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TLR9 Transcriptional Regulation in Response to Double-Stranded DNA Viruses

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The stimulation of TLRs by pathogen-derived molecules leads to the production of proinflammatory cytokines. Because uncontrolled inflammation can be life threatening, TLR regulation is important; however, few studies have identified the signaling pathways that contribute to the modulation of TLR expression. In this study, we examined the relationship between activation and the transcriptional regulation of TLR9. We demonstrate that infection of primary human epithelial cells, B cells, and plasmacytoid dendritic cells with dsDNA viruses induces a regulatory temporary negative-feedback loop that blocks TLR9 transcription and function. TLR9 transcriptional downregulation was dependent on TLR9 signaling and was not induced by TLR5 or other NF-kB activators, such as TNF-α. Engagement of the TLR9 receptor induced the recruitment of a suppressive complex, consisting of NF-kB and HDAC3, to an NF-kB cis element on the TLR9 promoter. Knockdown of HDAC3 blocked the transient suppression in which TLR9 function was restored. These results provide a framework for understanding the complex pathways involved in transcriptional regulation of TLR9, immune induction, and inflammation against viruses. The Journal of Immunology, 2014, 193: 000–000.

The TLR multicomponent inflammatory response must be tightly regulated to avoid tissue damage, chronic inflammation, and aberrant production of proinflammatory cytokines. Indeed, most known regulatory mechanisms target TLR-signaling pathways and, thus, broadly inhibit multiple aspects of the inflammatory response or regulate epigenetic changes on the promoter regions of inflammatory genes (10). Most studies of TLR modulation are based on post-TLR agonist activation, such as the modulation of TLR4 after exposure to its ligand, bacterial LPS. Overactivation of TLR4 with LPS leads to an acute systemic disease known as endotoxic shock. Endotoxin tolerance is a well-described phenomenon involving TLR4 modulation, whereby cells exposed to LPS become less responsive to a continued engagement of the TLR4 receptor. This may help to avoid the pathology associated with uncontrolled inflammation (11). Similar tolerogenic effects were reported with a wide range of agonist–TLR combinations (12, 13). Agonist tolerance can be mediated by a variety of mechanisms, including induction of negative regulators of TLR-signaling pathways, as well as by regulation of expression of TLRs and their signaling components (10). Changes in TLR expression can occur at transcriptional or posttranscriptional levels.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; HK, human female skin keratinocyte; HPV16, human papillomavirus type 16; EBV, human papillomavirus type 16 (HPV16), and HSV-2 (1–5).

Pat-tern-recognition receptors, including TLRs, are responsible for sensing microbial infection and tissue damage. They play a key role in host defense during pathogen infection by regulating and linking the innate and adaptive immune responses. TLRs 3, 7, 8, and 9 were demonstrated to recognize different forms of microbial-derived nucleic acid. TLR9 detects unmethylated CpG motifs found in the genomic DNA from a variety of viruses, such as EBV, human papillomavirus type 16 (HPV16), and HSV-2 (1–5). Although TLR9 signaling in hematopoietic cells was demonstrated to control infection of several DNA viruses, TLR9 is also expressed on epithelial cells, such as keratinocytes (6–8). Upon activation, TLR9 associates with the adaptor protein MyD88. This association promotes the recruitment of IRAK1 and IRAK4, which form a complex with TRAF6 to activate TAK1 and IKK. Activation of IKK leads to the degradation of IκB, which normally maintains NF-κB in the cytoplasm. Translocation of p50 and p65 into the nucleus allows for proinflammatory gene induction of cytokines such as TNF-α and pro–IL-1β. Specificity of NF-κB–triggered gene expression is achieved by several posttranslational modifications. Of the five known NF-κB family members, the p65 subunit is the most well studied. Numerous studies highlighted the diverse posttranslational modifications that exist, such as phosphorylation, monomethylation, ubiquitination, and acetylation, for p65 to achieve its full biological activity (9). p65 becomes transcriptionally active when acetylated at lysine residues by both p300 and PCAF. This complex was reported to be deacetylated by histone deacetylases (HDACs), which leads to attenuation of NF-κB transcriptional activity (10).

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Many studies use TLR expression as a means to assess innate immune responses (4, 14). Epidemiological and clinical studies documented a correlation between differences in TLR mRNA levels and disease progression or regression (15–20). Indeed, changes in TLR expression also could have positive or negative consequences in the regulation of both innate and adaptive immunity in different diseases (20). Despite this interest, understanding the mechanisms that control TLR transcription during the immune response remains limited. In this study, we examined the transcriptional regulation of human TLR9 expression during viral infection. To address this question, we used several dsDNA viruses and cellular models. We believe that tight control of TLR expression plays an essential role in the resolution of inflammation postinfection or tissue damage, both of which are critical for maintaining tissue homeostasis.

Materials and Methods

Cell culture procedures

NIH3T3, HEK 293T, HEK 293TT (for virus production), and RPMI8226 B cell lines were maintained as previously described (4). Primary human female skin keratinocytes (HKs) were grown as described (4, 21). Primary human B cells were purified and cultured as previously documented (1). Primary human plasmacytoid dendritic cells (pDCs) were purified and cultured as described (22). The Gen2.2 pDC cell line was obtained from the laboratory of Prof. J. Plumas (Université Joseph Fourier, Grenoble, France) and cultured as described (23).

Constructs. The plasmids used for HPV16 structural genes and control pseudovirus (PV) production, the target HPV6 genome, and GFP (for PV control) were kindly donated by the laboratories of Martin Müller and Angel Alonso (Deutsches Krebsforschungszentrum, Heidelberg, Germany). The NF-κB reporter plasmid was obtained from Becton Dickinson. The pCMV-pGL3 promoter and internal Renilla controls were purchased from Promega. The TLR9 promoter luciferase construct was described previously (14). TLR9 mutated promoters were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), as described previously (24). The NF-κB minimal promoters were cloned into pTAL-LUC vector (Becton Dickinson). The MyD88DN was described previously (25). Small hairpin RNA (shRNA) lentiviral constructs for TLR9 (shTLR9) and control were provided by the Procan Axe II CLARA platform (Centre Léon Berard, Lyon, France). Small interfering RNA (siRNA) for IKKα, IKKβ, or MyD88 was purchased from Ambion, and the sequence used was described previously (24).

Stimuli, inhibitors, and viral production. HSV-2 was kindly provided by the laboratory of Akiko Iwasaki (Yale University, New Haven, CT). HPV16 quasi virions (QsV) production, infection, and viral genome expression quantification of HPV16 were performed as described (26,27). The production of RfvB was described as previously published (27). All viruses were UV inactivated for 1 h. Bafilomycin, flagellin, TLR9 CpG, and Gpc 2006 ODN control were purchased from InvivoGen and used as indicated. The CpG motif from HPV16 was described previously (4). Trichostatin A (TSA) was kindly provided by the laboratory of Zdenko Viruses were UV inactivated for 1 h. Bafilomycin, flagellin, TLR9 CpG, and IL-6 secretion was tested using Quantikine ELISA kits (R&D Systems), as previously described (4).

Immunoblotting and immunoprecipitation. Biochemical analysis of the harvested cells was performed as described previously (25).

Transfections and luciferase assay. Cells were transiently transfected as described previously (4). siRNAs were transfected as described (25).

Quantitative RT-PCR. Total RNA was extracted from cells using the RNeasy Mini Kit (QIAGEN). cDNA was synthesized with the First Strand RT-PCR Kit (Montreal Biotech, Fermentas). The Mx3000P real-time PCR system (Stratagene) was used to perform qPCR with MESA GREEN qPCR MasterMix plus (Eurogentec). Primers were described previously (24, 31).

Abs. For cell ChIP assays, p65 (sc-7178) and p65 (sc-372) were purchased from Santa Cruz Technologies. TLR9, IKKα, IKKβ, p65, and p50 were obtained from Cell Signaling Technology, and the Li-neutralizing Ab was a kind gift from the laboratory of Martin Muller. AceH4 was obtained from Millipore, and control rabbit or mouse IgG was from Diagenode. HDAC1–3 (mouse or rabbit) were purchased from Santa Cruz Biotechnology. Peroxidase-conjugated anti-rabbit or anti-mouse secondary Abs were from Promega. β-Tubulin, β-actin, and LAMIN B1 Abs were purchased from Sigma. Secondary Abs for immunofluorescence used for cell staining were the TSA Plus Cyanine 3 System (NEL.744001KT), TSA Plus Cyanine 5 System (NEL.745001KT) (both from PerkinElmer), and Alexa Fluor 488 Donkey Anti-Rabbit IgG A21206 (Invirogen). Secondary Abs for immunofluorescence used for cellular staining were Alexa Fluor 488 or Alexa Fluor 594 Donkey Anti-Rabbit IgG (Invitrogen).

Duolink proximity ligation assay. Cells were fixed for 30 min with cold 4% paraformaldehyde. The blocking step was performed with Duolink Blocking Solution, according to the manufacturer’s instructions. Slides were incubated overnight at 4°C with primary Abs directed against p65 or HDAC3 and then with the appropriate DNA-linked secondary Abs. Duolink II Detection Reagents Orange was subsequently used, according to the manufacturer’s instructions (Eurogentec). Slides were analyzed using an inverted confocal microscope (LSM710; Leica).

Statistical analysis

GraphPad (version 5) software was used to calculate unpaired and paired p values.

Results

TLR9 is activated by the HPV16 genomic DNA virus

We reported TLR9-dependent recognition of oligonucleotide sequences found within the genome of HPV16 (4). As a first step to address the question of TLR9 regulation by dsDNA viruses, we felt it essential to corroborate these findings in primary HKs with HPV16 quasivirions that closely resemble the natural virus (24). To test the ability of HPV16 to activate TLR9, HKs were cotransfected with an NF-κB reporter gene, with or without shRNA for TLR9. HKs were then infected with UV-inactivated HPV16 quasivirions (HPV16) for 6 h. We observed NF-κB luciferase activity in cells infected with HPV16 or HSV-2 or stimulated with a commercial CpG motif (2006) (Fig. 1A). NF-κB activity was abrogated in cells treated with siTLR9 but not in cells treated with small hairpin (sh) scramble or with the TLR5–NF-κB ligand flagellin (Fig. 1A). No NF-κB activity was observed in cells treated with PV or Gpc controls. Human TLRs 1, 2, 4, 5, 6, and 10 are expressed on the cell surface, whereas TLRs 3, 7, 8, and 9 reside in the endoplasmic reticulum. In response to dsDNA, TLR9 moves, matures, and signals from the endosomal compartments (32). TLR9 maturation can be blocked by increasing the endosomal pH using bafilomycin A. Addition of bafilomycin A to HKs infected with HPV16 or stimulated with CpG 2006 abrogated NF-κB activity, as well as IL-8.
We showed recently that infection with non-UV–treated HPV16, or stimulation with CpG 2006 for 12 h did not induce IL-8, IL-6, or CCL20/MIP-3α secretion (Fig. 2C). However, infection with HPV16 for 6 h induced a period of unresponsiveness, because the subsequent addition of CpG 2006 for 12 h did not induce IL-8, IL-6, or CCL20/MIP-3α secretion (Fig. 2C). Taken together, these data show that TLR9 activation leads to a negative-feedback loop that transiently blocks TLR9 transcription and function. Upon recognition of the dsDNA, TLR9 recruits MyD88 via TIR–TIR domain interaction that promotes the phosphorylation and grouping of IKKs α/β, which, in turn, degrades IκB, releasing NF-κB into the nucleus to mediate gene expression. We asked whether MyD88 was required for the transient block in TLR9 transcription induced by HPV16. We observed that ectopic expression of a dominant-negative MyD88 mutant restored TLR9 transcription and protein levels in HKs infected with HSV-2 or HPV16 or stimulated with CpG 2006 (Fig. 2D), indicating that MyD88 was involved in this phenomenon. As reported previously, HKs treated with 16QsV did not alter luciferase activity (Fig. 2E), indicating that TLR9–MyD88–NF-κB signaling pathway by p65/p50 complex at site D

Our data indicate that dsDNA activates a TLR9–MyD88–signaling pathway (upstream of NF-κB) that transiently blocks the tran-
**FIGURE 2.** TLR9 expression is suppressed at 6 h and restored at 24 h postinfection with HPV16. (A) HKs were stimulated for 6, 8, 12, or 24 h with PV, HPV16, HSV-2, GpC, or CpG (2.5 μM), and TLR9 mRNA levels were determined by qPCR and normalized to the housekeeping gene GAPDH. TLR9 protein levels were determined by Western blotting using protein extracts 6 h posttreatment with HPV16, CpG (2.5 μM), or GpC. (B) HKs were transfected with the TLR9 promoter, and cells were stimulated 24 h later with HPV16, GpC, CpG (2.5 μM), or HSV-2 for 12 h. Cells were lysed, and luciferase activity was measured. (C) HKs were treated with HPV16 for 16 or 6 h (or were unstimulated) and washed, and CpG (2.5 μM) was added for 12 h. Supernatants were harvested, and IL-6, IL-8, and MIP-3α levels were measured by ELISA. (D) HKs were transfected with MyD88DN-Flag or Flag control vector with the TLR9 promoter, and cells were stimulated 24 h later with HPV16, 16QsV, CpG (2.5 μM), GpC, or HSV-2 (left panel). TLR9 and MyD88DN-Flag protein levels were examined by immunoblotting (right panel). (E) HKs were treated with siRNA for IKKα or IKKβ for 16 h and then transfected with TLR9 luciferase promoter. Sixteen hours posttransfection, cells were exposed to the indicated treatments for 8 h, and luciferase activity was measured. IKKα or IKKβ levels were determined by immunoblotting. Graphs show data from five independent experiments performed in triplicate. Error bars indicate SEM. ***p < 0.0001, unpaired Student t test.
FIGURE 3. TLR9 promoter regulation by HPV16 involves a change in the composition of the NF-κB complex. (A) Sequence mutations were made at NF-κB sites A, B, C, and D. (B) HKs were transfected with TLR9 promoter luciferase expression vectors containing either a WT or mutated promoter sequence on A, B, C, or D. Twenty-four hours later, cells were stimulated with PV or HPV16. Luciferase activity was measured 24 h later. (C) Sheared chromatin from HKs stimulated with CpG 2006 (2.5 μM) for 4 or 24 h was immunoprecipitated for ChIP analysis with Abs to p50 or p65. Site A, B, C, or D on the TLR9 promoter was amplified by qPCR to determine the specific binding to DNA. ChIP data are represented as percentage input (gene-specific)/percentage input (β-globin) amplified by qPCR. (D) ReChIP analysis was performed using HKs stimulated with CpG at 4 and 24 h on site D. (E) ChIP using anti-p65 and anti-p50 Abs from HKs infected with HPV16 or TNF-α for 6 or 36 h. (F) HKs were transfected with siRNA for MyD88. Twenty-four hours later, HKs were stimulated with HPV16 for 6 h, and ReChIP analysis for p65/p50 was performed. TLR9 mRNA levels were determined by qPCR and normalized to the housekeeping gene GAPDH. MyD88 levels were assessed by qPCR and normalized with the β2-microglobulin housekeeping gene. Graphs show data from three independent experiments performed in triplicate; error bars indicate SEM. ***p < 0.0001, unpaired Student t test.
scription of TLR9. We next wanted to determine which cis site(s) on the TLR9 promoter is involved. Previously, we identified three putative NF-κB sites on the TLR9 promoter that we termed site A (−3040), site B (−1237), and site C (−1148) (Fig. 3A). Site D (−403) was described previously (14, 24). We made three to six nucleotide mutations at site A, B, C, or D within the TLR9 Luc promoter (Fig. 3A (24). We observed that mutation of site D reduced the TLR9 basal activity (Fig. 3B). HK-TLR9 stimulation with HPV16 suppressed activity of the wild-type (WT), mutant A, B, and C, but not D (Fig. 3B). The same was observed with CpG 2006 (data not shown). Next, we used ChIP for p65 and p50 to determine which NF-κB family members bound to the site D region on the TLR9 promoter. In unstimulated cells, we observed that p65 and p50 bound to site D (Fig. 3C, left panel). However, at 24 h, p65 and p50 recruitment to site D was restored. In no case was binding of p65 or p50 seen at site A, B, or C (Fig. 3C). ReChIP experiments confirmed that p65/p50 heterodimers were recruited to site D on the TLR9 promoter in unstimulated cells or cells that were treated with CpG for 24 h (Fig. 3D). Because p65/p50 act as positive regulators, we deduced that this complex drives TLR9 transcription; TLR9 promoter activity was reduced in the steady-state when a site mutation was made at site D (Fig. 3B). In addition, TLR9 mRNA and protein were observed under the same experimental conditions (Fig. 2A). ChIP and ReChIP experiments showed that p65 alone was recruited to site D when HKs were treated with CpG for 4 h (Fig. 3C, 3D). In addition, recruitment of p65 only to site D was induced in HKs that were infected with HPV16 for 6 h (Fig. 3E), and it correlated with the suppression of TLR9 mRNA levels (Fig. 2A). Thus, we can consider that active p50/p65 complexes are lost and p65 transiently suppresses TLR9 transcription in response to CpG or HPV16 infection. In addition, the p65 recruitment to site D observed at 6 h postinfection with HPV16 was lost when HKs were treated with an siRNA MyD88 (Fig. 3F). These results were in conjunction with restoration of TLR9 mRNA levels (Fig. 3F). In summary, TLR9–MyD88 induction of p65 induces a negative-feedback loop on the TLR9 promoter at site D.

Transient repression of TLR9 transcription requires the recruitment of p65 and HDAC3. Posttranslational modifications of transcription factors are considered essential for their activity. p65 phosphorylation at serine 536 stimulates acetylation at lysine 310 and the interaction with the acetyltransferase CBP (33), which allows enhanced transcriptional activity of a variety of genes. We observed the recruitment of p65Lys310 in unstimulated HKs and cells that were infected with HPV16 for 24 h (Fig. 4A). However, binding of p65Lys310 at site D was greatly reduced in HKs 8 h postinfection with HPV16 (Fig. 4A). The loss of p65Lys310 was in conjunction with restoration of TLR9 mRNA levels (Fig. 3F). In summary, TLR9–MyD88 induction of p65 induces a negative-feedback loop on the TLR9 promoter at site D.

**FIGURE 4.** HPV16 stimulation in HKs induces p65 Lys310 at site D. HKs were treated with HPV16 for 8 or 24 h, and ChIP analysis was performed for p65 and p65 Lys310 (A) or for AceH4 (B) at site D on the TLR9 promoter. (C) HPV16, CpG (2.5 μM), and HSV-2 induce closing of the chromatin structure on the TLR9 promoter from site A to site D. ChIP using anti-AceH4 Abs was performed for site A, B, C, or D using HKs treated with GpC, CpG (2.5 μM), HPV16, or HSV-2 for 8 h. (D) HKs were treated with HPV16 or CpG for 8 h, with or without TSA, and ChIP using anti-AceH4 Abs was performed for site A, B, C, or D. (E) Cytoplasmic and chromatin extracts from HKs that had been treated with HPV16, with or without TSA, for 8 h were analyzed by immunoblotting for TLR9 and α-actin and for AceH4, respectively. (F) HKs were treated with HPV16 for 16 or 6 h (or were unstimulated) and washed, and CpG (2.5 μM), with or without TSA, was added for 12 h. Supernatants were harvested, and IL-8 levels were measured by ELISA. Graphs show data from four independent experiments performed in triplicate; error bars indicate SEM. ***p < 0.0001, unpaired Student t test.
FIGURE 5. HPV16 stimulation of HKs induces p65 and HDAC3 recruitment at site D on the TLR9 promoter. (A) ReChIP for p65 with HDAC1, HDAC2, or HDAC3 at site D on the TLR9 promoter was performed on HKs treated with CpG (2.5 μM) for 8 or 24 h. (Bi) Cytoplasmic and nuclear cell extracts were analyzed by Western blotting for the expression of p50, p65, p65 K310 acetylation, and HDAC3. β-tubulin or LAMIN B1 was used as a cytoplasmic or nuclear loading control, respectively. (Bii) Immunoprecipitation of p65/HDAC3 or p50 in chromatin fractions from HKs stimulated with HPV16 for 8 h. Duolink analysis of p65/HDAC3 in noninfected (NI) HKs (C) versus HKs infected with HPV16 for 6 h (D, upper panels). DAPI marks the nucleus, and the white dots represent p65/HDAC3 proximity ligation with < 40-nm distance. One of six fields examined for each section is shown. Original magnification ×60 and ortho Z slices (3.3 μM) and three-dimensional imaging shows nuclear expression in parallel to DAPI staining of the p65/HDAC3 proximity ligation, 40 nm. Scale bars, 10 μm. (D) p65/ HDAC3 proximity interaction localities were estimated by counting the number of red dots manually and using the Duolink Image tool (Olink, Biosciences) present in three field sections on three noninfected HKs versus HKs treated with HPV16 for 6 h (lower panel). (E) HKs were treated for 24 h with HDAC3 siRNA or scramble control, and cells were stimulated with HPV16 for 6 h and harvested for ReChIP analysis for p50 and p65. (G) TLR9 mRNA levels were determined by qPCR and normalized to the housekeeping gene GAPDH. (H) HKs were treated with HPV16 for 16 h or pretreated with HDAC3 siRNA or control (siRNAc) and stimulated or not for 6 h with HPV16 and washed, and CpG (2.5 μM) was added for 12 h. Supernatants were harvested, and IL-8 was measured by ELISA. Graphs show data from four independent experiments performed in triplicate; error bars indicate SEM. ***p < 0.0001, unpaired Student t test.
Acetylation also correlated with a loss of histone 4 acetylation (AcH4) on site D (Fig. 4B). Indeed, AcH4 binding was lost upstream from site D along the TLR9 promoter when HKs were treated with CpG, HPV16, or HSV-2 for 8 h (Fig. 4C). Reversible acetylation of histones and other proteins is mediated by the activity of HDACs. AcH4 at TLR9 promoter and TLR9 expression were restored in HKs treated with CpG or HPV16 for 8 h in the presence of the HDAC inhibitor TSA (Fig. 4D, 4E). We observed that infection with HPV16 for 6 h induced a period of unresponsiveness, because the subsequent addition of CpG 2006 for 12 h did not induce cytokine secretion (Fig. 2C). Addition of TSA abrogated the unresponsive state induced by HPV16 on TLR9 function, as measured by IL-8 secretion (Fig. 4F). AcH4 modifications are regulated by HDACs. We performed ReChIP experiments on cells that were stimulated for 8 h with CpG 2006 and observed that HDAC3, but not HDAC1 or HDAC2, was recruited to site D (Fig. 5A). To determine the cellular localization status of p50, p65, and HDAC3, we performed Western blot analysis on cytoplasmic and nuclear fractions from HKs that were stimulated with HPV16 for 6 h. Western blot analysis revealed the expression of total p50 and p65 in nonstimulated cells in both fractions (Fig. 5Bi). However, HPV16 infection led to a reduction in p50 and p65 levels in the nucleus, but no difference in HDAC3 nuclear levels between nonstimulated cells and HPV16-stimulated cells was observed (Fig. 5Bi). Coimmunoprecipitation experiments using chromatin extracts revealed that HDAC3/p65 interacted when cells were stimulated with HPV16 (Fig. 5Bi). To determine whether these two cellular proteins interacted in HPV16-infected HKs, we performed immunoprecipitation using DUOLINK technology, which determines the interactions penetrating the nucleus of the cells, emphasizing the importance of three-dimensional imaging. In addition, nuclear p65/HDAC3 interactions were lost in the presence of an siRNA for p65 (Fig. 5E). These data highlight the role of epigenetic modifications at histone 4 in the transient suppression of TLR9, as well as the requirement for HDAC3 to modify the action of p65 at site D. Knockdown of HDAC3 in HKs treated with HPV16 restored the p50/p65 complex, in conjunction with an increase in TLR9 mRNA (Fig. 5F, 5G). As shown in Fig. 2C, infection with HPV16 for 6 h induced a period of unresponsiveness, because the subsequent addition of CpG for 12 h did not induce IL-8. Pretreatment with HDAC3 siRNA permitted TLR9 to become responsive to CpG 2006 at 12 h poststimulation (Fig. 5H). These data indicate that the temporary suppression of TLR9 mediated by HDAC3 is essential to control cytokine production. The transient transcriptional inhibition of TLR9 by EBV in B cells and HSV-2 in pDCs requires the recruitment of HDAC3/p65. TLR9 expression and function in humans have been well documented in B cells and pDCs (34–36). We next determined whether the transient suppression of TLR9 also occurs in these cell types postinfection with EBV or HSV-2. PBMC purified human B cells (Supplemental Fig. 2) were stimulated with CpG or infected with UV-treated EBV. As expected, EBV infection in HEK293T cells exogenously expressing TLR9 activated the NF-κB reporter gene (Supplemental Fig. 3A). We observed a transient suppression of TLR9 mRNA (Fig. 6A) and protein levels (Supplemental Fig. 3B) in human B cells that occurred at 4 h and was restored at 24 h. To determine whether the reduced mRNA was due to a block in TLR9 transcription mediated by site D, WT and mutant TLR9 promoter constructs were transfected into a human B cell line and treated.

**FIGURE 6.** TLR9 expression is suppressed at 4 h and restored at 24 h postinfection with EBV and CpG on primary human B cells. (A) B cells were stimulated for 4, 8, or 24 h with EBV, and TLR9 mRNA levels were determined by qPCR and normalized to the housekeeping gene GAPDH. (B) Human B cells (RPMI8226) were transfected with the TLR9 promoter luciferase expression vectors containing either a WT or mutated promoter sequence on A, B, C, or D. Twenty-four hours later, cells were stimulated with CpG (2.5 μM), CpG, EBV, or TNF-α. Six hours later, luciferase activity was measured (reported as percentage compared with unstimulated cells). ReChIP for p65 with HDAC1, HDAC2, or HDAC3 (C) or ChIP with anti-AcH4 Abs (D) at site D on the TLR9 promoter was performed on B cells treated with EBV for 4 or 24 h. (E) B cells were treated with EBV at 4 or 24 h, with or without TSA, and ReChIP for p65/HDAC3 complex was performed.
with EBV, CpG, GpG, or TNF-α. To quantify our results, treated cells were divided by the basal activity of the TLR9 promoter and multiplied by 100 to give a percentage of promoter activity. B cells stimulated with CpG or EBV blocked TLR9 through site D (Fig. 6B). Transcriptional abrogation of TLR9 expression in HKs requires the recruitment of HDAC3/p65. We next determined whether the same complex was required to suppress TLR9 expression in human B cells. We observed that, at 4 h post-EBV infection in human B cells, HDAC3/p65 was recruited to site D on the TLR9 promoter (Fig. 6C), coinciding with the loss of AceH4 (Fig. 6D). The HDAC3/p65 complex was lost in the presence of TSA (Fig. 6C, 6E). CpG and stimulated primary pDCs also induced a transient suppression of TLR9 mRNA at 8 h posttreatment that was restored 24 h post-stimulation (Fig. 7A). Transfection of a pDC cell line (Gen2.2) with the TLR9 promoter revealed that transcriptional abrogation occurred at 8 h post-incubation with CpG 2006 and HSV-2 (Fig. 7B). Mutation of the TLR9 promoter at site D restored luciferase activity 6 h postaddition of CpG or HSV-2, although sites A, B, C, and D, and mutated site D (Dm) using protein lysates from primary pDCs stimulated with HSV-2 for 6 h. Bound proteins were assessed by immunoblotting for HDAC3, p65, p50, or input controls (10%).

Discussion
The importance of TLR-mediated induction of immune responses is matched by the need to regulate these responses to avoid pathological reactions. Therefore, TLR-mediated responses are tightly controlled by mechanisms including regulatory components of signaling pathways, subcellular relocalization of TLR proteins, and posttranslational modulation (10, 37, 38). The loss of TLR9 expression in our experiments resulted in a state of “unresponsiveness” in HKs infected with HPV16. TLR4 hyporesponsiveness

![Diagram of TLR9 transcriptional regulation](https://www.jimmunol.org/)

**FIGURE 8.** Model of the TLR9 transcriptional regulation in response to dsDNA viruses. At 0 h, TLR9 homeostatic expression is driven by p50/p65 binding on site D on the TLR9 promoter. Between 4 to 6 h infection with dsDNA viruses exposed CpG at 2.5 μM motifs activates the TLR9–MyD88 pathway that induces p65, as well as HDAC3 complexes. p65/HDAC3 complexes bind to site D and temporarily suppress TLR9 expression. TLR9 expression is restored 24 h later.
was reported in macrophages stimulated with LPS. Whitmore et al. (39) described that TLR4 levels remained unchanged, but LPS pretreatment led to induced histone deacetylation by recruiting AT3 and HDAC1 to the promoter region of IL-12p40, IL-6, and TNF-α. Furthermore, Foster et al. (11) showed that distinct patterns of TLR4–induced chromatin modifications were associated with transient silencing, which contributed to the phenomenon of “LPS tolerance.” Few studies have addressed the role of TLR stimulation on its transcriptional regulation. Measles virus infection of dendritic or epithelial cells leads to IFN-β induction, which positively induces the transcription of TLR3 (40). TLR7 and TLR8 transcriptional activity are induced in response to specific and nonspecific ligands (41). Cross-regulation of TLR mRNA levels in avian macrophages was recently reported by exposure to all of the tested agonists (42). For example, PAM (TLR2 ligand) treatment led to a transient downregulation of TLRs 3, 4, 5, and 7 and mRNA levels, whereas LPS induced transient downregulation of certain TLR1/2 family members, as well as TLR5 and TLR7. Similarly, complex and distinctive patterns of TLR mRNA regulation were observed with flagellin, R848, and CpG ODN (42). Peroval et al. (42) showed that the MAPK pathway was critical in the induction of inflammatory mediators, as well as in the control of cognate TLR expression. Shatz et al. (43) also showed that the p53 status in different cell lines influenced TLR mRNA levels. Despite the numerous data demonstrating alteration of TLR mRNA expression by ligand activation, few reports demonstrated the consequence or specific mechanism by which TLRs themselves are transcriptionally regulated. In addition, the importance of TLR transcriptional regulation is highlighted by the fact that several single nucleotide polymorphisms in TLR promoters predispose humans to several autoimmune and chronic inflammatory diseases, including asthma and Crohn’s disease (44–51). In this article, we provided novel insights on the transcriptional regulation of TLR9 with dsDNA viruses. In the steady-state, p50/p65 complexes already exist on the TLR9 promoter to drive its expression. We demonstrated that, prior to its activation, homeostatic transcription of TLR9 required p50/p65, Lys310, and AcH4 activity at site D on the TLR9 promoter. We went on to show that the HPV16 dsDNA genome is recognized by TLR9 and activates further NF-kB–dependent inflammatory responses. However, engagement of TLR9–MyD88 signaling by HPV16, EBV, or HSV-2 or by CpG motifs temporarily inhibited TLR9 expression. During this phase, HDAC3/p65 were recruited to site D on the TLR9 promoter. We observed that HDAC3 deacetylated H4, as well p65 Lys310, rendering it inactive at site D on the TLR9 promoter (Fig. 8). The temporary inhibition of TLR9 was abrogated when HDAC3 activity was blocked. The function of NF-kB is also known to be regulated by a variety of other posttranscriptional modifications, which can either enhance or inhibit transcriptional activity, in part, in a gene-specific manner. p65 can also be acetylated at different sites (Lys122, Lys123, Lys210, Lys221, and Lys310), with variable effects on its activity (52). Acetylation at Lys310, promoted by phosphorylation of Ser536, was shown to augment transcriptional activity without affecting DNA or IkB binding, presumably through generating a binding site for a coactivator protein. In contrast, acetylated Lys221 impedes association with IxBα, thereby enhancing DNA binding (53). How differential acetylation may influence dimer swapping of p50/p65 for HDAC3/p65 upon TLR9 engagement requires further investigation. A particularly intriguing aspect of the regulation described in this article is that NF-kB–mediated suppression of the TLR9 promoter was specific to the pathway activating NF-kB. Thus, TLR9–induced, but not TNFα–induced, NF-kB activation resulted in the assembly of the suppressive p65–HDAC3 complex on site D of the TLR9 promoter. Furthermore, we reported previously that non–UV–treated HPV16 (16QsV) also blocks TLR9 transcription. 16QsV abrogates TLR9 expression at 24 h without its restoration and using the viral oncoprotein E7, not MyD88, to suppress and escape immune recognition. These two divergent observations highlight two distinct inhibitory mechanisms of the TLR9 promoter—one that is essential in immune control and the other in immune escape—by the same oncovirus. Collectively, our data document a novel mechanism that affects TLR9 mRNA levels and outcome of TLR9 activation, with a level of complexity that was previously unappreciated.

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Disclosures

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