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Review:

Epstein-Barr virus interactions with the Bcl-2 protein family and apoptosis in human tumor cells*

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Abstract: Epstein-Barr virus (EBV), a human gammaherpesvirus carried by more than 90% of the world's population, is associated with malignant tumors such as Burkitt's lymphoma (BL), Hodgkin lymphoma, post-transplant lymphoma, extra-nodal natural killer/T cell lymphoma, and nasopharyngeal and gastric carcinomas in immune-compromised patients. In the process of infection, EBV faces challenges: the host cell environment is harsh, and the survival and apoptosis of host cells are precisely regulated. Only when host cells receive sufficient survival signals may they immortalize. To establish efficiently a lytic or long-term latent infection, EBV must escape the host cell immunologic mechanism and resist host cell apoptosis by interfering with multiple signaling pathways. This review details the apoptotic pathway disrupted by EBV in EBV-infected cells and describes the interactions of EBV gene products with host cellular factors as well as the function of these factors, which decide the fate of the host cell. The relationships between other EBV-encoded genes and proteins of the B-cell leukemia/lymphoma (*Bcl*) family are unknown. Still, EBV seems to contribute to establishing its own latency and the formation of tumors by modifying events that impact cell survival and proliferation as well as the immune response of the infected host. We discuss potential therapeutic drugs to provide a foundation for further studies of tumor pathogenesis aimed at exploiting novel therapeutic strategies for EBV-associated diseases.

Key words: Epstein-Barr virus, *Bcl* family members, Apoptosis, Drugs therapy
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1 Introduction

B-cell leukemia/lymphoma 2 (*Bcl-2*), an anti-apoptotic member of the *Bcl* family, was initially identified as a proto-oncogene at the breakpoint of the (14, 18) chromosomal translocation detected in human B-cell follicular lymphoma (Bakhshi *et al*., 1985). To date, 19 members of the *Bcl* family have been characterized. Based on their structural features and roles in regulating apoptosis, the *Bcl* family can be divided into three subgroups. (1) 'Multi-domain' anti-apoptotic members, containing multiple Bcl-2 homology (BH) domains (BH1–4) and promoting cell survival. This subgroup includes Bcl-2-like long, Bcl-xL, Bcl-2, Bcl-2-like 2, Bcl-w, myeloid cell leukemia sequence 1 (MCL-1), and Bcl-2 fetal liver (bfl-1). (2) 'Multi-domain' pro-apoptotic members, lacking only the BH4 domain characteristic of proteins that promote survival and induce cell death. This subgroup includes Bcl-2-associated X protein (Bax), Bcl-2-antagonist/killer (BAK), Bcl-2-associated agonist of cell death (Bad), and Bcl-2-related ovarian killer (Mtd/Bok). (3) 'BH3 domain-only' members (Puthalakath and Strasser, 2002), sensors of distinctive cellular stresses (Huang and Strasser, 2000; Puthalakath and Strasser, 2002) that share sequence homology only in the BH3 domain. This subgroup includes the death-promoting Bcl-2 homolog (BIK), Bcl-2-interacting protein (Bid), *p53*

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up-regulated modulator of apoptosis (PUMA/Bbc3), and the Bcl-2-interacting mediator of cell death (BIM).

Most studies have indicated that proteins of this gene family act mainly by forming among themselves a complex network of promiscuous homo- and heterodimers (Hsu *et al*., 1997). The anti-apoptotic proteins of the *Bcl-2* family induce oncogenesis by protecting cells from various apoptotic stimuli and triggers, such as DNA-damaging irradiation (Cory *et al*., 2003) and chemotherapeutic drugs, not by facilitating proliferation. However, evidence has demonstrated that over-expression of anti-apoptotic proteins (Bcl-w, Bcl-xL, and Bcl-2) interferes with the cell cycle by delaying the progression to S phase and inhibiting initiation of the cell cycle (Jamil *et al*., 2005; Zinkel *et al*., 2006). Pro-apoptotic members, such as Bax and BAK, were believed to undergo conformational changes and insert into the outer mitochondrial membrane as homo-dimerized multimers when death signals were received. This causes the release of apoptotic molecules from the inter-membrane space, such as the second mitochondria-derived activator of caspase (also known as SMAC), which leads to activation of caspase-9 (Riedl and Shi, 2004), and an apoptosis-inducing factor, which, in turn, leads to cell death. However, this pro-apoptotic activity is blocked via anti-apoptotic *Bcl-2* family members interacting with Bax and BAK. BH3-only proteins are transcriptionally or post-translationally activated by extracellular pro-apoptotic signals and intracellular damage. These proteins can then use their BH3 domains as ligands to activate Bax and BAK directly or to suppress the anti-apoptotic proteins of the *Bcl-2* family, such as Bcl-2 and Bcl-xL (Wei *et al*., 2001; Zong *et al*., 2001), to regulate apoptosis. Usually, the ratio of anti-apoptotic to pro-apoptotic proteins of the *Bcl-2* family determines whether a cell lives or dies (Cory *et al*., 2003; Danial and Korsmeyer, 2004).

Epstein-Barr virus (EBV), with a double-stranded DNA genome, was first discovered under electron microscopy in cultured Burkitt's lymphoma (BL) cells. EBV establishes a lifelong infection in more than 90% of the population, which makes it the most successful human virus. Interestingly, infection in childhood is usually asymptomatic, but in adolescence the result is often infectious mononucleosis. Furthermore, EBV participates in germinal center reaction (Spender and Inman, 2011) and resides in the latent phase. When infected B-lymphocytes receive a stimulus to divide into plasma cells and the EBV immediately-early genes *BRLF1* and *BZLF1* are expressed, the viral lytic reaction takes place (Thorley-Lawson and Gross, 2004; da Silva and de Oliveira, 2011). Much time has been devoted to clarifying the impact of EBV on the development of tumors and it has been found that EBV is associated with a great many malignancies, including post-transplant lymphoma disorders, BL, extra-nodal natural killer/T cell lymphoma, and nasopharyngeal and gastric carcinomas in immune-compromised patients.

In the two phases (latent and lytic) of the EBV life cycle (Rickinson and Kieff, 2007), EBV-infected cells express ~100 genes including, in the latent phase, EBV nuclear antigen 1 (*EBNA1*), *EBNA2*, *EBNA3A*, *EBNA3B*, *EBNA3C*, the latent membrane protein 1 (*LMP-1*), *LMP-2A*, *LMP-2B*, EBV-encoded small RNAs-1 (*EBER-1*), and *EBER-2*, and in the lytic phase, *BZLF1*, the EBV Bam HI fragment H rightward open reading frame 1 (*BHRF1*), and the EBV Bam H1 A fragment leftward reading frame 1 (*BALF1*) (Kalla and Hammerschmidt, 2012). Most of their functions have been characterized. For example, *EBNA1* is necessary for the transactivation of the C promoter (Cp) viral promoter (Altmann *et al*., 2006) as well as the latency and replication of the virus. In some microenvironments, *EBNA1* is thought to suppress cell death (Kennedy *et al*., 2003). *BARF1* and *BZLF1*, encoding viral transcription factors, switch the infection from latent to lytic phase (Feederle *et al*., 2000; Sinclair, 2003). EBV-expressed microRNAs (miRNAs) play a crucial role in development, the cell cycle, and immunity and contribute to cancer-associated pathology (Lee and Dutta, 2009; Forte and Luftig, 2011). However, the molecular interactions between EBV and host cells, especially between EBV and *Bcl-2* family proteins, are still poorly understood. The results of some studies suggest that elucidation of the relation between EBV and the *Bcl-2* family is important in understanding the pathogenesis of EBV-associated diseases. This review summarizes current knowledge on the interactions between EBV-encoded products and *Bcl-2* family members in the lytic and latent phases, and the details of the apoptotic pathway in EBV-infected cells, which contribute into the establishment of EBV latency and

carcinogenicity, as well as the effects of current therapeutic drugs.

2 Impact of EBV gene products on *Bcl*-family members during lytic infection

2.1 *BZLF1* down-regulates the expression of *Bcl-2* and *Bcl-xL*

The EBV immediate-early gene *BZLF1* encodes the transcription factor Zta (also called Zebra, Z, and EB1) and is related to the cellular activating protein-1 (AP-1). In latently infected B cells, its protein is a core regulator of the switch from the latent to the lytic phase (Countryman and Miller, 1985). After primary infection, *BZLF1* is expressed very early in B cells. However, its early expression does not immediately initiate the EBV lytic cycle but promotes the proliferation of resting memory and naive B cells (Kalla *et al*., 2010). It is also very important for maintaining cell survival during the lytic phase. Unexpectedly, *BZLF1* down-regulates the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL by down-regulating the major histocompatibility complex (MHC) class II-associated invariant chain (CD74) in $CD4^+$ T cells (Zuo *et al*., 2011). This result was confirmed in Akata-A3 cells (Zuo *et al*., 2011). In addition, *BZLF1*-mediated down-regulation of CD74 involves repression of activation of the 65-kDa (p65) member of the nuclear factor-kappa B (NF-κB) family, which can transactivate *Bcl-2* family genes (Lantner *et al*., 2007). NF-κB family members are crucial for inducing expression of a number of genes involved in immunity (Baldwin, 2001). Thus, the capacity of *BZLF1* to decrease the expressions of *Bcl-2* and *Bcl-xL* is essential for EBV to escape the host response during the lytic phase.

2.2 *BHRF1*

BHRF1, as a latent (Hayes *et al*., 1999; Kelly *et al*., 2009) and lytic gene encoding a 17-kDa component of the restricted early antigen complex (ER-A), is highly conserved in all EBV isolates. It contains three conserved BH domains, BH1–3, which are characteristic of the *Bcl-2* family. *BHRF1* and *Bcl-2* have a similar cellular distribution, both primarily localized in the mitochondrial (Hickish *et al*., 1994), endoplasmic reticulum, and nuclear membranes. Earlier studies showed that *BHRF1* is not responsible for the transformation of B lymphocytes induced by EBV and delays apoptosis during viral replication in vitro. However, lytic *BHRF1* transcripts and latent *BHRF1* transcripts have been found in EBV-positive B-cell lymphoma, T-cell lymphoma, and EBV-transformed tightly latent B-cell lines in vitro (Xu *et al*., 2001; Yu *et al*., 2001; Howell *et al*., 2005). These findings demonstrated that *BHRF1* protects various cell types from apoptosis induced by a wide range of external stimuli, tumor necrosis factor-α, activated monocytes, radiation, or anti-tumor necrosis factor receptor (TNFR) superfamily member 6 antibody. So BHRF1 is a viral homologue of the human cellular Bcl-2 protein in both structure and function. However, the anti-apoptotic activity of *BHRF1* is not exactly equivalent to that of *Bcl-2*. Studies of the three-dimensional (3D) structures showed that prenylated rab acceptor 1 (PRA1) expression regulates the anti-apoptotic activity of *BHRF1*, but not that of *Bcl-2* (Li *et al*., 2001). Since the activities of *BHRF1* and *Bcl-2* are modulated by specific mechanisms, such as diverse binding with distinctly different functions, *BHRF1* probably exerts its pro-survival function in a manner similar to that previously found for human anti-apoptosis *Bcl-2* members.

2.2.1 *BHRF1* and *Bok*

A pro-apoptotic member of the *Bcl-2* family, *Bok,* was first cloned from the ovarian complementary DNA (cDNA) library of a rat. Studies showed high expression of *Bok* messenger RNA (mRNA) in specific tissues, such as the testis, ovary, and uterus. The intracellular localization of Bok protein is either nuclear (involved in inducing apoptosis) (Bartholomeusz *et al*., 2006), cytosolic, or mitochondrial. Bok interacts only with EBV BHRF1, MCL-1, and bfl-1, but not other pro-apoptotic family members or anti-apoptotic Bcl-2 proteins, unlike other pro-apoptotic members (BIK, Bax, and BAK) (Hsu *et al*., 1997). Moreover, using a direct protein-protein interaction assay in vitro, Hsu *et al*. (1998) demonstrated that Bok-L alone, without Bok-S, interacts strongly with BHRF1. In a variety of cell types, *Bok* induces cell killing, but this is inhibited following co-expression with *BHRF1* and *MCL-1*, but not with *Bcl-2* or *Bcl-xL* (McCurrach *et al*., 1997; Rampino *et al*., 1997; Yin *et al*., 1997). These findings suggest

that *Bok* plays a unique role in apoptosis, and further indicate that *Bok*-expression may be a target for the EBV-encoding anti-apoptotic protein BHRF1 (Marchini *et al*., 1991).

Previous studies have shown that anti-apoptotic proteins are likely to bind to the cell death abnormality/apoptotic peptidase activating factor 1 (ced4/Apaf-1) homolog which activates downstream caspase (Wu *et al*., 1997; Zou *et al*., 1997). The hetero-dimerization partners of *Bok*, such as *BHRF1* and *MCL-1*, may involve in the common intrinsic pathway of apoptosis (Yakovlev *et al*., 2004). So it is likely that *Bok* exerts its pro-apoptotic function by a mechanism involving the formation of dimers with anti-apoptotic partners.

2.2.2 *BHRF1* directly counters BAK

BAK is an oligomeric mitochondrial membrane protein and has a redundant but essential function when the mitochondrial release of apoptogenic factors initiates apoptosis. In a lymphocyte cell line, EBV BHRF1 binds BAK and the cell dies when it receives signals from the messenger *BIM* (Desbien *et al*., 2009). Other studies showed that BHRF1 binds to full-length BAK (Theodorakis *et al*., 1996; Cross *et al*., 2008). In fact, BHRF1 changes its structure to accommodate the BAK domain and therefore keeps the BAK inactive (Kvansakul *et al*., 2010). However, in cytokine-deprived cells BHRF1 appears to inhibit apoptosis by binding BIM, but not BAK (see below). In addition, although BHRF1 does not bind with Bax directly and Bax can replace BAK in cell death, it has been shown to repress the activation of Bax and BAK to preserve mitochondrial function (Kvansakul *et al*., 2010). So why BHRF1 binds an apparently irrelevant protein is a conundrum.

2.2.3 *BHRF1* and *BOD*

The *Bcl-2*-related ovarian death gene (*BOD*) was first identified as an ovarian *Bcl-2*-related, *Bcl-2* homology (BH3) domain-only protein in an ovarian fusion cDNA library (Hsu *et al*., 1998). It has three variants (long, medium, and short), all of which contain a consensus BH3 domain without the other BH domains detected in channel-forming *Bcl-2* family members. In a yeast cell assay, Hsu *et al*. (1998) found that the C-terminal BH3 domain-containing region of *BOD* interacted strongly with EBV *BHRF1* and all known mammalian anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, Bcl-w/bcl-2-2, bfl-1, and MCL-1), but not with pro-apoptotic *Bcl-2* family proteins (Bok, Bax, Bad, and BAK). While studies on *BOD* are scarce, northern and southern blot analyses revealed that, unlike *Bok*, *BOD* is expressed in various tissues (mainly the spleen and kidney) and has been highly conserved during the evolution of mammals (Hsu *et al*., 1998). This suggests that, like BAD, it may function as an adaptor protein for upstream signals and induce cell-killing by hydro-isomerization with diverse anti-apoptotic Bcl-2 proteins in a variety of cell lines, especially in leukocytes. The exact role of *BOD* in the intracellular mechanism underlying apoptosis needs further study.

2.2.4 *BHRF1* interacts with BIK, Bcl-2, and Bcl-xL

BIK, a potent pro-apoptotic protein, shares only the BH3 domain and the C-terminal trans-membrane domains with other members of the *Bcl-2* family, such as Bax, Bcl-2, and Bcl-xL. Most data show that BIK promotes cell death and heterodimerizes with survival-promoting proteins. Evidence is accumulating that BIK interacts with the viral anti-apoptotic protein EBV BHRF1, and various cellular anti-apoptotic proteins, such as Bcl-2 and Bcl-xL (Boyd *et al*., 1995), and that this activity of *BIK* is inhibited following co-expression of EBV-*BHRF1*, *Bcl-2*, and *Bcl-xL* (Boyd *et al*., 1995). Further studies revealed that an 18-amino-acid region in the BH3 domain constitutes the critical heterodimerization domain. EBV BHRF1 is a viral homolog of the human cellular Bcl-2 protein both in structure and function (as noted above), indicating that BHRF1 might interact indirectly with Bcl-2 via BIK and prevent apoptosis during EBV replication, prolong the lifespan of EBV-infected cells, and potentiate viral persistence and spread.

2.2.5 *BHRF1* binds to *BIM*

BIM exists in three major isoforms (BIMEL, BIML, and BIMS) and is expressed in a variety of cell types, especially lymphocytes. Most reports show that *BIM* is a key regulator of life and death decisions (Anderton *et al*., 2008; Paschos *et al*., 2009). Thus, it is not surprising that EBV BHRF1 binds to the BH3-only peptide BIM and interacts strongly with its protein (Flanagan and Letai, 2008). Further study has

shown that BHRF1 blocks apoptosis by binding to a crucial, lethal fraction of the BIM, but not the general BIM pool (Desbien *et al*., 2009). Moreover, the structure of BHRF1 in complex with BIM confirms that BHRF1 can counteract BIM directly (Kvansakul *et al*., 2010). These findings may facilitate the exploitation of small-molecule inhibitors of BHRF1 to improve the poor prognosis in EBV-associated diseases, since BHRF1 confers strong chemoresistance and current small organic inhibitors of Bcl-2 do not target BHRF1.

2.2.6 *BHRF1* inhibits BH3-only proteins PUMA and Bid

PUMA (Nakano and Vousden, 2001) and Bid are both BH3-only proteins and are required for the induction of apoptosis. Accumulating evidence shows that BHRF1 is associated with PUMA and Bid, since it binds to them and interacts strongly with Bid protein (Flanagan and Letai, 2008; Kvansakul *et al*., 2010). EBV BHRF1 promotes cell survival by directly repressing pro-apoptotic *Bcl-2* family proteins including PUMA and Bid (Kvansakul *et al*., 2010). The structure of BHRF1 in complex with PUMA or Bid is still unclear. However, it is possible that BHRF1 changes its structure to accommodate the PUMA or Bid domain, because Bid is considered to be an activator, like BIM (Letai *et al*., 2002).

2.3 BALF1 associates with Bax and BAK

BALF1 protein is encoded by the smaller open reading frame of the EBV genome and has sequence homology with the *Bcl-2* family, especially in the functionally important BH1 and BH2 domains. Interestingly, compared to BHRF1, there is more similarity among BALF1, Bcl-2, and Bcl-xL. Bax and BAK, as *Bcl-2* family members, were found to associate with BALF1 in Henrietta Lacks (HeLa) cells. This association provides a possible mechanism for the anti-apoptotic function of BALF1. A recombinant green fluorescent protein-BALF1 fusion protein experiment also demonstrated the anti-apoptotic function of BALF1 in anti-Fas-treated HeLa cells. Similar results were found with anti-Fas plus interferon-γ (IFN-γ) when both cycloheximide and TNF-α or chloromethyl X-rosamine (CMXRos) staining were used to induce apoptosis (Marshall *et al*., 1999). However, a report showed that EBV BALF1 fails to protect cells from Bax-induced apoptosis in the DG75 B cell (EBV-negative BL lymphoma) (Bellows *et al*., 2002), so BALF1 lacks an anti-apoptotic function. Taken together, although the function of BALF1 is not yet clear, it remains possible that BALF1 interrupts the apoptotic pathway in EBV-infected cells by interacting with pro-apoptotic proteins, such as Bax and BAK.

2.4 *BARF1* activates *Bcl-2*

The EBV Bam HI-A rightward frame 1 (*BARF1*) gene is translated to form an early protein that is homologous to the intercellular adhesion molecule 1 (ICAM-1) and the human cloning stimulating factor I receptor (HCSF-IR). Previous studies suggested that BARF1 activity includes both an immunomodulatory function and oncogenicity. BARF1 protein modulation of the host immune response to the virus is achieved by the protein acting as a receptor of human colony-stimulating factor (Hcsf-1) (Strockbine *et al*., 1998) and as an inhibitor of α-interferon secretion from mononuclear cells (Cohen and Lekstrom, 1999). The oncogenic activity of the *BARF1* gene, specifically located in its N-terminal domain, can induce malignant transformation in the EBV-negative Louckes B cell line and aggressive tumors in new-born rats and rodent cells (Sheng *et al*., 2001). In addition, it can immortalize primary monkey kidney epithelial cells in vitro. To explain the mechanism of malignant transformation induced by *BARF1*, some reports suggested that the N-terminal domain of the *BARF1* gene is able to activate anti-apoptotic *Bcl-2* expression in rodent fibroblasts with deletion mutations (Sheng *et al*., 2001). A similar activation of *Bcl-2* expression was shown in *BARF1*-transfected, EBV-negative Akata cells (Sheng *et al*., 2003). These findings support the hypothesis that *BARF1* activates *Bcl-2* expression to resist apoptosis and that the cooperation of *Bcl-2* with *BARF1* is indispensable for inducing malignant transformation. However, the exact mechanism of this cooperation is not very clear. This study also suggested that the apoptosis induced by serum deprivation in Balb/c3T3 cells is mediated by myelocytomatosis oncogene (*c-MYC*) and is then blocked by the up-regulated expression of *Bcl-2* induced by *BARF1* (Sheng *et al*., 2001); but in the same cells, *LMP-1* does not activate Bcl-2 protein expression. If the association between *BARF1* and *Bcl-2* is more extensive than that between *BARF1* and *LMP-1*,

it may become a common target of treatment, clinical staging, and prognosis in certain tumors.

3 Impact of EBV gene products on *Bcl*-family members during latency

3.1 EBNA2 interacts with various *Bcl* family genes

EBV nuclear antigen 2 (EBNA2) is the earliest latent-cycle protein of EBV and is essential for B-cell immortalization proliferation and survival as well as chemotaxis. Pegman *et al*. (2006) demonstrated that in EBV-negative BL-derived cell lines EBNA2 up-regulates *bfl-1* expression by interacting with EBNA2-CBF-1 (Cp-binding factor 1, also known as RPR-JK) (Zimber-Strobl and Strobl, 2001; Hayward, 2004). These interactions involve receptors of the classical Notch pathway. EBNA2 also up-regulates most other anti-apoptotic proteins such as bfl-1, Bcl-xL, Bcl-2, and MCL-1 and induces expression of the pro-apoptotic proteins BIM and Bid in EBNA2-expressing cells (Kohlhof *et al*., 2009), which is differential in Notch1 or Notch2 IC-expressing cells. Further studies are necessary to confirm whether the Notch receptor is involved in the mechanism of EBNA2-up-regulated *Bcl* family gene expression. Such studies may provide a better understanding of the role of the EBNA2 in EBV-associated diseases.

3.2 EBNA3A and EBNA3C repress *BIM* expression

EBV nuclear antigens 3A, 3B, and 3C (EBNA3A, EBNA3B, and EBNA3C) are three of only six viral proteins encoded by latent EBV. Despite having the same gene structure (Bornkamm and Hammerschmidt, 2001), they have divergent functions. Genetic studies have revealed that both EBNA3A and EBNA3C (but not EBNA3B) are responsible for efficient immortalization in EBV-infected B cells (Tomkinson *et al*., 1993). Using recombinant EBVs established with a bacterial artificial chromosome (BAC) system, it was revealed that EBNA3A and EBNA3C cooperate to inhibit the activation of the tumor-suppressor gene *BIM*. The inhibition appears to be predominantly directed at the regulation of *BIM* mRNA levels (Anderton *et al*., 2008). This process may involve an epigenetic mechanism that can be initiated or maintained by interactions between EBNA3A and EBNA3C and suppressive marks on local chromatin. Covalent modifications to the N-terminal domains of histones can repress or silence a gene (Jaenisch and Bird, 2003; Suzuki and Bird, 2008). Clybouw *et al*. (2005) showed that EBV infection leads to the down-regulation of BIM protein expression, depending mainly on *BIMEL* expression. Most data indicate that EBNA3A and EBNA3C regulate the expression of *BIM* at the level of transcription (Anderton *et al*., 2008). Strong evidence was provided by the fact that EBNA3A and EBNA3C together inhibit the initiation of *BIM* transcripts (Paschos *et al*., 2012). What is more, EBNA3C is directly targeted to the *BIM* promoter (Paschos *et al*., 2012). Previous research has shown that in the 5′ regulator region of *BIM*, heritable epigenetic modifications initiated by EBNA3A and EBNA3C play a major role in determining the level of post-transcriptional *BIM* production expressed in EBV-infected B cells (Paschos *et al*., 2009).

3.3 *LMP-1*

LMP-1, an oncogene of EBV, encodes a 386-amino-acid integral membrane protein. Transfecting the *LMP-1* gene into human B cells causes many phenotypic changes characteristic of stimulated lymphocytes, including mediation of DNA synthesis, up-regulation of various cell surface stimulation markers, and increased cell size and production of adhesion molecules (Rowe *et al*., 1994). These changes are among many of the transformation-associated properties of EBV reproduced by *LMP-1* in various cell lines.

3.3.1 *LMP-1* specifically up-regulates *Bcl-2* expression

The association between *LMP-1* and *Bcl-2* has been a focus of research. Studies have suggested that the immortalization effect of *LMP-1* on B-lymphomas is mediated by the *Bcl-2* gene in many lymphoid malignancies, and that the mediation may be through cooperation between *Bcl-2* and *MCL-1*. Lu *et al*. (1997) suggested that *LMP-1*-induced apoptosis is specifically blocked by the abnormal expression of *Bcl-2* or co-expression of *LMP-1* and *Bcl-2* in epithelial cells (RHEK-1 cells). Down-regulation of *Bcl-2* expression is also directly induced by *LMP-1* when using antisense oligo-deoxynucleotides to

suppress *LMP-1* expression in an EBV-transformed B-cell line (Noguchi *et al*., 2001). Moreover, in BL cell lines in vitro, the latent viral gene *LMP-1* induces the expression of *Bcl-2* (Finke *et al*., 1992). A positive association between *LMP-1* and *Bcl-2* has been obtained in acquired immune deficiency syndrome (AIDS)-related primary brain lymphomas in vivo and in nasopharyngeal carcinoma (Carmilleri-Broet *et al*., 1995). Interestingly, reports have shown statistically significant co-expression of *LMP-1* and *Bcl-2* in pediatric cases although this association was not definite in classical Hodgkin's lymphoma cases. These results suggest that both proteins may play important complementary roles in the process of EBV-associated transformation. Up-regulation of *Bcl-2* expression induced by EBV *LMP-1* has been suggested as a B-cell-specific response (Rowe *et al*., 1994). In support of this argument, several reports have demonstrated that Bcl-2 protein expression cannot be activated by LMP-1 in human epithelial cell lines (Rowe *et al*., 1994) or in fibroblasts (Henderson *et al*., 1993). The finding supported that nasopharyngeal carcinoma (NPC) *Bcl-2* expression can be independent of *LMP-1* (Sarac *et al*., 2001), and that EBV up-regulation of apoptosis has no relation to *Bcl-2* expression. In EBV-associated non-Hodgkin lymphomas, the T cell type is predominant (Takano *et al*., 1997), which corresponds to the finding (Kim *et al*., 2004) that EBV does not induce the up-regulation of *Bcl-2* expression in classical Hodgkin's lymphoma. In addition, in EBV-positive natural killer cell lymphoma, *Bcl-2* expression is not directly mediated by *LMP-1* (Noguchi *et al*., 2001). The pathological mechanism of this specific response remains unclear but there are several possibilities. Firstly, some authors hypothesized that *LMP-1* up-regulates adhesion molecules, such as cell adhesion molecule 1 (CAM-1), by activating NF-κB, leading to the up-regulation of *Bcl-2* expression in B-cell lines (Rowe *et al*., 1994). However, the intracellular carboxyl-terminal cytoplasmic region of LMP-1 has been shown to interact with signaling molecules involved in the TNFR family-activated pathway, including the transmembrane protein CD40. This then activates the NF-κB/C-jun N-terminal kinase/AP-1 pathway and finally regulates *Bcl-2* expression (Noguchi *et al*., 2001). Secondly, the immune response of the brain can allow *EBNA2* and *LMP-1* over-expression in infected cells of AIDS-related primary brain lymphomas, but not in those of AIDS-related systemic lymphomas. Since the brain is immunologically privileged and does not elicit cytotoxic rejection, it allows *LMP-1* to transactivate *Bcl-2* (Carmilleri-Broet *et al*., 1995). Thirdly, alternatively, the suppression of *LMP-1*-induced apoptosis by *Bcl-2* or co-expression of *LMP-1* may be a coincidence. The pathogenesis might involve one aspect of EBV infection resulting in up-regulation of LMP-1 levels in epithelial target cells, and another aspect may be activation of *Bcl-2* expression by an EBV-independent mechanism. Therefore, it is possible that in epithelial cells and fibroblasts, EBV proteins other than LMP-1 may cause immortalization.

3.3.2 *LMP-1* mediates *MCL-1* expression

MCL-1 was first identified as a novel EBV gene active in early cell differentiation induced in a human myeloid leukemia cell line (Kozopas *et al*., 1993). *MCL-1* protects Chinese hamster ovary cells from apoptosis caused by *c-MYC* over-expression and heterodimerizes with Bax. Moreover, because in EBV-infected B cells the regulation of *MCL-1* by *LMP-1* occurs at an early stage, prior to up-regulation of *Bcl-2* and decreased MCL-1 levels, it was suggested that *MCL-1* functions as a rapid, crucial immediate-early response effector of cell survival (Wang *et al*., 1996). Kim *et al*. (2012) also showed that up-regulation of *MCL-1* by *LMP-1* contributes to survival in rituximab-treated B-cell lymphoma cells. Interestingly, the down-regulation of *MCL-1* expression is also blocked by *LMP-1* in response to apoptotic stimulation. This is supported by the finding that *LMP-1* promotes survival in the EBV-negative BL cell line BL41 (Wang *et al*., 1996). Therefore, the expression of *MCL-1* mediated by *LMP-1* is likely to play an important role in the immediate-early response in EBV infection. However, the significance of decreasing *MCL-1* by *LMP-1* (long-term expression) is still unclear and needs further study.

3.3.3 *LMP-1* drives the anti-apoptotic *bfl-1* gene

B-lymphocyte decision depends on activation of the NF-κB signaling pathway and the CD40 receptor and involves the participation of tumor necrosis factor receptor (TNFR)-associated factor 2. It is mediated via the carboxyl terminal activating region 2 (CTAR2) associated with the CTAR1 region of *LMP-1*. This

point was strongly supported by the finding that the expression of *bfl-1* represses apoptosis activated by the amino-terminal six-transmembrane domain (6TM) of *LMP-1* (Pratt *et al*., 2012). In this process, like the B-cell receptor, the 6TM of *LMP-1* activates an unfolded protein response (UPR), and then induces apoptosis, but the carboxy-terminal domain of LMP-1 activates the transcription of *bfl-1* to repress apoptosis by the UPR. These findings indicate that *bfl-1* contributes to the long-term survival of EBV-infected B cells in a way similar to the combined roles of CD40 and the B-cell receptor.

3.4 LMP2A increases the expression of *Bcl-xL* and *Bcl-2*

EBV latent membrane protein 2A (LMP2A) was identified in germinal center B cells (Babcock *et al*., 2000), but its transcripts are consistently found in all forms of EBV latency, including resting memory B cells, infectious mononucleosis, Hodgkin lymphoma, BL, and post-transplant lymphoproliferative disorder (Thorley-Lawson and Gross, 2004; Rickinson and Kieff, 2007). Thus, LMP2A is important in EBV-associated diseases. Many studies have demonstrated that it has a critical function to rescue cells from apoptosis by potentially altering the balance of pro-apoptotic and pro-survival *Bcl* family members, particularly by mediating the expression of *Bcl-xL* and *Bcl-2*. For example, using the model of *LMP2A* transgenic E mice and H-ras17 mice, it was demonstrated that LMP2A activates Ha-Ras, and in turn preferentially activates the phosphatidyl-inositol 3-kinase (PI3K)/Akt pathway, normally imparting an essential survival signal in response to B-cell receptor signaling. Importantly, both the PI3K/Akt and the Raf/ERK kinase (MEK)/extracellular-signal-related kinase (ERK) pathways, downstream of the Ras family, raise NF-κB, which is a critical mediator of Bcl-xL (Steelman *et al*., 2004). Therefore, LMP2A probably maintains cell survival by modulating *Bcl-xL* levels and *Bcl-2* expression patterns in the absence of B cell receptor signaling (Portis and Longnecker, 2004). Other studies have shown that LMP2A can bypass the intact *p53* pathway in the *c-MYC* model of lymphomagenesis (Bieging *et al*., 2009; 2010). Interestingly, the regulation was selective for *Bcl-2*, since the NF-κB inhibitor apparently affects the levels of *Bcl-2* only in LMP2A/HEL-Tg B cells, not in the *Bcl-2*-positive HEL-Tg B cell (Swanson-Mungerson *et al*., 2010).

3.5 MIR-BART5 suppresses *PUMA* expression

A microRNA (miRNA) is a new kind of small RNA (~22 nt in length), which negatively regulates gene expression by inducing mRNA degradation or repressing translation (Grundhoff *et al*., 2006). EBV was the first human virus to express miRNAs, such as MIR-BamHI A rightward transcripts 5 (*BART5*) (Pfeffer *et al*., 2004). Unlike cellular miRNAs (Grundhoff *et al*., 2006), the roles of most EBV miRNAs remain largely unknown. Previous studies suggested that EBV miRNAs are central mediators of viral gene expression, but recently experiments have demonstrated that *MIR-BART5* promotes host cell survival by targeting *PUMA* expression and contributes to the establishment of latent infection in nasopharyngeal carcinoma and EBV germinal center cells (Choy *et al*., 2008). Given that *MIR-BARTs* are abundantly expressed in epithelial cells latently infected with EBV but are less expressed in B cell lines (Cai *et al*., 2006), they may be important in epithelial carcinogenesis. Another recent study showed that *PUMA* is also mediated by cellular miRNAs, including miRNA 221/222, thereby inducing cell survival (Zhang *et al*., 2010). Hence, more experiments are required to understand fully whether cellular miRNAs and *MIR-BARTs* trigger the same sequence in the 3′-untranslated region of *PUMA* mRNA and whether other mechanisms are involved in mediating *PUMA* expression during the EBV infection process.

3.6 EBER-1 and EBER-2 up-regulate Bcl-2 protein expression

The EBV-encoded small RNAs EBER-1 and EBER-2 are small nuclear RNAs transcribed by RNA polymerase III and are the most abundant EBV transcripts expressed. Various studies have shown that EBV inhibition of apoptosis and up-regulation of the Bcl-2 protein are essential for the malignant phenotype (Marin *et al*., 1995; Komano *et al*., 1998). Previous reports also provided direct evidence that EBV up-regulates *Bcl-2* expression by repressing the activation of the double-stranded RNA-dependent protein kinase (PKR) (Komano *et al*., 1999). This allows *c-MYC* to exert its oncogenic function, and eventually results in the inhibition of apoptosis. Wong *et al*.

(2005) demonstrated that the EBER-induced up-regulation of *Bcl-2* expression involves the inactivation of PKR and might inhibit p38 mitogen-activated protein kinase (MAPK) and C-jun phosphorylation. The exact mechanism remains to be identified and may provide a novel treatment strategy for EBV-associated malignancies.

4 Drugs targeting *Bcl* family members in EBV-associated diseases

Increasing evidence points to a crucial function for the disruption of *Bcl* family proteins by EBV in regulating apoptosis in various cancers and in chemotherapy, and it is clear that exploiting this specific interaction is an appealing approach for new anticancer drugs. In recent years, therapeutic options for *Bcl* family members and EBV-encoded products have evolved and improved (Leber *et al*., 2010; Ghosh *et al*., 2012). However, therapies targeting *Bcl* family proteins in EBV-associated diseases have lagged behind.

Despite this, there have been important research findings in recent years. Early trials aimed mainly to exploit antisense-based strategies to repress the expression of *Bcl-2* or *MCL-1* by knocking down *LMP-1* expression. For example, antisense oligodeoxynucleotides were directed at *LMP-1* mRNA to knock down *LMP-1* expression and thereby suppress its function. This was associated with inhibition of *Bcl-2* expression in EBV-immortalized B cells (Kenney *et al*., 1998). With the development of *Bcl* family protein inhibitors for anticancer therapy, the first such inhibitor, G3139 (Genasense/oblimersen), was used in treating EBV-associated diseases (Loomis *et al*., 2003). It was shown that G3139 effectively suppresses Bcl-2 protein expression and has powerful anti-proliferative and pro-apoptotic effects in EBV-positive lymphoblastoid cells. Given that antisense oligodeoxynucleotides cause inflammatory responses and monotherapy most likely induces resistance in tumor cell clones due to the selection process in the tumor population, it is necessary and feasible to apply a combinatorial strategy in the treatment of cancer, especially in EBV-associated diseases. Such approaches have already been investigated in EBV-associated lymphoproliferative diseases using G3139 in combination with rituximab (Loomis *et al*., 2003). Preclinical and clinical trials have investigated the antitumor effects of this combinatorial therapy in mouse xenograft tumor models (Klasa *et al*., 2000; Miayake *et al*., 2000; Wachek *et al*., 2001) as well as its toxicity profile in human phase I testing (Jansen *et al*., 2000; Waters *et al*., 2000; Morris *et al*., 2002). This suggests a promising nontoxic and effective therapy for EBV-positive lymphoproliferative diseases. Abdulkarim *et al*. (2003) also demonstrated that treatment with Cidofovir combined with ionizing radiation leads to tumor remission without increasing toxicity in EBV-positive cells (Raji and C15) in nude mice. This approach is likely to greatly improve conventional cancer therapies. A recent exciting discovery showed that two new Bcl-2 inhibitors, HA14-1 and ABT-737, applied in combined treatments strongly induce apoptosis in EBV-associated diseases (Srimatkandada *et al*., 2008; Pujals *et al*., 2011). HA14-1 in combination with bortezomib (a proteasome inhibitor) synergistically enhances anti-proliferative and pro-apoptotic effects in EBV-positive lymphoproliferative diseases. This small molecule inhibitor has a high binding affinity to Bcl-2 and efficiently induces apoptosis by repressing BAK/Bcl-2 interaction (Azmi and Mohammad, 2009). ABT-737 allows Bax activation by a murine double minute-2 (MDM2) inhibitor by disrupting Bax/Bcl-2 interaction in latency III EBV-positive cells. ABT-737, a BH3 mimetic, represses the anti-apoptotic proteins Bcl-2, Bcl-w, and Bcl-xL and is the highest affinity drug reported to be currently in preclinical or clinical testing (Vogler *et al*., 2009). Another Bcl protein inhibitor, obatoclax, binds to all anti-apoptotic *Bcl* family proteins in vitro (Nguyen *et al*., 2007). These findings suggest that this strategy may have broader applications for other EBV-positive diseases with abnormal expression of Bcl proteins. The combination of EBV-based therapies to reduce expression and a *Bcl* family protein inhibitor to repress function or disrupt interaction of the protein may provide a 'double whammy' to tumor cells in the treatment of EBV-associated diseases.

5 Conclusions

Apoptosis plays a critical role not only in normal development but also in a variety of human disorders

including cancer, neurodegeneration, and autoimmunity (White, 1996). EBV interacts with various intracellular factors, especially *Bcl-2* family members, by impacting on multiple apoptotic pathways (Fig. 1). Moreover, the *Bcl-2* family member-mediated intrinsic apoptotic pathway is involved. From the results of recent studies, most *Bcl-2* family members are targeted by EBV-expressed products. In this complex network, EBV BHRF1, Bcl-2, and EBNA2 are prominent. Although the actions of EBV do not all occur in the same cell, it is still probable that the NF-κB pathway is a common target by which EBV

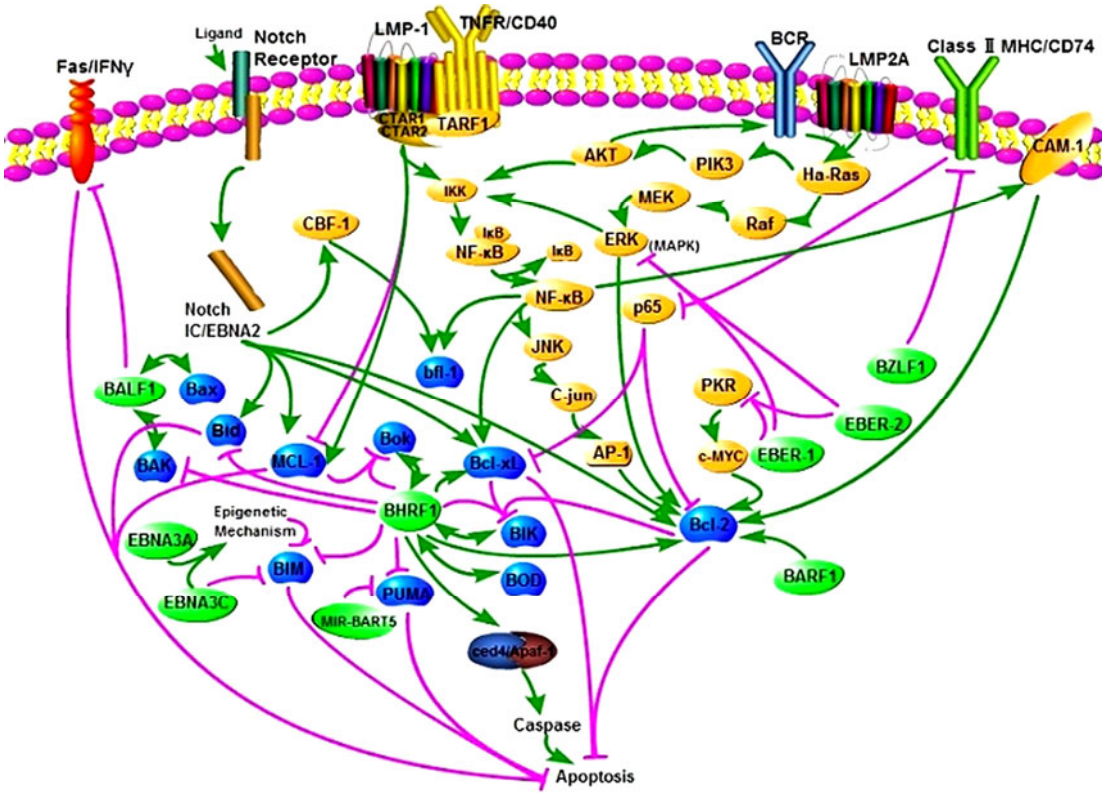

Fig. 1 Epstein-Barr virus interactions with the Bcl-2 protein family and apoptosis

EBV infection disrupts normal cell death and survival pathways in various human tumor cells, including lymphocytes, fibroblasts, and epithelial cells. Interconnected signaling pathways mediate apoptosis. Pro-apoptotic signals are mediated by *BZLF1*, down-regulating *Bcl-2* and *Bcl-xL* expression involving the repression of CD74 and p65. Pro-survival pathways occur mainly via EBV gene products (shown in light green). The activation of BHRF1 results in repression of the pro-apoptotic proteins BIM, PUMA, and Bid; the activation of *BARF1* causes the up-regulation of pro-survival Bcl-2 protein levels; *LMP-1* stimulation results in the CTAR1 and CTAR2 regions of LMP-1 interacting with TNFR/CD40, including TARF1, and then activates the NF-κB/c-JNK/AP-1 pathway and CAM-1, and finally up-regulates *bfl-1* and *Bcl-2* expression. In addition, the activation of LMP-1 inhibits or activates MCL-1; MIR-BART5 stimulation results in the repression of the pro-apoptotic protein PUMA; the activation of LMP2A results in activation of the Ha-Ras, PI3K/Akt pathway and the Raf/MEK/ERK pathway as well as NF-κB, and then increases the expression of *Bcl-xL* and *Bcl-2*. All of these result eventually in the inhibition of apoptosis. Unfortunately, the results of other interactions are not clear. BHRF1 is known to interact with the pro-apoptotic proteins Bok, BAK, BOD, and BIK. BALF1 interacts with Bax and BAK. Likewise, EBNA2 interacts with the pro-survival proteins Bcl2L1, Bcl-2, and MCL-1 and the pro-apoptotic proteins BIM and Bid. EBNA3A and EBENA3C inhibit *BIM* expression, and EBNA3C targets BIM directly. EBER-1 and EBER-2 up-regulate *Bcl-2* expression by inhibiting PKR or MAPK and C-jun. EBNA2, mimicking Notch-IC, activates CBF-1 to up-regulate *bfl-1*. EBV also impacts the apoptosis pathway via the FAS/IFN-γ receptor and by binding to ced4/Apaf-1. Interestingly, the *Bcl-2* family members, with which diverse EBV gene products interact, are essential in the 'decision' step of apoptosis, and then the activation of caspases mediates the 'execution' phase. These are both important steps in apoptosis

disturbs the normal apoptosis pathway, combined with BHRF1, Bcl-2, and ENBA2. These findings may bring about new insights for targeting therapy and prognosis of EBV-associated tumors.

Although it is not clear how these interactions proceed in detail, what we know already provides an exciting glimpse into how they contribute to the establishment of EBV latency and the formation of tumors. After infection, *BZLF1* is expressed immediately in the majority of primary B cells. Once the viral DNA is methylated, *BZLF1* initiates the lytic cycle. Surprisingly, *BZLF1* down-regulates the expression of *Bcl-2* and *Bcl-xL*. Coincidently, this action is neutralized by BHRF1 protein (Zuo *et al*., 2011). Obviously, the cooperation not only allows adequate time to synthesize and accumulate infectious viral progeny, but also enables *BZLF1* to efficiently evade CD^+ T cell responses. *BHRF1* is highly expressed in the first 24 h of infection as well as in the lytic phase, and can repress apoptosis by interacting with the pro-apoptotic protein directly and the pro-survival protein indirectly, which favors rapid transit through the cell cycle (Dawson *et al*., 1998). Thus, the anti-apoptotic function of *BHRF1* might contribute to the accomplishment of viral replication and assembly during the lytic phase. The second Bcl-2 homolog, *BALF1*, also appears to promote cell survival since it can associate with *Bax* and *BAK*. The development of events in the lytic phase is divided to three components: immediately-early, early, and late. *BHRF1*, *BALF1*, and *BARF1* are early genes, and only *BZLF1* is an immediately-early gene. The N-terminal 49 amino-acids of *BARF1* appear to be responsible for its transforming capacity (Sheng *et al*., 2001). However, viruses lacking the *BARF1* gene are not defective in their ability to immortalize B cells in vitro, suggesting that other mechanisms maybe involved. *BARF1* itself might induce cell transformation by cooperating with Bcl-2 protein, since *BARF1* activates anti-apoptotic *Bcl-2* in various cells. Thus, after infection, the infectious cell escapes immunosurveillance, survives to proliferate, and completes viral replication and assembly while accumulating infectious viral progeny.

About one week after infection, EBV ceases to express lytic genes, while latent genes are fully expressed. In fact, EBV might evade the host defenses more effectively by withdrawing into a latent status. During latency, several crucial latent genes, including *EBNA2*, *EBNA3A*, *EBNA3*, *LMP-1*, *LMP2A*, and *EBER*s, are expressed. Studies with EBV recombinants have shown that EBNA2 plays a crucial role in the transformation process. This effect may be at least partially relevant to the up-regulation of the anti-apoptotic protein bfl-1 induced by EBNA2, since the process of induction involves the Notch receptor, a pathway implicated in the progression of T-cell tumors in humans (Artavanis-Tsakonas *et al*., 1995). Like EBNA2, EBNA3A and EBNA3C are responsible for B cell transformation in vitro. They cooperate to repress pro-apoptotic protein BIM expression by an initial or sustained epigenetic mechanism, which causes aberrant large CpG islands utilized by DNA methylation. Therefore, EBV seems to contribute to the development of tumors by modifying the status of cellular DNA methylation, although the exact mechanism is unknown. As a classical oncogene, LMP-1 is another example of the transformation function in B cells. The effect may result from its pleiotropic functions. For example, LMP-1 can up-regulate the expression of the anti-apoptotic proteins Bcl-2, MCL-1, and bfl-1, and the process involves the induction of cell surface adhesion and the TNFR/CD40 pathway. This provides both growth and differentiation responses and is associated with activation of a number of signaling pathways. Studies have demonstrated that LMP-1 promotes genomic instability (Liu *et al*., 2004; 2005), which is strongly associated with cellular transformation. Interestingly, another transmembrane protein, LMP2A, appears to have transformation effects in epithelial cells but not in B cells. LMP2A rescues cells from apoptosis by mediating Bcl-2 and Bcl-xL protein expression. Similarly, NF-κB, downstream of the PI3K/Akt pathway, can mediate the expression *Bcl-xL* and *Bcl-2*. Scholle *et al*. (2000) demonstrated that LMP2A can transform epithelial cells and that this capacity is mediated, at least in part, by activating the PI3-kinase/Akt pathway, indicating that LMP2A-induced activation of the pro-apoptotic protein may contribute to the transformation of epithelial cells and the long-term survival of infectious B cells. In fact, the LMP2A immunoreceptor tyrosine-based activation motif is essential for repressing activation of the EBV lytic phase in B cells. In addition, MIR-BART5 appears to repress *PUMA* expression to promote infected cell survival. Intriguingly, cellular

miRNAs also mediate the expression of *PUMA* to induce host cell survival. This supports a role for MIR-BART5 in competing with host cellular factors to favor infected cell survival and the maintenance of EBV latency. As small RNAs, EBERs have also been shown to have oncogenic functions and may confer an apoptotic-resistant phenotype by up-regulating *Bcl-2* expression in EBV-associated malignant tumors. Thus, these latent genes favor the survival of infected cells, transform them, and maintain EBV latency synergistically. Finally, the EBV-mediated events generate an immortality-like environment for EBV to facilitate the establishment of viral latency and the formation of tumors.

Many questions remain. First, it is not clear which *Bcl* family proteins or other cellular factors are involved when the infected cell is killed to release viral progeny. Second, mRNAs may play a key role in immune evasion, because they are visible to the immune system in infected host cells, and they are highly expressed initially. Nevertheless, the exact mechanism and whether *Bcl* family members are involved remain a conundrum. Third, in addition to the *Bcl* family protein-mediated apoptotic pathway, it is still unknown which other pathways contribute to the progression of EBV-associated tumors. Fourth, the relationship between other EBV-encoded molecules and the *Bcl* family remains to be determined. Fifth, it is not clear which factors play a core role in the complex network. Although currently NF-κB, BHRF1, Bcl-2, and EBNA2 appear to be important components, other EBV-encoded molecules and Bcl proteins are still unknown. This is a fascinating field of study, and it can be expected that the fruits of new research will provide a better understanding of this complicated network in which the infected agents participate in carcinogenesis, uncovering new and significant information on EBV-associated tumor biology and target therapy.

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