**Short Communication**

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

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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 (Gagliá 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). 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 antiviral 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, which 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 with 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
Fig. 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 h 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 with those
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 the effects of BGLF5 in the context of productive EBV infection in B cells.

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) (Delecousse 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, rat CD2-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, 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 (Fig. S1, available in the online Supplementary Material) (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 (Fig. S2). Combining the two shRNAs (referred to as shBGLF5) reduced BGLF5 protein levels by 60–75% compared with lytically induced control AKBM cells (Figs 1a and S2). Also mRNA levels of BGLF5 were markedly reduced in productively infected AKBM-shBGLF5 cells, whereas levels of another EBV transcript, BLNL2a, remained unchanged (Fig. 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, 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 immediate-early transactivator BZLF1 and the early expressed rCD2-GFP reporter (Figs 1c and S3), both of which precede expression of the BGLF5 protein. In 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 the RNase activity of BGLF5, 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 (Figs 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 (Figs 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, BLNL2a 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 (Figs 2b, d and S4b). CD58, CD119, CD10 and CD45 were marginally affected and, therefore, the effect of BGLF5 silencing was difficult to evaluate (group I; Figs 2b, d and S4b), as was the case for CD71 (Fig. 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; Figs 2b, d and S4b). The phenotype for this latter group of proteins resembles that of the peptide-presenting HLA I and II complexes.
Fig. 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 silencing BGLF5 expression in EBV-producing B cells.
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 Fig. 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 percentage expression in lytically compared with latently infected cells, as determined in at least four independent experiments (mean ± s.d.; values for CD58, CD45 and CD119 based on two replicates).

We also included the non-classical HLA molecule CD1d in this analysis. CD1d molecules present lipid antigens to invariant natural killer T cells that express a semi-invariant T-cell receptor as well as natural killer cell markers. Invariant natural killer T 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 (Figs 2c, d and 5c). 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 reduced on BGLF5-silenced cells (group II; Fig. 2d) 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 the effects of BGLF5 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 confirmed induction of shutoff. The BGLF5-transfected cells displayed reduced surface expression of CD1d (Fig. 3a). This CD1d down-regulation was, however, less pronounced than that on B cells expressing all EBV gene products (Fig. 2c), reminiscent of the phenotype for HLA I (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 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; Ikawaki 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, WT BGLF5, or a catalytically inactive mutant, BGLF5\textsubscript{D203S}. TLR2 levels were reduced on WT BGLF5-expressing cells, but not on control cells (Fig. 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 (Fig. 1). While confirming that knockdown in EBV-producing B cells was sufficiently robust to observe a phenotype, this observation extends earlier studies in 293T cells transfected with a BGLF5 deletion mutant EBV bacterial artificial chromosome (Feederle et al., 2009a). Within the EBV genome, BGLF5 occurs in tandem with BGLF4, which codes for the EBV protein kinase, which 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 (Fig. 2). These findings are in agreement with promiscuous shutoff by γ-herpesvirus AE proteins, deduced from mRNA target analysis (Clyde & Glaunsinger, 2011) and metabolic labelling experiments (Rowe et al., 2007). Still, some gene products escape shutoff and TLR4 appears to be one of them (Fig. 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
Fig. 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
limited extent during EBV replication; the co-stimulatory 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 down-regulation 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, Fig. 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 with BNLF2a and BILF1 (Quinn et al., 2014). Along the same lines, we observed only partially rescued surface display of B-cell proteins upon BGLF5 knockdown during productive EBV infection (Fig. 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 -herpesvirus HSV-1 and the murine -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 -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.

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References


