Chapter 2

Silencing the shutoff protein of Epstein-Barr virus in productively infected B cells points to (innate) targets for immune evasion

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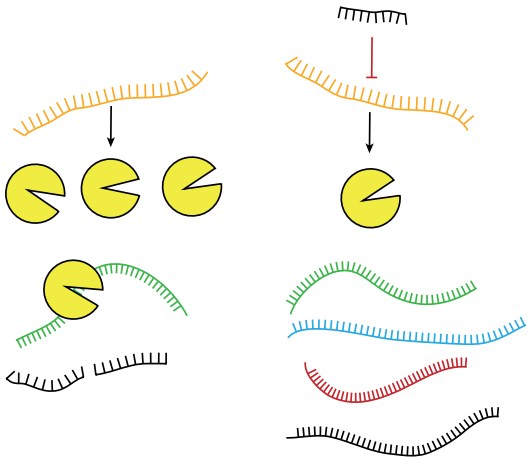


Summary

During productive infection with Epstein-Barr virus (EBV), a dramatic suppression of cellular protein expression is caused by the viral alkaline exonuclease BGLF5. Among the proteins downregulated by BGLF5 are multiple immune components. Here, we show that shutoff reduces expression of the innate EBV-sensing Toll-like receptor-2 and the lipid antigen-presenting CD1d molecule, thereby identifying these proteins as novel targets of BGLF5.

To silence BGLF5 expression in B cells undergoing productive EBV infection, we employed an shRNA approach. Viral replication still occurred in these cells, albeit with reduced late gene expression. Surface levels of a group of proteins, including immunologically relevant molecules such as CD1d and HLA class I and class II, were only partly rescued by depletion of BGLF5, suggesting that additional viral gene products interfere with their expression. Our combined approach thus provides a means to unmask novel EBV (innate) immune evasion strategies that may operate in productively infected B cells.

Herpesviruses are large enveloped DNA viruses that establish lifelong persistence in infected hosts. To achieve persistence, many herpesvirus gene products are dedicated to preventing elimination of virus-producing cells. For instance, members of all three herpesvirus subfamilies encode proteins that specifically interfere with antigen presentation to T cells (Griffin *et al.*, 2010). Prior to adaptive immunity, innate responses are elicited upon sensing of infection through pattern-recognition receptors, such as the Toll-like receptors (TLRs) (Iwasaki & Medzhitov, 2010;Paludan *et al.*, 2013). These innate antiviral responses are also subject to herpesvirus immune evasion (Feng *et al.*, 2013;Ning, 2011;Paludan *et al.*, 2013).

Productive infection by α- and γ-herpesviruses induces a global inhibition of protein synthesis resulting from enhanced mRNA degradation (Gaglia *et al.*, 2012). For the γ-herpesviruses, this shutoff is mediated by the viral alkaline exonuclease (AE) (Covarrubias *et al.*, 2009;Glaunsinger & Ganem, 2004;Rowe *et al.*, 2007). AE proteins are conserved throughout the herpesvirus family, reflecting their critical DNase function in processing of newly synthesized viral genomes; their additional RNase-based shutoff function is unique to γ-herpesviruses (Glaunsinger & Ganem, 2004;Rowe *et al.*, 2007). Shutoff appears broadly active and affects expression of most cellular proteins (Clyde & Glaunsinger, 2011). As such, herpesvirus-induced shutoff provides a general strategy to dampen anti-viral immune activation.

Additional, more specific, immune evasive mechanisms operating in herpesvirus-infected cells could be masked by the general effects of shutoff. Indeed, the first examples of T cell escape by dedicated herpesvirus immunoevasins were identified in the absence of shutoff: α-herpesvirus-encoded inhibitors of antigen presentation by HLA class I (HLA I) molecules were identified using shutoff-defective mutant viruses (Koppers-Lalic *et al.*, 2003;York *et al.*, 1994), and multiple HLA I evasion strategies were identified for the β-herpesvirus human cytomegalovirus that lacks a virus-encoded shutoff function (Barnes & Grundy, 1992). Cooperative targeting of a single immune pathway by multiple viral gene products has emerged as a common theme (Jones *et al.*, 1995;Ressing *et al.*, 2008). Compared to α-herpesviruses, shutoff by γ-herpesviruses has been discovered more recently. Consequently, less is known about the role of AE-mediated shutoff in immune evasion during productive γ-herpesvirus infection.

The prototypic human γ-herpesvirus, Epstein-Barr virus (EBV), naturally infects B cells, which form the latent virus reservoir in vivo (Rickinson & Kieff, 2007). For production of new viral progeny, EBV reactivates from a small percentage of latently infected B cells. The AE protein of EBV, BGLF5, is expressed during this lytic phase of infection. Earlier, we have reported that cellular expression of BGLF5 downregulates immunologically relevant proteins, such as HLA molecules and TLR9 (Rowe *et al.*, 2007;van Gent *et al.*, 2011;Zuo *et al.*, 2008), providing a means of general immune evasion. In this study, we aimed to evaluate BGLF5's effects in the context of productive EBV infection in B cells.

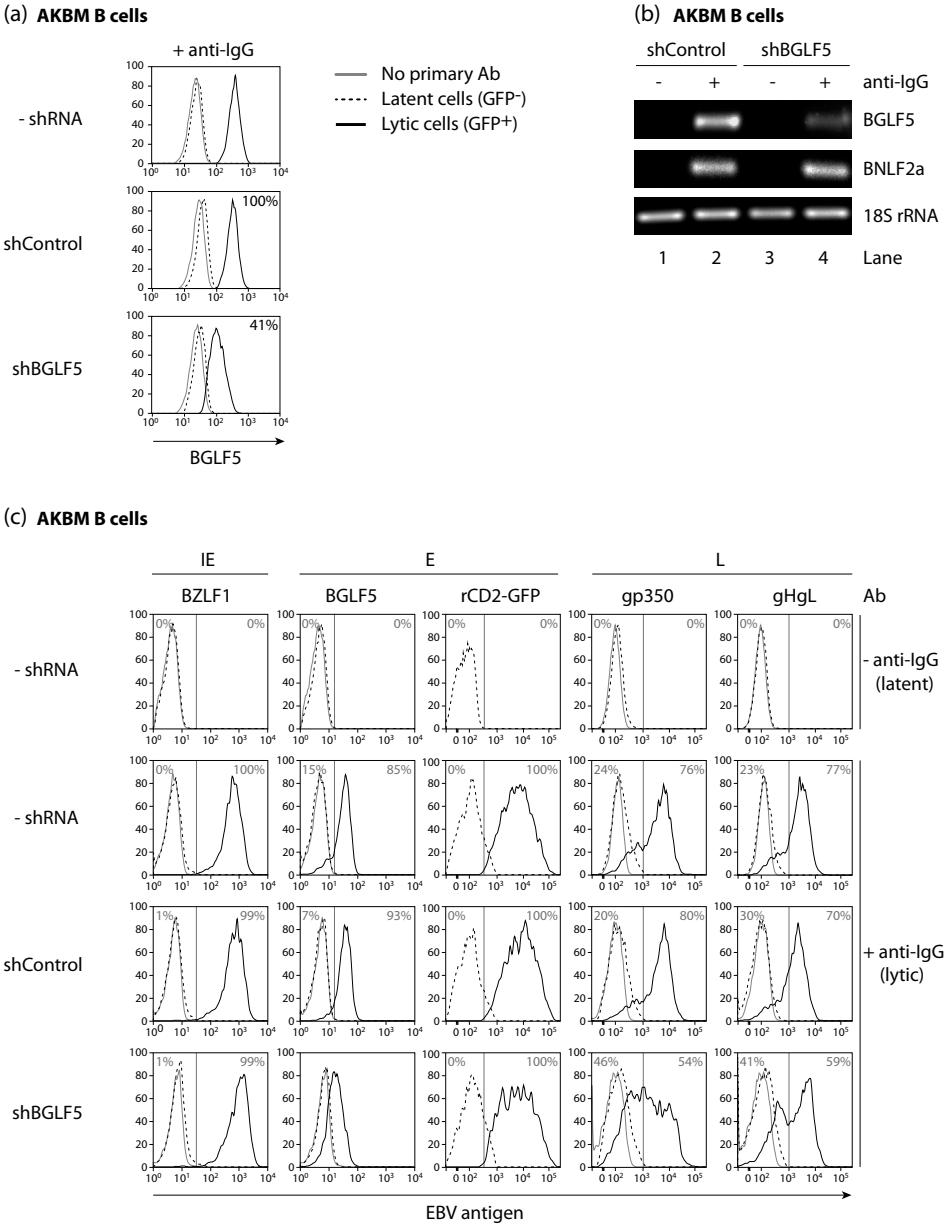


Figure 1 Silencing BGLF5 in productively EBV-infected B cells. AKBM B cells stably expressed no shRNA, a combination of two BGLF5-targeting shRNAs (shBGLF5), or an shRNA targeting Fas (shControl). By 20 hours of anti-human IgG treatment (+ anti-IgG), a population of cells had entered the EBV lytic cycle, with concomitant expression of the rCD2-GFP reporter. **(a)** Intracellular BGLF5 levels were determined by flow cytometry, with percentages indicating BGLF5 levels compared to those in shControl cells. In over 10 experiments, around 15-40% BGLF5 protein expression remained in lytic

AKBM-shBGLF5 cells. See **Figure S2** for further details. **(b)** Semi-quantitative RT-PCR analysis was performed to determine mRNA levels of BGLF5, an EBV control transcript (BNLF2a), or a cellular control RNA (18S ribosomal RNA). Almost pure populations of productively EBV-infected cells were obtained through magnetic sorting of lytically induced cells labeled with mouse-anti-rCD2 Ab and anti-mouse-magnetic beads (Miltenyi) (Ressing *et al.*, 2005). **(c)** Effects of shRNAs on progression through the replicative cycle were monitored by flow cytometric analysis of several EBV antigens (**Table S1, Figure S3**). Percentages indicate viral antigen-negative (left) and -positive cells (right) within the productively infected population (lytic cells); solid lines, lytic AKBM cells gated on expression of the rCD2-GFP reporter protein (GFP^+); dashed lines, latently EBV-infected cells (GFP^-); grey lines, no primary Ab.

Several approaches have been used to eliminate expression of individual herpesvirus genes from infected cells, one of which is based on the use of bacterial artificial chromosomes (BACs) (Delecluse *et al.*, 2008). Using this approach, deletion of BGLF5 was shown to perturb EBV replication in transfected 293T cells, resulting in reduced viral yields (Feederle *et al.*, 2009a). Studying EBV mutants during productive infection of B cells has been more difficult. Here, we have employed the EBV^+ Akata B cell line AKBM, in which cross-linking of the B cell receptor with anti-human IgG reactivates EBV in 10-40% of cells. Productively infected B cells can be identified and sorted on the basis of induced expression of a reporter protein, ratCD2-GFP (rCD2-GFP) (Ressing *et al.*, 2005). Using this system, we have elucidated several immune evasion mechanisms acting during the productive phase of EBV infection (Horst *et al.*, 2009;Ressing *et al.*, 2005;van Gent *et al.*, 2011;van Gent *et al.*, 2014). As our approach to suppress BGLF5-mediated shutoff during productive infection in B cells, lentivirus-delivered shRNAs were introduced into these EBV^+ AKBM cells.

Ten candidate shRNAs that target sites within the BGLF5 coding sequence were cloned into a lentiviral vector (**Figure S1**) (Lebbink *et al.*, 2011). Two of these considerably reduced BGLF5 levels in EBV-producing B cells, whereas the expression of a control protein, transferrin receptor (CD71), was not substantially affected (**Figure S2**). Combining the two shRNAs (referred to as shBGLF5) reduced BGLF5 protein levels by 60-75% compared to lytically induced control AKBM cells (**Figures 1a, S2**). Also mRNA levels of BGLF5 were markedly reduced in productively infected AKBM-shBGLF5 cells, whereas levels of another EBV transcript, BNLF2a, remained unchanged (**Figure 1b**). Thus, stable expression of specific shRNAs through lentiviral transduction substantially reduced BGLF5 levels during productive EBV infection in B cells.

AE proteins, through their conserved DNase function, are required for processing of replicated herpesvirus genomes in infected cells. We examined whether silencing of BGLF5 in B cells interfered with progression through the EBV replication cycle. Upon reactivation, immediate-early (IE), early, and late herpesvirus proteins are sequentially expressed (**Table S1**). Anti-IgG treatment of AKBM-shBGLF5 and control cells caused similar amounts of B cells to become positive for the IE transactivator BZLF1 and the early-expressed rCD2-GFP reporter (**Figures 1c, S3**), both of which precede expression of the BGLF5 protein. In

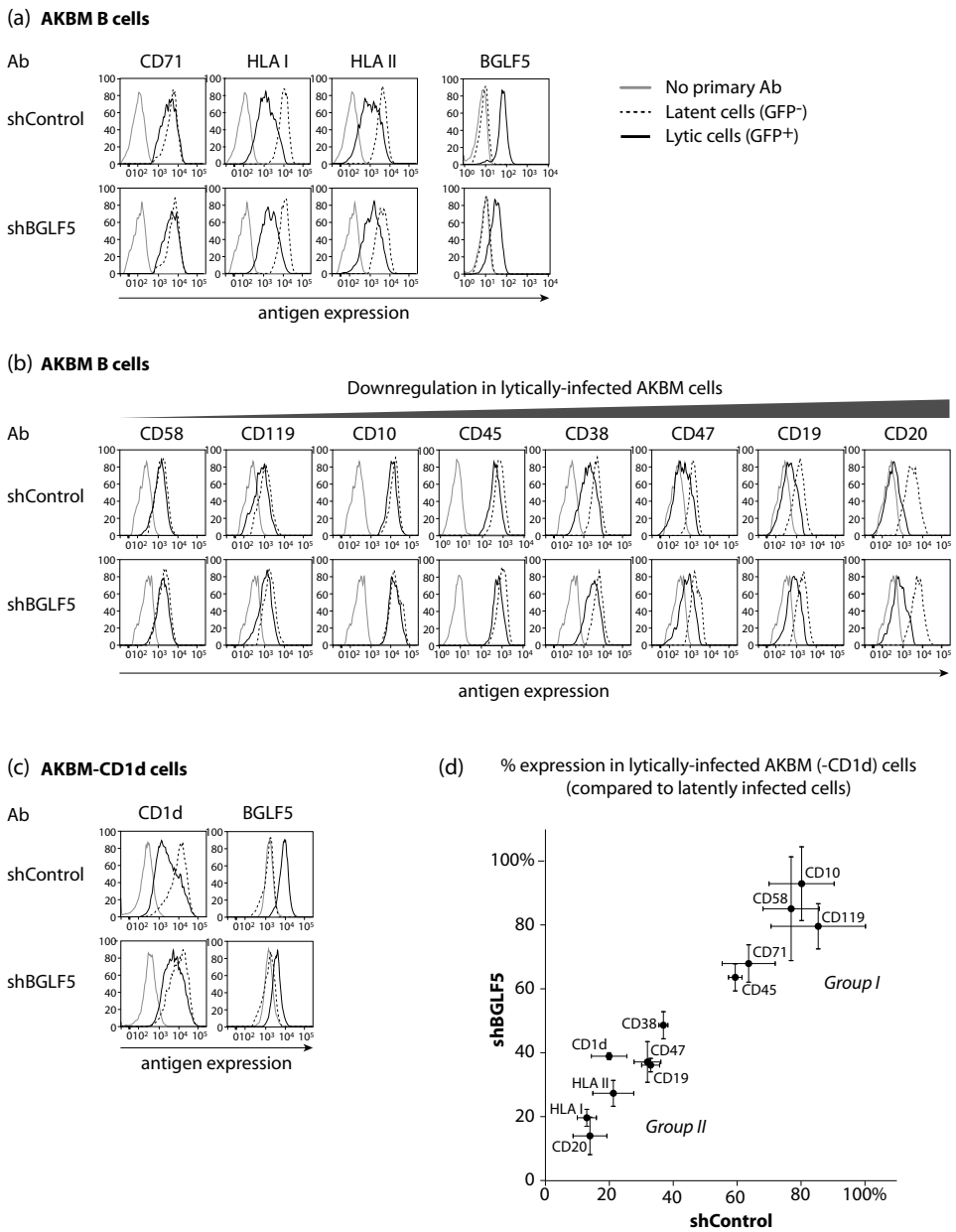


Figure 2 BGLF5 silencing rescues expression of surface proteins on EBV-producing B cells. EBV reactivation was induced in AKBM-shBGLF5 or -shControl cells. **(a,b)** Surface levels of the cellular antigens in **Table S2**, in **(b)** ordered by increasing downregulation from EBV-producing cells. **(c)** Surface levels of CD1d on AKBM cells that stably expressed human CD1d molecules after lentiviral transduction (AKBM-CD1d cells). The extent of BGLF5 silencing was visualized by intracellular staining. Solid lines,

productively EBV-infected (GFP^+) cells; dashed lines, latently infected (GFP^-) cells; grey, no primary Ab (dot plots in **Figure S4**). **(d)** Relative protein levels on productively versus latently EBV- infected shControl cells (horizontal axis) were plotted against those in shBGLF5 cells (vertical axis). Values were obtained by dividing geometric mean fluorescence intensities by background signals (isotype control or without primary antibody) and denoted as percentages expression in lytically compared to latently infected cells, as determined in at least four independent experiments (average ± SD; values for CD58, CD45, and CD119 based on two replicates).

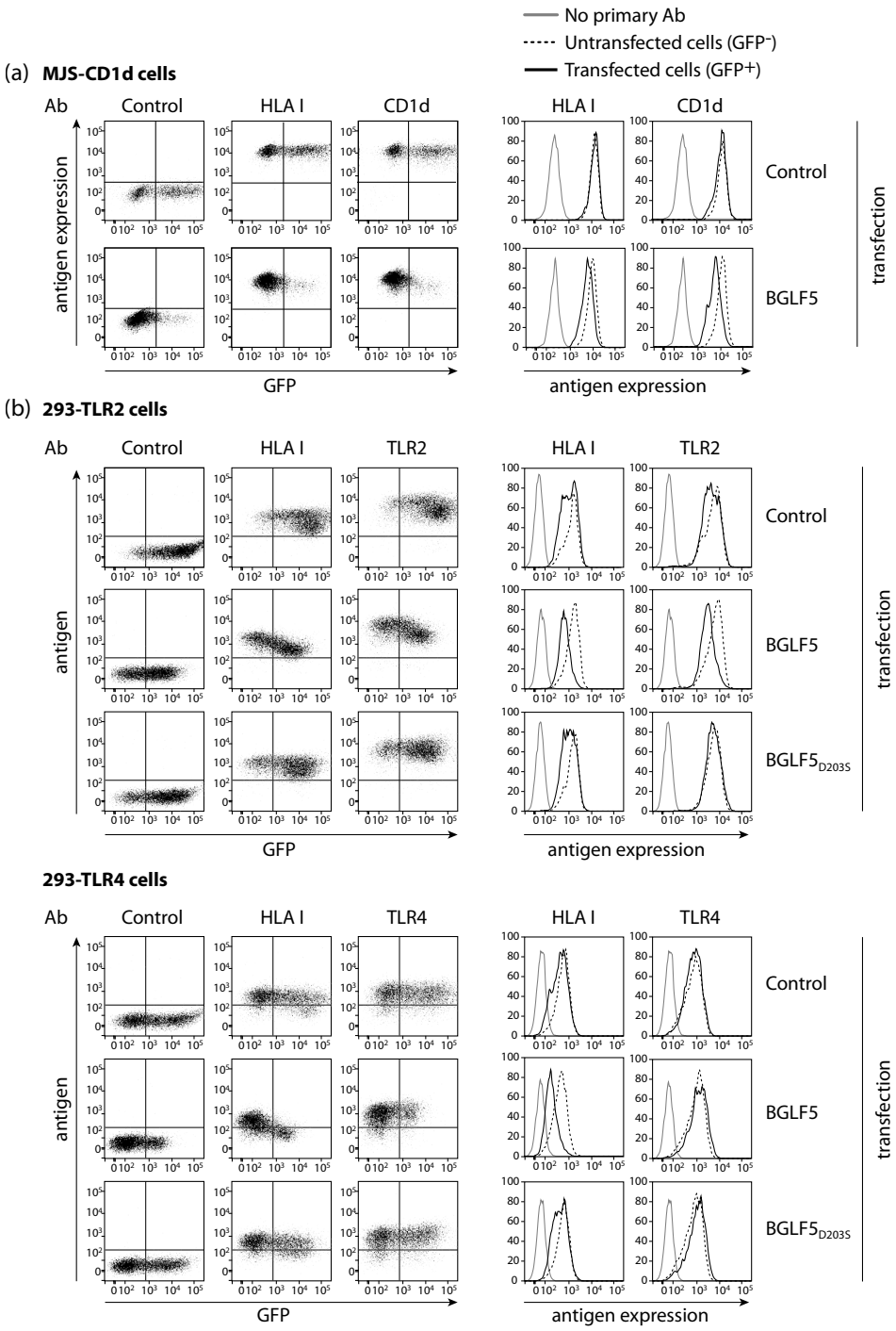
contrast, the proportion of AKBM-shBGLF5 cells expressing late proteins gp350, gH, and gL was substantially reduced. Thus, silencing BGLF5 appears to hamper entry into the late phase of productive EBV infection in B cells.

To evaluate the effects of shutoff, relying on BGLF5's RNase activity, in EBV-producing B cells, we examined the influence of BGLF5 silencing on the downregulation of various surface proteins. In control cells, EBV reactivation caused a minor reduction in CD71 surface levels, while HLA I and II were strongly downregulated (**Figures 2a and S4a**, upper panels), which is in line with earlier observations (Ressing *et al.*, 2005). In lytic AKBM-shBGLF5 cells, surface display of HLA I and II was partly rescued (**Figures 2a and S4a,** lower panels), supporting a contribution of shutoff to evasion from T cell detection during EBV replication in B cells. Still, levels of these antigen presenting molecules remained markedly reduced on BGLF5-silenced cells, which could reflect the specific effects on HLA I expression mediated by two dedicated EBV lytic cycle proteins, BNLF2a and BILF1 (Ressing *et al.*, 2008;Zuo *et al.*, 2009).

The analysis was extended to a panel of additional cellular proteins detectable at the surface of latently EBV-infected B cells (**Table S2**). Following viral reactivation, cellular display of the markers tested was reduced to varying degrees (**Figures 2b,d, S4b**). CD58, CD119, CD10, and CD45 were marginally affected and, therefore, the effect of BGLF5 silencing was difficult to evaluate (group I; **Figures 2b,d, S4b**), as was the case for CD71 (**Figure 2a**). Surface levels of another group of proteins, comprising CD38, CD47, CD19, and CD20, were strongly reduced during productive EBV infection of control cells and they remained downregulated in induced AKBM-shBGLF5 cells (group II, **Figures 2b,d, S4b)**. The phenotype for this latter group of proteins resembles that of the peptide-presenting HLA I and II complexes.

We also included the non-classical HLA molecule CD1d in this analysis. CD1d molecules present lipid antigens to invariant natural killer T (iNKT) cells that express a semi-invariant T cell receptor as well as NK cell markers. iNKT cells act at the interface of innate and adaptive immunity: they rapidly produce polarizing cytokines when activated, for instance in response to viral infection (Horst *et al.*, 2012a;Kinjo *et al.*, 2013). Induction of the EBV lytic cycle in AKBM-CD1d cells caused a dramatic decrease in surface appearance of human CD1d molecules (**Figures 2c,d, S4c**). Although CD1d expression was partly restored when BGLF5 was silenced, it remained far below the levels observed on latently infected cells. Thus, CD1d-restricted antigen presentation appears a novel target of EBV immune evasion, in part mediated by the shutoff protein BGLF5.

The combined data imply that B cell proteins whose surface display remains markedly


No primary Ab
Untransfected cells (GFP-)
Transfected cells (GFP+)
(a) MJS-CD1d cells
Ab
Control
HLA I
CD1d
antigen expression
GFP
antigen expression
Control
BGLF5
transfection
(b) 293-TLR2 cells
TLR2
antigen
BGLF5D203S
293-TLR4 cells
TLR4

reduced on BGLF5-silenced cells (**Figure 2d**, group II) are likely to be downregulated by additional EBV lytic phase proteins, for instance to effectuate reduced recognition of virus-producing B cells by the immune system.

To complement the studies performed in naturally infected AKBM-CD1d-shBGLF5 cells, we investigated BGLF5's effects on the non-classical antigen-presenting molecule CD1d in cells expressing BGLF5 in isolation. MJS-CD1d cells were transiently transfected with BGLF5 and reduction of GFP and surface HLA I levels cells confirmed induction of shutoff. The BGLF5-transfected cells displayed reduced surface expression of CD1d (**Figure 3a**). This CD1d downregulation was, however, less pronounced than that on B cells expressing all EBV gene products (**Figure 2c**), reminiscent of the phenotype for HLA I (**Figure 2a**) (Rowe *et al.*, 2007). These results show that BGLF5 reduces CD1d levels and that other viral factors, absent from the transfected MJS cells, are likely to add to the robust CD1d downregulation observed during productive EBV infection of B cells.

Earlier, we have found that expression of innate sensors, namely several TLRs, is reduced upon EBV reactivation in AKBM cells and that BGLF5 contributes to the downregulation of TLR9 (van Gent *et al.*, 2011). TLR2, 3, and 9 sense EBV particles (Gaudreault *et al.*, 2007;Iwakiri *et al.*, 2009;van Gent *et al.*, 2011), yet no evidence for TLR4-mediated recognition of EBV has been reported (Gaudreault *et al.*, 2007). Here, we monitored the influence of BGLF5 on TLR2 and TLR4. 293-TLR2 and 293-TLR4 cells were transiently transfected with the empty IRES-GFP vector, wild-type BGLF5, or a catalytically inactive mutant, $BGLF5_{D203S}$. TLR2 levels were reduced on wild-type BGLF5-expressing cells, but not on control cells (**Figure 3b**). In contrast, TLR4 levels were not affected by any of the transfected gene products. Thus, BGLF5-mediated shutoff appears to target TLR2, a pattern-recognition receptor sensing EBV.

To conclude, this study shows that lentivirus-delivered shRNAs can successfully be applied to our system for productive EBV infection of B cells to achieve stable silencing of BGLF5. A similar approach in EBV-transformed B-LCLs yielded around 75% knockdown of viral gene expression, which was sufficient to reveal a hierarchy in immune evasive properties of BNLF2a, BILF1, and BGLF5 (Quinn *et al.*, 2014).

In our system of EBV-producing AKBM cells, a comparable reduction of BGLF5 protein levels interfered with viral replication (**Figure 1**). While confirming that knockdown in EBV-producing B cells was sufficiently robust to observe a phenotype, this observation extends

Figure 3 BGLF5 contributes to downregulation of CD1d and TLR2. (a) MelJuSo cells were lentivirally transduced to stably express human CD1d molecules (MJS-CD1d). MJS-CD1d cells **(a)** and 293-TLR2/CD14 cells (Kurt-Jones *et al.*, 2002) or 293-TLR4/CD14/MD2 cells (Invitrogen) **(b)** were transiently transfected with a pcDNA3-IRES-nlsGFP vector without insert or encoding BGLF5 or the catalytic mutant $BGLF5_{D203S}$ (Horst *et al.*, 2012b). At 48 hours post-transfection, surface levels of HLA I, CD1d, TLR2, and TLR4 were determined by flow cytometry. Downregulation of GFP served as a measure for BGLF5's shutoff function. Dashed lines, untransfected GFP^- cells; solid lines, transfected GFP^+ cells; grey lines, secondary Ab only. Data shown are from one experiment representative of three independent experiments.

earlier studies in 293T cells transfected with a BGLF5 deletion mutant EBV BAC (Feederle *et al.*, 2009a). Within the EBV genome, *BGLF5* occurs in tandem with *BGLF4*, which codes for the EBV protein kinase that can regulate EBV late gene expression (El-Guindy *et al.*, 2014). Since BGLF4 is translated from a transcript encoding both BGLF4 and BGLF5, expression of both proteins was lost from BGLF5-deleted virus-producing 293T cells (Feederle *et al.*, 2009b). Likewise, the use of RNA interference to silence BGLF5 expression in B cells will target both BGLF5 and BGLF5+BGLF4 transcripts. When applying this approach to AKBM B cells, we have focused on the shutoff effects that are selectively induced by BGLF5.

During productive EBV infection, a broad range of B cell surface proteins is downregulated in the presence of BGLF5, and this effect is partly reversed upon silencing of BGLF5 (**Figure** 2). These findings are in agreement with promiscuous shutoff by γ-herpesvirus AE proteins, deduced from mRNA target analysis (Clyde & Glaunsinger, 2011) and metabolic labeling experiments (Rowe *et al.*, 2007). Still, some gene products escape shutoff and TLR4 appears to be one of them (**Figure 3**). Based on our current data, two groups of host surface proteins can be discriminated. The first group comprises proteins that are downregulated to a limited extent during EBV replication; the costimulatory molecules CD80 and CD86 can be included in this group (Ressing *et al.*, 2005). The second group is more strongly downregulated, likely by multiple EBV lytic proteins, and their surface levels remain substantially reduced when BGLF5 is silenced. For some proteins belonging to this latter group, a causative role for BGLF5 in their downregulation has been confirmed through transient transfection experiments, i.e. for HLA I and II (Rowe *et al.*, 2007), for TLR9 (van Gent *et al.*, 2011), and for CD1d and TLR2 (this study, **Figure 3**). These combined findings support the notion that BGLF5 contributes to EBV-induced immune evasion during productive infection of B cells.

A recent study in BGLF5-silenced B-LCLs revealed a minor role for BGLF5 in CD8+ T cell evasion when compared to BNLF2a and BILF1 (Quinn *et al.*, 2014). Along the same line, we observed only partially rescued surface display of B cell proteins upon BGLF5 knockdown during productive EBV infection (**Figure 2**). Residual downregulation could result from the ~25% BGLF5 protein expression that remained in induced AKBM-shBGLF5 cells and/or from additional EBV-encoded shutoff function(s), such as that recently reported to be exerted by BZLF1 (Park *et al.*, 2014). In vivo studies on the α-herpesvirus HSV-1 and the murine γ-herpesvirus MHV68 suggest that the immune evasive functions of shutoff mainly affect newly synthesized proteins induced by type I interferons (Murphy *et al.*, 2003;Pasieka *et al.*, 2008;Sheridan *et al.*, 2014). The above observations, together with the absence of shutoff from β-herpesviruses, point to a relatively small contribution of shutoff to immune evasion. This would provide a rationale as to why herpesviruses have acquired additional, specific immune evasion mechanisms to synergistically achieve the proper timing and extent of immune interference.

Analogous to all other herpesviruses studied, EBV encodes multiple gene products that act in concert to prevent T cell activation (Ressing *et al.*, 2008). Additional EBV strategies interfering with innate immunity continue to be identified (Ning, 2011). Here, we have added

CD1d and TLR2 to the target list of EBV BGLF5. Furthermore, we have identified a group of B cell surface proteins including CD1d whose expression is likely downregulated by EBV lytic phase proteins besides BGLF5.

Acknowledgements

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Supporting Information

Table S1. Viral antigens expressed during productive EBV infection

ORF	Name	Timing	Function	Ab clone
BZLF1	Z, Zta, ZEBRA	IE	Lytic cycle transactivator	BZ.1
BNLF2a		E	HLA I downregulation	N/A
BGLF5	EBV AE, DNase	E	Shutoff and exonuclease	311H
BLLF1	gp350	L	Major envelope glyco-protein, attachment to CR2	72A1
BXLF1	gp85/gH	L	Viral membrane fusion/entry	E1D1
BKRF2	gp25/gL	L	Chaperone of gH	E1D1
rCD2-GFP	rat CD2-GFP fusion protein	"E"	Lytic reporter, magnetic sorting	OX34 (α-ratCD2)

Table S2. Expression of cellular surface proteins by AKBM cells

CD#	Name	Function	Ab
Not expressed by latently EBV-infected AKBM cells			
CD1d		Non-classical HLA molecule, lipid presentation to iNKT cells	51.1.3
CD11b	Integrin alpha M	Complement receptor 3 subunit	BD550019
CD11c	Integrin alpha X	Complement receptor 4 subunit	BD559877
CD21	CR2	Complement receptor 2, B cell coreceptor component (with CD19), EBV B cell receptor	BC IM0473U
CD23	FcεRII	IgE receptor	BD347797
CD25		IL-2 receptor alpha-chain	BD341011
CD32		B cell coreceptor	RDI-CD32 abm-7PE
CD35	CR1	Complement receptor 1	BD559872
CD40		Costimulation	SC65263
CD44		Variety of lymphocyte functions	BD550989
CD54	ICAM-1	Intercellular adhesion molecule	BD555511
CD132	Common gamma chain (γc)	Common subunit of at least six interleukin receptors	BD555898
CD162	P-selectin glycoprotein ligand-1	Neutrophil recruitment	BD556055
CD181	CXCR1	IL-8 receptor	R&D-FAB330P
CD191	CCR1	Chemokine receptor, inflammatory responses	R&D-FAB145P
CD192	CCR2	Chemokine receptor, monocyte chemotaxis	R&D-FAB151P
CD282	Toll-Like Receptor 2	Pattern-recognition receptor	eBioscience 12-9024-82
CD284	Toll-Like Receptor 4	Pattern-recognition receptor	Biolegend 312802
Marginally downregulated during productive EBV infection (group I)			
CD10	Neprilysin	Zinc-dependent metalloprotease	BD340921
CD45	Leukocyte common antigen	Protein tyrosine phosphatase	BD555485
CD58	LFA-3	Adhesion molecule, antigen-presenting cell - T cell interaction	BD555921
CD71	Transferrin receptor	Iron uptake	BD555534
CD119	IFNγRα	Ligand binding α chain of IFNγ receptor	BD558934
Substantially downregulated during productive EBV infection (group II)			
CD19		B cell coreceptor component, reduces activation threshold	BD555415
CD20		Enables optimal B cell immune response	BD345793
CD38	Cyclic ADP ribose hydrolase	Multifunctional ectoenzyme, regulation of intracellular Ca2+ levels	BD347687
CD47	Integrin associated protein	Range of cellular processes	BD556047
	HLA I	Classical HLA molecule, peptide presentation to CD8+ T cells	W6/32
	HLA II	Classical HLA molecule, peptide presentation to CD4+ T cells	L243

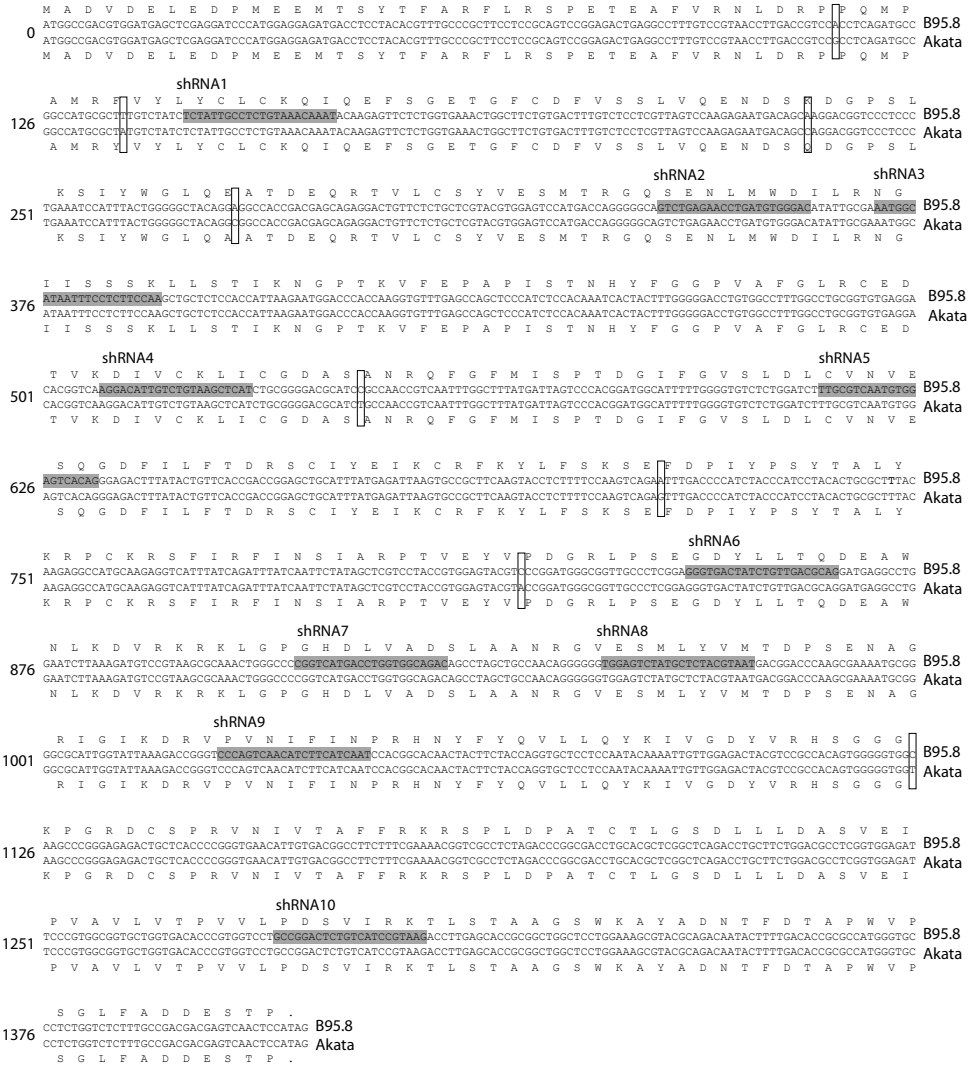

Figure S1 shRNA target sequences within EBV BGLF5. Nucleotide and amino acid alignments are depicted for the BGLF5 open reading frames from EBV strains B95.8 and Akata. Numbers indicate nucleotide positions and boxes show inter-strain differences. Ten candidate shRNA target sites within the BGLF5 coding sequence (in grey) were selected using a prediction algorithm (the Hannon lab website: http://cancan.cshl.edu/RNAi_central/main2.cgi) (Paddison *et al.*, 2004). As a control shRNA, a target sequence (tatgcagaggatgaaagattaa) within the cellular gene encoding the Fas receptor was used. shRNAs were cloned into a lentiviral vector derived from pSicoR (Jacks Lab, MIT), in which the U6 promoter was altered to allow for sticky cloning of shRNAs in between a BstXI and XhoI site, and in which an EF1α promoter was used to drive expression of a cassette encoding a puromycin resistance marker, the ribosome skipping peptide T2A, and mCherry. The resulting vectors were used for the generation of replication-deficient self-inactivating lentivirus stocks, as described (Lebbink *et al.*, 2011).

(a) AKBM B cells
+ anti-IgG
- shRNA
shRNA1
shRNA2
shRNA3
shRNA5
shRNA6
shRNA8
shRNA9
shRNA1+9
BGLF5
CD71
rCD2-GFP
No primary Ab
Latent cells (GFP-)
Lytic cells (GFP+)
(b) AKBM B cells
+ anti-IgG
- shRNA
shRNA1
shRNA2
shRNA3
shRNA5
shRNA6
shRNA8
shRNA9
shRNA1+9
0%
100%
37%
67%
75%
82%
131%
94%
54%
22%
0%
100%
105%
109%
94%
83%
87%
110%
100%
107%
BGLF5
CD71

Figure S2 Experimental selection of shRNA target sequences that silence BGLF5 protein expression. AKBM B cells were lentivirally transduced and puromycin selected (>95% mCherry-positive) to stably express single BGLF5-targeting shRNAs or a combination of two BGLF5 shRNAs (shRNAs #1 and #9, referred to as shBGLF5 in the remainder of the manuscript). Productive EBV infection was induced in these cells by treatment with anti-human IgG Abs (+ anti-IgG: 50 µg/mL; Cappel, MP Biochemicals) for 20 hours (Ressing *et al.*, 2005). Subsequently, cells were fixed, permeabilized, stained for intracellular expression of BGLF5 (Ab 311H; 'intracellular' staining; (Horst *et al.*, 2012b)), and analyzed by flow cytometry. The transferrin receptor (CD71; Ab BD555534) was taken along as a control protein and was visualized at the surface of unfixed and non-permeabilized cells ('surface' staining). Results of a representative experiment with seven out of the ten shRNAs tested are depicted as dot plots (a) and as overlay histograms (b). Solid lines, lytically induced AKBM cells gated on expression of the rCD2-GFP reporter protein (GFP+); dashed lines, latently EBV-infected cells (GFP-); grey lines, no primary Ab. Percentages denote BGLF5 protein expression levels in productively infected AKBM cells with BGLF5-targeting shRNAs compared to those of control cells without shRNA. Geometric mean fluorescence intensities of specifically stained cells (+ primary Ab + secondary APC-conjugated Ab) were divided by the background signal in the absence of primary Ab (second step only). The resulting fold inductions in lytic cells compared to latent cells were expressed as percentages with the expression in -shRNA control cells set at 100%. Over 10 such experiments have been performed with shBGLF5 (#1 + #9) yielding 60-85% silencing of BGLF5 protein expression. Of note, screening of the shRNAs specific for B95.8 BGLF5 by transient transfections in 293T cells (data not shown) had little predictive value for their efficacy during productive EBV infection in B cells, in our hands.

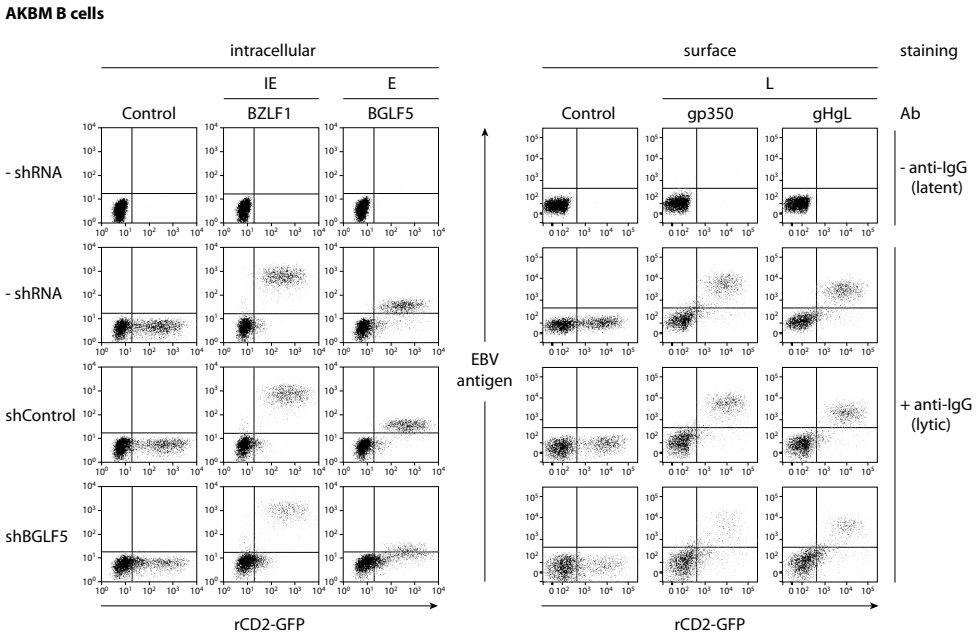

Figure S3 Silencing BGLF5 expression in AKBM B cells affects progression through the EBV replicative cycle. AKBM B cells stably expressing no shRNAs (-shRNA), a control Fas-targeting shRNA (shControl), or a combination of two BGLF5-specific shRNAs (shBGLF5) were treated with anti-human IgG for 20 hours. Entry into the EBV replicative cycle was monitored by flow cytometry. Abs used for intracellular stainings were directed against the IE transactivator BZLF1 and early (E)-expressed BGLF5. Abs used for surface stainings vizualised the late (L) EBV glycoproteins gp350 and gHgL. Dot plots depict the, early-expressed, rCD2-GFP lytic cycle reporter (horizontal axis) and the indicated viral antigens (vertical axis). Histograms of this experiment are presented in **Figure 1c.**

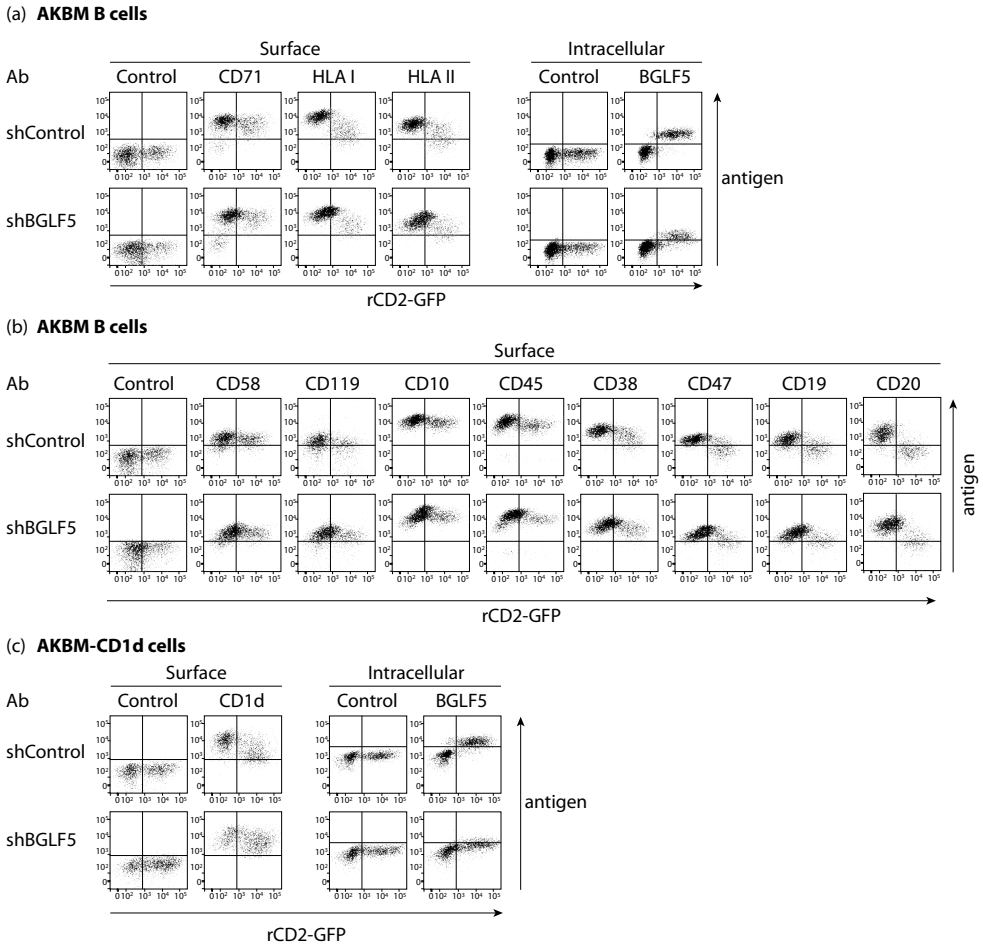

Figure S4 Silencing BGLF5 expression in AKBM B cells affects surface display of cellular antigens. AKBM B cells (a,b) and AKBM-CD1d cells (c) stably expressing a control shRNA targeting Fas (shControl) or a combination of two shRNAs targeting BGLF5 (shBGLF5) were treated with anti-human IgG for 20 hours to induce productive EBV infection. Intracellular BGLF5 levels and downregulation of various B cell surface markers were analyzed by flow cytometry and depicted as dot plots. Histograms of this experiment are presented in **Figure 2**.

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