Upregulation of IL-23 Expression in Patients with Chronic Hepatitis B Is Mediated by the HBx/ERK/NF-κB Pathway

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Uregulation of IL-23 Expression in Patients with Chronic Hepatitis B Is Mediated by the HBx/ERK/NF-κB Pathway

Limin Xia,*† Dean Tian,‡ Wenjie Huang,§ Hongwu Zhu,* Jing Wang,* Yongguo Zhang,* Hao Hu,* Yongzhan Nie,* Daming Fan,* and Kaichun Wu*

IL-23 is a newly discovered proinflammatory cytokine that contributes to the maintenance and expansion of Th17 cells. IL-23 has recently been identified as playing a critical role in a number of chronic inflammatory diseases. However, the regulatory mechanism of IL-23 in chronic hepatitis B (CHB) remains largely unknown. The aims of this study were to detect the expression of IL-23 in CHB patients and to explore the molecular mechanism of hepatitis B virus (HBV)-induced IL-23 expression. Serum levels and hepatic expression of IL-23 were significantly upregulated in CHB patients. A positive correlation was found between IL-23 expression and the histological activity index score, HBV DNA load, and serum alanine aminotransferase and aspartate aminotransferase levels. HBx protein increased IL-23 expression in a dose-dependent manner. It also aided in the nuclear translocation of NF-κB, which directly bound to the promoters of IL-23 subunits p19 and p40 to facilitate their transcription. NF-κB inhibitors blocked the effect of HBx on IL-23 induction, and NF-κB subunits p65 and p50 increased the augmented IL-23 expression. Inhibition of ERK1/2 activation and transfection with ERK dominant-negative plasmid significantly blocked the HBx-induced IL-23 expression. Furthermore, PI3K and Ras–MEK–MAPK inhibitors significantly decreased the ERK1/2 activation and IL-23 expression. Thus, we report a new molecular mechanism for HBV-induced IL-23 expression, which involves the activation of the ERK/NF-κB pathway by HBx, leading to the transactivation of the IL-23 p19 and p40 promoters. The Journal of Immunology, 2012, 188: 000–000.

Hepatitis B virus (HBV) infection is one of the leading causes of chronic liver disease, which affects >300 million people worldwide. Annually, HBV infection accounts for 1 million deaths worldwide due to cirrhosis, liver failure, and hepatocellular carcinoma (1, 2). Although there is no direct cytopathic effect on the hepatocytes, HBV infection induces immune cell infiltration, which leads to the formation of necroinflammatory foci and mediates the disease processes (3, 4). The recruitment and differentiation of immune cells to the inflammatory site is triggered and enhanced by proinflammatory cytokines, which play important roles in inducing liver injury (5, 6).

HBV encodes a small genome consisting of partially double-stranded, circular DNA that is encapsulated within an enveloped particle. The HBV genome encodes four overlapping open reading frames (S, C, P, and X) and expresses seven viral proteins including HBx, HBs, preS2, preS1, HBc, HBe, and HBp (2). Among these, HBx is considered one of the most important determinants of viral replication and viral-mediated pathogenesis (7, 8). The HBx protein is a small protein composed of 154 aa, and it is distributed in the cytoplasm and, to some extent, the nucleus. Distribution in the cytoplasm may activate the signal transduction cascade, but distribution in the nucleus activates specific transcription factors. HBx does not bind DNA directly but transactivates multiple transcription factors, including AP-1, AP-2, NF-κB, activating transcription factor (CREB/ATF), and HIF-1 (9–11). NF-κB has been evaluated because of its pivotal role in regulating inflammatory-related genes (12).

IL-23 is a heterodimeric cytokine belonging to the IL-12 cytokine family. It consists of two subunits: p40, which is shared with IL-12, and p19, which shares sequence homology with IL-12 p35 subunit but is distinct and leads to unique biological effects (13). IL-23 is predominantly produced by macrophages and dendritic cells (DCs) that are activated by bacterial products, the Sendai virus, and proinflammatory cytokines (14). The importance of IL-23 in inflammatory responses has been demonstrated in experimental autoimmune diseases. IL-23 selectively promotes IL-17 cytokine production by maintaining and expanding Th17 cell expansion/maintenance and promoting the production of other proinflammatory mediators, such as IL-8, IL-6, MCP-1, and G-CSF (15). IL-23 p19 knockout mice are resistant to experimental autoimmune encephalomyelitis and collagen-induced arthritis, which are associated with defects in IL-17 production (16, 17). In addition, multiorgan inflammation and premature death are observed in transgenic mice overexpressing the IL-23 p19 subunit (18). Previous studies have found that IL-12 family members, such as IL-12 and IL-27, are involved in chronic inflammation and liver damage from HBV and hepatitis C virus infections (19–22). However, the regulatory mechanism of IL-23 in chronic HBV infection remains largely unknown.

Recent studies have found that Th17 cells, which are expanded by IL-23, increase the severity of liver damage in patients with...
HBV (23–25), indicating that IL-23 may play an important role in promoting inflammatory injury in chronic hepatitis B (CHB). In this study, we evaluated the serum levels and hepatic expression of IL-23 in CHB patients and analyzed its correlation with HBV-mediated inflammatory damage. We also explored the possible molecular mechanism of HBV-induced IL-23 expression in hepatocytes.

Materials and Methods

Patients

CHB patients (n = 192) were recruited for this study. All patients were diagnosed based on described criteria (26) and were not taking antiviral therapy or immunosuppressive drugs within 6 mo of blood sampling. Individuals with concurrent hepatitis C virus, hepatitis D virus, HIV, autoimmune liver disease, or alcoholic liver disease were excluded. Sixty age-matched and sex-matched healthy individuals were enrolled as healthy controls (HCs). All patients and HCs were recruited from the Xijing Hospital at the Fourth Military Medical University (Shanxi, People’s Republic of China) between 2008 and 2009. Ethical approval was obtained from the Research Ethics Committee of Xijing Hospital, and written and informed consent was obtained from each patient. The basic characteristics of the enrolled subjects are listed in Table I.

Peripheral blood samples were collected from all enrolled subjects. Liver biopsy samples were collected from 60 CHB patients, and 16 healthy liver tissue samples were obtained from healthy donors whose livers were used

Table I. Clinical characteristics of the populations enrolled in the study

<table>
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<th>Parameter</th>
<th>HCs</th>
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<tr>
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<td>HBcAb IgM positive, n</td>
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</table>

Data are shown as median and range. ND, not determined.

FIGURE 1. Serum levels and the hepatic expression of IL-23 are increased in CHB patients. A, Serum IL-23 levels in CHB patients (n = 192) and HCs (n = 60) were analyzed by ELISA. B and C, Hepatic IL-23 expression in CHB patients (n = 60) and HCs (n = 16) was investigated with real-time PCR. D, Immunohistochemical analysis of IL-23 p19 expression in CHB liver and normal liver tissues. The IL-23 p19 protein in the tissue sections was detected with SP kit. The nuclei were then counterstained with hematoxylin. Original magnification ×40 (upper panels), ×200 (middle panels), and ×400 (lower panels). E, Statistical analysis of IL-23 p19 expression in CHB liver and normal liver tissues. *p < 0.001.
for transplantation. The degree of hepatic inflammation was graded using the modified histological activity index (HAI) described by Scheuer (27). The basic clinical data for the CHB patients with available liver biopsy samples are presented in Table II.

**Cell culture**

Human hepatocellular carcinoma cells (HepG2 and Huh-7), normal hepatocyte cells (Chang liver and HL-7702), and HepG2.2.15 cells were cultured in DMEM at 37°C in a 5% CO2 incubator. The medium was supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

**Real-time PCR**

Total RNA was extracted using TRIzol Reagent (Invitrogen), and reverse transcription was performed using an Advantage RT-for-PCR Kit (Takara) according to the manufacturer’s instructions. For the real-time PCR analysis, aliquots of double-stranded cDNA were amplified using a SYBR Green PCR Kit (Applied Biosystems). The cycling parameters were 95°C for 15 s, 55°C for 15 s, and 72°C for 15 s for 45 cycles. A melting curve analysis was then performed. Ct was measured during the exponential amplification phase, and the amplification plots were analyzed using SDS 1.9.1 software (Applied Biosystems). For the cell lines, the relative expression level (defined as fold change) of the target gene was determined by the equation $2^{-\Delta \Delta Ct} (\Delta Ct = \Delta Ct_{\text{target}} - \Delta Ct_{\text{control vector}})$. The expression level was normalized to the fold change detected in the corresponding control cells, which was defined as 1.0. For clinical CHB and normal liver tissue samples, the fold change of the target gene was determined by the equation $2^{-\Delta \Delta Ct} (\Delta Ct = \Delta Ct_{\text{tumor}} - \Delta Ct_{\text{nontumor}})$, and it was normalized to the average fold change in the normal liver tissues, which was defined as 1.0. All reactions were performed in duplicate. Primer sequences are listed in Table III.

**Immunohistochemistry**

In brief, after deparaffinization and hydration, the slides were treated with a diaminobenzidine solution until the color developed. The Labeled HRP was applied for 30 min at room temperature, and then, the slides were counterstained with hematoxylin, and the results were observed under a light microscope.

**Plasmid constructions**

All primers are shown in Table III. A 1348-bp IL-23 p19 promoter construct (−1269/+79) was generated from human genomic DNA, which corresponded to the sequence from −1269 to +79 (relative to the transcriptional start site) of the 5′-flanking region of the human IL-23 p19 gene. This construct was generated using F1 and R1 as the forward and reverse primers incorporating SacI and XhoI sites at the 5′ and 3′ ends, respectively. The PCR product was cloned into the SacI and XhoI sites of the pGGL3-Basic vector (Promega). The construct was confirmed by DNA sequencing. The 5′-flanking deletion constructs of the IL-23 p19 promoter (Δ(−1269/+79) p19 and Δ(−77/+79) p19) were similarly generated with the (−1269/+79) p19 construct as a template and the forward primers F2 and F3. Two NF-κB sites in the IL-23 p19 promoter were mutated with a QuikChange II site-directed mutagenesis kit (Stratagene). Truncated and mutated IL-23 p40 promoter constructs were cloned in the same way. The expression vectors containing the seven individual, viral structural genes of the HBV and NF-κB subunits (p65 and p50) were a generous gift from Dr. Wu Jianguo (Wuhan University, Wuhan, People’s Republic of China).

**Transient transfections**

The cells were plated at a density of 1 × 10⁵ cells/well in a 24-well plate. After 12–24 h, the cells were cotransfected with 0.6 μg of the expression vector plasmids, 0.18 μg of the promoter reporter plasmids, and 0.02 μg of the pRL-TK plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 5 h of transfection, the cells were washed and allowed to recover overnight in fresh medium supplemented with 1% FBS for 48 h. Serum-starved cells were used for the assay.

**Luciferase reporter assay**

The luciferase activity was detected with the Dual Luciferase Assay (Promega) according to the manufacturer’s instructions. The transfected cells were lysed in the culture dishes with a lysis buffer, and the lysates were centrifuged at maximum speed for 1 min in an Eppendorf microcentrifuge. The relative luciferase activity was determined by a Modulus TD20/20 Luminometer (Turner Biosystems), and the transfection efficiency was normalized by Renilla activity.

**ELISA**

The culture supernatants were assayed for IL-23 protein according to the manufacturer’s instructions (cat. no. D2300B; R&D Systems) at room temperature. The cells were transfected with 0.8 μg of the relative plasmids for 48 h. The supernatants were then collected and assayed for IL-23 using ELISA. The concentration of IL-23 was determined using a standard curve obtained with recombinant IL-23.

**Western blot analysis**

In brief, proteins from lysed cells were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked for 2 h at room temperature with a 1.5% blocking serum. Sections were incubated with an anti–IL-23 Ab (ab45420; Abcam) at 4°C overnight. The culture supernatants were assayed for IL-23 protein according to the manufacturer’s instructions. After 5 h of transfection, the cells were washed and allowed to recover overnight in fresh medium supplemented with 1% FBS for 48 h. Serum-starved cells were used for the assay.
blocked with 5% milk in TBST [120 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20] for 1 h at room temperature. Blots were incubated with a specific Ab overnight at 4°C. The membranes were then washed with PBS three times and incubated with an HRP-conjugated secondary Ab. Proteins were visualized using a Dura SuperSignal Substrate (Pierce).

EMSA

Biotin end-labeled oligonucleotides were synthesized and annealed to obtain dsDNA fragments. The oligonucleotide sequences are shown in Table II. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer’s protocol. DNA binding reactions were performed in 20-μl samples containing 20 fmol biotin-labeled oligonucleotides, 4 mg nuclear extracts, 2 μg d(l)-C, 2 μl 10× binding buffer, 0.1 mM EDTA, and 10% glycerol at room temperature for 20 min. For a competitor EMSA, 20-fold or 100-fold dilution of unlabeled oligonucleotide was added prior to the addition of the labeled probe. The samples were run on 6% nondenaturing polyacrylamide gels with 0.5× TBE buffer and transferred to a nylon membrane (Amersham Biosciences, Pharmacia, Buckinghamshire, U.K.) at 380 mA (~100 V) for 30 min. A chemiluminescent detection method using a luminal/enhancer solution and stable peroxide solution was used as described by the manufacturer, and the membranes were scanned by the Image Station 4000R (Kodak).

Chromatin immunoprecipitation assay

HepG2 cells transfected with relative plasmids were cross-linked using 1% formaldehyde at 37°C for 10 min. After washing with PBS, the cells were resuspended in 300 μl lysis buffer [50 mM Tris (pH 8.1), 10 mM EDTA, 1% SDS, and 1 mM PMSF]. DNA was sheared to small fragments by sonication. The supernatants were precleared using a herring sperm DNA/protein G–Sepharose slurry (Sigma-Aldrich). The recovered supernatants were incubated with anti-p50, p65, p52, c-Rel, RelB, or an isotype control IgG for 2 h in the presence of herring sperm DNA and protein G–Sepharose beads. The immunoprecipitated DNA was retrieved from the beads with 1% SDS and a 1.1 M NaHCO3 solution at 65°C for 6 h. DNA was then purified using a PCR purification kit (Qiagen), and real-time PCR was performed on the extracted DNA using IL-23 p19 and p40 promoter-specific primers (Table III).

Statistical analyses

Data are expressed as the means ± SD. Serum and hepatic IL-23 expression levels were compared between groups using the Student t test. Correlation analyses were evaluated using the Spearman rank correlation test. A p value <0.05 was considered statistically significant. Statistical analysis data were analyzed using SPSS software (version 11.0).

Results

Serum levels and hepatic expression of IL-23 are significantly upregulated in CHB patients

To determine the potential association between IL-23 and HBV infection, serum IL-23 levels from 192 CHB patients and 60 HCs (Table I) were evaluated by ELISA. Serum IL-23 levels were significantly increased in CHB patients compared with those of the HCs (Fig. 1A). Real-time PCR was used to detect hepatic IL-23 (subunits p19 and p40) mRNA expression in these patients and in HCs. Compared with the HCs, p19 and p40 mRNA levels were significantly increased in CHB patients (Fig. 1B, 1C). Furthermore, immunohistochemistry was also performed to detect the expression of IL-23 in the CHB patients. IL-23 was mainly visualized in hepatocytes and infiltrating inflammatory cells in CHB liver tissues. However, IL-23 could not be detected in normal liver tissues (Fig. 1D, 1E).

Increased IL-23 expression is positively correlated with liver injury in CHB patients

To determine whether serum IL-23 expression is correlated with liver injury, we determined the correlations between serum IL-23 levels and serum HBV DNA load, serum alanine aminotransferase (ALT) levels, or aspartate aminotransferase (AST) levels in CHB patients. Significant, positive correlations were found between serum IL-23 levels and HBV DNA load (R = 0.544, p < 0.001) and serum ALT (R = 0.508, p < 0.001) and AST (R = 0.442, p < 0.001) levels in CHB patients (Fig. 2A, 2B).

We subsequently analyzed the association between hepatic IL-23 mRNA expression and liver injury scores in CHB patients (Table II). Compared with patients with lower HAI scores, CHB patients with higher HAI scores had higher expression of hepatic IL-23 p19 mRNA (Fig. 2C). Further analysis found that hepatic IL-23 p19 mRNA levels were positively correlated with HBV DNA load (R = 0.54, 0.001) and serum ALT (R = 0.442, p < 0.001) levels in CHB patients (Table II).
HBV directly induces IL-23 expression in hepatocytes

The previous experiments suggest that IL-23 expression is significantly increased in CHB patients and positively correlated with liver injury and that the hepatocytes were one of the main cells that secreted IL-23 in CHB patients. However, the mechanism of IL-23 overexpression in hepatocytes remains unclear. To investigate whether HBV directly induces expression of IL-23, the plasmid pBlue-HBV, which contained the 1.3-fold HBV genome, was transfected into hepatocytes (HepG2, Huh-7, Chang liver, and HL-7702). Forty-eight hours after transfection, the protein levels in the supernatants and the mRNA levels in the cells were measured. Secretion of IL-23 significantly increased after transfection with pBlue-HBV (Fig. 3A). Accordingly, the mRNA levels of the p19 and p40 subunits were also increased in cells transfected with pBlue-HBV (Fig. 3B). We also compared the expression of IL-23 in HepG2 and HepG2.2.15 cells, which were stably transfected with the 1.3-fold HBV genome. IL-23 expression levels were much higher in the HepG2.2.15 cells than in the HepG2 cells (Fig. 3C, 3D), as assessed by ELISA and real-time PCR.

FIGURE 3. HBV induces IL-23 expression in hepatocytes. A. The hepatocytes (HL-7702, Chang liver, HepG2, and Huh-7) were transfected with pBlue-HBV, which contains the 1.3-fold HBV genome, and serum IL-23 levels were measured by ELISA. B. After the cells were transfected with pBlue-HBV, IL-23 mRNA levels (subunits p19 and p40) were detected by real-time PCR. C. Serum IL-23 levels from HepG2 and HepG2.2.15 cells were measured by ELISA. D. IL-23 mRNA levels in HepG2 and HepG2.2.15 cells were detected by real-time PCR. E. The cells (HL-7702, Chang liver, HepG2, and Huh-7) were transfected with HBV viral protein plasmids (pCMV-HBx, pCMV-HBs, pCMV-preS2, pCMV-preS1, pCMV-HBc, pCMV-HBe, or pCMV-HBp), and IL-23 protein levels were measured by ELISA. F. HBx transactivates the promoters of IL-23 p19 and p40. HepG2 cells were cotransfected with IL-23 p19 or p40 promoter luciferase reporter vectors [(−1269/+79) p19 or (−979/+106) p40] and seven HBV viral protein plasmids. Relative luciferase activities were determined by standard procedures. G. HepG2 cells were cotransfected with an increasing amount of plasmid expressing the HBx protein (pCMV-HBx) and reporter plasmids (−1269/+79) p19 or (−979/+106) p40. The luciferase activity of the mock pCMV-Taq group was designated as 1.00. The results are the mean ± SD of three experiments performed in duplicate. *p < 0.05.
HBx upregulates IL-23 expression and transactivates its promoter activity

To confirm further the effect of HBV on IL-23 expression, plasmids containing seven individual viral genes of HBV (pCMV-HBx, pCMV-HBs, pCMV-preS2, pCMV-preS1, pCMV-HBc, pCMV-HBe, and pCMV-HBp) or control plasmids (pCMV-Tag) were transfected into four different hepatocytes, respectively. IL-23 protein levels increased in cells transfected with pCMV-HBx, but the other viral proteins (S, preS2, preS1, HBc, HBe, and HBp) had no significant effects on IL-23 expression (Fig. 3E).

IL-23 is a heterodimeric cytokine composed of the p19 and p40 subunits. To determine whether HBx regulates the transcriptional activity of these subunits, a luciferase reporter plasmid containing the promoter for p19 or p40 was cotransfected with the seven viral protein levels increased in cells transfected with pCMV-HBx, but the other viral proteins (S, preS2, preS1, HBc, HBe, and HBp) had no significant effects on IL-23 expression (Fig. 3E).

FIGURE 4. The NF-κB sites in the IL-23 p19 and p40 promoters are involved in HBx-induced IL-23 expression. A and B, HepG2 cells were treated with three different NF-κB inhibitors after transfection with pCMV-HBx, and IL-23 expression was determined by ELISA and real-time PCR. The p19 and p40 mRNA levels in the untreated group were designated as 1.00. The results are the mean ± SD of three experiments performed in duplicate. C, HepG2 cells were treated with NF-κB inhibitors after cotransfection with pCMV-HBx and the reporter vector (−1269/+79) p19 or (−979/+106) p40. After 48 h, luciferase activity was measured. D, Effect of mutated NF-κB sites on the activity of the IL-23 p19 promoter. HepG2 cells were cotransfected with pCMV-HBx and NF-κB1 MUT-p19 or NF-κB2 MUT-p19. The left panel is a schematic representation of the reporter gene constructs. The bar graphs in the right panel represent the relative levels of luciferase activity in each of the transfected samples. E, The effect of the HBx protein on the truncated IL-23 p19 promoter. HepG2 cells were cotransfected with truncated IL-23 p19 promoter constructs and pCMV-HBx, and the relative luciferase activity was determined. F and G, Effect of the HBx protein on the mutated and truncated p40 promoter. The luciferase activity of the mock pCMV-Taq group was designated as 1.00. The results are the mean ± SD of three experiments performed in duplicate. *p < 0.05.
protein plasmids into HepG2 cells. The transcriptional activities of p19 and p40 were significantly increased by the HBx protein, but the other viral proteins (S, preS2, preS1, HBc, HBe, and HBp) had no significant effect on p19 and p40 promoter activities (Fig. 3F).

We next investigated the dose dependence of HBx on p19 and p40 promoter activities. Reporter plasmids containing the p19 or p40 promoter were cotransfected with an increasing concentration of pCMV-HBx plasmid into HepG2 cells. The transcriptional activities of p19 and p40 were significantly increased by the HBx protein, but the other viral proteins (S, preS2, preS1, HBc, HBe, and HBp) had no significant effect on p19 and p40 promoter activities (Fig. 3F).

The transcriptional inhibition (Fig. 4A) after transfection with pCMV-HBx. ELISA and real-time PCR showed that HBx-induced IL-23 expression was significantly inhibited by blocking the activation of NF-κB (Fig. 4A, 4B). The luciferase reporter assay also demonstrated that the transcriptional activities of p19 and p40 were gradually inhibited after NF-κB activation (Fig. 4C). Together, these data suggest that HBx is a critical regulator of IL-23 expression during HBV infection.

**NF-κB activity is required for HBx-induced IL-23 expression**

Recent studies have demonstrated that the p19 and p40 genes are transcriptionally regulated by NF-κB in murine macrophages and DCs (28–30). To determine whether the HBx-induced p19 and p40 expression was regulated through NF-κB, HepG2 cells were treated with three different NF-κB inhibitors (SN50, PDTC, and BAY) after transfection with pCMV-HBx. ELISA and real-time PCR showed that HBx-induced IL-23 expression was significantly inhibited by blocking the activation of NF-κB (Fig. 4A, 4B). The luciferase reporter assay also demonstrated that the transcriptional activities of p19 and p40 were gradually inhibited after NF-κB inhibition (Fig. 4C). These results suggest that NF-κB activation is required to activate the p19 and p40 promoters by the HBx protein.

The p19 promoter contains two putative NF-κB sites located from nt −884 to −874 (p19 NF-κB1) and from nt −90 to −80 (p19 NF-κB2). To investigate the roles of the two NF-κB binding sites in regulating p19 subunit expression via the HBx protein, these NF-κB sites were mutated (Table III). Mutating the NF-κB1 binding site effectively reduced the HBx-induced activation of the p19 promoter (Fig. 4D). To confirm further the role of the NF-κB2 binding site in regulating the p19 promoter by the HBx protein, a series of 5′ deletions in the p19 promoter were constructed and cotransfected with pCMV-HBx into HepG2 cells. Deletion from nt −1269 to nt −565 did not affect the HBx-induced p19 luciferase activity; however, deletion from nt −565 to nt −77 significantly decreased the HBx-induced p19 luciferase activity (Fig. 4E). These results suggest that the NF-κB2 binding site is required for the HBx-induced activation of the p19 promoter.

The p40 promoter contains only one NF-κB site, which is located from nt −131 to nt −122 (p40 NF-κB). Mutating the NF-κB binding site significantly reduced the HBx-induced transcription of the p40 promoter (Fig. 4F). Consistent with this result, a deletion from nt −979 to nt −268 did not affect the HBx-induced p40 promoter activity. A further deletion to nt −121 significantly decreased the HBx-induced p40 promoter activity (Fig. 4G). These results indicate that the NF-κB binding site located from nt −131 to nt −122 is required for the HBx-induced activation of the p40 promoter.

**The NF-κB subunit p65/p50 binds to the promoter of IL-23 subunits p19 and p40**

To determine whether NF-κB binds to the p19 promoter after transfection with pCMV-HBx, EMSA was performed using biotin-labeled NF-κB consensus oligonucleotides in the p19 promoter.

Table III. Primer sequences used in the study

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<th>Primer Sequences</th>
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<td>F3 (−77/−79)p19 sense</td>
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<tr>
<td>NF-κB2 MUT-p40 (−131/−122)</td>
<td>5'-GGAACCTCTCTAAATCTCTGTACGACATCGCTGCTG-3'</td>
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<tr>
<td>Primers used for ChIP essay</td>
<td></td>
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<tr>
<td>P19 NF-κB2 sense</td>
<td>5'-TGTTCCCAATACCGAGTGGTG-3'</td>
</tr>
<tr>
<td>P19 NF-κB2 antisense</td>
<td>5'-CTTGGAGGCTCGAAGAAGATT-3'</td>
</tr>
<tr>
<td>P40 NF-κB sense</td>
<td>5'-AGAGCGTCTTCTGCACCACAAG-3'</td>
</tr>
<tr>
<td>P40 NF-κB antisense</td>
<td>5'-GATGATTATGTTCTCTCTCTT-3'</td>
</tr>
<tr>
<td>Primers for real-time PCR</td>
<td></td>
</tr>
<tr>
<td>P19 sense</td>
<td>5'-CTTGGAGGCTCGAAGAAGATT-3'</td>
</tr>
<tr>
<td>P19 antisense</td>
<td>5'-AGAGCGTCTTCTGCACCACAAG-3'</td>
</tr>
<tr>
<td>P40 sense</td>
<td>5'-GATGATTATGTTCTCTCTCTT-3'</td>
</tr>
<tr>
<td>P40 antisense</td>
<td>5'-CTTGGAGGCTCGAAGAAGATT-3'</td>
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<td>GAPDH sense</td>
<td>5'-AGAGCGTCTTCTGCACCACAAG-3'</td>
</tr>
<tr>
<td>GAPDH antisense</td>
<td>5'-GATGATTATGTTCTCTCTCTT-3'</td>
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(-90 to -80) as a probe. The DNA binding activity of NF-κB was significantly increased in cells transfected with pCMV-HBx compared with cells transfected with the control plasmid. To determine the specificity of the NF-κB binding activity, nuclear extracts were incubated with the labeled NF-κB consensus oligonucleotides in the presence of either an unlabeled, wild-type NF-κB binding probe or a mutated probe. The wild-type NF-κB consensus oligonucleotides but not the mutated oligonucleotides showed significantly abrogated NF-κB complexes (Fig. 5A). Consistently, the addition of NF-κB inhibitors (SN50, PDTC, and BAY) inhibited the DNA binding activity of NF-κB. Similar results were found for the p40 promoter (Fig. 5B). These data indicate that NF-κB binds to the NF-κB binding site in the p19 and p40 promoters.

The NF-κB family consists of five structurally related proteins (p65, c-Rel, RelB, p50, and p52). We next performed a chromatin immunoprecipitation (ChIP) assay to confirm whether NF-κB binds to the p19 promoter in vivo. Chromatin fragments were prepared from HepG2 cells transfected with pCMV-HBx and immunoprecipitated with Abs against p65, c-Rel, RelB, p50, and p52 subunits, respectively. A 150-bp DNA fragment containing the NF-κB2 binding site in the p19 promoter was amplified by real-time PCR using specific primers. The NF-κB2 binding site at the p19 promoter was immunoprecipitated from the cells transfected with pCMV-HBx in the presence of an anti-p50 or anti-p65 Ab; however, they could not be immunoprecipitated in the presence of control plasmids and Abs against c-Rel, RelB, or p52 (Fig. 5C). These data indicate that the NF-κB subunits p65/p50 bind to the NF-κB2 binding site at the p19 promoter, and similar results were found for the p40 promoter (Fig. 5D).

The NF-κB subunit p65/p50 increases the transcriptional activities of the IL-23 p19 and p40 promoters

To investigate the effects of the p65 and p50 subunits of NF-κB on the expression of IL-23, HepG2 cells were cotransfected with plasmids expressing p65 and p50 (pCMV-p65 and pCMV-p50). IL-23 protein levels were measured by ELISA. The p65 and p50 proteins increased the expression of the IL-23 protein, and cotransfection with p65 and p50 further increased IL-23 protein levels (Fig. 6A). Furthermore, plasmids expressing p65 or p50 were cotransfected with the IL-23 (-1269+/79) p19 reporter plasmids into HepG2 cells, and the luciferase activity was measured. The transcriptional activity of p19 was highest when cells were transfected with both p65 and p50. However, mutating the NF-κB2 binding site in the IL-23 p19 promoter abolished the effect of the p50 and/or p65 protein on the activation of the IL-23 p19 promoter (Fig. 6B). Similar results were also found for the IL-23 p40 promoter (Fig. 6C). Therefore, these results indicate that the p65/p50 heterodimer induces IL-23 expression.

**FIGURE 5.** NF-κB subunits p65/p50 bind to the IL-23 p19 and p40 promoters directly. A and B, HepG2 cells were transfected with pCMV-HBx or the control plasmid pCMV-Taq for 48 h. Nuclear extracts were subjected to EMSA with the biotin-labeled (A) p19 NF-κB2 oligonucleotide or (B) NF-κB site of the IL-23 p40 promoter in the absence and presence of the indicated unlabeled or unlabeled-mutated competitors. In addition, three NF-κB inhibitors were involved in the indicated case. C and D, The ChIP assay showed the direct binding of p65/p50 to the IL-23 p19 and p40 promoters. Abs against p65, p50, p52, c-Rel, and RelB or a control IgG Ab pulled down the DNA fragment containing the NF-κB2 binding site in the p19 promoter and the NF-κB site in the p40 promoter. Real-time PCR was performed to detect the amount of immunoprecipitated products. The results are the mean ± SD of three experiments performed in duplicate. *p < 0.05.
HBx induces the translocation of the NF-κB subunit p65 into the nucleus

The above results suggest the involvement of NF-κB subunits p65/p50 in HBx-induced IL-23 expression. Therefore, we determined whether HBx induces p65 translocation. Confocal microscopy and Western blot were used to examine the effect of HBx on the translocation of p65 from the cytosol into the nucleus. Transfection with pCMV-HBx but not with pCMV-Taq caused a remarkable translocation of p65 from the cytosol into the nucleus after transfection with pCMV-HBx for 48 h. The cells were stained with an anti-p65 Ab, and immunofluorescence was monitored by confocal microscopy. Original magnification ×400. E, Levels of cytosolic and nuclear p65 proteins were determined by Western blot using an anti-p65 Ab after being transfected with pCMV-HBx. The results are the mean ± SD of three experiments performed in duplicate. *p < 0.05.

HBx-induced IL-23 expression is mediated by the ERK–p65/p50 signaling pathway

It is known that HBx can induce the phosphorylation of ERK1/2, JNK, and p38 MAPKs in various cell types (9). We sought to determine whether any of the MAPKs were involved in the induction of IL-23 expression by HBx. U0126, SP600125, and SB202190 have been used specifically to inhibit the activation of ERK1/2, JNK, and p38 MAPKs, respectively. Pretreatment of HepG2 cells with U0126 significantly reduced HBx-induced IL-23 protein expression. However, pretreatment of cells with SP600125 or SB202190 did not significantly affect HBx-induced IL-23 expression (Fig. 7A).

To determine whether HBx-induced IL-23 p19 and p40 promoter activities were dependent on ERK1/2 activity, HepG2 cells were cotransfected with IL-23 (−1269/+79) p19 or (−979/+106) p40 reporter plasmids and pCMV-HBx and treated with U0126, SP600125, and SB202190. The luciferase reporter assay showed that treatment of the cells with the U0126 but not the SP600125 or SB202190 significantly decreased the HBx-induced IL-23 p19 and p40 promoter activities (Fig. 7B).

We further determined the role of ERK1/2 in the HBx-induced activation of IL-23 p19 and p40 promoters by introducing a dominant-negative mutant of ERK1 (DN-ERK). This mutant blocks kinase activities by competing with endogenous kinases. HepG2 cells were cotransfected with pCMV-HBx (−1269/+79) p19 or (−979/+106) p40 reporter plasmids, and different concentrations of DN-ERK plasmids. The luciferase assay showed that DN-ERK significantly inhibited the transactivation of the p19 and p40 promoters by HBx (Fig. 7C).

Next, we investigated the role of the HBx protein in the activation of signal transduction pathways. The protein levels of phospho-ERK1/2, phospho-JNK, and phospho-p38 were measured by Western blot analysis in the transfected HepG2 cells. HBx expression significantly increased the phosphorylation level of the ERK1/2 protein (Fig. 7D). The EMSA assay showed that the inhibition of ERK1/2 phosphorylation by U0126 decreased the binding of NF-κB to the p19 and p40 promoters and inhibited IL-23 expression (Fig. 7E, 7F). Taken together, these results suggest that the HBx-induced IL-23 expression in hepatocytes might be mediated through the ERK–p65/p50 signaling pathway.

PI3K and Ras–MEK–MAPK pathway is involved in HBx-induced ERK1/2 activation and IL-23 expression

In the previous study, it was demonstrated that ERK1/2 activity could be regulated by the PI3K and Ras–MEK–MAPK pathway (31–33). To investigate the role of the PI3K and Ras–MEK–MAPK pathway in HBx-induced ERK1/2 activation and IL-23 expression, wortmannin and BAY43-9006 were used to inhibit
the activation of PI3K and Raf-1, respectively. ELISA assay showed that wortmannin and BAY43-9006 treatment significantly decreased IL-23 protein expression induced by HBx (Fig. 8A).

To determine whether HBx-induced IL-23 p19 and p40 promoter activities were dependent on activation of the PI3K and Ras–MEK–MAPK pathway, HepG2 cells were cotransfected with pCMV-HBx and pCMV-Taq. IL-23 protein levels were measured in the supernatant by ELISA. B, HepG2 cells were pretreated with inhibitors to ERK1/2, JNK, and p38 (U0126, SP600125, and SB202190) before being transfected with pCMV-HBx or the control plasmid pCMV-Taq. IL-23 protein levels were measured in the supernatant by ELISA. B, HepG2 cells were pretreated with inhibitors to ERK1/2, JNK, and p38 and cotransfected with pCMV-HBx and an IL-23 (−1269/+79) p19 or (−979/+106) p40 reporter plasmid. The luciferase activity of the mock pCMV-Taq group was designated as 1.00. C, HepG2 cells were cotransfected with pCMV-HBx, with increasing concentrations of DN-ERK and IL-23 (−1269/+79) p19 or (−979/+106) p40 reporter plasmids, and the luciferase activity was measured 48 h after transfection. The luciferase activity of the mock pCMV-Taq group was designated as 1.00. D, HepG2 cells were transfected with pCMV-HBx, and the phosphorylated and total ERK1/2, JNK, and p38 protein levels were analyzed by Western blot. E and F, HepG2 cells were pretreated with inhibitors to ERK1/2, JNK, and p38, and the nuclear extracts were isolated. EMSA was performed to measure the binding of NF-κB to the (E) p19 and (F) p40 promoters. The results are the mean ± SD of three experiments performed in duplicate. *p < 0.05.

Discussion
IL-23 has recently been identified as playing a critical role in a number of chronic inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis (34). IL-23 is essential for the survival and activation of Th17 cells, which produce proinflammatory cytokines, such as TNF-α, IL-17A, and IL-6. However, IL-23 expression and its role in CHB are still unknown. Recent studies have demonstrated that Th17 cells increase the severity of liver damage in CHB patients (23–25), indicating that IL-23 may play an important role in promoting inflammatory injury in CHB. In the current study, we found that serum levels and hepatic IL-23 expression were positively corre-
expression of both the p19 and p40 subunits. Several pieces of evidence indicate that IL-23 is closely associated with liver damage that is induced by HBV infection.

IL-23 is predominantly produced by macrophages and DCs in response to various stimuli. In this study, we found that IL-23 was upregulated in CHB patients. Furthermore, the immunohistochemistry results demonstrated that, besides inflammatory cells, hepatocytes also produce a significant amount of IL-23. These results are consistent with previous research because other colleagues and our group have also demonstrated that hepatocytes are capable of producing proinflammatory cytokines, such as TNF-α, IL-6, IL-8, IL-18, MIG, and IP-10 (35–39). To confirm further the direct effect of HBV on IL-23 expression, the plasmid pBlue-HBV was transfected into hepatocytes. The results from ELISA, real-time PCR, and the luciferase reporter assay demonstrated that HBV could directly induce IL-23 expression. Furthermore, we found that the viral protein HBx, which is encoded by HBV, transactivated the promoters of the IL-23 subunits p19 and p40.

Synthesis of biologically active IL-23 heterodimers requires the expression of both the p19 and p40 subunits. Several pieces of evidence have indicated that expression of the p19 and p40 genes are regulated by the NF-κB transcription factor. Recent studies have demonstrated that IL-23 p19 is mainly regulated by the NF-κB heterodimer (p65/c-Rel) that binds to the p19 promoter and that it is necessary for the TLR-induced IL-23 p19 expression in murine macrophages and DCs (28, 29). Similarly, c-Rel is required for LPS-induced IL-23 p40 gene transcription in macrophages (40). To determine the role of NF-κB in HBx-induced IL-23 p19 and p40 transcriptions, the NF-κB binding sites on the p19 and p40 promoters were mutated. Our results showed that the NF-κB binding sites in the p19 and p40 promoters are essential for regulating the transcription of IL-23 p19 and p40 by HBx.

NF-κB belongs to the Rel family of transcription factors, which regulate an exceptionally large number of genes, particularly those involved in immune and inflammatory responses (41, 42). The mammalian NF-κB family consists of five members: c-Rel, RelA (p65), RelB, p50, and p52. NF-κB subunits are able to homodimerize or heterodimerize to form transcription factors that bind to the promoters of target genes and regulate the expression of many cytokines, such as TNF-α, IL-1β, IL-2, IL-6, IL-8, CXCL9, CXCL10, and IL-12 p35 (43). In the current study, the EMSA assay revealed that NF-κB binds to the p19 and p40 promoters and is involved in HBx-induced IL-23 expression. The ChIP assay further confirmed that the NF-κB heterodimer p60/p50 binds to the IL-23 p19 and p40 promoters. Notably, the overexpression of wild-type p50 and p65 stimulated IL-23 expression, and the inhibition of NF-κB activation by the NF-κB inhibitor significantly suppressed IL-23 expression. These data indicate that NF-κB heterodimer p60/p50 activation and binding to the IL-23 subunit gene promoters are essential for HBx-induced IL-23 expression.

Previous studies have demonstrated that MAPK pathways are important regulators in proinflammatory cytokine production (44, 45). In mammals, three major MAPK pathways have been identified: ERK, JNK, and p38 MAPK. Studies have found that HBx can induce the phosphorylation of ERK1/2, JNK, and p38 MAPK in various cell types. To determine whether MAPK pathways are involved in HBx-induced IL-23 expression, different inhibitors (U0126, SP600125, and SB202190) of the MAPK pathways were used to block their activities. ELISA and the luciferase reporter assay showed that the U0126 significantly decreased HBx-induced IL-23 expression. It was demonstrated that U0126 could bind to MEK1/2 and indirectly affects ERK1/2 function (46). In accordance with previous research, we found that U0126 significantly decreased the phosphorylation of ERK1/2. Furthermore, EMSA assay showed that U0126 blocked the binding of NF-κB to the IL-23 p19 and p40 promoters. These results indicate that ERK1/2 activation is required for HBx-induced IL-23 expression. Previous studies have reported that ERK1/2 activity could be regulated by PI3K and Ras–MEK–MAPK pathway (31–33). In the current study, wortmannin and BAY43-9006 were used to inhibit PI3K and Ras–MEK–MAPK pathway, respectively. Consistent with
previous studies, wortmannin and BAY43-9006 significantly inhibit ERK activation. Furthermore, we also found that wortmannin and BAY43-9006 decreased IL-23 expression induced by HBx. These data suggest that HBx-induced IL-23 expression and ERK1/2 activation is mediated by PI3K and Ras–MEK–MAPK pathway. In summary, our findings demonstrate that IL-23 is significantly increased in CHB patients, and increased IL-23 expression is positively correlated with liver injury. Furthermore, viral protein HBx induces IL-23 expression in hepatocytes through the activation of the ERK/NF-κB pathway.

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Disclosures
The authors have no financial conflicts of interest.

References