Inducible Interleukin 32 (IL-32) Exerts Extensive Antiviral Function via Selective Stimulation of Interferon λ1 (IFN-λ1)*

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Background: Interleukin-32 participates in responses to viral infection, but how virus induces its expression and the mechanisms of its antiviral activities remain unclear.

Results: Interleukin-32 selectively stimulates IFN-λ1 via activating distinct regulatory elements in the promoter.

Conclusion: Interleukin-32 promotes IFN-λ1-mediated antiviral response.

Significance: Interleukin-32 is one immune response factor and plays an important role in the control of viral infection.

Interleukin (IL)-32 has been recognized as a proinflammatory cytokine that participates in responses to viral infection. However, little is known about how IL-32 is induced in response to viral infection and the mechanisms of IL-32-mediated antiviral activities. We discovered that IL-32 is elevated by hepatitis B virus (HBV) infection both in vitro and in vivo and that HBV induced IL-32 expression at the level of both transcription and post-transcription. Furthermore, microRNA-29b was found to be a key factor in HBV-regulated IL-32 expression by directly targeting the mRNA 3′-untranslated region of IL-32. Antiviral analysis showed that IL-32 was not sufficient to alter HBV replication in HepG2.2.15 cells. To mimic the viremic phase of viral infection, freshly isolated peripheral blood mononuclear cells were treated with IL-32γ, the secretory isoform, and the supernatants were used for antiviral assays. Surprisingly, these supernatants exhibited extensive antiviral activity against multiplex viruses besides HBV. Thus, we speculated that the IL-32γ-treated peripheral blood mononuclear cells produced and secreted an unknown antiviral factor. Using antibody neutralization assays, we identified the factor as interferon (IFN)-λ1 and not IFN-α. Further studies indicated that IL-32γ effectively inhibited HBV replication in a hydrodynamic injection mouse model. Clinical data showed that elevated levels of IFN-λ1 both in serum and liver tissue of HBV patients were positively correlated to the increased levels of IL-32. Our results demonstrate that elevated IL-32 levels during viral infection mediate antiviral effects by stimulating the expression of IFN-λ1.

IL-32 has been shown to be an important cytokine in diseases characterized by inflammation, such as rheumatoid arthritis (1) and inflammatory bowel disease (2). In human peripheral blood mononuclear cells (PBMCs), IL-32 can be stimulated by IFN-γ, lipopolysaccharide (LPS), and some exogenous pathogens such as Mycobacterium tuberculosis (3). Interleukin-32 has been described as an activator of the p38 mitogen-activated protein kinase (MAPK), NF-κB, and AP-1 signaling pathways, and it induces several cytokines, including IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α (4–6). Our previous study showed that the expression of this cytokine was up-regulated during influenza A infection (7), and elevated levels of IL-32 were found in serum from patients infected with HIV-1 (8). It has been newly reported that IL-32 is involved in HCV-related liver inflammation and fibrosis (9). Together, these findings suggest that this cytokine is relevant for viral infection in humans.

Additional experiments have been carried out to verify the impact of IL-32 on viral infection and replication; however, the mechanisms of IL-32 antiviral properties remain unclear. In one study, the specific knockdown of IL-32 gene expression in PBMCs led to a 4-fold increase in HIV-1 p24 production, indicating that endogenous IL-32 acted as a natural inhibitor of HIV-1 (4). New evidence has shown that IL-32 is capable of activating IFN-inducible antiviral effectors, such as protein kinase R (PKR) and myxovirus resistance protein (Mx), and it antagonized vesicular stomatitis virus in WISH cells and herpes simplex virus in epithelial Vero cells (10). However, IL-32 did not antagonize HCV replication in Huh7.5 cells (9).

IFN-λ1 belongs to the type III IFNs, which are a newly identified IFN family that includes IFN-λ1, IFN-λ2, and IFN-λ3 (also designated IL-29, IL-28A, and IL-28B, respectively). Type III IFNs bind to a distinct receptor complex composed of one exclusive chain and a second chain that is shared with the receptors for IL-10, IL-22, and IL-26 (11, 12). They are found to

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§ The abbreviations used are: PBMC, peripheral blood mononuclear cell; rhIL-32, recombinant human IL-32; HBV, hepatitis B virus; HBeAg, hepatitis B virus e antigen; HBsAg, hepatitis B virus surface antigen; HCV, hepatitis C virus; EV71, enterovirus 71; miRNA, microRNA; NF-κB, nuclear factor-κB; CREB, cAMP response element-binding protein; IRF, interferon regulatory factor; miroRNA, microRNA; HIV-1, human immunodeficiency virus type 1; Mx, myxovirus resistance protein; Ab, antibody; IκBα, inhibitor of NF-κBα; pgRNA, pregenomic RNA; HBx, hepatitis B virus x.
induce the activation of the JAK/STAT signaling pathways, activate the transcriptional factors γ-activated sequence and IFN-stimulated response element (13), and induce classical IFN-stimulated genes, such as 2′,5′-oligoadenylyl synthetase, Mx (14), and PKR (15). The type III IFNs are also capable of interfering with the multiplication of several human and murine viruses in vitro, including encephalomyocarditis virus, Sindbis virus (16), Apeu virus (17), HBV, HCV (18, 19), the human and murine cytomegaloviruses (20), and murine herpes simplex virus 2 (21). In addition, the type III IFNs exhibit antiviral features similar to those of the type I IFNs. In human primary liver cells, HVc infection induces IFN-αs production, which mediates viral infection (22). In this study, we identified a previously unrecognized mechanism whereby virus-induced IL-32 expression is mediated by microRNA. To more carefully explore IL-32 antiviral properties, we experimentally mimicked the physiological environment present during the viremic phase of viral infection. Our results demonstrate that IL-32 induced the expression of IFN-λ1 in PBMCs, leading to extensive antiviral activity during viral infection.

EXPERIMENTAL PROCEDURES

Ethics Statement—The use of serum and PBMC samples in this 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 at Wuhan University in accordance with its guidelines for the protection of human subjects. All participants gave written informed consent to participate in the study.

Cell Culture and Transfection—For cell culture, THP-1 (human acute monocytic leukemia cell line) and Jurkat (human T cell lymphoblast-like cell line) cells were cultured in RPMI 1640 medium, the human hepatoma cell lines (HepG2, Huh7, and Huh7.5.1) and HEK293 cells were grown in DMEM, and the human embryonal rhabdomyosarcoma (RD) cells were grown in minimum Eagle’s medium. RPMI 1640 medium, DMEM, and minimum Eagle’s medium were purchased from Invitrogen and were supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin. The cells were grown at 37°C in a 5% CO2 incubator.

HepG2, Huh7, and HEK293 cells were plated in 24- or 6-well plates and grown to ~80% confluence at the time of transfection. The cells (2 × 10^5) were cotransfected with 0.2 μg of DNA or 50 pmol of miRNA mimics/inhibitor using Lipofectamine 2000 reagent (Invitrogen).

Isolation of PBMCs and Electroporation—For PBMC isolation, 200 ml of venous blood was obtained from five healthy volunteers. The PBMCs were isolated by density centrifugation with an isolation solution of human lymphocytes (TBD Science) as described previously (23). The cells were washed twice in saline and suspended in culture medium (RPMI 1640 medium) supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin.

The transfection of PBMCs, Jurkat cells, and THP-1 cells was achieved by electroporation. A blended solution was prepared with a 1:4 volume of Solution 1 (20% ATP disodium salt, 12% MgCl2,6H2O) and Solution 2 (1.2% KH2PO4, 0.12% NaHCO3, 0.04% glucose, pH 7.4). The cells (10^6) were suspended in 100 μl of the blended solution with 4 μg of plasmid and vibrated lightly. The mixture was added into the cuvette (BTX, Taiwan), and electroporation was accomplished with Nucleofector (Amala, Germany). The cells were then washed into plates and cultured in RPMI 1640 medium.

ELISA for IL-32 and IFN-λ1 Measurement—IL-32 production in the culture supernatants was measured by ELISA as described in prior studies (24, 25). Briefly, the rIL-32α protein was expressed in Escherichia coli, purified by nickel-nitrilotriacetic acid His-Bind Resins, and used to generate a standard curve for ELISAs. The 96-well plates were precoated with commercial mAbs against IL-32 (KU32-56, 4 mg/ml; BioLegend, San Diego, CA) overnight at 4°C and blocked with 1% BSA for 1 h at 37°C. Serially diluted rIL-32α or other samples were then added to the wells and incubated at 37°C for 1 h. The plates were washed three times with phosphate-buffered saline with Tween 20 (PBST), and the biotinylated mAbs against IL-32 (KU32-52, 0.2 mg/ml; BioLegend) diluted in 1% BSA were then added for 1 h. The plates were washed three times with PBST and incubated with streptavidin-HRP (0.3 mg/ml; Upstate, New York, NY) diluted in 1% BSA for 30 min. After plate washing as described above, the 3,3′,5,5′-tetramethylbenzidine substrate (Thermo Scientific, Houston, TX) was added, and 50 ml of 2 N sulfuric acid was added to stop the enzyme reaction. The OD values were detected at 450 nm using a microplate reader. The IFN-λ1 levels in the cell medium supernatants and serum samples were measured using a commercial ELISA kit (eBioscience, San Diego, CA).

Construction of Plasmids—The fragment encoding luciferase was amplified by PCR from the pGL3-promoter and subcloned into a pcMV-Tag2A vector to generate pcMV-Tag2A-Luc. The 3′-UTR of IL-32 containing an intact microRNA (miR)-29b recognition sequence was amplified by PCR from genomic DNA, and the PCR product was subcloned into the pcMV-Tag2A-Luc vector immediately downstream of the luciferase gene to generate pcMV-Luc-IL-32-3′-UTR. A construct containing the IL-32 3′-UTR with a point mutation was synthesized with the QuikChange site-directed mutagenesis kit (Stratagene, LA Jolla, CA). A fragment of the IFN-λ1 promoter (−1903 bp) was amplified from human genomic DNA and subcloned into a pGL3-promoter vector. The truncation of the promoter (−728 bp) was amplified from this construct and subcloned into the same empty vector. Differently mutated IFN-λ1 promoter constructs were also generated from pGL3-IFN-λ1 (−728 bp) with the QuikChange site-directed mutagenesis kit. The constructs were confirmed by DNA sequencing. All primers used for construction are listed in supplemental Table 1 (P1–P18). All constructs for the IL-32y promoter, reporters, mutants, and promoter truncations were reported previously (7).

Luciferase Reporter Assay—When the samples were transfected with the reporter plasmids, each sample was cotransfected with 50 ng of a pRL-TK plasmid expressing Renilla luciferase to monitor the transfection efficiency (Promega, Madison, WI) as described previously (26). Twenty-four hours after transfection, the cells were serum-starved for an additional 24 h before they were harvested. The luciferase activities
were then measured and normalized to the Renilla luciferase activities. The assay results are expressed as relative luciferase activity.

**Reverse Transcription Reaction and Quantitative Real Time PCR**—Real time PCR for cytokine mRNA was performed as described previously (21). The total RNA was extracted with TRIzol reagent (Invitrogen), and the complementary DNA was generated with a reverse transcription system kit (Invitrogen). β-Actin was used as an endogenous control to normalize the amount of total mRNA in each sample. Murine GAPDH was used as internal control in animal experiments. Stem-loop reverse transcription for mature miRNA was performed as described previously to produce the complementary DNA (27). U6 RNA was used as an miRNA internal control for both human and mouse samples with shared primers.

Real time PCR was performed with a standard SYBR Green PCR kit protocol on a LightCycler 480II machine (Roche Applied Science). The primers used for the cytokines, miRNA, and endogenous controls are listed in **supplemental Table 1** (P19–P37). The primers for IL-32 were designed to bind a common sequence target on all six isoforms for measuring total IL-32 mRNA.

**Western Blot Analysis**—All protein extracts were prepared and quantified using a protein assay kit (Bio-Rad). Western blot analysis was performed, and sample loading was normalized with antibodies against β-actin. The immunoblots were visualized with a chemiluminescent HRP substrate (Millipore, Billerica, MA).

**Chromatin Immunoprecipitation Analysis**—ChIP analysis was performed as described previously (28). Briefly, the harvested cells were washed twice with PBS and lysed in lysis buffer (1% SDS, 10 mM Tris-HCl, pH 8.0, 1 mM PMSF, 50 mg/ml aprotonin and leupeptin) for 10 min on ice. 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 a DNA input control. The remaining supernatant was diluted 10-fold with dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) and incubated with antibodies against transcription factors overnight at 4 °C. The immunoprecipitated complexes were collected using protein A/G-agarose beads. The pellets were washed with dialysis buffer (2 mM EDTA, 50 mM Tris-HCl, pH 8.0). The samples were incubated at 67 °C for 5 h to reverse formaldehyde cross-links. The DNA was precipitated with ethanol and extracted three times with phenol/chloroform.

Finally, the pellets were resuspended in Tris-EDTA buffer and subjected to PCR amplification. The specific detection primers used are listed in **supplemental Table 1** (P38–P49). The PCR products were resolved by gel electrophoresis.

**Collection and Assessment of Clinical Samples**—One milliliter of serum was collected from each of 174 chronic HBV-infected patients before they received antiviral treatment. The patients spanned all chronic HBV infection phases according to the intracellular core-associated DNA in HBV was extracted and quantified by real time PCR as described previously (32). Bay-11-7082, which completely and specifically blocks the NF-κB signaling pathway by irreversibly inhibiting IkBα phosphorylation (30), was purchased from Sigma.

**Viruses Used and Measurement of Viral Infection**—The HepG2.2.15 cell line, which was derived from HepG2 cells and stably expresses HBV (Genotype D, Serotype ayw, U95551), was used as an HBV replication model (31). The Huh7.37 cell line, which also stably expresses HBV (Genotype B, Serotype ayw, JN406371), was constructed with Huh7 cells in this study. The stable cell lines were maintained in DMEM containing 400 µg/ml G418. The plasmid PN-HBV-1.3, which expresses HBV (Genotype C, Serotype adr, FJ899793), was a gift from Dr. Dongping Xu (Beijing 302 Hospital, Beijing, China).

HBV replication and expression were detected as described previously (32). Briefly, HBsAg and HBeAg levels in the culture supernatant of HBV-positive cells or patient sera were measured with commercial ELISA-based kits (Shanghai KeHua Biotech Co. Ltd., Shanghai, China). To detect HBV replication, the intracellular core-associated DNA in HBV was extracted and quantified by real time PCR as described in the manufacturer’s protocol (PG Biotech, Shenzhen, China). The primers used for RT-PCR are listed in **supplemental Table 1** (P50–P52). RT-PCR for HBV RNA was performed as described previously (33). Relative HBV pgRNA and X mRNA were detected with β-actin as an internal control. Primers P49 and P50 were specific for the 3.5-kb pgRNA detection. Primers P51–P54 were
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used for detection of X mRNA. Primers P51–P52 cover all four RNAs of HBV, including X (0.65 kb), S (2.1 kb), pre-S (2.4 kb), and C-E or pgRNA (3.5 kb), and primers P53 and P54 cover three RNAs of HBV, including S (2.1 kb), pre-S (2.4 kb), and C-E or pgRNA (3.5 kb), except the X mRNA. The value of real time PCR with P51 and P52 subtracted by that with P53 and P54 represents the level of X mRNA. Primers P53–P58 are listed in supplemental Table 1.

Human embryonal RD cells growing in plates were incubated with enterovirus 71 (EV71; C4 subtype, Xiangfan, Hubel, China) at a multiplicity of infection of 5 for 1 h with gentle shaking for 15 min. The cells were then removed from the medium, washed twice with PBS, and cultured in minimum Eagle’s medium for 4 h. These EV71-infected RD cells were then used in the antiviral experiments. When the antiviral treatment was finished, 100 μl of supernatant from the infected RD cells was dissolved in 1 ml of TRIzol reagent, and the viral mRNA was extracted and reverse transcribed into a cDNA library. Real time PCR was performed to measure the copies of the viral mRNA using the VP1 gene of the EV71 construct plasmid as the standard concentration. The primers used are listed in supplemental Table 1 (P59–P60).

The plasmid pNL4-3 is an HIV-based infectious vector, and upon transfection, this clone directs the production of infectious virus particles in a wide variety of cells (34). HEK293 cells transfected with this plasmid were used as an HIV replication and expression model. HIV p24 levels were measured with a p24 ELISA kit (provided by the mAb laboratory of Wuhan Institute of Virology, Chinese Academy of Sciences) and expressed as HIV replication levels.

The genotype 2a HCV virus JFH-1 was a gift from Dr. Takaji Wakita from the National Institute of Infectious Diseases of Japan. The Huh7.5.1 cells were infected with this virus and used as a model of HCV infection and replication (35). The numbers of HCV copies in the culture medium were detected by commercial HCV Copies Test real time PCR kits (Shanghai KeHua Biotech Co. Ltd.).

**Mouse Model of Hydrodynamic Injection**—All animal studies were approved by the Institutional Animal Care and Use Committee at Wuhan University. A mouse model of HBV infection by hydrodynamic injection was used in this study as described previously (36, 37). C57BL/6 mice (6–8-week-old male; The Jackson Laboratory) were each inoculated with 10 μg of pAAV-HBV-1.3 plasmid and/or 20 μg of pCMV-IL-32. The 30 μg of plasmid in a volume of 0.9% NaCl solution equivalent to 0.1 ml/g of mouse body weight was injected into the tail veins of mice within 5–8 s.

**Statistical Analysis**—The relationships between miR-29b and IL-32 mRNA expression, IL-32 and IFN-λ1 protein expression, and HBV DNA and IL-32 in patient samples were analyzed by Pearson’s correlation. All other comparisons were determined by the Student’s t test, and the data are expressed as means with S.D. All experiments in cell lines or PBMCs were reproducible and were carried out in triplicate. Each set was repeated at least three times, and a representative of similar results is shown. A p value <0.05 was considered statistically significant.

**RESULTS**

**IL-32 Expression Is Induced by HBV in Various Cell Types**—IL-32 levels in HepG2 and HepG2.2.15 cells (an HBV-positive stable cell line) were measured by real time PCR, ELISA, and Western blot. The IL-32 mRNA (Fig. 1A) and protein (Fig. 1, B and C) levels in HepG2.2.15 cells were significantly higher than those in HepG2 cells. HBV infection of PBMCs is a frequent event at all stages of viral infection (38), and HBV genome, transcripts, and antigens are detectable in immune cells (39). A large amount of X mRNA is expressed in PBMCs of highly viremic patients (33), and X protein stimulates PBMCs of infected patients and activates the immune response (40). It is reported that IL-32 mRNA is highly expressed in immune tissue rather than other tissue (6). Therefore, we electroporated Jurkat, THP-1, and freshly isolated PBMCs with the HBV-expressing plasmid pHBV-1.3 and analyzed IL-32 mRNA and protein levels. In PBMCs, the expression levels of HBV pgRNA and X mRNA are 16.2 and 33.9% less than levels in HepG2.2.15 cells, respectively, and are about 2-fold higher than the average levels in PBMCs from 10 patients. IL-32 was significantly up-regulated by HBV infection in these cells (Fig. 1, D–G). Remarkably, IL-32γ, the secreted IL-32, was detectable in the cell supernatants (Fig. 1, B and F). The expression of IL-32 was also increased in freshly isolated PBMCs after treatment with culture supernatants of HepG2.2.15 cells that contain HBV (8.5 × 10⁴ copies/ml), and this increment of IL-32 was reversed by human hepatitis B immunoglobulin, the HBV-neutralizing antibody (Fig. 1H).

**IL-32 Transcription Was Induced Mainly through NF-κB Activation**—NF-κB and cAMP response element sites are important cis-regulatory elements within the IL-32 promoter (7). To identify the cis-regulatory elements in the IL-32 promoter that are responsive to HBV, five truncation reporter plasmids and four mutant reporter plasmids were constructed such that the binding sites of the transcriptional factors were altered or deleted in the IL-32 promoter. The deletion or mutation of NF-κB binding sites had significant effects on the induction of the IL-32 promoter by HBV, and the cAMP response element site mutation also slightly reduced the HBV-induced promoter activity (Fig. 2A). ChIP assays were then performed with two pairs of primers to test the transcription factor binding activity on the two indicated regions within the IL-32 promoter. The results showed that the binding of the NF-κB p65 protein on both regions of the promoter was significantly strengthened by HBV (Fig. 2B). In contrast, the binding of NF-κB RelB and the CREB protein were not remarkably changed. Bay-11-7082, an NF-κB-specific inhibitor, almost completely abolished IFN-γ-stimulated IL-32 expression and greatly reduced the HBV-mediated IL-32 expression (Fig. 2C). Transfection of pHBV plasmid could induce the activity of the NF-κB reporter in Jurkat cells (Fig. 2D). Together, these results suggest that HBV stimulated IL-32 transcription mainly by activating the transcriptional factor NF-κB.

**miR-29b Is Involved in Regulating IL-32 Expression**—We have shown that HBV activated the IL-32 promoter to initiate the transcription of IL-32 (Fig. 2A). The activity of the IL-32 3′-UTR reporter was also markedly up-regulated by HBV (Fig. 2B).
indicating that IL-32 expression is regulated at both the transcriptional and translational levels. We next analyzed the 3'-UTR of IL-32, and specific miRNA recognition sites were identified.

miRNAs target the 3'-UTR of mRNAs and regulate the expression of numerous genes by catalyzing the cleavage of mRNA (41) or repressing mRNA translation (42). To identify miRNAs that may target and alter the expression of IL-32, we used several programs for miRNA targeting prediction, including PicTar, TargetScan, miRanda, and miRGen. We discovered over 20 high scoring candidate miRNAs that may target the IL-32 3'-UTR. Among these miRNAs, miR-233, miR-29a, miR-29b, and miR-29c were identified to be associated with HBV infection according to previous reports (43) and our microarray data (Fig. 3B). We transfected each of these four synthetic mature miRNAs, their inhibitors, and nonsense control miRNAs along with the IL-32 3'-UTR reporter plasmid into HepG2 cells and analyzed the luciferase activity. Results showed that only miR-29b and its inhibitor significantly affected the UTR reporter activity (Fig. 3C). To verify the binding of miR-29b to the predicted 3'-UTR site, a mutant of the predicted targeting site was generated from the IL-32 3'-UTR reporter plasmid (Fig. 3D). We found that miR-29b mimics significantly suppressed the activity of the IL-32 3'-UTR reporter, and miR-29b inhibitor increased this activity. Importantly, these effects were not observed in cells transfected with the IL-32 3'-UTR mutant construct plasmid (Fig. 3, C and E).

We next transfected synthetic miR-29b and its inhibitor into HepG2 and Huh7 cells. We found that IL-32 mRNA levels in miR-29b-transfected cells were significantly decreased com-
pared with those in cells transfected with the nonsense miRNA control (Fig. 3F). In contrast, transfection with the miR-29b inhibitor resulted in up to 2-fold increases in IL-32 mRNA levels compared with controls (Fig. 3G). The intracellular and extracellular proteins were also analyzed by Western blot and ELISA, respectively. Both intracellular (middle panel) and the secretory isoform (upper panel) of IL-32 were enhanced by miR-29b inhibitor in the presence of HBV. The secretory isoform, but not intracellular IL-32, was up-regulated by miR-29b inhibitor compared with the miRNA inhibitor nonsense control (Fig. 3H). Together, these data indicate that miR-29b targets IL-32 mRNA to regulate the expression of this gene.

**Suppression of miR-29b during HBV Infection Contributes to IL-32 Expression**—The relative expression of miR-29b in different cell lines was measured by real time PCR. The miR-29b levels in the HBV-positive cell lines HepG2.2.15 (Genotype D) and Huh7.37 (Genotype B) were markedly lower than in the control cell lines (Fig. 4A). The ratio of miR-29b in HepG2.2.15/HepG2 was lower than that in the microarray (Fig. 3B). We think the difference may come from the different approaches applied in the two experimental systems. Transfection of HBV-expressing plasmid pHBV-1.3 in HepG2 and Huh7 cells brought about similar suppressed miR-29b levels (Fig. 4B). Identical results were also observed in the presence of HBV Genotype C (Fig. 4C). In the mouse model of HBV infection, remarkably lower levels of murine miR-29b were found in the group of mice with virus infection (Fig. 4D). The expression of miR-29b and IL-32 in human PBMCs isolated from 17 chronic HBV patients and 10 healthy individuals was also detected by real time PCR. We discovered that the miR-29b levels in HBV

**FIGURE 2. IL-32 transcription was induced mainly through NF-κB activation.** A, pHBV or a vector control was individually co-transfected into HepG2 cells with luciferase (Luc) reporter plasmids, including the wild-type IL-32 promoter (-746/-125), truncated mutants, and site-specific mutants. The induction of luciferase activity was calculated compared with the vector control (fold change). CRE, cAMP response element. B, Jurkat cells were electroporated with pHBV or an empty vector. Forty-eight hours after transfection, ChIP assays were performed using 5 μg of anti-NF-κB p50, anti-NF-κB RelB, anti-CREB1, or anti-CREB2 antibodies. Normal rabbit IgG was used as a control. Immunoprecipitated DNA or control DNA was collected and amplified using specific primers. C, PBMCs were electroporated with pHBV or an empty vector. Four hours after transfection, the medium was removed and replaced by fresh RPMI 1640 medium, and simultaneously some samples as shown were treated with IFN-γ (100 units/ml) or Bay-11-7802 (10 μM). After 24 h, IL-32 levels in the supernatant were measured by ELISA and normalized to the vector control. D, Jurkat cells were electroporated with an NF-κB-Luc reporter plasmid and pHBV or empty vector. Luciferase activities were analyzed 48 h after electroporation. Rel. Lucif. Act, relative luciferase activity. *, p < 0.05. Error bars represent S.D.
patients were remarkably lower than those in healthy individuals (Fig. 4E and supplemental Table 2). Furthermore, a statistically significant negative correlation was observed between IL-32 mRNA and miR-29b in the PBMC samples from HBV patients (n = 17, r = −0.776, p < 0.01) (Fig. 4F). The RNA levels were also analyzed with HBV-infected liver tissues (n = 8). An inverse correlation between IL-32 mRNA and miR-29b existed among tissue samples (n = 8, r = −0.624, p < 0.05) (supplemental Table 3 and Fig. 4G). These data show that miR-29b levels are suppressed during chronic HBV infection, and this suppression contributed to HBV-induced IL-32 expression.

**Supernatants from rhIL-32-treated PBMCs Exhibit Antiviral Activity**—The antiviral effects of IL-32 have been described in infections with viruses such as influenza, (7), HIV
(4,8), herpes simplex virus 2, and vesicular stomatitis virus (10). Here, the antiviral effect of IL-32 on HBV replication was examined; however, neither HBV DNA nor HBe/s antigens were altered by transfection with pCMV-IL-32 or treatment with recombinant protein rhIL-32 (Fig. 5A). In the other three hepatocyte-derived cell lines, L02, Huh7, and Hep3B, no significant anti-HBV activity was found with IL-32 (Fig. 5B).

Because the secreted IL-32γ accumulated in sera of patients (supplemental Table 3) and was highly expressed in PBMCs, we speculated that IL-32γ may affect HBV replication indirectly. To mimic the physiological environment that exists in the viremic phase of HBV infection, freshly isolated PBMCs were treated with different concentrations of rhIL-32γ and the medium supernatants were collected and used for antiviral assays. The supernatants from rhIL-32γ-treated PBMCs were then incubated with pHBV-transfected Hep3B cells, and the replication of HBV was measured. Interestingly, our results showed that both HBV DNA and HBe/s antigen levels were significantly suppressed by the supernatant (Fig. 5C). We further discovered that the most effective antiviral activity could be achieved with rhIL-32γ concentrations of 5 ng/ml, the same dose as in the vesicular stomatitis virus antiviral study (10).

Surprisingly, we found that the supernatants from rhIL-32γ-treated PBMCs had universal antiviral properties as experiments were performed with three additional viruses to test the antiviral efficiency. For example, the replication of EV71 was hampered after incubation with these supernatants (Fig. 5D), and to study HIV replication, we transfected the PNL4-3 plasmid, an HIV-based infectious vector (34), into HEK293T cells. We found that the supernatant from rhIL-32γ-treated PBMCs inhibited HIV p24 expression (Fig. 5E). Although IL-32 did not antagonize HCV replication in Huh7.5 cells (9), the viral copies generated by HCV-infected Huh7.5.1 cells were reduced by nearly two orders of magnitude when incubated with supernatant from rhIL-32γ-treated PBMCs (Fig. 5F). Thus, rhIL-32γ-treated PBMCs secrete a factor that is capable of mediating antiviral activity against multiple viruses.

In the mouse model of HBV infection, the antiviral activity of IL-32γ was tested. The addition of pCMV-IL-32γ plasmid in the hydrodynamic injection successfully brought about the expression of human IL-32γ in mouse livers. The mRNA level relative to GAPDH reached up to $4.8 \times 10^{-3}$ on average, whereas the index of the empty vector group was lower than the former over 2 orders of magnitude. The secretion of hepatitis B s and e antigens and intracellular core protein was greatly inhibited in the group with human IL-32γ expression (Fig. 6, A and B). IL-32γ also effectively impaired viral DNA, pregenomic RNA, and HBV x (HBx) RNA (Fig. 6C).

**IL-32 Inhibits Viral Replication through the Induction of IFN-λ1 in PBMCs**—We hypothesized that IL-32 stimulates the expression and secretion of an unknown antiviral factor in PBMCs. Because type I and type III IFNs are known to have universal antiviral activities, we supposed that one or more IFN subtypes were produced and secreted by rhIL-32γ-treated...
PBMCs to achieve robust host responses against viral infection. To verify this, freshly isolated PBMCs were treated with 5 ng/ml rhIL-32 and mRNA levels of IFN-α, IFN-β, and IFN-λ1 were analyzed by real time PCR. The results showed that IFN-λ1 mRNA levels increased markedly, reaching a 30-fold increase. In contrast, the induction of IFN-α or IFN-β mRNA was not detectable after rhIL-32 treatment. PBMCs of six chronic HBV infectors were also isolated to perform the same experiment. IFN-λ1 was also selectively induced by rhIL-32, although it initially expressed at relatively higher levels in these patients than in healthy individuals (Fig. 7A and supplemental Table 4).

We next tested the induction of the IFN-β and IFN-λ1 promoters by IL-32γ. The results showed that IFN-λ1 promoter activity was induced significantly by IL-32γ. However, this induction was not observed for the IFN-β promoter (Fig. 7B). We then detected the IFN-λ1 protein in the supernatant of rhIL-32γ-treated PBMCs by ELISA and discovered that IFN-λ1 protein levels were dramatically higher than the control (Fig. 7C). The average IFN-λ1 protein concentrations reached up to 165 ± 15 pg/ml, which is an efficient antiviral dose according to reported data (22). We also assessed the effects of rhIL-32γ on IFN-λ1 expression in different cell lines. Interestingly, IFN-λ1 mRNA expression was significantly elevated in Jurkat and...
FIGURE 6. IL-32γ inhibits HBV in hydrodynamic injection mouse model. C57BL/6 mice (six males for each group) were treated by hydrodynamic injection of pAAV-HBV1.3 and pCMV-IL-32γ with empty vectors as a control. A, after 1, 3, and 7 days, blood was taken from fossa orbitalis and used for HBe/sAg measurement. B, on the 3rd and 7th days, the mice were sacrificed, and liver tissues were used for Western blot analysis of HBV core protein. C, HBV DNA, pregenomic RNA, and HBx mRNA in the 7th-day livers were analyzed by real time PCR. *, p < 0.05. Error bars represent S.D.
serum samples from a cohort of HBV patients compared with healthy individuals (Fig. 9C and supplemental Table 5).

We have provided evidence that recombinant IL-32 can induce IFN-α. To investigate this relationship in patients, IFN-α protein levels from the 174 serum samples from HBV patients were measured and subjected to a correlation analysis with IL-32 levels. A statistically significant correlation was observed between serum IL-32 and IFN-α protein levels ($n = 174$, $r = 0.778$, $p < 0.01$, Pearson's correlation) (Fig. 9D). This correlation was also observed among the HBV-infected liver tissues ($n = 8$, $r = 0.742$, $p < 0.05$) (Fig. 9E and supplemental Table 3).

To verify the relationship among miR-29b, IL-32, and IFN-α during HBV infection, the pHBV-1.3 plasmid was electroporated into PBMCs, and the miR-29b, IL-32, and IFN-α levels were detected simultaneously at the indicated time points. Our results showed that miR-29b levels dropped rapidly within 6 h after the electroporation, and IL-32 mRNA levels...
increased over the next 6 h followed by the appearance of elevated IFN-λ1 (Fig. 9F). The empty vector was used as a control, and no significant variations were observed. Additionally, to detect the effect of miR-29b on the expression of IL-32 and IFN-λ1, synthetic miR-29b or nonsense miRNA was co-electroporated along with pHBV-1.3 plasmid into PBMCs. Results showed that the supplement of exogenous miR-29b not only inhibited IL-32 induction but also impaired IFN-λ1 production (Fig. 9G). These data indicate that a suppressed level of miR-29b during HBV infection contributes to induction of both IL-32 and IFN-λ1.

DISCUSSION

In the current study, we demonstrate a previously unrecognized mechanism for HBV infection. A hypothetical model was drawn in light of our results to show the antiviral role of IL-32 and IFN-λ1 during chronic HBV infection. We have provided evidence here that HBV induces IL-32 expression through a complex multistage mechanism where both the activation of transcription factors and the suppression of miR-29b contribute to IL-32 production. In turn, elevated IL-32 exerts antiviral functions through the selective stimulation of IFN-λ1 produced by PBMCs.

Because HBV DNA, mRNA, and antigens are detectable in PBMCs in addition to hepatocytes of most infected patients (33, 38, 39), electroporation of immune cells with pHBV-1.3 plasmid is performed to imitate the presence of viral components. The viral RNA levels produced by this approach are lower than in HepG2.2.15 cells and are about 2-fold higher than the average level in PBMCs from patients. HBV infects PBMCs in most chronically infected patients in vivo (38), and HBx protein is expressed at high levels in PBMCs of some patients (33). Moreover, viremia appears frequently in patients. Viral components, including HBx protein, in serum can stimulate PBMCs and acti-
IL-32 Stimulates IFN-λ1 but Not IFN-α

vate the immune response (40). Multiple experiments in this study prove that HBV induces IL-32 expression in at least two types of cells, hepatocytes and PBMCs (Figs. 1 and 9).

The regulation of IL-32 transcription during viral infection has been reported previously (7). The HBx protein has been shown to activate the RAS-RAF-MAPK signaling cascade, resulting in the activation of transcription factors AP-1 and NF-κB (45, 46). This viral protein also induces the phosphorylation of IκBα, a 3–4-fold reduction in IκBα stability, and the concomitant nuclear accumulation of NF-κB DNA-binding complexes (47). In our study, we proved that the activation of the NF-κB signaling pathways during HBV infection contributed to IL-32 transcription.

MicroRNAs play important roles in various biological processes and are involved in various diseases. Bioinformatics analyses have suggested that miRNAs regulate the expression of more than 30% of all human genes. miRNAs are important mediators of HBV and HCV infection as well as liver disease progression and, therefore, could be potential therapeutic target molecules (43). In this study, we provided evidence showing that miR-29b targets the 3′-UTR of IL-32 mRNA, effectively down-regulating this gene (Fig. 3). miR-29b levels were found to be suppressed by HBV, and miR-29b and IL-32 mRNA levels were negatively correlated (Fig. 4). These findings suggest that suppressed miR-29b during HBV infection contributes to the elevated expression of IL-32.

Some evidence has shown that the secreted γ-isof orm of IL-32 exhibits antiviral properties during viral infection. For example, IL-32γ inhibited herpes simplex virus 2 and vesicular stomatitis virus by activating the IFN antiviral pathways, PKR-eIF-2α and MxA; however, specific blockade of type I IFN activity did not effectively inhibit IL-32-mediated antiviral function (10). In our study, IL-32γ did not directly affect HBV replication in HepG2.2.15 cells, which was consistent with previous HCV studies (9). However, the supernatants from rhIL-32γ-treated PBMCs showed extensive antiviral activity. Our results showed that IL-32 selectively stimulated IFN-λ1, but not IFN-α, expression in PBMCs through an IRF-independent mechanism. IL-32 inhibited HBV replication through the induction of IFN-λ1, and this antiviral effect was also effective against HBV, HCV, EV71, and HIV.

IFN-λ1 was shown to effectively block the replication of multiple human viruses with efficiency similar to that of IFN-α/β but does not require the expression of IFN-α/β or IFN-γ (13). Neutralization with IFN-λ1 Abs, but not IFN-α Abs, abolished the antiviral activity of the supernatants from IL-32γ-treated PBMCs (Fig. 7). Therefore, IFN-λ1 induced by IL-32γ appears to mediate the antiviral activities. The stimulation of IFN-λ1 expression likely plays a principal role in the antiviral mechanisms of IL-32. IL-32 is known to induce expression of TNF-α, which also has antiviral activity (6). A clear difference between the antiviral roles of these two cytokines is that TNF-α
enhances virus-induced expression of antiviral cytokines, but it cannot activate their expression without viral infection (48). In contrast, IL-32 selectively induces IFN-α expression in PBMCs, and this induction is independent of viral infection (Fig. 7, A–D).

Like the type I IFNs, IFN-α1 expression is regulated by viral infection. The induction of the IFN-β gene in response to viral infection requires the coordinated activation of the transcription factors NF-κB, AP-1, and IRF3/7 (49). A study indicates that the proximal promoter region of the IFN-α1 regulatory region is not sufficient for maximal gene induction. Remarkably, they found that these sites bind efficiently to NF-κB and function independently of the IRF3/7 binding sites. This group concluded that IFN-α1 gene expression requires NF-κB and proposed a model for IFN-α1 gene regulation in which IRF and NF-κB activate gene expression independently through spatially separated promoter elements (44). In our study, IL-32γ activates NF-κB but not IRF3/7 (Fig. 8) and stimulates IFN-α1 transcription in PBMCs. Furthermore, an essential cluster of homotypic NF-κB binding sites on the upstream region was identified in this study.

The antiviral activity of IL-32γ relies on the inducible expression of IFN-α1, and the process appears to be cell type-dependent. Like many cytokines, IL-32γ induced IFN-α1 expression in immune cells (Jurkat and THP-1) but not in hepatoma cells (HepG2 and HuH7) (Fig. 7D). This may explain why viral replication was not altered by IL-32γ in a hepatoma cell line. It is supposed that an unidentified receptor of IL-32 or a certain signaling pathway responsive to IL-32 exists in Jurkat cells, THP-1 cells, and PBMCs but not in HepG2 and HuH7 cells, leading to the diversity of IFN-α1 induction. However, IFN-α1 expression is induced in liver tissue with chronic hepatitis B (Fig. 9A), and the mRNA levels are positively correlated with IL-32 expression in HBV-infected liver cells (Fig. 9E). These results imply that IL-32 may contribute to IFN-α1 expression in hepatocytes in vivo.

Here, we demonstrate that HBV induces IL-32 expression via suppressing miR-29b and activating the IL-32 promoter. Furthermore, IFN-α1 is induced by IL-32γ in PBMCs, leading to profound antiviral activity. Human IL-32γ also efficiently inhibits HBV replication in an animal model (Fig. 6). Together, our findings suggest that inducible IL-32 exerts extensive antiviral function via selective stimulation of IFN-α1.

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