EBV-Positive Diffuse Large B-Cell Lymphoma of the Elderly

- A New Provisional Entity of Lymphoma in the WHO Classification

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Abstract

Epstein-Barr virus (EBV) positive diffuse large B-cell lymphoma (DLBCL) of the elderly, initially described in 2003, is a provisional entity in the 2008 World Health Organization classification system, and is defined as an EBV-positive monoclonal large B-cell proliferation that occurs in patients > 50 years of age and in whom there is no known immunodeficiency or history of lymphoma. These tumors are more common in Asia, but also occur in North America and Europe at a low frequency. These neoplasms exhibit a morphologic continuum, from polymorphous to monomorphous, but morphologic features do not correlate with prognosis as all patients have a clinically aggressive course. Most EBV-positive DLBCL of the elderly patients have an activated B-cell immunophenotype and are characterized by prominent NF-κB activation. Cytogenetic complexity is usually low. In this review, we comprehensively delineate the data emerging from analyses of EBV latency program, microRNA-mediated EBV viral oncogenesis, functional genomics of EBV and its biology, as well as differential diagnosis challenge for EBV-positive DLBCL of the elderly. It is hoped that the improved understanding of these tumors will lead to development of novel therapeutic approaches, enhance the effective clinical trials and improve the prognosis.
Introduction

The 2008 World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues has recognized a new provisional entity designated as Epstein-Barr virus-positive diffuse large B-cell lymphoma (DLBCL) of the elderly. This tumor is defined as an EBV-positive monoclonal large B-cell lymphoproliferative disorder arising in immunocompetent patients > 50 years.\(^1\) To denote patient age in the definition was a means of emphasizing the tendency of these tumors to arise in patients of advanced age, but this age cutoff is clearly arbitrary, and EBV-positive DLBCL of the elderly has been reported rarely in younger patients.

Since the initial description of 22 patients with EBV-positive DLBCL of the elderly in 2003 by Oyama et al,\(^2\) knowledge of EBV-mediated mechanisms of oncogenesis, microRNA profiles in EBV-positive lymphomas and novel efficient therapeutics for patients with these neoplasms have been developed. In this review, we discuss the epidemiology, clinicopathologic features, and differential diagnoses of EBV-positive DLBCL of the elderly. We also review novel molecular findings including host and virus-induced miRNAs, and some novel therapeutic approaches.

Epidemiology

The median age of patients with EBV-positive DLBCL of the elderly is 71 years (range, 50-91), however, younger patients can be affected.\(^3\) There is a slight male predominance with a male to female ratio of 1.4 to 1.\(^4\)-\(^10\) There is a higher prevalence of EBV-positive DLBCL among East Asians (8.7-11.4%)\(^11\)-\(^13\) compared with less than 5% in Western countries\(^5\),\(^6\) (Table 1). Meanwhile, Beltran et al\(^4\) has reported the highest frequency of EBV-positive DLBCL in a Peruvian population, 14.9%, using a criterion of ≥ 20% cells with positive EBV-
encoded RNA (EBER) expression. Of note, there are no uniform criteria for the percentage of EBV-positive cells in EBV-positive DLBCL and this is a limitation when assessing disease prevalence. The fact that differences in cutoffs for EBER positivity clearly impact the frequency of EBV positive DLBCL was respectively shown by Wada et al and others.\textsuperscript{13-15} These authors emphasize the need for establishing uniform criteria for EBV positivity, either >20%, >50%, or almost all tumor cells. Hofscheier et al interpreted EBER-positive DLBCL with <20% EBER as clonally unrelated EBV-transformed B-cells or secondary EBV infection in an established B-cell clone.\textsuperscript{7} The definitive criterion for EBV positivity in EBV-positive DLBCL is remained to be agreed.

Epidemiologic features correlate with geographical differences in the literature. In contrast with East Asian series, cases in Mexico display a relatively younger age at presentation, with predominantly nodal disease and rare expression of the EBNA2.\textsuperscript{7} Recent evidence casts a doubt on geographic and/or ethnic variation in the prevalence of EBV-positive DLBCL of the elderly. Contrary to earlier reports from the Far East, a report from Northern China showed a frequency of EBV positivity in only 8 of 212 (3.8%) in patients >50 years old with DLBCL (using a cutoff of >50% EBER positivity).\textsuperscript{9}

**Pathogenetic aspects**

From an immunologic viewpoint, aging can be characterized as a dysregulated relationship between inflammatory and inflammation-neutralizing processes, resulting in a low-grade chronic pro-inflammatory state.\textsuperscript{16} This imbalance might exert other potential effects promoting lymphomagenesis. Inflammation induces radical oxygen species which can cause dysregulation of critical oncogenic pathways, such as p53, retinoblastoma (Rb), NF-κB, and mitogen-activated protein (MAP) kinases.\textsuperscript{17}
It has been postulated that EBV-positive DLBCL of the elderly might be caused by the senescence of the immune system, as a part of the normal aging process, based largely on shared features with immunodeficiency-associated lymphoproliferative disorders (LPDs). These shared features include EBV infection, similar EBV latency pattern, morphologic similarities, and presence of monoclonal T-cell populations. Of note, immunosenescence is not simply the result of a progressive decline of all immune functions, but rather refers to a continuous remodeling process, whereby the adaptive immunity is preferentially affected compared with innate immunity. In this context, B-cell diversity decreases with age and is characterized by clonal expansions of B cells in vivo. Concurrently, the T-cell compartment shows characteristic modifications, such as: (i) a reduction of the absolute number of total T lymphocytes, including helper/inducer (CD4+) and suppressor/cytotoxic (CD8+) cell counts; (ii) a progressive decline of the naïve T-cell repertoire with a parallel decline in T-cell receptor repertoire diversity; and (iii) a progressive expansion of oligoclonal CD28- T cells, particularly among the CD8+ T-cell subset.

Particularly, Ouyang et al showed a significantly greater frequency of EBV-specific receptor-carrying cells within the CD8+ T-cell subset as well as a lower frequency of EBV antigen-specific interferon (IFN)-gamma-producing T cells in the elderly, suggesting clonal expansion of dysfunctional EBV-specific cells to age. Whether oligoclonal or monoclonal T-cell populations in patients with EBV-positive DLBCL of the elderly reflects a physiological T-cell response to EBV infection or antedate the development of the LPD stemming from an underlying deficiency in T-cell function is unknown.

EBV is a gamma-herpes virus that has a tropism for lymphocytes. The virus infects and replicates within epithelial cells and most viral particles are cleared by cytotoxic T cells.
However, EBV survives in a host cell by establishing latency as an episome (a double-stranded circular form of the genome) in memory B-cells. In latent infection, EBV uses only a limited number of genes to maintain its genome and evades the host immune reaction. Based on different patterns of viral gene usage, latency can be classified as type III, type II, or type I. Type III is characterized by the expression of all six EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and LP), three latent membrane proteins (LMP1, 2A and 2B), and EBER (Figure 1A-B). Type III latency is observed in patients with infectious mononucleosis or a subset of post-transplant lymphoproliferative disorders (PTLD). Type II latency is associated with expression of EBNA1, LMP1, LMP2A, and EBER and is commonly seen in classical Hodgkin lymphoma and a subset of PTLD. In type I latency, only EBNA1 and EBER are expressed; this pattern is characteristic of Burkitt lymphoma.

EBV-positive DLBCL of the elderly shares a similar EBV latency patterns (type II or III) with PTLD. Using reverse transcription polymerase chain reaction (RT-PCR) method, Nguyen-Van et al showed that many EBV-positive DLBCL of the elderly and EBV-positive PTLD resembling DLBCL shared features of EBV latency type III. A relatively high frequency of EBNA2 expression (28% to 32%) has been observed in several studies. However, Hofsheier et al found history of immunosuppression or immunodysfunction in 4/17 cases with EBNA2 expression, emphasizing that care should be taken in the diagnosis of EBNA2-positive DLBCL cases.

EBV-positive DLBCL of the elderly is characterized by prominent classical and alternative NF-κB pathway activation, with most cases displaying an activated B-cell (ABC) immunophenotype. Whereas NF-κB activation has been known in DLBCL, mostly in the ABC subtype, the association between NF-κB activation and the presence of EBV infection...
in DLBCL is much stronger. Interestingly, Montes-Moreno et al found NF-κB activation not only in the ABC but also in the germinal center B-cell (GCB) subtype of EBV-positive DLBCL, suggesting an additional role for EBV in NF-κB activation independent of the cell of origin. This finding could be related to c-Rel (a subunit of NF-κB transcription factor) amplification, which has been observed exclusively in the GCB immunophenotype of DLBCL on the basis of gene expression profiling.

The oncogenic mechanisms of EBV are thought to be attributable predominantly to LMP1 (Figure 2). LMP1 (MW 63kDa) is an integral membrane protein that consists of three different domains. The N-terminus cytoplasmic domain orientates LMP1 protein and binds to the plasma membrane. The long C-terminus cytoplasmic tail contains signaling activity. A six-transmembrane loop between the C- and N-terminus domains provides a platform for self-aggregation and oligomerization. LMP1 functionally mimics CD40, which is expressed constitutively on the B-cell membrane involving in B-cell activation and proliferation. Of note, the function of LMP1 is ligand-independent and is constitutively activated as a result of self-aggregation and oligomerization. The C-terminal tail has two distinct domains, C-terminal activation regions 1 and 2 (CTAR1 and 2), which activate the NF-κB pathway. LMP1 aggregates provide a platform for CTAR1 and CTAR2 to interact with downstream molecules (Figure 1-C). LMP1 can also activate the c-Jun N-terminal kinase (JNK)/AP-1, MAPK, and phosphatidylinositol 3-kinase (PI3K)-AKT pathways.

LMP1 also deregulates the cell cycle. Everly et al showed that LMP1 increases expression of cyclin-dependent kinase 2 (Cdk2), phosphorylates retinoblastoma (Rb) protein, and inhibits expression of p27Kip1. The p16INK4a-Rb pathway has been shown to be hindered by LMP1. LMP1 also increases expression of Bcl-2 to prevent latently EBV-infected cells from
undergoing apoptosis. Of note, virus and host transcriptome analyses have revealed an unexpected coordination between viral lytic reactivation and cellular pathways involved in B-cell expansion as well as tumor promotion, supporting a role for the lytic cycle in virus-mediated oncogenesis while further highlighting the complexity of interactions between EBV and host.

**Clinical features**

EBV-positive DLBCL of the elderly is characterized by higher age distribution and an aggressive clinical course with a median survival of two years in Asian patients (Figure 3A-B). Initial reports emphasized that EBV-positive DLBCL of the elderly commonly involved extranodal sites. Site of primary extranodal involvement include the skin, soft tissue, bones, nasal cavity, pharynx/hypopharynx, tonsils, tongue, lung, pleura, stomach, liver, spleen, peritoneum, cecum and bone marrow. Subsequently, several studies showed that lymph node involvement is very common, in up to 70% of patients.

EBV-positive DLBCLs, including EBV-positive DLBCL of the elderly, respond more poorly to treatment with a poorer outcome compared with patients who have EBV-negative DLBCL. Therefore, novel therapeutic agents are needed to treat patients with EBV-positive DLBCL of the elderly. Montes-Moreno et al compared the survival distributions of age-related EBV-positive DLBCL cases and a control cohort either selecting ABC-type DLBCL cases or patients > 60 years of age and subsequently confirmed that patients with EBV-positive DLBCL of the elderly have a poorer overall survival and progression-free survival than patients with ABC-type EBV-negative DLBCL in older European patients (Figure 3C-D). In comparison, a negative impact of EBV infection in DLBCL survival was not observed in the North America patients. There was a trend toward higher percentage of stage III/IV disease.
and bone marrow involvement (85% vs 57% and 46% vs 16%, p = 0.052), but no unique clinical features were found in EBV-positive DLBCL patients (Figure 3E-F). However, significantly worse survival is observed in EBV-positive DLBCL patients if CD30 expression is also present (Figure 3G-H).

Recently, Dojcinov et al\textsuperscript{18} expanded the spectrum of EBV-positive DLBCL. They analyzed 122 cases of EBV-positive B-cell LPDs in adults with no other identifiable cause of immunosuppression and classified them into four diagnostic categories: (1) reactive lymphoid hyperplasia; (2) polymorphic extranodal lymphoproliferative disease; (3) polymorphic nodal lymphoproliferative disease; and (4) DLBCL. In these four groups the five-year disease-specific survival decreases progressively (100%, 93%, 57%, and 25%, respectively) while the frequency of monoclonality increases (16%, 33%, 63%, and 56%, respectively). It needs to be emphasized that these categories are not fixed as patients can progress from reactive hyperplasia to polymorphic or monomorphic EBV-positive LPD.\textsuperscript{40} With respect to morphology, some EBV-positive DLBCL of the elderly display a mixed pattern with polymorphic and monomorphic areas\textsuperscript{8} or polymorphic cases eventually progress to the monomorphic subtype of EBV-positive DLBCL of the elderly\textsuperscript{5,41}.

Two additional entities have been described that further add to the spectrum of EBV-positive LPDs. EBV-positive mucocutaneous ulcer is a term used recently to designate small-volume extranodal polymorphic lymphoproliferative disease, particularly involving mucosal sites and skin, and characterized by an indolent clinical course and good prognosis.\textsuperscript{18,42} Plasmablastic lymphoma of the elderly is another newly recognized entity with a relatively indolent clinical course and better prognosis compared with other age-related EBV-positive B-cell LPDs\textsuperscript{24}. Plasmablastic lymphoma of the elderly shares features with age-related EBV-positive B-cell
LPDs, including older age, immunosenescence, EBV infection, HIV negativity, and an ABC immunophenotype, although contrasting features include a higher of extranodal infection and MYC translocations and EBV type I latency pattern.24

**Histopathologic Features**

Two morphologic subtypes of EBV-positive DLBCL of the elderly have been recognized, polymorphic and monomorphic. Both subtypes may include large transformed cells or immunoblasts as well as Hodgkin and Reed-Sternberg (HRS)-like giant cells and may demonstrate increased mitotic activity and areas of geographic necrosis.1 The polymorphic subtype displays a broad range of B-cell maturation, and lesions are composed of centroblasts, immunoblasts and plasmablasts with a variable component of admixed reactive cells, including small lymphocytes, plasma cells, histiocytes and epithelioid histiocytes.1 Montes-Moreno et al8 subdivided the polymorphic EBV-positive DLBCL of the elderly into three subgroups based on the relative proportion of large neoplastic cells and presence of HRS-like cells: (1) canonical large B-cell neoplasm (high density of large neoplastic cells and scattered cells with HRS-like features); (2) DLBCL with Hodgkin lymphoma-like features (lower density of neoplastic cells with HRS-like features); and (3) DLBCL with polymorphic LPD-like features (low density of neoplastic cells without HL-like features). The monomorphic subtype of EBV-positive DLBCL of the elderly is composed of monotonous sheets of large transformed B cells (Figure 4).1 Although this distinction is helpful for recognizing the spectrum, these morphologic variants do not impart clinical or prognostic significance.1 Cases of EBV-positive DLBCL of the elderly also can have a mixed pattern with intermingled polymorphic/and monomorphic areas8, suggesting that the subtypes represent two ends of a morphologic spectrum.
Immunophenotype

The neoplastic cells are of B-cell lineage as they express the pan B-cell antigens CD19, CD20, CD22, CD79a and PAX5 and are negative for pan T-cell antigents. Immunoglobulin light chain restriction may be difficult to demonstrate, except in cases with immunoblastic or plasmablastic features in which cytoplasmic Ig can be assessed. Plasmacytoid cases can be weakly positive or negative CD20.1 EBV-positive DLBCL of the elderly usually has an ABC immunophenotype being MUM1/IRF4+ and CD10- and usually BCL6-. BCL-2 and CD30 are usually positive; CD15 is negative.1,8 Ki-67 generally shows a high proliferation index.8 By definition, the neoplastic cells are EBV-positive and accordingly display EBER, LMP1 in > 90% and EBNA2 in approximately 15% and 30% of cases.1,43 The concordance rate between EBER and LMP1 can reach 80% to 90%, but is not uniformly consistent.

The proportion of EBV-positive DLBCL cases among all DLBCL patients increases with age,12,44 which is analogous to the ABC subtype that also increases with age.45 These findings are in accord with recent data indicating that aging is an important determinant of lymphoma biology.46 In an effort to explain the proportional increase of ABC DLBCL with age, Mareschal et al45 speculated that either a change in B-cell population during aging or on the putative pathological specificity of EBV-positive DLBCL of the elderly. Not surprisingly, most cases of EBV-positive DLBCL of the elderly displayed a striking shift to an ABC immunophenotype with prominent activation of NF-κB pathway.8

Differential Diagnosis

The major entities in the differential diagnosis of EBV-positive DLBCL of the elderly are summarized in Table 2. In part, EBV-positive DLBCL of the elderly is a diagnosis of
exclusion. If there is a known cause of immunodeficiency, these tumors are better classified as post-transplant LPDs, other iatrogenic immunodeficiency-associated LPDs, etc. Other forms of EBV-positive DLBCL such as lymphomatoid granulomatosis, DLBCL associated with chronic inflammation, primary effusion lymphoma, and plasmablastic lymphoma are also considered in the differential diagnosis. Reactive diseases such as infectious mononucleosis of the elderly and chronic active EBV infection also may be considered based on the morphologic findings but clinical history and serologic testing are helpful.

**Genetic and miRNA profile**

Only a few genetic studies on cases of EBV-positive DLBCL of the elderly have been performed. The immunoglobulin genes are monoclonally rearranged in most cases, with clonality of EBV also usually detectable using EBV terminal repeat regions probes and molecular techniques. IgH-mediated translocations are uncommon (~15%). However, these analyses were restricted to single loci (IGH, IGK, IGL, PAX5, C-MYC, BCL2 and BCL6). A single case with a t(9;14)(p13;q32) translocation involving the PAX5 gene has been reported. Given the presence of a low number of genomic aberrations in EBV-positive DLBCL of the elderly, it has been suggested that immunosenescence coupled with the EBV oncogenic properties are sufficient and additional chromosomal alterations are therefore usually not needed for lymphomagenesis.

EBV was the first virus in which miRNAs were identified and 44 mature EBV-miRNAs are currently known. A detailed list of EBV-miRNAs is summarized in Table 3. EBV-miRNAs can regulate viral and cellular targets, and they generally target host transcripts during latent infection (Table 3). Although viral miRNA constitutes only ~2% of all miRNA in EBV-positive DLBCL, the viral miRNAs share seed sequence homology with cellular miRNA. For
example, EBV-miR-BART5 and EBV-miR-BART1-3p share similarity with cellular miR-18 (miR-17-92 cluster) and miR-29a/b, respectively. Furthermore, viral miRNAs share seed sequence homology among themselves. Moreover, individual EBV-miRNAs can target multiple mRNAs, suggesting that many other targets are actually affected and await further identification.

Several studies have shown that EBV-miRNAs contribute to EBV-induced oncogenesis. EBV-miR-BART5 targets and degrades p53-up-regulated modulator of apoptosis, a pro-apoptotic protein. EBV-miR-BHRF1 promotes B-cell transformation and proliferation and prevents apoptosis. EBV-miR-BART9 and BART17-5p can downregulate BCL6 expression. Considering the fact that BCL6 represses NF-κB, downregulation of BCL6 by EBV-miRNA might indirectly contribute to NF-κB activation. Riley et al showed that numerous EBV-miR-Bam HI-A region rightward transcripts (BARTs) likely target at least 132 apoptotic mRNAs in latency type III infection, highlighting their collective influence on apoptosis regulation.

It also becomes evident that EBV-miRNAs have evolved to target multiple cellular pathways rather than a single pathway. Pathway analysis has predicted that there are several EBV-miRNA targeted genes in at least 20 specific pathways, including p53 feedback loops, B-cell activation, oxidative stress response, inflammation mediated by chemokines or the cytokine signaling pathway, the PI3K pathway and apoptosis (Figure 6).

Interestingly, cellular miRNAs are modulated by viral proteins. Mir-155 has been shown in DLBCL, especially in ABC subtype, and can be induced by LMP1 via the NF-κB pathway. Of note, miR-155 has been shown to co-target INPP5D (SHIP1), a key target implicated in
lymphomagenesis, with EBV-miRNAs.\textsuperscript{59,61} Mir-34a is induced by LMP1 via the NF-\kappa B pathway and promotes the growth of EBV-transformed cells.\textsuperscript{62} EBNA2 has been shown to induce miR-21 and downregulate miR-146a.\textsuperscript{63} Overexpression of miR-21 has been found to promote tumor progression and invasion in solid tumors via targeting PTEN.\textsuperscript{64,65} Mir-146a has been shown to inhibit the NF-\kappa B pathway in the NK/T-cell lymphoma cell lines.\textsuperscript{66} Therefore, EBNA2 can provide extra signals via miRNAs to enhance the NF-\kappa B and Akt pathways, thereby providing survival/proliferation benefits.

\textbf{Novel therapeutic approaches}

Novel therapeutic approaches need to be considered for patients with EBV-positive DLBCL of the elderly. Possible therapeutic approaches include: (1) EBV-specific adoptive immunotherapy; (2) microRNA-targeted therapy; (3) combination therapy based on EBV lytic phase induction followed by exposure of the tumor cells to anti-herpesvirus drugs; and (4) targeting specific signaling pathways, including the NF-\kappa B pathway (Table 4 and Figure 6).

EBV-specific adoptive immunotherapy, in which EBV-specific cytotoxic T-lymphocytes (CTLs) are \textit{ex vivo} activated and expanded and then reinfused seem promising.\textsuperscript{67} Using this approach, EBV-specific CTLs could be engineered to recognize and lyse tumor cell targets via chimeric receptors, while maintaining their ability to proliferate in response to EBV target antigens and accordingly destroy virus-infected cells.\textsuperscript{68} Optimal tumor eradication appears to require proper target antigen selection, co-stimulatory signalling and the ability of chimeric antigen receptor-modified T-cells to traffic, persist, and retain function following adoptive transfer.\textsuperscript{69} Recent evidence has suggested that both EBV-positive monomorphic PTLD that resemble DLBCL and EBV-positive DLBCL of the elderly share features consistent with type
III EBV latency, including immunodominant EBNA3A protein expression, indicating that such an approach may be feasible.\textsuperscript{19}

Given the aforementioned complex interplay between host and viral-encoded miRNAs in EBV-mediated oncogenesis, targeting miRNA as a therapeutic approach appears to be promising. Various approaches have been proposed: (1) anti-miRNA oligonucleotides are inhibitory molecules blocking the interactions between miRNAs and their target mRNAs by interacting directly to the target miRNA; (2) miRNA mask is a sequence with perfect complementarity to the binding site for a miRNA in the target mRNA, which can form a duplex with the target mRNA with higher affinity and block the access of the miRNA; (3) miRNA sponges are synthetic decoys that contain multiple binding sites for a miRNA of interest, preventing the interaction between miRNA and its targets; (4) small molecule inhibitors against specific miRNAs can specifically inhibit miRNA synthesis; (5) restoring activity of miRNAs with oncosuppressive functions; and (6) using miRNA to sensitize tumors to known and efficient cancer therapeutic modalities.\textsuperscript{70}

Another potential approach could be combining induction of EBV lytic phase with subsequent exposure to anti-herpesvirus drugs.\textsuperscript{71} Lytic phase inducers may refer to phorbol esters, DNA methylase transferase inhibitors, histone deacetylase (HDAC) inhibitors, proteasome inhibitors, B-cell receptor-blocking antibodies, chemotherapeutic drugs and cellular miRNAs.\textsuperscript{71,72} Although proof of principle of a combination therapy approach has been documented in a clinical trial,\textsuperscript{73} the optimal pharmacological lytic phase inducer remains to be determined. It has been recently shown that other HDAC inhibitors such as panobinostat appear to be efficient and potent for the treatment of EBV-associated lymphomas.\textsuperscript{74}
Given that the EBV-positive DLBCL of the elderly is an aggressive post-germinal center B-cell neoplasm characterized by prominent NF-κB activation, targeting the NF-κB pathway constitutes a rational therapeutic approach (Table 4). Bortezomib, a proteasome inhibitor, has been found to induce apoptosis in EBV lymphoblastoid cell lines and to reduce canonical and non-canonical activities of NF-κB pathway. A recent phase I/II clinical trial with bortezomib plus R-CHOP in DLBCL abrogated the adverse outcome in non-GCB and showed similar survival between GCB and non-GCB patients. Bay 11-7085, a pharmacological inhibitor of NF-κB activity, or NF-κB/p65 small interfering RNA (siRNA), can inhibit DLBCL growth in vitro via induction of apoptosis. In contrast with bortezomib, the latter strategies of NF-κB inhibition down-regulate expression of key NF-κB regulated gene products, including Bcl-2 and Bcl-XL. NF-κB essential modulator-binding domain peptide has been shown to inhibit NF-κB target gene expression and reduce tumor burden in an in vivo model as well.

Recent evidence indicates a critical function of PI3K-PDK1 signaling upstream of NF-κB in ABC-DLBCL cells. PI3K inhibition reduced NF-κB activity and decreased the expression of NF-κB target genes in ABC-DLBCL cell lines, providing a rationale for the pharmacological use of PI3K inhibitors for treating patients with ABC-type DLBCL. In addition, LMP1 has been implicated in the activation of the PI3K/Akt signaling pathway, which lies upstream of mTOR. Experimental evidence stemming either from dual PI3K/mTOR inhibitors or a single mTOR inhibitor further reinforces the putative exploitation of LMP1/LMP2A-activated PI3K/Akt/mTOR signaling pathway. Another attractive target for developing antiviral or antitumor strategies is EBNA1 owing to its essential role in maintaining the EBV genome and the consistent expression of EBNA1 in all proliferating EBV-positive cells. Sun et al showed that Hsp90 inhibitors block the outgrowth of EBV-infected malignant cells in vitro and in vivo through an EBNA1-dependent mechanism. Notably, Hsp90 inhibitors have been found to
suppress NF-κB activity. A CD30-directed antibody-drug conjugate could be a good option given frequent CD30 expression in EBV-positive DLBCL patients. A recent trial Brentuximab Vedotin in 2 cases of EBV-positive DLBCL did not show promising result, but small size trial might be not representative.

Conclusions
We have provided a comprehensive review of EBV-positive DLBCL of the elderly discussing its epidemiologic, pathogenetic and clinicopathologic features, differential diagnosis, and proposed some comments about potential novel therapeutic approaches for patients with this disease. Currently, an arbitrary cutoff of 50 years is used to define this entity in the current WHO classification, but patients younger than 50 years can also be affected. Although these tumors were originally reported as usually being extranodal, it is clear that nodal presentation is also seen. Most cases of EBV-positive DLBCL of the elderly have an activated B-cell immunophenotype. Assessment for EBV infection in tissue biopsy specimen can be easily performed by in situ hybridization analysis for EBER, but other methods of EBV detection including PCR can be used. Caution should be exercised before rendering this particular diagnosis as other causes of EBV-positive DLBCL must be excluded and there can be no known explanation for immunodeficiency. Clearly, a better understanding of the molecular genetic basis of EBV-positive DLBCL of the elderly is needed. Further studies are required to address the heterogeneous EBV latency status and miRNA expression profile of EBV-positive DLBCL of the elderly, as well as the role of the NF-κB pathway.
Acknowledgments

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Authorship Contributions:

C.Y.O., T.P., L.J.M., and K.H.Y. designed the study, analyzed data, and wrote the paper.

CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.
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Table 1. Frequency of EBV positivity with different cutoffs in B-cell lymphoproliferative disorder among different countries

<table>
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<tr>
<th>Country</th>
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<td>(2002-2007)</td>
<td>n/a</td>
<td>not specified</td>
<td>not specified</td>
<td>DLBCL</td>
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<tr>
<td>Saudi Arabia</td>
<td>16/217</td>
<td>7.4</td>
<td>not specified</td>
<td>not specified</td>
<td>DLBCL</td>
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</tbody>
</table>

*EBER positive rate was re-calculated in cases with an asterisk after excluding immunodeficiency-associated LPD (including PTLD and HIV-associated lymphoma), autoimmune-associated LPD, and non-DLBCL patients from each study.

** EBER: EBV-encoded RNA; LPD: lymphoproliferative disorder; S/I/A: Switzerland, Italy, and Austria; Ref: reference; n/a: not applicable; DLBCL: diffuse large B-cell lymphoma
<table>
<thead>
<tr>
<th>Entity</th>
<th>Site</th>
<th>Architecture</th>
<th>Immunophenotype</th>
<th>Specific findings</th>
<th>Light chain</th>
<th>Clonality</th>
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<tbody>
<tr>
<td>EBV-positive DLBCL, elderly</td>
<td>N (70%)</td>
<td>Effaced</td>
<td>Pan B markers⁺</td>
<td>Geographic necrosis</td>
<td>Monotypic</td>
<td>Monoclonal</td>
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<tr>
<td></td>
<td>E (30%)</td>
<td></td>
<td>CD10⁻/BCL6⁻/MUM1⁺, EBER⁺/CD30⁻</td>
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<tr>
<td>EBV-associated reactive atypical</td>
<td>N</td>
<td>Not effaced</td>
<td>Mixed B- and T-cell nature of</td>
<td>n/a</td>
<td>Polytypic</td>
<td>Polyclonal</td>
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<tr>
<td>lymphoproliferations/infectious</td>
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<td></td>
<td>HRS-like cells (CD3⁺/20⁺/30⁺)</td>
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<tr>
<td>mononucleosis in elderly</td>
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<td></td>
<td>EBER⁺</td>
<td></td>
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<tr>
<td>EBV-positive classical Hodgkin</td>
<td>N</td>
<td>Effaced</td>
<td>CD20+/CD79a⁻/Pax5⁺</td>
<td>HRS cells in reactive background</td>
<td>Polytypic</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>lymphoma</td>
<td></td>
<td></td>
<td>CD30⁺/CD15⁺, EBER⁻</td>
<td></td>
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<td>DLBCL, NOS, anaplastic variant</td>
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<td>Effaced</td>
<td>Pan B markers⁺</td>
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<td>Monoclonal</td>
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<td></td>
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<td>EBER⁺, CD30⁻</td>
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<tr>
<td>T-cell/histiocyte-rich large B-cell</td>
<td>N</td>
<td>Effaced</td>
<td>Pan B markers⁺</td>
<td>Scattered B cells in benign</td>
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<td>Monoclonal</td>
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<tr>
<td>lymphoma</td>
<td></td>
<td></td>
<td>EBER⁺, CD30⁻/CD15⁻</td>
<td>T-cells/histiocytes background</td>
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<td>Primary cutaneous DLBCL, leg</td>
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<td>Dermal</td>
<td>Pan B-cell markers⁺, EBER⁻</td>
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<td>Monoclonal</td>
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<td>type</td>
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<td>infiltration</td>
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<td>Immunophenotype</td>
<td>Molecular Markers</td>
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<tr>
<td>Lymphomatoid granulomatosis</td>
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<td>polymorphic</td>
<td>EBER⁺, LMP1⁻</td>
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<td>Angioinvasion, HRS-like cells in reactive background</td>
<td>Non-monoclonal</td>
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<tr>
<td>Plasmablastic lymphoma of the elderly</td>
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<td>Effaced</td>
<td>CD38⁺, CD138⁺, MUM1⁺, CD20⁻</td>
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<td></td>
<td>E (50%)</td>
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<td>Plasmablastic morphology</td>
<td>Monotypic n/a</td>
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<td>Angioimmunoblastic T-cell lymphoma</td>
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<td>FDC expansion</td>
<td>Large atypical cells</td>
<td>Polytypic Monoclonal</td>
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<td>Peripheral T-cell lymphoma, not otherwise specified</td>
<td>N</td>
<td>Effaced</td>
<td>CD3⁺/⁻ with partial loss of T-markers, CD30⁺/⁻, CD15⁺/⁻</td>
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<td></td>
<td></td>
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<td>Large atypical cells</td>
<td>Polytypic Monoclonal</td>
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<tr>
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<td></td>
<td>clear cytoplasm⁺/⁻</td>
<td>in T-cells</td>
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* Pan B-cell markers: CD19, CD20, CD22; Site N: nodal; Site E: extranodal; EBV: Epstein Barr virus; EBER: EBV-encoded RNA; DLBCL: diffuse large B-cell lymphoma; HRS: Hodgkin Reed-Sternberg; n/a: non-available

** References 1,25,42-44,62,84-88
Table 3. Viral and cellular targets and functions of EBV-miRNAs

<table>
<thead>
<tr>
<th>EBV-miRNA</th>
<th>Function of ebv-miR</th>
<th>Reference</th>
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<td><strong>Viral targets</strong></td>
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<tr>
<td>BALF5</td>
<td>EBV-miR-BART2</td>
<td>Inhibition of lytic cycle</td>
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<tr>
<td>EBNA2</td>
<td>EBV-miR-BART6</td>
<td>Maintenance of viral latency</td>
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<td>EBV-miR-BART-Cluster1</td>
<td>Growth control or escape from immunosurveillance</td>
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<td>LMP1</td>
<td>EBV-miR-BART19-5p</td>
<td>Prevent apoptosis</td>
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<tr>
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<td>EBV-miR-BART22</td>
<td>Growth control or escape from immunosurveillance</td>
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<td><strong>Cellular targets</strong></td>
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<tr>
<td>Dicer</td>
<td>EBV-miR-BART6</td>
<td>Promote latency</td>
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<tr>
<td>Bim</td>
<td>EBV-miR-BART-Cluster 1</td>
<td>Anti-apoptosis</td>
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<td>EBV-miR-BART-Cluster 2</td>
<td>Anti-apoptosis</td>
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<td>CXCL-11/I-TAC</td>
<td>EBV-miR-BHRF1-3</td>
<td>Anti-apoptosis</td>
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<td>PUMA</td>
<td>EBV-miR-BART5</td>
<td>Anti-apoptosis</td>
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<td>IPO7</td>
<td>EBV-miR-BART3-3p</td>
<td>Anti-inflammation</td>
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<td>TOMM22</td>
<td>EBV-miR-BART16-5p</td>
<td>Anti-apoptosis</td>
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<td>MICB</td>
<td>EBV-miR-BART2-5p</td>
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<td>BCLAF1</td>
<td>EBV-miR-BART17-5p</td>
<td>Anti-apoptosis</td>
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<td>CLEC2D (LLT1)</td>
<td>EBV-miR-BART3-3p</td>
<td>Immune evasion</td>
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<td>EBV-miR-BART1-3p</td>
<td>Immune evasion</td>
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<td>LY75 (DEC205)</td>
<td>EBV-miR-BART1-5p</td>
<td>Immune evasion</td>
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<td>CLEC7A (Dectin-1)</td>
<td>EBV-miR-BART4</td>
<td>Immune evasion</td>
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<td>CLEC2B (AICL)</td>
<td>EBV-miR-BART2-5p</td>
<td>Immune evasion</td>
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<td>NLRP3</td>
<td>EBV-miR-BART15</td>
<td>Immune dysregulation</td>
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Table 4. Differentially expressed genes in EBV-positive ABC DLBCL vs. EBV-negative ABC DLBCL

A. Genes upregulated in EBV-positive ABC-DLBCL

<table>
<thead>
<tr>
<th>Gene functional categories</th>
<th>No. of genes</th>
<th>Representative genes</th>
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<tbody>
<tr>
<td>NF-κB target</td>
<td>11</td>
<td>PRDM1, CYLD, FYN, TNFAIP3, FTH1, LITAF</td>
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<td>Cell cycle regulation</td>
<td>4</td>
<td>CYTIP, PPM1A, TMEM30A, MRPL41</td>
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<tr>
<td>Anti-apoptosis</td>
<td>4</td>
<td>MDM2, DNAJC5, SERPINB9, GSTO1</td>
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<tr>
<td>Tumor progression</td>
<td>5</td>
<td>LAMP1, PTP4A2, DYNLRB1, ASAP1, ERLEC1</td>
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<tr>
<td>Cell proliferation</td>
<td>3</td>
<td>TXN, PDXX, GLUL</td>
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<tr>
<td>Cell adhesion and cytoskeleton</td>
<td>6</td>
<td>GABARAP, WIPF, AOC3, DST, SLAMF7, Clorf38</td>
</tr>
<tr>
<td>Intracellular trafficking</td>
<td>4</td>
<td>TMED2, RAB27A, M6PR, FLOT1</td>
</tr>
<tr>
<td>Immune response</td>
<td>2</td>
<td>SAMHD1, GBP2</td>
</tr>
<tr>
<td>Cell metabolism</td>
<td>12</td>
<td>GPD2, P4HB, GNS, RPN2, APOL6, LASS6</td>
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<tr>
<td>Transcription factors</td>
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<td>ARNTL, TLE3</td>
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<td>Others and unknown</td>
<td>17</td>
<td>RPN2, SLC31A1, M6PR, SETD3, KIAA2013, TPCN2</td>
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B. Genes downregulated in EBV-positive ABC-DLBCL

<table>
<thead>
<tr>
<th>Gene functional categories</th>
<th>No. of genes</th>
<th>Representative genes</th>
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</thead>
<tbody>
<tr>
<td>Pro-apoptotic</td>
<td>2</td>
<td>TBX5, CUL3</td>
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<tr>
<td>Immune response</td>
<td>2</td>
<td>ENPP3, CD79B</td>
</tr>
<tr>
<td>Cell metabolism</td>
<td>3</td>
<td>GATM, MOCOS, GALNT6</td>
</tr>
<tr>
<td>ECM, adhesion, cytoskeleton</td>
<td>10</td>
<td>CDHR1, CDHR3, CDH11, TRIP6, CLDN10, SMAGP</td>
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<tr>
<td>Signaling and transcription factors</td>
<td>3</td>
<td>CHN2, STYXL1, SPIB</td>
</tr>
<tr>
<td>Others and unknown</td>
<td>49</td>
<td>SPINK5, MYO3A, PHF14, LRRC39, MAGEB3, MGP</td>
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</tbody>
</table>

* ECM: extracellular matrix
Figure legends

Figure 1. Schematic diagram of linear EBV genome (~172 Kb).

(A), In proportion to their size, largely unique (U1-U5), internal repeat (IR1-IR4) and terminal repeat (TR) sequence domains are demonstrated. Ori P (indicated in red), the origin for latent infection EBV episome replication, has plasmid maintenance and DNA replication activity. Location of exons for latent EBV-induced membrane proteins and nuclear proteins and the size of each gene are shown. Of note, LMP1 transcription has an opposite direction to the other gene transcripts. LMP2 can only be transcribed when EBV exists as episome because the gene spans across the TR. LMP2A is transcribed from the exon 1, whereas LMP2B is transcribed from the exon 2. EBNAs are transcribed from differently spliced primary EBNA transcript. In latency III, EBNA1 transcription begins from Cp or Wp, whereas it starts from Qp in latency I or II. EBNA-LP has variable sizes due to variable numbers of repetitive exons.98

(B), Schematic diagram of circular EBV genome. The U1-U5, IR1-IR4, TR, and Ori P sequence domains are indicated. Open reading frames for latent proteins are shown. Of note, the size of each open reading frame does not illustrate the size of each latent protein.98

(C), Amino acid sequence of LMP1. C-terminal activating regions (CTARs) are indicated in red. TRAF-binding motif in CTAR1 and TRADD-binding motif are indicated in blue with an underbar in the CTAR1 and CTAR2 domains, respectively. Numbers on the right denote the order of the amino acid sequence.99 Cp: C promoter, Qp: Q promoter, Wp: W promoter, LMP: latent membrane protein, EBNA: Epstein-Barr virus nuclear antigen, EBNA-LP: EBNA leader protein, EBER: Epstein-Barr virus-encoded RNA

Figure 2. Schematic diagram of EBV-mediated oncogenic signalling pathway activation in EBV-positive diffuse large B-cell lymphoma of the elderly. LMP1 has a six-transmembrane domain with a long cytoplasmic C-terminal chain. It can self-aggregate to activate itself constitutively and provide a platform to interact with downstream molecules. LMP1 provides a proliferation signal via activating...
NF-κB, PI3K/Akt, MEK-ERK, and JNK-AP-1 MAP kinase pathways. It also gives signal for cell cycle progression by enhancing cyclin-dependent kinase 2 and phosphorylation of retinoblastoma protein and by inhibiting p16 and p27. LMP1 can also activate bcl-2 to provide an anti-apoptotic signal. CTAR1 can directly interact with TRAF1, 2, 3, and 5 to activate NF-κB, PI3K/Akt, MEK-ERK, and JNK-AP-1 MAP kinase pathways. CTAR2 needs TRADD to interact with TRAF2 to activate NF-κB. Both canonical and non-canonical pathways of NF-κB are activated to give proliferation signal to the nucleus. There are 3 putative JAK3-binding motifs between CTAR1 and CTAR2. However, this finding was not reproduced by others.26-36 AP-1: activator protein 1, CTAR: C-terminal activation region, ERK: extracellular signal-regulated kinases, JAK3: Janus kinase 3, JNK: Jun amino-terminal kinases, MEK: MAPK (mitogen-activated protein kinase)/ERK kinase, PI3K: phosphatidylinositol 3-kinase, TRADD: tumor necrosis factor receptor type 1-associated DEATH domain, TRAF: tumor necrosis factor receptor-associated factor.

Figure 3. Geographical variations of overall survival and progression-free survival in EBV positive diffuse large B-cell lymphoma of the elderly. (A and B), EBV positive DLBCL of the elderly showed worse overall survival and progression-free survival in Korean patients13; (C and D), EBV positive DLBCL of the elderly were more aggressive compared to > 60 year-old patients with ABC subtype and GCB subtype in European patients8. (E and F), In North America, EBV positive DLBCL of the elderly showed no difference in overall survival and progression-free survival in comparison to EBV negative DLBCL patients100. (G and H), In North America, EBV’CD30+ DLBCL of the elderly showed significant difference in overall survival and progression-free survival in comparison to either EBV’CD30− or EBVD30+ DLBCL patients.

Figure 4. Morphologic variants and immunophenotypic profiling in EBV-positive diffuse large B-cell lymphoma of the elderly. (A-F), The monomorphic subtype is characterized by monotonous sheets of large transformed B cells. (G-L), Polymorphic DLBCL-like shows canonical large B-cell neoplasm morphology, characterized by a high density of large neoplastic cells and scattered cells with (Reed-
Sternberg) RS-like and Hodgkin-like features. (M-R), The polymorphic HL-like image, the image with DLBCL with Hodgkin lymphoma-like features, shows a lower density of neoplastic cells with RS-like and Hodgkin-like features. (S-X), The polymorphic PLPD-like subtype is DLBCL with polymorphic lymphoproliferative disorder (LPD)-like features. It is characterized by a low density of neoplastic cells without HL-like features. EBV-positive DLBCL of the elderly is predominantly the ABC subtype. Interestingly, the monomorphic case presented in this figure shows the GCB subtype (A-F). All polymorphic subtypes show the ABC-DLBCL molecular phenotype.

Figure 5. Gene expression profiling in the DLBCL ABC subtype was performed according to EBV status. The EBV-positive case shows a distinct gene expression pattern compared to cases with EBV negativity, with overexpression of multiple components of the NF-κB pathway.

Figure 6. Therapeutic modulation of EBV infection and associated signaling pathways. Strategies to inactivate EBV infection or EBV associated oncogenic pathways have been explored in lymphoma cells. Many therapeutic targets have been identified. (1) Conventional chemotherapeutic agents or radiation target DNA. (2) Different strategies disrupting miRNAs can be offered. (3) EBV-specific cytotoxic T-cells are engineered and re-infused to patients as immunoregulatory therapy approach. (4) Lytic cycle of EBV is induced by a couple of agents and anti-herpetic agents targeting virus in lytic cycle are added. Agents targeting (5) NF-κB pathway, (6) PI3K/Akt pathway, (7) PKC or (8) MAPK pathway can be tried. Monoclonal antibodies, Brentuximab vedotin and Rituximab, are available targeting, (9) CD30 and (10) CD20, respectively.
EBV Genome, 172 kb

CTAR1 (amino acid 194-232)
E H H H D D S L P H P Q Q A T D D S
G H E S D S N S N E G R H H L L V S G
A G
**PQQAT**: TRAF-binding motif

CTAR2 (amino acid 350-386)
S G H G G G D P H L P T L L L G S S G
S G G D D D D P H G P V Q L S Y Y D
**PVQLSYY**: TRADD-binding motif
Figure 3

Overall Survival

A

P = 0.026

EBV- (n=346)

EBV+ (n=34)

EBV-CD30+ (n=65)

EBV+CD30+ (n=14)

B

P = 0.018

EBV- (n=346)

EBV+ (n=34)

EBV+CD30+ (n=14)

Survival Time (months)

Survival Time (%)
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<th>MUM1</th>
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<th>EBER</th>
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Figure 4
EBV-positive diffuse large B-cell lymphoma of the elderly

Chi Young Ok, Thomas G. Papathomas, L. Jeffrey Medeiros and Ken H. Young