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Original Article

The hepatitis B virus pre-core protein p22 suppresses TNFα-induced apoptosis by regulating the NF-κB pathway

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Abstract: Objective: Cell apoptosis is strongly associated with hepatocellular carcinoma (HCC) progress. Thus, gaining a comprehensive understanding of the virus interfering with the apoptotic process is important for the development of effective anti-tumor therapies. The objective of this study is to explore the potential involvement of HBeAg-p22 (HBV-p22) in TNFα-induced apoptosis. Methods: Protein expression was detected using western blot. Cell viability and apoptosis were assessed by employing Cell Counting Kit-8 (CCK8) and flow cytometry, respectively. Evaluation of protein-protein interactions was accomplished through co-immunoprecipitation and glutathione-S-transferase (GST) pull-down assays. Results: In this study, it was shown that HBV-p22 inhibited apoptosis of human hepatoma cell lines after tumor necrosis factor-alpha (TNF-α) stimulation. Mechanistically, HBV-p22 suppressed Jun N-terminal kinases (JNK) signaling and enhanced nuclear factor kappa-B (NF-κB) signaling. Moreover, HBV-p22 interacted with I-kappa B kinase α (IKKα) and increased its phosphorylation. Conclusions: Collectively, HBV-p22, whereby the mechanism contributing to anti-apoptotic effect was regulation of the NF-κB pathway via enhancing the phosphorylation of IKKα.

Keywords: Hepatitis B virus, hepatocellular carcinoma, HBeAg-p22, NF-κB signaling, apoptosis, TNFα

Introduction

Chronic hepatitis B virus (HBV) infection represents a significant global health concern, and patients develop chronic active hepatitis, cirrhosis, and primary hepatocellular carcinoma in the absence of an effective immune response capable of virus clearance [1]. About 250 million people around the world are infected with hepatitis B virus for a long time. More than 780 thousand people die of hepatitis B related complications every year, including cirrhosis and liver cancer [2]. Existing studies suggest that immune imbalance induced by HBV infection may contribute to the development of hepatocellular carcinoma [3]. In general, HBV is a non-cytotoxic virus, which itself does not cause liver cell damage, and the liver damage accompanying HBV infection is mainly caused by the body's anti-HBV immune response. Natural killer cells (NK) are an important component of the innate immune system that can rapidly recognize and kill viruses, and the proportion of NK in the liver is five times of that in the blood [4]. It was noted that HBV inhibits the antiviral effects of NK cells and downregulates the immune response of T cells, which greatly promotes immune escape and persistent infection of HBV [5]. In recent years, efforts have been made to find ways to effectively inhibit HBV infection by studying the mechanisms of HBV replication.

The viral proteins that counteract immune defense, such as the hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) present in circulation, have been demonstrated to play a crucial role in the establishment of persistent hepatitis B infection [6, 7]. Following HBV infection, viral particles carrying the

relaxed circular DNA (rcDNA) genome are transported to the nucleus where they undergo conversion to episomal covalently closed circular DNA (cccDNA). This cccDNA assembles into a mini-chromosome and functions as a transcriptional template for the production of viral mRNA [8]. Four distinct groups of viral RNA are transcribed from cccDNA, namely pre-core messenger RNA (mRNA), pregenomic RNA (pgRNA), mRNA for the large envelope protein, and messenger RNA for the middle and major surface proteins [9].

HBeAg is a 17-kDa viral protein that is derived from the precursor protein, precore (p25). The p25 protein is a putative 25-kDa monomeric protein. It undergoes cleavage by signal peptidase to produce p22. The resulting p22 protein exhibits the ability to either translocate to the endoplasmic reticulum (ER) or backtrack into the cytoplasm [10]. Previous studies suggest that p22 is an intracellular HBeAg, which can be transported into the nucleus [11, 12]. However, the functions of p22 remain incompletely understood. HBV clearance was regulated by the immune response, which involves the release of incendiary cytokines, notably interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNFα) [13]. Under normal circumstances, normal cells can be removed by regulating cell death, but tumor cells often have a disorder in the cell death program, and enter a state of uncontrolled growth, thus obtaining "immortalization" [14]. TNFα has been demonstrated to perform a crucial function in regulating HBV infections and regulating cell apoptosis [15]. For example, TNFα promotes T cells to develop antiviral effector functions to effectively control HBV infection; in addition, TNFα is involved in the regulation of various signaling pathways associated with inflammation and apoptosis, such as the Jun N-terminal kinases (JNK) pathway, caspase-dependent signaling pathway, and nuclear factor kappa-B (NF-κB) signaling pathway [16]. It was shown that TNFα-mediated JNK activation promoted pro-apoptotic gene expression. In contrast, TNFα-induced NF-κB activation increased the transcription of anti-apoptotic genes in hepatocytes [16]. The present study aimed to investigate the role of HBeAg-p22 (HBV-p22) protein in TNFα-induced apoptosis of hepatoma cells.

Materials and methods

Cell culture and transfection

The HepG2 and Hep3B cells (human hepatoma cell lines) were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology. Cells were cultured routinely in a humidified incubator with Dulbecco's modified Eagle medium (Gibco; USA) supplemented with 10% FBS (Gibco; USA) and 1% penicillin-streptomycin at 37°C with 5% CO_2. All transfection experiments were conducted using Effectene Transfection Reagent (QIAGEN, China) and followed the manufacturer's instructions. The siRNA-IKKα was obtained from GenePharma (Shanghai, China). The previously described method was used to construct the pCMV-HBVp22-HA plasmid [17].

Cell viability assay

The assessment of cell viability was conducted by a CCK-8/WST-8 assay (Solarbio, China). The treated cells were moved to a 96-well plate and then treated with CCK8 reagent (10 μl). The reaction system was left to incubate for 45 minutes at 37°C. Ultimately, the relative cell viability was determined at 450 nm absorbance by using a Multimode microplate reader (Thermo Fisher Scientific, Inc.).

Flow analysis

Cell apoptosis was evaluated through the utilization of a V-FITC Apoptosis Detection kit (Solarbio, China). Briefly, cells (2×10^5) were incubated with 10 μL PE-Annexin V in the dark for 15 min. The populations of early and late apoptotic cells (Annexin V+/PI- and Annexin V+/PI+) were calculated with quadrant gates by flow cytometry (FACSVerse; BD Biosciences), and the flow cytometry data were analyzed using FlowJo v10 (FlowJo, LLC).

RNA analysis

Total RNA was extracted from cells using RNAisoPlus (TAKARA, Japan) and reverse transcribed into cDNA using the PrimeScript RT Master Mix cDNA synthesis system (Bio-Rad, USA). Quantification was performed using SYBR Green (Bio-Rad, USA). Expression of the gene of interest was normalized to the GAPDH RNA level. The following primers were used for

qPCR: IL-8-Forward: 5'-GAGAGTGATTGAGAGTGGACCAC-3'; IL-8-Reverse: 5'-CACAACCCTCTGCACCCAGTTT-3'; Ikbα-Forward: 5'-TCCACTCCATCCTGAAGGCTAC-3'; Ikbα-Reverse: 5'-CAAGGACACCAAAAGCTCCACG-3'.

Immunoblotting

Protein extraction from cells was accomplished using the lysis buffer (Solarbio, China), and protein content was quantified by utilizing the BCA protein quantitative kit (Beyotime Institute of Biotechnology, China). Subsequent to protein extraction and quantification, Western blot analysis was conducted in accordance with established protocols as previously described [21]. Primary antibodies are shown in Table S1.

Luciferase reporter assay

NF-κB-Luc luciferase reporter plasmid and pRL-CMVHepG2-Renilla luciferase reporter granulocytes were transfected with pCMV-HBVp22-HA plasmid (ratio of 15:1). After 24 hours, cells were treated with TNFα (10 ng/ml). Finally, luciferase activity was analyzed by the system of luciferase reporter assay.

Immunoprecipitation

After 48 hours, HepG2 cells were collected and subjected to lysis through treatment with a buffer (150 mM NaCl, 25 mM Tris-HCL, pH 7.4, 5% glycerol, 1% NP-40, 1 mM EDTA). Lysates were then incubated with Anti-Flag M2 affinity gel (Sigma) overnight at 4°C. After centrifugation, pellets were washed thrice with 1 ml of lysis buffer, subsequently suspended in 2X Laemmli SDS-PAGE buffer and detected by Western blot.

In vitro GST pull down assays

The expression and purification of recombinant GST-fused HBV-p22 protein were realized through E. coli BL21 (DE3). The Flag-IKKα, Flag-IKKβ, and Flag-IKKγ, were purified by Anti-Flag M2 affinity gel (Sigma).

GST pull-down assays were conducted by incubating immobilized HBV-p22-GST on glutathione-sepharose resin (BD Biosciences) with purified IKK proteins at 4°C overnight, with end-over-end rotation.

The unbound proteins were removed by washing the beads three times with pull-down buffer (40 mM Tris, pH 8.0, 0.01% IGEPAL CA630, 2 mM DTT, 50 mM NaCl, 20% glycerol, and 5 mM $MgCl_2$), followed by elution and subsequent separation using SDS-PAGE. Detection of the bound proteins was achieved by western blot.

Statistical analysis

The data were expressed as mean ± standard deviation (SD) and subject to analysis with the aid of GraphPad Prism. ImageJ 1.51p was used for the Quantification of immunoblots. Altered levels of protein were shown as normalized fold change by reference to the control value. The comparison test between multiple groups was performed by one-way ANOVA followed by post-hoc test (Tukey's). *P*-values <0.05 were seen as differences.

Results

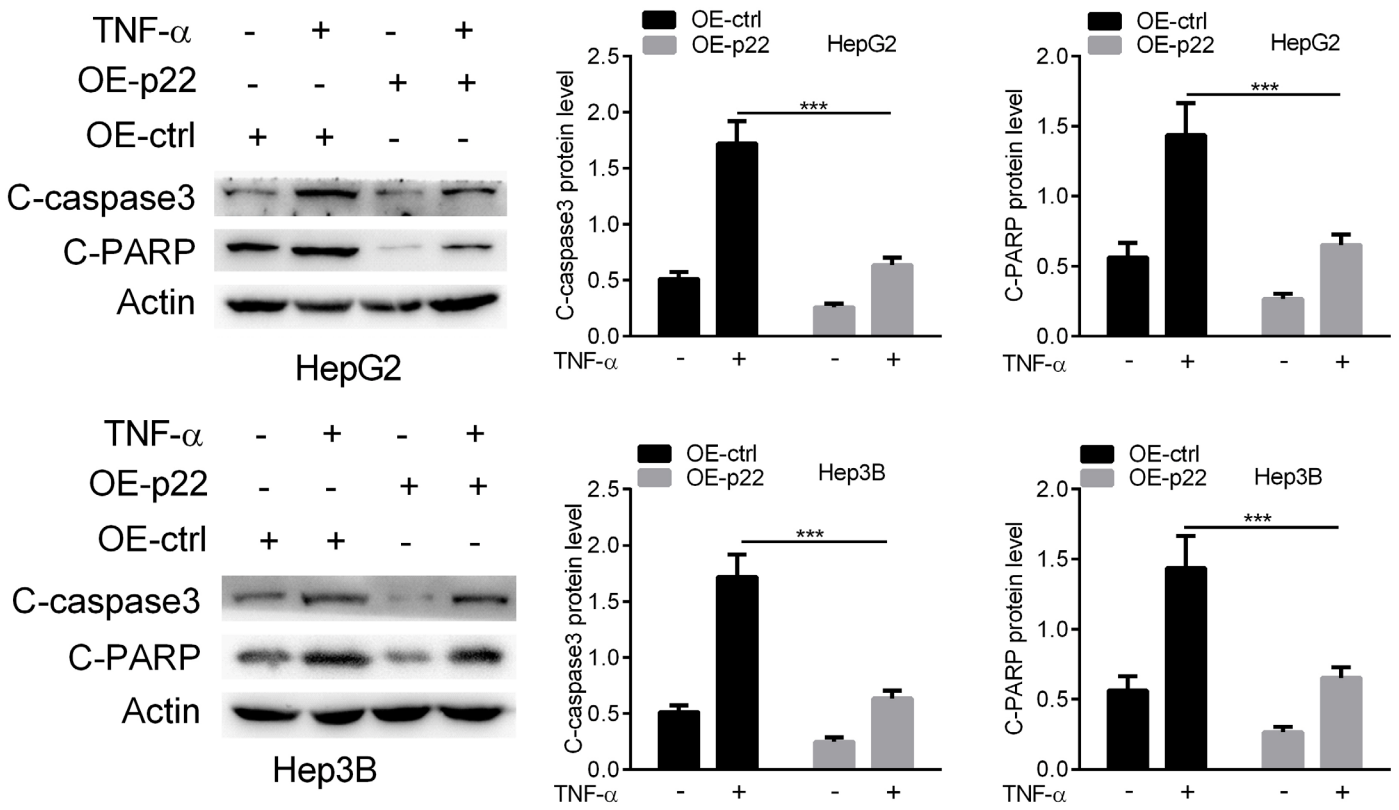
Overexpression of HBV-p22 inhibited TNFα-induced apoptosis

To investigate the contribution of p22 in TNFα-induced apoptosis, the overexpression plasmid pCMV-p22, which expressed HBV-p22 protein, was transfected into HepG2 and Hep3B cells. The western blot analysis demonstrated that the upregulated expression of p22 significantly decreased the expression level of cleaved-caspase 3 and cleaved-PARP under TNFα stimulation (**Figure 1A**). Annexin V/PI flow cytometric analysis yielded the same results (**Figure 1B**). Further, the CCK8 analysis was conducted to test whether p22 overexpression could modulate cellular sensitivity to TNFα. The results showed that p22-transfected cells were less sensitive to the cytotoxic effect of TNFα compared with the control (**Figure 1C**). Together, these results indicated that p22 overexpression inhibited TNFα-induced apoptosis in hepatoma cells.

Overexpression of HBV-p22 suppressed JNK activation

JNK-to-c-Jun signaling has been implicated in TNFα-induced apoptosis [18]. Therefore, the effect of p22 overexpression on JNK activation was examined. Treatment of HepG2 and Hep3B cells with TNFα increased phosphorylation of JNK, c-Jun, and MKK7. However, overexpression of p22 reduced the activation of these signaling molecules (**Figure 2A** and **2B**), indi-

A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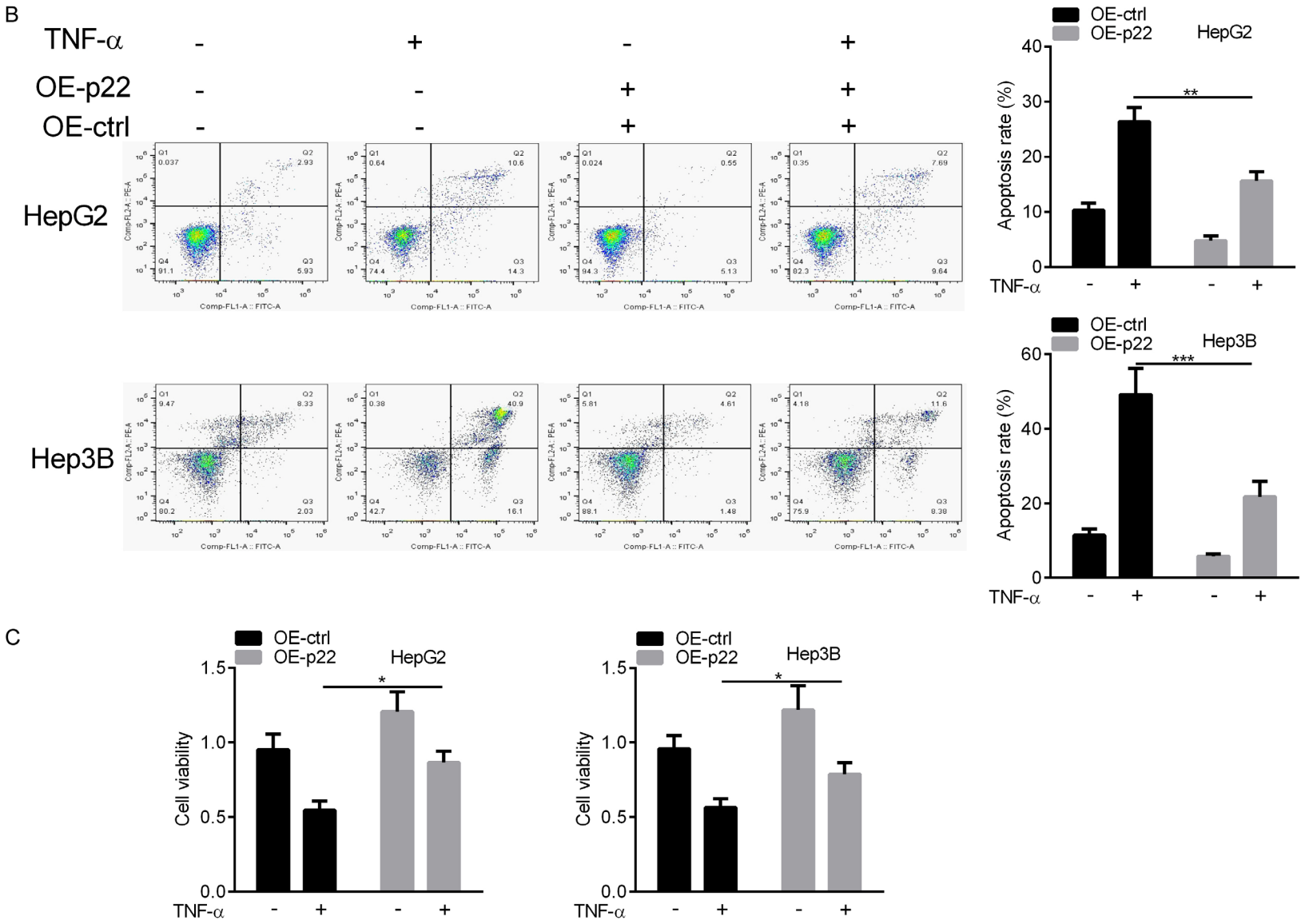


Figure 1. HBeAg-p22 (HBV-p22) overexpression inhibited tumor necrosis factor-alpha (TNF-α) induced apoptosis in hepatoma cells. A. HepG2 and Hep3B cells were transfected with the pCMV-HBVp22-HA (OE-p22) or the control empty vector (OE-ctrl), and then treated with or without TNFα for 1 h. The protein levels of Cleaved-caspase3 (C-caspase3), Cleaved-PARP (C-PARP), and p22-HA were determined using western blot. B. HepG2 and Hep3B cells were transfected with the pCMV-HBVp22-HA or the control empty vector, and then treated with or without TNFα for 1 h. Cell apoptosis was evaluated using flow cytometry. C. HepG2 and Hep3B cells were transfected with the pCMV-HBVp22-HA or the control empty vector, and then treated with or without TNFα for 1 h. Cell viability was measured by using CCK-8/WST-8 assay. The data are presented as the mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

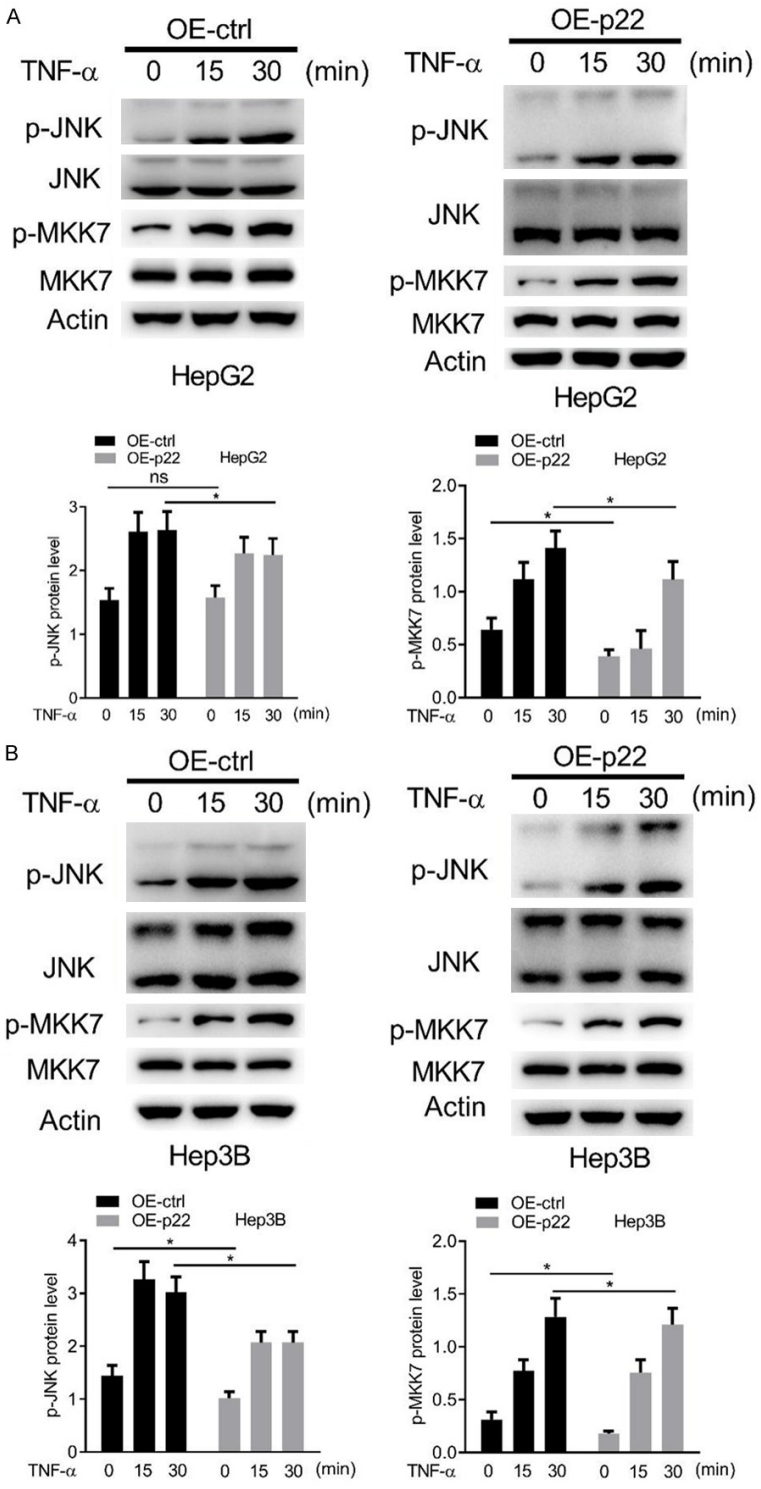


Figure 2. HBV-p22 overexpression attenuated TNFα-induced Jun N-terminal kinases (JNK) activation. A. HepG2 cells were transfected with the pCMV-HBVp22-HA or the control empty vector, and then treated with TNFα for 30 min. The protein levels of phosphorylated JNK (p-JNK), total JNK, phosphorylated MKK7 (p-MKK7), and total MKK7 were measured at 0, 15, and 30 min using western blot. B. Hep3B cells were transfected with the pCMV-HBVp22-HA or the control empty vector, and then treated with TNFα for 30 min. The protein levels of phosphorylated JNK (p-JNK), total JNK, phosphorylated MKK7 (p-MKK7), and total MKK7 were measured at 0, 15, and 30 min using western blot. *P<0.05; ^{ns}P>0.05.

cating that overexpression of p22 attenuated TNFα-induced JNK activation.

HBV-p22 positively regulated TNFα-induced NF-κB activation

NF-κB signaling has been shown to modulate the inhibition of JNK activation in TNFα-treated hepatocytes [19]. Next, the function of p22 on NF-κB signaling was investigated. The findings obtained from the NF-κB-responsive luciferase reporter assay demonstrated that in HepG2 cells, overexpression of p22 effectively augmented TNFα-stimulated NF-κB activation in a concentration-dependent manner (**Figure 3A**). As p65 phosphorylation was essential for NF-κB activation, we investigated the potential impact of p22 on the phosphorylation of p65. Consistently, it was found that overexpression of p22 increased phosphorylated p65 in TNFα-treated cells (**Figure 3B**). To verify these observations, the expression levels of TNFα-induced NF-κB target genes, IL-8 and IκBα were measured in hepatoma cells transfected with pCMV-p22 plasmid. The findings revealed that overexpression of p22 indeed enhanced protein and RNA levels of IL-8 and IκBα (**Figure 3C** and **3D**). The observed results strongly suggested that p22 enhanced NF-κB activation and NF-κB-driven transcription.

HBV-p22 interacted with IKKα

The I-kappa B kinase (IKK) complex is required for the release and nuclear translocation of NF-κB to activate the target gene's transcription [20]. Therefore, it is hypothe-

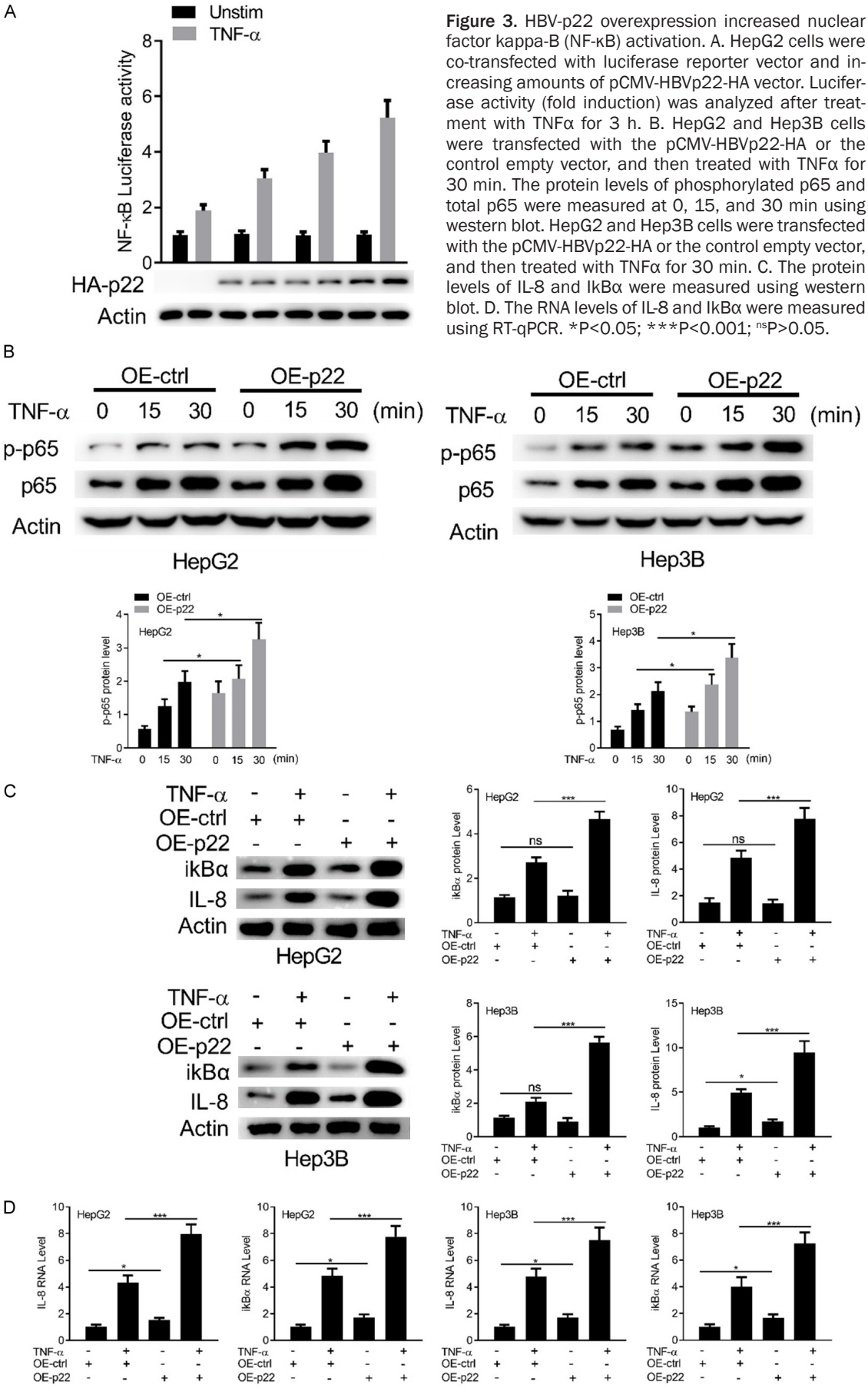


Figure 3. HBV-p22 overexpression increased nuclear factor kappa-B (NF-κB) activation. A. HepG2 cells were co-transfected with luciferase reporter vector and increasing amounts of pCMV-HBVp22-HA vector. Luciferase activity (fold induction) was analyzed after treatment with TNFα for 3 h. B. HepG2 and Hep3B cells were transfected with the pCMV-HBVp22-HA or the control empty vector, and then treated with TNFα for 30 min. The protein levels of phosphorylated p65 and total p65 were measured at 0, 15, and 30 min using western blot. HepG2 and Hep3B cells were transfected with the pCMV-HBVp22-HA or the control empty vector, and then treated with TNFα for 30 min. C. The protein levels of IL-8 and IkBα were measured using western blot. D. The RNA levels of IL-8 and IkBα were measured using RT-qPCR. *P<0.05; ***P<0.001; ^{ns}P>0.05.

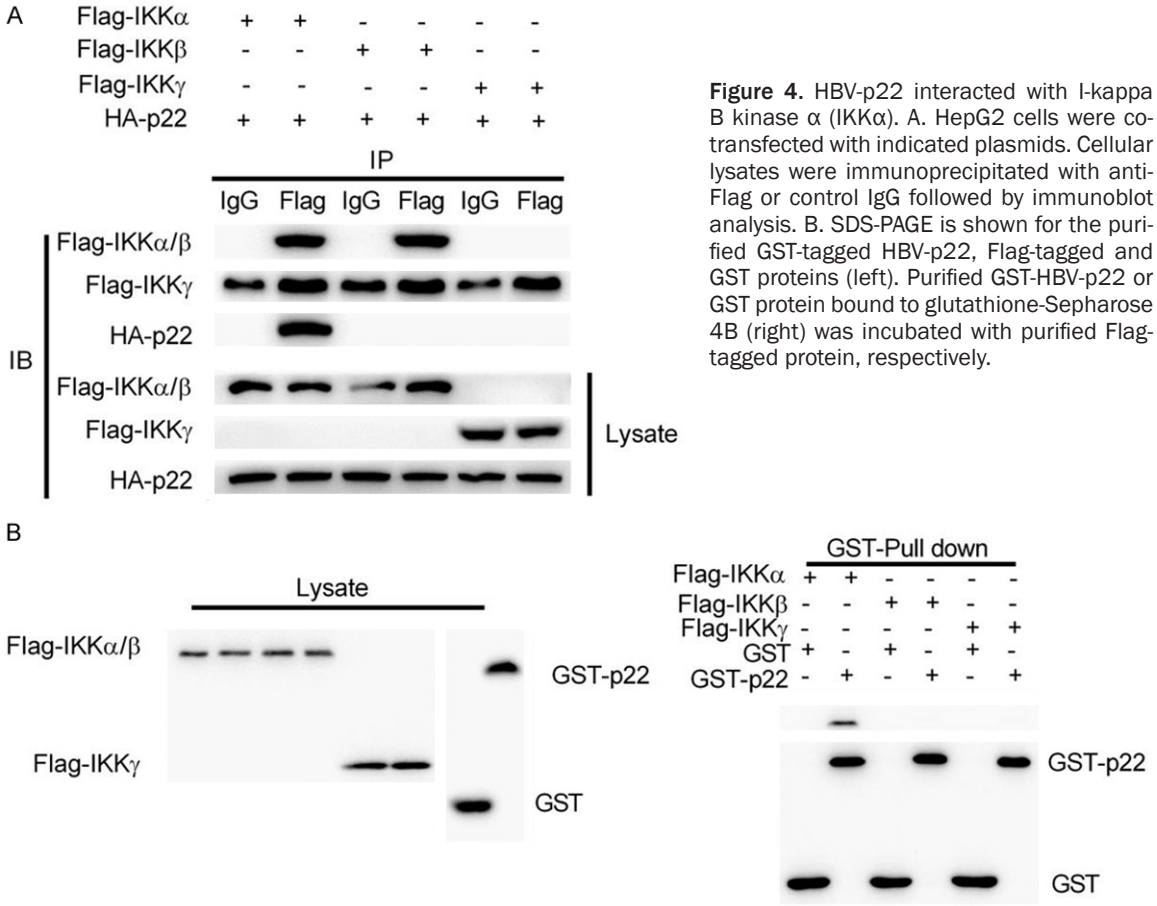
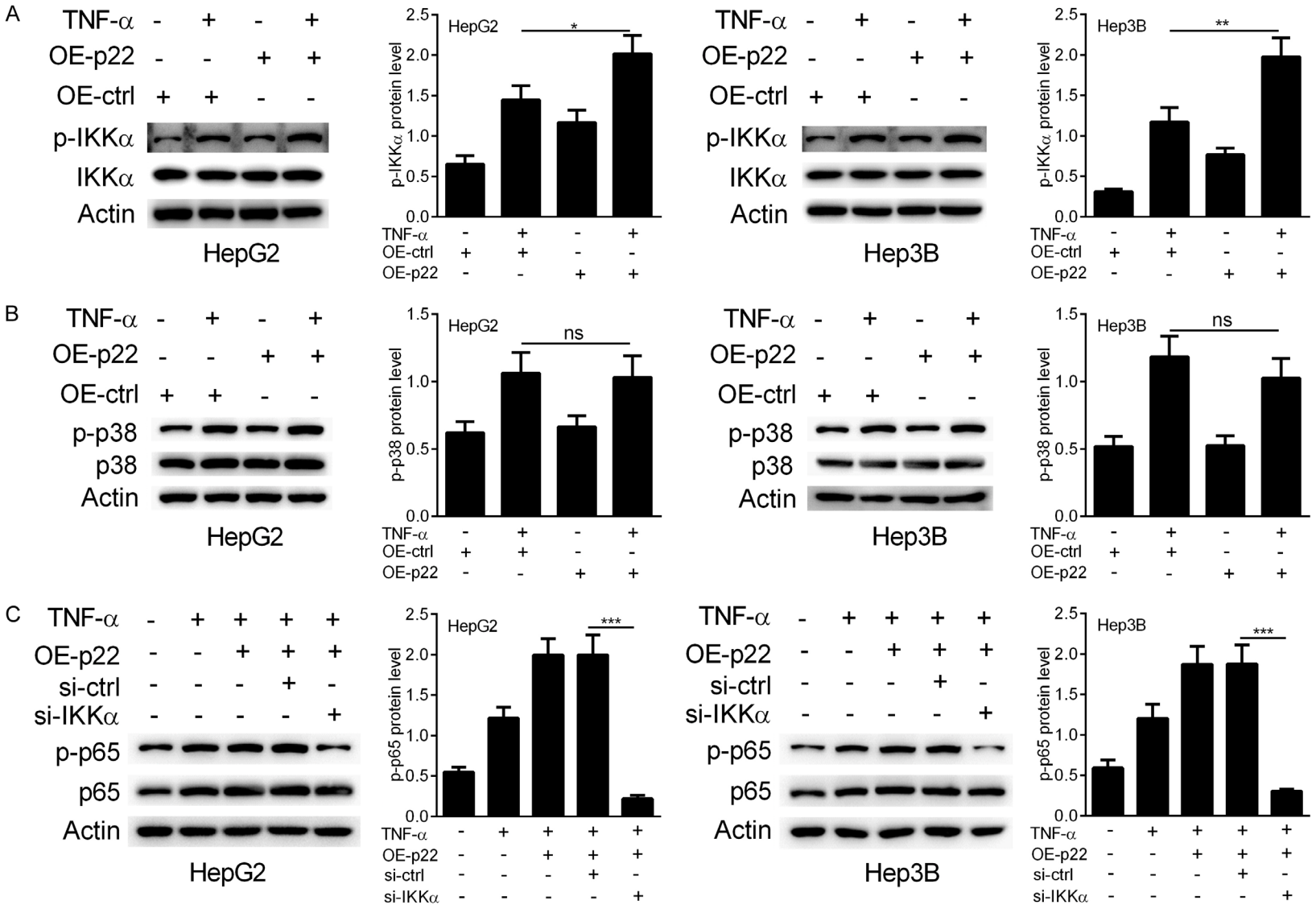


Figure 4. HBV-p22 interacted with I-kappa B kinase α (IKKα). A. HepG2 cells were co-transfected with indicated plasmids. Cellular lysates were immunoprecipitated with anti-Flag or control IgG followed by immunoblot analysis. B. SDS-PAGE is shown for the purified GST-tagged HBV-p22, Flag-tagged and GST proteins (left). Purified GST-HBV-p22 or GST protein bound to glutathione-Sepharose 4B (right) was incubated with purified Flag-tagged protein, respectively.

sized that there is an interplay between the HBV-p22 and IKK complex. Results from co-immunoprecipitation assays indicated that p22 did indeed interact with IKKα, but not with IKKβ or IKKγ (**Figure 4A**). An *in vitro* pull-down assay was conducted to rule out the possibility of artefact resulting from exogenous overexpression, whereby Flag-IKKα was expressed in HepG2 cells while GST-p22 was expressed and purified from E. coli. The results of GST-pull down showed that recombinant GST-p22 exhibited specific binding to Flag-IKKα, while GST alone failed to form such a complex (**Figure 4B**). These findings collectively support the notion that p22 interacts with IKKα in a dynamic and intricate manner.

HBV-p22 regulated TNFα-induced apoptosis depending on IKKα

Since IKKα activation was critical for NF-κB signaling, thus, it was tested whether the reduction of TNFα-induced apoptosis by HBV-p22 was through an IKKα-dependent manner. It was found that overexpression of HBV-p22 obviously increased TNFα-induced phosphorylation of endogenous IKKα (**Figure 5A**). In contrast, the phosphorylation of mitogen-activated protein kinase p38 was not significantly affected by HBV-p22 (**Figure 5B**), suggesting that the observed enhancement effect was restricted to the phosphorylation of IKKα. To determine that HBV-p22 promoted NF-κB signaling by regulation of IKKα, siRNA-mediated knockdown experiments was performed to downregulate IKKα expression and examine whether IKKα has an impact on the effect of HBV-p22 on NF-κB activation. Compared with si-ctrl transfection, si-IKKα group transfection inhibited the enhancement effect of HBV-p22 on NF-κB activation (**Figure 5C**). In addition, the apoptosis assay indicated that the knockdown of IKKα obviously blocked the protective effect of HBV-p22 on TNFα-induced apoptosis (**Figure 5D**). These findings together demonstrated that HBV-p22 regulated TNFα-induced NF-κB activation and apoptosis through enhancing IKKα phosphorylation.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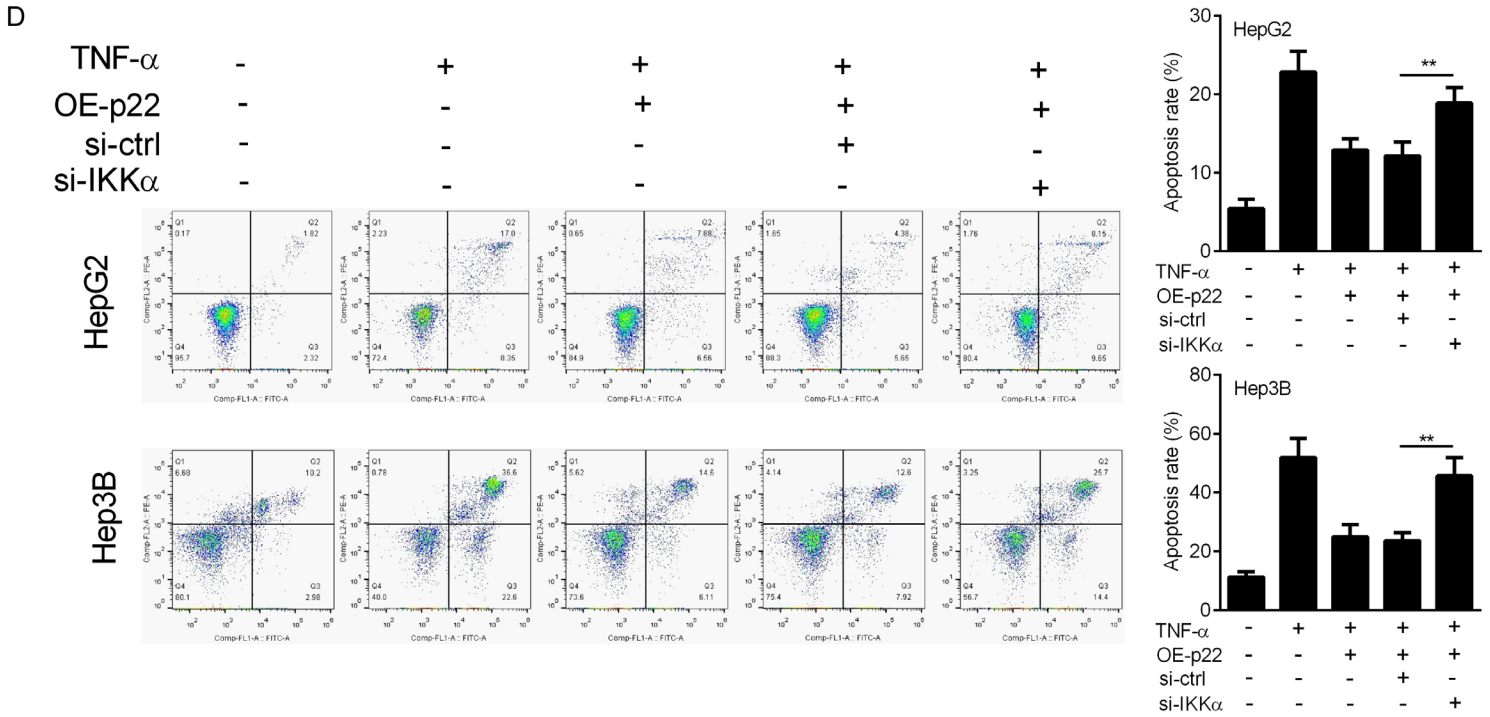

Figure 5. HBV-p22 regulated TNFα-induced NF-κB activation and apoptosis depending on IKKα. A. HepG2 and Hep3B cells were transfected with the pCMV-HBVp22-HA or the control empty vector, and then treated with or without TNFα for 1 h. The protein levels of phosphorylated IKKα and total IKKα were determined using western blot. B. HepG2 and Hep3B cells were transfected with the pCMV-HBVp22-HA or the control empty vector, and then treated with or without TNFα for 1 h. The protein levels of phosphorylated p38 and total p38 were determined using western blot. C. HepG2 and Hep3B cells were transfected with indicated plasmids and siRNA, and then treated with or without TNFα for 1 h. The protein levels of IKKα, phosphorylated p65, and total p65 were determined using western blot. D. HepG2 and Hep3B cells were transfected with indicated plasmids and siRNA, and then treated with or without TNFα for 1 h. Cell apoptosis was evaluated using flow cytometry. *P<0.05; **P<0.01; ***P<0.001; ^{ns}P>0.05.

Discussion

As both a serological indicator and nonstructural protein of HBV, HBeAg is commonly linked to the presence of hyper-viremia in individuals presenting with chronic HBV infection [21, 22]. Among all HBV proteins, HBV protein X (HBx) is the most widely studied. Evidence has suggested that HBX plays an important role in HCC progression [23]. For example, HBx modulated the DNA repair mechanism, DNA methylation, and non-coding RNA (ncRNA) signaling pathway of the host cells [24-26]. These activities may potentially lead to the progression of HCC.

These activities might contribute to the HCC progression. However, studies about HBV precore protein p22 remain scarce. In this study, the role of HBV-p22 in TNFα-induced apoptosis was identified. During infection, HBV regulated apoptosis signal transduction through various mechanisms to ensure its survival in host cells. The findings of the current investigation demonstrated that the presence of HBV-p22 resulted in the inhibition of apoptosis in hepatoma cells (**Figure 1**), which was in agreement with our prior investigation [27]. The activation of JNK signaling was required for TNFα-induced apoptosis in hepatoma cells [18]. In contrast, constitutive activation of JNK signaling has been linked to proliferation and resistance to antineoplastic agents in hepatoma cells [28]. HBV core protein (HBc) increased the secretion of interleukin-6 in hepatoma cells by enhancing the activation of JNK signaling [29]. The present investigation discovered that overexpression of HBV-p22 was found to suppress TNFα-induced JNK activation (**Figure 2**), suggesting the involvement of HBV-p22 in the control of JNK signaling. However, Bidisha et al. reported that HBV-p22 helped the virus evade the host immune response by blocking STAT1 signaling [30], indicating that the effect of HBV-p22 is likely to vary between different signaling pathways.

NF-κB has been demonstrated to play a crucial role in the development of chronic hepatitis and hepatocarcinogenesis [31]. It is widely believed that TNFα treatment can initiate the NF-κB pathway, which subsequently enhances the transcription of pro-inflammatory and anti-apoptotic target genes. TNFα activates caspase-8, which subsequently initiates the pro-apoptotic caspase-cascade. Meanwhile, caspase-8 is controlled by c-FLIP, a target gene antagonist regulated by NF-κB. HBx was reported to be implicated in the regulation of NF-κB signaling. For instance, the promoter activity of the Human Endogenous Retrovirus W family (HERV-W) envelope (env) was enhanced by HBx in HepG2 cells via the NF-κB pathway [32]. Furthermore, HBx regulated pro-inflammatory cytokines expression and ROS production by means of p38 phosphorylation and NF-κB activation [33]. Another HBV protein, HBc, was shown to promote the activity of the C5α receptor 1 (C5AR1) promoter via the NF-κB pathway in hepatoma cells [29]. In this study, it was observed that gene transcription of pro-inflammatory cytokines (IL-8 and c-FLIP), as well as TNFα-induced activation of the NF-κB were positively regulated by HBV-p22 (**Figure 3**). Our previous study also showed HBV-p22 enhanced the nuclear translocation of NF-κB after apoptosis induction [34], yet the molecular mechanisms remain unclear. The IKK complex contained three subunits, IKKα, IKKβ, and IKKγ, and HBV-p22 exhibits a selective interaction with IKKα rather than IKKβ and IKKγ. Importantly, after TNFα stimulation, it was discovered that the regulation of this interaction was time-dependent (**Figure 4**). A potential implication of this interactive process is the development of a novel regulatory mechanism for modulating IKKα activation, unlike the way in which A20 and CYLD control IKK activity following complete activation of IKKα [35, 36]. In the present study, we demonstrated the physical connection as well as functional interaction between HBV-p22 and IKKα, insinuating that the protective effect of HBV-p22 on TNFα-induced apoptosis was dependent on IKKα (**Figure 5**). These observations extended our knowledge and offered a novel perspective on the control of the NF-κB signaling pathway. However, how HBV-p22 affect IKKα remains a question. Further, whether HBV-p22 interacted with other regulators of the NF-κB pathway warrant further investigation.

Collectively, our findings have revealed the involvement of HBV-p22 in the downregulation of TNFα-induced NF-κB activation, which may facilitate the development of novel hepatitis B therapeutics and improve the effectiveness of TNFα-based immunomodulatory therapies. Although we have conducted a series of experi-

ments in cell culture, we have not yet verified the effect of HBV-p22 expression on the TNFα-induced NF-κB pathway in animal models or clinical environments. In the future, *in vivo* studies should be carried out to explore the effect of inhibiting HBV-p22 expression on the apoptosis of hepatocellular carcinoma, which will help to further elucidate the anti-apoptotic mechanism of HBV-p22.

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Disclosure of conflict of interest

None.

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Table S1. Primary antibodies

No	Antibodies	Dilution	Catalogue Number	Manufacturer
1	Actin	1:5000	cat. no. 3700S	Cell Signaling Technology, Inc.
2	Cleaved-PARP	1:2000	cat. no. 5625S	Cell Signaling Technology, Inc.
3	Cleaved-caspase3	1:2000	cat. no. 9664S	Cell Signaling Technology, Inc.
4	Phosphor-JNK	1:1000	cat. no. ab124956	Abcam
5	Total-JNK	1:2500	cat. no. ab237119	Abcam
6	Phosphor-c-Jun	1:1500	cat. no. ab32385	Abcam
7	Total-c-Jun	1:3000	cat. no. ab40766	Abcam
8	Phosphor-MKK7	1:1000	cat. no. ab278704	Abcam
9	Total-MKK7	1:2000	cat. no. ab52618	Abcam
10	HA-tag	1:3000	cat. no. 2367S	Cell Signaling Technology, Inc.
11	Phospho-NF-κB p65	1:1500	cat. no. 3033S	Cell Signaling Technology, Inc.
12	Total-NF-κB p65	1:2500	cat. no. 8242S	Cell Signaling Technology, Inc.
13	IKKα	1:2000	cat. no. 2682S	Cell Signaling Technology, Inc.
14	Phospho-IKKα	1:1000	cat. no. 14938S	Cell Signaling Technology, Inc.
15	IKKβ	1:3000	cat. no. 8943S	Cell Signaling Technology, Inc.
16	IKKγ	1:2500	cat. no. 2695S	Cell Signaling Technology, Inc.
17	Flag-tag	1:5000	cat. no. 14793S	Cell Signaling Technology, Inc.
18	GST-tag	1:5000	cat. no. 2622S	Cell Signaling Technology, Inc.
19	p38	1:2000	cat. no. ab170099	Abcam
20	Phospho-p38	1:2000	cat. no. ab178867	Abcam