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NLRX1 can counteract innate immune response induced by an external stimulus favoring HBV infection by competitive inhibition of MAVS-RLRs signaling in HepG2-NTCP cells

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Abstract

Introduction This study is aimed at the determination of the effect of the immune-regulatory factor NLRX1 on the antiviral activity of hepatocytes against an external stimuli favoring hepatitis B virus infection, and to explore its mechanism of action.

Methods A HepG2-NTCP model was established using the LV003 lentivirus. Cells were transfected using an overexpression vector and NLRX1 siRNA to achieve overexpression and

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interference of NLRX1 expression (OV-NLRX1, si-NLRX1). Levels of HBsAg and HBcAg were determined using Western blotting analysis and immunohistochemical analysis. The levels of hepatitis B virus DNA and hepatitis B virus cccDNA were determined by real-time quantitative polymerase chain reaction. The expression and transcriptional activity of IFN-α, IFN-β, and IL-6 were measured using real-time quantitative polymerase chain reaction, enzyme-linked immunosorbent assay, and promoter-luciferase reporter plasmids. Co-immunoprecipitation was used to determine the effect of NLRX1 on the interaction between MAVS and RIG-1. Western blotting was used to obtain the phosphorylation of essential proteins in the MAVS-RLRs signaling pathways.

Results NLRX1 promoted HepG2-NTCP cell hepatitis B virus infection. Compared to the control group, the levels of HBsAg, HBcAg, hepatitis B virus cccDNA, and hepatitis B virus DNA increased in the OV-NLRX1 group and decreased in the si-NLRX1. Co-immunoprecipitation results showed that NLRX1 competitively inhibited the interaction between MAVS and RIG-1, and inhibited the phosphorylation of p65, IRF3, and IRF7. Additionally, NLRX1 reduced the transcription activity and expression levels of the final products: IFN-α, IFN-β, and IL-6.

Conclusions NLRX1 can counteract innate immune response induced by an external stimuli favoring hepatitis B virus infection by competitive inhibition of MAVS-RLRs signaling in HepG2-NTCP cells. Inhibition of the MAVS-RLR-mediated signaling pathways leads to a decline in the expression levels of I-IFN and IL-6.

Keywords
Immuno-regulatory factors, NLRX1, hepatitis B virus, MAVS-RLRs, cytokines, antiviral capability

Introduction

Hepatitis B virus (HBV) infection causes life-threatening liver diseases such as cirrhosis and liver cancer.[1,2] Although the treatment of HBV infection with nucleoside analogs and interferon (IFN) yields excellent viral response, they do not eliminate the virus.[3,4] Effective regulation of the host's immune function is needed to inhibit virus replication and obtain lasting viral control. Recently, immune regulation has become an essential topic of research regarding the treatment of hepatitis virus infections.[5,6] Toll-like receptor (TLR) agonists inhibit HBV replication by promoting the production of IFN type 1 (I-IFN; IFN-α/IFN-β).[7] For example, GS-9620 is a TLR-7 agonist that is effective in reducing the spread of chimpanzee HBV DNA and groundhog HBsAg.[8] The development of vaccines to enhance the immune response is a recent strategy explored for treating chronic HBV infection.[9,10] Currently, most immune regulation studies have focused on TLRs and RIG-1-like receptors (RLRs).[11,12] The study demonstrated that retinoic acid-induced gene 1 (RIG-1), a RLR, can induce IFN-β production and viral replication in PK-15 cells after infection with porcine circovirus virus type 2.[13] The study also reported that RIG-I knockdown can heavily reduce the production of IL-6, IFN-α, and IFN-β in response to classical swine fever virus infection.[14]

Previous studies found that the encoding gene variation of NLRX1 (NLRX1 p.Arg707Cys) enhanced the susceptibility of host cells to HBV infection.[15] NLRX1 is the only NOD-like receptor (NLR) that localizes on the outer membrane of mitochondria.[16] NLRX1 has a dual role in immune regulation.[17] NLRX1 can inhibit MAVS and RIG-1 binding, which decreases the activation of NF-κB, IRF3, and IRF7 in the downstream MAVS-RIG-1 signaling pathway leading to reduced expression of I-IFN and interleukin (IL)-6 thus, suppressing innate immune response.[18,19] NLRX1 can also promote cell

autophagy by binding to the translation elongation factor (TUFM) of the mitochondrial protein Tu, and to the autophagy-related proteins Atg5-Atg12, and ATG16L1, which contribute to the survival of viruses.[20] However, when induced by poly I:C, tumor necrosis factor, or Shigella, NLRX1 promotes reactive oxygen species (ROS) production by binding to UQCRC2. ROS increased the activity of NF-κB and c-Jun n-terminal kinase and increased the expression of I-IFN, which promotes the antiviral capability of cells.[21]

Currently, no study has examined the role of NLRX1 in HBV infection. According to the study, we speculated that NLRX1 is involved in immune response regulation during hepatocytes against an external stimulus favoring HBV infection. Thus, this study aims to determine the effect of NLRX1 on HBV infection and the mechanism of action. This was done by changing the expression level of NLRX1 in HepG2 cells overexpressing Na + /taurocholate Co-transporting polypeptide (NTCP).

Materials and methods

Construction of a HepG2 cell line overexpressing NTCP

As previously reported,[22,23] the HepG2-NTCP cell line was successfully constructed. Briefly, the NTCP gene sequence (Human CHODL NM_001204174.1, NTCP cDNA) was obtained from NCBI. Polymerase chain reaction (PCR) was used to amplify NTCP cDNA, followed by insertion of the viral vector LV-003. The NTCP primers were provided by Forevergen Biotechnology (Guangzhou, China), and are exhibited in Table 1. The 293 T cells (provided by National Collection of Authenticated Cell Cultures, Shanghai, China, #SCSP-502) were co-transfected with LV-003-NTCP, packaged, and cultured for 48 h. The lentiviruses were recovered by centrifugating at 25,000 rpm for 1.5 h using a Beckman centrifuge (Fullerton, California, USA). The lentiviruses were used to infect HepG2 cells (provided by National Collection of Authenticated Cell Cultures, #SCSP-510), to which polybutadiene (6 mg/ml) was added for 48 h. The cells were cultured for 10 to 15 days in a medium containing puromycin (2 μg/ml) to form stable cell lines.

Overexpression and interference of NLRX1

The NLRX1 gene sequence (Human NLRX1, NM_001282143.1, 2928 nt) was extracted from NCBI. A target gene expression plasmid containing XbaI TCTAGA + 2928 bp + NotI GCGGCCGC was constructed using His tag (Forevergen Biotechnology, Guangzhou, China). NLRX1 siRNA was purchased from GenePharma (Suzhou, China), and the sequences of NLRX1 siRNA were 5′-GAGGAGGACU ACUACAACGAU-3′ (sense) and 5′-CUCCUCCUGAUGAUGUUGCUA-3′ (antisense). The plasmid and siRNA was transiently transfected into HepG2-NTCP cells for 48 h to achieve overexpression and interference of NLRX1 expression using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA, # L3000015).

HBV infection

The protocol of HBV serum collection and HBV infection was performed as previous studies.[24,25] In brief, 10 ml of serum samples from patients infected with HBV (HBV

Table 1. The amplification primers of genes in real-time quantitative polymerase chain reaction (RT-qPCR) assay.

	Upstream primers	Downstream primers
HBV DNA	5′-ATGGAGAACACAACATCAGG-3′	5′-GAGGCATAGCAGCAGGATG-3′
HBV cccDNA	5′-GTCTGTGCCTTCTCATCTGCC-3′	5′-ACAGCTTGGAGGCTTGAACAG-3′
IFN-α	5′-CCTGTGTGATGCAGGAGGAG-3′	5′-CCAGGCACAAGGGCTGTATT-3′
IFN-β	5′-TGCTCTCCTGTTGTGCTTCT-3′	5′-AGCCTCCCATTCAATTGCCA-3′
IL-6	5′-CTTCGGTCCAGTTGCCTTCT-3′	5′-TGGAATCTTCTCCTGGGGGT-3′
GAPDH	5′-GAGTCAACGGATTTGGTCGT-3′	5′-GACAAGCTTCCCGTTCTCAG-3′
NTCP	5′-ATGCTCTAGAGCCACCATGGAGGCCCAC-3′	5′-ATGGGGATCCCTAGGCTGTG CAAGGGGAG-3′
NLRX1	5′-CGCTGTTTTGACCTCCATAGAAGATTCTAGA ATGAGGTGGGGCCACCA-3′	5′-GCACCGGAGCGATCGCAGATCCTTCGCGGCCGCT CAGCTTCCAGAGCTTCCC-3′

DNA level >10^8 IU/ml) were collected in 2017, and then were centrifuged at 2000 × *g* for 30 min to obtain HBV serum. The patients were treated at the Department of Infectious Diseases of the Third Affiliated Hospital of Sun Yat-sen University.

For infection, 24-well plate was treated with 500 µl Collagen I solution (50 ug/ml) at 37 °C for 30 min, and the Collagen I solution was recovered. The HepG2-NTCP cells (1×10^6 cells/ml) were spread into a 24-well plate and cultured in DMEM with 10% FBS for 24 h, then medium was replaced with DMEM containing 10% HBV serum, 2% DMSO, and 4% PEG8000 and continue culture for 48 h prior to further experiments. The virus was applied to infect HPG2-NTCP cells at MOI = 300 (about 3×10^8 copies per well). In addition, for HBV infection of NLRX1 overexpression or interference HepG2-NTCP, NLRX1 overexpression plasmid or siRNA was transfected into HepG2-NTCP for 48 h, and then the cells were infected in the same manner as described above. This study was approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University for Human Study, and was conducted according to the principles of the Declaration of Helsinki (2013).

DNA extraction and PCR analysis

HBV serum-treated HepG2-NTCP cells were added with a lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl, 1% SDS) containing proteinase K (200 µg/ml) and allowed to stand at room temperature for 4 h at 65 °C for cell lysis. The DNA was extracted using a QIAGEN QIAamp DNA mini kit (Qiagen, Hilden, Germany, #51304) according to the instructions. And the levels of HBV DNA, HBV cccDNA were confirmed through PCR analysis.

Real-time quantitative PCR (RT-qPCR) assay

Total RNA was extracted from HBV serum-treated HepG2-NTCP cells by applying the TRIzol reagent (Invitrogen, # 10296010). Reverse transcription kit (Takara, Tokyo, Japan, #RR047Q) was adopted and used to synthesize cDNAs using RNA as a template. The levels of IFN-α, IFN-β, and IL-6 were determined by PCR with SYBR Green qPCR master Mix (DBI Bioscience, Shanghai, China, #DBI2044). GAPDH served as the internal reference. Gene levels were counted using $2^{-\Delta\Delta CT}$ method. The sequences of all primers are presented in Table 1.

Western blotting analysis

The HepG2-NTCP cells were treated with the lysis buffer, and the total protein concentration was determined using bicinchoninic acid assay. Equal amounts of total protein were separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, transferred to a polyvinylidene fluoride (PVDF) membrane, and blocked with 5% skim milk powder at room temperature for 1 h. The PVDF membrane was washed with tris buffered saline with Tween-20 (TBST) containing NaCl, Tris-HCl, and Tween-20, and incubated overnight at 4 °C using the primary antibody to the target protein, followed by washing two times with TBST. The PVDF membranes were incubated with the secondary antibody to the target protein for 1 h at room temperature, followed by

washing three times with TBST. The protein bands were observed with a chemiluminescence method (ECL, Forevergen, Guangzhou, China). Detailed information of primary antibodies are as follows: anti-HBsAg (1:1000; Santa Cruz, CA, USA, #sc-53299), anti-HBcAg (1:1000; Santa Cruz, #sc-23947), anti-NLRX1 (1:2000; Proteintech, Wuhan, China, #17215-1-AP), anti-NTCP (1:1000; Santa Cruz, #sc-518115), anti-MAVS (1:2000; Abcam, #ab31334), anti-RIG-1 (1:2000; CST, #3743 T), anti-p65 (1:500; Abcam, #ab7970), anti-p-p65 (1:1000; CST, #3033S), anti-IRF-3 (1:1000; CST, #4302), anti-p-IRF-3 (1:1000; CST, #29047), anti-IRF-7 (1:1000; CST, #13014), anti-p-IRF-7 (1:1000; CST, #12390), and anti-GAPDH (1:8000; Proteintech, 60004-1-Ig).

Immunohistochemistry (IHC) assay

HbsAg was monitored by IHC in HepG2-NTCP cells treated with HBV serum. After transfection with NLRX1 overexpression plasmid or siRNA, cells were collected at cell density of 5×10^6 cells/ml and added to sterile cell slippage for 3 h of culture at 37 °C. The cells were processed with 4% paraformaldehyde for 20 min, 0.5% Triton X-100 for 20 min, and 3% H_2O_2 for 15 min. After processing, cells were blocked with 5% normal goat serum (Invitrogen, #16210072) for 2 h, primary antibodies including anti-HBs (1:50, ab9193, Abcam, Cambridge, UK), anti-HBc (1:50, ab8639, Abcam) overnight at 4 °C, and goat anti-mouse or rabbit IgG secondary antibody (1:500, Abcam) for 1 h. After washing, cells were addressed with streptavidin-biotin-peroxidase (SABC, Boster, Wuhan, China, #SA1020) at 37 °C for 20 min and colored with diaminobenzidine hydrochloride (DAB, Boster, #AR1022). The color development under the light microscope was observed and recorded.

Enzyme-linked immunosorbent assay (ELISA) detection

A human IFN-α ELISA kit (Cusabio, Wuhan, China, #CSB-E08636 h), a human IFN-β ELISA kit (Cusabio, CSB-E09889 h), and a human IL-6 ELISA kit (Cusabio, #CSB-E04638 h) were used to detect the levels of IFN-α, IFN-β, and IL-6 in the supernatant of HBV serum-treated HepG2-NTCP cells, according to the supplied instructions.

Luciferase reporter gene experiment

Based on the predicted binding sites between NLRX1 and IFN-α, IFN-β, and IL-6 promoter regions, we constructed the IFN-α, IFN-β, and IL-6 plasmids with psiCHECK-2 vector. HepG2-NTCP cells (1×10^5 cells/well) in a 24-well plate were transfected with NLRX1 overexpression plasmid or siRNA and IFN-α, IFN-β, or IL-6 promoter fluorescent reporter plasmids using Lipofectamine 3000 for 48 h. Luciferase activity was confirmed using a Trans Detect Double-Luciferase Reporter Assay Kit (Transgen, Beijing, China, #FR201) in line with the manufacturer's instructions.

Detection of ROS levels

A ROS assay kit (Beyotime Biotechnology, Shanghai, China, #S0033S) was used to detect ROS levels before and after HBV infection of HepG2-NTCP cells. The in-situ

loading probe method was applied, cells were collected and examined using a flow cytometry. The parameters for detecting ratio of the positive DCF (ROS ratio) were set based on the parameters of FITC.

Co-immunoprecipitation (CO-IP) assay

CO-IP was used to examine the effect of NLRX1 on the binding of MAVS to RIG-1 in HepG2-NTCP cells. Lysate (RIPA buffer) was added to lyse the cells, and the supernatants were collected after centrifugation at 14,000 rpm for 15 min. Total protein was diluted to a concentration of 1 μg/μl with PBS. An amount of 1 μg of rabbit antibody (Anti-MAVS, Abcam, #ab31334; or Anti-Rabbit IgG Isotype Control, Boster, #BM4020) was added to 500 μl of total protein, the mixture was incubated at room temperature for 2 h. Then, 100 μl of Agarose-protein A was added, and the mixture was incubated at room temperature for 1 h. After centrifugation at 14,000 rpm for 5 s, the antigen–antibody complexes binding to the agarose beads were collected and washed three times with 800 μl of RIPA buffer. Finally, 60 μl of 2 × SDS was added, and the mixture was treated with boiling water for 10 min. SDS-PAGE and Western blotting were performed as previously described.

Statistical analysis

Normally distributed data were described by mean ± standard deviation (SD), and the Mann–Whitney U test was used to compare the two groups. A one-way analysis of variance was used to compare multiple groups. Data analysis was conducted with SPSS version 17.0 software, and values of $P < 0.05$ indicated statistical significance.

Results

Construction of HepG2 cells overexpressing NTCP

Preliminary experiments showed that the HBV infection rate of HepG2-NTCP cells increased, and the expression of NLRX1 was moderate in HepG2 cells.[26] HepG2-NTCP cells were constructed for the study. After infection of HepG2 cells with the LV003 lentivirus integrating the human NTCP gene, fluorescence microscopy expressed the percentage of GFP-labeled cells as >95% (Figure 1(A)). According to flow cytometry results, there was no difference in intracellular ROS fluorescence intensity before and after HBV serum treatment, suggesting that intracellular ROS did not increase after HBV serum treatment (Supplemental Fig. S1). Besides, Western blotting and RT-qPCR results indicated that the expression of NTCP in HepG2-NTCP cells increased compared to the control group (HepG2-NC) (Figure 1(B) and (C)). This result indicated that HepG2-NTCP cells were successfully constructed.

HBV replication in HepG2-NTCP cells

To prove that HepG2-NTCP cells can be infected by HBV and to judge the effect of NLRX1 in HBV serum treatment, HepG2-NTCP cells were examined after HBV

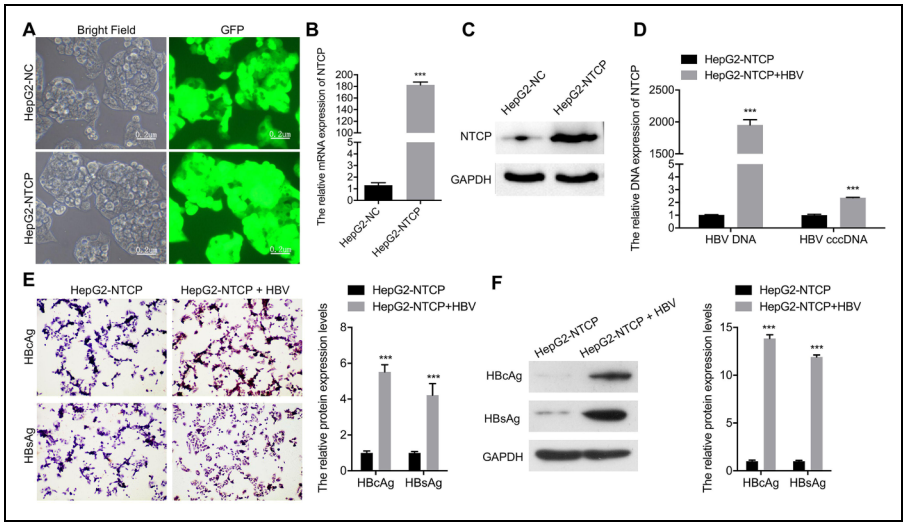

Figure 1. The successful construction of HepG2-NTCP cell lines. LV-003 (NC) and LV-003-NTCP lentiviruses were used to infect HepG2 cells for 48 h, and then cells were cultured for 10 to 15 days in a medium containing puromycin to form stable cell lines, HepG2-NC and HepG2-NTCP. (A) GFP expression was observed using fluorescence microscopy in HepG2-NC and HepG2-NTCP cells (magnification 200×). (B) The level of NTCP mRNA was determined by RT-qPCR in HepG2-NC and HepG2-NTCP cells. (C) The level of NTCP protein was confirmed using western blotting in HepG2-NC and HepG2-NTCP cells. (D) HBV DNA and HBV cccDNA levels were obtained using PCR in HepG2-NTCP cells after treatment with or without HBV serum for 48 h. Levels of HBsAg and HBcAg were determined by IHC (E, magnification 400×), and Western blot assays (F) in HepG2-NTCP cells after treatment with or without HBV serum for 48 h. All experiments were independently repeated for three times. Mann–Whitney *U* test was applied for the comparison of two sets of data.
HBV: hepatitis B virus; RT-qPCR: real-time quantitative polymerase chain reaction;
IHC: Immunohistochemistry.
***: $P<0.001$.

serum treatment. Western blotting and RT-qPCR showed that the levels of HBV DNA, HBV cccDNA, HBsAg, and HBcAg in HepG2-NTCP cells were higher than in the control group (Figure 1(D) to (F)), this suggested that HepG2-NTCP cells can be infected with HBV and can allow HBV replication and generation of virus particles after treated with HBV serum.

HBV serum treatment upregulated NLRX1 and increased the levels of cytokines in HepG2-NTCP cells

The expression of NLRX1 mRNA and proteins were higher than in the control group as discovered (Figure 2(A) and (B)), suggesting that NLRX1 is involved in the HBV infection process after treated by HBV serum. Further analysis showed that the levels of IFN-α, IFN-β, and IL-6 increased in HBV serum-treated HepG2-NTCP cells relative to that in HepG2-NTCP cells, especially IFN-α (Figure 2(C) and (D)), which suggests that antiviral cytokines are expressed in HepG2-NTCP cells after HBV serum treatment.

Effects of NLRX1 overexpression or knockdown on the antiviral capacity in HBV serum-treated HepG2-NTCP cells

To clarify the function of NLRX1, we altered the expression of NLRX1 in HepG2-NTCP cells (interference, si-NLRX1; overexpression, OV-NLRX1). Compared to the NC group, the expression of NLRX1 mRNA and protein decreased in the si-NLRX1

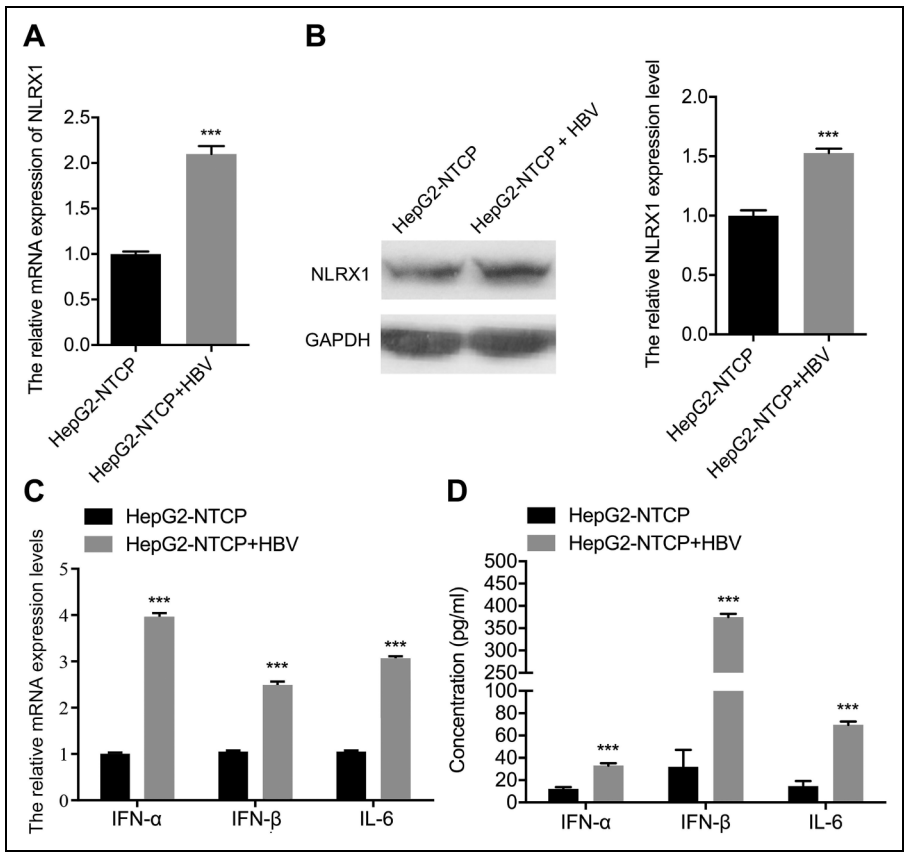

Figure 2. The effect of NLRX1 on HepG2-NTCP cells after HBV serum treatment. (A) RT-qPCR was used to measure the mRNA expression of NLRX1 in HepG2-NTCP cells after treatment with or without HBV serum for 48 h. (B) Western blotting was used to determine the expression of NLRX1 in HepG2-NTCP cells after treatment with or without HBV serum for 48 h. The levels of IFN-α, IFN-β, and IL-6 were determined by RT-qPCR (C), and ELISA (D) assays in HepG2-NTCP cells after treatment with or without HBV serum for 48 h. All experiments were independently repeated for three times. Mann–Whitney *U* test was applied for the comparison of two sets of data.

HBV: hepatitis B virus; RT-qPCR: real-time quantitative polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay.

***: $P < 0.001$.

group and increased in the OV-NLRX1 group (Figure 3(A) and (B)). Additionally, it was proven that compared to the NC group (HepG2-NTCP), the expression levels of HBV DNA, HBV cccDNA, HBcAg, and HBsAg decreased drastically in the si-NLRX1 group, and were increased in the OV-NLRX1 group (Figure 3(C) and (D)). IHC studies also showed that compared to the NC group, the numbers of HBsAg and HBcAg particles decreased in the si-NLRX1 group, while it increased in the OV-NLRX1 group (Figure 3(E)), suggesting that NLRX1 promotes HBV infection in HepG2-NTCP cells. Moreover, the result revealed that compared to the NC group, the levels of IFN-α, IFN-β, and IL-6 increased in the si-NLRX1 group and decreased in the OV-NLRX1 group (Figure 3(F) and (G)), this suggests that NLRX1 inhibits the anti-viral ability of HepG2-NTCP cells.

NLRX1 inhibits the interaction between RIG-I and MAVS

It is reported that the association of RIG-I-MAVS only occurred in Nlrx1−/− cells in the absence of viral infection and NLRX1 could reduce inflammatory response to infection by blocking RIG-I-MAVS and TRAF6-NF-κB pathways. To further explore the impact of NLRX1 on interaction between RIG-1 and MAVS, we adopted CO-IP analysis to determine the interaction between NLRX1 and MAVS, and between MAVS and RIG-1, which were mediated by NLRX1 in HepG2-NTCP cells. The results showed that MAVS-specific antibodies could precipitate NLRX1 and RIG-1. The silence of NLRX1 enhanced the interaction between MAVS and RIG-1, and weakened the interaction between NLRX1 and MAVS; overexpression of NLRX1 dramatically attenuated the interaction between MAVS and RIG-1, and increased the interaction between NLRX1 and MAVS (Figure 4). This result showed that NLRX1 competitively inhibits the interaction between MAVS and RIG-1 in HepG2-NTCP cells.

The effect of NLRX1 on activation of the MAVS-RLR downstream related signaling pathways in HepG2-NTCP cells after HBV serum treatment

Subsequently, we further explored the influences of NLRX1 on the MAVS-RLR-related signaling pathways in HBV serum-treated HepG2-NTCP cells. As presented in Figure 5(A) and (B), the phosphorylation levels of p65, IRF3, and IRF7 decreased in the NLRX1 silencing group compared with that in the NC group, and the expression of these three phosphorylated proteins decreased in the NLRX1 overexpression group with respect to that in the NC group. The result indicates that NLRX1 could inhibit the activation of p65, IRF3, and IRF7. Also, we discovered that the silence of NLRX1 induced the luciferase activities of IFN-α, IFN-β, and IL-6 promoter, and overexpression of NLRX1 attenuated luciferase activities of IFN-α, IFN-β, and IL-6 promoter in HBV serum-treated HepG2-NTCP cells (Figure 5(C)). Thus, we proved that NLRX1 overexpression could lower the transcriptional activation of IFN-α, IFN-β, and IL-6 in HBV serum-treated HepG2-NTCP cells.

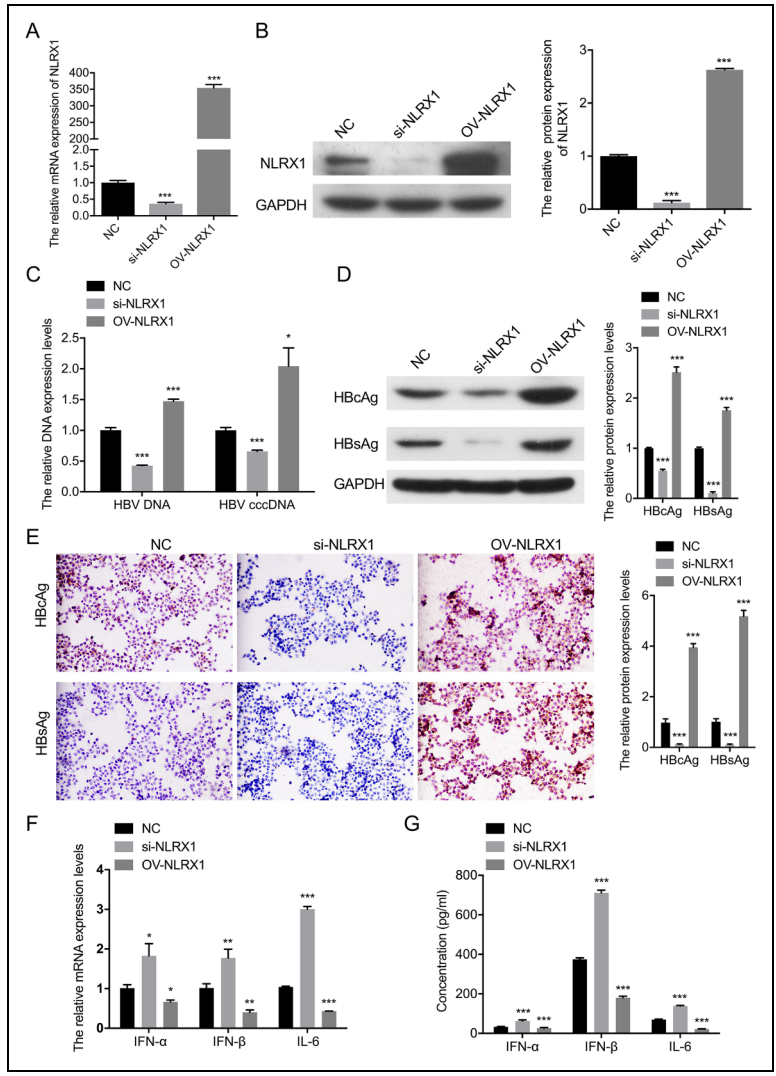

Figure 3. Analysis of the antiviral capability of HepG2-NTCP cells after interference or overexpression of NLRX1. The HepG2-NTCP cells were transfected with NLRX1-overexpressed plasmid or NLRX1 siRNA for 48 h, and then were treated with HBV serum for another 48 h prior further analysis. The level of NLRX1 was obtained using RT-qPCR (A) and western blotting (B). (C) Expression of HBV DNA and HBV cccDNA were detected by PCR. (D) Western blotting was conducted to examine the protein expressions of HBsAg and HBcAg. (E) HBcAg and HBsAg particles were observed by IHC staining (hematoxylin, silver-gray particles, magnification 400×). The levels of IFN-α, IFN-β, and IL-6 were determined using RT-qPCR (F) and ELISA (G). All experiments were independently repeated for three times. One-way ANOVA was applied for the comparison of three sets of data.

ANOVA: analysis of variance; RT-qPCR: real-time quantitative polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay.

*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

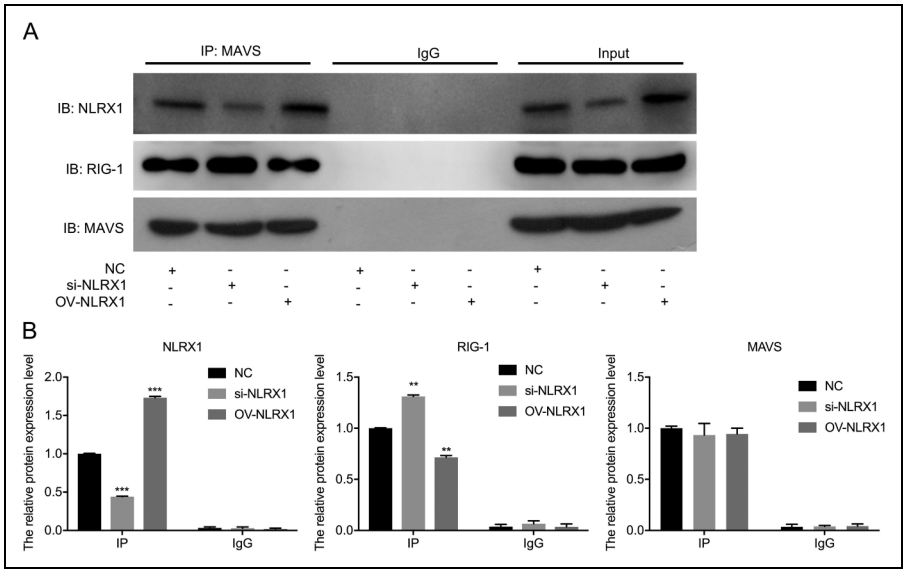

Figure 4. Verification of the interaction between MAVS and RIG-1 mediated by NLRX1. HepG2-NTCP cells were transfected with NLRX1-overexpressed plasmid or NLRX1 siRNA for 48 h prior CO-IP experiment. (A) CO-IP showed the interference or overexpression of NLRX1 on the interaction between MAVS and RIG-1. (B) The levels of RIG-1, MAVS, and NLRX1 were analyzed on the basis of the gray value. All experiments were independently repeated for three times. One-way ANOVA was applied for the comparison of three sets of data.
ANOVA: analysis of variance; CO-IP: co-immunoprecipitation.
**: $P < 0.01$; ***: $P < 0.001$.

Discussion

Researchers have studied NLRX1 with respect to innate immunity, but there are different opinions on its localization and function.[27,28] However, until now, there have been no studies on the role of NLRX1 in HBV infection. Previous research discovered that expression of the encoding gene variation of NLRX1 (NLRX1 p.Arg707Cys) was different between hepatitis B patients and those without HBV infection,[29] suggesting that NLRX1 affects HBV infection.[22,23,30] In the study, we adopted HBV serum to infect HepG2-NTCP cells susceptible to HBV infection. The result verified that NLRX1 promotes HBV infection in hepatocytes and reduced the production of antiviral cytokines by inhibiting the activation of the MAVS-RLR signaling pathway.

Both RIG-1 and melanoma differentiation-associated gene 5 (MDA5) belong to the RLR family, and both MAVS is the ligand.[31] The study reported that MDA5, affected the inhibiting HBV infection but had little effect on I-IFN.[32] We found that NLRX1 promoted the expression of HBV DNA, HBV cccDNA, HBcAg, and HBsAg in HepG2-NTCP cells infected with HBV. It inhibited the secretion of IFN-α, IFN-β, and IL-6. This result verifies that NLRX1 promotes HBV infection by inhibiting the expression of I-IFN and IL-6. Similarly, researchers reported that RIG-1 could recognize HBV

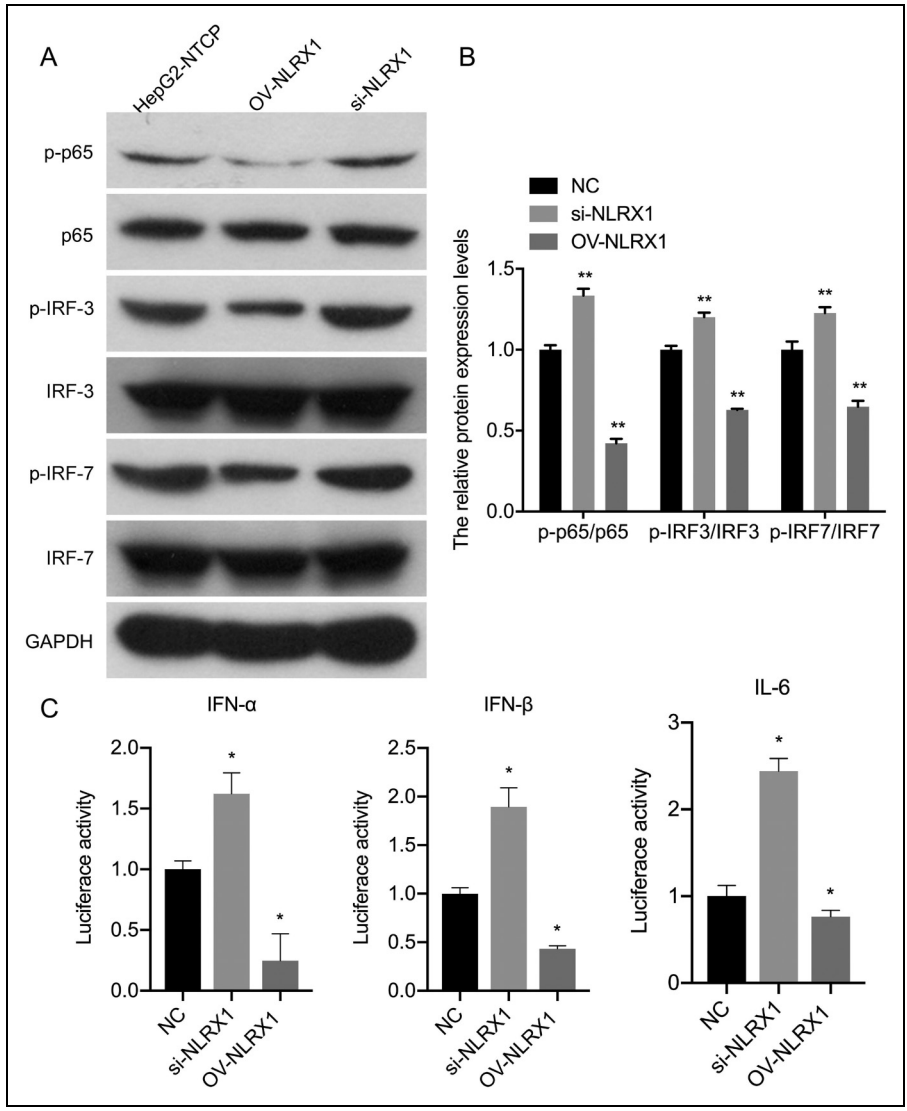

Figure 5. The effect of NLRX1 on MAVS-RLR-mediated downstream signaling pathways in HBV serum-treated HepG2-NTCP cells. The HepG2-NTCP cells were transfected with NLRX1-overexpressed plasmid or NLRX1 siRNA for 48 h, and then were treated with HBV serum for another 48 h prior further analysis. (A) Western blotting was used to analyze the protein level of p65, p-p65, IRF3, IRF7, p-IRF3, and p-IRF7. (B) The phosphorylation ratios of p65, IRF3, and IRF7 were analyzed on the basis of the gray value. (C) The effect of NLRX1 on the activity of IFN-α, IFN-β, and IL-6 promoters was calculated using a luciferase reporter assay. All experiments were independently repeated for three times. One-way ANOVA was applied for the comparison of three sets of data.

HBV: hepatitis B virus; ANOVA: analysis of variance.

*: $P < 0.05$; **: $P < 0.01$.

pgRNA and induce the production of III-IFN.[33] Therefore, we speculated that NLRX1 plays a regulatory role by interfering with MAVS-RIG-1-mediated signaling pathways.

NLRX1 can reduce the antiviral capability by inhibiting MAVS-RLRs signals,[34] but NLRX1 can also enhance the antiviral capability of cells by promoting the production of ROS.[35] Besides, the use of TLR/PRR agonists has anti-HBV effects. For instance, GS-9620, as an agonist of TLR7, can promote inhibition of HBV;[36] TLR dual-acting agonists can block the production of HBV;[37] GS-9688 (Selgantolimod), as potential TLR8 agonist, has a certain therapeutic effect on chronic hepatitis B.[38] According to this study, we verified that NLRX1 could inhibit the antiviral capability of HepG2-NTCP cells. Simultaneously, we found that ROS did not increase in HepG2-NTCP cells after HBV serum treatment. Thus, NLRX1 can counteract innate immune response induced by an external stimulus favoring HBV infection. Further experiments verified that NLRX1 inhibited the interaction between MAVS and RIG-1, and inhibited the phosphorylation of key molecules (p65, IRF3, IRF7) of the MAVS-RLR-mediated downstream NF-κB, IRF3, and IRF7 signaling pathways. Additionally, NLRX1 inhibited the transcription of IFN-α, IFN-β, and IL-6 after HBV serum treatment. Therefore, we demonstrated that the silence of NLRX1 could enhance the antiviral capacity of HepG2-NTCP cells against HBV by regulating the downstream signaling pathways of MAVS-RLRs.

RIG-1 is one of the most important channels in the innate immune system, which can be disrupted by HBV infection.[39] At present, compounds that activate the innate immune system, activator of TLR3, RIG1, MDA5, produced by YiSheng Biopharma (https://www.hepb.org/treatment-and-management/drug-watch/), and named as YS-HBV-002, was in preclinical study. Therefore, we speculated that inhibition of NLRX1 also can activate RIG-1 to exert the same role as YS-HBV-002 base on our conclusion.

However, HepG2-NTCP is not the nest model for innate immune studies, the HBV serum is not equal to purified HBV virus, therefore the conclusion needs to be confirmed in more relevant models, including immune-competent cells (HepaRG or primary human hepatocytes treated with purified HBV virus). We also speculated that the NLRX1 variation could affect the function of the protein, and the specific mechanism will be explored in future studies: how should the targeted inhibition of NLRX1 be implemented to exert an anti-HBV effect in patients; whether NLRX1 combined with immunomodulators should be used to treat patients with HBV infection.

Conclusions

In summary, this study confirmed that NLRX1 is an immune-regulatory factor that inhibits the antiviral capability of hepatocytes against an external stimulus favoring HBV infection. The inhibition of the activation of MAVS-RLR-mediated signaling pathways leads to decreased expression of I-IFN and IL-6, respectively. NLRX1 could be a potential target for HBV immunotherapy and improve the clinical antiviral response rate.

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Ethics statement

This study was approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University for Human Study, and was conducted according to the principles of the Declaration of Helsinki (2013).

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Supplemental material

Supplemental material for this article is available online.

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