

Exosomal Interferon-Induced Transmembrane Protein 2 Transmitted to Dendritic Cells Inhibits Interferon Alpha Pathway Activation and Blocks Anti-Hepatitis B Virus Efficacy of Exogenous Interferon Alpha

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The negative regulators in the interferon (IFN) signaling pathway inhibit intrahepatic immune response, resulting in suboptimal therapeutic response to IFN α treatment in chronic hepatitis B (CHB) patients. Identifying the key negative factors and elucidating the regulating mechanism are essential for improving anti-HBV (hepatitis B virus) efficacy of IFN α . From the Gene Expression Omnibus (GEO) database, we downloaded and analyzed gene expression profiles of CHB patients with different responses to IFN α (GSE54747), and found that innate immune status was associated with the IFN α -based therapeutic response in CHB patients. Through PCR array, we found higher baseline level of IFN-induced transmembrane protein 2 (IFITM2) mRNA and lower baseline level of IFN α mRNA in peripheral blood mononuclear cells (PBMCs) of CHB patients with suboptimal response to IFN α treatment. Increased IFITM2 protein was also found in the serum of IFN α nonresponsive patients. With further experiments, we found that overexpressing IFITM2 in Huh7 cells suppressed endogenous IFN α synthesis by inhibiting phosphorylation of extracellular signal-regulated kinase (ERK), TANK-binding kinase 1 (TBK1), and interferon regulatory factor 3 (IRF3); knocking out IFITM2 enhanced activation of the endogenous IFN α synthesis pathway, exhibiting better inhibition on HBV replication. We also found that IFITM2 protein was shuttled by exosomes to dendritic cells (DCs), the main source of endogenous IFN α . Exosome-mediated transport of IFITM2 inhibited synthesis of endogenous IFN α in DCs whereas the inhibitory effect was abolished when IFITM2 was knocked out. Furthermore, we demonstrated that both palmitoylation inhibitor and mutation on 70/71 sites of IFITM2 protein influenced its incorporation into exosomes. Mutated IFITM2 protein increased the effect of IFN α against HBV. **Conclusion:** Exosome-mediated transport of IFITM2 to DCs inhibits IFN α pathway activation and blocks anti-HBV efficacy of exogenous IFN α . The findings provide an explanation to the suboptimal response of CHB patients to IFN α treatment. (HEPATOLOGY 2019;69:2396-2413).

Hepatitis B virus (HBV) infects a population of approximately 240 million people worldwide. Persistent HBV infection leads to chronic hepatitis B (CHB) and increases risks of end-stage liver diseases, including cirrhosis and hepatocellular carcinoma (HCC).⁽¹⁾ Currently, nucleot(s)ide analogues and pegylated interferon (IFN) alfa (Peg-IFN α) are the recommended anti-HBV agents. Peg-IFN α has

Abbreviations: 2-BP, 2-bromopalmitate; CHB, chronic hepatitis B; CRISPR, clustered regularly interspaced short palindromic repeats; DAPI, 4',6-diamidino-2-phenylindole; DCs, dendritic cells; DEGs, differentially expressed genes; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; GEO, Gene Expression Omnibus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HDR, homology-directed repair; Hsp70, heatshock protein 70; IFITM2, interferon-induced transmembrane protein 2; IFN α , interferon alpha; IRF3, interferon regulatory factor 3; ISG, interferon-stimulated gene; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MX1, myxovirus resistance 1; OAS1, 2'-5'-oligoadenylate synthetase 1; p-, phosphorylated; PBMCs, peripheral blood mononuclear cells; Peg-IFN α , pegylated IFN α ; poly I:C, polyinosinic-polycytidylic acid; STAT, signaling transducer and activator of transcription; TBK1, TANK-binding kinase 1; wt, wild type.

the advantage of a finite duration of therapy and the potential to achieve a functional cure.⁽²⁾ However, the therapeutic outcome of Peg-IFN α on CHB patients is still suboptimal. Previous studies reported that sustained virological response rates (HBV DNA <2,000 IU/mL) were only 30% in hepatitis B e antigen (HBeAg)-positive and 43% in HBeAg-negative patients at 24 weeks after completion of a 48-week Peg-IFN α treatment.⁽³⁾ Improving the response to IFN α -based treatment remains an essential issue in the management of CHB patients.

Antiviral efficacy of IFN α is achieved through complex immune reactions. Innate immunity is the first line of defense to pathogens when the host is confronted with viruses. After the host recognizes the invading viral antigens, endogenous IFN α synthesis is triggered through the activation of mitogen-activated protein kinase (MAPK)-dependent phosphorylation of TANK-binding kinase 1 (TBK1) and IFN regulatory factor 3 (IRF3) subsequently.^(4,5) Endogenous IFN α binds with IFN α receptor, activating downstream pathways, especially the type 1 Janus kinase (JAK)/signaling transducer and activator of transcription (STAT) pathway. After activation, the homodimer or heterodimer of STATs is formed and translocated into the nucleus to bind with IFN-sensitive response elements, initiating transcription of IFN-stimulated genes (ISGs). Some ISGs encode

antiviral components, such as myxovirus resistance 1 (MX1), protein kinase R (PKR), or 2'-5'-oligoadenylate synthetase 1 (OAS1), to inhibit viral replication directly, whereas some ISGs encode proteins to exert immunomodulatory functions.^(6,7) These immunomodulatory ISGs contribute to different immune reactions such as pathogen recognition, immune cell activation, and endogenous IFN synthesis. Interestingly, some ISGs encode proteins which negatively impact the IFN system to impair host immune defense, which have been reported to be involved in the suboptimal efficacy of IFN in CHB patients.^(8,9) Identifying those key negative regulators and elucidating the mechanism are essential for improving anti-HBV efficacy of IFN α .

Exosomes, with diameters between 30 and 150 nm, are lipid bilayered natural nanoparticles produced by almost all types of cells. The exosome acts as an intercellular messenger by carrying and transporting functional proteins, lipids, and nucleic acids, mediating cell-to-cell communication.⁽¹⁰⁾ Numerous ISG-encoding proteins have been reported to be packaged into exosomes and shuttled to corresponding recipient cells, triggering downstream signaling.⁽¹¹⁾ The exosome-mediated ISGs play a vital role in regulation of IFN signaling pathways.

In this study, we found that exosome-mediated transport of interferon-induced transmembrane

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protein 2 (IFITM2) to dendritic cells (DCs) inhibited endogenous IFN α synthesis, resulting in suboptimal activation of the IFN pathway under exogenous IFN α treatment. IFITM2 belongs to the IFITM family, sharing a conserved transmembrane structure which influences their physical properties and biological roles. As a classical ISG, IFITM2 has been reported to inhibit some RNA virus replication while facilitating replication of some DNA viruses, indicating that IFITM2 may play diverse roles when the hosts are confronted with different pathogens.^(12,13) We also found that proper palmitoylation on 70/71 sites of IFITM2 protein was necessary for its shuttling to DCs by exosome. This study provides an explanation for the suboptimal response of CHB patients to IFN α treatment.

Materials and Methods

GENE EXPRESSION OMNIBUS DATA ACQUISITION AND ANALYSIS

Gene expression profiles of patients (GSE54747) were downloaded from the Gene Expression Omnibus (GEO) public database (<https://www.ncbi.nlm.nih.gov/geo/>).⁽¹⁴⁾ Data were analyzed by the DESeq2 package and presented as volcano plots by ggplot2 and heatmaps by Pheatmap. Differentially expressed genes (DEGs) were analyzed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) online database (<http://david.ncifcrf.gov/>).^(15,16)

ANALYSIS OF IFITM2 AND IFN mRNA IN PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood mononuclear cells (PBMCs) were separated from collected blood samples by Ficoll gradient centrifugation and lysed by TRIzol reagent (Life Technologies, Carlsbad, CA). Total RNA was isolated and reversely transcribed into complementary DNA with a PrimeScript RT Reagent Kit with genomic DNA (gDNA) Eraser (TaKaRa Bio Inc., Dalian, China). The RT2 Profiler PCR Array: Human IFN and Receptor Array (SA Biosciences, Frederick, MD) were used for transcriptional levels measurement.

DETECTION OF TRANSCRIPTION LEVEL OF FACTORS BY QRT-PCR

mRNA level was detected by qRT-PCR with Fast Start Universal SYBR Green Master Mix in a LightCycler 96 system (Roche Diagnostics GmbH, Mannheim, Germany). Primers are listed in Supporting Information S1.

QUANTIFICATION OF SERUM IFITM2 PROTEIN

Serum samples were collected and prepared for IFITM2 quantification using an enzyme-linked immunosorbent assay (ELISA) kit (Fine Test, Wuhan, China).

MODELING OF HBV INFECTION IN HEPATOCYTES AND DETECTION OF HBV REPLICATION INTERMEDIATES, PREGENOMIC RNA, AND HBV ANTIGENS

The HBV replicative plasmid, pHBV4.1, was transfected into Huh7 cells. Seventy-two hours after transfection, HBV replication intermediates were isolated and purified using DNase (Sigma-Aldrich, St Louis, MO) for Southern blotting.⁽¹⁷⁾ The filter was probed with a DIG Luminescent Detection Kit (Roche Applied Science, Basel, Switzerland) labeled full-length HBV gDNA (genotype D) to detect HBV replication intermediates. HBV RNA was extracted from culture supernatant with an HBV pregenomic RNA Extraction and detection Kit (Genome Precision, Beijing, China) and measured by a Roche LightCycler 96 system (Roche Diagnostics GmbH). HBeAg and hepatitis B surface antigen (HBsAg) in culture supernatant were detected by ELISA kits (Shanghai Shiye Kehua, Shanghai, China).

KNOCKOUT OF IFITM2 IN Huh7 CELLS AND VERIFICATION

The clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system including the IFITM2-CRISPR/Cas9 knockout and homology-directed repair (HDR) plasmids (Santa Cruz Biotechnology, Dallas, TX) were cotransfected into Huh7 cells, and stably transfected

cells were selected by puromycin. Immunofluorescent confocal microscopy was used to verify the knockout efficiency. Seventy-two hours after treatment, cells were fixed and blocked before incubating with primary rabbit anti-IFITM2 antibody (Cell Signaling Technology, Danvers, MA), followed by secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI). Images were captured with an Olympus confocal microscope (FV1000; Olympus, Tokyo, Japan).

OVEREXPRESSION OF IFITM2 IN Huh7 CELLS AND PROTEIN MEASUREMENT

Using specific primers, IFITM2 gene was amplified and inserted into the vector plasmid, pEGFP-N1, to construct an IFITM2-overexpressing plasmid. Seventy-two hours after transfection, total protein was extracted and subjected to western blotting. Membranes were blocked with 10% bovine serum albumin and incubated with the primary antibodies and corresponding horseradish peroxidase-labeled secondary antibodies, then visualized by an electrochemiluminescence western blotting substrate (Millipore, Billerica, MA).

DRUG TREATMENT AND ANALYSIS OF IFN α mRNA IN Huh7 CELLS

We administered either therapeutic IFN α (Kaiyin Bio, Beijing, China) directly or the endogenous IFN inducer, polyinosinic-polycytidylic acid (poly I:C; Amersham Biosciences, Piscataway, NJ), to cells to mimic IFN treatment, with final concentrations of 1,000 IU/mL and 50 μ g/mL, respectively. All treatments lasted for 48 hours before samples were collected and detected by qRT-PCR.

Chemical inhibitors U0126 and SB203580 (Selleck, Houston, TX) were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium to the required concentration.

IDENTIFICATION OF EXOSOMES IN CULTURE SUPERNATANT AND IDENTIFICATION OF IFITM2 IN EXOSOMES

Exosomes in culture supernatant of Huh7 cells were purified with exosome precipitation solution

(Macherey-Nagel GmbH & Co. KG, Bethlehem, Germany). Purified exosomes were resuspended, fixed with 2% paraformaldehyde, and negatively stained using uranyl acetate. The grids were visualized using a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan). IFITM2 in exosomes was detected through western blotting as described above.

INDUCTION OF DCs AND IDENTIFICATION OF EXOSOMES TRANSFERRED TO DCs

Mature DCs were induced from THP-1 cells as described.⁽¹⁸⁾

Purified exosomes were labeled with a PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich) before being added to the culture supernatant of DCs. An immunofluorescent confocal microscopy was used to identify the captured exosomes in DCs.

For IFITM2 visualization, exosomes-incubated DCs were treated with specific antibodies (Cell Signaling Technology) and observed as described above.

OVEREXPRESSION OF NONPALMITOYLATED IFITM2 IN Huh7 CELLS

Complete IFITM2 gene sequences were searched in the National Center for Biotechnology Information database, and palmitoylation sites in the sequence were predicted using online software (CSS-Palm). Point mutations were introduced by PCR to construct the IFITM2 mutant plasmids. The mutant plasmids were transfected into cells, and IFITM2 protein was detected.

Co-immunoprecipitation (Co-IP)/acyl-biotin exchange (ABE) was used to analyze the incorporated IFITM2 in exosomes, and the experiment was performed as mentioned.⁽¹⁹⁾

STATISTICAL ANALYSIS

Each experiment was repeated three times. Experimental data from all three independent experiments were included in the final analysis in SPSS software (version 18.00; SPSS, Inc., Chicago, IL). Measurement data are described by the means \pm SDs or the medians (interquartile range) according to their distribution characteristics and were analyzed with a *t*

test or a z test. A difference with a P value <0.05 was identified as significant.

ETHICS STATEMENT

Blood samples of patients were collected from a registered clinical trial (ChiCTR-OPC-16008586) with written informed consent, and blood samples of healthy subjects were collected from volunteers during physical examination with written informed consent. Liver tissues were acquired from the biobank of West China Hospital, Sichuan University (Chengdu, China). All procedures relevant to human samples were approved and monitored under the supervision of the West China Hospital Ethics Committee, Sichuan University.

Results

HOST IMMUNE STATUS IN CHB PATIENTS WAS CORRELATED WITH THERAPEUTIC RESPONSE OF IFN α

Transcriptomic array files of 15 CHB patients who have received treatment of Peg-IFN α plus adefovir dipivoxil for 48 weeks (GSE54747) were downloaded from the GEO database. The patients were grouped into responders ($N = 9$) and nonresponders ($N = 6$) according to their virological and biochemical responses. Raw data were compared by DESeq2, and results are shown in a volcano plot (Fig. 1A). DEGs were identified and screened in a heatmap (fold change, >1.2 ; $P < 0.05$), including 453 up-regulated and 151 down-regulated genes in responders compared to nonresponders (Fig. 1B). Using the online DAVID tool, three functional analyses were performed: molecular function, cell composition, and biological process. The top 20 items of each group are listed in Fig. 1C, showing that cascades related to immune response and defense of virus, such as innate and adaptive immune responsive pathways, were enriched. Gene Ontology analysis of GSE54747 indicated that therapeutic response of IFN in CHB patients was strongly correlated with intrahepatic immune status.

For further verification of the correlation between response to IFN α treatment in CHB patients and their initial immune status, we conducted a PCR array to measure transcription level of IFN genes and

IFN-related factors in CHB patients. It turned out the baseline transcriptional levels of these 10 IFN α subtypes were lower in nonresponders compared to responders (Fig. 1D, left panel). However, IFITM2 was rather higher in nonresponders (Fig. 1D, right panel). Consistently, the result of qRT-PCR exhibited a higher baseline level of IFITM2 mRNA and lower baseline level of IFN α 2 mRNA in PBMCs of CHB patients with suboptimal response to IFN α treatment (Fig. 1E, left and middle panels). Serum samples from the same subjects were collected to measure circulating IFITM2. Consistent with the level of IFITM2 mRNA in PBMC samples, serum IFITM2 level from the nonresponsive group was higher compared to that of the responsive group or healthy individuals (Fig. 1E, right panel). Taken together, higher IFITM2 expression is closely associated with decreased IFN α levels, which may contribute to nonresponsiveness to IFN α treatment in CHB patients.

IFITM2 INHIBITED THE ANTI-HBV EFFICACY OF IFN α

In both IFN responsive and nonresponsive patients, the PBMC mRNA and serum protein of IFITM2 were increased in HBV-infected subjects compared to healthy ones. In order to assess the correlation between HBV infection and IFITM2 expression, hepatic IFITM2 levels in CHB patients were detected by immunohistochemistry. Results showed that IFITM2 was significantly elevated in CHB patients compared to healthy people, whereas patients with higher viral load presented higher expression of hepatic IFITM2 expression (Fig. 2A).

Therefore, we hypothesized whether endogenous IFN α expression may be blocked by IFITM2 induced by HBV, resulting in insufficient stimulation of innate immunity to facilitate the antiviral capacity of exogenous IFN α . To verify this hypothesis, we utilized a CRISPR/Cas9 plasmid to specifically cleave the IFITM2 promoter gene in Huh7 cells, and relinked the gap with red fluorescent protein-labeled HDR plasmid to establish the cell line with IFITM2 knocked out (Huh7 IFITM2 $^{-/-}$). The new cell line was confirmed to be successfully constructed by immunofluorescent confocal microscopy assay. We observed the successful cleavage (red-labeled HDR) and attenuated IFITM2 expression (green) in the knock-out cells compared to original Huh7 cells (Fig. 2B).

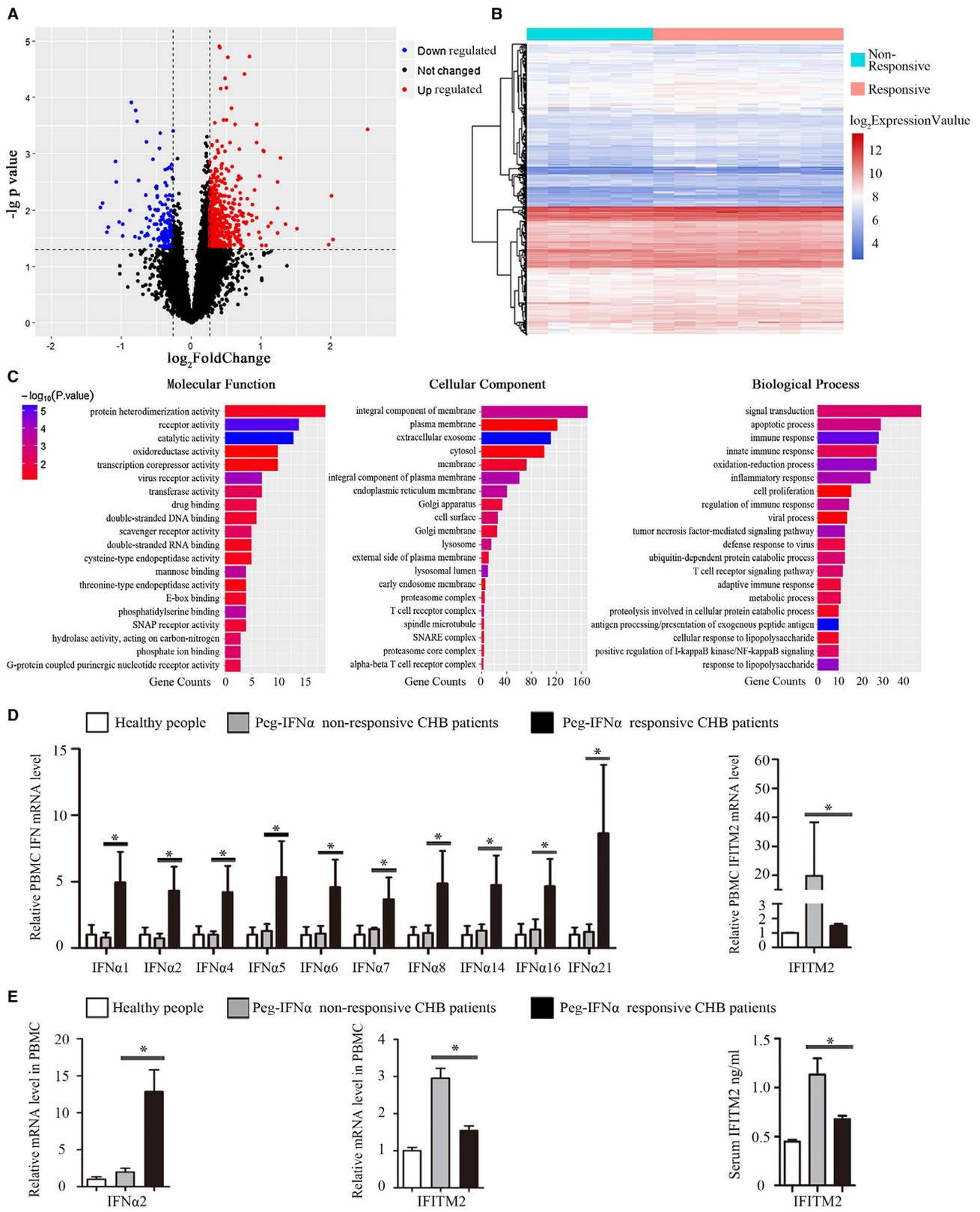


FIG. 1. Analysis of differentially expressed genes (DEGs) between interferon responsive and non-responsive CHB patients. (A) Volcano plot of DEGs with fold change >1.2 and $P < 0.05$. Blue points refer to down-regulated genes while red points refer to up-regulated genes in the responsive group compared to the non-responsive group. (B) Hierarchical clustering heatmap of the screened DEGs. (C) Gene Ontology analysis of the DEGs with enriched items of molecular function, cellular component and biological process. (D) HBV induced elevated baseline levels of endogenous IFN α and IFITM2 mRNA in CHB patients and healthy people. mRNA of PBMC from CHB patients and healthy people was reversely transcribed into cDNA to detect transcriptional levels of IFN genes or genes related to IFN α . (E) Transcriptional levels of IFN α 2 and IFITM2 in PBMCs of CHB patients and healthy people were measured by qRT-PCR, while serum IFITM2 level was quantified by ELISA.

When the HBV replicative plasmid, pHBV4.1, was transfected into Huh7 IFITM2^{-/-} cells, both IFITM2 transcription (mRNA level) and expression (protein level) could not be induced, confirming the establishment of an IFITM2^{-/-} cell line (Fig. 2C).

Then we transfected the wild-type (wt) and IFITM2^{-/-} Huh7 cells with pHBV4.1 plasmid and treated them with exogenous IFN α (working concentration, 1,000 IU/mL). Therapeutic responses were quite different in Huh7 and Huh7 IFITM2^{-/-} cells as expected. Viral proteins (HBsAg and HBeAg) were markedly decreased in culture supernatant of IFITM2^{-/-} cells. Even after IFITM2 expression was complemented in IFITM2^{-/-} cells by IFITM2 overexpressing plasmid, viral proteins could not be suppressed by exogenous IFN α . Furthermore, we also found that the knockout of IFITM2 resulted in a strong suppression of viral proteins, whereas rescue of IFITM2 expression could counteract the antiviral efficacy of IFN α (Fig. 2D). The same results were obtained when we detected HBV RNA in culture supernatant and HBV replication intermediates in hepatocytes (Fig. 2E,F), given that silencing IFITM2 relieved the suppression of endogenous IFN α production as well as anti-HBV efficacy of exogenous IFN α , suggesting that IFITM2 negatively regulated the antiviral effect of exogenous IFN α by affecting the IFN α pathway.

IFITM2 SUPPRESS ENDOGENOUS IFN α SYNTHESIS THROUGH THE EXTRACELLULAR SIGNAL-REGULATED KINASE/TBK1/IRF3 PATHWAY IN Huh7 CELLS

Because we have suggested that IFITM2 could affect host antiviral ability, we were interested in the mechanism underlying the process. When IFITM2 was overexpressed in Huh7 cells, the commonly effective treatment with 50 μ g/mL of poly I:C could not activate endogenous IFN α transcription (Fig. 3A).

TBK1 and IRF3, two essential activators of IFN α transcription, were measured to reflect the degree of IFN α activation by western blotting and immunofluorescent confocal assay. Overexpressing IFITM2 in Huh7 cells significantly reduced phosphorylation of TBK1 and IRF3 (Fig. 3B), and the nuclear localization of phosphorylated (p)-TBK1 and p-IRF3 was barely observed (Fig. 3C). These results implied that IFITM2 may attenuate the phosphorylation of TBK1/IRF3 to suppress endogenous IFN α synthesis.

Considering that the MAPK signaling pathway is crucial in the phosphorylation of TBK1 and IRF3, we detected the expression and phosphorylation of the three key proteins (c-Jun N-terminal kinase [JNK], p38, and extracellular signal-regulated kinase [ERK]) in the pathway. We observed that the phosphorylation of ERK and p38, but not JNK, were significantly inhibited after IFITM2 overexpression (Fig. 3D). For further investigation, we applied the chemical inhibitors U0126 to suppress ERK phosphorylation and SB203580 to suppress p38 phosphorylation, respectively. Phosphorylation of TBK1 and IRF3 was consistently decreased following inhibited ERK phosphorylation by 10 μ M of U0126 (Fig. 3E). However, inhibition of p38 phosphorylation exhibited no effect; no obvious alteration was observed in the downstream phosphorylation of TBK1 or IRF3 by bringing the treatment concentration of SB203580 to 10 μ M (Fig. 3F). These results suggested that IFITM2 attenuated ERK phosphorylation to down-regulate TBK1 and IRF3 phosphorylation, accounting for the impaired synthesis of endogenous IFN α .

IFITM2 FROM HBV-INFECTED Huh7 CELLS INHIBITED ERK/TBK1/IRF3 CASCADE IN COCULTURED DCs

To investigate whether hepatocyte-produced IFITM2 could be shuttled to DCs to amplify inhibitory

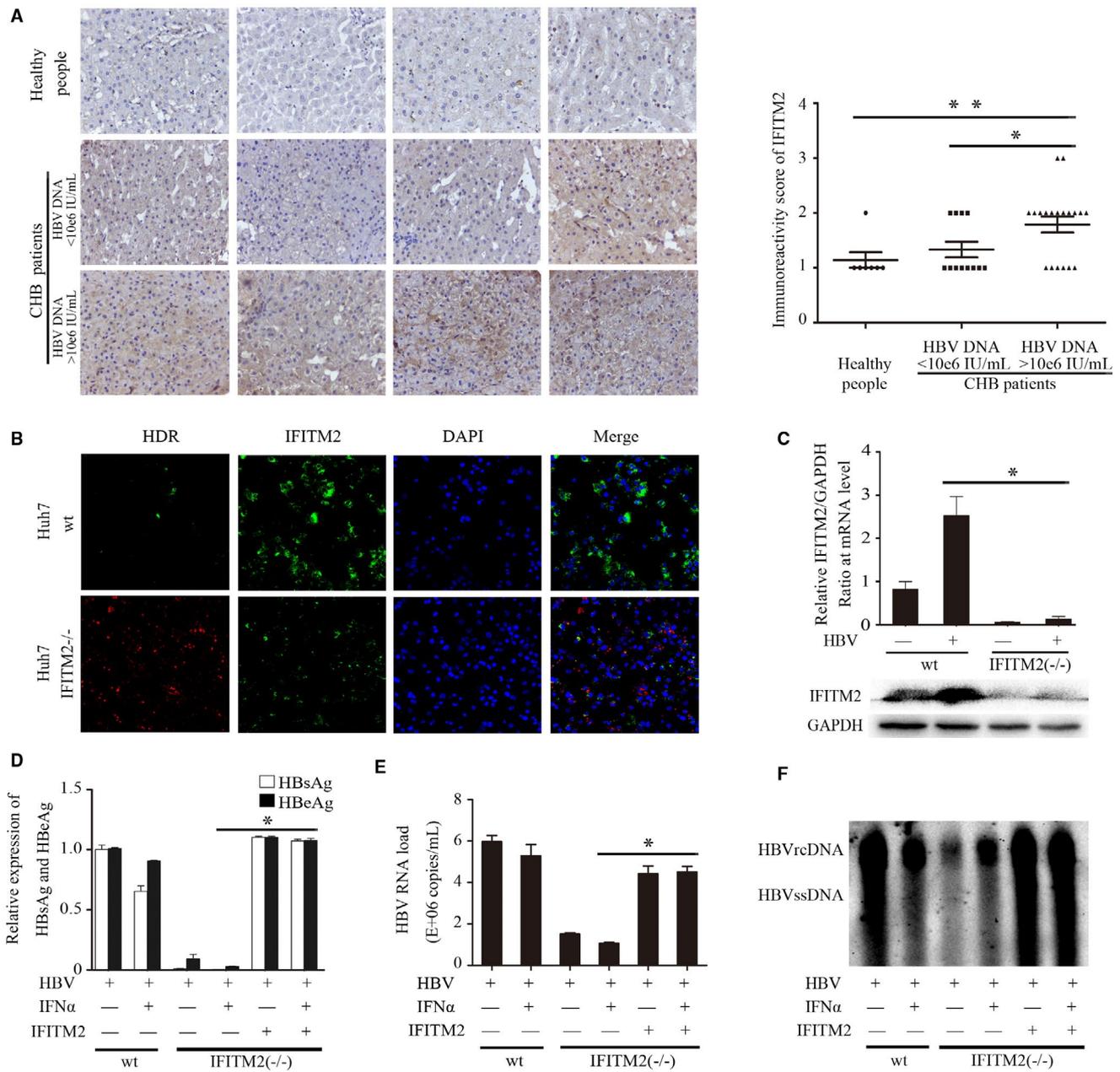


FIG. 2. The correlation between IFITM2 and the antiviral efficacy of IFN α . (A) Immunohistochemical staining of IFITM2 in liver tissues from healthy objects, CHB patients with low (<10e6 IU/mL) and high (>10e6 IU/mL) HBV load (representative images were shown). Quantification of positive IFITM2 expression was performed by ImageJ software. (B) Immunofluorescent confocal microscopy assay exhibited successful construction of IFITM2^{-/-} cell line with CRISPR-Cas9/HDR system. Successful cleavage of the IFITM2 promoter and relink by HDR were indicated by red. (C). HBV induced elevated levels of IFITM2 mRNA and protein in Huh7 cells but not in IFITM2 knockout cells. Transcriptional level of IFITM2 was measured by qRT-PCR. Translational level of IFITM2 was measured by western blot. (D-F) IFITM2 knockout facilitated anti-HBV efficacy of exogenous IFN α , while IFITM2 complementation by an overexpressing plasmid abolished the antiviral efficacy of IFN α . HBsAg, and HBeAg in supernatant of culture media were quantified by ELISA (D). HBV pgRNA in supernatant of culture media were purified and quantified by qRT-PCR (E). HBV replication intermediates in cells were purified and quantified by Southern blot (F).

function on the type I IFN signaling pathway, we used a Huh7 cells/DCs coculture model to mimic the real microenvironment in liver tissues. To this end, we induced differentiation of THP-1 cells into

mature DCs successfully (Fig. 4A). An IFITM2-overexpressing plasmid was transfected into Huh7 cells, which were then cocultured with DCs for 3 days. We found that the level of IFITM2 in DCs

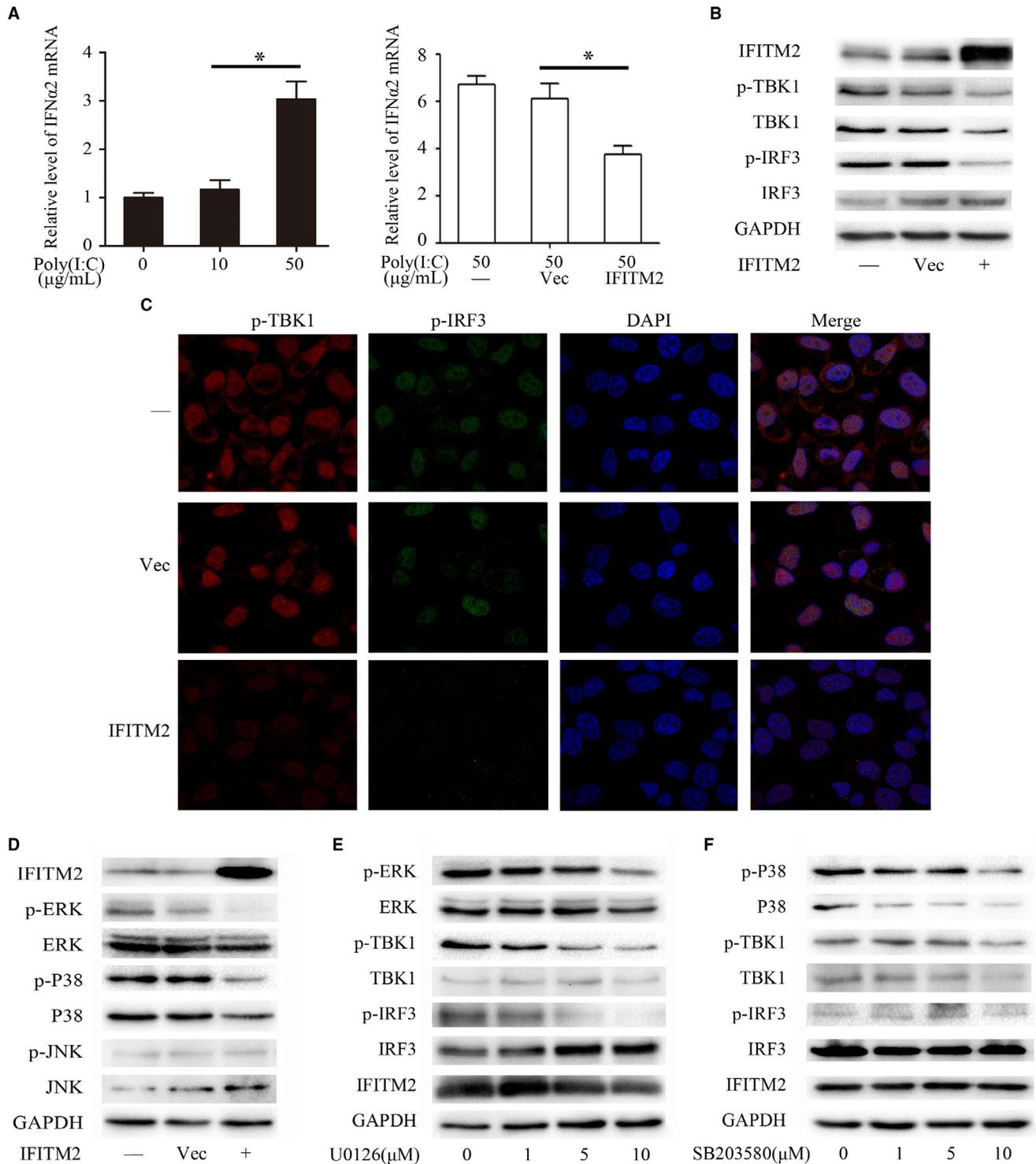


FIG. 3. IFITM2 inhibited the endogenous IFN α synthesis pathway through down-regulating phosphorylation of ERK, TBK1, and IRF3. (A) Transcriptional level of IFN α was measured by qRT-PCR. Poly(I:C) was applied to induce endogenous IFN α expression. Poly(I:C) at concentration of 50 μ g/mL effectively increased the mRNA level of endogenous IFN α in Huh7 cells. While in IFITM2-overexpressing Huh7 cells, Poly(I:C) at concentration of 50 μ g/mL could not increase the mRNA level of endogenous IFN α . (B) Western blot showed that overexpressing IFITM2 inhibited the phosphorylation of TBK1 and IRF3. (C) Immunofluorescence confocal microscopy assay showed reduced nuclear localization of p-TBK1 and p-IRF3 in Huh7 cells transfected with IFITM2 overexpressing plasmids. (D) Western blot showed that overexpressing IFITM2 inhibited the phosphorylation of ERK and p38 but not that of JNK in Huh7 cells. (E) Western blot showed that treatment with an ERK phosphorylation inhibitor (U0126) at concentrations of 10 μ M effectively inhibited the phosphorylation of TBK1 and IRF3 in Huh7 cells. (F) Western blot showed that treatment with a p38 phosphorylation inhibitor (SB203580) at concentrations of 10 μ M could not inhibit the phosphorylation of TBK1 and IRF3 in Huh7 cells.

cocultured with IFITM2-overexpressing Huh7 cells was obviously increased compared to control groups. Additionally, phosphorylation of ERK, TBK1, and IRF3 were significantly decreased in DCs, which was cocultured with IFITM2-overexpressing Huh7 cells, compared with that in cells cultured with empty-vector-expressing Huh7 cells (Fig. 4B). Even when exogenous IFN α was added, the decreased phosphorylation level of the three factors could not be recovered.

Moreover, Huh7 cells in the coculture model were harvested, and key factors in the JAK/STAT pathway were detected. Phosphorylation of STAT1 and STAT2 were significantly decreased after overexpressing IFITM2. In cells receiving exogenous IFN α , phosphorylation of STAT1 and STAT2 could not attain the same level as control groups, as long as IFITM2 was overexpressed. Downstream expression of the antiviral proteins, MX1 and OAS1, was also suppressed by

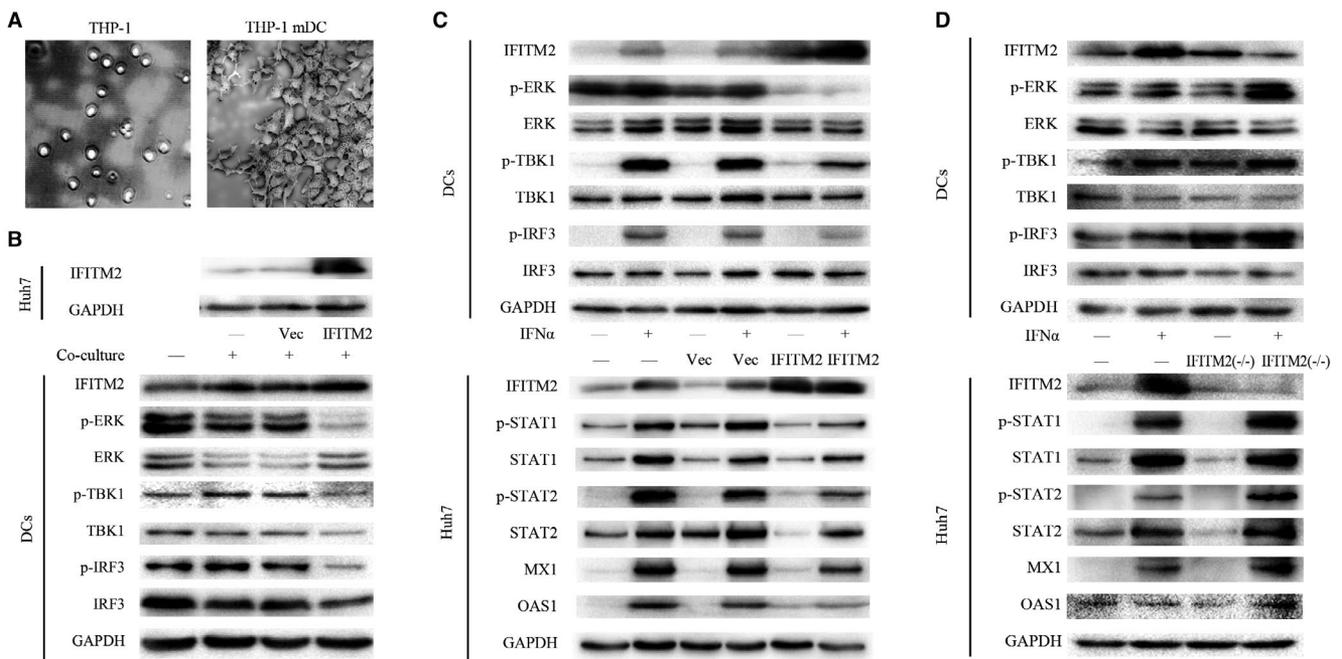


FIG. 4. IFITM2 in HBV-infected Huh7 cells inhibited the endogenous IFN α synthesis pathway in co-cultured DCs. (A) Successful induction of mature DCs with pseudopodia from THP-1 cells was visualized by microscopy. (B) Levels of IFITM2 and key factors in IFN α synthesis pathway were detected in DCs co-cultured with Huh7 cells by Western blot. When DCs were co-cultured with Huh7 cells transfected with IFITM2 overexpressing plasmid, IFITM2 increased in DCs while phosphorylated ERK, TBK1, and IRF3 decreased. (C) Western blot showed that overexpression of IFITM2 in Huh7 cells inhibited the activation of IFN α synthesis pathway (ERK, TBK1, and IRF3) in co-cultured DCs, and inhibited activation of Type I IFN signalling (STAT1 and STAT2) and production of antiviral proteins (MX1 and OAS1) in Huh7 cells, with the addition of 1000 IU/mL exogenous IFN α . (D) Western blot showed that knocking IFITM2 out in Huh7 cells increased the activation of IFN α synthesis pathway (ERK, TBK1, and IRF3) in co-cultured DCs, and increased activation of Type I IFN signalling (STAT1 and STAT2) and production of antiviral proteins (MX1 and OAS1) in Huh7 cells, with the addition of 1000 IU/mL exogenous IFN α .

IFITM2 overexpression even when exogenous IFN α was given (Fig. 4C). These results suggested that IFITM2 in hepatocytes influenced the synthesis of endogenous IFN α in DCs and that deficient endogenous IFN α synthesis in DCs could not facilitate the effective activation of the JAK/STAT pathway triggered by exogenous IFN α .

For further confirmation, we also performed these operations in Huh7 IFITM2^{-/-} cells cocultured with DCs. When exogenous IFN α was given to Huh7 IFITM2^{-/-} cells and cocultured DCs, phosphorylation of ERK, TBK1, and IRF3 in DCs markedly increased. Phosphorylation levels of STAT1 and STAT2 were also increased much more than that in IFN α -treated Huh7 cells. Moreover, expression of the downstream antiviral proteins, MX1 and OAS1, was increased substantially in Huh7 IFITM2^{-/-} cells compared to that in Huh7 cells under the same IFN α treatment (Fig. 4D). These results implied that the IFN α -synthesized pathway in DCs was strongly promoted after knocking out IFITM2 in hepatocytes, which would eventually facilitate exogenous IFN α -stimulated antiviral protein expression. Therefore, IFITM2 in hepatocytes may regulate both the synthesis of endogenous IFN α in DCs and, in turn, the therapeutic response to exogenous IFN α in hepatocytes.

IFITM2 IN HEPATOCYTES WAS TRANSFERRED TO DCs BY EXOSOMES

All the above findings directed us to ask how either IFITM2 or its inhibitory efficacy was transferred from hepatocytes to DCs. Considering that the conserved transmembrane domain of IFITMs has been reported to be involved in exosome-mediated transport, we first applied electron microscopy to confirm the existence of exosomes in the cultural supernatant of hepatocytes. We visualized particles with a classic cup-shaped structure and a diameter up to 60 nm in the culture supernatant of Huh7 cells (Fig. 5A). Then, we purified and labeled exosomes to coculture with DCs. After 6 hours, exosomes (labeled in green) were observed in the cellular area of DCs near their nuclei by immunofluorescent confocal microscopy (Fig. 5B). When DCs were incubated with exosomes from IFITM2-complemented Huh7 cells, IFITM2 was elevated in DCs compared to the group treated with exosomes from noncomplemented

cells. Moreover, colocalization of labeled IFITM2 proteins and exosomes were observed in DCs (Fig. 5C). These findings proved that exosomes derived from Huh7 cells could be recruited and internalized by DCs.

We next detected whether IFITM2 contained in exosomes was secreted from Huh7 cells. After the purified particles in the cultural supernatant of Huh7 cells were lysed, IFITM2 was identified as well as the two typical biomarkers of exosomes, CD63 and heatshock protein 70 (HSp70). Furthermore, when IFITM2 was overexpressed in the hepatocytes, the amount of IFITM2 in the exosomes was increased whereas the amount of exosomes remained constant (Fig. 5D). In addition, IFITM2 mRNA was not detected in exosomes produced by Huh7 cells (Supporting Information S2). When Huh7 cells were treated with a specific inhibitor of exosomal secretion, GW4869, the amount of IFITM2 in the exosomes along with the amount of exosomes released decreased in a dose-dependent pattern. Under 10- μ M GW4869 treatment, both secretion of exosomes and exosome-contained IFITM2 decreased the most, even with IFITM2-overexpressing plasmid transfected (Fig. 5E). For further verification, DCs were incubated with exosomes from IFITM2-overexpressing Huh7 cells. It turned out phosphorylation of the three key factors in the IFN α synthesis pathway (ERK, TBK1, and IRF3) was inhibited in DCs. Furthermore, when the corresponding exosomes were treated by 20% sodium dodecyl sulfate, they exhibited no inhibitory effect on the IFN synthesis pathway in DCs (Fig. 5F). These findings strongly suggested that IFITM2 protein could be transferred from hepatocytes to DCs in an exosome-mediated way.

PROPER PALMITOYLATION OF IFITM2 WAS REQUIRED FOR ITS LOCALIZATION IN EXOSOMES AND SUBSEQUENT ACTIVITIES IN REGULATING THE ANTIVIRAL EFFICACY OF IFN α

Using TMHMM and CSS-Palm software, we identified that two cysteine residues (C70/C71) in the transmembrane domain (amino acid sites 60-80) of IFITM2 had potential susceptibility to be palmitoylated (Fig. 6A,B). To examine whether palmitoylation

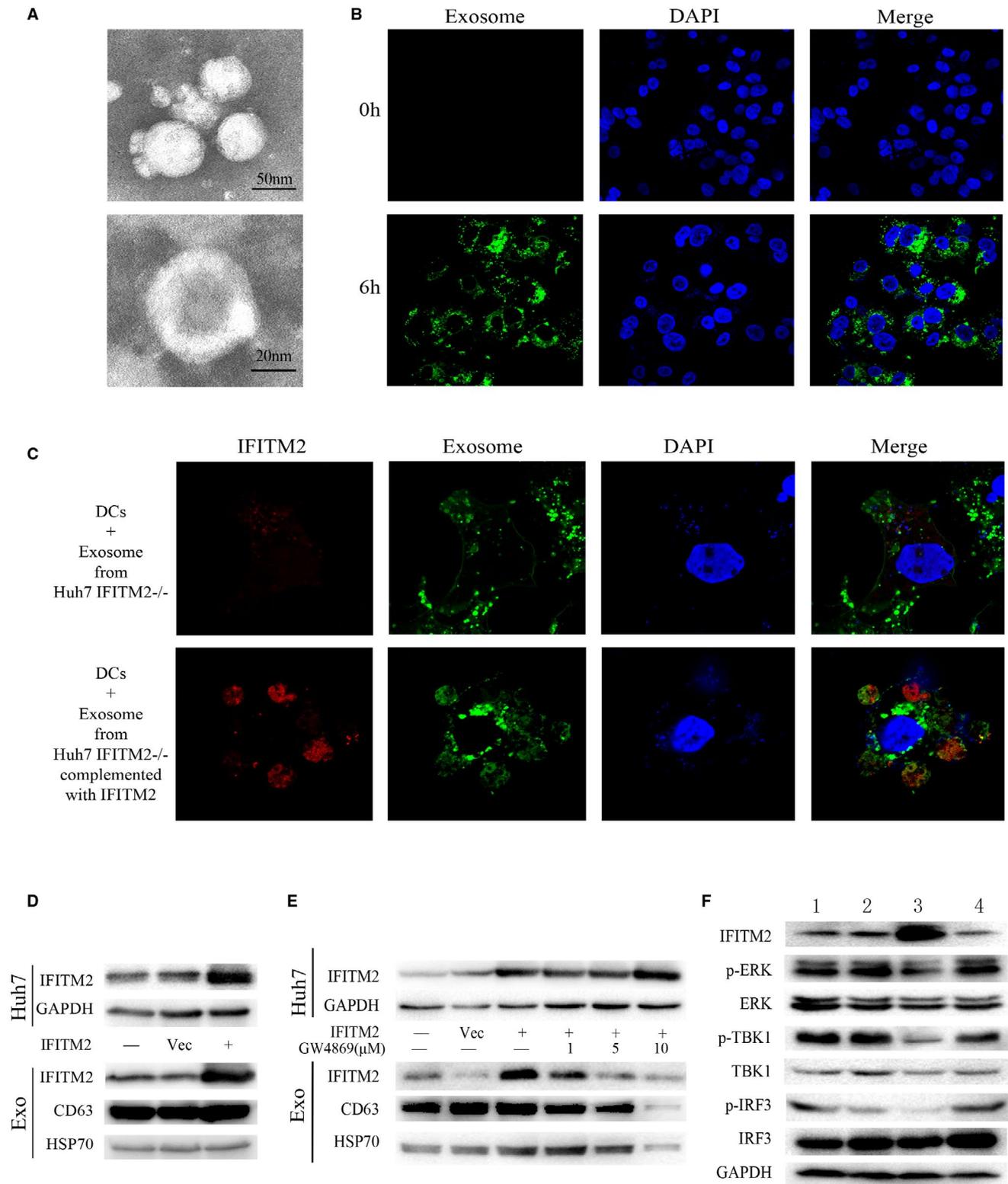


FIG. 5. Exosomes mediated the transport of IFITM2 from Huh7 to DCs. (A) Exosomes released from Huh7 cells were visualized by electron microscopy. (B) Exosomes (released from Huh7 cells, labelled as green) were engulfed in the DCs near their nuclei (blue) after co-culture for 6 hours. (C) Exosomes from Huh7 IFITM2^{-/-} cells and complemented cells (green) were engulfed in the DCs near their nuclei (blue) after co-culture. More IFITM2 was detected in DCs incubated with exosomes from IFITM2 complemented cells. (D) Western blot showed that overexpressing IFITM2 in Huh7 cells resulted in overexpressed IFITM2 in exosomes. IFITM2 level was detected in exosome from Huh7 cells transfected with IFITM2 overexpressing plasmid. CD63 and HSP70 are exosomal biomarkers. (E) Western blot showed that the exosome secretion inhibitor (GW4869) decreased the amount of exosomes from Huh7 cells, as well as exosome-harboring IFITM2. (F) The phosphorylated and total ERK/TBK1/IRF3 in DCs incubated with exosomes from Huh7 cells under different treatment were measured by Western blot. Exosomes are collected from: Lane 1, Huh7 cells; Lane 2, Huh7 cells transfected with vector plasmid; Lane 3, Huh7 cells transfected with IFITM2 overexpressing plasmid; Lane 4, Huh7 cells transfected with IFITM2 overexpressing plasmid and treated with 20%SDS.

is critical for incorporation of IFITM2 into exosomes, an effective palmitoylation inhibitor, 2-bromopalmitate (2-BP), was applied to Huh7 cells for 2 hours. Western blotting showed that the amount of IFITM2 incorporated into exosomes decreased noticeably after treatment with 200 μ M of 2-BP. We then purified palmitoylated IFITM2 by Co-IP/ABE assay, which showed that treatment with 200 μ M of 2-BP significantly inhibited the palmitoylation level of IFITM2 in hepatocytes (Fig. 6C). The consistent alteration of palmitoylated IFITM2 in hepatocytes and its expression in exosomes strongly suggested that the palmitoylation status of IFITM2 affected its incorporation into exosomes.

Next, we constructed three mutated IFITM2-overexpressing plasmids by introducing point mutations into 70/71 sites by PCR: C70A; C71A; C70A,C71A (Fig. 6B). When these mutated IFITM2 proteins were overexpressed in Huh7 IFITM2^{-/-} cells, it led to less incorporation of the mutated IFITM2 protein in exosomes compared to that of the WT IFITM2 in the same amount of exosomes, whereas the double-sites mutation (C70A,C71A) led to the lowest incorporation of IFITM2 in exosomes. In addition, neither the single (C70A; C71A) nor the double mutations (C70A,C71A) led to normal palmitoylation levels (Fig. 6D). Therefore, these two amino acid residues were critical to guarantee proper palmitoylation of IFITM2 and its incorporation into exosomes.

Then, we detected the factors of the type 1 IFN signaling pathway in Huh7 IFITM2^{-/-} cells and DCs coculturing system, while Huh7 IFITM2^{-/-} cells were being transfected with different types of mutated IFITM2 before coculturing. Level of cellular IFITM2 did not exhibit alteration in Huh7 IFITM2^{-/-} cells transfected with mutated or wt IFITM2. But phosphorylation of STAT1 and STAT2 was increased in cells restored with mutated IFITM2 compared to wt IFITM2. As a result, expression of the antiviral factors,

MX1 and OAS1, was increased in cells with mutated IFITM2. When the cytokine regulators in the endogenous IFN α synthesis pathway were detected in cocultured DCs, phosphorylation of ERK, TBK1, and IRF3 were all increased (Fig. 6E). These results implied that because the mutated IFITM2 could not be transported to DCs effectively, the inhibitory effect of IFITM2 on downstream pathways was attenuated. Consistently, levels of HBsAg and HBeAg in cultural supernatant and HBV replication intermediates in cells slightly decreased in Huh7 IFITM2^{-/-} cells restored with C70A-mutated IFITM2, moderately decreased in cells restored with C71A-mutated IFITM2, and substantially decreased in cells transfected with IFITM2 bearing double mutations (Fig. 6F). Based on all findings, we concluded that mutation at palmitoylation sites of IFITM2 inhibited its exosomal-mediated transportation, and failed to impair endogenous IFN α synthesis in DCs, followed by sufficient activation of the JAK/STAT pathway and antiviral factors in hepatocytes and eventually inhibited HBV (Fig. 7).

Discussion

For some CHB patients, the therapeutic efficacy of IFN α is suboptimal and the mechanism behind it is still not well understood. Activation of type 1 IFN signaling through the JAK/STAT pathway as well as transcription of ISGs are essential in the anti-HBV efficacy of IFN α . The negative regulators in the IFN α signaling pathway may play an important role in the suboptimal response to IFN α treatment in CHB patients. To identify the host factors related to different responses of IFN α in CHB patients, we downloaded and analyzed gene expression profiles of CHB patients with different responses to IFN α (GSE54747) from the GEO database, and found that innate immune status was associated with the

IFN α -based therapeutic response in CHB patients. In general, viral attacks would rapidly stimulate host immune defense once being recognized as exogenous invaders. However, HBV can utilize intrinsic materials to mimic itself as host components, inducing poor innate immune strike and helping HBV escape from immune surveillance.^(20,21) Previous study revealed that the innate immune pathway was not induced in CHB patients' liver, which could be reactivated following stimulation of Toll-like receptor (TLR)3 by

poly I:C or virus.⁽²²⁾ Yuan et al. also found that host factors played an important part in antiviral immunity, whereas HBV sensitivity to IFN α was more related to activation of cellular IFN pathway than the viral genotype.⁽²³⁾ Because the analysis of GEO data guided us to focus on host immunity, we applied PCR array to investigate factors concerning the innate immunity against virus. Consistently, our study demonstrated lower baseline level of IFN α mRNA in PBMCs of CHB patients with suboptimal response

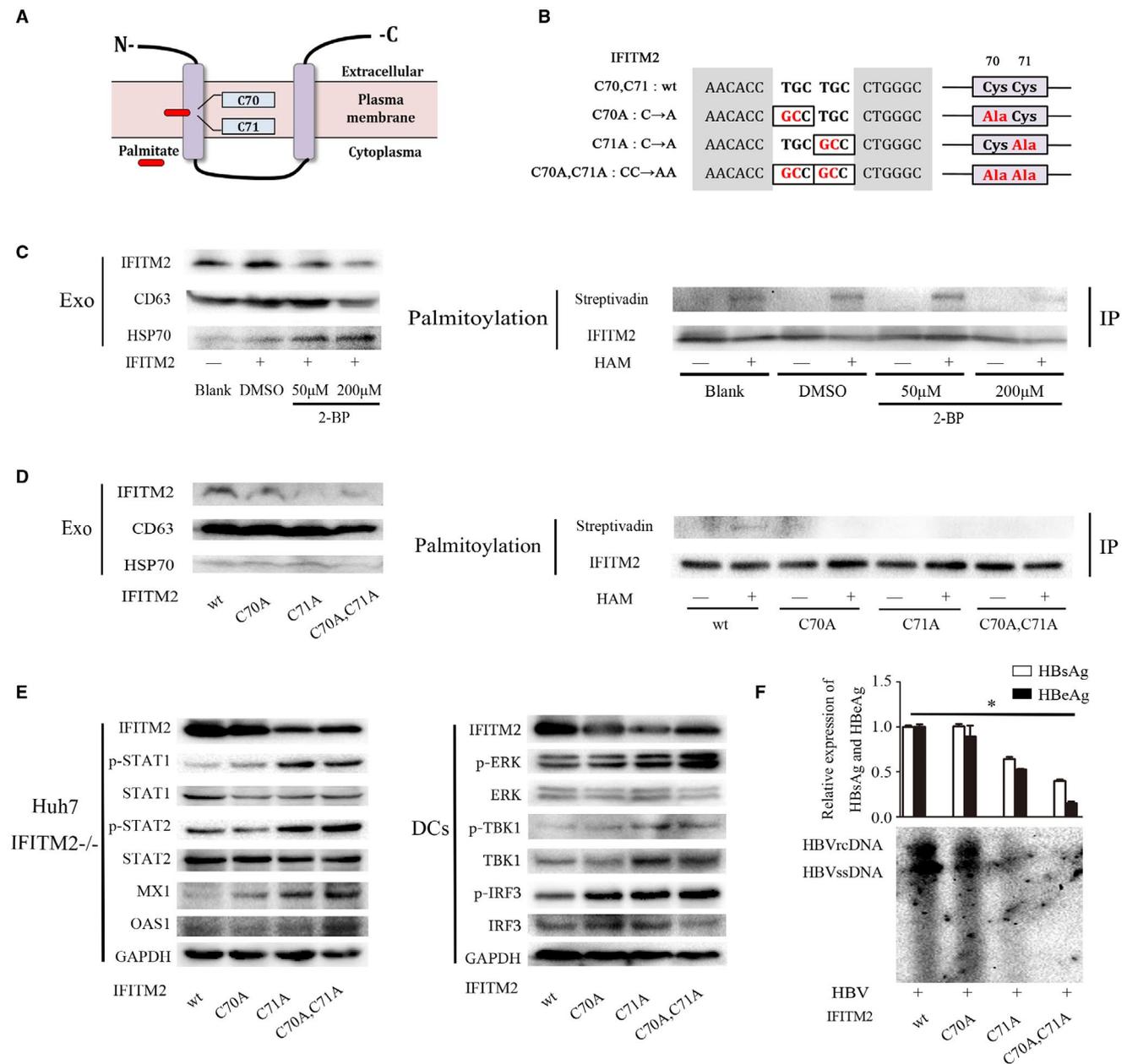


FIG. 6. Proper palmitoylation of IFITM2 is important for its incorporation into exosomes and its biological activities in regulating the antiviral efficacy of IFN α . (A) Cysteine residues 70 and 71 (C70, C71) in the transmembrane domain of IFITM2 were predicted by TMHMM and CSS-Palm software as potential palmitoylation sites. (B) Schematic diagram for the mutagenesis of IFITM2 proteins: C70A; C71A; C70A, C71A. (C) Western blot showed that palmitoylation inhibitor (2-BP, 200 μ M) decreased the incorporation of IFITM2 into exosomes and the palmitoylation of IFITM2 in Huh7 cells. IFITM2 protein level in exosomes from Huh7 cells treated with 2-BP at different concentrations (left panel); and palmitoylated IFITM2 in corresponding Huh7 cells (right panel). (D) Western blot showed that single mutation (C70A; C71A) and double mutations (C70A, C71A) led to decreased level of IFITM2 incorporation into exosomes and decreased IFITM2 palmitoylation in Huh7 cells. IFITM2 protein level in exosomes from Huh7 cells transfected with single mutation (C70A; C71A) and double mutations (C70A, C71A) of IFITM2 overexpressing plasmids (left panel), and palmitoylation degree in corresponding Huh7 cells (right panel). (E) Western blot showed decreased activation of IFN pathway in Huh7 IFITM2^{-/-} cells complemented with wild-type IFITM2 (wt) compared to cells complemented with mutated IFITM2 (C70A; C71A; C70A, C71A). The phosphorylation and total level of STAT1/STAT2 and expression of the antiviral proteins MX1 and OAS1 were detected in Huh7 IFITM2^{-/-} cells (left panel). The levels of IFITM2 and ERK/TBK1/IRF3 activation were detected in DCs co-cultured with complemented Huh7 IFITM2^{-/-} cells (right panel). (F) Mutated IFITM2 protein increased the effect of interferon- α against HBV. HBV replication in Huh7 IFITM2^{-/-} cells complemented with wild type IFITM2 was higher than that of cells complemented with mutated IFITM2. HBsAg and HBeAg were detected by ELISA (upper panel). HBV replication intermediates in corresponding cells were detected by Southern blot (lower panel).

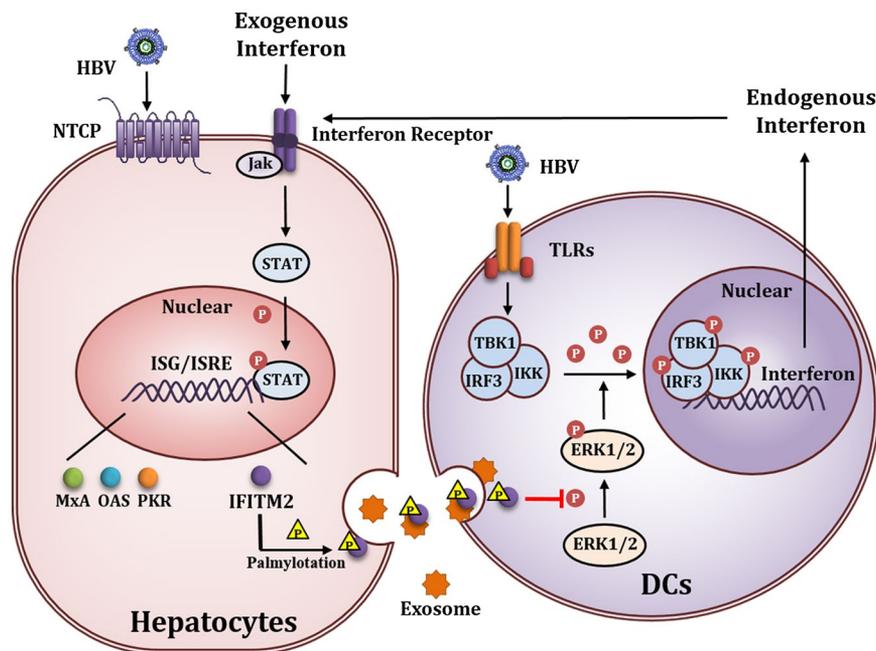


FIG. 7. A model describing the mechanism regarding the inhibitory effect of IFITM2 on Type I IFN signaling pathway. After palmitoylation, IFITM2 is incorporated into exosomes and transported to DCs, where it inhibits the phosphorylation of ERK1/2, sequentially inhibiting phosphorylation of TBK1 and IRF3 to attenuate the synthesis of endogenous IFN α .

to IFN α treatment, whereas there was an increased baseline level of the host immune regulator, IFITM2, indicating that an impaired immune pathway may be responsible for IFN α inefficiency (Fig. 1D,E). These findings suggested that IFITM2 might act as a key negative regulator in the host immunity against HBV. More studies were conducted to validate the negative role of IFITM2. We found higher hepatic expression of IFITM2 in CHB patients with higher viral

load and knocking out IFITM2 effectively inhibiting HBV replication irrespective of exogenous IFN α . Thus, higher baseline levels of IFITM2 may predict a suboptimal therapeutic response of IFN α in CHB patients.

We found that IFITM2 in Huh7 cells suppressed endogenous IFN α synthesis by inhibiting phosphorylation of key markers in the type I IFN signaling pathway (ERK, TBK1, and IRF3); knocking

out IFITM2 enhanced activation of the endogenous IFN α synthesis pathway, exhibiting better inhibition on HBV replication (Figs. 2D-F and 3A-C). The JAK/STAT pathway is one of the most important candidate pathways through which IFN works. Activation of JAK-STAT signaling leads to transcription of various downstream ISGs for antiviral activity. Previous studies have reported several negative regulators acting on the JAK/STAT pathway by inhibiting signaling transduction. For example, suppressor of cytokine signalling (SOCS)-1, SOCS-3, and ubiquitin-specific peptidase 18, which are encoded by ISGs, negatively regulated the JAK/STAT pathway to inhibit the antiviral efficacy of IFN α .⁽²⁴⁾ In this study, we identified that IFITM2 targets on the upstream of the type 1 IFN signaling pathway. Exogenous IFN α triggers the generation of endogenous IFN α to largely facilitate its activation on JAK/STAT signaling and production of antiviral proteins. Synthesis of endogenous IFN α is activated through phosphorylation of TBK1, IRF3, and IKK. To investigate further mechanisms, activation of three key factors in the MAPK cascade related to TBK1 and IRF3 phosphorylation, including ERK, p38, and JNK, were detected after overexpressing IFITM2. Overexpressing IFITM2 led to a significant reduction in ERK and p38 phosphorylation besides JNK phosphorylation. When specific inhibitors of these proteins were applied separately, only the ERK inhibitor blocked TBK1 and IRF3 phosphorylation (Fig. 3D-F). Upon activation, ERK could either directly regulate a number of substrates in the cytoplasmic compartment (MAPK-interacting kinase, ribosomal S6 kinase, focal adhesion kinase, adenosine monophosphate-activated protein kinase, etc.) or phosphorylate diverse transcription factors (FOS, MYC, early growth response 1, Ets-like kinase 1, JUN, etc.) in the nucleus.⁽²⁵⁾ Activated ERK has also been reported to suppress HBV replication by inhibiting hepatocyte nuclear factor (HNF)4 α and HNF1 α activity.⁽²⁶⁾ Counteracting the activation of ERK results in deficiency of multiple antiviral signaling downstream.⁽⁴⁾ Through attenuating phosphorylation and translocation of TBK1 and IRF3, IFITM2 inhibited the synthesis of endogenous IFN α . Therefore, insufficient endogenous IFN α could not facilitate the exogenous IFN α to effectively activate the JAK/STAT signaling pathway. Manipulating IFITM2 could avoid the

bypass pathways and provide us a better strategy to improve the therapeutic response of IFN α on the upstream of the type I IFN signaling pathway.

Because DCs are the most important source of endogenous IFN α , we focused on how the inhibitory function of IFITM2 affected the synthesis of endogenous IFN in DCs. We found that the IFITM2 protein was shuttled by exosomes to DCs, the main source of endogenous IFN α . Exosome-mediated transport of IFITM2 inhibited the synthesis of endogenous IFN α in DCs whereas the inhibitory effect was abolished when IFITM2 was knocked out (Fig. 4C,D). Exosomes mediate cell-to-cell communications by selectively sorted functional proteins, lipids, and RNAs it contains. In viral infection, exosomes participate in the spread of viral infection, modulation of immunity, and manipulation of the microenvironment.^(10,27) Among these functions, mediation of intercellular communication during innate and adaptive immunity is one main assignment.⁽²⁸⁾ In herpes simplex virus 1 infection, infected cells exported an important sensor of innate immunity, stimulator of IFN genes, to uninfected cells. This approach helped to trigger a type I IFN response in uninfected cells, suppressing virus and restricting infection.⁽²⁹⁾ Moreover, in HBV infection models, antiviral protein apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1-like 3G could be exported to neighboring cells by exosomes.⁽³⁰⁾ Increasing numbers of component proteins in exosomes were screened as biomarkers to assess the host immune microenvironment and improve personalized medicine.⁽¹¹⁾ Sharing highly conserved transmembrane structure with IFITM2, IFITM1 and IFITM3 have been reported to be transferred between cells by exosomes.⁽³¹⁾ Our study proved that exosomes were critical vehicles for the transportation of IFITM2 protein (Fig. 5A-F). Given that the inhibitory effect of IFITM2 on IFN α synthesis could be transferred from Huh7 cells to DCs by exosomes, the interference of this exosome-mediate transport could also be an effective way to improve the therapeutic efficacy of IFN α .

When investigating the mechanism of incorporating IFITM2 into exosomes, we demonstrated that both palmitoylation inhibitor and mutation on 70/71 sites of IFITM2 protein influenced its incorporation into exosomes. Mutated IFITM2 protein increased the effect of IFN α against HBV (Fig. 6A-F). For most functional proteins, proper posttranscriptional

modifications, such as phosphorylation, acetylation, and palmitoylation, are indispensable for their maintenance of activation status or proper location. Posttranslational palmitoylation is often associated with endosomal localization of proteins, especially for membrane proteins.⁽³²⁾ S-palmitoylation is a reversible posttranslational modification that acts by the addition of a saturated C16 acyl chain to cytosolic cysteines and is extensively associated with accurate localization of transmembrane proteins by a labile acyl-thioester linkage.⁽³³⁾ S-palmitoylation has been reported to be important for other members in the IFITM family in defending against viruses.⁽³⁴⁾ The transmembrane domain of the IFITM2 family is conserved and important for its biological activity. We analyzed the amino acid sequence of IFITM2 and found that 70/71 cysteines provided a possible binding area for palmitoylation, and discovered that proper palmitoylation was necessary for the incorporation of IFITM2 into exosomes and act effectively in negatively regulating the antiviral capacity of endogenous IFN α . This finding further clarified the mechanism of exosome-mediated transport of IFITM and presented it as a promising therapeutic target on the transport of IFITM2 and sequential inhibitory effect.

In conclusion, we identified a key negative regulator, IFITM2, acting on the upstream of type I IFN signaling (the synthesis of endogenous IFN α) against HBV. Exosome-mediated transport of IFITM2 from hepatocytes to DCs augments the inhibitory effect of IFITM2 on endogenous IFN α synthesis. The insufficient endogenous IFN α synthesis results in suboptimal therapeutic response of exogenous IFN α in CHB patients. This study suggests that baseline IFITM2 may be a marker for predicting the response to IFN α treatment in CHB patients. Down-regulating IFITM2 or interfering with its palmitoylation may be potential strategies to improve the therapeutic response to IFN α treatment.

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Supporting Information

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