Influenza A Virus Induces Interleukin-27 through Cyclooxygenase-2 and Protein Kinase A Signaling

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Background: Interleukin (IL)-27 is significantly elevated in influenza patients.

Results: IL-27 inhibits influenza A virus (IAV) replication and its expression is mediated by cyclooxygenase-2, protein kinase A (PKA), and the cAMP-response element-binding protein pathway.

Conclusion: IL-27 exerts antiviral function through activation of STAT1/2/3 and PKR phosphorylation.

Significance: IL-27 is one host immune factor produced in response to IAV infection.

We previously reported that IL-27, which belongs to the IL-12 family of cytokines, is elevated in the serum of patients infected with influenza A virus (IAV). Here, we show that the expression of IL-27 was significantly up-regulated in A549 human lung epithelial cells and human peripheral blood mononuclear cells infected with IAV. Additionally, IAV triggered IL-27 expression through protein kinase A and cAMP-response element-binding protein signaling, which was mediated by cyclooxygenase-2-derived prostaglandin E₂. IL-27 inhibited IAV replication by STAT1/2/3 phosphorylation and activated antiviral factor protein kinase R phosphorylation. Clinical analysis showed that IL-27 levels were significantly elevated in a cohort of patients infected with IAV compared with healthy individuals and that circulating IL-27 levels were tightly and positively correlated with prostaglandin E₂ levels. These results indicate that IL-27 expression is one host immune factor produced in response to IAV infection and that elevated IL-27 levels inhibit viral replication.

Influenza A virus (IAV) is a common pathogen that causes respiratory tract infections and acute severe pneumonia (1). Despite the use of vaccines and antiviral medications, IAV remains a cause of high morbidity and significant mortality worldwide (2). In response to viral infection, the host produces chemokines, proinflammatory cytokines, and immunoregulatory cytokines. Macrophages and dendritic cells produce large quantities of antiviral and immunostimulatory cytokines during IAV infection, which causes extravasation of blood mononuclear cells and antiviral and Th1 immune responses. The elevated levels of serum cytokines and chemokines that IAV induces contribute to increased disease severity (2–4). The broad range of cytokines and chemokines that are expressed during IAV infection include IL-1, -2, -8, -10, -15, -18, TNF-α, IFN-α/β, -γ, and MIP-1α/β (3). The role of other cytokines or chemokines have also been investigated (5). However, many other cytokines, some of which are still unknown, are also induced and play a role in the response to IAV infection. For example, it was recently reported that IL-32, a newly identified cytokine, is induced in response to IAV infection and is a potential target for screening of anti-inflammatory medications (6, 7). Further exploration of cytokines induced in response to IAV infection will increase the understanding of host-virus interactions and aid the advancement of antiviral research.

IL-27 is a recently identified cytokine member of the IL-6/12 family of type I cytokines. This cytokine consists of the heterodimeric IL-27/P28 chain and the IL-12p40-like subunit Epstein-Barr virus-induced 3 (EBI3) (8). IL-27R is composed of the T-cell cytokine receptor (TCCR/WSX-1) and the signal transducer glycoprotein 130. IL-27 binding of IL-27R activates the JAK-STAT signaling pathway, which drives rapid clonal expansion of naive but not memory CD4⁺ T cells. IL-27 also synergizes with IL-12 to trigger IFN-γ production of naive CD4⁺ T cells, promoting Th1 differentiation and T-bet induction (8, 9).

IL-27 has both pro- and anti-inflammatory properties and plays an important role in bridging innate and adaptive immunity (10). IL-27 has also been shown to regulate TNF-α, IFN-γ, IL-10, IL-17, and IL-21 production (11–13). In addition to the immune regulatory function, many studies report that IL-27 has both antiviral and antitumor properties (14). For example, IL-27 was shown to inhibit human immunodeficiency virus type-1 (HIV-1) replication in CD4⁺ T cells and monocytederived macrophages by inducing antiviral genes (15–17). Additionally, IL-27 inhibits hepatitis C virus (HCV) replication, suggesting a role for this cytokine in the immunotherapeutic response against HCV, as well as HCV/HIV co-infections (18, 19). Investigating the role of IL-27 in innate immune responses...
and regulation of putative antiviral functions will provide details about its potential as a mediator of microbial defense (16).

In the previous study, we conducted a protein antibody array and found that IL-27 is up-regulated in the serum of patients infected with IAV and down-regulated in response to cyclooxygenase (COX)-2 inhibition (20). In this study, we systematically explore the relationship between IL-27 and pathogenesis of IAV infection. We demonstrate that IL-27 is significantly up-regulated in IAV-infected A549 lung epithelial cells and freshly isolated PBMCs. We also show that IAV triggers IL-27/EBI3 promoter activity and IL-27 expression through a protein kinase A (PKA)-CREB signaling pathway and that this response is mediated by COX-2-derived prostaglandin E₂ (PGE₂). The elevated IL-27 levels induced by IAV ultimately have antiviral activity and inhibit IAV replication. We further demonstrate IL-27 is significantly elevated in serum of patients infected with 2009 pandemic H1N1 and seasonal H3N2 compared with that from healthy individuals, and that circulating IL-27 levels are closely associated with PGE₂ levels.

**EXPERIMENTAL PROCEDURES**

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

**Clinical Samples**—From 2007 to 2008, serum samples were collected from 110 healthy individuals and 85 patients who were confirmed H3N2 infection. Serum samples were collected during 2009 from 20 patients with confirmed 2009 pandemic H1N1 IAV infection. These serum samples were obtained with the assistance of the Hubei provincial Center for Disease Control and Prevention.

**Virus and Cell Culture**—The influenza virus strain A/Hong Kong/498/97 (H3N2) used in this study was provided by the China Center for Type Culture Collection. The virus stock was propagated in Madin-Darby canine kidney cells. Human lung epithelial cells (A549) were cultured in F12K medium (Invitrogen). All media were supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml of penicillin, and 100 mg/ml of streptomycin sulfate. All cell cultures were maintained at 37 °C in a 5% CO₂ incubator.

**Isolation, Viral Infection, and Inhibitor Treatment of PBMCs**—PBMCs were collected by density centrifugation of blood diluted with an equal volume of pyrogen-free saline over Histopaque (Sigma) as previously described (21). PBMCs were washed twice with PBS and resuspended in RPMI 1640 (Invitrogen) supplemented with 100 units/ml of penicillin and 100 μg/ml of streptomycin (Sigma). The cells were then infected with H3N2 (A/Hong Kong/498/97) at 37 °C in a 5% CO₂ incubator. ELISA was used to measure IL-27 levels in the supernatant 24 h postinfection.

In some cases, cells were treated with a specific inhibitor for 2 h before viral infection. Cells were incubated in the presence of the inhibitor throughout the experiment. ELISA was used to measure IL-27 in the supernatant 24 h postinfection.

For IFN-α neutralization and antiviral assays, freshly isolated PBMCs were treated with recombinant IL-27 for 2 h and culture supernatants were collected. Then IFN-α neutralization antibody or control IgG was added and incubated at 37 °C for 1 h. The neutralized culture supernatants were used for antiviral assays.

**Plasmids, Antibodies, Chemical Reagents, and Inhibitors**—The COX-2-expressing plasmid pCMV-tag2B-COX-2 has been previously described (22). The IL-27/EBI3 promoter (−1379/+ 142) was amplified from human genomic DNA by PCR with the following primers (MluI and BglII sites are underlined): 5’-CTACGGGTTCTGTACCCCTT3’ (sense), 5’-ATTAGATCTAGACATGATCCGCCAGTCC-3’ (antisense). The DNA fragment was then inserted into a pGL3-Basic vector (Promega) to generate an IL-27/EBI3 promoter and Luciferase gene fusion plasmid pIL-27/EBI3-Luc (−1379/+142). The primers for serial promoter truncation to generate pIL-27/EBI3-Luc (−464/+142), pIL-27/EBI3-Luc (−105/+142), and pIL-27/EBI3-Luc (+50/+142) are: pIL-27/EBI3-Luc (−464/+142): 5’-CTACGGGTTCTGTACCCCTT3’; pIL-27/EBI3-Luc (−105/+142): 5’-CTACGGGTGGGCTGTACCCCTT3’; pIL-27/EBI3-Luc (+50/+142): 5’-CTACGGGTGGGCTGCTCCACCGAGTTCC-3’, respectively. Mutated IL-27/EBI3 promoter constructs were also generated from pIL-27/EBI3-Luc (−1379/+142) with the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). COX-2 inhibitor NS-398, MEK inhibitor PD-98059, JNK inhibitor SP600125, ERK inhibitor U0126, p38 inhibitor SB203580, PI3K inhibitor LY294002, the PKA inhibitor H-89, PKC inhibitor GF109203, EGTA, BAPTA/AM, poly(I-C), and PGE₂ were all purchased from Sigma.

Polyclonal goat antibodies specific for phosphorylated PKR (sc-16565), phosphorylated STAT1 (sc-7988), and human β-actin (sc-1616); monoclonal mouse antibody specific for PKR (sc-100378), influenza A NP (sc-80481), and lamin A (sc-56137); and polyclonal rabbit antibodies specific for phosphorylated PKA (sc-32968), PKA (sc-98951), CREB (sc-58), STAT2 (C-20), STAT3 (SC-476), and COX-2 (sc-19999) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A polyclonal rabbit antibody specific for STAT1 (9172) and a polyclonal rabbit antibody specific for phosphorylated STAT2 (4441) and phosphorylated STAT3 (9145) were purchased from Cell Signaling (Danvers, MA). IFN-α neutralization antibody (21100-2) was purchased from PBL Interferon Source (Piscataway, NJ). Recombinant human IL-27 was purchased from R&D Systems (Minneapolis, MN).

**Transient Transfection and Luciferase Reporter Gene Assays**—Cells were plated at density of 4.0 × 10⁵ cells/plate and grown to a confluence of ~80% at the time of transfection. The plasmid pGL3-IL-27/EBI3 and pRL-TK (Promega, Madison, WI) were allowed to co-transfect into the cells with the Lipofectamine 2000 reagent over 24 h (Invitrogen). An internal control, pRL-TK, was used to calculate the transfection efficiency. Cells were serum starved for another 24 h before being harvested for luciferase activity assays. The assay results are expressed as relative luciferase activity.

**IL-27, PGE₂, IFN-α, and IL-35 ELISA**—The concentration of IL-27 in the serum samples and culture supernatants was measured with a LEGEND MAX™ Human IL-27 ELISA kit (BioLegend, San Diego, CA) according to the manufacturer’s instructions. The kit allows the specific detection of IL-27 with...
no detection of IL-35 or free P28 or EBI3. Serum PGE_{2} levels were measured using the Parameter™ Prostaglandin E_{2} Immunoassay kit (R&D Systems), according to the manufacturer’s instructions. IFN-α ELISA kit was purchased from R&D Systems and IL-35 ELISA kit was purchased from CUSABIO (Hubei, China).

**Bioinformatics Analysis**—Promoter analysis was performed by online software for genomict.

**Real-time PCR Assay**—Total cellular RNA was extracted with the RNeasy Mini Kit (Qiagen, Dusseldorf, Germany). EB13 and P28 RNA transcription levels were detected by LightCycler® 480 II (Roche Diagnostics) with the following primers: EB13, 5’-GAGCCAGGTACTACGTCCAAA-3’ (sense), 5’-GCTAGGCAGATCCCATCC-3’ (antisense); P28, 5’-CGCTTTGCGGAATCTCAC-3’ (sense), 5’-GGGCTATGGAAGGGCTGAA-3’ (antisense).

**Cell Activity Assay**—PBMCs viability and cytotoxicity induced by the chemical reagents were determined by CellTiter 96® AQueous One Solution Assay (Promega), according to the manufacturer’s instructions.

**Western Blot Analysis**—Protein extracts of cultured cells were prepared by suspending cells in lysis buffer (0.01% EDTA, 0.1% Triton X-100, and 10% proteinase inhibitors mixture), followed by sonication and centrifugation. The nuclear protein fractions were separated using a nuclear extraction kit (Chemi-con, Billerica, MA). The concentration of protein in the supernatant was quantified using a protein assay kit (Bio-Rad). Immunoblots were visualized with the ECL detection system (Pierce) and quantified by densitometric analysis to show fold-change.

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP assay on PBMCs cells infected with A/Hong Kong/498/97 (H3N2) was performed as described previously (23). The bound DNA fragments were amplified by PCR with the following primers for IL-27/EBI3: 5’-GCTATGCCTTTTCTCTGTGTA-3’ and 5’-GAGAAAAGCAGGAGGAAAGGACA-3’. For positive control, the primers for eNOS were: 5’-CTGTAGTTTTCTCATTGTCCTCC-3’ and 5’-TTCGCCCTCAGGCCCCCAAT-3’.

**Measurement of Influenza Virus Replication**—A549 cells were infected with A/Hong Kong/498/97 (H3N2) at 1 multiplicity of infection (m.o.i.). Viral titers were measured at various time points postinfection with a hemagglutination assay in U-shaped plates, as previously described (24). Relative RNA levels of IAV nucleoprotein (NP)-viral-RNA (vRNA), NP-cRNA, and NP-mRNA were detected with reverse transcription primers and real time PCR test primers. For reverse transcription: NP-vRNA, 5’-CTCACCGGAGTGACATCACTGAATT-ATCATG-3’; NP-cRNA, 5’-AGTAGAAACAAGGGTATTT-CTGTAGTTTCCCTA-3’ (sense) and 5’-GGAGGCCCTCTGTTGATTAGTGT-3’ (antisense); and γ-actin, 5’-TCTTGTCAGGTTGGAAG-GTC-3’ (sense) and 5’-AAATGCAAAACCGCTTCCACC-3’ (antisense).

**Statistical Analysis**—Statistical analysis was performed with the two-tailed Student’s t test for comparison between two groups. p < 0.05 was considered statistically significant.

**RESULTS**

**IAV Induces IL-27 Production in A549 Cells and PBMCs**—A549 cells were infected with IAV/Hong Kong/498/97 (H3N2) virus at 1 m.o.i. or stimulated with poly(I-C) (50 μg/ml), which was used to mimic the viral replication intermediate double-stranded RNA (22). After 24 h, cell culture supernatants were harvested and ELISA was used to measure IL-27 expression. Both IAV infection and poly(I-C) treatment significantly up-regulated IL-27 expression compared with mock infection (IAV: 328 ± 4 pg/ml versus mock: 175 ± 20 pg/ml, p < 0.05; poly(I-C): 246 ± 21 pg/ml versus mock 175 ± 20 pg/ml, p < 0.05) (Fig. 1A). These findings were validated in freshly isolated human PBMCs infected with IAV strain H3N2 (m.o.i. = 1) or stimulated with 50 μg/ml of poly(I-C), and these results were also compared with mock infection (IAV: 310 ± 92 pg/ml versus mock: 98 ± 26 pg/ml, p < 0.05; poly(I-C): 198 ± 38 pg/ml versus mock: 98 ± 26 pg/ml, p < 0.05) (Fig. 1B). In contrast, the heat-inactivated IAV did not induce IL-27 expression significantly (supplemental Fig. S1A). To determine the cellular source of IL-27 in response to IAV infection or poly(I-C) stimulation, PBMCs were separated based on adherence to the culture plate: non-adherent lymphocytes were removed, leaving adherent monocytes, and each cell type was then cultured separately. IAV infection or poly(I-C) stimulation resulted in no significant difference of IL-27 production between monocytes and lymphocytes (Fig. 1C). The PBMCs infected with IAV expressed IL-27 in a time-dependent manner. IL-27 was significantly up-regulated at 6 h post-infection (hpi) (from 84 ± 21 pg/ml at 0 hpi to 342 ± 13 pg/ml at 6 hpi, p < 0.05) and peaked at 12 hpi (411 ± 39 pg/ml; 0 hpi versus 12 hpi, p < 0.05). The concentration of IL-27 began to decrease shortly after 24 hpi (312 ± 27 pg/ml; 0 versus 24 hpi, p < 0.05) and decreased further at 48 hpi, but was still significantly elevated compared with 0 hpi (167 ± 18 pg/ml; 0 versus 48 hpi, p < 0.05) (Fig. 1D). The decrease of IL-27 expression was not caused by the decline of cell viability (supplemental Fig. S1B).

The mRNA of EB13 and P28, two subunits of matured IL-27 were also transcribed in a time-dependent manner. The mRNA levels of both EB13 and P28 were significantly up-regulated at 6 hpi. P28 mRNA reached the peak at 12 hpi. However, there was about 12 h delay for EB13 mRNA to peak. After EB13 and P28 reach the peak, they both began to decrease. Nevertheless, the expression levels were still significantly up-regulated compared with 0 hpi (Fig. 1E).

**A CREB Recognition Site Is Involved in IAV-induced IL-27/EBI3 Promoter Activation**—Because IL-27 consists of IL-27/EBI3 and P28 subunits, and EB13 is responsible for secretion of the entire complex (25), we investigated the mechanism by which IAV infection triggers the expression of IL-27 through IL-27/EBI3 promoter activation. Bioinformatics analysis (geno-matrix) determined that the IL-27/EBI3 promoter contains several cis-elements, including binding sites for NF-κB, CREB, and AP1 (Fig. 2A). These data were consistent with a previous study, showing that two NF-κB binding sites are located upstream (~84 and ~319) of the transcriptional start site of the IL-27/EBI3 promoter (26). However, the promoter reported in that study was short and lacked some cis-element information. We therefore generated a longer construct that included more cis-elements and a longer IL-27/EBI3 promoter region into a luciferase report system. We then transfected
these plasmids into A549 cells, infected the cells with IAV, and measured the luciferase activity. The luciferase assay results showed that truncation or mutation at the CREB site significantly reduced IL-27/EBI3 promoter activity induced by IAV (Fig. 2A). Additionally, when CREB expression was knocked-down with specific siRNA, IL-27/EBI3 promoter activity induced by IAV was significantly decreased (Fig. 2B). The ChIP assay showed that IAV stimulated the binding of CREB to the IL-27/EBI3 promoter. eNOS, whose expression is constitutive and CREB-dependent was used as a positive control. The binding of CREB to the eNOS promoter was not changed significantly by IAV infection (Fig. 2C). Together, these results indicate that CREB is required for activation of the IL-27/EBI3 promoter and IL-27 expression induced by IAV.

**PKA Is Required for IAV-mediated IL-27/EBI3 Promoter Activation and IL-27 Expression**—To further investigate the signal transduction pathway involved in the activation of CREB induced by IAV, we used several protein kinase-specific inhibitors to screen for the kinase involved. A549 cells were pretreated with inhibitors 2 h before transfection with pGL3-IL-27/EBI3 for 24 h and then IAV (m.o.i. = 1) was administered to the culture and cells were incubated for another 24 h. IL-27/EBI3 promoter activities were then tested. When PKA was specifically inhibited by H-89, the IAV-induced IL-27/EBI3 promoter activity was significantly decreased (Fig. 3A). No significant effect either on cell viability (supplemental Fig. S1C) or IAV expression (supplemental Fig. S1D) by the chemical inhibitors was observed. Because PKA is essential for IAV-mediated IL-27/EBI3 promoter activation, we next examined
Calcium influx has been reported to play an important role in mediating PKA/CREB activation (27). To determine whether calcium also affects CREB activation during IAV infection and regulates IL-27/EBI3 promoter activation and IL-27 expression, the cell-permeable calcium chelator EGTA or BAPTA/AM were administered to infected cells. Analysis of

whether the PKA-specific inhibitor H-89 blocked IL-27 expression in PBMCs infected with IAV. In the presence of H-89, IL-27 expression induced by IAV was down-regulated (from 330 ± 36 to 199 ± 23 pg/ml, p < 0.05) (Fig. 3B). Similar results were observed that IL-27 expression stimulated by IAV was down-regulated when PKA was knocked down by specific siRNA (supplemental Fig. S1E). What is the role of PKA during IAV infection? We then measured PKA expression after A549 cells were infected with IAV (m.o.i. = 1) for 24 h. eNOS, whose expression is constitutive and CREB-dependent was used as a positive control (Control/CREB).

Calcium influx has been reported to play an important role in mediating PKA/CREB activation (27). To determine whether calcium also affects CREB activation during IAV infection and regulates IL-27/EBI3 promoter activation and IL-27 expression, the cell-permeable calcium chelator EGTA or BAPTA/AM were administered to infected cells. Analysis of
the luciferase activity results showed that EGTA (0.5, 1, and 2 mM) or BAPTA/AM (2 mM) significantly reduced IL-27/EBI3 promoter activity resulting from IAV infection in A549 cells (supplemental Fig. S2, A and B). Additionally, EGTA (2 mM) reduced the expression of IL-27 from 289 ± 22 to 117 ± 38 pg/ml (p < 0.05), whereas BAPTA/AM (2 mM) reduced IL-27 levels to 192 ± 46 pg/ml (p < 0.05) (supplemental Fig. S2C). Also, EGTA (2 mM) and BAPTA/AM (2 mM) reduced the nuclear translocation of CREB in A549 cells (supplemental Fig. S2D). These data indicate that IAV activates CREB through the PKA signaling pathway, and leads to activation of the IL-27/EBI3 promoter and IL-27 expression. Additionally, calcium influx is upstream of PKA-CREB signaling pathway activation.

**IAV-induced IL-27 Expression through COX-2/PGE2-PKA Pathway**—COX-2 is hyperinduced during IAV infection and PGE2, the catalysis product of COX-2, mediates the PKA-CREB signaling pathway, and leads to activation of the IL-27/EBI3 promoter and IL-27 expression. Additionally, overexpression of COX-2 also stimulated both the nuclear translocation of CREB and PKA phosphorylation (Fig. 4, A and C). We then tested whether IL-27 expression and PKA-CREB signaling were dependent on COX-2 by administering the COX-2 selective inhibitor NS-398 during IAV infection of PBMCs or A549 cells. In the presence of the selective COX-2 inhibitor NS-398, IAV-induced IL-27 expression was significantly decreased compared with infected cells without the inhibitor (no inhibitor: 289 ± 22 pg/ml versus inhibitor: 168 ± 14 pg/ml, p < 0.05) (Fig. 4D). However, addition of PGE2 was able to compensate for NS-398-mediated inhibition, restoring expression of IL-27 (inhibitor: 168 ± 14 pg/ml versus inhibitor + PGE2: 243 ± 36 pg/ml, p < 0.05) (Fig. 4D). Similarly, the IAV-mediated nuclear translocation of CREB was blocked by NS-398, but was restored by addition of PGE2 (Fig. 4E). Furthermore, exogenous PGE2 also restored PKA phosphorylation following the inhibition of phosphorylation induced by addition of NS-398 during IAV infection (Fig. 4F).

To further investigate the function of PGE2 in signal transduction, PBMCs or A549 cells were treated with or without PGE2 and PKA inhibitor H-89. PBMCs were harvested 24 h after treatment and assayed for IL-27 expression, and A549 cells were collected for the nuclear translocation of CREB or PKA phosphorylation. Compared with cells not treated with PGE2, treated cells significantly expressed IL-27 (without PGE2: 98 ± 26 pg/ml versus with PGE2: 347 ± 51 pg/ml, p < 0.05) and the expression of PGE2-induced IL-27 was inhibited by the PKA-specific inhibitor H-89 (without inhibitor: 347 ± 51 pg/ml versus with inhibitor: 105 ± 56 pg/ml, p < 0.05) (Fig. 4G). Last, the PGE2-induced nuclear translocation of CREB or PKA phosphorylation was blocked by the inhibitor H-89 (Fig. 4, H and I).

Taken together, these data indicate that infection with IAV induces COX-2 expression and PGE2 accumulation. The elevated PGE2 levels then activate PKA-CREB signaling, which subsequently activates the IL-27/EBI3 promoter and IL-27 expression.

**IL-27 Inhibits IAV Replication through Activation of PKR**—To assess the biological function of IL-27 during IAV infection, A549 cells were treated with recombinant IL-27 (50 ng/ml) according to previous studies (15, 32). 12 h later, the cells were then infected with IAV (m.o.i. = 1). Viral NP gene expression levels, including vRNA, cRNA, and mRNA, were measured 3 h postinfection. Treating cells with 50 ng/ml of recombinant IL-27 led to a significant decrease in expression of the three types of viral RNA (Fig. 5A). Additionally, after A549 cells were treated with recombinant IL-27 and infected with IAV, viral titer was measured in supernatants with a hemagglutinin assay at 12, 24, 36, and 48 h postinfection. The viral titer significantly decreased when recombinant IL-27 was present (Fig. 5B). Taken together, these results indicate that IL-27 suppresses IAV replication.

To further explore the anti-IAV function of IL-27, we determined whether IL-27 affects the classic interferon-induced JAK-STAT signaling pathway and if IL-27 induces the expression of antiviral factors, similarly to how IL-27 induces factors to inhibit HIV and HCV replication (33). A549 cells were treated with 50 ng/ml of recombinant IL-27 for 12 h. Cells were then collected and the expression of JAK-STAT signaling factors and antiviral factors was analyzed. In response to recombinant IL-27 stimulation, IL-27 promoted STAT1/2 and STAT3 phosphorylation (Fig. 6, A–C). Additionally, IL-27 induced phosphorylation of PKR, an important interferon-induced antiviral effector and the downstream response factor of phosphorylated STAT1/2 and STAT3 (Fig. 6D).

A previous study reported that IL-27 stimulates IFN-α in PBMCs (16). To detect whether IFN-α is involved in IL-27-induced IAV inhibition, recombinant IL-27 protein was used to treat PBMCs for 2 h and then the culture supernatants were collected for the detection of IFN-α expression, or for antiviral and IFN-α neutralization assays. Data showed that IL-27 significantly induced IFN-α expression in PBMCs (from 117 ± 21 to 346 ± 38 pg/ml, p < 0.05) (Fig. 6E). The results were consistent with a previous report (16). For neutralization assays, IFN-α neutralization antibody or control IgG were incubated with the supernatants from the PBMCs culture at 37 °C for 1 h. The neutralized culture supernatants were subject to test of antiviral activity. Results indicated that the IgG-neutralized culture supernatants from IL-27-treated PBMCs inhibited IAV replication significantly. However, the inhibitory effect was partially reversed when IFN-α was neutralized (Fig. 6F), indicating that the antiviral effect of IL-27 is at least in part through the induction of IFN-α. Other pathways may also exist and contribute to IL-27-mediated antiviral function. The same trends were also observed in the phosphorylation levels of antiviral factor STAT1 and PKR (Fig. 6, G and H). Neutralization of IFN-α reduced the phosphorylation of STAT1 and PKR significantly. Based on these data, we concluded that IL-27 inhibits IAV replication by exerting an interferon-like function and through stimulation of IFN-α.

**Serum IL-27 Levels Are Significantly Elevated in Patients Infected with IAV**—To determine any clinical relevance and validate the laboratory findings, we measured IL-27 and PGE2 levels in clinical samples. We collected serum samples from 115
FIGURE 4. COX-2/PGE2 mediates IAV-induced CREB activation and IL-27 expression. A, IL-27 levels produced by A549 cells transfected with pCMV-tag2B-COX-2 or vector control. After 48 h, IL-27 levels were measured in the supernatant by ELISA. The results are expressed as mean ± S.E. (n = 3) from three independent experiments (*, p < 0.05). B, nuclear CREB levels in A549 cells transfected with plasmid pCMV-tag2B-COX-2 or vector control. Nuclear fractions were prepared 48 h post-transfection. Levels of COX-2 or nuclear CREB were determined by Western blot with the indicated antibody. The blot is representative of three experiments with similar results. Densitometric analysis relative to Actin or Lamin A levels were expressed as fold-change (*, p < 0.05). C, phosphorylated PKA in A549 cells transfected with pCMV-tag2B-COX-2 or vector control. Cells were harvested 48 h post-transfection and Western blot for COX-2 or phosphorylated PKA was measured by Western blot with indicated antibody. The blot is representative of three experiments with similar results. Densitometric analysis relative to Actin or PKA levels were expressed as fold-change (*, p < 0.05). D, IL-27 levels produced by freshly isolated PBMCs pre-treated with or without selective COX-2 inhibitor NS-398 (50 μM) for 2 h, then infected with IAV (m.o.i. = 1) for another 2 h, and treated with or without PGE2 (10 μM) for 24 h. IL-27 were measured in the supernatant by ELISA. Data are presented as mean ± S.E. from three independent experiments (*, p < 0.05). E, A549 cells were treated with or without NS-398 (50 μM) for 2 h, then IAV (m.o.i. = 1) was added and incubated for another 2 h and treated with or without PGE2 (10 μM) for 24 h, cells were then collected and nuclear fractions were prepared. Levels of nuclear CREB were determined by Western blot with CREB-specific antibody. The blot is representative of three experiments with similar results. Densitometric analysis relative to Lamin A levels was expressed as fold-change (*, p < 0.05). F, IL-27 produced by freshly isolated PBMCs pre-treated with or without H-89 (10 μM) for 2 h, followed by PGE2 (10 μM) treatment for 24 h. IL-27 in the supernatant was measured by ELISA. Data are expressed as mean ± S.E. from three independent experiments (*, p < 0.05). G, nuclear CREB in A549 cells pre-treated with or without H-89 (10 μM) for 2 h, followed by PGE2 (10 μM) treatment for 24 h. Cells were then collected and nuclear fractions were prepared. Levels of nuclear CREB were determined by Western blot with CREB-specific antibody. The blot is representative of three experiments with similar results. Densitometric analysis relative to Lamin A levels was expressed as fold-change (*, p < 0.05). H, phosphorylated PKA in A549 cells pre-treated with or without H-89 (10 μM) for 2 h, followed by PGE2 (10 μM) treatment for 24 h. Cells were then collected and the level of phosphorylated PKA were detected by Western blot with phospho-specific antibody. The blot is representative of three experiments with similar results. Densitometric analysis relative to PKA levels were expressed as fold-change (*, p < 0.05).
**COX-2 Mediates IL-27 Production during Viral Infection**

In this study, we found that IL-27 is significantly up-regulated in A549 cells and PBMCs infected with IAV (H3N2). The matured IL-27 consists of two subunits: EBI3 and P28. Our results indicated that the time of peak expression for IL-27/P28 mRNA is 12 h earlier than that for IL-27/EBI3 mRNA. And the time for matured IL-27 reaching a peak is exactly with the time of EBI3 peak expression. This finding is consistent with a prior report that showed that IL-27/P28 is induced more quickly than IL-27/EBI3 for about 12 h and P28 is poorly or not secreted without coexpression of EBI3 (25). The peak of EBI3 expression is at 24 h, a time point where IL-27 secretion is declining. One possible explanation for this is because of the competition by IL-35, one subunit of which is also EBI3. IL-35 may compete with IL-27. We found that at 24 h after IAV infection, there is a significant up-regulation of IL-35 (supplemental Fig. S2F). Because it has been previously reported that IL-27/P28 is up-regulated after IAV infection and that EBI3 is responsible for secretion of the IL-27 complex (25, 37, 38), we tested whether IAV triggers IL-27 expression by activation of the IL-27/EBI3 promoter. Viruses induce the production of host cytokines through multiple signaling pathways (3). Our results indicate that IAV activates IL-27/EBI3 promoter activity by enhancing CREB binding to the specific enhancer elements site within the IL-27/EBI3 promoter. Because CREB activation was a downstream effector in the cellular signaling pathway induced by IAV, we speculated that one or more kinases were involved in this cellular event. We therefore conducted experiments to identify these kinases and found that PKA is critical for activation of the IL-27/EBI3 promoter and IL-27 expression during IAV infection. PKA thus regulates cellular signal transduction through CREB activation (27, 28, 31). Additionally, a previous study showed that PKA and CREB are among the host factors required for influenza virus replication (39). Our data show that the PKA-CREB signaling pathway is also functional in IAV-mediated IL-27/EBI3 promoter activation and IL-27 expression. During the kinase inhibitor screening, we also found that when ERK was inhibited by inhibitor U0126, IL-27/EBI3 promoter activation induced by IAV was decreased. As ERK can also activate CREB signaling as PKA does, these findings somehow prove the results that CREB is critical for IAV-triggered IL-27/EBI3 promoter activation (40). Nevertheless, IL-27 also stimulates ERK phosphorylation (41, 42). Their complicated regulatory relationships need further investigation.

Activation of the PKA-CREB signaling pathway is mediated by two factors: calcium and cAMP. Briefly, calcium induces PKA-CREB activation through Rap1/B-Raf/ERK signaling, whereas cAMP-regulated PKA-CREB activation depends on the concentration of cAMP (27, 31). However, numerous extracellular signals affect the cellular concentrations of cAMP and calcium flux. Additionally, abundant cross-talk exists between cAMP- and calcium-induced signaling pathways, as well as convergence of common downstream effectors (43–45). A recent study reported that infection with the IAV accelerated extracellular Ca$^{2+}$ influx and that calcium/calmodulin proteins are host factors critical for IAV replication (39, 46). To determine whether a connection exists between the Ca$^{2+}$ influx induced by the virus and CREB activation, which subsequently stimulated IL-27/EBI3 promoter activation and IL-27 expres-

**DISCUSSION**

In this study, we show data supporting a previously unrecognized mechanism for IL-27 expression in response to IAV infection. In our previously published study, we conducted protein-antibody arrays and reported that IL-27 is one of the proteins up-regulated in serum of influenza patients and freshly isolated PBMCs infected with IAV, and down-regulated when IAV-infected PBMCs were treated with the COX-2 inhibitor NS-398 (20). PBMCs are mainly involved in immune system responses and play a major role in regulating host defense mechanisms against microbial infections (34). Cytotoxicity assays indicated that PBMCs could be infected by influenza virus and as a clean and efficient model, PBMCs are widely used in the research of immediate immune responses to viral and other microbe infections (21, 34–36).

**FIGURE 5. IL-27 inhibits IAV replication.** A, A549 cells were treated with the indicated dose of recombinant IL-27 (50 ng/ml) for 12 h before IAV A/Hong Kong/498/97 (H3N2) infection (m.o.i. = 1). Cells were collected 3 h post-infection and total RNA was isolated for detection of NP-specific mRNA, cRNA, and vRNA. Data are expressed as mean ± S.E. from three independent experiments (*, p < 0.05). B, A549 cells were treated with recombinant IL-27 (50 ng/ml) for 12 h before IAV infection (m.o.i. = 1). Cells were harvested at the indicated time point, and virus titer was measured by hemagglutination assay.
We administered a Ca\textsuperscript{2+} chelator to infected cells. The results indicate that the IAV-mediated increase in the cytosolic calcium concentration activated nuclear translocation of CREB, triggering IL-27/EBI3 promoter activation and IL-27 expression.

IAV infection hyperinduces COX-2 expression, a well known proinflammatory factor, and the COX-2 product PGE\textsubscript{2} stimulates PKA activation, therefore activating nuclear translocation of CREB (28, 29, 47, 48). We investigated a possible role of COX-2 and PGE\textsubscript{2} in IL-27 expression. The data presented here together provide details about a possible signaling pathway: 1) IAV infection first triggers COX-2 expression and PGE\textsubscript{2} accumulation; 2) increased PGE\textsubscript{2} levels then activate PKA-CREB signaling; and 3) the IL-27/EBI3 promoter is activated, resulting in IL-27 expression. Interestingly, we found that IL-27 expression is stimulated by COX-2, whereas a previous study showed...
that IL-27 directly suppressed COX-2, thus leading to an antitumor response (49). These results suggest a possible mechanism by which IL-27 is induced by COX-2, and the accumulated IL-27 then inhibits COX-2 in a cycle of feedback inhibition. It has been previously shown that IL-27 promotes anti-inflammatory immune responses (37), and our data indicate that IL-27 may mediate these responses by suppressing COX-2 expression.

Both calcium-PKA-CREB and COX-2/PGE2-PKA-CREB signaling pathways are activated during IAV infection, leading to IL-27/EBI3 promoter activation and IL-27 expression. Cellular signal transduction networks are inherently complex, and no single pathway exists independently. Within signaling networks, individual pathways participate in cross-talk and/or converge through common downstream effectors. In SARS-CoV infection, COX-2 expression is mediated by calcium (50). We do not know for certain whether calcium plays a role in IAV-mediated induction of COX-2 expression. If it does play a role, calcium would mediate the COX-2/PGE2-PKA-CREB signaling pathway and regulate IL-27/EBI3 promoter activation and IL-27 expression. Further study is needed to address this hypothesis.

The antiviral role of IL-27 has been demonstrated in HIV and HCV infections. IL-27 induces activation of both STAT1 and STAT2 (51). IL-27 inhibits viral replication through interferon-like effects and inducing the expression of multiple interferon-inducible genes (15, 16, 32). In this study, we identified an antiviral function of IL-27 and confirmed that IL-27 is also able to inhibit IAV infection by activating STAT1/2 and STAT3 signaling and ultimately activating PKR. This signaling cascade is the classic interferon pathway, with IL-27 exerting interferon-like effects. Besides, we demonstrated that IFN-α contributes in part to IL-27-mediated antiviral function.

To validate these findings clinically, we compared the level of IL-27 in serum samples from patients with the IAV and healthy individuals. We found that compared with healthy individuals, IL-27 levels were significantly elevated in the sera of patients infected with 2009 pandemic H1N1 or seasonal H3N2. These clinical findings indicate that up-regulation of IL-27 expression is a result of IAV infection and that the induction of IL-27 is not dependent upon viral strain or serotype. Additionally, the expression of IL-27 is associated with expression of PGE2, which is consistent with *in vitro* results showing that PGE2 stimulates IL-27 expression. In conclusion, the results of this study advance what is known about the host immune response to IAV infection and demonstrates the broad spectrum antiviral properties of IL-27, indicating the potential clinical use of IL-27 for antiviral therapy.

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