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*J Immunol* 2013; 190:5142-5151; Prepublished online 12 April 2013; doi: 10.4049/jimmunol.1201625

http://www.jimmunol.org/content/190/10/5142

Supplementary Material http://www.jimmunol.org/content/suppl/2013/04/12/jimmunol.1201625.DC1

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Hepatitis B Virus Surface Antigen Selectively Inhibits TLR2 Ligand–Induced IL-12 Production in Monocytes/Macrophages by Interfering with JNK Activation

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It is widely accepted that chronic hepatitis B virus (HBV) infection is the result of an ineffective antiviral immune response against HBV infection. Our previous study found that the hepatitis B surface Ag (HBsAg) was related to decreased cytokine production induced by the TLR2 ligand (Pam3csk4) in PBMCs from chronic hepatitis B patients. In this study, we further explored the mechanism involved in the inhibitory effect of HBsAg on the TLR2 signaling pathway. The results showed that both Pam3csk4-triggered IL-12p40 mRNA expression and IL-12 production in PMA-differentiated THP-1 macrophage were inhibited by HBsAg in a dose-dependent manner, but the production of IL-1β, IL-6, IL-8, IL-10, and TNF-α was not influenced. The Pam3csk4-induced activation of NF-κB and MAPK signaling were further examined. The phosphorylation of JNK-1/2 and c-Jun was impaired in the presence of HBsAg, whereas the degradation of IkB-α, the nuclear translocation of p65, and the phosphorylation of p38 and ERK-1/2 were not affected. Moreover, the inhibition of JNK and IL-12 production in response to Pam3csk was observed in HBsAg-treated monocytes/macrophages (M/MΦs) from the healthy donors and the PBMCs and CD14-positive M/MΦs from chronic hepatitis B patients. Taken together, these results demonstrate that HBsAg selectively inhibits Pam3csk4-stimulated IL-12 production in M/MΦs by blocking the JNK–MAPK pathway and provide a mechanism by which HBV evades immunity and maintains its persistence. The Journal of Immunology, 2013, 190: 5142–5151.

Hepatitis B virus (HBV) infection represents a major health problem worldwide. Over 350 million individuals are chronically infected with HBV and are at high risk of developing liver cirrhosis and hepatocellular carcinoma (1, 2). It has been reported that chronic infection with HBV occurs as a result of an ineffective antiviral immune response against the HBV infection (3). Although the role of adaptive immunity in HBV infection has been clearly demonstrated, the role of innate cellular immunity in infected hepatocytes and innate immune responses still remains to be clarified.

The significance of the innate immune response as a defense against microbial infections and its link to the adaptive immune responses have become increasingly recognized during the past few years. The activation of the innate immune response generally leads to the production of type I IFNs. The establishment of chronic HBV infection in humans indicates that HBV has evolved strategies to counteract at least some defense mechanisms to establish and maintain a persistent infection. A lack of the induction of type I IFNs has been observed in acute HBV patients (4). This is consistent with the results obtained in HBV-infected chimpanzees in which IFN-related genes were not induced during viral entry and expansion (5). Several studies have reported that plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs) were functionally impaired in chronic hepatitis B (CHB) patients (6). Further investigation indicated that HBV virions or hepatitis B surface Ag (HBsAg) could block TLR9-induced type I IFN production by pDCs and inhibit IL-12 production by mDCs (7, 8). Our previous study showed that the level of plasma HBsAg correlates closely with an impaired TLR2-induced IL-12 production in PBMCs from CHB patients (9), suggesting a role of HBsAg in HBV-mediated suppression of innate immune function of APCs.

HBsAg is the most abundant HBV protein in the liver and peripheral blood of CHB patients, can accumulate up to levels of 100 μg/ml in the peripheral blood, and typically outnumbers infectious virions by 1000:1 to 10,000:1 (10, 11). The high concentration of HBsAg in the bloodstream of CHB patients could theoretically contribute to the hampered immune response. Several studies have shown that HBsAg can suppress the release of LPS-induced cytokines in human monocytes by interfering with the TLR signal pathway in monocytes/macrophages (M/MΦs) (12, 13). These results suggest that HBsAg can alter the innate immune response, which may contribute to the establishment of chronic infections.

As the TLR2 molecule is mainly expressed on M/MΦs and the monocytes are the main APCs other than DCs among the PBMCs, we speculated that the effect of HBsAg on the TLR2-mediated signaling pathway and IL-12 secretion by PBMCs was mainly

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Received for publication June 13, 2012. Accepted for publication March 14, 2013. This work was supported by the National Science Foundation of Shanghai City (Grant 09ZR1426500), the National Natural Science Foundation of China (Grant 81071555), and the National Key Basic Research Program of China (Grant 2012CB519005).

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The online version of this article contains supplemental material.

Abbreviations used in this article: CHB, chronic hepatitis B; HBeAg, hepatitis B eAg; HBsAg, hepatitis B surface Ag; HBV, hepatitis B virus; mDC, myeloid dendritic cell; M/MΦ, monocyte/macrophage; pDC, plasmacytoid dendritic cell.

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associated with M/M$_{68}$. Therefore, in the current study, to further explore the underlying mechanism involved in the inhibitory effect of HBsAg on the TLR2 signaling pathway, PMA-differentiated THP-1 macrophages were used to examine the level of cytokine production and the activity of the related signaling pathways. Moreover, the inhibitory effect of HBsAg and other HBsAg-related mechanisms was also confirmed in M/M$_{68}$ from the peripheral blood of healthy donors and in the CD14-positive M/M$_{68}$ of CHB patients.

Materials and Methods

**Human subjects**

The samples used in this study were collected from 23 CHB patients who were admitted to Shanghai Changning Hospital. All of the patients were negative for other viral infections, including hepatitis C virus, HIV, CMV, and EBV (Supplemental Table 1). Blood samples from age- and sex-matched anonymous adult blood donors reporting to the Shanghai Red Cross blood center were used as healthy controls. This study was approved by the local ethics committee, and written informed consent was obtained from all of the participants.

**Reagents**

Plasma-derived HBsAg purified from the serum of HBV patients was kindly provided by Kehua Bio-Engineering of China. Briefly, the serum from CHB patients was inactivated with formaldehyde followed by sucrose density-gradient centrifugation and affinity chromatography to purify the Ags and was subsequently diluted in PBS. The plasma-derived HBsAg was then identified by using an HBsAg ELISA Kit (Kehua Bio-Engineering, Shanghai, China) and detected by Coomassie Brilliant Blue staining; HBsAg was also analyzed by transmission electron microscopy for particle size and morphology as described in our previous publications (8, 14). Moreover, SDS-PAGE was performed, and the HBsAg sample was analyzed by silver staining and Western blotting (Supplemental Fig. 1). To exclude the influence of LPS in the experiments described in this study, the concentration of endotoxin in the plasma-derived HBsAg used in our study was determined. The level of LPS in the purified HBsAg reagent was below the limitation of detection. Rabbit polyclonal anti-Lamin A/C, anti–Ibß-α, monoclonal anti–NF-κB p65, mouse monoclonal anti–phospho-1β-α, anti–phospho-p38, anti–phospho-JNK, anti–c-Jun, and anti–phospho-c-Jun Abs were purchased from Cell Signaling Technology. Mouse monoclonal anti–phospho-ERK was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal GAPDH, anti–β-actin, and γ-tubulin Abs were purchased from Sigma-Aldrich. Mouse monoclonal Anti-Lamin A/C was purchased from BD Biosciences. These Abs were detected using HRP-conjugated anti-mouse or HRP-conjugated goat-anti-rabbit (Santa Cruz Biotechnology) secondary Abs. SP600125, a specific JNK inhibitor, was purchased from Calbiochem (La Jolla, CA), and the TLR2 ligand Pam3csk4 (Cruz Biotechnology) secondary Abs. SP600125, a specific JNK inhibitor, was purchased from Invivogen.

**PBMC isolation and cryopreservation**

Plasma and PBMCs were separated by standard Ficoll-Hypaque (Accupin System-Histopaque; Sigma-Aldrich) density-gradient centrifugation from venous blood. Heparinized blood mixed with two volumes of prewarmed PBS (pH 7.4) was subjected to Ficoll-Hypaque density-gradient centrifugation at 800 $\times$ g for 15 min. Plasma samples were collected and stored at $-80^\circ$C until needed. The PBMCs were counted, resuspended in RPMI 1640 (Sigma-Aldrich) freezing media containing 10% DMSO (Sigma-Aldrich) and 10% PBS (FBS, Invitrogen), frozen at $-80^\circ$C overnight, and transferred to liquid nitrogen for storage until needed.

**Cell thawing after cryopreservation**

Cryopreserved cells were quick-thawed and immediately diluted with 10% FBS in RPMI 1640 that had been prewarmed to room temperature. The cells were centrifuged at 200 $\times$ g for 10 min, and the cell pellet was then gently resuspended and washed twice with an equal volume of RPMI 1640 medium containing FBS that had been prewarmed to room temperature. The cells were incubated at 37°C in 5% CO$_2$ for 2 h before use.

**Cells and cell culture**

THP-1, a human monocytic leukemia cell line, was obtained from the Institute of Biochemistry and Cell Biology at the Shanghai Institute for Biological Sciences. The cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 IU/ml), and streptomycin (100 $\mu$g/ml) (Life Technologies/BRL). To analyze the effect of HBsAg on THP-1 cells, the cells were differentiated with 50 nM PMA for 24 h, inoculated with 1, 5, or 25 $\mu$g/ml of HBsAg, and then stimulated with Pam3csk4 (1 $\mu$g/ml) for different lengths of time. As a negative control, an equivalent amount of BSA (purchased from Merck) was added to the cell-culture medium. Monocytes were isolated from PBMCs by positive immuno-magnetic selection using the mini-MACS system (StemCell Technologies) according to the manufacturer’s instructions. For preparation of human M/M$_{68}$, cultured monocytes at a density of 1 $\times$ 10$^7$ cells/ml were differentiated in the presence of 30 ng/ml GM-CSF (R&D Systems) for 6 d. The culture medium was changed twice during the differentiation period. Nonadherent cells were washed off with RPMI 1640 medium, and the remaining adherent cells were used for further assays.

**RNA isolation and quantitative real-time RT-PCR**

Total RNA from the lysed cells was extracted using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. Total RNA was treated with DNase I (Takara Bio) to remove the genomic DNA and then reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen Life Technologies) and random hexamer primers. Real-time PCR was performed in the Bio-Rad iCycler iQ detection system (Bio-Rad) using the SYBR(R) Premix Ex Taq (Takara Bio) according to the manufacturer’s instructions. The oligonucleotide primer sequences for IL-10, IL-12p40, and GAPDH were as follows: IL-10 sense, 5'-GGTACAACAAGCTGAGACCA-3'; IL-10 antisense, 5'-GAGCTCTTCTTCTTTGAGACC-3'; IL-12p40 sense, 5'-TGG AGT GCC AGG AGG ACA GTT G-3' and IL-12p40 antisense, 5'-TCT TGG GTG GGT CAG GTG TTG-3'; and GAPDH sense, 5'-GTT ATC GTG AGA GGA GTA CTC ATG AC-3' and GAPDH antisense, 5'-ATT CGCA CTA AGC TTC CGG TTC AGC-3'. The thermal cycling conditions were as follows: 5 min at 95°C followed by 45 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The threshold cycle for each reaction was converted to a DNA equivalent by reading standard curves that were generated by amplifying dilutions of a linearized plasmid containing a 188-bp GAPDH cDNA. The relative quantity of the target mRNA was normalized to the level of GAPDH mRNA (the internal control).

**ELISA**

The cells were treated with 50 nM PMA for 24 h. After washing, the cells were incubated with or without HBsAg for 24 h and stimulated with Pam3csk4 at the indicated concentration. Cell-culture supernatants were collected after 24 h, and the level of IL-12p40 in the supernatants was measured using an IL-12-p40 Human ELISA Kit (BioSource International) according to the manufacturer’s instructions. The level of IL-10 in the supernatants was measured with ELISA kit purchased from BD Biosciences.

**Cytometric bead array**

Supernatants were harvested from the stimulated cells. Before the assay, the samples were diluted 1:4. The levels of IL-1β, IL-6, IL-8, IL-10, IL-12, and TNF-α in the samples were detected simultaneously using the Cytometric Bead Array Human Inflammation Kit (BD Biosciences) according to the manufacturer’s instructions. The intensity of the fluorescence signal was detected using an FACScAria flow cytometer (BD Biosciences), and the data were analyzed using FCAP Array software (BD Biosciences). The linear range for the concentration of these six cytokines was 20–5,000 pg/ml in this detection system.

**Western blot analysis**

PMA-differentiated THP-1 cells were incubated with or without 20 µg/ml HBsAg before being stimulated with Pam3csk4. After 15 or 30 min of stimulation, cell lysates were prepared in SDS sample buffer (62.5 mM Tris·HCl [pH 6.8], 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue) with a mixture of protease inhibitors and PhosSTOP Phosphatase Inhibitor (Roche). Equal amounts of protein were loaded onto a gel, separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Roche). The membrane was blocked with 0.05% Tween 20 in PBS containing 5% skim milk and then incubated overnight with the primary Ab at 4°C. The membrane was then washed three times in 0.05% Tween 20 in PBS and incubated with an HRP-conjugated secondary Ab for 2 h at room temperature. The immunoblots were visualized using ECL (Western lightning; PerkinElmer).

**Cytometric bead assay cell signaling assay**

PMA-differentiated THP-1 cells were incubated with or without 20 µg/ml HBsAg before being stimulated with Pam3csk4. The phosphorylation of c-Jun, ERK, and p38 were simultaneously detected using the Cytometric Bead Array Cell Signaling Flex Set System (BD Biosciences) according to the manufacturer’s instructions. The oligonucleotide primer sequences for IL-10, IL-12p40, and GAPDH were as follows: IL-10 sense, 5'-GGTACAACAAGCTGAGACCA-3'; IL-10 antisense, 5'-GAGCTCTTCTTCTTTGAGACC-3'; IL-12p40 sense, 5'-TGG AGT GCC AGG AGG ACA GTT G-3' and IL-12p40 antisense, 5'-TCT TGG GTG GGT CAG GTG TTG-3'; and GAPDH sense, 5'-GTT ATC GTG AGA GGA GTA CTC ATG AC-3' and GAPDH antisense, 5'-ATT CGCA CTA AGC TTC CGG TTC AGC-3'. The thermal cycling conditions were as follows: 5 min at 95°C followed by 45 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The threshold cycle for each reaction was converted to a DNA equivalent by reading standard curves that were generated by amplifying dilutions of a linearized plasmid containing a 188-bp GAPDH cDNA. The relative quantity of the target mRNA was normalized to the level of GAPDH mRNA (the internal control).
HBsAg inhibits IL-12 in M/M₈₈ by blocking JNK activation

As our previous study found that serum HBsAg levels were conversely related to TLR2-induced cytokine secretion in PBMCs from CHB patients (9), we hypothesized that HBsAg might play an important role in interfering with the TLR2-related function of M/M₈₈.

We tested this hypothesis in the PMA-differentiated THP-1 macrophage model, because the TLR2 molecule is expressed mainly on M/M₈₈ among the PBMCs. To determine the time of pretreatment of HBsAg that will lead to the inhibition, the THP-1 macrophages treated with HBsAg for different times from 1 to 48 h were stimulated with Pam3csk4 for 6 h followed by quantitative RT-PCR analysis. The results showed that the inhibition of IL-12 transcription was observed in cells pretreated with HBsAg for 24 h and longer (Supplemental Fig. 2A). Thus, the cells were pretreated with 1, 5, or 25 μg/ml of plasma-derived HBsAg for 24 h and then stimulated with Pam3csk4 (1 μg/ml) for another 24 h. The supernatants of the cell cultures were collected and analyzed using a cytometric bead array for IL-1β, IL-6, IL-8, IL-12p70, and TNF-α. A dose-dependent inhibitory effect of HBsAg was observed for IL-12p70 only, whereas the production of IL-1β, IL-6, IL-8, and TNF-α was not significantly influenced by HBsAg treatment (Fig. 1A–D).

To confirm the results from the cytometric bead array assay, IL-12 p40 production and mRNA expression were assayed by ELISA and quantitative RT-PCR, respectively (Fig. 1E, 1F). As expected, both IL-12p40 production and mRNA expression were significantly reduced by HBsAg pretreatment in a dose-dependent manner, which is consistent with data from a previous study (9, 13). These results also suggest that the inhibition of IL-12 production might occur at the transcriptional level.

Interestingly, the production level of IL-10 was not affected in the THP-1 supernatants whether HBsAg was present (Fig. 1G), and the IL-10 mRNA expression did not increase in response to HBsAg pretreatment (Fig. 1H), which suggested that the suppression of IL-12 expression by HBsAg is not mediated by IL-10.

HBsAg did not interfere with the NF-κB signaling pathway

The cell-signaling pathways responsible for Pam3csk4-induced IL-12 production mainly include the NF-κB- and MAPK pathways. Pam3csk4 binds to TLR2, initiates activation of serial kinases, resulting in the phosphorylation and degradation of IκB-α, which leads to NF-κB release from IκB and translocation into the nucleus. Simultaneously, MAPKs are also activated by the interaction between Pam3csk4 and TLR2, which leads to the phosphorylation of ERK, p38, and JNK.

To investigate how HBsAg inhibited Pam3csk4-induced IL-12 production, we first analyzed the NF-κB pathway. Phosphorylation and degradation of IκB-α and NF-κB p65 nuclear translocation were assayed by Western blot and immunofluorescence staining, respectively. PMA-differentiated THP-1 cells were pretreated with 20 μg/ml HBsAg for 24 h and then stimulated with 1 μg/ml Pam3csk4 for 5, 10, 15, or 30 min. As shown in Fig. 2A and 2B, HBsAg did not interfere with NF-κB signaling, as assayed by phosphorylation and degradation of IκB-α induced by Pam3csk4, and there were no differences between HBsAg-pretreated THP-1 cells and the control cells with no pretreatment. In addition, immunofluorescence staining showed that nuclear translocation of NF-κB p65 was also not influenced in HBsAg-treated cells (Fig. 2C). This result was further verified by the subcellular fractionation assay. There was no difference in the Pam3csk4-induced accumulation of p65 in nuclear extracts prepared from the HBsAg-treated cells and the control cells (Fig. 2D). Taken together, these results indicate that HBsAg did not obviously interfere with the TLR2 ligand–induced NF-κB signaling pathway in THP1 macrophages under our experimental system.

HBsAg interfered with the JNK-1/2 MAPK pathway

Because HBsAg did not interfere with the NF-κB pathway, we next investigated the possible effect of HBsAg on the Pam3csk4-induced activation of ERK, p38, and JNK by Western blot.
PMA-differentiated cells were treated with 20 μg/ml HBsAg for 24 h followed by 1 μg/ml Pam3csk4 for 15 or 30 min. The levels of ERK, p38, and JNK phosphorylation were analyzed by Western blot. As shown in Fig. 3A, there was a basal level of ERK-1/2 phosphorylation in nonstimulated cells, and the phosphorylated form increased significantly with Pam3csk4 stimulation. However, pretreatment with HBsAg for 15 or 30 min did not lead to a reduction in ERK-1/2 phosphorylation. Similarly, p38 phosphorylation was not influenced by HBsAg treatment. However, the Pam3csk4-induced phosphorylation of the JNK-1/2 MAPKs was...
Abs were used to confirm appropriate fractionation. and subjected to Western blot analysis using anti-p65, anti-lamin A/C, and anti–

Because IL-12 production has been shown to be significantly impaired in HBsAg-pretreated cells compared with the control cells (Fig. 3B).

FIGURE 2. HBsAg does not interfere with the NF-κB signaling pathway. (A and B) PMA-differentiated THP-1 cells were pretreated with 20 μg/ml HBsAg for 24 h and then stimulated with 1 μg/ml Pam3csk4 for 5, 10, 15, or 30 min. The cell lysates were subjected to Western blot analysis using anti–IκB-α or anti–p-IκB-α Abs. β-actin or γ-tubulin was used as an internal control. (C) Following Pam3csk4 stimulation for 30 min, the cells were stained with an anti-p65 mAb for immunofluorescence (original magnification ×600). (D) Cytoplasmic and nuclear fractions were isolated and subjected to Western blot analysis using anti-p65, anti-lamin A/C, and anti–β-tubulin Abs. Anti–β-tubulin (cytoplasm) and anti-lamin A/C (nuclear) Abs were used to confirm appropriate fractionation.

To confirm the results obtained by Western blot analysis, we used the Cytometric Beads Array Cell Signaling Flex Set, a multipanel detection method based on Ab-anchored beads, to simultaneously measure the phosphorylation of c-Jun, ERK, and p38 (Fig. 3D). HBsAg inhibited Pam3csk4-induced c-Jun phosphorylation in a dose-dependent manner. However, no significant inhibitory effect was observed on the phosphorylation of ERK and p38. Taken together, these results strongly suggest that HBsAg can selectively interfere with the JNK-1/2 MAPK pathway.

To further validate the results observed in the PMA-differentiated THP-1 cell line, we cultured peripheral M/MΦs from healthy donors with GM-CSF in vitro. The cells were incubated with HBsAg for 24 h, stimulated with 1 μg/ml Pam3csk4 for 15 min, and then lysed and subjected to Western blotting. HBsAg significantly inhibited Pam3csk4-induced phosphorylation of JNK, but there was no effect on Pam3csk4-induced p38 phosphorylation (Fig. 5A, 5B). The cells treated with HBsAg also showed suppressed IL-12 induction and lower IL-12 p40 mRNA levels (Fig. 5C, 5D). Thus, the results obtained in human M/MΦs were consistent with what we observed in the THP-1 cell line.

The phosphorylation of JNK and the production of IL-12 are impaired in patients with chronic HBV infection

To evaluate whether the JNK activation and IL-12 production are impaired in CHB patients, we examined Pam3csk4-stimulated JNK activation and IL-12 production in M/MΦs from 23 individuals with chronic HBV infection. JNK phosphorylation in
PBMCs from both healthy controls and CHB patients was observed upon Pam3csk4 stimulation (Fig. 6A). However, the level of activated JNK in PBMCs and CD14+ M/MΦs was significantly attenuated in CHB patients compared with that in the healthy donors (Fig. 6B). Besides, IL-12 production in both PBMCs and CD14+ M/MΦs was also significantly attenuated in CHB patients when compared with the healthy donors (Fig. 6C, 6D). In conclusion, the impaired TLR2-induced JNK activation and IL-12 production were not only observed in the THP-1 cell line but also verified in the CD14+ M/MΦs from HBV-infected individuals.

FIGURE 3. HBsAg interferes with the JNK-1/2 MAPK pathway. (A–C) PMA-differentiated THP-1 cells were pretreated with 20 μg/ml HBsAg for 24 h and then stimulated with 1 μg/ml Pam3csk4 for 15 or 30 min. The cell lysates were subjected to SDS-PAGE, followed by Western blot analysis using anti-p-ERK, anti-p-p38, anti-p-JNK, anti-c-Jun, and anti-p-c-Jun Abs. GAPDH was used as an internal control. (D) PBMCs from patients and healthy controls were pretreated with different doses of HBsAg as described and then treated with Pam3csk4 (1 μg/ml) for 30 min. The phosphorylation levels of c-Jun, ERK, and p38 were determined using the Cytometric Bead Array Cell Signaling Flex Set. The columns represent the ratio of mean fluorescence intensity (MFI) to mock (means ± SD) collected from three independent experiments. Significant differences are indicated. *p < 0.05, **p < 0.01.

FIGURE 4. SP600125 inhibited JNK activation and IL-12 production in Pam3csk4-stimulated THP-1 cells. (A and B) PMA-differentiated THP-1 cells were treated with SP600125 at concentrations ranging from 0–50 μM for 2 h and then stimulated with Pam3csk4 1 μg/ml for 30 min. The cell lysates were analyzed by Western blot using anti-p-ERK, anti-p-p38, and anti-p-JNK Abs. (C) PMA-differentiated THP-1 cells were pretreated with 20 μg/ml HBsAg for 24 h and then treated with SP600125 for 2 h before stimulation with Pam3csk4 (1 μg/ml) for 24 h. IL-12p40 production was analyzed by ELISA. The columns represent the mean levels and SD of IL-12p40. Significant differences are indicated. *p < 0.05, **p < 0.01.
Discussion

There is a significant amount of HBsAg in the peripheral circulation of individuals with chronic HBV infection due to the secretion of these subviral particles from infected hepatocytes. This finding led to the speculation regarding a potential role of HBsAg in modulating cellular and immunological responses during HBV infection. These small subviral particles may induce T cell anergy and prevent Ab-mediated neutralization of HBV. However, the mechanism by which HBsAg blunts the host immune response to HBV is not yet clear.

Growing evidence points to reduced cytokine production in PBMCs from chronically HBV-infected patients upon LPS stimulation (18, 19). In our previous study, we demonstrated that the plasma level of HBsAg was associated with impaired cytokine production upon challenge with the TLR2 ligand (9). Because the TLR2 molecule is mainly expressed on M/MFs, these results indicated that there may be an interaction between HBsAg and M/MFs. In the current study, we demonstrated for the first time, to our knowledge, that HBsAg can selectively inhibit TLR2 ligand–induced IL-12 production in M/MFs by selectively blocking the JNK–MAPK pathway.

IL-12, as an immunoregulatory cytokine, favors a Th1 cell response that bridges innate and adaptive immunity (20–23). Monocyte-produced IL-12 also facilitates T cell proliferation and IFN-γ production, enhancing the generation of cytotoxic T lymphocytes and lymphokine-activated killer cells (24–26), all of which are important for eliminating intracellular viral infection. However, several viruses, including HIV and measles virus, prevent the establishment of a Th1 environment by inhibiting IL-12 production (27, 28). This suppression of Th1 polarization by viruses may represent an important mechanism for evading the host immune responses. The absence of an HBV-specific Th1 response is also thought to be one of the important factors in establishing a persistent HBV infection (29). In the current study, we demonstrated that HBsAg selectively inhibited IL-12 production in response to Pam3csk4 stimulation, which was caused by interfering with the JNK–MAPK pathway. Our results may help explain the impaired Th1 response observed in CHB patients and suggest a possible mechanism for HBV modulation of the Th1 and Th2 balance in the host by HBsAg-mediated regulation of the innate immune response.

Pam3csk4-induced cell signaling, including activation of NF-κB and MAPKs and multiple transcription factors, has been demonstrated to play an important role in the regulation of IL-12p40. The transcription factor C/EBP, in cooperation with the rel/NF-κB complex, was found to regulate the human IL-12 p40 gene. Zhu et al. (17) identified an AP-1–binding site in the murine IL-12p40 promoter, through which c-Jun and C/EBPβ cooperatively activate transcription of the IL-12p40 gene. In this study, we investigated the role of HBsAg in the regulation of the NF-κB and MAPK signaling pathways and Pam3csk4-stimulated IL-12 production. In this study, we show that HBsAg does not inhibit the activation of NF-κB, p38, or ERK. In contrast, HBsAg does inhibit the phosphorylation of JNK and its downstream transcriptional factor c-Jun. We also compared the inhibitory effect of cotreatment and pretreatment of HBsAg. The results showed that the Pam3csk4-induced JNK activation was inhibited, whereas NF-κB, ERK, and p38 activation were not affected, no matter whether HBsAg was present in the medium or removed when adding Pam3csk4 (Supplemental Fig. 2B, 2C). These results indicated that HBsAg can selectively target the JNK–MAPK pathway, although the underlying mechanism requires further elucidation.

In addition, the relationship between JNK activity and IL-12 expression in HBsAg-treated THP-1 cells was confirmed using

**FIGURE 5.** HBsAg pretreatment inhibited Pam3csk4-stimulated JNK activation and IL-12 induction in human M/MFs. (A) Human M/MFs pretreated with 1, 5, or 25 μg/ml HBsAg for 24 h were stimulated with 1 μg/ml Pam3csk4 for 15 min. The cell lysates were then analyzed by Western blot using anti-p-JNK and anti-p-p38 Abs. β-actin was used as a loading control. (B) Densitometry quantification for p-JNK and p-p38 expression normalized to β-actin. (C and D) Human M/MFs were pretreated with 1, 5, or 25 μg/ml HBsAg for 24 h and then stimulated with 1 μg/ml Pam3csk4 for 24 h. IL-12p40 production was analyzed by ELISA (C) or quantitative PCR (D). *p < 0.05, **p < 0.01.
The phosphorylation of JNK and the production of IL-12 are impaired in patients with chronic HBV infection. PBMCs isolated from healthy donors (n = 16) and CHB patients (n = 23) were stimulated with Pam3csk4 for 30 min for JNK activation analyzing or 22 h for IL-12 production by flow cytometry. (A) PBMCs and CD14+ M/MΦs were gated, and representative data of phosphorylation of JNK in PBMCs (upper panel) and M/MΦs (lower panel) were shown for CHB patients (blue histograms) and healthy donors (red histograms); the unstimulated control was shown in gray histograms. (B) Summary data of the mean fluorescence intensity (MFI) of p-JNK in PBMCs (left panel) and CD14+ M/MΦs (right panel) were shown for CHB patients in comparison with healthy donors. Each dot represents one individual donor. (C) PBMCs and CD14+ M/MΦs subsets were gated, and representative data of IL-12 production in CHB patients and healthy donors were shown. (D) Summary data of the percentage of IL-12+ cells in PBMCs (left panel) and CD14+ M/MΦs (right panel) of CHB patients and healthy donors. Each dot represents one individual donor. FSC-H, Forward scatter height; SSC-A, side scatter area.
a specific inhibitor of JNK activation, SP600125, which suggested that the attenuated JNK pathway might be responsible for the decreased IL-12 production induced by HBsAg. Therefore, we speculated that HBsAg might inhibit IL-12p40 production by specifically downregulating the activation of the JNK–MAPK pathway. This hypothesis was further tested in peripheral M/Ms from CHB patients and HBsAg-treated M/Ms from healthy donors. The mechanism by which HBsAg blocks the JNK–MAPK pathway, whether through directly targeting JNK or its upstream molecules or modulating JNK activity through the negative regulatory pathways in the cells, is unknown. Further investigation is required to elucidate the detailed mechanisms. The suppressed TLR2-induced JNK activation and IL-12 production were also observed in CHB patients and strongly supported the findings obtained in vitro cell lines. However, there is no statistical correlation between JNK activation and HBsAg serum titers or HBV-DNA levels. We speculate that this may be due to the coexistence of HBsAg, hepatitis B "e" Ag (HBeAg), and HBV virions in the serum of CHB patients. Besides HBsAg, HBeAg (30–32) and HBV virions (32) have been reported to be able to interfere with the TLR signaling, which may also influence JNK activation. The total effect of HBV in the CHB patients was more complicated than we expected.

The effect of HBsAg on TLR4 signaling pathway was also examined, and results showed that the LPS-induced phosphorylation of JNK and p38 and the degradation of IkB-α were inhibited by HBsAg; LPS-induced production of cytokines including IL-12, IL-6, and TNF-α was also found to be inhibited by HBsAg (Supplemental Fig. 3). These results suggested that the effect of HBsAg on TLR2 and TLR4 was different. The mechanism of how HBsAg universally inhibits TLR4-induced MAPK/NF-κB activation and selectively inhibits TLR2-mediated IL-12 production requires further investigation. Interestingly, Wu et al. (32) demonstrated that pretreatment of hepatocytes and nonparenchymal liver cells with HBV-Met cells supernatants, HBsAg, HBeAg, or HBV virions almost completely abrogated TLR4-induced antiviral activity, including the suppression of IFN-β production and expression of proinflammatory cytokines, as well as suppressed activation of IFN regulatory factor 3, NF-κB, and ERK 1/2. In the current study, using PBMCs and a human monocytic leukemia cell line, we found that HBsAg selectively inhibited TLR2 ligand-induced IL-12 production in M/Ms by interfering with JNK activation, and the inhibitory effect of HBsAg did not occur until 24 h after adding of HBsAg (Supplemental Fig. 2A, 2D). Recent studies also indicate that HBsAg can interfere with the function of APCs, including mDCs and M/Ms, which may consequently attenuate the adaptive immune response (7, 13). In addition to the mDCs, the function of pDCs was also influenced by HBsAg through the inhibition of the TLR9-activated signaling pathway (8, 14, 33). These results suggested that HBV has developed strategies to suppress the initial antiviral response in the liver. Once infected, persistent presence of large amount of HBsAg in peripheral blood may help the virus to evade the host immune responses, establishing a persistent HBV infection via multiple interactions between HBsAg and APCs, but not inhibiting the entire innate immune system.

In summary, the current study demonstrated that HBsAg selectively inhibited IL-12 production in M/Ms in response to Pam3csk4 stimulation by selectively interfering with the JNK–MAPK pathway. Therefore, we provide new insights into how HBsAg modulates the host innate immune response and may consequently hamper Th1 adaptive immunity, a condition that is favorable for persistent HBV infection. These results will not only deepen our understanding of the mechanisms of HBV infection but also help to provide new targets for prophylactic and therapeutic strategies for chronic hepatitis B.

Disclosures

The authors have no financial conflicts of interest.

References


