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Epstein-Barr Virus and the Pathogenesis of Lymphoma

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Abstract

Burkitt lymphoma (BL), first recognized by Denis Burkitt in 1958, is a high grade B cell malignancy particularly prevalent in young boys in tropical Africa and New Guinea. The high incidence of BL in areas of holo-endemic malaria prompted the search for a tumour-causing infectious agent transmitted by mosquitoes. This search, led by Anthony Epstein and co-workers, resulted in the discovery in cell lines derived from BL biopsies, of a gamma herpesvirus, later referred to as the Epstein-Barr virus (EBV). Subsequently, EBV was shown to be present in the tumour cells of patients with other forms of B cell lymphoma, such as Hodgkin lymphoma and post-transplant lymphoma, as well as in natural killer/T cell (NK/T) cell lymphomas and in several epithelial cancers such as nasopharyngeal carcinoma and gastric carcinoma. Understanding how EBV contributes to the development of these different forms of cancer has provided fundamental insights into the underlying mechanisms responsible for driving oncogenic processes as well as highlighting opportunities for prophylactic and therapeutic intervention. This chapter will summarise current knowledge of the role of EBV in lymphomagenesis, highlighting the importance of co-factors, including disorders of immunity, which can disrupt the delicate virus-host balance that otherwise ensures asymptomatic virus persistence in normal people.

Discovery of EBV in Burkitt lymphoma biopsies

In March 1961, Denis Burkitt gave a talk entitled 'The commonest children's cancer in tropical Africa: a hitherto unrecognised syndrome'. In the audience was Antony Epstein who later noted –" I was struck from the outset by the highly unusual nature of the new tumour and by the exciting and unprecedented findings on epidemiology which seemed to show that its distribution depended on climatic factors indicating that some biological agent might be involved in the aetiology" (Epstein, 1999). While listening Epstein formulated the hypothesis that "a biological agent must play a part in causation and … I immediately postulated a climate-dependant arthropod vector spreading a cancer-causing virus. It turned out later that it was a cofactor which was arthropod borne, but my idea focused correctly on the need to search for a viral cause". In discussions after the lecture Burkitt agreed to send fresh biopsies from some of his patients for analysis in Epstein's laboratory.

Following several years without success, in December 1963, a BL biopsy that had been delayed en route, finally reached Epstein's laboratory. In Epstein's own words "As usual the tissue was floating in transit fluid, but unusually this was cloudy. As it was getting late and the cloudiness was likely to be due to bacterial contamination, the feeling was that we could leave the laboratory for the weekend. But instead of discarding the specimen and going home I put a drop of the

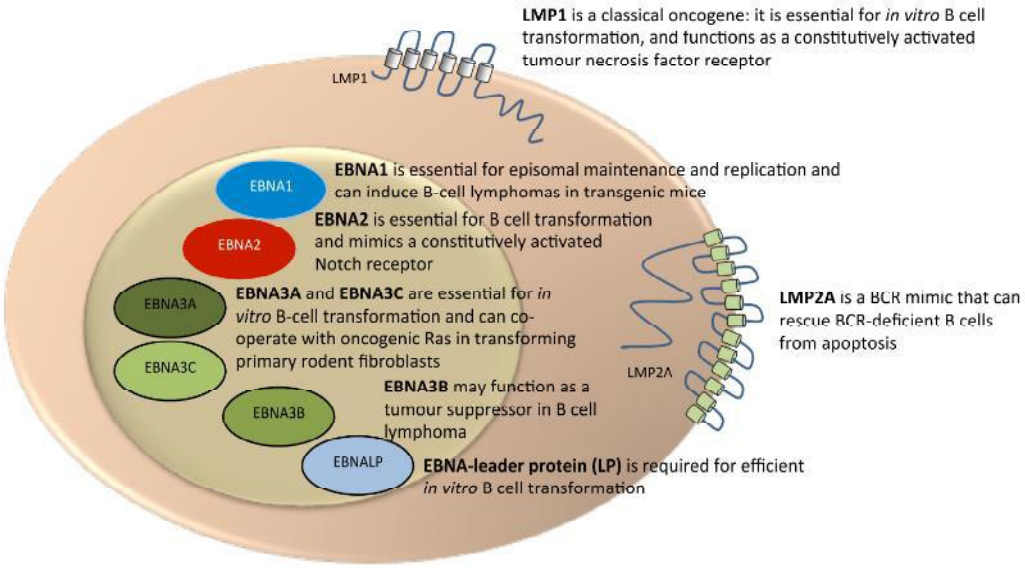

Figure 6.1. The functions of the major EBV latent proteins in B cell transformation/lymphomagenesis. Depicted with their known effects in B cells are the major EBV latent proteins including the Epstein-Barr nuclear antigens (EBNAs) and the latent membrane proteins, LMP1 and LMP2A.

cloudy fluid on a slide and examined it with the light microscope as a wet preparation. Rather than seeing the expected contaminating bacteria I was astonished to find that the cloudiness was due to large numbers of viable-looking free-floating tumour cells. " (Epstein *et al*., 1964). These cells grew when re-suspended in fresh culture medium, and so the first cell line from a human lymphoma was established (known as the EB1 cell line after Epstein and Yvonne Barr). In February 1964, Epstein prepared cells from the EB1 line for electron microscopy and was 'exhilarated to see unequivocal virus particles in a cultured lymphoma cell in the very first grid square I searched. I was extremely agitated in case the specimen might burn up in the electron beam- I switched off, I walked round the block in the snow without a coat, and when somewhat calmer I returned to record what I had seen. I recognized at once that I was looking at a typical member of the herpesvirus group' (Epstein, 2015).

Establishing the potent transforming ability of EBV in B cells

Following the discovery of EBV in BL, a critical question was whether the virus was transforming for B cells or merely a silent passenger. Volker Diehl co-cultivated cord blood lymphocytes with either an irradiated EBV-positive BL cell line, Jijoye, which produces virus particles, or Raji cells, which do not. He observed growth of the lymphocytes, but only after co-culture with Jijoye cells (Henle *et al*., 1967). Around the same time, John Pope showed that cell lines could be established from the peripheral blood lymphocytes of EBV-infected individuals (Pope, 1967). These experiments confirmed that EBV can potently transform B cells *in vitro*.

In 1984, the complete sequence of the B95.8 strain of EBV was published, heralding the advent of a post-genomic era in EBV research and one which led directly to the identification and functional characterization of EBV's most important genes (Baer *et al*., 1984). The EBV genes were broadly separated into the 'latent' genes, which were expressed in a phase of the virus life cycle in which there is no virus replication (latency), and the 'lytic' genes which are expressed during the stepwise progression from latency to virus replication and eventual virion assembly and release. We now know that it is the coordinated expression of the latent genes that is primarily responsible for the *in vitro* immortalization of B cells described above. In vitro immortalized B lymphocytes, which are commonly referred to as lymphoblastoid cell lines (LCL), express all latent genes (referred to as latency III or the 'growth programme') including six nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and EBNA-LP), two latent membrane proteins (LMP1 and LMP2), the non-coding Epstein-Barr-encoded RNA (EBER1 and EBER2), and a number of viral miRNA (Kerr *et al*., 1992; Pfeffer, 2004). The functions of the major latent genes are summarised in Figure 6.1.

Asymptomatic infection of B cells is widespread among human populations

The detection of antibodies to EBV proteins in BL patient sera had provided some of the initial evidence to support a pathogenic role for EBV in BL. However, the same antibodies were also present, albeit at lower levels, in most normal healthy people (Henle *et al*., 1969; Abate *et al*., 2015). EBV was also found to be the causative agent of infectious mononucleosis (IM) (Henle *et al*., 1968). Because transforming EBV is detectable at high levels in saliva from IM patients and at lower levels in healthy EBV-seropositive individuals, oral transfer was suspected to be the most likely mode of transmission (Niederman *et al*., 1976). Kissing is thought to be the major route of EBV transmission among adolescents and young adults. Transmission has also been suggested to occur through intercourse or oral sex (Crawford *et al*., 2002; Crawford *et al*., 2006; Higgins *et al*., 2007). However, the observation that the virus detectable in both male and female genital secretions (Sixbey *et al*., 1987; Israele *et al*., 1991; Woodman *et al*., 2005) is cell-associated rather than free infectious virus, argues against this (Thomas *et al*., 2006); deep kissing also carries the same risk of primary EBV infection irrespective of whether coitus has taken place (Thomas *et al*., 2006; Balfour *et al*., 2012; Balfour *et al*., 2013).

Although David Thorley-Lawson has elegantly shown that memory B cells are the site of EBV persistence in asymptomatic carriers (Babcock *et al*., 1998), how the virus gets into the memory B cell pool remains the subject of debate. In one model, memory B cells are directly infected with EBV (Kurth *et al*., 2000; Kurth *et al*., 2003). In the other model, proposed by Thorley-Lawson, EBV initially infects naïve B cells which then proliferate as a result of expression of the latency III pattern typical of LCL. The EBV-infected B cells enter germinal centres (GC) - B cell factories in which B cells undergo affinity maturation and class switch recombination. In the GC, the EBV-infected B cells express another form of latency, known as the 'default programme' or latency II; here the two latent membrane proteins, LMP1 and LMP2, are expressed together with EBNA1 driven by the viral Qp promoter (Babcock *et al*., 2000). LMP1 and LMP2 provide surrogate CD40 and B cell receptor (BCR) signals, respectively, which probably mediate the survival of EBV-infected GC B cells and their subsequent exit from the GC as memory cells (Gires *et al*., 1997; Caldwell *et al*., 1998). EBV-infected memory B cells shut down virus gene expression (latency 0) thereby avoiding recognition by the immune system (Babcock *et al*., 2000). One of the major functions of EBNA1 is to allow replication and segregation of viral episomes during cell division and so when memory B cells are required to proliferate, EBNA1 expression is switched on; this additional form of latency is known as latency I. The activation of EBV-infected memory B cells can lead to plasma cell differentiation which switches on the lytic cycle of EBV with subsequent production of new virions (Laichalk and Thorley-Lawson, 2004).

The origin of EBV-associated B cell lymphomas

Given that the B cell is the site of virus persistence in the asymptomatically infected host then we may think of the development of EBV-positive B cell lymphomas as rare accidents of EBV's ability to colonise the B cell system (Rickinson, 2014). Because malignancy is a relatively rare outcome of infection, co-factors must be involved in the oncogenic process. As we shall see, some of the co-factors are already known, especially in BL, where malaria and HIV, either alone or in combination, act as chronic immune stimulants.

Most EBV-associated B cell lymphomas display evidence of somatic hypermutation and so appear to have arisen from cells that have been through a GC reaction, but which have presumably not fully differentiated to the memory B cell stages (Timms *et al*., 2003; Bräuninger *et al*., 2006). The point at which the progenitor B cells are aberrantly arrested in the B cell differentiation pathway varies between the different EBV-associated B cell malignancies and is reflected in the differences in cellular and virus gene expression that characterise each type (Figure 6.2). We now consider in turn each of the major lymphoma subtypes associated with EBV and briefly outline how the virus is thought to contribute to each.

Defining the contribution of EBV to the pathogenesis of Burkitt lymphoma

Two years after the discovery of EBV, antibodies to EBV antigens were detected in the serum of African BL patients (Old *et al*., 1966). This was followed by reports of higher titres of EBV antibodies in the serum of BL patients compared with age-matched controls from the same area. In 1978, Guy de Thé and colleagues published the results of their serological observations in pre-BL samples taken from over 40,000 Ugandan children (de-Thé *et al*., 1978). Most of the children who subsequently developed BL had raised EBV antibody titres prior to diagnosis, leading the authors to conclude that 'BL develops in children who have had a long and heavy exposure to EBV' (de-Thé *et al*., 1978). In 1970, Harald zur Hausen used DNA hybridisation to identify the EBV genome in BL

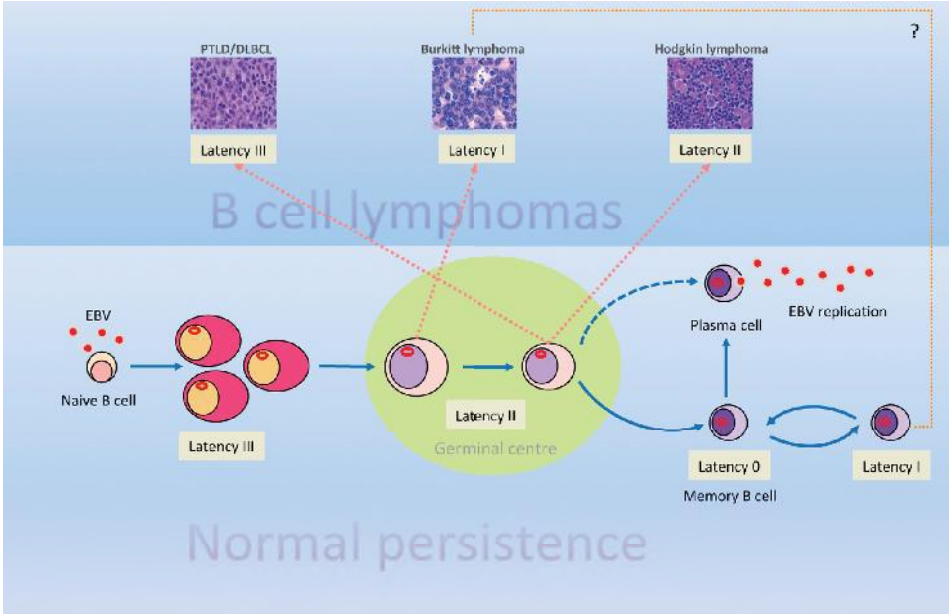

Figure 6.2. Origins of EBV-positive B cell lymphomas. Shown is the germinal centre model of EBV persistence depicting the transition of EBV-infected B cells through a germinal centre reaction and their subsequent differentiation to memory or plasma cells. EBV-infected memory B cells are the site of long-term latency and persistence, whereas virus replication occurs in plasma cells. The potential origin of some of the major EBV-associated B cell lymphomas is also shown. However, there is considerable uncertainty about the precise stage(s) from which each tumour derives.

(zur Hausen *et al*., 1970), and in 1974, Beverley Reedman working in George and Eva Klein's lab', used anti-complement immunofluorescence (ACIF) to show expression of an Epstein-Barr virus nuclear antigen, known as 'EBNA' for short; we now know this protein is EBNA1 (Reedman *et al*., 1974; Dillner *et al*., 1984; Dillner *et al*., 1985).

BL occurs not only in its African or 'endemic' form, but also in a 'sporadic' form at a much lower incidence throughout the world. BL also occurs with an increased incidence in people with HIV, usually occurring before CD4+ T cells counts rapidly decline. Almost all endemic BL is EBV-positive, whereas EBV-positive rates are lower in HIV-associated BL (~40%) and in sporadic BL (15-20%). However, irrespective of EBV status, all forms of BL harbour one of three reciprocal chromosome translocations involving the c-MYC gene on chromosome 8 and either the immunoglobulin heavy chain gene on chromosome 14, or the kappa or lambda light chain genes on chromosomes 2 and 22, respectively (Manolov and Manolova, 1972; Zech *et al*., 1976). These translocations bring c-myc under the transcriptional control of one of the immunoglobulin *loci* resulting in a constitutively elevated c-myc expression which maintains the high proliferative rate that characterises BL (Dean *et al*., 1983; Armelin *et al*., 1984; Einat *et al*., 1985). However, c-myc over-expression also induces apoptosis, and so the BL progenitor must acquire a second independent event that can overcome the apoptosis-inducing effects of c-myc (Evan *et al*., 1992; Milner *et al*., 1993).

The loss of virus episomes from EBV-positive BL cell lines was shown to result in diminished cell survival and provided the first evidence that EBV might provide an anti-apoptotic function to counteract c-myc induced apoptosis (Shimizu *et al*., 1994). Most EBV-positive primary BL and early passage BL cell lines express a latency I pattern of virus gene expression in which there is expression of EBNA1, EBERs and the viral miRNA. Although EBNA1 was shown to mediate some of the anti-apoptotic effects of EBV in BL cells, this virus protein cannot reconstitute complete protection from cell death and other studies suggest that the EBERs and the viral miRNAs have anti-apoptotic functions in BL (Komano *et al*., 1999; Kennedy *et al*., 2003; Vereide *et al*., 2013). In keeping with the requirement for the inhibition of apoptotic pathways, TP53 mutations are present in approximately 30%–40% of BL biopsies (Lindström and Wiman, 2002). Recent global genomic analysis have also identified recurrent mutations in the ID3, TCF3, and CCND3 genes, all of which are involved in the same pathway leading to the activation of proliferation

through cyclin D3 and survival through the induction of phosphoinositide 3-kinase (PI3K) signalling (Love *et al*., 2012; Richter *et al*., 2012; Schmitz *et al*., 2012). Mutations in MYC itself, and of ID3, TCF3 and TP53 have been shown to be more common in sporadic BL than in endemic BL, whereas mutations in other genes, including *ARID1A*, *CCNF* and *RHOA*, are less frequently seen in sporadic cases (Abate *et al*., 2015).

A minority of primary BL and BL cell lines express all the EBNAs with the exception of EBNA2 which is deleted leading to the loss of LMP1 expression (Kelly *et al*., 2002; Kelly *et al*., 2005). These unusual cases are known as 'Wp-restricted' BL because expression of the EBNAs is initiated from the Wp promoter as opposed to the Qp promoter that drives EBNA1 expression in conventional BL. It has been shown that Wp-restricted BL cell lines are more resistant to apoptosis than Qp-restricted BL lines, an effect that has been attributed to several possible mechanisms which include the down-regulation of the pro-apoptotic molecule, Bim by EBNA3A and EBNA3C, and the expression of an EBV-encoded homologue of bcl-2, known as BHRF1 (Anderton *et al*., 2007; Kelly *et al*., 2009).

It is now well-established that malaria and HIV are co-factors in BL development. Although both act in mechanistically different ways, the outcomes are similar - a sustained increase in EBV loads caused by the polyclonal activation of B-cells which increases the size of the EBV-infected B cell pool. In turn this probably makes the generation of EBV-infected B cells harbouring MYC translocations more likely (Rowe *et al*., 2014). Although malaria also impairs T-cell immunity to EBV this impairment is transient, whereas the increased EBV loads in circulating memory B cells associated with malarial infection are long-lived (Moss *et al*., 1983; Njie *et al*., 2009; Whittle *et al*., 1984). *P. falciparum* may also act more directly to induce BL since it has been shown to increase expression of the enzyme, activation-induced cytidine deaminase (AID) which can induce BL-associated mutations, including the characteristic translocations (Torgbor *et al*., 2014).

Post-transplant lymphoproliferative disorders: loss of EBV immunity takes centre stage

Although there had been some reports of an increased risk of lymphoma development in patients suffering from deficiencies of humoral or cell-mediated immunity, it was not until the late 1960s that the importance of immune surveillance in preventing lymphoma development was fully appreciated when several reports of lymphoma in iatrogenically immunosuppressed renal allograft recipients were published (Fialkow, 1967; Doak *et al*., 1968; Penn *et al*., 1969).

Raised antibody titres to the viral capsid antigen (VCA) of EBV were initially detected in transplant patients treated with cyclosporine, raising the possibility that EBV was involved (Nagington and Gray, 1980). Confirmation of EBV's involvement came when Dorothy Crawford identfied the 'EBNA' protein in post-transplant lymphoma tissues (Crawford *et al*., 1980), which was quickly followed by further reports documenting EBV in lymphomas arising in other solid organ transplant recipients, and in bone marrow transplant patients (Crawford *et al*., 1980; Hanto *et al*., 1981; Saemundsen Ak *et al*., 1981; Schubach *et al*., 1982; Hanto *et al*., 1983). Surprisingly, most lymphoproliferative diseases arising in bone marrow transplant recipients were not of recipient origin, but derived from the donor B cells (Schubach *et al*., 1982). Collectively, these tumours were termed 'post-transplant lymphoproliferative disorders' (PTLD) (Starzl *et al*., 1984). The incidence of PTLD was found to be high in the first weeks to months after transplantation when patients are intensely immunosuppressed. Thereafter, the incidence decreases, but rises again 4–5 years from transplantation (Leblond *et al*., 2001; Ghobrial *et al*., 2005a;). These data suggest that early-onset and late-onset tumours are aetiologically distinct. Indeed, early onset PTLD are typically oligoclonal and in many cases may be considered to be the *in vivo* equivalent of LCL - outgrowths of EBV-infected B cells expressing all latent genes (latency III). Late-onset PTLD is less likely to be associated with EBV, and overall is more likely than early-onset PTLD to be extranodal (Leblond *et al*., 1998; Ghobrial *et al*., 2005b). Monoclonal PTLD often occur as a type of B cell lymphoma known as diffuse large B cell lymphoma (DLBCL). A similar spectrum of disease is also observed in people with HIV (before the advent of highly active retroviral therapy) and in patients with some primary immunodeficiences (Palendira and Rickinson, 2015). Starzl and colleagues were the first to show that reduction or discontinuance of immunosuppression can lead to regression of PTLD (Starzl *et al*., 1984). An extension of this approach was pioneered by Cliona Rooney and others using the adoptive transfer of EBV-specific T cells (Rooney *et al*., 1995; Hislop *et al*., 2010). These and other immunotherapeutic anti-viral strategies are discussed elsewhere in this volume.

Early-onset PTLD are often thought of as EBV-driven malignancies. However, not all immunosuppressed patients will develop PTLD and so the pathogenesis of these tumours must be dependent upon other co-

factors. One striking example of this is the high incidence of early-onset PTLD in the transplanted organ suggesting that chronic antigen stimulation in the graft itself might be involved in the pathogenesis of these lesions. Indeed, T cells are required for the development of PTLD-like tumours in severe combined immunodeficient (SCID) mice, suggesting an important role for T cell help in the growth of PTLD (Johannessen *et al*., 2000).

EBV-positive DLBCL

As well as occurring during HIV infection and after post-transplant immunosuppressive therapy, EBV-positive DLBCL can also arise in patients without a prior history of immune suppression or lymphoma (Oyama *et al*., 2003; Oyama *et al*., 2007; Cohen *et al*., 2012; Cohen *et al*., 2014). In older patients senescence of the EBV-specific immune response associated with the normal ageing process is suspected; consistent with this these tumours often display a latency III pattern of virus gene expression (Ok *et al*., 2013; Ok *et al*., 2015). EBV-positive DLBCL has also been described in younger patients and in children who are not obviously immunodeficient. The precise nature of the defects in T cell immunity that allow the development of EBV-positive DLBCL have not been properly defined. In one study, compared to age-matched controls, patients with EBV-positive DLBCL showed a narrowed EBV-specific TCR-Vb repertoire, with reduced EBV-specific effector memory CD4+ and CD +8 T cell numbers (Cárdenas *et al*., 2015). However, more work is required to determine if EBV-specific immune responses are different between patients with EBV-positive and EBV-negative DLBCL, and if these defects remain after successful therapy.

Primary effusion lymphoma

Primary effusion lymphoma (PEL) is a B-cell lymphoma localised predominantly in body cavities (pleural, peritoneal, pericardial) as a neoplastic effusion rather than a solid tumour mass and without recognisable nodal involvement. PEL is characterised by infection of the neoplastic cells with human herpesvirus 8 (HHV8/KSHV) and often also with EBV (Horenstein *et al*., 1997). The tumour cells express markers of lymphocyte activation (CD30, CD38, CD71, HLA-DR) and plasma cell differentiation (CD138), exhibit clonal immunoglobulin gene rearrangements and show evidence of somatic hypermutation (Jenner *et al*., 2003; Schulz and Cesarman, 2015), consistent with a plasmablast origin. Although PEL occurs mostly in HIV-infected individuals, cases are also observed following iatrogenic immune suppression for organ transplantation, suggesting this neoplasm is strongly associated with loss of immune control (Boshoff and Weiss, 2001).

HHV8 is thought to be the primary causal agent in PEL because HHV8-encoded genes that can induce cellular proliferation, inhibit apoptosis and contribute to immune evasion are expressed by tumour cells (this is discussed elsewhere in this volume). However, the role of EBV in the pathogenesis of PEL is less well understood. EBV gene expression is largely restricted to the latency I programme, with expression of Qp-driven *EBNA1* and highly variable levels of the *EBER*s. Occasional low levels of *LMP1, LMP2A* and *BZLF1* expression are observed although this is highly variable between and within patients (Szekely *et al*., 1998; Trivedi *et al*., 2004). The Wp and Cp promoters are not used, and for this reason expression of *EBNA2* and the *EBNA3*s is not observed (Horenstein *et al*., 1997). Further study of EBV's contribution to the pathogenesis of PELs is required (Mack and Sugden, 2008).

DLBCL associated with chronic inflammation

DLBCL associated with chronic inflammation are EBV-positive tumours that occur in the context of long-standing chronic inflammation and are derived from late GC or post-GC B cells. They usually present as a tumour mass involving body cavities. Pyrothorax-associated lymphoma (PAL) is the prototypic form. PAL is associated with a history of chronic pyrothorax or chronic pleuritis due to the initiation of a therapeutic artificial pneumothorax for pleuropulmonary tuberculosis, which was used in the past as a surgical treatment for tuberculosis (Loong *et al*., 2010). The interval between the onset of chronic inflammation and malignant lymphoma is usually over 10 years, (median 37 years) with a median age of 65 to 70 years. Males are more susceptible to this lymphoma, with a male to female ratio of 4:1. Other EBV-positive DLBCL associated with chronic inflammation with similar features to PAL include those associated with metallic implants in bones and joints (Sanchez-Gonzalez *et al*., 2013), and chronic osteomyelitis or chronic venous ulcers (Copie-Bergman *et al*., 1997; Cheuk *et al*., 2005;).

The EBV gene expression profile of PAL is usually latency III. It has been suggested that the EBV-transformed B cells at the site of chronic inflammation are able to escape host immune surveillance through several different mechanisms: Cellular IL-10, an immunosuppressive cytokine (Kanno *et al*., 1997a; Kanno *et al*., 1997b), and IL-6 and IL-6 receptor are

produced by PAL cell lines and can promote tumour cell growth *in vitro* (Kanno *et al*., 1996). The down-regulation of HLA class I has also been observed (Kanno *et al*., 1999) and various mutations of CTL epitopes in EBNA3B, an immunodominant antigen for CTL responses, may also play a role in the immune escape of PAL (Kanno *et al*., 2000). A microarray analysis of PAL identified the interferon-inducible 27 (IFI27) as one of the most differentially expressed genes in PAL compared to regular DLBCL (Nishiu *et al*., 2004). Expression of IFI27 is in keeping with the role of chronic inflammation in this condition since it is known to be induced in B lymphocytes following stimulation with interferon-alpha (IFN-α), although the role of IFI27 in lymphomagenesis has yet to be resolved.

Hodgkin lymphoma

Although the incidence of the EBV-positive forms of Hodgkin lymphoma (HL) is also increased in immunosuppressed populations, most cases arise in apparently immunocompetent individuals. HL is distinguished histopathologically from the non-Hodgkin lymphomas, including DLBCL, by virtue of a particularly prominent tumour microenvironment that is especially rich in CD4+ T cells and other inflammatory cells. The tumour microenvironment is so extensive in many cases of HL that the malignant Hodgkin/Reed-Sternberg (HRS) cells are outnumbered and can represent as little as 1% or less of the total tumour mass.

In 1974, two reports were published documenting a significantly increased risk of HL in individuals with a prior history of IM (Connelly and Christine, 1974; Rosdahl *et al*., 1974). Once again the ACIF assay was employed, this time by Sibrand Poppema, who detected the 'EBNA' protein in the HRS cells of a single patient (Poppema *et al*., 1985). EBV DNA was then detected in whole HL biopsies and in HRS cells (Weiss *et al*., 1987; Weiss *et al*., 1989). Later, RT-PCR and immunohistochemistry showed that EBV-positive HRS cells express a latency II pattern of virus gene expression, characterised by high level expression of *LMP1* and *LMP2* (Wu *et al*., 1990; Pallesen *et al*., 1991; Murray *et al*., 1992; Deacon *et al*., 1993).

Ralf Kuppers showed that although HRS cells lack markers present on most B cells, they harbour clonal immunoglobulin rearrangements, are somatically hypermutated, and in some cases have mutations that prevent immunoglobulin expression (Kuppers *et al*., 1994). Thus, HRS cells are thought to be derived from GC B cells lacking a functional BCR and which must have escaped the apoptosis that would otherwise be their normal fate. There is compelling evidence to suggest that EBV can provide the necessary anti-apoptotic functions. Thus, the crippling mutations in immunoglobulin genes have so far almost always only been found in EBV positive cases (Bräuninger *et al*., 2006). Furthermore, EBV has been shown to immortalise BCR-negative GC B cells *in vitro*; an effect that is dependent upon BCR-like signalling provided by the viral *LMP2A* gene (Caldwell *et al*., 1998; Bechtel *et al*., 2005; Chaganti *et al*., 2005; Mancao *et al*., 2005). *LMP1* is also probably a major contributor to HRS cell survival because it has been shown to constitutively stimulate several pro-survival pathways, including NF-κB, STAT and PI3K/AKT and can activate an HRS-like transcriptional programme in normal GC B cells (Bargou *et al*., 1997; Dutton *et al*., 2005; Holtick *et al*., 2005; Vockerodt *et al*., 2008).

X-linked lymphoproliferative disease

Although IM is usually self-limiting, primary EBV infection may be associated with more severe symptoms, typically in immunodeficient patients, and most notably in a condition referred to as 'Duncan's syndrome', later to be called X-linked lymphoproliferative disease-1 (XLP1). Boys with XLP1 develop a severe, often fatal, form of IM usually within weeks following primary EBV infection, and those who survive have a increased risk of lymphoma (Purtilo *et al*., 1975; Seemayer *et al*., 1995). David Purtilo established the XLP registry at the University of Nebraska in 1980. In 1995 the registry had recorded data on 80 families including 272 boys; 75% of those with follow-up had died, most before 10 years of age (Seemayer *et al*., 1995). In 1998, the underlying defect in XLP1 patients was identified as loss of function mutations in the *SH2D1A* gene, resulting in defective forms of its gene product, the signalling lymphocyte activation molecule (SLAM)-associated protein (or SAP for short) (Coffey *et al*., 1998; Nichols *et al*., 1998; Sayos *et al*., 1998). As well as SLAM, SAP binds other proteins including 2B4, NTB-A, CD84, Ly108, and Ly986. XLP1 patients have impaired T-cell and NK cell engagement of EBV-infected B cells rather than a specific inability to cope with EBV itself (Hislop *et al*., 2010). SAP-defective T and NK cells are also resistant to apoptosis resulting in the massive proliferation of T and NK cells, excessive cytokine production, and hemophagocytic lymphohistiocytosis (HLH) (see below).

Spectrum of EBV-positive NK/T cell disease

The primary cellular targets of EBV during the natural life cycle of the virus are B lymphocytes and

oropharyngeal epithelial cells. However, EBV is seemingly able to infect T cells and NK cells. Because mature NK or T cells do not express the EBV receptor, the mechanism of infection is currently unknown.

The EBV-associated NK/T lymphoproliferative diseases (EBV+ NK/T-LPD) can be subdivided into distinct but overlapping clinical entities, which include: chronic active EBV (CAEBV), haemophagocytic lymphohistiocytosis (HLH), and hydroa vacciniforme (HV). EBV is also associated with a number of highly aggressive malignancies of NK/T cells, including extranodal NK/T cell lymphoma (ENKTL) and aggressive NK leukaemia (ANKL). These malignancies are extremely difficult to treat and as a result have a poor prognosis. Importantly, a proportion of these malignancies develop from the EBV+ NK/T-LPD.

Chronic active EBV

Chronic active EBV (CAEBV) is usually described as a chronic disease of childhood and young adulthood. Patients exhibit persistent IM-like symptoms including fever, hepatosplenomegaly, persistent hepatitis and extensive lymphadenopathy. This is coupled with elevated EBV-DNA loads in peripheral blood mononuclear cells, histological evidence of organ infiltration with EBV-infected cells and high levels of pro-inflammatory cytokines in the blood. CAEBV is considered a chronic disease, yet many patients develop severe, often fatal, complications including multi-organ failure, digestive tract ulceration/perforation, malignant lymphomas and HLH.

Although CAEBV occurs worldwide, the disease exhibits a distinct geographical distribution with increased frequency observed in East Asia and in native Americans from Central and South America and Mexico. The majority of cases occur in children and young adults with a mean onset of 11.3 years. There is no sex predilection and the frequency of T cell vs. NK cell infection is roughly equal (Kimura *et al*., 2003). However, the number of cases of CAEBV in adults is increasing. It is unclear if this is a true increase in incidence or merely increased awareness by clinicians. The virus is predominantly found in the T cells of adult cases. EBV infection of T cells is often associated with a worse outcome than infection of NK cells and this does appear to be borne out in the adult onset cases where the disease is more aggressive.

CAEBV was first described in 1978 and yet the pathogenesis of CAEBV is still poorly understood (Virelizier *et al*., 1978). A pathogenic role for EBV is suggested by the detection of monoclonal episomes in infected cells (Ohga *et al*., 1999; Oyoshi *et al*., 2003), which exhibit a latency II viral gene expression profile, including expression of LMP2-TR (LMP2-terminal repeat) (Fox *et al*., 2010). However, our understanding of the contribution of EBV to CAEBV is limited, partly due to the rarity of the condition and partly because of the inherent difficulty in infecting T cells or NK cells *in vitro*.

Individuals with CAEBV are unable to produce an effective immune response to control EBV. Patients frequently have elevated levels of pro- and anti-inflammatory cytokines including interleukin (IL)-1β, IFN-γ, IL-10, IL-13, IL-15, tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-β (Ohga *et al*., 2004; Kimura *et al*., 2005). One study of 11 CAEBV patients reported impaired NK cell activity, lymphokine activated killer activity and EBV-specific CTL activity (Wakiguchi *et al*., 1988), and another reported very low to undetectable EBV-specific CD8+ T cells (Sugaya *et al*., 2004). It has been suggested that adoptive T cell therapy may be a valid approach for CAEBV. Although the subset of viral genes expressed in CAEBV elicit only subdominant T cell responses, LMP2-TR specific CD8+ T cells were able to efficiently recognise and kill CAEBV cell lines (Fox *et al*., 2010) and autologous *ex vivo* expanded cytotoxic CD8+ T cells from CAEBV patients stimulated with LCLs were able to resolve mild/moderate CAEBV (Savoldo *et al*., 2002). The association of primary EBV infection with CAEBV and the strong racial predisposition suggests a genetic defect in the infected T- or NK cells, however no consistent mutations or chromosomal aberrations have been identified (Joncas *et al*., 1989; Fujieda *et al*., 1993; Sugaya *et al*., 2004).

CAEBV has also been well documented in the US. These cases differ from those observed in East Asia, because they are predominantly associated with EBV infection of B cells, but not T- or NK cells. The clinical course of the disease is milder, the mean age of clinical onset is 19 years. Unlike cases reported in East Asia, the US cases show a progressive loss of B cells, hypogammaglobulinaemia and reduced NK cell numbers and patients usually succumb to progressive lymphoproliferative disease or infection (Cohen *et al*., 2009).

In general, the prognosis for CAEBV is poor and the only cure is haematopoietic stem cell transplant. Even following transplant, at least 25% of patients will develop a B-, T- or NK cell malignancy.

Table 6.1. Mutations associated with primary haemophagocytic lymphohistiocytosis.

Primary HLH	Cytogenetic localisation	Mutated gene	Protein	Function
Familial HLH (FHL)				
FHL1	9q21.3-22	Unknown		Unknown
FHL2	10q21-22	*PRF1*	Perforin	Pore formation
FHL3	17q25	*UNC13D*	Munc13-4	Granule priming
FHL4	6q24	*STX11*	Syntaxin-11	Granule fusion
FHL5	19q13	*STXBP2*	Munc 18-2	Granule fusion
Chediak-Higashi syndrome	1q42-43	*LYST*	LYST	Granule trafficking
Griscelli syndrome type 2	15q21	*Rab27A*	Rab27A	Granule docking
XLP type 1	Xq25	*SH2D1A*	SAP	Signalling in T and NK cells
XLP type 2	Xq25	*BIRC4*	XIAP	Signalling pathways involving NF-κB

EBV-related haemophagocytic lympho-histiocytosis (EBV-HLH)

HLH is a life-threatening hyper-inflammatory syndrome that can be classified according to underlying etiology into primary (genetic) or secondary HLH. Primary HLH occurs during infancy and early childhood as the result of mutations affecting the perforin pathway which affect perforin-mediated cytotoxicity of T lymphocytes and NK cells (Table 6.1). Secondary HLH usually occurs in children and adults with no known underlying genetic defects and frequently as a complication of infection, malignancy, autoimmune or rheumatologic disease, immunosuppression or haematopoietic stem cell transplantation.

The diagnosis of HLH is based on fulfilling the diagnostic criteria described in the HLH-2004 protocol (Table 6.2) (Henter *et al*., 2007). Unfortunately, these diffuse criteria overlap with several other diseases, making the diagnosis of HLH challenging. Delayed diagnosis of EBV-HLH is associated with high mortality, usually within 2 months following the onset of symptoms. Therefore early recognition and prompt treatment is key.

EBV is recognised as the leading infectious cause of secondary HLH (EBV-HLH) (Janka *et al*., 1998; Rouphael *et al*., 2007). The highest incidence of EBV-HLH cases is observed in East Asia, including Japan, Taiwan, China and Vietnam where the disease is predominantly observed in children (mean 3.9 years ±2.8 years) (Su *et al*., 1989; Kawaguchi *et al*., 1993; Janka *et al*., 1998). In adults, EBV is associated with approximately 40% of HLH, however, in these cases the HLH usually arises as a complication of EBV-associated lymphomas including Hodgkin lymphoma and extranodal NK/T cell lymphoma. The viral load in peripheral blood mononuclear cells and plasma of EBV-HLH patients is usually very high and monoclonal EBV is found almost exclusively in the NK cells or T cells, the majority of which are clonal.

Secondary HLH is usually treated with the HLH-2004 protocol (etoposide, cyclosporine, dexamethasone) which is administered to eliminate the underlying

Table 6.2. Diagnostic criteria for haemophagocytic lympho-histiocytosis according to the HLH-2004 protocol.

A DIAGNOSIS OF HLH CAN BE MADE IF EITHER CRITERIA 1 OR 2 IS MET:
1. Molecular diagnosis consistent with primary HLH
2. Clinical and laboratory criteria (at least 5/8 criteria should be fulfilled)
Fever
Hepatosplenomegaly
Cytopenia ≥2-3 cell lines in peripheral blood (Haemoglobin <9 g/100mL, platelets <100 x 10^9/L, neutrophils <1,0 x 10^9/L)
Hypertriglyceridemia and/or hypofibrinogenemia (Fasting triglycerides ≥3,0 nmol/L, fibrinogen ≤1,5 g/L)
Haemophagocytosis in bone marrow, spleen, cerebrospinal fluid or lymph nodes. No sign of malignancy
Decreased or absent NK-cell activity
Ferritin ≥500 μg/L
sCD25 (soluble IL2 receptor) ≥2,400 U/mL

trigger of the HLH and to suppress the inflammation and activation of the immune cells. However, the only cure is haematopoietic stem cell transplant.

Like CAEBV, the role of EBV in the pathogenesis of HLH remains unclear. However, cases of both primary and secondary HLH present with the same clinicopathological condition, suggesting that EBV infection could mimic the effects of the genetic mutations observed in primary HLH. Indeed, the clinical manifestations of EBV-HLH and XLP-1 are difficult to distinguish without genetic analysis. As described earlier, the *SH2D1A* gene is mutated in XLP-1 patients (Table 6.1). The absence of SAP induces an exaggerated Th1 response to EBV infection leading to enhanced TNF-α and IFN-γ cytokine secretion, hypercytokinemia and macrophage activation (Veillette, 2010). However, EBV-HLH cases do not have such mutations. Instead, LMP1 inhibits the expression of SAP at the transcriptional level, resulting in enhanced TNF-α and IFN-γ cytokine secretion, akin to that observed in XLP-1 (Chuang *et al*., 2005).

Hydroa vacciniforme and Hydroa vacciniforme-like lymphoma

Hydroa vacciniforme is a rare chronic paediatric disorder characterised by photodermatosis of unknown etiology, characterised by sensivity to sunlight resulting in edema, vesicles, crusts and large ulcers which cause severe hypopigmented scarring and disfigurement of the skin. The disease usually occurrs in children aged 3-15 years from East Asia, Central and South America and Mexico and remits spontaneously by late adolescence. EBV is nearly always found in the infiltrating T cells or NK cells of the cutaneous lesions and in the blood, with high viral loads in patients with severe disease (Iwatsuki *et al*., 1999; Cho *et al*., 2001; Doeden *et al*., 2008). Classic HV is not however associated with haematologic abnormalities, chromosomal aberrations or mortality, but has been classified within the spectrum of CAEBV.

Hydroa vacciniforme-like lymphoma (HVLL) is defined by the World Health Organisation as an EBV-positive cutaneous T cell lymphoma that occurs mainly in children and adolescents with the same geographical distribution as HV. Here EBV is found predominantly in the malignant CD8 T cells, although infection of NK cells has been reported (Iwatsuki *et al*., 1999; Cho *et al*., 2001; Barrionuevo *et al*., 2002; Doeden *et al*., 2008). Cases of CD4 T cell infection are rare (Wu *et al*., 2007; Lee *et al*., 2012). Patients with HVLL usually have systemic symptoms including lymphadenopathy and hepatosplenomegaly and exhibit a slowly progressive relapsing course. HVLL is considered by some to derive from pre-malignant non-resolving HV over 2 to 10 years, although this point is contentious. The contribution of EBV to HV and the subsequent transformation to HVLL is completely unknown.

Extranodal NK/T-cell lymphoma, nasal type

Extranodal NK/T-cell lymphoma (ENKTL) is a rare but highly aggressive type of non-Hodgkin lymphoma associated with EBV. The lymphoma is marked by extensive necrosis and angioinvasion and usually presents in extranodal sites, predominantly within the upper aerodigestive tract (nasal cavity, nasopharynx, paranasal sinus and palate). Patients can also present with tumours in the skin, soft tissue, respiratory tract, gastrointestinal tract and testis and these are the primary sites of dissemination.

Like the EBV-associated T and NK cell infections described above, ENKTL shows a strong ethnic and geographic predilection. ENKTL occurs with the highest incidence in East Asian, Central and South American, and Mexican populations, accounting for 7% to 10% of non-Hodgkin lymphoma (20% - 30% of peripheral T cell lymphomas). In contrast, ENKTL only accounts for 1% of North American or European cases (Au, 2010). ENKTL usually occurs in adults (median age of presentation 40 to 50 years) with a stronger male predominance. However, it is important to note that the clinicopathological features are the same irrespective of ethnic origin.

ENKTL follows an aggressive clinical course with a poor prognosis. The efficacy of conventional anthracycline-based chemotherapy regimens is poor either with or without radiotherapy, with an overall 5 year survival of 6-25% for patients with advanced disease (Au *et al*., 2005; Lee *et al*., 2006; Pagano *et al*., 2006; Lim *et al*., 2008; Suzuki *et al*., 2010; Chauchet *et al*., 2012). It is thought the poor response to conventional anthracycline-based regimens may be, at least in part, associated with expression of the Multi-Drug Resistance gene (MDR1) encoding P-glycoprotein (Drenou *et al*., 1997).

Most cases of ENKTL are of NK lineage, as evidenced by positivity for CD56, CD2 and CD3ε, and lack of expression of CD4, CD8 and CD3s and a germline TCR. The remaining cases are of a cytotoxic T cell lineage with clonal TCR rarrangements (Pongpruttipan *et al*., 2012).

EBV is present in every malignant cell of ENKTL as clonal episomes that display a latency II gene expression pattern (including LMP2-TR).

Chromosome abnormalities are common; deletion of 6q21 is the most frequently observed alteration (Nakashima *et al*., 2005) and there is loss of expression of tumour suppressor genes including *PRDM1, ATG5, AIM1, FOXO3* and *HACE1* (Jiang *et al*., 2015). Mutations in TP53, MGA, STAT3, MSN, P21, K-ras, c-kit and β-catenin are also frequently observed (Quintanilla-Martinez *et al*., 2001). Gene expression profiling has highlighted the activation of several oncogenic pathways in NK/T cell tumours, including NF-κB, MAPK and JAK-STAT (Jiang *et al*., 2015). Activation of these pathways by LMP1 have been described in considerable detail in nasopharyngeal carcinoma and B cell lymphomas where LMP1 has been shown to drive the canonical and non-canonical NF-κB pathways (Eliopoulos *et al*., 1997; Blake *et al*., 2001; Baxendale *et al*., 2005), the three classic mitogen activated protein kinases (ERK-MAPK, p38 MAPK, JNK/SAPK) (Morris *et al*., 2009) and the JAK/STAT pathway (Roberts and Cooper, 1998; Dawson *et al*., 2008; Chen *et al*., 2010; Morris *et al*., 2016).

NK/T cell lymphomas also harbour alterations in genes encoding epigenetic modifiers. For example, a recent study of 105 ENKTL patients identified mutations in MLL2, ARID1A, EP300 and ASXL3 (Jiang *et al*., 2015). This is of interest because data is emerging which links EBV gene expression with the widespread epigenetic modification of the host cell genome in both EBV-associated lymphomas and carcinomas (Kaneda *et al*., 2012; Leonard *et al*., 2012; Kreck *et al*., 2013; Zhang *et al*., 2014; Niller *et al*., 2016; Peng *et al*., 2016; Ramayanti *et al*., 2016). The most complete study to date was performed in EBV-associated gastric carcinoma where EBV infection is associated with an extensively hypermethylated cellular epigenome (Cancer Genome Atlas Research, 2014). Several studies in both B cell and epithelial cell malignancies have identified the regulation of the cellular DNA methyltransferases DNMT1, DNMT3A and DNMT3B by EBV infection and by LMP1 or LMP2A (Tsai *et al*., 2006; Hino *et al*., 2009). Even transient EBV infection of epithelial cells leaves permanent epigenetic marks (Queen *et al*., 2013; Birdwell *et al*., 2014). Expression of LMP1 and LMP2A in NK/T cell lymphomas suggests that a hypermethylated epigenome might also be a feature of these tumours.

Conclusions

EBV was first identified as a potent transformer of B cells and to be causally associated with the development of Burkitt lymphoma. Since then EBV has been shown to be associated with a diverse range of different cancer types, including other types of B cell lymphoma and perhaps surprisingly a variety of different lymphoproliferations and malignanices of T and NK cells. Emerging evidence implicates chronic immune stimulation as an important co-factor in the development of many of these lymphomas. Unravelling the complex interplay between these factors and EBV infection and how this contributes to transformation will not only reveal new insights into the pathogenesis of these tumours, but is also likely to lead to the development of novel therapies.

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