

Review

Epstein–Barr virus, the germinal centre and the development of Hodgkin's lymphoma

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The relationship between Epstein–Barr virus (EBV) and the germinal centre (GC) of the asymptomatic host remains an enigma. The occasional appearance of EBV-positive germinal centres in some patients, particularly those with a history of immunosuppression, suggests that EBV numbers in the GC are subject to immune control. The relationship, if any, between lymphoid hyperplasia with EBV-positive germinal centres and subsequent or concurrent lymphomagenesis remains to be clarified. As far as the development of EBV-associated Hodgkin's lymphoma is concerned, the suppression of virus replication, mediated by LMP1 on the one hand, and the loss of B-cell receptor signalling on the other, appears to be an important pathogenic mechanism. A further important emerging concept is that alterations in the microenvironment of the EBV-infected B-cell may be important for lymphomagenesis.

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Introduction

The Epstein–Barr virus (EBV) infects most of the world's adult population. In most cases primary infection and virus persistence are asymptomatic, the virus having evolved a sophisticated strategy to exist long term in the B-cell pool. However, EBV can contribute to the development of several human B-cell lymphomas, which include Hodgkin's lymphoma (HL), Burkitt's lymphoma, and a subset of diffuse large B-cell lymphomas, and can potently transform resting B-cells *in vitro* (Young & Murray, 2003; Young & Rickinson, 2004; Oyama *et al.*, 2003, 2007). Two questions central to our understanding of the origins of EBV-associated B-cell lymphomas are (1) how the host and virus interact to allow benign persistent latent infection, and (2) how perturbation of this normal homeostasis leads to neoplastic transformation. This review will summarize current knowledge of how the EBV life cycle is regulated in the B-cells of the asymptomatic host. It will also discuss how the disruption of normal B-cell homeostasis can contribute to the development of B-cell lymphomas, focussing on several novel pathogenic mechanisms in EBV-associated HL, which include the suppression of the virus lytic cycle and the activation of collagen receptor signalling.

In vitro transformation of B-cells by EBV

Much of what we know of the role of EBV in B-cell transformation comes from the study of lymphoblastoid cell lines (LCLs), cell lines generated by the *in vitro* infection of resting B-cells. These *in vitro* transformed B-cells express a limited subset of viral gene products, the so-called latent genes. They include six nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and -LP), three latent membrane proteins (LMPs 1, 2A and 2B), two major groups of viral microRNA (miRNA) (Amoroso *et al.*, 2011) and the non-coding Epstein–Barr-encoded RNA (EBER); the latter of which is a target for *in situ*-based assays to detect EBV in tissue sections (Wu *et al.*, 1990). This pattern of virus latency is often referred to as latency III or the *growth programme* (Fig. 1). Experiments with recombinant EBV lacking individual latent genes have shown that EBNA2 and LMP1 are required for the *in vitro* transformation of B-cells, and have highlighted a critical role for EBNA-LP, EBNA3A and EBNA3C in this process (Rickinson & Kieff, 2001). EBNA3A and EBNA3C have recently emerged as potential oncogenes, which in combination repress transcription of pro-apoptotic and senescence-inducing genes (Anderton *et al.*, 2008; Skalska *et al.*, 2010; Maruo *et al.*, 2011). In

contrast, EBNA3B is dispensable for B-cell transformation and has recently been reported to have a potential tumour suppressor function *in vivo* (White *et al.*, 2012).

Latent EBV infection in the B-cells of normal carriers

Although the virus efficiently transforms B-cells *in vitro*, >90% of the human population carry EBV asymptomatically. Primary infection with EBV is usually also asymptomatic, but in some cases can result in infectious mononucleosis (IM) (Balfour *et al.*, 2013). Once infected, the individual will remain so for life.

The discovery that EBV is present in memory B-cells, where virus gene expression is probably completely silenced (Hochberg *et al.*, 2004), suggested a model in which the virus persists by hijacking the normal mechanism of memory B-cell homeostasis (Babcock *et al.*, 1998). However, the mechanism by which EBV gains access to the memory B-cell pool remains controversial. It has been proposed that the virus uses the growth programme to drive newly infected resting B-cells into the cell cycle so that they can differentiate into the resting memory state via a germinal centre (GC) reaction (Thorley-Lawson, 2001; Thorley-Lawson *et al.*, 2008; Thorley-Lawson & Gross, 2004) (Fig. 2). The GC is a region of secondary lymphoid tissue in which antigen-activated B-cells undergo proliferation, class switch recombination and somatic hypermutation, leading to antigen selection and affinity maturation (MacLennan, 1994; Klein & Dalla-Favera, 2008). In the GC, failure to successfully compete for antigen and T-cell help leads to apoptosis (Klein & Dalla-Favera, 2008).

EBV infection in GC B-cells

EBV-infected GC B-cells have been shown to express a more restricted pattern of virus gene expression than is observed in LCLs; in this form of latency, often referred to as latency II or the *default programme*, LMP1 and LMP2 are

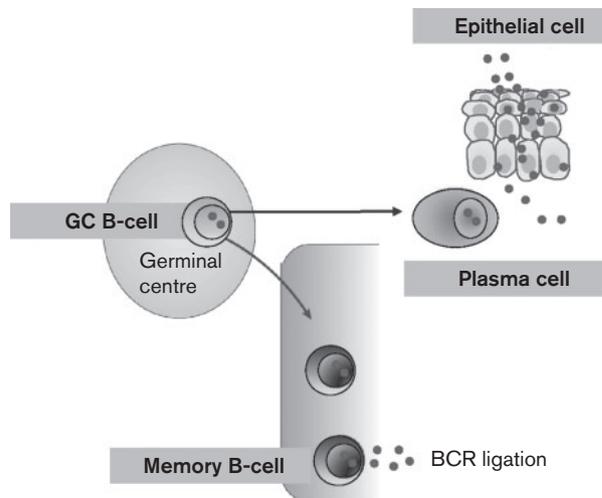


Fig. 2. The fate of EBV-infected GC B-cells. The normal fate of the non-malignant EBV-infected GC B-cell is unclear but like normal GC B-cells they are thought to be able to differentiate into either plasma cells or memory cells. This could be important for the normal viral life cycle because differentiation to memory cells is associated with latency and long-term persistence, whereas differentiation to plasma cells has been shown to induce the lytic cycle and is probably important for the replication of EBV in the oropharynx.

expressed, but the EBNAs, with the exception of EBNA1, are not (Babcock *et al.*, 2000; Thorley-Lawson & Gross, 2004; Roughan & Thorley-Lawson, 2009) (Fig. 1). LMP1 and LMP2 have been shown to possess, respectively, the CD40 and B-cell receptor (BCR) signalling functions necessary for GC survival and together are thought to contribute to the survival of EBV-infected B-cells in the GC and presumably their subsequent differentiation into memory B-cells (Casola *et al.*, 2004; Caldwell *et al.*, 1998; Gires *et al.*, 1997; He *et al.*, 2003; Panagopoulos *et al.*, 2004;

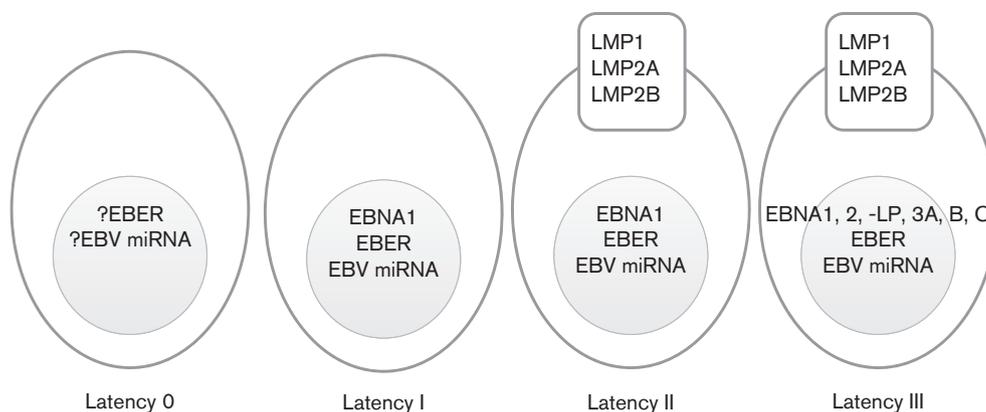


Fig. 1. Different forms of EBV latency. EBV, Epstein-Barr virus; EBNA, EBV nuclear antigen; LMP, latent membrane protein; EBV, EBV-encoded RNAs; miRNA, microRNA.

Swanson-Mungerson *et al.*, 2005; Vockerodt *et al.*, 2008). The default programme is also expressed in several EBV-associated cancers, including HL.

The frequency of EBV-infected GC B-cells in normal persistently infected individuals is very low; only around one-half of germinal centres from such individuals have been shown to be EBV-positive, on average harbouring only 3.5 EBV-infected cells per GC (Roughan *et al.*, 2010). In keeping with this, it has been shown that viral loads in GC B-cells isolated from persistently infected individuals are much lower than those found in memory B-cells from the same donors (Chaganti *et al.*, 2009). Previous studies that have used EBER *in situ* hybridization to document EBV-positive cells in the lymph nodes (Niedobitek *et al.*, 1992) and the tonsils (Hudnall *et al.*, 2005) of persistently infected individuals have shown that EBER-positive cells are mainly restricted to the extrafollicular areas, and are only rarely seen in GC. Similarly, most EBV-infected B-cells within the lymphoid tissues of patients with IM are located outside lymphoid follicles (Niedobitek *et al.*, 1992, 1997; Kurth *et al.*, 2000, 2003; Anagnostopoulos *et al.*, 1995; Chaganti *et al.*, 2009).

Lymphoid hyperplasia with EBV-positive germinal centres

Although EBV-positive GC B-cells are rare in the lymphoid tissues of healthy virus carriers and IM patients, a number of studies have reported instances of reactive lymphadenopathy in which lymphoid tissues display an expansion of EBV-positive cells within one or more GC (Dojcinov *et al.*, 2011; Niedobitek *et al.*, 1992; Martín *et al.*, 2011, 2012; Araujo *et al.*, 1999). This condition might represent an exaggerated form of the natural persistent state in which there is failure to control EBV infection in GC B-cells; in support of this possibility, eight cases of lymphoid hyperplasia with EBV-positive germinal centres were reported by Dojcinov *et al.* (2011), as part of the spectrum of adult late onset EBV-associated B-cell lymphoproliferations

which are presumed to have in common an age-related decline in EBV-specific immunity.

Lymphoid hyperplasia with EBV-positive germinal centres has also been reported in association with HL. For example, it has been found concurrently with a diagnosis of EBV-positive HL (Martín *et al.*, 2011, 2012), and at relapse following an initial diagnosis of EBV-positive HL (Martín *et al.*, 2011, 2012). Although this might support the possibility that lymphoid hyperplasia with EBV-positive germinal centres represents a stage in the evolution of HL, one cannot rule out the possibility that these are simply benign EBV-driven lymphoproliferations which share some of the histological characteristics of classical HL, including the presence of ‘Hodgkin/Reed–Sternberg (HRS)-like cells’ (Dojcinov *et al.*, 2011).

Our own analysis of five cases of lymphoid hyperplasia with EBV-positive germinal centres has revealed that the expansion of EBV-positive B-cells in germinal centres can be extensive and highly variable in adjacent follicles (Fig. 3, Table 1). Two of our five cases arose in patients with a history of immunosuppression (common variable immunodeficiency and human immunodeficiency virus (HIV) infection; cases 2 and 5, respectively; Table 1), and two further cases were found in association with EBV-positive HL (cases 1 and 4). Furthermore, while there are conflicting reports about latent gene expression in the EBV-infected GC B-cells of such cases, we did not observe LMP1 or LMP2A expression in EBER-positive germinal centres; this might suggest that the expansion of EBV-positive B-cells, at least in our cases, was antigen- rather than LMP1/2-driven.

Replication of EBV in B-cells

In LCLs, the virus can switch from latency to induce its replicative cycle. At any one time, LCLs contain a small proportion of cells that spontaneously enter the lytic cycle. The lytic cycle can be induced in these cells by various treatments including the cross-linking of the BCR (Kieff &

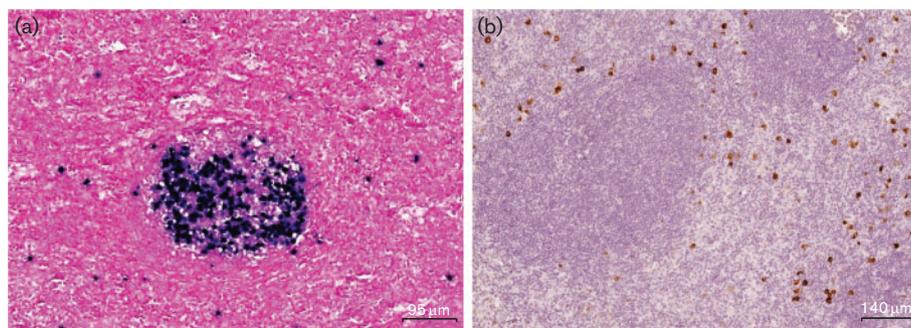


Fig. 3. Rare cases of reactive lymphadenopathy harbouring EBV-positive germinal centres. (a) EBER-positive cells in a GC with some scattered interfollicular EBER-positive cells in an enlarged cervical lymph node (case 1; see Table 1). The number of EBER-positive cells per GC was highly variable, including completely negative germinal centres. (b) Interfollicular EBER-positive cells express LMP1, while EBER-positive cells in GCs are LMP1-negative. A similar pattern was observed for LMP2A.

Table 1. Details of five patients with reactive lymphadenopathy containing EBV-positive GCs

+, Positive; -, negative; HPF, high power field.

Case no.	Sex	Age	Tissue	Diagnosis	Serology	EBER	LMP1/LMP2A IHC
1	M	27	Lymph node	EBV ⁺ classical HL. In retrospect considered as EBV-driven lymphoproliferation. Patient relapsed 4 years later with similar histology and gene expression findings	EBV IgG ⁺	Reactive lymphoid hyperplasia. EBER ⁺ cells in up to 4 GCs per section (range 2–40+ cells per GC) and EBER ⁺ interfollicular lymphocytes (range 24–63+ cells per High power field (HPF))	LMP1 and LMP2A expression detected in interfollicular lymphocytes but not in EBER ⁺ GC
2	M	49	Tonsil	Common variable immunodeficiency. EBV ⁺ classical HL 20 years earlier. In retrospect differential diagnosis EBV ⁺ HL or atypical reaction to EBV	EBV IgG ⁺ , IgM ⁻	Reactive lymphoid hyperplasia. EBER ⁺ cells in up to 2 GCs per section (range 22–133+ cells per GC). EBER ⁺ cells within and surrounding crypt epithelium. EBER ⁺ interfollicular lymphocytes (range 69–196+ cells per HPF)	LMP1, but not LMP2A, expression detected in interfollicular lymphocytes. EBER ⁺ GC LMP1 ⁻ and LMP2A ⁻
3	M	20	Lymph node	PET ⁺ enlarged lymph nodes left jaw angle and some smaller PET ⁺ nodes in neck and both axillae. No IM symptoms. Diagnosed as reactive lymphadenopathy	Not known	Reactive lymphoid hyperplasia. EBER ⁺ cells in up to 8 GCs per section (range 7–100+ cells per GC). EBER ⁺ interfollicular lymphocytes (range 0–15+ cells per HPF)	LMP1 and LMP2A expression detected in interfollicular lymphocytes but not in EBER ⁺ GC
4	F	23	Lymph node	EBV ⁺ classical HL 5 years earlier (with some EBER ⁺ GCs). In retrospect doubt about original HL diagnosis; differential diagnosis EBV reactive lymphadenopathy. Further cervical lymph node biopsy 1 year later shows same histology and EBER ⁺ GC	EBV IgG ⁺ , IgM ⁻	Reactive lymphoid hyperplasia. EBER ⁺ cells in up to 2 GCs per section (range 16–52+ cells per GC). EBER ⁺ interfollicular lymphocytes (range 0–58+ cells per HPF)	LMP1 and LMP2A undetectable in EBER ⁺ GC and in interfollicular cells. Subsequent cervical lymph node biopsy showed no LMP2A and LMP1 expression in EBER ⁺ GC. In this case interfollicular lymphocytes expressed both LMP1 and LMP2A
5	M	32	Tonsil	HIV ⁺ . Enlarged adenoid. Concurrent hairy leukoplakia	EBV IgG ⁺ , IgM ⁻	Reactive lymphoid hyperplasia. EBER ⁺ cells in up to 7 GCs per section (range 20–180+ cells per GC). EBER ⁺ interfollicular lymphocytes (range 2–5+ cells per HPF)	LMP1 and LMP2A undetectable in GC and interfollicular cells

Rickinson, 2001). Terminal differentiation into plasma cells can also initiate the viral replicative cycle (Anagnostopoulos *et al.*, 1995; Crawford & Ando *et al.*, 1986; Niedobitek *et al.*, 1997, 2000; Laichalk & Thorley-Lawson, 2005) (Fig. 2). Thus, the EBV lytic cycle can be triggered in B-cells by two distinct routes, either by activating BCR signalling or by terminal differentiation into plasma cells. The former probably represents an acute reactivation in response to stress that allows the virus to escape before the cell dies, whereas the latter is likely to be the natural route to EBV replication in tonsils (Laichalk & Thorley-Lawson, 2005).

The immediate-early EBV gene, *BZLF1*, provides the trigger for the induction of the lytic cycle and alone is sufficient to activate downstream lytic genes and viral replication (Countryman *et al.*, 1985; Takada *et al.*, 1986). The latent membrane proteins can regulate entry to the lytic cycle. Thus, while the virus lytic cycle can be activated by LMP2A, it is inhibited by LMP1 (Miller *et al.*, 1994; Schaadt *et al.*, 2005; Engels *et al.*, 2012; Adler *et al.*, 2002; Prince *et al.*, 2003).

EBV-associated HL

The association between EBV and a subset of classical HL was first established when it was shown that EBV DNA is present in the malignant HRS cells of this disease (Weiss *et al.*, 1989). EBV rates in HL tumours from North America and Europe have subsequently been shown to vary between 20 and 50 %, whereas much higher rates are observed in underdeveloped countries. EBV-positive rates are also generally higher: in males compared with females; in Asians and Hispanics compared with whites or blacks (Glaser *et al.*, 1997); and in the UK in South Asian children compared with non-South Asian children (Flavell *et al.*, 2001). In developed countries, the proportion of EBV-positive cases is higher in older people and in children, especially in those under 10 years of age, whereas the lowest rates of EBV-positive disease are found in young adults (Glaser *et al.*, 1997; Jarrett *et al.*, 1991; Armstrong *et al.*, 1998).

Although HRS cells are of B-cell origin, they lack a functional BCR, as well as the downstream components required for the transmission of the BCR signal. In some cases the loss of BCR expression can be explained by the presence of destructive IgV gene mutations (Bräuninger *et al.*, 2003; Küppers, 2005). Nearly all cases of HL harbouring these so-called 'crippling' mutations are EBV-positive (Bräuninger *et al.*, 2006). This observation suggests that HRS precursors with such mutations can survive and eventually undergo malignant transformation only if infected by EBV. In support of this, EBV has been shown to be able to rescue BCR-negative GC B-cells from apoptosis (Bechtel *et al.*, 2005; Mancao *et al.*, 2005; Chaganti *et al.*, 2005).

EBV-infected HRS cells are known to express LMP1 and LMP2. LMP1 could be especially important in the pathogenesis of HL since it constitutively activates several

of the pathways, including NF- κ B, JAK/STAT and phosphatidylinositol 3-kinase/AKT, which are known to be aberrantly activated in HRS cells (Bargou *et al.*, 1997; Kube *et al.*, 2001; Dutton *et al.*, 2005). The potential importance of LMP1 in the pathogenesis of HL is underscored by our observation that LMP1 expression in primary human GC B-cells, the presumed progenitors of HRS cells, can contribute up to one-quarter of the transcriptional changes observed when HL cell lines are compared with isolated GC B-cells (Vockerodt *et al.*, 2008).

Suppression of the EBV lytic cycle as a pathogenic mechanism in EBV-associated HL

We have hypothesized that the suppression of virus replication, which would otherwise lead to cell death, is an important pathogenic event in the development of EBV-associated lymphomas, such as HL. As described above, the EBV lytic cycle can be initiated in B-cells following either cross-linking of the BCR or their differentiation into plasma cells. We have found evidence which suggests that both mechanisms are disabled in EBV-infected HRS cells.

LMP1 suppresses plasma cell differentiation in EBV-infected B-cells

As described above, plasma cell differentiation leads to EBV replication in B-cells. Plasma cell differentiation is known to be controlled by the coordinated expression of B-cell-associated transcription factors. One of these transcription factors is BLIMP1 α . BLIMP1 α is encoded by the PRDM1 gene and is essential for plasma cell differentiation. We have shown that LMP1 can downregulate BLIMP1 α expression in primary human GC B-cells, an effect that was accompanied by the partial disruption of the BLIMP1 α transcriptional programme, including the aberrant induction of *myc*, the repression of which is required for terminal B-cell differentiation (Vrzalikova *et al.*, 2011; Lin *et al.*, 2000). Furthermore, this downregulation appeared to be important for the suppression of the virus lytic cycle since the re-expression of BLIMP1 α induced the lytic cycle in EBV-transformed B-cells. These observations are consistent with previous reports showing that LMP1 can block entry into the viral lytic cycle in B-cells (Adler *et al.*, 2002; Prince *et al.*, 2003). We have speculated that the disruption of normal BLIMP1 α functions could be an essential step in the pathogenesis of EBV-associated germinal centre-derived lymphomas, preventing not only the terminal differentiation of germinal centre-derived progenitors, but also virus replication (Fig. 4).

Absence of the BCR signalling machinery prevents BCR-mediated lytic cycle entry in HRS cells

The alternative route to virus replication in B-cells is induced following activation of the BCR. LMP2A is a BCR mimic and can induce the lytic cycle in B-cells. However, EBV-infected HRS cells do not have a functional BCR

receptor and lack expression of other critical downstream components of BCR signalling. We have shown that LMP2A and BCR signalling in primary human GC B-cells results in overlapping transcriptional changes, many of which are also observed when primary HRS cells are compared with normal GC B-cells. These data support the contention that LMP2A can provide a surrogate BCR-like signal in HRS cells. However, we also found that because HRS cells lack downstream BCR signalling components, LMP2A cannot induce the EBV lytic cycle in these cells (Vockerodt *et al.*, 2013). These observations have allowed us to develop a model in which LMP2A-expressing HRS cell progenitors lacking BCR signalling functions are protected from entry to the virus lytic cycle which in turn could lead to their positive selection during the evolution of EBV-associated HL.

Collagen receptor signalling in the pathogenesis of EBV-associated HL

As described above, LMP1 is reported to be expressed in the EBV-infected GC B-cells of normal healthy EBV carriers, where it is thought to provide a CD40-like survival signal. However, LMP1 is also reported to be an oncogene that can constitutively activate several cell signalling pathways known to be aberrantly expressed in HRS cells. In an attempt to resolve this apparent paradox, we revisited our previous analysis of the transcriptional changes induced by LMP1 in normal GC B-cells (Vockerodt *et al.*, 2008). During the course of this analysis, we noted that LMP1 upregulated expression of the discoidin domain receptor 1 (DDR1), a receptor tyrosine kinase (RTK) which can be activated by collagen (Cader *et al.*, 2013). This was of interest because HL tissues are frequently extensively infiltrated by

collagen. We were then able to show that short-term exposure to collagen was sufficient to activate DDR1 and promote the survival of lymphoma cells *in vitro* (Cader *et al.*, 2013). These observations are important because they suggest that the oncogenic activities of LMP1 may, at least in part, be dependent on cues from the microenvironment.

Conclusions

EBV is associated with a range of different B-cell malignancies; however, the contribution of the virus to each of these tumours is probably different. For example, in the case of Burkitt's lymphoma, EBV probably provides an anti-apoptotic signal which complements *myc* de-regulation. In contrast, in EBV-associated post-transplant lymphomas, in which T-cell control of EBV infection is severely impaired, the virus is thought to be the major driver of tumour cell growth and survival. In the case of HL, EBV is also thought to provide anti-apoptotic functions, this time facilitating the survival of BCR-negative HRS cells, or their precursors. Why EBV-infected HRS cells evolve to lose BCR functions is not known. Our studies suggest that this might be to avoid the induction of virus replication and the ensuing cell death which would otherwise be induced by cross-linking of the BCR. A further indication of the importance of suppressing virus replication in the pathogenesis of EBV-associated HL is provided by the observation that the viral oncogene, *LMP1*, can downregulate *BLIMP1 α* , thereby suppressing plasma cell differentiation and the alternative route to virus replication in B-cells.

Another unusual feature of HL not found to the same extent in other EBV associated B-cell lymphomas is the presence of an unusually florid microenvironment composed of numerous different non-malignant cell types and extracellular matrix components, such as collagen. This microenvironment contributes to disease pathogenesis not only by directly stimulating tumour cell growth and survival but potentially also by redirecting the functions of the EBV latent genes expressed by HRS cells. This is exemplified by the induction of the cell surface receptor DDR1 by LMP1; DDR1 is a growth- and survival-promoting tyrosine kinase that is activated in EBV-infected B-cells only in the presence of its ligand, collagen.

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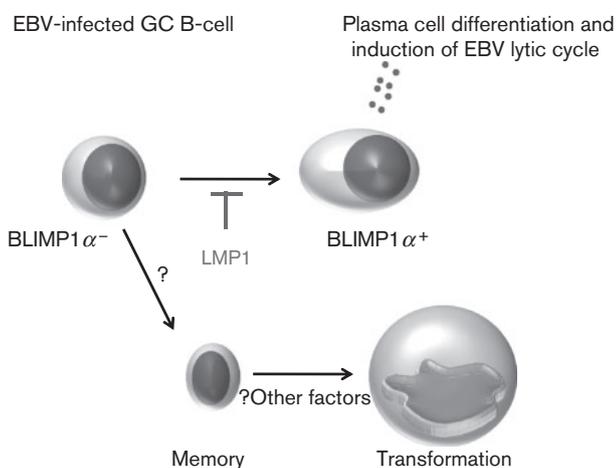


Fig. 4. Model in which LMP1 blocks plasma cell differentiation. *BLIMP1 α* is required for plasma cell differentiation. Plasma cell differentiation of EBV-infected B-cell leads to induction of the virus lytic cycle. LMP1 can suppress *BLIMP1 α* expression, thereby preventing terminal differentiation to plasma cells. LMP1 can also presumably drive cells into the memory B-cell pool.

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