one of the limitations of these technologies is their requirement for millions of cells, however, recent technologies such as CUT&RUN/Tag sequencing have resolved this issue. Additional adjustments to high-throughput sequencing technologies by ligating proximal chromatin regions (e.g., Hi-C) has enabled to study physical interactions between genomic loci. This technology has enabled the study of interactions between EBV episomes and host genomes that are consequential to host gene regulation (412, 413). There has been a large number of additional assays deployed to study specific aspects of EBV biology genome wide (414, 415). For example, assay for transposase-accessible chromatin using sequencing (ATAC-seq) has been developed to study chromatin accessibility and has been extensively used to study how EBV infection affects chromatin accessibility. It is important to note that most of these assays utilize bulk sample processing and therefore observations in heterogeneous cell populations should be carefully interpreted, as discussed above for bulk RNA-seq. Recently, the limits of some of these technologies have been pushed to the single cell level and integrated into scRNA-seq platforms to enable multiomics based investigation of EBV infection (409).

## Concluding marks

As the above studies demonstrate, EBV is a causative agent and/or associated with a plethora of diseases including cancer and autoimmunity. Despite decades of research, the underlying mechanisms governing how the interactions between EBV and host cells promote carcinogenesis are incompletely defined. As a result, effective and individualized treatments for EBV-associated diseases still remain either non-specific or lacking. Nevertheless, it is also obvious that EBV is heavily regulated by a variety of factors, and it extensively regulates cellular processes and the microenvironment. High-throughput methods are ideal for revealing complex networks of tissue and disease associations. As discussed, these technologies have their own limitations. Utilizing orthogonal and multiomics technologies can typically overcome some of these limitations. For example, one of the limitations of single-cell transcriptomics is the loss of spatial information during the tissue dissociation, which are important for understanding disease biology. Recent high resolution (down to the sub-cellular level) spatial transcriptomics methods can help overcome such issues, however, due to their recent development, they have not yet been employed to study EBV-associated diseases. Additional factors such as the microbiome might also be relevant to EBV-disease biology (416) and should be considered in designing tools and models. Rigorous computational modeling is also needed to accurately identify shared or tissue-specific signatures across EBV-associated diseases. Lastly, as discussed above, better animal models and drug delivery systems are needed in order to translate laboratory findings.

## Author contributions

All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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