Mechanisms of immune evasion in Epstein-Barr virus infection

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Chapter 4

Epstein-Barr virus BILF1 evolved to downregulate cell surface display of a wide range of HLA class I molecules through their cytoplasmic tail

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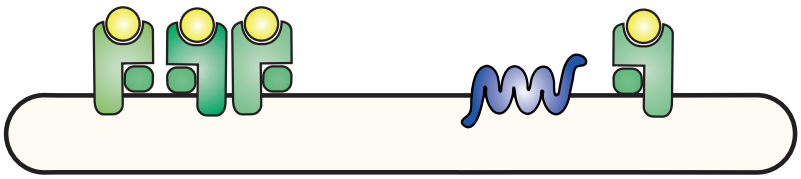

Abstract

Co-evolution of herpesviruses and their hosts has driven the development of both host antiviral mechanisms to detect and eliminate infected cells, and viral ploys to escape immune surveillance. Among the immune evasion strategies employed by the lymphocryptovirus (g_1-herpesvirus) EBV is the downregulation of surface HLA class I expression by the virally-encoded G-protein coupled receptor BILF1, thereby impeding presentation of viral antigens and cytotoxic T cell recognition of the infected cell. Here, we show EBV BILF1 to be expressed early in the viral lytic cycle. BILF1 targets a broad range of HLA class I molecules, including multiple HLA-A and -B types, and HLA-E. In contrast, HLA-C was only marginally affected. We advance the mechanistic understanding of the process by showing the cytoplasmic C-terminal tail of EBV BILF1 to be required for reducing surface HLA class I expression. Susceptibility to BILF1-mediated downregulation is in turn conferred by specific residues in the intracellular tail of the HLA class I heavy chain. Finally, we explore the evolution of BILF1 within the lymphocryptovirus genus. While the homolog of BILF1 encoded by the lymphocryptovirus infecting Old World rhesus primates shares the ability of EBV to downregulate cell surface HLA class I expression, this function is not possessed by New World marmoset lymphocryptovirus BILF1. This study therefore furthers our knowledge on the evolution of immunoevasive functions by the lymphocryptovirus genus of herpesviruses.

Introduction

Lymphocryptoviruses (LCVs) comprise a genus of the γ-herpesvirus subfamily whose members are only found in primates [1]. The LCV targeting humans, Epstein-Barr virus (EBV), is carried by more than 90% of adults worldwide [2, 3]. While infection is usually asymptomatic, primary encounter with the virus can present as infectious mononucleosis. EBV infection is also strongly associated with tumours of lymphoid and epithelial origin, reflecting the tropism of the virus for B cells and epithelial cells, and its potential for oncogenic transformation [4].

Despite the activation of a robust host T cell response upon primary infection, and the capacity for a memory T cell response thereafter, the virus persists for life even in immunocompetent individuals [5]. This is partly due to the ability of EBV, like all herpesviruses, to enter a state of latency in which protein expression is minimized, thereby limiting viral antigen display by the infected cell. Yet in order for EBV to spread to a new host, it must produce infectious virions by entering the replicative, or lytic, phase of its life cycle. In this phase, over 80 EBV-encoded proteins are expressed in a temporal cascade, with initial production of immediate early (IE) transactivators triggering induction of early genes, including enzymes required for viral replication [2]. This is followed by expression of late genes encoding virion structural components. The lytic phase thus creates ample viral antigens for proteasomal processing and HLA class I cell surface presentation, which can then recruit memory $CD8^+$ T cells capable of eliminating the infected cell. However, millions of years of co-evolution with their hosts have seen herpesviruses acquire active immune evasion mechanisms to thwart this host response and permit sufficient time for the lytically infected cell to generate new virus particles [6-8].

In the case of EBV, several such immune evasion strategies target the HLA class I antigen processing and presentation pathways [9]. The EBV host shutoff protein BGLF5 degrades mRNA, thus obstructing synthesis of new HLA class I molecules [10, 11]. Meanwhile, BNLF2a blocks entry of proteasome-generated peptides into the ER and subsequent loading onto HLA class I molecules by inhibiting the heterodimeric TAP complex [12, 13]. TAP transport is also hindered by the viral chemokine homolog, vIL-10, which reduces expression of the TAP1 subunit [14]. Another manner in which EBV can downregulate cell surface HLA class I expression and inhibit T cell recognition of infected cells is through BILF1, a viral G-protein coupled receptor (vGPCR) [15, 16].

Several poxviruses and herpesviruses encode GPCRs that were most likely pirated from their host by retrotransposition [17-19]. vGPCRs serve many functions, including the scavenging of host chemokines [20], cell-to-cell-adhesion [21] and the re-programming of intracellular signalling networks to promote efficient viral replication [22]. BILF1 is expressed during the EBV lytic cycle and was first identified as a potential vGPCR due to the presence of seven membrane-spanning domains and (limited) homology with known herpesviral GPCRs [23-25]. While it displays both structural and functional similarities to chemokine receptors,

EBV BILF1 modulates intracellular signalling pathways constitutively as an orphan receptor [24, 25].

Initial immune evasion ability was ascribed to EBV BILF1 as it reduced phosphorylation of the dsRNA-dependent protein kinase R [24]. It has also been found to heterodimerize with human chemokine receptors [26]. In the case of CXCR4 this results in impairment of ligand-induced receptor signalling [27]. Finally, EBV BILF1 decreases cell-surface levels of HLA class I [15]. This can be achieved by BILF1-mediated acceleration of endocytosis and subsequent lysosomal degradation of HLA class I molecules from the cell surface, or by diversion of newly synthesized HLA class I molecules from the normal exocytic pathway that allows proteins to travel from the ER to the cell surface ([16]). In turn, this leads to the inhibition of $CD8^+$ T cell recognition of infected cells.

Here, we further investigate the ability of EBV BILF1 to subvert the antigen presentation pathway. We assess the ability of BILF1 to target a range of specific HLA class I alleles and identify the protein domain present in BILF1 and specific amino acid residues in the HLA class I heavy chain (HC) that are required to facilitate downregulation of HLA class I from the cell surface. Finally, we examine the evolution of BILF1 by comparing the immunoevasive ability of EBV BILF1 with that of homologs expressed by LCVs infecting Old World rhesus and New World marmoset primates.

Results

EBV BILF1 is an early lytic cycle gene

Previous reports present conflicting information on the expression of BILF1 during the productive phase of EBV infection [24, 25, 35]. As the absence of a working anti-BILF1 antibody precluded the detection of BILF1 protein, we monitored the appearance of BILF1 mRNA in an EBV^+ Akata-derived B cell line following induction of the viral lytic cycle by cross-linking of the B cell receptor. BILF1 expression was strongly induced by 4 h and remained elevated up to 16 h post-induction (**Figure 1A**). The faint band observed for uninduced cells may indicate a low level of BILF1 expression, as has been described for other EBV^+ B cell lines under strict latency [24]. PAA inhibits the viral DNA polymerase and transcription of late lytic genes, and can thus be used to dissect the temporal profile of lytic gene expression. While expression of the gp42-encoding late gene BZLF2 was detectable after overnight lytic cycle induction, it was completely blocked by prior addition of PAA (**Figure 1B** upper panel). In contrast, BILF1 mRNA was still induced in the presence of PAA (middle panel). Together, these data show EBV BILF1 to be an early lytic cycle gene.

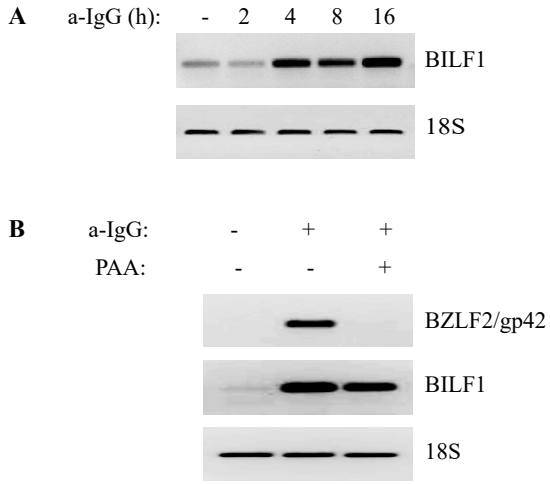

Figure 1 The BILF1 gene is expressed early in the EBV lytic cycle. EBV lytic cycle gene expression was induced in AKBM cells by cross-linking of the B cell receptor with α-IgG for the indicated time periods (hours) (A), or for 16 h in the presence or absence of PAA (B). RNA was isolated, cDNA generated, and expression levels of the indicated gene products determined by PCR and EtBr gel electrophoresis.

EBV BILF1 can selectively modulate cell surface expression of HLA class I alleles

To examine its effect on individual HLA class I alleles, cells with different HLA haplotypes were transduced to express EBV BILF1. In a lentiviral vector, the EBV BILF1 gene was cloned upstream of an internal ribosomal entry site that is followed by the gene encoding enhanced GFP. Transduced cells could thus be identified easily as a GFP^+ population. In addition, a FLAG-tag added to the BILF1 N-terminus made it possible to confirm surface expression of the viral protein. Cells were transduced with control GFP or EBV BILF1/GFP-encoding lentivirus and the presence of surface markers was analysed by flow cytometry after 7 days. Prior to staining, transduced and untransduced cells were mixed to allow comparison in a single assay.

Melanoma-derived Mel JuSo (MJS) cells [30], widely used in antigen presentation studies due to the expression of both HLA class I and II, displayed strong cell surface BILF1 expression after transduction with BILF1/GFP lentivirus (**Figure 2A**, upper row). Total surface HLA class I expression was reduced in BILF1/GFP-expressing cells compared to untransduced cells and cells expressing GFP alone (second row). However, BILF1 did not decrease cell surface HLA class II expression (third row). Through the use of alloantigen-specific antibodies, the effect of BILF1 on individual HLA class I types expressed by MJS cells was then evaluated. BILF1 reduced expression of HLA-A1 (fourth row) and mediated a particularly strong decrease in surface HLA-B8 expression (bottom row).

HeLa cells expressing EBV BILF1 after lentiviral transduction also displayed reduced surface levels of W6/32-reactive HLA class I (**Figure 2B**). Specific staining of both HLA-A68 and HLA-B15 showed that expression of both was decreased in $BILF1^+$ HeLa cells. Similarly, surface HLA class I levels in $BILF1^+$ HEK 293T cells were reduced compared to control cells (**Figure 2C**). Downregulation of HLA-B7 and a moderate decrease in HLA-A2 levels contributed to this effect (**Figure 2C**). Taking these data together, EBV BILF1 can thus target

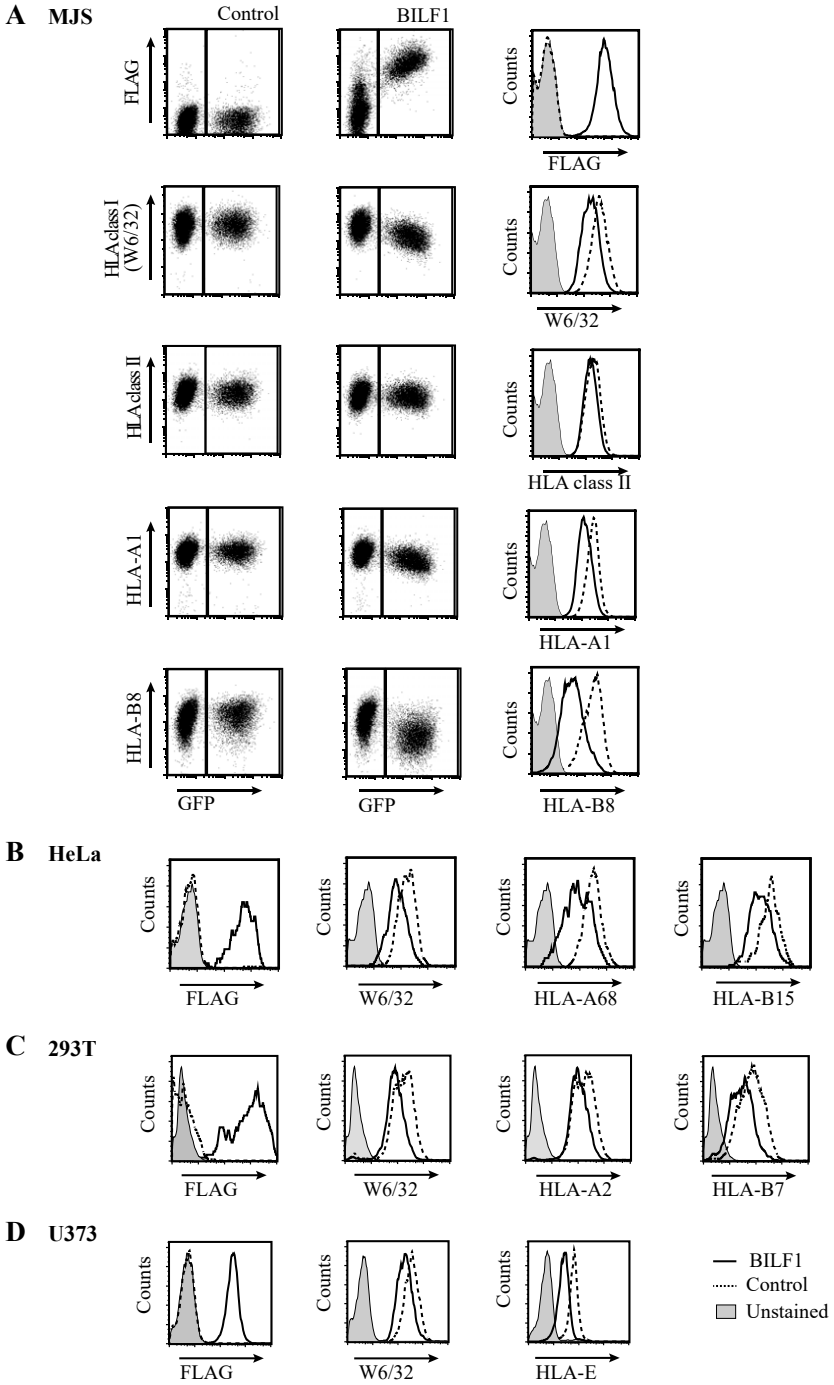
A MJS
Control
BILF1
FLAG
Counts
FLAG
HLA class I (W6/32)
W6/32
HLA class II
HLA class II
HLA-A1
HLA-A1
HLA-B8
GFP
GFP
HLA-B8
B HeLa
FLAG
W6/32
HLA-A68
HLA-B15
C 293T
FLAG
W6/32
HLA-A2
HLA-B7
D U373
FLAG
W6/32
HLA-E
BILF1
Control
Unstained

Figure 2 EBV BILF1 reduces surface expression of a broad range of HLA class I alleles. *A*, MJS cells were transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) replication-deficient lentivirus. After 7 days, surface expression of FLAG-tagged EBV BILF1, total HLA class I, HLA class II, HLA-A1 and HLA-B8 was determined by flow cytometry. Transduced and untransduced MJS cells were mixed before antibody staining to allow comparison in a single assay (left and middle columns). Surface levels of the indicated proteins were also compared between GFP^+ control cells and $BILF1/GFP^+$ cells (histograms, right column). *B*, HeLa; *C*, 293T and *D*, U373 cells were transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) lentivirus. Surface expression of FLAG-tagged EBV BILF1, total HLA class I or of the indicated HLA class I alleles was examined by flow cytometry after 7 days.

HLA-A and -B gene products, giving a reduction in expression of W6/32-reactive HLA class I molecules at the cell surface.

We next examined whether expression of the non-classical HLA-E is also affected by BILF1. U373 cells expressing endogenous HLA-E were lentivirally transduced to achieve BILF1 expression. BILF1 caused a decrease in cell surface levels of W6/32-reactive HLA class I, molecules (**Figure 2D**). Interestingly, $BILF1^+$ cells also displayed reduced levels of HLA-E, demonstrating that EBV BILF1 can target a wide range of HLA class I molecules to bring about a reduction in cell surface HLA class I expression.

Finally, we assessed whether EBV BILF1 can also downregulate cell surface levels of HLA-C. Although poorly expressed by many cell types, HLA-C is expressed at the surface of U937 myeloid cells and can be specifically detected using the human antibody WK4C11 [36]. N-terminally HA-tagged BILF1 was efficiently expressed in U937 cells following retroviral transduction (**Figure 3**, upper row), which led to a reduction in total surface HLA class I expression (second row). While surface expression of HLA-A3 (third row) and HLA-B18 (fourth row) was strongly reduced by BILF1, HLA-C was only marginally downregulated (bottom row). EBV BILF1 can therefore selectively decrease cell surface levels of HLA-A, -B and -E alleles, while only slightly affecting HLA-C.

The C-terminal tail of EBV BILF1 is required for downregulation of surface HLA class I

The C-terminal domain of GPCRs is known to interact with intracellular proteins including endocytic adaptors [37]. To assess whether the C-terminal domain of EBV BILF1 is involved in downregulation of surface HLA class I molecules, we generated a C-terminal deletion mutant, BILF1-ΔC19, which lacks the 19 most C-terminally-located amino acid residues (**Figure 4A**). This C-terminal deletion mutant retained some of the characteristics of its wt counterpart. EBV BILF1wt activates the transcription factor NFκB in a manner dependent on the integrity of the EKT motif in its third transmembrane domain [15]. BILF-ΔC19 expression also activated NFκB in HEK 293-kB luc cells following transient transfection (**Figure 4B**).

BILF1-ΔC19, containing an N-terminal FLAG-tag, was cloned into a lentiviral expression vector. MJS cells were then transduced with control, BILF1wt, or BILF1-ΔC19 lentivirus. Anti-FLAG staining showed that similar levels of BILF1wt and BILF1-ΔC19 were expressed at

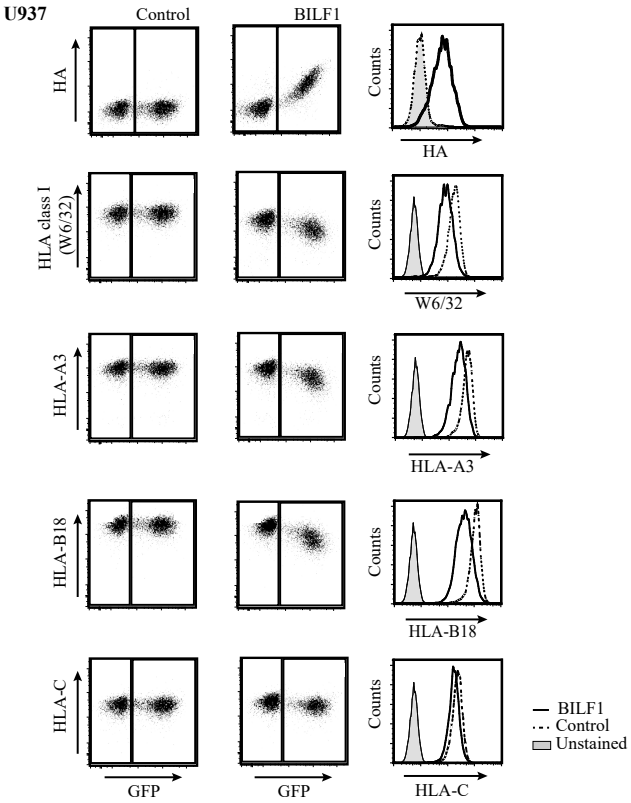

Figure 3 EBV BILF1 only marginally downregulates cell surface HLA-C expression. U937 cells were transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) replication-deficient retrovirus. Surface expression of HA-tagged EBV BILF1, total HLA class I, HLA-A3 and HLA-B18 and HLA-C was determined by flow cytometry. Surface levels of the indicated proteins were compared between BILF1$^-$/GFP$^-$ cells and BILF1$^+$/GFP$^+$ cells (histograms, right column).

the surface of transduced cells (**Figure 4C**). However, whereas MJS cells expressing BILF1 wt displayed a concomitant decrease in surface HLA-B8 expression, BILF1-ΔC19 was severely diminished in its ability to downregulate HLA-B8. Meanwhile, neither BILF1 wt nor BILF1-ΔC19 significantly altered the surface levels of HLA class II. This indicates that the C-terminal domain of EBV BILF1, specifically the 19 last C-terminal residues, is required to bring about the downregulation of HLA class I from the cell surface.

Inspection of the sequence of the BILF1 C-terminus identified two potential internalization motifs (highlighted in dashed-line boxes in **Figure 4A**). The first was QVTV, a putative Type II PDZ ligand sequence, conforming to the consensus sequence X-Φ-X-Φ, where X is any amino acid and Φ is a bulky hydrophobic residue [37, 38]. The second was a potential non–classical tyrosine-based motif, YFRRV, conforming to the consensus sequence Y-X-X-X-Φ [39, 40]. We thus generated two independent BILF1 point mutant proteins, substituting an alanine residue for either V299 of the putative Type II PDZ ligand sequence or Y303 of the proposed tyrosine based motif. The resulting point mutants were both expressed at the cell surface. However, the ability to downregulate HLA class I was retained in both cases, demonstrating that neither putative motif contributes to EBV BILF1-mediated downregulation of surface HLA class I (**Supplementary Figure I**).

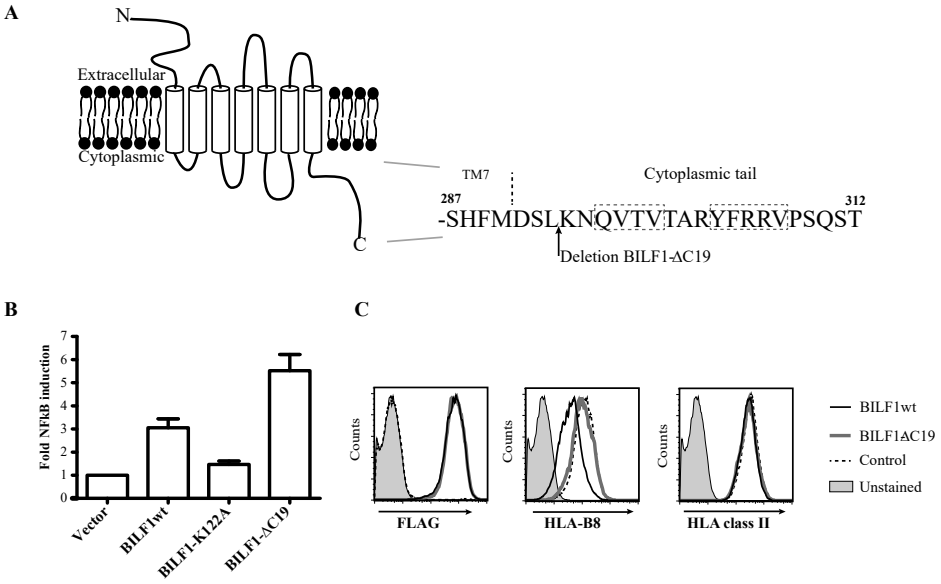

Figure 4 The EBV BILF1 C-terminal tail is required for HLA class I downregulation. *A*, Schematic representation of the 7-transmembrane EBV BILF1 vGPCR with the sequence of the 26 most C-terminally located amino acid residues described. The arrow indicates the point of truncation used to generate EBV BILF1-ΔC19. Dashed-line boxes indicate putative endocytosis/sorting motifs. *B*, EBV BILF1-ΔC19 activates intracellular signalling pathways. 293-NFκB-luciferase cells were co-transfected with control expression vector or constructs encoding EBV BILF1wt, EBV BILF1-K122A or EBV BILF1-ΔC19, and pGL3-*Renilla* (for normalizing transfection efficiency) luciferase. At 24 h post-transfection, cell lysates were assayed for firefly and *Renilla* luciferase activity. Data are presented as fold NFκB induction relative to cells transfected with vector alone. Results represent mean +/- SEM of a representative experiment performed in triplicate. *C*, MJS cells were transduced with GFP-encoding (control), EBV BILF1wt/GFP-encoding (BILF1wt) or EBV BILF1-ΔC19/GFP-encoding (ΔC19) replication-deficient lentivirus. After 7 days, surface expression of FLAG-tagged EBV BILF1, HLA-B8 and HLA class II was determined by flow cytometry.

The intracellular region of the HLA class I HC is required for downregulation by BILF1

Given the requirement for the C-terminal domain of BILF1 in bringing about a reduction of surface HLA class I, we further hypothesized that the intracellular region of the HLA class I HC might also be necessary. We therefore generated a form of the HLA-B8 HC from which the 24 most C-terminal amino acid residues were deleted, leaving only 6 intracellular residues (HLA-B8 short; **Figure 5A**). Full-length HLA-B8 and HLA-B8 short were cloned into a lentiviral expression vector that co-expressed the control protein truncated nerve growth factor receptor (trNGFR). The U373 cell line, which is susceptible to the effects of BILF1 on HLA class I (**Figure 2D**) and does not express endogenous HLA-B8 (**Figure 5B**), was then transduced with either HLA-B8 wt/trNGFR or HLA-B8 short/trNGFR lentivirus. Equivalent

surface levels of trNGFR and of wt and short HLA-B8 were observed in the respective U373 transductants (**Figure 5B**).

The U373-HLA-B8wt and U373-HLA-B8 short cell lines were next transduced with either control or BILF1 lentivirus. BILF1 functionality in both cell lines was confirmed by its ability to downregulate surface expression of the endogenously expressed HLA-B18 allele to a similar extent (**Figure 5C**).

Interestingly, BILF1 caused a decrease in HLA-B8wt levels in U373 cells, but failed to reduce surface levels of HLA-B8 short. Surface levels of trNGFR were not affected by BILF1, while surface expression of BILF1 itself was equivalent in U373-HLA-B8wt and U373-HLA-B8 short cells (**Supplementary Figure II**). These data therefore show that in addition to the C-terminal domain of BILF1, the intracellular region of the HLA class I HC is required for BILF1-mediated downregulation of HLA class I surface expression.

Having assessed the effect of BILF1 on a wide range of HLA class I alleles (**Figures 2** and **3**), we next examined whether the selective targeting of surface HLA-A, -B and -E, but not HLA-C, molecules could help pinpoint key residues in the HLA class I HC tail that facilitate BILF1-mediated downregulation (**Figure 6A**). Comparing the amino acid sequence of the BILF1-sensitive HLA-B intracellular tails with those of BILF1-resistant HLA-C showed that discriminating differences exist at positions 344, 351, 358 and 361 of the HLA-B alleles as indicated in **Figure 6A**. We therefore generated point mutations in HLA-B8 to examine whether the replacement of HLA-B8 residues with those found in the corresponding positions of the HLA-C intracellular tail would render the mutant HLA protein resistant to the effects of BILF1. The C-tail sequence of the HLA-B8 point mutants used is indicated in **Figure 6A**, lower panel.

U937 cells, which do not express endogenous HLA-B8 (data not shown), were transduced to express HLA-B8 wt or HLA-B8 point mutant proteins. Surface levels of endogenously expressed HLA-B18 were reduced by BILF1 to a similar extent in U937-HLA-B8 wt and U937-HLA-B8 point mutant cell lines (**Figure 6B**). However, while BILF1 downregulated cell surface HLA-B8 wt, it did not decrease cell surface HLA-B8 $Y_{344}C/D_{351}N/V_{358}E/T_{361}I$ expression (**Figure 6B**). This strongly indicated that one, some, or all of the HLA-B8 residues mutated in HLA-B8 $Y_{344}C/D_{351}N/V_{358}E/T_{361}I$ are required to facilitate targeting by BILF1.

In further analysis of HLA-B8 point mutants, surface expression of HLA-B8 $T_{361}I$ was efficiently decreased by BILF1, demonstrating that residue T361 is not essential for BILF1-mediated HLA-B8 downregulation. Indeed, as with HLA-B8 $Y_{344}C/D_{351}N/V_{358}E/T_{361}I$, surface levels of HLA-B8 $Y_{344}C/D_{351}N/V_{358}E$ remained unaffected by BILF1. Individual mutation of residues Y344, D351 and V358 has not allowed a single amino acid to be identified as being essential to targeting of HLA-B8 by BILF1 (data not shown). Rather, the possibility exists that each could play a contributory role.

In conclusion, the BILF1-mediated downregulation of HLA class I molecules requires their cytoplasmic domains; the amino acid residues at position 344, 351 and 358 appear to contribute essentially to the observed phenotype in the case of HLA-B8.

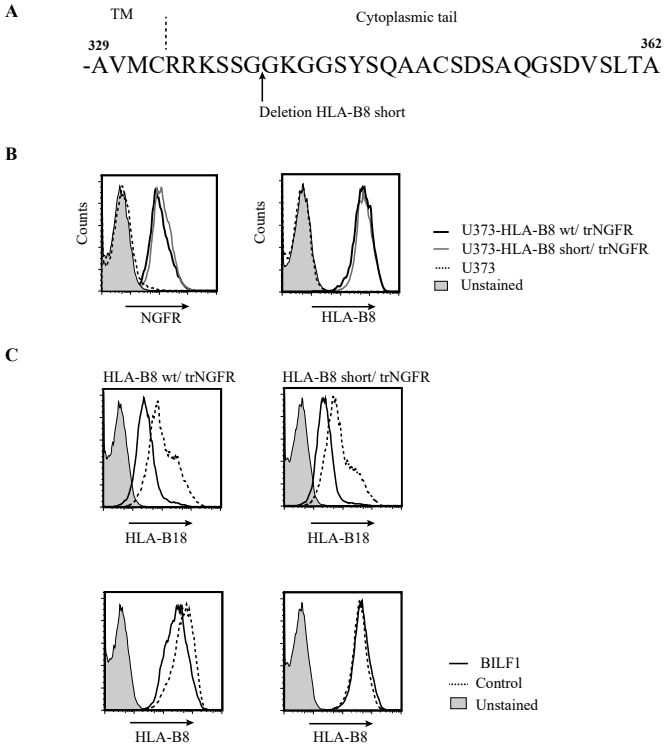

Figure 5 The cytoplasmic region of HLA class I HC is required for BILF1-mediated cell surface downregulation. *A*, Sequence of the HLA-B8 C-terminus. The arrow denotes the point of truncation used to generate HLA-B8 short. *B*, U373 cells were transduced with HLA-B8wt/trNGFR-encoding or HLA-B8 short/trNGFR-encoding replication-deficient lentivirus. Surface expression of HLA-B8 and trNGFR on transduced and untransduced cells was determined by flow cytometry. *C*, U373-HLA-B8wt and U373-HLA-B8 short cells were further transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) replication-deficient lentivirus. After 7 days, surface expression of HLA-B8 and the endogenous HLA-B18 allele was measured by flow cytometry.

Marmoset LCV BILF1 fails to downregulate cell surface MHC class I

BILF1 homologs are encoded by rhesus LCV (80.4% amino acid identity to EBV BILF1 in an NCBI blastp two-sequence comparison) and marmoset LCV (41% identity to EBV BILF1) (**Figure 7A**). Rhesus LCV BILF1 has previously been shown to mediate MHC class I downregulation [15], but the functionality of marmoset LCV BILF1 in this regard is unknown. Cloning the rhesus and marmoset LCV BILF1 genes into a lentiviral expression vector allowed us to directly compare the ability of the three LCV BILF1 homologs to downregulate surface HLA class I in MJS cells. Both EBV BILF1 and rhesus LCV BILF1 strongly decreased surface HLA-B8 in lentivirally transduced MJS cells, but marmoset LCV BILF1 failed to do so (**Figure 7B**). HLA class II was not affected by expression of any of the BILF1 proteins (data not shown).

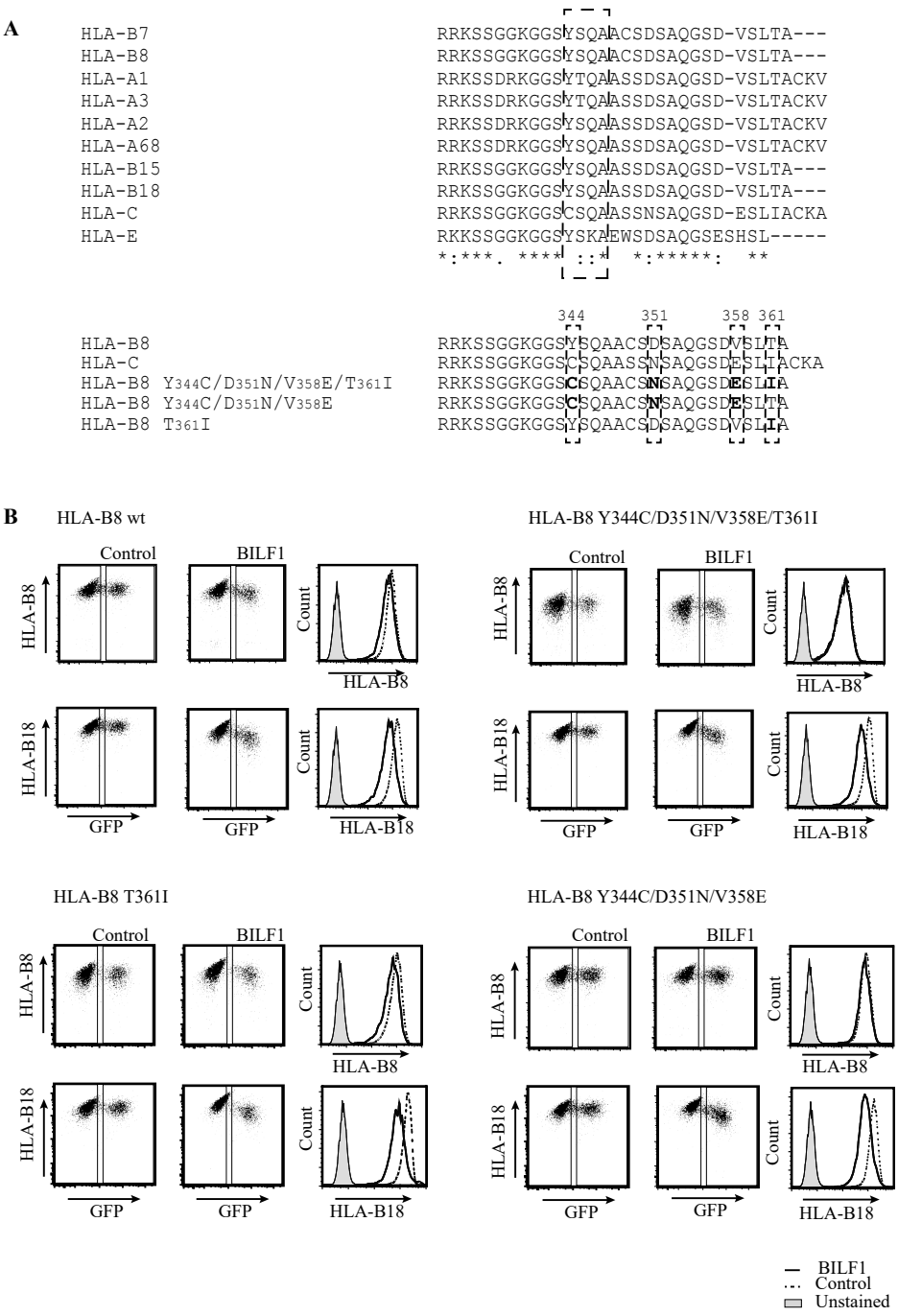
A
HLA-B7 RRKSSGGKGGSYSQAACSDSAQGSD-VSLTA---
HLA-B8 RRKSSGGKGGSYSQAACSDSAQGSD-VSLTA---
HLA-A1 RRKSSDRKGGSYTQAASSDSAQGSD-VSLTACKV
HLA-A3 RRKSSDRKGGSYTQAASSDSAQGSD-VSLTACKV
HLA-A2 RRKSSDRKGGSYSQAASSDSAQGSD-VSLTACKV
HLA-A68 RRKSSDRKGGSYSQAASSDSAQGSD-VSLTACKV
HLA-B15 RRKSSGGKGGSYSQAASSDSAQGSD-VSLTA---
HLA-B18 RRKSSGGKGGSYSQAASSDSAQGSD-VSLTA---
HLA-C RRKSSGGKGGSCSQAASSNSAQGSD-ESLIACKA
HLA-E RKKSSGGKGGSYSKAEWSDSAQGSESHSL-----
344 351 358 361
HLA-B8 RRKSSGGKGGSYSQAACSDSAQGSDVSLTA
HLA-C RRKSSGGKGGSCSQAASSNSAQGSDESLIACKA
HLA-B8 Y344C/D351N/V358E/T361I RRKSSGGKGGSCSQAACSNSAQGSDESLIA
HLA-B8 Y344C/D351N/V358E RRKSSGGKGGSCSQAACSNSAQGSDESLTA
HLA-B8 T361I RRKSSGGKGGSYSQAACSDSAQGSDVSLIA
B
HLA-B8 wt
HLA-B8 Y344C/D351N/V358E/T361I
HLA-B8 T361I
HLA-B8 Y344C/D351N/V358E
Control
BILF1
HLA-B8
HLA-B18
Count
GFP
BILF1
Control
Unstained

Figure 6 EBV BILF1-mediated HLA-B8 downregulation requires amino acid residues in the cytoplasmic region of the HLA-B8 molecule. *A*, *upper panel*, Amino acid sequence alignment of selected HLA class I heavy chain C-terminal tails. Alignments were generated using ClustalW2. An asterisk (*) indicates positions that have a single, fully conserved residue. A colon (:) indicates conservation between groups of strongly similar properties. A full stop (.) indicates conservation between groups of weakly similar properties. Sequences of the following subtypes were selected from the IMGT/HLA Database: A*01:01:01:01 (HLA-A1), A*02:01:01:01 (HLA-A2), A*03:01:01:01 (HLA-A3), A*68:01:01:01 (HLA-A68), B*07:02:01 (HLA-B7), B*08:01:01 (HLA-B8), B*15:03:01 (HLA-B15), B*18:01:01:01 (HLA-B18), C*01:02:01 (HLA-C), E*01:01:01:01 (HLA-E). The dashed box indicates a putative YXXA internalization motif present in HLA-A, -B and -E alleles but not HLA-C. *A, lower panel,* C-terminal tail amino acid sequences of HLA-B8 point mutants. Amino acids in the dashed boxes were subject to substitution. The numbers above the dashed boxes indicate the position of the residue in the HLA-B8 protein. *B,* U937 cells transduced to express HLA-B8 wild type, HLA-B8 $Y_{344}C/D_{351}N/V_{358}E/T_{361}I$, HLA-B8 $T_{361}I$, or HLA-B8 $Y_{344}C/D_{351}N/V_{358}E$ were transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) replication-deficient retrovirus. Surface expression of HLA-B8 and HLA-B18 was determined by flow cytometry. Surface levels of the indicated proteins were compared between $BILF1^-/GFP^-$ cells and $BILF1^+/GFP^+$ cells (histograms).

All three BILF1 homologs contained N-terminal FLAG tags, allowing their surface expression to be assessed (**Figure 7B**). Marmoset LCV BILF1 was efficiently expressed at the cell surface, albeit to a lesser extent than its EBV and rhesus LCV counterparts. Further analysis showed HLA-B8 levels to be reduced in the population of MJS cells expressing EBV or rhesus LCV BILF1 at an equivalent level to that obtained with marmoset LCV BILF1 (**Figure 7C**).

Although marmoset LCV BILF1 failed to reduce surface expression of MHC class I in human cells, it remained possible that the viral protein could be functional in this respect in marmoset cells. We thus transiently transfected primary marmoset fibroblasts with a vector offering co-expression of FLAG-tagged marmoset LCV BILF1 and GFP. Marmoset MHC class I on these cells was detectable using the W6/32 mAb. At 48 hours post-transfection, transfected cells were identified by GFP expression. No decrease in surface MHC class I was evident in cells transfected with the marmoset LCV BILF1-IRES-GFP vector (**Figure 7D**; left, upper panel). As a population of GFP^+ cells failed to express detectable levels of marmoset LCV BILF1 at the surface (middle, upper panel), a double-staining procedure was performed to more precisely assess MHC class I levels in cells clearly expressing marmoset LCV BILF1. This confirmed that GFP^+ marmoset cells containing high surface levels of FLAG-tagged marmoset LCV BILF1 did not undergo a downregulation of MHC class I (right, upper panel). EBV BILF1 also failed to reduce surface expression of marmoset MHC class I (**Figure 7D**, lower panels). This indicates that a species restriction exists with regard to EBV BILF1 function, and suggests that a sufficient level of homology with the host protein(s) required for EBV BILF1-mediated MHC class I downregulation may not be present in marmoset cells.

Given the importance of the C-terminal domain of EBV BILF1 in mediating its effect towards HLA class I, we examined finally whether this domain was itself sufficient to confer the ability to downregulate HLA class I to a heterotypic protein. We therefore generated a chimeric BILF1 protein in which the 19 most C-terminal amino acids of marmoset LCV BILF1 were removed and replaced by those of EBV BILF1 (marmoset LCV-EBV C-tail swap BILF1).

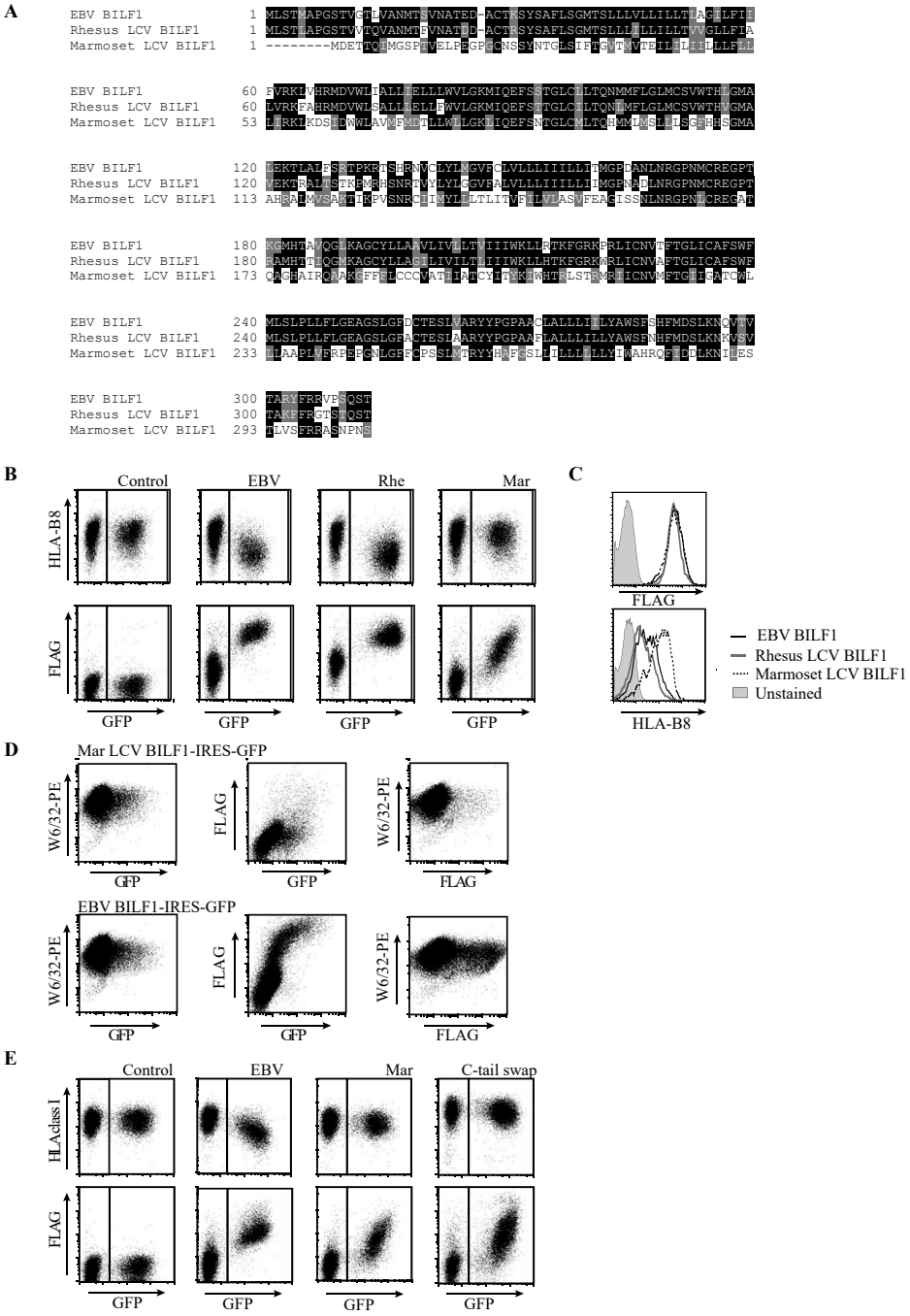
A
EBV BILF1
Rhesus LCV BILF1
Marmoset LCV BILF1
B
Control
EBV
Rhe
Mar
HLA-B8
FLAG
GFP
C
FLAG
HLA-B8
EBV BILF1
Rhesus LCV BILF1
Marmoset LCV BILF1
Unstained
D
Mar LCV BILF1-IRES-GFP
EBV BILF1-IRES-GFP
W6/32-PE
FLAG
GFP
E
Control
EBV
Mar
C-tail swap
HLAclass I
FLAG
GFP

Figure 7 MHC class I is not downregulated by the marmoset LCV BILF1 homolog. *A*, Amino acid sequence alignment of the BILF1 homologs encoded by EBV, rhesus LCV and marmoset LCV. Alignments were constructed using ClustalW2 and displayed using BOXSHADE version 3.21. *B*, MJS cells were transduced with GFP- (control), EBV BILF1/GFP- (EBV), rhesus LCV BILF1/GFP- (Rhe) or marmoset LCV BILF1 (Mar)-encoding replication-deficient lentivirus. After 7 days, surface expression of FLAG-tagged EBV BILF1 and HLA-B8 was determined by flow cytometry. Transduced and untransduced MJS cells were mixed before antibody staining to allow comparison in a single assay. *C*, Populations of transduced MJS cells expressing equivalent surface levels of FLAG-tagged EBV, rhesus LCV and marmoset LCV BILF1 were further analysed to examine their HLA-B8 surface expression levels. *D*, Marmoset primary fibroblasts were transfected with EBV or marmoset LCV FLAG-BILF1 genes in the pLV-IRES-GFP bicistronic vector. 48 h post-transfection, cells were stained with PE-conjugated W6/32 mAb, or with anti-FLAG and APC-conjugated anti-mouse antibody. Double staining of samples was performed to determine surface MHC class I expression on cells displaying cell surface FLAG-BILF1. *E*, MJS cells were transduced with GFP- (control), EBV BILF1wt/GFP- (EBV), marmoset LCV BILF1wt- (Mar), or marmoset LCV-EBV C-tail swap (C-tail swap)-encoding replication-deficient lentivirus. After 7 days, surface expression of FLAG-tagged EBV BILF1 and HLA class I was determined by flow cytometry. Transduced and untransduced MJS cells were mixed before antibody staining to allow comparison in a single assay.

This LCV BILF1 fusion gene was cloned into a lentiviral expression vector and human cells were transduced with control, EBV BILF1wt, marmoset LCV BILF1wt, or C-tail swap BILF1 lentivirus. C-tail swap BILF1 was expressed at the cell surface at a similar level to marmoset LCV BILF1wt in MJS cells but did not mediate downregulation of HLA class I (**Figure 7E**). This indicates that the intracellular C-terminal domain of EBV BILF1 is necessary but not alone sufficient for bringing about a reduction in surface HLA class I levels.

Discussion

Central to the execution of host anti-viral immunity is the action of $CD8^+$ T lymphocytes in detecting and eliminating virally infected cells. Herpesviruses have counter-evolved several strategies to thwart this system of immune surveillance by inhibiting the display of MHC class I:viral peptide complexes at the cell surface [7-9]. EBV inhibition of the HLA class I antigen presentation pathway during the viral lytic cycle is mediated through the concerted action of BGLF5 [10, 11], BNLF2a [12, 13], BILF1 [15, 16] and vIL-10 [14]. In understanding the dynamic process of multigenic immune evasion during the productive phase of the EBV life cycle, it is important to ascertain the temporal profile of individual gene expression. Previous studies have shown both BNLF2a and BGLF5 protein to be detectable approximately 3 h after lytic cycle induction [11, 41]. The vIL-10-encoding BCRF1 gene, in contrast, is expressed late in the lytic cycle [14]. Conflicting reports exist on the temporal expression of EBV BILF1 [24, 25, 35]. Here, we show BILF1 to be an early EBV lytic cycle gene, with transcripts first detectable by 4 h post induction. While this suggests that BILF1 protein is first expressed in conditions where BGLF5-mediated host-shutoff and BNLF2a-mediated TAP inhibition have

already been established, BILF1 may function to remove pre-existing HLA class I molecules from the cell surface. Furthermore, BNLF2a protein expression is transient, whereas the detection of BILF1 transcripts 16 h post-induction suggests that BILF1 is able to target HLA class I molecules presenting viral antigens late in the lytic cycle.

Although we have previously shown EBV BILF1 to downregulate HLA class I in human cells [15], the use of the pan-HLA class I-reactive mAb W6/32 could have masked selective targeting of specific HLA class I molecules. Other herpesvirus proteins targeting HLA class I for degradation have been found to demonstrate HLA class I type specificity. The Kaposi's sarcoma-associated herpesvirus (KSHV) E3 ligases K3 and K5 mediate ubiquitination of cell-surface HLA class I molecules, which are subsequently endocytosed and degraded by the lysosome [42-45]. While K3 is broadly reactive towards HLA-A, -B, -C and -E, K5 is selective for HLA-A and -B [45]. Meanwhile, HCMV US2 induces dislocation of newly synthesized HLA class I molecules from the ER to the cytosol for proteasomal degradation [46]. US2 targets HLA-A and certain HLA-B types, while surface expression of HLA-C, HLA-E and other HLA-B alleles is not affected [47].

Here, we employed a panel of alloantigen-specific mAbs to examine for the first time the effect of BILF1 on expression of individual HLA class I alleles in different cell types. Most cells express HLA-A, -B alleles, along with low levels of HLA-E, and in some cases HLA-C. HLA-A and -B molecules are most important for the presentation of viral peptides, a fact reflected in their high level of polymorphism. Surface expression of all HLA-A and -B alleles tested, was reduced by BILF1. Interestingly, the HLA-A2, -B7 and -B8 molecules shown here to undergo BILF1-mediated surface downregulation have been found to present epitopes derived from lytic cycle proteins, expressed by vaccinia virus infection of B cells, to EBV-specific $CD8^+$ T cells [48]. As some of the epitopes were from IE antigens, this provides further support to the idea that EBV BILF1 may target pre-existing HLA class I molecules for endocytosis upon its synthesis approximately 4 h after lytic cycle induction.

Additionally, it has been proposed that the repertoire of peptides bound by HLA-A2, -B7 and -B8 include those that do not require TAP for transport into the ER [49]. The EBV transmembrane protein LMP2 contains HLA-A2-restricted TAP-independent $CD8^+$ T cell epitopes [50], while a HLA-B7-restricted T cell clone specific for the EBV lytic cycle protein BMRF1 has been isolated from a TAP-deficient individual [51]. It is therefore possible that HLA class I molecules loaded with peptide in the presence of BNLF2a-mediated TAP inhibition during the lytic cycle may still be targeted by BILF1.

Different HLA class I molecules possess distinct peptide-binding specificity and will thus present a unique spectrum of viral peptides to $CD8^+$ T cells. A mechanism of immune escape confined to a narrow subset of HLA-A and -B alleles would not provide a selective advantage to the virus in transmission to a wide range of hosts. BILF1 likely mediates a broad-spectrum inhibition of HLA-A and -B surface expression that is particularly useful in a virus targeting a population with such high HLA-A and -B polymorphism.

HLA-C molecules are predominantly involved in regulating natural killer (NK) cell

function. HLA-C interacts with NK cell inhibitory receptors to prevent NK cell-mediated lysis. Interestingly, whereas a range of HLA-A and -B alleles were downregulated from the cell surface by EBV BILF1, HLA-C was only slightly affected. Through selective modulation of classical HLA class I molecules, BILF1 could target HLA-A and -B alleles presenting viral peptides to CD8+ T lymphocytes, while allowing the virally-infected cell to retain the inhibitory effect of HLA-C on NK cells. In this way, BILF1 could assist EBV evasion of both adaptive and innate immune mechanisms.

HLA-E is also best known for its role in regulating NK cell function. By presenting peptides derived from the HLA-A, -B and -C signal sequences and acting as a ligand for the CD94/NKG2A NK cell inhibitory receptor complex, HLA-E prevents killing by NK cells. EBV BILF1 did however decrease surface expression of HLA-E. Reducing surface HLA-E expression and removal of this inhibitory signal from the virally infected cell may therefore appear detrimental to virus survival. However, HLA-E has also been shown to bind viral antigens, including a BZLF1-derived peptide [52]. HLA-E:viral peptide complexes can then be recognized by $CD8^+$ T cells [53]. It may thus be advantageous for EBV to target HLA-E through BILF1, with the increased risk of NK cell attack being offset by other NK cell immune evasion tactics, such as the retention of HLA-C expression, and the action of miRNA BART-2 in reducing expression of the NK cell activating ligand MICB [54].

Previous studies have shown BILF1 to accelerate internalisation of HLA class I molecules, which are subsequently targeted for lysosomal degradation [15]. In an effort to gain further mechanistic insight into EBV BILF1-mediated HLA class I downregulation, we focussed on the role of BILF1 C-terminal tail, as this region of GPCRs is often involved in intracellular sorting and interaction with endocytic adaptor proteins [55]. While the C-tail is required for cell surface expression in the case of some GPCRs [56], our EBV BILF1 truncation mutant lacking the 19 most C-terminal residues was still detectable at the plasma membrane. The BILF1 deletion mutant was still functional with respect to modulation of intracellular signalling, as it retained the capacity to activate NFκB. However, BILF1-ΔC19 displayed a substantially abrogated ability to downregulate HLA class I surface expression relative to BILF1wt. Using a BILF1 deletion mutant lacking the 21 most C-terminal amino acid residues, a study by Zuo *et al.* indicated that the BILF1 C-tail is required for directing endocytosed HLA class I molecules to lysosomes [16]. The results of the present study are therefore in agreement with those of Zuo *et al.* in identifying a critical role for the BILF1 C-tail in reducing HLA class I levels.

As the C-tail of EBV BILF1 therefore likely contains motifs involved in the targeting of HLA class I to lysosomes, we scanned this domain for the short, linear amino acid sequences that typically mediate trafficking from the cell surface and sorting to lysosomes. Two candidate putative motifs related to protein endocytosis and intracellular sorting were identified. One, a type II PDZ ligand sequence, can be involved in regulating endocytosis, in addition to protein recycling [37]. The second, a non-classical tyrosine-based signal differs from the more common consensus motif by containing an extra residue between the tyrosine

and hydrophobic residue [37, 40]. Tyrosine-based motifs are recognized by clathrin adaptor proteins and can direct both protein endocytosis and lysosomal targeting [39]. In the case of both motifs, substitution of a key amino acid residue by alanine has been previously shown to functionally inactivate the signal [40, 57]. However, the failure of such point mutations to block the effect of BILF1 on HLA class I surface expression indicates that other structural determinants with the vGPCR C-tail are involved.

In addition to demonstrating the importance of the BILF1 C-tail, we found the intracellular portion of the HLA class I HC to be essential for BILF1-mediated downregulation. Other viral immunoevasins targeting the class I HC display a similar requirement. For instance, KSHV K3 catalyzes ubiquitination of lysine residues in the HC C-tail, thereby tagging the protein for internalisation and degradation [58]. However, the fact that BILF1 expression does not trigger ubiquitination of class I HC [15] points towards another basis for this dependence in the case of the EBV immunoevasin.

We identified three amino acid residues in the HLA-B8 intracellular tail that may be essential for BILF1-mediated downregulation. One of these, Y344, forms part of the tyrosine-based YXXA internalisation motif, which is conserved and required for constitutive endocytosis in the case of HLA-B27 [59]. This motif is present in all alleles tested in this study that were downregulated by BILF1, but absent from HLA-C (**Figure 6A**) and the HLA-B8 short mutant (**Figure 5A**). As it is reminiscent of the YXXΦ signal, the YXXA motif may also interact with clathrin adaptor proteins. However, the observation that the AP-2 is not required for BILF1-mediated HLA class I downregulation [16] would argue against the interaction of clathrin adaptor proteins with the HLA class I YXXA motif in facilitating the accelerated endocytosis or intracellular sorting directed by BILF1.

Thus, while the specific EBV BILF1 internalisation and sorting motifs and the intracellular adaptor protein(s) facilitating BILF1-mediated surface downregulation of HLA class I remain to be identified, our findings advance the molecular understanding of the process by identifying a critical domain in the viral effector protein and key amino acid residues in the host target proteins.

Besides EBV, other members of the LCV genus include *Callithricine herpesvirus 3* (marmoset LCV) and *Macacine herpesvirus 4* (rhesus LCV) [1]. These LCVs are prototypes for the New World and Old World primate LCVs, respectively, and display broadly similar biological properties to EBV. They can induce B cell growth transformation *in vitro*, possess inherent oncogenic potential *in vivo* and persistently infect their hosts. Rhesus LCV has a genetic repertoire that is identical to EBV, even though they are separated by an evolutionary distance of approximately 25 million years [60]. All EBV ORFs are accounted for in rhesus LCV and *vice versa*, with a similar relative genomic position. In contrast, marmoset LCV, which is estimated to have evolved 35 million years before EBV, has a similar, but less complete, genetic repertoire to EBV and rhesus LCV, suggesting a different evolutionary path [61, 62].

Notably, marmoset LCV lacks 14 genes encoded by the LCVs infecting higher-order primates [60-62]. None of these 14 genes are known to be essential for viral replication or B

cell immortalization, but they do include the immunomodulatory genes BNLF2a, vIL-10, the CSF receptor homolog BARF1 [63] and the EBERs [64]. Thus, the viral genes acquired during LCV evolution from New World hosts to Old World and human hosts may be less involved with intrinsic viral replication pathways during latent or lytic LCV infection, and may have evolved in order to survive in hosts with increasingly sophisticated host immunity, e.g. a more diverse MHC in humans and Old World versus New World hosts. While a BILF1 homolog is encoded by marmoset LCV, our results suggest the ability to downregulate cell surface MHC class I expression functionally evolved within the same related gene present in all LCV. Thus, LCV have made a concerted effort to target the host antigen presentation pathway and downregulate MHC by multiple evolutionary mechanisms, including (i) creation of novel immunomodulatory proteins (BNLF2a), (ii) acquisition of cellular homologs (vIL-10), and (iii) adaptation of novel functions into existing gene products (BILF1).

Materials and Methods

DNA Constructs

BILF1 wild type (wt) and mutant coding sequences were subcloned into the PstI/XhoI sites upstream of the internal ribosomal entry site (IRES) in the lentiviral expression vector pLV-IRES-GFP [28]. All were engineered to contain an N-terminal FLAG-tag. The EBV BILF1 wt sequence was subcloned from pcDNA5-FLAG-BILF1 and the EBV BILF1-K122A sequence from pcDNA3.1/TOPO-FLAG EBV BILF1 K122A (both gifts from H. Vischer, M. Smit, VU, Amsterdam, The Netherlands). The coding sequence of rhesus LCV BILF1 was amplified by PCR from pcDNA-Rhe LCV BILF1-IRES-GFP (a gift from M. Rowe, University of Birmingham, UK). The EBV BILF1 C-terminal truncation mutant ΔC19 was generated by PCR amplification of the wt sequence with the introduction of an early stop codon. To derive the EBV BILF1 point mutants, EBV BILF1-V_{299}A and EBV BILF1-Y_{303}A, the QuickChange site-directed mutagenesis kit (Stratagene) was used according to the manufacturer's instructions. The chimeric gene encoding a protein with amino acid residues 1-285 of marmoset LCV BILF1 fused to residues 293-312 of EBV BILF1 (BILF1 C-tail swap) was generated by PCR amplification using a reverse primer that encoded the 19 most C-terminal residues of the EBV BILF1 C-tail and annealed to nucleotides 830-855 of the marmoset LCV BILF1 coding sequence template.

The construct containing the N-terminally HA-tagged EBV BILF1 wt coding sequence in the retroviral expression vector pLZRS-IRES-GFP was a kind gift from J. Zuo and M. Rowe, University of Birmingham, UK.

The HLA-B8 wt sequence was PCR-amplified from pLZRS-HLA-B8-GFP (a gift from M. Heemskerk, LUMC, Leiden, the Netherlands) and subcloned into a lentiviral bidirectional vector, pCCLsin.PPT.pA.CTE.eGFP.mCMV.hPGK.NGFR.pre (kindly provided by L. Naldini, Milano, Italy), in which the human EF1A promoter replaced the minimal CMV-eGFP

cassette. The HLA-B8 short truncation mutant was generated by PCR amplification of the wt sequence with the introduction of an early stop codon.

To express HLA-B8 wt and C-tail mutants in U937 cells, cells were transduced with replication-deficient lentiviruses generated from the following lentiviral vector pSicoR-EF1a-Zeo-P2A (kindly provided by R.J. Lebbink, Utrecht, The Netherlands). The pSicoR-EGFP backbone vector (Addgene) was altered by removing the U6 promoter and replacing the CMV-EGFP cassette with the human EF1A promoter driving expression of the Zeocin resistance gene and the gene of interest. Zeocin and HLA-B8 genes were fused together by a self-cleaving 2A peptide derived from the porcine teschovirus-1 (P2A). The P2A peptide allows expression of both proteins from a single transcript. HLA-B8 wt was PCR-amplified from the bidirectional lentiviral vector mentioned above; the cytoplasmic tail mutants were generated by using reverse primers that encoded the mutations.

Restriction digests and sequence analysis verified the integrity of all gene sequences.

Cell Lines

The AKBM cell line is an EBV$^+$ Burkitt's lymphoma B cell line (Akata) stably transfected with the pHEBO-BMRF1p-rCD2-GFP reporter plasmid [29]. AKBM cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and 0.3 mg/ml hygromycin B. The MJS (Mel JuSo; HLA typing A*01, B*08) melanoma-derived cell line [30], the monocytic U937 cell line (A*03, B*18, Cw*01), and primary fibroblasts obtained from a common marmoset (*Callithrix jacchus*) (kindly provided by G. Koopman, BPRC, Rijswijk) were both maintained in RPMI (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (European Union approved; Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. 293T (A*02, B*07), 293-kB-luc ([31] ; a gift from G. B. Lipford, Coley Pharmaceutical Group), U373 (A*0201, B*18) and HeLa (A*68, B*15) cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. 293-kB-luc cells were cultured in the presence of 0.7 mg/ml geneticin.

Replication-deficient lentiviruses and retroviruses

Replication-deficient recombinant lentiviruses were generated by calcium phosphate co-transfection of HEK 293T cells with a pLV-CMV-IRES-eGFP, pCCLsin.PPT.pA.CTE.EF1A.hPGK.NGFR.pre or pSicoR-EF1a-zeocin-P2A lentiviral vector encoding the gene of interest, and pCMV-VSVG, pMDLg-RRE and pRSV-REV (kindly provided by R. Hoeben, LUMC, Leiden, the Netherlands) [32]. Replication-deficient recombinant retroviruses were produced by using the Phoenix amphotropic packaging system as described previously [13]. After 48 to 96 h, culture supernatants were harvested and frozen or filtered through a 0.45 mm pore filter. MJS, HeLa, 293T, U373 and U937 cell lines were infected with 1 ml lentivirus-containing medium in tissue culture dishes coated with 12 mg/ml retronection. Transduction efficiency

was examined by measuring GFP or surface NGFR expression. In the case of U937 cells transduced with pSicoR-EF1a-zeocin-P2A-based vectors, cells were selected using zeocin (400 μg/ml) to obtain pure populations.

Induction of EBV lytic cycle in AKBM cells, RNA isolation and RT-PCR

The EBV lytic phase was induced in AKBM cells by cross-linking surface IgG with 50 mg/ml goat $F(ab')_2$ fragments to human IgG (Cappel; MP Biomedicals, Solon, OH). Discrimination between IE and late lytic phases was achieved by inhibition of viral DNA replication and late lytic phase gene expression using phosphonoacetic acid (PAA). PAA (pH 7.4 in 100 mM Hepes) at a final concentration of 300 mg/ml was added 1 h prior to EBV lytic phase induction. Total RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNase (TURBO DNAsefree kit; Applied Biosystems), according to manufacturer's protocols. cDNA was synthesized using random hexamers and the Moloney murine leukemia virus reverse transcriptase (Finnzymes), and used for amplification with Taq DNA Polymerase. EBV BILF1 expression was measured using the primers 5'-GTATGGCGTTGGAGAAGACC-3' and 5'-TAATCAGCAGGAGTACCAGACA-3'; BZLF2/gp42 expression with the primers 5'- ATTCTACCTGTGGTAACTAGA-3' and 5'-TTAGCTATTTGATCTTTG-3'and 18S rRNA expression with the primers 5'-GTAACCCGTTGAACCCCATT-3' and 5'GATCCGAGGGCCTCACTAAAC-3'. DNA fragments of the expected length were visualized by 1% agarose gel electrophoresis and ethidium bromide (EtBr) staining.

Antibodies

The mouse monoclonal antibodies (mAbs) used in this study were: W6/32, which detects HLA class I molecules [33]; L243, which detects class II HLA-DR (American Type Culture Collection); anti-FLAG M2 (Sigma); and 3D12, detecting HLA-E (eBioscience). The rat mAb 3F10 (Roche) was used for detection of HA-tags. The human mAbs used were produced locally [34] and included VDK1D12, detecting HLA-A1; SN230G6 detecting HLA-A2; WIM8E5 detecting HLA-A68; VTM4D9 detecting HLA-B7; BVK 5B10 detecting HLA-B8; OUWF11 detecting HLA-B15, FVS4G4 detecting HLA-B18 and WK4C11 detecting HLA-C (Cw*01, Cw*03, Cw*08, Cw*12 and Cw*14). Additional antibodies used were Allophycocyanin (APC)-conjugated goat anti-mouse IgG (H+L) (Leinco Technologies); APC-conjugated goat anti-human IgG + IgM (H+L) (Jackson Immuno Research); APC-conjugated donkey anti-rat IgG $F(ab')_2$ (H+L) (Jackson Immuno Research); R-phycoerythrin (PE)-conjugated goat anti-mouse Ig $F(ab')_2$ (Dako); PE-conjugated goat anti-human IgM $F(ab')_2$ (Southern Biotech); biotinylated goat anti-mouse Ig (Dako) and APC-conjugated streptavidin (BD Pharmingen).

Flow Cytometry

Surface expression of specific molecules was determined using the indicated primary antibodies. Bound antibodies were detected using goat anti-mouse IgG-APC, or for staining of specific HLA class I alleles, goat anti-human IgG + IgM-APC. For HLA-E detection, a streptavidin-biotin-based three-step staining was performed. Double staining of marmoset fibroblasts, to examine co-expression of MHC class I and FLAG-BILF1 on single cells, was performed by consecutive incubation with anti-FLAG mAb, APC-conjugated anti-mouse Ab and PE-conjugated W6/32. Stained cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson) and using FlowJo software (TreeStar).

Transient transfections and luciferase assays

To examine activation of NF-κB by EBV BILF1wt, EBV BILF1-K122A and EBV BILF1-ΔC19, 293-κB-luc cells were seeded in a 96-well plate at a density of 2 x 10^5 cells/ml, 200 ml/well, 24 h before transfection with lipofectamine 2000 following the manufacturer's instructions. Cells were transfected with 70 ng of phRL-TK (constitutively expressing *Renilla* luciferase) and 160 ng of constructs encoding EBV BILF1 wt or mutants, or vector alone. NFκB-induced firefly luciferase and *Renilla* luciferase activity were assayed using the Luciferase Assay Reagent (Promega, Madison, WI) and Renilla Luciferase Assay System (both Promega), respectively, according to the instructions of the manufacturer. Luminescence was measured with the LB940 Mithras Research II microplate reader (Berthold Technologies). For transfection of primary marmoset fibroblasts, cells were seeded in a 6-well plate at a density of 2.5 x 10^5 per ml, 2 ml/well, 24 h before transfection with lipofectamine 2000 according to the manufacturer's instructions. Cells were transfected with 4 mg DNA and analyzed for expression of GFP and surface proteins after 48 h.

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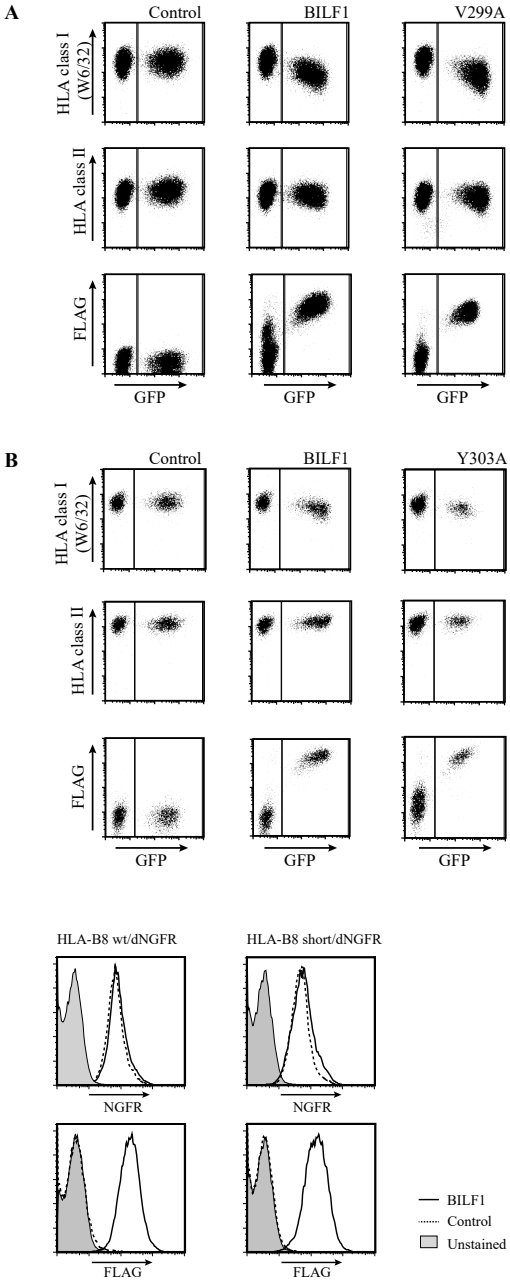
Supplementary Information



Supplementary Figure 1. C-tail point mutations fail to abrogate EBV BILF1-mediated downregulation of surface HLA class I. MJS cells were transduced with GFP- (control), EBV BILF1/GFP- (BILF1) and (*A*) EBV BILF1-V_{299}A/GFP- or (*B*) EBV BILF1-Y_{303}A/GFP-encoding lentivirus. After 7 days, surface expression of FLAG-BILF1, HLA class I and HLA class II was determined by flow cytometry. Transduced and untransduced MJS cells were mixed before antibody staining to allow comparison in a single assay.

Supplementary Figure 2. EBV BILF1 does not affect trNGFR levels in U373-HLA-B8 wt or U373 HLA-B8 short cells. U373-HLA-B8 wt and U373-HLA-B8 short cells were transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) lentivirus. After 7 days, surface expression of trNGFR and FLAG-BILF1 was measured by flow cytometry.

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