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Hepatitis B Virus Induces a Novel Inflammation Network Involving Three Inflammatory Factors, IL-29, IL-8, and Cyclooxygenase-2

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Chronic inflammation induced by hepatitis B virus (HBV) is a major causative factor associated with the development of cirrhosis and hepatocellular carcinoma. In this study, we investigated the roles of three inflammatory factors, IL-8, IL-29 (or IFN-A1), and cyclooxygenase-2 (COX-2), in HBV infection. We showed that the expression of IL-29, IL-8, and COX-2 genes was enhanced in HBV-infected patients or in HBV-expressing cells. In HBV-transfected human lymphocytes and hepatocytes, IL-29 activates the production of IL-8, which in turn enhances the expression of COX-2. In addition, COX-2 decreases the production of IL-8, which in turn attenuates the expression of IL-29. Thus, we proposed that HBV infection induces a novel inflammation cytokine network involving three inflammatory factors that regulate each other in the order IL-29/IL-8/COX-2, which involves positive regulation and negative feedback. In addition, we also demonstrated that COX-2 expression activated by IL-8 was mediated through CREB and C/EBP, which maintains the inflammatory environment associated with HBV infection. Finally, we showed that the ERK and the JNK signaling pathways were cooperatively involved in the regulation of COX-2. We also demonstrated that IL-29 inhibits HBV replication and that IL-8 attenuates the expression of IL-10R2 and the anti-HBV activity of IL-29, which favors the establishment of persistent viral infection. These new findings provide insights for our understanding of the mechanism by which inflammatory factors regulate each other in response to HBV infection.


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Abbreviations used in this article: ATF4, activating transcription factor 4; COX, cyclooxygenase; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; CRE, cAMP response element; DC, dendritic cell; HBV, hepatitis B virus; HBx, X protein of HBV; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN-A1, IFN regulatory factor 3; IFN-A2, IFN regulatory factor 7; ISRE, IFN stimulation response element; LIR, liver-enriched inhibitory protein; mCREB, mutant of CREB; 2′,5′-OAS, 2′-5′-oligoadenylate synthetase; PKR, dsRNA-activated protein kinase; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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COX-1 is constitutively expressed in almost all human tissues (15, 16). COX-2 is the inducible form of the enzyme, and its expression is significantly activated by inflammatory stimuli, resulting in increasing synthesis of prostanooids in inflamed tissues. Accumulated evidence has shown that viral proteins can stimulate COX-2 expression; for example, latent membrane protein 1 of the EBV (17), core and NS5A proteins of HCV (18), and HBx (19). COX-2 is overexpressed in liver cirrhosis, contributing to PG overproduction, which may be a major component of the inflammation and hyperdynamic circulation associated with HCC development in cirrhosis (20). Moreover, COX-2 and its downstream PG receptor EP1-mediated signaling pathway accelerate LPS-induced liver injury (21). Both IL-8 and COX-2 are stimulated by HBV proteins and associated with inflammatory processes. We need to elucidate whether there is a connection between these two proteins in the host inflammatory response to HBV infection.

IL-29 belongs to the IFN-λ gene family and is composed of three distinct genes: IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B) (22, 23). The IFN-α receptor complex consists of the unique ligand-binding chain IFN-αR, also known as IL-28R, and the accessory receptor chain IL-10R2. Although almost any cell type is able to express IFN-α after viral infection, dendritic cells appear to be major producers of IFN-α (24). IFN-αRs are expressed at variable levels in most cell types. Furthermore, IFN-α was shown to inhibit the replication of a number of viruses, including vesicular stomatitis virus, encephalomyocarditis virus, HCV, and HBV (25). Previous investigations have demonstrated elevated serum levels of IL-8 in patients with HCV infection and partial inhibition of IL-8 on the antiviral actions of IFN-α in vitro (26). However, the effect of IL-8 on the antiviral activity of IFN-α is still unclear.

Our previous studies have shown that influenza A virus infection activates IL-23, inducible NO synthase, and COX-2 expression by a complex mechanism, in which the three proximal inflammatory factors regulate each other. This prompted us to investigate the inflammatory cytokine network induced by viral infection and aroused our curiosity regarding the situation of HBV infection (27, 28). Because IL-8 is a key mediator in liver inflammation associated with HBV infection, the aim of this study was to investigate the role of IL-8 in the inflammatory response to HBV infection and identify the related molecular mechanism. In this study, we demonstrated that expression levels of IL-29, IL-8, and COX-2 were elevated in HBV patients and HBV-transfected hepatoma cells. In addition, we revealed a temporal correlation between HBV protein levels and IL-29, IL-8, and COX-2 levels. Because IL-29, IL-8, and COX-2 are all involved in regulation of inflammation, the question arises whether they are independent or not in the host inflammatory response to viral infection. Our results show that HBV infection activates the expression of IL-29, IL-8, and COX-2 by an unrecognized mechanism, in which these inflammatory factors regulate each other in the order IL-29/IL-8/COX-2 with positive regulation and negative feedback. Furthermore, we investigated the molecular mechanism that underlines the effects of IL-8 on the regulation of IL-29 and COX-2, respectively.

Materials and Methods

Clinical samples

Peripheral blood samples were obtained from 20 patients (13 males and 7 females with mean age 40.3 ± 12.7 y) with chronic hepatitis B admitted to RenMin Hospital (Wuhan University). All patients were confirmed HBV positive (but negative for HCV, HDV, and HIV), not suffering from any concomitant illness, and did not show any serological markers suggestive of autoimmune disease. To match for sex and age, 20 healthy individuals (16 males and 4 females with mean age 42.8 ± 11.5 y) with no history of liver disease were randomly selected as controls from the local blood donation center. The collection of blood samples for research was approved by the Institutional Review Board of the College of Life Sciences, Wuhan University, in accordance with guidelines for the protection of human subjects. Written informed consent was obtained from each participant.

Isolation of PBMCs

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

Generation of human immature dendritic cells

Monocytes were isolated from PBMCs by adhesion to plastic dishes for >2 h at 37°C as previously described (29). Immature dendritic cells (DCs) were generated from monocytes by culturing in RPMI 1640 medium containing 10% FBS, 1000 U/ml GM-CSF, 500 U/ml IL-4 (R&D Systems, Minneapolis, MN), and antibiotics for 7 d. Medium was changed 2, 4, and 6 d after culturing.

Reagents

Kinase inhibitors NS398, U0126, SB203580, SP600125, and GF109203 were purchased from Sigma Chemical Company (St. Louis, MO). All protein kinase inhibitors were dissolved in DMSO and used at a final concentration of 10 μM for NS398, U0126, and SB203580, 30 μM for SP600125, and 50 μM for GF109203. Escherichia coli LPS (No. L-2880; Sigma) dissolved in PBS was added at a final concentration of 1 μg/ml. Recombinant human IL-29 was purchased from eBioscience (San Diego, CA), and recombinant human IL-8 was purchased from R&D Systems. Ab against COX-2 was purchased from Cayman Chemical (Ann Arbor, MI). Abs specific for β-actin, ERK, phospho-ERK, phospho-JNK, IFN regulatory factor 3 (IRF3), IFN regulatory factor 7 (IRF7), IL-10R2, dsRNA-activated protein kinase (PKR), 2′,5′-oligoadenylate synthetase (2′,5′OAS), CREB, and C/EBP were purchased from Santa Cruz Bio-technology (Santa Cruz, CA). Ab specific for JNK was purchased from Cell Signaling Technology (Beverly, MA). Ab against IL-29 was obtained from R&D Systems.

Cell culture

The human hepatoma cell lines HepG2 and Huh7 were grown in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate at 37°C with 5% carbon dioxide. The HepG2.2.15 cell line was derived from HepG2 cells and stably expresses HBV (ayw) and was maintained in DMEM containing 400 μg/ml G418.

Plasmid construction

HBV-1.2, a plasmid carrying a greater-than-unit-length (129%) HBV genome (payw1.2; subtype ayw) and its control vector were described previously (30, 31) and were obtained from Dr. Robert Schneider (New York University Medical Center). The IFN stimulation response element (ISRE) luciferase reporter plasmid was a gift from Dr. Hongbing Shu (Wuhan University). The promoter regions of human IL-29 and IL-8 were amplified by genomic PCR: IL-29 sense, 5′-CGACGCCGCTCAATCCAAGATGATGAATGATGGCTGTCGTG-3′; IL-29 antisense, 5′-TAGTTAGCGCACTAGATAGATGAGAG-3′; IL-8 sense, 5′-ATCTTCAGGCGCTTCGTCGTCTGAA-3′; IL-8 antisense, 5′-ATAGTGAAACCCAGGCACCATTATTATT-3′. The PCR fragment was inserted into the MluI-KpnI and KpnI-XhoI sites of the luciferase vector [pGL3-IL-29(-1534/+38)-Luc and pGL3-IL-8(-1534–27)-Luc, respectively]. The luciferase reporter vector (pGL3) containing a COX-2 promoter region (~891/+9) and its site-specific mutants were reported previously (32). pdDNA-CREB–1–dominant negative mutant of CREB (mCREB) expresses CREB–1 dominant-negative mutant (S133A), as described previously (33, 34). Construction of the plasmid expressing the mutant of C/EBPβ (liver-enriched inhibitory protein; LIP) was described previously (35). Mutants of ERK1 and ERK2 were gifts from Dr. Michael P. Cobb (University of Texas Southwestern Medical Center, Dallas, TX), and mutants of JNK were gifts from Dr. Michael Karin (University of California at San Diego, San Diego, CA). The plasmid expressing the IL-29 gene, pEF-SPFL-IL-29, and its vector plasmid pEF-SPFL were obtained from Dr. Sergei V. Kotenko (University of Medicine and Dentistry of New Jersey). An IL-8 vector was constructed by RT-PCR amplification of the open reading frame of human hepatoma cells. To create the IL-8 encoding vector, the IL-8 gene was amplified using the primers IL-8 sense 5′-TTTGAATCATGACTCTGCAAGGCTGCGTGCT-3′ and IL-8 antisense 5′-GGCTCGAGTTATGATTACTCTGAGCCCTCTTCAAA-3′, in which...
BamHI and XhoI sites were introduced, respectively. The PCR product was cloned into BamHI and XhoI sites of pCMV-tag2B to generate the plasmid pCMV-tag2B-IL-8, in which IL-8 was tagged with FLAG. The resulting construct was confirmed by DNA sequencing. CREB, activating transcription factor 4 (ATF4), CREBα, and CREBβ short hairpin RNA (shRNA) plasmids were constructed by ligating the corresponding pairs of oligonucleotides (Invitrogen) to pSilencerTM 2.1- U6 neo (Ambion, Austin, TX). The sequences of 64 nt primers were as follows: CREB sense, 5'-GATCGAGTTCAAGAGACTCGTAGTAGAAGTTGGCCTTTTTTGGAAA-3', CREB antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3'; CREBα sense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3', CREBβ antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3'; CREBα antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3', CREBβ antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3'; CREBβ antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3', CREBβ antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3'.

IL-8 small interfering RNA (siRNA) and negative control were synthesized by Ribo Biotech (GuangZhou Ribo Biotech) based on the sequence described previously (36). IL-29 shRNA, its negative control (shRNA-control) and positive control (shRNA-GAPDH) were from GenPharma (Shanghai GenePharma) and prepared by ligation of the corresponding pairs of oligonucleotides to PGPU6/GFP/Neo. The shRNA-IL-29 target sequence was 5'-GCCACATTGGCAGGTTCAAAT-3'; CREBβ antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3'; CREBβ antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3'; CREBβ antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3'.

Luciferase assay
HepG2 cells were cotransfected with reporter plasmids and their corresponding expression plasmids. Cells were lysed with luciferase cell culture lysis reagent (Promega) and random primers (Invitrogen, Carlsbad, CA); it was treated with DNase I and reverse transcribed with MLV reverse transcriptase (Promega).反转录产物用DNA模板，和RNase-free水到完成的20体积。Real-time PCR包括10mL DNA浓稀释液，1mL的SYBR Green PCR master mix, 1mL of DNA diluted template, and RNA-free water to complete the 20 mL volume. Real-time primers were as follows: IL-29 sense, 5'-TACGAA-3'; IL-8 antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3'; CREBβ antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3'; CREBβ antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3'; CREBβ antisense, 5'-AGCTTTTCCAAAAGGGTGACGATATTCAAGAGATACACTATCCATTCTACGCTTTTCTTTTTTTGGAAA-3'.

Assay for HBV protein expression
Forty-eight hours postinfection, levels of HBsAg and HBeAg proteins in cell culture media were determined by ELISA using an HBV HBsAg Ag diagnostic kit (Shanghai KeHua Biotech), respectively.

Analysis of HBV DNA by real-time PCR
Capsid-associated DNA was extracted as described previously, with modifications. Equivalent amounts of HepG2 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 mL 1 M MgCl2 and 10 mL DNase (10 mg/mL) were added and incubated for 2 h at 37°C. Viral cores were then precipitated by adding 35 mL (0.5 M) EDTA and 225 mL 35% polyethylene glycol and incubating them at 4°C for at least 30 min, after which the cores were concentrated by centrifugation and the pellet was resuspended in 10 mL Tris, 100 mM NaCl, 1 mM EDTA, 1% SDS, and 20 mL proteinase K (25 mg/ml) and incubated overnight. Viral DNA released from lysed cores was extracted with phenol and chloroform, precipitated with isopropanol, and resuspended in Tris-EDTA.

Resuspended capsid-associated HBV DNAs were quantified by real-time PCR as described by the manufacturer (PG Biotech, Shenzhen, China).

Primers used in real-time PCR were as follows: P1, 5'-ATCTTGGCTGTACCACTGTTCTAC-3'; P2, 5'-ACATGGGGAAAAAGCTTGCAGA-3'. The probe was 5'-TGGCGATTTGACTGAGGTTTGG-3'. The primer set was designed by a Roche LC480.
Nuclear extraction

Cells were incubated in serum-free media for 24h, washed twice with cold PBS, and scraped into 1 ml cold PBS. Cells were harvested by centrifugation (15 s) and incubated 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 EDTA, and 200 mM sucrose) for 5 min at 4°C with flipping of the tube. The crude nuclei were collected by centrifugation (30 min); pellets were rinsed with buffer A, resuspended in one packed cell volume of 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 shaking platform for 30 min at 4°C. Nuclei were centrifuged (5 min), and 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). Cocktail protease inhibitor tablets were added to each type of buffer. Nuclear extracts were snap-frozen in liquid nitrogen and stored at −70°C until use.

Western blot analysis

Whole-cell lysates were prepared by lysing cells with PBS, pH 7.4, containing 0.01% Triton X-100, 0.01% EDTA, and 10% mixture protease inhibitor (Roche). Protein concentration was determined by the Bradford assay (Bio-Rad). Cultured cell lysates (100 μg) were electrophoresed in 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Amersham). Nonspecific sites were blocked with 5% nonfat dried milk before being incubated with an Ab used in this study. Protein bands were detected using SuperSignal Chemiluminescent (Pierce, Rockford, IL).

Statistical analysis

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

Results

IL-29, IL-8, and COX-2 expression was upregulated in patients with HBV infection

To determine the effects of HBV infection on the expression of inflammatory factors, we measured and compared IL-29, IL-8, and COX-2 mRNA levels in PBMCs of 20 patients with chronic hepatitis B and 20 healthy individuals by real-time PCR. Results showed that IL-29 mRNA levels were significantly higher in patients with chronic hepatitis B than those in healthy individuals (means ± SEM: 0.9405 ± 0.43984 versus 2.06397 ± 0.96463, p < 0.01) (Fig. 1A). IL-8 mRNA levels were also much higher in patients than in healthy individuals (means ± SEM: 0.9405 ± 0.43984 versus 2.06397 ± 0.96463, p < 0.01) (Fig. 1B). COX-2 mRNA levels were higher in patients than in healthy individuals (means ± SEM: 2.045 ± 0.6266 versus 4.414 ± 2.87483, p < 0.001) (Fig. 1C). These results suggested that HBV infection may result in the upregulation of IL-29, IL-8, and COX-2 expression.

HBV activates the expression of IL-29, IL-8, and COX-2 and the production of PGE₂

To verify the above results from clinical analysis, we further determined and compared IL-29, IL-8, COX-2, and PGE₂ levels in human hepatoma HepG2.2.15 cells that carry an integrated HBV genome and human hepatoma HepG2 cells without the HBV genome. Results from real-time PCR analyses showed that relative mRNA levels of IL-29, IL-8, and COX-2 were much higher in HepG2.2.15 cells than those in HepG2 cells (Fig. 2A). The production of IL-29, IL-8, and PGE₂ was also upregulated in culture supernatants of HepG2.2.15 cells compared with that in culture supernatants of HepG2 cells.
supernatants of HepG2 cells (Fig. 2B). Western blot analyses also showed that COX-2 protein level was increased in HepG2.2.15 cells (Fig. 2C).

To determine whether HBV directly activates the expression of IL-29, IL-8, and COX-2, we performed an additional experiment. HepG2 cells and Huh7 cells were cotransfected with plasmid pHBV-1.2 (which carries 1.2-fold length of the HBV genome and has the ability to produce mature HBV virions) or its parental plasmid pRL-TK without the HBV genome, along with the reporter plasmids pGL3-IL-29-Luc, pGL3-IL-8-Luc, or pGL3-COX-2-Luc, respectively. Results from luciferase activity analyses indicated that IL-29, IL-8, and COX-2 promoter activities were stimulated in the presence of HBV in both hepatoma cells, HepG2 (Fig. 2D, left panel) and Huh7 (Fig. 2D, right panel).

The effects of HBV infection on the expression of IL-29, IL-8, and COX-2 proteins or the production of PGE2 were further assessed. HepG2 and Huh7 cells were transfected with pHBV-1.2. The culture supernatants and the cell pellets of transfected cells were harvested at 12, 24, 36, 48, and 72 h posttransfection. The production of IL-29, IL-8, and PGE2 was measured in culture supernatants. Results showed that the level of IL-29 protein increased as transfection time increased and reached the peak at 12 h posttransfection in HepG2 cells (Fig. 2E, left panel) and at 24 h posttransfection in Huh7 cells (Fig. 2E, right panel). The level of IL-8 protein increased as transfection time increased and reached a peak at 36 h posttransfection in HepG2 cells (Fig. 2E, left panel) and at 48 h posttransfection in Huh7 cells (Fig. 2E, right panel).

The levels of PGE2 were accumulated as transfection time increased in both cells (Fig. 2E). In addition, the expression of COX-2 protein was examined in cell lysates by Western blot analyses, which showed that COX-2 protein levels increased as the transfection time increased and reached in both HepG2 (Fig. 2F, left panel) and Huh7 (Fig. 2F, right panel) cells. These results suggested that HBV activates IL-29, IL-8, and COX-2 expression and PGE2 production.

**FIGURE 3.** Determination of the role of IL-29 in the regulation of IL-8 expression in blood monocyte-derived DCs and hepatoma cells. A and B, DCs were generated from monocytes, which were isolated from PBMCs, and then incubated with recombinant human IL-29 protein at different concentrations as indicated for 24 h. Cell lysates and culture supernatants were prepared and collected. IL-8 mRNA (A) and protein (B) were detected by real-time PCR and ELISA, respectively. C and D, HepG2 cells (left panels) or Huh7 cells (right panels) were incubated with recombinant human IL-29 protein at different concentrations as indicated for 24 h. Cell lysates and culture supernatants were prepared and collected. IL-8 mRNA (C) and protein (D) were detected by real-time PCR and ELISA, respectively. E, HepG2 cells (left panel) or Huh7 cells (right panel) were cotransfected with reporter pGL3-IL-8-Luc and pEF-SPL-IL-29 or pEF-SPLFL. IL-8 promoter activity was determined by luciferase activity assay at 48 h posttransfection. Data shown are mean ± SE; n = 3. *p < 0.05. F, HepG2 or Huh7 cells were transfected with pEF-SPL-IL-29 at different concentrations as indicated. IL-8 protein (left and middle panels) and IL-29 protein (right panel) in culture supernatants were detected by ELISA at 48 h posttransfection, respectively. Data shown are mean ± SE; n = 3. *p < 0.05.
concentrations for 24 h. Results from real-time PCR analyses showed that relative IL-8 mRNA levels were increased as the concentrations of IL-29 increased in both HepG2 cells (Fig. 3C, left panel) and Huh7 cells (Fig. 3C, right panel). In an add-on, ELISA results indicated that IL-8 protein levels were increased as the concentrations of IL-29 increased in both HepG2 cells (Fig. 3D, left panel) and Huh7 cells (Fig. 3D, right panel).

To confirm further the effect of IL-29 on IL-8 expression, we tested the role of IL-29 in the regulation of IL-8 gene promoter activity and protein expression in human hepatoma cells. HepG2 and Huh7 cells were cotransfected with the reporter plasmid pGL3-IL-8-Luc and pEF-SPLF-IL-29 or its control pEF-SPLF. Results showed that IL-8 promoter was activated in the presence of IL-29 in HepG2 cells (Fig. 3E, left panel) and Huh7 cells (Fig. 3E, right panel). The effect of IL-29 on the expression of IL-8 protein was then investigated in cells transfected with pEF-SPLF-IL-29 at different concentrations. ELISA results revealed that IL-8 protein levels were increased as IL-29 concentrations increased in the supernatants of transfected HepG2 and Huh7 cells (Fig. 3F).

**HBV activates IL-8 expression through IL-29**

The effect of IL-29 on the expression of IL-8 in hepatoma cells was further evaluated using an additional approach, RNA interference (RNAi). Cells were transfected with shRNA-control, shRNA-GAPDH, and shRNA-IL-29, respectively. ELISA results showed that IL-29 protein released in the supernatants of transfected cells was reduced by treatment with shRNA-IL-29 but not affected by shRNA-control or shRNA-GAPDH (Fig. 4A, top panel), indicating shRNA-IL-29 was specific and effective. In addition, we showed that GAPDH protein level was decreased in the presence of shRNA-GAPDH (Fig. 4A, bottom panel).

The effects of shRNA-IL-29 on the activity of IL-8 gene promoter and the expression of IL-8 protein in HBV-transfected HepG2 and Huh7 cells were further investigated. Cells were cotransfected with pHBV-1.2, pGL3-IL-8-Luc, and shRNA-IL-29 or shRNA-control. Luciferase activity assays showed that IL-8 promoter activity was decreased in HepG2 cells (Fig. 4B, left panel) and Huh7 cells (Fig. 4B, right panel) in the presence of shRNA-IL-29. ELISA results indicated IL-8 protein level was also reduced in the presence of shRNA-IL-29 in both HepG2 and Huh7 cells (Fig. 4C). These results suggested that activation of IL-8 regulated by HBV may require IL-29.

**IL-8 inhibits IL-29 expression during HBV infection**

We also investigated the role of IL-8 in the regulation of IL-29 expression mediated by HBV. HepG2 and Huh7 cells were treated with recombinant human IL-8 protein at different concentrations. Results from RT-PCR showed that IL-29 mRNA levels were reduced as the concentrations of IL-8 increased in both HepG2 cells (Fig. 5A, left panel) and in Huh7 cells (Fig. 5A, right panel). Similarly, IL-29 protein levels were also decreased when the concentrations of IL-8 protein increased in both cell types (Fig. 5B). These results suggest that IL-8 represses the expression of IL-29 mRNA and protein.

To determine the effect of IL-8 on the activity of IL-29 promoter in the presence of HBV, HepG2 and Huh7 cells were cotransfected with pCMV-tag2B-IL-8 at different concentrations, pHBV-1.2, and pGL3-IL-29-Luc. Results from the luciferase activity assay demonstrated that the activity of IL-29 promoter was decreased in both HepG2 cells (Fig. 5C, left panel) and Huh7 cells (Fig. 5C, middle panel) as the concentration of IL-8 increased (Fig. 5C, right panel). Similarly, the effect of IL-8 on the expression

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**FIGURE 4.** Analysis of the role of IL-29 in the regulation of IL-8 expression mediated by HBV. A. Determination of the effectiveness and specificity of shRNA-IL-29 on IL-8 gene expression. HepG2 cells were transfected with shRNA-control, shRNA-GAPDH, and shRNA-IL-29, respectively. IL-8 protein in culture supernatants was detected by ELISA (top panel). GAPDH in cell lysates was detected by Western blot (bottom panel). B. HepG2 cells (left panel) or Huh7 cells (right panel) were cotransfected with pGL3-IL-8-Luc, shRNA-IL-29, or shRNA-control, and pHBV-1.2. Luciferase activity was measured at 48 h posttransfection. Data shown are mean ± SE; n = 3, *p < 0.05. C. HepG2 cells (left panel) or Huh7 cells (right panel) were transfected with shRNA-IL-29 at different concentrations as indicated, and pHBV-1.2. IL-8 protein released in the culture supernatants was measured by ELISA at 48 h posttransfection. Data shown are mean ± SE; n = 3, *p < 0.05.
of IL-29 protein regulated by HBV was also determined. HepG2 and Huh7 cells were cotransfected with pCMV-tag2B-IL-8 at different concentrations and pHBV-1.2. Results from Western blot analysis showed that IL-29 protein in cell supernatants was detected by ELISA. C, HepG2 and Huh7 cells were cotransfected with pCMV-tag2B-IL-8 at different concentrations as indicated, pGL3-IL-29-Luc, and pHBV-1.2. Luciferase activity was measured at 48 h postinfection in HepG2 cells (left panel) and Huh7 cells (middle panel). IL-8 protein was determined by ELISA (right panel). D, HepG2 cells and Huh7 cells were cotransfected with pCMV-tag2B-IL-8 at different concentrations and pHBV-1.2. Forty-eight hours posttransfection, IL-29 proteins were detected by Western blot for HepG2 cells (left panel) or by ELISA for Huh7 cells (right panel). E, HepG2 cells were transfected with shRNA-control, shRNA-GAPDH, siRNA-control, or siRNA-IL-8. IL-8 protein in culture supernatants was detected by ELISA (top panel). GAPDH in cell lysates was detected by Western blot (bottom panel). F, HepG2 cells (left panel) and Huh7 cells (right panel) were cotransfected with pHBV-1.2 and siRNA-IL-8 or siRNA-control. The level of IL-29 mRNA was examined by real-time PCR. G, HepG2 and Huh7 cells were cotransfected with pHBV-1.2 and siRNA-IL-8 or siRNA-control. The level of IL-29 protein was detected by Western blot for HepG2 cells (left panel) or by ELISA for Huh7 cells (right panel). Data shown are mean ± SE; n = 3. *p < 0.05.

FIGURE 5. Determination of the effect of IL-8 on the regulation of IL-29 expression mediated by HBV. A, HepG2 cells (left panel) and Huh7 cells (right panel) were incubated with recombinant human IL-8 protein at different concentrations as indicated for 24 h. IL-29 mRNA in cell lysates was detected by real-time PCR. B, HepG2 cells (left panel) and Huh7 cells (right panel) were incubated with recombinant human IL-8 protein at different concentrations as indicated for 24 h. IL-29 protein in cell supernatants was detected by ELISA. C, HepG2 and Huh7 cells were cotransfected with pCMV-tag2B-IL-8 at different concentrations as indicated, pGL3-IL-29-Luc, and pHBV-1.2. Luciferase activity was measured at 48 h postinfection in HepG2 cells (left panel) and Huh7 cells (middle panel). IL-8 protein was determined by ELISA (right panel). D, HepG2 cells and Huh7 cells were cotransfected with pCMV-tag2B-IL-8 at different concentrations and pHBV-1.2. Forty-eight hours posttransfection, IL-29 proteins were detected by Western blot for HepG2 cells (left panel) or by ELISA for Huh7 cells (right panel). E, HepG2 cells were transfected with shRNA-control, shRNA-GAPDH, siRNA-control, or siRNA-IL-8. IL-8 protein in culture supernatants was detected by ELISA (top panel). GAPDH in cell lysates was detected by Western blot (bottom panel). F, HepG2 cells (left panel) and Huh7 cells (right panel) were cotransfected with pHBV-1.2 and siRNA-IL-8 or siRNA-control. The level of IL-29 mRNA was examined by real-time PCR. G, HepG2 and Huh7 cells were cotransfected with pHBV-1.2 and siRNA-IL-8 or siRNA-control. The level of IL-29 protein was detected by Western blot for HepG2 cells (left panel) or by ELISA for Huh7 cells (right panel). Data shown are mean ± SE; n = 3. *p < 0.05.
cells (Fig. 5F, right panel). Western blot results showed that IL-29 protein was stimulated by treatment with siRNA-IL-8 in HepG2 cells (Fig. 5G, left panel), and ELISA results indicated that IL-29 protein was increased in Huh7 cells treated with siRNA-IL-8 (Fig. 5G, right panel). These results indicate that IL-8 is able to inhibit the expression of IL-29 and demonstrate that IL-8 has an inhibitory effect on the expression of IL-29 protein.

**IL-8 stimulates COX-2 expression in human PBMCs and hepatoma cells**

The above results demonstrated that HBV stimulates the expression of IL-8 and COX-2 proteins. The next question we asked is whether the two proteins interact with each other or act as independent effectors in the progression of chronic inflammation induced by HBV infection. Previous study showed that IL-8 enhances amyloid-β (Aβ1–42)-induced expression of COX-2 in cultured human microglia (40). In this study, we investigated the role of IL-8 in COX-2 expression in human PBMCs and hepatoma cells.

Human PBMCs were treated with recombinant human IL-8 protein for 24 h, and COX-2 mRNA and protein levels were determined. Results from real-time PCR indicated that COX-2 mRNA level was increased as the concentrations of IL-8 increased (Fig. 6A). Western blot analysis showed that COX-2 protein expression was stimulated by IL-8 in a dose-dependent manner (Fig. 6B). The production of PGE₂ was also enhanced by treatment with IL-8 in a concentration-dependent fashion (Fig. 6C).

In addition, HepG2 cells and Huh7 cells were also treated with recombinant human IL-8 protein for 24 h. Similar results were obtained from the treated hepatoma cells. In treated HepG2 cells, the levels of COX-2 mRNA (Fig. 6D, left panel), COX-2 protein (Fig. 6E, left panel), and PGE₂ (Fig. 6F, left panel) were stimulated by IL-8 in a dose-dependent manner. In treated Huh7 cells, the levels of COX-2 mRNA (Fig. 6D, right panel), COX-2 protein (Fig. 6E, right panel), and PGE₂ (Fig. 6F, right panel) were enhanced by IL-8 in a concentration-dependent fashion.

To confirm the effect of IL-8 on COX-2 expression, we further investigated the role of pCMV-tag2B-IL-8 in the activation of the COX-2 gene promoter activity, mRNA expression, and protein production in HepG2 and Huh7 cells. Cells were cotransfected with pGL3-COX-2-Luc, and pCMV-tag2B-IL-8 or pCMV-tag2B. Results of luciferase activity assay, semiquantitative RT-PCR, and Western blot showed that the levels of COX-2 promoter activity (Fig. 6G), mRNA expression (Fig. 6H), and protein production (Fig. 6I) were enhanced in cells transfected with pCMV-tag2B-IL-8. These results demonstrate that IL-8 stimulates COX-2 expression.

**IL-8 enhances COX-2 expression regulated by HBV**

To investigate the role of IL-8 in the regulation of COX-2 during HBV infection, the RNAi approach was applied. To test the effect of siRNA-IL-8 on the regulation of COX-2 expression mediated by HBV infection, HepG2 and Huh7 cells were cotransfected with pGL3-COX-2-Luc, pHBV-1.2, and siRNA-IL-8. The results demonstrated that IL-8 stimulates COX-2 expression.

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**FIGURE 6.** Determination of the effect of IL-8 on the regulation of COX-2 expression mediated by HBV. A–C, PBMCs were incubated with recombinant human IL-8 protein at different concentrations as indicated or treated with 100 ng/ml LPS as positive control for 24 h. Cell lysates and culture supernatants were prepared and collected. The levels of COX-2 mRNA (A), COX-2 protein (B), and PGE₂ (C) were detected by real-time PCR, Western blot, and ELISA, respectively. Data shown are mean ± SE; n = 3. *p < 0.05. D–F, HepG2 cells (left panels) or Huh7 cells (right panels) were incubated with recombinant human IL-8 protein at different concentrations as indicated for 24 h. Cell lysates and culture supernatants were prepared and collected. COX-2 mRNA (D), COX-2 protein (E), and PGE₂ (F) were detected by real-time PCR, Western blot, and ELISA, respectively. Data shown are mean ± SE; n = 3. *p < 0.05. G–I, HepG2 cells (left panels) and Huh7 cells (right panels) were cotransfected with pGL3-COX-2-Luc and pCMV-tag2B-IL-8 or pCMV-tag2B for 48 h. COX-2 promoter activity (G), COX-2 mRNA (H), and COX-2 protein (I) were determined by luciferase activity analysis, semiquantitative RT-PCR, and Western blot analysis, respectively. Data shown are mean ± SE; n = 3. *p < 0.05.
Luciferase activity analysis results indicated that COX-2 promoter activity was stimulated by HBV and inhibited by siRNA-IL-8 in both HepG2 cells (left panel) and Huh7 cells (right panel). In addition, the levels of COX-2 mRNA (Fig. 7B) and protein (Fig. 7C) were activated by HBV and also reduced by siRNA-IL-8 in both cell types. These results suggest that IL-8 facilitates the expression of COX-2 activated by HBV infection.

**COX-2 inhibits IL-8 expression induced by HBV**

In a previous study, we have reported that inducible NO synthase negatively regulates IL-32 expression mediated by influenza A virus.

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**FIGURE 7.** Analysis of the role of IL-8 in the regulation of COX-2 expression mediated by HBV. (A) Analysis of the effect of siRNA-IL-8 on the regulation of COX-2 expression mediated by HBV. HepG2 cells (left panel) and Huh7 cells (right panel) were cotransfected with siRNA-IL-8 or siRNA-control, pGL3-COX-2-Luc, and HBV-1.2 for 48 h. COX-2 promoter activity in transfected cells was determined by luciferase activity assays. Data shown are mean ± SE; n = 3. *p < 0.05. (B and C) HepG2 cells (left panels) and Huh7 cells (right panels) were cotransfected with siRNA-IL-8 or siRNA-control and HBV-1.2. Forty-eight hours posttransfection, total RNA extracts (B) and protein extracts (C) were prepared, and the levels of mRNA and proteins of COX-2 and β-actin were determined by semiquantitative RT-PCR and Western blot, respectively.

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**FIGURE 8.** Determination of the effect of COX-2 on the regulation of IL-8 expression mediated by HBV. A and B, PBMCs were treated with the inhibitor of COX-2, NS398, at different concentrations as indicated or treated with 100 ng/ml LPS as a positive control. Cell lysates and culture supernatants were prepared and collected 48 h posttreatment. The levels of IL-8 mRNA (A) and IL-8 protein (B) were detected by real-time PCR and ELISA, respectively. C and D, HepG2 cells (left panels) and Huh7 cells (right panels) were transfected with pHBV-1.2 for 12 h and then treated with NS398 at different concentrations as indicated. Thirty-six hours posttreatment, the mRNA levels (C) and protein levels (D) of IL-8 and β-actin were examined by semiquantitative RT-PCR and ELISA, respectively. Data shown are mean ± SE; n = 3. *p < 0.05.
virus infection (28). In this study, we speculated that there is also a feedback regulation between IL-8 and COX-2 during HBV infection. The effect of COX-2 on IL-8 expression induced by HBV was then investigated in this study. Human PBMCs were transfected with pHBV1.2 and treated with NS398 (an inhibitor of COX-2) at different concentrations for 24 h. Results from real-time PCR and ELISA showed that the levels of IL-8 mRNA (Fig. 8A) and protein (Fig. 8B) were increased in cells treated with NS398 in a concentration-dependent manner. These results suggested that COX-2 has a negative effect on IL-8 expression induced by HBV.

**FIGURE 9.** Analysis of the roles of IL-8 in the regulation of ISRE and IRF3/7. A, The role of IL-8 in the activation of ISRE. HepG2 cells were cotransfected with pCMV-tag2B-IL-8 at different concentrations and the reporter pISRE-Luc. ISRE promoter activity was measured by luciferase activity assays at 48 h posttransfection. Data shown are mean ± SE; n = 3. *p < 0.05. B–D, The effects of IL-8 and siRNA-IL-8 on the expression of IRF3. HepG2 cells were cotransfected with pCMV-tag2B-IL-8 or pCMV-tag2B and siRNA-IL-8 or siRNA-control. Forty-eight hours posttransfection, IRF3 mRNA (B) and IRF3 protein (C, D) were examined by real-time PCR and Western blot, respectively. Data shown are mean ± SE; n = 3. *p < 0.05.

**FIGURE 10.** Determination of the effect of IL-8 on IL-10R2 expression. A and B, The effect of pCMV-tag2B-IL-8 on IL-10R2 expression. HepG2 cells were transfected with pCMV-tag2B-IL-8 at different concentrations. Forty-eight hours posttransfection, the levels of IL-10R2 mRNA were examined by real-time PCR (A) and semiquantitative RT-PCR (B), respectively. C, The effect of pCMV-tag2B-IL-8 on IL-10R2 expression. HepG2 cells were transfected with pCMV-tag2B-IL-8 at different concentrations. Forty-eight hours posttransfection, the levels of IL-10R2 and β-actin proteins were examined by Western blot using Ab to IL-10R2 or β-actin, respectively. D–F, The effect of siRNA-IL-8 on IL-10R2 expression. HepG2 cells were transfected with siRNA-IL-8 or siRNA-control. Forty-eight hours posttransfection, the levels of IL-10R2 mRNA and protein were examined by real-time PCR (D), semiquantitative RT-PCR (E), and Western blot (F), respectively. Data shown are mean ± SE; n = 3. *p < 0.05.
To confirm the effect of COX-2 on IL-8 expression, HepG2 cells and Huh7 cells were also transfected with pHBV1.2 and treated with NS398 at different concentrations for 24 h. Both semi-quantitative RT-PCR (Fig. 8C) and ELISA (Fig. 8D) results showed that the levels of IL-8 mRNA and protein were enhanced by NS398 in a dose-dependent fashion in HepG2 cells (Fig. 8C, 8D, left panels) and Huh7 cells (Fig. 8C, 8D, right panels). Thus, our results demonstrate that COX-2 acts as a negative regulator of IL-8 expression during HBV infection.

**FIGURE 11.** Determination of the effects of CREB and C/EBP on the expression of COX-2 regulated by IL-8. A. Diagrams of COX-2 core promoters, which includes the wild-type COX-2 promoter (WT, -891/+9), a COX-2 promoter with a C/EBP mutated (Mut1, -124/+132), a COX-2 promoter with a CRE mutated (Mut2, -53/+59), and COX-2 promoter with a double mutant of C/EBP and CRE (Mut3, -124/+132 and -53/+59). B. The effect of IL-8 on the activation of COX-2 promoter and its mutants. HepG2 cells were cotransfected with pCMV-tag2B-IL-8 or its control and plasmids containing the luciferase reporter gene under the control of different COX-2 promoters, WT, Mut1, Mut2, and Mut3. Forty-eight hours posttransfection, COX-2 promoter activity was determined by luciferase activity assay. Data shown are mean ± SE; n = 3. *p < 0.05. C. The effects of mCREB and LIP on the activation of COX-2 promoter and its mutants. Cells were cotransfected with pCMV-tag2B-IL-8, pGL3-COX-2-Luc, and different amounts of mCREB and LIP, respectively. Forty-eight hours posttransfection, COX-2 promoter activity was determined by luciferase activity assay. Data shown are mean ± SE; n = 3. *p < 0.05. D and E. The effects of siRNA on the expression of COX-2. HepG2 cells were cotransfected with pCMV-tag2B-IL-8 along with siRNA specific to CREB, ATF4, C/EBPa, and C/EBPβ. The levels of COX-2 mRNA (D) and protein (E) were examined by semiquantitative RT-PCR and Western blot, respectively. Results represent means of three independent experiments. F. The role of IL-8 in the expression of CREB. Cells were transfected with pCMV-tag2B-IL-8 for different times as indicated. Protein extracts were prepared, and the levels of CREB protein were determined by Western blot. G. The role of IL-8 in the translocation of CREB. HepG2 cells were transfected with pCMV-tag2B-IL-8 for different times as indicated. Protein extracts were prepared from cytosol and nucleus of transfected cells. CREB protein in nucleus (top panel) and cytosol (bottom panel) was detected by Western blot using Ab to CREB. The blot is a representative of three experiments with similar results. H. The role of IL-8 in the translocation of C/EBP. HepG2 cells were transfected with pCMV-tag2B-IL-8 for different times as indicated. Protein extracts were prepared from whole cell and nucleus of transfected cells. C/EBP protein in nucleus (top panel) and whole cell (bottom panel) was detected by Western blot using Ab to C/EBP. The blot is a representative of three experiments with similar results.

IL-8 inhibits the activity of ISRE on IL-29 promoter and the expression of IRF3/7 genes

Previous studies showed that IL-29 (IFN-λ1) gene expression was mediated by spatially separated promoter elements that independently interact with IFN regulatory factor and NF-κB. Both NF-κB and IRF3/7 were required for transcriptional regulation of the IL-29 gene (41). NF-κB and IRF3/7 also bind to the NF-κB element and ISRE of the IL-29 promoter in IFN-α–primed cells (42). Additionally, we demonstrated that IL-8 inhibits IL-29
expression during HBV infection. Thus, we speculated that IL-8 may also regulate IL-29 expression through IRF3/7.

To demonstrated this speculation, HepG2 cells were cotransfected with pCMV-tag2B-IL-8 at different concentrations and with a reporter, pISRE-Luc. Results of the luciferase assay showed that ISRE activity was decreased in the presence of IL-8 in a dose-dependent manner (Fig. 9A), suggesting IL-8 plays a negative role in the regulation of ISRE activity.

To investigate the effects of IL-8 on the expression of IRF3 and IRF7, two approaches were used: overexpression of IL-8 and knockdown of IL-8. HepG2 cells were cotransfected with pCMV-tag2B-IL-8 or pCMV-tag2B and siRNA-IL-8 or siRNA-control. The effects of IL-8 on the expression of IRF3 and IRF7 mRNAs were determined by real-time PCR, which showed that the levels of IRF3 mRNA (Fig. 9B) and IRF7 mRNA (Fig. 9E) were reduced in cells transfected with pCMV-tag2B-IL-8 and increased in cells treated with siRNA-IL-8. These results suggested that IL-8 inhibits the transcription of IRF3 and IRF7 genes.

The effects of IL-8 on the expression of IRF3 and IRF7 proteins were determined by Western blot using Abs to IRF3, IRF7, or β-actin (as a control), respectively. The results showed that protein levels of IRF3 (Fig. 9C) and IRF7 (Fig. 9F) were reduced in cells transfected with pCMV-tag2B-IL-8. However, protein levels of IRF3 (Fig. 9D) and IRF7 (Fig. 9G) were increased in cells treated with siRNA-IL-8. Taken together, these results demonstrate that IL-8 inhibits the expression of IRF3 and IRF7.

**IL-8 inhibits the expression of IL-29 receptor, IL-10R2**

IL-10R2 is one of the receptor chains of IFN-λ3, which is also called “type III IFN.” It is a member of a newly identified IFN family composed of three members: IL-29, IL-28A, and IL-28B. IL-28R1 and IL-10R2 bind to the same receptor pair to activate downstream signaling pathways.

To investigate the regulatory effect of IL-8 on the expression of IL-10R2 gene, HepG2 cells were transfected with pCMV-tag2B-IL-8. Results of real-time PCR (Fig. 10A) and semiquantitative RT-PCR (Fig. 10B) and Western blot (Fig. 10C) showed that the levels of IL-10R2 mRNA and protein were decreased as the concentrations of IL-8 increased. In addition, HepG2 cells were treated with siRNA-control or siRNA-IL-8. Results of real-time PCR (Fig. 10D), semiquantitative RT-PCR (Fig. 10E), and Western blot (Fig. 10F) showed that the levels of IL-10R2 mRNA and protein were increased in the presence of siRNA-IL-8. These results demonstrate that IL-8 has an inhibitory effect on IL-10R2 expression in HepG2 cells.

**CREB and C/EBP recognition sites are required for the activation of COX-2 regulated by IL-8**

Regulation of COX-2 gene expression relies on many consensus cis-elements, including the CREB and C/EBP binding sites in the promoter (43, 44). Our above results suggested that IL-8 activates the expression of COX-2. We next investigated the roles of cis-regulatory elements in the activation of COX-2 expression regulated by IL-8 using a mutation analysis approach. A C/EBP binding site mutant (Mut1), a CAMP response element (CRE) binding site mutant (Mut2), and a C/EBP binding site plus CRE binding site double mutant (Mut3) were generated by site-specific mutagenesis. Reporter plasmids were then constructed, in which the luciferase gene was under the control of wild-type, Mut1, Mut2, and Mut3 promoter of COX-2 gene, respectively (Fig. 11A). HepG2 cells were cotransfected with pCMV-tag2B-IL-8 and each of the reporters. Results from luciferase activity analysis indicated that Mut1, Mut2, and Mut3 resulted in the reduction of COX-2.

**FIGURE 12.** Analysis of the effects of ERK and JNK signaling pathways in the expression of COX-2 regulated by IL-8. A, The effects of inhibitors of signaling components on the regulation of COX-2 promoter activity mediated by IL-8. Cells were cotransfected with pCMV-tag2B-IL-8 and pGL3-COX-2-Luc for 12 h and then treated with DMSO (control) or inhibitors of signaling components U0126, SB203580, SP600125, and GF109203 for 36 h. The cells were lysed, and COX-2 promoter activity was measured by luciferase activity assays. Data shown are mean ± SE, n = 3, *p < 0.05. B, The effects of the mutants of ERK1/2 and JNK on the activity of COX-2 promoter. HepG2 cells were cotransfected with pCMV-tag2B-IL-8 and pGL3-COX-2-Luc, and each of the three dominant-negative mutants of ERK1 (mERK1), ERK2 (mERK2), and JNK (mJNK). Forty-eight hours posttransfection, COX-2 promoter activity was determined by luciferase activity assays. Data shown are mean ± SE, n = 3, *p < 0.05. C, The effect of IL-8 on the phosphorylation of ERK. HepG2 cells were transfected with pCMV-tag2B-IL-8 or pCMV-tag2B for 48 h. Proteins were then prepared from transfected cells, and the levels of phospho-ERK1/2 and nonphospho-ERK in cell lysates were detected by Western blot using Ab to phospho-ERK (P-ERK) or ERK, respectively. The blot is a representative of three experiments with similar results. D, The effect of IL-8 on the phosphorylation of JNK. HepG2 cells were transfected with pCMV-tag2B-IL-8 or pCMV-tag2B. At 48 h posttransfection, proteins were prepared from transfected cells, and the levels of phospho-JNK and nonphospho-JNK in cell lysates were detected by Western blot using Ab to phospho-JNK (P-JNK) or JNK, respectively. The blot is a representative of three experiments with similar results.
promoter activity to the basal level (Fig. 11B). These results indicated that C/EBP and CRE recognition sites were required for activating COX-2 expression regulated by IL-8.

The roles of CREB and C/EBP in the activation of COX-2 mediated by IL-8 were also examined by introducing two dominant-negative mutants, mCREB and LIP, of CREB and C/EBP. HepG2 cells were cotransfected with pCMV-tag2B-IL-8, pGL3-COX-2-Luc, and each of the two mutants at different concentrations. Luciferase activity analysis results showed that COX-2 promoter activity was stimulated by IL-8 (Fig. 11C, lane 2 versus lane 1), but such activation was repressed by mCREB (Fig. 11C, lanes 3, 4, and 5) and LIP (Fig. 11C, lanes 6, 7, and 8) in a dose-dependent manner. These results again suggested that C/EBP and CRE recognition sites were required for regulating COX-2 mediated by IL-8.

The roles of CREB and C/EBP in the activation of COX-2 mediated by IL-8 were further determined by the RNAi approach. HepG2 cells were cotransfected with pCMV-tag2B-IL-8 and siRNA specific to CREB, ATF4, C/EBPx, and C/EBPβ, respectively. Semiquantitative RT-PCR results showed that the level of COX-2 mRNA was increased in the presence of IL-8 (Fig. 11D, lane 2 versus lane 1), and such activation was attenuated by siRNA-CREB (Fig. 11D, lane 3), siRNA-C/EBPx (Fig. 11D, lane 5), and siRNA-C/EBPβ (Fig. 11D, lane 6), but not by siRNA-ATF4 (Fig. 11D, lane 4). Western blot also showed that the level of COX-2 protein was enhanced by IL-8 (Fig. 11E, lane 2 versus lane 1). Such regulation was affected by siRNA-CREB (Fig. 11E, lane 3), siRNA-C/EBPx (Fig. 11E, lane 5), and siRNA-C/EBPβ (Fig. 11E, lane 6), but not by siRNA-ATF4 (Fig. 11E, lane 4). These results confirm that CREB and C/EBP play important roles in the activation of COX-2 regulated by IL-8.

We next investigated the effect of IL-8 on the expression of CREB protein. HepG2 cells were transfected with pCMV-tag2B-IL-8 for different times. CREB or β-actin proteins were then determined by Western blot, which showed that CREB protein expression was activated in the presence of IL-8 in a time-dependent manner, whereas the expression of β-actin protein remained unchanged (Fig. 11F). It has been demonstrated that localization to the nucleus is essential for CREB and C/EBP functions. Thus, we further examined whether IL-8 was involved in the translocation of CREB and C/EBP from cytosol to nucleus. Results showed that in the presence of IL-8, the level of CREB protein in the nucleus was gradually increased while the level of CREB protein in the cytosol remained relatively unchanged as transfection time increased (Fig. 11G). Furthermore, we observed a similar effect of IL-8 on the translocation of C/EBP from cytosol to nucleus, whereas the levels of C/EBP protein in the whole-cell lysates remained relatively unchanged (Fig. 11H). Taken together, these results demonstrated that during HBV infection, IL-8 stimulates the translocation of CREB and C/EBP from cytosol to nucleus, resulting in the activation of COX-2 expression.

IL-8 stimulates COX-2 expression through activating ERK and JNK signaling pathways

Members of the MAPK family have been shown to regulate the activities of several transcription factors that are important in the
proinflammatory response (45). Regulation of COX-2 expression depends on different kinase activators in various cell types (46, 47). Based on our findings that IL-8 activates COX-2 expression and such regulation required C/EBP and CREB, we further investigated the roles of the members of the MAPK family in the regulation of COX-2 expression mediated by IL-8. HepG2 cells were cotransfected with pcMV-tag2B-IL-8 and pGL3-COX-2-Luc and then treated with DMSO (as a control), U0126 (ERK-specific inhibitor), SB203580 (p38 MAPK-specific inhibitor), SP600125 (JNK-specific inhibitor), and GFI09203 (protein kinase C-specific inhibitor), respectively. Results of luciferase activity analysis showed that in IL-8–transfected cells, COX-2 promoter activity was increased in control cells (DMSO), decreased by the treatment of U0126, SB203580, and SP600125, but not affected by GFI09203 (Fig. 12A). These results suggested that ERK and JNK signaling pathways might be involved in the activation of COX-2 expression regulated by IL-8.

The effects of ERK and JNK signaling pathways on the activation of COX-2 induced by IL-8 were further investigated using two different approaches. First, three dominant kinase-inactive mutants, mERK1, mERK2, and mJNK, were introduced, whose expression blocks kinase activity by competing with endogenous kinases (48, 49). HepG2 cells were cotransfected with pcMV-tag2B-IL-8, pGL3-COX-2-Luc, and each of the three kinase mutants, respectively. Results showed that COX-2 promoter activity was enhanced in the presence of pcMV-tag2B-IL-8 (Fig. 12B, lane 2 versus lane 1), but such activation was reduced in the presence of mERK1 (Fig. 12B, lane 3), mERK2 (Fig. 12B, lane 4), and mJNK (Fig. 12B, lane 5). Second, the phosphorylation status of ERK1/2 and JNK was examined in HepG2 cells by Western blot analyses using Abs specific to phospho-ERK, ERK, phospho-JNK, or JNK, respectively. Results showed that both the levels of phosphorylated ERK (Fig. 12C) and phosphorylated JNK (Fig. 12D) were increased in the presence of pcMV-tag2B-IL-8, whereas ERK and JNK proteins remained relatively unchanged with or without IL-8. Taken together, these results suggested that IL-8 activates ERK and JNK signaling pathways, resulting in the stimulation of COX-2 expression.

IL-29 inhibits HBV replication, and IL-8 plays a negative role in anti-HBV activity of IL-29

It has been shown that IL-29 initially binds to IL-28R1 and causes a conformational change that subsequently allows IL-10R2 to bind to IL-29. The receptor complex is then activated and induces the expression of many IFN-stimulated genes that in turn induces an antiviral state in target cells. Protein kinase regulated by dsRNA (PKR) and 2′,5′-OAS is well known to be induced by IL-29 stimulation. Upon binding to dsRNA, 2′,5′-OAS catalyzes the formation of 2′-5′-linked oligoadenylate and activates RNase L, which breaks down viral and cellular RNA (50). PKR is also activated by dsRNA, which leads to the phosphorylation of its substrate, eukaryotic translation initiation factor 2α, inhibits the guanosine nucleotide exchange factor, eukaryotic translation initiation factor 2β, and halts viral replication (51).

Our results demonstrating that IL-8 inhibits the expression of IL-29 and IL-10R2 led us to consider that such inhibition could result in impairing the formation of the antiviral state. To assess this speculation, HepG2 cells were cotransfected with pEF-SPFL, pEF-SPFL-IL-29, or pcMV-tag2B-IL-29 plus pcMV-tag2B-IL-8. Results of real-time PCR showed that the levels of PKR mRNA (Fig. 13A) and 2′,5′-OAS mRNA (Fig. 13C) were enhanced in the presence of IL-29 but were reduced in the presence of both IL-29 and IL-8. Results of Western blot showed that the levels of both PKR protein (Fig. 13B) and 2′,5′-OAS protein (Fig. 13D) were also increased in the presence of IL-29 but reduced in the presence of both IL-29 and IL-8.

To investigate the effects of IL-29 and IL-8 on HBV protein expression and the viral DNA replication, HepG2 cells were cotransfected with pEF-SPFL-IL-29 or pEF-SPFL and pcMV-tag2B-IL-8 or pCMV-tag2B. Levels of HBV Ags, HBcAg and HBsAg, and HBV capsid-associated DNA were determined in transfected cells. Results showed that in cells transfected with pEF-SPFL-IL-29, the levels of HBcAg (Fig. 13E) and HBsAg (Fig. 13F) were reduced, and the level of HBV DNA (Fig. 13G) was significantly inhibited. In addition, results indicated that in cells transfected with both pEF-SPFL-IL-29 and pcMV-tag2B-IL-8, the levels of HBcAg (Fig. 13E) and HBsAg (Fig. 13F) were relatively unchanged, whereas the level of HBV DNA (Fig. 13G) was slightly reduced. Taken together, these results demonstrate that IL-29 inhibits HBV replication, and IL-8 plays an negative role in the anti-HBV activity of IL-29. On the basis of this study, we propose a hypothetical model (Fig. 14) according to which hepatitis B virus infection triggers production of IL-29, IL-8, and COX-2 resulting in a host inflammatory response.

Discussion

Generally, the inflammatory response is beneficial to the host. Inflammatory cytokines are normally released in response to infection as a defense mechanism, but excessive or inappropriate cytokine release can lead to clinical pathology (19). Cytokines, chemokines, and growth factors are involved in the progression of chronic hepatitis B and C (20). Under certain conditions, a number of cytokines are secreted in an autoenhancing manner, suggesting a positive feedback loop that sustains the expression of these cytokines. IL-8 is one of the most potent growth factors secreted by monocytes and macrophages and is involved in the progression of chronic hepatitis B and C (21). IL-8 activates ERK and JNK signaling pathways, resulting in the activation of COX-2 expression.

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FIGURE 14. A proposed model for the regulation of an inflammatory cytokine network mediated by HBV infection. HBV infection induces a regulatory loop of inflammatory cytokine network (I), in which three inflammatory cytokines regulate each other in the order IL-29/IL-8/COX-2, which involves positive regulation and negative feedback. IL-29 activated by HBV stimulates the expression of IL-8 (II) that in turn represses IL-29 expression (III). Moreover, IL-8 activated by HBV infection through IL-29 stimulates the ERK and JNK signaling pathways (IV) and enhances the translocation of C/EBP and C/EBP transcriptional factors from cytosol to nucleus, resulting in the activation of COX-2 expression (V) that triggers PGE2 production and finally inflammatory responses (VI). COX-2 also inhibits the production of IL-8 (VII). In addition, IL-29 activates the expression of PKR and 2′,5′-OAS (OAS) (VIII) that in turn inhibits HBV replication (IX). Also, after activation by IL-29, IL-8 negatively feeds back to inhibit the expression of IL-29 (X), resulting in the repression of PKR and 2′,5′-OAS expression and thus enhancing HBV replication.
HBV induces a novel inflammatory network

diverse kinds of cellular stimuli, including viral infection, and function to stimulate host responses aimed at controlling cellular stress and minimizing cellular damage. However, when the damage is not repaired, excessive immune cell infiltration and persistent cytokine production can be provoked. In fact, the host immune responses to hepatitis viruses are fairly weak and are unable completely to downregulate and clear the infection, resulting in chronic stimulation of the Ag-specific immune response in persistently infected patients. The continuous expression of cytokines and recruitment of activated lymphomononuclear cells to the liver affects many cellular pathways and ultimately results in fibrosis, cirrhosis, and/or HCC. Many studies have suggested that hepatitis viral proteins play direct roles in interfering with cytokine production. For example, HBx has been shown to interact directly with mediators including IL-8, ICAM-1, and the MHC factor and regulate their function in the inflammatory process. IL-8 has been shown to be associated with HBV-infected patients, and higher expression levels were observed in liver cirrhosis and HCC than in healthy individuals (52, 53). Moreover, IL-8 levels in patients with chronic hepatitis B were significantly higher than in healthy controls, and large fluctuations in IL-8 concentrations in patients’ sera associated with hepatic flares were observed. For most patients, the peak serum level of IL-8 preceded the onset of the flare of liver inflammation (the alanine transaminase peak), either simultaneous with or immediately after a sharp increase in viral load (11). Thus, IL-8 is thought to be an important inflammatory mediator, responsible for maintaining the inflammatory environment associated with HBV infection that may play a role in the pathogenesis of liver damage. We focused the current study on the role of IL-8 in the inflammatory cytokine network induced by HBV infection.

Using an RNAi approach, we recently demonstrated that IL-29 was upregulated and COX-2 was downregulated, respectively, after knocking down IL-8 in HBV-infected HepG2 or HuH7 cells. Furthermore, we demonstrated that HBV infection resulted in activation of IL-29, IL-8, and COX-2 by a heretofore unrecognized mechanism. Based on these results, we proposed a model (Fig. 14) of the activation of IL-29, IL-8, and COX-2 by HBV infection, resulting in a host inflammatory response. The model indicates that HBV infection stimulates an inflammatory cytokines network including at least three inflammatory factors. HBV stimulates the expression of IL-29 (I), IL-29 then upregulates the production of IL-8 (II), which in turn attenuates IL-29 production (III). Also, IL-8 activates ERK and JNK signaling pathways (IV) and enhances the binding of CREP and C/EBP to COX-2 promoter (V) resulting in the activation of COX-2 gene, which in turn stimulates PEG2 production and induces inflammation (VI). COX-2 also inhibits the production of IL-8 (VII). In addition, IL-29 enhances the production of PKR and 2’5’OAS (VIII), which inhibit HBV replication (IX). Moreover, IL-8 has a negative effect on the anti-HBV activity of IL-29 (X).

Although IL-8 inhibits the antiviral actions of type I IFN in vitro (8, 54), little is currently known regarding the effect of IL-8 on the antiviral response of type III IFN. Our study provides new knowledge regarding the antiviral response of type III IFN. IL-29 is able to inhibit the replication of a number of viruses, including vesicular stomatitis virus, encephalomyocarditis virus, HCV, and HBV. We further extended the antiviral activity of IL-29 on HBV in HepG2 cells and demonstrated that IL-8 attenuated the antiviral activity of IL-29. We also provided evidence that IL-29 expression was decreased by IL-8 through the inhibition of the expression of IRF3 and IRF7. IL-10R2, one of the receptor chains of IL-29, was also downregulated by IL-8 in HepG2 cells. Additionally, IL-8 was found to attenuate the antiviral responsiveness of IL-29 by impairing induction of antiviral genes (PKR and 2’5’OAS) and inhibiting HBV replication. These results indicate that HBV inhibited the antiviral effect of type III IFN by altering IL-8 levels to favor the establishment of persistent HBV infection.

IL-8, as a classic proinflammatory cytokine, also plays an important role in the inflammatory process. In the current study, we identified a novel IL-8 downstream proinflammatory factor, COX-2, in the presence of HBV infection. Although adult hepatocytes do not express COX-2 under normal conditions, the level of COX-2 in hepatocytes increases during chronic inflammatory liver diseases (20, 55). COX-2 is also overexpressed in liver cirrhosis and may contribute to HCC development. After being induced by HBV infection, IL-8 may trigger proinflammatory factor expression, including COX-2, and subsequent host inflammatory responses to maintain the inflammatory status for the development of liver damage. CREB and C/EBP have been reported to mediate COX-2 activation induced by diverse stimulating agents. We demonstrated in this study that CREB and C/EBP are also essential for IL-8–induced COX-2 expression because mutations in binding sites, negative mutants, and specific siRNA of CREB and C/EBP eliminated IL-8 function in the activation of COX-2. Based on the findings that IL-8 activates COX-2 expression and CREB and C/EBP are involved in such regulation, we further investigated the roles of different MAPKs on the activation of COX-2 regulated by IL-8. Our results demonstrated that ERK and JNK were involved in the activation of COX-2 regulated by IL-8 because inhibition of each of those kinases abolished the expression of COX-2 activated by IL-8.

This novel notion that HBV activates IL-29, IL-8, and COX-2 expression and that these three inflammatory cytokines regulate each other in the order IL-29→IL-8→COX-2, which involves positive regulation and negative feedback, expands our understanding of relevant, highly pathophysiological processes caused by HBV. IL-8 may be a key mediator in the cytokine network induced by HBV, which not only impairs the antiviral activity of IL-29 and favors the establishment of persistent viral infection but also induces the high expression of proinflammatory factor COX-2 to maintain the inflammatory environment associated with HBV infection (Fig. 14). This study may allow the rational development of immunotherapeutic strategies that enhance viral control while limiting or blocking liver inflammation.

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Disclosures
The authors have no financial conflicts of interest.

References


