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Latency and lytic replication in Epstein–Barr virus-associated oncogenesis

Christian Münz

Institutions: University of Zurich

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Münz, Christian

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Latency and lytic replication in the oncogenesis of the Epstein–Barr virus

Christian Münz

Viral Immunobiology, Institute of Experimental Immunology, University of Zürich, Zürich, Switzerland.

e-mail: christian.muenz@uzh.ch

Abstract

Epstein–Barr virus (EBV) was the first tumor virus identified in humans. It is primarily associated with lymphomas and epithelial cell cancers. These tumors express latent EBV antigens and the oncogenic potential of individual latent EBV proteins has been extensively explored. Nevertheless, it was presumed that the pro-proliferative and anti-apoptotic functions of these oncogenes allow the virus to persist in humans; however, recent evidence suggests that cellular transformation is not required for virus maintenance. Vice versa, lytic EBV replication was assumed to destroy latently infected cells and thereby inhibit tumorigenesis, but at least the initiation of the lytic cycle has now been shown to support EBV-driven malignancies. In addition to these changes in the roles of latent and lytic EBV proteins during tumourigenesis, the function of non-translated RNAs has become clearer, suggesting that they might mainly mediate immune escape rather than cellular transformation. In this Review, these recent findings will be discussed with respect to the role of EBV-encoded oncogenes in viral persistence and the contributions of lytic replication as well as non-translated RNAs in virus-driven tumor formation. Accordingly, early lytic EBV antigens and attenuated viruses without oncogenes and miRNAs could be harnessed for immunotherapies and vaccination.

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Epstein–Barr virus (EBV) establishes latent and persistent infections in humans, and is associated with several cancers. In this Review, Münz discusses the evidence for EBV persistence without B cell transformation and the role of early abortive lytic replication, as well as non-translated RNAs in EBV-driven tumourigenesis.

Introduction

Epstein–Barr virus (EBV; also known as human herpesvirus 4 (HHV4)) is a large double-stranded DNA virus that belongs to the γ-herpesviridae subfamily[1]. It was originally identified in 1964 by Sir Anthony Epstein and co-workers in Burkitt's lymphoma which is still the most common childhood tumor in Sub-Saharan Africa[2,3]. EBV is also the most growth transforming and the most widely distributed human pathogen. It can readily transform human B cells into indefinitely growing lymphoblastoid cell lines (LCLs) in the culture dish[4,5]. Despite this high tumorigenic potential (Box 1), the vast majority of the >95% of the human adult population that carry EBV as a persistent infection never develop EBV-associated malignancies[6]. EBV research has been driven by this fascinating conundrum ever since its discovery.

EBV is transferred via saliva exchange, and therefore symptomatic primary infection or infectious mononucleosis was referred to as 'kissing disease' in the Anglo-Saxon world[7]. In submucosal secondary lymphoid tissues like the tonsils, the virus infects its primary host target cell — the human B cell — by binding to complement receptors 1 and 2 as well as MHC class II as a co-receptor[8,9]. How the virus is transferred across the mucosal epithelium that separates saliva from secondary lymphoid tissues remains unclear, despite the fact that EBV-associated carcinomas (for example, nasopharyngeal carcinoma and the ~10% EBV-positive gastric carcinomas) clearly indicate that EBV can infect epithelial cells[10]. However, polarized epithelia cannot be infected with virus particles from the apical surface that lines the oropharynx[11]. Moreover, virus seems to appear in blood B cells earlier than detectable shedding into the saliva, possibly from epithelial cells[12]. Furthermore, the epigenetic modifications that render the viral genome susceptible for the induction of lytic replication after it circularizes into an episome in the nucleus of infected cells seems to take approximately two weeks in B cells[13], raising the possibility that the virus infection would be stuck in the rapidly turning over mucosal epithelium before it could be shed into the submucosal secondary lymphoid tissues. Importantly, this epigenetic modification might strongly depend on the cellular context. Lytic cycle reactivation might be less dependent on DNA methylation in epithelial cells and this epigenetic modification of the viral genome could also differ in its kinetics from B cells in this cell type[14-17]. Nevertheless, transcytosis of EBV across polarized oral epithelia cell cultures has been demonstrated[18]. These considerations suggest that infectious EBV particles are transported across mucosal epithelia to infect B cells first.

In B cells, EBV persists by B cell transformation into immortalized proliferating LCLs *in vitro* and by establishing latency with only non-translated RNA expression from the viral DNA in

memory B cells *in vivo*[8,19]. The eight viral proteins that are expressed in LCLs in addition to the non-translated RNAs that are found during persistence in memory B cells were named the latent EBV proteins and primarily studied in the context of EBV-driven oncogenesis[20]. Based on the detection of intermediate expression levels of only three latent EBV proteins during the B cell differentiation stage of germinal center B cells that could result from naïve B cells after their activation by the eight latent EBV proteins and precede memory B cell development[21], it was suggested that the virus induces oncogenesis to drive infected B cells into differentiation in order to gain access to the memory B cell pool for persistence. However, recent evidence suggests that expression of all eight latent EBV proteins and B cell transformation by these proteins might not be required for EBV persistence and latency[22]. Furthermore, not only these eight latent EBV proteins, but also early lytic EBV proteins could enhance viral oncogenesis[23].

In this Review, I will discuss the evidence for EBV persistence without B cell transformation and the role of early abortive lytic replication as well as non-translated RNAs in EBV-driven tumor formation. These are timely topics as the field gears up to develop an EBV-specific vaccine and the identity of the infection programs and their antigens that should be targeted is hotly debated. Moreover, attenuated viruses, including virus-like particles, are considered as vaccine candidates[24], but the new roles of viral oncogenes in persistence, and of lytic EBV antigens and non-translated RNAs in tumorigenesis could also point towards attenuated viruses without the respective genes as viable vaccine candidates.

Epstein–Barr virus replication

EBV can replicate by two means — vertical B cell proliferation or lytic virion production. Latent EBV proteins stimulate host cell proliferation and EBV DNA replicates within these cells. Alternatively, EBV can produce infectious virions during lytic replication; however, the latter might be mainly required for transmission, whereas latent infection is the default program of infection in B cells and could spread EBV in the infected host. Within tonsillar B cells, latent EBV protein encoding genes are predominantly expressed and cause activation, proliferation and resistance to cell death. These genes of the latent EBV infection encode 8 EBV proteins, 2 Epstein–Barr virus encoded small RNAs (EBERs) that are not translated and 25 pre-miRNAs that give rise to at least 44 miRNAs[20,25]. All of these can be found in LCLs, naïve tonsillar B cells of healthy virus carriers and nearly all tonsillar B cells of individuals with infectious mononucleosis[21,26,27] (Figure 1). The respective viral gene expression program is called latency III. Presumably after activation from EBV latency III, B cells enter the germinal center reaction and only 3 latent EBV proteins can be found in centroblasts and centrocytes[21]. These are

Epstein–Barr nuclear antigen 1 (EBNA1) and the two latent membrane proteins (LMP1 and LMP2). Their expression in the so-called latency IIa program is thought to ensure that EBV-infected B cells survive the germinal center reaction to gain access to the memory B cell pool, in which EBV persists without viral protein expression in latency 0[19]. Only during homeostatic proliferation is EBNA1transiently expressed in memory B cells, and this pattern is called latency I[28]. These latent EBV infection programs in B cells of healthy virus carriers represent the premalignant states of EBV-associated B cell lymphomas. Accordingly, Burkitt's lymphoma expresses latency I, Hodgkin's lymphoma expresses latency IIa and some, but not all diffuse large B cell lymphomas express latency III[6,10]. EBV replicates in latency I, II and III via the proliferation of activated B cells. Only from latency 0 and I, and after extensive methylation of the viral genome, can lytic replication with its expression of >80 viral genes be efficiently induced, because the immediate early transcription factor BZLF1 that cooperates with the BRLF1 transcription factor to initiate infectious particle production prefers methylated CpG sequences[13,15]. It is thought that stimulation of the B cell receptor of EBV-infected B cells expressing latency 0 or I programs leads to lytic reactivation[29]. The resulting plasma cell differentiation stimulates BZLF1 expression via the plasma cell-associated transcription factors XBP1 and BLIMP1[30,31] (Figure 1). Lytic EBV gene products then further stimulate plasma cell differentiation with B cell receptor down-regulation and complement secretion[32]. In healthy EBV carriers, lytic replication is found in plasma cells only[33]. Basolateral infection of mucosal epithelial cells by plasma cell-released virus might lead to an additional replication round for more efficient EBV shedding into the saliva. This epithelial cell infection presumably occurs via virus binding to $\alpha_v\beta$ integrins and the ephrin A2 receptor[34-36]. Terminal epithelial cell differentiation has also been shown to trigger lytic replication via BLIMP1-mediated BZLF1 expression[31]. Furthermore, during uncontrolled lytic EBV replication in the tongue epithelium (a condition called oral hairy leukoplakia), EBV replication could only be found in BLIMP1-positive cells [37]. Thus, most of the EBV life cycle in healthy EBV carriers is confined to B cells, in which the virus establishes premalignant latent gene expression patterns that are also found in EBV-associated lymphomas.

Transformation and oncogenesis

EBV infection is sufficient to transform human B cells in cell culture. The resulting LCLs resemble EBV-associated B cell lymphomas that develop under immune suppression, for example, during HIV-1 co-infection, due to old age or after iatrogenic immune suppression during transplantation[6]. In addition, EBV infection is thought to drive infected B cells through

their activation into the germinal center reaction, where additional mutations can arise via the machinery that diversifies the B cell receptor in this reaction. Some of these somatic mutations substitute for the down-regulation of some of the latent EBV antigens in tumors like Hodgkin's and Burkitt's lymphoma[6].

What are the functions of the respective EBV gene products that give the virus its oncogenic abilities? Many of the respective proteins (for example, the six nuclear antigens or EBNAs and the two membrane proteins or LMPs), are like Swiss Army Knives with many functions. Therefore, I will only highlight their main effects during B cell transformation, which has been suggested to result from the desire of the virus to activate and differentiate host cells into long-lived memory B cells. The EBNA1 protein is required to initiate viral genome replication during latent infection prior to mitosis and then it anchors the viral episomes to condensed host chromatin during cell division for correct distribution of the 10–40 viral genomes per infected B cell to the daughter cells[38]. However, its host chromatin binding activity also mediates some growth transforming activity[39]. Accordingly, EBNA1 expression in murine B cells induces tumors with some similarities to Burkitt's lymphoma[40]. EBNA2 induces the transcription of the cellular oncogene *MYC* and compromises lytic EBV replication by inducing Tet methylcytosine dioxygenase 2 (*TET2*) expression, thereby blocking methylation sites for BZLF1 binding[16,17,41]. The EBNA leader peptide (EBNA-LP) cooperates with EBNA2 for viral oncogene, like LMP1, expression[42]. EBNA3A and EBNA3C rescue infected cells that are driven into a proliferative state by EBNA2-dependent *MYC* expression via the down-regulation of the proapoptotic BIM and $p16^{INK4a}$ proteins that respond to the hyperproliferation of the infected cells[43]. Furthermore, they prevent transition into lytic replication by suppression of *BLIMP1* expression[44]. By contrast, EBNA3B ensures sufficient immune cell infiltration between EBV transformed B cells to restrict these to a level that most EBV carriers do not develop lymphomas[45]. The two latent membrane proteins replace signals that are required for EBV transformed B cells to survive the germinal center reaction[46]. LMP2 signals constitutively, similar to the B cell receptor, which needs to be engaged by antigen on follicular dendritic cells as signal 1 in order for germinal center B cells to not undergo apoptosis[47]. When expressed in murine B cells, LMP2 can even replace the B cell receptor and B cells that inactivate their receptor through somatic hypermutation can still survive[48]. Thus, LMP2 provides a strong survival signal for B cells. By contrast, LMP1 mimics $CD4^+$ T helper cells in the germinal center by constitutively signaling in a manner similar to CD40 that is engaged by these helper T cells via their CD40 ligand[49]. Expressing *LMP1* in murine B cells leads to aggressive lymphomagenesis[50,51]. The germinal center differentiation of EBV-infected B cells also leads them into a dangerous environment for the acquisition of

additional, growth transforming mutations. Indeed the translocation of *MYC* into the B cell receptor loci, a hallmark of Burkitt's lymphoma, seems to originate from germinal centers and is likely initiated by activation induced deaminase (AID), an enzyme that is expressed at these sites for B cell receptor diversification[52,53]. Thus, EBV encodes at least two sets of proteins that combine pro-proliferative and anti-apoptotic functions (pro-proliferative EBNA2 plus anti-apoptotic EBNA3A and EBNA 3C; and pro-proliferative LMP1 and anti-apoptotic LMP1 and LMP2). The classical view has been that these latent EBV proteins are necessary and sufficient for both tumor formation and activation of infected B cells to drive their differentiation into the long-lived memory B cell pool of EBV persistence. In the following sections, I will discuss how the sequential expression of the protein groups of latency III might allow latency 0 to branch off prior to full transformation for an alternative pathway to EBV persistence, and how lytic EBV replication and the viral non-translated RNAs contribute to viral oncogenesis. These new models could explain recent studies that demonstrate persistence without prior establishment of latency III and decreased EBV-driven tumor formation without lytic EBV protein and EBV miRNA expression.

Persistence without transformation

The above linear differentiation model from latency III to II and then to latency 0 or I is also called the germinal center model of EBV persistence[20]. It was originally proposed on the basis of successive down-regulation of latent EBV protein expression along the path of B cell differentiation, suggesting that EBV drives this differentiation through its oncogenes[21]. By contrast, persistence without transformation suggests that EBV can reach the memory B cell pool without latency III protein expression as a prerequisite, and outside of the germinal center. Indeed, even under conditions in which germinal centers are disorganized, like during infectious mononucleosis[26], latency 0 expressing B cells start circulating in the peripheral blood pool[54]. It was postulated that massive clonal expansion of infected memory B cell populations would allow for the establishment of this pool for EBV persistence[26]. Since the germinal center model is based on the cross-sectional analysis of EBV latency patterns in B cell differentiation stages and not fate mapping of latency III infected cells, establishment of latency 0 outside of germinal centers cannot be completely excluded. Along these lines, LCLs do not automatically differentiate into memory B cells with latency 0.

With the advent of recombinant EBV technology[55], it has become possible to delete genes from the EBV genome and compromise complete B cell transformation and latency III gene expression. This allows for the investigation if then all other latency programs are also

abolished. This was recently queried using EBV deficient in EBNA3A and EBNA3C. As discussed above, these are essential latent EBV gene products that rescue EBV infected cells from cell death that is induced by EBNA2-driven proliferation[43]. Indeed, it is quite difficult to establish EBNA3A deficient LCLs[56], and BIM as well as $p16^{INK4a}$ expression arrest proliferation of EBNA3C deficient LCLs[57,58]. Despite this, $p16^{INK4a}$ overexpression and a block in complete EBV latency III protein expression with LMP1 and LMP2, EBNA3A or EBNA3C deficient EBV establishes persistence in mice with reconstituted human immune system components (HIS mice)[22]. This persistence was associated with EBNA2-driven proliferation during the first month of infection, which then, switched to EBV latency 0 persistence with only non-translated *EBER* expression after three months[22]. The observed absence of EBV latency III seems to be caused by a combination of EBNA3A or EBNA3C deficiency and immune control of rare completely virus transformed B cells, because in a HIS mouse model with less immunocompetence, LMP-expressing EBNA3C-negative lymphomas can be observed at lower frequency compared to wild-type EBV infection[59]. These findings suggest that EBV persistence might be achieved with minimal or no EBV latency III infection. This points to an alternative route to EBV latency 0 (Figure 1). Nevertheless, the combination of both the germinal center and the persistence without transformation pathways might render access to the memory B cell compartment for EBV more robust in humans, whose immune responses most likely pose greater obstacles to EBV persistence than those of HIS mice.

The observed EBNA2-driven proliferation prior to EBV latency 0 persistence points towards a distinct stage of B cell infection by EBV from which persistence might develop. Indeed, EBV gene expression is orchestrated during the first three weeks of B cell infection by EBV, as has been established by in vitro infection studies. Immediately after infection, the two viral *BCL2* homologues *BHRF1* and *BALF1*, which are usually considered lytic EBV gene products, are transiently expressed to prevent apoptosis[60] (Figure 2). EBNA2 then starts driving proliferation of the infected B cells within the first three days through *MYC* expression among other factors[61]. The resulting rapid cell division (8–10h doubling time) activates the DNA damage response[61] with an increase in *BIM* and $p16^{INK4a}$ tumor suppressor gene expression, which is inhibited by EBNA3C and to a lesser extent EBNA3A[57,58,62]. The pro-proliferative and anti-apoptotic gene expression programs induced by EBNA2, EBNA3A and EBNA3C dominate the first two weeks of B cell infection by EBV and are to a large extent regulated by viral superenhancers that are targeted by the viral nuclear antigens[63,64]. This infection program is also called latency IIb[65,66] and has been observed in infectious mononucleosis and post-transplant lymphoproliferative disease patients[67,68]. Only after two to three weeks are the LMPs

sufficiently expressed to exert their pro-proliferative (LMP1) and anti-apoptotic (LMP1 and LMP2) functions[69], resulting in complete EBV latency III expression with an LCL doubling time of 24h. This time period is also needed for epigenetic modifications of the viral episome as a prerequisite of lytic EBV replication[13]. Therefore, between the 3 days of *EBNA2* expression and the 2–3 weeks of *LMP1* expression, EBV infected B cells might exit this latency III program into latency 0 persistence (Figure 2) in the absence of EBNA3C and to a lesser extent EBNA3A. This might allow the establishment of latent EBV infection for the priming of protective immune responses without the threat of overt lymphomagenesis.

Oncogenesis with lytic replication

Recent evidence suggests that latent EBV infection, especially latency III, is not the only contribution of this tumor virus to its associated malignancies. It was observed that BZLF1 deficient EBV causes fewer B cell lymphomas in HIS mice[23,70] (Figure 3). BZLF1 is the immediate early transcription factor for the activation of lytic EBV replication[71]. Is this observation just due to an increased viral titer or is there a novel oncogenic effect of lytic cycle genes? In these studies, early lytic EBV gene expression was primarily observed in the absence of late structural EBV proteins. This early lytic EBV gene expression includes the immediate early transcription factors BZLF1 and BRLF1, as well as proteins for viral DNA replication, immune evasins and anti-apoptotic proteins[71]. This is a fairly common observation, with often less than half of the BZLF1 and BRLF1 expressing cells progressing to complete lytic EBV replication[32,72]. Accordingly, LCLs deficient in the catalytic DNA polymerase subunit BALF5 caused lymphomas more efficiently in immunodeficient mice[73]. Thus, it is most likely not increased B cell infection due to infectious EBV particle production, but rather a conditioning of the tumor microenvironment by abortive early lytic EBV replication that is responsible for the observed increased tumorigenesis (Figure 4). Along these lines, it was observed that more TNF-α, CCL5 and IL-10 are produced by LCLs with higher levels of spontaneous lytic EBV reactivation[74]. These might inhibit the immune control by cytotoxic lymphocytes and recruit immunosuppressive myeloid cells[75]. Indeed, monocytes attracted by CCL5 into the Hodgkin's lymphoma microenvironment support tumor growth in a xenograft model through their immune suppressive activities[76]. Contrary to loss of *BZLF1*, mutations in three suppressive elements of the *BZLF1* promotor renders the respective EBV more lytic[77]. This ZV, ZV′ and ZIIR triple mutant presents with increased lymphoma formation in HIS mice[78] (Figure 3). Furthermore, a natural variant of the *BZLF1* promoter was found in EBV viruses that are more often associated with nasopharyngeal carcinoma, EBV positive gastric carcinoma, Burkitt's lymphoma and EBV-

positive B cell lymphomas of individuals with AIDS[79] (Figure 5). This *BZLF1* promotor V3 variant demonstrates elevated induction of lytic EBV replication upon B cell receptor cross-linking or treatment of EBV infected cells with ionomycin, which stimulates the transcription factor NFAT. Indeed, the variation in the *BZLF1* V3 promotor generates a NFAT binding side and the increased lytic replication can be blocked with the NFAT inhibitor cyclosporin. In addition to polymorphisms in the *BZLF1* promotor, polymorphisms in the *BZLF1* gene might also account for higher lytic EBV replication. Along these lines, the M81 EBV strain isolated from a nasopharyngeal carcinoma sample and three EBV isolates from gastric carcinomas induced increased spontaneous lytic EBV replication in B cells and epithelial cells[80,81]. M81 BZLF1, but not BZLF1 from EBV B95-8 that was isolated from an American individual with infectious mononucleosis, was able to induce this elevated lytic replication in the M81 EBV background, when provided in trans[80]. Thus, BZLF1 activity and the resulting early lytic EBV replication might condition the microenvironment for increased EBV-associated tumor formation.

A role for lytic EBV replication in EBV-associated tumor formation is further substantiated by deletions in EBV BART miRNAs, which were found to be enriched in EBV-associated NK/T cell and diffuse large B cell lymphomas[73] (Figure 3). These viruses are thought to promote higher levels of lytic EBV replication due to up-regulation of *BZLF1* and *BRLF1* expression that are suppressed by one of the BART miRNAs[82]. The increased lytic replication might also contribute to circulating cell-free plasma EBV DNA loads, which are indicative of EBV-associated tumors in a variety of clinical settings[83]. This plasma viral load, rather than peripheral blood cell-associated EBV titers, have been found to correlate with nasopharyngeal carcinoma[84], post-transplant lymphoproliferative disease[85], diffuse large B cell lymphoma[86], NK/T cell lymphomas[87] and Hodgkin's lymphoma[88]. The risk for Hodgkin's lymphoma is also increased following primary EBV acquisition with infectious mononucleosis[89-91]. Infectious mononucleosis is characterized by elevated virus shedding into the saliva, high antibody titers against structural EBV proteins and massive expansion of lytic EBV antigen specific $CD8^+$ T cells[92] (Figure 5). These are all parameters of elevated lytic EBV replication and, thus, inefficient immune control of productive infectious viral particle production might contribute to the increased risk for Hodgkin's lymphoma after infectious mononucleosis. Finally, EBV-associated post-transplant central nervous system lymphoma was cured by pharmacological inhibition of lytic EBV replication in a small number of individuals using a combination of Zidovudine, Rituximab and Dexamethasone[93]. Altogether, lytic EBV replication increases EBV-associated lymphomagenesis in preclinical in vivo models, virus strains with increased lytic EBV replication are enriched in EBV-associated malignancies and plasma viral loads correlate with some of

these diseases. Moreover, inefficiently controlled lytic replication predisposes for Hodgkin's lymphoma and in one EBV-associated tumor setting, inhibition of lytic EBV replication seems to have been therapeutically beneficial for the affected patients. Thus, lytic EBV replication might contribute to virus-associated tumorigenesis, possibly by conditioning the tumor microenvironment.

Non-translated RNAs and tumorigenesis

The non-translated RNAs expressed by EBV include the two EBERs and ~44 miRNAs[25]. Originally, both were suggested to promote EBV-driven tumorigenesis[94-99]. By contrast, and as discussed above, viruses with deletions in some of the BART miRNAs were found to be associated with diffuse large B cell and NK/T cell lymphomas[73]. This region in which deletions were found contains 22 pre-miRNAs and an additional three are located adjacent to the viral *BHRF1* gene encoding a BCL2 homologue[25] (Figure 6). The resulting ~44 miRNAs are grouped into either BHRF1 or BART miRNAs. The BHRF1 miRNAs are expressed during EBV latency III infection and its associated tumors, and two of the three pre-miRNAs are expressed during lytic EBV replication[100,101] (Figure 1). By contrast, the BART miRNAs are expressed in all EBV infection programs, including EBV latency I and II, albeit at lower levels during latency I[101,102]. In addition to the regulation of lytic replication via down-regulation of *BZLF1* and *BRLF1* by BART miRNAs[82], they have also been described to limit *EBNA2*, *LMP1* and *LMP2* expression[103-106]. In addition, BHRF1 miRNAs optimize the timing of *EBNA-LP* and *BHRF1* expression for optimal B cell transformation[107,108] and suppress sumoylation that is required for efficient lytic replication induction[109]. Finally, both BART and BHRF1 miRNAs attenuate B cell receptor signaling and thereby desensitize infected B cells to lytic EBV replication induction[110]. Therefore, both BART and BHRF1 miRNAs contribute to suppression of lytic EBV replication and optimize B cell transformation by EBV[96,97].

The B95-8 strain of EBV[4,5] lacks many of the BART miRNAs but readily transforms human B cells, and viruses with deletions in the same region are enriched in diffuse large B cell lymphomas[73]. Along these lines, complete loss of all BART miRNAs from the B95-8 virus does not significantly alter its infection in HIS mice[111]. By contrast, loss of BHRF1 miRNAs either alone or in addition to BART miRNA deletion attenuates B95-8 EBV infection in HIS mice[99,111]. Interestingly, BHRF1 miRNAs are not necessary for B cell transformation, but the contribution of these miRNAs to immune escape seems to be crucial for the in vivo phenotype in HIS mice (Figures 4 and 6). Depletion of $CD8^+$ T cells restores viral loads and tumorigenicity of miRNA deficient EBV[111]. Along these lines, BHRF1 miRNAs target *CXCL11*, which encodes a

chemokine that attracts CD8+ T cells via the CXCR3 chemokine receptor into sites of inflammation and tumorigenesis[112,113]. Furthermore, they also down-regulate the transporter associated with antigen processing (TAP) complex that is required for antigenic peptide import into the endoplasmic reticulum and loading onto MHC class I molecules for CD8+ T cell recognition[114]. In particular, TAP2 levels are down-regulated by BHRF1 miRNAs, which also destabilizes TAP1 levels and results in lower surface expression of some MHC class I molecules as well as diminished recognition of miRNA deficient LCLs by EBV-specific CD8+ T cell clones[111,114]. Thus, both BART and BHRF1 miRNAs of EBV optimize virus-mediated B cell transformation and block lytic replication, but BHRF1 miRNAs also promote immune escape from CD8+ T cell responses. This later function seems dominant in vivo during EBV infection of HIS mice, because CD8+ T cell depletion restores viral loads and tumorigenesis of miRNA deficient EBV.

Similar to BART miRNAs, EBERs are highly expressed in all EBV infection programs[25]. They are the most abundant viral transcripts with more than one million copies per EBV infected cell[115]. Due to their high abundance, in situ hybridization against EBERs still constitutes the gold standard for detecting EBV infected cells. In contrast to the miRNAs, they are confined to the nucleus and seem to interact with various RNA binding proteins, including La and L22[115-117]. Similar to BART miRNAs, EBERs seem to optimize B cell transformation by EBV, but possibly only in certain EBV strains[94,118-120]. Transgenic overexpression of EBERs leads to lymphoproliferations and less frequently B cell lymphomas[95]. However, EBER deficient B95-8 EBV infection in HIS mice did not alter viral loads or tumorigenesis[121]. Thus, as for BART miRNAs, EBERs seem to improve B cell transformation, but their absence does not significantly alter EBV infection and tumorigenesis in HIS mice.

In summary, miRNA and EBER deficient viruses have helped reveal the immune escape function of the BHRF1 miRNA cluster and show that BART miRNA and EBER deficiency seems to have little impact on EBV infection and immune control in HIS mice. This is consistent with BART miRNAs being often partially deleted in EBV isolates. By contrast, the conservation and high expression of EBERs among all EBV viruses remains enigmatic.

[H1] Conclusions

Recent studies have changed our view on the tumorigenesis of the most common human tumor virus, namely EBV. As outlined in this Review, complete B cell transformation does not seem to be a prerequisite for EBV persistence, lytic EBV proteins play a role during virus associated tumorigenesis and viral miRNAs serve an important immune escape function during infection.

The contribution of lytic EBV antigen expression to virus-induced lymphoma and carcinoma formation reveal similarities to Kaposi sarcoma-associated herpesvirus (KSHV; also known as human herpesvirus 8 (HHV8)), the other oncogenic human γ-herpesvirus[122]. Some of the KSHV-associated malignancies, like Kaposi sarcoma, seem to depend on lytic replication of this virus[123]. Targeting of early lytic antigen expression might provide a promising novel strategy for the treatment of both EBV and KSHV associated tumors[124].

These three new characteristics of EBV associated lymphomas and carcinomas might also suggest mechanisms to attenuate and render EBV more immunogenic for vaccination. An *EBNA3*C, *BZLF1* and miRNA deficient virus (Δ*3CZmiR* EBV) might combine minimal oncogenicity with increased $CD8^+$ T cell recognition. Such a virus would allow for EBNA2-dependent viral antigen expression but compromise anti-apoptotic *EBNA3C* expression as well as the downstream pro-proliferative LMP1 and anti-apoptotic LMP1 and LMP2 functions. Furthermore, it would remove any tumor promoting early lytic EBV protein expression. Finally, such an Δ*3CZmiR* EBV would make the expressed EBV antigens (presumably EBNA1, EBNA2, EBNA3A, EBNA3B and EBNA-LP) more visible to $CD8^+$ T cells due to efficient antigen processing for MHC class I presentation and attraction of these T cells into the tumor microenvironment through the CXCR3 ligands CXCL9, CXCL10 (both EBNA3B-driven) and CXCL11 (no longer inhibited by BHRF1 miRNAs). The strong dependency on cytotoxic lymphocytes, including the T cells that such an attenuated EBV would elicit for natural immunity to EBV[125], has so far made it difficult to develop vaccines against this virus. Most of the vaccines currently in use mainly elicit protective antibody responses and the use of EBV itself, even in an attenuated form (as has been used for the vaccination against the varicella zoster α-herpesvirus[126]), has been considered too risky due to the oncogenic potential of EBV. Accordingly, new vaccination strategies that are being explored are either based on recombinant viral vectors that elicit EBV-specific immune control by cytotoxic lymphocyte populations[127], or are based on novel recombinant viral glycoprotein formulations that stimulate more potent EBV-specific antibody responses than those usually observed in healthy EBV carriers[128-130]. Irrespective of the efficacy of these new EBV vaccine candidates, a better understanding of EBV-driven cellular transformation and its immune control, which has in part emerged from the use of HIS mice as a preclinical in vivo model for this virus, should allow us to more efficiently interfere with EBV pathologies and also to refine EBV-specific vaccination strategies in the future.

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Competing interests

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References

1 Farrell, P. J. Epstein-Barr Virus and Cancer. *Annu Rev Pathol* **14**, 29-53 (2019).

2 Epstein, M. A., Achong, B. G. & Barr, Y. M. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet* **1**, 702-703 (1964).

3 Epstein, M. A., Henle, G., Achong, B. G. & Barr, Y. M. Morphological and biological studies on a virus in cultured lymphoblasts from Burkitt's lymphoma. *J Exp Med* **121**, 761-770 (1964).

4 Miller, G. & Lipman, M. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. *Proc Natl Acad Sci U S A* **70**, 190-194 (1973).

5 Miller, G. & Lipman, M. Comparison of the yield of infectious virus from clones of human and simian lymphoblastoid lines transformed by Epstein-Barr virus. *J Exp Med* **138**, 1398-1412 (1973).

6 Cesarman, E. Gammaherpesviruses and lymphoproliferative disorders. *Annu Rev Pathol* **9**, 349-372 (2014).

7 Luzuriaga, K. & Sullivan, J. L. Infectious mononucleosis. *N Engl J Med* **362**, 1993-2000 (2010).

8 Young, L. S., Yap, L. F. & Murray, P. G. Epstein-Barr virus: more than 50 years old and still providing surprises. *Nat Rev Cancer* **16**, 789-802 (2016).

9 Chesnokova, L. S., Valencia, S. M. & Hutt-Fletcher, L. M. The BDLF3 gene product of Epstein-Barr virus, gp150, mediates non-productive binding to heparan sulfate on epithelial cells and only the binding domain of CD21 is required for infection. *Virology* **494**, 23-28 (2016).

10 Kutok, J. L. & Wang, F. Spectrum of Epstein-Barr virus-associated diseases. *Annu Rev Pathol* **1**, 375-404 (2006).

11 Tugizov, S. M., Berline, J. W. & Palefsky, J. M. Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells. *Nat Med* **9**, 307-314 (2003).

12 Dunmire, S. K., Grimm, J. M., Schmeling, D. O., Balfour, H. H., Jr. & Hogquist, K. A. The Incubation Period of Primary Epstein-Barr Virus Infection: Viral Dynamics and Immunologic Events. *PLoS Pathog* **11**, e1005286 (2015).

13 Woellmer, A., Arteaga-Salas, J. M. & Hammerschmidt, W. BZLF1 Governs CpG-Methylated Chromatin of Epstein-Barr Virus Reversing Epigenetic Repression. *PLoS Pathog* **8**, e1002902 (2012).

14 Wille, C. K. *et al.* 5-hydroxymethylation of the EBV genome regulates the latent to lytic switch. *Proc Natl Acad Sci U S A* **112**, E7257-7265 (2015).

15 Wille, C. K. *et al.* Viral genome methylation differentially affects the ability of BZLF1 versus BRLF1 to activate Epstein-Barr virus lytic gene expression and viral replication. *J Virol* **87**, 935-950 (2013).

16 Lu, F. *et al.* Coordinate Regulation of TET2 and EBNA2 Controls the DNA Methylation State of Latent Epstein-Barr Virus. *J Virol* **91**, e00804-17 (2017).

17 Wille, C. K., Li, Y., Rui, L., Johannsen, E. C. & Kenney, S. C. Restricted TET2 Expression in Germinal Center Type B Cells Promotes Stringent Epstein-Barr Virus Latency. *J Virol* **91**, e01987-16 (2017).

18 Tugizov, S. M., Herrera, R. & Palefsky, J. M. Epstein-Barr virus transcytosis through polarized oral epithelial cells. *J Virol* **87**, 8179-8194 (2013).

19 Babcock, G. J., Decker, L. L., Volk, M. & Thorley-Lawson, D. A. EBV persistence in memory B cells in vivo. *Immunity* **9**, 395-404 (1998).

20 Thorley-Lawson, D. A. EBV Persistence--Introducing the Virus. *Curr Top Microbiol Immunol* **390**, 151-209 (2015).

21 Babcock, J. G., Hochberg, D. & Thorley-Lawson, A. D. The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. *Immunity* **13**, 497-506. (2000).

22 Murer, A. *et al.* EBV persistence without its EBNA3A and 3C oncogenes in vivo. *PLoS Pathog* **14**, e1007039 (2018).

23 Ma, S. D. *et al.* A new model of Epstein-Barr virus infection reveals an important role for early lytic viral protein expression in the development of lymphomas. *J Virol* **85**, 165-177 (2011).

24 van Zyl, D. G. *et al.* Immunogenic particles with a broad antigenic spectrum stimulate cytolytic T cells and offer increased protection against EBV infection ex vivo and in mice. *PLoS Pathog* **14**, e1007464 (2018).

25 Skalsky, R. L. & Cullen, B. R. EBV Noncoding RNAs. *Curr Top Microbiol Immunol* **391**, 181-217 (2015).

26 Kurth, J. *et al.* EBV-infected B cells in infectious mononucleosis: viral strategies for spreading in the B cell compartment and establishing latency. *Immunity* **13**, 485-495. (2000).

27 Hartung, A. *et al.* EBV miRNA expression profiles in different infection stages: A prospective cohort study. *PLoS One* **14**, e0212027 (2019).

28 Hochberg, D. *et al.* Demonstration of the Burkitt's lymphoma Epstein-Barr virus phenotype in dividing latently infected memory cells in vivo. *Proc Natl Acad Sci U S A* **101**, 239-244 (2004).

29 Tovey, M. G., Lenoir, G. & Begon-Lours, J. Activation of latent Epstein-Barr virus by antibody to human IgM. *Nature* **276**, 270-272 (1978).

30 McDonald, C., Karstegl, C. E., Kellam, P. & Farrell, P. J. Regulation of the Epstein-Barr virus Zp promoter in B lymphocytes during reactivation from latency. *J Gen Virol* **91**, 622-629 (2010).

31 Reusch, J. A., Nawandar, D. M., Wright, K. L., Kenney, S. C. & Mertz, J. E. Cellular differentiation regulator BLIMP1 induces Epstein-Barr virus lytic reactivation in epithelial and B cells by activating transcription from both the R and Z promoters. *J Virol* **89**, 1731-1743 (2015).

32 Ersing, I. *et al.* A Temporal Proteomic Map of Epstein-Barr Virus Lytic Replication in B Cells. *Cell Rep* **19**, 1479-1493 (2017).

33 Laichalk, L. L. & Thorley-Lawson, D. A. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. *J Virol* **79**, 1296-1307 (2005).

34 Chen, J. *et al.* Ephrin receptor A2 is a functional entry receptor for Epstein-Barr virus. *Nat Microbiol* **3**, 172-180 (2018).

35 Zhang, H. *et al.* Ephrin receptor A2 is an epithelial cell receptor for Epstein-Barr virus entry. *Nat Microbiol* **3**, 1-8 (2018).

36 Hutt-Fletcher, L. M. The Long and Complicated Relationship between Epstein-Barr Virus and Epithelial Cells. *J Virol* **91**, e01677-16 (2017).

37 Buettner, M. *et al.* Lytic Epstein-Barr virus infection in epithelial cells but not in B-lymphocytes is dependent on Blimp1. *J Gen Virol* **93**, 1059-1064 (2012).

38 Frappier, L. Ebna1. *Curr Top Microbiol Immunol* **391**, 3-34 (2015).

39 Hammerschmidt, W. & Sugden, B. Epstein-Barr virus sustains Burkitt's lymphomas and Hodgkin's disease. *Trends Mol Med* **10**, 331-336 (2004).

40 AlQarni, S. *et al.* Lymphomas driven by Epstein-Barr virus nuclear antigen-1 (EBNA1) are dependant upon Mdm2. *Oncogene* **37**, 3998-4012 (2018).

41 Kempkes, B. & Ling, P. D. EBNA2 and Its Coactivator EBNA-LP. *Curr Top Microbiol Immunol* **391**, 35-59 (2015).

42 Szymula, A. *et al.* Epstein-Barr virus nuclear antigen EBNA-LP is essential for transforming naive B cells, and facilitates recruitment of transcription factors to the viral genome. *PLoS Pathog* **14**, e1006890 (2018).

43 Styles, C. T., Paschos, K., White, R. E. & Farrell, P. J. The Cooperative Functions of the EBNA3 Proteins Are Central to EBV Persistence and Latency. *Pathogens* **7**, E31 (2018).

44 Styles, C. T. *et al.* EBV epigenetically suppresses the B cell-to-plasma cell differentiation pathway while establishing long-term latency. *PLoS Biol* **15**, e2001992 (2017).

45 White, R. E. *et al.* EBNA3B-deficient EBV promotes B cell lymphomagenesis in humanized mice and is found in human tumors. *J Clin Invest* **122**, 1487-1502 (2012).

46 Thorley-Lawson, D. A. Epstein-Barr virus: exploiting the immune system. *Nature Reviews Immunology* **1**, 75-82 (2001).

47 Cen, O. & Longnecker, R. Latent Membrane Protein 2 (LMP2). *Curr Top Microbiol Immunol* **391**, 151-180 (2015).

48 Caldwell, R. G., Wilson, J. B., Anderson, S. J. & Longnecker, R. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. *Immunity* **9**, 405-411 (1998).

49 Kieser, A. & Sterz, K. R. The Latent Membrane Protein 1 (LMP1). *Curr Top Microbiol Immunol* **391**, 119-149 (2015).

50 Kulwichit, W. *et al.* Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. *Proc Natl Acad Sci U S A* **95**, 11963-11968 (1998).

51 Zhang, B. *et al.* Immune surveillance and therapy of lymphomas driven by Epstein-Barr virus protein LMP1 in a mouse model. *Cell* **148**, 739-751 (2012).

52 Ramiro, A. R. *et al.* AID is required for c-myc/IgH chromosome translocations in vivo. *Cell* **118**, 431-438 (2004).

53 Torgbor, C. *et al.* A multifactorial role for P. falciparum malaria in endemic Burkitt's lymphoma pathogenesis. *PLoS Pathog* **10**, e1004170 (2014).

54 Babcock, G. J., Decker, L. L., Freeman, R. B. & Thorley-Lawson, D. A. Epstein-barr virus-infected resting memory B cells, not proliferating lymphoblasts, accumulate in the peripheral blood of immunosuppressed patients. *J Exp Med* **190**, 567-576. (1999).

55 Delecluse, H. J., Hilsendegen, T., Pich, D., Zeidler, R. & Hammerschmidt, W. Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. *Proc Natl Acad Sci U S A* **95**, 8245-8250 (1998).

56 Hertle, M. L. *et al.* Differential gene expression patterns of EBV infected EBNA-3A positive and negative human B lymphocytes. *PLoS Pathog* **5**, e1000506 (2009).

57 Paschos, K., Parker, G. A., Watanatanasup, E., White, R. E. & Allday, M. J. BIM promoter directly targeted by EBNA3C in polycomb-mediated repression by EBV. *Nucleic Acids Res* **40**, 7233-7246 (2012).

58 Skalska, L. *et al.* Induction of p16(INK4a) is the major barrier to proliferation when Epstein-Barr virus (EBV) transforms primary B cells into lymphoblastoid cell lines. *PLoS Pathog* **9**, e1003187 (2013).

59 Romero-Masters, J. C. *et al.* An EBNA3C-deleted Epstein-Barr virus (EBV) mutant causes B-cell lymphomas with delayed onset in a cord blood-humanized mouse model. *PLoS Pathog* **14**, e1007221 (2018).

60 Altmann, M. & Hammerschmidt, W. Epstein-Barr virus provides a new paradigm: a requirement for the immediate inhibition of apoptosis. *PLoS Biol* **3**, e404 (2005).

61 Nikitin, P. A. *et al.* An ATM/Chk2-mediated DNA damage-responsive signaling pathway suppresses Epstein-Barr virus transformation of primary human B cells. *Cell Host Microbe* **8**, 510-522 (2010).

62 Maruo, S. *et al.* Epstein-Barr virus nuclear antigens 3C and 3A maintain lymphoblastoid cell growth by repressing p16INK4A and p14ARF expression. *Proc Natl Acad Sci U S A* **108**, 1919-1924 (2011).

63 Ma, Y. *et al.* CRISPR/Cas9 Screens Reveal Epstein-Barr Virus-Transformed B Cell Host Dependency Factors. *Cell Host Microbe* **21**, 580-591 e587 (2017).

64 Jiang, S. *et al.* The Epstein-Barr Virus Regulome in Lymphoblastoid Cells. *Cell Host Microbe* **22**, 561-573 (2017).

65 Klein, E., Nagy, N. & Rasul, A. E. EBV genome carrying B lymphocytes that express the nuclear protein EBNA-2 but not LMP-1: Type IIb latency. *Oncoimmunology* **2**, e23035 (2013).

66 Price, A. M. & Luftig, M. A. To be or not IIb: a multi-step process for Epstein-Barr virus latency establishment and consequences for B cell tumorigenesis. *PLoS Pathog* **11**, e1004656 (2015).

67 Kurth, J., Hansmann, M. L., Rajewsky, K. & Kuppers, R. Epstein-Barr virus-infected B cells expanding in germinal centers of infectious mononucleosis patients do not participate in the germinal center reaction. *Proc Natl Acad Sci U S A* **100**, 4730-4735 (2003).

68 Oudejans, J. J. *et al.* Detection of heterogeneous Epstein-Barr virus gene expression patterns within individual post-transplantation lymphoproliferative disorders. *Am J Pathol* **147**, 923-933 (1995).

69 Price, A. M. *et al.* Analysis of Epstein-Barr virus-regulated host gene expression changes through primary B-cell outgrowth reveals delayed kinetics of latent membrane protein 1-mediated NF-kappaB activation. *J Virol* **86**, 11096-11106 (2012).

70 Antsiferova, O. *et al.* Adoptive transfer of EBV specific $CD8^+$ T cell clones can transiently control EBV infection in humanized mice. *PLoS Pathog* **10**, e1004333 (2014).

71 McKenzie, J. & El-Guindy, A. Epstein-Barr Virus Lytic Cycle Reactivation. *Curr Top Microbiol Immunol* **391**, 237-261 (2015).

72 Chiu, Y. F. & Sugden, B. Epstein-Barr Virus: The Path from Latent to Productive Infection. *Annu Rev Virol* **3**, 359-372 (2016).

73 Okuno, Y. *et al.* Defective Epstein-Barr virus in chronic active infection and haematological malignancy. *Nat Microbiol* **4**, 404-413 (2019).

74 Arvey, A. *et al.* The tumor virus landscape of AIDS-related lymphomas. *Blood* **125**, e14-22 (2015).

75 Walens, A. *et al.* CCL5 promotes breast cancer recurrence through macrophage recruitment in residual tumors. *Elife* **8**, e43653 (2019).

76 Casagrande, N. *et al.* CCR5 antagonism by maraviroc inhibits Hodgkin lymphoma microenvironment interactions and xenograft growth. *Haematologica* **104**, 564-575 (2019).

77 Yu, X., McCarthy, P. J., Wang, Z., Gorlen, D. A. & Mertz, J. E. Shutoff of BZLF1 gene expression is necessary for immortalization of primary B cells by Epstein-Barr virus. *J Virol* **86**, 8086-8096 (2012).

78 Ma, S. D. *et al.* An Epstein-Barr Virus (EBV) mutant with enhanced BZLF1 expression causes lymphomas with abortive lytic EBV infection in a humanized mouse model. *J Virol* **86**, 7976-7987 (2012).

79 Bristol, J. A. *et al.* A cancer-associated Epstein-Barr virus BZLF1 promoter variant enhances lytic infection. *PLoS Pathog* **14**, e1007179 (2018).

80 Tsai, M. H. *et al.* Spontaneous lytic replication and epitheliotropism define an Epstein-Barr virus strain found in carcinomas. *Cell Rep* **5**, 458-470 (2013).

81 Tsai, M. H. *et al.* The biological properties of different Epstein-Barr virus strains explain their association with various types of cancers. *Oncotarget* **8**, 10238-10254 (2017).

82 Jung, Y. J., Choi, H., Kim, H. & Lee, S. K. MicroRNA miR-BART20-5p stabilizes Epstein-Barr virus latency by directly targeting BZLF1 and BRLF1. *J Virol* **88**, 9027-9037 (2014).

83 Kanakry, J. & Ambinder, R. The Biology and Clinical Utility of EBV Monitoring in Blood. *Curr Top Microbiol Immunol* **391**, 475-499 (2015).

84 Lin, J. C. *et al.* Quantification of plasma Epstein-Barr virus DNA in patients with advanced nasopharyngeal carcinoma. *N Engl J Med* **350**, 2461-2470 (2004).

85 Ruf, S. *et al.* Comparison of six different specimen types for Epstein-Barr viral load quantification in peripheral blood of pediatric patients after heart transplantation or after allogeneic hematopoietic stem cell transplantation. *J Clin Virol* **53**, 186-194 (2012).

86 Morishima, S. *et al.* Increased T-cell responses to Epstein-Barr virus with high viral load in patients with Epstein-Barr virus-positive diffuse large B-cell lymphoma. *Leuk Lymphoma* **56**, 1072-1078 (2015).

87 Suzuki, R. *et al.* Prospective measurement of Epstein-Barr virus-DNA in plasma and peripheral blood mononuclear cells of extranodal NK/T-cell lymphoma, nasal type. *Blood* **118**, 6018-6022 (2011).

88 Kanakry, J. A. *et al.* The clinical significance of EBV DNA in the plasma and peripheral blood mononuclear cells of patients with or without EBV diseases. *Blood* **127**, 2007-2017 (2016).

89 Hjalgrim, H. *et al.* Characteristics of Hodgkin's lymphoma after infectious mononucleosis. *N Engl J Med* **349**, 1324-1332 (2003).

90 Hjalgrim, H. *et al.* HLA-A alleles and infectious mononucleosis suggest a critical role for cytotoxic T-cell response in EBV-related Hodgkin lymphoma. *Proc Natl Acad Sci U S A* **107**, 6400-6405 (2010).

91 Hjalgrim, H. *et al.* Infectious mononucleosis, childhood social environment, and risk of Hodgkin lymphoma. *Cancer Res* **67**, 2382-2388 (2007).

92 Taylor, G. S., Long, H. M., Brooks, J. M., Rickinson, A. B. & Hislop, A. D. The immunology of Epstein-Barr virus-induced disease. *Annu Rev Immunol* **33**, 787-821 (2015).

93 Dugan, J. P. *et al.* Complete and durable responses in Primary Central Nervous System Post-Transplant Lymphoproliferative Disorder with Zidovudine, Ganciclovir, Rituximab and Dexamethasone. *Clin Cancer Res* **24**, 3273-3281 (2018).

94 Yajima, M., Kanda, T. & Takada, K. Critical role of Epstein-Barr Virus (EBV)-encoded RNA in efficient EBV-induced B-lymphocyte growth transformation. *J Virol* **79**, 4298-4307 (2005).

95 Repellin, C. E., Tsimbouri, P. M., Philbey, A. W. & Wilson, J. B. Lymphoid hyperplasia and lymphoma in transgenic mice expressing the small non-coding RNA, EBER1 of Epstein-Barr virus. *PLoS One* **5**, e9092 (2010).

96 Feederle, R. *et al.* The members of an Epstein-Barr virus microRNA cluster cooperate to transform B lymphocytes. *J Virol* **85**, 9801-9810 (2011).

97 Feederle, R. *et al.* A viral microRNA cluster strongly potentiates the transforming properties of a human herpesvirus. *PLoS Pathog* **7**, e1001294 (2011).

98 Seto, E. *et al.* Micro RNAs of Epstein-Barr virus promote cell cycle progression and prevent apoptosis of primary human B cells. *PLoS Pathog* **6**, e1001063 (2010).

99 Wahl, A. *et al.* A Cluster of Virus-Encoded MicroRNAs Accelerates Acute Systemic Epstein-Barr Virus Infection but Does Not Significantly Enhance Virus-Induced Oncogenesis In Vivo. *Journal of Virology* **87**, 5437-5446 (2013).

100 Xing, L. & Kieff, E. cis-Acting effects on RNA processing and Drosha cleavage prevent Epstein-Barr virus latency III BHRF1 expression. *J Virol* **85**, 8929-8939 (2011).

101 Amoroso, R. *et al.* Quantitative studies of Epstein-Barr virus-encoded microRNAs provide novel insights into their regulation. *J Virol* **85**, 996-1010 (2011).

102 Vereide, D. T. *et al.* Epstein-Barr virus maintains lymphomas via its miRNAs. *Oncogene* **33**, 1258-1264 (2014).

103 Skalsky, R. L. *et al.* The viral and cellular microRNA targetome in lymphoblastoid cell lines. *PLoS Pathog* **8**, e1002484 (2012).

104 Riley, K. J. *et al.* EBV and human microRNAs co-target oncogenic and apoptotic viral and human genes during latency. *EMBO J* **31**, 2207-2221 (2012).

105 Skalsky, R. L., Kang, D., Linnstaedt, S. D. & Cullen, B. R. Evolutionary conservation of primate lymphocryptovirus microRNA targets. *J Virol* **88**, 1617-1635 (2014).

106 Lung, R. W. *et al.* Modulation of LMP2A expression by a newly identified Epstein-Barr virus-encoded microRNA miR-BART22. *Neoplasia* **11**, 1174-1184 (2009).

107 Bernhardt, K. *et al.* A Viral microRNA Cluster Regulates the Expression of PTEN, p27 and of a bcl-2 Homolog. *PLoS Pathog* **12**, e1005405 (2016).

108 Poling, B. C., Price, A. M., Luftig, M. A. & Cullen, B. R. The Epstein-Barr virus miR-BHRF1 microRNAs regulate viral gene expression in cis. *Virology* **512**, 113-123 (2017).

109 Li, J., Callegari, S. & Masucci, M. G. The Epstein-Barr virus miR-BHRF1-1 targets RNF4 during productive infection to promote the accumulation of SUMO conjugates and the release of infectious virus. *PLoS Pathog* **13**, e1006338 (2017).

110 Chen, Y., Fachko, D., Ivanov, N. S., Skinner, C. M. & Skalsky, R. L. Epstein-Barr virus microRNAs regulate B cell receptor signal transduction and lytic reactivation. *PLoS Pathog* **15**, e1007535 (2019).

111 Murer, A. *et al.* MicroRNAs of Epstein-Barr Virus Attenuate T-Cell-Mediated Immune Control In Vivo. *MBio* **10**, e01941-18 (2019).

112 Pfeffer, S. *et al.* Identification of virus-encoded microRNAs. *Science* **304**, 734-736 (2004).

113 Xia, T. *et al.* EBV microRNAs in primary lymphomas and targeting of CXCL-11 by ebv-mir-BHRF1-3. *Cancer Res* **68**, 1436-1442 (2008).

114 Albanese, M. *et al.* Epstein-Barr virus microRNAs reduce immune surveillance by virus-specific $CD8^+$ T cells. *Proc Natl Acad Sci U S A* **113**, E6467-E6475 (2016).

115 Lerner, M. R., Andrews, N. C., Miller, G. & Steitz, J. A. Two small RNAs encoded by Epstein-Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosus. *Proc Natl Acad Sci U S A* **78**, 805-809 (1981).

116 Fok, V., Friend, K. & Steitz, J. A. Epstein-Barr virus noncoding RNAs are confined to the nucleus, whereas their partner, the human La protein, undergoes nucleocytoplasmic shuttling. *J Cell Biol* **173**, 319-325 (2006).

117 Fok, V., Mitton-Fry, R. M., Grech, A. & Steitz, J. A. Multiple domains of EBER 1, an Epstein-Barr virus noncoding RNA, recruit human ribosomal protein L22. *RNA* **12**, 872-882 (2006).

118 Wu, Y., Maruo, S., Yajima, M., Kanda, T. & Takada, K. Epstein-Barr virus (EBV)-encoded RNA 2 (EBER2) but not EBER1 plays a critical role in EBV-induced B-cell growth transformation. *J Virol* **81**, 11236-11245 (2007).

119 Swaminathan, S., Tomkinson, B. & Kieff, E. Recombinant Epstein-Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates in vitro. *Proc Natl Acad Sci U S A* **88**, 1546-1550 (1991).

120 Gregorovic, G. *et al.* Cellular gene expression that correlates with EBER expression in Epstein-Barr Virus-infected lymphoblastoid cell lines. *J Virol* **85**, 3535-3545 (2011).

121 Gregorovic, G. *et al.* Epstein-Barr viruses deficient in EBER RNAs give higher LMP2 RNA expression in lymphoblastoid cell lines and efficiently establish persistent infection in humanized mice. *J Virol* **89**, 11711-4 (2015).

122 McHugh, D. *et al.* Infection and immune control of human oncogenic gamma-herpesviruses in humanized mice. *Philos Trans R Soc Lond B Biol Sci* **374**, 20180296 (2019).

123 Martin, D. F. *et al.* Oral ganciclovir for patients with cytomegalovirus retinitis treated with a ganciclovir implant. Roche Ganciclovir Study Group. *N Engl J Med* **340**, 1063-1070 (1999).

124 Bilger, A. *et al.* Leflunomide/teriflunomide inhibit Epstein-Barr virus (EBV)- induced lymphoproliferative disease and lytic viral replication. *Oncotarget* **8**, 44266-44280 (2017).

125 Damania, B. & Münz, C. Immunodeficiencies that predispose to pathologies by human oncogenic gamma-herpesviruses. *FEMS Microbiol Rev* **43**, 181-192 (2019).

126 Sullivan, N. L. *et al.* Understanding the immunology of the Zostavax shingles vaccine. *Curr Opin Immunol* **59**, 25-30 (2019).

127 Rühl, J. *et al.* Heterologous prime-boost vaccination protects from EBV antigen expressing lymphomas. *J Clin Invest* **129**, 2071-2087 (2019).

128 Bu, W. *et al.* Immunization with Components of the Viral Fusion Apparatus Elicits Antibodies That Neutralize Epstein-Barr Virus in B Cells and Epithelial Cells. *Immunity* **50**, 1305-1316 e1306 (2019).

129 Kanekiyo, M. *et al.* Rational Design of an Epstein-Barr Virus Vaccine Targeting the Receptor-Binding Site. *Cell* **162**, 1090-1100 (2015).

130 Snijder, J. *et al.* An Antibody Targeting the Fusion Machinery Neutralizes Dual-Tropic Infection and Defines a Site of Vulnerability on Epstein-Barr Virus. *Immunity* **48**, 799-811 (2018).

131 Parkin, D. M. The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* **118**, 3030-3044 (2006).

132 Bouvard, V. *et al.* A review of human carcinogens--Part B: biological agents. *Lancet Oncol* **10**, 321-322 (2009).

133 Cohen, J. I., Fauci, A. S., Varmus, H. & Nabel, G. J. Epstein-Barr virus: an important vaccine target for cancer prevention. *Sci Transl Med* **3**, 107fs107 (2011).

134 Totonchy, J. & Cesarman, E. Does persistent HIV replication explain continued lymphoma incidence in the era of effective antiretroviral therapy? *Curr Opin Virol* **20**, 71-77 (2016).

135 McLaughlin, L. P., Gottschalk, S., Rooney, C. M. & Bollard, C. M. EBV-Directed T Cell Therapeutics for EBV-Associated Lymphomas. *Methods Mol Biol* **1532**, 255-265 (2017).

136 Smith, C. *et al.* Effective treatment of metastatic forms of Epstein-Barr virus-associated nasopharyngeal carcinoma with a novel adenovirus-based adoptive immunotherapy. *Cancer Res* **72**, 1116-1125 (2012).

137 Ansell, S. M. *et al.* PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med* **372**, 311-319 (2015).

138 Olsson, T., Barcellos, L. F. & Alfredsson, L. Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nat Rev Neurol* **13**, 25-36 (2017).

139 Pender, M. P. *et al.* Epstein-Barr virus-specific T cell therapy for progressive multiple sclerosis. *JCI Insight* **3**, 124714 (2018).

140 Sokal, E. M. *et al.* Recombinant gp350 vaccine for infectious mononucleosis: a phase 2, randomized, double-blind, placebo-controlled trial to evaluate the safety, immunogenicity, and efficacy of an Epstein-Barr virus vaccine in healthy young adults. *J Infect Dis* **196**, 1749-1753 (2007).

Highlighted references

18 Murer, A. *et al.* EBV persistence without its EBNA3A and 3C oncogenes in vivo. *PLoS Pathog* **14**, e1007039 (2018).

This reference demonstrates that latency III does not need to be established for EBV to gain access to persistence in latency 0.

19 Ma, S. D. *et al.* A new model of Epstein-Barr virus infection reveals an important role for early lytic viral protein expression in the development of lymphomas. *J Virol* **85**, 165-177 (2011).

This reference shows that EBV deficient in lytic gene expression does not efficiently establish lymphomas in HIS mice.

56 Ma, Y. *et al.* CRISPR/Cas9 Screens Reveal Epstein-Barr Virus-Transformed B Cell Host Dependency Factors. *Cell Host Microbe* **21**, 580-591 e587 (2017).

57 Jiang, S. *et al.* The Epstein-Barr Virus Regulome in Lymphoblastoid Cells. *Cell Host Microbe* **22**, 561-573 e564 (2017).

These two references describe systematic approaches to characterize host cell factors and superenhancer regulation during B cell transformation by EBV.

70 Okuno, Y. *et al.* Defective Epstein-Barr virus in chronic active infection and haematological malignancy. *Nat Microbiol* **4**, 404-413 (2019).

This reference demonstrates that EBV isolates with BART miRNA deficiencies and higher lytic EBV gene expression are enriched in EBV pathologies.

99 Murer, A. *et al.* MicroRNAs of Epstein-Barr Virus Attenuate T-Cell-Mediated Immune Control In Vivo. *MBio* **10**, e01941-18 (2019).

102 Albanese, M. *et al.* Epstein-Barr virus microRNAs reduce immune surveillance by virus-specific $CD8^+$ T cells. *Proc Natl Acad Sci U S A* **113**, E6467-E6475 (2016).

These two references demonstrate that EBV miRNAs mainly serve the role of immune escape from $CD8^+$ T cell responses.

Glossary terms

Latency: virus persistence without virion production.

Abortive lytic replication: early lytic viral gene expression without virion production.

EBNA: Epstein–Barr virus nuclear antigen that is expressed during latent infection with oncogenic function.

LMP: Epstein–Barr virus encoded latent membrane protein that mimics signals that B cells have to receive in germinal centers for their survival and that contribute to viral oncogenesis.
BZLF1: An immediate early lytic transcription factor that initiates lytic EBV replication from fully methylated viral DNA.

Box 1: Clinical aspects of Epstein–Barr virus infection
Epstein–Barr virus (EBV) is a WHO class I carcinogen[131,132]. It is estimated to cause 1–2% of all tumors in humans and ~200,000 new cancers per year[133]. Epithelial cancers like nasopharyngeal carcinoma and the ~10% of gastric carcinoma that are associated with EBV outnumber in incidence the EBV-associated lymphomas, which include Burkitt's, Hodgkin's, diffuse large B cell, NK/T cell and primary effusion lymphoma[6,10]. The B cell lymphomas emerge either spontaneously or during immune suppression, for example, during HIV-1 co-infection[134]. Although B cell depleting therapy and EBV specific T cell transfer can often therapeutically address EBV associated B cell lymphomas[135], the therapeutic options for the epithelial cell cancers, especially at an advanced disease stage, are often limited. However, adoptive EBV specific T cell transfer is currently being explored for nasopharyngeal carcinoma[136]. For Hodgkin's lymphoma, immune checkpoint blockade of PD-1 has also shown promising results[137]. Thus, EBV causes a variety of tumors due to failing immune control, some of which can be treated by restoring EBV specific T cell responses by adoptive transfer or blocking of inhibitory receptors.

By contrast, other EBV associated pathologies seem to result from too strong immune responses, which do not efficiently clear the virus. These immunopathologies include symptomatic primary EBV infection or infectious mononucleosis, EBV associated hemophagocytic lymphohistiocytosis (HLH) and possibly the autoimmune disease multiple sclerosis[7,125,138]. The symptoms of these diseases might be caused by the efficient stimulation of T cell mediated cytokine production by latently EBV infected B cells, in the absence of efficient cytotoxic elimination of infected cells. In multiple sclerosis, adoptive transfer of EBV specific T cells has been tried to eliminate this T cell stimulating EBV reservoir, with promising initial results[139]. In addition, vaccination against EBV will probably be further explored in EBV seronegative adolescents to prevent infectious mononucleosis[140].

Figure legends

Figure 1: Models of latent Epstein–Barr virus infection to reach viral persistence.

Epstein–Barr virus (EBV) persists in circulating memory B cells without viral protein expression (latency 0). Only during homeostatic proliferation of these memory B cells is *EBNA1* transiently expressed. After transfer across the mucosal epithelium from the saliva, the virus infects B cells in secondary lymphoid tissues like the tonsils. This infection leads to EBNA2-dependent proliferation of infected cells. Infected memory B cells may differentiate directly into latency 0 after infection. Alternatively, it drives naïve B cells into full latency III (during which *EBNA1*, *EBNA2*, *EBNA3A-C*, *EBNA-LP*, *LMP1* and *LMP2* are expressed) transformation and this activation leads to their differentiation via latency IIa (during which *EBNA1*, *LMP1* and *LMP2* are expressed) expressing germinal center B cells to latency 0 memory B cells. This germinal center differentiation pathway is thought to provide premalignant precursors of the EBV-associated diffuse large B cell lymphoma, Hodgkin's lymphoma and Burkitt's lymphoma. From circulating memory B cells, EBV reactivates lytic replication upon plasma cell differentiation and primary effusion lymphomas (PELs) are EBV associated plasmacytomas. This lytic reactivation most likely allows epithelial cell infection from the basolateral side for efficient shedding into the saliva and virus transmission. This epithelial cell infection gives rise to EBV associated carcinomas, for example, nasopharyngeal carcinoma (NPC). Expression of the viral non-translated RNAs (EBERs, BART and BHRF1 miRNAs) is also depicted.

Figure 2: Persistence without transformation.

Upon B cell infection by Epstein–Barr virus (EBV) the viral BCL2 homologues *BHRF1* and *BALF1* are expressed during the first three days to ensure survival of the host cell. Then EBNA2 drives cellular proliferation through the viral oncogene *MYC* and cooperates with EBNA-LP for *LMP1* and *LMP2* expression. The resulting apoptosis induction by p16INK4a and BIM is blocked by EBNA3A and EBNA3C. After several weeks, *LMP1* and *LMP2* expression activates NF-κB transcription and this completes B cell transformation. EBV persistence in memory B cells without viral gene expression can be reached after transformation through differentiation in germinal centers, or directly from the EBNA2-induced B cell proliferation outside of germinal centers.

Figure 3: Oncogenesis with lytic replication.

Conditions that lead to higher *BZLF1* expression and thus induction of lytic Epstein–Barr virus (EBV) replication increase virus driven tumorigenesis. These include elevated *BZLF1*

expression due to loss of BART miRNA-mediated suppression (Δ*BART*), *BZLF1* promotors that increase expression (ZV, ZV′, ZIIR and V3) or polymorphisms in the *BZLF1* coding sequence (M81 *BZLF1*). Suppression of *BZLF1* expression (Δ*BZLF1*) inhibits virus induced lymphoma formation. Lytic replication driven by the *BZLF1* gene of the B95-8 virus isolate causes an intermediary phenotype.

Figure 4: Potential functions of lytic EBV antigens and non-translated RNAs during Epstein–Barr virus driven tumor formation.
Early, most likely abortive lytic Epstein–Barr virus (EBV) replication, might condition the tumor microenvironment for EBV associated malignancies through attraction of monocytes via CCL5 and their differentiation into immune suppressive tumor associated macrophages (TAMs). These TAMs and early lytic EBV replication seem to promote IL-10 production to suppress protective cytotoxic lymphocyte responses, including $CD8^+$ T cells. In addition, EBV encoded miRNAs compromise the attraction of these cytotoxic lymphocytes into the tumor microenvironment by down-regulating *CXCL11* expression and also inhibit antigen presentation on MHC class I molecules to these $CD8^+$ T cells. Thus, early lytic EBV replication and viral miRNAs seem to collaborate to render the microenvironment of EBV associated malignancies immune suppressive.

Figure 5: Lytic replication in clinical manifestations of Epstein–Barr virus infection.
The association of varying degrees of lytic Epstein–Barr virus (EBV) replication with EBV associated malignancies (lymphomas and carcinomas), overt lytic EBV replication in the tongue epithelium (oral hairy leukoplakia) and immune pathologies (infectious mononucleosis). These associations are based on the enrichment of viral strains with enhanced lytic replication with the respective tumors, detection of serum viral loads in affected patients and decreased tumorigenesis of certain lymphomas (post-transplant lymphoproliferative disease, diffuse large B cell lymphoma and primary effusion lymphoma) upon lytic replication-incompetent EBV infection of preclinical in vivo models.

Figure 6: Non-translated RNAs in the Epstein–Barr virus genome.
Schematic depiction of the 172 kb Epstein–Barr virus (EBV) genome showing the location of the two Epstein–Barr virus encoded small RNAs (EBERs), the BHRF1 (BHRF1 miR) and BART miRNAs (BART miR). The locations of the latent EBV genes encoding EBNA-LP, EBNA2, EBNA3A-3C and EBNA1, as well as LMP1 and LMP2 are also shown. The loci of the viral BCL2

homologues BHRF1 and BALF1, as well as the immediate early transcription factors for lytic EBV replication, BZLF1 and BRLF1, are depicted.

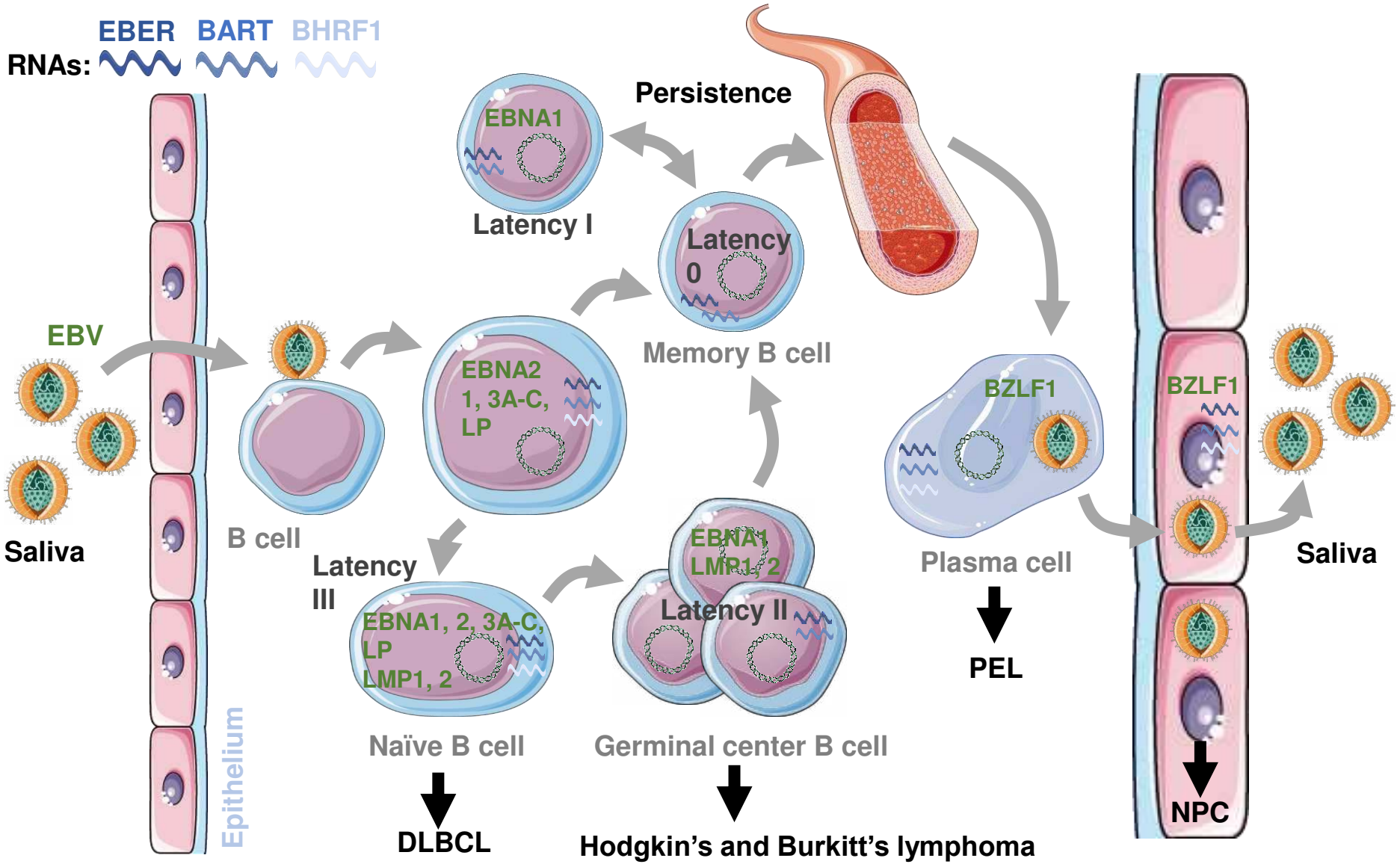
EBER BART BHRF1
RNAs:
EBNA1
Latency I
Persistence
Latency 0
Memory B cell
EBV
Saliva
B cell
EBNA2 1, 3A-C, LP
Latency III
EBNA1, 2, 3A-C, LP LMP1, 2
Naïve B cell
DLBCL
EBNA1 LMP1, 2
Latency II
Germinal center B cell
Hodgkin's and Burkitt's lymphoma
BZLF1
Plasma cell
PEL
BZLF1
Saliva
NPC
Epithelium

Figure 1

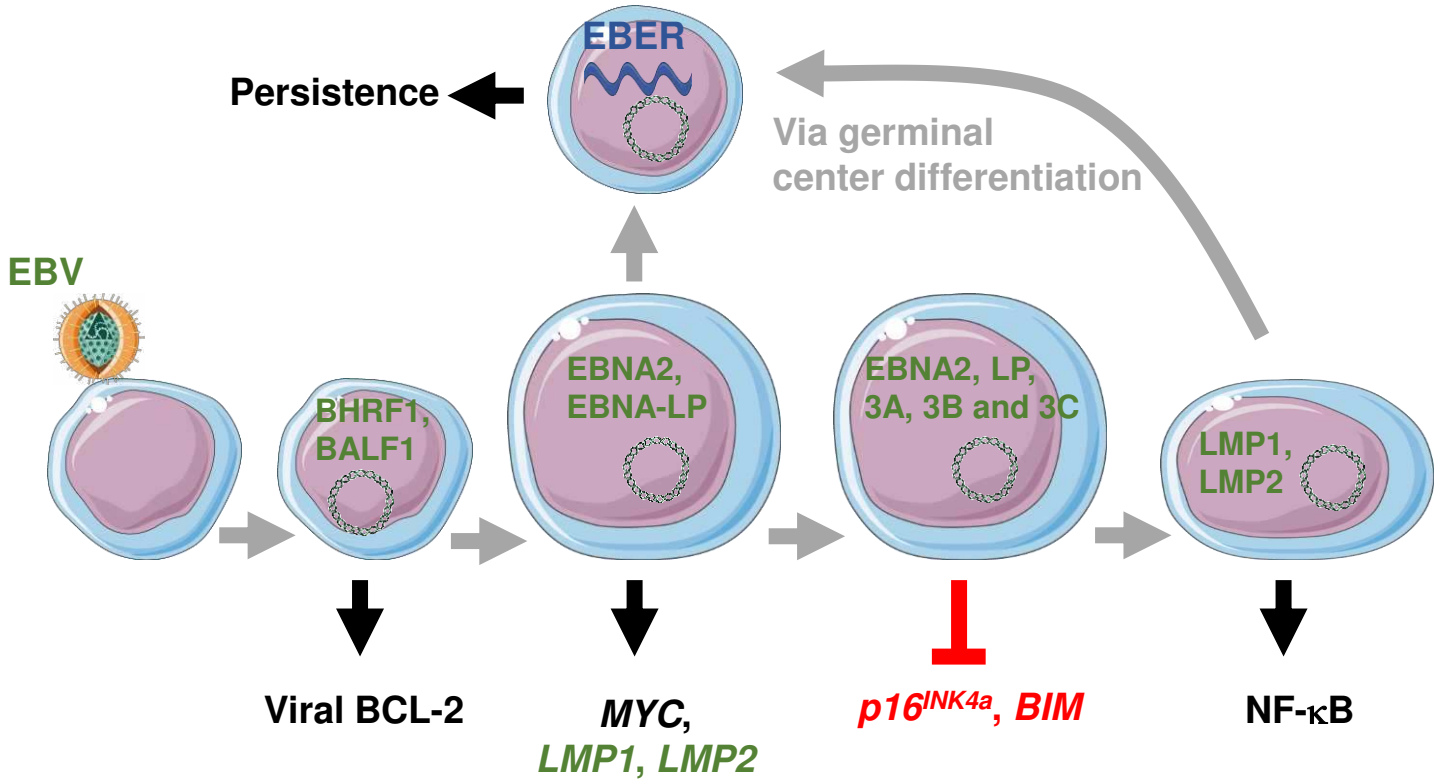
EBER
Persistence
Via germinal
center differentiation
EBV
BHRF1,
BALF1
EBNA2,
EBNA-LP
EBNA2, LP,
3A, 3B and 3C
LMP1,
LMP2
Viral BCL-2
MYC,
LMP1, LMP2
p16INK4a, BIM
NF-κB

Figure 2

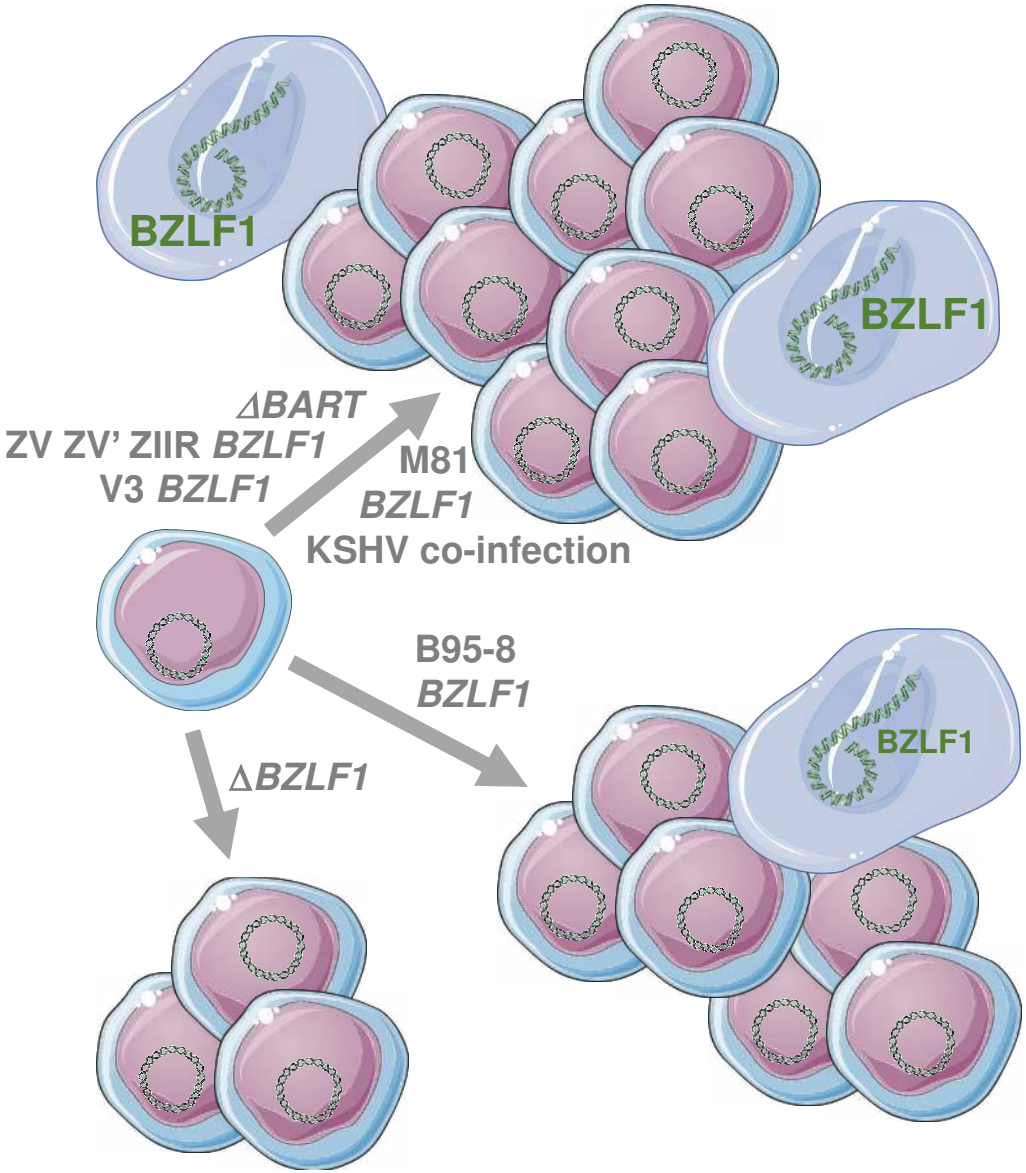
BZLF1
BZLF1
ΔBART
ZV ZV' ZIIR BZLF1
V3 BZLF1
M81
BZLF1
KSHV co-infection
B95-8
BZLF1
ΔBZLF1
BZLF1

Figure 3

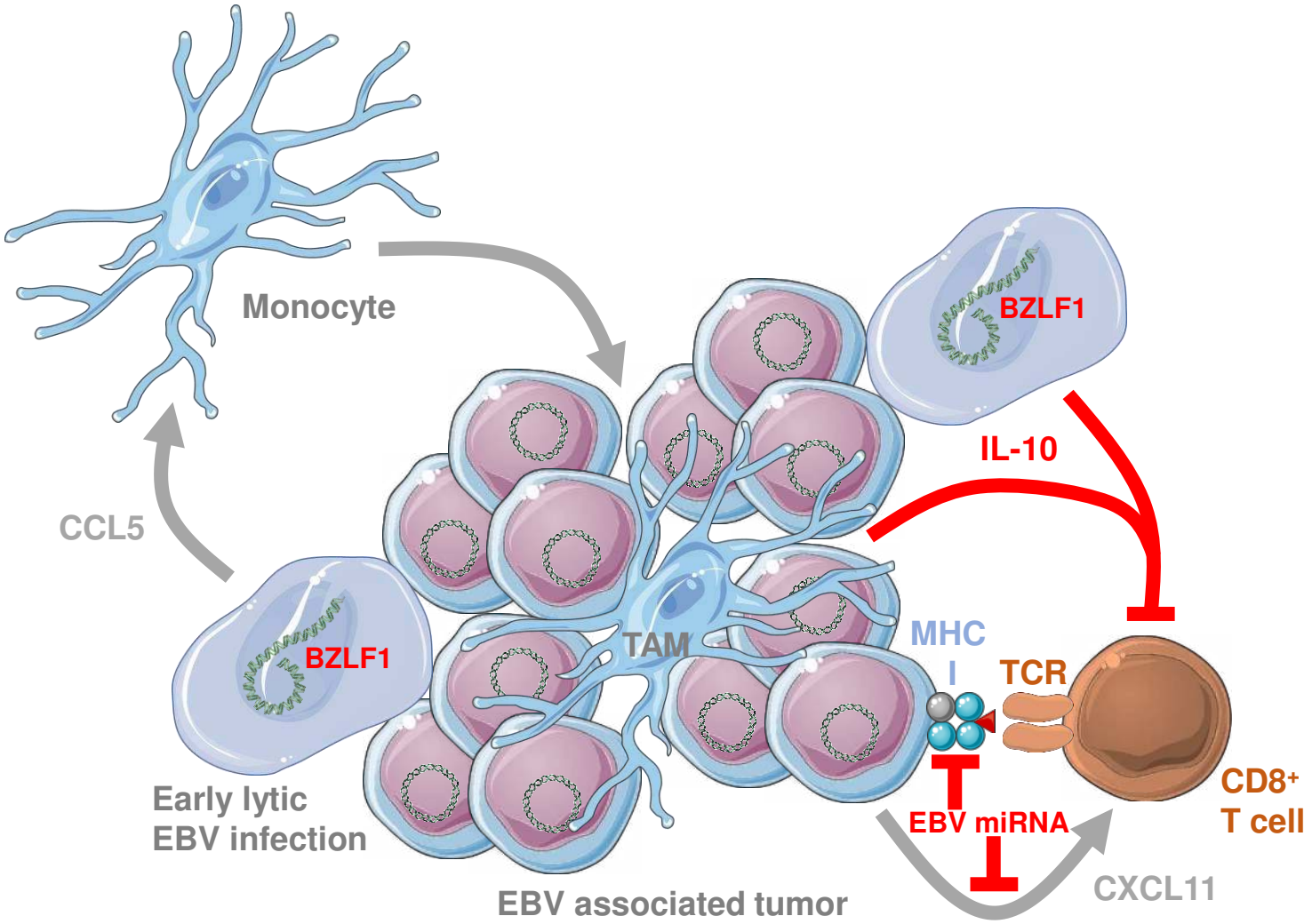
Monocyte
BZLF1
IL-10
CCL5
BZLF1
TAM
MHC
I
TCR
Early lytic
EBV infection
CD8+
T cell
EBV miRNA
EBV associated tumor
CXCL11

Figure 4

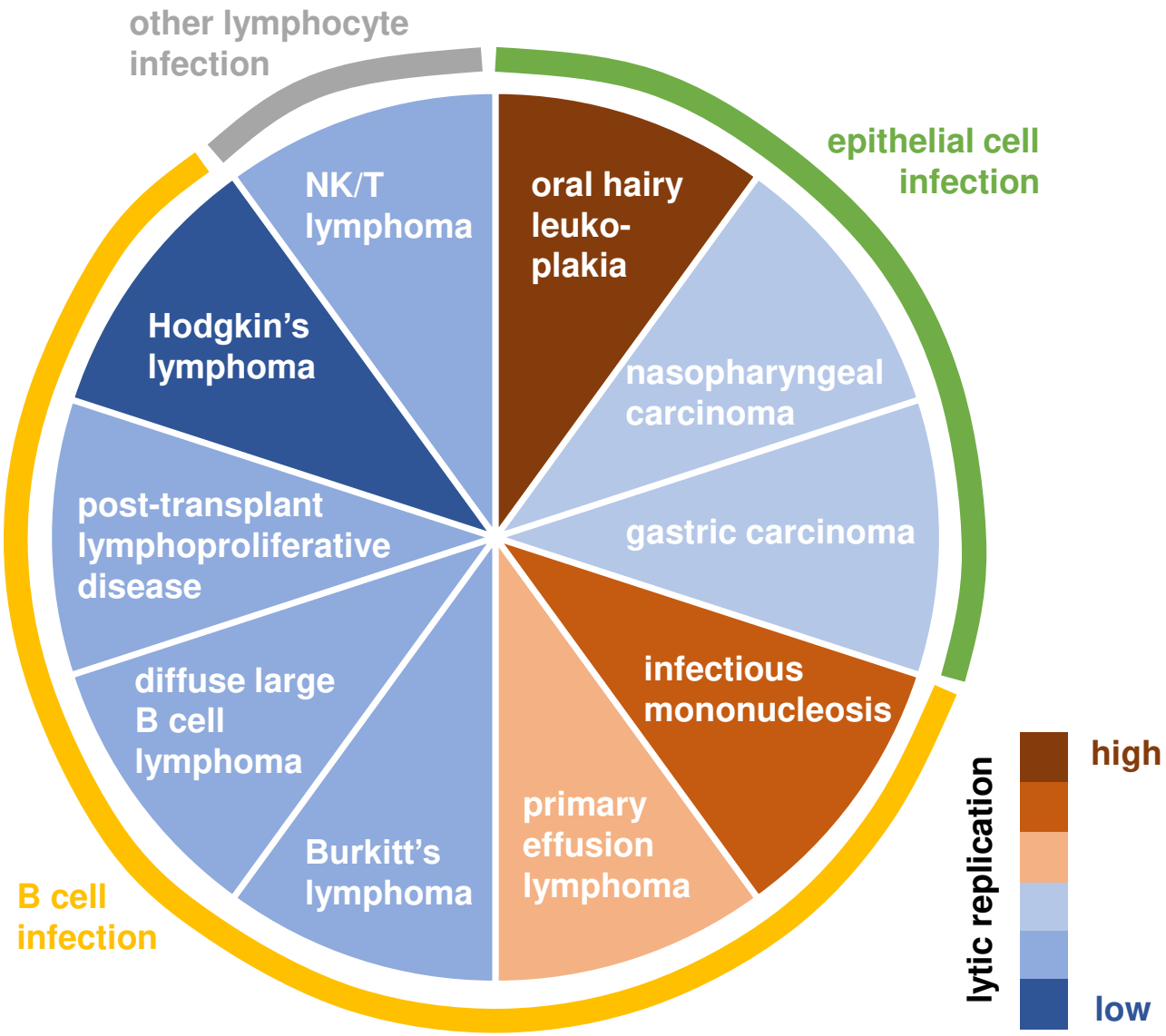
other lymphocyte infection
epithelial cell infection
B cell infection
NK/T lymphoma
oral hairy leuko-plakia
Hodgkin's lymphoma
nasopharyngeal carcinoma
post-transplant lymphoproliferative disease
gastric carcinoma
diffuse large B cell lymphoma
infectious mononucleosis
Burkitt's lymphoma
primary effusion lymphoma
lytic replication
high
low

Figure 5

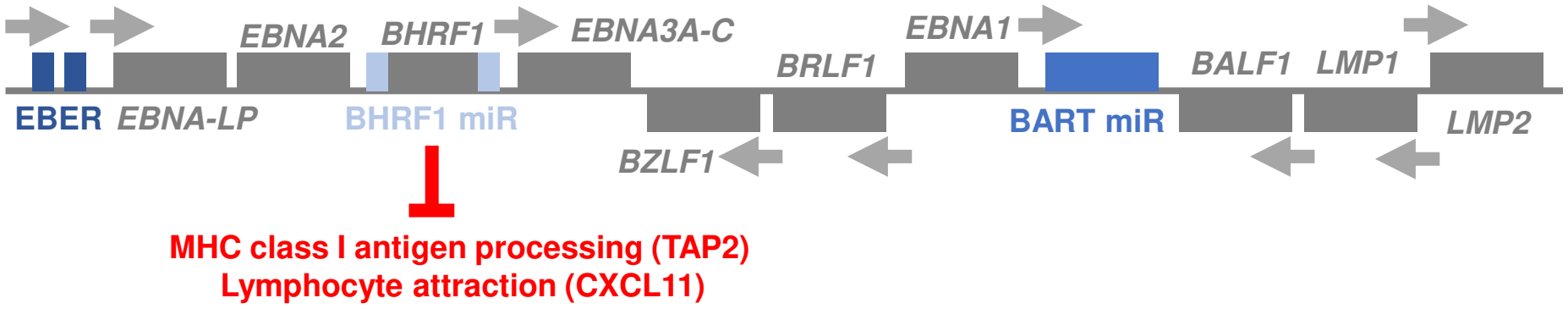
EBNA2
BHRF1
EBNA3A-C
EBNA1
BRLF1
BALF1
LMP1
EBER
EBNA-LP
BHRF1 miR
BART miR
LMP2
BZLF1
MHC class I antigen processing (TAP2)
Lymphocyte attraction (CXCL11)

Figure 6