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The T Antigen Locus of Merkel Cell Polyomavirus Downregulates Human Toll-Like Receptor 9 Expression

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Establishment of a chronic infection is a key event in virus-mediated carcinogenesis. Several cancer-associated, double-stranded DNA (dsDNA) viruses act via their oncoproteins to downregulate Toll-like receptor 9 (TLR9), a key receptor in the host innate immune response that senses viral or bacterial dsDNA. A novel oncogenic virus, Merkel cell polyomavirus (MCPyV), has been recently identified that causes up to 80% of Merkel cell carcinomas (MCCs). However, it is not yet known whether this oncogenic virus also disrupts immune-related pathways. We find that MCPyV large T antigen (LT) expression downregulates TLR9 expression in epithelial and MCC-derived cells. Accordingly, silencing of LT expression results in upregulation of mRNA TLR9 levels. In addition, small T antigen (sT) also appears to inhibit TLR9 expression, since inhibition of its expression also resulted in an increase of TLR9 mRNA levels. LT inhibits TLR9 expression by decreasing the mRNA levels of the C/EBPβ transactivator, a positive regulator of the TLR9 promoter. Chromatin immunoprecipitation reveals that C/EBPβ binding at a C/EBPβ response element (RE) in the TLR9 promoter is strongly inhibited by expression of MCPyV early genes and that mutation of the C/EBP RE prevents MCPyV downregulation of TLR9. A survey of BK polyomavirus (BKPyV), JC polyomavirus (JCPyV), KI polyomavirus (KIPyV), MCPyV, simian virus 40 (SV40), and WU polyomavirus (WUPyV) early genes revealed that only BKPyV and MCPyV are potent inhibitors of TLR9 gene expression. MCPyV LT targeting of C/EBP transactivators is likely to play an important role in viral persistence and potentially inhibit host cell immune responses during MCPyV tumorigenesis.

Merkel cell polyomavirus (MCPyV) is a small, nonenveloped, double-stranded DNA (dsDNA) polyomavirus identified by digital transcriptome subtraction (1) from Merkel cell carcinoma (MCC), a rare and aggressive form of human skin cancer (2). MCPyV has a 5.4-kb genome which includes the early and late genes expressed during the viral life cycle (3). Three early viral transcripts, large T antigen (LT), small T antigen (sT), and 57KT, are generated by alternative splicing of early region (T antigen locus), whereas the viral structural capsid proteins are encoded by the late region (4, 5). MCPyV LT is a multifunctional protein that plays a key role in carcinogenesis as well as the viral life cycle (6, 7). LT has an LXCXE domain present in many viral oncoproteins that mediates their interaction with the tumor suppressor retinoblastoma. The C terminus of LT has origin-binding and helicase domains which are essential for viral replication (3, 6). In approximately 80% of MCCs, MCPyV DNA is integrated in the host genome and has mutations at the 3′ end of the T antigen locus, resulting in the translation of truncated forms of LT lacking helicase function (3, 8). These signature truncation mutations result in the loss of LT viral DNA replication as well as growth-inhibitory functions, which appear to be important for MCPyV-mediated carcinogenesis (3, 9, 10). In contrast, although early gene locus mutations affect LT in MCC tumors, sT is encoded as an intact protein. sT indirectly promotes viral DNA replication (6) and, unlike MCPyV LT or simian virus 40 (SV40) sT, directly transforms rodent fibroblasts (11). Silencing the expression of sT or LT in MCPyV-positive MCC cell lines shows that both viral proteins are required for tumor cell survival and proliferation (7, 11).

In addition to their ability to promote cellular transformation, oncogenic viruses have developed mechanisms to target cellular pathways related to innate and adaptive immune surveillance (12–17). It is believed that these properties facilitate the establishment of a persistent/chronic infection, a key step in virus-mediated carcinogenesis.

Innate immunity is a critical intracellular barrier against invading microbes. This defense system recognizes infections through a repertoire of pattern recognition receptors (PRRs) (18). Toll-like receptors (TLRs) are the best-studied PRRs. TLR activation by pathogen components produces an array of bioactive molecules such as antimicrobial peptides, cytokines, and chemokines, which are important for the clearance of infection (19). Viral or bacterial dsDNA, for example, is sensed by the intracellular viral DNA immune sensor, TLR9, in the form of nonmethylated CpG motifs (20). TLR9 subfamily members reside within endosomal compartments of the cell (21, 22). Upon ligand binding, TLR9 induces the transcription factor NF-κB in cells of the immune system, leading to increased production of inflammatory mediators (23). Some dsDNA oncogenic viruses, such as Epstein-Barr virus (EBV), hepatitis B virus (HBV), and the mucosal high-risk human papillomavirus 16 (HPV16), inhibit the expression of TLR9 (24–28).

In the past 5 years, eight new human polyomaviruses, including MCPyV, have been discovered (29, 30). Little is known about how these viruses overcome innate immune responses to establish persistent infections. Here, we present evidence that MCPyV via LT induces downregulation of TLR9 through targeting of C/EBPα

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TABLE 1 Primers used for cloning

Name	Sequence
Forward primers	
BKPyVLT F	ATGGATAAAGTTCTTAACAGGG
JCPyVLT F	ATGGACAAAGTGCTGAATAGG
KIPyVLT F	ATGGATAAAACTTTATCTAGAGAA
WUPyVLT F	ATGGATAAAACTTTGTCCAGAAA
SV40PyVLT F	ATGGATAAAGTTTTAAACAGAGA
MCPyVLT WT F	ATGGATTTAGTCCTAAACAGG
MCPyVLT Trunc	ATGGATTTAGTCCTAAACAGG
MCPyVST F	ATGGATTTAGTCCTAAACAGGA
C/EBP RE F[a]	AGGCCCCTGCAGAACTCTGGAGATG
Reverse primers	
BKPyVLT R	TTATTTTGGGGGTGGTGTTTT
JCPyVLT R	TTATTTTGGGGGAGGGGTCT
KIPyVLT R	TTATTCAAACCACTTTTCTAGTAT
WUPyLT R	TTAAGCATTGTGTTTTTCAAGTA
SV40PyLT R	TTATGTTTCAGGTTCAGGGG
MCPyV WTLT R	ATTCTGGTACTTTTTCTCAATAA
MCPyVLT Trunc	CAGGACAGAAATGCTATGTAA
MCPyVST R	CTAGAAAAGGTGCAGATGCA
C/EBP RE R[a]	GCTGGGGGGCAGGGGCTTCTCCA

[a] Primers used for deletion of C/EBP responsive element (−570/−1).

TABLE 2 RT-PCR primers used to check the expressions of polyomaviruses

Name	Sequence
Forward	
BKPyV F	AATCTGCTGTTGCTTCTTCATCAC
JCPyV F	GGGATCCTGTGTTTTCATCATCAC
KIPyV F	CTTCTGGTGCACTTCTTTTTGGAG
WUPyV F	GCTTT GTAGGGGTTTCTCCAGG
SV40PyV F	AATGTTGAGAGTCAGCAGTAGCC
MCPyV F	ACACCCTTGGAGCAAATTCCAG
Reverse	
BKPyV R	TCACCCTGACAAAGGGGGCGAC
JCPyV R	CCACCCTGATAAAGGTGGGGAC
KIPyV R	TAAAGGAGGAAATGAGGAAAGCATG
WUPyV R	CAAAGGGGGAAATGAGGAAAAAATG
SV40PyV R	TCCTGATAAAGGAGGAGATGAAGA
MCPyV R	CGAAGCTGAATCCTCCTGATCTC

and C/EBPβ transactivators. We also show that among different human polyomaviruses, MCPyV is one of the most efficient in downregulating TLR9 expression. The fact that different tumor-associated viruses share the ability to target the TLR9 pathway underscores a potentially important role for this function in virus-driven carcinogenesis.

MATERIALS AND METHODS

Expression vectors. Full-length MCPyV early and LT genes were a kind gift from D. A. Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA). Early genes of BK polyomavirus (BKPyV), JC polyomavirus (JCPyV), KI polyomavirus (KIPyV), simian virus 40 (SV40), WU polyomavirus (WUPyV), and MCPyV and small T antigen (sT) of MCPyV were amplified by PCR using primers described in Table 1. The viral open reading frames were cloned into the retroviral vector pLXSN (Clontech, Palo Alto, CA) and into the mammalian expression vector pcDNA3 (Invitrogen). C/EBPβ and C/EBPα were cloned in pcDNA3. The C-terminally truncated form of MCPyV LT was generated by modifying the codon 534 AAG to TAA by site-directed mutagenesis and cloned into pcDNA3 or pLXSN. The pcDNA3-LMP-1, pGL3-TLR9 delta NF-κB response element (RE), and pcDNA-6-57 KT constructs have been previously described (3, 24). The TLR9 promoter luciferase constructs, full length (−3227/−1) or deleted (−1017/−1, −640/−1, −290/−1, −240/−1, −160/−1, −130/−1, −1017a/−1, and −1017b/−1), were a kind gift from Fumihiko Takeshita (Yokohama City University Graduate School of Medicine, Kanagawa, Japan) (31). The −640/−625 deletion mutant of the TLR9 promoter lacking the C/EBP RE was obtained by PCR using primers described in Table 1 and the TLR9 deletion mutant (−640/−1) promoter as the template. After PCR, the −570/−1 deletion mutant was cloned into the pGL3 basic vector. The NF-κB reporter plasmid was obtained from BD Clontech.

Cell culture procedures. RPMI-8226 cells (kindly provided by Christophe Caux, Centre Leon Berard, Lyon, France) and MCPyV-positive MCC cells (MKL-1) were cultured in RPMI 1640 medium (Invitrogen Life Technologies, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum (PAA, Pasching, Austria), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Pen Strep; Gibco, Invitrogen), 2 mM L-glutamine (PAA), and 1 mM sodium pyruvate (PAA). Human embryonic kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal calf serum (PAA) and all the supplements mentioned above. NIH 3T3 and Phoenix cells were cultured according to the previously described protocol (32). Naturally immortalized keratinocytes (NIKS) were grown together with NIH 3T3 feeder cells in FAD medium, containing Ham's F-12 medium (PAA), DMEM (Gibco), 2% fetal calf serum (PAA), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Pen Strep; Gibco, Invitrogen), adenine (Sigma), 10 ng/ml human epidermal growth factor (R&D), 5 mg/ml insulin (Sigma-Aldrich), 400 μg/ml hydrocortisone (Sigma), 10 mg/ml ciprofloxacin hydrochloride (Euromedex), and 20 mg of cholera toxin (List Biological Laboratories). Epilife medium (Cascade) supplemented with growth factors (Cambrex) was used when NIKS were grown in the absence of feeder cells. All cells were cultured at 37°C with 5% CO_2.

Retroviral infections. Retroviral transduction of NIKS with the early genes from BKPyV, JCPyV, KIPyV, SV40, WUPyV, and MCPyV cloned in pLXSN was performed as previously described (32). After viral transduction, NIKS were selected in medium containing 1 mg/ml G418 (PAA).

Transient-transfection and luciferase assays. The different types of cells (1×10^5) were transiently transfected with firefly luciferase pGL3 vector (0.5 μg), *Renilla* luciferase pGL3 vector (0.05 μg), and DNA expression vectors (0.5 μg, unless differently indicated in figure legends) by using FuGENE6 reagents (Roche) according to the manufacturer's protocols. Forty-eight hours after transfection, cells were lysed in 80 μl of 1× passive lysis buffer (Promega) for 30 min at 4°C. Luciferase activity of 30-μl aliquots from each of the cell lysates was measured using the dual luciferase reporter assay system (Promega) according to the manufacturer's protocols. Luciferase activity of lysates was normalized by *Renilla* activity and expressed as relative luciferase activity.

RT-PCR and QRT-PCR. Reverse transcription-PCR (RT-PCR) and quantitative real-time PCR (QRT-PCR) were performed as previously described (33). Briefly, total RNA was extracted from cells using the Absolutely RNA Miniprep kit (Stratagene) and reverse transcribed to cDNA by using RevertAid H Minus Moloney murine leukemia virus (M-MuLV) reverse transcriptase (MBI; Fermentas) according to the manufacturer's protocol. The primer sequences used for RT-PCR and QRT-PCR are listed in Tables 2 and 3, respectively.

Silencing of LT and sT expression. NIKS (1×10^5 cells) expressing MCPyV early genes were seeded in a 2-ml total volume of cell culture medium per well in 6-well plates (B&D), 24 h before transfection. Transfection using Interferin (Polyplus Transfection) was done according to the manufacturer's protocol. Small interfering RNAs (siRNAs) targeting the sequences 5′-ATGTTTGATGAGGTTGACGAGGCT-3′ and 5′-AAG TTGTCTCGCCAGCATTGT-3′ were used to knock down the MCPyV LT

TABLE 3 Primers used for qRT-PCR

Name	Sequence
Forward	
C/EBPα F	CTAGAGATCTGGCTGTGGGG
C/EBPβ F	CACCTGCAGTTCCAGATCG
hTLR9 F	CGTCTTGAAGGCCTGGTGTTG
SPINK1 F	GGGAAGAGAGGCCAAATGTT
IL-13 F	GTACTGTGCAGCCCTGGAAT
IL-24 F	GCCTCTCAAATGCAGATGGT
CSF3R F	GACCTGGAGGATGGAACAGA
GAPDH F	CGTCTTGAAGGCCTGGTGTTG
Reverse	
C/EBPα R	TCATAACTCCGGTCCCTCTG
C/EBPβ R	CTCGTTGCTGTTCTTGTCCA
hTLR9 R	CTGGAAGGCCTTCGTTTTAGTGA
SPINK1 R	GGCCCAGATTTTTGAATGAG
IL-13 R	TTTACAAACTGGGCCACCTC
IL-24 R	GGGCACTCGTGATGTTATCC
CSF3R R	AGGTCTTGCCAATGTGCTTT
GAPDH R	CTGGAAGGCCTTCGTTTTAGTGA

and MCPyV sT, respectively. Scrambled siRNA was used as a negative control. Four days after transfection, cells were collected and processed for RNA or protein extraction.

Lentiviral short hairpin RNAs (shRNAs) targeting both LT and sT (PanT) for knockdown or sT alone (sT) were generated as reported previously (11). MKL-1 cells were transduced with sT, PanT, or scrambled (Scr) shRNA lentiviruses with 1 μg/ml of Polybrene. Infected cells were selected for 4 days at day 2 posttransduction with 1 μg/ml puromycin. Cells were harvested for mRNA and protein extraction at day 10 postinfection.

IB and antibodies. Whole-cell lysates were prepared, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (IB) were performed according to previously described protocols (33, 34). Protein concentrations were measured with bicinchoninic acid (BCA) assay reagent; 40 to 80 μg of protein extracts was used for SDS-PAGE and immunoblot analyses, and IB was performed according to the previously described method. The antibodies used for IB were β-actin (C4; MP Biomedicals), MCPyV LT (CM2B4; Santa Cruz), hTLR9 (Cell Signaling), C/EBPα (Cell Signaling), C/EBPβ (Santa Cruz), antitubulin (Sigma), and LT and sT (CM8E6) (6).

TLR9 ligands and ELISA. The different types of cells (0.5×10^5 cells/well of 96-well plates) were seeded in 200 μl of Epilife growth medium (R&D), for 24 h at 37°C. After that, cells were stimulated in triplicate with TLR9 ligand CpG oligonucleotides (ODN2006CpG; Sigma), and medium alone was used as a negative control. After 24 h of stimulation, supernatant was harvested, and MIP3α, interleukin-8 (IL-8), and IL-6 levels were measured using the Quantikine (R&D Systems, Minneapolis, MN) and DuoSet (R&D Systems) enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's protocols.

Quantitative ChIP. NIKS expressing early genes of MCPyV and pLXSN (2×10^6 cells for each chromatin immunoprecipitation [ChIP]) were formaldehyde fixed and processed for ChIP using the Shearing ChIP kit (Diagenode) and the OneDay ChIP kit (Diagenode) according to the manufacturer's protocols. Quantitative PCR analysis of the eluted ChIP fractions from antibody-bound and input chromatin was performed using primers flanking the TLR9 C/EBP RE (−640/−625) (C/EBP ChIP forward, 5′-TCACTGGGGGCAGGGTGG-3′, and C/EBP ChIP reverse, 5′-TGGCGGGCAGACTGGACA-3′). The amounts of amplified DNA found binding to C/EBPα and C/EBPβ antibodies were expressed as a percentage of levels of the same DNA fragment in the input sample (100%).

Statistical analyses. The Student *t* test was applied to check the statistical significance of the obtained data. *P* values relating to each experiment are indicated in the figure legends; *P* values of <0.05 and >0.01 were considered significant and indicated with a single asterisk; *P* values of <0.01 were considered highly significant and were indicated with a double asterisk. Error bars in the graphs represent the standard deviations (SDs).

RESULTS

MCPyV LT downregulates TLR9 promoter activity. Several dsDNA oncogenic viruses, including EBV, HBV, and the mucosal high-risk HPV16, are able to downregulate the expression of TLR9 (24–28). To determine whether MCPyV also affects TLR9 expression, we first performed transient-transfection experiments in RPMI-8226 cells that express TLR9 protein. These cells have been successfully used in previous studies to characterize the impact of HPV16 oncoproteins on TLR9 promoter activity (27). The TLR9 promoter, cloned in front of a luciferase reporter gene (31), was introduced in RPMI-8226 cells together with increasing amounts of MCPyV early region DNA (Fig. 1A). Expression of MCPyV early proteins efficiently reduced TLR9 promoter activity similarly to LMP-1, an EBV oncoprotein known to repress TLR9 expression (Fig. 1A) (24). This MCPyV activity was still present after deletion of the LT C terminus as occurs in Merkel carcinoma cells (Fig. 1A) (3). The early region of MCPyV has the potential to encode LT, sT, and 57KT (3). We therefore sought to determine whether inhibition of TLR9 promoter activity could be ascribed to one or more of these viral early proteins. Transient-transfection experiments in RPMI-8226 cells with pcDNA3-LT showed that LT was able to inhibit TLR9 promoter activity (Fig. 1B). Mutation in the early gene MCPyV region (earlym57KT), which eliminates 57KT expression, did not affect MCPyV early region activity on the TLR9 promoter (Fig. 1C). Further, overexpression of 57KT in RPMI-8226 cells did not inhibit TLR9 promoter activity (Fig. 1D). However, we found that LT when expressed together with other early genes was more efficient in downregulating TLR9 promoter than when it was expressed alone (Fig. 1). Together, these data indicate that MCPyV, similarly to several other oncogenic viruses, downregulates TLR9 promoter activity and that this event is in part mediated by the LT antigen isoform.

MCPyV downregulates TLR9 signaling in epithelial and Merkel cells. We next determined whether expression of MCPyV early genes in the spontaneously immortalized keratinocytes (NIKS) downregulates TLR9. These cells retain many features of primary keratinocytes and represent an appropriate experimental model for MCC since the Merkel cells are believed to be derived from cells of epithelial histogenesis (35). The entire MCPyV early region or LT alone was cloned into the retroviral vector pLXSN and transduced into NIKS. TLR9 mRNA levels were lower in cells expressing the MCPyV early genes or LT than in the mock cells (pLXSN) (Fig. 2A). Similarly to what has been observed in transient-transfection experiments in RPMI-8226 cells, inhibition of TLR9 expression was more evident in cells expressing the entire early region than in cells expressing LT alone (Fig. 2A). Immunoblotting performed using the total protein extracts from the same cells confirmed the quantitative RT-PCR data, showing that, similarly to HPV16 oncoproteins, MCPyV early proteins or LT alone decreased TLR9 protein levels (Fig. 2B). Also, in the immunoblot TLR9 protein levels were slightly lower in cells containing the entire early region than in the cells expressing LT alone (Fig. 2B).

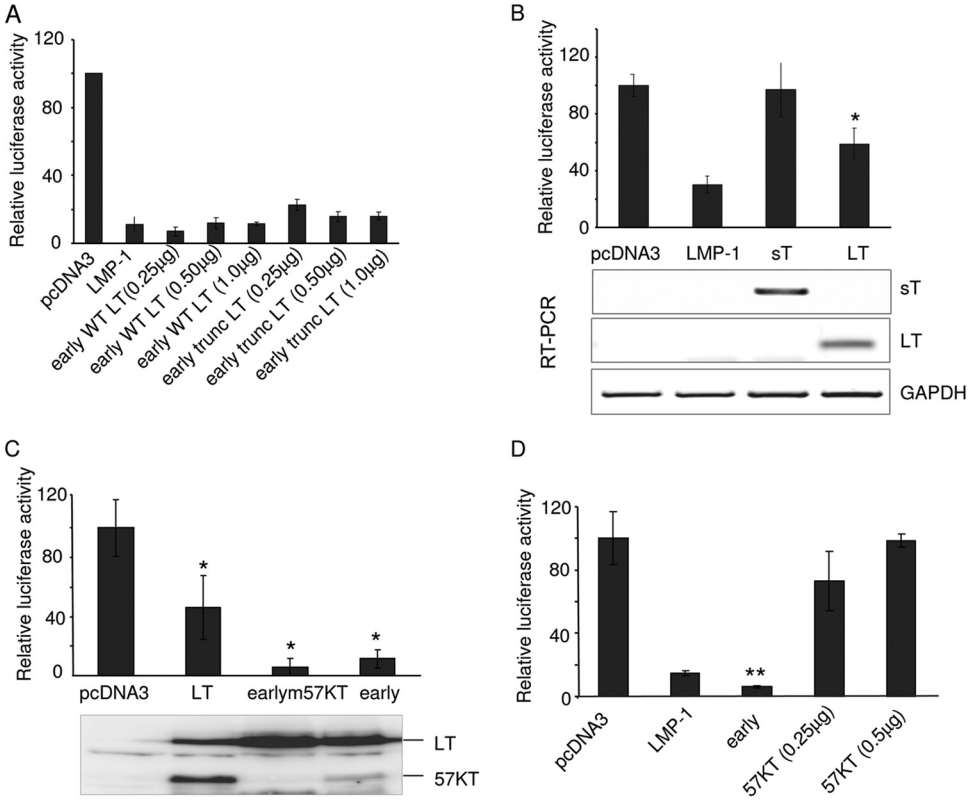

FIG 1 The LT antigen of MCPyV downregulates TLR9 promoter activity. (A) RPMI-8226 cells were cotransfected with a pGL3-based luciferase reporter construct carrying the TLR9 promoter fused to the firefly luciferase gene (pGL3-TLR9), with a pGL3-*Renilla* construct, and with growing amounts (0.25 μg, 0.50 μg, and 1 μg) of MCPyV early genes (early), harboring wild-type LT (early WT LT) or truncated LT (early trunc LT) cloned in mammalian expression vector pcDNA3. The pcDNA3 vector alone and that expressing LMP-1 were used as a negative control and a positive control, respectively. Firefly luciferase activity was measured after 48 h of transfection, and values were normalized to *Renilla* luciferase activity and expressed as relative luciferase activity. Results (± SDs) are representative of three independent experiments performed in triplicate. (B) pGL3-TLR9 was cotransfected in RPMI-8226 cells with pcDNA3-MCPyV-sT (sT) or pcDNA3-MCPyV-LT (LT). After 48 h of transfection, cells were collected and processed for luciferase assay and for RNA extraction. The histogram (upper panel) shows the levels of luciferase activity calculated as explained for panel A. The difference in TLR9 promoter activity in cells transfected with LT compared to pcDNA3 was statistically significant ($P = 0.02$), whereas sT did not vary significantly ($P = 0.81$). The expression levels of MCPyV sT and LT mRNA in the same cells were evaluated by RT-PCR (lower panel). (C) The graph shows the luciferase activity of the TLR9 promoter in RPMI-8226 cells, in the presence of pcDNA3-MCPyV LT (LT), pcDNA3-MCPyV early (early), or pcDNA3-MCPyV early genes lacking 57KT (earlym57KT) (upper panel). Immunoblotting with the anti-LT CM2B4 antibody shows the expression of MCPyV LT and 57KT in the same cells (lower panel). The difference between the values of the control (pcDNA3) and other values is statistically significant ($P < 0.05$ and $P > 0.01$). (D) RPMI-8226 cells were cotransfected with pGL3-TLR9 and pcDNA3 expressing the different viral genes. For the pcDNA6-MCPyV-57KT (57KT), increasing amounts (0.25 μg and 0.50 μg) of DNA were used. TLR9 promoter luciferase activity was measured after 48 h of transfection, and the histogram was obtained as explained for panel A. The difference between the values of pcDNA3 and early is statistically significant ($P < 0.01$).

Silencing the expression of LT by siRNA significantly rescued the expression of TLR9 in NIKS transduced with the recombinant retrovirus expressing MCPyV early genes (Fig. 2C). In addition, silencing the sT expression also resulted in a slight increase of TLR9 mRNA levels, indicating that sT may also cooperate with LT in the inhibition of TLR9 expression (Fig. 2C). In agreement with the findings in NIKS and RPMI-8226 cells, QRT-PCR revealed that TLR9 is weakly expressed in the MCPyV-positive MCC cell line, MKL-1 (Fig. 2D). When both LT and sT in MKL-1 cells were knocked down by a PanT shRNA, significant increases of TLR9 mRNA levels were detected (Fig. 2D). Also in these cells, silencing the expression of sT alone resulted in a modest increase of TLR9 mRNA levels.

We also examined whether MCPyV hampered functional TLR9 signaling. NIKS or NIKS expressing the MCPyV early proteins were exposed to TLR9 ligand CpG oligonucleotides. The CpG ligand led to an increase in secreted IL-6, IL-8, and MIP3α levels in nontransduced NIKS but not in NIKS cells expressing the MCPyV early gene region (Fig. 2E). In addition, TLR9 ligand did not stimulate cytokine secretion in MCPyV-positive Merkel cells (MKL-1). Indeed, the levels of secreted IL-8 in unstimulated MKL-1 cells were below the detection levels and did not increase upon CpG stimulation.

MCPyV inhibits TLR9 expression by downregulating C/EBPβ. The TLR9 promoter and its transcription factors have been previously characterized (31). To identify specific TLR9 REs that are targeted by MCPyV, we performed transient-transfection experiments in RPMI-8226 cells using deletion mutants of the TLR9 promoter cloned in front of the luciferase reporter gene (Fig. 3A). Deletion of the region between nucleotides −640 and −290 abolished the MCPyV-mediated inhibition of TLR9 promoter activity (Fig. 3B). This region includes REs recognized by several cellular transcription factors, including NF-κB and C/EBP (Fig. 3A) (31). Activation of the NF-κB signaling pathway by EBV and HPV16

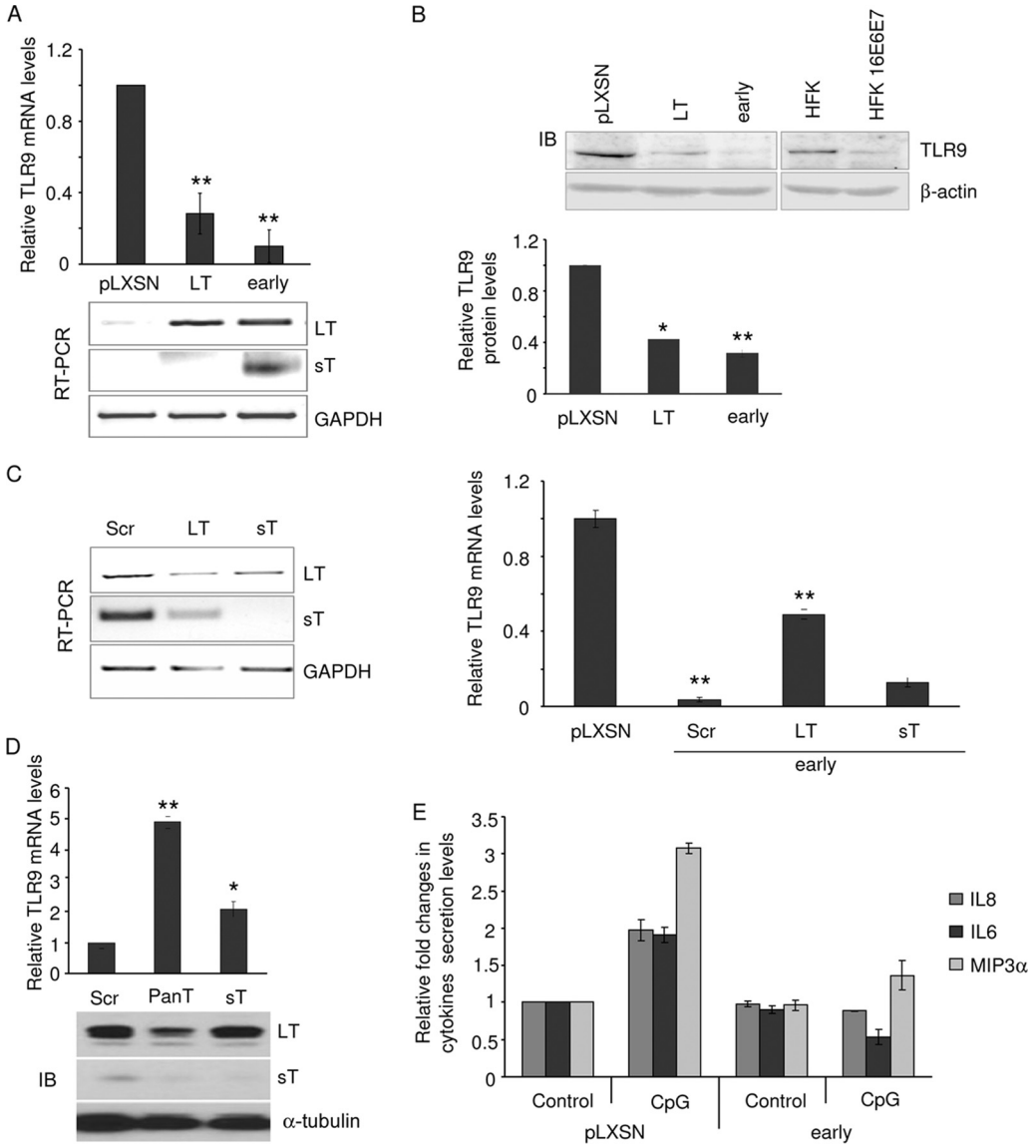

FIG 2 TLR9 signaling is altered by MCPyV in epithelial cells. (A and B) NIKS stably expressing MCPyV LT (LT) or early genes (early) or NIKS carrying the empty retroviral pLXSN vector (pLXSN) were generated by retroviral infection and processed for RNA or total protein extraction. (A) After reverse transcription, the endogenous TLR9 mRNA levels were measured by QRT-PCR and normalized to the levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results (± SDs) are representative of three independent experiments performed in duplicate (upper panel). Differences in the TLR9 mRNA levels between NIKS expressing LT, early genes, and NIKS pLXSN were statistically significant ($P = 0.006$ for pLXSN versus LT and 0.002 for pLXSN versus early). The expression of MCPyV LT and sT was determined by RT-PCR (lower panel). (B) Total protein extracts (80 μg) were separated by SDS-PAGE and analyzed for TLR9 and β-actin protein levels by immunoblotting. Human foreskin keratinocytes (HFK) alone and those transduced with HPV16E6E7 (HFK16E6E7) were included as negative and positive controls, respectively (upper panel). The graph (lower panel) displays the quantification of the Western blot results for TLR9 protein levels in NIKS expressing pLXSN, MCPyV LT (LT), and early genes (early). The band intensities were quantified with ImageJ software, and TLR9 protein levels were normalized on the β-actin levels. The results (± SDs) shown are representative of three independent experiments. The differences in the TLR9 protein levels between NIKS expressing LT and early genes and NIKS pLXSN were significant ($P = 0.01$ for pLXSN versus LT and 0.001 for pLXSN versus early). (C) NIKS retrotransduced with MCPyV early genes were transfected with siRNA against MCPyV LT or sT and scrambled (Scr) siRNA as a negative control. At 48 h after transfection, the expression levels of MCPyV LT and sT were measured by RT-PCR (left panel), and TLR9 mRNA levels were determined by QRT-PCR (right panel). Results (± SDs) are representative of two independent experiments performed in duplicate. The difference in the TLR9 mRNA levels between NIKS pLXSN and NIKS early transfected with Scr was statistically significant ($P = 0.002$). The difference in the levels of TLR9 transcript between Scr and LT siRNA-transfected cells was also statistically significant ($P = 0.003$), whereas the TLR9 mRNA level did not vary significantly between cells treated with sT siRNA and Scr ($P = 0.06$). (D) Knockdown of both LT and sT (PanT) or sT alone (sT) in MCPyV-positive MCC cells (MKL-1) was achieved by transduction with lentivirus-based shRNA. Scrambled shRNA (Scr) was used as a negative control. Cells were collected and processed for total RNA and protein extraction. After reverse transcription, TLR9 mRNA levels were determined by QRT-PCR and normalized to the levels of the housekeeping gene GAPDH (upper panel). Results (± SDs) are representative of two independent experiments performed in duplicate. Differences in the levels of TLR9 mRNA between cells treated with Scr, PanT, and sT shRNAs were significant ($P = 0.001$ for Scr versus PanT; $P = 0.02$ for Scr versus sT). In the same cells, the levels of MCPyV LT and sT were evaluated by immunoblotting analysis with the indicated antibodies (lower panel). (E) NIKS retrotransduced with pLXSN or with MCPyV early genes were stimulated with TLR9 ligand CpG oligonucleotides for 24 h, and secretion of the chemokines IL-8, IL-6, and MIP3α was monitored by ELISA as explained in Materials and Methods. Values were expressed in term of fold change to nontreated cells.

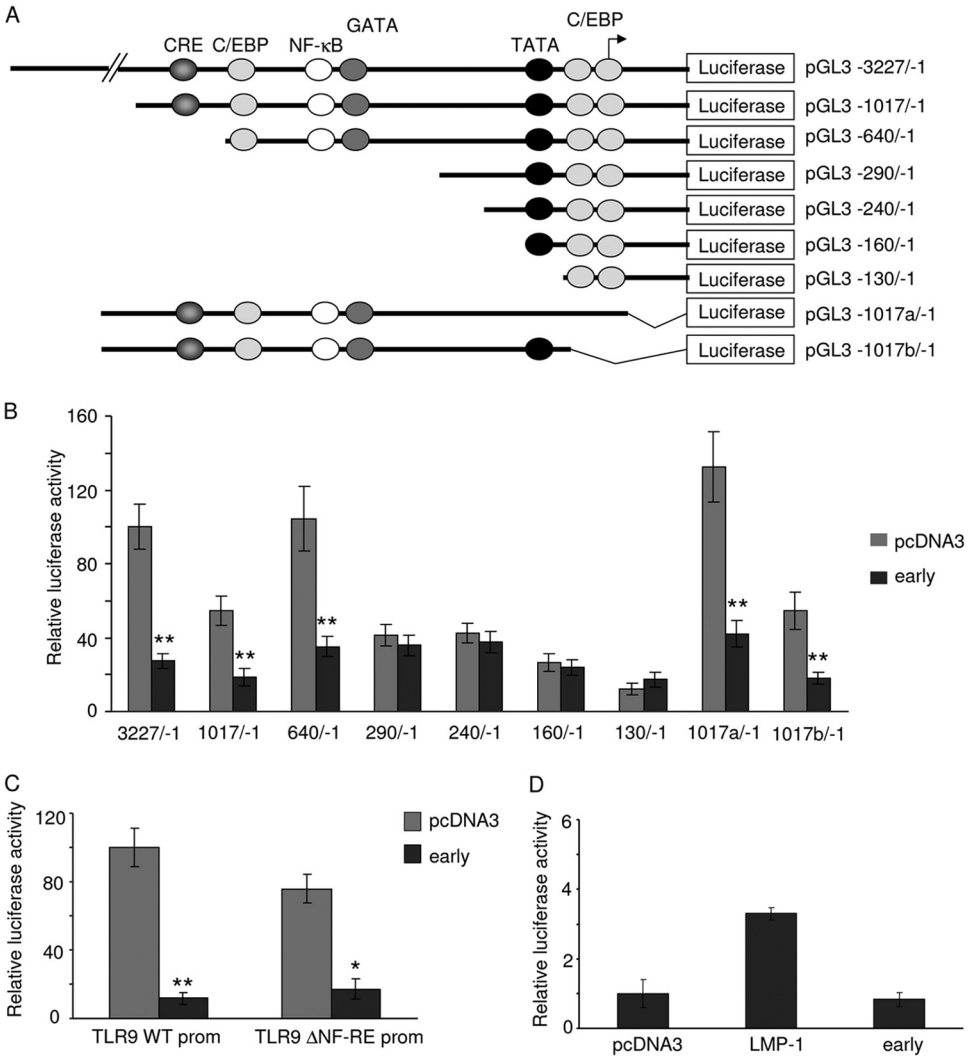

FIG 3 The NF-κB pathway is not involved in MCPyV-mediated downregulation of TLR9. (A) Schematic representation of TLR9 promoter full length (−3227/−1) and deletion mutants. (B) Transfection in RPMI-8226 cells was conducted as explained for Fig. 1A. Cells were cotransfected with full-length TLR9 promoter (pGL3-TLR9) or with each of the deletion mutants depicted in panel A and with pcDNA3 or pcDNA3-MCPyV early genes (early). After 48 h, cells were processed for the luciferase assay. The luciferase values were normalized to the *Renilla* luciferase activity, and results are shown in terms of relative fold change. Results (± SDs) are representative of three independent experiments performed in triplicate. The differences in TLR9 promoter activities in cells transfected with the early genes (early) compared to the pcDNA3 were significant ($P < 0.05$) for the following deleted promoters: 3227/−1, 1017/−1, 640/−1, 1017a/−1, and 1017b/−1. (C) RPMI-8226 cells were cotransfected with TLR9 promoter wild type (TLR9 WT prom) or mutated on the NF-κB RE (−413/−403) (TLR9 ΔNF-RE prom) and with pcDNA3 empty vector or pcDNA3-MCPyV early genes (early). The luciferase activity was measured as described for panel B. (D) The histogram shows the relative fold activation of luciferase activity in RPMI-8226 cells cotransfected with pGL3-NF-κB construct and with pcDNA3-LMP1 (LMP-1) or with pcDNA3-MCPyV early genes (early). The pcDNA3 empty vector was used as a negative control. Values (± SDs) shown are the means of three independent experiments performed in triplicate.

oncogenic viruses contributes to TLR9 promoter inhibition (24, 27). Mutations of NF-κB RE located in this region of the TLR9 promoter, however, did not interfere with the ability of MCPyV to repress TLR9 promoter activity (Fig. 3C). In addition, expression of the MCPyV early genes, in contrast to EBV LMP-1 expression, did not lead to activation of the NF-κB signaling pathway (Fig. 3D and data not shown).

Members of the C/EBP family positively activate transcription of several TLR genes, for example, TLR8 and TLR9 (31, 36). Therefore, we next evaluated whether MCPyV inhibits TLR9 expression by targeting C/EBP transcription factors. We generated a C/EBP RE deletion mutant of the TLR9 promoter (Fig. 4A) and determined its activity in transient-transfection assays. Deletion of the C/EBP RE significantly decreased TLR9 promoter activity (Fig. 4B). Unlike other promoter constructs, no repression was found for the C/EBP RE mutant promoter during coexpression of MCPyV early genes (Fig. 4B). Consistent with this, MCPyV early gene expression downregulated C/EBPα and C/EBPβ mRNA levels in NIKS (Fig. 4C). Immunoblotting revealed reduced levels of C/EBPα and C/EBPβ proteins in NIKS expressing the MCPyV early region compared with the empty pLXSN vector (Fig. 4D). Additionally, mRNA levels of genes known to be regulated by C/EBPβ, i.e., SPINK1, IL-13, IL-24, and CSF3R (37), are strongly downregulated in cells expressing the MCPyV early genes in com-

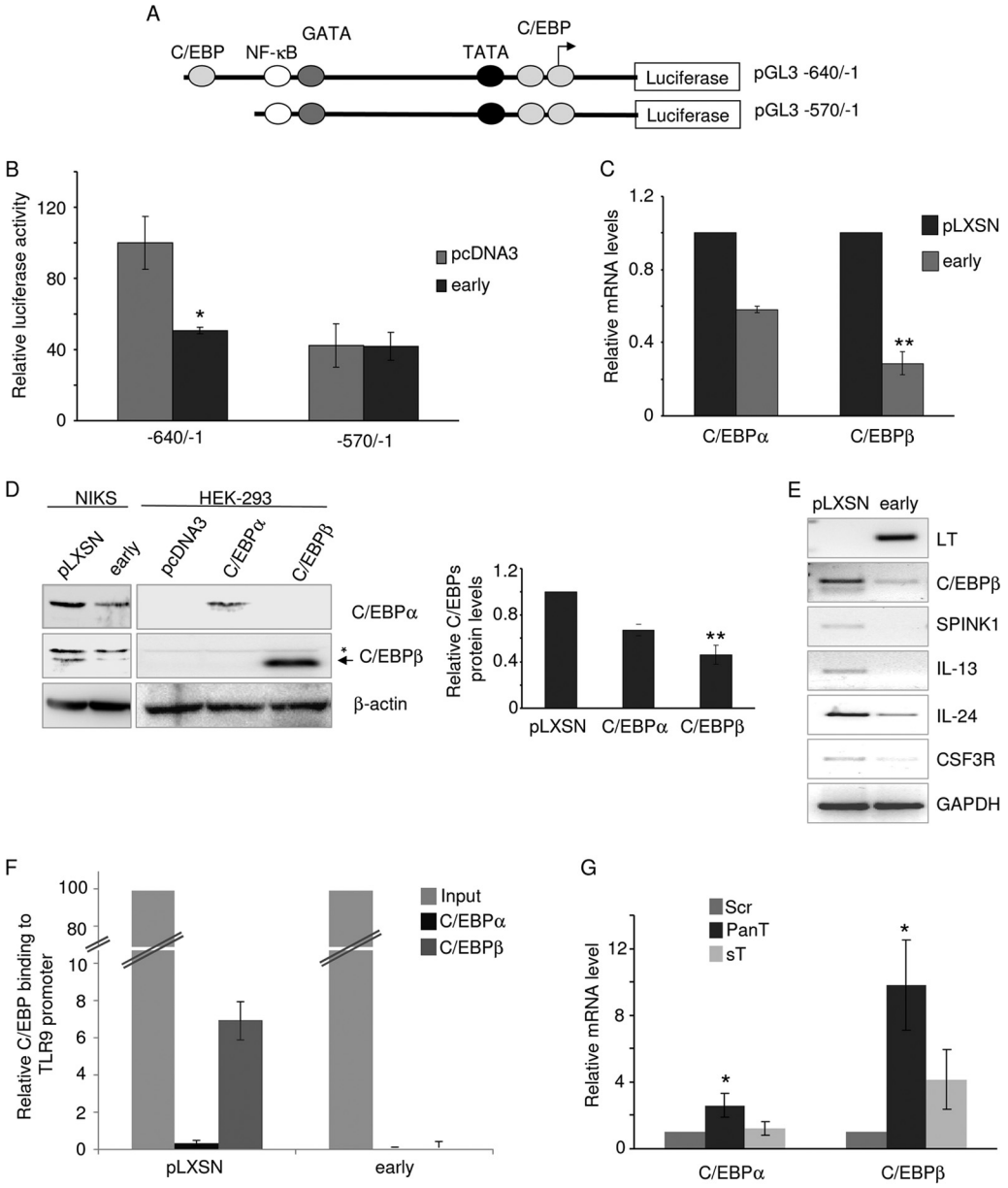

FIG 4 MCPyV hampers TLR9 expression by downregulating C/EBP transcription factors. (A) Schematic representation of a deletion mutant of the TLR9 promoter (pGL3−570/−1) lacking the C/EBP RE. (B) Luciferase activity of the pGL3−640/−1 and pGL3−570/−1 promoters was measured in RPMI-8226 cells after 48 h of cotransfection with pcDNA3 alone or with pcDNA3-MCPyV early genes (early). Firefly luciferase values were normalized to the *Renilla* luciferase values. Results (± SDs) are representative of three independent experiments performed in triplicate. (C to E) NIKS transduced with pLXSN or with MCPyV early genes (early) were processed for total RNA and protein extraction. (C) After reverse transcription, QRT-PCR was performed to measure the mRNA levels of C/EBPα and C/EBPβ. mRNA values obtained for of C/EBPα and C/EBPβ were normalized to GAPDH levels. The results (± SDs) are representative of three independent experiments performed in duplicate. Differences in the C/EBPβ levels between NIKS pLXSN and early were significant with a *P* value of 0.001, whereas the level of C/EBPα did not change significantly ($P = 0.08$). (D) Total extracts (40 μg) were analyzed by immunoblotting for the protein levels of C/EBPα, C/EBPβ, and β-actin (left panel). The graph (right panel) shows the quantification of the C/EBPα and -β protein levels in NIKS transduced with pLXSN or MCPyV early genes (early), which were measured as described in the legend to Fig. 2B. Protein extracts from HEK293 cells transfected with pcDNA3, pcDNA3-C/EBPα, and pcDNA3-C/EBPβ were also loaded as a positive control. The asterisk and the arrow indicate, respectively, a nonspecific band and the specific band for C/EBPβ. The differences in the C/EBPβ protein levels between NIKS/early and NIKS/pLXSN were statistically significant ($P = 0.007$). (E) RT-PCR was performed to measure the expression levels of C/EBPβ downstream genes in NIKS pLXSN or expressing MCPyV early genes (early). (F) NIKS pLXSN or NIKS early were fixed for ChIP analysis, which was performed by using C/EBPα and C/EBPβ antibodies. IgG was used as a negative control. The histogram represents the relative amounts of DNA bound to C/EBPα and C/EBPβ expressed as a percentage of the input (100%). (G) MCPyV-positive MCC cells (MKL-1) were treated with lentivirus-based shRNA to knock down both LT and sT (PanT), sT alone (sT), or shRNA scrambled (Scr). Cells were collected and processed for total RNA. After reverse transcription, C/EBPα and C/EBPβ mRNA levels were determined by QRT-PCR and normalized to the levels of the housekeeping gene GAPDH. Results (± SDs) are representative of two independent experiments performed in triplicate. Differences in the levels of C/EBPα or C/EBPβ between cells treated with Scr or with PanT shRNAs were significant ($P = 0.01$ for C/EBPα and 0.02 for C/EBPβ). Nonsignificant differences were observed in the levels of C/EBPα and C/EBPβ between Scr and sT shRNA-treated cells ($P = 0.26$ and 0.08, respectively).

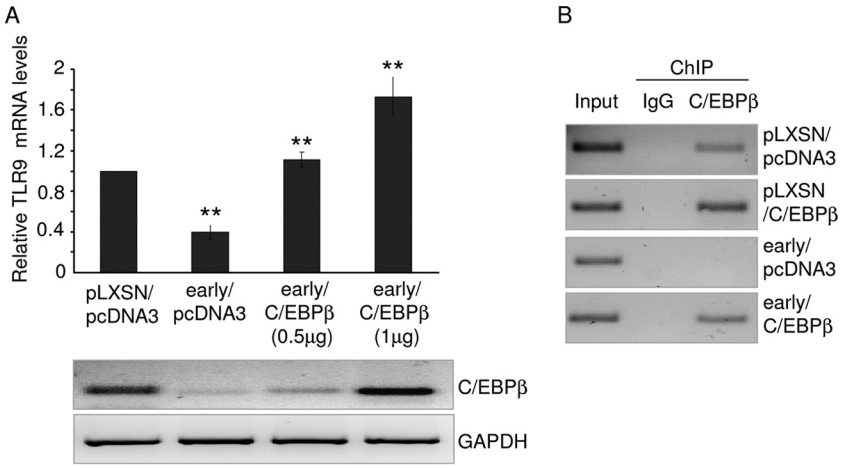

FIG 5 The ectopic expression of C/EBPβ transcription factor in NIKS expressing MCPyV early genes rescues TLR9 mRNA levels. (A) NIKS stably expressing the MCPyV early genes (early) and pLXSN were transfected with increasing amounts (0.5 μg or 1 μg) of pcDNA3-C/EBPβ vector. After 48 h of transfection, cells were processed for total RNA and reverse transcribed into cDNA. TLR9 mRNA levels were measured by QRT-PCR and normalized to the levels of the housekeeping gene GAPDH. Results (± SDs) are representative of three independent experiments performed in duplicate (upper panel). Differences in the levels of TLR9 between NIKS early and pLXSN were significant ($P = 0.008$). Moreover, the differences between NIKS early transfected with pcDNA3 alone and those with pcDNA3-C/EBPβ were also statistically significant ($P < 0.02$). In the lower panel, the expression levels of C/EBPβ in the transfected NIKS were determined by RT-PCR. (B) NIKS transduced with pLXSN and MCPyV early genes (early) were transfected with pCDNA3-C/EBPβ at the concentration of 1 μg or with the pcDNA3 empty vector as a negative control. Forty-eight hours posttransfection, cells were fixed and processed for ChIP analysis. The binding of C/EBPβ to TLR9 promoter under the different conditions was determined by PCR using primers flanking the C/EBP binding sites.

parison to mock cells (Fig. 4E). ChIP experiments showed that C/EBPβ efficiently binds to the TLR9 C/EBP RE, whereas C/EBPα binding is not appreciably detected either in the presence or in the absence of MCPyV early gene expression (Fig. 4F). Expression of MCPyV early genes in NIKS strongly inhibited binding of C/EBPβ to the C/EBP RE (Fig. 4F). Silencing of T antigen expression in MCPyV-positive MCC cells led to a significant increase of C/EBPα and C/EBPβ mRNA levels (Fig. 4G), which is consistent with the effects of MCPyV LT knockdown on TLR9 expression in epithelial and Merkel cancer cells (Fig. 2). In contrast, inhibition of sT expression in the same cells resulted in a reduced but still significant increase in mRNA levels of C/EBPα or C/EBPβ (Fig. 4G). Reexpression of C/EBPβ in cells containing the MCPyV early region restored TLR9 expression (Fig. 5A). Accordingly, reactivation of TLR9 expression in these cells correlated with recruitment of C/EBPβ to TLR9 promoter (Fig. 5B). Together, these data show that the MCPyV LT-induced TLR9 downregulation is mainly mediated by loss of C/EBPβ expression.

Members of the polyomavirus family have different efficiencies in downregulating TLR9 expression. Eleven different human polyomaviruses have been isolated so far (38). To determine whether TLR9 downregulation is common to most human polyomaviruses, we cloned early gene regions from four additional human polyomaviruses (BKPyV, JCPyV, WUPyV, and KIPyV) and simian virus 40 (SV40) into the retroviral vector pLXSN or into the pcDNA3 expression vector. We found that all tested polyomavirus early regions inhibited TLR9 promoter luciferase activity in RPMI-8226 cells, although BKPyV, SV40, and MCPyV were most efficient in this inhibition (Fig. 6A). JCPyV showed the lowest activity, though this might be due to the lowest levels of expression of its LT and sT genes. To corroborate these findings, we transduced NIKS cells with the different recombinant retroviruses and determined LT and sT expression by RT-PCR (Fig. 6B). QRT-PCR revealed that TLR9 mRNA levels were significantly decreased in cells expressing the early genes from BKPyV, JCPyV, and MCPyV (Fig. 6C). Unlike RPMI-8226 cells, SV40 and WUPyV early region expression did not affect the TLR9 mRNA levels in NIKS cells (Fig. 6C).

DISCUSSION

Little is known about MCPyV targeting of innate immune defenses or the importance of this targeting to viral persistence (39). In this study, we show that MCPyV, similar to the human tumor viruses HBV, EBV, and HPV16, inhibits the expression of TLR9. However, the mechanism for the TLR9 suppression by MCPyV is distinct from those of the other oncogenic viruses. HPV16 and EBV both downregulate TLR9 through the activation of the NF-κB signaling pathway (24, 27). Here, we show that MCPyV inhibits TLR9 expression by downregulating the mRNA levels of the transcription factor C/EBPβ.

Many studies have highlighted the importance of C/EBP family members in the regulation of immune-related pathways. These transcription factors positively regulate the expression of TLR8 and TLR9 (31, 36). In addition, C/EBPβ plays a key role in the interferon signaling network by interacting with other transcription factors such as NF-κB, SP1, and STAT3 to regulate IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) cytokine transcription (40). Consistent with this, C/EBPβ$^{-/-}$ mice display a high susceptibility to infection with certain agents such as *Candida albicans* and *Salmonella enterica* serovar Typhimurium (40).

Additional well-characterized properties of the C/EBPs include their ability to induce differentiation and to inhibit cellular proliferation in a broad spectrum of cell types (41). Expression of C/EBPα and C/EBPβ decreases proliferation in normal and transformed cells via repression of the E2F complex and deregulation of CDK2 and CDK4 functions (42, 43), suggesting a tumor-suppressing activity of C/EBPs (41). It is likely that MCPyV downregulates the expression of C/EBPs, leading to inhibition of TLR9

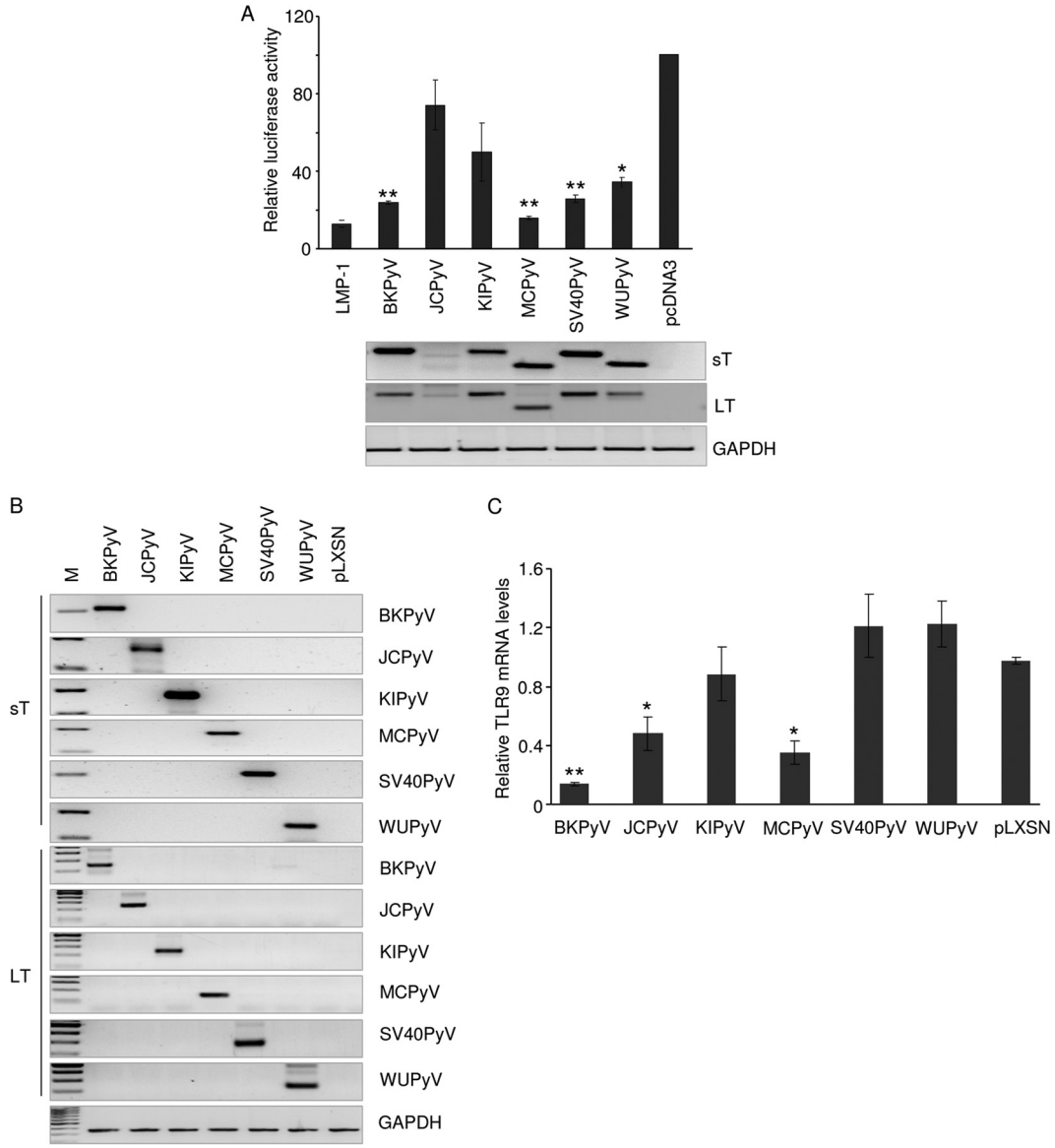

FIG 6 Different members of the polyomavirus family downregulate TLR9 expression with different efficiencies. (A) RPMI-8226 cells were cotransfected with pGL3-TLR9 and with BKPyV, JCPyV, KIPyV, MCPyV, SV40, and WUPyV early genes cloned in pcDNA3. pcDNA3 empty vector and pcDNA3-LMP-1 were used as negative and positive controls, respectively. After 48 h of transfection, cells were analyzed for luciferase activity of pGL3-TLR9 vector. Firefly luciferase values were normalized to the *Renilla* luciferase values and shown as relative luciferase activity to the empty vector (upper panel). Values (± SDs) shown are the means of three independent experiments performed in triplicate. The differences in TLR9 promoter activities in cells transfected with the early genes of BKPyV, MCPyV, SV40, and WUPyV compared to the pcDNA3 were significant with *P* values of 0.002, 0.008, 0.009, and 0.02, respectively. The RT-PCR shows the expression of sT and LT from the indicated polyomaviruses (lower panel). (B and C) NIKS stably expressing the early genes of the indicated polyomaviruses were generated by retroviral transduction as described in Materials and Methods. After selection, cells were collected and processed for RNA extraction. (B) The expression of the different sTs and LTs was evaluated by RT-PCR. GAPDH was used as a loading control. (C) TLR9 mRNA levels were measured by QRT-PCR and normalized to GAPDH levels. Results (± SDs) are representative of three independent experiments performed in duplicate. Differences in the levels of TLR9 mRNA between NIKS expressing the different polyomavirus early genes and NIKS pLXSN were significant only in the case of BKPyV ($P = 0.005$), JCPyV ($P = 0.02$), and MCPyV ($P = 0.01$).

expression as well as the expression of other cellular genes under the control of this transcription factor.

In addition to MCPyV, other viruses also target C/EBPs or inhibit C/EBP activities (44–47). Cutaneous HPV8, which has been associated with nonmelanoma skin cancer in epidermodysplasia verruciformis patients, suppresses C/EBPβ-induced expression of MIP3-α (CCL20) via the E7 oncoprotein, impairing migration of Langerhans cells to sites of infection (46). In addition, it has been shown that C/EBPβ negatively regulates the life cycle of JCPyV (45).

Our study does not provide insights about the MCPyV mechanism involved in the downregulation of C/EBPβ. However, we analyzed the C/EBPβ promoter and identified a putative *cis* element for the transcription factor c-fos. Interestingly, gene expression profile array validated by QRT-PCR showed that MCPyV early gene-expressing cells had lower levels of c-fos mRNA than

did mock cells (data not shown). Thus, it is likely that MCPyV may inhibit C/EBPβ expression by downregulating c-fos mRNA levels.

We observed MCPyV-mediated downregulation of TLR9 in immortalized NIKS and in RPMI-8226 B cells as well as in the MCC-derived cell line MKL-1. LT plays a major role in the inhibition of TLR9 expression. In addition, we present evidence that sT is able to repress TLR9 transcription, since silencing of its expression led to an increase of the levels of TLR9 transcripts in cells expressing the MCPyV early region. It is not yet clear whether sT can be directly involved in the inhibition of TLR9 expression using a mechanism distinct from LT, or whether it can indirectly act by promoting stabilization of LT protein (48) and the potentiation of the various functions of LT. Various studies, including ours, have shown that several oncogenic viruses inhibit TLR9 expression, highlighting the importance of this function in virus-mediated carcinogenesis (24, 27, 28). However, the precise biological significance of the TLR9 downregulation remains to be elucidated. Hasan et al. have previously shown that the HPV16 genome contains CpG motifs recognized by TLR9 (27). It is likely that MCPyV also contains specific CpG motifs that lead to TLR9 engagement and the activation of the cellular innate immune response. However, upon expression of the viral oncogenes TLR9 mRNA levels are rapidly downregulated. Thus, inhibition of TLR9 expression by MCPyV oncoproteins may be required after viral entry to facilitate subsequent steps of the viral life cycle leading to persistence of infection. Downregulation of TLR9 also may be linked to specific events involved in cellular transformation. In support of this, Fathallah et al. observed that EBV infection of primary B cells resulted in a rapid decrease of TLR9 mRNA, which became more pronounced upon immortalization (24).

Among the human polyomaviruses that we examined, MCPyV is the most efficient at downregulating TLR9 expression. However, our data show only that all recombinant retroviruses tested express similar mRNA levels of sT and LT from the different polyomaviruses but do not provide information about corresponding LT protein levels. Our data do not exclude the possibility that LT protein levels in cells expressing the early genes of SV40 or WUPyV are not sufficiently high to induce TLR9 downregulation.

Our initial data indicate that, with the exception of SV40, other polyomaviruses were able to inhibit C/EBPβ expression, although to a lesser extent than MCPyV. However, the C/EBPβ mRNA levels did not tightly correlate with the downregulation of TLR9 expression, suggesting that the different polyomaviruses may use different mechanisms in inhibiting TLR9 transcription (data not shown).

In summary, the present study underscores the importance of deregulation of immunorelated pathways in the life cycles of cancer-associated viruses.

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