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

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Abstract | Epstein–Barr virus (EBV) was the first tumour virus identified in humans. The virus is primarily associated with lymphomas and epithelial cell cancers. These tumours 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 tumorigenesis, the function of non-coding 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-coding RNAs in virus-driven tumour formation. Accordingly, early lytic EBV antigens and attenuated viruses without oncogenes and microRNAs could be harnessed for immunotherapies and vaccination.

Burkitt's lymphoma
The B cell tumour in which Epstein–Barr virus was discovered and that expresses *EBNA1* as the only viral gene in the context of *MYC* translocations into the immunoglobulin locus.

Infectious mononucleosis
Immunopathological primary Epstein–Barr virus infection with massive $CD8^+$ T cell lymphocytosis.

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https://doi.org/10.1038/s41579-019-0249-7

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]. The virus was originally identified in 1964 by Sir Anthony Epstein and co-workers in Burkitt's lymphoma, which is still the most common childhood tumour 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 such as 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, the 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 2 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. However, 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.

Box 1 | **Clinical aspects of Epstein–Barr virus infection**

Epstein–Barr virus (EBV) is a WHO class I carcinogen[132,133]. EBV is estimated to cause 1–2% of all tumours in humans and ~200,000 new cancers per year[134]. Epithelial cancers such as nasopharyngeal carcinoma and the ~10% of gastric carcinomas that are associated with EBV outnumber in incidence the EBV-associated lymphomas, which include Burkitt's lymphoma, Hodgkin's lymphoma, diffuse large B cell lymphoma, natural killer (NK)/T cell lymphoma and primary effusion lymphoma[6,10]. The B cell lymphomas emerge either spontaneously or during immune suppression, for example during HIV-1 co-infection[135]. Although B cell-depleting therapy and EBV-specific T cell transfer can often therapeutically address EBV-associated B cell lymphomas[136], 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[137]. For Hodgkin's lymphoma, immune checkpoint blockade of PD-1 has also shown promising results[138]. Thus, EBV causes various tumours owing 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 immune responses that are too strong, which do not efficiently clear the virus. These immunopathologies include symptomatic primary EBV infection or infectious mononucleosis, EBV-associated haemophagocytic lymphohistiocytosis and, possibly, the autoimmune disease multiple sclerosis[7,126,139]. 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[140]. In addition, vaccination against EBV will probably be further explored in EBV-seronegative adolescents to prevent infectious mononucleosis[141].

In B cells, EBV persists by B cell transformation into immortalized proliferating LCLs in vitro and by establishing latency with only non-coding 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-coding 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 only three latent EBV proteins during the B cell differentiation stage of germinal centre B cells that could result from naive 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-coding RNAs in EBV-driven tumour formation. These are timely topics as the field gears up to develop an EBV-specific vaccine and the identity of the infection programmes and their antigens that should be targeted is hotly debated. Moreover, attenuated viruses, including virus-like particles, are considered vaccine candidates[24], but the new roles of viral oncogenes in persistence and of lytic EBV antigens and non-coding RNAs in tumorigenesis could also point towards attenuated viruses without the respective genes as viable vaccine candidates.

Latency
Virus persistence without virion production.

Germinal centre
The location of activated naive B cell differentiation with B cell receptor affinity maturation due to somatic hypermutation, in which centroblasts and centrocytes (activated and resting germinal centre B cells) need to receive signals via their B cell receptor engaging antigen on follicular dendritic cells (signal 1) and T cell help via CD40 (signal 2), in order to survive.

Abortive lytic replication
Early lytic viral gene expression without virion production.

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

Latent membrane proteins
An Epstein–Barr virus-encoded latent membrane protein that mimics signals that B cells have to receive in germinal centres for their survival and that contribute to viral oncogenesis.

Epstein–Barr virus replication

EBV can replicate by two means — infected 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 programme of infection in B cells and seems to be sufficient to 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 eight EBV proteins, two EBV-encoded small RNAs (EBERs) that are not translated and 25 pre-microRNAs (pre-miRNAs) that give rise to at least 44 miRNAs[20,25]. All of these can be found in LCLs, naive tonsillar B cells of healthy virus carriers and nearly all tonsillar B cells of individuals with infectious mononucleosis[21,26,27] (FIG. 1). The respective viral gene expression programme is called latency III. Presumably after activation from EBV latency III, B cells enter the germinal centre reaction and only three latent EBV proteins can be found in centroblasts and centrocytes[21]. These proteins are Epstein–Barr nuclear antigen 1 (EBNA1) and the two latent membrane proteins (LMP1 and LMP2). Their expression in the so-called latency IIa programme is thought to ensure that EBV-infected B cells survive the germinal centre reaction to gain access to the memory B cell pool, in which EBV persists without viral protein expression in latency 0 (REF.[19]). Only during homeostatic proliferation is *EBNA1* transiently expressed in memory B cells, and this pattern is called latency I (REF.[28]). These latent EBV infection programmes 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 (REFS[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 programmes leads to lytic reactivation[29]. The resulting plasma cell differentiation stimulates *BZLF1* expression via the plasma cell-associated transcription factors XBP1 and BLIMP1 (REFS[30,31]) (FIG. 1). Lytic EBV gene products then further stimulate plasma cell differentiation with B cell receptor downregulation 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

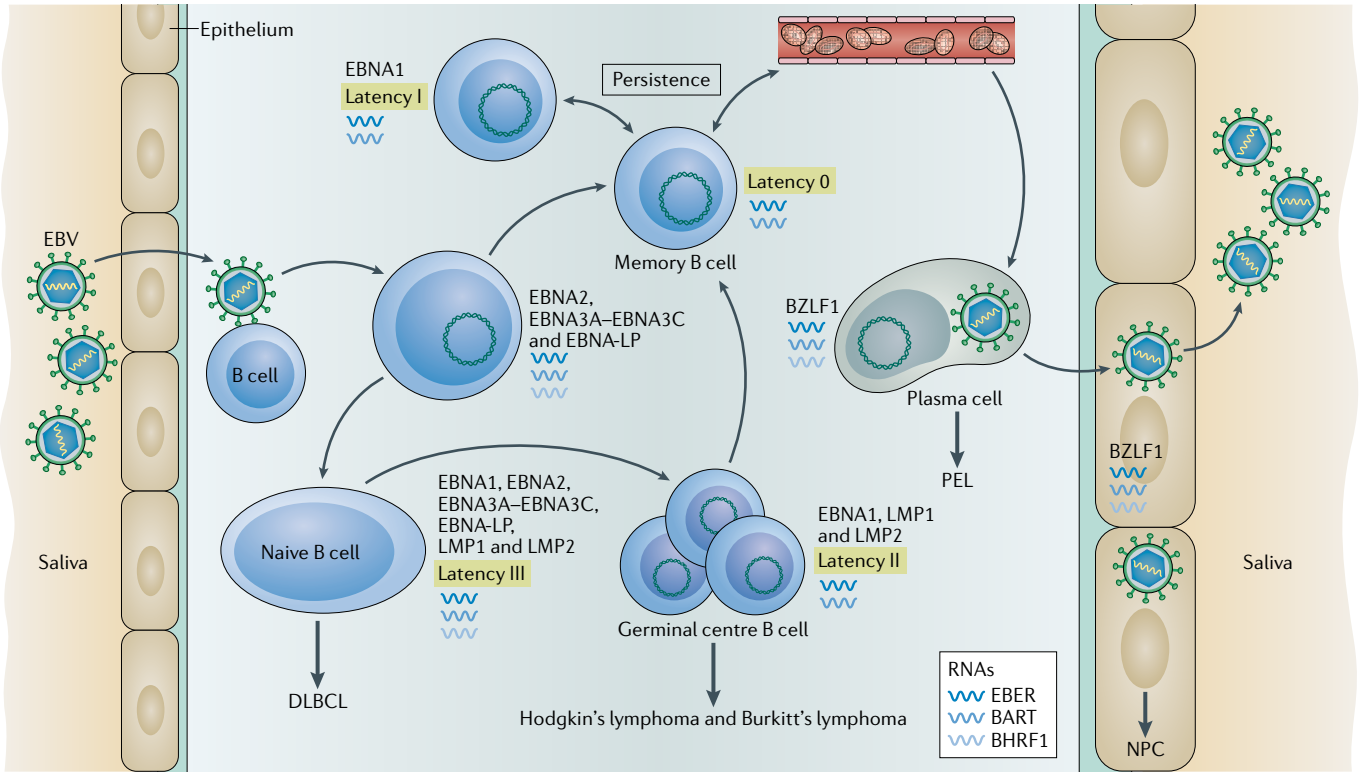

Fig. 1 | **Models of latent Epstein–Barr virus infection to reach viral persistence.** Epstein–Barr virus (EBV) persists in circulating memory B cells without viral protein expression (latency 0). Only during homeostatic proliferation of these memory B cells is *EBNA1* transiently expressed. After transfer across the mucosal epithelium from the saliva, the virus infects B cells in secondary lymphoid tissues such as the tonsils. This infection leads to Epstein–Barr nuclear antigen 2 (EBNA2)-dependent proliferation of infected cells. Infected memory B cells may differentiate directly into latency 0 after infection. Alternatively, EBV drives naive B cells into full latency III transformation (during which *EBNA1*, *EBNA2*, *EBNA3A–EBNA3C*, *EBNA-LP*, *LMP1* and *LMP2* are expressed) and this activation leads to their differentiation via latency IIa-expressing germinal centre B cells (in which *EBNA1*, *LMP1* and *LMP2* are expressed) to latency 0 memory B cells. This germinal centre differentiation pathway is thought to provide premalignant precursors of the EBV-associated diffuse large B cell lymphoma (DLBCL), Hodgkin's lymphoma and Burkitt's lymphoma. From circulating memory B cells, EBV reactivates lytic replication upon plasma cell differentiation and elevated lytic EBV replication can also be found in the EBV-associated plasmacytoma primary effusion lymphoma (PEL). 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-coding RNAs (EBV-encoded small RNAs (EBERs), BART and BHRF1 microRNAs) is also depicted. EBNA-LP, EBNA leader peptide; LMP, latent membrane protein.

into the saliva. This epithelial cell infection presumably occurs via virus binding to αvβ 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 centre reaction, where additional mutations can arise via the machinery that diversifies the B cell receptor in this reaction. Some of the somatic mutations that are thought to be introduced in the germinal centre reaction substitute for the downregulation of some of the latency III EBV antigens in tumours such as Hodgkin's lymphoma and Burkitt's lymphoma[6].

The functions of the respective EBV gene products give the virus its oncogenic abilities. Many of the respective proteins (the six nuclear antigens or EBNAs and the two membrane proteins or LMPs), however, are like a Swiss army knife 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

BZLF1
An immediate early lytic transcription factor that initiates lytic Epstein–Barr virus replication from fully methylated viral DNA.

infection prior to mitosis and then 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 tumours 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 expression, including *LMP1* (REF.[42]). EBNA3A and EBNA3C rescue infected cells that are driven into a proliferative state by EBNA2-dependent *MYC* expression via the downregulation of the pro-apoptotic 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 at which 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 centre reaction[46]. LMP2 constitutively engages signalling similar to the B cell receptor, which needs to be engaged by antigen on follicular dendritic cells as signal 1 in order for B cells to not undergo apoptosis in germinal centres[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 cell help in the germinal centre by constitutively signalling 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 centre 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 centres 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 EBNA3C, 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 tumour 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-coding 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 tumour formation without lytic EBV protein and EBV miRNA expression.

Persistence without transformation

The above linear differentiation model from latency III to latency II and then to latency 0 or I is also called the germinal centre model of EBV persistence[20]. This model was originally proposed on the basis of successive downregulation 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

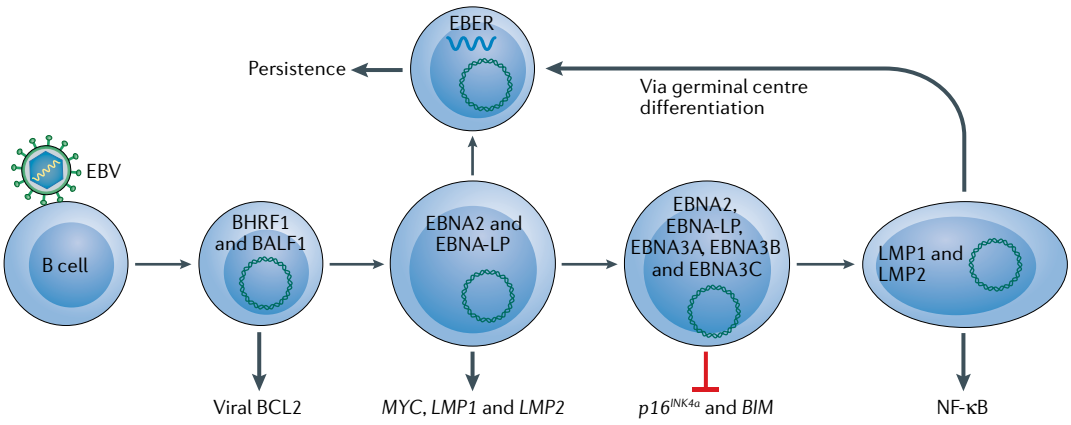

Fig. 2 | **Persistence without transformation.** Upon B cell infection by Epstein–Barr virus (EBV), the viral BCL2 homologues *BHRF1* and *BALF1* are expressed during the first 3 days to ensure survival of the host cell. Then, Epstein–Barr nuclear antigen 2 (EBNA2) drives cellular proliferation through the viral oncogene *MYC* and cooperates with EBNA leader peptide (EBNA-LP) for *LMP1* and *LMP2* expression. The resulting apoptosis induction by $p16^{INK4a}$ and BIM is blocked by EBNA3A and EBNA3C. After several weeks, *LMP1* and *LMP2* expression activates nuclear factor-κB (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 centres, or directly from the EBNA2-induced B cell proliferation outside germinal centres. EBER, EBV-encoded small RNA; LMP, latent membrane protein.

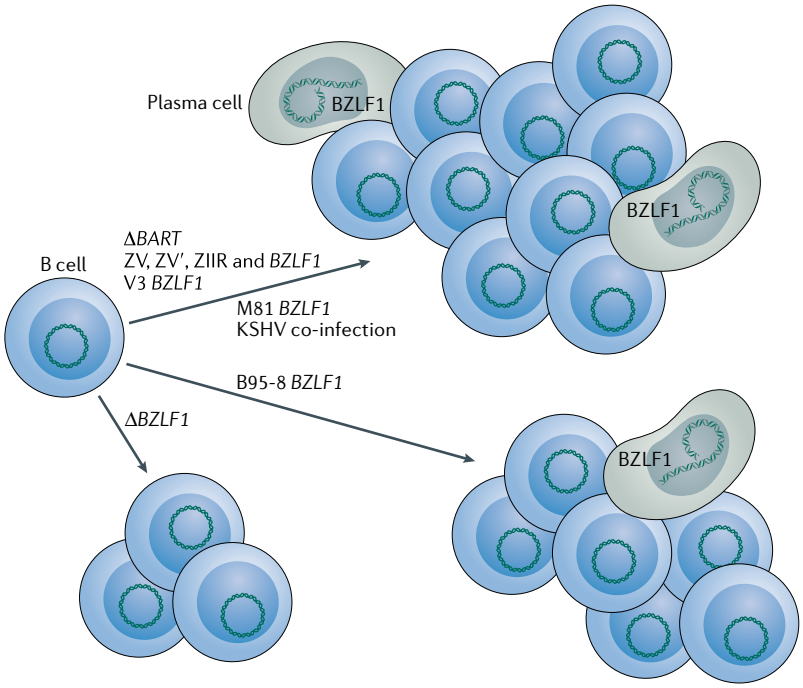

Fig. 3 | **Oncogenesis with lytic replication.** Conditions that lead to higher *BZLF1* expression, and thus induction of lytic Epstein–Barr virus (EBV) replication, increase virus-driven tumorigenesis. These include elevated *BZLF1* expression due to loss of BART microRNA-mediated suppression (Δ*BART*), *BZLF1* promoters that increase expression (ZV, ZV′, ZIIR and V3), polymorphisms in the *BZLF1* coding sequence (M81 *BZLF1*) and Kaposi sarcoma-associated herpesvirus (KSHV) co-infection. 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.

reach the memory B cell pool without latency III protein expression as a prerequisite, and outside the germinal centre. Indeed, even under conditions in which germinal centres are disorganized, such as during infectious mononucleosis[26], latency 0 expressing B cells start circulating at increased frequency compared with persistent infection 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]. As the germinal centre 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 germinal centres 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 enables investigation into whether all other EBV latency programmes that presumably differentiate from this transformation programme are also abolished in the absence of essential latency III genes. Latency 0 persistence without latency III transformation 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 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-coding *EBER* expression after 3 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, *LMP1*-expressing EBNA3C-negative lymphomas can be observed at lower frequency compared with 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 (FIG. 1). Nevertheless, the combination of both the germinal centre and the persistence without transformation pathways might increase the efficacy of EBV in setting up persistence in memory B cells 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 genes are sequentially expressed during the first 3 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] (FIG. 2). EBNA2 then starts driving proliferation of the infected B cells within the first 3 days through *MYC* expression among other factors[61]. The resulting rapid cell division (8–10 h doubling time) activates the DNA damage response[61] with an increase in *BIM* and $p16^{INK4a}$ tumour 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 programmes induced by EBNA2, EBNA3A and EBNA3C dominate the first 2 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 programme is also called latency IIb[65,66] and has been observed in infectious mononucleosis and post-transplant lymphoproliferative disease patients[67,68]. Only after 2–3 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 24 h. 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 programme into latency 0 persistence (FIG. 2) in the absence of EBNA3C and, to a lesser extent, EBNA3A. This might

HIS mice
In the context of this review, immunodeficient mice with reconstituted human immune system compartments after transfer of human CD34⁺ haematopoietic progenitor cells or human cord blood mononuclear cells.

Superenhancers
Often distal genetic elements that strongly increase gene promoter activity.

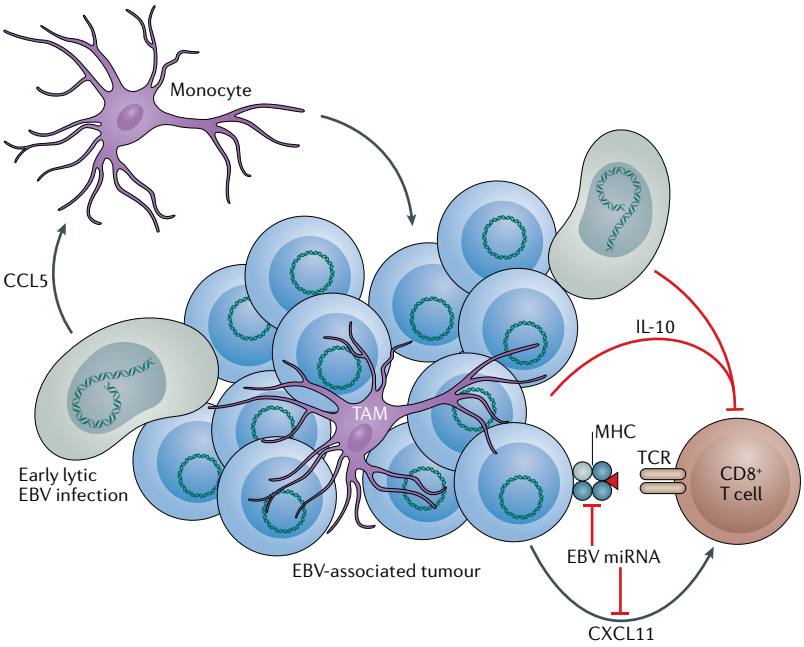

Fig. 4 | **Potential functions of lytic Epstein–Barr virus antigens and non-coding RNAs during Epstein–Barr virus-driven tumour formation.** Early, most likely abortive, lytic Epstein–Barr virus (EBV) replication might condition the tumour microenvironment for EBV-associated malignancies through attraction of monocytes via CCL5 and their differentiation into immune-suppressive tumour-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 microRNAs (miRNAs) compromise the attraction of these cytotoxic lymphocytes into the tumour microenvironment by downregulating *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. TCR, T cell receptor.

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 tumour virus to its associated malignancies. It was observed that BZLF1-deficient EBV causes fewer B cell lymphomas in HIS mice[23,70] (FIG. 3). BZLF1 is the immediate early transcription factor for the activation of lytic EBV replication[71]. Therefore, this observation might just be due to an increased viral titre, or perhaps there is 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 observation is fairly common, with often fewer 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 tumour microenvironment by abortive early lytic EBV replication that is responsible for the observed increased tumorigenesis (FIG. 4). Along these lines, it was observed that more tumour necrosis factor, CCL5 (also known as RANTES) 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 tumour growth in a xenograft model through their immune suppressive activities[76]. Contrary to loss of *BZLF1*, mutations in three suppressive elements of the *BZLF1* promoter render the respective EBV more lytic (resulting in more infected cells entering early lytic gene expression)[77]. This ZV, ZV′ and ZIIR triple mutant presents with increased lymphoma formation in HIS mice[78] (FIG. 3). Furthermore, a natural variant of the *BZLF1* promoter was found in EBVs 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] (FIG. 5). This *BZLF1* promoter V3 variant demonstrates elevated induction of lytic EBV replication upon B cell receptor crosslinking or treatment of EBV-infected cells with ionomycin, which activates the transcription factor NFAT. Indeed, the variation in the *BZLF1* V3 promoter 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* promoter, 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 tumour formation.

A role for lytic EBV replication in EBV-associated tumour 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] (FIG. 3). These viruses are thought to promote higher levels of lytic EBV replication owing to upregulation of *BZLF1* and *BRLF1* expression that are suppressed by one of the BART miRNAs[82]. Furthermore, co-infection with Kaposi sarcoma-associated herpesvirus (KSHV; also known as human herpesvirus 8 (HHV8)) stimulates lytic EBV cycle induction and thereby increases lymphomagenesis in HIS mice with hallmarks of primary effusion lymphoma, a plasmacytoma that is often infected by both EBV and KSHV[83]. The increased lytic replication might also contribute to circulating cell-free plasma EBV DNA loads, which are indicative of EBV-associated tumours in various clinical settings[84]. This plasma viral load, rather than peripheral blood cell-associated EBV titres, have been found

to correlate with nasopharyngeal carcinoma[85], post-transplant lymphoproliferative disease[86], diffuse large B cell lymphoma[87], NK/T cell lymphomas[88] and Hodgkin's lymphoma[89]. The risk for Hodgkin's lymphoma is also increased following primary EBV acquisition with infectious mononucleosis[90–92]. Infectious mononucleosis is characterized by elevated virus shedding into the saliva, high antibody titres against structural EBV proteins and massive expansion of lytic EBV antigen-specific CD8$^+$ T cells[93] (FIG. 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 in a small number of individuals by combining pharmacological inhibition of lytic EBV replication with zidovudine, rituximab and dexamethasone[94]. 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 tumour 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 tumour microenvironment.

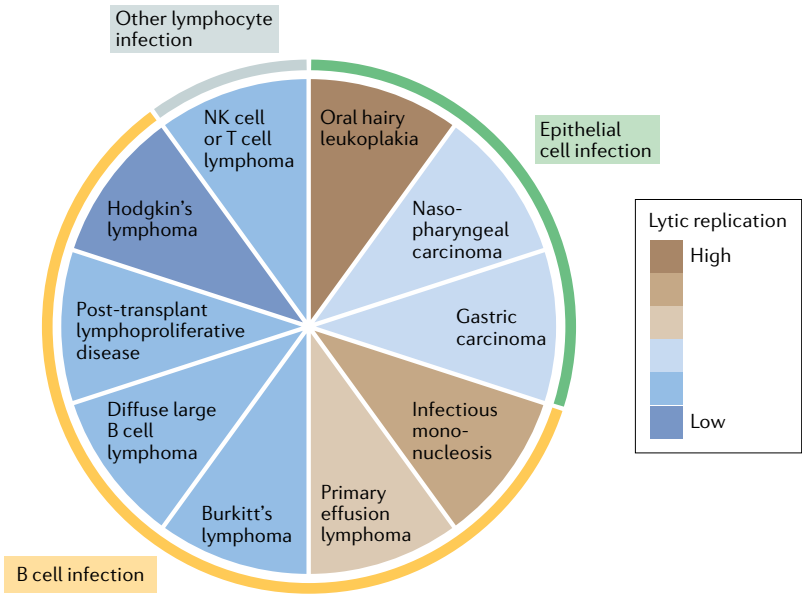

Fig. 5 | **Lytic replication in clinical manifestations of Epstein–Barr virus infection.** The association of varying degrees of lytic Epstein–Barr virus (EBV) replication with EBV-associated malignancies (lymphomas and carcinomas), overt lytic EBV replication in the tongue epithelium (oral hairy leukoplakia) and immune pathologies (infectious mononucleosis). These associations are based on the enrichment of viral strains with enhanced lytic replication with the respective tumours, 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. NK, natural killer.

Non-coding RNAs and tumorigenesis

The non-coding RNAs expressed by EBV include the two EBERs and ~44 miRNAs[25]. Originally, both were suggested to promote EBV-driven tumourigenesis[95–100]. 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] (FIG. 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 tumours, and two of the three pre-miRNAs are expressed during lytic EBV replication[101,102] (FIG. 1). By contrast, the BART miRNAs are expressed in all EBV infection programmes, including EBV latency I and II, albeit at lower levels during latency I[102,103]. 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[104–107]. In addition, BHRF1 miRNAs optimize the timing of *EBNA-LP* and *BHRF1* expression for optimal B cell transformation[108,109] and suppress sumoylation that is required for efficient lytic replication induction[110]. Finally, both BART and BHRF1 miRNAs attenuate B cell receptor signalling and thereby desensitize infected B cells to lytic EBV replication induction[111]. Therefore, both BART and BHRF1 miRNAs contribute to suppression of lytic EBV replication and optimize B cell transformation by EBV[97,98].

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 substantially alter its infection in HIS mice[112]. By contrast, loss of BHRF1 miRNAs either alone or in addition to BART miRNA deletion attenuates B95-8 EBV infection in HIS mice[100,112]. 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 (FIGS 4,6). Depletion of CD8$^+$ T cells restores viral loads and tumorigenicity of miRNA-deficient EBV[112]. 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 tumourigenesis[113,114]. 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[115]. In particular, TAP2 levels are downregulated 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[112,115]. 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 latter function

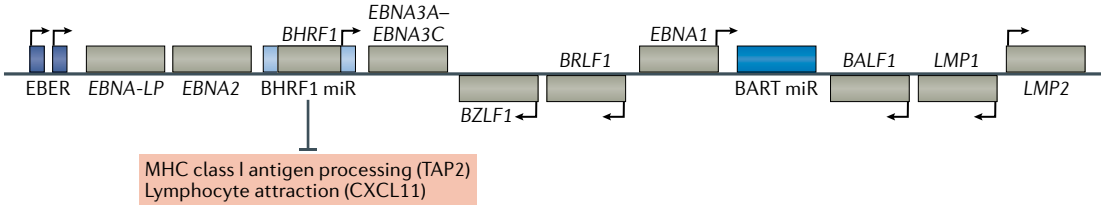

Fig. 6 | **Non-coding RNAs in the Epstein–Barr virus genome.** Schematic depiction of the 172-kb Epstein–Barr virus (EBV) genome showing the location of the two EBV-encoded small RNAs (EBERs) and the BHRF1 and BART microRNAs (miRs). The locations of the latent EBV genes encoding Epstein–Barr nuclear antigen (EBNA) leader peptide (EBNA-LP), EBNA2, EBNA3A–EBNA3C and EBNA1 as well as latent membrane protein 1 (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.

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 programmes[25]. These RNAs are the most abundant viral transcripts, with more than 1 million copies per EBV-infected cell[116]. Owing to their high abundance, in situ hybridization against EBERs still constitutes the gold standard for detecting EBV-infected cells. In contrast to the miRNAs, EBERs are confined to the nucleus and seem to interact with various RNA binding proteins, including La and L22 (REFS[116–118]). Similar to BART miRNAs, EBERs seem to optimize B cell transformation by EBV, but possibly only in certain EBV strains[95,119–121]. Transgenic overexpression of EBERs leads to lymphoproliferations and, less frequently, B cell lymphomas[96]. However, EBER-deficient B95-8 EBV infection in HIS mice did not alter viral loads or tumorigenesis[122]. Thus, as for BART miRNAs, EBERs seem to optimize 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.

Conclusions

Recent studies have changed our view on the tumorigenesis of the most common human tumour 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 have 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 reveals similarities to KSHV, the other oncogenic human γ-herpesvirus[123]. Some of the KSHV-associated malignancies, such as Kaposi sarcoma, seem to depend on lytic replication of this virus[124]. Targeting early lytic antigen expression might provide a promising novel strategy for the treatment of both EBV and KSHV-associated tumours[125].

These three new characteristics of EBV-associated lymphomas and carcinomas might also suggest mechanisms to attenuate and render EBV more immunogenic for vaccination. An *EBNA3C*, *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, such a virus would remove any tumour-promoting early lytic EBV protein expression. Finally, such a Δ*3CZmiR* EBV would make the expressed EBV antigens (presumably EBNA1, EBNA2, EBNA3A, EBNA3B and EBNA-LP) more visible to $CD8^+$ T cells owing to efficient antigen processing for MHC class I presentation and attraction of these T cells into the tumour microenvironment through the CXCR3 ligands CXCL9, CXCL10 (both EBNA3B-induced[45]) and CXCL11 (no longer inhibited by BHRF1 miRNAs[113,114]). The strong dependency on cytotoxic lymphocytes, including the T cells that such an attenuated EBV would elicit with essential features of immunity to EBV[126], 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[127]), has been considered too risky owing 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[128] 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[129–131]. 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.

Published online 2 September 2019

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Acknowledgements

Research in C.M.'s laboratory is supported by Cancer Research Switzerland (KFS-4091-02-2017), KFSP-Precision^{MS} of the University of Zürich, the Vontobel Foundation, the Baugarten Foundation, the Sobek Foundation, the Swiss Vaccine Research Institute, the Swiss Multiple Sclerosis Society, Roche, ReiThera and the Swiss National Science Foundation (310030B_182827 and CRSII5_180323).

Competing interests

The author declares no competing interests.

Peer review information

Nature Reviews Microbiology thanks P. Farrell, S. Kenney and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.