The curious case of the tumour virus: 50 years of Burkitt's lymphoma

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Abstract | Burkitt's lymphoma (BL) was first described 50 years ago, and the first human tumour virus Epstein–Barr virus (EBV) was discovered in BL tumours soon after. Since then, the role of EBV in the development of BL has become more and more enigmatic. Only recently have we finally begun to understand, at the cellular and molecular levels, the complex and interesting interaction of EBV with B cells that creates a predisposition for the development of BL. Here, we discuss the intertwined histories of EBV and BL and their relationship to the cofactors in BL pathogenesis: malaria and the *MYC* translocation.

EBNA1
(EBV nuclear antigen 1). Responsible for replicating and tethering the viral genome to ensure its segregation.

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Burkitt's lymphoma (BL) was first described in 1958 as a prevalent lymphosarcoma of young children in tropical Africa[1]. The earliest recorded description that matches a BL tumour was from 1910 (REF. 2) (for an informed and interesting summary of early literature and general clinical references, see REF. 3). BL is most prevalent in a central band of sub-Saharan Africa referred to as the lymphoma belt[4]. Consequently, the tumour was originally called the African lymphoma and subsequently endemic BL (see BOX 1 for a discussion of BL nomenclature). Before the spread of AIDS, BL was the most common tumour of children in equatorial Africa.

It was quickly realized that the lymphoma belt coincided with the distribution of hyperendemic and holoendemic malaria[5], leading to the suggestion that malaria or some other infectious agent carried by mosquitoes was responsible for the tumour. Not long after, a herpes-like virus, subsequently named Epstein–Barr virus (EBV), was discovered in cultures of the tumour[6] and serological and immunofluorescence studies[7] demonstrated the uniqueness of EBV. The long, intertwined history of EBV and BL had begun (TIMELINE). Several years later, a consistent cytogenetic lesion that resulted in the translocation and constitutive activation of the *MYC* oncogene was detected in BL[8–10]. The stage was set and the main players were in place: *MYC*, EBV and malaria must cooperate to cause BL. Today, it is sobering to realize how much we still do not know about the mechanism by which these factors conspire to produce BL.

The EBV tumour-virus hypothesis

Evidence from tumours. The discovery in 1964 of EBV in BL tumours created a paradigm for the idea that human cancer could be caused by viruses. Early on the hypothesis looked solid. Almost 100% of BL in equatorial Africa carried the virus[11], which is still the most compelling argument for a direct role for EBV in BL pathogenesis. Furthermore, children infected early in life who produced the highest antibody titres to the virus were at highest risk for developing the tumour[12]. The tumour cells were latently infected and carried the viral genome as a clonal, extra-chromosomal episome[13]. They also expressed EBNA (EBV nuclear antigen), a serologically defined, putative tumour antigen, in their nuclei[4], although, as discussed below, this antigen was later shown to be composed of six components, of which only one, EBNA1, was expressed in EBV-positive BL (Supplementary information S1 (table)). Subsequently, EBV was firmly linked to other proliferative diseases, including acute infectious mononucleosis, nasopharyngeal carcinoma, Hodgkin's disease, immunoblastic lymphoma in individuals who are immunosuppressed, a subset of gastric carcinomas, rare T- and NK-cell lymphomas and leiomyosarcoma (reviewed in REFS 15–17). EBV is therefore found in several human cancers that tend to reflect the known tissue tropism of the virus for B cells and epithelial cells.

The EBV growth transcription programme. The next observation that seemed to confirm the EBV tumour-virus hypothesis was the finding that EBV was a potent transforming virus in culture for the same cell type that develops into BL, the B lymphocyte[18]. EBV is probably the most efficient transforming virus in culture: it can convert >50% of all target cells (the resting B cell) into continuously proliferating, latently infected lymphoblastoid cell lines (LCLs) within a few days. This finding was a huge breakthrough, because it allowed the production of essentially limitless material for the study of latent infection. Intensive study revealed that LCLs express nine viral

Box 1 | **Endemic BL versus EBV-positive BL**

Burkitt's lymphoma (BL) was originally described in tropical Africa and >90% of BL tumours contain Epstein–Barr virus (EBV). Based on its high prevalence (1–20 cases per 10^5 individuals)[93] and assumed geographical localization, BL was referred to as endemic BL (eBL). However, the term endemic became a misnomer when it was realized that BL can be found worldwide. Worldwide, BL has a lower prevalence (~0.01 cases per 10^5 individuals in the United States and Europe)[93], and the frequency of EBV-positive tumours is variable (less than 25% in some areas, such as the United States and Europe). Consequently, BL that was found outside of tropical Africa was called sporadic BL (sBL). However, EBV-negative BL also occurs rarely in Africa. Currently, therefore, eBL describes all BL in tropical Africa and sBL describes all BL in the rest of the world, irrespective of EBV status. Mechanistically this does not make sense, as EBV-positive BLs are likely to be related diseases with similar aetiologies, whereas EBV-negative BLs are likely to be distinct (see the table). For this reason, we do not use the term eBL in this Review, but instead refer to EBV-positive BL.

	EBV-positive BL	EBV-negative BL	References*
Age distribution	Peaks at 6–8 years old	All	3,11,93,113
Site	Jaw, eye and abdomen	Abdomen	3,11,93,113,114
Location	Extranodal	Lymph nodes; extranodal	3,11,93,113,114
Likely cofactors	Malaria and EBV	Unknown	3,11,93
***MYC* translocation**	Yes	Yes	115,116
Usual *MYC* break point	>100 bp upstream of first exon	Between exons 1 and 2	115,116
Usual immunoglobulin break point	VDJ region	Switch region	115,116
Somatic hypermutations	Yes	Yes	115,116
Surface phenotype	Germinal centre ($CD10^+$, $CD77^+$, $CD23^-$, $BCL6^+$ and $PAX5^+$)	Germinal centre ($CD10^+$, $CD77^+$, $CD23^-$, $BCL6^+$ and $PAX5^+$)	32,43,117
Likely precursor	Memory B cell	Germinal centre B cell	40,44

*The table contains generalizations to which there are exceptions, particularly in the form and structure of the translocations. For a more detailed and considered discussion, see the cited references. These data compare equatorial African BL, which is predominantly EBV positive, with BLs in Europe and/or the United States, which are predominantly EBV negative. Only limited information is available from other world regions.

latent proteins and several non-coding RNAs that are under the control of the master transcription factor EBNA2 (reviewed in REF. 19). Together, these cooperate to drive the newly infected resting cell to resemble an antigen-activated B lymphoblast[20]. This is termed the growth transcription programme[21,22] (also referred to as latency 3; see Supplementary information S1 (table)) and provided the first inkling that the biology of EBV was intimately wrapped up with that of the B lymphocyte. In addition, some of the latent genes (notably *LMP1* (latent membrane protein 1)) behaved as oncogenes in classic transformation assays, which further implicated EBV in BL causation[23]. Early reports claimed that LCL-like cells were prevalent in the blood of acutely infected individuals[24] (although later studies failed to corroborate this finding[25,26]), and it was firmly established that infected individuals mount a potent cytotoxic T-cell (CTL) response to these latent proteins (reviewed in REF. 27). Furthermore, if the immune response is suppressed, LCL-like cells that express the growth programme can give rise to lymphoma (reviewed in REF. 28).

Taken together, these findings seemed to show that EBV persisted in healthy carriers in the form of transformed proliferating lymphoblasts that were constantly kept in check by the immune response. We now know, however, that EBV persists in resting memory B cells and that lymphoma in individuals who are immunosuppressed does not resemble BL and is probably due to a rare, aberrant event in which a B cell is infected under conditions in which it is unable to differentiate into a resting memory B cell[22,29] (discussed below).

Is EBV really a tumour virus?

MYC translocation, not EBV, defines BL. The first crack in the EBV–BL story came with the realization that BL can arise without EBV[11,30] (BOX 1). In fact BL occurs worldwide, although at a much lower frequency than in the lymphoma belt, and in some parts of the world <25% of BLs are EBV positive[11]. More recently AIDS-associated BL, in which most tumours are EBV negative, has also been described[11]. In all three types of BL, the *MYC* proto-oncogene is activated through translocation into an immunoglobulin (Ig) locus[9,10]. The consistent feature of BL is therefore translocated, activated *MYC*, not the presence of EBV.

The EBV EBNA1-only programme. The EBV–BL story was further unravelled by a series of observations that led to the current model of EBV persistence. The first important insight was simple: BL tumour cells look different from LCL cells[31]. BL cells are small and round, almost like resting B cells (despite the fact that BL is actually the fastest growing tumour known), whereas LCL cells are large and irregular, as expected for an activated B lymphoblast. Furthermore, the cell-surface phenotypes of LCLs and BL tumour

EBNA2
(EBV nuclear antigen 2). Transcription factor that is responsible for transactivating the promoters of the viral genes expressed in the growth programme. Functional homologue of Notch[IC]. Upregulates *MYC* expression

Lymphoblast
An activated lymphocyte.

LMP1
(Latent membrane protein 1). Provides a constitutive T-helper cell signal that may help to rescue Epstein–Barr virus infected germinal-centre cells and drive them into the memory compartment. *LMP1* downregulates BCL6 and induces AID (activation-induced cytidine deaminase).

Box 2 | **Is EBV-positive BL derived from a germinal centre or a memory B cell?**

Epstein–Barr virus (EBV)-positive Burkitt's lymphoma (BL) is often spoken of as a tumour of the germinal centre (GC) because it bears phenotypic[32,43,117] markers of the GC and expresses a subset of GC-related genes[118] (H. Stein, personal communication). However, tumours undergo intensive selection and can exhibit phenotypes that are not expressed in the cell of origin. Furthermore, EBV-positive BL grows in extranodal locations, and therefore a GC origin would require the tumour cell to migrate out of the lymph node while expressing at least some GC properties.

If, as recently suggested[119,120], GC cells do not express *MYC*, then where does the translocation occur? Activation induced cytidine deaminase (AID) is highly expressed in GCs and is essential for the translocation. So *MYC* may be expressed in GCs, but only transiently and at low levels, such that it has gone undetected. EBNA2 (EBV nuclear antigen 2) may also turn on *MYC* expression, which may increase the probability of a translocation before the GC. Furthermore, AID expression is not limited to the GC, and EBV-latent proteins induce AID[62,64] expression.

The translocation in EBV-positive BL is not consistent with a GC origin[121], but suggests an early B-cell precursor. However, the patterns and rate of somatic hypermutations in EBV-positive BL[122] are identical to those found in EBV-positive memory B cells in the peripheral blood[39], linking EBV-positive BL to the latently infected memory cell.

Finally, EBV-positive GC B cells *in vivo* express the default programme, not the EBNA1-only programme of EBV-positive BL[21,22]. The EBNA1-only programme is limited to dividing memory cells[40], linking EBV-positive BL to the latently infected post-GC memory B cell.

Murine plasmacytoma (PC) could help resolve the question of BL origin. PC is a tumour of plasma cells that also has an *Myc*–immunoglobulin translocation[123]. The translocation could occur in the GC for both BL and PC, but in a cell type that is already committed to enter either the memory- (BL) or plasma-cell (PC) pool. Thus, EBV-positive BL could arise from a GC cell that left the lymph node on its way to becoming a resting memory B cell but continues to proliferate owing to deregulated *MYC* and would therefore express the EBNA1-only programme.

cells are markedly different from each other. BL cells express classic markers of the germinal centre (GC) (BOX 1), whereas LCL cells express activation markers and cell-adhesion molecules[32]. Furthermore, EBV-positive BL tumour cells do not express any of the EBV latent proteins that are involved in driving B-cell proliferation, such as LMP1 and EBNA2. Of the nine latent proteins found in LCL cells only EBNA1, the genome-tethering protein, is consistently expressed in EBV-positive BL[32]. This expression profile, called the EBNA1-only transcription programme[22], (also referred to as latency 1; Supplementary information S1 (table)) could have been caused by tumour-growth selection against the expression of the other latent proteins. This interpretation was rejected, however, when Schaefer *et al.*[33] made the seminal observation in 1995 that EBNA1 in EBV-positive BL was expressed from the Q promoter (Qp), a different promoter from that used in LCL cells, which use Cp and Wp[33]. The fundamental transcriptional biology of the virus in EBV-positive BL cells was therefore different from that of LCL cells. Furthermore, if none of the growth-promoting genes (such as EBNA2 and LMP1) were active in EBV-positive BL, how could EBV drive tumour growth? One possibility is that researchers had been misled by the growth-promoting aspects of EBV biology, and that the real contribution of the virus was to make the tumour cells resistant to the apoptosis that should have been induced by deregulated *MYC* (discussed below).

The EBV 'default' transcription programme. The EBV–BL story became even more complex when viral latent gene expression was analysed in Hodgkin's disease and nasopharyngeal carcinoma[34,35]. In these tumours, yet another pattern was observed when Qp-derived EBNA1 was expressed with LMP1 and LMP2, but the rest of the latent proteins were again absent. This expression pattern was called the default transcription programme[22] (also referred to as latency 2; see Supplementary information S1 (table)). So now there were three different EBV transcription programmes that were expressed in three different tumour types, none of which, at that time, had been observed in B cells during a normal infection (outside of tumour cells). It seemed inconceivable that the virus could have evolved a specific biology for each tumour — so what was going on?

EBV persistence in vivo. Most of the early work on EBV was performed on LCL- and BL-derived cell lines. Starting in 1995 (REF. 26), analysis of the virus *in vivo* produced another set of conflicting observations. As with the tumours, the starting point was simple: EBV infects and persists for life in >90% of human adults[36] without causing disease (it is usually benign). How could such an efficient transforming virus be so benign? Detailed analysis of acute and persistent infection revealed that latently infected cells in the blood were not like LCL cells[26]. Instead they were small, resting memory cells ($CD20^+$, $CD27^+$, $CD5^-$, $CD10^-$ and IgD^- cells)[25,37] (reviewed in REFS 17,22) that had undergone isotype switching (CSR) and somatic hypermutation (SHM), consistent with antigen selection[38,39]. These resting memory cells expressed none of the viral latent proteins (the latency-transcription programme, which is also referred to as latency 0; see Supplementary information S1 (table)) but, just like EBV-positive BL cells, they expressed the EBNA1-only programme when they divided. Importantly, this cell division is not driven by the viral growth-promoting genes, but by the cell as part of normal memory B-cell homeostasis[40].

By every criterion measured so far, these latently infected memory cells are indistinguishable from normal memory B cells[38,39]. To all intents and purposes

Germinal centre
The region in a lymph node where antigen-activated B cells proliferate, actively mutate and isotype switch their immunoglobulin genes.

Activation marker
A molecule expressed on the surface of lymphocytes when they become lymphoblasts.

Isotype switching
The process by which immunoglobulins change their isotype.

Somatic hypermutation
The process by which immunoglobulins have their affinity for antigen altered by mutations that target the antibody-combining site.

Antigen selection
The process by which B cells that have undergone somatic hypermutation of their immunoglobulins in a germinal centre compete to bind antigen.

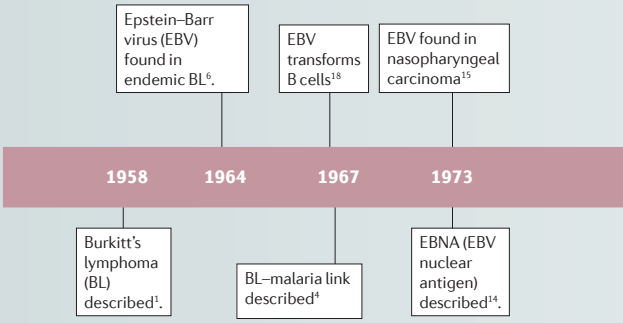
these are normal memory cells that are maintained by homeostasis, rather than by virus-induced proliferation. The cells in which EBV persists in the blood are therefore benign, and curiously, more closely resemble EBV-positive BL than LCL cells. Perhaps EBV-positive BL was not a tumour of an LCL cell, but of a latently infected memory B cell (BOX 2)?

The role of *MYC*

The mechanism of the* MYC *translocation. The structure of the chromosomal translocations in BL have been reviewed extensively[9,10,30]. The translocation essentially places the proto-oncogene *MYC* under the control of one of the Ig loci, such that *MYC* becomes constitutively activated in mature B cells. An important step forward in understanding how the translocations are produced has come from recent studies on the enzyme AID (activation-induced cytidine deaminase)[41,42]. AID is highly expressed in GCs and, for many years, it was thought that BL was a tumour of the GC (BOX 2). The GC has long been thought of as a prime target for oncogenic transformation because it is the region of the lymph node where antigen-activated B cells migrate to undergo rapid proliferation, CSR and SHM as they transit to become plasma or memory cells. Both CSR and SHM are AID dependent[43]. AID activation leads to the deamination of cytidine residues, resulting in U:G mismatches that are processed to produce the double stranded (ds) DNA breaks that are essential for CSR, but that can also occur during SHM. In an interleukin-6 (IL-6) transgenic model it was shown that aberrant AID expression causes Ig–*MYC* translocations that resemble those found in EBV-positive BL[44]. Both translocated genes had undergone SHM, suggesting that dsDNA breaks that are induced by AID and arise during SHM are intermediates in the translocation process. In addition, aberrant SHMs might contribute to lymphomagenesis by mutating non-Ig genes, including *MYC*[45].

The role of the* MYC *translocation. As a result of the translocation of *MYC* to an Ig locus, MYC RNA is constitutively expressed in the B cells and MYC protein can accumulate. In mouse transgenic models, constitutive activation of MYC alone causes B-cell lymphoma, although these tumours are usually derived from early B cells[46] and do not resemble BL[47]. For the induction of BL-like disease, it seems that the transgene must consist of a *Myc*–Ig translocation[47]. This implies that subtleties in the structure of the translocation are also important in the development of BL. MYC is a sequence-specific DNA-binding transcription factor that acts in B cells as a major transcriptional hub that controls a hierarchy of multiple sub-hubs. These hubs directly or indirectly regulate the transcription of ~15% of all genes[48], particularly those that are involved in cell-cycle progression, growth (including metabolism, ribosome biogenesis, protein synthesis and mitochondrial function)[49], proliferation and apoptosis[48]. MYC also regulates a network of microRNAs that are involved in the modulation of tumorigenesis[50,51]. The net effect of MYC activation is cell growth, uncontrolled proliferation, increased genomic instability[52] and, possibly, a reduction in the immunogenicity of the tumour cells[53,54].

Timeline | **The evolving role of EBV in BL***



*Events above the line support the EBV tumour virus hypothesis, whereas events below the line do not.

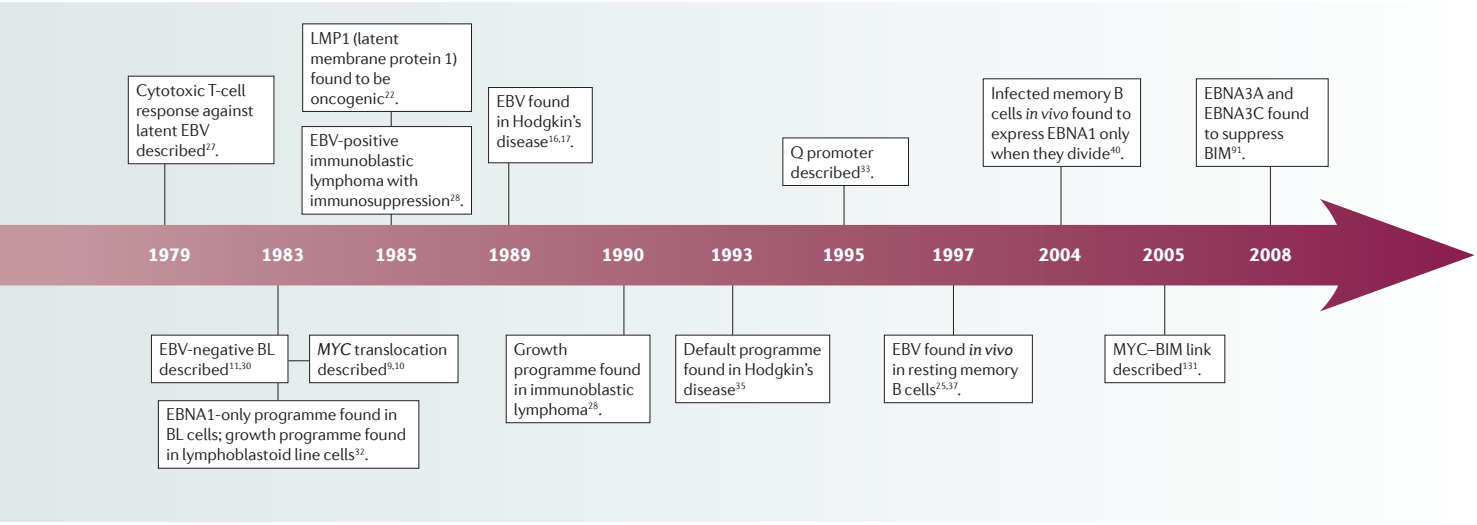
The role of EBV

In normal cells, the potent proliferative activity of MYC is counterbalanced by increased sensitivity to apoptosis, which is mediated by both the p53 and BIM pathways. For lymphoma outgrowth, at least one of these pathways must be suppressed (BOX 3), and therefore the key molecular events in BL pathogenesis are the activation of MYC growth-promoting activity (through translocation) and the inactivation of MYC apoptosis-inducing activity. But how might EBV be involved? The transforming genes of EBV are not expressed in EBV-positive BL, so they cannot contribute directly to tumour growth. Could EBV be responsible for the ablation of the apoptotic activity of MYC? It has been suggested that EBV proteins (particularly EBNA1 and the EBV-encoded RNAs) that are expressed in BL may possess anti-apoptotic properties and contribute to increased tumorigenicity[55–57]. However, these effects are modest[58] and the studies have mostly been performed in EBV-negative revertants of BL lines. EBV-negative revertants can be obtained from BL lines which means that the continuous presence of EBV is not necessary to ablate the apoptotic activity of MYC. There must be a more fundamental mechanism at work, with the EBV gene products of the EBNA1-only programme providing an added level of protection at best. Before proposing an alternative mechanism for how EBV could confer resistance to the induction of apoptosis by MYC, the mechanism by which EBV establishes persistent latent infection in memory B cells must be discussed.

The mechanism of EBV persistence. EBV uses normal B-cell biology to establish infection, persist and replicate[21,22] (FIG. 1). Here we will focus on aspects of EBV biology that shed light on the pathogenesis of EBV-positive BL. Specifically, we will discuss how EBV uses the GC to allow B cells that are activated by EBV infection to differentiate into resting memory cells, in which EBV persists. EBV spreads through saliva to enter the epithelium of Waldeyer's ring, where it infects resting, naive B cells and causes them to express the growth programme[22]. From

AID
(Activation-induced cytidine deaminase). An enzyme that is essential for catalysing isotype switching and somatic hypermutations, and is highly expressed in germinal centres.

Waldeyer's ring
The ring of lymphoid tissue at the back of the mouth that includes the adenoids and the five tonsils (lingual and paired palatine and tubal).



in vitro studies we know that the EBV genome circularizes when it enters the nucleus of the infected cell[59] and Wp is activated, allowing expression of EBNA2 in the quiescent environment of a resting B cell[60]. EBNA2 then turns on the growth programme[22], which involves a switch to viral Cp (discussed below). This initiates cellular activation, which drives the newly infected tonsillar naive B-cell lymphoblast to begin proliferating. The newly infected naive B lymphoblasts are thought to enter a follicle, where they switch to express the default programme[22] and undergo the GC differentiation process[21,22] (J. Roughan and D.A.T.-L., unpublished observations). This is the programme that is expressed in Hodgkin's disease and, as discussed below, has the potential, through LMP1 and LMP2, to provide the signals that are necessary to allow differentiation of the latently infected B cell into the memory compartment[61–65] (FIG. 1), where viral protein expression is shut down (the latency transcription programme begins) and the virus can persist for the lifetime of the host.

The key to understanding the potential role for EBV in BL development might lie in the transition from the growth programme to the default programme. Upon arrival in the follicle, the newly infected EBV lymphoblast, which is unable to differentiate because EBNA2 is driving growth, must receive a signal (or signals) that turns off both EBNA2 and the growth programme. This would allow the infected cell to assume a GC phenotype and differentiate through the GC into memory. But how could this transition be mediated?

The Notch signalling pathway. How EBNA2 is turned off remains unknown, but a clue comes from the observation that EBNA2 must interact with the cellular DNA-binding protein CBF1 (also known as RBPJκ; recombination signal binding protein)[66] to bind Cp and regulate gene expression. This suggests a mechanism, based on the known role of CBF1 in the Notch signalling pathway of developmental biology, for the regulation of EBNA2 expression (reviewed in REFS 67,68) (FIG. 2).

During *Drosophila* development, ligand interaction with the Notch receptor causes proteolytic cleavage of Notch, releasing the intracellular domain ($Notch^{IC}$). $Notch^{IC}$ translocates to the nucleus and binds to the transcription factor Su(H) (Suppressor of Hairless) to function as a direct transcriptional co-activator of Notch target genes, leading to cellular growth and the inhibition of differentiation (FIG. 2a). Hairless is a potent antagonist of this pathway and is thought to directly displace $Notch^{IC}$ from Su(H). Subsequent interaction of the co-repressors Groucho and CtBP (carboxy-terminal-binding protein)[69] with Hairless leads to transcriptional silencing of genes through the recruitment of histone deacetylases (HDACs) and histone methyltransferases (reviewed in REF. 70) (FIG. 2b). *Drosophila* Su(H) is equivalent to human CBF1, and EBNA2 is a functional homologue of $Notch^{IC}$ (REF. 71) (FIG. 2a). It is has also been proposed that EBNA3A and EBNA3C are functional homologues of Hairless because they repress EBNA2-mediated transcriptional activation of Cp by antagonizing the interaction of EBNA2 with CBF1 (REFS 72,73) and because they directly recruit CtBP[74,75] and HDACs[76] (FIG. 2b).

Taken together these similarities to Notch signalling suggest a pathway that might regulate EBV-driven proliferation and differentiation. Infection of the B cell leads to the LCL phenotype and migration into the follicle. Expression of EBNA2 mimics $Notch^{IC}$ and therefore blocks differentiation and allows cellular proliferation by an interaction with CBF1. At the same time, EBNA2, through its interaction with CBF1, activates the major EBV latent promoter Cp, leading directly to the expression of EBNA3A and EBNA3C. Similar to Hairless, EBNA3A and EBNA3C bind CBF1 and displace EBNA2, leading to growth arrest. At the same time they recruit HDACs and CtBP complexes, thereby inactivating Cp and blocking further production of EBNA2. CtBP recruitment leads to deacetylation and methylation of histones that are assembled into chromatin in and around the Cp promoter, leading to its stable silencing. The silent chromatin state could then trigger DNA

Follicle
The region of lymph nodes where naive B cells migrate, proliferate and expand to form a germinal centre if they encounter antigen.

EBNA3A and EBNA3C
(EBV nuclear antigen 3A and 3C). Negative regulators of EBNA2 that are thought to be involved in the transition from the growth programme to the default programme through the epigenetic silencing of the C and W promoters. EBNA3A and EBNA3C are functional homologues of the *Drosophila* gene *Hairless* and downregulate BIM.

Box 3 | **Escaping MYC-induced apoptosis — a central role for BIM**

As a fail-safe mechanism to prevent unscheduled cell division, the potent proliferative activity of MYC is counterbalanced by increased sensitivity to apoptosis[124]. When *MYC* is overexpressed, the tumour suppressor p53 is activated, predominantly through CDKN2A (E3 ubiquitin-protein ligase; also known as $p14^{ARF}$), which in turn interacts with and antagonizes MDM2, the negative regulator of p53 (REF. 125) (see the table). Apoptosis is then induced, primarily by p53-mediated transactivation of pro-apoptotic genes, such as *PMAIP1* (phorbol-12-myristate-13-acetate-induced protein 1; also known as *NOXA*), *BBC3* (BCL2-binding component 3; also known as *PUMA*) and *BAX* (BCL2-associated X protein)[126]. For Burkitt's lymphoma (BL) to develop, the sensitivity to MYC-induced apoptosis must be suppressed. Approximately 30% of Epstein–Barr virus (EBV)-positive BL tumours carry p53 mutations, and those with wild-type p53 frequently overexpress *MDM2* or lose $p14^{ARF}$ (REF. 126). In many BLs the $p14^{ARF}$–MDM2–p53 axis is compromised. However, a second MYC-activated pathway to apoptosis that involves BCL2L11 (Bcl2-like protein 11; also known as BIM) also exists.

BIM is a major regulator of life-and-death decisions in mature B cells[127]. BIM is thought to initiate apoptosis by binding to and inactivating pro-survival BCL2-family members, such as BCL2 and MCL1 (myeloid leukaemia cell differentiation protein 1)[127]. Loss of even a single allele of BIM accelerates B lymphomagenesis[128] and deletions or methylation of the *BIM* locus are found in various human B lymphomas[129,130]. Two mutants of MYC (P57S and T58A) that are commonly observed in BL tumours retain their ability to stimulate proliferation and thereby activate p53 on overexpression, but do not promote apoptosis because they do not induce *BIM* expression. By contrast, BL tumours that carry MYC that can induce *BIM* expression generally have genetic lesions in the $p14^{ARF}$–p53 pathway[131,132]. It seems that deregulated MYC can trigger apoptosis through multiple, independent mechanisms that act cumulatively; inactivation of any one of several MYC effectors of apoptosis ($p14^{ARF}$, 19^{ARF}, p53 or BIM) can allow cell proliferation and initiate lymphomagenesis[131,132].

MYC defect	Apoptotic mechanism	Outcome
Deregulated wild-type MYC	$p14^{ARF}$–p53 and BIM	Apoptosis
Deregulated wild-type MYC	BIM only	B lymphoma
Deregulated mutant MYC (codons 57 and 58)	$p14^{ARF}$–p53	B lymphoma
Wild-type MYC activated by translocation	$p14^{ARF}$–p53	EBV-positive BL

methylation (reviewed in REF. 77), which could spread downstream and also engulf Wp, until an 'insulator' sequence is reached. This fits well with the observation that the region which encompasses Cp and Wp is epigenetically silenced and exhibits CpG methylation in the EBNA1-only programme, both in EBV-positive BL[78,79] and in the latently infected memory B-cell reservoir *in vivo*[80].

Ablation of EBNA2 activity allows the cells to assume a GC phenotype and express the default programme. LMP1 and LMP2 have been shown, in transgenic mouse and *in vitro* experiments, to possess all of the signalling capacity (including the induction of AID) that is necessary to allow the latently infected B cell to survive and exit the GC as a resting memory B cell in the absence of antigen or T-cell help[61,63,64,81–84]. However, recent studies[39] (J. Roughan and D.A.T.-L., unpublished observations) have challenged the validity of these observations to *in vivo* infection. These studies suggest that the findings in transgenic mice[82,84,85] might be more relevant to disease pathogenesis than to the mechanism of viral persistence. LMP1 and LMP2 probably only play auxiliary parts in normal persistence (rather than controlling or taking over the GC process), which could guarantee the survival of the latently infected cell in the highly competitive environment of the GC. The mechanisms that activate LMP1 and LMP2 expression in the default programme *in vivo* when EBNA2 is absent are unclear, but probably involve triggering by cytokines such as IL-4, IL-10 and IL-21 (REFS 86–88). The absence of this cytokine milieu alone might be sufficient to cause the cessation of LMP1 and LMP2 expression once the cell leaves the GC to become a memory cell.

In the model described above, EBV-induced proliferation *in vivo* is a transient event and long-term persistence is associated with the latency programme, rather than the growth programme. Specifically, EBV-induced growth is a self-regulating balance between the EBNA2 and EBNA3 proteins that normally leads to rapid extinction of proliferation. It follows that the phenomenon of *in vitro* immortalization that is observed in LCLs is a biological artefact in which the balance has been shifted slightly in favour of EBNA2 and proliferation. This is presumably due to the powerful selection pressure of *in vitro* growth and to the absence of signals that are associated with the lymphoid microenvironment and are required to signal the shift from proliferation to differentiation. Thus, we must concede that all observations on the behaviour of EBV made with established LCLs should be interpreted in the light that they might be artefacts of the immortalization process; they may not relate to the behaviour of the virus *in vivo* and thus need to be verified by *in vivo* studies.

Resetting the apoptotic threshold in EBV-positive BL. The role of EBNA3A and EBNA3C as transcriptional repressors suggests a mechanism for how EBV might decrease the apoptotic sensitivity to deregulated *MYC*, which leads to an increased risk for BL. Of the two pro-apoptotic pathways activated by MYC (p53 and BIM; BOX 3), there is no evidence to suggest that EBV directly impairs the p53-mediated pathway in latently infected B cells[89,90]. However, EBNA3A and EBNA3C functionally interact to inhibit the expression of the

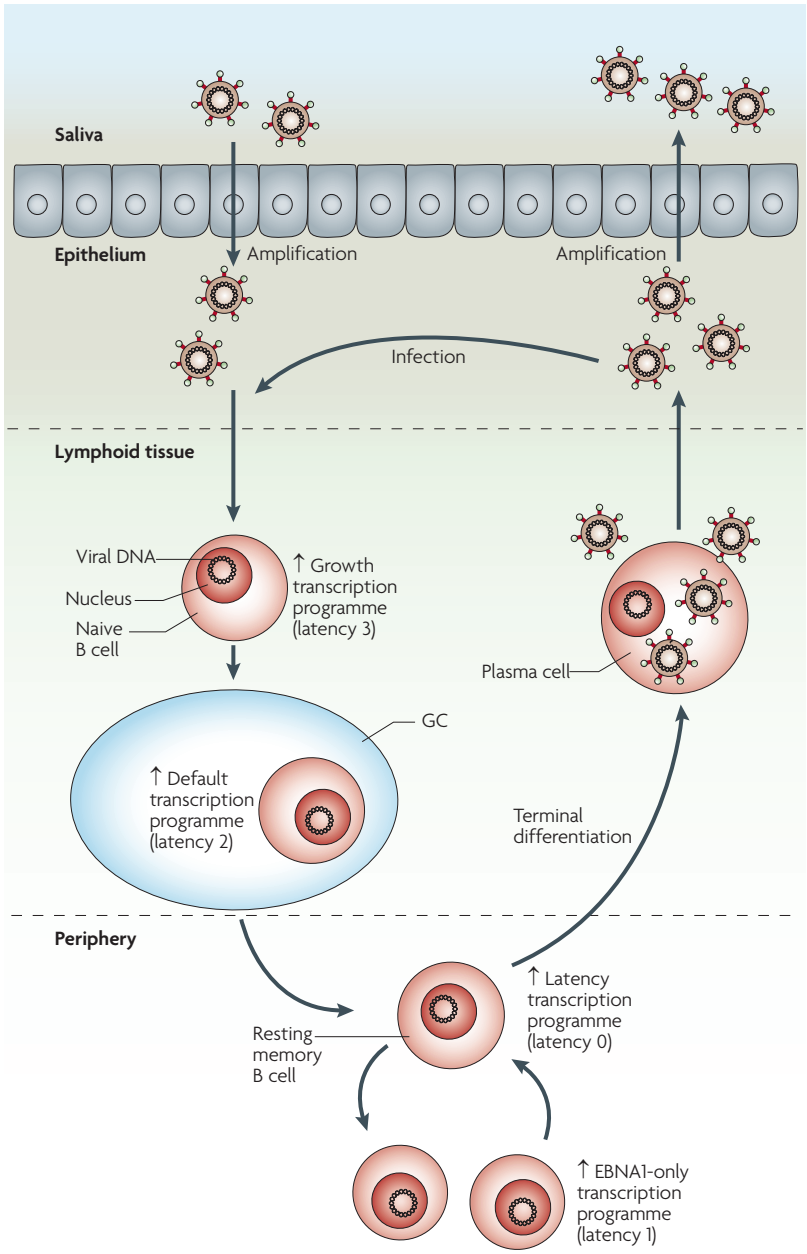


Figure 1 | **The current model of how EBV establishes and maintains persistent infection.** Epstein–Barr virus (EBV) spreads through saliva contact. It enters the epithelium, where it probably initiates a lytic infection that leads to amplification of the virus. The virus then infects naive B cells in the underlying lymphoid tissues and drives them to become activated lymphoblasts using the growth transcription programme (see Supplementary information S1 for details of different transcription programmes). Three of the growth-programme proteins (EBNA3A (EBV nuclear antigen 3A), EBNA3B and EBNA3C) negatively autoregulate the growth programme. This allows the cell to migrate into the follicle, initiate a germinal centre (GC) reaction and establish the default transcription programme. The default programme provides rescue or survival signals that allow the cell to exit the GC as a resting memory B cell. The latency transcription programme then begins, in which all viral protein expression is turned off. These cells are maintained by normal memory B-cell homeostasis. When they occasionally divide they express the EBNA1-only programme. The memory cells eventually return to the tonsil, where they occasionally undergo plasma-cell differentiation, which triggers viral replication[133–135]. The resulting virus may be shed into saliva for spread to other hosts or may infect other B cells. Notably, only the dividing memory B cells express the same transcription programme as that found in EBV-positive Burkitt's lymphoma (BL) cells — the EBNA1-only programme.

pro-apoptotic protein BCL2L11 (Bcl2-like protein 11; also known as BIM) (BOX 3) in these cells[91] through a mechanism that involves HDACs and DNA methyltransferases (K. Paschos and M.J.A., unpublished observations). This is the same mechanism that was postulated above for autoregulation of viral Cp and Wp, which repress the growth programme and terminate proliferation by the newly infected blast, allowing it to enter a follicle and switch to the default programme. In summary, a consequence of the self-regulation of EBV might be the suppression of BIM as the latently infected cell transits from the growth programme to the default programme (FIG. 3), which would create a B-cell environment in which translocation of wild-type *MYC* would be tolerated. The repression of BIM by EBNA3A and EBNA3C could be fortuitous, but more likely occurs because EBNA2 constitutively transactivates *MYC*[92]. EBNA3A and EBNA3C may have evolved to suppress BIM and so prevent apoptosis that is induced by EBNA2 through *MYC* to allow transient EBV-induced growth and proliferation.

As the repression of BIM transcription by EBNA3A and EBNA3C involves epigenetic mechanisms, repression may persist or, in the case of BL, may even be selected for in progeny cells after the expression of the EBNA3 proteins has been extinguished. The implication is that, unlike the other cells in the GC, the EBV-infected cells can tolerate a translocated *MYC* without undergoing apoptosis as they differentiate into the memory compartment (FIG. 3), even if the EBV proteins that caused the condition are no longer expressed. The regulatory function that is required to establish EBV latency and persistence thus increases the risk of EBV-positive BL, and it is predicted that in EBV-positive BL cells, chromatin modifications and DNA methylation will suppress the transcription of BIM epigenetically and prevent its induction by deregulated MYC.

MYC translocation need not occur in the GC for this mechanism of BL pathogenesis — it could occur any time between the activation of EBNA3A and EBNA3C and the final transcriptional quiescence of the latently infected memory cell. However, the only infected B cells *in vivo* that express the BL transcription programme, the EBNA1-only programme, are the resting memory B cells in the periphery as they enter into the cell division that is related to homeostasis[40]. Thus, if we assume that the *MYC* translocation occurs owing to the expression of AID in the GC and is tolerated because of prior BIM suppression by EBV, then an EBV-positive BL tumour could arise from a GC cell that has left the lymph node but cannot become a resting memory cell because of the constitutive activation of *MYC* (FIG. 3). This would explain why EBV-positive BL tumours grow extranodally and only express a small subset of GC-related genes (BOX 2). The viral transcriptional machinery responds to the signals that are associated with leaving the GC and entering the memory compartment. Consequently, latent protein expression is turned off, with the exception of the expression of EBNA1 Q–K, which continues because the cell fails to enter a resting state and keeps

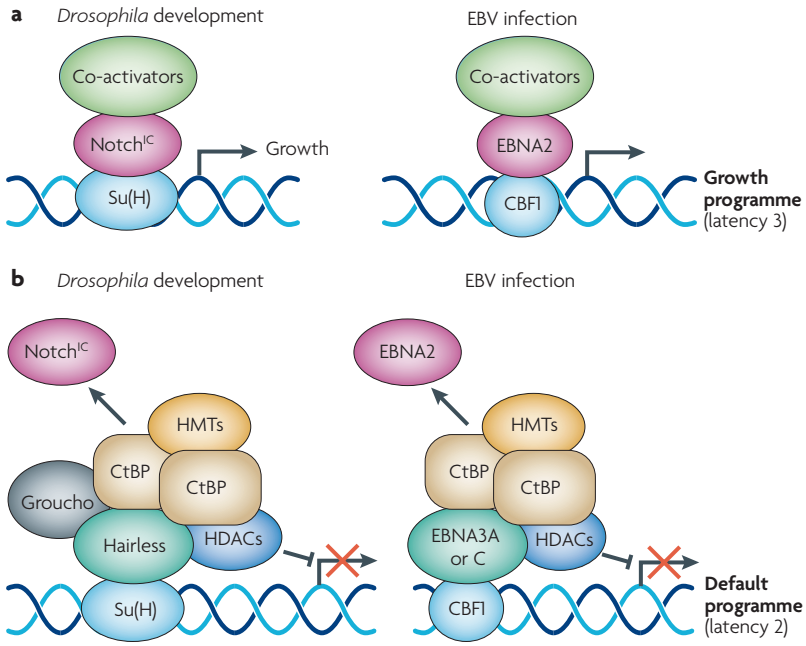


Figure 2 | **EBV uses the Notch system to autoregulate its growth. a** | In *Drosophila* development, the intracellular domain of Notch ($Notch^{IC}$) binds to DNA through Suppressor of Hairless (Su(H)) to initiate cell growth and inhibit differentiation. In a B cell that is newly infected with Epstein–Barr virus (EBV), EBNA2 (EBV nuclear antigen 2), the functional homologue of $Notch^{IC}$, is expressed and binds to DNA through CBF1 (also known as RBPJκ; recombination signal binding protein), which is equivalent to *Drosophila* Su(H) (Suppressor of Hairless), to activate the viral C promoter (Cp). This leads to expression of the growth programme, in which all the latent proteins are expressed. This expression programme drives the newly infected cell to become activated and begin proliferating. **b** | In *Drosophila*, cells stop growing and initiate differentiation through expression of Hairless, which competes with $Notch^{IC}$ for Su(H) binding. The binding of Hairless to Su(H) leads to the recruitment of Groucho and CtBP (carboxy-terminal-binding protein), which silence genes epigenetically through the recruitment of histone deacetylases (HDACs) and histone methyltransferases (HMTs). Similarly, EBNA3A, EBNA3C and probably EBNA3B can displace EBNA2 and may recruit CtBP co-repressor complexes and HDACs to epigenetically silence Cp, allowing the B cell to stop proliferating, undergo the transition to the default programme and differentiate into a memory cell through the germinal centre. Although the precise mechanism by which Cp is silenced *in vivo* has not yet been established, removal of EBNA2 and co-activators is essential to extinguish the growth programme.

proliferating. This would explain why EBV-positive BL cells express the EBNA1-only programme. It also explains why the rate of SHM is nearly identical in EBV-positive BL (6.1 per 100 bp)[45] and EBV-positive memory B cells (6.0 per 100 bp)[39], both of which are significantly higher than the rate of SHM in normal, EBV-negative memory B cells (5.2 per 100 bp). The higher rates of SHM in EBV-positive memory B cells might indicate that the virus causes extended expression of AID, which may in turn increase the likelihood of a *MYC* translocation.

The role of malaria

As EBV-positive BL is extremely uncommon outside of Africa, B cells in which both EBV and the *MYC* translocation occur must either be rare and/or efficiently eliminated. A crucial factor must dramatically increase the frequency of these events in areas where there is a high incidence of EBV-positive BL. The most likely candidate is malaria[5] (reviewed in REF. 93). It should be noted, however, that most children in equatorial Africa, all of whom are infected with EBV and malaria, do not develop BL. So even in these children, the coincidence of EBV and the *MYC* translocation must be a rare event.

Of the three key players in EBV-positive BL, malaria remains the orphan. The broad generalizations and speculations about the *MYC* translocation and EBV have been winnowed and clarified such that we are now beginning to understand their role at the molecular level. This is not true of malaria, however, for which speculations remain general and unsubstantiated. Indeed, there is little information about the biology of EBV in young children in general, and in those with malaria in particular. We do know, however, that the children in the African malaria belt with the highest antibody responses to EBV are the ones who are most likely to develop BL[12]. Why this is so remains a mystery. That malaria is itself the cofactor, and not simply coincident with some other agent, comes from studies which indicate that malarial parasite burdens correlate with EBV-positive BL incidence[94] and that consistent prophylaxis for malaria might lower the risk for EBV-positive BL[95,96].

Most thinking about the role of malaria in EBV-positive BL to date has tended to centre on its profound impact on the immune system (reviewed in REF. 93) and how this might increase EBV load[97–99]. Malaria is immunosuppressive for T-cell responses, including those directed against EBV[100,101], and is a polyclonal activator of B cells that can reactivate EBV in culture[102]. Unsurprisingly, the levels of EBV infection are higher in patients with malaria than in healthy individuals[97]. Increased viral loads have been interpreted to be the malaria-associated risk factor for EBV-positive BL. However, it should be noted that allograft patients, who are highly immunosuppressed, also carry high loads of EBV[29]. These patients also occasionally succumb to EBV lymphomas, but these are immunoblastic lymphomas, rather than BLs, and they usually express the growth programme[28], not the EBNA1-only programme. Put simply, increased EBV loads alone do not result in BL.

In the context of understanding the possible role of malaria-induced immunosuppression in BL, AIDS BL might be informative[103]. BL arises in patients with AIDS independently of their immunological status. However, as immunosuppression becomes profound, EBV loads increase and patients become susceptible to immunoblastic lymphoma, just as with allograft patients[104]. This suggests that immunosuppression and high viral loads alone produce immunoblastic lymphoma, but that these are not sufficient to cause BL. The additional cofactor that is required for AIDS BL is probably the chronic antigenic stimulation associated with repeated, opportunistic infections, and this is precisely what occurs in malaria. Children in holoendemic areas are chronically infected with *Plasmodium falciparum* such that the parasite is present in up to 90% of children under the age of 5 (REF. 105). Furthermore, parasitaemia levels peak in the first year of life, when

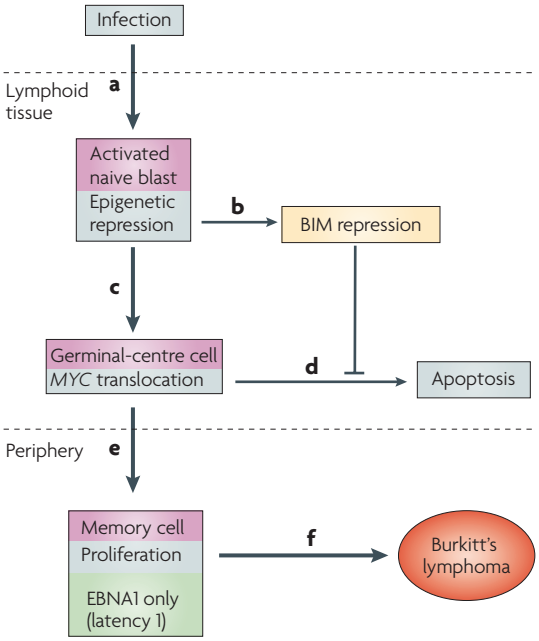


Figure 3 | **Schematic of the proposed mechanism of EBV-positive Burkitt's lymphoma pathogenesis.** **a** | A naive B cell that is newly infected with Epstein–Barr virus (EBV) in the tonsil expresses the growth transcription programme that is driven by EBNA2 (EBV nuclear antigen 2) to become a B blast (latency 3). Proliferation of the infected B blasts is driven in part by EBNA2 activation of MYC. **b** | EBNA2 activates the transcription of EBNA3A and EBNA3C, which in turn induce the epigenetic repression of BCL2L11 (Bcl2-like protein 11; also known as BIM), thereby allowing the cell to tolerate activated MYC. **c** | In parallel, EBNA3A and EBNA3C autoregulate cell growth through epigenetic repression of the growth-programme (FIG 2), allowing the cell to differentiate into a germinal-centre (GC) cell with a repressed *BIM* gene. **d** | The *MYC* translocation occurs at the GC stage, which leads to uncontrolled proliferation. Normally this would induce apoptosis through the activation of BIM. However, the prior repression of BIM allows the cell to survive and continue proliferating. **e** | The infected GC cell then follows its normal behaviour and leaves to become a memory cell. During this transition the virus also follows its normal behaviour (FIG 1) for EBNA1, which is required to replicate the viral genome when the cell proliferates. **f** | MYC-driven proliferation prevents the latently infected cell from becoming a resting memory cell, allowing it to grow as a Burkitt's lymphoma tumour that expresses the viral EBNA1-only programme (latency 1).

EBV infection also occurs[12]. A potentially fruitful line of investigation might be to look for a link between the chronic B-cell stimulation of malaria and prolonged or atypical expression of AID, which would lead to a higher occurrence of spontaneous *MYC* translocations (discussed above). At the same time, the higher levels of EBV infection caused by malaria would simultaneously produce a higher throughput of EBV-infected cells through the GC into memory. Consequently, more cells could tolerate the increased number of *MYC* translocations.

Conclusions

The 50-year history of BL and EBV study has taught us that the interaction of effectors in tumour development is rarely as simple as it seems.

We now know that EBV is not simply a B-cell transforming virus: it uses multiple aspects of B-cell biology and different combinations of latent gene expression to progress from initial infection through the GC to long-term persistence in memory B cells. The different patterns of latent protein expression observed in the EBV-associated lymphomas represent their origin from different stages in the virus life cycle. Specifically, EBV-positive BL is probably derived from a GC cell that has left the lymph node to become a resting memory cell, but continues to proliferate owing to deregulated MYC. Thus, like its normal infected counterpart, the proliferating memory cell, EBV-positive BL expresses the EBNA1-only programme.

We also now know that it is the deregulation of *MYC*, through a translocation, that is essential for BL development, not EBV. The GC is the most likely location for the *MYC* translocation event, which implies that it occurs before the development of full-blown lymphoma once the GC cell has left the lymph node in an attempt to become a memory cell. Definitively identifying the cell in which the translocation occurs will be crucial for our understanding of BL pathogenesis. For BL to develop, however, other changes must arise in the cell that allow the deregulated expression of *MYC*. EBV provides one, but not the only, way of achieving this: BL can occur with or without EBV.

Nevertheless, EBV is clearly associated with, and is a risk factor for, most BLs. But how? We now know that the EBV transforming genes do not have a direct role in the growth of EBV-positive BL. However, our current understanding of EBV biology has opened up another possibility — the EBV transforming genes may play an early part in predisposing or making the infected B cell susceptible to subsequent steps in BL lymphomagenesis. There is still much to be understood about how EBV could achieve this. We need more information on the precise mechanism by which EBV represses BIM, as there is evidence that this may involve both transcriptional[91] and post-transcriptional[106] regulation. To further develop models of BL pathogenesis, it will be important to determine the methylation status of BIM, the integrity of the CDKN2A (E3 ubiquitin-protein ligase; also known as $p14^{ARF}$)–p53 pathway and the nature of *MYC* mutations in samples that are derived from primary EBV-positive BL biopsies. Furthermore, we should be open to the possibility that EBV might contribute different mechanisms to BL lymphomagenesis, depending on the particular evolution of each tumour. In this context, we need to further clarify the extent to which viral genes that are expressed in BL have a role in tumour development and survival. Of particular relevance is the discovery that EBV encodes for a large number of micro-RNAs, the functions of which are poorly understood. Furthermore, the small subset of

EBV-positive BLs that retain expression of the EBNA3 proteins[107] require more scrutiny as, in addition to modulating transcription, these factors interact with regulators of cell-cycle checkpoints[108,109]. Finally, it is worth exploring whether the EBV growth programme increases genomic instability[110] as a prerequisite for subsequent BL development.

Ultimately, we need to know more about how the *MYC* translocation drives growth so aggressively and how EBV creates a permissive environment to tolerate this translocation. But we also need to keep in mind the importance of malaria in BL pathogenesis. We know almost nothing about the molecular basis for the role of malaria in BL pathogenesis. Until this mechanism is uncovered, we cannot develop a complete understanding of EBV-positive BL pathogenesis. Indeed, we cannot even be sure that it is malaria, and not some other factor with a similar geographical distribution (such as infection by arboviruses[111] or exposure to the plant *Euphorbia tirucalli*[112]) that is responsible. This point has serious clinical implications. If malaria is the driving force behind EBV-positive BL in equatorial Africa, then the elimination or control of malaria could also reduce the incidence of BL in Africa by ~99%.

In conclusion, a model of EBV-positive BL is emerging in which cells that are newly infected with EBV accumulate genetic and epigenetic changes, which predispose the cells to tolerate the subsequent *MYC* translocation events long after viral gene expression has been silenced. As these cells progress from new infection, through the GC, where the translocation occurs, they alone of all the GC cells can tolerate deregulated *MYC*, survive and leave the GC to become a memory cell that expresses the full BL phenotype. There is still much that we do not know, but perhaps this model will provide a framework for future studies, including an understanding of the role of malaria.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
MYC
Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
Plasmodium falciparum
UniProtKB: http://www.uniprot.org
BCL2L11 | CtBP | Groucho | IL-4 | IL-6 | IL-10 | IL-21 | RBPJκ | Su(H)

FURTHER INFORMATION

David A. Thorley-Lawson's homepage: http://www.tufts.edu/sackler/immunology/thorley-lawson/index.html

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