EBI2 expression in B lymphocytes is controlled by the Epstein–Barr virus transcription factor, BRRF1 (Na), during viral infection

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Abstract

Epstein–Barr virus-induced gene 2 (EBI2) is an important chemotactic receptor that is involved in proper B-cell T-cell interactions. Epstein–Barr virus (EBV) has been shown to upregulate this gene upon infection of cell lines, but the timing and mechanism of this upregulation, as well as its importance to EBV infection, remain unknown. This work investigated EBV’s manipulation of EBI2 expression of primary naive B cells. EBV infection induces EBI2 expression resulting in elevated levels of EBI2 after 24 h until 7 days post-infection, followed by a dramatic decline (P=0.027). Increased EBI2 expression was not found in non-specifically stimulated B cells or when irradiated virus was used. The EBV lytic gene BRRF1 exhibited a similar expression pattern to EBI2 (R²=0.4622). BRRF1-deficient EBV could not induce EBI2. However, B cells transduced with BRRF1 showed elevated expression of EBI2 (P=0.042), a result that was not seen with transduction of a different EBV lytic transfection factor, BRLF1. Based on these results, we conclude that EBI2 expression is directly influenced by EBV infection and that BRRF1 is necessary and sufficient for EBI2 upregulation during infection.

INTRODUCTION

Epstein–Barr virus (EBV), a human B-lymphotropic herpes virus, infects over 90% of adults [1–3]. Primary EBV infection usually occurs in childhood [4]. Although most infections are asymptomatic, delayed infection into adolescence commonly causes infectious mononucleosis [5, 6]. In this case, the virus is spread via saliva and infects permissive epithelial cells of the oropharynx [7]. During this time, the virus also infects mucosal B cells. Once B cells are infected, the virus enters a stage of latency. This results in the expansion of an lymphoblastoid cell lines (LCL)-like population of B lymphocytes in the tonsils [8]. This also allows for the virus to avoid immune detection and remain with the host for the rest of their life, persisting in the B-lymphocyte population. Infection with EBV is also associated with and suspected of being responsible for several types of cancers [9]. It is a strongly associated agent of Burkitt’s lymphoma, as well as various types of non-Hodgkin’s lymphomas, nasopharyngeal carcinoma and immunoproliferative disorders, and is associated as an environmental trigger in autoimmune diseases such as systemic lupus erythematosus [10–14]. Post-infection (p.i.), EBV is adept at controlling lymphocyte gene expression, likely manipulating EBV-induced gene 2 (EBI2) expression, among others, as a means of controlling B-lymphocyte migration. However, after a time, the virus lowers the expression of most viral genes and enters a latent state.

Following the establishment of latency, it is possible for EBV to reactivate due to environmentally induced stimulation. This is a problem of particular concern during tissue and organ transplants and can result in post-transplant lymphoproliferative disease [15]. Key to this process of reactivation are the various EBV lytic transcription factors, mainly BZLF1, BRLF1 and BRRF1, that assist in initiating the cascade of transcription needed to achieve viral lytic replication [16, 17]. These and various other EBV genes can also be transiently expressed early after infection. This transient expression of EBV genes before latency is often called pre-latency [18]. Pre-latency is thought to be important for viral immune evasion as various pre-latently expressed proteins assist in immune avoidance by suppressing intracellular and extracellular detection.

EBI2 is a member of the rhodopsin-like subfamily of seven-transmembrane G protein-coupled receptors. Sequence alignments have grouped EBI2 with lipid receptors, and it shows...
EBV controls expression of EBI2 upon infection. This control could affect EBV-infected cells in several ways. These include B-cell chemotaxis, improper positioning of B cells within the follicle and immune deficiency or other disruptions to cell haematopoiesis and function [26]. Altered positioning and chemotaxis of B cells is also thought to be necessary for the development and progression of lymphomas [27]. Understanding how EBV controls lymphocyte positioning could allow for improved control of infection and response to EBV-induced malignancies. To enhance our understanding of EBV infection, we investigated the expression profile of EBI2 during EBV infection and potential viral mechanisms responsible for tampering with EBI2 expression.

RESULTS

Time course of EBI2 expression after EBV infection

EBI2 is vital in directing migration of primary B cells to areas in secondary lymph tissue where they can potentially detect antigenic proteins [28]. To investigate the way EBI2 expression was influenced by viral infection, naive human B cells were infected with EBV. Levels of EBI2 expression were measured and compared to uninfected control naive B cells (Fig. 1). EBV-infected cells showed, on average, a threefold higher relative mRNA expression of EBI2 than uninfected cells. When the EBI2 expression found in infected B cells is compared to the expression in cell lines that contain EBV, there is a significant difference of gene expression. Freshly isolated naive B cells demonstrated a much higher expression of EBI2 when compared to the expression observed in the EBV-positive LCL (P=0.012) and Raji cell lines (P=0.027), which are primarily in a latent state.

To determine the pattern of EBI2 expression during the course of EBV infection, isolated naive B cells were infected with EBV and EBI2 expression was measured at intervals over a 21-day period of time. An expression time line was constructed starting with pre-infection and ending 21 days p.i. Between 3 and 6 h p.i., there was a significant decrease in EBI2 expression (P=0.002). There is also a significant increase in expression at 24 h p.i. (P=0.027) (Fig. 1). This heightened level of EBI2 expression persisted for as long as 7 days p.i. At this point, there was a large variance in the samples. This could be indicative of EBV induction of EBI2 ending prior to this time in some samples received from different donors, while others are still maintained. However, by days 8 and 9 p.i., the levels of EBI2 expression have decreased to levels equal to or lower than those observed in uninfected control naive B cells (Fig. 1).

Non-specific gene regulation in infected cells was controlled for by measuring the expression of the REEP5 gene at every time point. This gene expression was stable throughout the time course of infection, except that it decreased between 3 and 6 h p.i., as did EBI2. This suggests that some genes are downregulated immediately upon EBV infection, and the initial dip in EBI2 may be part of an overall pattern of gene expression that is separate from the increase seen by 24 h.

To determine if increased RNA expression corresponded to increased protein levels, naive B cells were infected with EBV and proteins were harvested at 24 h p.i. Western blots were performed to determine the ratio of EBI2 protein between the control and infected cells (Fig. 2). At 24 h p.i., the EBV-infected cells displayed more than twice the amount of protein compared to uninfected cells (P=0.015).

Cell activation alone is not sufficient to increase EBI2 expression

It is important to establish if EBI2 expression could possibly be induced by a mechanism such as B-cell activation due to viral infection. For this purpose, isolated naive B cells were treated with imiquimod. Imiquimod binds to Toll-like receptor 7 (TLR7), which normally recognizes ssRNA, and leads to B-cell activation [29]. We used imiquimod to stimulate naive B cells instead of using other methods, such as CD40 and IL-4, because we wanted to investigate whether the TLR pathway could be responsible for the induction of EBI2 [30, 31]. The imiquimod treatment activated the B cells, as shown by increased IL-1 and IL-6 transcription after treatment, but no increase in EBI2 expression was noted (Fig. 2).

To further establish that upregulation of EBI2 expression was due to EBV genes expressed during infection, naive B cells were treated with UV-irradiated EBV. Media containing EBV was UV irradiated as described and then used to treat naive B cells. The irradiation inactivates viral particles, preventing replication after entering the cell due to DNA damage. After incubation with the UV-irradiated EBV, EBI2 expression was measured. The relative mRNA expression in the B cells treated with the UV-irradiated EBV did not differ from that found in uninfected naive B cells (Fig. 2).

**BRF1 demonstrates a similar pattern of expression as EBI2 during EBV infection of B cells**

EBV uses various viral proteins to regulate cellular gene expression [32, 33]. Significant regulation of EBI2 was observed beginning between 3 and 6 h p.i., indicating that
an immediate early or early gene product would most likely be responsible for regulating EBI2 expression. Various EBV genes that are expressed during this period were selected as candidates for investigation. These included BARF1, BHRF1, BRRF1, BMLF1, LMP1, LMP2, BRLF1 and BZLF1. Analysis was first begun on these genes, with the intention of examining other viral genes if strong correlation values could not be found upon comparing the expression pattern of these EBV genes to EBI2.

Expression of these genes at three different time points during naive B-cell infection by EBV was measured using reverse-transcriptase quantitative PCR (RT-qPCR) and a pattern of expression was established for each one (Fig. 3). These time points were chosen due to the distinct difference of EBI2 expression observed during EBV infection. Epstein–Barr virus nuclear antigen 1 (EBNA1) was used to control for viral gene expression as it is expressed throughout EBV infection. It was also chosen as the control to standardize for viral infection, and even though the m.o.i. used was the same for each experiment, there is still variation observed in the samples treated with EBV. Therefore, all viral gene levels are relative to EBNA1 in this experiment. Upon comparing the expression patterns of the viral genes to the expression pattern of EBI2, BRRF1 demonstrated a similar expression pattern with the highest $R^2$ value (Fig. 3). Linear regression analysis comparing EBI2 gene expression to the mRNA expression of the various viral genes revealed that BRRF1 shared more similarity than any other gene screened ($R^2=0.866$). Between 3 and 6 h p.i., there is a significant decrease in expression ($P=0.033$) and by 12 h p.i., expression had increased to a level similar to that observed at 3 h p.i. These results suggest a possible connection between BRRF1 and EBI2 expression.
BRRF1 induces a heightened expression of EBI2 in B lymphocytes during EBV infection

BRRF1 is an early lytic gene product [17, 34] encoding a transcription factor (Na), which plays an important part in regulating between latent and lytic EBV infection [35]. In high enough concentrations, BRRF1 presence alone has been shown to induce EBV lytic gene expression [35]. To determine if upregulation of EBI2 was caused by BRRF1, naive B lymphocytes were infected with BRRF1-deficient (BRLF1/BRRF1 knockout, R-KO) EBV. The R-KO EBV was used and described in previous studies [17, 35]. To summarize, the R-KO EBV strain is a bac engineered B95.8...

Fig. 2. EBI2 protein expression is highly upregulated only by infection with infectious EBV. (a) EBI2 protein levels correlate with RNA levels. Twenty-four hours p.i. of naive B cells infected with EBV demonstrate heightened protein levels compared to uninfected naive B cells. (b) EBI2 protein signal measured was twofold higher than the uninfected cells as observed by Western blot (*P=0.015), n=4. EBI2 was normalized to β-actin, which was used as a loading control. (c) Both Toll-like receptor 7 stimulation with imiquimod and incubation with UV-irradiated EBV showed expression levels of EBI2 similar to unstimulated naive B cells, while EBV-infected naive B lymphocytes demonstrated significantly higher levels of EBI2 mRNA expression (**P=0.028), n=3. (d) IL-1 and IL-6 mRNA expression was measured in all imiquimod-treated samples. Heightened mRNA levels indicate successful imiquimod stimulation compared to unstimulated cells. Error bars indicate standard error, n=3.
Epstein–Barr virus (V01555). It lacks the ability to express both the BRLF1 and BRRF1 lytic genes resulting in the absence of the subsequent gene products, Rta and Na, respectively. Without the BRLF1 gene, it would be predicted that the virus would not be able to induce expression of various genes that depend on Rta response elements in their promoters including BMLF1, BMRF1, BALF2, BaRF1 and BLRF2 [36]. Rta is one of the main proteins involved in EBV reactivation and is thought to be essential for viral reactivation and lytic cycle induction, along with Zta. Na is known to act with Rta as a co-activator and assists in inducing transcription of BZLF1 and the subsequent protein synthesis of Zta [17, 35]. It has been hypothesized that it helps regulate Rta transcriptional effects [37].

The EBI2 mRNA expression was measured at 0 and 24 h p.i. in naive B lymphocytes with R-KO EBV (Fig. 4). The mRNA was extracted from cells and quantified by qPCR. Following infection of naive B lymphocytes with Na- and Rta-deficient R-KO EBV, no significant change in expression of EBI2 was detected at 24 h p.i. (Fig. 4). To verify that our R-KO infection assays were not yielding low EBI2 levels of expression due to low R-KO EBV infection rate, we measured the mRNA expression of EBNA1 in samples that were EBV or R-KO EBV infected at 24 h p.i. (Fig. 4). If our R-KO EB2 expression was a result of low viral infection, we would expect the measured EBNA1 expression to be less than that measured in samples from EBV-infected B cells. However, our results show that EBNA1 expression is not lower in

Fig. 3. BRRF1 demonstrates a similar pattern of expression to EBI2 in EBV infected B cells. (a) Several EBV early genes were screened using qPCR to measure relative mRNA expression, n=5. (b) BRRF1 demonstrated the most similar pattern of expression to that of EBI2. This correlation ($R^2=0.4622$) suggests that there might be a connection between the expression of BRRF1 and EBI2. (c) BZLF1 and BRLF1 expression patterns were also compared to EBI2 expression. The resulting correlation plot demonstrates that these other EBV lytic genes demonstrate lower correlation values with EBI2 mRNA expression.
samples from R-KO-infected B cells. This suggests that lower levels of EBI2 expression are not a result of a poor R-KO infection.

To confirm our findings from the R-KO EBV assay and verify that low EBI2 levels were a result of the lack of BRRF1 and not due to the lack of expression of other viral genes, such as BRLF1, we first transduced naive B lymphocytes with a lentivirus containing the BRRF1 gene (pUltra+BRRF1). The relative EBI2 expression was measured at 0 and 24 h (Fig. 4a). Heightened expression of EBI2 was observed at 24 h post-transduction (\(P=0.042\)) when naive B lymphocytes were transduced with pUltra+BRRF1. Second, to test whether BRRF1 was specifically causing EBI2 upregulation and confirm that the increased levels of EBI2 expression were not the product of transactivation by a viral DNA-binding gene, the viral gene BRLF1 was transduced into naive B cells (pUltra+BRLF1). The relative EBI2 mRNA expression was measured at 0 and 24 h post-transduction. In contrast with BRRF1, there was not a significant increase in EBI2 expression after treatment with BRLF1 (Fig. 4).

It was probable that most of our blood donor volunteers had previously been infected with EBV. It was not expected that EBV reactivation would contribute to the heightened EBI2 expression levels observed since EBV genomes are only present in approximately 1 in \(10^6\) circulating B cells. However, to verify that latent EBV from prior infection was not interfering with the results from our pUltra+BRRF1- and pUltra+BRLF1-transduced samples, BZLF1 was measured in the EBV-infected naive B cells and the lentivirus-transduced naive B cells. BZLF1 expression was greater, estimated at \(2 \times 10^4\) fold higher, in EBV-infected B-cell samples compared to the pUltra+BRRF1-transduced B-cell samples. In most cases, BZLF1 expression was not
detectable in B cells transduced with the BRRF1-expressing lentivirus. This would suggest that potential reactivation of EBV by the pUltra+BRRF1 in EBV-positive primary B cells is not responsible for the resulting increase in EBI2 expression observed.

It could also be suggested that perhaps high expression of an EBV lytic gene could non-specifically induce EBI2 expression. To investigate this possibility, we measured BRRF1 transcripts in pU+BRRF1-transduced, EBV-infected and control samples. We found that there was about three-fold more BRRF1 expression in pU+BRRF1-transduced samples compared to EBV-infected samples. This difference can be attributed to the effectiveness of the human ubiquitin promoter used to drive expression of BRRF1 post-transduction compared to the BRLF1 and BRRF1 promoter used by wild-type EBV. These results combined with the lack of upregulation in cells transfected with BRLF1 using the same promoter led us to conclude that BRRF1 expression alone can induce EBI2 and that EBI2 expression is not a result of non-specific binding or latent EBV reactivation.

DISCUSSION

Regulation of EBI2 is crucial to B-cell chemotaxis in secondary lymph tissue [28, 38]. Upregulation of EBI2 generally occurs during cellular migration in secondary lymph tissue [28]. When expressed, EBI2 allows the cell to follow a 7α,25-OHC gradient [21, 38, 39]. 7α,25-OHC is produced by stromal cells of secondary lymph tissue and is the only identified natural ligand for the EB2 chemokine receptor. B cells upregulate EBI2 at key times to manoeuvre the cell away from the follicular region to the outer and inter-follicular regions [24]. It has been suggested that these movements allow the naive B cells to potentially be exposed to any antigens that are present in that region (Fig. 5) [28]. Downregulation of EBI2 allows for the naive B cells to return to the follicular area and migrate to the T-cell zone for a time before exiting the secondary lymph tissue. The migration of B cells through this pattern allows for exposure to areas with different antigens as well as the possibility to be primed by T cells.

EBV is known to manipulate cellular genes to avoid the immune system, control viral replication and prevent apoptosis [40–42]. EBV had been previously shown to influence EBI2 [43–45]. Various studies have researched EBI2 expression during EBV infection; however, it has yet to be determined if the virus induces EBI2 or if heightened EBI2 expression is a result of the cellular response to viral infection [20, 46–48]. Previously, it has been hypothesized that EBI2 expression is a result of the immune response to viral infection since EBI2 has been shown to be induced during EBV latency program I. Normally, only EBNA1 is highly expressed [20, 43]. It has also been hypothesized that the increase of EBI2 expression observed during EBV infection is a result of viral manipulation to promote a successful persisting EBV infection, allowing for the virus to directly infected cells to areas that would provide a better survival niche for viral persistence [22, 49]. The results of this study provide evidence for the later explanation of why heightened EBI2 expression is observed during EBV infection.

It is possible that the EBV-induced upregulation of EBI2 could have been the result of non-specific activation, as a cellular response to EBV infection. However, the B-cell-stimulating agent imiquimod did not cause an upregulation in EBI2 expression, nor did incubation with inactivated EBV. These findings indicate that EBI2 upregulation is not likely due to cellular activation by TLR7, and that viral tegument or surface proteins are not responsible for the upregulation of EBI2.

Screening for viral gene candidates that could be responsible for the regulation of EBI2 post-EBV infection found that BRRF1 shares a similar mRNA expression pattern as EBI2. BRRF1 encodes a viral transcription factor, Na. Na is responsible for assisting in the activation of viral lytic genes in various latently infected epithelial cells [35] and has been found to associate with human TNF receptor-associated factor 2 in a yeast two-hybrid assay [50]. Due to its function as a transcription factor and its expression pattern being similar to that of EBI2, BRRF1 seemed the best candidate of those screened. To ascertain if BRRF1 expression could directly influence EBI2 expression, the BRRF1-deficient R-KO EBV was used to infect isolated naive B cells. R-KO EBV has been used in several studies and has the R gene, BRF1, knocked out as well as the promoter of BRRF1 resulting in EBV that cannot express BRRF1 [35]. R acts with BZLF1 to activate latent EBV and can bind to various EBV promoters [16, 51, 52]. The results show no upregulation of EBI2 expression upon infection with the BRRF1-negative EBV. This would suggest that BRLF1 or BRRF1 was inducing EBI2 expression. To further verify that BRRF1 could induce EBI2 expression, the pUltra+BRRF1 lentivirus was used to treat isolated naive B cells. This resulted in a significant increase in EBI2 expression. These results demonstrate that the expression of BRRF1 was necessary and sufficient to induce EBI2 expression. While the mechanism that BRRF1 uses to induce this expression in B cells is unknown, it is possible that Na acts as a transcription factor at the EBI2 promoter site. Further research needs to be performed in order to ascertain if this is the case.

It is probable that cell migration would be influenced by the EBV-induced regulation of EBI2. With a heightened concentration of EBI2 on the surface of the infected B cells, it is no stretch to infer that EBI2 might override other migratory signalling pathways and direct the cell to follow a 7α,25-OHC gradient, which is the natural ligand of chemottractant receptor EBI2 (see Fig. 5b) [21, 39]. 7α,25-OHC is only produced by stromal cells of secondary lymph tissue [53]. Following this gradient would cause infected B cells to migrate and remain in the outer follicular and inter-follicular regions of secondary lymph tissue. This type of control over cell migration could allow the virus to avoid immune detection until latency has been established, assisting in immune evasion.
It is also possible that increased levels of EBI2 might influence B-cell proliferation. Benned-Jensen et al. [54] found that overexpression of EBI2 in antibody-stimulated murine B cells resulted in increased proliferation. By increasing the proliferation of infected B cells during a specific time, it would increase the chances of viral persistence in the infected host. The results of this study emphasize the importance of EBI2 regulation during viral infection. It is possible that the use of EBI2 antagonists, or inverse agonists, could be used as EBV antiviral treatment options or to disrupt the viral life cycle in an animal model [54–56].

During our study, we have established that EBI2 is controlled during EBV infection of B cells. Different EBV expression profiles result in different levels of EBI2 expression. Higher levels of EBI2 mRNA expression result in higher levels of EBI2 protein. We have further established that EBI2 is induced by an EBV gene, BRRF1. Potential research for the future can address to what degree heightened expression of EBI2 possibly changes migration of EBV-infected B cells. It would also be beneficial to verify the mechanism used by BRRF1 to induce EBI2 expression.

**Fig. 5.** Proposed effects on B-cell migration during EBV infection due to modulation of EBI2 expression. (a) This illustration demonstrates the regular pattern of naive B-cell migration in the lymph node. (1) CXCR5 is expressed, allowing cells to follow a CXCL13 gradient and enter the follicular region. CXCR5 is constitutively expressed to assist in cell migration. (2) EBI2 is upregulated and the cells follow a 7α,25-OHC gradient to the outer inter-follicular region. (3) Downregulation of EBI2 allows the naive B cells to return to the follicular area and (4) upregulation of CCR7 permits the cell to follow the CCL21 chemokine gradient to the T-cell zone. If B cells remain inactivated, they will leave via the cortical sinus of the lymph node. If they become activated, they will be directed back to the follicular and germinal centre area. (b) This figure depicts the predicted pattern of migration during EBV infection of naive B cells. (1) Upon entry into the lymph node, the B cell will follow the 7α,25-OHC gradient to the outer and inter-follicular region of the lymph node. (2) The cell will be unable to migrate to the follicular region.
METHODS

Generation and harvesting of viral stock

Viral stock was generated using the Bac B95-8 EBV producing HEK 293 cell line as previously constructed and described by Delecluse et al. [57]. To summarize, the strain has been engineered with an F factor origin of replication, partitioning proteins A and B, chloramphenicol-resistance gene, hygromycin-resistance gene and an EGFP reporter. Cells were seeded in T-75 flasks (Corning) to attain a confluence of 50 to 60%. The cells were then transfected with pUltra+BZLF1, pUltra+BRRF1 and pUltra+BRLF1 plasmids using calcium-phosphate transfection. Media was changed 16 h post-transfection to RPMI and cells were allowed to incubate for 7–10 days. Viruses were harvested in the RPMI media, filtered using a 0.45 µm filter, collected in 15 ml conical vials and stored at −80 °C. The titre of Epstein–Barr viral infectious units was determined using green Raji cell assays following a previously described protocol [17].

To summarize, 2×10^6 Raji cells were suspended in 0.5 ml of viral supernatant. The cells were allowed to incubate for 3 h and then 1.5 ml of RPMI media was added to the wells. At day 2 p.i., sodium butyrate and phorbol-12-myristate-3-acetate (PMA; ACROS) were added to a final concentration of 3 mM sodium butyrate and 50 ng of PMA ml⁻¹. The cells were allowed to incubate for another 16–24 h and then the GFP-positive cells were quantified by fluorescence microscopy.

The BRLF1- and BRRF1-deficient Epstein–Barr virus (R-KO EBV) was kindly given to us by Dr Henri-Jacques Delecluse from the German Cancer Research Center, having been described and used in previous studies [17, 35, 58]. For specific information on the construction and testing of this virus, the reader is referred to previous publications by Hong et al. [17], Hagemeier et al. [35] and Feederle et al. [59]. R-KO EBV viral stocks were generated by the same method previously described for B95-8 EBV production. The titre of viral infectious units was determined using green Raji cell assays following a previously described protocol [17] and as described.

The lentiviruses pUltra+BZLF1, pUltra+BRRF1 and pUltra+BRLF1 DNA were grown in Escherichia coli DH5α cells and extracted using plasmid extraction kits (Qiagen). The plasmid DNA was then transfected into the PHX cell line using calcium-phosphate transfection. The supernatant was harvested and filter sterilized using a 0.45 µm filter. Lentiviral vector concentrations were quantified by placing 100 µl of lentiviral media with 1 ml of Raji cells at a concentration of 1×10^6 cells ml⁻¹. Cells were allowed to incubate for 48 h and then counted by fluorescence microscopy to determine the lentivirus titre.

UV irradiation of EBV

EBV supernatants in 15 ml centrifuge conical tubes were exposed to ultraviolet light (200–280 nm) in a biosafety cabinet hood for a period of 4 h. The UV-irradiated virus was then used to infect isolated B cells.

Human B-cell isolation

Human naive B cells were isolated using lymphocyte separation media (Cellgro) and EASYSEP negative selection magnetic separation (STEMCELL Technology). PBMCs were isolated from 30 ml of peripheral blood collected from healthy volunteers after informed consent using lymphocyte separation medium (Cellgro). PBMCs were then resuspended in PBS +2% FBS with 1 mM EDTA added. Naive B cells were separated from other lymphocytes by magnetic cell separation using the Human B cell enrichment kit (STEMCELL Technology) following the EASYSEP protocol.

Infection assays and RNA extraction

Using standard 12-well plates, 2×10^6 naive B cells were placed in each well in 1 ml of medium. EBV viral stock was added to each well at an m.o.i. of 15. An equal volume of media without EBV was added to the negative controls. Cells were collected by centrifugation. Using the RNAquous-Micro RNA extraction kit (Ambion) and procedure, RNA was extracted and suspended in elution solution. Since in naive B cells high rates of infection with EBV are difficult to obtain, we used the highest m.o.i. that was practical. Using an m.o.i. of 15, we typically obtained infection of about 15–25% of naive B cells. All viral infection experiments were repeated at least three or more times in duplicate.

For infection with R-KO EBV, isolated naive B cells at a concentration of 2×10^6 cells ml⁻¹ were incubated with R-KO EBV supernatant at an m.o.i. of 15. At each time point, samples were pelleted and treated as previously described for RNA extraction.

For lentiviral transduction, either pUltra+BRRF1 or pUltra+BRLF1, at an m.o.i. of 15, was added to isolated naive B cells at a concentration of 2×10^6 cells ml⁻¹. At 24 h p.i., the cells were pelleted and treated as previously described for RNA extraction.

Quantification of gene expression by qPCR

Reverse-transcriptase quantitative PCR (RT-qPCR) was performed using StepOne Plus software and equipment with Power SYBR Green PCR master mix (Applied Biosystems). Samples were analysed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. As a control for non-specific gene regulation, levels of the REEP5 gene were also measured using RT-qPCR. qPCR primers for all target genes were designed using Primer Express 3.0 (Applied Biosystems). Primer sequences as well as the consistency between experiments can be found in Fig. 6.

Statistical analysis

In most cases, two-tailed paired t-tests and an alpha value of 0.05 were used to determine significant differences in relative mRNA expression levels. Since our experiments were run and analysed in tandem, it allows us to use the paired tests when doing our statistical analysis when comparing variable conditions in the same experiment. There was one exception when determining the significance of the EBI2
expression between the pUltra+BRRF1-transduced samples and the pUltra+BRLF1-transduced samples 24 h post-transduction: some of the samples were lost during harvesting and new samples were transduced and harvested. Due to this event, the experiment was analysed using Welch’s unpaired t-test, and an alpha value of 0.05 was still considered significant. To analyse correlations between the expression of EBV genes and EBI2, linear regression analysis was performed using R statistical analysis software. In this analysis, EBI2 expression was compared to the expression of each gene in turn and $R^2$ values calculated [EBI2 ~ (EBV gene of interest)].

LCL cell line generation

PBMCs were re-suspended at $2 \times 10^6$ cells ml$^{-1}$ in complete RPMI. Then 5 ml of cells suspended in media and 5 ml of B95-8 EBV cell culture supernatant were placed together in a T-25 flask with cyclosporin A or actinomycin D. Cells were incubated for 3 weeks, pipetted weekly to break up cell clumps. LCL cell lines are maintained in complete RPMI and passaged frequently.

Lentivirus generation

The lentiviral vector pUltra+BRRF1 was constructed using pUltra, a third-generation lentivirus obtained from Addgene. The BRRF1 gene was PCR amplified from wild-type B95.8 EBV using forward (TCTAGAATGGCAGTAGTAACAGAGGAATG) and reverse (TGATCATATTGTTATACAGAT) primers with XbaI and BclI restriction site extensions added, respectively. BRRF1 was then cloned into pUltra cut with XbaI and BclI restriction enzymes. The ligated pUltra+BRRF1 was then transfected into E. coli DH5α cells. Using a BRRF1-specific forward primer and a pUltra-specific reverse primer, the colony containing the complete pUltra+BRRF1 plasmid was verified by sequencing. The plasmid used for transfecting EBV producing HEK cells pUltra+BZLF1 was produced using pUltra with the BZLF1 gene cloned into the construct. Forward (GTGACTCTACGACAGAGGAAG) and reverse (GAATTCAGAGTGATAGCAGAAG) primers with SalI and EcoRI restriction site extensions were added, respectively. The PCR-amplified BZLF1 gene was cloned into pUltra cut with SalI and EcoRI. The ligated pUltra+BZLF1 was transfected into E. coli DH5α cells and verified as described previously using a BZLF1-specific forward primer and a pUltra-specific reverse primer. The lentiviral vector pUltra+BRLF1 was constructed using the same pUltra lentivirus as aforementioned. The BRLF1 gene was PCR amplified from wild-type B95.8 EBV using forward (TCTAGAATGGCTGGGACAGAGGAGGAG) and reverse (TGATCATATTGTTATACAGAT) primers with SalI and EcoRI restriction site extensions added, respectively. The PCR-amplified BRLF1 gene was cloned into pUltra cut with SalI and BclI. The ligated pUltra+BRLF1 was then transfected into E. coli DH5α cells and verified as described previously using a BRLF1-specific forward primer and a pUltra-specific reverse primer. All primers for cloning the desired the PCR products were designed using Primer 3 software. All lentiviral constructs were verified by sequencing and expression of the cloned genes were verified by RT-qPCR.

![Fig. 6. Primers and consistency of RT-qPCR analysis.](http://www.microbiologyresearch.org)
Western blot

One millilitre of naive B cells at a concentration of 2 × 10^6 cells ml⁻¹ was infected with EBV at an m.o.i. of 15. At the time of sample collection, the cells were pelleted and resuspended in lysis buffer (Thermo Scientific). Cells were then vortexed and passed through a 25-gauge needle, followed by incubation in Laemmli sample buffer (BIO RAD) and 5 % 2-mercaptoethanol (Sigma) for 5 min at 95 °C. The samples were then subjected to electrophoresis in a 12 % polyacrylamide gel and transferred to a nitrocellulose membrane (Thermo Scientific). Blots were blocked with 2.5 % (w/v) non-fat dry milk. EBI2 polyclonal goat IgG obtained from Santa Cruz Biotechnology at a diluted 1:1000 was used as the primary Ab. Rabbit anti-goat IgG-HRP obtained from Santa Cruz Biotechnology at a diluted 1:1000 was used as the secondary Ab. ECL Plus (GE Healthcare) lumigen reagents and C-DiGit blot scanner (LI-COR) were used to image the Western blot. For measuring protein loading, blots were stripped using stripping buffer (100 mM β-mercaptoethanol, 2 % SDS, 62.5 mM Tris/HCl, pH 6.7) and reprobed using goat anti-actin (Abcam) as the primary antibody followed by secondary antibody and visualization as described for EBI2.

Funding information

This work was funded by a Mentoring Environment Grant from Brigham Young University for Dr Poole.

Acknowledgements

We would like to thank Dr Henri-Jacques Delecluse from the German Cancer Research Center for his generous gift of the BRLF1 knockout (R-KO) EBV strain. We would also like to thank Dr Brent Nielsen and Dr William Young University for Dr Poole.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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