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Original Article

IL2-inducible T-cell Kinase is Required for HBV-induced Type T Interferon Expression and Antiviral Response

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Abstract

Background and Aims: Hepatitis B is a vaccine-preventable liver infection caused by the hepatitis B virus (HBV), and is seen as a serious global health problem. HBV infection induces the expression of type I interferon (IFN), including IFN-α and IFN-β, which have anti-HBV activities, and have been used for HBV treatment. IL2-inducible T-cell kinase (ITK) is a tyrosine kinase, which regulates T-cell differentiation and activation, while its precise effects on type I IFN production during HBV infection remain unknown. ***Methods:*** We monitored the ITK expression in peripheral blood mononuclear cells (PBMCs) from healthy donors and patients with acute and chronic HBV infection. We used ITK inhibitor ibrutinib to treat hepatocytes and evaluated the type I IFN expression after HBV infection. We also administrated ibrutinib to mice and evaluated its effect on HBV infection *in vivo*. We generated ITK, suppressor of cytokine signaling 1 (SOCS1) knockout and ITK/SOCS1 double knockout cells using CRISPR, and monitored the HBV-induced type I IFN production. ***Results:*** ITK and type I IFN were upregulated in patients with acute HBV infection. Inhibition of ITK by ibrutinib suppressed HBV-induced expression of type I IFN mRNA in mice. ITK knockout cells had decreased IRF3 activation but promoted the expression of SOCS1. ITK negatively regulated SOSC1 expression. The down regulation of type I IFN in ITK knockout cells after HBV stimulation was abolished in the absence of SOCS1. ***Conclusions:*** ITK regulated HBV-induced expression of type I IFN mRNA by modulating SOCS1.

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Keywords: HBV; ITK; Type I IFN; SOCS1.

Abbreviations: ALT, alanine aminotransferase; BCA, bicinchoninic acid; cccDNA, covalently closed circular DNA; ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HRP, horseradish peroxidase; IFN, interferon; IRF, IFN regulatory factor; ITK, IL2-inducible T-cell kinase; PVDF, polyvinylidene difluoride; PBMCs, peripheral blood mononuclear cells; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation; SOCS1, suppressor of cytokine signaling 1; STING, stimulator of IFN genes; TBK1, TANK-binding kinase 1; TCR, T-cell receptor.

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Introduction

Hepatitis B is the most common serious liver infection in the world.[1] In China, there are approximately 74 million people who are hepatitis B virus (HBV) carriers, accounting for one-third of the world's total. Approximately 260,000 patients die from hepatitis B-related liver diseases annually.[2] Hepatitis B is caused by the hepatitis B virus (HBV),[1] a DNA virus belonging to the *Hepadnaviridae* family.[3,4] After entering the cells via endocytosis, the HBV envelope fuses with the endosome membrane, resulting in the release of nucleocapsids, translocation to the nucleus and formation of covalently closed circular DNA (cccDNA),[5] which functions as the template for transcription and translation. During the life cycle of HBV, viral nucleic acids (DNA and RNA) induce the production of interferon (IFN), a group of signaling proteins.[6] The viral DNA in the cytoplasm is recognized by cytosolic DNA sensors and activates downstream factors including stimulator of IFN genes (STING) and TANK-binding kinase 1 (TBK1), resulting in IFN regulatory factor (IRF) activation and IFN expression.[7] IFNs have antiviral activity and can also amplify immune response. For example, IFN-α could induce cells to release factors restricting HBV entry,[8] decrease transcription of viral RNA from the HBV cccDNA[9] and has been used to treat HBV for decades.[10] IFN-γ reduces HBV cccDNA by inducing deamination and subsequent cccDNA decay.[11] Therefore, IFNs are widely used for HBV treatment and understanding the mechanisms of IFN induction and enhancing IFN production are important for HBV treatment.[12,13]

IL2-inducible T-cell kinase (ITK) is a member of the tyrosine kinase expressed in hepatocellular carcinoma (Tec) family.[14] It has roles essential for T-cell development and adaptive immunity, and also regulates cytokine production and transcription factor expression. For example, ITK-mediated signals control the expression of Eomesodermin (EOMES),[15] which is a master regulator of $CD8^+$ effector and memory T cells and is critical for T-cell-mediated immune responses against pathogens.[16] ITK has been detected in other tissues including the liver and lung. Until now, the precise role of ITK in IFN production during HBV infection remains unknown. In this study, we explored the effects of ITK on type I IFN during HBV infection.

Methods

Patient samples

Patients with acute (n=20) and chronic (n=20) HBV infection attending Quanzhou First Hospital Affiliated to Fujian

Medical University were enrolled. The study was approved by the ethics committee of Quanzhou First Hospital Affiliated with Fujian Medical University, and informed consent was obtained from each patient. Blood samples were collected from patients and healthy donors (n=20) to purify peripheral blood mononuclear cells (PBMCs) by ficoll-hypaque density gradient centrifugation.

Mouse infection

Eight-week old wild-type C57BL/6 mice were purchased from GemPharmatech (Nanjing, China). All mouse experiments were approved by the ethics committee of Quanzhou First Hospital Affiliated to Fujian Medical University. Mice were injected with 5×10^{10} viral genome AAV/HBV in 200 μL phosphate-buffered saline in the tail vein. Some mice were treated with 10 mg/kg ibrutinib (CAS: 936563-96-1; Sigma, St. Louis, MO, USA) for 24 h before infection. 14 days post-infection, samples were harvested for analysis.

Cell culture

Huh-7 cells were cultured in completed Dulbecco's minimum Eagle's medium. ITK knockout and ITK/SOCS1 double knockout Huh7 cells were generated by Crispr-Cas9 as described previously.[17] The plasmids containing gRNA targeting ITK or SOCS1 were produced by Genescript (Nanjing, China) and transfected into Huh-7 cells by Lipofectamine 2000 (Thermo Fisher Scientific Corp., Waltham, MA, USA).

Dual luciferase assay

HEK293T cells were transfected with IFNβ-luc luciferase reporter together with other plasmids as indicated, using Lipofectamine 2000 following the manufacturer's protocol. Luciferase signals were quantified with a dual luciferase reporter assay system.

Enzyme-linked immunosorbent assay (ELISA)

Serum alanine aminotransferase activity and serum levels of HBsAg were determined using commercial ELISA kits (R&D Biosystem, Minneapolis, MN, USA) following the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was performed as described previously.[18] Briefly, total RNA from cells was extracted using PureLink RNA Mini Kit (Thermo Fisher Scientific Corp.) and then reverse-transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific Corp.). Real-time PCR was performed with PowerUp SYBR Green Master Mix and performed using QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific Corp.). The sequences of primers used in this study are listed in Table 1.

Western blotting

Western blotting was performed following a standard protocol described previously.[18] Briefly, total proteins were extracted from cells by RIPA lysis buffer (Abcam, Cambridge, UK) and protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Abcam). Equal amounts of protein (25 μg) resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat milk for 1 hour at room temperature and then incubated with primary antibodies for overnight at 4°C. The next day, membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Antibodies used in this study included anti-TBK1 (108A429; Santa Cruz Biotechnology Inc., Dallas, TX, USA), anti-β-actin (C-4; Sigma), anti-phospho-TBK1 (D52C2; Cell Signaling, Danvers, MA, USA), anti-ITK (D3H5; Cell Signaling), suppressor of cytokine signaling 1 (anti-SOCS1, A156; Cell Signaling), anti-phospho-SOCS1 (Cell Signaling), anti-IRF-3 (D83B9; Cell Signaling), anti-HBsAg (ab20402; Abcam). Anti-β-actin was used as an internal control.

Statistical analysis

Data were reported as means ± standard deviation (SD). Statistical analysis was performed with two-tailed unpaired Student's t-tests and/or one way analysis of variance in GraphPad Prism software (La Jolla, CA, USA). P-values <0.05 were considered statistically significant.

Results

Upregulation of ITK in HBV infection

We compared the transcription profile of different kinases in PBMCs isolated from heathy donors and patients with HBV infection. We found that ITK was significantly upregulated in PBMCs isolated from patients with HBV infection (Fig. 1A). We further confirmed the significant upregulation of ITK1 in PBMCs isolated from patients with acute HBV infection but not chronic HBV infection using RT-PCR (Fig. 1B). We also detected significantly increased IFN-β expression in PBMCs isolated from patients with acute HBV infection but not in PBMCs isolated from patients with chronic HBV infection (Fig. 1C). Correspondingly, we detected a positive correlation between ITK and IFN-β expression in patients with acute HBV infection (Fig. 1D).

Inhibition of ITK suppressed HBV-induced production of IFN-β

Next, we used a luciferase system to explore whether IKT induced IFN-β production. We cotransfected plasmid expressing ITK and plasmids expressing different IFN-β inducers including MAVS, STING, TBK1, and IRF3. The activity of human IFN-β promoter was monitored. As shown in Figure 2A, transfection of MAVS, STING, TBK1, and IRF3 induced luciferase activity and cotransfection of ITK together with these inducers promoted luciferase activity, showing that ITK promoted IFN-β production. We further analyzed mRNA expression of IFN-α and IFN-β in primary hepatocytes after AAV-HBV infection. We observed significantly increased mRNA level of IFN-α and IFN-β at 1h and 3h post AAV-HBV infection while the expression of IFN-α and IFN-β was suppressed by ITK inhibitor ibrutinib (Fig. 2B). We also detected increased IFN-β levels in the cell culture supernatant after AAV-HBV infection, while ibrutinib significantly decreased the amount of IFN-β (Fig. 2C). We then compared the expression of IFN-α and IFN-β in wild-type (WT) and ITK knockout Huh7 cells. As shown in Figure 2D, both IFN-α and IFN-β mRNA levels were upregulated in WT Huh7 cell at 1 and 3 h after AAV-HBV infection. In contrast, IFN-α and IFN-β mRNA levels in AAV-HBV-infected ITK knockout Huh7 cells were significantly decreased compared with AAV-HBV-infected WT Huh7. Taken together, the data showed that ITK promoted AAV-HBV-induced type I IFN expression.

ITK was required for type I IFN production and antiviral immunity against AAV/HBV infection in vivo

We pre-treated mice with the ITK inhibitor ibrutinib before AAV/HBV infection. Fourteen days post infection, significantly

Table 1. Primer sequences

Gene	Sequence
Mouse *IFN-α*	
	Forward: AGTCCATCAGCAGCTCAATGAC
	Reverse: AAGTATTTCCTCACAGCCAGCAG
Mouse *IFN-β*	
	Forward: AGCTCCAAGAAAGGACGAACAT
	Reverse: GCCCTGTAGGTGAGG TTGATCT
Human *ITK*	
	Forward: GAAGTGGAGGTGCTGTTCTCAG
	Reverse: CTTCAGGTTCCCAAAGTGGTCG
Human *IFN-α4*	
	Forward: GTTCCAGAAGGCTCAAGCCATC
	Reverse: TAGGAGGCTCTGTTCCCAAGCA
Human *IFN-β*	
	Forward: CTTGGATTCCTACAAAGAAGCAGC
	Reverse: TCCTCCTTCTGGAACTGCTGCA
Mouse *IL-6*	
	Forward: TACCACTTCACAAGTCGGAGGC
	Reverse: CTGCAAGTGCATCATCGTTGTTC
Mouse *TNF-α*	
	Forward: GGTGCCTATGTCTCAGCCTCTT
	Reverse: GCCATAGAACTGATGAGAGGGAG
Mouse *IL-1β*	
	Forward: TGGACCTTCCAGGATGAGGACA
	Reverse: GTTCATCTCGGA GCCTGTAGTG
Mouse *IFN-γ*	
	Forward: CAGCAACAGCAAGGCGAAAAAGG
	Reverse: TTTCCGCTTCCTGAGGCTGGAT
Mouse *Eomes*	
	Forward: CCACTGGATGAGGCAGGAGATT
	Reverse: GTCCTCTGTCACTTCCACGATG
Mouse *Socs1*	
	Forward: AGTCGCCAACGGAACTGCTTCT
	Reverse: GTAGTGCTCCAGCAGCTCGAAA

elevated serum level of HBsAg (Fig. 3A) and increased serum alanine aminotransferase (ALT) (Fig. 3B) were detected in AAV/HBV-infected mice treated with ibrutinib. We continued to compare the serum level of IFN-α and IFN-β between untreated and ibrutinib-treated mice, finding that the ibrutinib-treated mice had significantly reduced serum IFN-α and IFN-β levels after AAV/HBV infection (Fig. 3C). We also detected significantly enhanced expression of inflammatory cytokine mRNAs including IL6, TNF-α and IL1β in liver tissue from ibrutinib-treated mice after AAV/HBV infection (Fig. 3D). In contrast, the mRNA level of IFN-gamma and Eomes was significantly decreased in liver tissue from ibrutinib-treated mice (Fig. 3E). Collectively, the results show that ITK had a role in the anti-viral immunity against AAV/HBV infection in mice.

ITK regulated the stability of IRF3 through SOCS1

Next, we explored the mechanisms of ITK regulation of type I IFN production during HBV infection. We compared the expression of nuclear IRF3 and phosphorylation of TBK1 between WT and ITK deficient Huh7 cells after HBV genomic DNA treatment. As shown in Figure 4A, we detected IRF3 in the nuclei of WT Huh7 cells 30 m after treatment, indicating the HBV genome DNA treatment activated IRF3. In contrast, nuclear IRF3 level in ITK knockout Huh7 cells was significantly decreased. We further evaluated the phosphorylation of TBK1, the upstream factor of IRF3 activation. We detected similar levels of p-TBK1 and TBK1 in WT and ITK knockout Huh7 cells, indicating ITK did not regulate IRF3 through

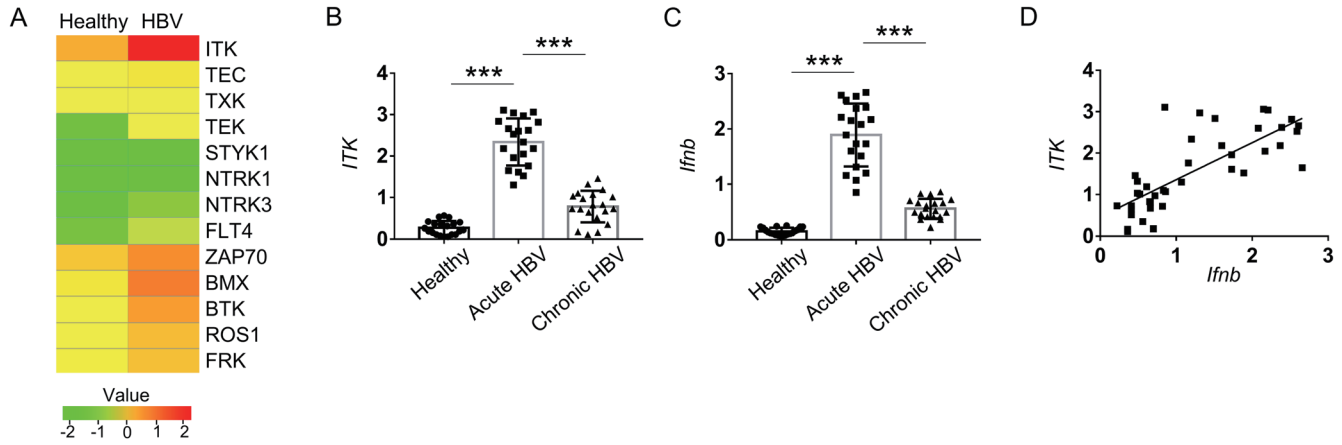

Fig. 1. ITK expression is associated with HBV infection. (A) Heatmaps show the genes differentially expressed in PBMCs from healthy donors and patients with HBV infection. (B, C) qRT-PCR analysis of ITK and Ifnb in PBMCs from healthy donors and patients with HBV infection. Actin was the loading control and used for relative normalization. (D) Correlation analysis of overlapped expression between ITK and Ifnb in PBMCs from healthy donors and patients with chronic HBV infection. ***p<0.0005. PBMCs, peripheral blood mononuclear cells; ITK, IL2-inducible T-cell kinase; IFN, interferon; HBV, hepatitis B virus; qRT-PCR, quantitative real-time polymerase chain reaction.

TBK1. ITK was shown to interact with SOCS1 in a STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) network (Fig. 4B). We further evaluated whether ITK regulated SOCS1 by monitoring SOCS1. Phosphorylated SOCS1 was not detected in ITK knockout Huh7 cells after treatment (Fig. 4C). In contrast, we detected significantly increased levels of SOCS1 protein in ITK knockout Huh7 cells. Interestingly, there was no difference of SOCS1 mRNA between WT and ITK knockout Huh7 cells after HBV genomic DNA treatment (Fig. 4D). We continued to evaluate effects of SOCS1 on type I IFN expression in WT and ITK knockout Huh7 cells. We generated ITK/SOCS1 double knockout Huh7 cells. AAV-HBV infection induced IFN-α and IFN-β expression in WT Huh7 cells, but the expression of both cytokines was impaired in ITK knockout Huh7 cells (Fig. 4E). In contrast, we detected similar levels of IFN-α and IFN-β mRNA in SOCS1 knockout and SOCS1/ITK double knockout Huh7 cells, indicating that the effects of ITK deficiency on IFN-α and IFN-β expression dependent on SOCS1.

Discussion

We explored the effects of ITK on HBV-induced production of type I IFN. First, we detected the upregulation of ITK in HBV

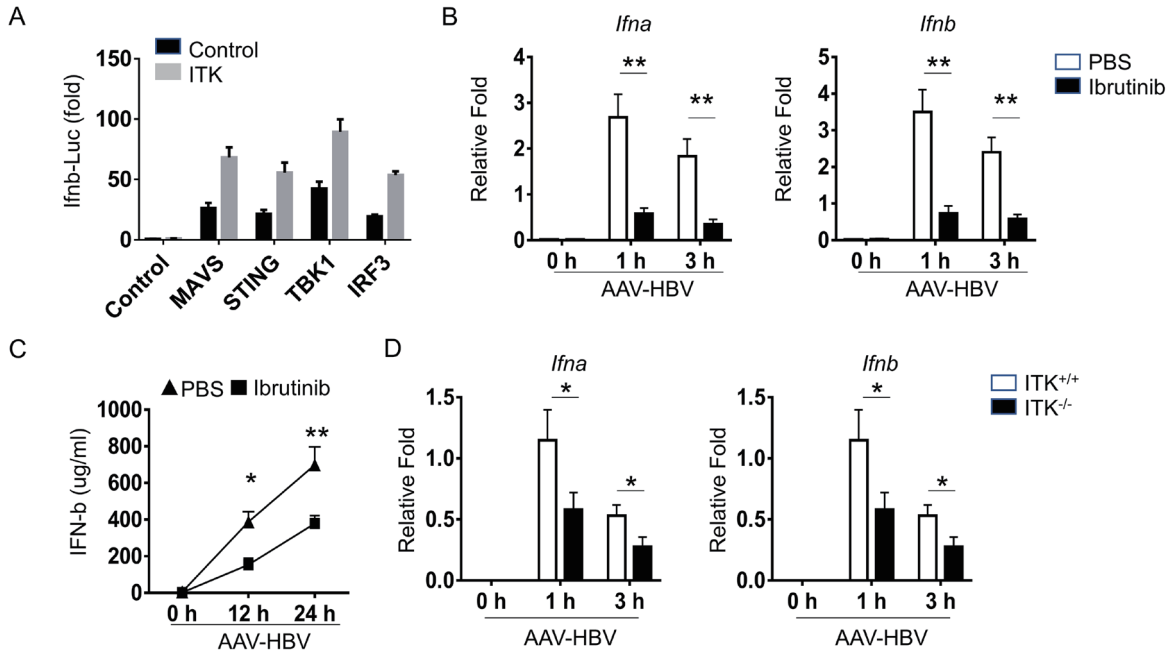

Fig. 2. Ibrutinib inhibits the expression of IFN-I in hepatocytes. (A) HEK293T cells were transfected with IFNb luciferase reporters along with indicated plasmids Readouts are shown relative untransfected cells. (B, C) Expression of IFN-I in mouse primary hepatocytes stimulated with Ibrutinib for the indicated time and measured by qRT-PCR (B) and ELISA (C). (D) qRT-PCR shows the induction of Ifna and Ifnb in ITK KO Huh7 generated by crisper. Expression is fold-relative to Actb mRNA. *p<0.05; **p<0.01. ITK, IL2-inducible T-cell kinase; IFN, interferon; qRT-PCR, quantitative real-time polymerase chain reaction.

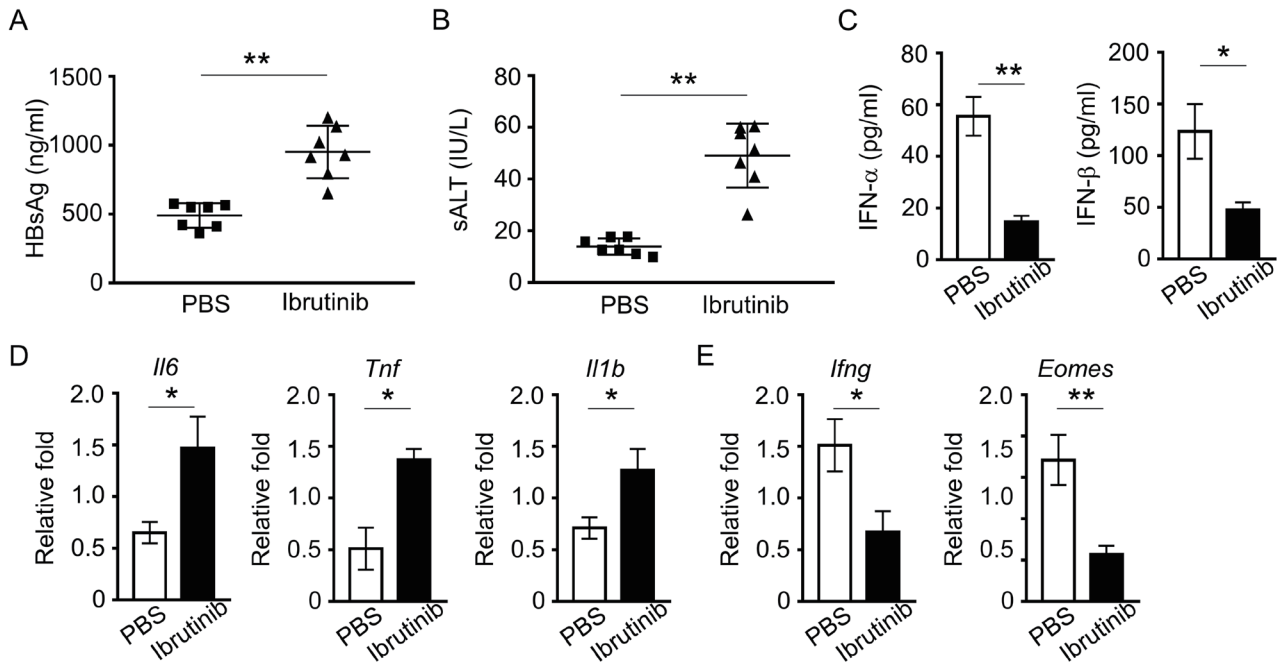

Fig. 3. ITK is required for IFN-I induction and antiviral immunity. (A, B) C57BL/6 mice were pretreated with 10 mg/kg ibrutinib for 24 h and infected with AAV/HBV at 5×10^{10} viral genome equivalents by tail-vein injection. Blood samples were collected 14 days post infection. Serum HBsAg and ALT levels were measured by ELISA. (C) Type I interferon expression in the serum of HBV-carrier mice was measured by ELISA on day 14. (D, E) Induction of proinflammatory cytokines and T-cell-derived cytokines in liver tissue from HBV-carrier mice was measured by qRT-PCR on day 14. *p<0.05; **p<0.01. ITK, IL2-inducible T-cell kinase; IFN, interferon; HBV, hepatitis B virus; qRT-PCR, quantitative real-time polymerase chain reaction; ALT, alanine aminotransferase; ELISA, enzyme-linked immunosorbent assay.

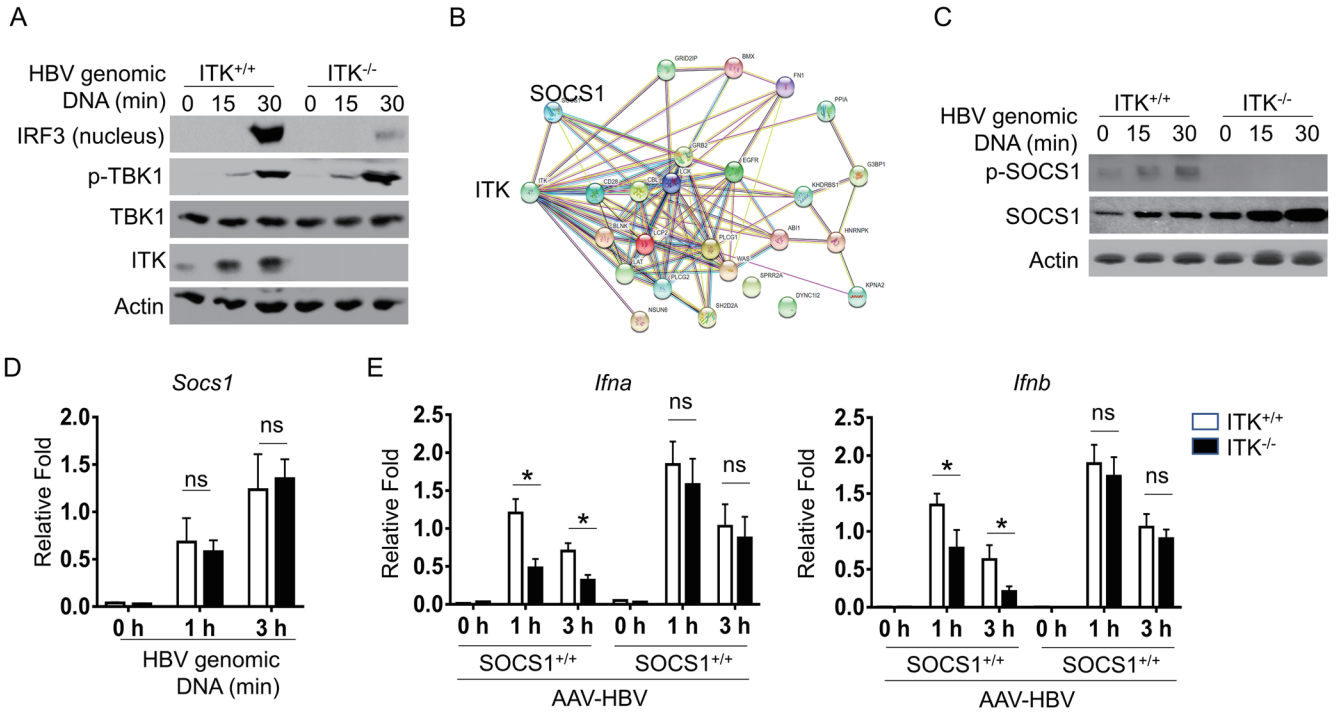

Fig. 4. ITK deficiency suppresses the stability of IRF3 via SOCS1. (A) ITK KO Huh7 was generated by CRISPR, and treated with HBV genomic DNA (1 μg/mL) for the indicated times. Aactivation of TBK1 and IRF3 was determined by western blotting. (B) Proteins interacting with ITK are shown in the STRING Interaction Network. (C) ITK KO Huh7 was treated with HBV genomic DNA (1 μg/mL) for the indicated times. Immunoblotting of phosphorylated (P-) and total SOCS1 in whole-cell lysates. (D) mRNA level of Socs1 in the cells was evaluated by qRT-PCR. (E) ITK and SCOS1 double knockout Huh7 cells were generated by CRISPR-CAS9, infected with AAV-HBV for the indicated times, and assayed by qRT-PCR. Expression is fold-relative to the Actb mRNA level. *p<0.05. ITK, IL2-inducible T-cell kinase; IRF, IFN regulatory factor; TBK1, TANK-binding kinase 1; SOCS1, suppressor of cytokine signaling 1; HBV, hepatitis B virus; qRT-PCR, quantitative real-time polymerase chain reaction.

patients and the positive correlation between ITK and IFN-β. Second, we demonstrated that the production of IFN-α and IFN-β was decreased in ibrutinib-treated primary hepatocytes and in ITK knockout Huh7 cells after AAV/HBV infection. Third, we found ibrutinib treatment decreased type I IFN production, promoted immune cell infiltration into liver, enhanced inflammatory cytokine production, and decreased IFN-γ production in AAV/HBV-infected mice. Last, we demonstrated that ITK regulated the type I IFN expression during AAV/HBV infection through regulating SOCS1.

IFNs are a family of cytokines including types I, II, and III.[19,20] IFN-α and IFN-β are type I IFNs that are synthesized by many cells during viral infection. We found AAV/HBV infection induced robust expression of IFN-α and IFN-β in primary hepatocytes and Huh7 cells. After viruses enter cells, viral nucleic acid sensors such as cyclic GMP-AMP synthase/stimulator of IFN genes (cGAS/STING), Toll-like receptors, and retinoic acid inducible gene I (RIG-1) recognize viral DNA and RNA and activate downstream factors such as TBK1. Phosphorylation and activation of IRFs finally results in IFN production.[21] We consistently demonstrated that HBV genomic DNA treatment activated TBK1 and IRF3.

ITK is a tyrosine kinase that regulates the T-cell receptor (TCR) signaling pathway.[14] Activation of ITK leads to the activation of MAPK, NF-κB and JNK signaling pathway.[22] ITK deficiency in humans causes primary immunodeficiency, lymphopenia, and higher susceptibility to infection.[23–25] ITK deficient mice have altered T-cell development.[26,27] In this study, we observed upregulated ITK level in PBMC from patients with acute HBV infection, which was positively correlated to type I IFN production. Interestingly, we further found ITK promoted the activity of IFN-β promoter in the luciferase system. Our findings suggest the potential regulation of HBV-induced expression of type I IFN by ITK. Ibrutinib is an inhibitor that irreversibly inhibits ITK. We demonstrated that ibrutinib suppressed type I IFN expression after AAV/HBV infection and promoted the inflammation and HBV antigen burden in serum. Type I IFNs have a role in HBV infection. IFN-α has been shown to inhibit HBV replication through multiple mechanisms, including preventing the core particle formation, promoting the decay of core particles, and degrading pregenomic RNA and cccDNA.[28] Therefore, IFN-α has been has been used to treat chronic hepatitis B infection. We demonstrated that ibrutinib-treated mice had significantly increased serum level of HBsAg, which can be explained by the decreased levels of IFN-α and IFN-β.

The expression of type I IFN is driven by IRF transcription factors. With the detection of nucleic acid by sensors, the downstream factor TBK1 phosphorylates IRF3 and IRF7. Phosphorylated IRF3 and IRF7 are translocated into the nucleus and positively regulate the expression of IFN-α and IFN-β. We demonstrated that HBV genomic DNA treatment induced the nuclear translocation of IRF3 in Huh7 cells. The nuclear translocation of IRF3 was significantly decreased in ITK knockout Huh7 cells. Interestingly, ITK did not affect the activation of TBK1, the upstream factor of IRF3, indicating ITK may not regulate the activation of IRF3 through TBK1. The decreased IRF3 level in the nucleus can be explained by the instability of IRF3. Interestingly, ITK interacted with SOCS1. We detected upregulated SOCS1 in ITK knockout Huh7 cells after HBV genomic DNA treatment, suggesting ITK negatively regulated SOCS1 expression. SOCS proteins have been shown to target IRF7 for degradation, resulting in suppressed type I IFN production.[29] We speculate that SOCS1 targeted IRF3 for degradation in our study. In ITK knockout Huh7 cells, there was increased expression of SOCS1, which resulted in decreased protein level of IRF3. Unfortunately, we did not test the total amount of IRF3 in our study, it is necessary to measure the total amount of IRF3 in ITK-deficient cells.

SOCS1 is induced by JAK-STAT signaling pathway and functions as inhibitor of cytokine signaling. SOCS1 can be phosphorylated by the SRC family of non-receptor tyrosine kinases and then dimerize.[30] In this study, we demonstrated that ITK knockout Huh7 and WT Huh7 cells had similar levels of SOCS1 mRNA. In contrast, phosphorylated SOCS1 was not detected, but increased SOCS1 protein was detected in ITK knockout Huh7 cells after HBV genomic DNA treatment. There results suggest that ITK phosphorylated SOCS1 directly or indirectly, which promoted degradation of SOCS1. A study by Sepulveda *et al*.[31] demonstrated that SOCS1 bound to ITK, but the biological significance remains unknown. Additional study is needed to further characterize the phenomena identified here. For example, we need to test whether ITK can directly phosphorylate SOCS1, and how phosphorylation promotes the degradation of SOCS1. ITK is activated by TCR,[32] and how ITK is activated in hepatocytes needs further investigation.

Conclusion

ITK regulated HBV-induced type I IFN production through SOCS1.

Funding

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (ML, ZS), acquisition of data (ML, RG, DZ, JL, MZ), analysis and interpretation of data (ML, RG, DZ, JL, MZ), drafting of the manuscript (ML, RG, DZ, JL, MZ, ZS), critical revision of the manuscript for important intellectual content (ZS), administrative, technical, or material support (ZS), and study supervision (ZS). All authors have made a significant contribution to this study and have approved the final manuscript.

Ethical statement

The study was approved by the ethics committee of Quanzhou First Hospital Affiliated with Fujian Medical University, and informed consent was obtained from each patient. All mouse experiments were approved by the ethics committee of Quanzhou First Hospital Affiliated to Fujian Medical University.

Data sharing statement

The datasets used during the current study are available from the corresponding author on reasonable request.

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