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EBV Latent Membrane Protein 1 Is a Negative Regulator of TLR9

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EBV infects most of the human population and is associated with a number of human diseases including cancers. Moreover, evasion of the immune system and chronic infection is an essential step for EBV-associated diseases. In this paper, we show that EBV can alter the regulation and expression of TLRs, the key effector molecules of the innate immune response. EBV infection of human primary B cells resulted in the inhibition of TLR9 functionality. Stimulation of TLR9 on primary B cells led to the production of IL-6, TNF-α, and IgG, which was inhibited in cells infected with EBV. The virus exerts its inhibitory function by decreasing TLR9 mRNA and protein levels. This event was observed at early time points after EBV infection of primary cells, as well as in an immortalized lymphoblastoid cell line. We determined that the EBV oncoprotein latent membrane protein 1 (LMP1) is a strong inhibitor of TLR9 transcription. Overexpression of LMP1 in B cells reduced TLR9 promoter activity, mRNA, and protein levels. LMP1 mutants altered in activating the NF-κB pathway prevented TLR9 promoter deregulation. Blocking the NF-κB pathway recovered TLR9 promoter activity. Mutating the NF-κB cis element on the TLR9 promoter restored luciferase transcription in the presence of LMP1. Finally, deletion of the LMP1 gene in the EBV genome abolished the ability of the virus to induce TLR9 downregulation. Our study describes a mechanism used by EBV to suppress the host immune response by deregulating the TLR9 transcript through LMP1-mediated NF-κB activation.  The Journal of Immunology, 2010, 185: 000–000.

EBV, Epstein-Barr virus; LMP1, latent membrane protein 1; qPCR, quantitative PCR; RT, reverse transcriptase; WT, wild-type.

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Abbreviations used in this paper: CTAR, C-terminal activation region; IKK, IkB kinase; LCL, lymphoblastoid cell line; LMP1, latent membrane protein 1; qPCR, quantitative PCR; RT, reverse transcriptase; WT, wild-type.

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EBV survival, although how EBV escapes from the innate immune response is still to be studied (15).

The innate immune response is the first line of defense against infection. Innate immunity uses several pattern recognition receptors to sense pathogen-associated molecular patterns. The TLRs are the most studied pattern recognition receptors, and activation by pathogen components leads to robust immune response (19). TLR2 and TLR4 recognize Gram-positive and -negative bacteria cell wall products, respectively. TLR5 recognizes a structural epitope of bacterial flagellin, whereas TLR3, TLR7, and TLR8 have been demonstrated to recognize different forms of nucleic acid derived from viruses. TLR9 recognizes dsDNA sequences from bacteria or viruses in the form of nonmethylated CpG oligonucleotides. TLR activity has been intensely investigated in the B cells, the host cell of EBV infection. B cells express a variety of TLRs such as 1, 2, 6, 7, 9, and 10 (20). Furthermore, TLR7 and TLR9 expression and functionality were historically characterized in B cells (21, 22). Classically, B cell activation was shown to require BCR cross-linking and CD40 signaling. Yet, a third signal needs to be delivered coupled by BCR-TLR7 or -TLR9 in response to R848 or CpG, respectively, to produce optimal Ab responses to T cell-dependent Ags (23). In terms of viral recognition, it has been shown that TLR9 is required for the innate immune response to human DNA viruses including HSV-2 (21, 24) and EBV (25), and the involvement of TLR9 in the antiviral immune response to the murine γ-herpesvirus MHV-68 (a murine model of EBV infection) was demonstrated in a recent study (26). However, the direct effect of EBV infection in B cells on the TLR9-mediated innate immune response has not been elucidated.

We show that EBV escapes the innate immune response by downregulating TLR9 transcription. Most importantly, the virus exerts this activity by using its major oncoprotein LMP1 via activation of NF-κB signaling.

Materials and Methods
Cell culture and EBV infection
RPMI 8226 cells were a kind gift from the laboratory of Christophe Caux (Centre Leon Berard, Lyon, France). PBMCs were freshly isolated by density-gradient centrifugation using Ficoll Hypaque (r). B cells were purified from the PBMCs using anti-CD19–coated nanoparticle (EasySep; StemCell Technologies, Vancouver, British Columbia, Canada) or using the B cell isolation kit II from Miltenyi Biotec (Auburn, CA). The purity of the cells was assessed by flow cytometry (FACSCalibur, BD Biosciences Pharmingen, San Diego, CA, and FACSLRQ) using an anti-CD19 (BD Pharmingen). In total, 10 PBMC donors were used for this study in which primary B cells and corresponding LCLs were generated. B cells and RPMI 8226 cells were infected with recombinant EBV expressing GFP, as described previously (27). In addition, purified B cells were also infected for 5 d with a recombinant EBV deleted of LMP1 (gene EBV (EBV-LMP1)) also expressing the GFP, as described previously (28). Primary B cells, infected B cells, and RPMI 8226 cells were maintained in culture in RPMI 1640 medium (GIBCO; Invitrogen life Technologies, Cergy-Pontoise, France) supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mM l-glutamine, and 1 mM sodium pyruvate (PAA, Pasching, Austria). HEK293 T cells were obtained from American Type Culture Collection and maintained in DMEM supplemented as described earlier.

TLR ligands
CpG2006-G and GpC2006 were purchased from Sigma-Aldrich (St. Louis, MO). PamC2ysKKK was purchased from EMRC (Tubingen, Germany) micromembrane and used at 4 mM. Polycl (R) and R848 were purchased from Invivogen and used at 10 μM and 10 μg/ml, respectively. Repurified LPS was used at 500 ng/ml (Invivogen); Flagellin (Salmonella muenchen) was from Calbiochem (San Diego, CA) and used at 50 ng/ml.

Retroviral infections
RPMI 8226 cells were transduced using retroviral supernatant generated by transfection of pLNSX and pLNSX LMP1 (gift from L. Young, University of Birmingham, Birmingham, U.K.) into phoenix cells using the method previously described (29).

RNA extraction and reverse transcription
Total RNA was extracted from cells (RNeasy; Qiagen [Courtaboef Cedex, France] or Macherey Nagel [Hoeorch, France]). One microgram of RNA was reverse transcribed using the first strand cDNA synthesis kit according to the manufacturer’s protocol (Fermentas, St. Leon-Rot, Germany).

Quantitative PCR
The reverse transcriptase (RT) reaction was diluted according to detection sensitivity, and 5 μl of the diluted sample was added to a 15-μl PCR mixture containing 4 μM of each primer and 8.75 μl Mesa green quantitative PCR (qPCR) Master Mix (Eurogentec, Angers, France). PCR was conducted in a 40 × 3000P real-time PCR system (Stratagene, La Jolla, CA). Two sets of PCR assays were conducted for each sample using the following primers specific for TLR9: forward: 5’-CTG CTT GAA GCC CTG GTG TG TGA-3’; reverse: 5’-CTG CAA GCC CTT GGT TTT AGT GA-3’. Housekeeping genes β2-microglobulin or β-actin were used. The sequence of the LMP1 primers was: forward: 5’-CCC CCT CTC CTC TTC CAT AG-3’; reverse: 5’-GCC AAA GAT GAA CAC CAG AA-3’. Amplification specificity was assessed for each sample by melting curve analysis, and the size of the amplicon checked by electrophoresis (data not shown). Relative quantification was performed using standard curve analysis. TLR9 expression data were normalized with β2-microglobulin or β-actin and are presented as a ratio of gene copy number per 100 copies of β2-microglobulin or β-actin ± SD.

Western blotting
Whole-cell lysates were prepared using lysis buffer (20 mM Tris/HCl, pH 8, 200 mM NaCl, 0.5% Nonidet P40, 1 mM EDTA, 10 mM NaF, 1 mM PMSF, 1 μg/ml leupeptin, and 1 mM Na3VO3). The protein concentration was measured using BCA (BC Assay reagent; Uptima Interchim, Montluçon, France). Adequate quantity of proteins (between 15 and 25 μg) was used for SDS-PAGE analysis and immunoblotting.

Luciferase assay
Transient transfection of the reporter plasmid TLR9 luciferase or NF-κB luciferase was performed as previously described (30, 31).

ELISA
Cells were seeded at 2 × 105/200 μl/well and stimulated for 24 h. Supernatant was harvested, and IL-6 (R&D Systems, Minneapolis, MN) and TNF-α (Bender MedSystems, Vienna, Austria) were measured following the manufacturer’s protocol. Protein concentration of three wells per cell type was measured to normalize cytokine secretion. The IgG secretion was evaluated in supernatants harvested 120 h after CpG and GpC stimulation using the Human IgG ELISA Quantitation kit (Bethyl Laboratories, Montgomery, TX).

Conofocal staining
Primary B cells and LCL from the same donor were fixed using cold acetone/ methanol (v/v) and then saturated using 5% BSA (PAA) in 0.05% TBS-Tween–20 at room temperature for 1 h. Slides were mounted using Vectra shield mounting media with DAPI. Analysis of the slides was performed using a Leica confocal TCS SP5 System (Leica, Wetzlar, Germany).

Plasmid constructs
The NF-κB luciferase and Renilla reporter gene constructs were purchased from BD Clontech (Palo Alto, CA). The pGL3-TLR9 promoter was obtained from Fumihiko Takeshita (31). The NF-κB site (−413GGG-GAGTGGCCC−403) was mutated to (−413CTCGAGTGCCC−403) using the QuikChange site-directed mutagenesis kit (Stratagene) according to manufacturer’s instructions. pcDNA3-LMP1 (15) was obtained from E. Kieff (Harvard Medical School, Boston, MA). The expression vectors for LMP1 and LMP1 mutants: LMP1AAXAx (Mut 1), LMP1 378 STOP (Mut 2), and LMP1AXAXAx3/78STOP (Mut 3) were a kind gift from L. Young (University of Birmingham) and described by Eliopoulos and colleagues (32). NF-κB superrepressor (pN-InvkB, which lacks the sequence that codes for the first N-terminal 36 aa, was obtained from E. Kieff and cloned into pcDNA3. The dominant negative constructs Iκκα–DN (pCR3-Flag-IKKα-κα) and Iκκβ–DN (pCR3-Flag-IKKβ-κα) were obtained from D. Goeddel (Tullirak, San Francisco, CA).

Abs
The following Abs were used: anti-TLR9 (2254; Cell Signaling Technology, Beverly, MA), anti-LMP1 Ab (S12; a gift from Georges Mosialos, Alexandra Fleming Institute, Varkiza, Greece), anti-rabbit/mouse IgG conjugated to HR (W401B and W40213; Promega, Madison, WI),
Alexa Fluor 488 mouse anti-Human CD19 (BD Pharmingen), Alexa Fluor 488 mouse IgG1 κ isotype control (BD Pharmingen), and anti-GFP (Roche, Basel, Switzerland).

Results

EBV infection hampers TLR9 pathway in B cells

To address whether EBV affects TLR-regulated pathways, primary human B cells (Fig. 1A) were first infected with recombinant EBV virus GFP gene (27). The efficiency of EBV infection was evaluated by monitoring GFP expression using confocal microscopy (Supplementary Fig 1). Additional evidence of viral infection was seen in viral gene expression, unscheduled proliferation, and B cell immortalization into LCL (data not shown).

We next determined the functionality of TLR2-9 pathways in noninfected primary B cells and in LCL derived from the same batch of primary B cells by adding their specific ligands and monitoring the secretion of IL-6. We identified the optimal TLR ligand concentrations for maximal IL-6 secretion poststimulation in primary B cells (data not shown). No significant difference in R848-induced TLR7 activation was detected in primary B cells or LCL, indicating that EBV has no effect on the TLR7 activity (Fig. 1B). Primary B cells, as well as LCL, were refractory to LPS-induced TLR4 stimulation. Poly(I:C) marginally activated the TLR3 pathway in primary cells; however, this activity was inhibited in LCL. In contrast, the levels of secreted IL-6 were strongly decreased in LCL in comparison with primary cells on engagement of TLR2 (pam2Cys4K), TLR5 (Flagellin), and TLR9 (CpG; \( p < 0.006 \), Student nonpaired t test; Fig. 1B).

Previous work has highlighted the importance of TLR9 in controlling EBV or murine γ-herpesvirus in animal experimental models (26); thus, we focused our study on the characterization of the events involved in the alteration of the TLR9 pathway. The EBV-mediated inhibition of TLR9 pathway was further confirmed using serial dilutions of CpG, whereas no significant changes in IL-6 levels were observed in primary cells and LCL on exposure to GpC control oligos (Fig. 1C).

To further demonstrate the impairment of TLR9 functionality after EBV infection, we monitored TNF-α and IgG secretion on TLR9 stimulation. As shown in Fig. 1D, TNF-α secretion was severely altered in EBV-infected LCL compared with primary B cells. Similarly, a significant reduction of IgG secretion was also observed in LCL after stimulation with TLR9 ligand (Fig. 1E).

FIGURE 1. EBV affects TLR-mediated cytokine production in B cells. A, Purified primary B cells from PBMCs were extracted from several donors. B cell purity was between 96 and 98% as measured by flow cytometry. B, TLR-induced secretion of IL-6 in primary B cells and in EBV-immortalized LCLs. Levels of secreted IL-6 were measured by ELISA from primary B cells and LCLs (>5 wk infection), 24 h poststimulation with different TLR ligands. C, Secretion of IL-6 in primary B cells and LCLs stimulated with increasing concentrations of TLR9 ligand CpG 2006. GpC was used as a control for CpG stimulation. D, TLR9-induced secretion of TNF-α in B cells and LCLs. Secretion of TNF-α was measured by ELISA from primary B cells and LCLs, 24 h poststimulation with CpG. E, Production of IgG from primary B cells and LCLs was measured by ELISA, 120 h poststimulation with CpG. Data for IgG levels were normalized by dividing values of stimulated cells by nonstimulated cells. Results shown represent the SD of the mean of four experiments performed in B cells purified by CD19+ and three experiments performed with negative selection of B cells.
Taken together, our data demonstrated that EBV inhibits TLR2, TLR5, and TLR9 signaling pathway in B cells.

**EBV downregulates TLR9 expression**

To elucidate the mechanisms underlying the EBV-induced TLR9 inhibition, we measured the TLR9 expression levels in LCLs compared with primary B cells by RT-qPCR. As shown in Fig. 2A, a significant reduction of TLR9 mRNA was observed in LCLs in comparison with primary B cells. This inhibition of TLR9 mRNA expression correlated with a lower level of TLR9 protein in LCLs (Fig. 2B). In these cells, infection with EBV was monitored by GFP expression (Supplementary Fig. 1).

TLR9 downregulation observed in LCLs might be a consequence because of the immortalization of B cells induced by EBV or, alternatively, associated with a specific EBV function. To evaluate whether EBV has a direct role in TLR9 downregulation, we infected the immortalized EBV-negative RPMI 8226 B cell line, which expressed high levels of TLR9 (30, 31), with EBV. We observed that EBV infection led to downregulation of TLR9 mRNA and protein levels in RPMI 8226 cells (Figs. 2C, 2D), providing evidence for a direct involvement of EBV in the event. In addition, the EBV-induced TLR9 protein downregulation was observed at early stages of infection in B cells, before immortalization (Fig. 2E). Together, these data show that EBV plays a direct role in downregulating TLR9 expression.

**LMP1 negatively regulates TLR9 expression in B cells**

We have previously reported that the main oncoproteins of HPV16, namely, E6 and E7, suppress TLR9 levels in primary keratinocytes and B cells (30). Therefore, we hypothesized that EBV may use its main oncoprotein LMP1 to suppress the TLR9 transcript in B cells. RT-qPCR analysis showed that LMP1 expression correlates with a decrease in expression of TLR9 mRNA (Fig. 3A). Indeed, the appearance of a few copies of LMP1 at 24 h is associated with a significant decrease of TLR9 mRNA. A further decrease of TLR9 mRNA occurred when a high expression of LMP1 is observed at 48 h and 5 d postinfection (Figs. 2E, 3A). LMP1 protein expression was confirmed by Western blotting (data not shown). To determine the direct role of LMP1 in TLR9 downregulation, we transduced the RPMI 8226 B cell line with recombinant retrovirus expressing LMP1 and, as a control, with an empty retrovirus (pLNSX). LMP1 expression resulted in a decrease of TLR9 mRNA (Fig. 3B). In addition, infection with a recombinant EBV lacking LMP1 (EBV ΔLMP1) in primary B cells had no effect on TLR9 protein expression (Fig. 3C). Thus, LMP1 plays a central role in EBV-mediated downregulation of TLR9.

**LMP1 inhibits TLR9 promoter activity**

LMP1 may decrease TLR9 by affecting the stability of TLR9 transcript or directly inhibiting TLR9 transcription. To discriminate between these two possibilities, we determined whether LMP1...
FIGURE 3. LMP1 downregulates TLR9 expression. A, Expression of TLR9 and LMP1 during viral infection. Expression of TLR9 (top panel) and LMP1 (bottom panel) mRNAs was monitored by qPCR at different time points postinfection with EBV in primary B cells. The levels of TLR9 and LMP1 expression were normalized to housekeeping genes mentioned in Materials and Methods. B, Expression of TLR9 mRNA in RPMI 8226 cells transduced with LMP1. Expression of TLR9 mRNA in RPMI 8226 transduced with retrovirus expressing pLNSX-LMP1 (LMP1) or the vector alone was analyzed by qPCR, and the level of expression was normalized to the β2-microglobulin. C, TLR9 protein levels in not infected human primary B cells, infected with EBV defective for LMP1 (ΔEBV), or WT EBV (EBV). Protein expression was determined by immunoblotting for TLR9, LMP1 (asterisk indicates background band in the WB, which comigrates with LMP1), GFP (marker of viral infection), and β-actin. Results shown here represent the SD of the mean of four experiments performed with negative selection of B cells.

protein influences the activity of the TLR9 promoter. The TLR9 promoter cloned in front of luciferase reporter gene was introduced in RPMI 8226 B cells together with increasing amounts of LMP1 expression plasmid. High basal luciferase activity was detected in these cells in absence of LMP1. (However, the expression of the oncprotein LMP1 correlated with a strong inhibition of TLR9 promoter activity in a dose-dependent manner [Fig. 4A], indicating that LMP1 can alter the transcription of the TLR9 gene.) Similarly, transient transfection of the TLR9 reporter vector in LMP1 transduced RPMI 8226 cells led to a significant decrease of the promoter activity compared with the control cells (Fig. 4B). Similar results were obtained in a different cell system using HEK293 T cells (Fig. 4C). These data correlated with LMP1 ability to increase the NF-κB luciferase activity in the same dose-dependent manner (Fig. 4C, bottom panel). The relative low level of NF-κB activation observed with high level of LMP1 (0.5 and 1.0 μg) could result from a saturation effect because of a large amount LMP1 protein in the cells on NF-κB signaling. Indeed, no further significant decrease of TLR9 promoter activity was observed between 0.2 or 0.5 and 1.0 μg (p = 0.12, NS, Student nonpaired t test). In summary, these data show that the oncprotein LMP1 downregulates TLR9 promoter activity in B cells and nonhematopoietic cells.

LMP1-mediated NF-κB activation is required for the downregulation of TLR9 promoter activity

Many cis-acting elements that regulate TLR9 transcription have been characterized along the -3277 bp region of the TLR9 promoter (30, 31). In particular, Takeshita et al. (31) first identified NF-κB as being one of the suppressive transcription factors potentially associated with TLR9 downregulation. Therefore, we next determined whether the LMP1-induced TLR9 downregulation is mediated by its ability to activate NF-κB signaling. We used LMP1 mutants in CTAR1 and/or CTAR2 (Fig. 5A) that are respectively responsible for 30 and 70% of NF-κB activity induced by wild-type (WT) LMP1 (12). Mut 1 and Mut 2 LMP1 mutants retain part of the WT ability to activate NF-κB promoter, whereas Mut 3 was completely inactive (Fig. 5B). The impairment of the three LMP1 mutants to activate NF-κB promoter tightly correlated with their inability in TLR9 downregulation (Fig. 5C). NF-κB activation appears to be important in inhibiting TLR9 expression. To corroborate this observation, we determined whether inhibition of NF-κB signaling results in increase of TLR9 promoter activity in LCL. Indeed, overexpression of dominant-negative mutants of IKKα or IKKβ resulted in high level of TLR9 promoter activity (Fig. 5D, top panel) coinciding with a successful inhibition in the NF-κB pathway (Fig. 5D, bottom panel). Similar data were obtained when the NF-κB pathway was blocked by expressing the IκB superrepressor ΔN-IκBα, an IκBα deletion mutant that is not phosphorylated by the IKK complex, thereby promoting the sequestering of NF-κB p65 in the cytoplasm (Fig. 5E) (33). Thus, LMP1-induced TLR9 downregulation occurred via the activation of NF-κB signaling pathway.

We speculated that LMP1 exerts its inhibitory effect on the TLR9 promoter by activating NF-κB, which would, in turn, suppress TLR9 transcription. The promoter of TLR9 gene contains a NF-κB binding site at the position −413 to −403 within the −3327 bp sequence, and overexpression of the NF-κB p65 resulted in the inhibition of TLR9 promoter activity (31). We performed three point mutations on the NF-κB site within the full-length TLR9 promoter. RPMI B cells or LCLs were cotransfected with either the WT or the mutated TLR9 luciferase reporter plasmid together or not with an LMP1 expression plasmid (Fig. 5F, 5G, respectively). The effect of LMP1 on the mutated TLR9 promoter was drastically reduced. Furthermore, transfection of the mutated construct in LCLs from three donors revealed once again that NF-κB activity by LMP1 is required to suppress TLR9 promoter activity of the luciferase reporter gene. In all, these data strongly show the involvement of NF-κB as a negative regulator in the LMP1-mediated inhibition of TLR9.

Discussion

Understanding the importance of host innate immune responses in the context of EBV infection will allow us to determine how EBV
can persist to promote cellular transformation. Whereas viral escape during the adaptive immune response toward EBV infection has been well studied, the direct effect on the innate immune response has not been elucidated (15). Our study reveals that EBV is able to efficiently target TLR9-regulated pathways. Primary B cell infection with EBV resulted in lower levels of IL-6, TNF-α, and IgG secretion on TLR9 engagement with CpG. This impairment of TLR9 pathway functionality in EBV-infected cells correlated with a decrease of TLR9 mRNA and protein levels in comparison with noninfected primary B cells. Our observations in terms of TLR9 mRNA levels confirmed the findings of Martin and colleagues (34), who observed that both UV-inactivated and untreated EBV downregulated the expression of TLR9 in B cells. This impairment of TLR9 pathway functionality in EBV-infected cells correlated with increased LMP1-mediated NF-κB activation. HEK293 T cells were cotransfected with increasing concentrations of LMP1 and the TLR9-luciferase reporter plasmid (top panel). To control for LMP1 ability to activate the NF-κB pathway, HEK293 T cells were transiently transfected with NF-κB luciferase reporter plasmid with increasing concentrations of LMP1 (bottom panel). The luciferase activity was measured 48 h posttransfection. LMP1 expression was evaluated by immunoblotting in both cases but represented as a control for TLR9 promoter versus LMP1 (middle panel). Results shown here represent the SD of the mean of six experiments performed.

Several studies highlight the importance of the role played by TLR9 in anti-EBV immunity (25, 26). We demonstrated that the inhibition of TLR9 expression by EBV is mainly mediated by its major oncoprotein LMP1, as EBV mutant lacking LMP1 is impaired in its ability to downregulate TLR9. In the 95% of the human population asymptomatic for EBV infection, the virus has evolved to evade the immune system to persist “normally” within the host, and LMP1 expression is tightly controlled and regulated. LMP1 can contribute to the immune suppression at earlier stage of infection by blocking IFN-α secretion and increasing the transcription of the anti-inflammatory cytokine IL-10 (16). Yet, deciphering the precise role of LMP1 in EBV-associated carcinogenesis has proved a wide task because it can activate many pathways. What can be noted is that signaling pathways associated to B cell proliferation and LMP1-induced NF-κB signaling has a significant role in EBV latency and persistence. Our data show that LMP1 is able to inhibit TLR9 transcription in primary and immortalized B cells by activating NF-κB pathways. In fact, inhibition of the NF-κB in EBV-infected cells by different means at earlier time points after EBV infection with either UV-inactivated or live virus.
resulted in an increase of TLR9 expression. In addition, LMP1 deletion mutants lacking the well-characterized domains CTAR1 and CTAR2 showed a reduced efficiency in downregulating TLR9 pathways (12) showed a reduced efficiency in downregulating TLR9. Together, these data showed the implication of NF-κB in the mechanism used by LMP1 to downregulate TLR9 expression. However, the precise contribution of NF-κB pathways in this event remains to be elucidated. The characterization of the TLR9 promoter revealed the presence of several transcription factors sites including four NF-κB sites (30). It has been previously reported that the NF-κB cis element at position -413 bp plays a negative role in regulating TLR9 levels (31). Our data suggest that LMP1 may use the same NF-κB cis element to inhibit TLR9 transcription. In fact, a deletion mutant of TLR9 promoter containing only the first 700 bp upstream of the ATG and including the NF-κB cis element still resulted in TLR9 repression by EBV (unpublished data).

Data from our group have showed that the major oncoproteins, E6 and E7, from carcinogenic HPV type 16 inhibit the expression of TLR9. Thus, the ability to downregulate TLR9 expression appeared to be a shared property between DNA tumor viruses. Interestingly, the low-risk HPV types that are normally associated with benign cervical lesions are not able to alter TLR9 expression, suggesting that TLR9 downregulation is an event exclusively associated with carcinogenic viruses (30). Whether EBV promotes TLR9 downregulation, as a means to exist within the host or promote carcinogenesis, needs to be addressed. We observed that
initial infection up to 120 h with EBV did decrease TLR9 mRNA levels compared with primary B cells (Fig. 2E). However, in LCLs (minimum 5 wk in culture), we noted a further decrease in TLR9 expression (data not shown) in which decreasing the dose of the TLR9 ligand conferred a wider decline in IL-6 levels between primary B cells and LCLs (Fig. 1C). This subtle difference observed might indicate the difference between the virus escaping TLR9 recognition to coexist at the initial stages of EBV infection versus type III latency (LCL) in which TLR9 expression is further reduced. Iskra et al.'s recent study (35) highlights the role of TLR9 agonist CpG2006 in synergistically increasing proliferation and activation of B cells by EBV at an early time point postinfection. Thus, EBV uses TLR9 to infect and induce proliferation; yet, our study demonstrates that later it downregulates TLR9 expression to escape the immune response.

In addition to TLR9, we have observed that EBV infection of primary B cells led to inhibition of TLR2 transcription and functionality (Fig. 1B, Supplementary Fig. 2). Interestingly, it has recently been shown that the nonstructural EBV protein, dUTPase, is recognized by TLR2, leading to NF-κB activation (36). It is not surprising that we have observed that the virus efficiently downregulates both TLR2 and TLR9 mRNA, as both receptors are activated by two distinct EBV components, similarly to HSV-2 (37). Furthermore, EBV-encoded small nonpolyadenylated RNA-ase are recognized by RIG-I and TLR3, enhancing the induction of type I IFN (38). We observed that TLR3 responses for IL-6 secretion were low in primary B cells and abolished in LCLs. Discrepancies between our findings and those reported in terms of TLR3 activity could deviate from the cellular models used, and the time points postinfection chosen for expression and functionality studies (39, 40). Most strikingly, TLR5 functionality was halted in our EBV-infected cells; although there are no reports correlating TLR5 to EBV, this observation merits further investigation.

In summary, our data showed that LMP1, in addition to its key role in cellular transformation, is able to efficiently target innate sensors, although the possibility that additional viral protein may synergize with LMP1 remains to be investigated. This study reveals a mechanism used by EBV to escape innate immune TLR9 recognition, and it highlights the importance of immune deregulation mediated by tumor-inducing viruses.

Acknowledgments


S1. **Confocal monitoring of EBV infection.** A, For confocal staining, LCL were directly fixed on coverslides and mounted using a mounting reagent containing DAPI. B, LMP1 staining was performed by using anti LMP1, and a secondary PE-conjugated anti-mouse was used. As negative control, LCL were incubated with secondary antibody only (data not shown).

S2. **TLR gene expression in primary B cells and LCL.** Primary B-cells and EBV-infected LCLs were stimulated with ligands for TLR family members (TLR1 to TLR10, with exception of TLR4 and 8) and TLR mRNA expression was determined by RT-PCR (left panel) with the quantification normalized to GAPDH (right panel).