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IL-27, a Cytokine, and IFN-α1, a Type III IFN, Are Coordinated To Regulate Virus Replication through Type I IFN

Yanhua Cao,* Rui Zhang,* Wei Zhang,* Chengliang Zhu,* Yi Yu,* Yu Song,* Qing Wang,* Lan Bai,* Yingle Liu,*‡ Kailang Wu,*‡ and Jianguo Wu*‡,‡

IL-27, a member of the IL-12 family, plays a critical role in the control of innate and adaptive immune responses. IFN-α1, a member of the type III IFN family, shows antiviral abilities. In this study, we investigated the effects of IL-27 and IFN-α1 on the replication of hepatitis B virus (HBV), a major pathogen associated with a high risk for cirrhosis, liver failure, and hepatocellular carcinoma. We revealed that HBV infection activates IL-27 expression and IFN-α1 production and demonstrated that viral-activated IL-27 and IFN-α1 are coordinated to inhibit HBV replication. Initially, HBV infection upregulates IL-27 expression, which, in turn, stimulates IFN-α1 production through regulating ERK1/2 signaling and by enhancing NF-κB nuclear translocation to bind to the IFN-α1 promoter. Moreover, IL-27-activated IFN-α1 upregulates IFN-α1 receptor (IL-28R1 and IL-10Rβ) activity, resulting in the activation of the STAT1/2 pathway, which, in turn, induces the expression of IFN-stimulated genes, including IFN-inducible dsRNA-activated protein kinase, oligoadenylate synthetase 1, and IFN-induced GTP-binding protein 1 and, finally, inhibits HBV protein expression and viral capsid–associated DNA replication. More interestingly, we also revealed that type I IFN (IFN-α) is also involved in the downregulation of HBV replication mediated by IL-27. Thus, we identified a previously unknown mechanism by which IL-27 and IFN-α1 are coordinated to regulate virus replication through type I IFN. The Journal of Immunology, 2014, 192: 691–703.

Cytokines play a critical role in controlling the innate and adaptive immune responses. IL-27, a member of the IL-12 family, is a heterodimeric anti-inflammatory cytokine composed of EBV-induced protein 3 (EBI3) and IL-27p28 protein (1). IL-27p28 is a four-helical cytokine requiring association with EBI3 to secrete efficiently and activate functionally. The signal-transduction receptor complex for IL-27 is composed of the cytokine receptor IL-27Rα (WSX-1 or alternatively T cell cytokine receptor [TCCR]) and the gp130 protein (2).

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Abbreviations used in this article: CHB, chronic hepatitis B virus; COX-2, cyclooxygenase-2; DC, dendritic cell; EBI3, EBV-induced protein 3; HBV, hepatitis B virus; HCV, hepatitis C virus; IA V , influenza A virus; ISG, IFN-stimulated gene; ISRE, IFN stimulation response element; mERK, mutant ERK; Mx1, IFN-induced GTP-binding protein 1; NC, negative control; OAS1, oligoadenylate synthetase 1; PKA, protein kinase A; PKR, IFN-inducible dsRNA-activated protein kinase; rh, recombinant human; sh, short hairpin; si, small interfering; siRNA, small interfering RNA; TCCR, T cell cytokine receptor.

IL-27 is produced mainly by activated macrophages and dendritic cells (DCs) and is a cytokine that regulates Th function during autoimmune and pathogen-induced immune responses (3, 4). In early studies, IL-27 was initially described as a proinflammatory cytokine that promoted Th1 responses (1, 5). However, subsequent studies in multiple models of infectious and autoimmunity diseases confirmed anti-inflammatory roles for IL-27 in Th1, Th2, and Th17 responses (6). Recent work showed that IL-27 can induce T cells to produce the anti-inflammatory cytokine IL-10 (7–9). Overexpressing IL-27 decreases regulatory T cell frequencies, induces spontaneous inflammation, and activates proliferation of naive human B cells and CD4+ T cells (10, 11). IL-27 has therapeutic potential through various mechanisms: inhibiting replication of HIV (12) and hepatitis C virus (HCV) (13), enhancing antitumor immune responses (14), directing antiproliferative effects on tumors (15–17), and reducing the inflammation associated with experimental autoimmune arthritis (18, 19). We recently demonstrated that during influenza A virus (IAV) infection, IL-27 is strongly induced through cyclooxygenase-2 (COX-2)– and protein kinase A (PKA)–signaling pathways (20).

Upon detecting viral components, host cells activate antiviral responses to prevent viral infection by activating TLRs and IFN cascades. Type I IFNs (IFN-α/β) play an essential role in both the innate immune response and the induction of adaptive immunity against viral infections. Viral infections trigger the production of IFN-α/β (21), which leads to the activation of several hundred IFN-stimulated genes (ISGs). These genes encode a variety of antiviral proteins and cytokines, leading to host protection from further viral infections (22).

IFN-α1 (also called IL-29) belongs to the type III IFN (IFN-λ) family, which is composed of three members: IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B) (23, 24). PBMCs and DCs are the major producers of IFN-λ when infected by viruses or...
stimulated by polyinosinic-polycytidylic acid or LPS (25). It was reported that NF-κB and IRF3/7 are required for induction of IFN-α expression (26). Although IFN-α binds to distinct receptors, IL-28R1 and IL-10Rβ (24), it activates signal transduction pathways by a manner similar to that activated by type I IFN (23, 24) through inducing tyrosine phosphorylation of STAT proteins (27). IFN-α also shows antiviral abilities, similar to those of type I IFN (28), by inhibiting the replication of a number of viruses, including HCV, hepatitis B virus (HBV), encephalomyocarditis virus, IAV, and vesicular stomatitis virus (23, 24, 28-31).

HBV is a major human pathogen chronically infecting >350 million individuals worldwide and is associated with a high risk for developing cirrhosis, liver failure, and hepatocellular carcinoma (32, 33). HBV replication is noncytopathically inhibited by type I IFN (IFN-α/β) (34), type II IFN (IFN-γ) (35), and type III IFN (IFN-λ) (30). Our previous study (36) showed that HBV enhances IL-27 expression both in vivo and in vitro. We also demonstrated that HBV induces a novel inflammation network involving three inflammatory factors: IFN-λ1, IL-8, and COX-2 (37). Although IFN-α is administered to HBV-infected patients (38), some patients fail to respond to the treatment (39, 40). Thus, a new or improved immunotherapy is needed to treat HBV infection.

In this study, we investigated the effects of IL-27 and IFN-λ1 on HBV replication. We revealed that HBV infection upregulates IL-27 expression, which, in turn, stimulates IFN-λ1 production through regulating ERK1/2 signaling and by enhancing NF-κB nuclear translocation to bind to the IFN-λ1 promoter. Moreover, we demonstrated that IL-27-activated IFN-λ1 regulates IFN-λ1 receptors (IL-28R1 and IL-10Rβ), resulting in activation of the STAT1/2 pathway, which, in turn, induces the expression of ISGs, including IFN-inducible dsRNA-activated protein kinase (PKR), oligoadenylate synthetase 1 (OAS1), and IFN-induced GTP-binding protein 1 (Mx1), in a manner similar to type I IFN and, finally, inhibits HBV protein expression and viral capsid-associated DNA replication. Our study revealed a novel mechanism by which IL-27 and IFN-λ1 are coordinated to regulate virus replication through type I IFN.

Materials and Methods

Clinical samples

Peripheral blood samples were obtained from 30 patients with chronic HBV (CHB; 22 males and 8 females; mean age 47 ± 14.6 y) who were admitted to Rennin Hospital of Wuhan University. All patients were confirmed HBV positive (but negative for HCV, hepatitis D virus, and HIV), had no evidence of concomitant illness, and showed no seroconversion of HBV positive (but negative for HCV, hepatitis D virus, and HIV), had no evidence of concomitant illness, and showed no seroconversion. A control group, consisting of 30 healthy individuals (19 males and 11 females; mean age 47 ± 17 y) who had no history of liver disease and who were sex- and age-matched with the patient samples, was randomly selected from blood donation centers in Wuhan, China.

Written informed consent was obtained from each patient. The study was conducted according to the principles of the Declaration of Helsinki and 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.

Isolation of PBMCs

PBMCs were isolated by density centrifugation of fresh peripheral venous blood samples diluted 1:1 in pyrogen-free PBS over Histopaque (Haoyang Biotech). Cells were washed twice in PBS and resuspended in culture medium (RPMI 1640) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml).

Generation of human immature DCs

Monocytes were isolated from PBMCs by adherence to plastic dishes for >2 h at 37°C, as described previously (41). To derive immature DCs, monocytes were cultured at 1×10^5 in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 50 ng/ml GM-CSF, and 20 ng/ml IL-4 (PeproTech, Rocky Hill, NJ) for 7 d. Medium was changed 2, 4, and 6 d after culturing.

Reagents

Recombinant human (rh)IL-27 and rhIFN-λ1 protein were purchased from R&D Systems (Minneapolis, MN). Protein A/G was from Santa Cruz Biotechnology (Santa Cruz, CA). Abs specific for p-STAT1, p-STAT2, PKR, OAS1, p-ERK, ERK1/2, IL-28R1, IL-10Rβ, NF-κB p50, IRF3, and IFN-γ were purchased from Thermo Scientific (Rockville, MD). A specific for MxA was purchased from ProteinTech Group (Chicago, IL). Abs specific for lamin A/C were purchased from Epitomics (Burlingame, CA). Abs specific for GAPDH and β-actin were purchased from CWBIO (Beijing, China). Flow cytometry Abs specific for CD4, CD8, CD19, CD20, and CD25 were purchased from BD Biosciences (Franklin Lakes, NJ). Abs specific for IFN-α/βR1, IFN-βR1, IL-12Rβ1, and IL-10Rβ were purchased from R&D Systems. Neutralizing type I IFN receptor (IFNAR) chain 2 Ab (CD118) and neutralizing IFN-λ1 Ab were purchased from R&D Systems.

The inhibitors used in this study were purchased from Sigma-Aldrich (St. Louis, MO): U0126 (iERK; 10 μM), H-89 (iPKA; 10 μM), LY294002 (iPI3K; 10 μM), SP600125 (iJNK; 10 μM), and SB203580 (iP38; 10 μM). All inhibitors were dissolved in DMSO.

Cell culture

Human hepatocytes, HepG2 and HuH7 cells, were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate at 37°C with 5% CO2. Chloroform-exposed HBV (ayw, one of HBV subtypes) were derived from HepG2 cells and were maintained in DMEM containing 400 μg/ml G418.

Plasmid construction

The DNA fragment carrying HBV genomic DNA (ayw subtype) was amplified from HepG2.2.15 cells that stably express HBV (ayw, one of HBV subtypes) were derived from HepG2 cells and were maintained in DMEM containing 400 μg/ml G418.

Luciferase assay

HepG2 cells were transfected with reporter plasmids and then treated with rhIL-27 protein. Cells were lysed with luciferase cell culture lysis reagent (Promega, Madison, WI). Cell lysates and luciferase assay substrate (Promega) were mixed, and the light intensity was detected by a luminometer (Turner T20/20).

ELISA

IFN-λ1 release in culture supernatants was quantitated using a Human IFN-λ1 Platinum ELISA kit (ebioscience, San Diego, CA), according to the manufacturer’s instructions.

Real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen), following the manufacturer’s instructions. Real-time quantitative RT-PCR was performed using the Roche LC480 and SYBR RT-PCR kits (DBI Bioscience, Ludwigshafen, Germany) in a reaction mixture of 20 μl SYBR Green PCR master mix, 1 μl DNA diluted template, and RNase-free water to complete the 20 μl volume. Real-time PCR was carried out as follows: step 1, preincubation, 95°C, 5 min; step 2, amplification and quantification, 95°C, 10 s, 39°C, 15 s, 72°C 15 s, with a single fluorescence measurement, for 40 cycles; step 3, melting curve, 60-95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement; and step 4, cooling, 40°C. Real-time PCR primers were designed by Primer Premier 5.0 and their sequences were as follows: IFN-α sense: 5'-TTTCCCTCTCCTTGAAGGACAG-3′, IFN-α antisense: 5'-GCTCATATTCTCTGTCGCA-3′; IFN-β sense: 5'-TGGGAGGCTTGAATCTGCCTCA-3′, IFN-β antisense: 5'-TTCCCTGGCCTTACGATTTGAGCA-3′; IFN-γ sense: 5'-GGCCCTCCAGGACCTCCACG-3′, IFN-γ antisense: 5'-GTAGATGACCCATTGCTAG-3′; R-IFN-β sense: 5'-CAACAGGAGGAGGAGGAGGAG-3′, R-IFN-β antisense: 5'-CCAGGACCTCCAGGACCTCCACG-3′; PKR sense: 5'-AAAGGGCCAGAAGGGAAAG-3′, PKR antisense: 5'-GAGTATGACCCATTGCTAG-3′, OAS1 sense: 5'-CCCTGCTCAATAGGAGGACCCACG-3′, OAS1 antisense: 5'-GAGGCTCTACCAAGATGTTGAC-3′, OAS1 antisense: 5'-ACCAAGAGGAGGCTCAGCAT-3′, Mx1
HBV protein–expression assay

HepG2 cells and HuH7 cells were transfected with plasmid pHBV1.3 and treated with rhIL-27 protein. HepG2.2.15 cells also were treated with rhIL-27 protein. At 24 h posttransfection, HBBeAg and HBsAg protein levels in cell culture media were determined by ELISA using an HBV BeAg and HBsAg diagnostic kit (KeHua Biotech, Shanghai, China), respectively.

HBV DNA assay by real-time PCR

Capsid-associated DNA was extracted as described previously (42), with modifications. Equivalent amounts of posttreatment cells were homogenized in 1 ml lysis buffer (50 mM Tris [pH 7.5], 0.5% Nonidet P-40, 1 mM EDTA, and 100 mM NaCl) and mixed gently at 4°C for 1 h. Next, 10 μl 1 M MgCl₂ and 10 μl DNase (10 mg/ml) were added and incubated for 2 h at 37°C. Viral cores were precipitated by adding 35 μl 0.5 M EDTA and 225 μl 35% polyethylene glycol and incubating them at 4°C for 30 min, after which the cores were centrifuged by centrifugation, and the pellet was resuspended in resuspending buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1% SDS, and 25 μg/ml protease K) and incubated overnight. Viral DNA released from lysed cores was extracted with phenol and chloroform, precipitated with isopropanol, and resuspended in Tris-EDTA buffer.

HBV DNA quantified by real-time PCR performed in a Light Cycler 480 real-time PCR instrument (Roche, Basel, Switzerland) using the following primers:

- Primer P1: 5’-AGAAAACAACATAGGCCTCAT-3’ and primer P2: 5’-TGCCCCATGCTTAGATCTTG-3’
- Primer P: 5’-TGTTGCGTCACCA-TGTTGTTGG-3’
- Primer P1 distal: 5’-CTGCTTCG-3’
- Primer P1 proximal: 5’-CTCGCTCCTGGAGATGGTGATGGG-3’
- Primer P2 distal: 5’-CTGCTGCTG-3’

The PCR reaction was performed as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. Plasmid pHBV1.3 was diluted over a range of 10⁻⁵ to 10⁻⁹ and used as a standard, and all samples were analyzed in triplicate.

Nuclear extraction

HepG2 cells and HuH7 cells were incubated in serum-free media for 24 h and then treated with rhIL-27 protein at 20 ng/ml or PBS as a negative control for 3 h. Cells were washed with cold PBS and scraped into 1 ml cold PBS, followed by centrifugation at 2000 g for 5 min in a microcentrifuge and incubation in two packed-cell volumes of buffer A (10 mM HEPES [pH 8], 0.5% Nonidet P-40, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 200 mM sucrose) for 10 min on ice, with flipping of the tube. Nuclei were collected by centrifugation (12,000 × g for 30 s). Pellets were rinsed with buffer A, resuspended in buffer B (20 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, and 1.0 mM DTT), and incubated on a rocking platform at 4°C for 30 min. Nuclei were clarified by centrifugation (12,000 × g for 10 min), and the supernatants were diluted 1:1 with buffer C (20 mM HEPES [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 20% glycerol, and 1 mM DTT). A mixture of protease inhibitors (Roche) was added to each type of buffer. Nuclear extracts were stored at −70°C until use.

Western blotting

Whole-cell lysates were prepared by lysing cells with PBS (pH 7.4) containing 0.01% Triton X-100, 0.01% EDTA, and 10% protease inhibitor mixture (Roche). Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA). The samples from cell lysates were separated on 12% SDS polyacrylamide gels and transferred electrically to nitrocellulose membranes (Amersham, Piscataway, NJ). Nonspecific sites were blocked by 5% skim milk before being incubated with an Ab in this study. Protein bands were detected using SuperSignal Chemiluminescent substrate (Pierce, Rockford, IL).

Chromatin immunoprecipitation assay

HepG2 cells were treated for 3 h with rhIL-27 protein at 20 ng/ml or PBS as a negative control. Formaldehyde was added to the culture medium to a final concentration of 1%. The cells were washed twice with PBS, scraped, and lysed in lysis buffer (1% SDS, 10 mM Tris-HCl [pH 8], 10% protease inhibitor mixture, and 50 μg/ml both aprotinin and leupeptin) for 10 min at 4°C. The lysates were sonicated on ice, and the debris was removed by centrifugation at 12,000 × g for 15 min at 4°C. One fourth of the supernatant was used as DNA input control. The remaining sample was diluted 10-fold with dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl [pH 8], and 150 mM NaCl), followed by overnight incubation at 4°C with Ab against NF-κB p65/p50, IRF3, or nonspecific IgG, respectively. Immunoprecipitated complexes were collected using protein A/G-Sepharose. The pellets were washed four times with dialysis buffer (2 mM EDTA and 50 mM Tris-HCl [pH 8]). After washing, the precipitates were eluted in elution buffer (1% SDS and 0.1 M NaHCO₃) at room temperature. Supernatants were transferred to clean tubes, and RNA A was added to destroy bound RNA in the sample. Samples were incubated at 65°C for 5 h to reverse formaldehyde-cross links, and DNA was precipitated with ethanol and extracted twice with phenol/chloroform. Finally, pellets were resuspended in Tris-EDTA buffer and subjected to PCR amplification using the following primers: IFN-α1 proximal: 5’-GGCCACTTTGCGCTAAAGTCCCA-3’ (sense) and 5’-GGACCACTTTGAGCTGGAGG-3’ (antisense); IFN-α1 distal: 5’-TTTAAAGGCGAGTTCAGG-3’ (sense) and 5’-TTCACTTGGTCAGCACACCT-3’ (antisense); and IRF7 5’-CATAGTGAGACCCCCTATTCTCAA-3’ (sense) and 5’-ACTGCGTTTTTACTATGTGGTACCCG-3’ (antisense).

Immunofluorescence

HepG2 and HuH7 cells were treated with rhIL-27 protein (20 ng/ml) or PBS as a negative control. At 3 h posttreatment, cells were fixed with 4% paraformaldehyde for 15 min, washed three times with PBS, permeabilized with PBS containing 0.5% Triton X-100 for 5 min, washed three times with PBS, and finally blocked with PBS containing 4% BSA for 1 h at room temperature. The cells were then incubated with the primary Ab overnight at 4°C, followed by incubation with Alexa Fluor 492–labeled secondary Abs (Proteintech Group) for 1 h. Mounting was done with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA), and the cells were visualized by confocal laser microscopy (FluoView FV1000; Olympus, Tokyo, Japan).

Flow cytometry

HepG2 and HuH7 cells were Fc blocked by treatment with 1 μg human IgG/10⁶ cells for 15 min at 4°C prior to staining. A total of 5 × 10⁵ to 10 × 10⁶ cells in cold PBS buffer (supplemented with 2% BSA) was incubated with 1 μg/ml specific Ab for 1 h at 4°C. Cells were washed with cold PBS buffer (supplemented with 2% BSA) twice, and then 2.5 × 10⁵ cells/sample were analyzed by flow cytometry using a FACSCalibur (Beckman Coulter).

Statistical analysis

All experiments were reproducible and were carried out in triplicate. Each set of experiments was repeated at least three times with similar results, and representative experiments are shown. The results are presented as means. ANOVA test for paired samples was used to determine statistical significance. Differences were considered statistically significant at p < 0.05.

Results

IL-27 represses HBV protein expression and capsid-associated DNA replication

We reported previously that IL-27 protein was elevated in patients with HBV infection and that HBV enhances IL-27 expression in several cell types, including THP1, HepG2, HuH7, and HEK293T cells (36). A correlation between HBV infection and IL-27 expression was also shown (43). In this study, we explored the impact of IL-27 on HBV replication. HepG2.2.15 cells, which carry HBV genomic DNA (ayw subtype) on their chromosome, were treated with different concentrations of rhIL-27, as indicated. In addition, HepG2 cells and HuH7 cells were transfected with pHBV-1.3, which carries HBV genomic DNA (ayw subtype) on its chromosome, and then were treated with rhIL-27 at different concentrations, as indicated. ELISA results showed that HBV HBsAg protein (Fig. 1A) and HBsAg protein (Fig. 1B) were downregulated by rhIL-27 in a dose-dependent fashion in HepG2.2.15, pHBV-1.3-transfected HepG2, and pHBV-1.3-transfected HuH7 cells. Similarly, real-time PCR results revealed that HBV capsid–associated DNA (Fig. 1C) was downregulated by rhIL-27 in a concentration-dependent manner in the reporter system.
all three cell types. We also showed that the cell viability was not affected under these experimental conditions (Supplemental Fig. 1A), indicating that the reduction in HBV protein expression and capsid-associated DNA replication were not due to alterations in cell viability caused by rhIL-27. These results suggested that IL-27 has inhibitory effects on HBV protein expression, as well as on HBV capsid–associated DNA replication.

**IL-27 activates ISG expression by activating STAT1/2**

Upon detection of viral components, host cells are known to activate antiviral responses via TLRs and the IFN cascade. In the IFN antiviral response, IFN-\(\alpha\) initially binds to IFNAR1 and IFNAR2, thereby activating the receptor complex, resulting in induction of the phosphorylation of STAT1/2 and the expression of ISGs, which, in turn, cause an antiviral state in target cells (21).

To explore the regulatory mechanism involved in the downregulation of HBV replication mediated by IL-27, we first evaluated the effect of IL-27 on the phosphorylation status of STAT1/2 proteins. HepG2 cells were treated with rhIL-27 for different times, as indicated, or were treated with IFN-\(\alpha\) protein as a positive control. Western blot results showed that p-STAT1 and p-STAT2 proteins were upregulated by rhIL-27 and rhIFN-\(\alpha\), respectively, whereas STAT1, STAT2, and \(\beta\)-actin proteins remained relatively unchanged under the same conditions (Fig. 2A, Supplemental Fig. 2A). These results suggested that, similar to type I IFN (IFN-\(\alpha\)), IL-27 has a stimulatory effect on the activation of STAT1/2.

Viral infection triggers the production of type I IFN (IFN-\(\alpha/\beta\)), which leads to the activation of several hundred ISGs through ISRE on the promoters of ISGs. It was reported that IL-27 can exert IFN-like function in liver cells and contribute to the antiviral response in these cells (44). To investigate the role of IL-27 in regulating the activity of ISRE, HepG2 and Huh7 cells were transfected with the reporter pISRE-Luc, in which expression of the luciferase gene is under the control of ISRE, and the expression of capsid-associated DNA was assessed by real-time PCR. *\(p < 0.05$.

**FIGURE 1.** Evaluation of the effects of IL-27 on HBV protein expression and viral capsid–associated DNA replication. HepG2.2.15 cells were treated with rhIL-27 at different concentrations, as indicated. HepG2 cells and Huh7 cells were transfected with plasmid pHBV-1.3 and treated with rhIL-27 protein at 6 h posttransfection. At 24 h posttreatment, cell culture supernatants were collected, and HBV capsid–associated DNA was extracted. The levels of secreted HBeAg (A) and HBsAg (B) were determined by ELISA, and the expression of capsid-associated DNA (C) was assessed by real-time PCR. *\(p < 0.05$.

**IL-27 expression and IFN-\(\alpha\) production are highly correlated in patients infected with HBV, and IL-27 upregulates IFN genes in vitro**

We showed previously that HBV upregulates IL-27 expression both in vivo and in vitro (36) and demonstrated that HBV induces a novel inflammation network involving three inflammatory factors: IFN-\(\alpha\), IL-8, and COX-2 (37). However, the correlation of IL-27 and IFN-\(\alpha\) has not been studied. In this study, we evaluated...
the expression status of IL-27 and IFN-λ1 in patients with CHB infection and determined the correlation between IL-27 expression and IFN-λ1 production during HBV infection.

PBMCs were isolated from patients with HBV infection (n = 30) and healthy individuals (n = 30). Total mRNA were extracted from isolated PBMCs and assessed by real-time PCR. Results showed that the mRNA levels of IFN-λ1 and the two subunits of IL-27 (p28 and EBI3) were significantly higher in patients with CHB than in healthy individuals (mean ± SEM, IFN-λ1: 8.379 ± 0.875 versus 1.325 ± 0.0877, p < 0.05; p28: 5.648 ± 0.4068 versus 2.297 ± 0.1936, p < 0.05; EBI3: 5.891 ± 0.2983 versus 2.656 ± 0.1238, p < 0.05) (Fig. 3A). Further analysis revealed that elevated levels of IFN-λ1 were tightly correlated with increased levels of p28 and EBI3 in the PBMCs of patients with CHB (Fig. 3B). These results revealed that IFN-λ1, p28, and EBI3 are upregulated in PBMCs of HBV-infected patients and that IFN-λ1 expression and IL-27 production are highly correlated in vivo. However, we do not know whether an unknown factor (HBV infection or type I IFNs) causes the observed simultaneous induction of IL-27 and IFN-λ1.

In addition, we evaluated the effect of IL-27 on IFN expression and antiviral protein production. PBMCs were treated with rhIL-27 for different times, as indicated. Real-time PCR results showed that IL-27 activates the expression of ISGs (OAS1 > Mx1 > PKR) in a time-dependent manner at 24 h posttreatment in PBMCs (Fig. 3C). In addition, IL-27 also stimulates the expression of ISGs (OAS1 > Mx1 > PKR) in a time-dependent manner at 24 h posttreatment in PBMCs (Fig. 3D).

IFN-α and IFN-λ1 are involved in the repression of HBV replication mediated by IL-27

IFN-α mediates the antiviral response by binding to a common heterodimeric receptor consisting of two subunits (IFNAR1 and IFNAR2) (45), whereas IFN-λ1 regulates its biological function through binding to a distinct receptor complex consisting of two subunits (IL28R1 or IFN-λ1R1 and IL10R2) (24). However, IFN-λ1 can activate signal-transduction pathways similar to those activated by IFN-α (23, 24), and it displays antiviral activity similar to IFN-α (28). Because our results revealed that IL-27 upregulates the expression of IFN-α, IFN-β, and IFN-λ1, we further evaluated the effects of IFN-α/β and IFN-λ1 on IL-27 function.

The roles of IFN-α/β and IFN-λ1 in IL-27 activity were investigated initially using an RNA-interfering approach. The efficiencies of small interfering RNA (siRNA) molecules were evaluated in HepG2 cells transfected with small interfering (si)–negative control (NC), si–IFNAR1, si–IFNAR2, short hairpin (sh)–NC, and sh–IFN-λ1. Real-time PCR results indicated that IFNAR1 mRNA and IFNAR2 mRNA were significantly downregulated in the presence of si–IFNAR1 and si–IFNAR2, respectively (Supplemental Fig. 3A). Flow cytometry results showed that IFNAR1 and IFNAR2 proteins were not affected by the treatment with si–IFNAR1 and si–IFNAR2 (Supplemental Fig. 3B). Similarly, real-time PCR and ELISA results revealed that both IFN-λ1 mRNA (Supplemental Fig. 3C, left panel) and IFN-λ1 protein (Supplemental Fig. 3C, right panel) were significantly downregulated in the presence of sh–IFN-λ1. These results indicated that si–IFNAR1, si–IFNAR2, and sh–IFN-λ1 are specific and efficient.
The role of IFN-\(\alpha\) in the regulation of HBV replication was determined by knockdown and overexpression of IFN-\(\alpha\). HepG2 cells were cotransfected with pHBV1.3 and sh–IFN-\(\alpha\) or treated with rhIFN-\(\alpha\). Results showed that HBV HBeAg protein, HBsAg protein, and capsid-associated DNA were upregulated by sh–IFN-\(\alpha\) but downregulated by rhIFN-\(\alpha\), suggesting that IFN-\(\alpha\) represses HBV replication.

Next, the effect of IFN-\(\alpha/\beta\) on the expression of IFN-\(\lambda\) induced by IL-27 was determined. Cells were treated with rhIL-27, si-IFNAR, or the neutralizing Ab specific to IFNAR2 (anti-IFNAR2). In addition, IFN-\(\lambda\) mRNA and protein (Fig. 4D) were upregulated by rhIL-27, but such activation was repressed by si-IFNAR2 or anti-IFNAR2. These results suggested that type 1 IFN is involved in the activation of IFN-\(\lambda\) induced by IL-27.

Next, we evaluated the effects of IFN-\(\lambda\) and IFNAR on the regulation of IL-27 antiviral activity. HepG2 cells were cotransfected with pHBV1.3 and sh–IFN-\(\lambda\) or si-IFNAR and then treated with rhIL-27. ELISA results showed that HBV HBeAg protein, HBsAg protein, and capsid-associated DNA were downregulated by IL-27, and such repression was suppressed by sh–IFN-\(\lambda\) (Fig. 4E) and si-IFNAR (Fig. 4F). These results indicated that IFN-\(\lambda\) and IFN-\(\alpha\) are involved in the downregulation of HBV replication mediated by IL-27.

Moreover, HepG2 cells were transfected with pHBV1.3, treated with anti-IFNAR2 or with neutralizing Ab specific to IFN-\(\lambda\) (anti–IFN-\(\lambda\)) and incubated with rhIL-27. Results revealed that HBV HBeAg protein (Fig. 4G, left panel), HBsAg protein (Fig. 4G, middle panel), and capsid-associated DNA (Fig. 4G, right panel) were downregulated by IL-27, but this repression was suppressed by anti-IFNAR2 or by anti–IFN-\(\lambda\). These results confirmed that IFN-\(\alpha\) and IFN-\(\lambda\) are involved in the repression of HBV replication mediated by IL-27.

IL-27 specifically activates IFN-\(\lambda\) expression in immature DCs and hepatocytes

We revealed that IL-27 induces an antiviral response similar to IFN-\(\alpha\), showed that IFN-\(\lambda\) expression is well correlated with IL-27 expression in HBV-infected patients, and demonstrated that IFN-\(\lambda\) is involved in regulating the anti-HBV function of IL-27. These facts suggested that there is a close correlation between IL-27 and IFN-\(\lambda\) and prompted us to investigate the role of IL-27 on the expression and function of IFN-\(\lambda\).

Human immature DCs, HepG2 cells, and Huh7 hepatocytes were treated with rhIL-27 for different times or at different concentrations, as indicated. Real-time PCR results showed that IFN-\(\lambda\) mRNA was upregulated by IL-27 in a time-dependent fashion (Fig. 5A) and in a dose-dependent manner (Fig. 5C) in DCs, HepG2 cells, and Huh7 cells. ELISA results indicated IFN-\(\lambda\) protein was enhanced by IL-27 in a time-dependent manner (Fig. 5B) and in a dose-dependent fashion (Fig. 5D) in all three cell types. Similarly, results also indicated that IFN-\(\alpha\) mRNA and IFN-\(\beta\) mRNA were upregulated by IL-27 in a time-dependent fashion (Supplemental Fig. 4A) and in a dose-dependent manner (Supplemental Fig. 4B). These results demonstrated that IL-27 activates IFN-\(\lambda\) expression in human immature DCs and hepatoma cells.

IL-27 mediates its biological function through binding to a specific receptor (IL-27R) consisting of TCCR (WSX-1 or IL-27R\(\alpha\)) and a common receptor subunit of the IL-6 family cytokine (gp130) (3, 46–48). Like other class I cytokine receptor chains, IL-27R\(\alpha\) heterodimerizes with gp130 to form a functional IL-27R (2).

The role of IL-27 in the activation of IFN-\(\lambda\) was evaluated further by an RNA-interference approach, using siRNA specific to IL-27R (si–WSX-1). To determine the efficiency of siRNAs, HepG2 and Huh7 cells were transfected with si–WSX-1 or its control si–NC. Real-time PCR results (Supplemental Fig. 3D) and
FIGURE 4. Blocking IFN-α/β and IFN-λ1 signals suppresses the host anti-HBV effect mediated by IL-27. (A) HepG2 cells were cotransfected with pHBV1.3 and sh–IFN-λ1 (or sh-NC). At 24 h posttreatment, the levels of HBeAg protein and HBsAg protein of HBV in culture supernatants were measured by ELISA, and the levels of HBV capsid–associated DNA in transfected cells were assessed by real-time PCR. (B) HepG2 cells were cotransfected with pHBV1.3 and then treated with rhIFN-λ1 protein (20 ng/ml) for 24 h. The levels of HBeAg protein and HBsAg protein of HBV in culture supernatants were measured by ELISA, and the levels of HBV capsid–associated DNA in transfected cells were assessed by real-time PCR. (C and D) HepG2 cells were treated or not with neutralizing Ab to IFNAR2 (10 μg/ml) for 30 min or were transfected with si-IFNAR (or si-NC) for 24 h before rhIL-27 (50 ng/ml) treatment. IFN-λ1 expression was determined by real-time PCR (C) and ELISA (D) 24 h post-IL-27 treatment. (E and F) HepG2 cells were cotransfected with pHBV1.3 and si-IFNAR1/2 (or si-NC) or sh–IFN-λ1 (or sh-NC) and then treated with rhIL-27 protein (50 ng/ml) at 12 h posttransfection. At 24 h posttreatment, the levels of HBeAg protein and HBsAg protein of HBV in culture supernatants were measured by ELISA, and the levels of HBV capsid–associated DNA in transfected cells were assessed by real-time PCR. (G) HepG2 cells were transfected with pHBV1.3 and treated with neutralizing Ab to IFNAR2 (10 μg/ml) or neutralizing Ab or IFN-λ1, which was added 30 min before rhIL-27 (50 ng/ml) treatment. The HBeAg and HBsAg levels were determined by ELISA, and the levels of capsid-associated DNA in transfected cells were assessed by real-time PCR. Data are mean + SE (n = 3). *p < 0.05, **p < 0.01.
flow cytometry results (Supplemental Fig. 3E) showed that IL-27Ra RNA and protein were downregulated by si–WSX-1, indicating that it is effective. HepG2 and Huh7 cells were transfected with si–WSX-1 or si-NC and treated with rhIL-27 protein or PBS at 20 ng/ml for different times, as indicated. Cell culture supernatant was collected, and total mRNA was extracted. IFN-α1 protein levels were assessed by ELISA (A), and IFN-α1 mRNA levels were determined by real-time PCR (B). Data shown are mean ± SE (n = 3). (C and D) DCs (left panels), HepG2 cells (middle panels), and Huh7 cells (right panels) were treated with rhIL-27 protein or LPS at 20 ng/ml. At 24 h post-treatment, cell culture supernatant was collected, and total mRNA was extracted. IFN-α1 protein levels were assessed by ELISA (C), and IFN-α1 mRNA levels were determined by real-time PCR (D). (E and F) HepG2 cells and Huh7 cells were transfected with si–WSX-1 or si-NC, rhIL-27 protein (50 ng/ml) was added at 12 h posttransfection, and IFN-α1 expression was assessed by real-time PCR (E) and ELISA (F) 24 h posttreatment. Data are mean ± SE (n = 3). *p < 0.05.

HepG2 and Huh7 cells were treated with PBS, rhIL-27, or IFN-α. Cytoplasm extracts and nuclear extracts were prepared, and proteins were detected by Western blot using specific Abs. Results revealed that, in the presence of IL-27, the p65, p50, and IRF7 proteins were decreased in the cytoplasm and increased in the nucleus of HepG2 and Huh7 cells, whereas the IRF3 and GAPDH proteins were relatively unchanged in the cytoplasm, and the IRF3 and lamin proteins were relatively unchanged in the nucleus of both cell types (Fig. 6A, Supplemental Fig. 2C). In addition, immunofluorescence assays clearly showed that, in the absence of IL-27, the p65 and p50 proteins were distributed mainly in the cytosol of cells; however, in the presence of IL-27, the majority of the p65 and p50 proteins were translocated to the nucleus of cells (Fig. 6B). These results indicated that IL-27 enhances NF-κB p65 and NF-κB p50 translocation from the cytosol to the nucleus.

Moreover, the effect of NF-κB on IFN-α1 expression regulated by IL-27 was verified further by assessing the ability of p65 and p50 binding to the IFN-α1 promoter. Results from chromatin immunoprecipitation assays showed that IL-27 enhances the binding of p65 and p50 to the distal (Fig. 6C, left panel) and proximal (Fig. 6C, middle panel) NF-κB binding sites on the IFN-α1 expression.
promoter, as well as stimulates the binding of IRF7 to the ISRE sites on the IFN-αβ receptor (Fig. 6C, right panel). These results demonstrated that IL-27 activates IFN-αβ expression by enhancing the binding of transcription factor NF-κB and IRF7 to the promoter of IFN-αβ gene.

**ERK1/2 and NF-κB are involved in the activation of IFN-αβ regulated by IL-27**

Next, we investigated the molecular mechanism and signaling pathway involved in the activation of IFN-αβ regulated by IL-27. First, the effects of different inhibitors of signaling components on IL-27–activated IFN-αβ expression were evaluated. Cells were treated with DMSO (negative control), U0126 (ERK1/2 inhibitor), LY294002 (PI3K inhibitor), H89 (PKA inhibitor), SP600125 (JNK inhibitor), or SB203589 (p38 inhibitor). RT-PCR results revealed that IFN-αβ mRNA was upregulated by IL-27, and this activation was not affected by DMSO or LY294002, was slightly reduced by H89, SP600125, and SB203589, and was significantly inhibited by U0126 (Fig. 7A). We also noticed that these inhibitors had no significant effect on cell viability under the experimental conditions used (Supplemental Fig. 1B). These results revealed that the ERK1/2-signaling pathway is involved in the activation of IFN-αβ expression regulated by IL-27.

Second, the role of ERK1/2 signaling in the IL-27–activated expression of IFN-αβ was evaluated further by introducing two dominant kinase-inactive mutants, mERK1 and mERK2, which block ERK activity by competing with endogenous kinases (49). HepG2 and Huh7 cells were transfected with the plasmids expressing mERK1 or mERK2 and then treated with rhIL-27. Results revealed that IFN-αβ mRNA (Fig. 7B) and IFN-αβ protein (Fig. 7C) were stimulated by IL-27, but such activation was blocked by mERK1 and mERK2 in both cell types. These results further supported that ERK1 and ERK2 are involved in the activation of IFN-αβ expression regulated by IL-27.

Third, the role of IL-27 in the phosphorylation of ERK1/2 was determined. HepG2 and Huh7 cells were treated with rhIL-27 for different times, as indicated. Results showed that p-ERK protein was upregulated by IL-27 in both HepG2 cells and Huh7 cells, whereas ERK and β-actin proteins were relatively unchanged under the same conditions (Fig. 7D, Supplemental Fig. 2D), suggesting that IL-27 stimulates the phosphorylation of ERK protein.

Finally, the effects of IL-27 and ERK1/2 on the nuclear translocation of NF-κB p65 and p50 were investigated. HepG2 and Huh7 cells were transfected with plasmids expressing mERK1 or mERK2 and then treated with rhIL-27. Results revealed that nuclear translocation of p65 and p50 was enhanced by IL-27; however, such activation was reduced, in part, by mERK1 and mERK2 in all cell types (Fig. 7E, Supplemental Fig. 2E), suggesting that the role of NF-κB in IFN-αβ expression is regulated by ERK1/2. Taken together, these results demonstrated that the ERK1/2-signaling pathway and NF-κB protein are involved in the activation of IFN-αβ regulated by IL-27.

**IL-27 enhances the expression of IFN-αβ receptors IL-28R1 and IL-10Rβ**

IFN-αβ and IFN-α/β share the same JAK/STAT-signaling pathway in the induction and expression of a common set of ISGs. However, IFN-αβ binds to a distinct receptor complex consisting of the unique ligand-binding subunit IL-28R1 (also known as IFN-λ1) and the accessory receptor subunit IL-10Rβ (also known as IFN-AR2). To investigate the roles of IL-27 in the expression of IL-28R1 and IL-10Rβ, HepG2 and Huh7 cells were incubated with PBS or rhIL-27. Real-time PCR and Western blot results showed that IL-28R1 and IL-10Rβ mRNAs (Fig. 8A) and IL-28R1 and IL-10Rβ proteins (Fig. 8B, Supplemental Fig. 2F) were upregulated by IL-27 in both HepG2 cells and Huh7 cells. To evaluate the biological relevance of IL-27–activated IL-28R1 and IL-10Rβ expression, we determined whether IL-28R1 and IL-10Rβ proteins were expressed on the cell surface of IL-27–stimulated cells. Thus, FACS analyses of cell surface expression of IL-28R1 and IL-10Rβ proteins were carried out. Flow cytometry results revealed that IL-28R1 and IL-10Rβ proteins were stimulated on the cell surface by IL-27 (Fig. 8C). Thus, these results demonstrated that IL-27 activates the expression of the IFN-αβ receptors IL-28R1 and IL-10Rβ. Taken together, we propose a hypothetical model (Fig. 9) that HBV infection could activate the expression of IL-27 and IFN-λ1, and they inhibit the HBV replication through type 1 IFN.

**Discussion**

Cytokines play a critical role in the control of the innate and adaptive immune responses. Members of the IL-12 cytokine family (IL-12, IL-23, IL-27, and IL-35) are heterodimeric, which allows unique functional interactions not shared by other cytokine families. Despite
many shared structural features and molecular partners, members of the IL-12 family mediate surprisingly diverse functions. IL-27 is a heterodimeric anti-inflammatory cytokine composed of EBI3 and p28, which regulates Th cell function during autoimmune and pathogen-induced immune responses (1, 3, 4). IL-27 exhibits various therapeutic capabilities, such as regulating antiviral ability (12, 13), enhancing antitumor immune responses (14), directing anti-proliferative effects on tumors (15–17), and reducing the inflammation associated with experimental autoimmune arthritis (18, 19).

Serum IL-27 triggers an earlier immune response to prevent hepatic injury in different clinical-pathologic stages of HBV-infected patients (50). The receptor complex for IL-27 is composed of the cytokine receptor IL-27Rα (WSX-1 or TCCR) and gp130 (2).

We recently reported that IL-27 is elevated in the serum of patients infected with IAV and demonstrated that IL-27 is highly induced through COX-2- and the PKA-signaling pathways during IAV infection in human lung epithelial cells (A549) and human PBMCs infected with IAV and revealed that activated IL-27...
inhibits IAV replication by activating STAT1/2 phosphorylation and PKR phosphorylation (20). We also showed that IL-27 protein levels were elevated in patients with HBV infection and that HBV enhances the expression of IL-27 in several cell types, including THP-1, HepG2, Huh7, and HEK293T cells (36). A correlation between HBV infection and IL-27 expression was also reported (43). These results suggested that IL-27 plays an important role in viral infections. However, the function of IL-27 in HBV replication and infection has not been reported.

This study explores the effect of IL-27 on HBV replication. Our results showed that IL-27 represses the expression of HBV proteins and the replication of HBV capsid–associated DNA in HepG2.2.15, HepG2, and Huh7 cells in a dose-dependent fashion, suggesting that IL-27 plays an inhibitory role in HBV replication. IL-27 was reported to exhibit antiviral activity by inhibiting replication of HIV and HCV (12, 13) and to produce IFN-inducible antiviral proteins by eliciting a type I IFN-like response (51, 52), in which IFN-α initially binds to the type I IFN receptor, IFNAR1/2, and induces the phosphorylation of STAT1/2 and the expression of ISGs, which, in turn, initiate an antiviral state in target cells.

After determining that IL-27 inhibits HBV replication, we investigated the mechanism involved in such regulation. We revealed that phosphorylation of STAT1 and STAT2 was stimulated by IL-27 and IFN-α proteins, respectively, suggesting that IL-27 and IFN-α have a similar effect on the activation of STAT1/2. It is known that IFN-α activates several hundred ISGs through ISRE. Our results showed that ISRE activity was stimulated by IL-27, indicating that IL-27 also plays a positive role in the regulation of ISRE activity. IFN-α is essential for the induction of adaptive immunity against viral infections by stimulating a variety of antiviral proteins and cytokines, leading to protection of the host from further viral infections. In this study, we revealed that the expression of IFN-regulated antiviral proteins Mx1, OAS1, and PKR is enhanced by IL-27 and stimulated by IFN-α. Thus, our data demonstrated that IL-27 displays an IFN-α–like ability to induce the expression of IFN-regulated antiviral proteins.

Our results indicated that both IFN-λ1 mRNA and IFN-λ1 protein were significantly upregulated by IL-27, but this activation was repressed by anti-IFNAR2 Ab, indicating that IFN-α is involved in the activation of IFN-λ1 mediated by IL-27. These results were consistent with previous findings that showed that neutralization of type I IFN Ab blocks the IL-27–induced antiviral state (52).

Chronic viral hepatitis is the leading cause of liver disease and may play a role in the pathogenesis of lesions characteristic of cirrhosis and hepatocellular carcinoma. HBV and HCV are the two major causes of chronic viral hepatitis. Therapeutic IFN-α is used widely to treat HBV and HCV infections with considerable clinical success (38, 53). However, IFN-α is not effective for all infected
investigated the correlation of IL-27 and IFN-α/β. Cause both IL-27 and IFN-α/β play roles in antiviral responses, we demonstrated that ERK-signaling pathway is required for the activation of IFN-α1. In this study, we demonstrated that the ERK1/2 phosphorylation and revealed that, in the presence of IL-27, p-ERK is increased, but ERK protein is relatively unchanged. In addition, expression of IFN-α1 was elevated in HBV-infected patients and IFN-α1 mRNA levels were significantly higher in PBMCs of HBV patients with CHB. These results suggested that expression of IFN-α1 in patients with HBV infection and evaluated the correlation between IL-27 and IFN-α1 expression during HBV infection. Our results showed that p28, EB13, and IFN-α1 mRNA levels were significantly higher in PBMCs isolated from patients with CHB compared with healthy individuals. We also revealed that elevated IFN-α1 levels were tightly correlated with the elevated p28 and EB13 levels in the PBMCs of patients with CHB. These results suggested that expression of IFN-α1 was elevated in HBV-infected patients and that IFN-α1 expression was highly correlated with IFN-α1 expression.

IFN-α1 is able to inhibit HBV and HCV replication (28, 30). DCS are the major producers of IL-27 (1) and IFN-α1 (25). Because both IL-27 and IFN-α1 play roles in antiviral responses, we investigated the correlation of IL-27 and IFN-α1 function. We showed that overexpression of IL-27 stimulates IFN-α1 expression in human immature DCS and hepatoma cells, whereas knockdown of IFN-α1 reduces the IL-27–mediated anti-HBV effect. These results suggested that the antiviral ability of IL-27 was mediated by IFN-α1 to some extent.

The mechanism underlying IL-27–mediated regulation of IFN-α1 expression was evaluated further. It was reported that NF-κB and IRF3/7 are required for LPS-induced activation of IFN-α1 and that the organization of IFN-α1 regulatory sequences appears to be different from that of IFN-β (26). In this study, we evaluated the effect of IL-27 on the activities of NF-κB and IRF3/7 by assessing the role of IL-27 in the nuclear translocation of NF-κB and IRF3/7. Our results revealed that NF-κB p65, NF-κB p50, and IRF7 proteins are accumulated in the nucleus and decreased in the cytosol in the presence of IL-27, whereas the levels of IRF3 and lamin A proteins are relatively unchanged in the cytosol and the nucleus. We also showed that NF-κB p65 and NF-κB p50 were distributed mainly in the cytosol in the absence of IL-27, but they translocated to the nucleus in its presence. In addition, IL-27 enhances the binding of NF-κB to the IFN-α1 promoter. These results demonstrated that IL-27 activates IFN-α1 expression through enhancing nuclear translocation of NF-κB and the binding of this transcription factor to the IFN-α1 promoter and, thus, stimulates IFN-α1 expression.

To explore the signaling pathways involved in IL-27–mediated IFN-α1 expression, two approaches were used. First, we evaluated the effects of IL-27–regulated signaling components on IFN-α1 expression. The results showed that IFN-α1 expression is enhanced by IL-27; such activation was not affected by DMSO or LY294002; was slightly reduced by H89, SP600125, and SB203589; and was significantly repressed by U0126. These results suggested that ERK1/2 is required for the activation of IFN-α1 expression regulated by IL-27. Moreover, expression of IFN-α1 stimulated by IL-27 is blocked by two dominant ERK mutants: mERK1 and mERK2. Second, we determined the effect of IL-27 on ERK1/2 phosphorylation and revealed that, in the presence of IL-27, p-ERK is increased, but ERK protein is relatively unchanged. In addition, nuclear translocation of NF-κB is induced by IL-27, but such regulation is partially blocked by mERK1 and mERK2. Thus, we demonstrated that the ERK-signaling pathway is required for the activation of IFN-α1 regulated by IL-27.

IFN-α1 and IFN-α/β share the same JAK/STAT-signaling pathway, which leads to the expression of a common set of genes. However, IFN-α1 binds to a distinct receptor complex consisting of IL-28R1 (IFN-αR1) and IL-10Rβ (IFN-αR2). In this study, we demonstrated that IL-27 activates the expression of IL-28R1 and IL-10Rβ in hepatocytes and suggested that IL-27 plays a role in magnifying the antiviral responsiveness of IFN-α1.

In conclusion, we revealed that HBV infection activates cytokine (IL-27) and type III IFN (IFN-α1) expression and demonstrated that viral-activated IL-27 and IFN-α1 are coordinated to inhibit HBV replication through regulating type I IFN (IFN-α/β). On one hand, HBV infection activates IL-27 expression, which, in turn, stimulates IFN-α1 production through regulating ERK1/2 signaling and enhancing NF-κB nuclear translocation and binding to the IFN-α1 promoter. On the other hand, IL-27–activated IFN-α1 activates its receptors (IL-28R1 and IL-10Rβ), resulting in activation of the STAT1/2 pathway, which, in turn, induces the expression of antiviral proteins (PKR, OAS1, and Mx1) by a manner similar to type I IFN and, finally, inhibits HBV protein expression and viral DNA replication.

FIGURE 9. A novel mechanism by which a cytokine limits virus replication through regulating a type III IFN in a manner similar to type I IFN. During HBV infection, the virus activates cytokine (IL-27) expression, which, in turn, stimulates type III IFN (IFN-α1) production through regulating ERK1/2 signaling and enhancing transcription factor (NF-κB) nuclear translocation and its binding to IFN-α1 promoter. In contrast, IL-27–activated IFN-α1 upregulates the production of IFN-α1 receptors (IL-28R1 and IL-10Rβ), resulting in activation of the STAT1/2 pathway, which, in turn, induces the expression of antiviral proteins (PKR, OAS1, and Mx1) by a manner similar to type I IFN and, finally, inhibits HBV protein expression and viral DNA replication.

Disclosures
The authors have no financial conflicts of interest.
References

Supplemental Figure 1. Evaluation of the effect of rhIL-27 protein and protein inhibitors treatment on cell viabilities. (A) HepG2 cells (left panel) and Huh7 cells (right panel) were treated with rhIL-27 or PBS at 100 ng/ml for 24 h or 48 h, respectively, and then added with MTS reagents for 2 h. (B) HepG2 cells and Huh7 cells were treated with DMSO, U0126, LY294002, H89, SP600125, and SB203589, respectively, and then added with MTS reagents for 2 h. The percentages of cell activities were measured by MTS assay according to the manufacturer’s procedure.
Supplement Figure 2. Western blot band densitometry analyses. (A) Cells were treated with rhIL-27 for different time, or treated with PBS or rhIFN-α, respectively. Phosphorylated STAT1 (p-STAT1) and phosphorylated STAT2 (p-STAT2) proteins
were detected by Western blot and band densitometry analyses. (B) PKR, Mx1, and OAS1 proteins were detected by Western blot and band densitometry analyses. (C) HepG2 and Huh7 cells were treated with PBS, rhIL-27, or IFN-α. Cytoplasm extracts and nuclear extracts were prepared and NF-κB p65, NF-κB p50, IRF3, and IRF7 proteins were detected by Western blot and band densitometry analyses. (D) HepG2 and Huh7 cells were treated with rhIL-27 for different times, as indicated. The phosphorylated ERK protein levels were detected by Western blot and band densitometry analyses. (E) HepG2 and Huh7 cells were transfected with plasmids expressing mERK1 or mERK2, and then treated with rhIL-27. Cytoplasm extracts and nuclear extracts were prepared and NF-κB p65 and NF-κB p50 proteins were detected by Western blot and band densitometry analyses. (E) HepG2 and Huh7 cells were incubated with PBS or rhIL-27. The IL-28R1 and IL-10Rβ protein levels were detected by Western blot band densitometry analyses. Protein bands signals were quantified by image pro plus 6.0 and presented as the fold of band densitometry relative to that of control sample. The data presented as mean + SEM of triplicates.
Supplemental Figure 3. Determination of the efficiency of si-IFNAR1, si-IFNAR2, sh-IFNλ1, and si-WSX-1. (A and B) HepG2 cells were transfected with si-IFNAR or si-NC. (A) The IFNAR1 and IFNAR2 mRNA levels were determined by real-time PCR. (B) The IFNAR1 and IFNAR2 proteins were detected by flow cytometry analysis. (C) HepG2 cells were transfected with sh-IFNλ1 or sh-NC. The IFNλ1 mRNA level was determined by real-time PCR (left panel) and the IFNλ1 protein level was determined by ELISA (right panel). (D and E) HepG2 and Huh7 cells were transfected with sh-WSX-1 or sh-NC. (D) The WSX-1 mRNA level was determined by real-time PCR. (E) The WSX-1 protein level was determined by flow cytometry.
Supplemental Figure 4. Analysis of the effects of IL-27 on the expression of IFN-α and IFN-β. (A) HepG2 cells, Huh7 cells, and DCs were treated with rhIL-27 or PBS at different times, as indicated. The IFN-α and IFN-β mRNA levels were determined by real-time PCR. (B) HepG2 cells, Huh7 cells, and DCs were treated with rhIL-27 protein or LPS at different concentrations, as indicated. The IFN-α and IFN-β mRNA levels were determined by real-time PCR. Data shown are means ± SE; n = 3. *p < 0.05