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EVII Acts as an Inducible Negative-Feedback Regulator of NF-κB by Inhibiting p65 Acetylation

Xiangbin Xu,*,† Chang-Hoon Woo,*,‡,† Rachel R. Steere,*, Byung Cheol Lee,*,‡ Yuxian Huang,*,§ Jing Wu,*,§ Jinjiang Pang,*,† Jae Hyang Lim,*,†,‡ Haidong Xu,*,† Wenhong Zhang,§ Anuhya S. Konduru,*,§,†,‡ Chen Yan,§ Michael T. Cheeseman,*,‡,† Steve D. M. Brown,*,‡ and Jian-Dong Li*,†,‡

Inflammation is a hallmark of many important human diseases. Appropriate inflammation is critical for host defense; however, an overactive response is detrimental to the host. Thus, inflammation must be tightly regulated. The molecular mechanisms underlying the tight regulation of inflammation remain largely unknown. Ecotropic viral integration site 1 (EVII), a proto-oncogene and zinc finger transcription factor, plays important roles in normal development and leukemogenesis. However, its role in regulating NF-κB–dependent inflammation remains unknown. In this article, we show that EVII negatively regulates nontypeable Haemophilus influenzae- and TNF-α–induced NF-κB–dependent inflammation in vitro and in vivo. EVII directly binds to the NF-κB p65 subunit and inhibits its acetylation at lysine 310, thereby inhibiting its DNA-binding activity. Moreover, expression of EVII itself is induced by nontypeable Haemophilus influenzae and TNF-α in an NF-κB–dependent manner, thereby unveiling a novel inducible negative feedback loop to tightly control NF-κB–dependent inflammation. Thus, our study provides important insights into the novel role for EVII in negatively regulating NF-κB–dependent inflammation, and it may also shed light on the future development of novel anti-inflammatory strategies.


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Abbreviations used in this article: AML, acute myelogenous leukemia; BAL, bronchoalveolar lavage; CBP, CREB protein; ChIP, chromatin immunoprecipitation; CBP, C-terminal binding protein; EVII, ecotropic viral integration site 1; EVII MT, ecotropic viral integration site 1 N782I mutation; HDAC, histone deacetylase complex; HMEC-1, human middle ear endothelial cell line; IKK, IkB kinase; IKKβ CA, constitutively active form of IkB kinase β; MEF, mouse embryonic fibroblast; MOI, multiplicity of infection; MPO, myeloperoxidase; NTLH, nontypeable Haemophilus influenzae; oligo, oligonucleotide; OM, otitis media; PMN, polymorphonuclear neutrophil; Q-PCR, quantitative RT-PCR; siRNA, small interfering RNA; WB, Western blot; WT, wild-type.

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inflammation must be tightly regulated. The inflammatory response can be controlled at multiple levels (18), but the underlying molecular mechanisms still remain largely unknown, partly because of the complexity of the inflammatory response and the multitude of components involved. The transcription factor NF-κB is activated by inflammatory stimuli, such as bacteria and TNF-α, and plays a critical role in mediating inflammatory responses by regulating the expression of proinflammatory mediators, including cytokines, chemokines, and adhesion molecules (19). NF-κB is activated via phosphorylation and degradation of IκB by the IκB kinase (IKK) enzyme complex (20–23), which, in turn, leads to the nuclear translocation of NF-κB and the subsequent transcription of NF-κB–dependent genes, such as TNF-α, IL-1β, and IL-8. Acetylation of p65, an important posttranslational modification, plays a critical role in the regulation of the nuclear function of NF-κB, which leads to changes in its biological activity, such as alterations in DNA-binding activity and transcriptional activity (24–28). EVI1 acts as both transcriptional activator and repressor to recruit the HDACs and histone acetyltransferase. EVII itself can also be acetylated by p300/CBP-associated factor at lysine residues (12, 29). However, the role of EVI1 in controlling the acetylation of other molecules is unknown. Moreover, the role of EVI1 in regulating the activation of NF-κB, a key regulator for proinflammatory responses, has yet to be determined.

Nontypeable Haemophilus influenzae (NTHi), a Gram-negative bacterium, is an important human pathogen in both children and adults (30). In children, it causes OM, one of the most common childhood infections and the leading cause of conductive hearing loss in the United States (31, 32). In adults, it exacerbates chronic obstructive pulmonary disease, the fourth leading cause of death in the United States (33, 34). Despite the need for prophylactic measures, the development of a vaccine to prevent NTHi infections has been difficult and remains a great challenge. Moreover, inappropriate antibiotic treatment contributes to the worldwide emergence of antibiotic-resistant strains of NTHi. Therefore, there is an urgent need to develop alternative therapeutic strategies for the treatment of NTHi infections, based on understanding the molecular pathogenesis of these infections. Like most other bacterial infections, NTHi infection is characterized by inflammation, which is mainly mediated by NF-κB–dependent upregulation of proinflammatory mediators (35–38).

Based on the essential involvement of NF-κB in NTHi-induced inflammatory responses and the upregulation of EVI1 by inflammatory stimuli in our preliminary gene-profiling studies, we hypothesized that EVI1 negatively regulates NTHi-induced inflammation via inhibition of NF-κB activity. In this article, we show that EVI1 negatively regulates NTHi-induced NF-κB activation and the subsequent inflammatory response by regulating the acetylation of NF-κB p65 subunit at lysine 310, thereby inhibiting the DNA-binding activity of NF-κB to κB sites. Given the important role of NF-κB in host immune and inflammatory responses against bacterial infections, the current studies provide novel insights into a previously unidentified role for EVII in the regulation of NF-κB and may also lead to the development of novel therapeutic strategies for controlling overactive inflammatory responses.

Materials and Methods

Reagents and Abs

Recombinant mouse TNF-α was purchased from Roche (Mannheim, Germany). Anti-phospho-IκBα, anti-IκBα, anti–acetyl-p65 (Ly310), anti-phospho-p65 S536, anti-IKKβ, anti-EVI1, and anti-CtBP2 Abs were purchased from Cell Signaling (Danvers, MA). Anti-actin, anti-p65, anti-EVI1, anti-tubulin, and anti-TFIID were purchased from Santa Cruz (Santa Cruz, CA). Anti-Flag was purchased from Sigma-Aldrich (St. Louis, MO), and anti–acetyl-lysine was purchased from Upstate (Lake Placid, NY).

Mice and animal experiments

Junbo mutant mice were generated by N-ethyl-N-nitrosourea mutagenesis screen (39, 40), as previously reported (13). Genotyping was performed by single-nucleotide polymorphism-genotyping assay on tail-derived genomic DNA.

For the NTHi-induced lung inflammation model in WT and Junbo (Jbo+) mice, anesthetized mice were inoculated intratracheally with NTHi, and saline was inoculated as control. The inoculated mice were sacrificed by i.p. inoculation of 100 mg/kg sodium pentobarbital at 9 or 24 h after NTHi inoculation. For histological analysis, dissected lung was inflated and fixed with 10% buffered formaldehyde, embedded in paraffin, and sectioned at 5-μm thickness. Sections were then stained and inspected. For real-time PCR (RT-PCR), BAL (BAL) was performed by cannulating the trachea with sterilized PBS. Cells from BAL fluid were stained with Hemacolor (EM Science) after cyto-centrifugation (Thermo Electronic). To assess the mRNA expression of proinflammatory mediators, total RNA was extracted from the lung of NTHi- and saline-inoculated mice at the time points indicated above, and real-time quantitative RT-PCR (qRT-PCR) was performed, as described previously (41). All animal experiments were approved by the Institutional Animal Care and Use Committee at University of Rochester and Georgia State University.

Bacteria strain and culture

A clinical isolate of NTHi strain 12 was used in in vitro cell culture experiments and in vivo animal experiments (42). Bacteria were grown on chocolate agar plate at 37°C in an atmosphere of 5% CO₂ overnight and inoculated in brain heart infusion broth supplemented with 3.5 μg NAD/mL and hemin. After overnight incubation, bacteria were subcultured into 5 ml fresh brain heart infusion broth, and the log-phase NTHi, monitored by measurement of OD value, was washed and suspended in PBS for in vitro cell experiments or in isotonic saline for in vivo animal experiments. In vitro experiments, the cells were treated with NTHi at a multiplicity of infection (MOI) of 1:25 for various times, as indicated.

Cell culture

Human airway epithelial A549 cells, human middle ear epithelial cells (HMEEC-1), and mouse macrophage RAW 264.7 cells were maintained, as described previously (41, 43, 44). Both p65 knockout (p65Δ/Δ) mouse embryonic fibroblasts (MEFs), as well as p65Δ/Δ MEFs reconstituted with p65 WT or p65 K310R constructs, were grown in DMEM supplemented with 10% FBS. All cells were cultured under standard conditions (5% CO₂ in air in a humidified environment at 37°C).

Plasmids, transfections, and luciferase reporter assay

Flag-tagged full-length EVI1 (1–1052) was kindly provided by Dr. Giuseppe Nucifora (University of Illinois at Chicago, Chicago, IL) (12). The N782I point mutation of EVI1 (EVI1 MT) was constructed using a Quik-Change Site-Directed Mutagenesis Kit (Stratagene). p65 WT, p65 Δ310R, a constitutively active form of IκKB (IKKβ CA), a transdominant-negative mutant form of IκBo (IκBo S32/36A), and NF-κB–luciferase reporter were described previously (45). Cells were cotransfected with NF-κB–luciferase reporter plasmid and various expression plasmids, as indicated in the figure legends. Empty vector was used as a control and was also added where necessary to ensure an equivalent amount of input DNA. All transient transfections were carried out in triplicate using a TransIT-LT1 reagent (Mirus), following the manufacturer’s instructions. At 40 h after transfection, cells were inoculated with NTHi for 5 h before cell lysis for luciferase assay, as described previously. Data from all experiments are presented as the relative luciferase activity (mean ± SD) from at least three independent sets of experiments, each with triplicate measurements.

RNA-mediated interference

Human and mouse EVI1 small interfering RNA (siRNA) oligonucleotides (oligo) were synthesized by Dharmacon and Santa Cruz. siRNA was transfected into A549 cells and MEF cells using Lipofectamine 2000 reagent (Invitrogen), following the manufacturer’s instructions.

RNA isolation and real-time Q-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen), following the manufacturer’s instructions. TaqMan reverse-transcription reagents (Applied Biosystems) were used for the reverse-transcription reaction. In brief,
Results

**EVII negatively regulates bacteria-induced NF-κB–dependent inflammatory response in vitro**

To test our hypothesis, we first investigated the role of EVII in NTHi-induced NF-κB luciferase promoter activity using an EVII siRNA knockdown approach. First, we confirmed the efficiency of EVII-specific siRNA in reducing EVII expression in A549 cells. As expected, the expression of EVII at both mRNA and protein levels was markedly reduced by EVII siRNA (Supplemental Fig. 1A, 1B). Next, we determined whether EVII knockdown affects NTHi-induced NF-κB activation in epithelial cells. As shown in Fig. 1A, NTHi-induced activation of NF-κB was greatly enhanced by EVII siRNA in human airway epithelial A549 cells, suggesting that EVII acts as a negative regulator of NTHI-induced NF-κB activation in epithelial cells. Next, we sought to determine the generalizability of negative regulation of NF-κB activation by EVII by assessing its effect on NF-κB activation induced by TNF-α, a commonly used inflammation inducer. Similar to NTHi-induced NF-κB activation, TNF-α–induced NF-κB activation was also markedly enhanced by EVII siRNA in A549 cells (Supplemental Fig. 1C). We next confirmed the role of EVII in negatively regulating NTHi-induced NF-κB activation by overexpressing wild-type (WT) EVII. Overexpression of EVII markedly reduced NTHi-induced NF-κB activation in A549 cells (Fig. 1B), HMEEC-1 cells (Fig. 1C), and mouse macrophage RAW 264.7 cells (Supplemental Fig. 1D). Similar results were also observed in TNF-α–induced NF-κB activation in A549 cells (Supplemental Fig. 1E). Together, these results demonstrate the negative role of EVII in regulating NTHI- and TNF-α–induced NF-κB activation.

Previously, a missense change in the C-terminal zinc finger region of the **Evil** gene was found to cause chronic middle ear inflammatory disease in mice (13). Thus, we generated EVII N782I mutation (EVII WT), a human homolog of mouse EVI1 N763I mutation (13), and determined its role in NTHI-induced NF-κB activation by coexpressing the EVII WT. Consistent with our results obtained with EVII siRNA, overexpressing EVII WT also markedly enhanced NTHI-induced NF-κB activation in A549 cells (Fig. 1D) and RAW 264.7 cells (Supplemental Fig. 1F), indicating that EVII WT indeed acts as a dominant-negative mutant. Similar results were also observed in TNF-α–induced NF-κB activation by coexpressing EVII WT in A549 cells (Supplemental Fig. 1G). Collectively, it is evident that EVII is a negative regulator of NF-κB activation induced by bacterial pathogen NTHI, as well as by proinflammatory cytokine TNF-α.

Having identified EVII as a negative regulator of NF-κB activation, we next sought to determine whether EVII also negatively regulates NF-κB–dependent transcription of several key proinflammatory mediators. As shown in Fig. 1E and Supplemental Fig. 1H, EVII siRNA greatly enhanced NTHI-induced mRNA expression of TNF-α, IL-1β, IL-8, MCP-1, and ICAM-1 in A549 cells. Similarly, TNF-α–induced mRNA expression of TNF-α, IL-1β, IL-8, MCP-1, and ICAM-1 was also potently enhanced by knockdown of EVII in A549 cells (Supplemental Fig. 1I, 1J). To further confirm the negative effect of EVII on NTHI- and TNF-α–induced upregulation of proinflammatory mediators, we evaluated the effects of overexpression of EVII WT and EVII MT expression plasmids on NTHI- and TNF-α–induced mRNA expression of TNF-α, IL-1β, and IL-8. As shown in Fig. 1F–H and Supplemental Fig. 1K, overexpressing EVII WT significantly reduced NTHI- and TNF-α–induced mRNA expression of TNF-α, IL-1β, and IL-8, whereas overexpressing EVII MT significantly increased NTHI-induced mRNA expression of TNF-α, IL-1β, and IL-8, similar to the findings with EVII.
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To further determine the in vivo biological role for EVI1 in neg- 

EVI1 negatively regulates bacteria-induced inflammation in 

ory epithelial cells and macrophages in vitro. 

inflammation in a variety of human cell types, including respira-

tory epithelial cells and macrophages in vitro. 

FIGURE 1. EVI1 negatively regulates bacteria-induced NF-κB-dependent inflammatory response in airway epithelial and middle ear epithelial cells. A549 or HMEEC-1 cells were cotransfected with NF-κB-luciferase reporter plasmid together with EVI1 siRNA (A), EVI1 WT (B, C), or EVI1 MT (D) construct for 40 h and treated or not with NTHi for 5 h. NF-κB-dependent promoter activity was measured by luciferase assay. A549 or HMEEC-1 cells were transfected with EVI1 siRNA (E), EVI1 WT (F, G), or EVI1 MT (H) construct for 40 h and treated or not with NTHi for 5 h. Total RNA was extracted, and mRNA expression of TNF-α, IL-1β, and IL-8 was measured by real-time Q-PCR assay. Data represent mean ± SD of at least three independent experiments, and each experiment was performed in triplicate. *p < 0.05 versus control, †p < 0.05 versus NTHi alone.

siRNA. Taken together, these results confirmed our hypothesis that EVI1 is indeed a negative regulator of bacterial pathogen NTHi- and proinflammatory cytokine TNF-α-induced NF-κB-dependent inflammation in a variety of human cell types, including respiratory epithelial cells and macrophages in vitro.

**EVI1 negatively regulates bacteria-induced inflammation in mouse model in vivo**

To further determine the in vivo biological role for EVI1 in neg-

atively regulating inflammation, we explored the consequences of EVI1 mutations using Junbo mutant mice (Jbo/*) generated from an N-ethyl-N-nitrosourea mutagenesis screen (13). Age- and sex-
matched WT and Jbo/* mice were intratracheally inoculated with NTHi, and the inflammatory response in the lung of infected mice was monitored 9 or 24 h after NTHi inoculation. As shown in Fig. 2A, mRNA expression of NF-κB-regulated proinflammatory markers such as TNF-α, IL-1β and MIP-2 was markedly enhanced in lungs of Jbo/* mice compared with that of WT littermate controls and was further enhanced with NTHi inoculation. Consistent with these results, histopathological analysis of the lung of NTHi-inoculated mice exhibited enhanced leukocyte infiltration in peribronchial and interstitial areas in Jbo/* mice compared with that in WT mice (Fig. 2B). Moreover, as shown in Fig. 2C, MPO activity, a key index of neutrophil activity, was also markedly enhanced in Jbo/* mice compared with that in WT mice after NTHi inoculation. Similar results were also observed in the analysis of BAL fluids (Fig. 2D, 2E). Next, we confirmed whether EVI1-mediated negative regulation of inflammatory response is dependent on the negative regulatory effect of EVI1 on NF-κB by assessing the effect of EVI1 siRNA on a transdominant-negative mutant of IκBα (IκBα S32/36A)-mediated inhibition of proinflammatory gene expression. As shown in Supplemental Fig. 1L, NTHi- and TNF-α-induced NF-κB-dependent promoter activity was enhanced by EVI1 siRNA and inhibited by overexpressing IκBα S32/36A. Moreover, EVI1 knockdown using EVI1 siRNA no longer enhanced NTHi- or TNF-α-induced NF-κB-dependent promoter activity in cells overexpressing IκBα S32/36A. Consistent with the findings from the luciferase assay, NTHi- and TNF-α-induced mRNA expression of TNF-α was enhanced by EVI1 knockdown and inhibited by overexpressing IκBα S32/36A. EVI1 siRNA no longer enhanced NTHi-induced mRNA expression of TNF-α and IL-8 in IκBα S32/36A-overexpressing cells, suggesting that EVI1 negatively regulates NTHi-induced TNF-α mRNA expression via inhibition of NF-κB (Supplemental Fig. 1M). Together, these results demonstrate that EVI1 is indeed a negative regulator of inflammatory response induced by bacterial pathogen NTHi and proinflammatory cytokine TNF-α in vitro and in vivo by negatively regulating NF-κB signaling. 

**EVI1 negatively regulates bacteria-induced activation of NF-κB by inhibiting its DNA-binding activity, likely independently of p65 nuclear translocation**

We next sought to determine how EVI1 inhibits NF-κB–dependent inflammation. IKKs are part of the upstream NF-κB signal-transduction cascade. The IκBα (inhibitor of κB) protein inactivates the NF-κB transcription factor by masking the nuclear localization signals of NF-κB proteins and keeping them sequestered in an inactive state in the cytoplasm (20–22). Specifically, IKKs phosphorylate IκBα (23). This phosphorylation results in the degradation and dissociation of IκBα from NF-κB, which, in turn, leads
to determine whether EVI1 inhibits NTHi-induced NF-κB activation by regulating nuclear translocation of p65 by performing WB analysis of the nuclear extract. As shown in Fig. 3D, EVI1 exhibited no inhibitory effect on NTHi-induced p65 translocation, which was further confirmed by p65 immunofluorescence staining (Fig. 3E). Similarly, EVI1 also exhibited no inhibitory effect on TNF-α–induced p65 translocation (Supplemental Fig. 2A, 2B). Thus, these results led us to conclude that the negative regulation of NF-κB activation by EVI1 may occur at a level further downstream of p65 nuclear translocation.

Because the DNA-binding activity of the NF-κB complex is critical for NF-κB to exert its transcriptional regulatory activity, we next investigated the effect of EVI1 on NTHi-induced DNA-binding activity of NF-κB using EMSA. As shown in Fig. 3F, overexpressing EVI1 significantly decreased NTHi-induced DNA-binding activity of NF-κB. Moreover, NTHi- and TNF-α–induced DNA-binding activity of NF-κB was further enhanced and remained sustained with EVI1 knockdown using EVI1 siRNA, as assessed by ChIP assays (Fig. 3G, Supplemental Fig. 2C). Taken together, these data suggest that EVI1 negatively regulates NTHi-induced NF-κB activation by inhibiting its DNA-binding activity, likely independent of p65 nuclear translocation.

**NTHi induces direct interaction of EVI1 with p65**

We found that EVI1 negatively regulates NTHi-induced NF-κB activation and subsequent inflammatory responses by inhibiting DNA-binding activity of the NF-κB complex. Next, we sought to determine whether NTHi induces interaction between EVI1 and p65, the major subunit of the NF-κB complex, by performing coimmunoprecipitation analysis of the interaction between EVI1 and p65. As shown in Fig. 4A, NTHi markedly increased the interaction of EVI1 with p65 in A549 cells. This interaction was confirmed by reverse immunoprecipitation, as indicated in Fig. 4B. To further confirm the interaction of EVI1 with p65, triple immunostaining with Abs against DAPI, EVI1, and p65 was performed. As shown in Fig. 4C and Supplemental Fig. 3A, the majority of EVI1 is localized in the nucleus, whereas p65 is mainly localized in the cytoplasm in the absence of NTHi but translocated to the nucleus and colocalized with EVI1 in the nucleus in response to NTHi. We next determined, by coimmunoprecipitation analysis, whether endogenous EVI1 interacts directly with endogenous p65 and whether such a direct interaction is further enhanced upon NTHi and TNF-α treatment. As shown in Fig. 4D, endogenous EVI1 directly interacts with endogenous p65, and both NTHi and TNF-α treatment markedly enhanced their direct interaction. Because EVI1 was shown to interact directly with the known transcriptional repressor CtBP2 via two CtBP-binding consensus motifs (10, 11), we determined whether NTHi induces the formation of p65:CtBP2 repressor complexes and EVI1 knockdown using EVI1 siRNA inhibits their interaction, thereby resulting in enhanced activation of NF-κB. As shown in Supplemental Fig. 3B, protein–protein interaction between p65 and CtBP2 was observed in cells treated with NTHi, and EVI1 knockdown using EVI1 siRNA inhibited their interaction, similar results were observed in cells treated with TNF-α (data not shown). However, the data with regard to TNF-α was not as definitive as that with NTHi. Thus, these results suggest that NTHi or TNF-α induces direct physical interaction of EVI1 with p65 in the nucleus, and EVI1 promotes the association of CtBP2 with p65, which, in turn, may lead to inhibition of NF-κB activation.

**EVI1 negatively regulates NTHi-induced NF-κB activation via inhibition of p65 acetylation at lysine 310**

Having demonstrated that EVI1 directly interacts with p65 and inhibits NTHi-induced DNA-binding activity, the molecular mechanism underlying the inhibition of DNA-binding activity of p65
Posttranslational modifications, particularly acetylation, were shown to play a critical role in NF-κB activation by enhancing the DNA-binding activity of p65 to the κB site (45). Because EVI1 was known to potentially recruit both HDACs and p300/pCAF (12), it is logical to hypothesize that EVI1 may negatively regulate NTHi-induced DNA-binding activity of NF-κB by inhibiting acetylation of p65. Thus, we determined whether EVI1 inhibits NTHi-induced p65 acetylation by

![Image](http://www.jimmunol.org/)

**FIGURE 3.** EVI1 negatively regulates bacteria-induced activation of NF-κB by inhibiting its DNA-binding activity, likely independently of p65 nuclear translocation. (A) A549 cells were cotransfected with NF-κB–luciferase reporter plasmid together with IKKβ CA, and NF-κB–dependent promoter activity was measured by luciferase assay. A549 cells transfected either with EVI1 siRNA (B) or EVI1 WT construct (C) were treated with NTHi for the time indicated and analyzed by WB analysis with the indicated Abs. (D) A549 cells cotransfected with p65 and EVI1 WT or control vector were treated with NTHi, and nuclear extracts were analyzed by WB analysis with the indicated Abs. (E) A549 cells cotransfected with EVI1 and p65 were treated with NTHi and immunostained with DAPI and anti-p65 Abs. Cells were visualized by fluorescence microscopy (original magnification ×200). (F) A549 cells cotransfected with p65 and EVI1 WT or control vector were treated with NTHi, and DNA-binding activity of NF-κB was assessed by EMSA. (G) A549 cells transfected with EVI1 siRNA or control siRNA were treated with NTHi for the times indicated in the figure; ChIP assay was conducted using Ab against p65 and analyzed by PCR using specific primers for the IL-8 promoter sequences spanning the κB-binding sites. Data are representative of at least three or more independent experiments. *p < 0.05 versus control, #p < 0.05 versus IKKβ CA alone.

![Image](http://www.jimmunol.org/)

**FIGURE 4.** EVI1 negatively regulates bacteria-induced NF-κB activation via direct interaction with p65. A549 cells cotransfected with Flag-EVI1 and p65 were treated with NTHi for the times indicated, and the proteins were immunoprecipitated with anti-Flag Ab (A) or anti-p65 Ab (B). Immunoprecipitations were analyzed by WB analysis with the indicated Abs. (C) A549 cells cotransfected with Flag-EVI1 and p65 were treated with NTHi and then triple immunostained with DAPI, anti-p65, and anti-Flag Abs. Slides were visualized by fluorescence microscopy (original magnification ×600). (D) A549 cells were treated with NTHi or TNF-α for the times indicated; the proteins were then immunoprecipitated with anti-p65 Ab and analyzed by WB analysis with the indicated Abs. Data are representative of at least three independent experiments.
assessing the effect of EVI1 overexpression in p65-overexpressing cells. Cells were first transfected with p65 WT, with or without EVI1, and NTHi-induced p65 acetylation was measured (Fig. 5A, 5B). Interestingly, as shown in Fig. 5A, overexpression of EVI1 markedly inhibited NTHi-induced p65 acetylation. Because acetylation of p65 at lysine 310 (p65 K310) is required for full transactivation activity of the NF-κB complex, we next determined whether EVI1 inhibits the acetylation of p65 at K310 (24). As shown in Fig. 5B, the acetylation of p65 at K310 induced by NTHi is markedly reduced by overexpressing EVI1. To further confirm the functional involvement of p65 K310, A549 cells were transfected with EVI1 siRNA, alone or together with p65 WT or p65 K310R (a p65 mutant at K310), and NF-κB luciferase activity was assayed. As shown in Fig. 5C, knockdown of EVI1 using EVI1 siRNA markedly enhanced NF-κB activation in cells cotransfected with WT p65 but not with p65 K310R. To further confirm the functional involvement of p65 K310 in the inhibition of NF-κB activation by EVI1, we assessed the effect of EVI1 knockdown on NTHi-induced NF-κB activity in p65^−/− MEF cells that were reconstituted with either WT p65 expression plasmid or p65 K310R mutant. The expression of p65 WT and p65 K310R in p65^−/− cells was first confirmed by WB analysis (Fig. 5D, lower panel). As shown in Fig. 5D (upper panel), EVI1 knockdown markedly enhanced NTHi-induced NF-κB activation in p65^−/− MEF cells that were reconstituted with WT p65 expression plasmid but not with p65 K310R mutant.

Next, we determined whether EVI1 regulates endogenous acetylation of p65 by assessing the effect of EVI1 siRNA on NTHi- and TNF-α–induced acetylation of endogenous p65. As shown in Supplemental Fig. 4, NTHi-induced acetylation of endogenous p65 and knockdown of EVI1 using EVI1 siRNA further enhanced and led to the sustained acetylation of p65. It was previously reported that acetylation of p65 is dependent on p65 phosphorylation (51). Thus, we further determined whether EVI1 regulates acetylation of p65 by regulating p65 phosphorylation. As shown in Supplemental Fig. 4A, NTHi-induced p65 acetylation was further enhanced by EVI1 siRNA, whereas phosphorylation of p65 at Ser536 remained unaffected by EVI1 siRNA. Similar results were also found in TNF-α–treated cells (Supplemental Fig. 4B). Taken together, our data demonstrate that EVI1 acts as a negative regulator of NTHi-induced DNA-binding activity of NF-κB by inhibiting acetylation of p65 at the K310 site, thereby leading to the inhibition of NF-κB–dependent inflammation.

Expression of EVI1 itself is also induced by NTHi both in vitro and in vivo

Negative-feedback regulation plays a critical role in preventing overactive and detrimental inflammatory response in a variety of human inflammatory diseases, including infectious diseases (52). It is well known that many genes involved in the inflammatory response undergo changes in expression pattern in response to inflammatory stimuli, such as bacteria. Because EVI1 negatively regulates the TNF-α– and NTHi-induced NF-κB–dependent inflammatory response, we hypothesized that EVI1 itself may also be induced by NTHi, which, in turn, may lead to the inhibition of the NTHi-induced inflammatory response, thereby preventing an overactive inflammatory response. To test our hypothesis, we determined whether NTHi induces EVI1 expression. As shown in Fig. 6A, EVI1 expression at the mRNA level was markedly upregulated by NTHi in A549 cells. The induction of EVI1 by NTHi was also confirmed at the protein level by performing WB analysis (Fig. 6B). Moreover, upregulation of EVI1 by NTHi was also observed in the lung of WT mice inoculated with NTHi (Fig. 6C). Together, these data suggest that EVI1 itself is also induced by NTHi, which, in turn, leads to inhibition of the NTHi-induced inflammatory response. EVI1 is induced by NTHi via an IKKβ-p65–dependent mechanism, thereby unveiling a novel negative-feedback loop of NF-κB–dependent inflammation.

Because our data indicate that EVI1 is induced by NTHi, a potent inducer for inflammation, we sought to determine whether NTHi-induced EVI1 expression is also controlled by the IKKβ–NF-κB pathway. Interestingly, as shown in Fig. 7A, NTHi-induced EVI1

FIGURE 5. EVI1 negatively regulates bacteria-induced NF-κB activation via inhibition of p65 acetylation at lysine 310. (A and B) A549 cells cotransfected with EVI1 and p65 were treated with NTHi; the cell lysates were immunoprecipitated with anti-p65 Ab and analyzed by WB analysis with the indicated Abs. (C) A549 cells were cotransfected with NF-κB–luciferase reporter plasmid together with EVI1 siRNA and p65 WT or p65 K310R constructs, and NF-κB–dependent promoter activity was measured by luciferase assay. *p < 0.05 versus p65 WT alone. †p < 0.05 versus p65 WT with EVI1 siRNA. (D) p65^−/− MEF cells reconstituted with vector, p65 WT, or p65 K310R were cotransfected with NF-κB–luciferase reporter plasmid together with EVI1 siRNA or control vector. Cells were treated with NTHi, and cell lysates were analyzed by WB analysis with anti-p65 and tubulin Abs (lower panel). NF-κB–dependent promoter activity was measured by luciferase assay (upper panel). *p < 0.05 versus NTHi in p65^−/− MEF cells, †p < 0.05 versus NTHi in p65^−/− MEF cells reconstituted with p65 WT, ‡p < 0.05 versus NTHi treatment with EVI1 siRNA in p65^−/− MEF cells reconstituted with p65 WT. For WB, data are representative of three independent experiments. For luciferase assay, data represent the mean ± SD of at least three independent experiments, and each experiment was performed in triplicate.
expression was markedly inhibited by overexpressing a dominant-negative mutant form of IKKβ in A549 cells. We next investigated the role of p65 in NTHi-induced EVI1 expression. As shown in Fig. 7B, no induction of EVI1 expression by NTHi was observed in p65−/− MEF cells. In contrast, NTHi markedly induced EVI1 expression in p65−/− MEF cells that were reconstituted with p65 WT construct (Fig. 7B). Thus, these data unveil a novel EVI1-dependent negative-feedback loop controlling NF-κB–dependent inflammation.

Discussion

In the current study, we provide direct evidence that EVI1 acts as a negative feedback regulator of NTHi-induced NF-κB–dependent inflammation in human respiratory epithelial cells in vitro and in a mouse model of lung inflammation in vivo. Interestingly, expression of EVI1 itself is also induced by NTHi in an NF-κB–dependent manner, which, in turn, leads to the inhibition of acetylation of p65 at K310 site, thereby leading to the decreased DNA-binding activity of NF-κB and subsequent inhibition of the inflammatory response (Fig. 7C). This study will provide new insights into the novel role of EVI1 in the regulation of NF-κB–dependent inflammatory response through a negative feedback loop and may also lead to the development of new therapeutic interventions for controlling inflammation.

Of particular interest in this study is the identification of EVI1 as a novel negative-feedback regulator of NF-κB–dependent inflammatory response in vitro and in vivo. EVI1 was originally identified as a proto-oncogene and zinc finger transcription factor with important roles in normal development and leukemogenesis. As a proto-oncogene, the aberrant expression of EVI1 is associated with AML (2, 4), myelodysplastic syndrome (5), and chronic myelogenous leukemia (3). High expression of EVI1 is detectable in ~8% of AML cases and is a poor prognostic indicator (4, 53). As a transcription factor, EVI1 has the potential to interact with both corepressors and coactivators, and it is involved in many signaling pathways for both corepression and coactivation of cell cycle genes (6–9). It was also demonstrated that Smad3 interacts with the first zinc finger domain of EVI1. As a consequence, EVI1 disturbs the TGF-β–signaling pathway, which is known to be a negative regulator for cellular growth and differentiation (54). However, there has been no report demonstrating a role for EVI1 in regulating NF-κB activity and the subsequent inflammatory response. In the current study, we show that EVI1 negatively regulates NTHi- and TNF-α–induced NF-κB activation and inflammatory response in vitro and in vivo. This finding is in line with the previous study demonstrating that the loss of function mutation in the second zinc finger domain of Evil in the Junbo mouse is associated with the predisposition to chronic OM (13). Interestingly, human EVI1 MT that is homologous to the point mutation of Junbo mouse indeed acts as a dominant-negative mutant in our in vitro studies. Thus, our results unveil a previously unrecognized role for EVI1 as a negative feedback regulator of NF-κB–dependent inflammation in upper respiratory inflammatory diseases.

In the current study, the generalizability of our finding that EVI1 acts as a negative regulator of NF-κB–dependent inflammation was further confirmed by evaluating the negative regulation of TNF-α–induced NF-κB–dependent inflammation by EVI1. Because we found that EVI1 inhibits inflammation by directly targeting NF-κB, it seems reasonable to postulate that EVI1 may act as a negative regulator of NF-κB–dependent inflammation induced by other inflammatory stimuli as well.

Another major interesting finding in this study is that EVI1 inhibits NTHi-induced DNA-binding activity of NF-κB by directly interacting with p65, as well as by inhibiting the acetylation of p65. The NF-κB family of transcription factors consists of five members in mammalian cells: RelA (p65), RelB, c-Rel, p50/p105, and p52/p100. NF-κB is a dimeric transcription factor consisting of homo- or heterodimers of Rel-related proteins (55). The most important heterodimer, consisting of two subunits, RelA/p65 and p50, is involved in the regulation of a variety of physiologic and pathologic processes, including proliferation, differentiation, survival, tumorigenesis, and inflammation (55). In the inactive state, NF-κB resides in the cytoplasm and forms a multiprotein complex with an inhibitory subunit, IκB. Upon activation by external stimuli, the inflammatory signal converges on and activates a set of IκB kinases, known as the IκK complex, which phosphorylates NF-κB, targeting it for proteasomal degradation. Once released from the IκB complex, NF-κB translocates to the nucleus, where it binds to DNA and promotes the transcription of proinflammatory genes.
mediators, including cytokines, chemokines, and adhesion molecules, thereby playing a critical role in mediating the inflammatory response (19). Our studies indicate that EVI1 negatively regulates NTHi-induced NF-κB activation via a mechanism independent of IκBα phosphorylation or degradation (Fig. 3B, 3C) and p65 nuclear translocation (Fig. 3D, 3E) but dependent on the inhibition of DNA-binding activity of the NF-κB complex (Fig. 3F, 3G).

Recent studies suggested that degradation of IκBα and nuclear translocation of NF-κB are insufficient to induce a maximal NF-κB-dependent transcriptional activity. Rather, the NF-κB complex must undergo additional posttranslational modifications. Among all known posttranslational modifications of p65, acetylation of p65 was shown to play a critical role in controlling the duration and strength of NF-κB signaling and regulating various biological functions of NF-κB, including DNA binding, transcription, and association with the inhibitor IκBα (38, 51, 56). p300 and CBP were shown to play major roles in the acetylation of p65, whereas HDAC3 appears to be critical for the deacetylation of p65. Endogenous p65 is acetylated in a stimulus-coupled manner after activation of cells with NTHi or other stimuli (45). Interestingly, our data demonstrate that EVI1 directly binds to p65 and inhibits activation of cells with NTHi or other stimuli (45). Interestingly, HDAC3 appears to be critical for the deacetylation of p65. En-

functions of NF-κB and strength of NF-κB–dependent inflammatory response (19). Our studies indicate that EVI1 negatively regulates NF-κB–dependent inflammatory response by inhibiting p65 acetylation. Of additional interest in this study is that EVI1 itself is markedly induced by NTHi in an NF-κB–dependent manner, which, in turn, leads to the inhibition of the NTHi-induced inflammatory response, thereby preventing an overactive inflammatory response. The inflammatory response triggered by bacteria is a protective attempt by the host to remove the injurious stimuli and to initiate the healing process, but an excessive inflammatory response is detrimental to the host, because of severe tissue damage (57, 58). To avoid an overactive and detrimental inflammatory response in infectious disease, the bacteria-induced inflammatory response must be tightly regulated. The host has evolved a variety of strategies to prevent a detrimental inflammatory response during bacterial infections. Bacteria-induced negative-feedback regulation is a paradigm in information transfer from membrane to nucleus. Sci. STKE 1999: RE1.

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Disclosures

The authors have no financial conflicts of interest.

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