TLR9 Expression and Function Is Abolished by the Cervical Cancer-Associated Human Papillomavirus Type 16[1]

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Cervical cancer development is linked to the persistent infection by high-risk mucosal human papillomaviruses (HPVs) types. The E6 and E7 major oncoproteins from this dsDNA virus play a key role in the deregulation of the cell cycle, apoptosis, and adaptive immune surveillance. In this study, we show for the first time that HPV type 16 (HPV16), the most carcinogenic type among the high-risk subgroup, interferes with innate immunity by affecting the expression of TLRs. Infection of human primary keratinocytes with HPV16 E6 and E7 recombinant retroviruses inhibits TLR9 transcription and hence functional loss of TLR9-regulated pathways. Similar findings were achieved in HPV16-positive cancer-derived cell lines and primary cervical cancers, demonstrating that this event occurs also in an in vivo context. Interestingly, E6 and E7 from the low-risk HPV type 6 are unable to down-regulate the TLR9 promoter. In addition, E6 and E7 from the high-risk HPV type 18, which are known to persist less competently in the host than HPV16, have reduced efficiency compared with HPV16 in inhibiting TLR9 transcription. Furthermore, a CpG motif derived from the HPV16 E6 DNA sequence activated TLR9, indicating this virus is able to initiate innate responses via the receptor it later down-regulates. This study reveals a novel mechanism used by HPV16 to suppress the host immune response by deregulating the TLR9 transcript, providing evidence that abolishing innate responses may be a crucial step involved in the carcinogenic events mediated by HPVs. *The Journal of Immunology,* **2007, 178: 3186–3197.**

Human papillomaviruses (HPVs)[3] are double-stranded circular DNA viruses, of which over 90 different types have been fully characterized so far. They can be classified in general and divided into mucosal and cutaneous types based on their tissue tropism (1). The mucosal HPV types are well characterized and are classified into two groups: low-risk (LR) HPVs (e.g., types 6 and 11) that are mainly associated with benign genital warts, and high-risk (HR) HPVs (e.g., types 16 and 18) that are the etiological agents of cervical cancer (2), affecting ~500,000 women worldwide (3). Among the HR types, HPV16 is the most prevalent type in premalignant and malignant cervical lesions (4, 5). The product of two early genes, *E6* and *E7*, are mainly responsible for the carcinogenic activity of the virus (6). They can promote the transformation of the infected cell (2) by altering the regulation of fundamental cellular events, such as apoptosis and the cell cycle. E6 and E7 exert their functions by inducing the degradation of two important tumor suppressors, p53 and retinoblastoma (pRb), respectively (6). Persistence of the viral infection is considered a key event in virus-induced carcinogenesis. As shown by its high prevalence in cervical carcinoma, HPV16 is likelier to persist and to cause progression into cervical intraepithelial neoplasia (CIN) than other HR HPV types (7–10). It has been suggested that immune deregulation may be the other key event required for cancer development. In fact, rapid clearance by the immune system will never lead to carcinogenesis. HPV is a nonlytic virus that is permissive for viral replication only in epidermal keratinocytes. The ability of the virus to influence the immune system is therefore limited to the localized environment of the infected epidermis (11). Furthermore, localized activation of the adaptive immune response to HPV is dependent on cross-presentation of viral Ags to APCs such as Langerhans cells (LCs) in the skin (12–15). LC precursors migrate from the dermis in response to MIP3α secreted by keratinocytes (13). Although several studies have shown that HPV16 E6 and E7 play a key role in the evasion of adaptive immune surveillance (16–19), the relationships between innate immunity and HPV infection have been less investigated so far. Mammalian TLRs play a key role in host defense during pathogen infection by regulating and linking the innate and adaptive immune responses (20–22). TLRs belong to a family of receptors that recognize pathogen-associated molecular patterns and are expressed on immune and nonimmune cells, endowing them with the capacity to sense pathogen-derived products and to alert the immune system (23). Ligand specificity has been elucidated for most TLRs. TLR2 and 4 recognize Gram-positive and Gram-negative bacterial cell wall products, respectively. TLR5 recognizes a structural epitope of bacterial flagellin, while TLR3, 7, and 8 have been demonstrated to recognize different

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[3] Abbreviations used in this paper: HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; HPK, human primary keratinocyte; HR, high risk; LC, Langerhans cell; LR, low risk; VLP, virus-like particle.

Table I. *Forward primers for TLR9 deletions*

−2923	CGA CGC GTC GTG AGT GGG CGT GCA GCC TCA CGC GG G TCT
−2952	CGA CGC GTG GGG CCC CTT CCT CCA CCT CCA AAG CCA GA
−2253	CGA CGC GTC GTG AGT GGG CGT GCA GCC TCA CGC GG G TCT
−1451	CGA CGC GTT GAT CCG GTG TGC TAG CAG TTA AAA AAT GT

forms of microbial-derived nucleic acid, whereas viral or bacteria dsDNA-derived CpG motifs activate TLR9 (24). To date in man, only HSV (25) has been shown to activate TLR9, and there has been much speculation as to whether TLR9 is able to recognize and induce immune response in vivo to dsDNA viruses overall (26–28). This may be due to viral immune strategies used against TLRs that may favor viral replication and thus survival in the host (29–37).

In this study, we show for the first time that HPV16 E6 and E7 oncoproteins interfere with the activation of the innate immune response. We show that activation of the TLR9 pathway by CpG motifs is impaired severely in human keratinocytes expressing HPV16 E6 and E7 oncoproteins. This event is due to the ability of the viral oncoproteins to down-regulate TLR9 mRNA. This phenomenon was also observed in HPV16-positive cancer-derived cell lines and in primary cervical cancers. TLR9 promoter down-regulation was less significant for HR HPV 18 compared with HPV 16 and was completely absent in cells expressing E6 and E7 from the LR HPV 6. Thus, the efficiency of HPV16 in persisting appears to correlate with its ability to down-regulate the transcription of TLR9.

Materials and Methods

Constructs

The retroviral vector pBabe-puro has been previously described (38), and pLXSN was obtained from BD Clontech. The constructs pLXSN-HPV16E6/E7 and pLXSN-HPV6E6/E7 were a gift from D. Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA). To generate the pLXSN-HPV18E6/E7, pLXSN-HPV16E6, pBabe-HPV16E7, and pLXSN-HPV6E6 constructs, viral open reading frames were amplified by PCR upstream and downstream of the different genes to introduce suitable enzyme sites for cloning into pLXSN or pBabe vectors. The TLR9 promoter luciferase construct and its deletions have been previously described (39), as has the TLR9 construct (40). The forward primers used to make the new TLR9 promoter deletions are listed in Table I and prepared as described previously (39). The NF-κB reporter plasmid was obtained from BD Clontech.

TLRs ligands

Peptidoglycan (Fluka) was used at 10 μg/ml and LPS (Sigma-Aldrich) at 50 ng/ml. R848 (InvivoGen) was reconstituted in DMSO to give a stock solution at 10 mM, and a 10 μM final concentration was used to stimulate the cells. Poly(deoxyinosinic-deoxycytidylic acid) (InvivoGen) was used at 10 μg/ml. Flagellin from *Salmonella munchen* (Calbiochem) was used at 50 ng/ml. Uridine (Sigma-Aldrich) was diluted to give a 4.5 mM final concentration. Synthetic phosphodiester oligodeoxynucleotides were synthesized by InvivoGen and used at 10 μg/ml (2006 and GC 2006). CpG motifs from HPV16 E6 and E7 were designed based on motif activity described previously (41–43).

Cell culture

293 and cervical cancer-derived cell lines, HeLa, SiHa, C33a, and CaSki, were obtained from American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine (Invitrogen Life Technologies), and 1 mM sodium pyruvate (Sigma-Aldrich). KG cells expressing the HPV16 genome were grown as described previously (44). RPMI8226 B cell line was obtained from the Centre Léon Bérard and grown in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with the same components as listed above. NIH 3T3 and Phoenix cells were cultured as described previously (45). Primary human foreskin and embryonic keratinocytes were isolated and grown together with NIH 3T3 feeder cells in *FAD* medium (Cascade) as previously described (45), or when in the absence of feeder cells, keratinocytes were grown in *EPI*-LIFE medium (Cascade) supplemented with growth factors (Cambrex), 100 U/ml penicillin G, 100 μg/ml streptomycin (Invitrogen Life Technologies), and 10 ng/ml human epidermal growth factor (R&D Systems). Cells were cultured at 37°C with 5% CO_2.

Retroviral infections

High-titer retroviral supernatants (>5 × 10^6 IU/ml) were generated by transfection of pLXSN and pBabe constructs into Phoenix cells and used to infect primary human keratinocytes as described previously (45). After completion of selection (corresponding to 100% death of uninfected cells), infected keratinocytes were cultured in medium containing neomycin (for cells infected with pLXSN constructs) or puromycin (for cells infected with pBabe constructs).

RT-PCR

Total cellular RNA was extracted from cells using the RNA extraction kit (Qiagen). cDNA was synthesized with the First Strand cDNA Synthesis kit (MBI Fermentas). The primer sequences used for the RT-PCR are indicated in Table II.

Immunofluorescence

Keratinocytes were divided into a 12-well plate containing poly-lysine-coated coverslips. The following day the coverslips were washed in PBS,

Table II. *Primers used for RT-PCR for TLR1-10 and HPV oncogenes*

	Forward		Reverse
TLR		TLR	
hTLR1F	CCCATTCCGCAGTACTCCAT	hTLR1R	TTTTCCTTGGGCCATTCCA
hTLR3F	TGGTTGGGCCACCTAGAAGTA	hTLR3R	TCTCCATTCCTGGCCTGTG
hTLR4F	CTGCAATGGATCAAGGACCA	hTLR4R	TTATCTGAAGGTGTTGCACATTCC
hTLR5F	TGCCTTGAAGCCTTCAGTTATG	hTLR5R	CCACCACCATGATGAGAGCA
hTLR6F	CCCTCAACCACATAGAAACG	hTLR6R	GAGATATTCCACAGGTTTGG
hTLR7F	TTACCTGGATGGAAACCAGCTACT	hTLR7R	TCAAGGCTGAGAAGCTGTAAGCTA
hTLR8F	AACTTTCTATGATGCTTACATTTCTTATGAC	hTLR8R	GGTGGTAGCGCAGCTCATTT
hTLR9F	TGAAGACTTCAGGCCCAACTG	hTLR9R	TGCACGGTCACCAGGTTGT
hTLR10F	TTTGATCTGCCCTGGTATCTCA	hTLR10R	AGTTGTTCTTGGGTTGTTTTCCTAAC
HPV		HPV	
HPV16E6F	CCCACAGGAGCGACCCAGAAAGTTACC	HPV16E6R	CCCATCTCTATATACTATGCATAAATCCC
HPV6E7F	ATGCATGGCAGGCACGTGACCCTGAAG	HPV6E7F	TTGGGGGCGCAGATGGGGCACACGATGTTC
HPV16E7F	See Gheit et al. (48)	HPV16E7R	See Gheit et al (48)
HPV186F	See Gheit et al. (48)	HPV18E6R	See Gheit et al. (48)

fixed with 3.7% paraformaldehyde, and stained for TLR3 and 9 (Imgenex). Briefly, primary Abs were diluted 1/50 with 0.1% saponin in PBS, added to cells, and incubated for 1 h at room temperature. Cells were washed in 0.1% saponin/PBS, and the secondary Ab-Alexa 488 conjugate (1/500 dilution) was added and incubated for 30 min in the dark at room temperature. Cells were washed, the coverslips were mounted onto slides using a 1/10 dilution of 4′,6′-diamidino-2-phenylindole (nuclear stain; Invitrogen Life Technologies) in fluoromount (Southern Biotechnology Associates), and protein expression was detected by direct fluorescence microscopy. Photographs were taken at ×40 magnification using IRIS imaging.

Immunoblot analysis

Biochemical analysis of harvested cells was performed as described previously (46). Briefly, harvested cells were lysed in mild lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 0.5 mM PMSF, 1% aprotinin, 20 mM NaF, and 0.3 mM orthosodium-vanadate. Between 50 and 80 μg of total cellular protein (determined by the Bradford assay; Bio-Rad) was used for SDS-PAGE and immunoblotting (Invitrogen Life Technologies). After incubation with primary Abs, proteins were detected with peroxidase-conjugated goat anti-rabbit or anti-mouse secondary Abs (Jackson ImmunoResearch Laboratories) and ECL (Amersham Biosciences).

Luciferase assay

Cells were transiently transfected with the TLR9 luciferase construct using FuGene (Roche) as described previously (39). Cells were cotransfected with HPV constructs where indicated. Promega Dual Glo reporter assay reagents were used to read luminescence. HEK 293 and primary keratinocytes were transfected with NF-κB luc as described previously (47). Each experiment was repeated three times in triplicate; results generally deviated by $<$10% of the mean value.

ELISA

To measure secreted cytokines, cells were seeded at 4×10^4 cells/96-well in 200 μl of EPI-LIFE growth medium. The following day the cells were stimulated in triplicate with TLR ligands for 24 h, and supernatants were collected for analysis of IL-8 or MIP3α secretion using Quantikine ELISA kits obtained from R&D Systems. After sample handling, noninfected and infected cells from each donor were trypsinized and counted (5 wells per donor). Cytokine secretion was normalized by dividing the number of infected cells by noninfected cells.

Histological and immunohistochemical analysis

Biopsies of normal and cancer cervical tissues were taken from patients. Samples were snap frozen with liquid nitrogen and stored at −80°C until required. Sections of 5-μm thickness were cut and either stained with H&E for histology or used for immunostaining. Staining was performed using biotin-labeled goat anti-mouse IgG and ABComplex AP amplification (DakoCytomation). The number of positive cells was determined by counting under ×40 magnification in five to six different fields of epidermis.

Genotyping

Tumor samples were genotyped using multiplex PCR with HPV type-specific primers for amplification of viral DNA and array primer extension for typing (48).

Results

TLR-regulated pathways are altered in keratinocytes expressing HPV16 E6 and E7 oncoproteins

Human keratinocytes are naturally infected by HPV (49–53). We therefore selected foreskin or embryonic human primary keratinocytes (HPKs) as an in vitro model to determine the impact of HPV16 E6 and E7 on TLR-regulated pathways. HPKs isolated from three different donors were infected with recombinant retrovirus expressing HPV16 E6 and E7 oncoproteins. As a control, HPKs from one donor were also infected with an empty retrovirus (pLXSN). Expression of the viral genes was determined by RT-PCR (Fig. 1*A*). As a first step, we characterized the functionality of TLR2–9 pathways in HPKs and pLXSN-HPKs by adding their specific ligands and monitoring the secretion of two cytokines, IL-8 and MIP3α (Fig. 1, *B* and *C*, respectively). As previously mentioned, LC precursors migrate from the dermis in response to MIP3α secreted by keratinocytes. Serial dilutions of TLR ligands were used to activate cells, and supernatants were collected at 24, 48, and 72 h poststimulation. Fig. 1, *B* and *C*, represents cytokine secretion 24 h poststimulation at optimal TLR ligand concentration for IL-8 and MIP3α, respectively. The addition of the TLR9 ligand CpG oligo 2006 led to a clear increase in secreted cytokines in noninfected and pLXSN-infected cells, while a moderate increase was observed in the presence of the other ligands for TLR3, 4, and 5. No activity was observed for TLR2. Strikingly, no cytokine secretion was observed in HPV16 E6/E7-infected cells when stimulated with the ligand for TLR9, indicating that the viral proteins are able to strongly inhibit the TLR9 pathway. These data were also confirmed by performing a luciferase assay for NF-κB activity in the same cells. In fact, luciferase activity was only detected in mock cells but not in keratinocytes expressing HPV16 E6 and E7 when stimulated with the CpG oligo 2006 (Fig. 1*D*). In contrast to TLR9 pathway inhibition, TLR3 and 5 pathways were clearly up-regulated in HPV16 E6/E7 HPKs in comparison to mock cells. In addition TLR8 activity was observed only in infected cells. Up-regulation of TLR3-mediated pathways via viral infections has also previously been observed for influenza A, SIV, and measles (29, 54–56).

In summary, these data show that HPV16 E6 and E7 alter several TLR pathways, and in particular they strongly inhibit TLR9 activity.

TLR9 is down-regulated in HPV16 E6/E7 keratinocytes

Since HPV is a dsDNA virus, it is likely that down-regulation of the TLR9 pathway in HPV16 E6/E7 HPKs may play a key role during the natural infection. Therefore, we focused our study on the characterization of the events involved in the alteration of the TLR9 pathway.

As a first step, we performed immunoblotting for TLR9 expression to determine whether the loss of functional TLR9 pathways in HPV16 E6/E7 HPKs was due to altered TLR9 expression. As an additional control, we also checked the protein levels of TLR3 because its regulated pathway appears to be up-regulated in HPV16 E6/E7 HPKs (Fig. 2*A*). TLR9 was detected in total extracts from mock cells (pLXSN), while no positive signal was observed in HPV16 E6/E7 keratinocytes. In contrast, TLR3 protein levels were elevated in HPV16 E6/E7 keratinocytes in comparison to control cells (Fig. 2*A*). Thus, the expression levels of TLR3 and TLR9 correlate with the functionality of their regulated pathways.

In agreement with the endosomic TLR3 and 9 localization (57, 58), immunofluorescence staining with TLR3 or 9 Ab showed cytoplasmic staining in control cells, while no TLR9 staining was detected in HPV16 E6/E7 HPKs, confirming our Western blot data (Fig. 2*B*).

Several independent studies have demonstrated that HPV16 E6 and E7 interact with p53 and retinoblastoma (pRb), respectively, and promote their degradation via the proteasome pathway (6). Therefore, we next determined whether a similar mechanism was responsible for the strong decrease in TLR9 levels in HPV16 E6/E7 HPKs. The addition of a specific proteasome inhibitor on HPV16 E6/E7 keratinocytes did not restore the normal levels of TLR9 protein, indicating that the viral proteins did not alter the TLR9 protein stability (Fig. 2*C*).

An alternative hypothesis for the down-regulation of TLR9 is that HPV16 E6 and E7 proteins can alter its mRNA levels. To test this hypothesis, we determined the mRNA levels of TLRs by RT-

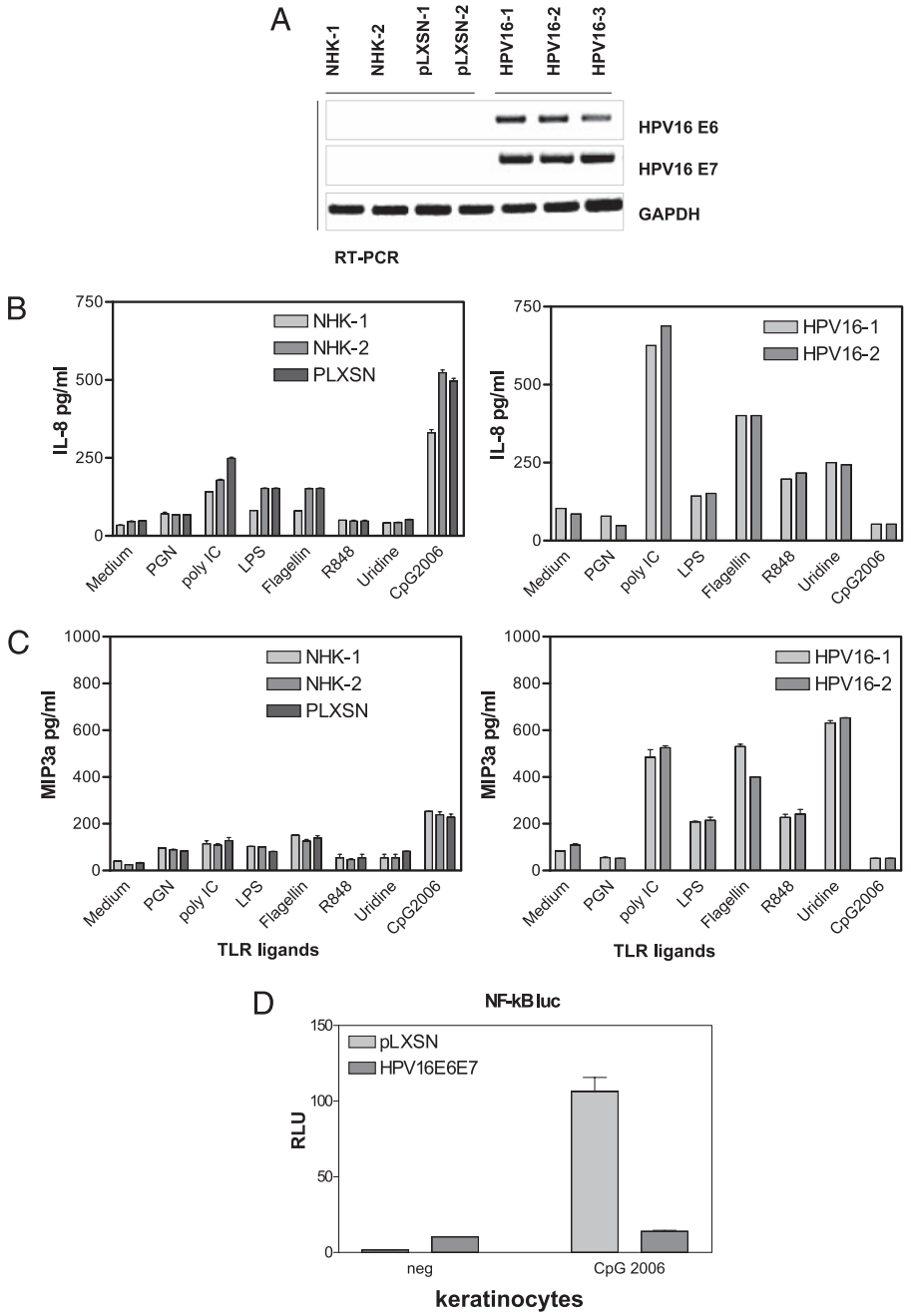

FIGURE 1. TLR-regulated pathways are altered in keratinocytes expressing HPV16 E6 and E7. *A*, RT-PCR analyses. Total RNA was extracted from primary (normal human keratinocytes, NHK)-, pLXSN-, or HPV16 E6/E7-infected keratinocytes. After reverse transcription, PCR was performed using HPV16 E6- or E7-specific primers. As positive control, GAPDH was amplified at the exponential phase of 20 cycles. *B* and *C*, Functionality of TLR-regulated pathways. Primary keratinocytes, noninfected and infected with pLXSN or HPV16E6E7 retrovirus, were stimulated for 24 h with TLR ligands as indicated in the figure. Measuring IL-8 (*B*) and MIP3α (*C*) levels in culture medium by ELISA monitored the activation of the TLR-regulated pathways. *D*, Transient expression of NF-κB luciferase in primary keratinocytes infected with pLXSN or pLXSNHPV16E6/E7 stimulated with CpG oligo 2006 for 6 h. Experiments were repeated three times in triplicate. Results shown here represent the SEM of one of three experiments performed.

-PCR in control and HPV16 E6/E7 HPKs. We observed that HPKs express TLR2–7 and 9, whereas no mRNA was detected for TLR1, 8, and 10. This TLR expression pattern was identical in three different donors, and the transduction with empty retrovirus (pLXSN) did not alter the expression profile of the TLRs (Fig. 3*A*). In contrast, several changes in TLR mRNA levels were observed in HPV16 E6/E7-infected keratinocytes (Fig. 3*A*). In particular, TLR8 mRNA was clearly up-regulated, which is consistent with its activity as shown in Fig. 1, *B* and *C*. No TLR9 transcript was found in any of the three HPV16 E6/E7 keratinocyte lines generated independently using primary cells from different donors (Fig. 3*A*).

Two different scenarios can be envisaged in the alteration of TLR expression induced by HPV16. E6 and E7 may act directly on cellular pathways involved in TLR expression regulation or, alternatively, they may promote the secretion of soluble autocrine factors that in turn influence the TLR expression. To discriminate between these two hypotheses, we exposed primary keratinocytes infected with empty retrovirus (pLXSN) to the culture medium of pLXSN or HPV16 E6/E7 HPKs. The supernatant of control cells did not lead to any TLR expression alteration. In contrast, culture medium of HPV16 E6/E7 cells induced up-regulation of TLR8 in pLXSN-HPKs, while no changes in the levels of TLR9 mRNA were observed (Fig. 3*B*). These data showed that the two viral proteins are directly involved in TLR9 down-regulation, whereas the increase in TLR8 levels is most probably linked to the secretion of an autocrine factor(s).

E6 and E7 of the HR HPV18 have reduced efficiency in down-regulating TLR9 expression

Several epidemiological studies have shown that HPV16 persists more than the other mucosal high-risk HPV types (7, 10) and causes progression into CIN (8). We tested the ability of the mucosal high-risk type HPV18 to down-regulate TLR9, which is, after

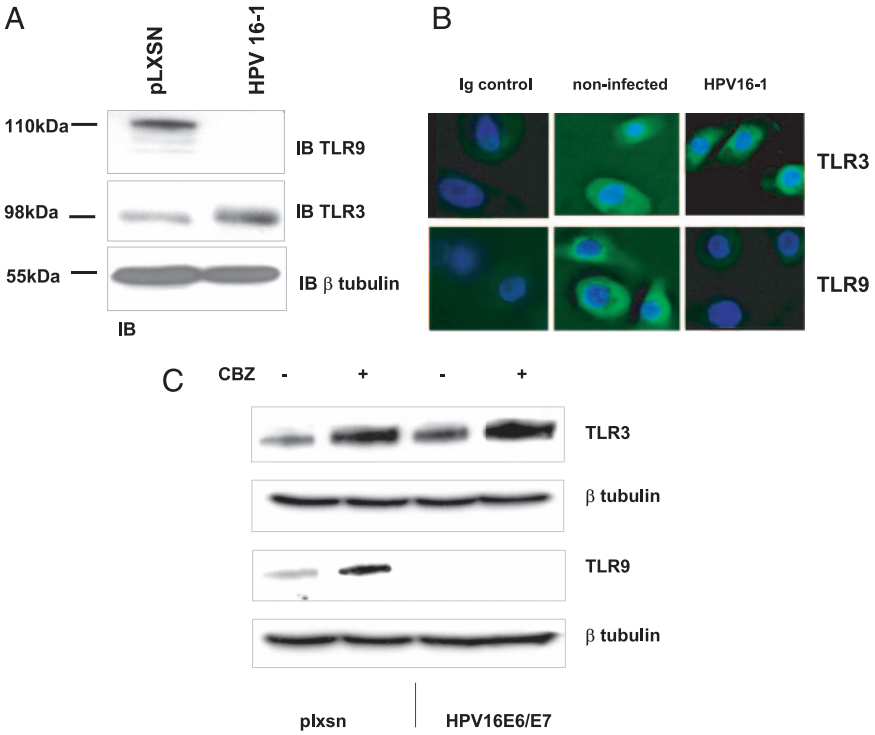

FIGURE 2. TLR9 is down-regulated in HPV16 E6/E7 keratinocytes. *A*, Determination of TLR3 and -9 levels by immunoblotting using 80 μg of total protein extracts from keratinocytes infected with empty retrovirus (pLXSN) or expressing HPV16 E6 and E7. *B*, Immunofluorescence staining of primary and HPV16 E6/E7 keratinocytes. For the immunofluorescence staining, keratinocytes were grown on coverslides, fixed, and incubated with anti TLR3 and TLR9 Abs, and a secondary FITC-conjugated anti-rabbit Ab was used. As negative control, primary keratinocytes were incubated with secondary Ab only (*left panel*). *C*, Determination of TLR3- and -9-protein stability in presence of the proteasome inhibitor CBZ. Cells were cultured for 24 h in the absence (−) or presence (+) of CBZ. Posttreated cells were harvested, and TLR3 and -9 levels were examined by immunoblotting as described in *A*.

HPV16, the second most frequently detected type in cervical cancers worldwide (4, 5). Primary keratinocytes from three different donors were infected with HPV18 E6/E7 recombinant retrovirus and the TLR pattern expression was determined by RT-PCR (Fig. 3*C*). A fourth independent infection with HPV16 E6 and E7 was produced in another laboratory and used as a control. In these three HPV18 E6/E7-infected cell lines, TLR9 mRNA levels were slightly reduced or similar to the control cells. In addition, no TLR8 up-regulation was observed (Fig. 3*C*). In summary, HPV18 has clearly reduced efficiency, in comparison to HPV16, to alter the expression pattern of TLRs.

HPV16 E6 and E7 down-regulate the TLR9 promoter

HPV16 may decrease TLR9 by two distinct mechanisms; E6 and/or E7 can affect the stability of TLR9 transcript or can directly inhibit TLR9 transcription. To discriminate between these two possibilities, we have determined whether HPV16 E6 and E7 proteins influence the activity of the TLR9 promoter. The isolated TLR9 promoter cloned in front of luciferase reporter gene was introduced by transient transfection in the B cell line RPMI8226 that expresses high levels of endogenous TLR9 (39). High basal luciferase activity was detected in these cells after transient transfection, but HPV16 E6 and E7 inhibited luminescence in a dose-dependent manner (Fig. 4*A*), indicating that E6 and/or E7 can block directly the transcription of the TLR9 gene. Subsequently, we checked in transient transfection experiments whether the inhibition of TLR9 expression could be ascribed to HPV16 E6 or E7. Fig. 4*B* shows that both HPV16 proteins have the ability to inhibit the TLR9 promoter, although E6 appeared to be slightly more efficient than E7. We also compared the efficiency of HR HPV18 and LR HPV6 in repressing TLR9 transcriptional activity. Since HPV6 E7 is not effectively transcribed by the polycistronic E6/E7 mRNA, we separately cloned HPV6 E7 in retroviral vectors (pLXSN or pBabe). HPV18 E6/E7 inhibited TLR9 transcription less efficiently than HPV16 E6/E7 (Fig. 4*C*). In addition, E6 and E7 of the low-risk HPV6 did not have any effect on TLR9 transcription (Fig. 4*C*). Similarly to what was observed in primary keratinocytes, expression of the two viral proteins in RPMI8226 cells led to a decrease in endogenous TLR9 levels (Fig. 4*D*). Transient expression of transfected constructs was controlled by RT-PCR for E7 expression (Fig. 4*E*).

Although the *cis*-acting elements previously described to regulate TLR9 transcription (39) are found in the regions −700 to −1 of the TLR9 promoter, in this study, the deletions made in the TLR9 promoter showed that the element(s) involved in the HPV16-mediated inhibition is/are located within a region of ~2 kb upstream of this region (Fig. 5, *A* and *B*). We also observed that E6 or E7 might have the ability to inhibit TLR9 transcription potentially via distinct elements (Fig. 5*B*). Therefore, we made further deletions in the 2-kb portion to determine which region of the promoter is required by HPV16 to inhibit TLR9 transcription. We chose to look for *cis*-acting elements that were previously characterized by Takeshita et al. (39) responsible for TLR9 transcription, including AP-1, NF-κB, PU-BOX, SP-1, CRE, and C/EBP (39). As TLR9 suppression by HPV16E6 and/or E7 did not affect this region, we looked to see whether we could identify the same *cis*-acting elements in the 2-kb region of interest. We have identified three other NF-κB sites along the promoter as well as three PU-BOX and one SP-1 site (Fig. 5*C*). Asterisked deletions are those constructed by Takeshita et al. (39). Deletion of the 5′ end from −3227 to −2923 resulted in a 7-fold increase in luciferase activity, a further 2-fold increase was observed from −2923 to −1451 (Fig. 5*D*). However, a significant increase of 17-fold was observed from −1451 to −1017, indicating the removal of *cis*-acting elements involved in the HPV16 suppression of the TLR9 promoter. Interestingly, the removal of a total of three NF-κB binding sites coincided with higher fold increase in luciferase activity from the region −3227 to −2923 and −1451 to −1017. These data show that the inhibition of the TLR9-regulated pathway observed in HPV16 E6/E7 keratinocytes is due to down-regulation of the TLR9 transcript in the region of −3227 to −2923 and −1451 to −1017.

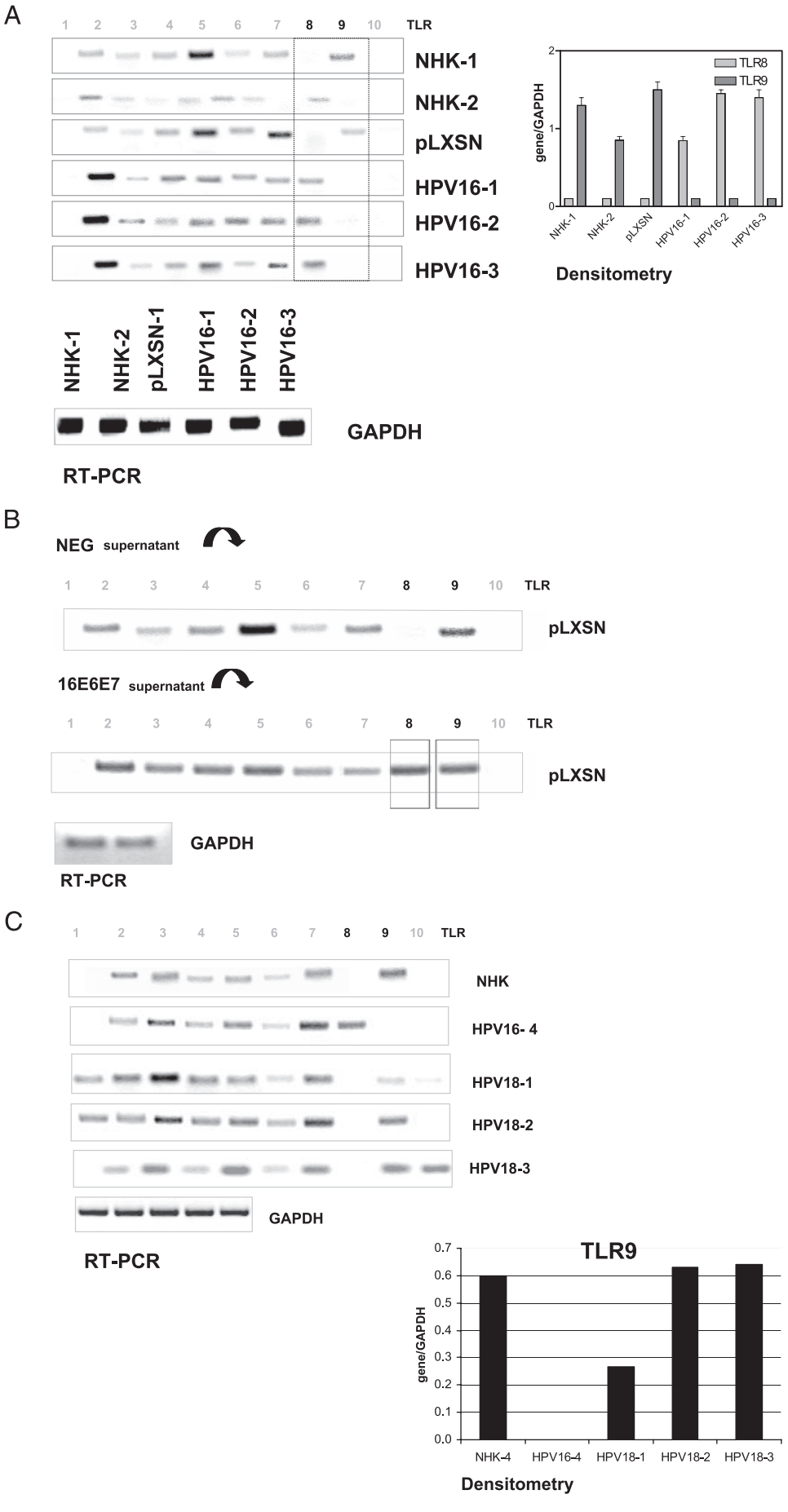

FIGURE 3. E6 and E7 of HPV16 and HPV18 down-regulate TLR9 mRNA levels with different efficiency. *A*, Determination of TLR mRNAs in primary, pLXSN, and HPV16 E6/E7 keratinocytes. The mRNA levels of different TLRs were examined by RT-PCR. HPV16-1, -2, and -3 cells were obtained by three independent infections of keratinocytes from three different donors. Densitometry levels were determined using the Bio-Rad phosphoimaging software. *B*, Effect of autocrine factors induced by HPV16 E6 and E7 on TLR expression in primary keratinocytes. Supernatants from pLXSN or HPV16 E6/E7 keratinocytes were transferred into a plate containing pLXSN keratinocytes seeded at 60% confluency. After 24 h, cells were harvested for RNA extraction, and RT-PCR was performed for TLR expression. *C*, Determination of mRNA levels of the different TLRs in HPV18 E6/E7 keratinocytes. The mRNA levels of different TLRs were examined by semiquantitative RT-PCR in pLXSN and HPV18 E6/E7 keratinocytes. HPV18-1, -2, and -3 cells were obtained by three independent infections of keratinocytes from three different donors. Densitometry levels were determined as described in *A*.

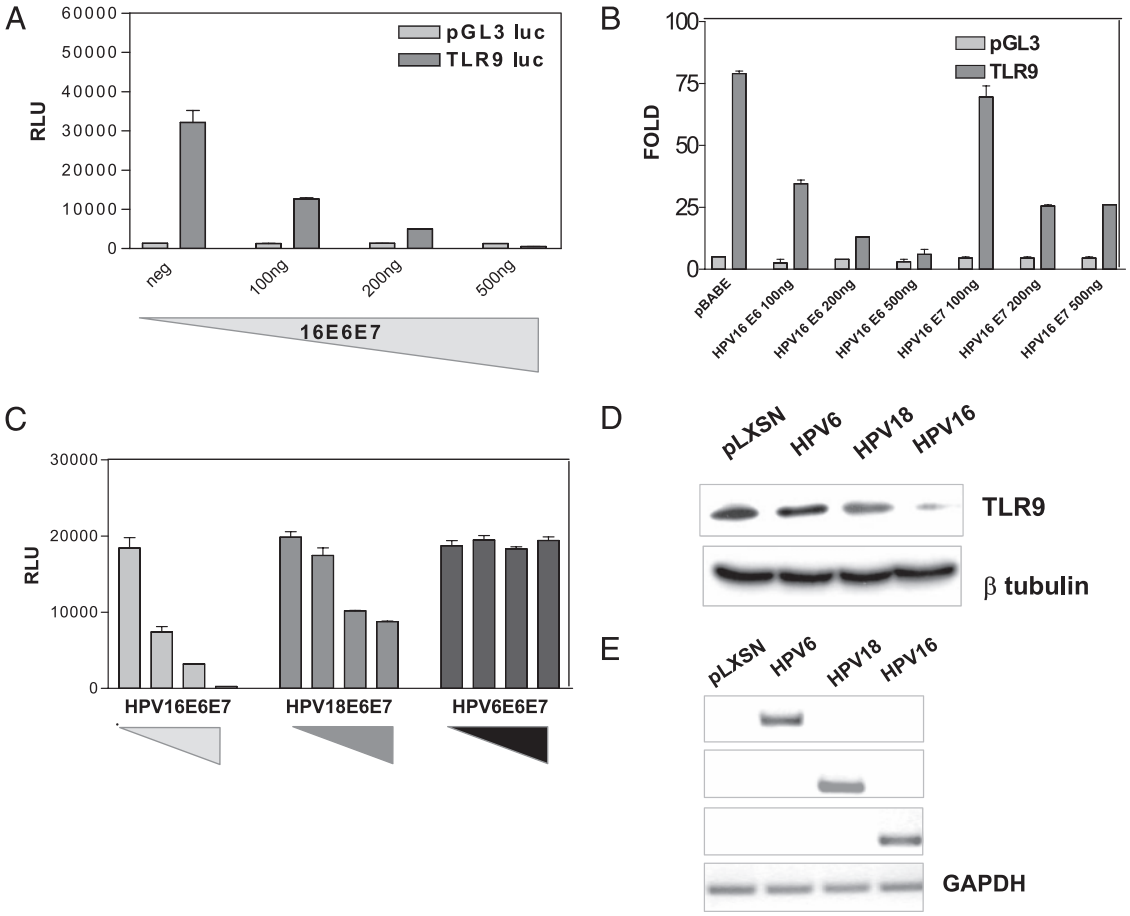

FIGURE 4. HPV16 E6 and E7 down-regulate the TLR9 promoter in RPMI8226 B cells. *A*, Impact of HPV16 E6 and E7 on TLR9 promoter activity. RPMI8226 B cells were cotransfected with TLR9 promoter cloned in front of the luciferase reporter gene with increasing concentrations of pLXSN HPV16 E6/E7 or as indicated. After 48 h, cells were harvested, and luciferase activity was measured. As a control, the vector containing only the luciferase gene (pGL3) was cotransfected with pLXSN HPV16 E6/E7. The data are the mean of three independent experiments performed in triplicate. *B*, Effect of HPV16 E6 or E7 on TLR9 promoter activity. RPMI8226 B cells were cotransfected with TLR9 promoter/luciferase construct with increasing concentrations of pLXSN HPV16 E6 or E7 as indicated. After 48 h, cells were harvested, and luciferase activity was measured as described in *A*. The data are the mean of three independent experiments. *C*, Efficiency of E6 and E7 from other mucosal HPV types in inhibiting TLR9 promoter. RPMI8226 B cells were cotransfected with TLR9 promoter/luciferase construct with increasing concentrations of pLXSN HPV6, HPV16, or HPV18 E6/E7 as previously used and processed as described in *A*. The data are the mean of three independent experiments. *D*, Determination of TLR9 endogenous levels in RPMI8226 B cells by immunoblotting. Fifty micrograms of total protein extracts from RPMI8226 B cells infected with empty retrovirus (pLXSN) or expressing HPV16 E6 and E7 was used. *E*, RT-PCR control of E7 expression in RPMI8226 B cells transfected as described in *D*.

TLR9 is down-regulated in HPV16-positive cervical cancer-derived cell lines and in human cervical cancers

To corroborate our data, we analyzed the levels of TLR9 in three cell lines derived from cervical cancer, HeLa, SiHa, and CaSki. The first line is positive for HPV18, whereas SiHa and CaSki contain integrated HPV16 DNA. RT-PCR analysis showed that TLR9 is weakly expressed in SiHa and completely down-regulated in CaSki cells (Fig. 6*A*). In addition, TLR9 expression in HeLa cells was more pronounced than SiHa, confirming the lower efficiency of HPV18 in inhibiting TLR9 transcription. In addition, in transient transfection experiments, the TLR9 promoter activity resembled the levels of endogenous mRNA in these cervical cancer-derived cell lines. In contrast, high TLR9 promoter activity was detected in the cervical cancer cell line negative for HPV (C33a) (Fig. 6*B*). Furthermore, immunoblotting showed a reduction in TLR9 protein levels from cervical cell lines infected with HPV16, with CaSki cells expressing the least amount (Fig. 6*C*). C33a, however, expressed a high level of TLR9 protein. TLR9 expression was also analyzed in normal and cancer cervical tissue by immunohistochemistry. To substantiate our data shown for E6 and E7 only expressing cells (integrated form of the virus), we also examined TLR9 expression in cells that express the entire genome (44). We observed that TLR9 mRNA levels were also decreased in cells that maintain the HPV16 genome as a multicopy in the episome (Fig. 6*D*).

Tumor and normal tissue biopsies were taken from 12 patients with cervical cancer and snap frozen. After pathological analysis and HPV typing, sections were stained by immunohistochemistry for TLR9. We selected tissues that were negative for HPV (3 patients) and gave a normal histological profile as a control and HPV16-positive samples (4 patients) with tumor formation for TLR9 staining. In agreement with the endoplasmic localization of TLR9, basal cells of normal epidermis showed strong cytoplasmic staining. Nuclear staining, most likely nonspecific, was also observed. No cytoplasmic staining was observed in HPV16-positive cancer cells (representative staining are shown in Fig. 6*E*). Quantification of the cytoplasmic staining clearly showed that TLR9 expression was strongly down-regulated in cancerous as compared with normal tissue, fully confirming the in vitro data.

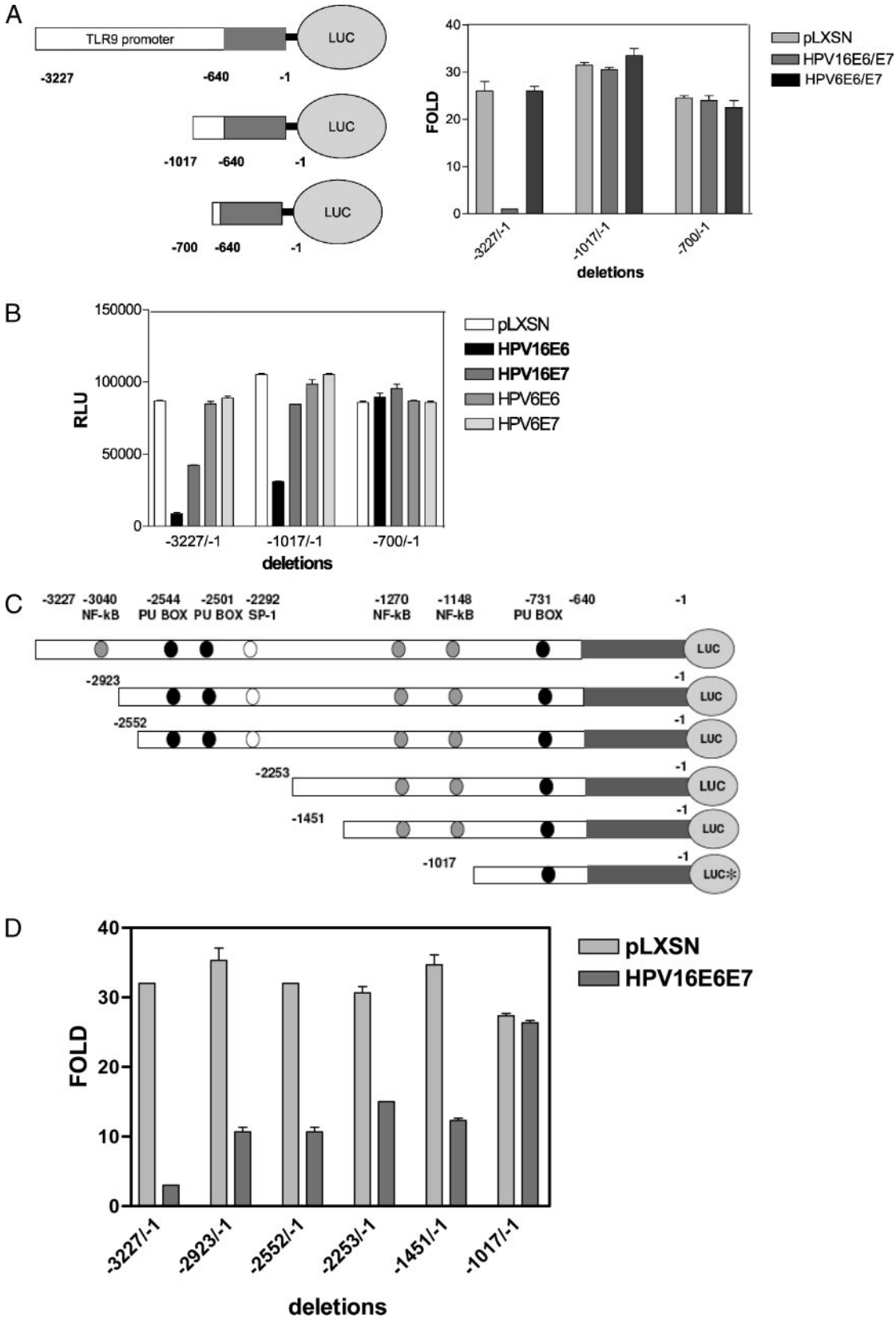

FIGURE 5. Deletion in TLR9 promoter abolished the HPV16 E6/E7 inhibition. *A*, Schematic representation of TLR9 deletion mutants. The constructs shown in *A* were cotransfected with pLXSN HPV16 E6/E7 in RPMI8226 cells. After 48 h, cells were processed as described in the legend of Fig. 4*A*. *B*, The constructs shown in *A* were cotransfected with 500 ng of pLXSN HPV16 or HPV6 E6 or E7 in RPMI8226 cells. After 48 h, cells were processed as described in the legend of Fig. 4*A*. *C*, Schematic representation of new TLR9 deletions. *D*, The constructs shown in Fig. 5*C* were cotransfected with 500 ng of pLXSN or HPV16E6/E7 in RPMI8226 cells. After 48 h, cells were processed as described in the legend of Fig. 4*A*.

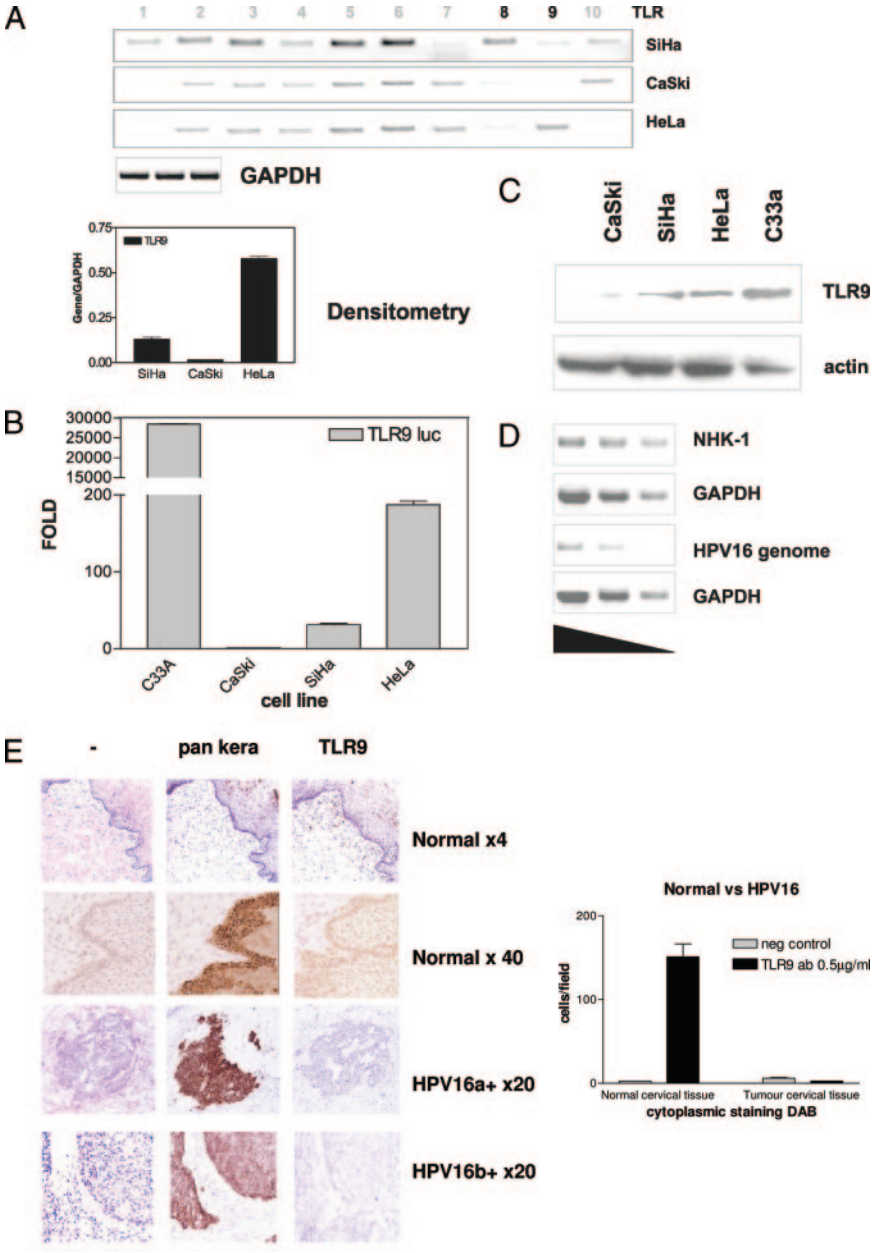

FIGURE 6. TLR9 is down-regulated in HPV16-positive cervical cancers and derived cell lines. *A*, Determination of TLR mRNAs in cervical cancer-derived cell lines. The mRNA levels of different TLRs were examined by RT-PCR. *B*, Efficiency of cervical cancer-derived cell lines to inhibit the TLR9 promoter. SiHa, HeLa, C33a, and CaSki cells were transfected with the TLR9 promoter/luciferase as described in the legend of Fig. 4*A*. The data are the mean of three independent experiments. *C*, TLR9 protein expression reflects promoter deregulation by HPV16 plus cancer-derived cell lines. Western blot analysis was performed using 50 μg of protein. *D*, Determination of TLR9 mRNA in KG cells expressing episomal HPV16. The mRNA levels of TLR9 were examined by semi-quantitative RT-PCR. Serial fold dilutions from 1/5 to 1/125 are shown. *E*, Biopsies from patients with cervical cancer were stained for normal and tumoral histology. After pathological analysis, sections were stained by immunohistochemistry for pan keratin (NovaCastra) or TLR9. *Left panel*, Two representative staining from different HPV16-positive patients (*a* and *b*) are shown. *Right panel*, Five random ×20 field sections were counted for cells positive for TLR9-cytoplasmic staining. We observed that cells in the basement membrane from normal tissue expressed TLR9. No staining was seen in the tumor biopsy from the same patient.

CpG motifs derived from HPV16 activate TLR9

We have shown that the oncoproteins E6 and E7 from HPV16 can down-regulate the TLR9 receptor at the transcriptional level; we speculated that this dsDNA virus may use TLR9 deregulation as a strategy to avoid viral recognition. Analysis of the HPV16 8-kb genome revealed the presence of 109 CpG motifs, of which 70% were defined as stimulatory (41–43). As a preliminary experiment, CpG motifs in the E6 and E7 regions of the HPV6 and 16 genomes were identified and selected based on stimulatory predictions (Fig. 7*A*). Of the three stimulatory CpG motifs selected, one was found to activate at high levels the NF-κB promoter via TLR9 (Fig. 7*B*). These data were confirmed in RPMI8226 cells transfected also with the NF-κB luc promoter, and similar activity was observed (data not shown). No activity with control CpG motifs designed based on the E6 and E7 DNA sequence from HPV6 was observed as well as the GC 2006 negative control (data not shown). The CpG motif 16E62p stems from the HPV16 E6-encoding sequence. This motif was also shown to be active on noninfected primary keratinocytes transfected with the reporter gene NF-κB luc but not in HPV16 E6- and E7-expressing keratinocytes (Fig. 7*C* and data not shown). These initial data indicate that TLR9 is able to recognize DNA motifs from HPV16.

Discussion

Cancer is the second most common cause of death in industrialized countries, and 15% of these cancers are due to human tumor viruses, of which approximately one-third are HPV associated (59). However, the majority of HPV infections do not lead to cytological anomalies or cancer because they are cleared by the immune system in a relatively short time (6–18 mo). Only a small percentage of infections promote the development of low- and/or high-grade CIN, which may regress or progress to invasive carcinoma (60). Thus, as the failure of the immune response is a key component for the development of these cancers, enhancing immunity is imperative to prevent viral cellular transformation. Several studies have already shown that E6 and E7 from HPV16 and 18 are able to efficiently subvert the immune response by binding and blocking IFN regulatory factor-3 and -1 regulatory signaling pathways, respectively, and thus the induction of the antiviral IFN response

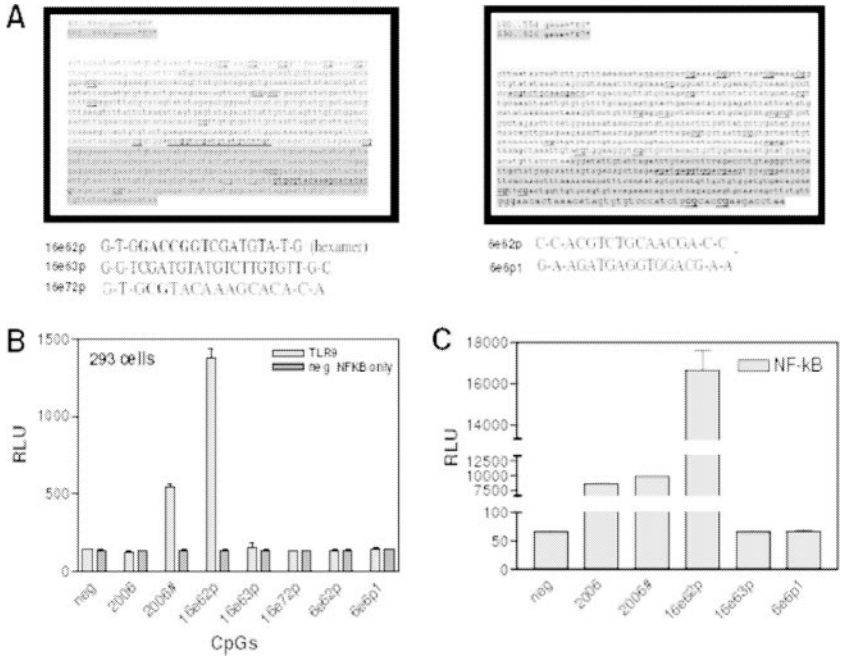

FIGURE 7. HPV16-derived CpG motifs activate TLR9. *A*, *Left panel*, HPV16 E6/E7 stimulatory sequences, 16E62p and 16e63p, were designed based on the first overlapping sequence. *Right panel*, HPV6 E6/E7 stimulatory sequences. *B*, HEK293 cells at 70% confluency were transiently transfected with TLR9 and NF-κB luciferase in a 10-cm dish, and 6 h posttransfection, cells were trypsinized and divided into a 48-well dish. The following day, cells were stimulated for 6 h with CpG oligos, and luciferase activity was measured as outlined in Fig. 4*A*. *C*, Primary keratinocytes at 70% confluency were transiently transfected with NF-κB luciferase in a 6-cm dish, and 6 h posttransfection, cells were trypsinized and divided into a 48-well dish. The following day, cells were stimulated for 6 h with CpG oligos in triplicate, and luciferase activity was measured as outlined in Fig. 4*A*.

(11, 19, 61–70). HPV16 E5-mediated immune evasion also involves suppressing the expression of the MHC class I and Ag processing via the TAP pathway, reflecting the lack of Ag presentation to CTLs (11, 16). In this study, we show that immune deregulatory events may occur before those previously described, in that HPV16 has the ability to interfere with the first response to infectious agents via TLRs. HPV16 E6 and E7 directly inhibit TLR9-mediated pathways by down-regulating the transcription of the TLR9 gene. The fact that TLR9 levels were reduced in HPV16-positive cervical cancer-derived cell lines and cervical tumors provides important evidence for the *in vivo* relevance of this phenomenon.

It is now evident that the majority of HPV types classified as HR for their phylogeny and association with cervical cancers display similar *in vitro*-transforming activity to HPV16 mediated by E6 and E7 (71–75). Despite the similar behavior of the HR HPV types in in vitro experimental models, their in vivo carcinogenic potential is clearly different. Certainly, also their efficiency in escaping immune surveillance contributes to the carcinogenic potential of specific HR HPV types. HPV16 is definitely more aggressive than HPV18, being present in ~50% of cervical cancers worldwide, compared with 20% for HPV18 (4, 5). Our data clearly show that E6 and E7 from HPV18 have a reduced activity to down-regulate TLR9 levels in comparison to HPV16, reflecting their in vivo carcinogenicity. Interestingly, the LR HPV type 6 that is normally associated with benign lesions did not affect TLR9 transcription.

Deletions within the TLR9 promoter indicate that the binding elements involved in the TLR9 repression mediated by HPV16 E6 and/or E7 are 2 kb upstream of the −700 to −1 regulatory region as previously identified (39). As shown in Fig. 5*C*, a number of putative binding sites for these transcription factors were found were found in the 2-kb region. Deletion of the PU-BOX and the SP-1 sites provoked a 2-fold increase in the presence of HPV16E6 and E7; however, removal of the three NF-κB sites at position −3040, −1270, and −1148 reconstituted the TLR9 luciferase activity back to normal levels as compared with the negative control. Furthermore, Takeshita et al. (39) identified NF-κB as being one of the suppressive transcription factors' associated with TLR9 down-regulation. Over expression of NF-κB along with the regulatory region of TLR9 reporter gene resulted in reduced luciferase activity. In addition it has been reported that NF-κB levels are up-regulated in HPV16E6/E7 infected keratinocytes (76) as well as HPV16-positive patients with oral cancer (77). Carefully reanalyzing of the TLR9 promoter between −3221 and −1017 needs to be performed as well as generating point mutations in the sites already determined as potential regulators. This will enable the precise identification of new *cis*-elements and clarify the role of NF-κB induced TLR9 down-regulation, in the context of HPV16-induced cancers.

In addition to TLR9 down-regulation, HPV16 may evade the innate immune system by other means. TLR immune evasion in mice has been previously described for HPV16L1 virus-like particles (VLPs). Yang et al. (18) described immune activity by HPV16L1 VLPs mediated by TLR4-MyD88 in B cell- and bone marrow-derived dendritic cells, in contrast to responses with bone marrow-derived dendritic cells where immune responses were absent when stimulated with natural HPV16VLPL1 variants (19).

Similarly to what we have observed for HPV16, it is becoming clear that many other viruses have adopted ways to escape TLR recognition. Vaccinia viral proteins A56R and A42R are key players in blocking TLR adaptor and IL-1R-associated kinase-2-mediated responses, respectively (78). Chronic hepatitis C infection leads to expression and functional impairment of TLR2 (32, 79, 80). Respiratory syncytial and measles viruses are also able to block IFN-mediated responses induced via TLR-dependent and -independent mechanisms (81).

In contrast to TLR9 down-regulation, our findings show that TLR3, 5, and 8 pathways are activated in HPV16 E6/E7 HPKs in comparison to primary cells. The stimulation of these pathways correlated with an increase in mRNA of the corresponding TLRs. We still do not understand the biological significance of up-regulation of TLR3, 5, and 8, but it may be an attempt by the infected cells to counteract the TLR9 down-regulation and to initiate immune responses.

Furthermore, we have identified a CpG motif from HPV16 E6 that was able to stimulate TLR9 responses; we are currently investigating whether other TLR9-stimulating motifs are present in the rest of the HPV16 genome. The mechanism by which viral CpG-DNA motifs enter the endosomal compartment, where TLR9 is expressed, is presently unclear. We speculate that, during infection, disassembly of the viral capsid in the endosome results in

viral DNA release, and this may initiate TLR9 signaling (82). Although at this point, no E6 and E7 would be present, it has been shown that as early as 8 h post infection E6/E7 transcripts can be detected that could then repress TLR9 transcription and interfere with TLR9 signaling (82). An alternative hypothesis could be that TLR9 down-regulation might help to establish persistent infections. In the nonproductive persistent phase, HPV replicates as extra chromosomal plasmids with ~100 copies/cell. Upon cell division, papillomavirus genomes are attached to mitotic chromosomes to facilitate successful segregation to the nuclei of the daughter cells (83). Segregation of viral plasmids depends on tethering to chromosomes and is regulated by phosphorylation. If some viral genomes are lost from mitotic chromosomes during mitosis, they may end up in the cytoplasm and could be transported to the endosome to potentially activate TLR9 signaling.

Understanding TLR biology holds future promise for the prevention of infectious diseases, cancer, and autoimmune diseases because there is also strong evidence for immune clearance of viral infections in therapy. In summary, we show that HPV16 targets innate immunity by down-regulating the expression of TLR9. Full elucidation of the molecular mechanism of HPV16-induced TLR9 down-regulation will have an important impact on the development of novel therapeutic strategies for HPV and possibly other viral infections. In addition, these data shed light on the ability of HPV16 to escape TLR responses, which may lead to its persistence in the host and which may also explain the lack of TLR recognition to viruses in vivo.

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Disclosures

The authors have no financial conflict of interest.

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