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IL-32: A Host Proinflammatory Factor against Influenza Viral Replication Is Upregulated by Aberrant Epigenetic Modifications during Influenza A Virus Infection

Wei Li,¹ Wei Sun,¹ Li Liu, Fang Yang, Yongkui Li, Yanni Chen, Jiali Fang, Wenjing Zhang, Jianguo Wu, and Ying Zhu

Our previous studies with clinical data analysis have shown that the proinflammatory factor IL-32 is activated in response to influenza virus infection. However, little is known about how influenza virus induces IL-32 production, and the role of IL-32 in the host immune responses during viral infection remains unclear. In this study, we show that IL-32 production is stimulated by influenza A virus or dsRNA in human PBMCs from healthy volunteers. We demonstrate that the NF- κ B and CREB pathways play key roles in the activation of IL-32 production in response to influenza virus infection in A549 human lung epithelial cells. We then show that aberrant epigenetic modifications in the *IL32* promoter are important in the transcriptional regulation of IL-32 expression. Interestingly, one CpG demethylation within the CREB binding site increases the binding of CREB to the promoter, which is followed by *IL32* transcriptional activation in influenza A virus-infected cells. Overexpression assays combined with RNA interference show that DNA methyltransferases DNMT1 and DNMT3b are critical for *IL32* promoter methylation and gene silencing before viral infection. We have demonstrated the anti-influenza virus function of IL-32. Assays for each of the six IL-32 isoforms (α , β , γ , δ , ϵ , and ζ) during influenza virus infection indicated that all the isoforms have antiviral activity, with different inhibitory rates, and that the effect of IL-32 γ is strongest. Our results indicate that the elevated IL-32 levels triggered by influenza virus infection in turn hamper viral replication. *The Journal of Immunology*, 2010, 185: 5056–5065.

Our previous studies have demonstrated that IL-32 is an important proinflammatory cytokine, which is induced by influenza virus and HIV infections in humans (1–3). It is also stimulated by other exogenous pathogens, such as *Mycobacterium tuberculosis* (4). Besides its role in acute infections, IL-32 has biological activities associated with chronic inflammatory diseases, including rheumatoid arthritis and obstructive pulmonary disease (5, 6). IL-32 has been identified as a critical regulator of endothelial function, and it can act synergistically with nucleotide-binding oligomerization domain containing 1 and nucleotide-binding oligomerization domain containing 2 ligands to stimulate IL-1 β and IL-6 release in a caspase-1-dependent

manner (7, 8). Recently, a specific IL-32 α -binding protein, neutrophil proteinase 3, has been shown to enhance IL-32 activity by cleaving it into separate domains, and the separated N-terminal IL-32 γ domain displayed the highest level of biological activity of all the separate IL-32 domains (9, 10). TNF- α -induced IL-32 is reported to be positively regulated via the Syk/PKC δ /JNK pathway in rheumatoid synovial fibroblasts (11).

The synergism between IL-32 and other well-characterized players in innate immunity has been documented (12). IL-32 not only contributes to host responses through the induction of proinflammatory cytokines but also directly affects specific immunity by causing the differentiation of monocytes into macrophage-like cells (13). Furthermore, an anti-HIV effect of IL-32 has recently been reported (2, 14). IL-32 appears to possess antiviral activity due to induction of IFN- α (15). IL-32 is transcribed as six alternatively spliced variants, and the biological activity of each individual isoform remains unknown (16). The γ isoform of IL-32 is reported to be the most biologically active (17).

DNA methylation and histone modifications are the major epigenetic mechanisms implicated in the regulation of gene transcription. In mammalian cells, the somatic DNA methylation patterns of a given cell are established during gametogenesis and early embryogenesis by three known DNA methyltransferases (DNMTs): DNMT1, 3a, and 3b (18). In virus-associated epigenetic modifications, the SV40 T Ag activates DNMT1 and uses DNMT3b to inactivate tumor suppressor genes (19, 20). Hepatitis B virus X (HBx) specifically represses insulin-like growth factor 3 expression through its de novo methylation by DNMT3a and by inhibiting *trans*-acting transcription factor 1 (SP1) binding by recruiting methyl CpG-binding protein 2 (MeCP2) to the newly methylated SP1-binding element. Thus, viral infections appear to participate in epigenetic changes involved in the stimulation of gene expression. However, it is not yet clear how influenza virus mediates the methylation levels of the proinflammatory cytokine

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Abbreviations used in this paper: Act., relative DNMT activity; 5'-aza-CdR, 5-aza-2'-deoxycytidine; ChIP, chromatin immunoprecipitation; CRE, cAMP response element; Δ , mutant-type; DNMT, DNA methyltransferase; HBx, hepatitis B virus X; HDAC2, histone deacetylase 2; IKK, inhibitor of κ B kinase; IRAK2, IL-1R-associated kinase 2; MDCK, Madin-Darby canine kidney; met-, methylated; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, Z-LLL-CHO; MOI, multiplicity of infection; NP, nucleoprotein; n.s., nonspecific; PBST, phosphate buffered saline-Tween; siRNA, small interfering RNA; SP1, *trans*-acting transcription factor 1; TRAF6, TNFR-associated factor 6; vRNA, viral RNA.

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gene *IL32* and which DNMTs are responsible for the observed epigenetic modifications.

In this study, we analyzed the upregulation of *IL32* expression by determining the signal pathway activation and DNA demethylation involved in the cellular response to influenza virus infection. To our knowledge, our results show that influenza virus activates IL-32 expression by a novel and complex mechanism, in which the NF- κ B and CREB pathways are activated, together with site-specific demethylation within the cAMP response element (CRE) in the *IL32* promoter region. We also examined each of the six IL-32 isoforms (α , β , γ , δ , ϵ , and ζ) during influenza virus infection. Our results demonstrate that all the isoforms display antiviral activity, with different inhibition rates, and that the effect of IL-32 γ is the most significant.

Materials and Methods

Ethics statement

All research involving human participants was approved by the Institutional Review Board of the College of Life Sciences, Wuhan University, in accordance with its guidelines for the protection of human subjects. Written informed consent was obtained from each participant.

Isolation of PBMCs and stimulation of IL-32 production

Venous blood was drawn into heparin tubes from the antecubital vein of five healthy volunteers. The PBMC fraction was obtained by density centrifugation of the blood diluted 1:1 in pyrogen-free saline over Histopaque (Sigma, St. Louis, MO). The cells were washed twice in saline and suspended in culture medium (RPMI 1640) supplemented with penicillin (100 U/ml) and streptomycin (100 μ g/ml).

To flat-bottom 96-well plates was added 5×10^5 PBMCs in a 100- μ l volume, which was then incubated with either 100 μ l culture medium (mock control) or 100 μ l stimulus: influenza A virus (H5N1; multiplicity of infection [MOI] = 1) or dsRNA [poly(I:C); 50 μ g/ml]. After incubation at 37°C for 24 h, the cells were lysed with 1% Triton X-100 (Bio-Rad Laboratories, Hercules, CA) and two cycles of freeze-thaw. The IL-32 concentrations were then determined.

Virus and cell culture

Influenza virus strain A/chicken/Hubei/327/2004 (H5N1) was used as described previously (1, 21). Two other influenza virus strains used in this study, A/Yamagata/120/86 (H1N1) and A/Hong Kong/498/97 (H3N2), were provided by the China Center Type Culture Collection. Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's MEM, and human lung epithelial cells (A549) were cultured in F12K medium.

Plasmids and reagents

A human DNMT1 full-length cDNA clone (pcDNA3.1-DNMT1) was a gift from Dr. Moshe Szyf (McGill University, Montreal, Quebec, Canada). Human DNMT3a and DNMT3b full-length cDNA clones (pcDNA3-DNMT3a and pcDNA3-DNMT3b, respectively) were gifts from Dr. Arthur D. Riggs (Beckman Research Institute, Duarte, CA). Small interfering RNA (siRNA) plasmids specifically directed against RIG-I, MAVS, IL-1R-associated kinase 2 (IRAK2), TNFR-associated factor 6 (TRAF6), IKK α , inhibitor of κ B kinase (IKK) β , IKK γ , p50, p65, CREB, DNMT1, DNMT3a, DNMT3b, MECP2, and histone deacetylase 2 (HDAC2) were constructed by ligating the corresponding pairs of oligonucleotides to pSilencer 2.1-U6 neo (Ambion, Austin, TX) based on the target sequences described previously (22–31).

The luciferase reporter vector (pGL3) containing the *IL32* promoter (–746/+25), pIL32-Luc, and a *Renilla* internal control vector, pRL-TK (Promega, Madison, WI), were documented in our previous study (1). Truncated *IL32* promoter constructs were generated from pIL32-Luc using the following primers (MluI and HindIII sites are underlined): 5'-GTTACGCGTGGCAAGG-GCGTGGTTG-3' (–501, sense); 5'-GTTACGCGTGGAGCTAGAAGAG-3' (–407, sense); 5'-GTTACGCGTCTTGGCCAGAG-3' (–336, sense); 5'-GTTACGCGTGGACAGGGTCCAAATTCC-3' (–98, sense); 5'-GTTAAGC-TTAGGGAAAGTCCAGACTCGGG-3' (+25, antisense). Differently mutated *IL32* promoter constructs were also generated from pIL32-Luc with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, LA Jolla, CA). The specifically mutated sites are indicated in Fig. 4A. All six isoforms of human IL-32 cDNA were amplified by RT-PCR and cloned into the pCMV-tag2A vector to generate different IL-32 isoform-expressing plasmids (Flag2A-

IL32 α , Flag2A-IL32 β , Flag2A-IL32 γ , Flag2A-IL32 δ , Flag2A-IL32 ϵ , and Flag2A-IL32 ζ) using different sense primers and the same antisense primer (EcoRI and HindIII sites are underlined), as follows: 5'-CAGCGAATT-CAATGTGCTTCCCGAAGG-3' (sense primer for isoforms α , β , γ , and ϵ); 5'-CAGCGAATTCAATGAAGAAGCTGAAGG-3' (sense primer for isoform δ); 5'-CAGCGAATTCAATGCAAAATGCAGAAT-3' (sense primer for isoform ζ); 5'-GTCGAAGCTTTCATTTGAGGATTGGG-3' (antisense primer for all isoforms). All the constructs were confirmed by DNA sequencing.

Carbobenzoyl-L-leucyl-L-leucyl-L-leucinal, Z-LLL-CHO (MG132), 5-aza-2'-deoxycytidine (5'-aza-CdR), and synthetic poly(I:C) were purchased from Sigma. IL-32 γ protein was purchased from R&D Systems (Minneapolis, MN). Four Abs against IL-32 were used in the experiments. A commercial mAb KU32-52 (BioLegend, San Diego, CA) and a rabbit polyclonal Ab, produced in this study using the recombinant IL-32 α protein, were used in the neutralization assays. mAb KU32-56 (BioLegend) and KU32-52 (BioLegend) were used for the measurement of IL-32 by ELISA. Polyclonal Ab (R&D Systems) was used for Western blot analysis only.

Transient transfection and luciferase assay

Cells were plated at a density of 4.0×10^5 cells per well in 24-well or 6-well plates and grown to ~80% confluence at the time of transfection. The plasmids were co-transfected into the cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, the cells were serum starved for another 24 h before they were harvested. The luciferase activities were then measured, and *Renilla* luciferase activities were determined as the internal control for transfection efficiency, as previously described (1, 2, 32). The assay results are expressed as relative luciferase activity.

Measurements of IL-32 expression

Total RNA extraction, reverse transcription, and the primers for *IL32* and β -actin used in the semiquantitative RT-PCR assay have been described previously (1, 33). IL-32 production in the culture supernatants or cell lysates was measured by ELISA as described in prior studies (15, 34). Briefly, the rIL-32 α protein was expressed in *Escherichia coli* and purified by Ni-NTA His \cdot Bind Resins and was used to generate a standard curve in ELISA assays. The 96-well plates were precoated with commercial mAb to IL-32 (KU32-56, 4 μ g/ml; BioLegend) overnight at 4°C and blocked with 1% BSA (Roche, Basel, Switzerland) for 1 h at 37°C. Then, serially diluted rIL-32 α or other samples were added to wells and incubated at 37°C for 1 h. The plates were washed three times with phosphate buffered saline-Tween (PBST), and biotinylated mAb of IL-32 (KU32-52, 0.2 μ g/ml; BioLegend) diluted in 1% BSA was then added, followed by 1 h of incubation. After that, the plates were washed three times with PBST and

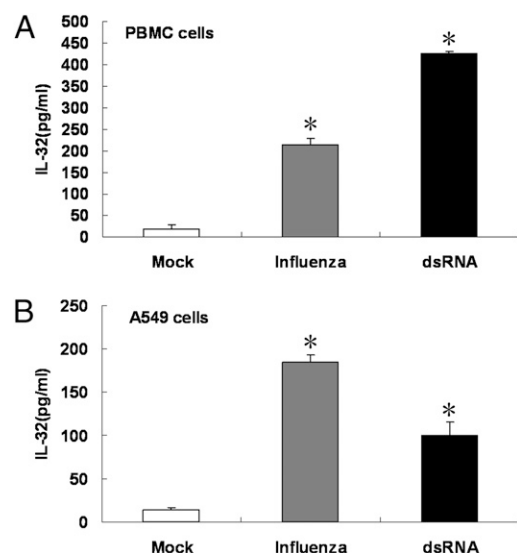


FIGURE 1. Stimulation of IL-32 production in PBMCs and A549 cells by influenza virus or dsRNA. **A**, Freshly isolated PBMCs were stimulated with influenza A virus (H5N1; MOI = 1) or dsRNA [poly(I:C); 50 μ g/ml]. **B**, A549 cells were infected with influenza A virus (H5N1; MOI = 0.1) or treated with dsRNA [poly(I:C); 50 μ g/ml]. IL-32 concentrations were measured in cell lysates by ELISA 24 h postinfection or treatment. Data are presented as mean \pm SEM; $n = 5$; * $p < 0.05$.

incubated with streptavidin–HRP (0.3 μ g/ml; Upstate, New York, NY) diluted in 1% BSA for 30 min. Followed by washes of the plates as described above, 3',3',5,5'-tetramethylbenzidine substrate (Thermo Scientific, Houston, TX) was added and then 50 μ l 2 N sulfuric acid was added to stop the enzyme reaction. The plates were subjected to detect OD values at 450 nm using a microplate reader.

Chromatin immunoprecipitation analysis

Forty-eight hours after infection with influenza virus (H5N1) in A549 cells, chromatin immunoprecipitation (ChIP) analysis was performed as described previously (35). The Abs used in ChIP assays are as follows: anti-p50, anti-p65, anti-p52, anti-RelB, anti-CREB1, anti-CREB2, anti-DNMT1, and negative control rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-DNMT3a and anti-DNMT3b were purchased from Cell Signaling Technology (New York, NY). The bound DNA fractions were PCR amplified using the following primers: Chip1, 5'-GGGCGTGGTTGTGCTCAGC-3' and 5'-GGGGCCGAAGTCACTGG-3', amplifying the region spanning nucleotides -463 to -315 of the *IL32* promoter region; Chip2, 5'-GGATCCCACTGGCTGACG-3', and 5'-GAGCCAGGAATGGGCTGC-3', amplifying the region spanning nucleotides -38 to +96. The resulting PCR products were separated electrophoretically on agarose gels.

Electrophoretic mobility shift assay

Nuclear fractions were extracted from 1×10^7 cells, and the DNA-binding reactions were performed at room temperature for 30 min with 1.5×10^5 counts per minute of double-stranded oligonucleotide probes that had been end-labeled with [γ - 32 P]ATP using T4 polynucleotide kinase (Takara, Dalian, China), 2 μ g nuclear extract, and 1 μ g poly(I:C) (Amersham Biosciences, Piscataway, NJ) in a final volume of 20 μ l. The probes used in the EMSA analysis were based on the human *IL32* promoter sequence: wild-type NF- κ B(a), 5'-GGAGGAGGTGGGAGGCCAGCCAGGC-3'; mutant-type NF- κ B(a), 5'-GGAGGAGGTctcAGGCCAGCCAGGC-3'; wild-type NF- κ B(b), 5'-GAGCCCGTAGGGGAGGCCCAAGATTGC-3'; mutant-type NF- κ B(b), 5'-GAGCCCGTactGAGCCCAAGATTGC-3'; wild-type NF- κ B(c), 5'-GTACTCAGGAGTTTCCCCAGCCAG-3'; mutant-type NF- κ B(c), 5'-GTA-CTCactAGTTTCCCCAGCCAG-3'; wild-type CRE, 5'-CTTGGCTGACGTCACCTTCC-3'; mutant-type CRE, 5'-CTTGGCTGcaGctgCCTTCC-3'. The resulting complexes were resolved on a 5% native polyacrylamide gel and exposed to x-ray film.

DNMT activity assay

Protein samples (cell lysates or nuclear extracts) from A549 cells were prepared, and 20 μ g of each sample was used for the measurement of DNMT

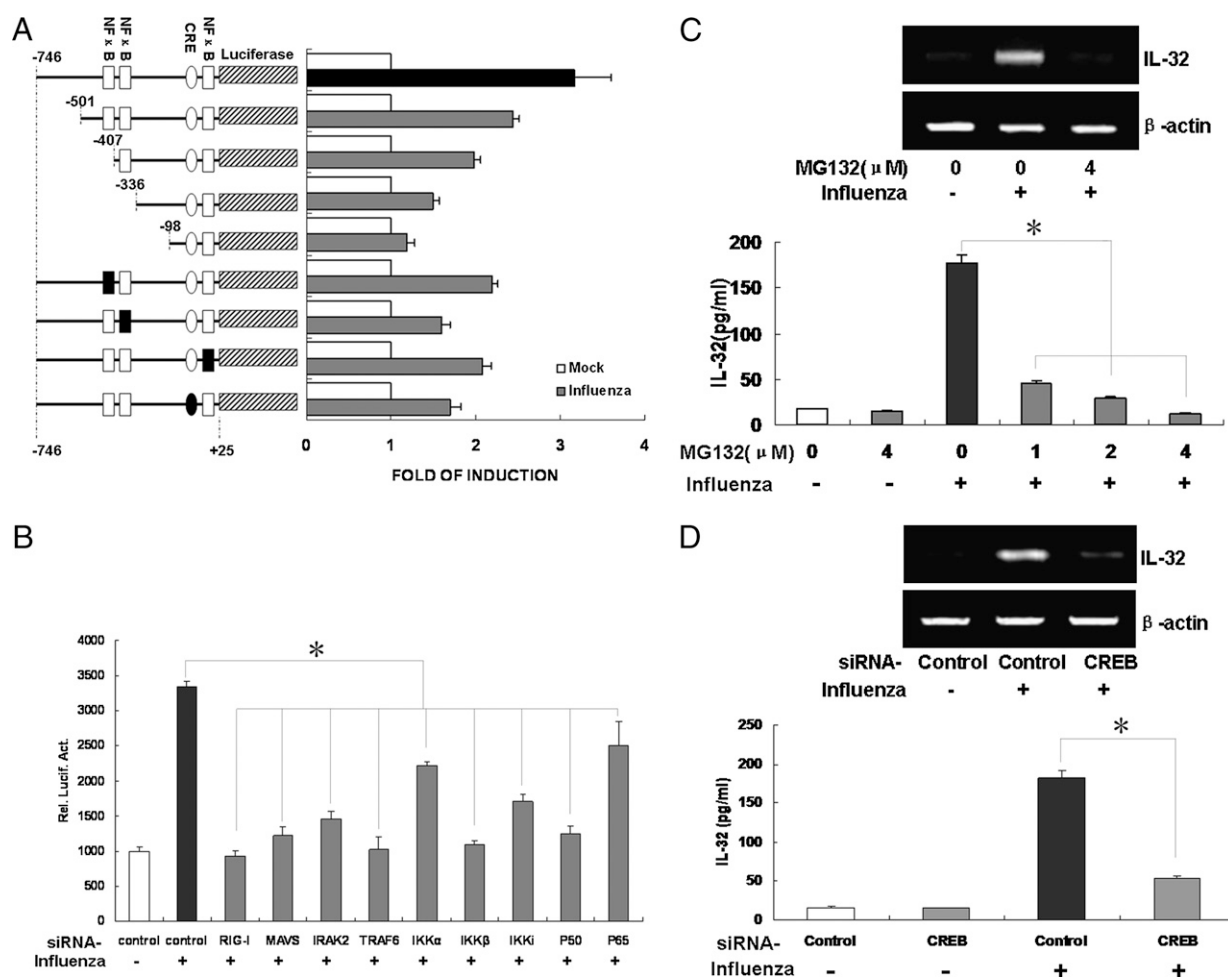


FIGURE 2. NF- κ B and CRE cis-acting elements are involved in the induction of IL-32 expression during influenza virus infection. **A**, A549 cells were infected with or without influenza A virus (H5N1; MOI = 0.1) and then transfected with constructs containing *IL32* full-promoter, four promoter truncations, three NF- κ B binding site mutants, and one CREB binding site mutant generated from the *IL32* full-promoter plasmid (pIL32-Luc). pRL-TK was co-transfected as internal control. Luciferase activity was measured 48 h after transfection. **B**, Plasmids pIL32-Luc and pRL-TK were co-transfected with control siRNA, siRNA against RIG-I, MAVS, IRAK2, TRAF6, IKK α , IKK β , IKK γ , p50, or p65, respectively, into A549 cells, which were then infected with or without influenza A virus (H5N1; MOI = 0.1) as indicated. Luciferase activity was measured 48 h after transfection. **C**, A549 cells were infected with or without influenza A virus (H5N1; MOI = 0.1) and then treated with MG132 at the indicated concentrations. Measurements of IL-32 in the culture supernatants (bottom panel) and RT-PCR for *IL32* and β -actin in the cell lysates (top panel) were performed after 48 h. **D**, A549 cells were infected with or without influenza A virus (H5N1; MOI = 0.1) and transfected together with control siRNA or CREB siRNA. Measurements of IL-32 in the culture supernatants (bottom panel) and RT-PCR for *IL32* and β -actin in the cell lysates (top panel) were performed after 48 h. Data shown are mean \pm SE; $n = 3$; * $p < 0.05$.

activity with the EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit (Epigentek, Brooklyn, NY) according to the manufacturer's protocol.

Bisulfite modification and sequencing analysis

Bisulfite treatment was performed 48 h after influenza virus infection. Briefly, 2 μ g genomic DNA extracted from A549 cells was denatured in 0.2 M NaOH. Sodium bisulfite and hydroquinone were added to final concentrations of 3.1 M and 0.5 mM, respectively, and the samples were incubated at 55°C for 16 h. After purification with a DNA Clean-Up System (Promega, Madison, WI), the DNA samples were desulfonated in 0.3 M NaOH, precipitated with ethanol, and dissolved in 30 μ l Tris-EDTA buffer. The modified DNA (20 ng) was PCR amplified with the following primers specific for *IL32* promoter regions: region I, 5'-TAG-GAAATTATTTTAAAGGTAAGGG-3' and 5'-TAACCCCTTCTAAACCA-AAACAAAAC-3'; region II, 5'-AGAAGGGTTAGAAGGATTGTT-AG-3' and 5'-AAAACCTCCCTAAATACAAAAAATA-3'. The resulting PCR products were cloned into the pGEM-T Easy vector (Promega) and subjected to sequencing analysis.

Measurement of influenza virus replication

MDCK cells were infected with influenza A virus (H5N1; MOI = 0.1). The viral titers were measured 48 h or at indicated time points postinfection with a hemagglutination assay in U-shaped 96-well plates, as described

previously (21). Total RNA extraction, reverse transcription, and real-time PCR were conducted to detect the relative RNA levels of nucleoprotein (NP)-mRNA, NP-cRNA, and NP-viral RNA (vRNA), as described previously (21).

Neutralization of the antiviral effect by IL-32 Ab

For antiviral neutralization assay, MDCK cells were infected with influenza A virus (H5N1; MOI = 0.1) and treated with or without IL-32 γ (40 ng/ml) and polyclonal Ab against IL-32 (5 μ g/ml). Three hours postinfection, total RNAs were isolated from the infected cells. The relative RNA levels of NP-specific mRNA, cRNA, and vRNA were measured by real-time RT-PCR and normalized to the level of the corresponding γ -actin mRNAs. For non-antiviral neutralization assay, see Supplemental Fig. 1.

Results

Influenza virus induces the production of IL-32 in PBMCs

In a previous study, we found that influenza virus infections in human patients are associated with increased serum levels of the proinflammatory cytokine IL-32 (1). In this study, we found influenza virus infection or the dsRNA treatment in both PBMCs and A549 lung epithelial cells resulted in the activation of IL-32

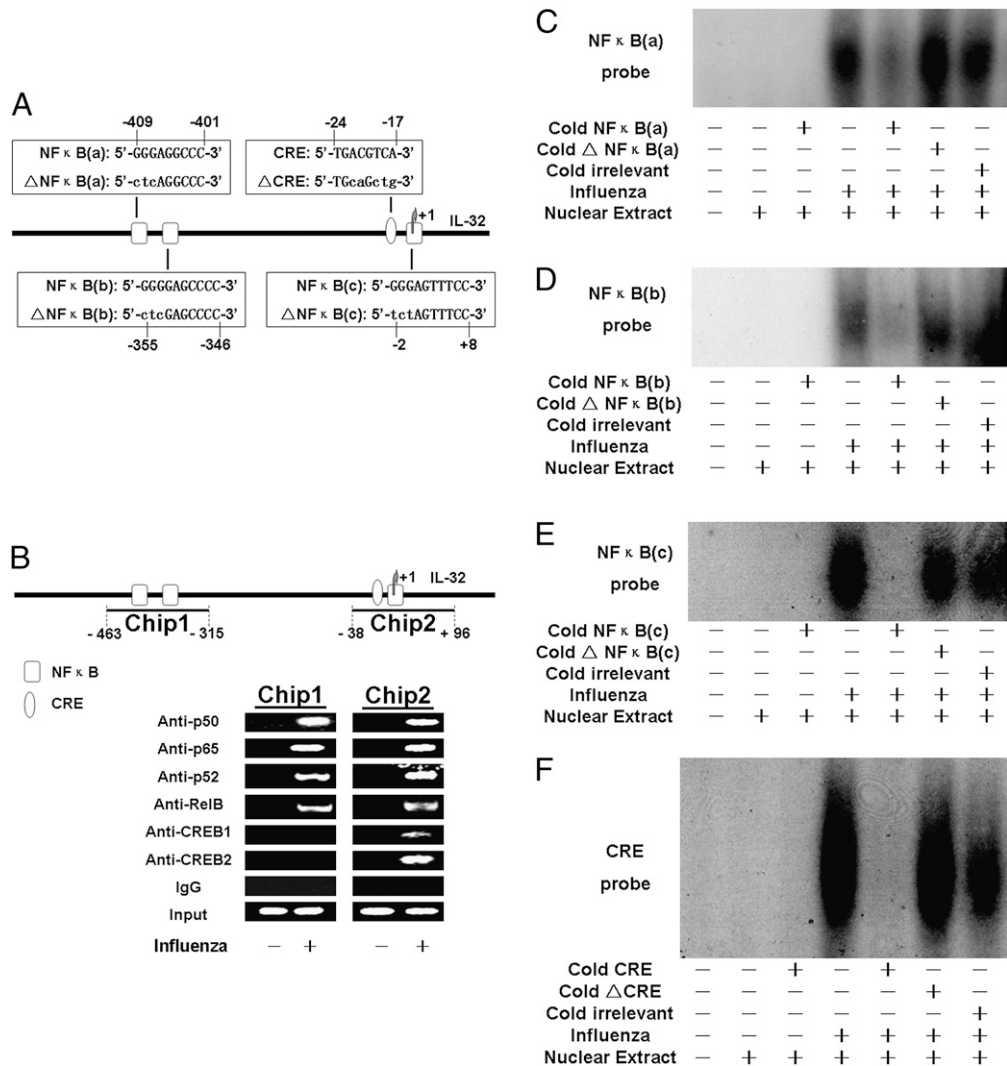


FIGURE 3. Roles of one CRE and three NF- κ B binding sites in the *IL32* promoter during influenza infection. *A*, Schematic diagram of the three possible NF- κ B binding sites and one possible CRE site in the *IL32* promoter. Small letters indicate mutations in the conserved sequences used in this study. *B*, ChIP assay of the *IL32* promoter in A549 cells infected with or without influenza A virus (H5N1; MOI = 0.1) for 48 h. The exact locations of the PCR products are indicated as Chip1, Chip2, and lines under the simplified genomic structure of *IL32*. +1, transcription initiation site. *C–F*, A549 cells were infected with influenza A virus (H5N1; MOI = 0.1) for 48 h. Nuclear extracts were prepared, then NF- κ B (*C–E*) and CREB (*F*) DNA binding activities were determined by EMSA. As specific or nonspecific competitors, unlabeled (cold) wild-type, mutant-type (Δ), or irrelevant double-stranded oligonucleotides (100-fold) were used.

expression. As shown in Fig. 1, a significant increase in IL-32 production was observed when freshly isolated PBMCs were stimulated with influenza A virus (MOI = 1) or viral replicative intermediate dsRNA.

NF- κ B and CRE cis-acting elements are involved in the induction of IL-32 expression by influenza virus

To investigate the transcriptional regulation of IL-32 expression, we analyzed the possible consensus *cis* elements in the *IL32* promoter using software from <http://www.gene-regulation.com>. Four promoter truncations, three NF- κ B binding site mutants, and one CREB binding site mutant were generated from the *IL32* full-promoter plasmid (pIL32-Luc). A549 cells were transfected with plasmids containing the luciferase reporter gene under the control of different *IL32* promoters in the presence or absence of influenza virus (Fig. 2A). The results of the luciferase assays show that the *cis* elements NF- κ B(a), NF- κ B(b), NF- κ B(c),

and CRE were all required for the activation of *IL32* expression triggered by influenza virus.

We also found that the *IL32* promoter activity was suppressed by siRNA plasmids directed against several cellular factors (RIG-I, MAVS, IRAK2, TRAF6, IKK α , IKK β , IKK γ , p50, and p65), all involved in the NF- κ B signaling pathway (Fig. 2B), and that IL-32 expression was downregulated by MG132 (NF- κ B inhibitor) in a dose-dependent manner (Fig. 2C). RT-PCR and ELISA analyses also showed that IL-32 was downregulated at the mRNA and protein levels by siRNA directed against CREB (Fig. 2D). All these results demonstrate that NF- κ B and CREB signaling pathways are involved in the upregulation of IL-32 expression in response to influenza virus infection.

Increased binding of NF- κ B and CREB to IL32 promoter in response to influenza virus infection

To confirm that influenza virus transcriptionally regulates IL-32 expression, we investigated the localization of the NF- κ B subunits

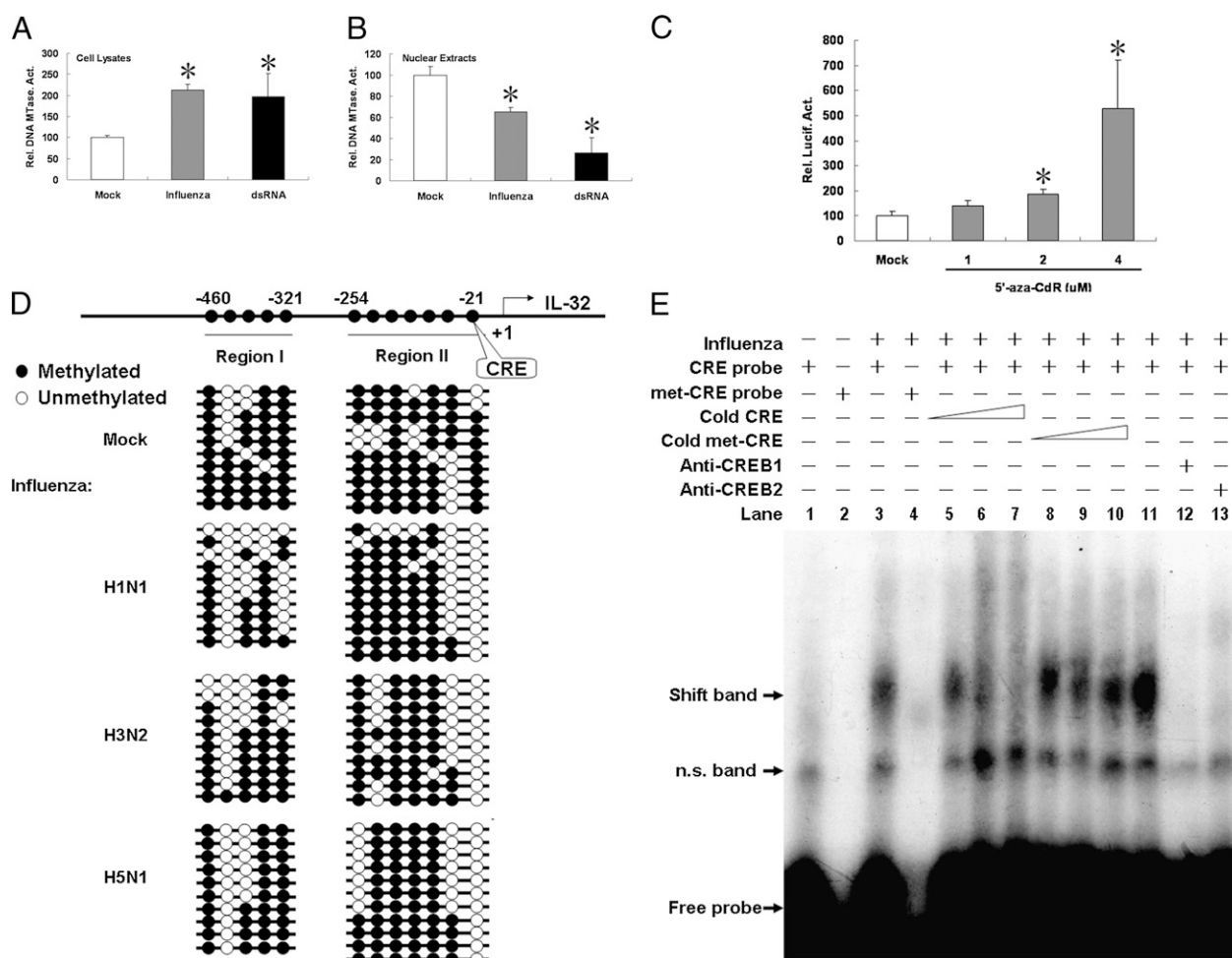


FIGURE 4. Epigenetic modification of the *IL32* promoter in influenza virus-infected A549 cells. **A** and **B**, DNMT activity assay of whole-cell lysates (**A**) and nuclear extracts (**B**) of A549 cells infected with influenza A virus (H5N1; MOI = 0.1) or treated with dsRNA [poly(I:C); 50 μ g/ml] for 48 h. The observed DNMT activities were normalized to that in the mock-treated cells. Rel. DNA MTase. **C**, A549 cells were co-transfected with pIL32-Luc and pRL-TK in the presence or absence of different concentrations of 5'-aza-CdR for 48 h. The luciferase activity was then measured. **D**, Bisulfite sequencing of the endogenous *IL32* promoter region in A549 cells 48 h postinfection with influenza A virus strain H1N1, H3N2, or H5N1 (MOI = 0.1 for all). The -21 CpG site occurs precisely in the consensus sequence of the CRE. Open circle, unmethylated; solid circle, methylated. **E**, EMSA using a labeled 20-bp unmethylated wild-type double-stranded oligonucleotide (CRE probe) containing the CRE (-30 to -11) and the same double-stranded oligonucleotide methylated in vitro with *SssI* methyltransferases (met-CRE probe). Gel shift assays were performed with nuclear extract isolated from A549 cells infected with or without influenza A virus (H5N1; MOI = 0.1). Unlabeled unmethylated (cold CRE) or methylated (cold met-CRE) wild-type double-stranded oligonucleotides were used as competitors. To determine the specific CREB binding on the probe, anti-CREB1 and anti-CREB2 Abs were used. Data shown are mean \pm SE; $n = 3$; * $p < 0.05$. Act., relative DNMT activity; met-, methylated; n.s., nonspecific; triangle, concentrations of the competitors were increased from 1-, 10-, to 100-fold relative to that of the labeled probes.

and CREB isoforms at endogenous *IL32* gene loci in A549 cells infected with influenza virus. ChIP with Abs directed against p50, p65, p52, RelB, CREB1, and CREB2 showed that the NF- κ B subunits were recruited to two *IL32* promoter regions (nucleotides -463 to -315 and nucleotides -38 to +96) by influenza virus, whereas CREB was only recruited to one region (nucleotides -38 to +96; Fig. 3A, 3B). EMSA also confirmed the binding of NF- κ B to recognition sites NF- κ B(a) (Fig. 3C), NF- κ B(b) (Fig. 3D), and NF- κ B(c) (Fig. 3E), and CREB to the CRE recognition site (Fig. 3F) in vitro. These results suggest that influenza virus promotes NF- κ B and CREB binding to the promoter of *IL32*, initiating its transcription.

Demethylation of the *IL32* promoter by influenza virus upregulates *IL-32* expression

To determine whether influenza virus initiates *IL32* transcription by demethylation, we examined the influence of influenza virus infection or dsRNA treatment on the DNMT activity in A549 cells. Notably, we found that influenza virus or dsRNA promoted the total cellular DNMT activity (Fig. 4A), whereas it reduced the DNMT activity in the nuclear extract (Fig. 4B).

The demethylating agent 5'-aza-CdR has previously been used to investigate the role of methylation in gene expression (36, 37). In this study, we used reporter assays to investigate whether demethylation increases the activity of the *IL32* promoter. A549 cells were transfected with pIL32-Luc, and 5'-aza-CdR was added to a final concentration of 1, 2, or 4 μ M (Fig. 4C). The results show that treatment with 5'-aza-CdR stimulated the luciferase activities in a dose-dependent manner.

To obtain more-precise information about the methylation status of the *IL32* promoter, high-sensitivity mapping of the methylated cytosines was performed by bisulfite modification. As shown in Fig. 4D, two *IL32* promoter regions (nucleotides -460 to -321 and nucleotides -254 to -21 with respect to the transcription initiation site) contain five and seven putative CpG methylation sites, respectively. Two pairs of PCR primers were used to amplify the

bisulfite-modified genomic DNA. After the amplification of the region, the PCR products were cloned, and the individual plasmids were sequenced. The results show that the percentage of methylated CpGs was significantly lower in the influenza A virus-infected A549 cells (for H1N1, 58.3%; for H3N2, 65%; and for H5N1, 67.7%) than in the mock-infected cells (80%), indicating that influenza A virus induces the demethylation of the *IL32* promoter. More importantly, the CRE motif (nucleotides -24 to -17) in the *IL32* promoter, which contains a CpG site, became 100% demethylated (from 20% demethylation) in response to influenza A virus infection.

We then tested the influence of methylation on the affinity of CREB for its binding motif, CRE. We performed EMSA using a CRE probe and the corresponding methylated CRE probe, as described in *Materials and Methods*. The specificity of CREB binding was verified with an unlabeled CRE competitor and an unlabeled methylated CRE competitor and by reducing the shift band densities with anti-CREB1 or anti-CREB2 Abs (Fig. 4E). The results of the EMSA indicated that the methylation of the oligonucleotides inhibited the formation of the CREB protein-DNA complex (lanes 1–4) and that increased cold probe significantly reduced band densities due to competition with radiolabeled probe (lanes 5–7), whereas methylated cold probe did not affect the band densities (lanes 8–10). These data further support the notion that influenza virus-induced DNA demethylation promotes the binding of CREB to the *IL32* promoter. Taken together, these results suggest that the hypomethylation of the *IL32* promoter and the demethylation of its CRE site in influenza virus-infected cells activate *IL32* transcription.

DNMT1 and DNMT3b are responsible for the hypomethylation of the *IL32* promoter by influenza virus

To identify the genes with key roles in the methylation of the *IL32* promoter, we co-transfected A549 cells with pIL32-Luc and an siRNA plasmid directed against DNMT1, DNMT3a, DNMT3b,

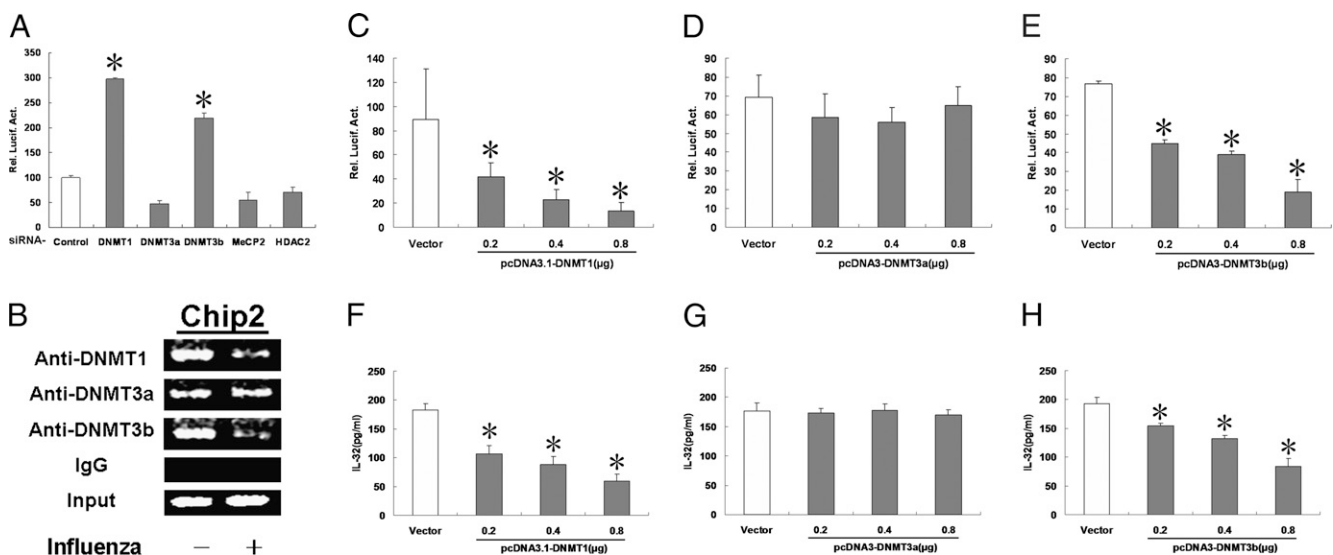


FIGURE 5. DNMT1 and DNMT3b are responsible for influenza virus-induced hypomethylation of the *IL32* promoter region. **A**, Plasmids pIL32-Luc and pRL-TK were co-transfected into A549 cells with control siRNA, DNMT1 siRNA, DNMT3a siRNA, DNMT3b siRNA, MECP2 siRNA, or HDAC2 siRNA. Luciferase activity was measured 48 h after transfection. **B**, ChIP analysis using Abs directed against DNMT1, DNMT3a, or DNMT3b in A549 cells for 48 h in the presence or absence of influenza A virus (H5N1; MOI = 0.1) infection. **C–E**, Plasmids pIL32-Luc and pRL-TK were co-transfected into A549 cells with different amounts of pcDNA3.1-DNMT1 (**C**), pcDNA3-DNMT3a (**D**), pcDNA3-DNMT3b (**E**), or the control vector. Luciferase activity was measured 48 h after transfection. **F–H**, A549 cells were pretreated with influenza A virus (H5N1; MOI = 0.1) for 12 h and transfected with different amounts of pcDNA3.1-DNMT1 (**F**), pcDNA3-DNMT3a (**G**), pcDNA3-DNMT3b (**H**), or the control vector. The IL-32 concentrations in the culture supernatants were determined 48 h after transfection. Data shown are mean \pm SE; $n = 3$; * $p < 0.05$.

MECP2, or HDAC2. As shown in Fig. 5A, the *IL32* promoter activity was upregulated by knocking down the expression of DNMT1 and DNMT3b. We then investigated the recruitment of the three DNMTs to the *IL32* promoter with a ChIP assay, using primers for Chip2 (Fig. 5B). Our results show that the binding of DNMT1 and DNMT3b to the *IL32* promoter decreased when the cells were infected with influenza virus, whereas the binding of DNMT3a remained constant.

We then investigated which of the DNMTs is responsible for the regulation of *IL32* expression by transfecting A549 cells with the DNMT expression vectors pcDNA3.1-DNMT1, pcDNA3-DNMT3a, and pcDNA3-DNMT3b. The results of luciferase assays show that the activity of the *IL32* promoter was repressed dose dependently by the overexpression of DNMT1 (Fig. 5C) and DNMT3b (Fig. 5E) in vitro, whereas DNMT3a (Fig. 5D) had very little effect. These findings were confirmed by examining the influence of DNMT1 (Fig. 5F), DNMT3a (Fig. 5G), or DNMT3b (Fig. 5H) overexpression individually on influenza virus-induced

IL-32 production in vivo. These findings, combined with the previous finding that the *IL32* promoter is demethylated in influenza virus-infected cells, suggest that DNMT1 and DNMT3b play key roles in *IL32* promoter methylation and that the inactivation of DNMT1 and DNMT3b is responsible for the hypomethylation of the promoter in response to influenza virus infection.

IL-32 affects influenza A virus production by inhibiting viral transcription and replication

To assess the effects of the IL-32 isoforms on influenza A virus production, MDCK cells were transfected with plasmids encoding one of the isoforms of IL-32 (α , β , γ , δ , ϵ , or ζ) or the control vector. The results of ELISA analysis show that IL-32 γ is the only secreted isoform of IL-32 (Fig. 6A), whereas IL-32 α , β , δ , ϵ , and ζ are cell associated (Fig. 6B), and confirm the expression of the IL-32 isoforms.

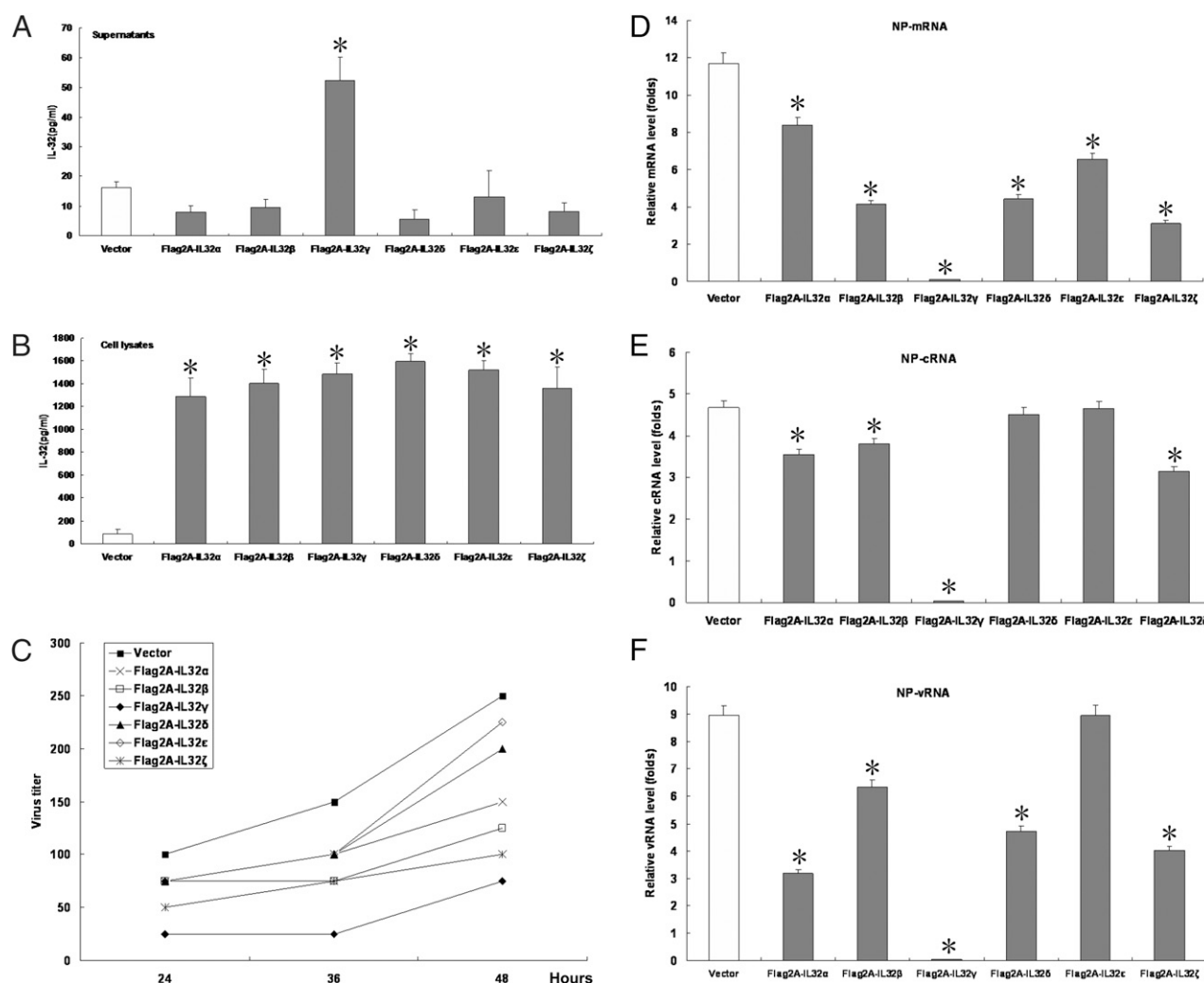


FIGURE 6. IL-32 inhibits influenza virus replication. **A** and **B**, Plasmid Flag2A (empty vector) and constructs individually encoding the six IL-32 isoforms (Flag2A-IL32 α , Flag2A-IL32 β , Flag2A-IL32 γ , Flag2A-IL32 δ , Flag2A-IL32 ϵ , Flag2A-IL32 ζ) were transfected into MDCK cells. ELISA analysis of the IL-32 concentrations in the supernatants (**A**) and cell lysates (**B**) was performed 48 h after transfection. **C**, Determination of the effects of IL-32 isoforms on virus production. MDCK cells were transfected with plasmids encoding one of the six IL-32 isoforms. The cells were infected with influenza A virus (H5N1; MOI = 0.1) 48 h after transfection. At the indicated time points postinfection, the viral titers in the culture supernatants were measured by hemagglutination assay. **D–F**, Determination of the roles of the IL-32 isoforms in influenza viral RNA production. Constructs for each of the six IL-32 isoforms were transfected into MDCK cells. The cells were infected with influenza A virus (H5N1; MOI = 0.1) 48 h after transfection. Total RNAs were isolated from the infected cells 3 h postinfection. The relative RNA levels of NP-specific mRNA (**D**), cRNA (**E**), and vRNA (**F**) were measured by real-time RT-PCR and normalized to the level of the corresponding γ -actin mRNAs. Data shown are mean \pm SE; $n = 3$; * $p < 0.05$.

Forty-eight hours after transfection, the MDCK cells were infected with virus (H5N1) at MOI = 0.1. Empty vector-transfected MDCK cells were used as the negative control. The supernatants of the cell cultures were harvested 24, 36, and 48 h postinfection, serially diluted, and assayed for virus titer using the hemagglutination test. The results show that the virus titers were reduced in cells expressing all six IL-32 isoforms in the order IL-32 γ > IL-32 ζ > IL-32 β > IL-32 α > IL-32 δ > IL-32 ϵ compared with the titer of the control MDCK cells (Fig. 6C). We have examined cell viability after overexpressing the six different IL-32 constructs in MDCK cells by trypan blue exclusion assay. No significant difference in cell survival was found between vector control and each of the isoforms. These results suggest that virus production is potently inhibited by IL-32 γ , moderately blocked by IL-32 ζ , IL-32 β , and IL-32 α , and slightly affected by IL-32 δ and IL-32 ϵ .

To determine the effect of the IL-32 isoforms on viral transcription and replication, we measured the production of three different forms of influenza A virus RNA (mRNA, cRNA, and vRNA), using an approach described previously (21). Forty-eight hours after transfection with vectors encoding the IL-32 isoforms, MDCK cells were infected with influenza A virus at MOI = 0.1, and the total RNA was isolated 3 h postinfection, when sufficient viral RNA transcription and replication had occurred. Viral mRNA, cRNA, and vRNA were reverse transcribed using the corresponding primers, and the quantity of cDNA products was measured by real-time PCR. Data from the real-time PCR show that the levels of NP-specific mRNA (Fig. 6D), cRNA (Fig. 6E), and vRNA (Fig. 6F) were reduced in the presence of all six IL-32 isoforms. The suppressive effect of IL-32 γ was extremely significant compared with that of the control cells. These results suggest that IL-32, and especially IL-32 γ , has inhibitory effects on the production of influenza A virus mRNA, cRNA, and vRNA. Furthermore, we also found that commercial IL-32 γ protein had the inhibition role on influenza A virus production (Fig. 7A, 7B). To examine whether IL-32 Ab neutralized the antiviral activity, a polyclonal Ab developed in this study was added along with IL-32 γ protein into the culture supernatant of MDCK cells infected by influenza A virus; results showed that the Ab was able to neutralize the antiviral activity partially (Fig. 7C). To validate the antiviral neutralization assay, a non-antiviral neutralization assay was performed by measuring IL-32 γ -induced TNF- α expression. Results indicated that IL-32 Ab could inhibit IL-32 γ -induced TNF- α expression (Supplemental Fig. 1).

Discussion

To our knowledge, we show that influenza virus activates IL-32 expression by a novel and complex mechanism, in which the NF- κ B and CREB pathways are activated, together with site-specific demethylation within the CRE in the *IL32* promoter region. We also examined each of the six IL-32 isoforms (α , β , γ , δ , ϵ , and ζ) during influenza virus infection. Our results demonstrate that all the isoforms have antiviral activity, with different inhibition rates, and that the effect of IL-32 γ is the most significant. The results extend our understanding of previous clinical finding that IL-32 is elevated in patients infected by influenza A virus and shed light on how proinflammatory factors respond to viral infection.

Mechanistically, there are a number of ways in which DNA methylation can repress transcription. Many of the *trans* factors known to bind to sequences containing CpG dinucleotides do not bind when the CpG doublets are methylated (38). For a general understanding of the transcriptional regulation of IL-32, it is important to determine the pivotal region that regulates IL-32 expression by DNA methylation. Our results reveal that the *IL32* promoter is methylated and transcriptionally inactivated before

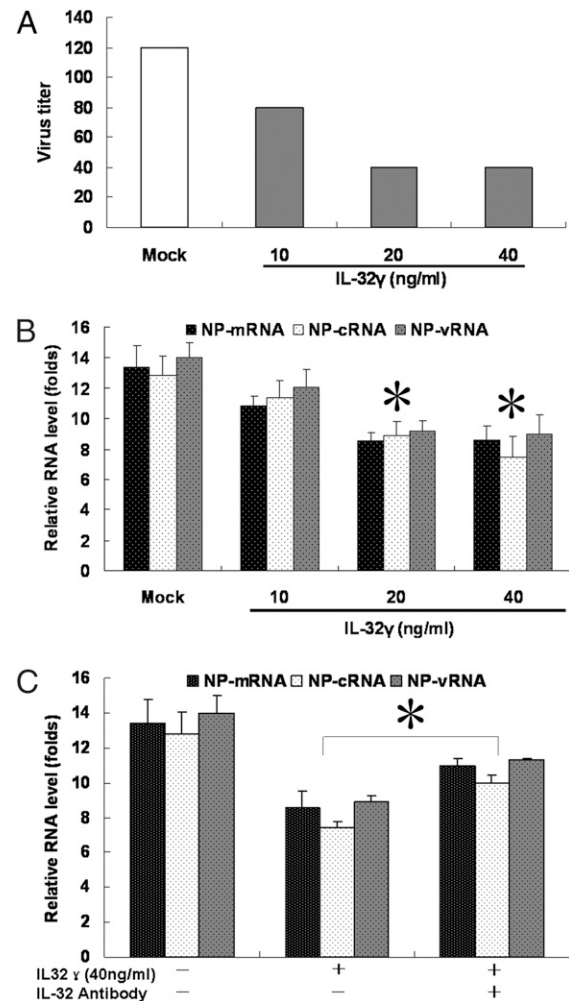


FIGURE 7. IL-32 Ab relieves the IL-32 γ -inhibited influenza virus replication. **A** and **B**, Determination of the effects of commercial IL-32 γ protein on virus production and viral RNA production. MDCK cells were infected with influenza A virus (H5N1; MOI = 0.1) and treated with or without IL-32 γ at indicated concentrations: 48 h postinfection, the viral titers in the culture supernatants were measured by hemagglutination assay (**A**); or 3 h postinfection, total RNAs were isolated from the infected cells (**B**). The relative RNA levels of NP-specific mRNA, cRNA, and vRNA were measured by real-time RT-PCR and normalized to the level of the corresponding γ -actin mRNAs. **C**, Neutralization of the antiviral effect of IL-32 γ protein by IL-32 Ab. MDCK cells were infected with influenza A virus (H5N1; MOI = 0.1) and treated with or without IL-32 γ protein and IL-32 polyclonal Ab (5 μ g/ml). Three hours postinfection, total RNAs were isolated from the infected cells. The relative RNA levels of NP-specific mRNA, cRNA, and vRNA were measured by real-time RT-PCR and normalized to the level of the corresponding γ -actin mRNAs. Data shown are mean \pm SE; $n = 3$; * $p < 0.05$.

viral infection in A549 cells. The hypomethylation of the *IL32* promoter and the demethylation of one CpG within the CREB binding site led to increased binding of CREB to the promoter, followed by *IL32* transcriptional activation in influenza A virus-infected cells. We investigated the role of the three DNMTs in the epigenetic modification of the *IL32* promoter. An overexpression assay combined with RNAi indicated that DNMT1 and DNMT3b are critical for *IL32* promoter methylation. A ChIP assay showed that the binding of DNMT1 and DNMT3b to the *IL32* promoter is reduced in influenza virus-infected cells, which is consistent with the finding that the promoter region is hypomethylated. Because DNMT1 and DNMT3b were demonstrated to play key roles in

IL32 promoter methylation, their reduced affinities for the promoter might be responsible for the demethylation of the CpG site in the CRE and the increased binding of the transcription factor CREB.

To our knowledge, in this study, we identified a previously unrecognized novel mechanism that controls IL-32 expression during influenza A virus infection, in which both aberrant epigenetic modifications and multiple transcription factors are involved. We observed the demethylation-linked recruitment of transcription factors CREB1 and CREB2 to the *IL32* promoter region during viral infection. To clarify the relationship between the binding of transcription factors and the epigenetic modifications involved in the regulation of IL-32 expression, which plays an important role in the airway inflammation induced by influenza virus infection, we introduced an approach combining transcription factors exploration and epigenetic analysis to study the transcriptional regulation of IL-32 expression. The notion that influenza virus activates IL-32 expression through the NF- κ B and CREB pathways as well as through the aberrant epigenetic state of *IL32*, including the hypomethylation and demethylation of CRE in the *IL32* promoter, extends our understanding of the relevant highly pathophysiological processes caused by the influenza virus. It may also facilitate the development of novel therapeutic strategies to control airway inflammation.

A previous study demonstrated that one viral protein, HBx, promotes aberrant epigenetic modifications in hepatocarcinogenesis by the transcriptional regulation of DNMTs (39). DNMT1 is activated by the Ras-AP-1 signaling pathway (40), which can be turned on by HBx in the cytoplasm. In this study, we found that DNMT activity was increased in whole-cell lysates by influenza virus infection but was reduced in the nuclear extracts, suggesting that influenza virus reduces the methylation of the *IL32* promoter by suppressing DNMT activity in the nucleus. The effects of influenza virus infection on the individual expression levels of DNMT1, DNMT3a, and DNMT3b at different cellular locations require further clarification.

IL-32 has been identified as a proinflammatory factor in airway inflammation during influenza virus infection. However, very little is currently known about the mechanism by which influenza virus upregulates IL-32 expression. HIV-induced IL-32 has been demonstrated to control HIV-1 production (2, 14). In this study, we examined the influence of all six IL-32 isoforms on influenza virus replication. We have identified the anti-influenza function of IL-32; that the different IL-32 isoforms exert different degrees of inhibition on influenza virus replication; and that IL-32 γ has the strongest effect. This is consistent with the fact that IL-32 γ is the most biologically active isoform (10, 17).

IL-32 γ is the only variant that has a signal sequence, whereas the other IL-32 isoforms lack putative signal sequences (16). The presence of a signal sequence and the lack of a transmembrane region suggest that IL-32 γ is secreted via the classical endoplasmic reticulum/Golgi-dependent pathway (41). Our results from the overexpression of each IL-32 isoform in MDCK cells also showed that only IL-32 γ was detectable in the culture supernatant, though a large portion of IL-32 γ was detected intracellularly. Addition of the exogenous IL-32 γ protein into the supernatant was also observed to inhibit the influenza A virus production significantly, but the inhibition efficiency seems to be not as strong as the transfected IL-32 γ -expressing plasmid. This could be due to the commercial IL-32 γ protein, which was purified from bacteria that lack eukaryotic posttranslational modifications. The fact that IL-32 γ -inhibited viral replication or IL-32 γ -induced TNF- α production is only partially neutralized by IL-32 Ab added into the culture supernatant suggests that more efficient neutralizing Abs need to be developed further. The mechanism underlying the

inhibition of influenza virus replication by the IL-32 isoforms also requires further investigation. In conclusion, this study shows that the elevated IL-32 levels observed during influenza virus infections may inhibit viral replication, which is one of the protective mechanisms of the host during influenza virus infection.

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Disclosures

The authors have no financial conflicts of interest.

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