Hepatitis B Virus–Induced Imbalance of Inflammatory and Antiviral Signaling by Differential Phosphorylation of STAT1 in Human Monocytes

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It is not clear how hepatitis B virus (HBV) modulates host immunity during chronic infection. In addition to the key mediators of inflammatory response in viral infection, monocytes also express a high-level IFN-stimulated gene, *CH25H*, upon response to IFN-α exerting an antiviral effect. In this study, the mechanism by which HBV manipulates IFN signaling in human monocytes was investigated. We observed that monocytes from chronic hepatitis B patients express lower levels of IFN signaling/stimulated genes and higher levels of inflammatory cytokines compared with healthy donors. HBV induces monocyte production of inflammatory cytokines via TLR2/MyD88/NF-κB signaling and STAT1-Ser727 phosphorylation and inhibits IFN-α–induced *stat1*, *stat2*, and *ch25h* expression through the inhibition of STAT1-Tyr701 phosphorylation and in an IL-10–dependent, partially autocrine manner. Further, we found that enhancement of STAT1 activity with a small molecule (2-NP) rescued HBV-mediated inhibition of IFN signaling and counteracted the induction of inflammatory cytokines. In conclusion, HBV contributes to the monocyte inflammatory response but inhibits their IFN-α/β responsiveness to impair antiviral innate immunity. These effects are mediated via differential phosphorylation of Tyr701 and Ser727 of STAT1. ***The Journal of Immunology*****, 2019, 202: 2266–2275.**

Although an effective vaccine has been in use for decades, over 240 million people in the world are chronically infected with hepatitis B virus (HBV), and new infections continue to occur (1–3). HBV is an infection in which chronic liver inflammation coexists with impaired antiviral immunity. The functionality of many immune cells is impacted, including monocytes, but the molecular details of such impairment are not well understood.

HBV infects hepatocytes of the human liver and initiates virus replication to release HBV virions, HBV e Ag (HBeAg), and HBV surface Ag (HBsAg). The serum levels of HBsAg, HBeAg, and HBV DNA may fluctuate over time and are a reflection of disease activity to define the natural course of chronic HBV infection (4–6). During HBV infection, the virus exposes a number of pathogen-associated molecular patterns to be recognized by pattern recognition receptors located on the cell membrane, such as TLR. Circulating monocytes express a variety of pattern recognition receptors, so it is likely that the function of these cells will be affected. Monocytes represent ∼10% of leukocytes in human peripheral blood and also reside in or pass through the liver as precursors of dendritic cells and macrophages. The frequency and number of $CD14^+/CD16^+$ monocytes in immune active chronic hepatitis B (CHB) patients were significantly increased (7), and their positive correlation with alanine amino transferase (ALT) suggested a potential role contributing to liver inflammation (8). However, the role of HBV infection in monocytes remains controversial. It has been reported that HBsAg induces TNF-α and IL-10 production of monocytes (9, 10), suggesting that HBsAg can potentially trigger TLR activation through its association with CD14 (11). The arginine-rich domain of the core protein was shown to bind the macrophage-like cell line, THP-1, in a heparin sulfate–dependent manner and trigger signaling through TLR-2 (12). But others have reported that exposure of monocytes to HBsAg suppresses LPS-induced TNF and IL-1β production (13).

Type I IFNs (IFN-I), produced by cells infected with viruses and some sentinel cells of the innate immune system, play a crucial role in defense against viral infections by inducing the expression of IFN-stimulated genes (ISGs) such as cholesterol 25-hydroxylase (*ch25h*) (14–17). *CH25H* can be expressed at high levels in monocytes and macrophages activated by IFN-I via STAT1 and synthesizes 25HC to exert an antiviral effect (18–20). Monocytes interact with the virus to produce proinflammatory cytokines and IFN-I and to initiate immune responses to control infection.

Most vertical transmission of HBV from mother to child often leads to chronic infection, whereas horizontal transmission between adults often leads to self-limited acute infection. It has been

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Abbreviations used in this article: ALT, alanine amino transferase; CHB, chronic hepatitis B; CH25H, cholesterol 25-hydroxylase; HBeAg, HBV e Ag; HBsAg, HBV surface Ag; HBV, hepatitis B virus; HBVcc, cell-cultured HBV; HSA, human serum albumin; IFN-I, type I IFN; ISG, IFN-stimulated gene; KO, knockout; 2-NP, 2-(1,8-naphthyridin-2-yl)phenol; qRT-PCR, quantitative real-time PCR; WT, wild type.

demonstrated that Kupffer cells from nontransgenic offspring of HBV transgenic mothers that have been exposed to HBeAg in utero express a higher level of PD-L1 to exhaust the function of CTLs. But Kupffer cells from nontransgenic adult mice that were never exposed to HBeAg generate efficient immunity to control HBV infection (21). More recently, we reported that both HBV and HBsAg induce immune suppressive monocytes to initiate regulatory NK cell differentiation, resulting in T cell inhibition (22). We thus postulated that HBV develops sophisticated strategies to counteract this defense, as it established de novo infection.

We now show that the HBV-induced antagonism between inflammation and antiviral immunity can be studied in vitro. HBV activates TLR2/NF-κB signaling and STAT1-Ser727 phosphorylation to induce the production of inflammatory cytokines and to make a concerted attack on the IFN signaling pathway manifest both as reduced transcription of STAT1 and STAT2 genes and in aberrant STAT1 phosphorylation.

Materials and Methods

Human subjects

Thirty-six treatment-naive CHB patients in hospital, which were identified as immune active phase based on sera levels of HBV DNA, HBeAg, HBsAg, and ALT, and 30 healthy donors were enrolled in this study (Table I). All studies were conducted according to the experimental practices and standards that were approved by the Medical Ethics Committee of The First Hospital of Jilin University (approval code 2015-125). Written informed consent was obtained from all adult participants, and no children were involved in this study. There were no hepatitis D virus coinfected in any of the patients. All experiments were carried out in accordance with the approved guidelines and regulations.

Reagent

HBsAg was purchased from Meridian (catalog no. R36100), which was purified from human plasma with the purification >95% and dissolved in the following buffer: 0.02 M sodium phosphate, pH 7.2. Human serum albumin (HSA) purified from healthy person serum was purchased from ProSpec (catalog no. PRO-354). Human TLR2 Ab (catalog no. MAB2616), human TLR4 Ab (catalog no. AF1478), and human recombinant IFN-α (catalog no. 11200-1) were purchased from R&D Systems. Anti-human CD282 (TLR2) FITC was purchased from eBioscience (catalog no. 11-9922-41). Human IL-10 Ab (catalog no. MAB2171), human IL-1β/IL-1F2 Ab (catalog no. MAB201), and human TNF-α Ab (catalog no. MAB210) were purchased from R&D Systems. Abs against MyD88 and p-STAT2 (Tyr690) were purchased from Abcam. Protease/Phosphatase Inhibitor Cocktail and Abs against p-STAT1 (Tyr701 and Ser727), STAT1, STAT2, p-IκBα, IκBα, and TRAF6 were purchased from Cell Signaling Technology (Danvers, MA). CH25H Ab was purchased from Santa Cruz catalog no. sc-293256). BAY-11-7085 was purchased from Medchem Express (catalog no. HY-10257). 2-(1,8-naphthyridin-2-yl)phenol (2-NP) was purchased from Sigma-Aldrich (catalog no. N2040).

Purification of cell-cultured HBV

HepG2 cells and HepG2.2.15 cells purchased from American Type Culture Collection were maintained in DMEM medium (Corning Life Science, Lowell, MA) with 10% FBS, penicillin, streptomycin, and glutamine. HBV particles were collected and purified from the supernatant of HepG2.2.15 cells as described previously with modification (23, 24). Briefly, HepG2.2.15 cells and HepG2 cells were cultured in 75T flasks in DMEM complete medium to induce HBV replication and virion production; supernatant was collected every other day with culture medium replenishment for 18 d. The pooled supernatant was mixed with polyethylene glycol (PEG)-8000 powder (Sigma-Aldrich, final concentration of 10%) and gently rotated at 4°C for overnight; HBV particles were then precipitated by centrifugation at 10,000 × *g* for 45 min at 4°C and redissolved in serum-free DMEM medium with 1% vol of the original supernatant samples. HBV virion stocks, which were measured using the COBAS AmpliPrep/COBAS TaqMan assay (Roche Molecular Diagnostics, Grenzach, Germany) for viral load and ELISA for encoded proteins were aliquoted and stored at −80°C. The supernatant of HepG2 cell has been treated with PEG8000 and concentrated in the same way as cell-cultured HBV (HBVcc) stock, then used as mock control.

Limulus amebocyte assay for LPS contamination

Endotoxic contamination of HBsAg and HBVcc was assessed using QCL-1000 chromogenic end point assay (Combrex, Cottonwood, AZ) and found to be <1 pg/ml.

Cell isolation and purification

PBMCs were freshly isolated from peripheral blood of healthy individuals and CHB patients by Ficoll density gradient separation. Monocytes were then purified by magnetic cell sorting with CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the $CD14^+$ cells was equal or >95% determined by flow cytometry.

Cell culture

Purified monocytes were cultured with HBsAg, HBVcc, and/or IFN-α (concentrations are given in the figure legends) in 96-well plates containing 200 μl of complete medium (RPMI 1640, 10% FBS, penicillin, streptomycin, and glutamine) at 37°C in the presence of 5% CO_2 for 12 or 24 h. Cells were collected for quantitative real-time PCR (qRT-PCR), Western blot, FACS analysis, and the supernatants were harvested for cytokine detection by ELISA.

RNA extraction and qRT-PCR

Total RNA was extracted from cells using EasyPure RNA Kit (TransGen Biotech, Beijing, China) and then converted to first-strand cDNA using TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech). Real-time PCR was performed with Power SYBR Green PCR Master Mix (TransGen Biotech) in an ABI StepOne Plus Real-Time PCR system (Applied Biosystems). Housekeeping gene GAPDH was used as an internal control for quantitation, respectively. The sequences of gene-specific primers used for qRT-PCR are listed in Supplemental Table I.

Immunoblotting

Immunoblotting was carried out as described previously (25). Briefly, cells were collected and lysed by adding lysis buffer (RIPA buffer) together with Protease/Phosphatase Inhibitor Cocktail on ice for 30 min and tapping tubes every 10 min. Protein concentration was quantified by Coomassie Plus protein assay Reagent (Thermo Fisher Scientific). The quantitation of immunoblotting band intensity was carried out with ChemiDoc XRS+ Molecular Imager software (Bio-Rad). Samples were separated by SDS–PAGE and transferred to PVDF membranes. After blocking in PBS containing 0.1% Tween-20 and 5% skim milk, the blots were probed with relative Abs.

STAT1 knockout and mutation

STAT1 knockout (KO) of THP1 cells was carried out by CRISPR/Cas9 as described previously (26, 27). Briefly, 293T cells were cotransfected with GagPol, VSV-G expression plasmid, and plentiCRISPRv2-STAT1 plasmids using ViaFect Transfection Reagent (Promega). Supernatant was harvested 2 d after transfection and added to THP1 cell; transduced bulk cells were selected with puromycin selection marker. Immunoblotting was done to ensure STAT1 KO results, and DNA sequencing was performed to further confirm the results of gene KO. Single guide RNA sequences are shown in Supplemental Table I. The expression constructs of STAT1 were generated by cloning the sequence of the coding region into a Flag-tagged VR1012 expression vector (STAT1 wild type [WT]). Site-directed mutagenesis of STAT1 (Y701A and S727A) was generated using mutations primers by Quik Change PCR (TransGen). All constructs were confirmed by DNA sequencing, and plasmids were prepared using Endo-Free Plasmid Kits (Ω). STAT1-KO THP1 cells were differentiated with 0.5 μg/ml of PMA for 3 h. To remove the residual effect of PMA, the medium was replaced with complete RPMI 1640 overnight, then transfected with the plasmids of STAT1 WT and mutation (STAT1-S727A and STAT1-Y701A).

Flow cytometry

Purified monocytes were cultured with HBsAg or HBVcc, resuspended in staining buffer, and preincubated with FcR blocking reagent (Miltenyi Biotec) for 15 min at 4°C. The cells were stained with anti-human CD14-PE, anti-human CD282 (TLR2) FITC Abs, and incubated for 15 min at 4°C in the dark. The cells were washed with staining buffer, then resuspended in PBS containing 1% paraformaldehyde. For intracellular STAT staining, cells were first fixed/permeabilized and subsequent STAT staining performed according to the manufacturer's protocol (Cytofix Buffer and Permeabilization Buffer III; BD Biosciences). The intracellular Abs included STAT1 (pY701) Alexa Fluor 488 (4a; BD Biosciences), STAT1

(pS727) PE (clone K51-856 [RUO] BD Biosciences). All data were acquired with an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star) software (Ashland, OR).

Cytokine measurements

IL-1β, IL-10, and TNF-α were quantified in supernatants using ELISA kits (R&D Systems) according to the manufacturer's recommended protocol.

Statistical analysis

Statistical analysis was performed using a two-tail unpaired Student *t* test. Correlation coefficient: Pearson correlation analysis was used to determine *R*-values. A p value <0.05 was considered statistically significant. Statistical analysis and Pearson correlation analysis were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

Table I. Characteristics of study population

Clinical Data	Healthy Controls (n = 30)	Chronic HBV Patients (n = 36)
Sex (male/female)	16/14	19/17
Age (y)	35.3 ± 9.9	34.47 ± 14.13
ALT (U/l)	32.6 ± 5.5	391.4 ± 319.7
HBsAg (IU/ml)	Negative	8441.5 ± 4598.5
HBeAg (S/CO)	Negative	173.2 ± 311.3
HBeAb (S/CO)	Negative	11.6 ± 15.5
HBV DNA (log10 IU/ml)	Negative	6.6 ± 1.1

Data are shown as average ± SD.
HBeAb, HBV e Ab; S/CO, signal to cutoff ratio.

Results

Peripheral monocytes express lower levels of IFN signaling/stimulated genes and higher levels of inflammatory cytokines in CHB patients

We first compared the difference of antiviral and inflammatory gene expression in monocytes between healthy donors and CHB patients (Table I). Monocytes were purified to determine the mRNA expression of IFN signaling/stimulated genes and inflammatory cytokines by qRT-PCR, and the relative fold induction was normalized to a housekeeping gene (GAPDH). We observed that the mRNA expression levels of *stat1* (Fig. 1A), *stat2* (Fig. 1B), and *ch25h* (Fig. 1C) were significantly lower in CHB patients than in healthy individuals. However, the mRNA expression levels of *IL-1β* (Fig. 1D), *TNF-α* (Fig. 1E), and *IL-10* (Fig. 1F) in CHB patients were significantly higher than in healthy individuals. Among CHB patients, our statistical analysis showed (Supplemental Fig. 1) that the serum levels of HBV DNA and HBsAg correlate negatively with the expression levels of *stat1*, *stat2*, and *ch25h* positively with the expression levels of *IL-1β*, *TNF-α*, and *IL-10* in monocytes. *IL-10* correlates negatively with the expression levels of *stat1*, *stat2*, and *ch25h*. These results indicate that the inflammatory cytokines (*IL-10*) may inhibit the expression of IFN signaling/stimulated genes in monocytes of CHB patients.

HBV and IFN-α inversely modulate ISGs expression of monocytes

Next, we investigated HBV effect on the antiviral gene expression. Purified monocytes from healthy donors were cultured with increasing concentrations of HBsAg (0, 0.2, 2, 20 μg/ml), HBVcc (0, 10^5, 10^6, 10^7 copies/ml), or IFN-α (0, 1, 10, 100 ng/ml) using HSA, the supernatant of non-HBV transfected cells (HepG2), and vehicle of IFN-α as controls, respectively. The mRNA expression levels of *stat1*, *stat2*, and *ch25h* were examined by qRT-PCR, and the relative fold induction was normalized to HSA, HepG2, or vehicle of IFN-α. We observed that both HBsAg and HBVcc caused a dose-dependent decrease in expression of *stat1* (Fig. 2A), *stat2* (Fig. 2B), and *ch25h* (Fig. 2C); IFN-α induced a dose-dependent increase in the expression of *stat1* (Fig. 2D), *stat2* (Fig. 2E), and *ch25h* (Fig. 2F). A total of 10^7 copies/ml of HBVcc, 20 μg/ml of HBsAg, and 10 ng/ml of IFN-α significantly modulated expression of these genes and were used as the standard

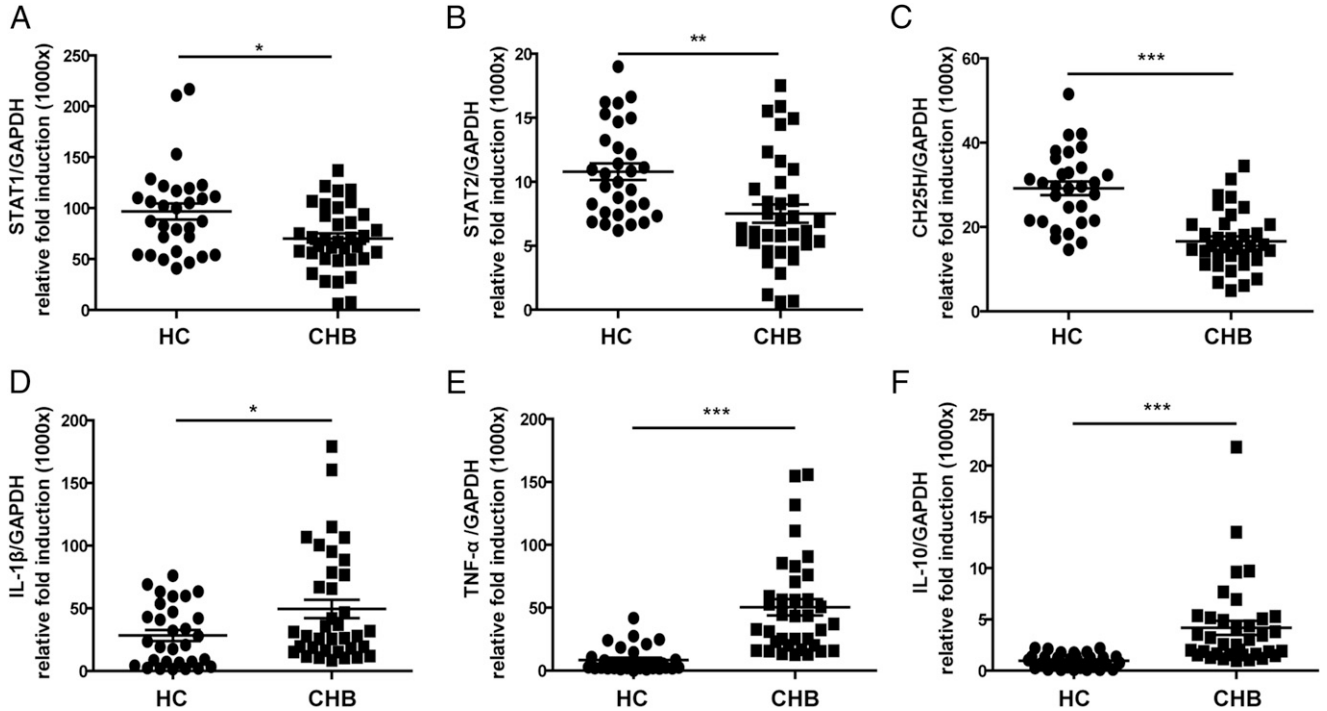

FIGURE 1. Monocytes express lower levels of IFN signaling/stimulated genes and higher levels of inflammatory cytokines on chronic HBV infection. Monocytes were purified from PBMC of 30 healthy individuals (HC) and 36 CHB patients. The mRNA expression levels of *stat1* (**A**), *stat2* (**B**), *ch25h* (**C**), *IL-1β* (**D**), *TNF-α* (**E**), and *IL-10* (**F**) were determined by qRT-PCR. Error bars represent the SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

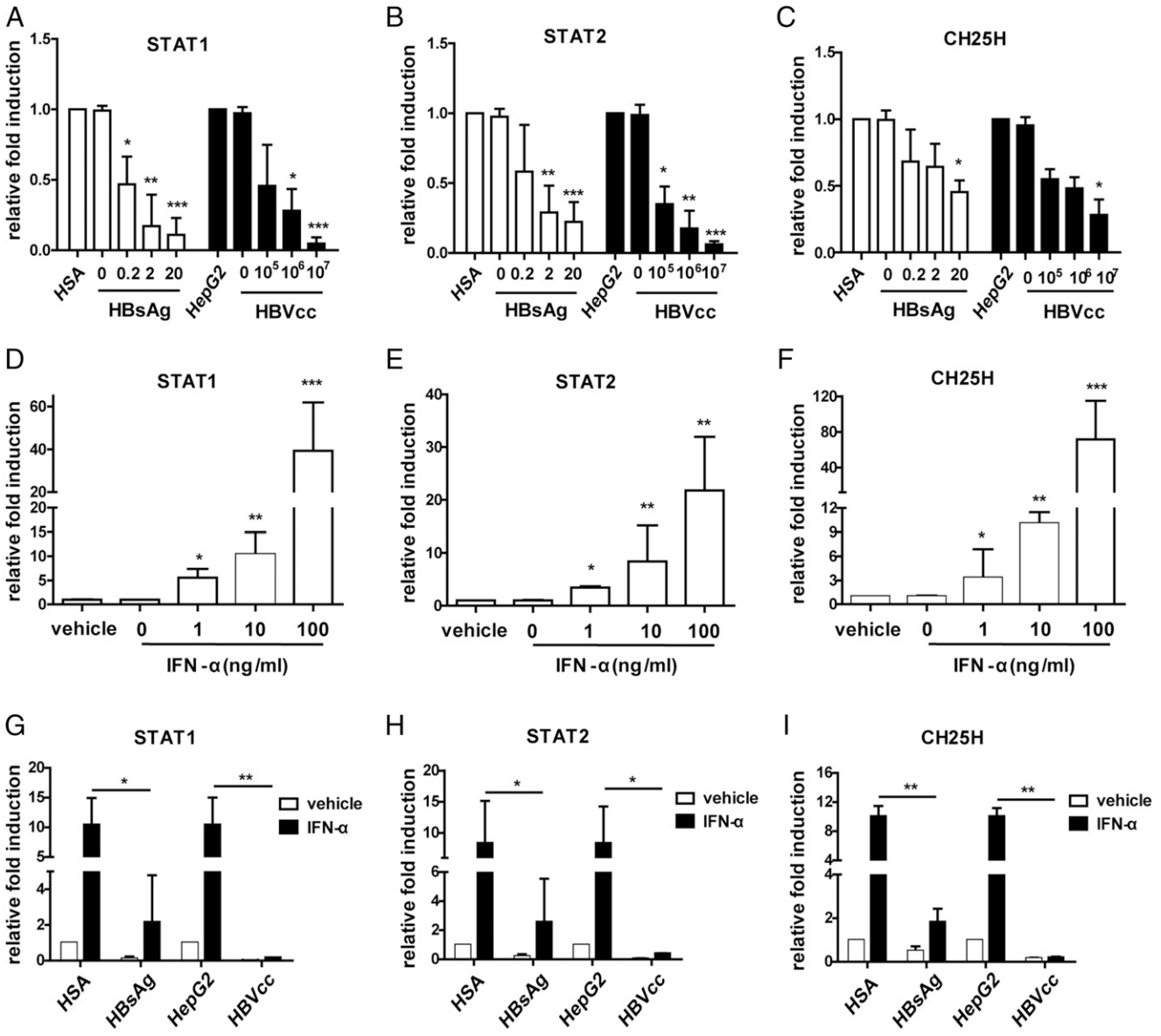

FIGURE 2. HBV and IFN-α inversely modulate ISGs' expression of monocytes. Purified monocytes from healthy donors (n = 8) were cultured with increasing concentrations of HBsAg (0, 0.2, 2.0, 20.0 μg/ml) or HBVcc (0, 10^5, 10^6, 10^7 copies/ml) or IFN-α (0, 1, 10, 100 ng/ml) for 12 h. Cells were collected to assay the mRNA expression of *stat1*, *stat2*, and *ch25h* by qRT-PCR (**A**–**F**). Purified monocytes from healthy donors were cultured with or without IFN-α (10 ng/ml) in presence of HBsAg (20 μg/ml) or HBVcc (10^7 copies/ml). Cells were collected to assay the mRNA expression of (**G**) *stat1*, (**H**) *stat2*, and (**I**) *ch25h* by qRT-PCR. Error bars represent the SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

concentrations in the following experiments. Although HBVcc or HBsAg were added into monocytes cultured with IFN-α, both HBsAg and HBVcc significantly inhibited IFN-α–induced mRNA expression levels of *stat1* (Fig. 2G), *stat2* (Fig. 2H), and *ch25h* (Fig. 2I). Also, cells were collected for RNAseq. Self-organizing heat maps confirmed that HBsAg and HBVcc inhibited IFN-α–induced ISG expression (e.g., CH25H, TRIM6, TRIM58, APOBEC3B, APOBEC3D, STAT1, STAT2, etc. Supplemental Fig. 2A).

HBV induces inflammatory response of monocytes via TLR2/MyD88/NF-κB signaling pathway

We further investigated whether HBV has direct effects on the inflammatory response of monocytes, which might account for the abnormalities of monocytes from CHB patients. Fresh monocytes from healthy donors were purified and cultured with increasing concentrations of HBsAg or HBVcc. As expected, both HBsAg (0, 0.2, 2, 20 μg/ml) and HBVcc (0, 10^5, 10^6, 10^7 copies/ml) caused a dose-dependent increase in mRNA expression and secretion of IL-1β (Fig. 3A), TNF-α (Fig. 3B), and IL-10 (Fig. 3C). To determine if HBV activates MyD88/NF-κB signaling in monocytes, the protein expression of MyD88, TRAF6, and NFκB1, and IκBα phosphorylation was detected by Western blot. As shown in Fig. 3D, both HBsAg and HBVcc also induced the protein expression of MyD88, TRAF6, and promotes IκBα phosphorylation and IκBα degradation compared with mock control (HSA and the supernatant of HepG2 cell). Moreover, when monocytes were treated with the inhibitor of NF-κB (BAY-11-7085) and cultured with HBVcc or HBsAg, we observed that BAY-11-7085 inhibited HBsAg- and HBVcc-induced production of IL-1β, TNF-α, and IL-10 (Fig. 3E).

Previous reports showed that TLR2 expression of monocytes was reduced in patients with HBeAg-positive chronic HBV infection but increased in HBeAg-negative CHB, compared with

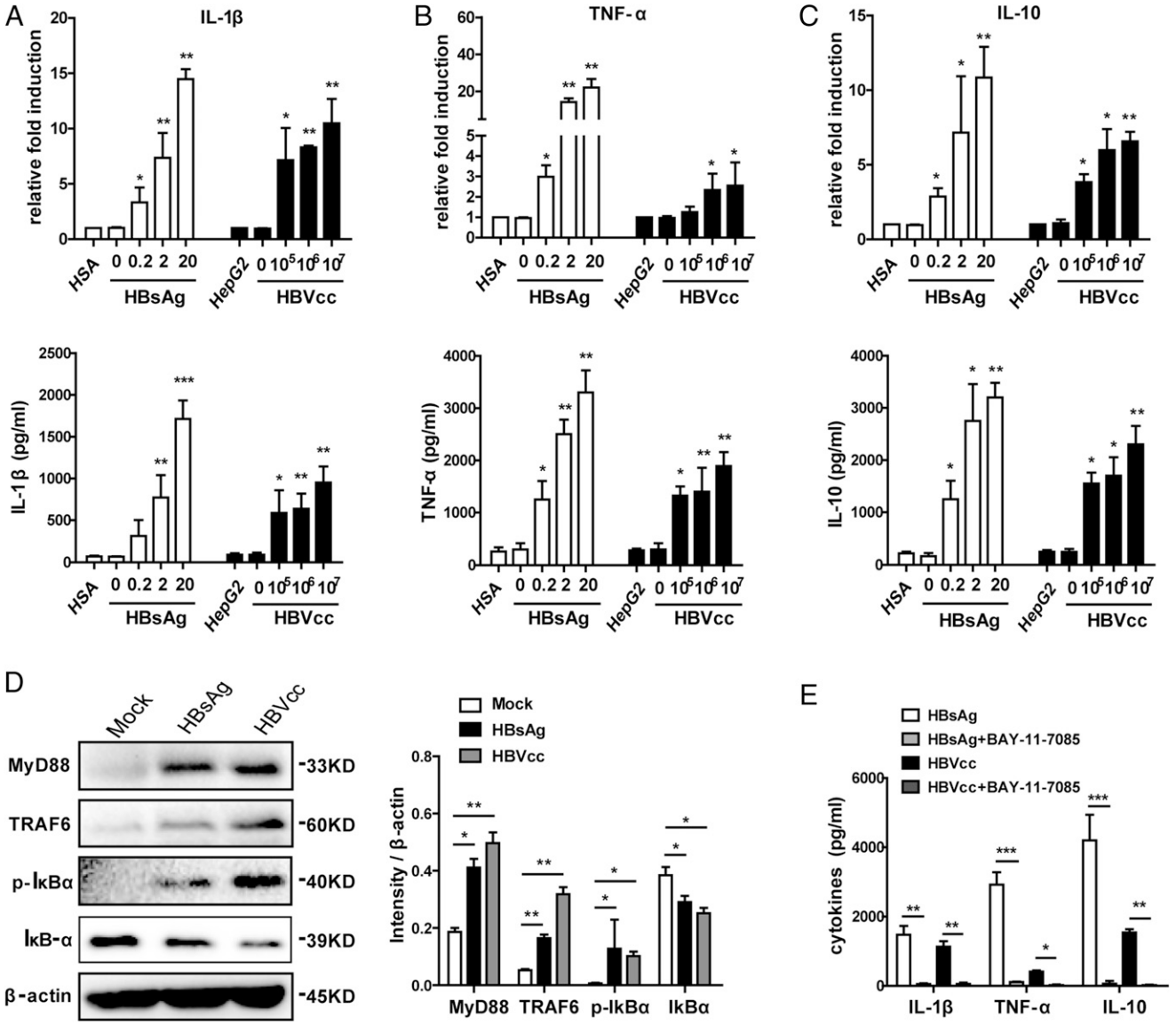

FIGURE 3. HBV activates MyD88/TRAF6/NF-κB signaling. Purified monocytes from healthy HBV-negative blood donors ($n = 6$) were cultured in the presence of increasing concentrations of HBsAg (0, 0.2, 2.0, 20.0 μg/ml) or HBVcc (0, 10^5, 10^6, 10^7 copies/ml) for 24 h; the mRNA expression level and production of IL-1β (**A**), TNF-α (**B**), and IL-10 (**C**) were determined by real-time RT-PCR and ELISA, and the relative fold induction was normalized to HSA or HepG2. Purified monocytes from healthy HBV-negative blood donors were cultured with HBsAg (20 μg/ml) or HBVcc (10^7 copies/ml) for 12 h. Western blots were performed for the expression of MyD88, TRAF6, IκBα, and IκBα phosphorylation. β-actin was used as loading control (**D**). Purified monocytes were treated with 5 μM BAY-11-7085 in the presence of HBsAg (20 μg/ml) or HBVcc (10^7 copies/ml) for 24 h; cytokine production of IL-1β, TNF-α, and IL-10 were detected by ELISA (**E**). Error bars represent the SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

controls (28). In our study population, these findings were confirmed as Fig. 4A. We thus investigated whether HBsAg is different from HBeAg in its ability to upregulate TLR2 expression of monocytes, resulting in inflammatory cytokines production. Purified monocytes from healthy donors were cultured with HBsAg and HBVcc (without encoded protein contamination), and we observed that both HBsAg and HBVcc upregulated TLR2 expression of monocytes on both mRNA (Fig. 4B) and protein level (Fig. 4C) but not other TLRs compared with HSA and HepG2 controls. We thus tested if HBV-induced inflammatory response is mediated via TLR2 signaling. Purified monocytes were pretreated with anti-TLR2 or anti-TLR4 Ab and then stimulated with HBsAg or HBVcc. We observed that blockade of TLR2 significantly inhibited HBsAg- and HBV-induced cytokine expression and secretion of IL-1β (Fig. 4D), TNF-α (Fig. 4E), and IL-10 (Fig. 4F).

HBV and IFN-α, respectively, induce inflammatory and antiviral gene by differential STAT1 phosphorylation

STAT1 is a key component of the transcription factor complex in the IFN signaling pathway (29) and also plays a role in the inflammatory response (30). We investigated STAT1 phosphorylation in response to HBV and IFN-α. Purified monocytes were treated with IFN-α and/or HBVcc/HBsAg, and the effect of HBV and IFN-α on the phosphorylation of STAT1–Tyrosine 701 and STAT1–Serine 727 was determined by Western blot. We observed that both HBsAg and HBVcc activated STAT1 Serine 727 phosphorylation but not STAT1 Tyrosine 701 phosphorylation compared with mock (Fig. 5A). IFN-α activated STAT1 Tyrosine 701 phosphorylation and CH25H protein expression, and both HBsAg and HBVcc inhibited IFN-α–induced STAT1 Tyrosine 701 phosphorylation and CH25H protein expression compared with IFN-α (Fig. 5B). Further, we found that IFN-α induced STAT2 protein

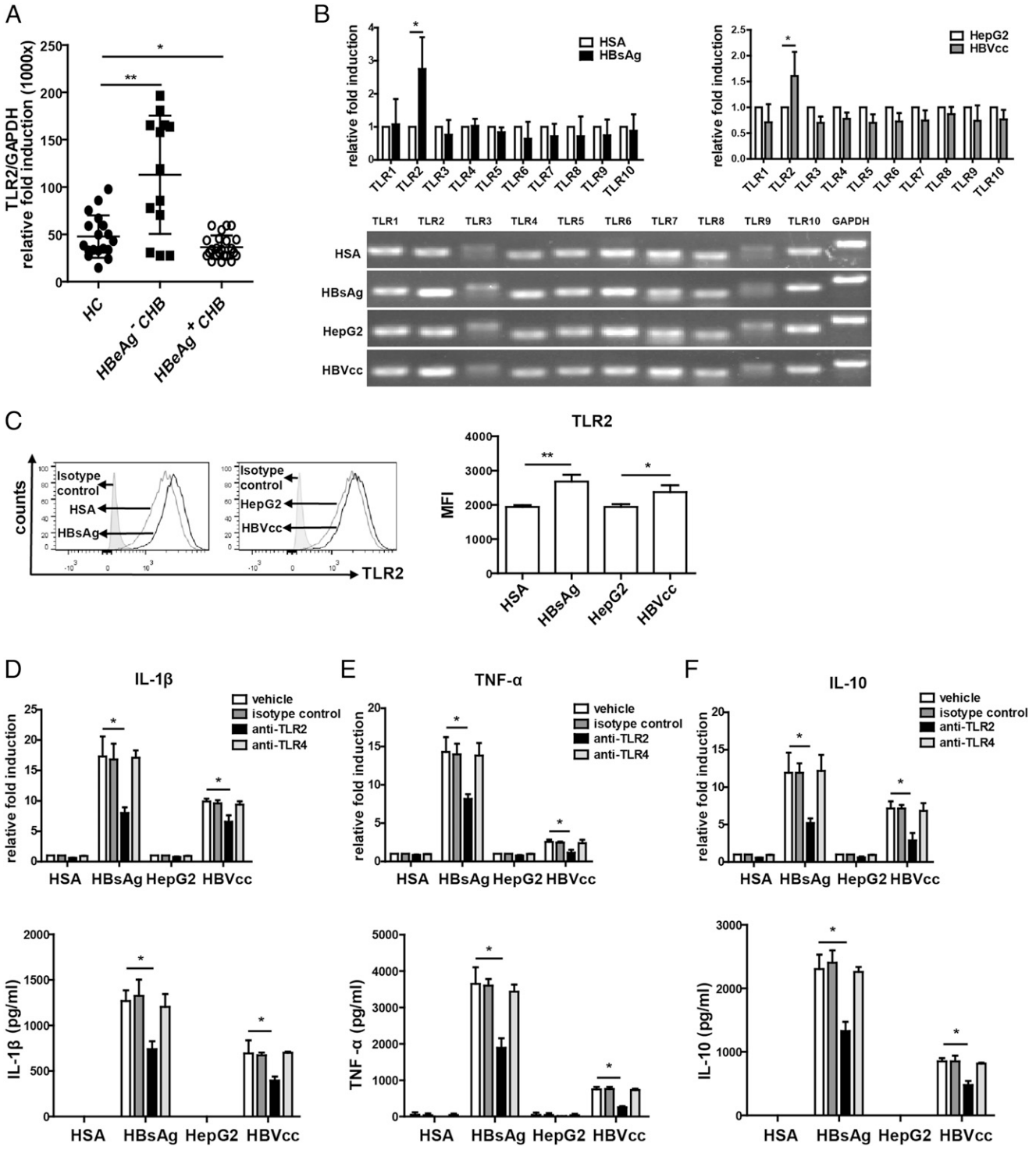

FIGURE 4. HBV induces inflammatory cytokines production via TLR2 signaling. Monocytes were purified from PBMC of 18 healthy individuals (HC), 13 HBeAg-negative CHB and 23 HBeAg-positive CHB. The mRNA expression levels of TLR2 were determined by real-time RT-PCR (**A**). Purified monocytes from healthy HBV-negative blood donors (n = 10) were cultured with HBsAg (20.0 μg/ml) or HBVcc (10^7 copies/ml) for 12 h, the mRNA expression level of TLR1-TLR10 were determined by real-time RT-PCR and agarose gel electrophoresis (**B**), and cell surface expression of TLR2 were analyzed by flow cytometry (**C**). Purified monocytes were pretreated with anti-TLR2 Ab (0.5 μg/ml) or anti-TLR4 Ab (5 μg/ml) for 1 h, and then stimulated with HBsAg or HBVcc for 24 h, the cytokine expression and production of IL-1β (**D**),TNF-α (**E**) and IL-10 (**F**) were determined by real-time RT-PCR and ELISA, the relative fold induction was normalized to HSA or HepG2. Error bars represent the SD. *$p < 0.05$, **$p < 0.01$.

expression and phosphorylation, but HBsAg and HBVcc did not inhibit IFN-α–induced STAT2 (Y690) phosphorylation. We next constructed the plasmids encoding STAT1 WT and two mutations (STAT1-S727A and STAT1-Y701A) and then transfected them into STAT1-deficient THP1 cells. The cells were stimulated with IFN-α or HBsAg. The results showed that HBsAg induced the phosphorylation of STAT1–Serine 727 and *IL-1β* expression in STAT1 WT transfected cells, but the mutation STAT1-S727A shut down HBsAg-induced phosphorylation of STAT1–Serine 727 and *IL-1β* expression (Fig. 5C). Conversely, IFN-α induced the phosphorylation of

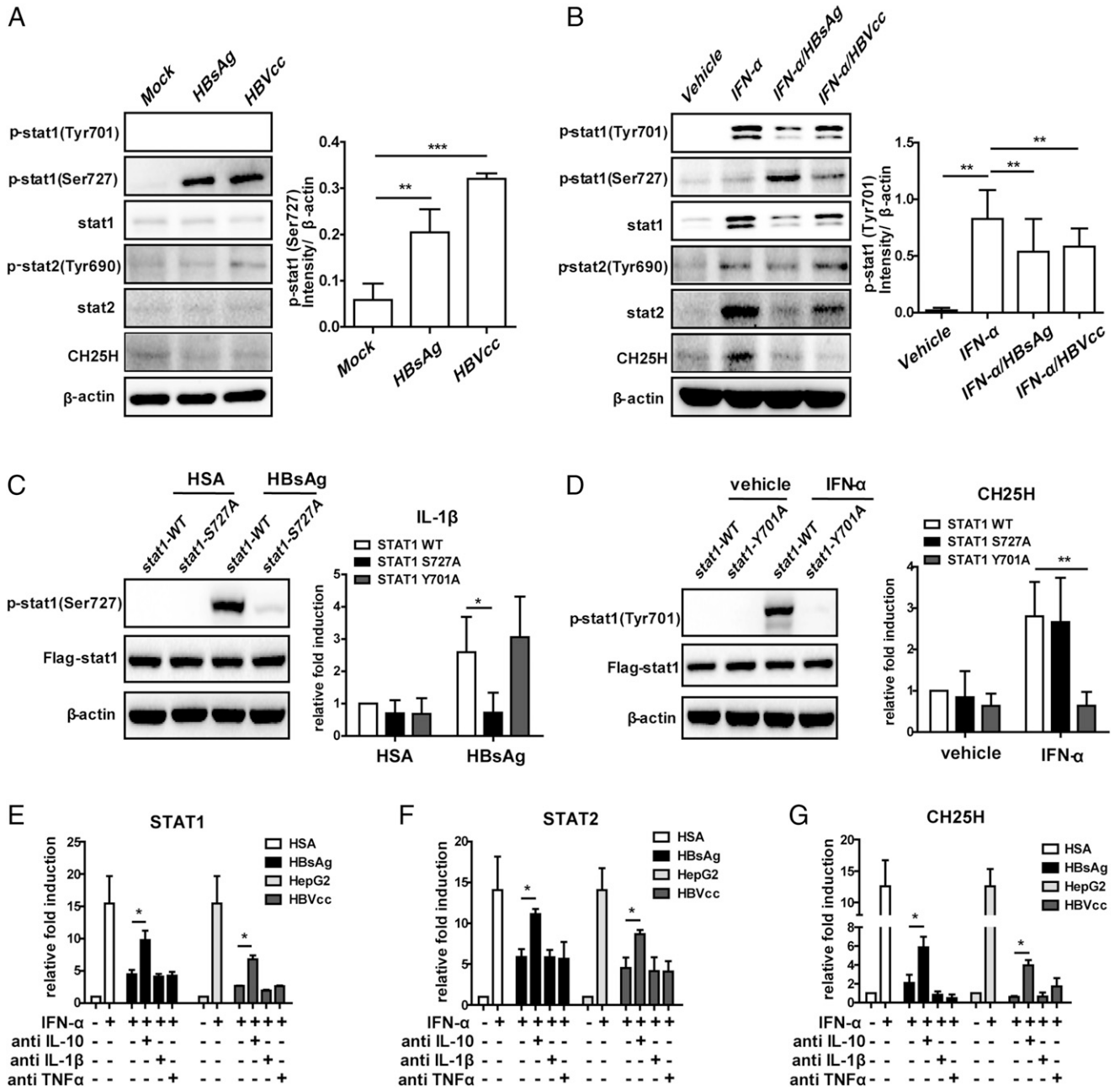

FIGURE 5. HBV activates STAT1-ser727 phosphorylation and inhibits IFN-α–induced STAT1-Tyr701 phosphorylation. Purified monocytes from healthy donors ($n = 6$) were stimulated for 12 h with HBsAg (20 μg/ml) or HBVcc (10^7 copies/ml) (**A**) or with IFN-α (10 ng/ml) in the presence of HBsAg (20 μg/ml) or HBVcc (10^7 copies/ml) (**B**). Western blots were performed for STAT1, STAT2, CH25H expression and phosphorylation of STAT1-Tyr701, STAT1-ser727, and STAT2-Tyr690; β-actin was used as loading control. STAT1 KO THP1 cells were seeded in 24-well plate and differentiated with 0.5 μg/ml of PMA for 3 h. To remove the residual effect of PMA, the medium was replaced with complete RPMI 1640 overnight, then transfected with STAT1-WT, STAT1-S727A, or STAT1-Y701A expression plasmids as indicated. After 24 h, Cells were treated with HBsAg (20 μg/ml) or HSA for 12 h, Cells were collected to examine phosphorylation of STAT1-ser727 and the mRNA expression levels of *IL-1β* by Western blot and qRT-PCR, respectively (**C**). Cells were treated with IFN-α (10 ng/ml) or vehicle for 12 h; phosphorylation of STAT1-Tyr701 and the mRNA expression levels of *CH25H* were detected as well (**D**) ($n = 3$). Purified monocytes were treated with neutralization Abs of IL-10 (0.5 μg/ml), IL-1β (1 μg/ml), or TNF-α (0.5 μg/ml) for 1 h and then stimulated with IFN-α (10 ng/ml) in the presence of HBsAg (20 μg/ml) or HBVcc (10^7 copies/ml); cells were collected to assay the mRNA expression of (**E**) *stat1*, (**F**) *stat2*, and (**G**) *ch25h* by qRT-PCR. Error bars represent the SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

STAT1–Tyrosine 701 and *CH25H* expression in STAT1 WT transfected cells, but the mutation of STAT1-Y701A–shut off IFN-α induced the phosphorylation of STAT1–Tyrosine 701 and *CH25H* expression (Fig. 5D). These results indicate that HBV induces the inflammatory response by activation of STAT1–Serine 727 phosphorylation, whereas IFN-α induces the antiviral effect by activation of STAT1–Tyrosine 701 phosphorylation.

Given that HBV induces the production of IL-1β, TNF-α, and IL-10 and inhibits IFN-α–induced mRNA expression of *stat1*, *stat2*, and *ch25h* in monocytes, we next tested if HBV inhibits IFN-α–induced IFN signaling gene expression through inflammatory cytokine production. The Abs were respectively added into monocytes cultured with IFN-α in the presence of HBVcc or HBsAg to neutralize IL-10, IL-1β, and TNF-α. We observed that

neutralization of IL-10 but not IL-1β or TNF-α partially restored the mRNA expression levels of IFN-α–induced *stat1* (Fig. 5E), *stat2* (Fig. 5F), and *ch25h* (Fig. 5G), which were inhibited by HBsAg or HBVcc. These data suggest that HBV-induced IL-10 production of monocytes partially inhibited IFN-α–induced ISG expression in an autocrine manner.

2-NP rescues IFN signaling and inhibits the inflammatory cytokines production

As an enhancer of STAT1, 2-NP has been reported to specifically enhance STAT1-dependent gene expression and the induction of an endogenous STAT1 target gene by specifically prolonging the tyrosine phosphorylation of STAT1 (31), and we confirmed this in Supplemental Fig. 3B. We thus investigated whether 2-NP affects IFN-α–induced IFN signaling gene expression and HBV-induced inflammatory cytokines. Purified monocytes were pretreated with increasing concentrations of 2-NP for 1 h and then stimulated with HBsAg or HBVcc in the presence or absence of IFN-α. The results, shown as Supplemental Fig. 3C, were that 2-NP causes a dose-dependent increase of *ch25h* expression but not *stat1* nor *stat2*. A total of 45 μmol/l of 2-NP was used as the optimal concentration in the following experiments. We further observed that 2-NP rescues the inhibition of STAT1-dependent gene (*ch25h*) expression by HBV (Fig. 6C) but not STAT1 (Fig. 6A) or STAT2 (Fig. 6B). 2-NP also inhibits inflammatory cytokines in monocytes, including IL-1β (Fig. 6D), TNF-α (Fig. 6E), and IL-10 (Fig. 6F). These results imply that the combination of an agent that acts like 2-NP with IFN-α warrants careful consideration as a potential therapeutic strategy in chronic HBV infection.

Discussion

Circulating monocytes are a clinically accessible cell population that differentiates into tissue macrophages resident or dendritic cells and may directly be modified during the natural course of viral infection (32). Monocytes are key mediators of inflammatory and antiviral response in viral infection (33). In this study, we observed the lower expression of IFN signaling/stimulated genes and higher expression of inflammatory cytokines genes in peripheral monocytes from CHB patients (Fig. 1), suggesting mutual antagonism between the inflammatory and antiviral response. In addition, the serum levels of HBsAg and HBV DNA correlate negatively with IFN signaling/stimulated genes and positively with the expression of inflammatory cytokines in monocytes. These data imply that HBsAg and HBV may suppress the antiviral response and induce the inflammatory response in the monocytes of CHB patients.

To analyze this phenomenon, peripheral blood monocytes from healthy donors were exposed to HBV and/or IFN-α; we found that HBsAg/HBV and IFN-α inhibit or induce *stat1*, *stat2*, *ch25h* expression, respectively, and HBsAg/HBV inhibits IFN-α–induced *stat1*, *stat2*, *ch25h* expression in monocytes (Fig. 2). To the best of our knowledge, this is the first description of HBV-impaired antiviral response on monocytes. The nature of innate immunity induced by HBV remains controversial. One study showed that HBV cannot activate host innate antiviral responses in the liver (34), whereas another showed that HBV induces IFN-I and type III IFN, suggesting that the host can sense the HBV infection (35–37). At present, these conflicting conclusions are difficult to reconcile. STAT1 and STAT2 are key components of the transcription factor complex in the IFN signaling pathways.

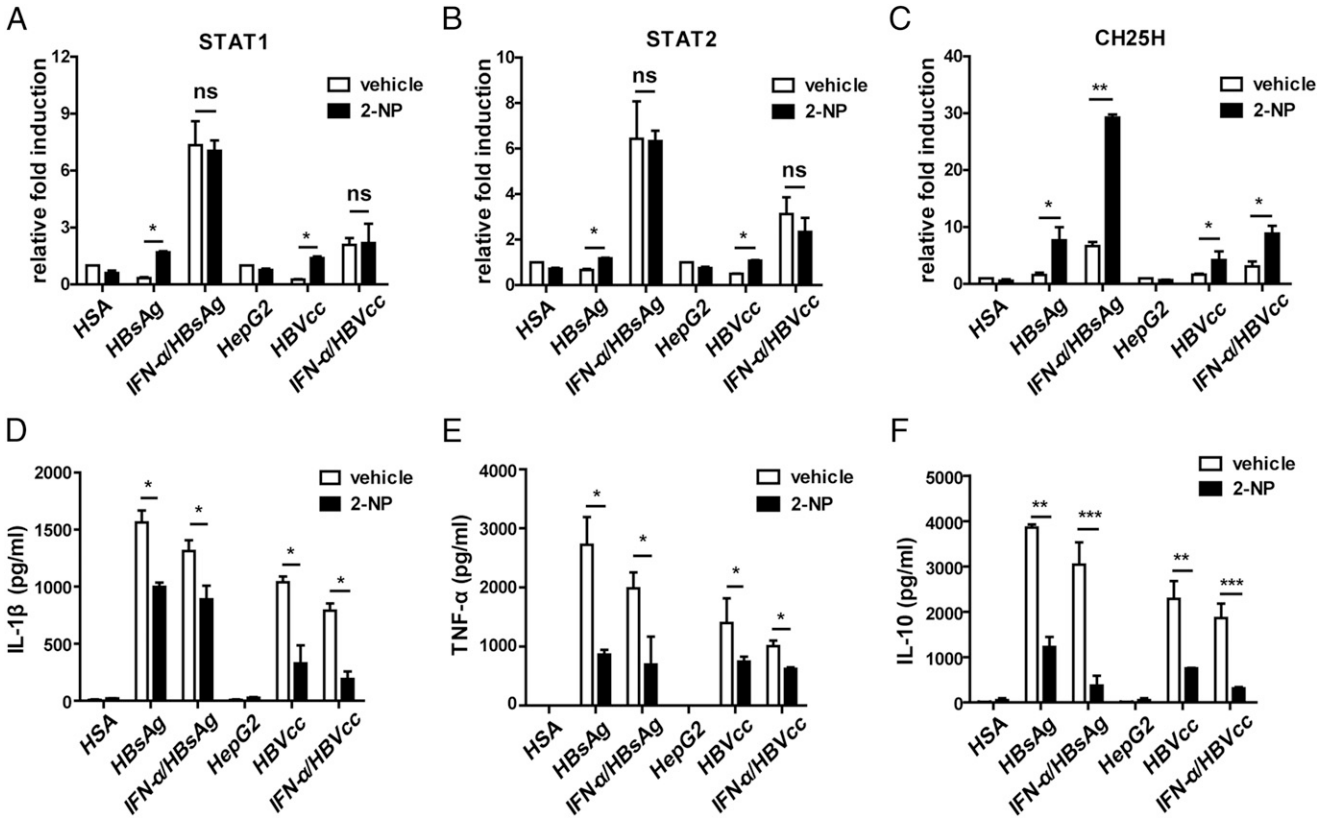

FIGURE 6. 2-NP rescues IFN signaling and inhibits the inflammatory cytokines production. Purified monocytes from healthy donors (n = 6) were pretreated with 2-NP (45 μmol/l) for 1 h, then stimulated with HBsAg (20 μg/ml) or HBVcc (10^7 copies/ml) and IFN-α (10 ng/ml) for 12 h. Cells were collected to assay the mRNA expression of (**A**) *stat1*, (**B**) *stat2*, and (**C**) *ch25h* by qRT-PCR, and the relative fold induction was normalized to HSA or HepG2. The supernatants were collected at 24 h; the productions of IL-1β (**D**), TNF-α (**E**), and IL-10 (**F**) were detected by ELISA. Error bars represent the SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The response of immune and nonimmune cells to type I and III IFNs triggers the formation of heterotrimers containing STAT1, STAT2, and the IFN regulatory factor 9 (38). STAT1 is the most essential transcriptional factor involved in the induction of *CH25H* (39). As shown previously, *CH25H* expression in response to viral infections or IFNs was almost completely abrogated in STAT1-deficient macrophages (40). HCV-induced *CH25H* expression synthesizes 25HC to inhibit HCV RNA replication by preventing formation of the viral replication factory in human hepatocytes (41). IFN-α induces the transfer of resistance to HBV from non-permissive liver nonparenchymal cells to permissive hepatocytes via exosomes, which were rich in molecules with antiviral activity (42). IFN-α induces *CH25H* expression in monocytes, and its product, 25HC, may inhibit HBV replication primarily at the level of transcription or RNA translation in hepatocytes. Our data further reveal that HBV and HBsAg inhibit IFN-α–induced *CH25H* expression of monocytes, which may be relevant to the poor response rate of IFN-based clinical therapy in chronic HBV patients. These findings show that the application of either HBV or HBsAg reproduces the effects of in vivo HBV infection in terms of IFN signaling/stimulated genes such as *stat1*, *stat2*, *ch25h*.

HBV is well known to subvert monocyte function, but molecular mechanisms are not fully defined. TLRs are key sensors in HBV recognition (43, 44). TLR2 detects several microbial pathogen-associated molecular patterns either in its homodimer form or in a heterodimer with TLR1 or TLR6 and subsequently activates TRAF6 and NF-κB in a MyD88-dependent manner (45). In this study, we found that both HBsAg and HBVcc upregulated TLR2 expression, whereas blockade of TLR2 suppressed the HBV-induced inflammatory response (Fig. 4), confirming the specificity of the effect. Because the inflammatory response was likely to be driven via NF-κB (46, 47), we confirmed that HBV induces the increased expression of *MyD88*, *TRAF6*, and *NFκB1*, promotes IκBα phosphorylation, and that inhibition of NF-κB suppressed HBV-induced inflammatory cytokine production (Fig. 3). We consistently found that serum from CHB patients significantly induced inflammatory cytokines production and inhibited IFN-α–induced *stat1*, *stat2*, *ch25h* expression in monocytes compared with serum from healthy individuals (Supplemental Fig. 2B, 2C), Therefore we argue the NF-κB pathway was engaged. Our findings identify a novel mechanism by which HBV induces inflammatory response via TLR2/MyD88/NF-κB signaling pathway in peripheral monocytes.

Previous studies have demonstrated that TLR2, TLR4, and TLR9 induce STAT1-Ser727 phosphorylation (48, 49) and that TLR-induced STAT1-Ser727 phosphorylation is independent of IFN-I production (30). TRAF6 appears to have a critical role not only in transducing the canonical MyD88 signal transduction pathway leading to NF-κB nuclear translocation but also interacting with components of the JAK/STAT pathway. TRAF6 may act as the proximal bridge for the recruitment of the STAT1 serine kinase into the MyD88 canonical pathway to NF-κB activation (30). In the current study, we found that HBV activates the STAT1-Ser727 phosphorylation and inhibits IFN-α–induced STAT1-Tyr701 phosphorylation in monocytes. However, HBV polymerase was shown to suppress IFN-α–induced STAT1-Ser727 phosphorylation in human hepatic cell lines (50), suggesting that HBV manipulates monocytes in a way distinct from its effects on hepatocytes. It has been reported that HBV represses IFN/JAK/STAT signaling by attenuating the phosphorylation and nucleus translocation of STAT1 to facilitate HBV replication (51). We consistently found that the expression level of STAT1-Ser727 was significantly higher in monocytes from CHB patients than those from healthy individuals, but the expression level of STAT1-Tyr701 was not significantly different (Supplemental Fig. 3A). Moreover, we also identified that HBV inhibits IFN-α–induced *stat1*, *stat2*, *ch25h* expression via an autocrine effect of secreted IL-10 partially (Fig. 5). These in vitro data reproduce our in vivo findings, which showed *IL-10* correlates negatively with the expression of IFN signaling/stimulated genes in CHB patients. It has been demonstrated that HBsAg stimulates monocytes to produce IL-10, which then suppress IFN-α production by plasmacytoid dendritic cells (10). IFN-α increases the sensitivity of monocytes to IL-10 by upregulating the expression of IL-10R1, and IL-10 induces a higher level of STAT3 phosphorylation in IFN-primed cells (52). These findings suggest that HBV and IFN-α differentially target the phosphorylation of STAT1 to determine the inflammatory response and antiviral effect in monocytes and that HBV induces the antagonism between inflammation and antiviral immunity in an IL-10–dependent, partially autocrine manner.

It remains controversial whether chronically HBV-infected patients have impaired immunity to infection with viruses (e.g., influenza A virus, Sendai virus, etc.) that induce strong IFN-I responses. Luangsay et al. (53) reported that HBV can inhibit the innate response triggered by a super infection with Sendai virus in dHepaRG cells. However, another studies showed that Sendai virus and poly I:C–induced IFN-I responses and ISG expression were not suppressed in HBV-infected liver (54–56). Moreover, although HBV infection may not induce any IFN-I response in hepatocytes, they nevertheless activate inflammatory response of macrophages (56). In our study we investigated that HBV manipulated IFN signaling in human monocytes, not hepatocytes. In exposure of blood monocytes to poly I:C and HBVcc/HBsAg, we found that poly I:C induced *stat1*, *stat2*, *ch25h*, and IFN-β mRNA expression, whereas HBsAg and HBVcc inhibited the effect of poly I:C (H. Song and Z. Tu, unpublished observations). However, whether it reflects the impaired immunity to infection with viruses (e.g., influenza A virus, Sendai virus, etc.) in chronic HBV-infected patients needs to be further explored.

In summary, we showed that HBsAg and HBVcc have similar effects in inducing both proinflammatory and IL-10 responses but inhibiting the induction of ISGs and that these effects are likely integrated through an effect of HBV on STAT1 phosphorylation. In the presence of HBV, STAT1 phosphorylation on tyr701 is impaired, but phosphorylation on ser727 is increased. This suggests HBV may increase the abundance of signaling molecules in the NF-κB pathway but conversely decreases the abundance of STAT1 and STAT2 as well as ISGs. These data argue that HBV impairs STAT1/2–mediated signaling at multiple levels, subverting host antiviral immunity. We further conclude that monocytes display two distinct and mutually antagonistic modes of activation in response to HBV and IFN-α and that HBV imposes a bias, which emphasizes inflammation at the expense of IFN-α– induced antiviral defense.

Disclosures

The authors have no financial conflicts of interest.

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