The Journal of Immunology

EBV Latent Membrane Protein 1 Is a Negative Regulator of TLR9

Ikbal Fathallah, Peggy Parroche, Henri Gruffat, Claudia Zannetti, Hanna Johansson, Jiping Yue, Evelyn Manet, Massimo Tommasino, Bakary S. Sylla and Uzma A. Hasan

J Immunol 2010; 185:6439-6447; Prepublished online 27 October 2010;
doi: 10.4049/jimmunol.0903459
http://www.jimmunol.org/content/185/11/6439

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

EBV Latent Membrane Protein 1 Is a Negative Regulator of TLR9

Ikbal Fathallah,*,[1] Peggy Parroche,*,†,‡,[1] Henri Gruffat,‡,§ Claudia Zannetti,*,†,‡ Hanna Johansson,* Jiping Yue,* Evelyn Manet,‡,§ Massimo Tommasino,* Bakary S. Sylla,* and Uzma A. Hasan*,†,‡

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: 6439–6447.**

Epstein-Barr virus belongs to the human γ-herpesvirus type 4 family (HHV4) and infects >95% of the adult population with a specific tropism for B and epithelial cells (1). When primary infections occur at young ages, EBV may cause a benign lymphoproliferative disease known as infectious mononucleosis. Chronic EBV infections are associated with a set of severe human diseases including Burkitt's lymphoma, Hodgkin's disease, posttransplant lymphoma, nasopharyngeal, and some forms of gastric carcinomas (2). EBV also induces lymphoma development in genetically predisposed immune deficiency families affected by the X-linked lymphoproliferative syndrome (3–5). In vitro, EBV can efficiently infect human resting B lymphocytes and transform them into indefinitely proliferating lymphoblastoid cell lines (LCLs). In these cells, several latent viral genes are expressed including the latent membrane protein 1 (LMP1) (2). LMP1 is essential for EBV-induced B cell immortalization and long-term proliferation, and it is the only latent protein that displays in vitro transforming properties (6). LMP1 is a plasma membrane aggregating protein that mimics the activated form of TNFR, CD40, or CD30 and exerts its transforming functions via a long C-terminal cytosolic domain (7–9). Accordingly, LMP1 can activate several signal transduction pathways, such as MAPK, PI3K and JAK3/STAT, and NF-κB (8, 10–13). EBV LMP1 C-terminal activation region 1 (CTAR1) and CTAR2 both activate NF-κB (14). NF-κB is a transcription factor with the prototype being p65/Rel A, which is kept inactive in the cytoplasm of quiescent cells by its inhibitor IκB. NF-κB–inducing agents, including proinflammatory cytokines, lead to activation of IκB kinase complex (IKKα/β/γ), which, in turn, phosphorylates and promotes degradation of IκB. This event results in NF-κB nuclear translocation and expression of genes encoding key players of cell survival, cellular proliferation, and immune response. NF-κB is constitutively activated in many types of viral and nonviral-associated human cancers.

The normal life cycle of EBV infection and the function of the LMP1 in relation to the adaptive immune response are subtle and foremost, very complex. The virus must be able to persist for life in the host without doing much harm to ensure its survival (6, 15, 16). For instance, EBV targets preferentially circulating memory B cells expressing low levels of MHC class I and costimulatory molecules to avoid presentation to the immune cells (17). In addition, EBV also has the ability to downregulate the expression of MHC molecules in proliferating B cells (15). During latent infection, a small number of EBV Ags are expressed at a low copy number, which leads to low T cell $CD8^+$ epitope presentation (15, 18). Thus, escaping adaptive immune responses play a key role in

*International Agency for Research on Cancer, 69008 Lyon; †Innate Immunity and Cancer Team, INSERM U851, Faculté de Médecine Lyon Sud, 69495 Pierre Benite; ‡Institut Fédératif de Recherche 128 BioSciences Gerland-Lyon Sud, 69366 Lyon Cedex 7; and §Laboratoire de Biologie Moléculaire des γ-Herpesvirus INSERM U758, F-69007 Lyon, France

[1]I.F. and P.P. contributed equally to this work.

Received for publication October 23, 2009. Accepted for publication September 16, 2010.

This work was supported by grants from La Ligue contre le Cancer (Comité de Savoie to I.F. and P.P.; Comité du Rhône et de la Drome to B.S.S.); Association pour la Recherche contre le Cancer (to P.P. and U.H.; Grants 4914 and 3420); Government Français and DEMINAP Région Rhône-Alpes (to M.T. and U.A.H.); International Agency for Research on Cancer postdoctoral fellowship program (to J.Y.); Agence Nationale pour la Recherche (Programme MIE 2009); and Réseau INSERM Herpesvirus et Cancer.

Address correspondence and reprint requests to Dr. Uzma A. Hasan, Faculty of Medecine, Lyon Sud, 165 Chemin du Grand Revoyet, 69495 Pierre Benite, France. E-mail address: uzma.hasan@inserm.fr

The online version of this article contains supplemental material.

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

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 (σ). 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 FAC LSRII) 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 the LMP1 gene ($EBV_{\Delta LMP1}$) 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). Pam2CysKKKK was purchased from EMC (Tubingen, Germany) microcollection and used at 4 nM. Poly(I:C) 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 [Courtabouef Cedex, France] or Macherey Nagel [Hoerdt, 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′-CGT CTT GAA GGC CTG GTG TTG A-3′; reverse: 5′-CTG GAA GGC CTT GGT TTT AGT GA-3′. Housekeeping genes β_2-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 CAG CAC 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 β_2-microglobulin or β-actin and are presented as a ratio of gene copy number per 100 copies of β_2-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 Na_3VO_3). 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 2×10^5/200 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).

Confocal 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 ($^{-413}$GGGGAGTGCCC^{-403}) was mutated to ($^{-413}$CTCGAGTGCCC^{-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: LMP1AxAxA (Mut 1), LMP1 378 STOP (Mut 2), and LMP1AxAxA/378STOP (Mut 3) were a kind gift from L. Young (University of Birmingham) and described by Eliopoulos and colleagues (32). NF-κB superrepressor ΔN-IκBα, which lacks the sequence that codes for the first N-terminal 36 aas, was obtained from E. Kieff and cloned into pcDNA3. The dominant negative constructs IKKα–DN (pcRK5-Flag-IKKα-KA) and IKKβ–DN (pcRK5-Flag-IKKβ-KA) were obtained from D. Goeddel (Tulirak, 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 HRP (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. 1*A*) were first infected with recombinant EBV virus GFP gene (27). The efficiency of EBV infection was evaluated by monitoring GFP expression using confocal microscopy (Supplemental 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. 1*B*). 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. 1*B*).

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. 1*C*).

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. 1*D*, 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. 1*E*).

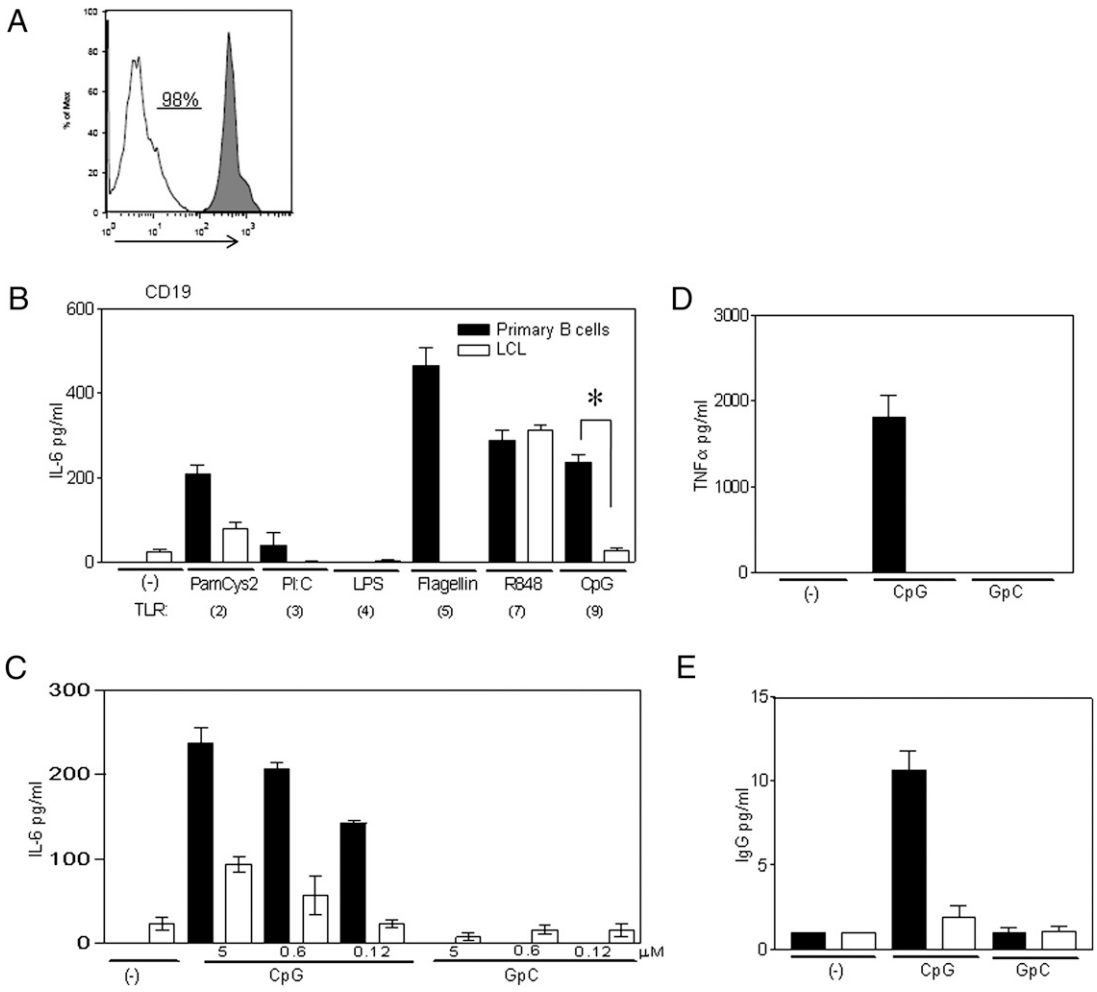

FIGURE 1. EBV affects TLR-mediated cytokine production in B cells. *A*, Purified primary B cells from PBMCs were extracted from several donors. B cell purity was between 96 and 98% as measured by flow cytometry. *B*, TLR-induced secretion of IL-6 in primary B cells and in EBV-immortalized LCLs. Levels 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 LCL compared with primary B cells by RT-qPCR. As shown in Fig. 2*A*, 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. 2*B*). In these cells, infection with EBV was monitored by GFP expression (Supplemental 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. 2*C*, 2*D*), 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. 2*E*). 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. 3*A*). 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. 2*E*, 3*A*). 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. 3*B*). In addition, infection with a recombinant EBV lacking LMP1 ($EBV_{\Delta LMP1}$) in primary B cells had no effect on TLR9 protein expression (Fig. 3*C*). 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

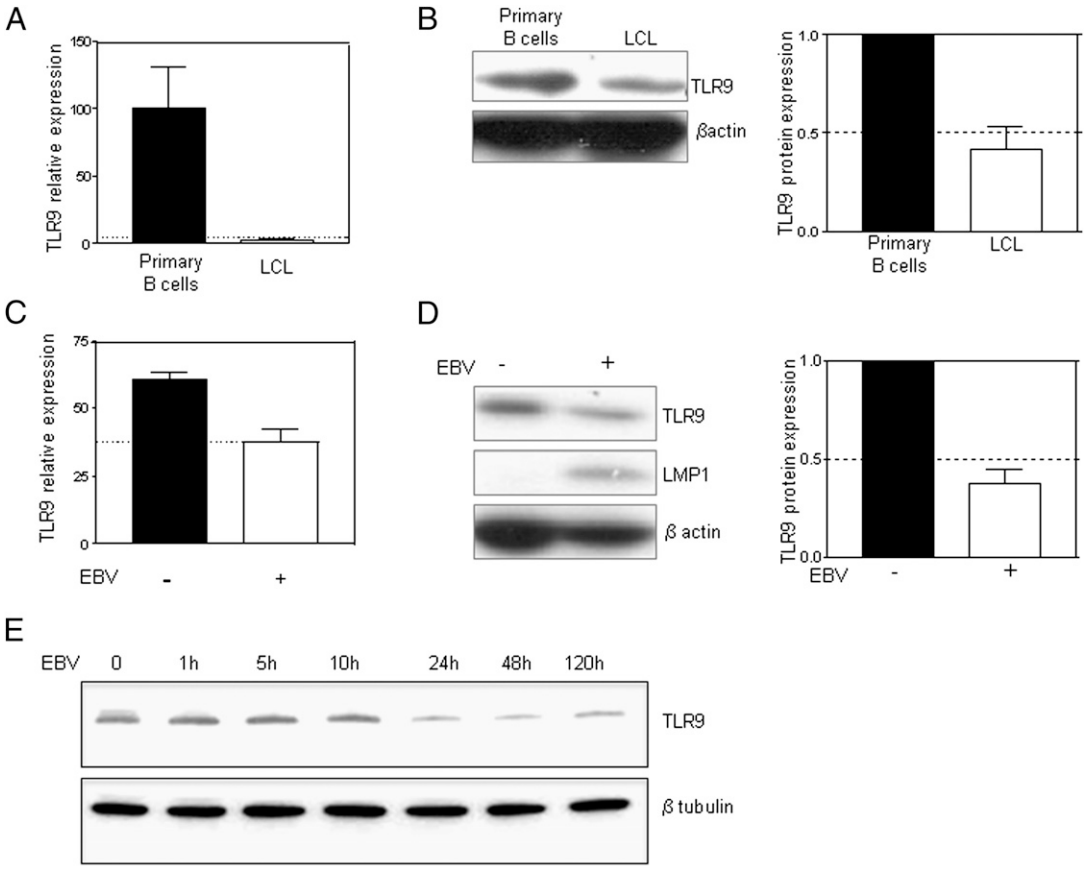

FIGURE 2. EBV downregulates TLR9 mRNA and protein expression in B cells. *A*, Total RNA was extracted from primary B cells and LCL. After reverse transcription, qPCR was performed using TLR9-specific primers, and the level of TLR9 mRNA was normalized to β_2-microglobulin levels. *B*, The effect of EBV infection on TLR9 protein expression in primary B cells was monitored by immunoblotting (*left*) and normalized to β-actin levels (*right*). *C*, mRNA expression of TLR9 in an immortalized B cell line (RPMI 8226) not infected or infected with EBV. The level of TLR9 mRNA was determined by qPCR in RPMI 8226 cells infected with EBV >2 mo postinfection compared with noninfected cells. *D*, Protein expression of TLR9 in RPMI 8226 cells infected with EBV. Immunoblotting (*left panel*) and quantification of TLR9 protein expression relative to β-actin (*right panel*) are presented. *E*, Early kinetics of EBV infection to measure the effects on TLR9 protein expression. TLR9 protein in EBV-infected primary B cells was monitored by immunoblotting at different time points postinfection.

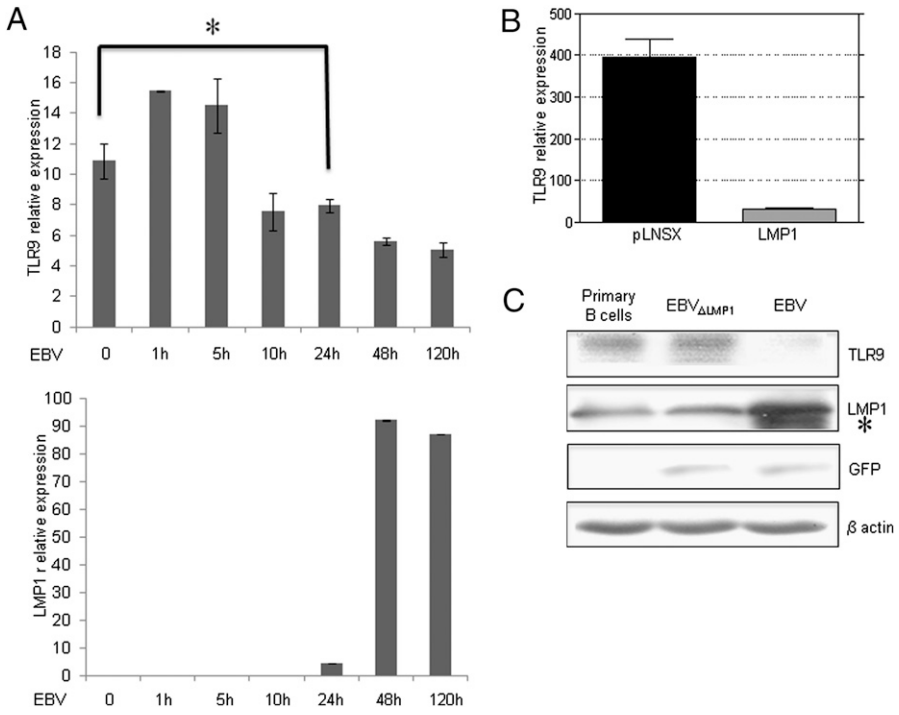

FIGURE 3. LMP1 downregulates TLR9 expression. *A*, Expression of TLR9 and LMP1 during viral infection. Expression of TLR9 (*top panel*) and LMP1 (*bottom panel*) mRNAs was monitored by qPCR at different time points postinfection with EBV in primary B cells. The levels of TLR9 and LMP1 expression were 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 β_2-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 in B cells purified by CD19$^+$ and three 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 oncoprotein LMP1 correlated with a strong inhibition of TLR9 promoter activity in a dose-dependent manner [Fig. 4*A*], 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. 4*B*). Similar results were obtained in a different cell system using HEK293 T cells (Fig. 4*C*). These data correlated with LMP1 ability to increase the NF-κB luciferase activity in the same dose-dependent manner (Fig. 4*C*, *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 oncoprotein 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. 5*A*) 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. 5*B*). The impairment of the three LMP1 mutants to activate NF-κB promoter tightly correlated with their inability in TLR9 downregulation (Fig. 5*C*). 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. 5*D*, *top panel*) coinciding with a successful inhibition in the NF-κB pathway (Fig. 5*D*, *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. 5*E*) (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. 5*F*, 5*G*, 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

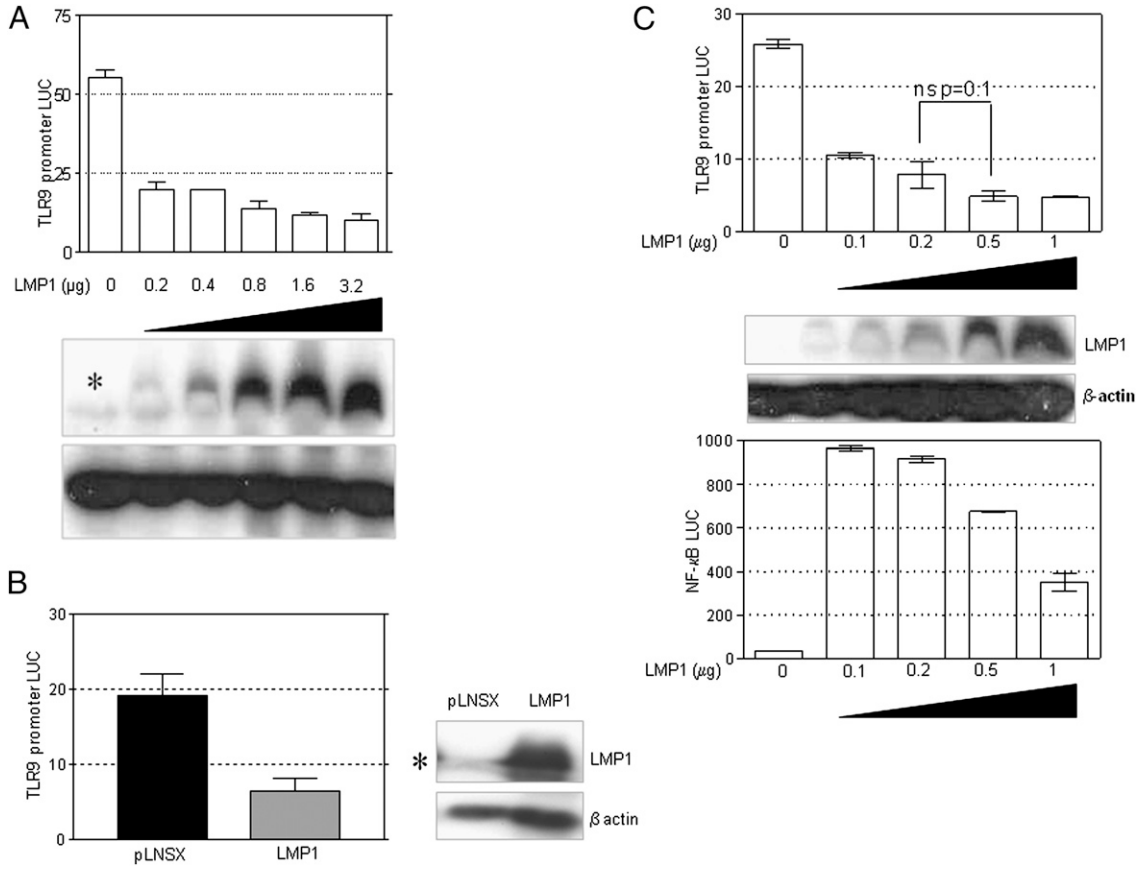

FIGURE 4. LMP1 negatively regulates TLR9 promoter activity. *A*, RPMI 8226 cells were transiently transfected with TLR9 promoter coupled to the luciferase gene with increasing concentrations of the LMP1 expression vector. After 48 h, cells were harvested, and luciferase activity was measured. The data are the mean of three independent experiments performed in triplicate (*top panel*). LMP1 protein expression was monitored by immunoblotting as shown (*bottom panel*). *B*, LMP1 downregulates TLR9 promoter in RPMI 8226 cells stably expressing LMP1. RPMI 8226 cells were transduced with LMP1 and transfected with TLR9 luciferase promoter. TLR9 promoter activity was monitored as described earlier (*left panel*), and the expression of LMP1 was controlled by immunoblotting (*right panel*). Asterisk indicates that the band seen in the pLNSX vector control is a nonspecific band that comigrates with LMP1, observed when using the S12 Ab. *C*, LMP1-induced downregulation of TLR9 promoter activity correlates 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.

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. They also showed an increase in TLR7 functionality in EBV-infected B cells. However, we did not detect any alteration in TLR7 function (Fig. 1*B*), although mRNA levels were reduced (Supplemental Fig. 2). The discrepancies between our findings and those of Martin and colleagues could be because of the time course of assessing TLR7 functionality and expression, in which Martin et al. looked at proliferation alteration by TLR7, not cytokine secretion. In addition, most of their studies were performed at earlier time points after EBV infection with either UV-inactivated or live virus.

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

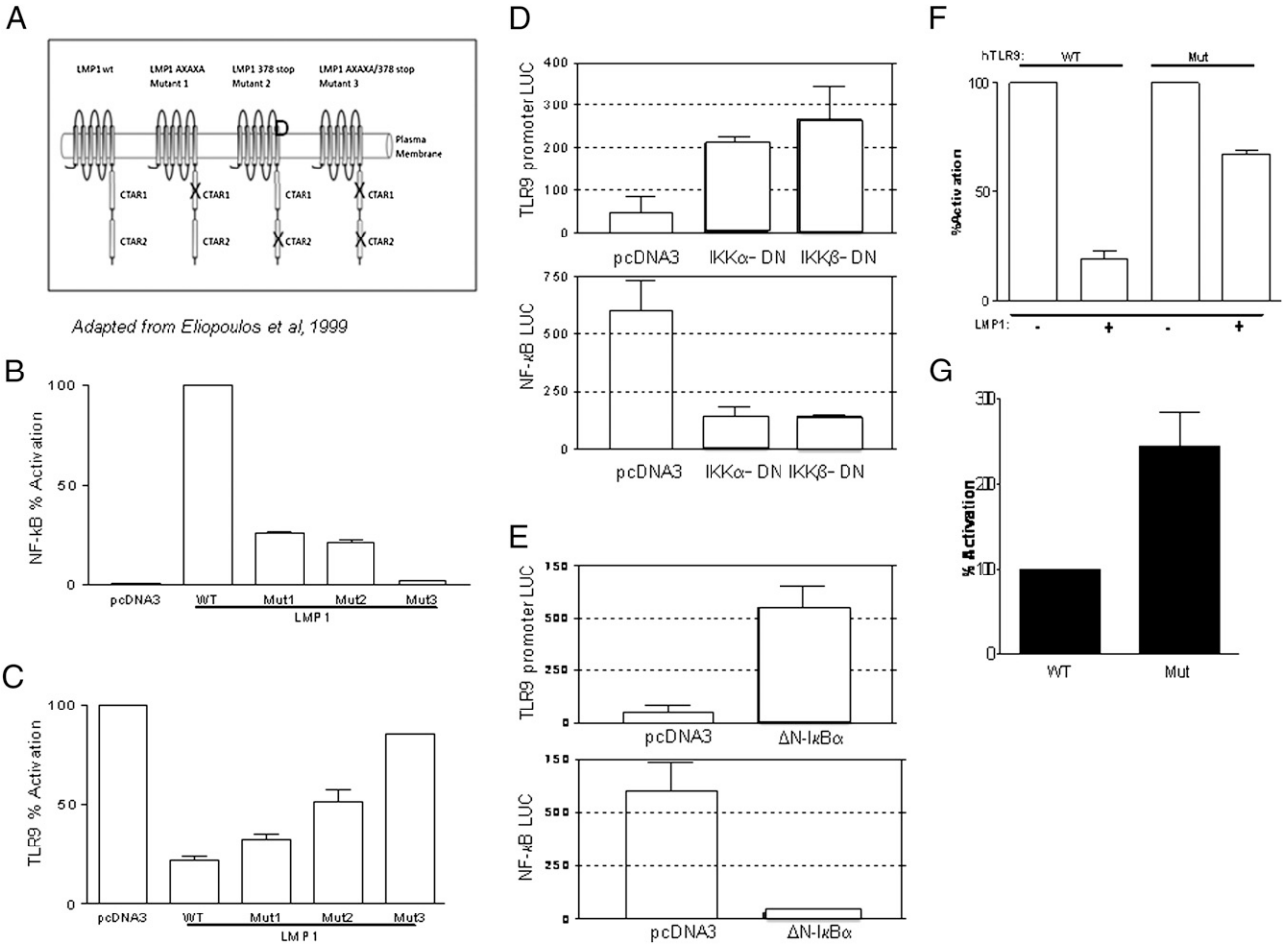

FIGURE 5. TLR9 downregulation is mediated by NF-κB signaling induced by LMP1. *A*, Schematic representation of LMP1 WT and mutations made in the NF-κB activator domains of CTAR1 and CTAR2. Mut 1 is within the CTAR1 domain (aas 196–231), has a triple PxQxT3AxAxA point mutation, which abrogates TNFR-associated factor binding. Mut 2 in the CTAR2 domain of LMP1 contains a stop codon at aa 378 that abolishes the interaction of TRADD and RIP. Mut 3 contains both. This research was originally published in The Journal of Biological Chemistry. Aristides G. Eliopoulos, Neil J. Gallagher, Sarah M. S. Blake, Christopher W. Dawson, and Lawrence S. Young. Activation of the p38 mitogen-activated protein kinase pathway by Epstein-Barr virus-encoded latent membrane protein 1 coregulates interleukin-6 and interleukin-8 production. J. Biol. Chem. 1999; 274: 16085–16096. © The American Society for Biochemistry and Molecular Biology. *B*, HEK293 T cells were transfected with expression plasmids for WT LMP1, and its mutants, together with NF-κB promoter luciferase reporter plasmid and the relative activation, are presented. *C*, LMP1 mutants less potent for NF-κB activity are partially defective in inhibiting TLR9 promoter activity. LMP1 (WT) and the three LMP1 mutants were transfected in RPMI8226 cells together with TLR9 promoter luciferase reporter plasmid, and luciferase activity was monitored. Data for *B* and *C* are reported as percentage of luciferase activity normalized by dividing relative luciferase units obtained for LMP1 WT for *C* and empty vector for *D*. LMP1 and mutants were monitored for expression by Western blotting using the S12 Ab (data not shown). *D*, Blocking NF-κB activation increases TLR9 promoter activity. Plasmids expressing dominant-negative forms of IKKα or IKKβ and vector control were cotransfected with either the TLR9 (*top panel*) or NF-κB promoter (*bottom panel*) in LCLs, and luciferase activity was measured 48 h post-transfection. *E*, NF-κB superrepressor ΔN-IκBα and vector control were cotransfected with either the TLR9 (*upper panel*) or NF-κB promoters (*lower panel*) in LCL, and luciferase activity was measured 48 h posttransfection. Results shown here represent the SD of one of three experiments performed. RPMI 8226 B cells (*F*) or LCLs (*G*) were cotransfected with the TLR9 WT or with mutated at the NF-κB promoters ± LMP1; luciferase activity was measured 48 h posttransfection.

resulted in an increase of TLR9 expression. In addition, LMP1 deletion mutants lacking the well-characterized domains CTAR1 and CTAR2 important for the activation of NF-κB 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 repressed 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. 2*E*). 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. 1*C*). 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. 1*B*, Supplemental 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 thus 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 RNAase 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

We thank Annick Rivoire for her administrative aid and E. Kieff, T. Gilmore, G. Mosialos, and L. Young for contributing reagents. We thank Dr. W. Hammerschmidt for the gift of the $HEK293_{EBV}$ and $HEK293_{EBV\Delta LMP1}$ cells. We also thank the team of Christine Delprat at Lyon Sud for providing PBMCs for B cell purification and Nadege Goutagny at the Centre Leon Berard, Lyon, France, for continual availability and advice.

Disclosures

The authors have no financial conflicts of interest.

References

1. Parkin, D. M., F. Bray, J. Ferlay, and P. Pisani. 2005. Global cancer statistics, 2002. *CA Cancer J. Clin.* 55: 74–108.
2. Kieff, E. D., and A. B. Rickinson. 2007. Epstein-Barr virus and its replication. In *Fields Virology*, 5th Ed. . D. M. Knipe, and P. M. Howley, eds. Philadelphia : Lippincott Williams &Wilkins, p. 2603–2655.
3. Coffey, A. J., R. A. Brooksbank, O. Brandau, T. Oohashi, G. R. Howell, J. M. Bye, A. P. Cahn, J. Durham, P. Heath, P. Wray, et al. 1998. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat. Genet.* 20: 129–135.
4. Lamartine, J., K. E. Nichols, L. Yin, M. Krainer, F. Heitzmann, A. Bernard, S. Gaudi, G. M. Lenoir, J. L. Sullivan, J. E. Ikeda, et al. 1996. Physical map and cosmid contig encompassing a new interstitial deletion of the X-linked lymphoproliferative syndrome region. *Eur. J. Hum. Genet.* 4: 342–351.
5. Purtilo, D. T., R. S. Strobach, M. Okano, and J. R. Davis. 1992. Epstein-Barr virus-associated lymphoproliferative disorders. *Lab. Invest.* 67: 5–23.
6. Kaye, K. M., K. M. Izumi, and E. Kieff. 1993. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc. Natl. Acad. Sci. USA* 90: 9150–9154.
7. Cahir McFarland, E. D., K. M. Izumi, and G. Mosialos. 1999. Epstein-barr virus transformation: involvement of latent membrane protein 1-mediated activation of NF-kappaB. *Oncogene* 18: 6959–6964.
8. Eliopoulos, A. G., and L. S. Young. 2001. LMP1 structure and signal transduction. *Semin. Cancer Biol.* 11: 435–444.
9. Mosialos, G., M. Birkenbach, R. Yalamanchili, T. VanArsdale, C. Ware, and E. Kieff. 1995. The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* 80: 389–399.
10. Dawson, C. W., G. Tramountanis, A. G. Eliopoulos, and L. S. Young. 2003. Epstein-Barr virus latent membrane protein 1 (LMP1) activates the phosphatidylinositol 3-kinase/Akt pathway to promote cell survival and induce actin filament remodeling. *J. Biol. Chem.* 278: 3694–3704.
11. Gires, O., F. Kohlhuber, E. Kilger, M. Baumann, A. Kieser, C. Kaiser, R. Zeidler, B. Scheffer, M. Ueffing, and W. Hammerschmidt. 1999. Latent membrane protein 1 of Epstein-Barr virus interacts with JAK3 and activates STAT proteins. *EMBO J.* 18: 3064–3073.
12. Huen, D. S., S. A. Henderson, D. Croom-Carter, and M. Rowe. 1995. The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-kappa B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. *Oncogene* 10: 549–560.
13. Izumi, K. M., and E. D. Kieff. 1997. The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to mediate B lymphocyte growth transformation and activate NF-kappaB. *Proc. Natl. Acad. Sci. USA* 94: 12592–12597.
14. Sylla, B. S., S. C. Hung, D. M. Davidson, E. Hatzivassiliou, N. L. Malinin, D. Wallach, T. D. Gilmore, E. Kieff, and G. Mosialos. 1998. Epstein-Barr virus-transforming protein latent infection membrane protein 1 activates transcription factor NF-kappaB through a pathway that includes the NF-kappaB-inducing kinase and the IkappaB kinases IKKalpha and IKKbeta. *Proc. Natl. Acad. Sci. USA* 95: 10106–10111.
15. Levitsky, V., and M. G. Masucci. 2002. Manipulation of immune responses by Epstein-Barr virus. *Virus Res.* 88: 71–86.
16. Middeldorp, J. M., and D. M. Pegtel. 2008. Multiple roles of LMP1 in Epstein-Barr virus induced immune escape. *Semin. Cancer Biol.* 18: 388–396.
17. Miyashita, E. M., B. Yang, G. J. Babcock, and D. A. Thorley-Lawson. 1997. Identification of the site of Epstein-Barr virus persistence in vivo as a resting B cell. *J. Virol.* 71: 4882–4891.
18. Babcock, G. J., L. L. Decker, M. Volk, and D. A. Thorley-Lawson. 1998. EBV persistence in memory B cells in vivo. *Immunity* 9: 395–404.
19. Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *Int. Immunol.* 17: 1–14.
20. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdörfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* 168: 4531–4537.
21. Bowie, A. G., and I. R. Haga. 2005. The role of Toll-like receptors in the host response to viruses. *Mol. Immunol.* 42: 859–867.
22. Finberg, R. W., J. P. Wang, and E. A. Kurt-Jones. 2007. Toll like receptors and viruses. *Rev. Med. Virol.* 17: 35–43.
23. Pasare, C., and R. Medzhitov. 2005. Control of B-cell responses by Toll-like receptors. *Nature* 438: 364–368.
24. Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki. 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J. Exp. Med.* 198: 513–520.
25. Lim, W. H., S. Kireta, G. R. Russ, and P. T. Coates. 2007. Human plasmacytoid dendritic cells regulate immune responses to Epstein-Barr virus (EBV) infection and delay EBV-related mortality in humanized NOD-SCID mice. *Blood* 109: 1043–1050.
26. Guggemoos, S., D. Hangel, S. Hamm, A. Heit, S. Bauer, and H. Adler. 2008. TLR9 contributes to antiviral immunity during gammaherpesvirus infection. *J. Immunol.* 180: 438–443.
27. Delecluse, H. J., D. Pich, T. Hilsendegen, C. Baum, and W. Hammerschmidt. 1999. A first-generation packaging cell line for Epstein-Barr virus-derived vectors. *Proc. Natl. Acad. Sci. USA* 96: 5188–5193.
28. Dirmeier, U., B. Neuhierl, E. Kilger, G. Reisbach, M. L. Sandberg, and W. Hammerschmidt. 2003. Latent membrane protein 1 is critical for efficient growth transformation of human B cells by Epstein-Barr virus. *Cancer Res.* 63: 2982–2989.
29. Caldeira, S., I. Zehbe, R. Accardi, I. Malanchi, W. Dong, M. Giarrè, E. M. de Villiers, R. Filotico, P. Boukamp, and M. Tommasino. 2003. The E6 and E7 proteins of the cutaneous human papillomavirus type 38 display transforming properties. *J. Virol.* 77: 2195–2206.
30. Hasan, U. A., E. Bates, F. Takeshita, A. Biliato, R. Accardi, V. Bouvard, M. Mansour, I. Vincent, L. Gissmann, T. Iftner, et al. 2007. TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. *J. Immunol.* 178: 3186–3197.
31. Takeshita, F., K. Suzuki, S. Sasaki, N. Ishii, D. M. Klinman, and K. J. Ishii. 2004. Transcriptional regulation of the human TLR9 gene. *J. Immunol.* 173: 2552–2561.

32. Eliopoulos, A. G., S. M. Blake, J. E. Floettmann, M. Rowe, and L. S. Young. 1999. Epstein-Barr virus-encoded latent membrane protein 1 activates the JNK pathway through its extreme C terminus via a mechanism involving TRADD and TRAF2. *J. Virol.* 73: 1023–1035.
33. Natoli, G., A. Costanzo, F. Guido, F. Moretti, A. Bernardo, V. L. Burgio, C. Agresti, and M. Levrero. 1998. Nuclear factor kB-independent cytoprotective pathways originating at tumor necrosis factor receptor-associated factor 2. *J. Biol. Chem.* 273: 31262–31272.
34. Martin, H. J., J. M. Lee, D. Walls, and S. D. Hayward. 2007. Manipulation of the toll-like receptor 7 signaling pathway by Epstein-Barr virus. *J. Virol.* 81: 9748–9758.
35. Iskra, S., M. Kalla, H. J. Delecluse, W. Hammerschmidt, and A. Moosmann. 2010. Toll-like receptor agonists synergistically increase proliferation and activation of B cells by epstein-barr virus. *J. Virol.* 84: 3612–3623.
36. Ariza, M. E., R. Glaser, P. T. Kaumaya, C. Jones, and M. V. Williams. 2009. The EBV-encoded dUTPase activates NF-kappa B through the TLR2 and MyD88-dependent signaling pathway. *J. Immunol.* 182: 851–859.
37. Sato, A., M. M. Linehan, and A. Iwasaki. 2006. Dual recognition of herpes simplex viruses by TLR2 and TLR9 in dendritic cells. *Proc. Natl. Acad. Sci. USA* 103: 17343–17348.
38. Samanta, M., D. Iwakiri, T. Kanda, T. Imaizumi, and K. Takada. 2006. EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN. *EMBO J.* 25: 4207–4214.
39. Iwakiri, D., L. Zhou, M. Samanta, M. Matsumoto, T. Ebihara, T. Seya, S. Imai, M. Fujieda, K. Kawa, and K. Takada. 2009. Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3. *J. Exp. Med.* 206: 2091–2099.
40. Samanta, M., D. Iwakiri, and K. Takada. 2008. Epstein-Barr virus-encoded small RNA induces IL-10 through RIG-I-mediated IRF-3 signaling. *Oncogene* 27: 4150–4160.