Epstein-Barr Virus Provides a New Paradigm: A Requirement for the Immediate Inhibition of Apoptosis

Markus Altmann, Wolfgang Hammerschmidt
Department of Gene Vectors, GSF-National Research Center for Environment and Health, München, Germany

DNA viruses such as herpesviruses are known to encode homologs of cellular antiapoptotic viral Bcl-2 proteins (vBcl-2), which protect the virus from apoptosis in its host cell during virus synthesis. Epstein-Barr virus (EBV), a human tumor virus and a prominent member of γ-herpesviruses, infects primary resting B lymphocytes to establish a latent infection and yield proliferating, growth-transformed B cells in vitro. In these cells, 11 viral genes that contribute to cellular transformation are consistently expressed. EBV also encodes two vBcl-2 genes whose roles are unclear. Here we show that the genetic inactivation of both vBcl-2 genes disabled EBV’s ability to transform primary resting B lymphocytes. Primary B cells infected with a vBcl-2-negative virus did not enter the cell cycle and died of immediate apoptosis. Apoptosis was abrogated in infected cells in which vBcl-2 genes were maximally expressed within the first 24 h postinfection. During latent infection, however, the expression of vBcl-2 genes became undetectable. Thus, both vBcl-2 homologs are essential for initial cellular transformation but become dispensable once a latent infection is established. Because long-lived, latently infected memory B cells and EBV-associated B-cell lymphomas are derived from EBV-infected proapoptotic germinal center B cells, we conclude that vBcl-2 genes are essential for the initial evasion of apoptosis in cells in which the virus establishes a latent infection or causes cellular transformation or both.

Introduction

Apoptosis is a mechanism used by the infected cell as part of its antiviral response; cells can commit suicide as a direct response to viral infection prior to viral maturation. Therefore, regulating apoptosis during infection is a high priority for many viruses that frequently encode Bcl-2-like mechanisms to do so [1]. Cellular Bcl-2 was originally discovered as an oncogenic protein in follicular lymphomas and up to now many Bcl-2 family members have been identified that share distinct domains of high sequence homology. Bcl-2 family members include apoptosis-inhibitory proteins, such as Bcl-2 and Bcl-XL, or those that mediate proapoptotic functions exemplified by Bax. All viral Bcl-2 (vBcl-2) homologs identified so far block apoptosis. The major role of vBcl-2 proteins is therefore to prevent premature death of the host cell during virus production, which would otherwise reduce the amount of progeny virus [2,3]. Adenovirus E1B 19K, for example, is a minimal version of the cellular Bcl-2 prototype, which prevents premature cell death during productive infection. Bcl-2 and E1B 19K can functionally substitute for each other in the suppression of apoptosis during virion synthesis and oncopgenic transformation [1,3]. While members of the γ-herpesvirus family carry one vBcl-2 gene in general, Epstein-Barr virus (EBV) encodes two Bcl-2 homologs, BALF1 and BHRF1. The BHRF1 protein clearly resembles Bcl-2 in its antiapoptotic function during in vitro assays [4], while the role of BALF1 is controversial [5,6]; however, no function has been assigned to either BALF1 or BHRF1 in the context of viral infection [1,7]. Interestingly, vBcl-2 homologs have not been recognized to contribute to viral latent infection directly [2,8,9], probably because viral latency can only be studied with very few viruses. In addition, functions involved in viral “fitness” often overlap with specific functions, which contribute directly to initiation and maintenance of viral latency in vivo [10–12].

EBV provides a unique in vitro model, which permits the dissection of viral contributions to latent infection. EBV can infect all cells of the B-cell lineage, but its main targets in vivo are naïve B cells and B cells that undergo affinity maturation during a germinal center reaction to establish long-term latent infections in memory B cells [13,14]. In vivo and in vitro EBV’s latent state is characterized by the absence of virus synthesis and maintenance of the viral genome as plasmids in the infected cell. In vitro, EBV infects resting human B lymphocytes and transforms them into lymphoblastoid cell lines (LCLs), a process that is termed growth transformation and a hallmark of this virus. In LCLs, 11 so-called latent genes are consistently expressed. These

Citation: Altmann M, Hammerschmidt W (2005) Epstein-Barr virus provides a new paradigm: A requirement for the immediate inhibition of apoptosis. PLoS Biol 3(12): e404.
are the EBV nuclear antigens EBNA1, EBNA2, EBNA-LP, EBNA3A, -B, -C, and the latent membrane proteins LMP1, LMP2A, and -B, and two noncoding RNAs [15]. Genetic and biochemical experiments demonstrate that several of these viral proteins directly contribute to growth transformation and latency of infected cells in vitro and in vivo, although only a subset of these 11 genes are expressed in the human host [16]. Typically, latent genes of EBV mimic cellular functions. For example, the viral proteins LMP1 and LMP2A target physiological signaling pathways, which are engaged by the B-cell and CD40 receptors upon contact with antigen and T helper cells, respectively. LMP1 and LMP2A are also expressed in EBV-positive B-cell lymphomas such as Hodgkin’s disease and posttransplant lymphomas [13], which are two of several malignancies with which EBV has been associated [16,17]. LMP1 indirectly inhibits apoptosis by upregulating several cellular antiapoptotic genes [18], presumably through the induction of the NF-κB pathway [19–21]. LMP1 promotes cell proliferation [18,22] and scores as an oncogene in transgenic animals [23]. In LCLs, but not in EBV-associated tumors, LMP1 and LMP2A are transcriptionally upregulated by EBNA2, which is the first latent viral gene expressed after infection of B cells in vitro. EBNA2 is the key viral factor, which is essential for in vitro growth transformation of resting primary B cells [24,25], along with LMP1 [26], EBNA1 [15], EBNA3A, and -C [27]. This minimal set of five latent EBV genes has been regarded as sufficient to yield growth-transformed LCLs in vitro [15], although the requirements for EBNA3A, EBNA1, and LMP1 appear to be less stringent [28–30]. None of the known latent gene products of EBV directly regulate apoptosis, and little is known about viral genes expressed very early in primary B cells upon viral infection. We analyzed the two vBcl-2 genes of EBV—BHRF1 and BALF1—and demonstrate that their gene products in addition to the known latent EBV genes are essential for the process of B-cell transformation. The vBcl-2 homologs are maximally expressed initially after infection but are neither expressed nor required once a latent infection is established. The very early expression of vBcl-2 genes prevents EBV-infected B cells from undergoing spontaneous programmed cell death and is mandatory to establish a latent infection and cause cellular transformation. We conclude that EBV employs a biologic strategy—characterized by viral genes expressed transiently and those that are expressed stably—to initiate and maintain a latent infection.

**Results**

**vBcl-2 Genes Are Essential for Growth Transformation of Resting B Cells but Dispensable for Proliferating B Blasts**

In order to gain insight into the function of BALF1 and BHRF1, we constructed several viral mutants, which carry singly or dually inactivated alleles of BALF1 and BHRF1 (Figure 1A and 1B). Mutant virus stocks were generated as described [31]. Surprisingly, virus production was not impaired even in the case of the dually inactivated vBcl-2 mutants, and transcomplementation of either BALF1 or BHRF1 in virus-producing cells did not yield higher titers (data not shown). Viral stocks were quantified by infecting Raji cells, an established B-cell line [29]. Because our recombinant EBVs encode green fluorescence protein (GFP), we could measure the concentration of GFP-transducing virions as “green Raji units” (GRUs), which were in the range of $10^4$–$10^5$/ml GRUs similar to wild-type 2089 EBV stocks [32]. Primary B cells were infected with serial virus dilutions to determine the number of GRUs required statistically to give rise to clonal LCLs [33]. Two GRUs were sufficient in the case of 2089 wild-type EBV, and the single BALF1- or BHRF1-mutants were equally capable of yielding clonal LCLs but at a slightly higher virus dose (Figure 1B–1D). Two independent viral mutants with inactivated vBcl-2 genes failed to generate LCLs, even at a dose of $10^4$ GRUs per well (Figure 1C and 1D).

Reconstruction of the BALF1 allele in the revertant BALF1+/ BHRF1+ virus reconstituted EBV’s capacity to growth transform human B lymphocytes (Figure 1C and 1D).

We determined whether infection of preactivated human B cells might overcome the failure of the vBcl-2 mutants to establish growth-transformed LCLs from resting primary B cells. B blasts, which can be generated from resting primary B cells by cocultivation on CD40 ligand-expressing feeder cells in conjunction with IL-4, proliferate in vitro and express cellular antiapoptotic Bcl-2 family members, which are upregulated through the CD40 and JAK/Stat-induced signaling pathways [34,35]. The continued proliferation of B blasts is totally dependent on both activating stimuli. Upon removal of either CD40L or IL-4 signals, the B blasts cease to proliferate immediately and undergo apoptosis within 10 d (data not shown). Limiting dilution assays with activated B blasts as target cells and serially diluted BALF1+/BHRF1+ 2636 virus (Figure 1B) resulted in clonal LCLs, which proliferated in the absence of either CD40L or IL-4 signals, indicating that preactivated B cells compensate for the functional defect of a vBcl-2− virus (Figure 1E). Once established, these LCLs infected with vBcl-2+ EBV proliferated normally and did not show an apparently different phenotype when compared with wild-type EBV-infected LCLs (data not shown).

**vBcl-2 Genes Are Maximally Expressed Very Early After EBV Infection of Primary B Cells**

Eleven EBV genes have previously been identified as being constitutively expressed in established LCLs. Neither BALF1 nor BHRF1 is among this group, however [15,36]. Latent EBV genes such as EBNA2 and LMP1 encode key regulators of cellular proliferation, which are required to initiate and maintain proliferation of lymphoblastoid cells in vitro [22,24–26,37]. Therefore, we set out to analyze the expression kinetics of BALF1 and BHRF1 in infected primary B cells. As shown in Figure 2, upon infection with the B95.8 prototype EBV strain, BALF1 and BHRF1 transcripts were readily detected within 24 h postinfection (p.i.) but not in cells infected at 4 °C or in uninfected cells (Figure 2A and 2E). Whereas EBNA2 is constitutively expressed in established latently infected LCLs (data not shown [15]), the expression of both BALF1 and BHRF1 rapidly decreased, and their transcripts were weakly or not detected by RT-PCR 3 wk p.i. (Figure 2A and 2E). EBNA2 transcripts could be detected 1 d p.i. in wild-type 2089 EBV or 2636 BALF1+/BHRF1+ mutant infected primary B cells (Figure 2B). Similarly, vBcl-2 transcripts are readily detectable in cells infected with an EBNA2− 2491 mutant EBV (Figure 2C), indicating that EBNA2 and vBcl-2 genes are independently expressed very early after infection.
As expected, BALF1 and BHRF1 were not expressed in other latently EBV-infected cells (Figure 2D) but readily detectable in cells that spontaneously support the lytic phase of EBV's life cycle (B95.8 cells in Figure 2A and 2D) and maximally expressed in cells in which the lytic phase was induced (Figure 2D). Because both vBcl-2 genes were induced during EBV's lytic phase, and several reports indicate that the lytic expression of BHRF1 is strictly dependent on the immediate-early transcriptional activator BZLF1 ([15,38] and references therein), we wanted to learn whether initial expression of BALF1 or BHRF1 in newly infected primary B cells is also regulated by BZLF1. BZLF1 encodes a molecular switch protein, which is instrumental in inducing the lytic phase in latently EBV-infected cells ([15] and references therein). The EBV mutant 2809, which is BZLF1− [39], was used to infect primary B cells, and the expression of BALF1 and BHRF1 was assessed by RT-PCR. Again, vBcl-2 transcripts were readily detectable in cells infected with the BZLF1− 2809 EBV mutant, indicating that BZLF1 does not regulate BALF1 and BHRF1 expression (Figure 2C). As expected, EBNA2 expression was also BZLF1 independent (Figure 2B).

Cell Cycle Activation Is Abrogated in vBcl-2– EBV-Infected Cells

We questioned whether vBcl-2 gene products might be critical for cell cycle entry similar to EBNA2 [15,40]. Stocks of the BALF1−/BHRF1− 2636 virus were used to infect primary B cells at a multiplicity of infection (MOI) of 0.5 GRU with the EBNA2− mutant 2491 or wild-type EBV 2089 serving as controls. For direct comparison, a fraction of the cells was left uninfected. The cell cycle status of the cells was analyzed by 5-bromo-2′-deoxyuridine (BrdU)-incorporation and fluorescence-activated cell-sorting (FACS) analysis, which revealed cycling cells in wild-type EBV-infected cells as early as day 3 p.i. (Figure 3). Uninfected cells did not enter the cell cycle nor did the cells infected with the EBNA2− or the BALF1−/BHRF1− 2636 mutants. These latter infected cells all showed a rapid increase in the fraction of cells with a subG1 DNA content indicating that the inactivation of both vBcl-2 genes prevented cell cycle entry similar to EBNA2− EBV, which lacks the key regulator of EBV's latent genes (Figure 3).

B-Cell Apoptosis Is Delayed in vBcl-2− EBV-Infected Primary B Cells

Given that EBNA2 is a pivotal mediator of EBV's known latent genes, it is important to know if EBV's vBcl-2 genes, which are required to establish latency (see Figure 1), depend on any of EBNA2's functions. We tested whether primary B cells infected with the EBNA2− mutant, which is capable of expressing both vBcl-2 genes (see Figure 2C), showed a phenotype different from cells infected with a mutant null for vBcl-2. Because both viral mutants are deficient in inducing cellular proliferation (see Figure 3), we could concentrate on effects related to apoptotic markers independent of global cellular activation (Figure 4). Toward this end, we infected primary B cells with the EBNA2− 2491 or the BALF1−/BHRF1− 2636 viruses (or wild-type 2089 EBV as a positive control) and determined the occurrence of GFP+/PI− EBV-infected cells by FACS (Figure 4C). To monitor the numbers of GFP+ cells over a period of 8 d, calibration beads were used as an internal reference (Figure 4A). By three-parameter FACS analysis, GFP+ B cells were also analyzed for binding of Annexin-V and propidium iodide (PI) uptake as indicators of early apoptosis and loss of membrane integrity, respectively (Figure 4D). In parallel, uninfected cells with typical lymphocytic characteristics by forward and sideward scatter criteria (Figure 4B) were compared for their Annexin-V and PI staining. As shown in Figure 4D and 4E, uninfected primary B cells as well as those infected with BALF1−/BHRF1− 2636 mutant rapidly lost their viability. As early as day 3 p.i., about 70% of the BALF1−/BHRF1− virus-infected cells were Annexin-V+/PI+ comparable to uninfected cells. By day 5, few lymphocytes had survived and none were found on day 8 (Figure 4E). In stark contrast, all primary B cells infected with the EBNA2− mutant 2491, which expressed BALF1 and BHRF1 transcripts immediately after infection (see Figure 2C), were alive on day 3 p.i., about 15% survived until day 5, and a few percent of the cells were GFP+/Annexin-V/PI− 8 d p.i. (Figure 4D and 4E). Thus, EBV's Bcl-2 homologs support initial B-cell survival and rescue EBV-infected B cells, which would otherwise succumb rapidly to spontaneous apoptosis.

Discussion

vBcl-2 Genes Are Essential for Growth Transformation of Primary B Lymphocytes

Some viruses encode proteins that interfere with the host's apoptotic machinery to ensure short-term cell survival and to yield progeny before the cell lyses and dies [3,41,42]. Similarly, γ-herpesviruses encode vBcl-2 genes, whose gene products are antiapoptotic Bcl-2 family members and act during productive infection or contribute to viral virulence [10,12]. Only EBV is known to encode two Bcl-2 homologs, BALF1 and BHRF1. BHRF1 was shown to be dispensable for B-cell growth transformation [43,44], but its product does have antiapoptotic functions [4,45]. The function of the BALF1 gene product is controversial [5,6]. Our experiments

Figure 1. Construction and Genealogy of EBV Mutants and Their Efficiency of B-Cell Growth Transformation

(A) Construction of the loss of function mutation in BALF1 and BHRF1. (B) Wild-type (WT) 2089 EBV is based on the genome of the EBV strain B95.8. The BALF1 and BHRF1 genes were inactivated through insertional mutagenesis with antibiotic resistance genes. Two vBcl-2 mutants 2636 and 2765 were constructed independently on the basis of the singly inactivated viruses, and the BALF1−/BHRF1− mutant 2636 was reverted to BALF1+/BHRF1+. (C) The numbers indicate the number of GRUs of the virus stocks, which were required to yield one clonal LCL for each virus noted in (B). (D) Efficiency of B-cell growth transformation. The different singly and dually vBcl-2− EBV virus mutants were compared with wild-type 2089 EBV for their efficiencies to growth transform primary B cells in limiting dilution assays. Forty-eight wells with 10^4 target B cells per well were infected with each virus dilution, and wells with proliferating cells were recorded 6 wk p.i. The data are graphed to identify the number of GRUs of the different virus isolates required to yield one LCL. The horizontal line at 30 wells positive for 48 wells plated identifies for a Poisson distribution the required number of GRUs shown on the x-axis. Infection of primary B cells with the vBcl-2− mutants 2636 and 2765 did not yield stable LCLs even with up to 10^4 GRUs per 10^5 cells per well. (E) Comparison of the efficiency of wild-type 2089 EBV and the BALF1−/BHRF1− mutant 2636 to yield stable, clonal LCLs in limiting dilution assays with primary B cells or activated B blasts as target cells. Activated B blasts readily gave rise to LCL clones with the BALF1−/BHRF1− mutant 2636 in contrast to the situation in (D), although about 200 GRUs were statistically required to establish clonal lines, which all could be further expanded and characterized (data not shown). DOI: 10.1371/journal.pbio.0030404.g001
Viral Bcl-2 Homologs in Latent Infection

A

B

C

D

E
indicate that (i) both BALF1 and BHRF1 belong to the same genetic complementation group and are functionally redundant (see Figure 1). Either BALF1 or BHRF1 is (ii) essential to establish latently infected LCLs (see Figures 1 and 3) but (iii) dispensable for their continued proliferation (see Figure 1E) and (iv) generation of virus progeny (data not shown). In newly infected primary B cells, (v) both vBcl-2 genes are expressed immediately but transiently after infection (see Figure 2), in contrast to the group of 11 latent EBV genes, which are constitutively expressed [15,46]. Surprisingly, BALF1 and BHRF1 proteins (vi) prevent spontaneous programmed cell death in newly infected resting primary B cells (see Figure 4). This newly identified function of vBcl-2 ensures successful infection of primary B cells, gives rise to latent infection, and supports growth transformation of resting B cells in vitro.

This finding seems to be without precedent because vBcl-2 homologs have not been recognized to contribute to viral latent infection directly [2,8,9], probably because herpesviral latency can only be studied with a few viruses. Similar to EBV, long-term latency of the murine γ-herpesvirus MHV-68 is narrowly confined to cells expressing a B-cell phenotype [47,48]. Infection of mice with MHV-68 provides a tractable and authentic animal model to study herpesviral latency in the context of its natural host. A viral mutant deficient for the MHV-68 vBcl-2 gene M11 was found compromised in reactivating from latency [12], whereas a more recent paper identified a vBcl-2 associated deficit in establishment of latency [10]. Although the molecular basis for these observations remains unclear, both seem to be consistent with vBcl-2 protecting latently infected B cells from apoptotic death. Similar to our findings, no role for vBcl-2 of MHV-68 was apparent during acute viral replication in vivo or viral virulence [10–12].

How Are vBcl-2 Genes Regulated in Newly Infected Primary B Cells?

Experimental data clearly demonstrate that BALF1 and BHRF1 are maximally expressed in primary B cells within 24 h p.i. (see Figure 2A). Because the expression of BALF1 and BHRF1 is also prominent during EBV's lytic phase (see Figure 2D), we wondered whether their expression might be regulated by the viral immediate-early gene BZLF1. BZLF1 is a transcription factor and acts like a molecular switch to induce the lytic phase in latently EBV-infected cells ([15] and references therein). Unexpectedly, the expression of both vBcl-2 genes was found to be independent of BZLF1 (see Figure 2C). Thus, we do not know the mechanisms regulating the expression of both BALF1 and BHRF1, but we would like to speculate that both genes are directly and spontaneously expressed from the transducing EBV genome. The herpesviral DNA delivered to the nucleus of the infected cell is unmethylated, coated with polyamines [49], and not in a
chromatin-like state [50], which should permit universal access of the transcriptional machinery to many viral promoter elements. Modifications of the EBV genomic DNA over time will alter its accessibility, which might cause a rapid decline in the expression of BALF1 and BHRF1 when the virus eventually establishes a genuine latent infection (see Figure 2A).

vBcl-2 Homologs Are Likely to Be Involved in In Vivo Latency and Oncogenesis of EBV-Associated Tumors

EBV-associated B-cell lymphomas originate from germinal center B cells, which are inherently prone to apoptosis as they undergo affinity maturation and somatic hypermutation in their B-cell receptor genes, events characteristic of this stage.
Viral Bcl-2 Homologs in Latent Infection

A. CaliBRITE beads gate

B. Lymphocyte gate
day 1  day 8

C. GFP+ gate
uninfected  infected

D. Annexin-V

- day 1
- day 3
- day 5
- day 8
- post infection
- uninfected
- BALF1+/BHRF1-
- EBNA2-
- 2089 wild-type EBV

E. Plot of infected cells over time
- AnnexinV+/PI-
- AnnexinV+/PI+FP+
Figure 4. Apoptosis of Primary B Cells

Primary B cells were infected with the EBNA2Δ mutant 2491, the BALF1Δ/BHRF1Δ mutant 2636, or wild-type 2089 EBV, at an MOI of 0.1, or the cells were left uninfected. Cells were analyzed by FACS at days 1, 3, 5, and 8 p.i. (A) Total cellular events were collected until 3 × 10^5 EBV particles were added per cell, which was a constant value. These beads, which are indicated by red circles in the FACS diagrams in (A) and (C), were as an internal volume reference corresponding to 3 × 10^5 cells plated initially. (B) Uninfected cells that fulfilled the criteria of lymphocytes according to their forward (FSC) and side scatter (SSC) characteristics were gated. Lymphocytes, annexin-V positive, annexin-V negative, or annexin-V positive/PI positive cells were present in this gate as expected, but only a few lymphocytes were still present in this gate 8 d after cell preparation when the cells had been left uninfected. Similarly, EBV-infected lymphocytes were selected according to the same scatter criteria (data not shown). (C) EBV-infected GFP+ cells were gated as indicated. The example shows an uninfected and a 2491 EBNA2Δ EBV-infected B-cell sample with 3 × 10^6 EBV particles added 1 d p.i. (D) Uninfected primary B cells within the lymphocyte gate or EBV-infected GFP+ lymphocytes were analyzed by FACS for Annexin-V and PI staining at different time points p.i. The absolute numbers of Annexin-V+ cells allowed the calculation of surviving cells at each time point p.i. Uninfected cells indicate the rate and kinetics of spontaneous apoptosis of primary B cells ex vivo. Primary B cells infected with the BALF1Δ/BHRF1Δ mutant 2636 died as rapidly as uninfected cells. Only B cells infected with the EBNA2Δ mutant 2491, which is vBcl-2-, survived considerably longer. Primary B cells infected with wild-type and (SSC) characteristic parameters were gated as indicated. One day after B-cell infection, the percent of GFP+ cells (EBV-infected cells) was calculated as the ratio of the number of GFP+ cells present in the lymphocyte gate in (D). Annexin-V+ cells (uninfected control to determine spontaneous programmed cell death), or Annexin-V- cells at each time point was calculated. One representative experiment out of three is shown.

Viral Bcl-2 Homologs in Latent Infection

of B-cell differentiation. It is unclear whether EBV infects naïve B cells prior to their germlinal center passage [51,52] or germinal center B cells directly [53]. Our data indicate in either case that BALF1 and BHRF1 are involved in the in vivo survival of EBV-infected CD77+ germinal center B cells. These cells normally are highly sensitized to apoptotic signals, and most will be eliminated via programmed cell death [54]. EBV is expected to provide an important signal or signals to prevent apoptosis common to germinal center B cells because a fraction of EBV-infected and malignant tumor cells contain only nonfunctional B-cell receptor genes and would have been expected to undergo programmed cell death. EBV’s vBcl-2 proteins likely constitute this survival signal, which would be superseded by the eventual expression of LMP2A [55,56] and/or other vBcl-2 proteins likely constitute this survival signal, which would be superseded by the eventual expression of LMP2A [55,56] and/or other vBcl-2 proteins likely constitute this survival signal, which would be superseded by the eventual expression of LMP2A [55,56] and/or other vBcl-2 proteins likely constitute this survival signal, which would be superseded by the eventual expression of LMP2A [55,56] and/or other vBcl-2 proteins likely constitute this survival signal, which would be superseded by the eventual expression of LMP2A [55,56] and/or other vBcl-2 proteins likely constitute this survival signal, which would be superseded by the eventual expression of LMP2A [55,56] and/or other vBcl-2 genes or the gene in the EBV genome respectively. The BHRF1 gene was constitutively expressed in a recent review [57]. To promote the integration of EBV strain cloned onto the F-factor plasmid p2089 [32]. The deletion mutants were constructed on the basis of the wild-type B95.8 center passage, we favor the notion that during this passage...
irradiated CD4-CD8- feeder layer in the absence of IL-4 and infected with serially diluted virus stocks as described above. Fifty percent of the volume of the cell culture media was exchanged on a weekly basis, and the cells were cultivated for 6 wk, during which the CD4-CD8-feeder cell layer had completely disintegrated.

RT-PCR analysis. RNA was extracted from primary B cells with the RNeasy Mini Kit (Qiagen, Valencia, California, United States); 5 μg of RNA was reverse transcribed with the Superscript III First Strand Synthesis Kit (Invitrogen, Carlsbad, California, United States) according to the manufacturer's protocol in a total volume of 20 μl. To monitor cellular DNA contamination of the RNA preparation, PCR reactions were performed with primers for the abundant cellular transcripts HPRT and β-actin. PCR reactions for β-actin were an initial template denaturation of 4 min at 94°C, with amplification for 25 cycles (1 min at 94°C, 1 min at 61°C, 1 min at 72°C), followed by a final elongation for 10 min at 72°C. PCR reactions for HPRT were 4 min at 94°C, with amplification for 25 cycles (1 min at 94°C, 1 min at 54°C, 1 min at 72°C), followed by a final elongation for 10 min at 72°C. Two out of 20 μl of the cDNA reaction were used as template. Unspliced PCR amplification products indicative of cellular DNA contamination were not detected (see Figure 2). PCR reactions for cDNA detection of three EBV genes were as follows: ERNA2 (an initial template denaturation of 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 61°C, 1 min at 72°C; followed by a final elongation for 10 min at 72°C); BHFR1 (an initial template denaturation of 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 61°C, 1 min at 72°C; followed by a final elongation for 10 min at 72°C); and BALK1 (an initial template denaturation of 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 61°C, 1 min at 72°C; followed by a final elongation for 10 min at 72°C). Oligonucleotide primer sequences for the EBV genes ERNA2, BALK1, and BHFR1 are provided in Figure 2E together with the nucleotide coordinates of the EBV strain B95.8. The primer sequences for HPRT cDNA detection were 5′-GAGCTTGGTATAGAAGCTGTC-3′ and 5′-CCAAAAGTCAACTTGGACCTC-3′; β-actin cDNA was amplified with the primers 5′-CAGCCCTGTCCTGCTCACGGAGGCG-3′ and 5′-AGCGAAGCTTGCAATAGTGTGA-3′.

Cell cycle analysis and analysis of apoptosis. Primary B cells (4 × 10^6) were infected with 4 × 10^3 GRUs of the different virus stocks in 20 ml of culture medium in order to obtain an MOI of 0.1. The cells were kept on plastic, and at days 1, 3, 5, and 8 one-fourth of the culture (5 ml) was harvested and further analyzed for cell cycle status and apoptosis. Infection experiments were carried out with 2089 EBV, the ERNA2 deletion mutant 2491, and the different mutant virus stocks (see Figure 1); for a negative control the cells were left uninfected. To determine the fraction of apoptotic cells, Annexin-V staining was performed with the Annexin-V-APC kit (BioVision, Mountain View, California, United States), according to the manufacturer's protocol. For each time point, 5 ml of each sample was harvested, spun, and the cells were stained with 5 μl of APC-coupled Annexin-V and 5 μl of PI at a final concentration of 10 μg/ml. As an internalization standard, a 1000 μM 6-FAM-DNA affinity purified Biotin-DNA (Becton-Dickinson, Palo Alto, California, United States) were added to yield a final concentration of 2 × 10^9 beads/ml. The beads are very small, resulting in a high intensity in the sideward scatter channel. Since the beads also display a very bright APC fluorescence, their characteristics do not interfere with the cells to be analyzed but allow their unbiased detection and quantification. FACScan analysis was carried out in a FACS-Calibur machine (Becton-Dickinson). The 3 × 10^5 BD CaliBRITE Beads were set as a volume standard to 3 × 10^5 cells plated initially to determine the absolute number of cells at any given time point without interference from the dynamics of the cell culture. To exclude dead cells, only cells within the lymphocyte gate were analyzed (see Figure 4B).

Cells infected with the different recombinant virus stocks express GFP as early as day 1 p.i. The GFP gate was set such that only cells with a GFP signal brighter than primary B cells infected with the GFP+ prototypic B95.8 EBV strain scored positive as infected GFP+ cells (see Figure 4C). EBV-infected GFP+ cells in the lymphocyte gate were analyzed for their Annexin-V and PI staining. In uninfected samples, only cells that fulfilled the criteria of lymphocytic cells by forward and sideward scatter criteria were analyzed for both Annexin-V and PI staining. The number of Annexin-V+ PI cells in the infected samples and the uninfected control were set to 100% at day 1 p.i. To analyze the cell cycle status of the infected primary B cells in comparison to the uninfected controls, the samples were incubated with the thymidine analog BrdU for 2 h prior to FACS analysis at each time point. The cell proliferation assays were immediately performed with the BrdU Flow Kit (BD Biosciences Pharmingen, San Diego, California, United States). The cells were stained with an APC-coupled BrdU-specific antibody after fixation and permeabilization, and the cellular DNA was counterstained with the DNA intercalating dye 7-AAD according to the manufacturer's protocol. FACS analysis was performed until 3 × 10^5 cells were analyzed. The recorded data were gated for cells in the G1, S, G2/M phases of the cell cycle and for cells with a subG1 DNA content. The total of all events was set to 100%.

**Supporting Information**

**Accession Numbers**

UniProtKB/T ReMBL (http://www.expasy.org/uniprot) accession numbers for the proteins are Bax (Q07812), Bcl-XL (Q07817), Bcl-2 (P10415), p57 receptor (P25024), LMP1 (P09230), LMP2A (Q77714), and LMP2B (Q8AZK5); for the virus, E1B 19K (P03247); for the genes, BALK1 (Q777A6) and BHFR1 (Q777H0); for EBV nuclear antigens, EBNAL1 (P03211), EBNAL2 (Q09923), EBNA3A (Q8AZJ8), EBNA3B (Q777E8), EBNA3C (Q777E7), and EBNA-LP (Q8AZK7).

**Acknowledgments**

We thank Bill Sugden for helpful discussions, suggestions, and comments on the manuscript. We are also extremely grateful to Andreas Moosmann for providing us with B blasts and technical advice. Our work was supported by SFB455 of the Deutsche Forschungsgemeinschaft, the Bayerische Forschungsstiftung, and the Sanderstiftung and by a National Institutes of Health Public Health Service Grant CA70723.

**Competing interests.** The authors have declared that no competing interests exist.

**Author contributions.** WH conceived and designed the experiment, performed the experiments, wrote the data, MA and WH analyzed the data, MA and WH contributed reagents/materials/analysis tools, WH wrote the paper.

**References**

1. Gacconi A, White E (2002) Viral homologs of BCL-2: Role of apoptosis in the regulation of the virus infection. Genes Dev 16: 2465–2478.
2. Boya P, Pauleau AL, Poncect D, Gonzalez-Polo RA, Zamzami N, et al. (2004) Viral proteins targeting mitochondria: Controlling cell death. Biochim Biophys Acta 1670: 178–189.
3. Benedict CA, Norris PS, Ware CF (2002) To kill or be killed: Viral evasion of human B cells from programmed cell death. Proc Natl Acad Sci U S A 99: 8479–8483.
4. Bellows DS, Howell M, Pearson C, Hazlewood SA, Hardwick JM (2002) Epstein-Barr virus BHRF1 protein: A viral version of Bcl-2 protects human B cells from programmed cell death. Proc Natl Acad Sci U S A 99: 8479–8483.
5. Marshall WL, Yim C, Graf T, Sage DR, et al. (1999) Epstein-Barr virus encodes a novel homolog of the bcl-2 oncogene that inhibits apoptosis and associates with Bax and Bcl-2. J Biol Chem 274: 5181–5186.
6. Isken PW, Chen Y, Hardwick JM (2004) Viral modulation of cell death provide new links to old pathways. Curr Opin Cell Biol 15: 700–705.
7. Hilleman MR (2004) Strategies and mechanisms for host and pathogen survival in acute and persistent viral infections. Proc Natl Acad Sci U S A 101: 11566–11566.
8. Roush A, Marcellus RC, Branton PE (1999) Viruses and apoptosis. Annu Rev Microbiol 53: 577–628.
9. de Lima BD, May JS, Marques S, Simas JP, Stevenson PG (2003) Murine gammaherpesvirus 68 bcl-2 homologue contributes to latency establishment in vivo. J Gen Virol 84: 31–40.
10. Group A, Capsid SB, Speck SH, Virgin HW (2002) Antibody to a lytic cycle viral protein decreases gammaherpesvirus latency in B-cell-deficient mice. J Virol 76: 11469–11468.
11. Kieff E, Rickinson AB (2001) Epstein-Barr virus latent infection and disease. In: Farber EN, Roizman B (eds) The Baltimore herpesvirus family, 2nd ed. Academic Press, San Diego, CA, pp 105–251.
12. Kieff E, Rickinson AB (2001) Epstein-Barr virus and its replication. In: Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, et al. (eds) Fields' virology, 4th ed. Philadelphia: Lippincott–Williams and Wilkins. pp 2511–2573.
