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# EBV Lytic-Phase Protein BGLF5 Contributes to TLR9 Downregulation during Productive Infection

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Viruses use a wide range of strategies to modulate the host immune response. The human gammaherpesvirus EBV, causative agent of infectious mononucleosis and several malignant tumors, encodes proteins that subvert immune responses, notably those mediated by T cells. Less is known about EBV interference with innate immunity, more specifically at the level of TLR-mediated pathogen recognition. The viral dsDNA sensor TLR9 is expressed on B cells, a natural target of EBV infection. Here, we show that EBV particles trigger innate immune signaling pathways through TLR9. Furthermore, using an in vitro system for productive EBV infection, it has now been possible to compare the expression of TLRs by EBV<sup>-</sup> and EBV<sup>+</sup> human B cells during the latent and lytic phases of infection. Several TLRs were found to be differentially expressed either in latently EBV-infected cells or after induction of the lytic cycle. In particular, TLR9 expression was profoundly decreased at both the RNA and protein levels during productive EBV infection. We identified the EBV lytic-phase protein BGLF5 as a protein that contributes to downregulating TLR9 levels through RNA degradation. Reducing the levels of a pattern-recognition receptor capable of sensing the presence of EBV provides a mechanism by which the virus could obstruct host innate antiviral responses. *The Journal of Immunology*, 2011, 186: 1694–1702.

Epstein-Barr virus is a ubiquitous human herpesvirus that targets B lymphocytes and persistently infects >90% of adults worldwide (1). In the majority of cases, persistent infection remains subclinical. However, EBV can induce lymphoproliferative disorders in immunocompromised patients and is also strongly associated with several malignant tumors of lymphoid and epithelial origin, such as Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (2). During childhood, primary infection is typically asymptomatic, but if delayed until adolescence, it manifests as infectious mononucleosis in ~50% of cases (3). This syndrome arises because of vigorous, nonspecific T cell activation producing a large number of atypical lymphocytes (2). A subsequent virus-specific T cell response reduces the number of infected

B cells, although fails to completely eradicate the virus. Instead, EBV persists for life in memory B cells, where up to nine viral proteins are expressed in the latent stage of the viral life cycle. This reduction in the number of viral Ags presented to the immune system aids latently infected B cells in escaping destruction. Transmission to another host requires the production of new virions, which is facilitated by occasional viral reactivation and is characterized by the expression of up to 80 viral proteins during the lytic phase. During this replicative phase of infection, dedicated immune evasion molecules encoded within the viral genome are thought to extend the life span of virus-producing cells. Indeed, the past years have witnessed the identification of a number of EBV immunoevasins targeting adaptive immune responses, particularly the T cell-mediated response (4).

A compelling paradox of primary EBV infection is the failure of the apparently robust T cell response to result in viral clearance. High T cell numbers may suggest that prior innate immune responses were triggered by viral infection. However, were EBV to subvert the innate immune response upon primary infection, this could result in a poorly tailored adaptive immune response, possessing insufficient antiviral specificity in its early stages, and thereby allowing a number of infected B cells to escape elimination and establish a persistent infection. To examine this hypothesis, we set out to investigate whether EBV can modulate innate immune responses.

Successful elimination of most pathogens requires cross-talk between the innate and adaptive arms of immunity. TLRs occupy a pivotal position in this regard, detecting the presence of microbial pathogens through recognition of pathogen-associated molecular patterns (5). TLRs are responsible for host detection of several herpesviruses (6). For instance, TLR2 senses human CMV (7), HSV-1 (8), as well as EBV (9, 10). TLR3 detects viral dsRNA, including the noncoding EBV EBER molecules (11), and also dsRNA intermediates produced during viral replication, as seen with Kaposi's sarcoma-associated herpesvirus (12). In addition, TLR7 and 9, expressed to high levels by plasmacytoid dendritic

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Abbreviations used in this article: BL, Burkitt's lymphoma; CpG, deoxy-cytidylate-phosphate-deoxyguanylate; Cys, cysteine; DC, dendritic cell; IRF, IFN regulatory factor; Met, methionine; NP-40, Nonidet P-40; ODN, oligodeoxyribonucleotide; PAA, phosphonoacetic acid; pDC, plasmacytoid dendritic cell; TfR, transferrin receptor.

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cells (pDC), appear to play a role in the recognition of EBV (13–15). TLR9 is activated by DNA sequences containing unmethylated deoxy-CpGs flanked by two 5' purines and two 3' pyrimidines, which are abundant in certain dsDNA viral genomes, including those of herpesviruses (16, 17). Indeed, TLR9 also recognizes HSV-1 (18) and -2 (19), and mouse CMV (20).

TLR triggering upon ligand recognition activates intracellular signaling networks that ultimately lead to the production of a wide range of immunoregulatory molecules, some of which possess direct antiviral activity, while others serve to orchestrate the adaptive immune response (21–23). This is exemplified by TLR9 signaling in B cells: in response to CpG, purified human B cells display up-regulation of the T cell costimulatory molecules CD80 and CD86, as well as HLA class II molecules (16, 24). TLR9 signaling in B cells also directly induces CXCR3 chemokines capable of attracting and activating Th1 cells (25). Furthermore, while pDCs are considered more important in this regard, some studies provide evidence of a role for TLR9 activation in B cells in production of IL-12, tilting the T cell response toward Th1 polarity (26).

In the current study, we examined whether TLRs are differentially expressed by EBV<sup>-</sup> and EBV<sup>+</sup> B cells during the latent and lytic phases of infection. The fact that reactivation occurs in only a small percentage of EBV-infected cells (1–5%) has limited studies on productive EBV infection so far. To overcome this, we used a strategy to select populations of productively EBV-infected BL cells based on the inducible expression of a lytic phase reporter following viral reactivation (27). To our knowledge, this is the only system that allows isolation of lytically EBV-infected B cells (AKBMs) to high purity for subsequent studies. Previously, this system proved essential in the identification of EBV lytic-phase genes interfering with the machinery of adaptive immune responses (27, 28). Importantly, phenotypes of, for example, HLA class I downregulation observed at the surface of lytically induced AKBM cells, recapitulated those reported for the small fraction of EBV<sup>+</sup> B-LCL that spontaneously reactivate (29). This demonstrates that the AKBM system, although unique in its ability to allow isolation of a pure population of lytically infected cells, remains representative of productive EBV infection of B cells. In this study, we use this system to explore the effects of EBV infection on innate immunity, and specifically on TLRs.

## Materials and Methods

### Cells

Human embryonic kidney 293 cells constitutively expressing functional human TLR genes were purchased from InvivoGen (San Diego, CA), with the exception of 293 cells stably transfected with human TLR9 (30) (a gift from G.B. Lipford, Coley Pharmaceutical Group, Wellesley, MA) and 293 cells expressing a TLR9-CFP fusion protein (31) (provided by P. Massari, Boston University School of Medicine, Dept. of Infectious Diseases, Boston MA, USA). 293T cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (European Union approved; Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cell lines stably expressing TLRs were cultured in selective antibiotics as recommended. The B cell lines BJAB, AKBM, AK31, 2A8, and Mutu I clone 3 and clone 9 (provided by J. Sixbey, St. Jude Children's Research Hospital, Departments of Infectious Diseases and Virology & Molecular Biology, Memphis, TN) (32) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (Standard Quality, European Union approved; PAA Laboratories, Pasching, Austria), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. In addition, AK31 and AKBM cells were cultured in the presence of 1 mg/ml geneticin or 0.3 mg/ml hygromycin B, respectively.

Primary human B cells were prepared from peripheral blood donated by adult volunteers. Mononuclear cells were enriched by centrifugation on Ficoll-Paque (GE Healthcare, Waukesha, WI). B cells were positively selected from PBMCs by immunomagnetic sorting using CD19 Microbeads and LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany), according

to the manufacturer's protocols. The purity of the population was assessed by monitoring CD19 expression.

### Antibodies

The following specific mouse mAbs were used: anti-TLR9 (Imgenex IMG-305A, clone 26C593.2), antihuman transferrin receptor (TfR) H68.4 (Roche Diagnostics, Indianapolis, IN) for Western blot analysis, and anti-TfR CD71 (BD Pharmingen, San Diego, CA) for flow cytometry; 72A1 (American Type Culture Collection, Middlesex, U.K.) recognizing the BLLF1-encoded gp350/220 late membrane Ag; OX34 specific for the extracellular domain of rat CD2 (provided by M. Rowe, University of Birmingham Medical School, Birmingham, U.K.); and a PE-conjugated Ab directed against human CD54/ICAM-1 (555511; BD Pharmingen, San Diego, CA). The rat mAb 3F10 is directed against an influenza virus-derived HA-tag (Roche Diagnostics). HRP-conjugated secondary anti-species Abs (DakoCytomation, Glostrup, Denmark), and secondary goat anti-mouse allophycocyanin-conjugated Ab (Leinco Technologies, St. Louis, MO) were used for Western blot analysis and flow cytometry, respectively.

### Induction of the EBV lytic cycle

To study latent and lytic phases of EBV infection, we used EBV<sup>+</sup> B cells that express a rat CD2-GFP reporter protein during lytic phase, designated AKBM cells. The derivation of the AKBM cells and the isolation of populations in the EBV lytic phase have been described previously (27). In brief, the human EBV<sup>+</sup> BL cell line Akata was stably transfected with a reporter plasmid (pHEBO-prBMRF1-ratCD2-GFP). The EBV lytic phase can be induced by cross-linking surface IgG on AKBM cells with 50 µg/ml goat F(ab')<sub>2</sub> fragments to human IgG (Cappel; MP Biomedicals, Solon, OH). To isolate EBV<sup>+</sup> B cells in the lytic phase, cells were stained with rat CD2-specific Ab (OX34) and were positively selected by magnetic cell sorting with anti-mouse IgG<sub>2a/b</sub> microbeads and MS columns (Miltenyi Biotec), according to the instructions of the manufacturer. Sorted populations of >90% purity were obtained in this manner. Discrimination between (immediate) early and late stages of the lytic phase 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) was added 1 h prior to induction of the EBV lytic phase at a final concentration of 300 µg/ml. To exclude any secondary effects of PAA, Ab treatment used for induction of the EBV lytic phase, or magnetic cell sorting, we used a subclone of Akata that has lost its EBV genome (AK31 cells) in parallel. These AK31 cells constitutively express pEGFPN1-rat CD2 in part of the population.

### RNA isolation and PCR

Total RNA was extracted from 1 to 3 × 10<sup>6</sup> cells using TRIzol reagent (Invitrogen Life Science, Carlsbad, CA) or RNeasy Plus Mini kit (Qiagen Benelux B.V., The Netherlands) and treated with DNase (TURBO DNase-free kit; Applied Biosystems, Foster City, CA), according to manufacturer's protocols. One microgram of RNA was used for cDNA synthesis using random hexamers and the Moloney murine leukemia virus reverse transcriptase (Finnzymes, Espoo, Finland). To confirm the absence of genomic DNA, the reactions were also performed without reverse transcriptase. The resulting cDNA was used diluted 1:10 with water, and 1–2 µl was used for amplification with Taq or Pfu DNA Polymerase as indicated in Table I. TLR-specific primer sequences and the corresponding number of amplification cycles for semiquantitative PCR analysis are listed in Table I. DNA fragments of expected length were visualized by 1% agarose gel electrophoresis and ethidium bromide staining.

Quantitative PCRs were performed in duplicate using a final concentration of 200 nM probe and 300 nM primers (18S rRNA), or a TaqMan Gene Expression Assay (TLR9), according to the instructions of the manufacturer (Applied Biosystems). The amplifications were performed on an ABI/PRISM 7500 sequence detector system (Applied Biosystems). 18S rRNA was amplified using the forward primer 5'-AGTCCCTGCCCTTTGTACACA-3', the reverse primer 5'-GATCCGAGGGCCTCACTAAAC-3', and the probe 5'-CGCCCGTCGCTACTACCGATTGG-3', labeled at the 5' end with the reporter dye JOE and at the 3' end with the quencher dye carboxytetramethylrhodamine (TAMRA). Gene expression was normalized to the housekeeping gene 18S rRNA, and calculations were performed using the comparative Ct method (User Bulletin number 2, ABI Prism 7700 Sequence Detection System, P/N 4303859) to assess the difference in TLR9 mRNA levels in AK31/AKBM cells treated with or without anti-IgG.

### Transient transfections

For assessment of the effects of BGLF5 on TLR9 protein levels, 293T cells were seeded at a density of 2.5 × 10<sup>5</sup> cells/ml, 2 ml/well, in a 6-well plate. After 24 h, cells were transfected with or without 0.125 µg pUNO-hTLR9-



HA (InvivoGen), and 0.625  $\mu\text{g}$  ( $5\times$ , relative to the quantity of pUNO-TLR9-HA transfected), 1.25  $\mu\text{g}$  ( $10\times$ ), 2.5  $\mu\text{g}$  ( $20\times$ ), or 3.75  $\mu\text{g}$  ( $30\times$ ) pcDNA3-BGLF5-HA-IRES-nlsGFP (BGLF5) or 3.75  $\mu\text{g}$  ( $30\times$ ) pcDNA3-BNLF2a-IRES-nlsGFP (BNLF2a) using Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen). The total amount of DNA was maintained at 4  $\mu\text{g}$  in each case using pcDNA3-IRES-nlsGFP (provided by E. Reits, Academic Medical Center, Amsterdam, The Netherlands). Lysates were generated 40 h posttransfection, as described below.

### Western blotting

Western blot analysis was performed as described previously (27). In brief, for the detection of TLR9, HA-tagged BGLF5, and TIR, postnuclear lysates were generated using 0.5% Nonidet P-40 (NP-40) buffer (0.5% NP-40, 50 mM Tris HCl [pH 7.5], 5 mM  $\text{MgCl}_2$ , 10  $\mu\text{M}$  leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride). Lysates were denatured in reducing sample buffer (final concentration: 2% SDS, 50 mM Tris-HCl [pH 8.0], 10% glycerol, 5% 2-ME, and 0.05% bromophenol blue), and solubilized proteins equivalent to  $2 \times 10^5$  cells (for 293T cells) or  $1 \times 10^6$  cells (for AK31 and AKBM cells) were separated by NaDodSO<sub>4</sub> (SDS)-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare). Proteins of interest were detected by incubating the membranes with specific Abs followed by HRP-conjugated specific secondary Abs (DakoCytomation). Bound HRP-labeled Abs were visualized using ECL Plus detection kit (GE Healthcare). For quantification of the protein expression levels, scanned films were analyzed with Quantity One software (Bio-Rad).

### Pulse-chase analysis

For pulse-chase experiments, cells were cultured in methionine (Met)- and cysteine (Cys)-free RPMI 1640 medium (Lonza BioWhittaker, Fisher Scientific, Pittsburgh, PA) for 60 min at 37°C (starvation) prior to metabolic labeling for 30 min with [<sup>35</sup>S]Met/Cys (250  $\mu\text{Ci/ml}$ ; [<sup>35</sup>S] Redivue Promix, a mixture of >70% [<sup>35</sup>S]Met and <30% [<sup>35</sup>S]Cys; Amersham, Buckinghamshire, U.K.) (pulse). Incorporation of the label was terminated by replacing the medium with culture medium supplemented with 1 mM Met and 0.1 mM Cys (chase). Precleared postnuclear NP-40 cell lysates were subjected to immunoprecipitation with TLR9- or TIR-specific Abs for 2–4 h at 4°C. Immune complexes were washed with a buffer containing 0.5% NP-40, 5 mM EDTA, 50 mM Tris-HCl (pH 7.4), and 150 mM NaCl, boiled for 5 min in reducing sample buffer, and subjected to SDS-PAGE. Dried gels were exposed to a phosphorimaging screen, which was scanned with Personal Molecular Imager FX (Bio-Rad) and analyzed with Quantity One software.

### EBV stimulation of BJAB

BJAB were seeded in a 48-well plate at a density of  $5 \times 10^5$  cells/ml and stimulated for 18 h with CpG oligodeoxyribonucleotide (ODN) 2006 type B (10  $\mu\text{g/ml}$ ; InvivoGen) or various amounts of EBV B95.8 purified virus particles ( $1 \times 10^4$ ,  $5 \times 10^4$ ,  $10 \times 10^4$ ,  $25 \times 10^4$ , or  $50 \times 10^4$  virus particles/ml; lot number 106-099, Advanced Biotechnologies, Columbia, MD). Surface expression of CD54 was determined by flow cytometry.

### Flow cytometry

Cell surface expression of specific molecules was determined by using fluorochrome-conjugated Ab or indirectly by using unlabeled primary Ab together with goat anti-mouse allophycocyanin-conjugated Ab. GFP expression in AK31 cells, AKBM cells upon the EBV lytic-phase induction, or 293T cells upon transient transfection was measured without further staining. Cells were fixed with 2% paraformaldehyde and analyzed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ), using CellQuest Pro (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR).

### TLR reporter assays

To examine activation of NF- $\kappa\text{B}$  by EBV particles, 293T and 293-TLR9-CFP cells were seeded in 96-well plates at a density of  $2 \times 10^5$  cells/ml, 200  $\mu\text{l/well}$ , 24 h before transfection with lipofectamine 2000 following the manufacturer's instructions. Cells were transfected with 15 ng NF- $\kappa\text{B}$ -luciferase (a gift from P. Moynagh, NUI Maynooth, Ireland), 80 ng of pRL-TK (constitutively expressing *Renilla* luciferase) and 135 ng of pcDNA3.1 to give a total amount of 230 ng DNA/well. Forty hours posttransfection, cells were stimulated with TNF- $\alpha$  (10 ng/ml), CpG oligodeoxyribonucleotide (ODN) 2006 type B (InvivoGen, 10  $\mu\text{g/ml}$ ) or various amounts of EBV B95.8 purified virus particles ( $5 \times 10^4$ ,  $10 \times 10^4$ ,  $20 \times 10^4$  or  $40 \times 10^4$  virus particles per ml) for a further 6 h. NF- $\kappa\text{B}$ -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, Bad Wildbad, Germany).

### Degradation of in vitro-transcribed mRNA

The pCR2.1-TOPO-TLR9-HA plasmid was linearized with SpeI restriction enzyme and used for in vitro transcription with T7 polymerase (Invitrogen) according to the manufacturer's protocol. Assays were performed in reaction buffer (250 mM NaCl and 20 mM Tris, pH 7.5, supplemented with 10 mM  $\text{MnCl}_2$  or 5 mM EDTA in the presence or absence of 10  $\mu\text{M}$  BGLF5 as described previously (33). The samples were incubated at 37°C for 30 min and stopped by the addition of EDTA to a final concentration of 50 mM before analysis on a 1.5% agarose gel stained with ethidium bromide. Gels were visualized with Gel Doc XR (Bio-Rad) and quantified using Quantity One software (Bio-Rad).

## Results

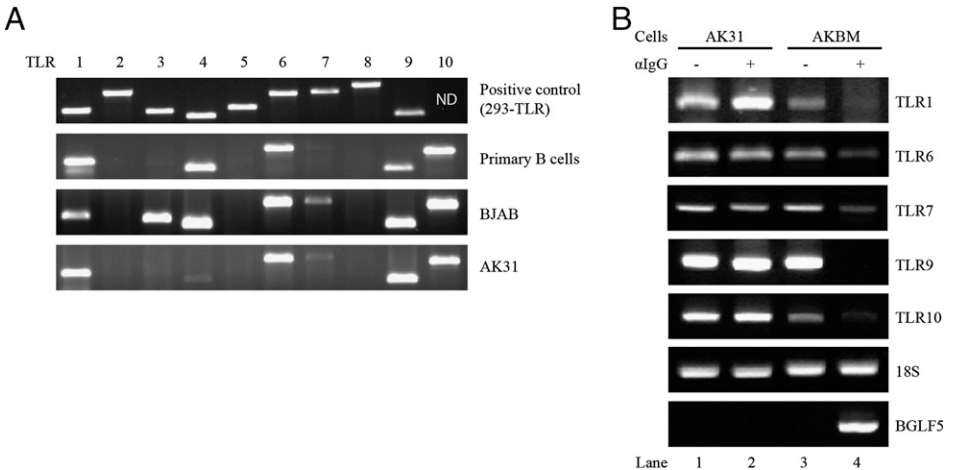
### Differential expression of TLRs by B cells during the latent and lytic phase of EBV infection

To examine whether EBV affects TLR expression in B cells, we initially compared the TLR expression profile of primary B cells to that of two EBV<sup>−</sup> BL cell lines, BJAB and AK31. AK31 is the EBV<sup>−</sup> counterpart of the AKBM cell line used for EBV reactivation studies. RT-PCR was used to monitor expression of TLR 1–10 mRNAs (Fig. 1A, Table I), with 293 cells transfected to express individual TLRs serving as positive controls for TLR detection. Primary B cells, BJAB, and AK31 displayed common expression of TLRs 1, 6, 9, and 10. Furthermore, low levels of TLR7 were observed in both BL cell lines, whereas this signal was very weak in primary B cells. Expression of TLRs 2, 5, and 8 was not detectable in any of the B cell types tested, although low levels of TLR2 have been reported to be expressed on naive and memory B cells (34). Primary B cells and the two EBV<sup>−</sup> BL lines differed only in levels of TLR3 (only present in BJAB) and TLR4 (hardly detectable in AK31). Thus, the TLR expression profile of primary B cells is largely retained in the EBV<sup>−</sup> BL cells, setting the stage for studies on the effect of EBV on expression of TLR1, 6, 7, 9, and 10.

We next assessed whether TLR expression in B cells is altered by EBV infection. To this end, we compared the Akata-derived EBV<sup>−</sup> AK31 to EBV<sup>+</sup> AKBM cells that display a latency I phenotype and to AKBM cells undergoing lytic EBV replication. AKBM cells express a rat CD2–GFP reporter only after induction of the lytic phase by cross-linking of surface IgG (27). Cells receiving anti-IgG treatment for 16 h were immunomagnetically sorted for surface rat CD2 expression to high purity. As a control, AK31 cells constitutively expressing rat CD2–GFP were treated in an identical manner to exclude any secondary effects of anti-IgG Ab treatment or immunomagnetic sorting.

RT-PCR was then conducted to investigate the effects of latent and lytic EBV infection on a range of TLRs expressed by Akata-derived cells, reflecting those detected in primary B cells. We found that latent infection leads to reduced expression of TLRs 1 and 10 (Fig. 1B, compare lanes 1 and 3), whereas levels of TLRs 6, 7, and 9 were not downregulated by latent EBV gene expression. This phenotype was reproduced in another pair of EBV<sup>−</sup> BL cells (Mutu I; see Supplemental Fig. 1). When replicating EBV, AKBM cells displayed reduced mRNA expression of TLRs 1, 6, 7, 9, and 10, with abrogation of TLR9 expression being most pronounced (Fig. 1B, compare lanes 3 and 4). The detection of the EBV lytic cycle transcript BGLF5 confirmed induction of the productive phase of the viral life cycle in AKBM cells treated with anti-human IgG. As a control indicating that the differences in signal were not the result of differences in RNA extraction efficiencies, the level of 18S RNA was similar for all cells tested. Thus, these data show that EBV modulates TLR mRNA levels during both the latent and lytic stages

**FIGURE 1.** TLR mRNAs are differentially expressed during latent and lytic phases of EBV infection. *A*, cDNA was prepared from human primary B cells and EBV<sup>−</sup> BJAB and AK31 BL cells. Expression levels of TLR1–10 were determined by PCR analysis. cDNA from 293 cells stably expressing individual TLRs was used as positive control for specific TLR detection. *B*, EBV<sup>−</sup> AK31 and EBV<sup>+</sup> AKBM cells were incubated with (+) or without (−) anti-IgG for 16 h. Cells treated with anti-IgG were immunomagnetically sorted for surface rat CD2 expression to >95% purity. cDNA was generated and expression levels of the indicated gene products were determined by PCR analysis. ND, not determined.



of infection, with TLR9 expression during productive infection undergoing a particularly strong inhibition.

*TLR9 expression during the EBV lytic phase is strongly reduced at both the RNA and protein level*

To determine the extent of the observed striking reduction in TLR9 expression in the lytic phase, we performed real-time PCR analysis on cDNA generated from EBV<sup>−</sup> AK31 as well as latent and lytic EBV<sup>+</sup> AKBM cells (Fig. 2*A*). TLR9 mRNA levels, normalized to 18S RNA levels, were reduced by 86% in AKBM cells during EBV lytic phase compared with latently infected or uninfected cells; anti-IgG treatment of AK31 cells had no such effect. These real-time PCR results therefore confirm and extend our RT-PCR data (Fig. 1).

To examine whether TLR9 mRNA levels correlated with TLR9 protein expression, Western blot analyses on cell lysates of EBV<sup>−</sup> AK31 and EBV<sup>+</sup> AKBM cells were performed. TfR was used as a control and its expression was not affected by cross-linking of surface IgG in either AK31 or AKBM cells (Fig. 2*B*). However, although TLR9 was detected in cell lysates of EBV<sup>−</sup> AK31 cells and EBV<sup>+</sup> AKBM cells during the EBV latent phase, it was virtually absent from lysates of AKBM cells during the EBV lytic phase (Fig. 2*B*, compare lanes 1–3 to 4). Because the EBV<sup>−</sup> AK31 cells were examined under the same conditions as the EBV<sup>+</sup> AKBM cells, the reduction of TLR9 expression during EBV lytic cycle is a specific feature of the virus, exhibited during the productive phase of infection. Such a dramatic decrease in the

steady-state levels of a cellular protein have not previously been observed after induction of the lytic cycle for 16–20 h. Notably, despite displaying reduced mRNA expression, steady-state levels of HLA class I H chain, HLA class II  $\alpha$ - and  $\beta$ -chains, TAP1 and TAP2 all remain unaffected at this time (27, 28).

To probe whether the dramatic reduction in TLR9 protein relative to other cellular proteins could be explained by a shorter half-life, we performed pulse-chase analyses to compare the relative stabilities of TLR9 and TfR protein. 293 cells stably expressing TLR9 were metabolically labeled for 30 min before immunoprecipitating TLR9 and TfR at the indicated chase time points. Both TLR9 and TfR were detected to similar levels up to at least 480 min after pulse-labeling (Fig. 2*C*). Quantification of protein levels revealed almost identical stabilities for TLR9 and TfR proteins (Fig. 2*D*). Thus, despite displaying equivalent stability, steady-state levels of TLR9 protein are dramatically reduced after one day of productive EBV infection, whereas TfR levels remain largely unaltered.

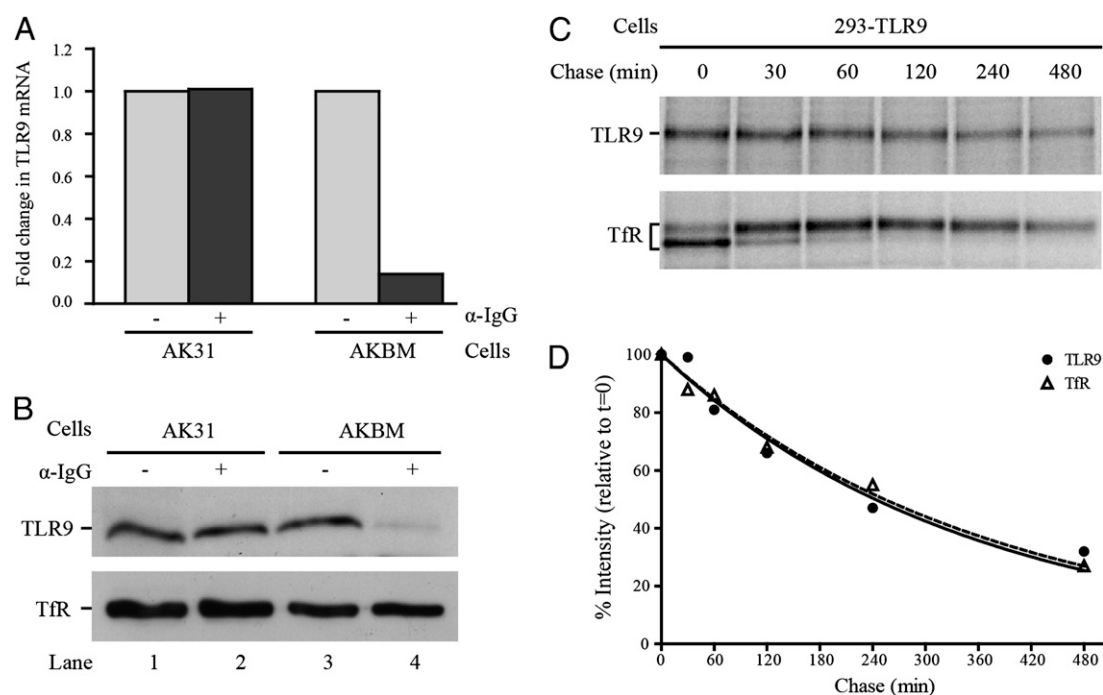
*TLR9-mediated intracellular signaling is activated by EBV*

To explore the functional relevance of the strong reduction in TLR9 protein seen in lytically infected cells, we examined whether TLR9 can recognize EBV. BJAB B cells were monitored by flow cytometry for surface expression of the activation marker CD54 (ICAM-1) upon overnight stimulation with increasing concentrations of EBV particles or the known TLR9 activating ligand CpG. Virus particles caused a dose-dependent increase in CD54

Table I. Primer sequences for detection of TLR expression by RT-PCR analysis

	Forward (5'–3')	Reverse (5'–3')	No. of Cycles
TLR			
1	AAAAGAAGACCTTGAGGGCC	TCTGAAGTCCAGCTGACCCCT	40 <sup>a</sup>
2	AACCCTAGGGGAAACATCTCT	GGAATATGCAGCCTCCGGAT	40 <sup>a</sup>
3	AAATTGGGCAAGAAGTCAACAGG	GTGTTTCCAGAGCCGTGCTAA	45 <sup>a</sup>
4	TACAAAAATCCCGACAACCTC	AGCCACCAGCTTCTGTAAACT	40 <sup>b</sup>
5	TGCATTAAGGGGACTAAGCCTC	AAAAGGGAGAAGCTTTAGGGACT	40 <sup>a</sup>
6	TTGACAGTTTGGAGACTTTCCC	TGAACCTCTGGTGAGTTCTG	45 <sup>a</sup>
7	TCCAGTGTCTAAAGAAGCTGG	TGGTATATATACCACACATCCC	40 <sup>b</sup>
8	TAATAGGCTCAAGCACATCCC	TCCCAGTAAAACAAATGGTGAG	45 <sup>a</sup>
9	GTGCCCCACTTCTCCATG	GGCACAGTCATGATGTTGTTG	38 <sup>a</sup>
10	TGACCACAATTCATTGACTACTC	TTGAATACTTTTGGGCAAGCACC	40 <sup>a</sup>
Controls			
18S	GTAACCCGTTGAACCCCATTT	GATCCGAGGGCCTCACTAAAC	25 <sup>a</sup>
GAPDH	GGATGATGTTCTGGAGAGCC	CATCACCATCTTCCAGGAGC	32 <sup>a</sup>
BGLF5	GGGACATATTGCGAAATGGC	CACTGTGGCGGACGTAGTC	30 <sup>b</sup>

<sup>a</sup>Amplified with Taq DNA polymerase.  
<sup>b</sup>Amplified with Pfu DNA polymerase.



**FIGURE 2.** TLR9 expression is reduced during EBV lytic phase both at the mRNA and protein level. **A**, EBV<sup>+</sup> AKBM cells were cultured for 16 h with or without anti-IgG and subjected to immunomagnetic sorting to isolate productively infected cells. To exclude any secondary effects of Ab treatment and/or immunomagnetic sorting, EBV<sup>-</sup> AK31 cells were treated in the same manner. Levels of TLR9 mRNA were determined by quantitative real-time PCR analysis and normalized to 18S RNA expression. Data are expressed relative to TLR9 mRNA levels in non-IgG-treated cells. SD of Ct values in 18S and TLR9 amplification replicates was <2% in each case. **B**, Protein expression of TLR9 and Tfr in postnuclear lysates of the cells described in **A** was determined by Western blot analysis. **C**, 293-TLR9 cells were labeled for 30 min with [<sup>35</sup>S]Met/Cys and chased for the times indicated. TLR9 and Tfr were immunoprecipitated from cell lysates and all samples were analyzed by SDS-PAGE. **D**, The relative stabilities of TLR9 and Tfr proteins were evaluated by quantification of the experiment depicted in **C**. The small shift in mobility observed for Tfr is explained by maturation of its N-linked glycans over time.

expression, with the highest concentration used providing a similar induction to that observed with CpG (Fig. 3A), suggestive of TLR activation by EBV.

As BJAB cells express a range of TLRs other than TLR9 (Fig. 1A), we determined whether TLR9 in particular can detect EBV. 293 cells stably expressing TLR9 (293-TLR9) and control 293T cells were transfected with an NF- $\kappa$ B-inducible firefly luciferase reporter gene. Stimulation with TNF $\alpha$  verified the responsiveness of the reporter system to NF- $\kappa$ B activation in both cell lines (data not shown). Addition of CpG to 293-TLR9 cells confirmed the functionality of the TLR9 receptor (Fig. 3B). Interestingly, addition of EBV particles to the TLR9-expressing cells activated NF- $\kappa$ B in a dose-dependent manner. This effect required TLR9, because the highest concentration of EBV particles used failed to trigger signaling through NF- $\kappa$ B in control 293T cells. The absence of detectable viral latent or lytic gene expression in 293T cells following culture in the presence of EBV (data not shown) renders involvement of infection unlikely. These combined data demonstrate that TLR9 can sense the presence of EBV, highlighting the significance of TLR9 downregulation during the EBV lytic cycle.

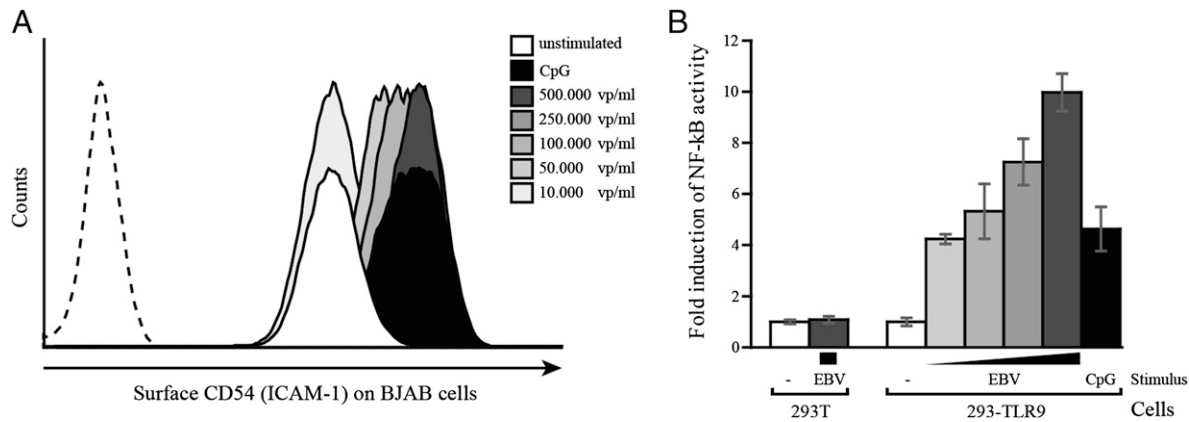
#### *The EBV lytic-phase protein BGLF5 reduces TLR9 expression through mRNA degradation*

To identify the EBV gene responsible for TLR9 downregulation, we first determined the stage of the lytic phase at which TLR9 protein expression is decreased. PAA inhibits viral DNA replication and late lytic phase gene expression and was used in this study to discriminate early from late EBV protein expression (28). Irrespective of the PAA-mediated block of late lytic phase gene expression, TLR9 protein levels were reduced in EBV<sup>+</sup> AKBM cells

during the lytic phase when compared with anti-IgG-treated EBV<sup>-</sup> AK31 cells (Supplemental Fig. 2). As a control, no reduction in  $\beta$ -actin levels was observed under the same conditions. Thus, an EBV gene product expressed (immediate) early during EBV lytic cycle causes the reduction in TLR9 expression.

Previously, we described a block in de novo protein synthesis occurring during productive EBV infection, referred to as host shutoff (28). The EBV protein responsible for virus-induced host shutoff was identified as the early lytic-phase protein BGLF5, the viral alkaline exonuclease that also causes enhanced mRNA turnover. Recently, we have shown that recombinant BGLF5 acts not only as a DNase in vitro, but also as an Mn<sup>2+</sup>-dependent RNase, mediating the degradation of GFP mRNA (33). In this paper, we addressed the question of whether BGLF5 could degrade TLR9 mRNA. We observed that in vitro transcribed TLR9 mRNA was strongly reduced upon addition of recombinant BGLF5, provided Mn<sup>2+</sup> ions were present (Fig. 4A, compare lanes 1 and 3). No degradation was seen in the presence of EDTA (Fig. 4A, compare lanes 2 and 4). As BGLF5 DNase activity is also dependent on the presence of bivalent ions, remaining plasmid DNA was also degraded by recombinant BGLF5 in Mn<sup>2+</sup>-containing buffer. Quantification of RNA levels demonstrated that EBV BGLF5 degraded TLR9 mRNA in vitro (Fig. 4B).

To examine whether BGLF5 expressed in cells can reduce levels of TLR9, 293T cells were transiently transfected with TLR9 in combination with increasing amounts of BGLF5, or another EBV lytic phase protein BNLF2a (35), used as a control. Both BGLF5 and BNLF2a were expressed from a bicistronic vector that also encodes GFP. At 40 h posttransfection, cellular GFP expression was measured by flow cytometry as an indication of transfection efficiency. On average, cells cotransfected with the controls, vector



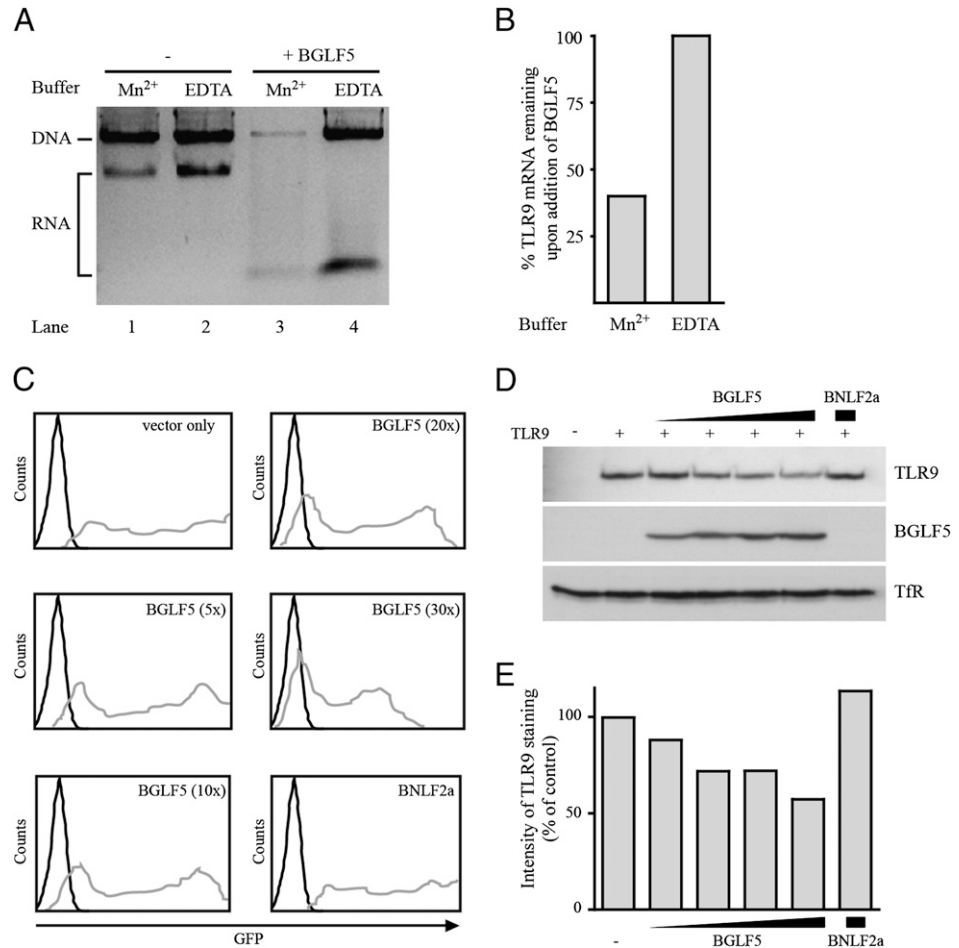
**FIGURE 3.** EBV particles activate TLR9 signaling. *A*, BJAB B cells were stimulated for 18 h with or without CpG-ODN2006 (10  $\mu$ g/ml) or increasing amounts of purified EBV particles, as indicated. Surface expression of CD54 (ICAM-1) was determined by flow cytometry. The dashed line represents unstained control cells. *B*, 293T and 293-TLR9-CFP cells, transiently cotransfected with NF- $\kappa$ B-luciferase and phRL-TK (constitutively expressing *Renilla* luciferase) were stimulated for 6 h with or without CpG-ODN2006 (10  $\mu$ g/ml) or increasing amounts of purified EBV particles (see *Materials and Methods*). Cell lysates were assayed for firefly and *Renilla* (for normalizing transfection efficiency) luciferase. Results (mean  $\pm$  SD) are presented relative to cells treated with medium alone (–).

alone or BNL2a, were >95% GFP<sup>+</sup> (Fig. 4C). In the case of BGLF5 cotransfections, we have previously seen that GFP is susceptible to host shutoff. Thus, the decreasing GFP levels observed with increasing doses of BGLF5 are indicative of BGLF5-mediated enhanced turnover of GFP mRNA.

TLR9 protein was detected by Western blot analysis of postnuclear cell lysates (Fig. 4D). Coexpression of BGLF5 led to decreasing levels of TLR9 protein in a dose-dependent manner. TfR,

used as a control protein, was found to be expressed to similar extents in all samples. Quantification of the Western blot data indicated a reduction in TLR9 protein levels of up to 50% with the highest concentration of BGLF5 coexpressed, when normalized for TfR protein levels (Fig. 4E). The specificity of BGLF5-mediated downregulation of TLR9 was demonstrated by the failure of BNL2a to cause a reduction in TLR9 expression. Thus, BGLF5 can degrade TLR9 mRNA and, thereby, decrease levels of

**FIGURE 4.** EBV lytic phase protein BGLF5 degrades TLR9 mRNA and reduces TLR9 protein expression. *A*, Following in vitro transcription, TLR9 mRNA was incubated with or without recombinant BGLF5 protein for 30 min at 37°C in the presence of either Mn<sup>2+</sup> or EDTA. TLR9 mRNA and linearized plasmid DNA were visualized with ethidium bromide in a 1.5% agarose gel. *B*, RNA bands were quantified using Quantity One software; the bars depict the percentage TLR9 mRNA that remains after incubation with BGLF5, with TLR9 levels in buffer alone set at 100%. *C*, 293T cells were transiently cotransfected with pUNO-TLR9-HA and pcDNA3-IRES-GFP, pcDNA3-BNL2a-IRES-GFP, or increasing amounts of pcDNA3-BGLF5-IRES-GFP. Forty hours post-transfection, GFP expression was measured by flow cytometry. *D*, Expression of TLR9-HA, BGLF5-HA, and TfR was examined by Western blot analysis using postnuclear lysates of the cells described in *C*. *E*, The results of the TLR9 immunoblot depicted in *D* were quantitated using Quantity One software and normalized to TfR levels. The bars represent the percentage of TLR9 expression upon cotransfection with BGLF5 or control BNL2a, with the level of TLR9 alone set at 100%.





TLR9 protein, providing compelling evidence that this EBV lytic cycle product contributes to downregulation of TLR9 expression observed during productive viral infection.

## Discussion

Although our understanding of evasion from adaptive immune responses by EBV has increased greatly over recent years (4), less is known about the interactions of the virus with innate immunity. In this study, we examined the interplay between EBV and TLRs. We show that expression of TLRs 1, 6, 7, 9, and 10 is downregulated in human B cells undergoing productive EBV infection. In particular, TLR9 protein levels were dramatically decreased. We demonstrate that TLR9 signaling is activated in the presence of EBV particles, revealing the reduction of TLR9 levels to be a potentially useful immune evasion strategy used by the virus. We established that the EBV early protein BGLF5 degrades TLR9 mRNA *in vitro*, providing a mechanism for its contribution to TLR9 downregulation. To our knowledge, this is the first demonstration of an EBV lytic cycle gene product directly affecting expression levels of a pattern-recognition receptor capable of alerting the immune system to viral infection.

Although the expression of several TLRs was altered during productive EBV infection, the striking reduction in TLR9 at both RNA and protein levels was of particular interest because the motif recognized by this receptor is widely present in various herpesvirus genomes, including EBV. This would supply an abundance of potential TLR9-activating ligands both during primary infection and virus production. In fact, a number of recent publications indicate that TLR9 is involved in detecting EBV, leading to production of IL-8 in primary monocytes and IFN- $\alpha$  in pDCs (13, 14). As for B cells, we show in this paper that stimulation of BJAB cells with EBV increases surface CD54 expression. Likewise, Iskra et al. (36) found upregulation of CD80 on primary B cells following coculture with EBV. Although EBV recognition by monocytes and pDCs appears to depend on TLR9 acting in tandem with TLRs 2 and 7, this may be different for B cells that do not or only weakly express TLRs 2 and 7. In line with this, we now demonstrate that stimulation with EBV particles leads to NF- $\kappa$ B activation in 293 cells only when TLR9 is expressed, but not in its absence, thereby establishing that TLR9 in isolation can specifically recognize EBV. Most likely, this TLR9-mediated recognition of EBV does not require prior cellular infection as we did not detect signs of infection of 293T cells (data not shown). Indeed, this is in agreement with recent findings showing that pDCs can recognize UV-irradiated EBV particles in a TLR9-dependent fashion (13). As opposed to infection not being required for TLR9 recognition of EBV, the presence of viral DNA as well as endosomal maturation are necessary (13).

In latently infected EBV<sup>+</sup> BL cells, the expression levels of several TLRs were also found to be altered. Observing decreased expression of TLRs 1 and 10 in both the Akata and Mutu I model systems of latency suggests that EBV latency-associated gene products may modulate TLR expression. A recent report indicated decreased TLR9 and increased TLR7 expression within hours after primary EBV infection of naive B cells that had most likely entered latency III (15). We did not detect decreased TLR9 mRNA expression in either latently infected Akata or Mutu I cells and observed only a modest increase in TLR7 in latently infected Akata cells. One explanation for this discrepancy could be the different stages of latency examined, with both AKBM and Mutu I cells existing in latency I. Another possible reason for the decrease in TLR9 mRNA seen by Martin et al. (15) could be due to the actions of EBV lytic cycle gene products that undergo transient expression upon primary infection. An example of this latter phenomenon is provided by the immediate but transient expression of the viral

bcl-2 homolog genes, BALF1 and BHRF1, upon infection of primary B cells by EBV (37). Thus, it is tempting to speculate that transient expression of BGLF5 upon primary infection could mediate downregulation of TLR9.

In our search for the EBV modulator of TLR9 expression during productive infection, we focused on (immediate) early lytic-phase genes, as inhibition of late viral gene expression failed to block the downregulation of TLR9 observed during productive EBV infection. An appropriate candidate was BGLF5, which appears 3–6 h after lytic cycle induction. In this study, we find that expression of BGLF5 in isolation is sufficient to cause an ~50% decrease in TLR9 steady-state protein levels in transfected cells. The extent of TLR9 downregulation achieved in 293T cells upon expression of BGLF5 (Fig. 4C, 4D) is less than that observed upon induction of the viral lytic cycle in AKBM cells (Fig. 2B). However, it should be noted that upon cotransfection of 293T cells, levels of BGLF5 protein approximated those observed during lytic infection of B cells, although expression of TLR9 far exceeded that present in latently infected AKBM cells (data not shown). Thus, higher relative levels of TLR9 expressed in transfected 293T cells may have resulted in a less robust BGLF5-mediated downregulation. In addition, other EBV lytic cycle gene products may contribute to the stronger decrease of TLR9 seen in productively infected cells (further discussed below). Finally, in addition to identifying BGLF5 as an EBV protein contributing to decreased levels of TLR9 during productive infection, we offer a mechanistic explanation for this effect by demonstrating the ability of BGLF5 to degrade TLR9 mRNA *in vitro*.

During productive EBV infection, the decrease in TLR9 mRNA levels is more pronounced than the downregulation of other cellular components tested, including  $\beta_2$ -microglobulin transcripts, suggesting that a further mechanism operates to diminish TLR9 expression at this stage in the viral life cycle. One possibility involves downregulation of TLR9 transcription, potentially through activation of NF- $\kappa$ B (38) induced by viral gene products. More remarkable than the decrease in TLR9 mRNA levels is the strong downregulation of steady-state TLR9 protein levels seen at 16 h postinduction of the lytic cycle in AKBM cells. At this time, although mRNA expression and surface protein levels of, for instance, HLA molecules are both diminished, no reduction in total protein levels is detected by Western blot analysis. Therefore, the dramatic reduction in total TLR9 protein levels in lytically infected cells, combined with metabolic labeling experiments showing TLR9 protein to have stability similar to that of the Tfr, raise the prospect that EBV also targets TLR9 posttranslationally. The existence of viral strategies other than host shutoff targeting TLR9 would provide an intriguing parallel to EBV-mediated subversion of adaptive Ag presentation pathways: BNLF2a and BILF1 combine with BGLF5 to sabotage HLA class I Ag presentation (35, 39), whereas gp42 complements the effect of BGLF5 on HLA class II-restricted T cell recognition (28, 40).

A fundamental role for TLRs in controlling viral disease is supported by the correlation between severe disease upon viral infection and loss-of-function mutations in TLRs themselves (41). In addition, HSV encephalitis can arise from a deficiency in UNC-93B, a protein required for translocation of the “nucleotide-sensing” TLRs (TLRs 3, 7/8, and 9) to endolysosomes, which is critical for signaling (42–45). Interestingly, our preliminary data indicate that expression of UNC-93B transcripts is also suppressed during productive EBV infection. The recent identification of the first virus-encoded proteins that subvert TLR signaling further emphasizes the importance of TLRs in the antiviral response. Among these, vaccinia virus A46R can interact with the Toll-like IL-1R-domain-containing adaptors that associate with TLRs, thus reducing downstream



IFN-regulatory factor (IRF) and NF- $\kappa$ B activation (46). The hepatitis C virus protease NS3-4A cleaves the Toll-like IL-1R-domain containing adaptor-inducing IFN- $\beta$ , thereby inhibiting the antiviral effects of TLR3 signaling (47). Direct targeting of TLRs has also been reported, with E6 and E7 of human papillomavirus causing diminished TLR9 levels through negatively regulating promoter activity, thereby functionally impairing TLR9 signaling (48).

For EBV, some mechanisms by which viral gene products can thwart components of the innate immune response have emerged in recent years. For instance, the nonpolyadenylated untranslated RNAs, EBER-1 and -2, expressed during latency, inhibit the activation of the dsRNA-stimulated protein kinase R, thereby providing resistance to IFN-induced apoptosis (49). Various immunoevasive capabilities have also been assigned to the immediate-early transactivator BZLF1, including association with the NF- $\kappa$ B subunit p65, resulting in its nuclear location while impairing its transcriptional ability (50), inhibition of IRF7, thereby hampering type I IFN production during viral reactivation (51), and downregulation of the TNFR gene promoter activity compromising the effects of TNF- $\alpha$  on an infected cell (52). More recently, the EBV tegument protein LF2 was shown to block dimerization of IRF7, whereas the virion-associated kinase BGLF4 was found to curtail IRF3 transactivation ability (53, 54). These latter examples indicate that EBV particles can begin countering the host innate immune response upon cellular entry without the need for prior gene expression.

In this paper, we demonstrate the ability of the EBV early lytic-cycle gene product BGLF5, expressed following viral reactivation in B cells, to reduce expression of TLR9, capable of recognizing pathogen-associated molecular patterns present in the EBV genome. The biological relevance of this effect is underscored by the demonstration that recognition of EBV particles by TLR9 leads to activation of the transcription factor NF- $\kappa$ B. This constitutes a novel manner in which EBV can thwart the host immune response, thus extending the time window for the virus to establish persistent infection and to produce new virions.

**Note added in proof.** Since resubmission of our manuscript, Fathallah et al. (55) reported that EBV LMP1, expressed during latency III, can downregulate TLR9 through NF- $\kappa$ B-dependent inhibition of TLR9 transcription.

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## Disclosures

The authors have no financial conflicts of interest.

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