Hepatitis B Virus Particles Activate Toll-Like Receptor 2 Signaling Initially Upon Infection of Primary Human Hepatocytes

Zhenhua Zhang,1,2 Martin Trippler,1 Catherine I. Real,1 Melanie Werner,1 Xufeng Luo,1 Stefan Schefczyk,1 Thekla Kemper,2 Olympia E. Anastasiou,1,2 Yvonne Ladiges,1 Juergen Treckmann,4 Andreas Paul,4 Hideo A. Baba,5 Lena Allweiss,3 Maura Dandri,3 Guido Gerken,1 Heiner Wedemeyer,1 Joerg F. Schlaak,6 Mengji Lu,2 and Ruth Broering1

BACKGROUND ANDAIMS: Todate, conflicting data exist as to whether hepatitis B virus (HBV) has the ability to induce innate immune responses. Here, we investigated cellular changes after the first contact between HBV and primary human hepatocytes (PHH) in vitro and in vivo.

APPROACH AND RESULTS: The exposure of PHH to HBV particles resulted in nuclear translocation of NFκB, followed by the expression and secretion of inflammatory cytokines (IL [interleukin] 1B, IL6, and TNF [tumor necrosis factor]). Ultraviolet irradiation of viral particles suppressed HBV infectivity but not the induction of cytokines in PHH, suggesting that the inoculum contains the immune-inducing agent. Purified HBV particles on the whole, which were prepared from HBV DNA-positive and protein-rich fractions after heparin column separation, still had immune-inducing capacity in PHH. The HBV-induced gene expression profile was similar to that induced by toll-like receptor 2 (TLR2) ligand Pam3Cys, but different from those induced by the viral sensors TLR3 or TLR7-9. Treatment of PHH with both HBV particles and Pam3Cys led to phosphorylation of ERK (extracellular signal–regulated kinase), JNK, and p38 mitogen-activated protein kinases as well as NFκB (nuclear factor kappa B). Finally, HBV-induced gene expression could be neutralized by TLR2-specific antibodies. Of note, pretreatment with an HBV entry inhibitor attenuated the TLR2-mediated response to HBV, suggesting a receptor binding–related mechanism. In liver-humanized uPA/severe combined immunodeficient (SCID)/beige mice challenged with HBV in vivo, immune induction could only marginally be seen.

CONCLUSIONS: PHHs are able to sense HBV particles through TLR2, leading to an activation of anti-HBV immune responses in vitro. These findings challenge the previously described stealth properties of HBV. (Hepatology 2020;72:829-844).

Chronic hepatitis caused by hepatitis B virus (HBV) infection is among the most frequent causes for liver-related morbidity and mortality. Persistent infections mostly occur in perinatal and childhood infections as well as in immunocompromised

Abbreviations: ACTB, β-actin; CCL, C-C motif chemokine ligand; CXCL, chemokine (C-X-C motif) ligand; ELISA, enzyme-linked immunosorbent assay; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; ICG, immunocytotoxicity; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IL, interleukin; MOI, multiplicity of infection; NFκB, nuclear factor kappa B; NTCP, sodium-taurocholate co-transporting polypeptide; PC3, Pam3Cys; PEG8000, polyethylene glycol 8000; PHH, primary human hepatocyte; PreC/pg, precore/pregenomic; RT-PCR, reverse-transcription polymerase chain reaction; SCID, severe combined immunodeficient; TLR, toll-like receptor; TNF, tumor necrosis factor; USB, uPA/SCID/beige; UV, ultraviolet.

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patients, whereas 95% of immunocompetent adults spontaneously resolve acute HBV infections.\(^{(1,2)}\) The natural history of persistent HBV infection is represented by different phases: hepatitis B e antigen (HBeAg)-positive chronic infection, HBeAg-positive chronic hepatitis, HBeAg-negative chronic infection, HBeAg-negative chronic hepatitis, and a hepatitis B surface antigen (HBsAg)-negative phase. Patients with chronic HBV infection are at increased risk of progression to cirrhosis and/or hepatocellular carcinoma, depending on host and viral factors.\(^{(3)}\) The efficiency of innate immune responses in the liver likely facilitates systemic immune or tolerance induction as well as viral immune evasion, and therefore affects the pathogenesis of HBV infection.

Both innate and adaptive immune systems are involved in the clearance of viral infections. The innate response is important to control viral replication early after infection and to orchestrate virus-specific adaptive immune responses.\(^{(4,5)}\) In the case of HBV infection, it has been demonstrated that adaptive immune responses are needed for efficient control of infection.\(^{(6,7)}\) Investigation of acute HBV infection in chimpanzees revealed that HBV fails to induce enhanced expression of cellular genes related to the entry and expansion of the virus, implicating the lack of innate immune response following HBV infection.\(^{(8,9)}\) However, due to the low numbers of cells infected at the beginning of natural infection, the changes of cellular gene expression may not be detected by the methods used in earlier studies.\(^{(8,9)}\) Indeed, an early induction of innate immune responses, represented by the development of natural killer cell responses, has been observed in patients with acute HBV infection.\(^{(10)}\) Thus, HBV may activate innate immune responses in the early phase of infection.

High species specificity of HBV limits the number of experimental models. Cell cultures that support physiological HBV infection are especially rare. Primary human hepatocytes (PHHs) have been shown to fully support the HBV life cycle in vitro.\(^{(11)}\) We have previously characterized the toll-like receptor (TLR) signaling and antiviral responses in primary human hepatocytes,\(^{(12)}\) indicating that PHHs exhibit functional TLR1-9 signaling. The most potent TLR signaling pathways in PHHs are those sensing primarily bacterial structures, namely, TLR1, TLR2, TLR5, and TLR6. More importantly, in PHHs, interferon responses can only be mediated by TLR3\(^{(12)}\) and RIG-I (retinoic acid-inducible gene I)/MDA5 activation.\(^{(13)}\) Thus, PHHs harbor distinct immunological properties and likely affect hepatic and systemic immune responses. In the present study, we addressed the question of whether and how PHHs respond to infection with cell culture-derived HBV particles in vitro.

**Experimental Procedures**

**ISOLATION AND CULTURE OF HUMAN HEPATOCYTES**

PHHs were regularly prepared from nontumor tissue obtained from fresh liver resections (n = 20), reaching 95% purity, as described in detail by Werner et al.\(^{(14)}\) Patients provided written documentation of informed consent. The study conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved.
by the institutional review board (Ethics Committee) of the medical faculty at the University of Duisburg-Essen. Hepatocytes were seeded into collagen I–coated culture plates (24-well) using Dulbecco’s modified Eagle’s medium Ham’s F12 (Biochrom, Berlin, Germany) supplemented with 10% inactivated fetal bovine serum (FBS; Biochrom), 2 mM l-glutamine (Biochrom), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Biochrom) and cultured overnight. To enable long-term culture, maintenance medium was used 1 day after preparation (Williams medium [Biochrom], 10% FBS [Biochrom], 2% DMSO [Sigma Aldrich, Darmstadt, Germany], 1% l-glutamine, insulin [5 µg/mL, Sigma Aldrich], 1% HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; Biochrom], 2% nonessential amino acid [Biochrom], hydrocortisone hemisuccinate [2.5 µg/mL, Sigma Aldrich], and epidermal growth factor [55 ng/mL, Sigma Aldrich]). All PHH experimental settings, except gene arrays, included biological triplicates and were repeated with at least three PHH preparations from different liver donors.

GENERATION AND INFECTION OF HUMAN LIVER CHIMERIC MICE

Animals were maintained under specific pathogen-free conditions in accordance with institutional guidelines under approved protocols. Human hepatocytes, purchased from Thermo Fisher Scientific (Waltham, MA), were used to generate human liver chimeric mice (uPA/SCID [severe combined immunodeficient]/beige, USB) as previously described.(15) Mice were inoculated with a single intraperitoneal injection of HBV-containing mouse serum (1 × 10⁷ HBV DNA copies/mouse, genotype D, HBeAg-positive) or were challenged with the equivalent amount of noninfectious mouse serum.(15,16) Mice were sacrificed 3 hours or 6 hours after inoculation (group size n = 4). Liver specimens were snap-frozen in 2-methylbutane and stored at −80°C for gene expression analyses.

HBV PREPARATION AND PHH INFECTION

The cell line HepG2.117, stably transfected with an HBV-coding plasmid, was cultured as described elsewhere.(17) HBV particles (genotype D, serotype ayw, HBeAg-positive) were prepared from cell culture supernatants by overnight precipitation with 6% polyethylene glycol 8000 (PEG8000; Sigma, Darmstadt, Germany) at 4°C, concentrated by centrifugation (12,000g for 60 minutes at 4°C) and stored at −80°C. A mock control was produced by precipitating supernatants of HepG2 cells under the same conditions. For the infection and immune induction experiments, a total of five different HBV preparations with 10⁹ genome equivalents/mL were used. One day following preparation, PHHs were treated with purified HBV particles at different MOIs (multiplicity of infection). To increase infectivity and virus cell contact, the medium was supplemented with 4% polyethylene glycol PEG8000. After 24 hours, cells were washed 3 times with phosphate-buffered saline (PBS) and were further cultured in PHH maintenance medium; the medium was changed every second day. If indicated, PEG8000-precipitated HBV particles were additionally purified using Hi Trap heparin column (GE Healthcare Life Sciences, Freiburg, Germany) according to the manufacturer’s instructions. The fractions were analyzed using Bradford reagent (BioRad, Munich, Germany) and QiaAmp DNA Blood Kit (Qiagen, Hilden, Germany) to identify virus-containing fractions. Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Schwerte, Germany) were used to remove low molecular-weight contaminant for buffer exchange, desalting, and sample concentration. A myristoylated preS1 peptide, representing the N-terminal 47 amino acids of the large HBsAg (also known as myrcludex B [MyrPharma, Burgwedel, Germany]) as well as a nonmyristoylated control peptide were purchased from Peptides & Elephants GmbH (Hennigsdorf, Germany) and used in an HBV entry inhibition assay. Brefeldin A (1x; BioLegend, London, United Kingdom) was applied to inhibit intracellular protein transport to the Golgi complex, leading to accumulation of secretory proteins in the endoplasmic reticulum.

HBsAg AND HBeAg DETECTION

HBV-infected PHHs were cultured for 10 days. Cell culture supernatants were collected at different time points, snap frozen, and stored at −80°C. HBeAg and HBsAg were detected with the ARCHITECT chemiluminescent micro particle immunoassays and the ARCHITECT immunoassay analyzer (Abbott Diagnostics, Wiesbaden, Germany), according to the manufacturer’s instructions.
ENZYME-LINKED IMMUNOSORBENT ASSAY

Cell culture supernatants from HBV-treated PHHs were harvested 24 hours after exposure. IL6 and tumor necrosis factor (TNF) enzyme-linked immunosorbent assay (ELISA) were performed according to the manufacturer’s instructions (R&D Systems, Wiesbaden, Germany).

IMMUNOCYTOCHEMISTRY

The procedure of immunocytochemical (ICC) staining was previously described by Werner et al. Briefly, cells were fixed with precooled −20°C methanol/acetone (1:2) for 10 minutes at 4°C, and washed and permeabilized with 0.3% Triton X-100 in PBS for 30 minutes. Cells were blocked in 5% normal donkey serum for 1 hour, and incubated with the following primary antibodies overnight at 4°C: anti-albumin (1:100; R&D Systems), anti-HBsAg (1:100, Aviva Systems Biology, San Diego, CA), anti-nuclear factor kappa B (NFκB; 1:400, Cell Signaling Technology, Danvers, MA), and anti-IL6 (1:200, BioLegend). Cells were washed with PBS and incubated with fluorescent conjugated secondary antibodies for 1 hour: donkey anti-mouse Alexa Fluor 488 (1:500; Thermo Fisher Scientific), donkey anti-rabbit Alexa Fluor 488 (1:2000; Abcam, Cambridge, United Kingdom), and donkey anti-mouse Alexa Fluor 594 (1:500; Thermo Fisher Scientific). Nuclei were counterstained with Fluoroshield mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Steinheim, Germany).

QUANTITATIVE REVERSE-TRANSITION POLYMERASE CHAIN REACTION

Total RNA was extracted with QIAzol Lysis Reagent and the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. One-step quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed using QuantiFast SYBR Green PCR Kit (Qiagen) with 100-200 ng of total RNA. Primer sequences or information on commercially available primer sets (QuantiTec Primer Assay; Qiagen) are described in Supporting Table S1. Expression levels were normalized to ACTB (β-actin).

Expression levels of human and murine genes in the USB mouse liver tissue were determined as previously described. PCR measurements were performed on the ViiA 7 Real-Time PCR System with probes and primers (Supporting Table S1) from the TaqMan Gene Expression Assay System and the Taqman Fast Advanced Master Mix (Thermo Fisher Scientific). Expression levels were normalized to the mean of two human (GAPDH [glyceraldehyde 3-phosphate dehydrogenase] and RPL30 [ribosomal protein L30]) or two murine (Eef2 [eukaryotic translation elongation factor 2] and Actb) housekeeping genes.

AFFYMETRIX’S GeneChip

PHHs were treated with HBV (MOI 1,000) or TLR ligands Pam3Cys (PC3; 2 µg/mL), poly(I:C) (50 µg/mL), Guardiquimod (20 µg/mL), R848 (10 µg/mL), and CpG ODN2216 (30 µg/mL) for 6 hours (ligands, InvivoGen, Toulouse, France). Sample preparations and GeneChip arrays were performed as recently described. RNA samples were preprocessed with the Affymetrix 3’ IVT Express Kit (Santa Clara, CA). Samples were hybridized on Human Genome U219 Array Plates using the AffymetrixGeneTitan MC Instrument (Affymetrix). The average array signal intensity of 100 was defined as the cutoff. Gene expression signals that were induced by HBV treatment (≥ 1.5-fold) were compared with TLR activation signatures using MultiExperiment Viewer.

WESTERN BLOT

Western blot analysis of phosphorylated and unphosphorylated forms of NFκB and mitogen-activated protein kinases was performed as previously described.

STATISTICAL ANALYSIS

Data are expressed as mean ± SEM. Statistically significant differences between two groups were determined with the Student t test, including Welch’s correction if indicated. Statistical significance was set at the level of Pot < 0.05.
Results

PRIMARY HUMAN HEPATOCYTES SUPPORT HBV INFECTION IN VITRO

PHHs have been described as a suitable culture model that efficiently supports HBV infection. (11) We isolated human hepatocytes from liver tissues (donors, n = 3) that were obtained after resection and exposed these cells to cell culture-derived HBV particles (MOI 1,000). The hepatocytes were intensively washed 24 hours after HBV exposure. PHHs began to secrete HBsAg (Supporting Fig. S1A) and HBeAg (Supporting Fig. S1B) from day 6 following infection. The HBV antigen levels further increased until day 10. To test whether the release of antigens depends on active viral replication, HBV particles were irradiated with ultraviolet (UV) light of different intensities (20, 100, and 500 J/cm²) before infection. The irradiation of viral particles led to dose-dependent suppression of HBsAg (Supporting Fig. S1A) and HBeAg (Supporting Fig. S1B) levels in the cell culture supernatant. At day 10, RNA had been extracted and HBV mRNA expression was assessed by quantitative RT-PCR. The UV irradiation clearly suppressed the expression of precore/pregenomic RNA (PreC/pg) and total HBV mRNA transcripts (HBV mRNA), determined by detection of the HBV-encoded X antigen region, which belongs to all HBV-coding open reading frames (Supporting Fig. S1C). The HBsAg was additionally visualized by ICC, 6 hours after 100% infection of PHHs, stained for albumin, showed high positivity for HBsAg, indicating complete infection (Supporting Fig. S1D). Furthermore, the PHHs remained HBsAg-positive and albumin-positive until day 7, although the intensity of albumin and HBsAg signals were dampened in comparison to that at 6 hours after infection. These results clearly show that PHHs efficiently support HBV infection in vitro and represent a feasible tool to analyze immune events early during infection.

HBV INDUCES THE EXPRESSION OF INFLAMMATORY CYTOKINES INITIALLY AFTER INFECTION OF PHH

In a previous study, the immunological properties of PHHs have been described, (12) indicating that PHHs can be stimulated through the TLR1-9 system and may play a relevant role in the hepatic innate immunity. Although HBV has been described as a stealth virus, (9) innate immune induction in HBV-exposed hepatocytes cannot be ruled out and has been addressed as follows. PHHs of three different donors were infected with cell culture-derived HBV particles, and induction of immune genes was analyzed by quantitative RT-PCR 3 hours, 6 hours, and 24 hours after infection. Gene expression of IL1B, IL6, and TNF increased initially following exposure to HBV, reaching maximum expression levels 3 hours after stimulation (Fig. 1A). Secretion of IL6 and TNF was shown by ELISA measurements using supernatants of PHHs (donors, n = 3) exposed to HBV particles for 24 hours (Fig. 1B). Furthermore, HBV dose-dependently (MOI 62.5-1,000) induced the expression of IL1B, IL6 and TNF, whereas the mock control (MOI 500/1,000 equivalents) did not affect cytokine gene expression (Supporting Fig. S2A). UV irradiation of the inoculum that was sufficient to inhibit viral replication (Supporting Fig. S1A-C) dose-dependently enhanced IL1B, IL6, and TNF expression (Fig. 1C). These data indicate that the inoculum harbors the immunostimulatory pattern. Furthermore, immune responses significantly increased with the loss of infectivity (Fig. 1C), suggesting a very early event of immune control. An induction of cytokine gene expression was also observed after treatment with HBV particles that were purified using a heparin column fractionation and dialysis steps (Fig. 1D), to remove any cellular and viral debris or scattered antigens. The purified HBV particle appeared to be more potent than the PEG precipitate, especially when no PEG was added to the culture condition (Fig. 1D). Finally, intracellular ICC staining revealed NFκB nuclear translocation in albumin-positive PHHs 2 hours after HBV treatment (Fig. 1E). In addition, intracellular accumulation of IL6 (Supporting Fig. S2B) was observed in PHHs treated with HBV and brefeldin A for 24 hours. These data suggest that the HBV particle on the whole induced a time-dependent and dose-dependent induction of inflammatory genes in PHHs. The overall robustness of our model is underlined by the pairwise analysis of cytokine expression between mock and HBV treatment in all PHH preparations (n = 15) that have been used for RNA analysis within this study (Supporting Fig. S2C).
HBV-INFECTED CYTOKINE PROFILE IN PHH IS COMPARABLE TO A PC3-MEDIATED INNATE IMMUNE RESPONSE

To further characterize the HBV-induced immune response in PHHs, gene chip analysis was performed using PHHs (donor, n = 1) treated with HBV (MOI 1,000) or TLR ligands PC3, poly(I:C), Gardiquimod, R848, and CpG ODN2216 for 6 hours. Gene expression signals that were induced by HBV (>1.5-fold) were compared with TLR-induced gene expression using MultiExperiment Viewer. Signal intensities and identifications of selected genes are given in Supporting Table S2. Hierarchical clustering of the 380 selected genes revealed that HBV particles induced a gene expression profile that showed the highest similarity to that induced by TLR2 ligand PC3 (Fig. 2). Poly(I:C) induced a strong but distinct gene expression profile, whereas TLR7-9 activation induced only weak alterations of the selected genes, showing high comparison with the negative control.

FIG. 2. Hierarchical clustering of gene expression signatures indicates a close relation between HBV-induced and TLR2/3-induced responses in PHHs. PHHs (1 donor) were treated with HBV (MOI of 1,000) or TLR ligands (PC3 [2 µg/mL]; poly(I:C) [50 µg/mL]; Gardiquimod, [20 µg/mL]; Resiquimod [10 µg/mL]; and ODN2216 [30 µg/mL]) for 6 hours. Total RNA was extracted, processed, and exposed to the Affymetrix Human Genome U219 Array Plate according to the manufacturer’s instructions. The average array signal intensity of 100 was defined as the cutoff. Gene expression signals that were induced by HBV (>1.5-fold) were visualized using MultiExperiment Viewer. Abbreviations: Gdq, Gardiquimod; R848, Resiquimod.
(Fig. 2). These data present an HBV-induced immune response, and therefore question the stealth properties of HBV.

To compare HBV-altered and PC3-altered gene expression in more detail, additional gene chip array data were generated (PHH donors, n = 2). Again, the average array signal intensity of 100 was defined as the cutoff. Interestingly, 155 valid genes were up-regulated 2-fold or greater by HBV and/or PC3 treatment (Fig. 3A). Detailed information on up-regulated and down-regulated gene sets is given in Supporting Table S3. The Venn diagram (Fig. 3B) indicates the number of equally or distinctly induced genes, so an overlay of 96 commonly regulated genes was determined. Molecular interactions among regulated genes were analyzed using the search tool for the retrieval of interacting genes/proteins (STRING). Networks of up-regulated genes are given in Supporting Fig. S3. STRING analyses of regulated genes included Kyoto Encyclopedia of Genes and Genomes annotations, indicating only inflammatory and immunity processes, and are listed in Table 1. Neither down-regulated genes nor those induced solely by HBV or PC3 led to significant pathway annotations. The gene chip data were verified in PHHs (donors, n = 5) treated with PEG8000-precipitated HBV particle or the mock control as well as with TLR2 ligand PC3 for 6 hours. Gene expression of selected genes (highlighted in yellow, Fig. 3A) was determined by quantitative RT-PCR, indicating a high reproducibility in this experimental setup (Fig. 3C). These data indicate that the cytokine expression profile in HBV-infected PHHs is comparable to that induced by TLR2 activation. Furthermore, TLR2 signal transduction was determined by western blot analysis; PHHs were treated with PEG8000-precipitated HBV particles, the mock control, and PC3 for 30 minutes; and protein lysates were prepared and separated solely by gel electrophoresis. Phosphorylation of mitogen-activated protein kinases (ERK [extracellular signal–regulated kinase], JNK, and p38) and NFκB could be seen in HBV-treated and PC3-treated PHHs (Fig. 3D). No differences could be observed between HBV and PC3 treatment. The expression of interferon genes or interferon-stimulated genes could neither be detected initially (array data) nor at later time points after HBV infection. Interferon genes are often expressed at very low levels; therefore, expression of more sensitive response genes like ISG15 (interferon-stimulated gene 15) and IFIT1 (interferon-induced protein with tetratricopeptide repeats 1) was analyzed at day 3 and day 7 to test whether viral replication is accompanied by interferon responses (Supporting Fig. S4). No HBV-induced changes could be seen for ISG15 and IFIT1 gene expression. All together, these data clearly indicate that HBV exposure in PHHs mediated TLR2-like inflammatory immune responses but did not induce expression of interferon response genes.

**HBV-MEDIATED IMMUNE INDUCTION IN PHHs DEPENDS ON TLR2 AND REDUCES HBV REPLICATION**

To further investigate the relation between HBV and TLR2 activation, PHHs were treated with neutralizing antibodies against TLR2 and an isotype control antibody 2 hours before treatment with HBV or PC3. Both the PC3-mediated and HBV-mediated induction of IL1B, IL6, and TNF gene expression could be significantly neutralized by blocking of TLR2 (Fig. 4A–C). Interestingly, blockade of HBV-mediated TLR2 activation by neutralizing antibodies enhanced viral replication, determined by expression of viral mRNA transcripts (preC/pg RNA and HBV mRNA), but did not affect the secretion of HBsAg or HBeAg into the cell culture supernatant (Fig. 4D–F). These data demonstrate that HBV particles harbor a TLR2-activating molecular pattern that mediates inflammatory antiviral responses.

**INHIBITION OF HBV ENTRY BY MYRISTOYLATED preS1 PEPTIDE SUPPRESSES THE HBV-INDUCED IMMUNE RESPONSE**

To determine whether the binding of HBV to its receptor, sodium-taurocholate co-transporting polypeptide (NTCP) is required for TLR2 activation. We used a myristoylated polypeptide according to the HBV preS1 sequence, described as an HBV entry inhibitor, to analyze whether HBV-receptor binding influences the HBV-mediated immune induction. PHHs from three different donors were pretreated with myristoylated (20 µg/mL) or nonmyristoylated (20 µg/mL) preS1 peptides for 30 minutes before treatment with cell culture-derived HBV particles (MOI of 250) or the mock control. Total
FIG. 3. HBV-induced cytokine profile in PHHs is comparable to a P3C-mediated response. PHHs (2 donors) were treated with HBV (MOI of 1,000) or P3C (2 µg/mL); n = 1) for 6 hours. Total RNA was extracted, processed, and exposed to the Affymetrix Human Genome U219 Array Plate according to the manufacturer's instructions. The average array signal intensity of 100 was defined as the cutoff. (A) Gene expression signals that were induced by HBV and/or P3C (≥ 2 fold) were hierarchically clustered and visualized using MultiExperiment Viewer. (B) The Venn diagram indicates the number of equally or distinctly induced genes. To validate the array data set, PHHs (5 donors) were treated with mock (NP, non-particle) control, HBV (MOI of 1,000), and P3C (2 µg/mL) for 6 hours. The total RNA was extracted, and expression of selected genes (highlighted in yellow) was determined by quantitative RT-PCR. (C) Copy numbers were normalized to ACTB expression (mean ± SEM). (D) Western blot analysis was performed to characterize TLR downstream signals in PHHs treated with mock control, HBV (MOI of 1,000), or P3C (2 µg/mL) for 30 minutes. *P < 0.05; **P < 0.01; ***P < 0.001. Abbreviations: BCL2A1, B cell lymphoma 2A1; CES1P1, carboxylesterase 1 pseudogene 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAP3K8, mitogen-activated protein kinase kinase kinase 3; NP, RND1, Rho family GTPase 1; STAT5A, signal transducer and activator of transcript 5A; TRAF1, tumor necrosis factor receptor–associated factor 1.
**Table 1. KEGG Pathway Annotation for Genes Commonly Up-regulated by HBV and P3C**

| Pathway Description                              | Counts | FDR             | Gene IDs                                                                 |
|--------------------------------------------------|--------|-----------------|--------------------------------------------------------------------------|
| Cytokine–cytokine receptor interaction           | 18     | $3.36 \times 10^{14}$ | CCL2, CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CCL8, CCR7, CD40, CX3CL1, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCR7, IL1B, IL8 |
| Chemokine signaling pathway                      | 16     | $3.36 \times 10^{14}$ | CCL2, CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CCL8, CCR7, CX3CL1, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, LYN |
| TNF signaling pathway                            | 12     | $8.23 \times 10^{12}$ | CCL2, CCL5, CX3CL1, CXCL1, CXCL2, CXCL3, CXCL5, IL1B, MAP3K8, NOD2, PTGS2, TRAF1 |
| Salmonella infection                             | 10     | $3.28 \times 10^{10}$ | CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CX3CL1, CXCL2, CXCL3, CXCL5, IL1B, IL8, IL8, NOS2 |
| NFκB signaling pathway                           | 10     | $6.14 \times 10^{10}$ | BCL2A1, CCL4, CCL4L1, CD40, CXCL2, IL1B, IL8, IL8, PTGS2, TRAF1 |
| Rheumatoid arthritis                             | 9      | $1.11 \times 10^{8}$ | CCL2, CCL3, CCL3L1, CCL5, CXCL1, CXCL5, CXCL6, IL1B, IL8 |
| TLR signaling pathway                            | 9      | $4.52 \times 10^{8}$ | CCL3, CCL3L1, CCL4, CCL4L1, CX3CL1, CXCL2, CXCL3, IL1B, IL8, MAP3K8 |
| NOD-like receptor signaling pathway              | 7      | $2.77 \times 10^{7}$ | CCL2, CCL5, CXCL1, CXCL2, IL1B, IL8, NOD2 |
| Chagas disease (American trypanosomiasis)        | 7      | $9.77 \times 10^{6}$ | CCL2, CCL3, CCL3L1, CCL5, IL1B, IL8, NOS2 |

Note: A false discovery rate greater than $1.43 \times 10^{6}$ was suggested as being significant.

Abbreviations: FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes.

**Figure 4.** Neutralization of TLR2 blocks HBV-mediated immune induction and leads to enhanced HBV mRNA transcription. PHHs (3 donors) were pretreated with TLR2 neutralizing antibodies or an isotype control antibody for 2 hours. Cells were then treated with HBV (MOI of 1,000), PC3 (2 µg/mL), or mock control for 6 hours. Total RNA was extracted, and gene expression of IL1B (A), IL6 (B), and TNF (C) was determined by quantitative RT-PCR (mean ± SEM). Cells treated with neutralizing antibodies for 2 hours were infected with HBV (MOI of 1,000) and cultured for 10 days. Supernatants were collected at different time points and analyzed for HBsAg (D) and HBeAg (E). (F) Total RNA was extracted at day 10, and expression of HBV mRNA transcripts (PreC/pg RNA and HBV mRNA) was determined by quantitative RT-PCR. Copy numbers were normalized to ACTB expression (mean ± SEM). *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant. Abbreviations: IsoAB, isotype control antibody; nABTLR2, neutralizing antibodies against TLR2; ns, not significant.
RNA was extracted after 6 hours, and gene expression was analyzed by quantitative RT-PCR. First, treatment of PHHs with myristoylated preS1 peptide alone did not induce the gene expression of \( \text{IL1B} \) (Fig. 5A-C) or \( \text{IL6} \) (Fig. 5D-F), whereas pretreatment with myristoylated preS1 before HBV exposure led to significantly decreased levels of \( \text{IL1B} \) and \( \text{IL6} \) gene expression. The inhibition of HBV entry by the myristoylated preS1 peptide resulted in complete suppression of HBsAg release, determined at day 6, day 8, and day 10 after infection (Fig. 5G). Although HBV infection was totally blocked by the myristoylated preS1 peptide, cytokine induction by HBV particles was only partially affected, suggesting that binding to the entry receptor NTCP facilitates, but is not required for, TLR2 receptor activation. Additionally, the intracellular HBV replication process is unlikely involved in HBV-induced immune responses.

**HBV INOCULATION OF HUMANIZED USB MICE DOES NOT INDUCE A SYNCHRONIZED EARLY INNATE IMMUNE RESPONSE IN HUMAN HEPATOCYTES**

Our presented data indicate that HBV particles induced innate immune responses in PHHs *in vitro*. To investigate these early immune events in acute HBV

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**FIG. 5.** Pretreatment with HBV entry inhibitor suppresses HBV-induced TLR2 response. PHHs (3 donors, PHH I-III) were pretreated with myristoylated (20 µg/mL) and control (20 µg/mL) preS1 peptides 30 minutes before treatment with mock control and cell culture-derived HBV (HepG2.117) at an MOI of 250. Total RNA was extracted after 6 hours, and gene expression of \( \text{IL1B} \) (A,C,E) and \( \text{IL6} \) (B,D,F) was determined by quantitative RT-PCR. Copy numbers were normalized to \( \text{ACTB} \) expression (mean ± SEM). (G) Secretion of HBsAg was measured at day 6, day 8, and day 10 after infection, to indicate the antiviral effect of the myristoylated peptide (mean ± SEM). Abbreviations: ctrl, control; myr, myristoylated.
infection *in vivo*, the liver chimeric USB mouse model was applied. These mice were challenged with HBV through intraperitoneal injection of HBV-containing mouse serum (1 × 10⁷ HBV DNA copies [estimated MOI of 1]) and control mouse serum. Quantitative PCR analyses of USB liver tissues 3 hours and 6 hours after injection revealed that infection with physiological HBV titers only slightly elevated the hepatic expression of human *CCL2* (C-C motif chemokine ligand 2), *CXCL8* (chemokine [C-X-C motif] ligand 8)/IL8, and *CXCL10* (Fig. 6A), which, based on the model used, could originate exclusively from human hepatocytes. In contrast, expression of these genes was significantly induced in PHHs (n = 3, PHHs I-III) treated with HBV for up to 6 hours *in vitro* (Fig. 6B). In the USB model, murine parenchymal and nonparenchymal cells also tended to respond to HBV inoculation, as indicated by the slightly elevated expression of murine cytokine genes (Supporting Table S4). However, the generally low gene expression levels combined with the high variation determined between individual samples and mice did not allow us to achieve significance in the current data set.

**Discussion**

One of the main controversies in the field of HBV is the question of whether it is stealthy or cunning. In the present study, early immune events were analyzed...
after exposure of PHHs to cell culture-derived HBV particles. A time-dependent and dose-dependent induction of inflammatory genes was shown in hepatocytes, initially following infection. Hösel et al. already described HBV-induced cytokines, including IL6 and IL1B, in human parenchymal and nonparenchymal liver cell cultures. They suggested that Kupffer cell contaminations are the driver of immune response in HBV-exposed PHH cultures. However, there was no clear evidence to exclude that hepatocytes also responded to HBV exposure. ICC staining in the present study clearly demonstrates, at the single-cell level, that PHHs respond to HBV exposure by NFκB activation and IL6 production. Furthermore, we were able to attribute the HBV-induced immune response to TLR2 signaling, suggesting an HBV binding-related activation. Accordingly, HBV-induced production of inflammatory cytokines in monocytes depends on TLR2/MyD88/NFκB signaling. Although the initial immune responses were transient in vitro, it is likely that this innate signaling in vivo is one of the first steps in a line of defense mechanisms leading to HBV clearance.

Pathogen recognition receptors play a central role in innate immunity and have been recognized to affect chronic viral hepatitis. PHHs of high purity and retaining functionality respond to TLR1-9 activation with NFκB translocation into the nucleus, leading to secretion of inflammatory factors. However, interferons were only produced in TLR3-activated PHHs, suggesting a distinct response pattern in TLR-activated PHHs compared with classical representatives of the innate immune system. Stimulation of human peripheral blood mononuclear cells with the TLR1-9 ligands showed interferon responses after activating TLR3 and TLR7-9. HBV has evolutionarily evolved, evading strategies to subvert the innate immune system of the liver, which is of relevance for understanding the mechanisms leading to chronicity of HBV infection. The question of whether HBV is a stealthy or cunning virus has not been solved since Wieland and Chisari formed this statement. Although Sato et al. identified RIG-I activation in transduced cell culture models, physiological infection with HBV virus fails to induce hepatic interferon signatures. Very recent publications demonstrated that HBV harbors stealth characteristics, when focusing on interferons and interferon-mediated antiviral effects. HBV infection of differentiated HepaRG(NTCP) and PHHs as well as a model for liver biopsies showed neither activation nor inhibition of antiviral signaling. The gene set analyzed by Mutz et al. (IFIT1, GBP1 [guanylate binding protein 1], TLR3, DNMT3A [DNA methyltransferase 3 alpha], PML [promyelocytic leukemia], STAT2 [signal transducer and activator of transcription 2], and IL10) indicated the nonresponse features of PHHs to HBV infection. Accordingly, the expression of none of these genes was altered in the model presented here (Supporting Table S3). The lack of interferon signatures in HBV infection has previously been dedicated to restricted STING (stimulator of interferon genes)/cGAS (cyclic guanosine monophosphate-adenosine monophosphate synthase) signaling in hepatocytes. More recently, it has been shown that naked, relaxed, circular HBV DNA is sensed in a cGAS-dependent manner in hepatoma cell lines and primary human hepatocytes. However, this sensing is abolished during HBV infection, suggesting escape mechanisms. Although intrinsic innate recognition of HBV structures failed in primary hepatocytes, it has been shown that macrophages exhibit elevated IL1B, IL6, and TNF gene expression signatures following exposure to HBV particles. Cheng et al. suggested that whole HBV particles, but not single components like HBV DNA, RNA or antigens, drive the inflammatory response in macrophages. Interestingly, Song et al. showed that whole HBV particles as well as HBsAg, purified from human plasma, activate TLR2 signaling in monocytes. This leads to suggest that naturally occurring HBsAg, but not a recombinant one, harbors TLR2-activating potential. Here, HBV particles on the whole efficiently triggered TLR2-mediated inflammatory responses in hepatocytes. This transient inflammatory response induced by the first contact between PHH and HBV, in the initial phase of infection, underlines the cunning characteristics of this virus, which appear to evade this signaling later during infection.

The identification of a TLR2-mediated antiviral action against HBV infection and woodchuck hepatitis virus infection increases the importance of TLR2 in the hepatic immune responses against HBV. We demonstrate that HBV particles could activate the TLR2 signaling in hepatocytes, suggesting a notable role for the first line of defense against HBV. Indeed, TLR2 activation decreased HBV replication in our experiment, at least on the transcriptional level.
The antiviral action may be mediated by direct activation of cellular genes but also by cytokines produced by PHHs. The inflammatory cytokines IL1B, IL6, and TNF cytokines have already been described to have antiviral activity against HBV in vitro. Interferon-independent antiviral mechanisms as well as the orchestration of an effective adaptive immune response in the initial phase of infection appear to determine the outcome of HBV infection.

The impact of TLR2 on chronic HBV infection has been studied intensively in patients, describing a close relation between hepatic TLR2 and HBeAg expression. TLR2 expression on hepatocytes, Kupffer cells, and peripheral monocytes is significantly reduced in patients with HBeAg-positive chronic HBV infection, in comparison with HBeAg-negative patients and uninfected controls. Furthermore, TLR2 expression in HBeAg-negative carriers is significantly increased in comparison to uninfected controls. HBeAg seroconversion during antiviral (Peg-IFN) treatment is associated with elevated TLR2 expression on monocytes. Furthermore, TLR2-associated IL6 production at baseline of Peg-IFN therapy might already predict HBeAg seroconversion. A recent publication indicated that TLR2 activation occurs in cultured monocytes after exposure to HBV particles in vitro. Song et al. further showed that monocytes, derived from patients chronically infected with HBV, exhibit elevated expression of inflammatory cytokines. The role of HBeAg has not been addressed in that publication, but it might be suggested that increased TLR2 expression in HBeAg-negative patients results in increased inflammatory responses. The present work leads to suggest that the receptor binding-related immune responses, induced by purified HBV particles, occurred independently of the presence of HBeAg. The mechanism by which the cunning HBV evades the TLR2signaling cascade in vivo remains unclear.

Whether HBV infection induces hepatic immune responses in vivo could neither be shown nor ruled out. The experimental approach chosen for the in vivo study varies greatly from the experimental in vitro settings. Although higher amounts of input virus (minimum MOI of 62.5) were used for immune induction in cultured PHHs, an estimated MOI of 1 was used to infect human liver chimeric mice. Here, the infection kinetic is generally slow and depends on the spreading of the virus from a few initially infected hepatocytes. This is in stark contrast to the in vitro approach, in which inoculation and infection happens simultaneously in all of the hepatocytes. Given that TLR2 induction is a transient effect following initial infection in only a few hepatocytes at a particular time in vivo, inflammatory gene induction is likely to be missed by quantitative RT-PCR measurements. However, we cannot rule out that by applying higher MOI, a larger proportion of human cells could be simultaneously engaged by HBV infection. The modest induction of murine immune genes seen here is in accordance with our previous work, in which murine hepatocytes showed immune responses after HBV exposure. Furthermore, Kupffer cells and monocytes are likely to show TLR2 induction after HBV exposure. In the humanized mouse liver, where only hepatocytes are of human origin, we could not differentiate whether this induction was derived from murine hepatocytes or nonparenchymal liver cells. Thus, we propose that hepatic immune activation depends on both the intrinsic activation of the infected hepatocyte and the interplay of parenchymal and nonparenchymal liver cells. Further studies focusing on the single-cell level are needed to investigate early immune induction in HBV-inoculated USB mice.

In conclusion, the present data suggest that TLR2 is involved in the recognition of HBV during the early infection process and initiates innate immune signaling in PHHs, at least in vitro. However, the complexity and dynamics of the virus-host interplay occurring in vivo augment the difficulties in discerning the mechanisms involved in immune induction and evasion in HBV infection. Our observations highlight the need to dissect such mechanisms at the single-cell level, taking into account spatial and temporal events that may be determinant in understanding and successfully treating chronic HBV.

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for providing the liver samples. R.B. and M.L. were responsible for the original draft. J.F.S., G.G., and H.W. were responsible for the study review and editing. R.B., M.L., G.G., and H.W. were responsible for receiving the funding. R.B. and J.L. were responsible for the study supervision.

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Authors names in bold designate shared co-first authorship.

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