

# Hepatitis B Virus Suppresses Toll-like Receptor–Mediated Innate Immune Responses in Murine Parenchymal and Nonparenchymal Liver Cells

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We have previously shown that Toll-like receptor (TLR)-activated murine nonparenchymal liver cells [(NPC); Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC)] can suppress hepatitis B virus (HBV) replication. Therefore, the aim of this study was to investigate whether HBV has the ability to counteract the TLR-mediated control of its replication. Freshly purified murine hepatocytes and NPCs obtained from C57BL6 mice were stimulated by TLR 1-9 ligands in the presence or absence of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), HBV virions, or supernatants from HBV-producing HBV-Met cells, and HBV replication was suppressed by anti-hepatitis B virus X protein (HBx) small interfering RNA (siRNA) in HBV-Met cells. Supernatants were collected and tested for antiviral cytokines by viral protection assay. HBV gene expression and replication was analyzed by southern blot. RNA and proteins were analyzed by quantitative reverse transcription polymerase chain reaction (RT-PCR) or western blot and enzyme-linked immunosorbent assay, respectively. Pretreatment of hepatocytes and NPCs with HBV-Met cells supernatants, HBsAg, HBeAg, or HBV virions almost completely abrogated TLR-induced antiviral activity, which correlated with suppression of interferon beta (IFN- $\beta$ ) production and subsequent interferon-stimulated gene induction as well as suppressed activation of interferon regulatory factor 3 (IRF-3), nuclear factor kappa B (NF- $\kappa$ B), and extracellular signal-regulated kinase (ERK) 1/2. In HBV-infected HBV-Met cells, TLR stimulation did not induce antiviral cytokines in contrast to primary hepatocytes. TLR-stimulated expression of proinflammatory cytokines [tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-6 (IL-6)], and activation of IRF-3 was suppressed after up-regulation of HBV replication in HBV-Met cells. Accordingly, suppression of HBV replication by siRNA led to activation or expression of proinflammatory transcription factors and cytokines. **Conclusion:** Our data indicate that HBV can suppress the TLR-induced antiviral activity of liver cells. This has major implications for the interaction between HBV and the immune system. (HEPATOLOGY 2009;49:1132-1140.)

The hepatitis B virus (HBV) is a hepatotropic DNA virus that can lead to chronic hepatitis, which can be complicated by the development of liver cirrhosis and hepatocellular carcinoma. Current approved therapeutic strategies for treatment HBV include

interferon-alpha (IFN- $\alpha$ ) and nucleoside and nucleotide analogs.<sup>1,2</sup> However, only a minority of patients that are treated with these agents show a long-term sustained response with “eradication” [for example, hepatitis B surface antigen (HBsAg) loss] of the virus.

*Abbreviations:* EMCV, encephalomyocarditis virus; ERK, extracellular signal-regulated kinase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; KC, Kupffer cells; LSEC, liver sinusoidal endothelial cells; MxA, myxovirus resistance protein A; NF- $\kappa$ B, nuclear factor kappa B; NPC, nonparenchymal liver cells; RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering RNA; TGF, transforming growth factor; TIR, Toll/Interleukin-1 receptor; TLR, Toll-like receptor; TNF, tumor necrosis factor.

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Received June 27, 2008; accepted November 17, 2008.

J.F.S. is supported by the Deutsche Forschungsgemeinschaft (DFG) SCHL 377/2-2, M. Lu is supported by DFG (Lu 669/2-1, GRK1045/1, and Lu 669/5-1). D. Yang and J. Wu are supported by the National Key Basic Research Program of China (2005CB522901) and the National Natural Science Foundation of China (20571646).

Therefore, it is necessary to gain further insights into the interaction between HBV and the immune system. It has been shown that type I and type II interferons that are produced by cells of the innate and adaptive immune system are able to suppress HBV replication in livers from HBV-transgenic mice.<sup>3,4</sup> The effector mechanisms that have been associated with IFN-induced suppression of HBV replication include myxovirus resistance protein A (MxA) and proteasome-mediated activities.<sup>5,6</sup> Additional data suggest a role for guanosine triphosphate-binding proteins, signaling molecules, and various other proteins in the control of HBV replication.<sup>7</sup> HBV can counteract these antiviral effector mechanisms by inhibiting proteasome activities in a hepatitis B virus X protein (HBx)-dependent manner and by suppressing MxA expression at the promoter level.<sup>8,9</sup>

Other mechanisms possibly contributing to the development of chronic HBV infection include viral factors, mutational escape, and immunomodulatory effects of HBV proteins, such as hepatitis B e antigen (HBeAg) and HBsAg. By using T cell receptor transgenic (Tg) and T cell receptor  $\times$  hepatitis B core/HBeAg double-transgenic and triple-transgenic pairs, it has been demonstrated that HBeAg elicits tolerance in hepatitis B core/HBeAg-specific T cells and thereby prevents anti-hepatitis B core seroconversion.<sup>10</sup> In addition, HBsAg has also been shown to suppress the CD8 T cell response, which is characterized by altered human leukocyte antigen/peptide tetramer reactivity in chronically infected patients.<sup>11</sup>

Nonparenchymal liver cells [NPC; in other words, Kupffer cells (KC) and sinusoidal endothelial cells (LSEC)] play an important role in mediating early innate and adaptive immune responses. Their role in chronic HBV infection, however, is not well understood. Recent data from our group suggested that the local innate immune system of the liver can control HBV, because Toll-like receptor (TLR)-stimulated NPCs produce large amounts of antiviral cytokines (in particular IFN- $\beta$ ) that suppress HBV replication.<sup>12</sup> In this paper, we have addressed the question of whether HBV can counteract the TLR-mediated control of its replication by the innate immune system.

## Materials and Methods

**Mice.** Eight-week-old to 10-week-old inbred C57BL/6 mice were kept in the animal facilities at the University Hospital of Essen. All animals received hu-

mane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health.

**Reagents.** Agonists for TLR 1/2 (palmitoyl-3-cysteine-serine lysine-4, Pam3CSK4), TLR 2 (heat-killed preparation of *Listeria monocytogenes*), TLR 4 (lipopolysaccharide from *Escherichia coli* 011:B4 strain), TLR 5 (*S. typhimurium* flagellin), TLR 6/2 ([S-(2,3-bisphosphatidyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe, Pam2CGDHPKSF [FSL1]), TLR 7 (single-stranded RNA40), TLR 8 (Gardiquimod), and TLR 9 (CpG oligonucleotides, ODN1826) were purchased from InvivoGen (San Diego, CA). The TLR 3 agonist polyinosine-polycytidylic acid was obtained from Amersham Biosciences (Buckinghamshire, England). HBsAg was purchased from Rhein Biotech (Düsseldorf, Germany). HBeAg was obtained from Jena Biosciences (Jena, Germany).

**Purification of HBV Virions.** HBV virions were isolated from HBV-Met cells supernatants by HiTrap heparin columns (Amersham Biosciences, Freiburg, Germany) as described previously.<sup>13</sup>

**Isolation and Culture of Parenchymal and Nonparenchymal Liver Cells.** Primary murine hepatocytes and NPCs were prepared and cultured as described previously.<sup>12</sup> For stimulation with TLR ligands, hepatocytes and NPCs were cultured at a density of  $0.5 \times 10^6$  cells/mL in six-well culture plates in the presence of 1  $\mu$ g/mL Pam3CSK4,  $10^8$  cells/mL heat-killed preparation of *Listeria monocytogenes*, 100  $\mu$ g/mL polyinosine-polycytidylic acid, 10  $\mu$ g/mL lipopolysaccharide from *Escherichia coli* 011:B4 strain, 1  $\mu$ g/mL flagellin, 1  $\mu$ g/mL FSL, 10  $\mu$ g/mL single-stranded RNA40, 10  $\mu$ g/mL gardiquimod, or 2  $\mu$ M ODN1826, respectively. Then, culture supernatants were collected or total RNA and nuclear or total protein were isolated and kept at  $-80^\circ\text{C}$ .

**HBV-Met Cell Culture and Transfection of Small Interfering RNAs.** The HBV-Met cell line (provided by S. Wieland and F. Chisari, La Jolla, CA) was cultured as described previously.<sup>14</sup> Transfection of HBV-Met cells was carried out on day 1 of culture as previously described.<sup>15</sup> Briefly, 20 nM small interfering RNA (siRNA) and 5  $\mu$ L lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) were applied in a final volume of 1.5 mL Opti-

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22773

Potential conflict of interest: Nothing to report.

MEM per well. After 6 hours, the supernatants were replaced by fresh culture medium. As small interfering hepatitis B protein X target sequence, 5'-AAGAG-GACTCTTGGACTCTCA-3' (nt1656-1676) was used.

**Virus Protection Assay.** Supernatants collected from resting and stimulated hepatocytes and NPCs were analyzed for the presence of IFN by virus protection assay as described previously.<sup>16</sup> A unit of IFN was defined as the ability to protect 50% of the cells from cell death.

**HBsAg and HBeAg Chemiluminescent Microparticle Immunoassay.** Levels of HBsAg and HBeAg in cell supernatants were determined using the Architect system/Abbott HBsAg and HBeAg chemiluminescent microparticle immunoassay kit according to the manufacturer's instructions (Abbott, Wiesbaden, Germany).

**Analysis of HBV Replicative Intermediates and HBV RNA.** Analysis of HBV replicative intermediates and HBV RNA was performed as described previously.<sup>17</sup> For quantification, blots were exposed to Kodak imaging screens and the signal intensities were quantified by using a Storage Phosphor Screen Cyclone (Packard Instruments, Waltham, MA).

**RNA Isolation and Real-Time Reverse Transcription Polymerase Chain Reaction.** Total RNA was isolated from  $1 \times 10^6$  to  $10 \times 10^6$  cells using Trizol (Gibco), following the manufacturer's protocol. One-step reverse transcription polymerase chain reaction (RT-PCR) with real-time detection was carried out with the QuantiTect SYBR Green RT-PCR Kit (Qiagen) on the Rotor-Gene 2000 real-time amplification system (Corbett Research, Mortlake, Australia) according to the instructions of the manufacturer. The expression of cytokines and interferon-stimulated genes (ISG) was detected by commercial Quantitec Primer Assays (Qiagen, primer sequences not available).

**Nuclear, Cytosolic and Total Protein Extraction.** Extraction of nuclear, cytosolic, and total proteins was carried out as described previously.<sup>18</sup>

**Enzyme-Linked Immunosorbent Assay.** Detection of mouse IFN- $\beta$ , nuclear factor kappa B (NF- $\kappa$ B) (p65), and phosphorylated extracellular signal-regulated kinase (ERK) 1/2 was carried out using commercially available kits (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

**Western Blot.** Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis or native-polyacrylamide gel electrophoresis in Tris/glycine buffer (25 mM Tris and 250 mM glycine) and electroblotted onto nitrocellulose transfer membranes followed by immunoblotting with rabbit polyclonal antibodies to interferon regulatory factor (IRF)-3 (FL-425) (Santa Cruz Biotechnology, Heidelberg, Germany). The analysis of

western blot was carried out by using a Biostep gel documentation system (Scie-Pls, Southam, UK) and the Phoretix 1D advanced v4.01 software (Phoretix International, Newcastle, UK).

**Statistical Analysis.** Data are expressed as mean  $\pm$  standard error of the mean. Differences between any two groups were determined by Wilcoxon's test;  $P < 0.05$  was considered statistically significant.

## Results

**Supernatants from HBV-Infected HBV-Met Cells Suppress the TLR-Induced Antiviral Activity of Parenchymal and Nonparenchymal Liver Cells Against Encephalomyocarditis Virus and HBV.** Because previous data from our group suggested that TLR-3-activated and TLR-4-activated NPCs can potently suppress HBV replication,<sup>12</sup> we investigated whether HBV has the ability to counteract this antiviral activity. Therefore, primary hepatocytes and NPCs were isolated from C57BL/6 mice and incubated for 24 hours with supernatants from differentiated HBV-Met cells cultured for 11 days containing high amounts of HBV. After replacement of the supernatants by supernatants from HBV-Met cells from day 2 of culture that contain low amounts of HBV, the cells were stimulated with TLR 3 and TLR 4 ligands for 20 hours. Then, the supernatants were assayed for their antiviral activity against encephalomyocarditis virus (EMCV) and HBV using a virus protection assay or HBV-Met cells, respectively.

Preincubation of hepatocytes and NPCs with HBV-bearing culture medium led to suppression of the TLR-induced antiviral activity against EMCV (Fig. 1A). Similarly, TLR-3-mediated and TLR-4-mediated antiviral activities of NPCs and hepatocytes against HBV were completely abrogated (Fig. 1B).

**Purified HBV and Recombinant HBV-Proteins Suppress the TLR-Induced Antiviral Activity of Parenchymal and Nonparenchymal Liver Cells Against EMCV and HBV.** To exclude a nonspecific effect of HBV-Met cell supernatants and determine possible components of HBV that may mediate the inhibitory effect on TLR-induced antiviral activity, hepatocytes and NPCs were stimulated with TLR 3 and TLR 4 ligands with or without preincubation (24 hours) with HBV-Met cell supernatants, purified HBV virions, HBsAg, HBeAg, or an HBV mix (HBV virions + HBsAg + HBeAg), respectively. Then, the supernatants were assayed for their antiviral activity against EMCV and HBV using a virus protection assay or HBV-Met cells as target cells, respectively.



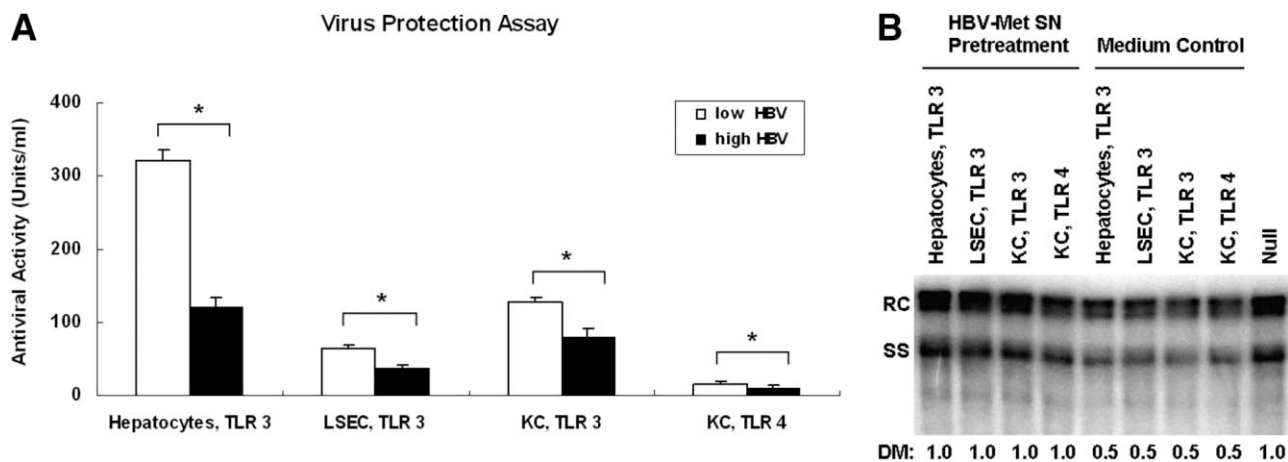


Fig. 1. Supernatants from HBV-Met cells suppress the TLR-induced antiviral activity of parenchymal and nonparenchymal liver cells against EMCV and HBV. Hepatocytes and NPC (KC, LSEC) were stimulated with TLR-3 and TLR-4 ligands for 20 hours with or without preincubation with supernatants from HBV-Met cells (low HBV levels: HBV-Met SN from day 2, containing 8.21 IU/mL HBsAg and 24.14 S/CO reactive HBeAg); high HBV levels: HBV-Met SN from day 11, containing 169.97 IU/mL HBsAg and 119.4 S/CO reactive HBeAg) for 1 day. Then, the supernatants were used (A) in an antiviral assay (EMCV) or (B) added to differentiated HBV-Met cells with 1:2 dilutions for 2 days. In A, data are shown as mean antiviral units/mL  $\pm$  SD (\* $P < 0.01$ ). (B) HBV replication in HBV-Met cells was analyzed by southern blot. The bands shown correspond to the relaxed circular double-stranded (RC) and single-stranded (SS) HBV DNA replicative intermediates. Densitometric values (DM) are given as ratio sample/untreated control.

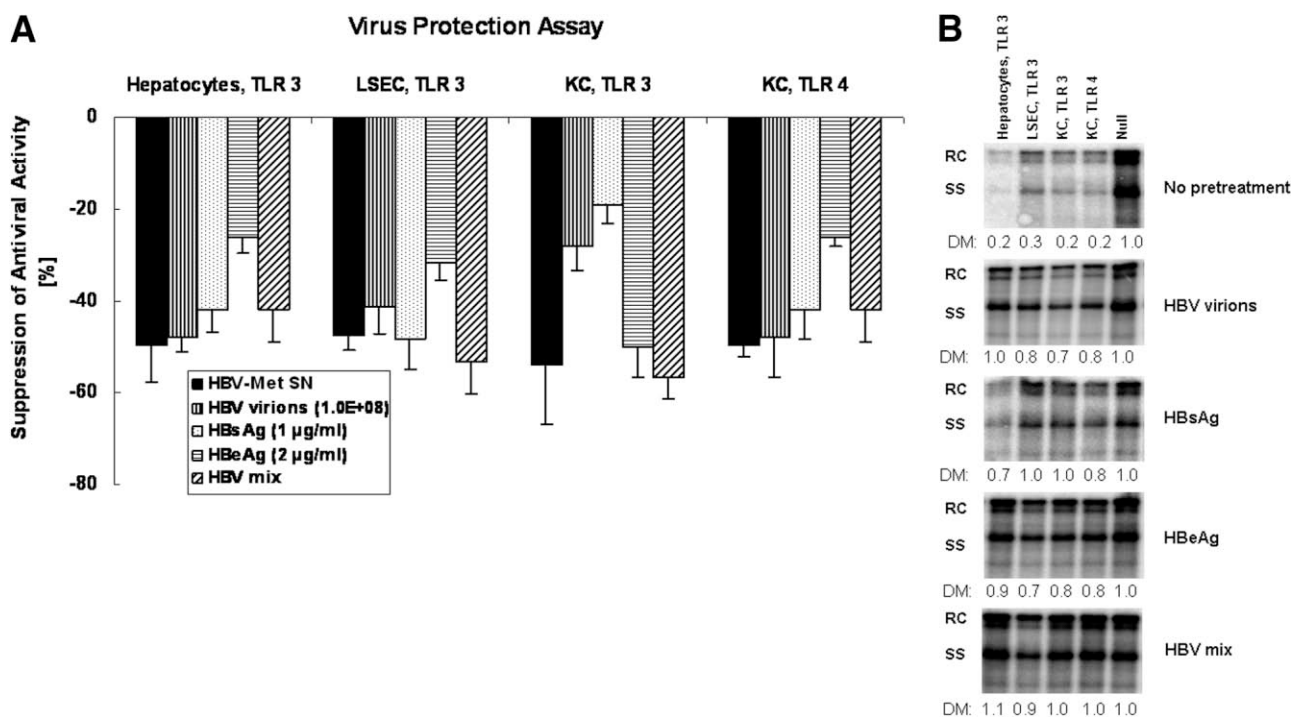


Fig. 2. Purified HBV and recombinant HBV proteins suppress the TLR-induced antiviral activity of parenchymal and nonparenchymal liver cells against EMCV and HBV. Hepatocytes and NPC (KC, LSEC) were stimulated with TLR-3 and TLR-4 ligands for 20 hours (A, B), 6 hours (C), or 2 hours (D) with or without preincubation with supernatants from HBV-Met cells (HBV-Met SN, day 11), HBV virions (1.0E + 08), HBsAg (1  $\mu$ g/mL), HBeAg (2  $\mu$ g/mL), or an HBV mix [HBV virions (1.0E + 08) + HBsAg (1  $\mu$ g/mL) + HBeAg (2  $\mu$ g/mL)] for 24 hours, respectively. Then, the supernatants were (A) used in an antiviral assay (EMCV) or (B) added to differentiated HBV-Met cells with 1:2 dilutions for 2 days. (A) Data are shown as mean antiviral units/mL  $\pm$  standard deviation (\* $P < 0.01$ ). (B) HBV replication in HBV-Met cells was analyzed by southern blot. The bands shown correspond to the relaxed circular double-stranded (RC) and single-stranded (SS) HBV DNA replicative intermediates. Densitometric values (DM) are given as ratio sample/untreated control.

As shown in Fig. 2A, all HBV preparations had strong suppressive effects on TLR-induced antiviral activities against EMCV, although the magnitude was dependent on cell type, stimulus, and the preparation used for pre-treatment. The HBV mix and HBV-Met cell supernatants had very similar suppressive effects on TLR-3-induced and TLR-4-induced antiviral activity of hepatocytes and NPCs against EMCV. Purified HBV virions had only moderate suppressive effects on TLR-3 stimulation of KCs, whereas HBeAg was less potent in suppressing the antiviral activity of TLR-3-stimulated hepatocytes or LSEC and TLR-4-stimulated KCs. HBsAg had only moderate suppressive effects on TLR-3-stimulated KCs.

These observations could be confirmed for HBV, although the individual patterns differed in terms of magnitude. TLR-induced antiviral activities of supernatants from hepatocytes and NPCs against HBV were significantly reduced by preincubation with HBeAg, whereas only moderate suppression was seen with virions or HBsAg. Interestingly, the combination of virions, HBsAg, and HBeAg completely abrogated the TLR-mediated antiviral activities of hepatocytes and NPCs against HBV (Fig. 2B), suggesting that these three viral components may act synergistically in this system.

**HBV Suppresses TLR-Induced IFN- $\beta$  Production, ISG Expression, and Induction of Transcription Factors in Parenchymal and Nonparenchymal Liver Cells.** To determine the effect of HBV on TLR responses in hepatocytes and NPCs, we examined the ability of various HBV preparations and recombinant HBV proteins to inhibit TLR-induced production of IFN- $\beta$ , ISG expression (MxA, IP10), and activation of transcription factors (NF- $\kappa$ B, ERK). The cells were stimulated with TLR-3 and TLR-4 ligands with or without preincubation with supernatants from HBV-Met cells, purified HBV virions, HBsAg, HBeAg, or an HBV mix for 24 hours. ISG expression was examined by quantitative RT-PCR, and IFN- $\beta$  production and activation of transcription factors was determined by enzyme-linked immunosorbent assay.

Preincubation with HBV or recombinant HBV proteins led to suppression of TLR-induced production of IFN- $\beta$  (Fig. 3A), subsequent ISG expression (Fig. 3B), and activation of NF- $\kappa$ B and ERK (Fig. 3C). The magnitude of this suppressive effect was dependent on cell type, stimulus, and HBV preparation or antigen used. Interestingly, preincubation with HBV virions and HBeAg led to complete suppression of TLR 4-induced ISG expression and ERK activation in KCs. These results suggest that HBV may inhibit both TLR-induced Toll/Interleukin-1 receptor domain-containing adaptor inducing IFN- $\beta$ /IRF-dependent IFN- $\beta$  production as well

as MyD88/NF- $\kappa$ B/ERK-dependent production of proinflammatory cytokines. As shown for the suppression of ISG expression (MxA) in LSEC, this effect occurs in a dose-dependent manner (Fig. 3D) and is not mediated by control proteins such as bovine serum albumin (Fig. 3D) or hepatitis C virus (HCV) pseudoparticles (data not shown).

**Modulation of TLR Responses in HBV-Met Cells by HBV.** Because the experiments described were focused on the effect of exogenously administered HBV or HBV proteins on liver cells, we next studied the impact of integrated HBV on immune responses. For this purpose, we used the HBV-Met cell system.<sup>7</sup> In this cell line, HBV replicative intermediates are only detectable after induction of cell differentiation. Therefore, we compared the TLR responses in undifferentiated (low levels of HBV) and differentiated HBV-Met cells (high levels of HBV).

Our data show that the TLR-induced expression of pro-inflammatory and anti-inflammatory cytokines is strongly modulated after induction of HBV replication in HBV-Met cells, mostly (depending on the stimulus) favoring the production of the anti-inflammatory cytokine transforming growth factor beta (TGF- $\beta$ ) while suppressing production of the proinflammatory cytokines tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-6 (IL-6). TNF- $\alpha$  is reduced in the presence of high levels of HBV after stimulation with TLR-2 through TLR-4 and TLR-7 through TLR-9 ligands, whereas it is enhanced after stimulation with TLR-1, TLR-5, and TLR-6 ligands (Fig. 4A). High levels of HBV suppress TLR-induced expression of IL-6 after stimulation with TLR-1 through TLR-5 and TLR-7 through TLR-9 ligands (Fig. 4B). In contrast, high levels of HBV enhance the TLR-induced expression of the anti-inflammatory cytokine TGF- $\beta$  via stimulation with TLR-1 through TLR-4, TLR-6, and TLR-9 ligands, whereas reduced TGF- $\beta$  production is found after stimulation with TLR-5, TLR-7, and TLR-8 ligands (Fig. 4C). Interestingly, in HBV-Met cells that contain high levels of HBV, TLR-3 stimulation led to a strong suppression of IRF-3 nuclear translocation (Fig. 4D), indicating that integrated HBV also has an inhibitory effect on the TLR-activated IRF pathway.

**Integrated HBV Down-Regulates the TLR Response of Hepatocytes.** To rule out that the modulation of TLR signaling in differentiated HBV-Met cells is caused by hepatocyte differentiation and not by HBV, we suppressed HBV gene expression and replication in these cells by transfecting them with specific siRNAs against HBx. Seven days after transfection, the cells were stimulated with TLR ligands.

To determine the efficiency of the siRNA knockdown, we examined HBsAg and HBeAg levels in cell culture

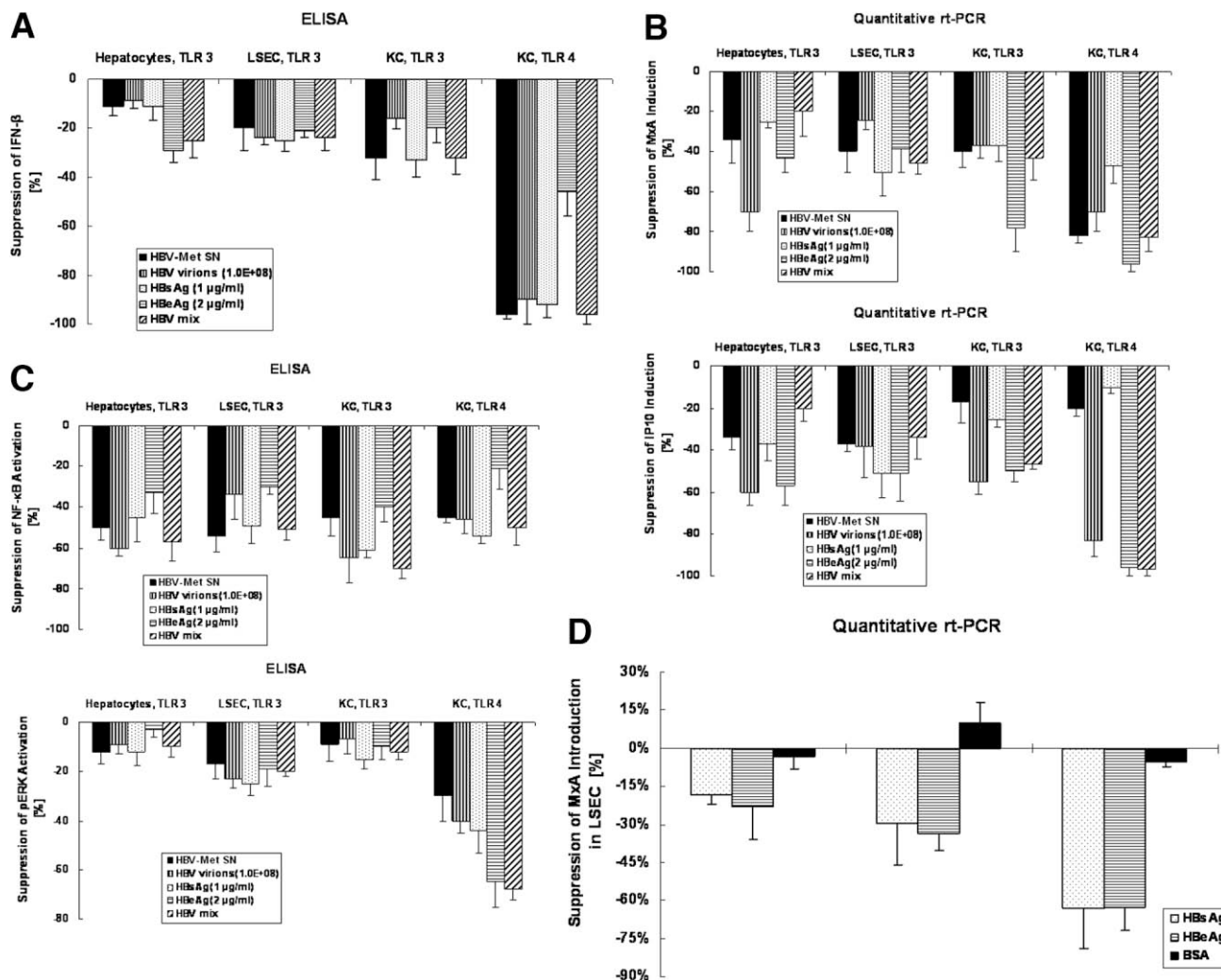


Fig. 3. HBV suppresses TLR-induced IFN- $\beta$  production, ISG expression, and induction of transcription factors in parenchymal and nonparenchymal liver cells. Hepatocytes and NPC (KC, LSEC) were stimulated with TLR-3 and TLR-4 ligands with or without preincubation with supernatants from HBV-Met cells (HBV-Met SN, day 11), HBV virions (1.0E + 08), HBSAg (1  $\mu$ g/mL), HBeAg (2  $\mu$ g/mL), or an HBV mix [HBV virions (1.0E + 08) + HBSAg (1  $\mu$ g/mL) + HBeAg (2  $\mu$ g/mL)] for 24 hours, respectively. Then, (A) supernatants were harvested after 6 hours and assayed for the presence of IFN- $\beta$  by enzyme-linked immunosorbent assay. (B) Total RNA was extracted after 6 hours, and expression of MxA and IP10 was analyzed by quantitative RT-PCR. (C) After 2 hours, total protein was isolated and assayed for the presence of activated ERK  $\frac{1}{2}$ , and nuclear protein was isolated to analyze the amount of translocated NF- $\kappa$ B (p65) by enzyme-linked immunosorbent assay. (D) LSECs were stimulated with TLR-3 ligands with or without preincubation with different concentrations of HBSAg and HBeAg. Total RNA was extracted after 6 hours, and expression of MxA was analyzed by quantitative RT-PCR. Data are shown as mean  $\pm$  standard deviation suppression of the TLR response of hepatocytes and NPC induced by pretreatment with HBV or HBV proteins.

medium by chemiluminescent microparticle immunoassay. Both proteins were significantly reduced (HBSAg: >70%; HBeAg: >50%) on day 7 after transfection (Fig. 5A). Only after suppression of HBV TLR 3-mediated activation of IRF-3 (dimer) was detectable (Fig. 5B). In accordance with the previous data, TLR-mediated activation of NF- $\kappa$ B and ERK was enhanced after suppression of HBV replication (Fig. 5C). Finally, HBx siRNA treatment resulted in enhanced TLR-mediated induction of proinflammatory cytokines (TNF- $\alpha$ , IL-6) and ISGs (IP10) as well as suppressed production of the antiinflammatory cytokine TGF- $\beta$  (Fig. 5D).

## Discussion

In this article, we demonstrate that HBV can suppress the innate immune response elicited by TLR stimulation of hepatocytes and nonparenchymal liver cells. The data suggest that HBV-bearing supernatants, purified HBV virions, and recombinant HBSAg or HBeAg can induce this effect. The inhibitory effect is at least partially mediated by suppression of IRF-3 activation resulting in down-regulation of IFN- $\beta$  production and subsequent ISG expression, which leads to reduced TLR-mediated induction of antiviral activity against EMCV and HBV.

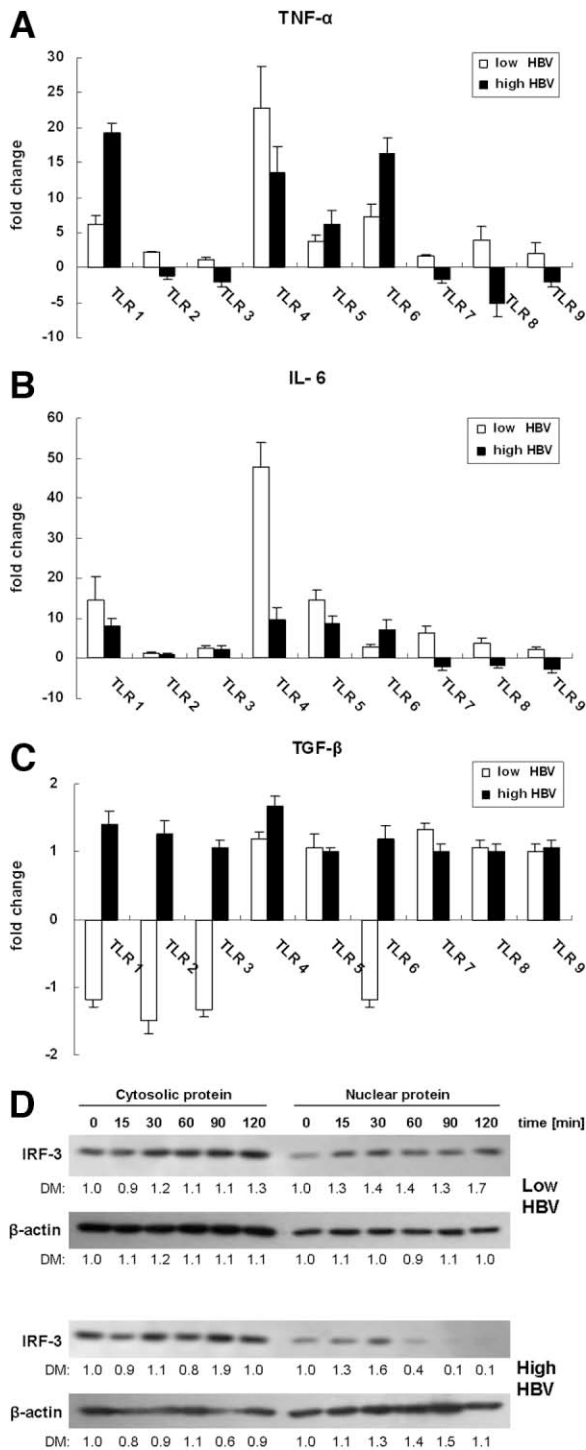


Fig. 4. Modulation of TLR responses in HBV-Met cells by HBV. Undifferentiated (day 2, low HBV levels) and differentiated HBV-Met cells (day 11, high HBV levels) were stimulated with TLR 1-9 ligands for 6 hours. Total RNA was extracted, and expression of TNF- $\alpha$  (A), IL-6 (B), and TGF- $\beta$  (C) was analyzed by quantitative RT-PCR. Data are shown as fold change compared with unstimulated cells. (D) Cytosolic proteins and nuclear proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblotting using anti-mouse IRF-3 antibodies. Densitometric values (DM) are given as ratio sample/untreated control.

In addition, activation of NF- $\kappa$ B and ERK are also suppressed, which results predominantly, depending on the stimulus used, in down-regulation of the proinflammatory cytokines IL-6 and TNF- $\alpha$  and up-regulation of the anti-inflammatory cytokine TGF- $\beta$ .

In our view, these data have major implications for the pathogenesis of HBV infection and, in particular, the interaction of HBV with the innate immune system.

Recent studies that were mainly focused on the role of the adaptive immune system in HBV infection demonstrated that clearance of HBV is typically associated with vigorous, polyclonal, and multispecific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses.

However, only little is known about the role of the innate immune system in HBV infection. The innate immune system detects so-called pathogen-associated molecular patterns via the TLR system. It is involved in the regulation of innate immune responses via proinflammatory signaling cascades such as the NF- $\kappa$ B, c-Jun-N-terminal kinase, ERK, and interferon pathways in the liver and regulates antiviral and antibacterial responses, hepatic injury, and wound healing.<sup>19,20</sup> TIR (Toll/Interleukin-1 receptor) domain-containing adaptors such as MyD88 (myeloid differentiation primary response gene 88), TIR domain-containing adaptor protein, Toll/interleukin-1 receptor domain-containing adaptor inducing IFN- $\beta$ , and Toll/interleukin-1 receptor domain-containing adaptor inducing IFN- $\beta$ -related adaptor molecule are regulating the TLR signaling pathways. Differential utilization of these TIR domain-containing adaptors provides specificity of individual TLR-mediated effects. TLR-3, TLR-7, TLR-8, and TLR-9 are known to detect viral components, leading to cytokine induction via NF- $\kappa$ B activation and release of type I and type II IFNs.

More recent data suggest that activation of the local innate immune system of the liver through the TLR system can effectively suppress HBV replication *in vivo* and *in vitro*.<sup>12,21</sup> Wieland et al.<sup>22</sup> reported, however, that genes characteristic for the innate immune response are not detectable during HBV entry and expansion. They suggested that HBV may act as a "stealth virus" and does not induce genes of the innate immune system during acute infection. This is in contrast to other hepatotropic viruses such as HCV, where a strong type I IFN-mediated innate immune response is observed and maintained after infection.<sup>23,24</sup>

Our data support another hypothesis that postulates that HBV has developed strategies to suppress the initial antiviral response that is elicited by the innate immune system of the host. In accordance with this hypothesis, Cheng et al.<sup>25</sup> demonstrated that recombinant HBsAg inhibits lipopolysaccharide from *Escherichia coli* 011:B4 strain/TLR 4-induced NF- $\kappa$ B activation leading to re-



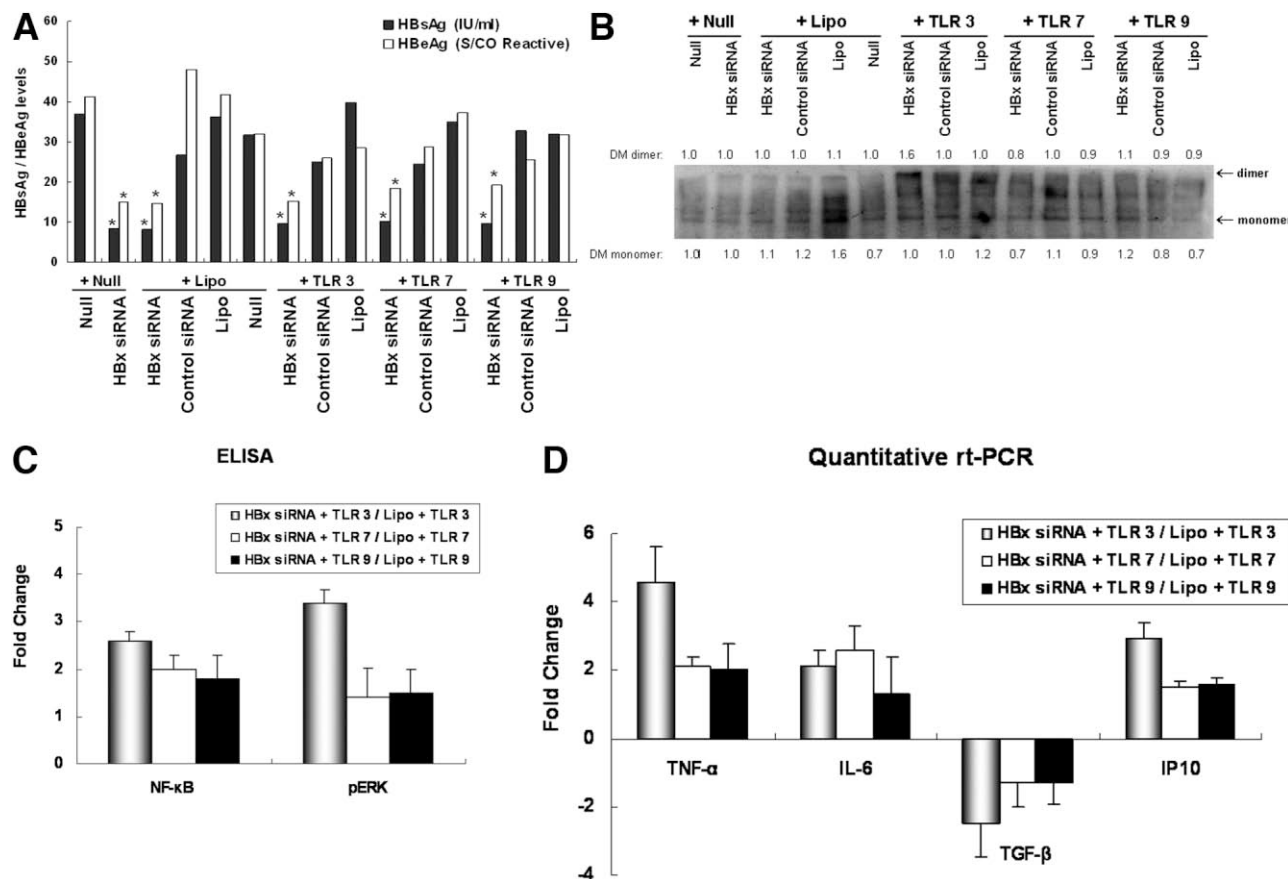


Fig. 5. Integrated HBV down-regulates the TLR response of hepatocytes. HBV-Met cells were transfected with HBx siRNA, green fluorescent protein siRNA (control siRNA), or lipofectamine 2000 on day 1 followed by stimulation with TLR-3, TLR-7, and TLR-9 ligands for 20 hours (A), 2 hours (B), 2 hours (C), or 6 hours (D) 7 days posttransfection. (A) HBsAg and HBeAg levels in cell culture medium were analyzed by chemiluminescent microparticle immunoassay. Significant levels of suppression ( $P < 0.05$ ) are indicated by the asterisk. (B) The cell extracts were subjected to native polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting using anti-mouse IRF-3 antibodies. Densitometric values (DM) are given as ratio sample/untreated control. (C) Total or nuclear protein was isolated, and the amount of NF- $\kappa$ B and pERK was detected by enzyme-linked immunosorbent assay. (D) Total RNA was extracted, and expression of cytokines TNF- $\alpha$ , IL-6, TGF- $\beta$ , and antiviral genes IP10 were analyzed by quantitative RT-PCR. Data are shown as fold change of siRNA pretreatment compared with lipofectamine-treated controls.

duced cyclooxygenase-2, IL-18, and IFN- $\gamma$  production in the human monocytic cell line human acute monocytic leukemia cell line-1.<sup>25</sup> Locarnini et al.<sup>26,27</sup> have shown that TLR-2 expression is reduced in liver cells (hepatocytes and KCs) and peripheral blood monocytes of HBeAg-positive patients, and in HepG2 cells expressing HBeAg, which indicates that HBeAg also may have a suppressive effect on the TLR system. Interestingly, they also reported lower TNF- $\alpha$  levels in HBeAg-positive patients compared with HBeAg-negative patients, which correlates with our *in vitro* data.

Previous data suggested that HBV may not be the only virus that targets the TLR system and thereby attenuates the antiviral response of the innate immune system. It has been demonstrated that two vaccinia virus open reading frames, termed A46R and A52R, that share amino acid sequence similarity with the TIR domain, suppress the activation of NF- $\kappa$ B by multiple TLRs, including TLR-3

and TLR-4.<sup>28,29</sup> The HCV NS3/4A serine protease has been shown to inhibit the virus-induced retinoic acid inducible gene and TLR-3 signaling, leading to suppressed IRF-3 and NF- $\kappa$ B activation.<sup>30</sup> The HCV NS5A protein was found to modulate the TLR-MyD88-dependent signaling pathway in macrophage cell lines.<sup>31</sup> In addition, it has been shown that infectious cell culture-produced HCV impairs TLR-9-induced production of IFN- $\alpha$  by plasmacytoid dendritic cells.<sup>32</sup>

In patients with chronic hepatitis B, HBV-specific immune responses are either absent or narrowly focused, which has been associated with impaired function of antigen-presenting cells.<sup>33</sup> Hasebe et al.<sup>34</sup> demonstrated that liver dendritic cells from HBV transgenic mice produced significantly lower levels of IL-12, TNF- $\alpha$ , and IL-6 compared with liver dendritic cells from normal C57BL/6 mice.<sup>34</sup> Conversely, mature dendritic cells from HBV patients that were successfully treated with adefovir and ex-



perienced a profound drop in viral load exhibited an increased capacity to produce TNF- $\alpha$  and IL-12 after 3 to 6 months of therapy.<sup>35</sup> However, only little is known about the immunosuppressant effects of HBV on Kupffer cells and sinusoidal endothelial cells. They are exposed to HBV virions and proteins in the liver sinusoid, which may be taken up by these cells through receptor-mediated endocytosis or phagocytosis.<sup>36</sup>

In conclusion, our data indicate that HBV can counteract the antiviral response of the local innate immune system of the liver by antagonizing TLR-mediated induction of proinflammatory cytokines. This is of relevance for our understanding of the interaction of HBV and the innate immune system and may, at least in part, explain why HBV appears to act as a "stealth virus" in the initial phase of the infection.

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