Mechanisms of B-cell oncogenesis induced by Epstein-Barr virus

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Abstract

Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus, which asymptotically infects majority of the world population. In immune-compromised conditions, EBV can trigger human cancers of epithelial and lymphoid origin. The oncogenic potential of EBV is demonstrated by in-vitro infection and transformation of quiescent B-cells into lymphoblastoid cell lines (LCLs). These cell lines, along with primary infection using genetically engineered viral particles coupled with recent technological advancements have elucidated the underlying mechanisms of EBV-induced B-cell lymphomagenesis.
Introduction

Epstein-Barr virus (EBV), also known as human herpes virus 4 (HHV4), is highly immunogenic with >95% of the world population found to be seropositive (82). Primary infection occurs in oropharyngeal epithelial cells; however, EBV predominantly infects B-lymphocytes. Within the immune-competent host, virus persists in naïve memory B-cells in a non-pathogenic state for the lifetime of the host. Intermittently, these virus infected memory B-cells differentiate into plasma cells ensuing in lytic-cycle activation promoting infection of other resting B-lymphocytes (29). In the immunocompromised host like post-operative organ-transplant and HIV-infected patients, EBV-infection demonstrated a strong association with several B-cell lymphomas (9). In addition, the list includes, endemic/sporadic Burkitt’s lymphoma (eBL/sBL), diffuse large B-cell lymphoma (DLBCL), classical Hodgkin lymphoma (cHL), primary central nervous system lymphoma (PCNSL), primary effusion lymphoma (PEL) and plasmablastic lymphoma (92, 97). These lymphomas exhibit a distinct expression pattern of latent genes. For example, EBV associated post-transplant lymphoproliferative disorder (PTLD), PCNSL and a fraction of DLBCL typically express a full repertoire of latent genes encoding six nuclear (EBNA1, -2, -3A, -3B, -3C, and -LP) and three membrane (LMP1, -2A, and -2B) proteins along with several untranslated RNAs, recognized as latency-III program (44). HL and BL are characterized by a more restricted pattern of latent gene expression. While HL is associated with EBNA1, LMP1 and LMP2 expressions (latency-II), BL predominantly expresses EBNA1 (latency-I) (23, 40). Both the coding and non-coding viral transcripts with varying potency, simultaneously affect multiple signaling cascades accompanied with genetic/epigenetic alterations leading to various EBV-driven B-cell lymphomas. The latency patterns of EBV gene expression in different B-cell lymphomas are summarized in Table-1.

Studies indicate that EBV also affects the lymphoma microenvironment, in which the latent oncoproteins manipulate cell machineries favoring the lymphoma cells for an immune escape and proliferation (86, 106). The interaction between EBV-infected lymphoid cells and the tumor microenvironment offers promising therapeutic targets.
The transforming ability of EBV was discovered soon after its discovery from BL patient’s samples (107). The process of transformation of primary B-cells \textit{in-vitro} has been used to establish EBV transformed lymphoblastoid cell lines (LCLs) over many decades for genetic studies. These LCLs contain donor specific genetic alterations. The viral gene expression pattern in LCLs is similar to that of B-lymphoblasts isolated from patients having PTLDs, PCNSLs and a fraction of DLBCLs (44, 82). Therefore, LCLs are being used as a surrogate \textit{in-vitro} model for studying the EBV-induced B-cell transformation process and subsequent lymphoma development. Since culturing cells in laboratory conditions for long time may introduce further genomic instability, LCLs at early passage would be a better choice for functional validation and follow-up investigation into clinical samples.

**Recombinant EBV Bacmids**

Using a Bacterial Artificial Chromosome (BAC) system, the whole viral genome can be easily propagated in Escherichia coli (27, 42). Additionally, any desired mutations can be introduced into a specific viral gene locus. A number of labs across the globe utilized this strategy delineating the precise function of a particular viral gene in B-cell transformation, or maintenance of outgrowth of transformed B-cell blasts. While, in most cases, the B95.8 EBV-strain was utilized for the generation of BAC clones, there are examples where researchers used EBV-DNA from a BL-line AKATA (42). The EBV-BAC clones, typically maintained in an epithelial cell background (HEK293/T) under antibiotic selection, are induced by either over-expressing an immediate early viral-protein, BZLF1 (78) or treating cells with chemical inducers - a protein kinase C inhibitor, tetradecanoyl phorbol acetate (TPA), plus a HDAC inhibitor, sodium butyrate (25, 100). Occasionally, an immunosuppressive drug (FK506) is also used to facilitate the infection (33).

In our system, a GFP-cassette was introduced to examine viral-infection and to sort infected cells from uninfected populations (27). In other systems several B-cell antigens are used to validate viral infection and the subsequent B-cell immortalization. These markers include surface antigen B-cell activation markers CD23, CD40, CD44 or the intracellular B-cell proliferation marker Ki-67 (27).
CD40 plays an important role during B-cell activation by providing survival signals through its interaction with the CD40 ligand (CD154) expressed on the surface of activated T-cells (36).

Interestingly, LMP1 functionally mimics CD40 receptor-mediated signaling pathways and profoundly contributes to the formation of B-cell blasts (61). The early events of EBV-infection in primary B-lymphocytes provide a model for B-cell activation and downstream signaling processes as well as the specific contributions of individual viral genes during B-cell transformation.

**B-cell transformation**

EBV-mediated B-cell transformation is associated with global alteration of both viral and cell gene expressions (30, 81). During initial infection of primary B-cells, almost all the genes including lytic and latent are expressed. While the DNA within the viral particle is unmethylated, in latently infected B-cells progressive methylation of the viral-DNA regulates promoter usage and transcriptional repression (3). In cell, the viral-DNA is associated with nucleosomes, collectively contributing to the restricted viral gene expression (2), while, during initial phase of infection the entire viral-DNA is accessible to the cellular transcription machinery and thus, many viral genes are simultaneously expressed (104). Importantly, during latent infection EBV undergoes intermittent lytic replication ensuring newer infection of the surrounding B-cells. Additionally, lytic antigens are also closely associated with B-cell transformation (45) and accordingly removal of important lytic genes significantly affect B-cell transformation (2, 32, 46, 102).

Besides differential viral gene expression pattern, cellular gene expressions along with global epigenetic landscape are also largely affected (30, 81). For example, a drastic reduction of heterochromatin marks associated with transcriptional activation was observed during the initial phase of infection in quiescent B-lymphocytes (30). In contrast, EBV-infection leads to a global increase in promoter methylation of tumor suppressor genes (TSGs), leading to aberrant proliferation and transformation of the infected B-cells (81).
EBV transforming antigens

Using various genetically engineered EBV and in-vitro infection models, five viral latent antigens - EBNA2, EBNALP, EBNA3A, EBNA3C and LMP1 are shown to be essential for efficient B-cell transformation (44, 82, 107). Other latent antigens and several non-coding RNAs also influence B-cell transformation and subsequent maintenance of B-cells outgrowth. Below we will discuss how modern genetic engineering strategies and in-vitro infection, or transformed LCL-model progressively revealed the importance of viral transcripts in B-cell transformation, and subsequent development of B-cell lymphoma. Table-2 and Fig-1 elucidate the major mechanisms associated with EBV-latent transcripts.

EBNA1 – Since EBNA1 is essential for DNA replication and maintenance of the viral latent genome, its expression expectedly has been demonstrated in all forms of latency programs (107). EBNA1 binding to viral episomal origin of replication (OriP) recruits numerous cellular proteins including DNA replication machinery ensuring appropriate duplication of viral genome during each cell-cycle. While, in latency-III, EBNA1 expression is maintained by the Cp-promoter, in latency-I, its expression is regulated by the Qp-promoter (93). EBNA1 can coordinate the switch between different latency programs through promoter selection coupled with extensive epigenetic regulation (22). A genome wide ChIP-seq analysis demonstrated that a chromosome insulator protein CTCF is involved in regulating EBNA1 mediated promoter switch and silencing of the Qp promoter in latency-III associated B-cells (93).

Moreover, EBNA1 can induce transcription of various cellular genes (8, 26), and contribute to the altered regulation of telomeres on cell chromosomes (41). The Glycine–Alanine repeat region of EBNA1 responsible for resistance to proteasome mediated degradation plays an important role in regulation of MHC class-II presentation to cytotoxic T-lymphocytes (CTLs) (55, 70). This repetitive region also causes an indirect activation of c-Myc expression by PI3-kinase (PI3K)-signaling pathway (24). EBNA1 binding with ubiquitin-specific protease USP7 influences p53 and Mdm2 expression. This results in regulation of anti-apoptotic activity, possibly through promoting Survivin expression...
Despite these critical activities, using recombinant virus, EBNA1 was shown to be not essential for in-vitro B-cell transformation. However, EBNA1 expression enhanced the capability of the virus to drive B-cell transformation and the severity of associated lymphomas (34).

**EBNA2 and EBNALP** – EBNA2 along with EBNALP are the first latent genes expressed after B-cell infection (44). EBNA2 represents the major viral transcription factor responsible for activating the expression of the entire repertoire of latent transcripts together with several host genes through employing cell transcription factors - RBP-Jκ and EBF1 (57). EBNALP simultaneously assists EBNA2 mediated transcriptional activity through blocking the NCoR and RBP-Jκ occupancy at the genome (76, 112). However, genome wide ChIP-sequencing analyses in LCLs demonstrated that only one third of the EBNALP-sites are co-localized with EBNA2-sites, indicating the complicated nature of B-cell transformation induced by EBV-infection (76, 112). EBNA2, most prominently contributes to the B-cell proliferation through transcriptional activation of approximately 300 cell genes such as MYC and RUNX3 transcription (105, 110). Importantly, this transcriptional activation is regulated through super-enhancers, characterized by dense clusters of several transcription factors coupled with enhanced signals for H3K27ac histone activation mark (113). In contrast, EBNALP sites were occupied by RNA polymerase II, histone acetylase (HAT) p300, transcription factors such as SP1, PAX5, BATF, IRF4, PU.1, CTCF, RBPJ, NF-κB along with several histone activation marks including H3K4me3, H3K27ac, H2Az and H3K9ac [(64), and reviewed in (44)].

**EBNA3 family proteins** – The EBNA3 family of proteins consisting of EBNA3A, -3B and -3C, are transcription factors that precisely regulate host gene transcription and B-cell proliferation particularly in immunosuppressive setting [reviewed in (1, 6)]. It is believed that EBNA3 gene family begun from cyclic duplications of an ancestral gene. Initial studies revealed that EBNA3A and EBNA3C, but not EBNA3B cooperate with oncogenic Ha-Ras for transformation and immortalization of rat embryonic fibroblasts (31, 71). Later, genetic studies revealed that EBNA3A and EBNA3C are necessary for B-cell transformation, whereas EBNA3B is dispensable (10, 96). An added complication to this idea came from a more recent finding that EBNA3B functions rather as a tumor suppressor in...
a humanized-mouse model NOD/SCID/γc-/- through assisting T-cell surveillance (101). In fact, tumors induced by EBNA3B knockout virus demonstrated a lack of T-cell infiltrate and related activation of the chemokine CXCL10 (101). In contrast, EBNA3A and EBNA3C cooperatively act as predominant viral oncoproteins through regulating cellular gene transcription. Although functionally diverse, EBNA3-proteins share significant sequence similarity (~30% at the N-terminal domain) and selection of cellular binding partners (6). Despite the sequence similarity, EBNA3C depletion can only be rescued by EBNA3C itself to maintain LCLs outgrowth (63). This phenomenon is also true for EBNA3A (63).

Initial experiments described that EBNA3 proteins negatively regulate EBNA2 mediated gene transcription through interaction with RBP-Jκ (99). Later, EBNA3A and EBNA3C were shown to interact with a long list of cellular proteins, and transcription factors involved in regulating multiple cell-signaling pathways. Additionally, although the functional relevance is still not clear in terms of B-cell lymphomagenesis, EBNA3C can form a complex with both EBNA3A and EBNA3B (72). The interacting partners for EBNA3C include transcription factors, chromatin modulators - both histone deacetylase and histone acetylase enzymes, cell-cycle proteins involving G1-S and G2-M transitions, metastasis suppressor, post-translational modifiers, E3-ubiquitin ligase, ubiquitin specific proteases, unfolded protein response (UPR) regulator, cell tumor suppressors and oncoproteins [(74, 75), also reviewed in (1, 6, 83)]. Similar to EBNA3C, EBNA3A also interacts with numerous cellular proteins, such as transcription insulators, cell-cycle regulators, members of the ubiquitin protease complex, chaperones and a number of proteins with unknown functions connecting to EBV-induced B-cell lymphomagenesis [reviewed in (1, 6)]. These viral proteins do not have specific binding sequence similarities but regions associated with them are found to be occasionally functionally overlapping, indicating that both EBNA3A and EBNA3C employ complex oncogenic mechanisms have collaborative activities. Importantly, we and others using various genetically engineered BACmids expressing EBNA3C mutants, as well as trans-complementation assays validated the in-vitro biochemical studies and demonstrated the importance of these binding regions during initial infection or maintenance of LCLs outgrowth (31, 45). For example, earlier EBNA3C was shown to
form a complex with Chk2 and thereby manipulates the G2/M phase of the cell-cycle (12). Later, using a conditional knockout virus, EBNA3C was shown to block ATM/Chk2-dependent DNA damage response during the initial phase of viral infection in B-lymphocytes (67). Utilizing a similar strategy, both EBNA3A and EBNA3C were shown to concomitantly repress pro-apoptotic BIM (BCL2L11) and senescence inducing p16\(^{INK4A}\) and p14\(^{ARF}\) (CDKN2A) by recruiting extensive epigenetic modifications (38, 63, 72, 105).

EBNA3A and EBNA3C block B-cell differentiation to a plasma cell phenotype through transcriptional activation of the cyclin-dependent kinase inhibitor p18\(^{INK4c}\) and the master transcriptional regulator of plasma cell differentiation BLIMP-1 (91). This helps to establish a long-term latency and subsequent lymphoma development. Although EBNA3A and EBNA3C share similar oncogenic properties, genome wide ChIP-sequencing analyses in LCLs revealed limited co-localization with a number of cellular transcription factors (38, 87). Most significantly these two viral proteins regulate transcription of many important cellular genes through recruitment of IRF4/BATF complex (38, 87). In response to metabolic stress, EBNA3C but not EBNA3A, activates autophagosome formation through transcriptional induction of several autophagy regulators including ATG3, ATG5 and ATG7 (5). Moreover, similar to EBNA2 and EBNALP, EBNA3C among EBNA3-proteins acts as a potent regulator of viral gene transcription (28, 68, 111). EBNA3C mediated co-activation of EBNA2 requires PU.1 site, but not RBPJ\(\kappa\) binding sites, in the LMP1 promoter (111).

Overall, the EBNA3-proteins directly influence B-cell transformation and B-cell lymphoma development through targeting key cell signaling cascades including cell-cycle, apoptosis, and autophagy. This involves direct protein-protein interaction, recruitment of chromatin remodeling factors (HATs, HDACs, histone modification enzymes), translational control (miRNAs) and the protein degradation machinery (chaperones, protease and ubiquitin ligases) (1, 6). Over the last decade, employment of various technological developments including genetically modified EBV either knockout for each EBNA3 proteins or conditionally expressed, global transcriptomic and ChIP-seq
analyses successfully demonstrated the importance of these proteins and offer potential therapeutic
expansion against multiple B-cell lymphomas where EBNA3 proteins were expressed.

**Latent membrane proteins** – The transcripts of latent membrane proteins – LMP1, LMP2A
and LMP2B are generated from a common viral locus with convergent and overlapping primary
transcripts (50). LMP1 represents one of the major EBV-encoded oncoproteins mimicking CD40
receptor signaling pathway (108). It is essential for EBV-induced B-cell transformation through
activation of multiple cellular pathways such as the NF-κB, JNK and p38 cascades (17, 18, 51, 95).
Using LCLs generated with either wild-type or CTCF binding domain knockout virus, it was
demonstrated that CTCF plays an important role in regulating transcription of LMPs from OriP region
and maintenance of episome copy number during EBV latency (11). Unlike the nuclear antigens,
LMPs particularly regulate the host immune response and thereby contributes to activation and
proliferation of the infected B-cells leading to B-cell lymphomas in absence of immune surveillance
(108). Using LMP1 knockout virus infection in humanized mice model, it has been clearly shown that
activated T-cells can substitute the requirement of LMP1 expression in EBV-induced B-cell
lymphomas by providing a source of CD40-signaling. However, compared to the LMP1 knockout
virus, the wild-type virus can drive the formation of B-cell lymphomas more efficiently in this model
(61, 108). LMP1 expression level varies in different EBV-associated B-cell lymphomas. For example,
many EBV-induced AIDS related lymphomas is associated with low LMP1 expression (53, 61),
portrayed as a strategy for immune escape from activated CTLs as LCLs with the highest level of
LMP1 expression was demonstrated to enhance MHC class-I expression and subsequent killing by
CTLs (108). Besides CD40 signaling, LMP1 also regulates cellular apoptosis through activation of the
NF-κB pathway by elevating anti-apoptotic Bcl2 expression (95, 109). Importantly, unlike the tumor
necrosis factor receptor (TNFR), LMP1-mediated NF-κB activation is largely mediated via IRAK1 and
TRAF6; IRAK1 is essential for both p38 activation and p65/RelA phosphorylation (59, 65, 66). LMP1
also modulates autophagy and UPR network affecting its own expression (35, 52, 53). Interestingly,
LMP1-induced pro-apoptotic polycomb complex protein Bmi-1, is further recruited by EBNA3C for
transcriptional repression of other genes (15, 40). Moreover, LMP1 expression is also controlled by EBNA3C in an EBNA2/RBP-Jκ dependent manner (111).

LMP2B is a truncated isoform of LMP2A. While, both LMP2A and LMP2B contain 12 transmembrane domains, LMP2B lacks the N-terminal cytoplasmic signaling domain (56). Although in B-lymphocytes, LMP2A is tyrosine phosphorylated by the Src family kinase (such as Lyn, Syk), in epithelial cells it is mediated by the C-terminal Src kinase, which is triggered by epithelial cell adhesion to extracellular matrix proteins (88). Through this domain, LMP2A acts as a functional homolog of B-cell receptor (BCR) and thereby promoting B-cell survival (103). The importance of this cytoplasmic domain was demonstrated by using an activation motif LMP2A mutant or the Syk inhibitor or Syk-specific small interfering RNA (23). LMP2A is absolutely necessary for growth transformation of germinal center derived B-cells, which are BCR negative (62). Unlike LMP1, LMP2A does not cause any adverse effect on B-cell maturation through activation of immune surveillance (98). LMP2B negatively regulates LMP2A functions (80) and switches latent to lytic activation through depletion of LMP2A-mediated BCR cross-linking and restoration of Ca\(^{2+}\) mobilization (79).

Interestingly, although none of these LMPs are essential to induce B-cell lymphomas in a humanized mouse model, absence of LMPs caused a significant decline in the propensity of lymphoma development, indicating a plausible role in the initial phase of tumor growth (60). Interestingly, LMP2A can rescue LMP1 induced damage in the germinal center, promote cell-cycle progression through accelerating c-Myc activity and p27\(^{kip1}\) degradation (20, 62, 98).

**Noncoding viral transcripts** – In addition to nuclear and membrane associated proteins, EBV also expresses a variety of noncoding RNAs (ncRNAs) upon infecting B-cells, namely the EBV encoded nonpolyadenylated RNAs (EBER1 and EBER2) and numerous miRNAs [reviewed in (90)]. Although most of these ncRNAs are not essential for B-cell transformation, they help with immune evasion and are abundantly expressed in the different types of latency programs, providing tools for viral detection in numerous EBV-associated malignancies. Overall, a somewhat contradictory role for EBERs in EBV-mediated B-cell transformation has been established (47). For example, expression of
EBERs increase colony formation, induce growth of B-cells and block PKR-dependent eIF2α phosphorylation, resulting in blockage of eIF2α-mediated inhibition of protein synthesis and resistance to IFNα-induced apoptosis (84). EBERs also interact with several important cellular partners. For example, EBER1 interaction with ribosomal protein L22 regulates protein translation, EBER-mediated gene expression and PKR-dependent apoptosis (16, 21). Interaction of EBERs with RIG-I, AU-rich element binding factor 1 and pattern-recognition receptors activates the host innate immune responses (84, 94). In addition, EBER2 specifically recruits PAX5 to regulate LMP2A expression which was also confirmed using an EBER2 mutant virus that showed lower LMP2A expression (54). Additional studies suggested that EBER1 and several viral miRNAs are exported from the infected cell in exosomes with functions related to activities in the surrounding cells (73).

Although EBV miRNAs are abundantly expressed in infected B-lymphocytes, sometimes as high as cell miRNAs, their precise role in B-cell transformation is not clear. Three BHRF1 and about forty BART region miRNAs are expressed from different regions of the viral episome [reviewed in (90)]. While BART miRNAs are expressed in nearly all EBV associated B-cell lymphomas, BHRF1-encoded miRNA expressions are relatively restricted to different latency programs (37, 77). Expectedly, these viral miRNAs regulate expression of a number of cellular genes. Although expendable, B-cells infected with recombinant virus lacking viral miRNAs of the BHRF1 cluster resulted in a drastic reduction in their efficiency to support B-cell survival, proliferation and transformation (19). Moreover, during early phase of infection viral miRNA expression levels are significantly higher compared to transformed LCLs (39). In addition to their central role in immune evasion during early phase of viral infection of the nascent B-cells, many important cellular targets have been identified for BART and BHRF1 miRNAs particularly influencing apoptosis and B-cell proliferation (19). For example, while BHRF1 miRNAs are required for proficient B-cell transformation through targeting multiple tumor suppressor proteins such as PTEN and p27KIP1; BART miRNAs block expression of many tumor suppressor genes, including, DICE1, PUMA, PTEN, and BCL2L11 to promote epithelial cell survival (4, 7, 43, 48).
Future Perspective

The ease of attaining EBV transformed LCLs from practically any genetic background has led these cells to be used as a powerful tool for numerous investigations as discussed above. Additionally, studies with LCLs have generated huge public resources on a genome-wide scale highlighting critical regulation by multiple cell and viral transcription factors coupled with epigenetic alterations. Studies revealed critical contribution of each viral oncoprotein and described the intricate nature of B-cell transformation and subsequent B-cell lymphoma development. Importantly, these LCLs are also being used as a preclinical model system for pharmacogenomic studies envisaging drug response due to genetic predispositions along with epigenetic variations. Although LCLs are helpful for primary evaluation of a drug response and identification of biomarkers [reviewed in (69)], experiments on human cancer cell line model such as NCI-60 panel (the National Institute of Health, USA) (89) and humanized mouse model (103) systems coupled with information from various omics datasets are also essential for subsequent validation prior to clinical trials. A number of LCL collections from diverse genetic backgrounds are now available for pharmacogenomics studies. Particularly, LCLs from National Institute of General Medical Science (NIGMS) and National Human Genome Research Institute (NHGRI) including the LCLs used for the ‘HapMap Project’ (14) have been extensively used. LCLs along with Next-generation sequencing information from the ENCODE, and the 1000 Genomes Project (13) have also been submitted into the NHGRI collection. ‘Biobanking’ (49) is another strategy to maintain large LCL collections from population based cohorts. In the coming years, LCLs would serve an important model system providing the foundation of ‘personalized medicine’ (Fig. 2).

EBV was discovered more than 50 years ago and still remains the most frequent persistent asymptomatic virus infection in humans suffering from several B-cell malignancies, particularly in an immune-compromised scenario. Nonetheless, great progress has been made in understanding the underlying oncogenic mechanisms by which EBV contributes to the development of different B-cell lymphomas. The comprehensive understanding of EBV biology gathered particularly in the last
decade will certainly allow us to improve many aspects of clinical care regarding patients suffering from EBV-associated B-cell lymphomas. There are great opportunities to offer early diagnosis of different EBV-associated lymphomas differentially expressed viral latent antigens, immunotherapy to specifically target EBV-infected B-lymphocytes, and chemotherapy targeting potential cell pathways as above-discussed.
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Fig. 1. Salient features of EBV latent transcripts during B-cell transformation followed by B-cell lymphoma development. After initial infection of oropharyngeal epithelial cells, EBV primarily infects the naïve B-lymphocytes. Subsequently the infected B-cells are growth transformed, expressing a subset of viral genes – 6 nuclear antigens (EBNAs), 3 membrane proteins (LMPs) and several non-coding RNAs (EBERs and BARTs). EBNA1 binds to the episome origin of replication to allow viral genome replication. EBNA2 transcriptionally activates a number of viral (red) and cellular (black) genes through recruiting cell transcription factors (TFs) like RBP-Jκ and induces cell growth. EBNALP promotes EBNA2 mediated gene transcription. EBNA3 proteins (EBNA3A, EBNA3B and EBNA3C) modulate viral gene and Notch signaling by blocking EBNA2 association with RBP-Jκ. Both EBNA3A and EBNA3C recruit several epigenetic modifications (such as polycomb repressor complex PRC2) to transcriptionally repress BIM, BLIMP-1 and p15, p16 and p18 expressions and inhibit B-cell to plasma cell differentiation. Through epigenetic control EBNA3C transactivates ATG3, ATG5 and ATG7 expressions and thereby promoting autophagosome formation. EBNA3A and EBNA3C enhance miR221/222 transcription, which in turn block p27 and p57 translations. EBNA3C employs several mechanisms to block p53 mediated apoptotic activities. For example, EBNA3C recruits Mdm2 E3 ligase activity and stabilizes Gemin3 to enhance p53 degradation, and competes with ING4 and ING5 binding to block p53-dependent apoptosis. EBNA3C enhances Pim-1 mediated p21 phosphorylation and degradation. Both EBNA3A and EBNA3C interact with Chk2 and facilitate G2-M transition. In response to DNA damage signals, EBNA3C enhances E2F1 degradation thereby blocking E2F1 mediated apoptosis. EBNA3C binds to E2F6 to block E2F1 mediated transcription. EBNA3C forms complexes and enhances the kinase activities of CyclinD1/CDK6, CyclinD2/CDK6 and CyclinA/CDK2 and augments pRb phosphorylation. EBNA3C recruits IRF4 to block Bcl6 expression and enhances IRF8 degradation. EBNA3C increases ubiquitin-proteasomal mediated degradation of hyperphosphorylated pRb, p27 and Bcl6, which facilitates G1-S transition of cell-cycle. LMP1 mimics CD40 signaling, prevents apoptosis by upregulating bcl-2 and A20. LMP1, through interacting with
tumor necrosis factor receptors (TNFR)-associated factors (TRAFs) and TNFR-associated death domain protein (TRADD), constitutively induces NF-κB signaling pathway. LMP1 also activates JAK/STAT, ERK MAPK, IRF and Wnt signaling pathways. LMP2A blocks B-cell receptor (BCR) signaling, while LMP2B regulates LMP2A functions. EBV noncoding RNAs, EBERs (EBER1 and EBER2) regulate innate immune response and block apoptosis. EBER2 recruits PAX5 to the terminal repeat (TR) region of nascent viral transcript, which helps for viral lytic replication. BARTs mediate evasion of T- and NK-cells during infection of B-cells in peripheral blood lymphocytes.

**Fig. 2. Systematic strategy for studying EBV-induced B-cell transformation and lymphomagenesis.** Burkitt’s lymphoma (BL) cell line Akata or marmoset cell line B95.8 are used to generate virus particles and subsequent infection to nascent B-lymphocytes in the absence or presence of a immunosuppressive drug FK506. Addition of FK506 facilitates the transformation process though inhibiting T-cell mediated immune-surveillance. Alternatively, the whole virus genome is cloned into Bacmid and maintained in epithelial cells (HEK293 or HEK293T). In order to pinpoint the function of viral latent genes and respective domains, genetically engineered BACmids are used to transform naïve B-cells. B-cells infected with wild-type virus are eventually growth transformed expressing latency III program with a full panel of viral latent transcripts, resembling EBV associated lymphomas in HIV-infected population. Several biochemical assays and high-throughput strategies are employed to delineate the underlying mechanism of B-cell transformation and subsequent B-cell lymphoma development. Additionally, these LCLs are used to study EBV-induced B-cell lymphomagenesis in humanized mouse model. Since the LCLs possess donor specific genetic variations, they can provide an ideal *in-vitro* model to study pharmacogenomics leading to futuristic ‘personalized medicine’.
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Table 1: EBV associated B-cell lymphomas and gene expression patterns

| Lymphomas                                      | Latent gene expression                                                                 | Latency program |
|------------------------------------------------|----------------------------------------------------------------------------------------|-----------------|
| Post-transplant B-lymphoproliferative disorder| EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNALP, LMP1, LMP2A, LMP2B, EBER1, EBER2, miRNAs - BHRF1 and BARTs | III             |
| HIV linked B-lymphoproliferative disorder      | EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNALP, LMP1, LMP2A, LMP2B, EBER1, EBER2, miRNAs - BHRF1 and BARTs | III             |
| Primary central nervous system lymphoma         | EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNALP, LMP1, LMP2A, LMP2B, EBER1, EBER2, miRNAs - BHRF1 and BARTs | III             |
| Endemic Burkitt’s lymphoma (eBL)                | EBNA1, EBER1, EBER2, and BART miRNAs                                                   | I               |
| Sporadic Burkitt’s lymphoma (sBL)               |                                                                                       |                 |
| HIV linked Burkitt’s lymphoma                   |                                                                                       |                 |
| Classical Hodgkin’s lymphoma (cHL)              | EBNA1, LMP1, LMP2A, EBER1, EBER2, and BART miRNAs                                     | II              |
| HIV linked Hodgkin’s lymphoma                   |                                                                                       |                 |
| Diffuse large B cell lymphoma (DLBCL), NOS (not otherwise specified) | EBNA1, LMP1, LMP2A, EBER1, EBER2, and BART miRNAs or all transcripts                  | II or III       |
| Diffuse large B cell lymphoma (DLBCL), PAL (pyothorax-associated lymphoma) | EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNALP, LMP1, LMP2A, LMP2B, EBER1, EBER2, miRNAs - BHRF1 and BARTs | III             |
| Diffuse large B cell lymphoma (DLBCL), HIV linked| EBNA1, EBER1, EBER2, and BART miRNAs or EBNA1, LMP1, LMP2A, EBER1, EBER2, and BART miRNAs or all transcripts | I or II or III  |
| Primary effusion lymphoma (PEL)                 | EBNA1, EBER1, EBER2, and BART miRNAs                                                   | I               |
| Plasmablastic lymphoma                          | EBNA1, EBER1, EBER2, and BART miRNAs                                                   | I               |
Table 2: Impact of EBV latent antigens on B-cell transformation and subsequent lymphoma development

| EBV latent proteins | Function related to B-cell lymphomagenesis |
|---------------------|-------------------------------------------|
| EBNA1               | Regulates viral DNA replication and transcription of a number of viral and cellular genes; facilitates p53 degradation and thereby promotes overall oncogenesis. |
| EBNA2               | One of the key viral transcription factors. In association with EBNALP, EBNA2 regulates transcription of several viral and cellular gene expressions; essential for B-cell transformation. |
| EBNALP              | Transcriptional co-activator of EBNA2 mediated transcription of both viral and cellular genes; bypasses cell innate immune response; essential for B-cell transformation. |
| EBNA3A              | Along with EBNA3C, repress BIM and p14, p15, p16 and p18 gene transcription through epigenetic regulation; inhibits B-cell to plasma cell differentiation; essential for B-cell transformation. |
| EBNA3B              | Viral encoded tumor suppressor protein. |
| EBNA3C              | Along with EBNA3A, repress BIM and p14, p15, p16 and p18 gene transcription through epigenetic regulation; facilitates G1-S and G2M transition of cell-cycle; hijacks ubiquitin-proteasomal pathway; inhibits p53, E3F1 and Bim mediated apoptosis; activates autophagy; essential for B-cell transformation. |
| LMP1                | Functionally mimics CD40 signaling pathway; one of the major transcriptional regulator, constitutively activates NF-kB, JAK/STAT, ERK MAPK, IRF and Wnt signaling pathways; stimulates bcl-2 and a20 expression to block apoptosis; essential for B-cell transformation. |
| LMP2A               | Functionally mimics BCR signaling pathway; blocks apoptosis; EBV latency regulation |
| LMP2B               | Regulates LMP2A functions. |
| EBERs               | Most abundant non-coding viral RNAs present in all form of latency programs; affect innate immune response and gene expressions; blocks PKR dependent apoptosis. |
| miRNAs              | Transcribed from BART and BHRF1 loci; maintains latently infected B-cells through blocking cellular apoptosis. |
