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Epstein-Barr Virus Evades CD4⁺ T Cell Responses in Lytic Cycle through BZLF1-mediated Downregulation of CD74 and the Cooperation of vBcl-2

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Abstract

Evasion of immune T cell responses is crucial for viruses to establish persistence in the infected host. Immune evasion mechanisms of Epstein-Barr virus (EBV) in the context of MHC-I antigen presentation have been well studied. In contrast, viral interference with MHC-II antigen presentation is less well understood, not only for EBV but also for other persistent viruses. Here we show that the EBV encoded BZLF1 can interfere with recognition by immune CD4⁺ effector T cells. This impaired T cell recognition occurred in the absence of a reduction in the expression of surface MHC-II, but correlated with a marked downregulation of surface CD74 on the target cells. Furthermore, impaired CD4⁺ T cell recognition was also observed with target cells where CD74 expression was downregulated by shRNA-mediated inhibition. BZLF1 downregulated surface CD74 via a post-transcriptional mechanism distinct from its previously reported effect on the CIITA promoter. In addition to being a chaperone for MHC-II αβ dimers, CD74 also functions as a surface receptor for macrophage Migration Inhibitory Factor and enhances cell survival through transcriptional upregulation of Bcl-2 family members. The immune-evasion function of BZLF1 therefore comes at a cost of induced toxicity. However, during EBV lytic cycle induced by BZLF1 expression, this toxicity can be overcome by expression of the vBcl-2, BHRF1, at an early stage of lytic infection. We conclude that by inhibiting apoptosis, the vBcl-2 not only maintains cell viability to allow sufficient time for synthesis and accumulation of infectious virus progeny, but also enables BZLF1 to effect its immune evasion function.

Introduction

Successful persistence of viral infection depends on the establishment of a balance between host immune responses and viral immune evasion. Epstein-Barr virus (EBV), which is carried by more than 90% of the adult human population worldwide, is a prime example of a persistent virus that is generally harmless, but which can cause serious disease including various tumours [1]. A number of immunoevasins of EBV have recently been identified as acting at different points along the MHC class I (MHC-I) presentation pathway to modulate recognition by CD8⁺ T cell responses [2–7]. However, with regards to evasion of the MHC class II (MHC-II) antigen presentation pathway, EBV is less well understood. Virus-specific CD4⁺ T cell responses, which include some clones with cytotoxic activity, are broadly distributed against numerous proteins encoded by the EBV genome; both latent protein antigens [8] and the larger number of lytic protein antigens [9,10]. These observations indicate a need for EBV to also modulate MHC-II antigen presentation pathways, particularly during in EBV lytic cycle.

Mechanisms for interfering with the MHC-II antigen presentation pathway have been reported for other herpesvirus; for example, US2, US3 and pp65 of cytomegalovirus [11–13] and glycoprotein B (gB) of herpes simplex virus type 1 [14]. However, EBV has no homologues to these immune evasion genes of CMV, and there is no evidence that the homolog of HSV-1 gB protein encoded by the BALF4 gene of EBV targets the MHC-II pathway. An unrelated EBV glycoprotein, gp42, has however been shown to associate with MHC-II molecules and to inhibit antigen presentation to CD4⁺ T cells [15,16].

More recently, the immediate-early EBV gene BZLF1, which encodes a transcription factor initiating EBV lytic cycle, was reported to be a potential modulator of MHC-II antigen presentation [17]. Ectopic expression of BZLF1 in Raji cells inhibited the expression of MHC-II molecules, apparently through repression of CIITA transcription. However, interpretation of these data is complicated by the fact that Raji is an EBV-carrying B cell line in which expression of BZLF1 can initiate virus lytic cycle and, therefore, the expression of other viral genes that may be responsible for modulating MHC-II expression. One such candidate is the early antigen, BGLF5, which has been shown to induce global mRNA degradation and thereby to reduce expression of various host proteins, including MHC-I and MHC-II [2,3]. In addition, as the level of expression of MHC molecules does not necessarily reflect the degree of T cell recognition, it is important to assay antigen presentation using functional T cell recognition.
MHC-II Evasion Mediated by EBV BZLF1

Author Summary

Epstein-Barr virus (EBV) is a herpesvirus and an important human pathogen that can cause diseases ranging from non-malignant proliferative disease to fully malignant cancers of lymphocytes and epithelial cells. The persistence of EBV in healthy individuals relies on the balance between host immune responses and viral immune evasion. As CD4+ immune T cell responses include both helper and cytotoxic functions, viral mechanisms for interfering with MHC class II antigen presentation to CD4+ T cells have the potential to greatly influence the outcome of viral infections. Our work on Epstein-Barr virus provides a new paradigm for viral immune evasion of MHC-II presented antigen by targeting CD74. CD74 is a dual function protein; it serves as a surviving receptor as well as a chaperone for MHC-II antigen presentation. Therefore, downregulation of CD74 as a T cell evasion strategy comes at the cost of potentially inducing cell death. However, EBV also encodes a vBcl-2 to attenuate the toxicity associated with reduced CD74, thus enabling the immune-impairment function to be effected. We expect that future studies will identify other viruses utilizing a similar strategy to evade CD4+ immune T cell responses.

In this study, we demonstrated for the first time that BZLF1 does indeed impair recognition by EBV-specific CD4+ T cells. However, this was not the result of downregulation of MHC-II α/β chain molecules as expected, but through downregulation of the invariant chain (Ii, or CD74) which serves as a chaperone to ensure correct loading of antigenic peptide fragments. As CD74 also mediates cell survival, its downregulation by BZLF1 induced cell death. A pivotal role was also identified for a vBcl-2 (BHRF1) which serves as a chaperone and 1D are from one representative experiment. In three independent experiments, recognition of newly processed BXL2 peptides from virus-pulsed targets (Figure 1C, lower histogram) and by EBNA1-specific CD4+ effectors (Figure 1D; upper histograms). In contrast, recognition of newly processed BXL2 peptides from virus-pulsed targets (Figure 1C, lower histogram) and by EBNA1-specific CD4+ effectors (Figure 1D; upper histograms) was impaired in GFP+ cells expressing BZLF1 protein. The results illustrated in Figs. 1C and 1D are from one representative experiment. In three independent experiments, recognition of newly processed BXL2 antigen by LEK+ CD4+ T cells was reduced by 40–60%, and recognition of pre-existing EBNA1 peptide by SNP+ CD4+ T cells was reduced by 25–40% following expression of BZLF1. These experiments demonstrate that BZLF1 expression in LCLs leads to impaired EBV-specific CD4+ effector T cell recognition.

In the BXL2-specific CD4+ T cell recognition assays, there was very low but consistent recognition of BZLF1-expressing LCLs that had not been pulsed with EBV particles (bottom histogram, Figure 1C). This suggested that even within the short time-frame of this experiment the expression of BZLF1 can trigger lytic cycle and produce small but sufficient amounts of virus antigen for MHC-II processing. This conclusion was supported by further assays where expression of BZLF1 led to clear recognition not only by BZLF1-specific CD4+ effectors, but also by CD4+ effectors specific for the BMRF1 early antigen or the gp350 late antigen (Figure S1). Immunoblot analysis of the LCL targets showed that induction of BZLF1 led to expression of EBV lytic proteins (Figure 1E). Thus, whilst the data in Figure 1 are consistent with BZLF1 interfering with CD4+ T cell responses, further experiments with EBV-negative targets were necessary to avoid complications arising from BZLF1 initiation of the cascade of lytic cycle expression.

Effect of BZLF1 on CD4+ T cell recognition of MHC-II presented antigen

To investigate the effect of BZLF1 on MHC-II antigen presentation in EBV-negative target cells, we developed a strategy that allowed analysis of transiently transfected target cells. As intercellular antigen transfer is never detectable for EBNA1 [20], we chose this as the target antigen. To increase the sensitivity of the assay, a mutant form of EBNA1 (Cyto-EBNA1) was used as its cytosolic location renders its processing to MHC-II molecules more efficient than wild-type nuclear EBNA1 [20].
EBV-negative MJS melanoma cells expressing MHC-II DRB5*01 were transfected with cyto-EBNA1 expression plasmid together with IRES-GFP plasmid vectors for BZLF1 or other EBV lytic genes. The cultures were then assayed for antigen presentation to MHC-II DRB5*01-restricted CD4+ T cells specific for the ‘SNP’ EBNA1-derived peptide. T cell recognition was measured by the release of IFN-γ from the effector cells. The results of 5 independent experiments are summarised in Figure 2A. Mock transfected cells were not recognised, but good recognition was seen with target cells co-transfected with cyto-EBNA1 and control IRES-GFP vector. Recognition of cyto-EBNA1 was not significantly affected by co-transfection of BZLF2 or BALF4 vectors expressing the EBV lytic proteins gp42 or gp110, respectively. However, recognition of cyto-EBNA1 was substantially and reproducibly reduced by 60–80% when the target cells were co-transfected with BZLF1.

Replicate aliquots of cells analyzed by immuno-blots confirmed expression of BZLF1 in the cells transfected with the BZLF1-GFP plasmid (Figure 2B). There was no obvious change of MHC-II DR in any of the target cells. Whilst the levels of EBNA1 were similar in the cells co-transfected with cyto-EBNA1 and IRES-GFP, BZLF2-GFP or BALF4-GFP plasmids, there was an unexpected and marked reduction of EBNA1 expression when co-transfected with the BZLF1-GFP plasmid (Figure 2B).

Two colour flow cytometry analysis of viable cells stained for surface MHC-II DR suggested that none of the EBV lytic genes tested reduced the level of cell surface DR molecules on GFP+ cells compared to the expression on untransfected GFP− cells in the same cultures (Figure 2C). Notably, there were relatively few GFP+ cells in the cultures transfected with BZLF1-GFP, typically less than 1% compared with 15% in cultures transfected with control IRES-GFP plasmid. This indicated a toxic effect of BZLF1 in these cells, which could account for the reduced EBNA1 target antigen expression (Figure 2B) and also the reduced T cell recognition (Figure 2A).

BZLF1 downregulates anti-apoptotic Bcl-2 family members

Toxicity of BZLF1 was confirmed in other cell types, including an EBV-negative subclone of the Akata Burkitt’s lymphoma, Akata-A3. Here, we used the DOX-inducible pRTS-CD2-BZLF1 expression plasmid, which drives the expression of BZLF1 together with a non-functional neuronal growth factor receptor (NGFR) and green fluorescent protein (GFP) as markers of DOX-induced expression. (B) BZLF1KO-LCLs transfected with pRTS-CD2-BZLF1 or pRTS-CD2-control vector were treated with DOX for 24 h. The induced plasmid-containing cells were separated using MACSelect LNGFR MicroBeads. Both the unlabelled cells and the labeled cells were collected, and then analyzed by flow cytometry. The flow-through cells were NGFR−/GFP−, indicating that they lacked the transfected plasmid and/or DOX treatment failed to induce expression from pRTS-CD2-BZLF1 (dotted line, top histogram). The bound and eluted cells were NGFR+/GFP+, which indicated that expression of BZLF1 from pRTS-CD2-BZLF1 had been induced (dashed line, bottom histogram). (C) These sorted cells were pulsed with or without concentrated EBV B95.8 virus particles and then were co-cultured with ‘LEK’ CD4+ effector T cells (specific for a BXL2F2-derived peptide), for a further 18 h before assaying T cell recognition by IFN-γ ELISA. (D) Unpulsed targets were also assayed with ‘SNP’ CD4+ effector T cells (specific for a EBNA1-derived peptide). All results are expressed as IFN-γ release in pg/ml, and error bars indicate standard deviation of triplicate cultures. (E) The pRTS-CD2-control and pRTS-CD2-BZLF1 transfected LCLs were analyzed, before and after DOX induction, by SDS-PAGE and immuno-blotting with antibodies specific for BZLF1, BMRF1, BALF2, EBNA1 or calregulin as a loading control.

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as in Figure 1. Akata-A3 cultures transfected with either pRTS-CD2-BZLF1 or pRTS-CD2-control plasmids were enriched by CD2 selection to around 80% purity. Following treatment with DOX, the percentage of GFP+ cells was monitored over a period of 72 h. For the first 24 h of DOX treatment, both control- and BZLF1-transfected cultures showed a drop in the percentage of GFP+ cells such that by 72 h these BZLF1 cultures contained only around half the percentage of GFP+ cells as the control cultures (Figure 3A). These results indicate that BZLF1 is toxic to Akata-A3, but with slower kinetics than in MJS cells. The slower kinetics of toxicity in Akata-A3 allowed examination of cellular protein expression at 48 h after DOX induction. The GFP+ cells from control and BZLF1-transfected cultures were purified on a Mo-Flow cell sorter. The BZLF1 expressing Akata-A3 cell lysate was serially diluted with Akata-A3 cell lysate to give equivalent BZLF1 content of 100%, 50%, 10%, 2%, 1% or 0% and was analyzed by SDS-PAGE alongside a cell lysate from a spontaneous lytic LCL containing 5% BZLF1+ cells. The samples were immunoblotted with antibodies specific for BZLF1 or calregulin as a loading control. (C) Total cell lysates of the purified GFP+ populations from control- and BZLF1-transfected cultures were analyzed by SDS-PAGE and immunoblotting with antibodies specific for BZLF1, Bcl-2, Bcl-xL, or calregulin as a loading control. (D) Bcl-2 and Bcl-xL transcripts in total RNA isolated from induced and sorted cells were measured by QRT-PCR assay, which was normalized to measured GAPDH transcripts.

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EBV vBcl-2 attenuates BZLF1 toxicity

EBV encodes a well-characterised Bcl-2 homolog, BHRF1, as an early lytic cycle protein [21]. We therefore investigated whether the toxicity of BZLF1 could be reversed by this vBcl-2. To address this possibility, we first examined the MJS line as it appears to be particularly sensitive to BZLF1. In the representative experiment shown in Figure 4A, MJS cells were transfected with IRES-GFP, BZLF1-GFP, or with BZLF1-GFP and a BHRF1 expression plasmid. Cultures were sampled at various time points post-transfection for analysis of GFP expression by flow cytometry. In the IRES-GFP control transfectants, about 10% of the viable cells were GFP+ at 8 h, rising to around 17% by 30 h. In marked contrast, MJS cells transfected with BZLF1-GFP showed only about 4% GFP+ viable cells at 8 h post-transfection, and thereafter the percentage of GFP+ cells gradually fell at later time points. However, when a BHRF1 expression vector was co-transfected with the BZLF1-GFP plasmid, the percentage of viable GFP+ cells continued to increase to about 10% by 30 h.

Figure 3. BZLF1 downregulates Bcl-2 family members. (A) EBV-negative Akata-A3 cells transfected with pRTS-CD2-control (solid line) or pRTS-CD2-BZLF1 (dotted line) were induced with DOX and analyzed by flow cytometry at the indicated times for the percentage of cells expressing GFP. Error bars indicate standard deviation of three independent induction experiments. (B) At 48 h after DOX induction, the GFP+ cells from control and BZLF1-transfected cultures were purified on a Mo-Flow cell sorter. The BZLF1 expressing Akata-A3 cell lysate was serially diluted with Akata-A3 cell lysate to give equivalent BZLF1 content of 100%, 50%, 10%, 2%, 1% or 0% and was analyzed by SDS-PAGE alongside a cell lysate from a spontaneous lytic LCL containing 5% BZLF1+ cells. The samples were immunoblotted with antibodies specific for BZLF1 or calregulin as a loading control. (C) Total cell lysates of the purified GFP+ populations from control- and BZLF1-transfected cultures were analyzed by SDS-PAGE and immunoblotting with antibodies specific for BZLF1, Bcl-2, Bcl-xL, or calregulin as a loading control. (D) Bcl-2 and Bcl-xL transcripts in total RNA isolated from induced and sorted cells were measured by QRT-PCR assay, which was normalized to measured GAPDH transcripts.

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As in the previous set of experiments with Akata-A3 transfectants, the toxicity in MJS cells was mediated by physiological levels of BZLF1 (Figure 4B). Interestingly, the partial reversal of toxicity by co-expressed BHRF1 was achieved by levels of the vBcl-2 that were substantially lower than the levels observed in lytically-infected LCLs (Figure 4B). BHRF1-mediated protection from BZLF1-induced toxicity was also observed in other EBV-negative B cells (Figure S2). These results suggest that both the toxicity of BZLF1 and its reversal by BHRF1 are phenomena that are likely to be physiologically relevant during normal lytic cycle in EBV-infected cells.

**BZLF1 causes marked downregulation of CD74 but not MHC-II DR molecules at the cell surface**

The protection afforded by BHRF1 in MJS cells enabled us to re-examine the effect of BZLF1 on MHC-II antigen presentation without the confounding effect of induced cell death. MJS cells co-transfected with BHRF1 together with either IRES-GFP or BZLF1-GFP were examined for expression of cellular proteins in viable GFP+ sorted subpopulations at 48 hr post-transfection. Immuno-blot analysis of cell lysates showed that, as with the Akata-A3 cells (Figure 3C), BZLF1 also downregulated Bcl-2 and Bcl-xl protein expression in MJS cells (Figure 4C).

Interestingly, whilst the total cellular level of MHC-II DRx molecules was only slightly reduced following BZLF1 expression, the level of invariant chain, CD74, was markedly downregulated (Figure 4C). In BZLF1-transfected cultures, cell surface MHC-II DR expression was reproducible slightly elevated by around 10–20% in the GFP+ gated cells (Figure 4D, top right histogram); in contrast, there was a marked reduction in the expression of CD74 (about 50%) on the surface of GFP+ cells compared with GFP- cells (Figure 4D, lower right panel). One possible explanation for the discordance between total and cell surface MHC-II DR levels may be an altered localisation of MHC-II molecules when CD74 is downregulated. Analysis of control transfections showed that the levels of cell surface MHC-II DR (Fig 4D, top left histogram) and CD74 (bottom left histogram) were indistinguishable between GFP+ and GFP- gated cells in the same culture.

Similar experiments performed with the Akata-A3 cell line using the DOX inducible vector (Fig 5A) revealed that expression of BZLF1 in these B cells gave similar results to those seen with MJS cells.

To study the surface levels of MHC-II DR and CD74 in the context of lytic cycle in normal EBV-transformed normal B cells, we examined selected LCL cultures that showed a clear subpopulation of cells spontaneously in lytic cycle. These LCLs were stained for surface DR or CD74 on viable cells, then were fixed and permeabilized for staining of intracellular BZLF1. The representative result in Figure 5B shows that the BZLF1+ cells spontaneously entering lytic cycle also showed a slight elevation of surface MHC-II DR (around 20%) and a clear reduction of surface CD74 (typically, 30–40%) compared with the BZLF1- latently infected population in the same LCL culture, i.e. mirroring the results obtained with BZLF1-transfected cells in previous experiments. Interestingly, when these same LCL cultures were co-stained for VCA to analyse the minor subpopulation of lytically-infected cells that have progressed to late lytic cycle, both MHC-II DR and CD74 were seen to be downregulated (Figure 5C).

**BZLF1 downregulates CD74 post-translationally**

As both MHC-II and CD74 can be transcriptionally regulated by CIITA [22], and BZLF1 was previously reported to transcriptionally repress the CIITA promoter [17], we investigated whether BZLF1 in our experiments might be modulating CD74 transcription via CIITA. Using HEK293 as a model CIITA-/MHC-II-/CD74- line, we overexpressed CIITA from a heterologous promoter, which in turn induced MHC-II and CD74 protein expression. However, when BZLF1 was co-expressed, surface expression of MHC-II DR was slightly elevated and CD74 was markedly downregulated (Figure S3), exactly as seen in our earlier experiments with MJS and B cells. This suggests that BZLF1 is modulating CD74 expression by a CIITA-independent mechanism. Furthermore, in Akata-A3 B cells, whilst MHC-II DR transcripts were slightly reduced following BZLF1 expression, no reduction in CD74 transcripts was observed (Fig 6A). Pulse-chase metabolic labeling experiments with 35S-methionine, revealed that BZLF1 has no effect on CD74 translation or protein maturation (Figure 6B). The exact mechanism of CD74 regulation was not actively pursued further, although experiments performed for
other reasons (e.g. Figure S4) indicated that BZLF1 may be regulating trafficking of CD74 to or from the plasma membrane.

**Inhibition of immune CD4^+ T cell recognition by BZLF1 is retained when toxicity is attenuated by BHRF1.**

We next revisited whether BZLF1 retains the ability to impair CD4^+ T cell recognition when its toxicity is attenuated by BHRF1. MJS cells were co-transfected with cyto-EBNA1 target antigen plasmid and control-IRES-GFP or BZLF1-GFP plasmid vectors without (Figure 7A) or with (Figure 7B) BHRF1 expression plasmid. As in earlier experiments, immune CD4^+ T cell recognition of the processed EBNA1 target was substantially impaired by expression of BZLF1 in the absence of BHRF1 (Figure 7A, histogram), which correlated with a clear reduction in the amount of EBNA1 antigen expression (Figure 7A, blots). However, when co-expressed with BHRF1 the BZLF1 had no significant effect on the levels of EBNA1 target antigen (Figure 7B, blots), and the immune CD4^+ T cell recognition was inhibited to an even greater extent than when BHRF1 was not expressed (Figure 7A, histogram).

These data demonstrate that BZLF1 can indeed interfere with MHC-II antigen presentation to cause a substantial impairment of CD4^+ effector T cell recognition.

**Downregulation of CD74 is a mechanism for impaired CD4^+ T cell recognition.**

As BZLF1 impairs T cell recognition without downregulating MHC-II DR expression, we considered it likely that the downregulation of CD74 might be responsible by qualitatively altering the MHC-II/peptide complexes available at the cell surface. To directly test this, we first generated an MJS line in which CD74 was over-expressed from a strong heterologous promoter, to see if we could maintain high levels of surface CD74 after BZLF1 expression and reverse the impaired CD4^+ T cell recognition. However, despite massive over-expression of total cellular CD74, the amount of CD74 at the cell surface was barely increased; and the ability of BZLF1 to downregulate surface CD74 was unaffected (Figure S4). This is consistent with BZLF1 downregulating surface CD74 by a post-translational trafficking mechanism, but the experiment was otherwise uninformative.

We next carried out the reverse experiment, asking whether downregulation of CD74 by itself was sufficient to impair CD4^+ T cell recognition. Again, we first tested this in MJS cells, using an shRNA inhibition approach. Successful knockdown of CD74 in MJS cells was associated with considerable cell death. However, using MJS-BHRF1 cells, we achieved efficient knockdown of CD74 and retained cell viability (Figure 8A and 8C). Importantly, knockdown of CD74 was associated with significant impairment of CD4^+ T cell recognition by CD4^+ effector T cells (Figure 8E, left histogram). The specificity of this effect in LCLs was demonstrated by the observation that CD74 knockdown had no effect on CD8^+ effector T cell recognition (Figure 8E, right histogram).

**Figure 5. Downregulation of CD74, and not MHC-II DR, is a consistent effect of BZLF1.**

(A) EBV-loss Akata-A3 cells transfected with pRTS-CD2-BZLF1 or pRTS-CD2-control were induced with DOX for 24 h, then stained for MHC-II DR or CD74 as in Figure 4D. Histograms show the surface MHC-II DR and CD74 expression on GFP^+ cells from pRTS-CD2-control transfection (solid line) and pRTS-CD2-BZLF1 transfection (dashed line). The shaded histogram is the isotype control staining. (B) An LCL with a subpopulation of spontaneously lytic cells was first stained for surface MHC-II DR or CD74 then fixed, permeabilized and stained with anti-BZLF1 followed by FITC-conjugated anti-mouse IgG. Histograms show the surface MHC-II DR or CD74 expression on latent BZLF1^+ cells (solid line) and lytic BZLF1^+ cells (dashed line). The shaded histogram is the isotype control staining. (C) The same LCL with a subpopulation of spontaneously lytic cells was first stained for surface MHC-II DR or CD74 as before, then were fixed, permeabilized and stained with anti-VCA followed by FITC-conjugated anti-mouse IgG. Histograms show the surface MHC-II DR or CD74 expression on VCA^+ cells (solid line) and late lytic VCA^+ cells (dashed line). The shaded histogram is the isotype control staining.
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Figure 6. BZLF1 downregulates CD74 post-translationally. (A) DRα and CD74 transcripts in total RNA isolated from DOX-induced and anti-NGFR bead-sorted control or BZLF1 transfected Akata-A3 cells, were measured by qRT-PCR assay, which was normalized to measured GAPDH transcripts. (B) DOX induced and anti-NGFR bead-sorted control or BZLF1 transfected Akata-A3 cells were metabolically labeled for 15 min with 35S-methionine and chased for the indicated time periods. After lysis in NP-40 detergent buffer, samples were immunoprecipitated with mouse anti-CD74. Samples were separated by 12% SDS/PAGE gel, dried and exposed to autoradiography (left panel). Aliquots of the whole cell lysates before chasing were analyzed (right panel) and served as a loading control.

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Discussion

We have demonstrated that the EBV-encoded BZLF1 protein can interfere with recognition by immune CD4+ effector T cells, but it comes at a cost of toxicity. During EBV lytic cycle, this toxic property of BZLF1 can be overcome by expression of the vBcl-2 but it comes at a cost of toxicity. During EBV lytic cycle, this toxic effect of BZLF1 would downregulate both CD74 and MHC-II. However, from the present work, it is clear that BZLF1 selectively downregulates CD74. Transcription of CD74 is not solely regulated by CIITA [28], which could potentially explain discordant regulation of CD74 and MHC-II genes. Nevertheless, we found that the dominant mechanism by which BZLF1 downregulates CD74 was in fact post-translational.

Although BZLF1 can inhibit CIITA transcription [17], we consistently found cell surface MHC-II DR to be slightly elevated in BZLF1 expressing EBV-negative cells (Figure 4D, 5A). This mirrors what is observed following synchronous induction of lytic cycle in EBV-positive B cells, where cell surface MHC-II DR is initially elevated although it then falls between 12 to 24 h post-induction to a level that is around 40% of that in latently infected cells [29]. In the present study, we also observed that the MHC-II DR is slightly elevated in BZLF1+ cells in spontaneously lytic LCLs (Figure 5B), but that the level of surface DR was reduced in the minor population of cells expressing late viral capsid antigen (Figure 5C). Together, these results suggest that the initial rise in surface MHC-II DR expression in lytically infected cells is likely to be due to BZLF1 expression, while the later reduction in MHC-II DR in lytic cycle may be due to BGLF5, which acts as a host shutoff protein and contributes to immune evasion [2,3], and/or a delayed effect of BZLF1-mediated inhibition of CIITA.

CD74 is a polypeptide involved in the transport and peptide loading of MHC-II molecules [23–30]. Newly synthesized MHC-II α and β chains complex with CD74 (invariant chain) in the endoplasmic reticulum. A cytosolic di-leucine-targeting motif of CD74 directs MHC-II complexes to the endocytic pathway, either directly from the trans-Golgi network or via rapid internalization from the cell surface. The majority of CD74 at the cell surface is physically associated with MHC-II molecules [31] and most, if not all, of immature MHC-II molecules (complex of α chain, β chain and invariant chain) reach the cell surface before entering the peptide-loading compartment [32]. CD74 is...
rapidly turned over at the cell surface. Downregulation of surface CD74 by BZLF1 may therefore indicate a reduction of available immature MHC-II complexes for processing and uptake of antigenic peptides in the endosomes, and would account for the marked effect of BZLF1 expression on the MHC-II antigen processing pathway. Indeed, when we targeted expression of CD74 through shRNA, the knockdown of CD74 itself was sufficient to inhibit CD4+ T cell recognition (Figure 8).

In addition to serving as a chaperone for MHC-II, CD74 has been reported to play an essential role in B cell maturation [33], which involves activation of transcription mediated by p65 member of the NF-κB family [34]. These two functions of CD74 are genetically separable and map to different regions of the protein [35]. More recently CD74 has been identified as a receptor for MIF, and to promote cell survival and proliferation [26]. Binding of MIF to CD74 triggers activation of the p65 member of the NF-κB family, which in turn trans-activates Bcl-2 family genes, thereby providing the cells with increased survival capacity [27]. Furthermore, antibodies that block MIF/CD74 interaction cause growth inhibition and induction of apoptosis in B-cell lines [36]. BZLF1 is known to inhibit NF-κB p65 activity [37] and is toxic for all CD74+ cell lines used in our experiments, a phenomenon that correlated with downregulation of the Bcl-2 and Bcl-xl anti-apoptotic proteins (Figure 3C, 4C). It is notable that we observed no toxicity of BZLF1 in the epithelial cell line, HEK-293, which lacks expression of MHC-II and CD74.

The kinetics of the toxicity of BZLF1 in the EBV-negative Akata-A3 B cell line is such that BZLF1-transfected cells survive only 2 days (Figure 3A). This contrasts with what is observed during the normal physiological process of lytic cycle in the EBV-positive

Figure 8. Downregulation of CD74 is sufficient to impair CD4+ T cell recognition. (A) MJS-BHRF1 cells were transduced with control shRNA or CD74 shRNA lentivirus and selected with puromycin. The stable cell lines were stained with PE-conjugated anti-DR or with PE-conjugated anti-CD74, then analyzed by flow cytometry. Histograms show the surface MHC-II DR or CD74 expression on control shRNA cells (solid line) and CD74 shRNA cells (dashed line). The shaded histogram indicates isotype control staining. (B) The control shRNA and CD74 shRNA expressing MJS-BHRF1 cells were co-transfected with a cytoEBNA1 expression vector and DRB5*01 β chain expression plasmids. At 24 h post-transfection, the cells were assayed for recognition by ‘SNP’ CD4+ effector T cells specific for a EBNA1 peptide. Error bars for the IFN-γ release in the histograms indicate standard deviation of triplicate cultures. (C) Total cell lysates of the MJS-BHRF1 target cell transfections, were analyzed by immuno-blotting with antibodies to BHRF1, CD74, DRα, EBNA1, or calregulin as a loading control. (D) LCLs transformed with B95.8 EBV were transduced with control shRNA or CD74 shRNA lentivirus and selected with puromycin. The stable cell lines were stained with PE-conjugated CD74, then analyzed by flow cytometry. Histograms show the surface CD74 expression on control shRNA cells (solid line) and CD74 shRNA cells (dashed line), with isotype control antibody background staining shaded grey. (E) Control shRNA and CD74 shRNA expressing LCLs were assayed for recognition by EBNA1-specific ‘SNP’ CD4+ effector T cells (left graph) and ‘HPV’ CD8+ effector T cells (right graph).

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parental Akata line, where cell viability is maintained for at least 4 days after expression of BZLF1 [29]. EBV encodes two vBcl-2 homologs, both of which are expressed early following initiation of lytic cycle by EBV. The best characterized vBcl-2 is BHRF1, a potent anti-apoptotic protein that clearly enhances survival of B lymphocytes [21] and whose molecular mechanisms are beginning to be elucidated [30]. In contrast, it is unclear whether the second vBcl-2, BALF1, actually functions to modulate apoptosis [39]. In the present study, we showed that BHRF1 alone is able to moderate BZLF1 toxicity to an extent that is consistent with the enhanced survival period of cells entering lytic cycle. In this study we have shown for the first time that the MHC-II antigen presentation is impaired during lytic infection of normal B cells (Figure 1). About 80 antigens are expressed in the EBV lytic cycle, representing a large pool of potential target antigens as reflected in the broad repertoire of EBV-specific CD4+ T responses identified, including some clones with cytotoxic activity to these EBV antigens [9,10]. Therefore, impairment of MHC-II antigen presentation is likely to be crucial for the lytic cycle cells to survive long enough to generate infectious virus progeny. In addition to T cell responses to newly-synthesized early and late antigens expressed at the time of initiation of lytic cycle, a process that can be sustained for several days before cell death occurs [29], its immune-evasion functions may be reflected in the broad repertoire of EBV-specific CD4+ T cell responses identified, including some clones with cytotoxic activity to these EBV antigens [9,10]. Therefore, impairment of MHC-II antigen presentation is likely to be crucial for the lytic cycle cells to survive long enough to generate infectious virus progeny. In this context, we are interested to note that recognition of EBNA1, which is expressed during both latent and lytic infection, by specific CD4+ T cells is also impaired following expression of BZLF1 and induction of lytic cycle (Figure 1D).

As BZLF1 is the first EBV antigen to be expressed during lytic cycle, a process that can be sustained for several days before cell death occurs [29], its immune-evasion functions may be paramount in EBV's strategy for attenuating anti-viral responses. In this context, the impairment of the MHC-II antigen presentation pathway by BZLF1 adds to other previously reported immune-modulating properties of BZLF1, notably; inhibition the IFN-gamma signaling pathway [40] and TNF-alpha activation [41,42] by down-regulation of IFN-γ receptor and TNF-R1. However, with regards to modulation of MHC-II antigen presentation it is likely that multiple EBV genes will cooperate to evade immune CD4+ T cell responses, as is seen with MHC-I antigen presentation to CD8+ T cells [43]. The exonuclease/host shut-off protein, BGLF5, expressed in early lytic cycle may contribute by degrading MHC-II mRNA transcripts [2,3] and the late BZLF2 glycoprotein, gp42, may contribute by binding to MHC-II molecules and sterically inhibiting recognition by the T cell receptor of immune CD4+ T cells [16].

In summary, this work provides a new paradigm for viral immune evasion of MHC-II presented antigen. Targeting CD74 expression is sufficient to substantially impair MHC-II presentation of antigenic peptides even when levels of MHC-II DR molecules are barely affected. However, as CD74 also serves as an important regulator of cell survival, fresh insight is provided as to the role of vBcl-2 during lytic cycle in B cells. It is widely accepted that BHRF1 prolongs cell survival during lytic cycle to allow sufficient time for production and accumulation of new infectious virions. Now, we suggest that BHRF1 also plays a pivotal role in enabling BZLF1 to attenuate recognition by CD4+ T cell responses.

Materials and Methods

Plasmids and transfection

A derivative of the DOX-dependent expression vector pRTS-1 [44] was kindly provided by Dr J. Mautner, Munich; BZLF1 and a reverse BZLF1 sequence as control were introduced into the vector by standard DNA cloning procedures to create vectors pRTS-CD2-BZLF1 and pRTS-CD2-control. The EBV lytic genes BZLF1, BZLF2, BALF4 were also subcloned into the EcoRI/NotI sites of pCDNA3-IRES-NSL-GFP vector. All plasmids were verified by restriction digest and sequence analysis. The pCDNA3-cyto-EBNA1 plasmid was described previously [20]. Transient transfection of MJS cells with plasmid DNA was routinely performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Targets for the T cell recognition assay clone were generated by co-transfection of HLA-DRB5*01 MJS cells with a cyto-EBNA1 expression plasmid and IRES-GFP, BZLF2-GFP, BALF4-GFP or BZLF1-GFP expression plasmids.

Stable transfection and establishment of pBZLF1-tet cell lines

LCIs were established using the reference B95.8-based recombinant lacking the BZLF1 gene (BZLF1KO) [45]. A doxycycline (DOX)-inducible BZLF1 expression vector, pRTS-CD2-BZLF1, or control vector with the reverse BZLF1 sequence (pRTS-CD2-control) were introduced into LCLs or Akata-A3 by electroporation of 10 μg plasmid DNA into 10^7 cells in OptiMem medium (Invitrogen) at 280 V and 960 μF using a Biorad electroporation apparatus. Transfected cells were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS). After 24 h, the transfected cell population was enriched by staining with OX34 antibody to rat CD2, and positively selected by magnetic cell sorting with anti-mouse IgG2a/b Microbeads and LS columns (Miltenyi Biotech) according to the manufacturer’s guidelines. Cells were thereafter expanded and maintained in RPMI 1640 medium supplemented with 10% FCS. BZLF1 expression was induced by addition of 200 ng/ml DOX for 24 h, and the induced cells were positively selected by magnetic cell sorting with anti-NRFR Microbeads and LS columns (Miltenyi Biotech). The purity of the sorted cells was checked with Beckman Coulter XL flow cytometer.

MJS-DRB5*01 and MJS-BHRF1 cells

The MJS (Mel JuSol) melanoma-derived cell line [46] was maintained in RPMI 1640 medium (Gibco BRL) supplemented with 10% FCS. HLA-DRB5*01 expressing MJS cells were generated by transduction with a DRB5*01 retrovirus vector; a HLA-DRB5*01 β chain gene was cloned into retroviral expression plasmid pQXIN (Clontech) by standard methods. Vesicular stomatitis virus-pseudotyped retrovirus particles were produced in GP2-293 cells co-transfected with the pSVS-G envelope vector. Virus in the culture supernatant at 72 h was concentrated by ultracentrifugation and used to infect 5 x 10^5 target cells overnight. Infected cells were selected with G418 (Invitrogen).

BHRF1 retroviral constructs were engineered by cloning the cDNA encoding BHRF1 into the pLZRS retroviral vector. Immediately downstream from the inserted BHRF1 gene lies an IRES sequence and the marker gene, a truncated nerve growth factor (NGFR). Vesicular stomatitis virus-pseudotyped retrovirus particles were produced as above and used to transduce MJS cells. Transduced cells were magnetically sorted using MACS NGFR-specific beads as directed by the manufacturer (Miltenyi Biotech).

CD74 knockdown by shRNA lentivirus

The lentivirus plasmids containing a sequence of CD74-specific shRNA or a sequence of scrambled shRNA were purchased from Santa Cruz Biotechnology. Vesicular stomatitis virus-pseudotyped lentivirus particles were produced in FT-293 cells co-transfected...
with the pVSV-G and Gag-Pol expressing vectors. Virus in the culture supernatant at 72 h was concentrated by ultracentrifugation and used to infect 5 x 10^5 target cells overnight. Infected cells were selected with puromycin (Sigma).

**Metabolic labelling and immunoprecipitation**

Cells were starved by culturing 10^5 in 15 ml methionine-free RPMI medium supplemented with 10% dialysed FCS for 1 h at 37°C, then labeled for 15 min with 200 μCi of ^35S protein labeling mix (PerkinElmer) in a final volume of 1 ml. After two washes with chase medium (normal RPMI medium supplemented with 10% FCS), the cells were resuspended at 2 x 10^6 cells/ml and chased at 37°C for the times indicated. Samples containing 2 x 10^6 cells were lysed in 400 μl of NP-40 buffer (0.5% Nonidet P-40, 5 mM MgCl_2 and 50 mM Tris-HCl, pH 7.5) with protease inhibitor cocktail (Sigma) at 4°C for 45 min. Nuclei and insoluble debris were removed by centrifugation, and the supernatants were preclreated, first with 1.2 μl normal mouse serum and 20 μl Dynabeads Protein A (Invitrogen) for 2 h at 4°C, and then with 20 μl Dynabeads Protein A and 20 μl Dynabeads Protein G at 4°C overnight. The precleared lysates were immunoprecipitated for 2 h with 1 μg of mouse anti-CD74 and 20 μl Dynabeads Protein A plus 20 μl Dynabeads Protein G, before washing the beads four times with NET buffer (0.5% NP-40, 150 mM NaCl2, 5 mM EDTA and 50 mM Tris-HCl, pH 7.5) and eluting by boiling in reducing gel sample buffer for 5 min. Finally, the samples were separated by SDS-PAGE on 12% Bis-Tris NuPage mini-gels with MOPS running buffer (Invitrogen). The capacity of CD4+ and CD8+ T cell clones to recognize target LCls or MJS cells was measured by IFNγ ELISA (Endogen). Briefly, 10^5 effector T cells were incubated for 18 h at 37°C in V-bottom microtest plate wells with 10^5 target cells, before assaying the supernatants for IFN-γ release by ELISA (Endogen) in accordance with the manufacturer’s recommended protocol.

**QRT-PCR assay**

Total RNA was isolated from cultured cell lines using QIAGEN RNaseasy kit and treated with DNase I (Turbo DNA-free kit; Ambion). Quantitative reverse-transcription polymerase chain reaction (QRT-PCR) assays for DRA, CD74, Bcl-2 and Bcl-xI were performed with TaqMan® Gene Expression Assays (Applied biosystem), duplexed with GAPDH assays for normalization.

**Supporting Information**

**Figure S1** BZLF1-expressing LCls can be recognized by EBV lytic antigen specific CD4 T cells. The pRTS-C2D2-BZLF1 and pRTS-C2D2-control vector transfected BZLF1KO LCls were induced by treatment with DOX for 24 h. The induced cultures were assayed for recognition by various CD4+ effector T cell clones specific for different EBV lytic cycle antigens. The CD4+ T cells used in this figure were: ‘VKF’ effectors specific for amino acid residues 11–25 of BZLF1 protein and restricted through DRB3*01; ‘VKL’ effectors specific for amino acid residues 136–150 of BMRF1 protein and restricted through DRB1*01; ‘LDL’ effectors specific for amino acid residues 61–81 of gp350 protein and restricted through DRB1*01. Induced BZLF1KO LCls and effector T cells were co-cultured for 18 h, and culture supernatants were tested for the release of IFN-γ as a measure of T cell recognition. All results are expressed as IFN-γ release in pg/ml, and error bars indicate standard deviation of triplicate cultures. (TIF)

**Figure S2** The toxicity of BZLF1 in EBV negative DG75 B cells can be attenuated by BHRF1. EBV negative DG75 B cells were transfected with pMAX-GFP expression plasmid alone (solid line), together with pCDNA-BZLF1 expression plasmid (dotted line) or together with pCDNA-BZLF1 and pSG5-BHRF1 expression plasmids (dashed line). All transfection plasmid mixes were bulked to a constant amount of DNA with control vector. Cells were harvested at indicated time points after transfection for analysis of GFP expression by flow cytometry. All results are...
expressed as the percentage of GFP+ cells, and error bars indicate standard deviation of three independent transfections.

**Figure S3** CD74 can be downregulated by BZLF1 in a CIITA-promoter independent manner. 293-CIITA cells were generated by transduction with a retrovirus vector. Retroviral constructs were engineered by cloning the cDNA encoding CIITA (accession number EAW5172) into the pLZRS retroviral vector. Immediately downstream from this gene was an IRES and the marker gene, truncated nerve growth factor (ΔNGFR). Vesicular stomatitis virus-pseudotyped retrovirus particles were produced in GP2-293 cells co-transfected with the pSV-G envelope vector. Virus in the culture supernatant at 72 h was concentrated by ultracentrifugation and used to infect 5 × 10^5 target cells overnight. Transduced cells were magnetically sorted using MACS NGFR-specific beads as directed by the manufacturer (Miltenyi Biotech). (A) 293 cells transduced with a control NGFR retrovirus or with a CIITA-IRES-NGFR retrovirus were stained with PE-conjugated anti-DR or with CD74 MAb followed by PE conjugated anti-mouse IgG2a antibody, then analyzed by flow cytometry. Solid lines show the surface MHC-II DR or CD74 expression in 293-CIITA cells. The shaded histogram indicates isotype control staining. (B) Cell lysates prepared from 293 control and 293-CIITA cells were analyzed by immunoblotting using antibodies specific for CIITA, DRα2 chain, CD74 or calreculin as a loading control. (C) 293-CIITA cells transfected with either IRES-GFP or BZLF1-GFP expression plasmids were stained with PE-conjugated anti-DR (C) or with CD74 MAb followed by PE conjugated anti-mouse IgG2a antibody (D), then analyzed by flow cytometry. Histograms show the surface MHC-II DR or CD74 expression on GFP- cells (solid line) and GFP+ cells (dashed line). The shaded histogram indicates isotype control staining.

**Figure S4** Downregulation of CD74 by BZLF1 cannot be reversed when the CD74 is over expressed from a CMV promoter. MJS cells with CMV promoter-driven CD74 overexpression were generated by transduction with a retrovirus vector. CD74 cDNA was cloned into retroviral expression plasmid pQcxIIH (Clontech) by standard methods. Vesicular stomatitis virus-pseudotyped retrovirus particles, including PQcxIIH empty vector and PQcxIIH-CD74 were produced in GP2-293 cells co-transfected with the pSV-G envelope vector. Virus in the culture supernatant at 72 h was concentrated by ultracentrifugation and used to infect 5 × 10^5 target cells overnight. Infected cells were selected with Hygromycin (Invitrogen). (A) Cell lysates of MJS-PQcxIIH and MJS-CD74 cell lines were analyzed by immunoblotting with antibodies to DRα2, CD74, or calreculin as a loading control. (B) MJS-PQcxIIH and MJS-CD74 were stained with PE-conjugated anti-DR or with PE-conjugated anti-CD74, then analyzed by flow cytometry. Histograms show the surface MHC-II DR or CD74 expression on control MJS-PQcxIIH cells (solid line) and MJS-CD74 cells (dashed line). The shaded histogram indicates isotype control staining. (C) MJS-PQcxIIH and MJS-CD74 cells were co-transfected with BHRF1 and either IRES-GFP or BZLF1-GFP expression plasmids were stained with PE-conjugated anti-DR or with GFP+ population from IRES-GFP transfected cells (solid line) and GFP+ population from the BZLF1-GFP transfected cells (dashed line). The shaded histogram indicates isotype control staining.

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**Author Contributions**

Conceived and designed the experiments: JZ GST MR. Performed the experiments: JZ WAT. Analyzed the data: JZ MR. Contributed reagents/materials/analysis tools: TAH LF HML ADH. Wrote the paper: JZ MR.

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