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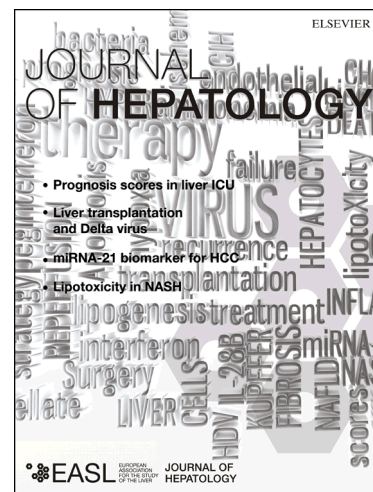
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Early Inhibition of Hepatocyte Innate Responses by Hepatitis B Virus

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43

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46 immunity.

47

48 **List of abbreviations:**49 Bac, baculovirus; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein; HBV, hepatitis B
50 virus; HCV, hepatitis C virus; KC, IFN, interferon; ISG, interferon stimulated genes; Kupffer cells;
51 LSEC, liver sinusoidal endothelial cells; MDA, melanoma differentiation-associated gene; MOI,
52 multiplicity of infection; NLR, NOD-like receptor; PEG, polyethylene glycol; PHH, primary human
53 hepatocytes; PRR, pathogen recognition receptor; RIG, retinoic-acid-inducible protein; RLR, RIG-
54 like receptor; SV, Sendai virus; TLR, toll-like receptor; VGE, virus genome equivalent.

55

56

Abstract

Background & Aims: The outcome of Hepatitis B virus infection may be influenced by early interactions between the virus and hepatocyte innate immune responses. To date, the study of such interactions during the very early step of infection has not been adequately investigated.

Methods: We used the HepaRG cell line, as well as primary human hepatocytes to analyze, within 24 hours of exposure to HBV, either delivered by a physiologic route or baculovirus vector (Bac-HBV), the early modulation of the expression of selected antiviral/pro-inflammatory cytokines and interferon-stimulated-genes. Experiments were also performed in presence or absence of innate receptor agonists to investigate early HBV-induced blockade of innate responses.

Results: We show that hepatocytes themselves could detect HBV, and express innate genes when exposed to either HBV virions or Bac-HBV. Whereas Bac-HBV triggered a strong antiviral cytokine secretion followed by the clearance of replicative intermediates, a physiologic HBV exposure led to an abortive response. The early inhibition of innate response by HBV was mainly evidenced on TLR3 and RIG-I/MDA5 signaling pathways upon engagement with exogenous agonist, leading to a decreased expression of several pro-inflammatory and antiviral cytokine genes. Finally, we demonstrate that this early inhibition of dsRNA-mediated response is due to factor(s) present in the HBV inoculum, but not being HBsAg or HBeAg themselves, and does not require *de novo* viral protein synthesis and replication.

Conclusions: Our data provide strong evidence that HBV viral particles themselves can readily inhibit host innate immune responses upon virion/cell interactions, and may explain, at least partially, the “stealthy” character of HBV.

Introduction

Viral hepatitis represents a major health problem worldwide, with hundreds of millions of chronic carriers who have a high risk of developing liver cirrhosis and hepatocellular carcinoma [1]. To establish and maintain persistent replication, HBV has evolved multiple strategies to evade the host innate and adaptive immune responses [2]. To restore immune control of the virus, virus-mediated inhibitory mechanisms could be defeated/unlocked in an interventional therapeutic perspective. A better knowledge of the underlying molecular mechanisms responsible for virus-induced blockade of immune responses is crucial before envisaging such strategies to improve the success of current antiviral treatments [3].

Most viruses are detected early after infection by both immune or/and infected cells via pathogen recognition receptors (PRR), including Toll like receptors (TLR) and RIG-I like receptors (RLR) and NOD-like receptors (NLR) [4]. It is still unclear if HBV is recognized by the innate immune system and/or if the virus can actively suppress or avoid early antiviral responses that drive the control of HBV infection [2, 5, 6]. The few data obtained in acutely infected humans, chimpanzees and woodchucks have shown that during the natural course of HBV infection, the activation of innate responses is predominantly weak or absent [7-10]. In particular, a seminal work performed in chimpanzees, has shown that HBV does not induce a strong modulation of gene expression in the liver of infected animal as compared to HCV [10]. Following this work HBV was qualified as a “stealth virus” [11] as opposed to HCV. A stealth virus can be a virus that does not induce measurable innate responses because of lack of detection by PRR, or a virus that is able to actively inhibit nascent innate responses. In this respect, some other data suggest that, during chronic infection, HBV could negatively regulate host immune responses by interfering with TLR expression and signaling pathways [12-14], or by inhibiting IFN response [15-19]. Underlying molecular mechanisms could involve several distinct HBV proteins as recently reviewed [2, 5, 6].

However, to date no very early kinetic interaction study between HBV and hepatocytes has been performed to determine if the virus could i) be initially detected by host cells, ii) modulate host immune gene expression, and then iii) inhibit innate responses. Only one study, performed in primary human hepatocyte (PHH) cultures showed that HBV could induce the transient production of IL-6, thus suggesting that the virus could be initially “sensed”, at least by liver macrophages (i.e. present in PHH cultures), and lead to the production of pro-inflammatory cytokines. However, HBV could, 24 hour (hr) after the onset of infection, inhibit the production of IL-6 by a yet unknown mechanism [20].

To better characterize the early interplay between HBV and hepatocyte innate immunity in terms of recognition and evasion, we used a non-transformed human hepatocyte cell line, differentiated HepaRG (dHepaRG), which is permissive for persistent HBV infection and is devoid of contamination by immune cells [21-23]. We showed that hepatocytes themselves could “sense” HBV and initiate an antiviral response, when delivered to cells by baculoviral transduction, as previously observed [24], but also during a more physiologic infection with HBV virions. While an efficient antiviral response was observed against HBV replication, when launched by baculovirus, this response was abortive in the context of a physiological HBV infection. In this case, the suppression of innate responses was exerted by viral component(s) already present within the inoculum not requiring *de novo* viral synthesis. This active suppression of pathogen-sensing pathways in the very early phase of infection, which prevented the establishment of a competent innate immunity, correlated with the development of a persistent infection *in vitro*.

Materials and methods

HBV and Sendai viral inocula

HBV inoculum was either concentrated from filtered HepG2.2.15 (wild type virus) or K6 (HBx negative virus) [25] supernatants by PEG precipitation as previously described [22], or partially purified by heparin chromatography [26], then concentrated using centrifugal filters devices (Amicon Ultracel 100K, Millipore). A mock “HBV-negative” inoculum (mock control) was generated by depletion of Dane particles, HBsAg and HBeAg using centrifugal filters devices (Amicon Ultracel 10K, Millipore). After DNA extraction (QIAmp Ultrasens Virus kit, Qiagen), HBV inoculum was titrated by qPCR with forward 5’-GCTGACGCAACCCCACT-3’ and reverse 5’-AGGAGTTCCGCAGTATGG-3’ probes using a standard curve from a quantified HBV encoded plasmid. All preparations were tested for the absence of endotoxin (Lonza Verviers, Belgium). Sendai virus (Cantell strain; titer: 4000 HAU/mL) was obtained from Charles River Laboratories (Bois des Oncins, France) and used according to recommendations.

HBV virion and viral protein purification

Dane particles were purified from the PEG precipitated HBV inoculum by sequential ultracentrifugation through a cushion of sucrose first, then on a sucrose density gradients at 35000 rpm for 16 hr at 4°C in a Beckman SW41Ti Rotor. Collected fractions were tested for sucrose density, HBV DNA (qPCR), HBcAg (western blot with anti-HBc (Dako)), HBeAg and HBsAg (ELISA). The overall purity of preparation was investigated by SDS-PAGE and SYPRO-Ruby Protein Gel Staining (Life Technologies). The concentrations of HBsAg and HBeAg were measured by commercial immunoassay kits, according to the manufacturer’s protocols (Autobio Diagnostics Co., China). One NCU, i.e. unit used in “HBeAg detection and relative quantification ELISA” from Autobio, is equivalent to 13.33 ng.

Human hepatocyte culture and HBV infection

The human liver progenitor HepaRG cells were cultured and infected as previously described [21, 22]. Primary human hepatocytes were prepared from surgical liver resections as previously described [27]. They were infected similarly to differentiated HepaRG.

Baculovirus vectors and cell transduction

Two baculoviruses were used in this study: a 1.1x genome-length HBV recombinant baculovirus vector (Bac-HBV) and a control baculovirus expressing GFP instead of HBV pgRNA. Baculoviral transduction of mammalian cells was performed as previously described [24].

Cell stimulation

Cells (10^6 per well) were stimulated with TLR agonists (Invivogen) and harvested after 6 hr (except for RIGI/MDA5 after 24 hr) for the analysis of IL-6 protein production by ELISA : TLR1/2 (pam3CSK4, 0.8 μ g/ml), TLR3 (poly(I:C), 10 μ g/ml), TLR4 (LPS 0.4 μ g/ml), TLR5 (flagellin, 0.1 μ g/ml), TLR6 (FSL-1, 0.1 μ g/ml), TLR7/8 (ssRNA, 10 μ g/ml), RIGI/MDA5 (transfection of poly(I:C) with the reagent Iyovec, 0.2 μ g/ml). For the cytokine gene expression analysis following poly(I:C) stimulation, cells were harvested after 3 hr of stimulation.

Nucleic acid purification, RTqPCR, and qPCR

Total RNA or DNA were respectively purified with the Nucleospin RNA II or MasterPure™ DNA Purification kits according to manufacturer's instructions (Macherey Nagel or Epicentre). cDNA was obtained after reverse transcription using the SuperScript® III Reverse Transcriptase (Life technologies) and real time quantitative qPCR (the sequence of primer pairs were listed in **Table 1**) was performed using the EXPRESS SYBR® GreenER™ qPCR Supermix Universal (Life technologies), and run on the MyiQ Biorad machine. Relative mRNA expression was analyzed with q-base software (Biogazelle, Belgium) using the comparative cycle threshold ($2^{-\Delta\Delta C_t}$) method with 2 housekeeping genes (RPLP0 and β -actin) previously tested for their stability in the HepaRG cells and the PHH, and normalized to the control conditions (=1). The relative HBV mRNA level was quantified using the same primer pairs used for the HBV PCR quantification.

Analysis of cytokine production

At selected time points, cell culture supernatants were harvested and tested for the secretion of IL-6, IFN- λ 1/3 (R&D system), IFN- α and IFN- β (PBL Interferon Source) according to the manufacturer's instructions.

UV inactivation

HBV and mock inocula were irradiated or not at room temperature on a UV Transilluminator (Appligene) delivering 3.3 mw/cm² for 30, 60, or 90 min. The efficacy of HBV replication with UV-inactivated inocula was analyzed after exposure to HepaRG cells by RT-qPCR and secretions of HBeAg and HBsAg were analyzed by ELISA (see **Fig. Sup 3A**).

Statistical analysis

Statistical analysis was performed using the Dixon and the nonparametric Mann-Whitney tests using the GraphPad Prism software. For all tests, a p value ≤ 0.05 (*), ≤ 0.005 (**), or ≤ 0.0005 (***) was considered as significant.

Results

IL-6 production during vector-mediated or physiologic HBV infection of differentiated HepaRG (dHepaRG) cells

To start examining the modulation of hepatocyte innate immunity during persistent or resolving HBV infection *in vitro*, dHepaRG cells were either infected with recombinant HBV or transduced with Bac-HBV, a recombinant baculovirus carrying 1.1 HBV genome unit, which is able to launch synchronized and strong intracellular HBV replication [24, 28]. The former infection model leads to a persistent infection which can last for months in dHepaRG cells [22], whereas the latter leads to a strong initial replication peaking at 24 hr post-transduction (p.t.) followed by a non-cytopathic, interferon-driven, elimination of HBV replicative intermediates (referred as “clearance of viral replication” hereafter) [24].

In transduced-dHepaRG cells, the rapid and transient synthesis of HBV RNA correlated with a strong secretion of IL-6 (≈ 1500 pg/ml), which peaked at 24 hr p.t., remained high until day-3 p.t., before returning to baseline at day-6 p.t. (**Fig. 1A**). The elimination of replication intermediates in those cells was very fast, and due to the antiviral effect of secreted IFNs[24] and likely other inflammatory cytokines, including IL-6 which has been recently shown to exhibit direct antiviral activity[20]. Differentiated HepaRG transduced with an identical amount (i.e. 100 pfu/cell) of control baculovirus (Bac-GFP) did not lead to significant release of IL-6, thus showing that IL-6 secretion was the consequence of the synthesis of HBV replicative intermediates. In sharp contrast, cells infected with either a low (**Fig. 1B**) or high (**Fig. 1C**) dose of HBV virions showed only a weak (< 200 pg/ml) and transient secretion of IL-6 (detection only at 24 hr post infection (p.i.); undetectable after), and was not associated with an inhibition of HBV replication, as intracellular HBV RNA started to increase at day-3 or day-6 according to initial multiplicity of infection. It is worth noting that both transduction with Bac-HBV (or its control Bac-GFP) and a high multiplicity of infection with HBV led to non-significantly different amounts of initially nuclear-delivered baculoviral and/or HBV DNA (**Fig. 1D**), and HBV RNA in cells (**Fig. 1A and 1C**). These results

supported the relevance of the comparison between baculovirus-mediated and physiologic HBV infection, in terms of correlation between initial response and outcome of infection. However in the case of viral transduction, the maximal accumulation of HBV RNA occurred shortly (i.e. before 24 hr p.t.) after the onset of infection and was associated with a strong production of IL-6, whereas in the case of natural infection with a high multiplicity of infection, HBV RNA started to accumulate only at day-3 after a lag phase. These results suggest that a “physiological” HBV infection does not induce a significant innate response in dHepaRG cells, as measured here by IL-6 secretion, and inevitably leads to persistence, whereas during an experimental, baculovirus-mediated infection, a strong and rapid production of HBV replication intermediates induces a strong innate response, leading to the elimination of replicative intermediates *in vitro*.

Detailed analysis of the modulation of innate gene expression during vector-mediated or physiologic HBV infection of dHepaRG cells

One main advantage of *in vitro* HBV infectious models, over biopsy-based approach in chimpanzees [10], is that the hepatocyte innate responses to HBV can be monitored by sensitive RT-qPCR to detect very early and subtle variations in the expression of host innate genes in a tight time course-dependent manner. To this end, we demonstrated that a productive HBV infection, measured by HBeAg and HBsAg quantification, was obtained several days post-infection in PHH and dHepaRG cells with an initial inoculation time as short as 2 hr (**Fig. Sup 1A-D**). We confirmed that infection is more efficient in PHH than in dHepaRG (**Fig. Sup 1A-B versus 1C-D**), and that the yield of infection is dependent upon the initial multiplicity of infection (m.o.i.; i.e. amount of virus-genome-equivalent (vge)/cell) (**Fig. Sup 1E-F**).

Rather than going for pan-genomic analysis, we have chosen to focus on the expression of some antiviral/pro-inflammatory cytokines (IFN- α , IFN- β , IL-29, IL-6, IL-1 β) and two prototypic interferon stimulated genes (ISGs; ISG56 and OAS1). Following Bac-HBV transduction, the expression of all antiviral/pro-inflammatory cytokine genes was significantly up-regulated, IFN- β

being the most induced transcript (**Fig. 2A**). Corroborating this increased gene expression at RNA level, a measurable secretion of IFN- α (20 pg/ml), IFN- β (30 pg/ml), and IL-6 (500 pg/ml) was observed at 24 hr p.t. in the supernatant of Bac-HBV-transduced cells (compared to the Bac-GFP control) (**Fig. 2B**). Again in sharp contrast, following a natural HBV infection, a weak but significant gene induction of type-I IFN, IL-29 and IL-6 expression was observed between 4 hr to 8 hr p.i., which rapidly returned to baseline within 24 hr p.i. No induction of ISG (ISG56 and OAS1) expression was detected in HBV-infected cells (**Fig. 2C**). As a result of this weak increase of gene expression, there was no detectable secretion of cytokines in the supernatant of HBV-infected cells (**Fig. 2D**). In PHHs, as for HepaRG cells, a weak and transient innate response was also observed upon HBV infection (**Fig. 2E**), despite a higher infectivity rate (**Fig. Sup 1C-D**), and was similarly associated with the establishment of a persistent infection (**Fig. Sup 1C-D**).

Therefore, a strong intracellular HBV replication, experimentally launched by Bac-HBV transduction, could be easily sensed by innate receptors and induced potent antiviral responses, which in turn led to the elimination of replicative intermediates. In contrast, during physiological HBV infection, a modest and transient response, with no detectable production of cytokines in cell supernatant, that can be qualified as abortive response, was observed and associated with the persistence of replication *in vitro*. Since such responses could be measured at least at the level of gene expression, it does indicate that HBV was “sensed” by cells, but the virus seemed to rapidly disarm this nascent response. The next step was to determine whether HBV is able to inhibit the hepatocyte innate responses experimentally engaged by “exogenous” ligands of PRRs.

HBV actively represses dsRNA-mediated innate responses in hepatocytes shortly after the onset of infection

To investigate whether HBV could rapidly repress hepatocyte innate responses, dHepaRG cells were infected with HBV at low or high multiplicity for 24 hr, then stimulated by various PRR agonists known to induce the production of IL-6 in these cells (*unpublished data* and [29]), to

measure the induction of the innate response. While stimulation of TLR5 or TLR6 receptors led to a stronger secretion of IL-6 in HBV-infected cells in comparison to controls (production of IL-6 in control mock-infected cells represented 100%), stimulations of TLR2, TLR4 or TLR7/8 led to similar IL-6 protein secretion. Interestingly, stimulation of dsRNA sensors (TLR3 and RIG-I/MDA5) with either poly(I:C) or transfected poly(I:C), led to significantly less IL-6 protein secretion by HBV infected cells (**Fig. 3A**). Moreover, analysis of the induction of selected host innate gene expression in poly(I:C)-stimulated cells also showed a strong inhibition reaching 60-70% at the highest multiplicity of infection for IL-6 gene expression, but also for type I and type III IFNs (IFN- β , IL-29) and OAS1 (i.e. used as a prototypic ISG) in HBV-infected hepatocytes (**Fig. 3B**). Although this HBV-mediated inhibition of TLR3, RIG-I/MDA5 pathways by poly(I:C) seemed to be dependent on the amount of virus used to initially inoculate dHepaRG cells, it seemed that a very low quantity of HBV virus (i.e. 1 vge/cell) was sufficient to significantly repress the expression of IFN- β , IL-29 and IL-6 in infected hepatocytes (**Fig. 3B**). Similar observations were also made in HBV-infected PHHs at both RNA (IFN- β , IL-29, IL6, OAS1) and, more importantly, at level of secreted proteins (IL-6, IFN- λ and IFN- β) following poly(I:C) stimulation (**Fig. 4A and 4B**). The latter suggests that an inhibition observed at RNA level translates into a stronger inhibitory phenotype at the level of secreted cytokines, thus strengthening the relevance of the results. To functionally confirm this inhibition, we used two types of assays. First, we could observe a significant inhibition of IFN- β gene 24 hr following Sendai virus (SV) super-infection in dHepaRG cells previously infected with HBV, compared to cells not infected with HBV but “super-infected” by SV in a similar manner (**Fig. 5A and B**). Second we extended Bac-HBV mediated intracellular replication in super-infection experiments (**Fig. Sup 2**); indeed we had previously shown that HBV intracellular replication launched by Bac-HBV delivery was abortive due to a strong IFN response [30]. Pre-infection of cells with HBV was capable to block IFN response and therefore extend Bac-HBV-mediated replication. Both assays suggested that HBV could suppress a strong induction of the IFN response, in a context of super-infection. Finally, the repressive effect

of HBV on the dsRNA-mediated innate response observed after only 24 hr of inoculation with HBV was maintained after 12 days of infection when HBV infection is well established, which suggests that HBV is capable to induce a long lasting inhibition of the dsRNA-mediated innate response in infected cells (**Fig. 3C**).

The very early inhibition of the dsRNA-mediated innate response in hepatocytes does not require de novo viral protein synthesis, but is initiated by viral proteins associated with HBV virions

So far the inhibition of the dsRNA-mediated innate response in hepatocytes was measured after 24 hr of inoculation with the virus. To determine whether this inhibition could be set even earlier, we shortened the inoculation time down to 2 hr. Cells were inoculated with HBV for either 2, 4, 8, or 24 hr, then stimulated for 3 additional hour with poly(I:C) after removal of the viral inoculum. In this setting, the HBV-mediated inhibition of IFN- β and IL-29 gene expression and to a lesser extent of OAS1 and IL-6 gene expression seemed to occur very rapidly after the virus addition; in fact, as early as 2 hr after virus inoculation. Interestingly, the inhibition of expression of IFN- β and IL-29 upon poly(I:C) stimulation reached its maximum after only 4 hr of viral exposure (**Fig. 6A**), which suggests that no viral proteins were required for this very early inhibition.

To demonstrate that no viral replication (i.e. no *de novo* production of replication intermediates and viral proteins) was needed, a UV-inactivated HBV inoculum, deficient in triggering a productive infection (**Fig. 6B**) was used. We stimulated non-UV and UV-inactivated HBV inoculated cells (at 24 hr post-exposure) with poly(I:C), to measure the impact on induction of dsRNA-mediated innate gene expression (**Fig. 6A**). In both non-UV and UV-HBV exposed cells showed a comparable strong inhibition of the induction of innate gene expression (IFN- β , IL-29, IL-6 and OAS1), demonstrating that an HBV factor (or an host-factor associated with HBV virions) present in the inoculum was sufficient to mediate a prompt and strong repression of the TLR3 and RIG-I/MDA5 signaling pathways. Similarly UV-inactivated HBV, as well as HBx-negative HBV could block Sendai-mediated IFN responses (**Fig. 5C**).

To identify which viral determinants could be responsible for the suppressive effect on the innate response, we purified each viral component of the HBV inoculum (i.e. infectious viral Dane particles, subviral particles (HBsAg), and HBeAg) (**Fig. Sup 4A**). Differentiated HepaRG cells were exposed with either a full inoculum free of non-enveloped capsids (called HBV) (**Fig. Sup 4B**), or to the various purified viral components including infectious Dane particles purified by a double cushion/gradient methodology to insure purity (**Fig. Sup 4C**), subviral particles SVPs (i.e. HBsAg), and HBeAg for 24 hr. The concentration of infectious particles, SVPs and HBeAg was harmonized to that of full inoculum as follow: HBsAg at 5 ng/10⁶ cells, HBeAg at 4.4 NCU/10⁶ cells, and HBV DNA at 100 vge/cell. After 24 hr of exposure to HBV or various viral components, the impact on IL-6, IFN- β , IL-29, and OAS1 gene expression following poly(I:C) stimulation was evaluated. While IL-6 expression was significantly inhibited by each viral component, a strong inhibition of type I/III IFNs and OAS1 gene expression was only observed with the HBV full inoculum and purified infectious Dane particles (**Fig. 6B**). To further exclude the implication of HBeAg and HBsAg in this phenotype, we also used recombinant antigens produced in *Pichia Pastoris* (HBeAg and HBsAg from commercial source) or produced in HepaRG cells (**Fig. Sup 5**) and performed similar experiments to find that neither HBeAg nor HBsAg, yet used at a 10x higher equivalent MOI as compared to full HBV inoculum or purified Dane particles, were capable to inhibit the induction of IFN- β expression after poly(I:C) stimulation (**Fig. 6C**). To further demonstrate that the very early inhibitory phenotype was associated with the entry of infectious particles themselves, we treated cells with Myrcludex[®], a peptide capable to block in a specific manner HBV entry into cells [31]. Indeed, we found that a concentration of as low as 100nM of Myrcludex[®] could prevent viral entry and replication (**Fig. Sup 3B**), and revert HBV-mediated inhibition of IFN- β gene induction (**Fig. 7**). This latter data further demonstrate that a UV-resistant viral component within HBV infectious virions, is responsible for the early repression of IFN responses.

Discussion

In this study, we used two relevant hepatocyte culture models, based on “immunocompetent” PHH and HepaRG cells, the latter being also devoid of non-parenchymal or liver resident immune cells, to decipher how HBV early interactions with hepatocytes may lead to the establishment of a persistent infection or, on the contrary, to the elimination of HBV replicative intermediates.

Collectively, our results showed that HBV could rapidly interfere with the hepatocyte antiviral responses mediated by the host viral recognition system, in particular by innate receptors detecting dsRNA PAMPs. When HBV strongly replicates following Bac-HBV transduction, a strong antiviral response that could not be counteracted by the virus and led to the elimination of replicative intermediates, was observed. However, in the context of a more physiologic HBV infection with a recombinant virus, HBV induced only a transient and modest increase of interferon and pro-inflammatory gene expression, which was associated *in fine* to a persistent infection. We provide evidence that HBV actively impair dsRNA-mediated recognition, including heterologous Sendai virus in the context of super-infection, during the very early phase of infection. Because inhibition of the hepatocyte innate response already occurred within the first 24 hr of infection and was maintained until at least 12 days after infection, which is considered as a “persistent” infection in cell culture [22], our results may in part explain why HBV was previously considered as a stealth virus[10]. Furthermore, we showed that HBV components and/or host factors associated with HBV present in the viral inoculum were necessary and sufficient to suppress the innate response driven by the dsRNA sensors. Following this very early inhibition, neo-synthesis of viral proteins in infected cells beginning a few hours post-infection, and thereafter, may be responsible for amplification of the inhibitory phenotype and its maintenance over time.

By studying the modulation of interferon and pro-inflammatory gene expression few hours after exposure to the virus, we have confirmed that HBV can be initially sensed by hepatocyte PRRs. The fact that HBV could be sensed *in vitro*, i.e. in primary hepatocyte cultures, was previously shown by Hösel and coworkers [20]. Our detailed kinetic studies enabled to detect an early, weak, transient,

yet reproducible, up-regulation of the expression of downstream genes, which were not reported previously due to the late monitoring after the onset of infection, when the virus has already established inhibitory strategies.

While it is not yet established which sensors are involved in the detection of HBV virions and intracellular intermediates of replication, our data clearly demonstrate the particular ability of the virus to efficiently and promptly inhibit, within 2 hours of viral exposure and in the absence of *de novo* viral protein synthesis, the TLR3 and RIG-I/MDA5-mediated innate response. While HBeAg and SVPs containing HBsAg alone seemed sufficient to down-regulate the IL-6 response at a comparable level to infectious Dane particles and the whole HBV inoculum, neither HBeAg nor HBsAg could repress the type I/III IFNs and ISGs response when cells were exposed to a complete HBV inoculum, which contained the same viral protein concentration. Only viral components of the infectious Dane particles (i.e. HBcAg, viral polymerase, HBV genome, or host-associated proteins) [32] seemed to exert a specific inhibition on IFN response. Further studies will be needed to determine which of these viral or host components composing the Dane particle could rapidly impact on the IFN response and by which mechanisms. Recently, Wu and coworkers [14] showed, in primary murine hepatocytes, a significant suppression of NFkB activity and to a lesser extent of IFN- β secretion in TLR3 stimulated primary murine hepatocytes, LSEC and Kupffer cells exposed to human HBV or secreted HBV antigens (e.g. HBeAg, HBsAg) that had been produced by an immortalized hepatocyte cell line derived from primary mouse hepatocyte cultures (HBV-met cells) [33]. However, in this setting, the consequences on HBV infection could not be studied, as murine hepatocytes are not susceptible to HBV. In addition, the authors observed the inhibition of the IL-6 response in the HBV-met cells following TLR4, TLR7 and TLR9 stimulation, which implies that human HBV could suppress the murine hepatocyte innate responses [14]. In our model of human hepatocyte infection by HBV, none of these sensors was modulated by HBV, at least early after the onset of the infection. The mechanism described here may be hepatocyte specific, while HBV may

adopt other suppressive mechanisms on immune cells as described elsewhere in human monocyte and dendritic cell populations [2, 34-39].

Our results shed light on the early interplay between HBV and hepatocytes and demonstrate a suppressive effect of HBV on hepatocyte innate responses (i.e. dsRNA-mediated) that occurs before the onset of viral replication (i.e. no viral protein neo-synthesis) and therefore may contribute to the establishment of a persistent infection. This inhibitory process was timely and quantitatively adjusted to HBV, which is, due to the nature of its genome and life cycle, a weak inducer of innate responses, but noteworthy strong enough to counteract strong inflammation induced by the Sendai virus or enable extension of HBV intracellular replication mediated by a Bac-HBV virus, which is otherwise abortive due to a strong IFN response [30]. Further studies are now required to decipher the inhibitory mechanisms mediated by the different components of HBV during the very early phase of infection in the liver microenvironment. The understanding of the host-virus interactions and the mechanisms that underlie the regulation of innate responses of parenchymal and non-parenchymal liver cells is an essential step for the development of future treatment intervention targeting specifically the HBV-induced repression of innate responses in the infected liver.

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Table**Table 1. Sequences of Human primer pairs used for RT-qPCR**

	Forward	Reverse
IFN-α	GTGAGGAAATACTTCCAAAGAATCAC	TTCATGATTTCTGCTCTGACAA
IFN-β	GCCGCATTGACCATGTATGAGA	GAGATCTTCAGTTTCGGAGGTAAC
IL-29	GTGGTGCTGGTGACTTTGG	CTCCTGTGGTGACAGAGATTG
IL-6	ACCCCTGACCCAACCACAAAT	AGCTGCGCAGAATGAGATGAGTT
IL1-β	AATCTGTACCTGTCTGCGTGTT	TGGGTAATTTTGGGATCTACACTCT
ISG56	AGCCAACATGTCCTCACAGAC	CTTCTACCACTCGTTTCATGC
OAS1	AGGTGGTAAAGGGTGGCTCC	ACAACCAGGTCAGCGTCAGAT
β-ACTIN	TGGCATTGCCGACAGGATGC	TCTGCTGGAGGTGGACAGCGA
RPLP0	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCCAAAGGAGAAG

Figure legends

Figure 1. Kinetics of HBV replication and IL-6 secretion in transduced or infected dHepaRG

cells. Differentiated HepaRG cells were either transduced with Bac-HBV (A), or infected with a low dose (100 vge/cell) (B), or high dose (1000 vge/cell) (C) of HBV. Cell supernatants were harvested at the indicated time points to measure IL-6 protein secretion (left axis) and compared to the control media (Bac-GFP or mock-HBV) (A, B, C). The relative HBV mRNA expression in these cells was quantified in parallel by RT-qPCR (right axis). Results are represented as the mean \pm sem of 2 independent experiment (A, B, C). Baculoviral and/or HBV DNA were also quantified in nuclear extract at 24h post-transduction or infection by qPCR using primers specific to baculovirus (i.e. IE-1 gene) backbone or HBV (D).

Figure 2. Kinetics of the relative mRNA expression of cytokine and ISG after either Bac-

HBV-transduction or HBV-infection in human hepatocytes. Bac-HBV-transduced cells (A and B) and HBV-infected dHepaRG cells (C and D) or HBV-infected primary human hepatocytes (PHH) (E) were harvested at the indicated time points (2, 4, 8, or 24 hr), and RNA extracted and subjected to RT-qPCR. The fold induction of the relative mRNA expression level of the cytokines (A, C and E) was normalized to housekeeping genes and compared to either Bac-GFP or mock controls. The supernatants from these cells were also harvested at the indicated time point to measure the secreted proteins and compared to the control media (Bac-GFP or mock) (B and D). Results are given as a mean \pm sem of at least 3 independent experiments (n= 3 to 7 when required to shown significance) and differences were considered as statistically significant to the control conditions (Bac-GFP or mock) when the p value was ≤ 0.05 (*) or ≤ 0.005 (**). The results for the relative mRNA expression for PHH are given as a mean \pm sem of 2 independent experiments (E).

Figure 3. Cytokine expression following the stimulation of PRR in HBV-infected dHepaRG

cells. The induction of the innate response was first evaluated through IL-6 secretion in the supernatant of cells infected with HBV (low =100 vge/cell or high =1000 vge/cell) for 24 hr, then stimulated with indicated PRR agonists for 6 hr (A). The induction of the relative innate gene expression level in poly(I:C) 3 hr-stimulated cells was analyzed by RT-qPCR in dHepaRG cells infected for 24h with increasing amount of virus (1, 10, 100, or 1000 vge/cell) (B), or after 12 days of infection (100-1000 vge/cell) (C). The mRNA expression level for each gene was normalized to housekeeping genes and relatively compared to the control mock-infected cells. The percentage of activation of each gene was then compared to the stimulated control mock-infected cells (=100%). Results are given as a mean \pm sem of at least 3 independent experiments (n=3 to 15) and differences

were considered as statistically significant to the control condition when the p value was ≤ 0.05 (*), ≤ 0.005 (**), or ≤ 0.0005 (***).

Figure 4. Cytokine expression and secretion in HBV-infected PHH following TLR3 stimulation. PHH were infected for 24 hr with HBV (low =100 vge/cell, high =1000 vge/cell) and stimulated with poly(I:C) for 3 hr. The induction of the innate response was first evaluated through the induction of the relative gene expression level of several innate genes (IL-6, IL-29, IFN- β , OAS1) (A), and protein secretion of IL-6, IFN- λ , and IFN- β by ELISA . (B) The mRNA expression level for each gene was normalized to housekeeping genes and relatively compared to the control mock-infected cells. The percentage of activation of each gene was then compared to the stimulated control mock-infected cells (=100%). Results are given as a mean \pm sem of at least 3 independent experiments (n=3 to 6) and differences were considered as statistically significant to the control condition when p value was ≤ 0.05 (*).

Figure 5. HBV can inhibit the innate response triggered by a super-infection with Sendai virus in HepaRG cells. (A) dHepaRG cells were infected at different multiplicity of infection (m.o.i.) (between 1 and 500 HAU/cell) of Sendai virus (SV), and expression of IFN- β gene analyzed by RTqPCR 24 hr post infection. (B) dHepaRG cells were either mock or infected for 24 hr with HBV at two different doses (100 or 1000 vge/cell), and then mock or super-infected with Sendai virus at a m.o.i. of 1 or 10 HAU/cell. The induction of the innate response was evaluated through the induction of the relative gene expression level of IFN- β gene. The condition mock-infection followed by super-infection with SV at a m.o.i. of 10 was set as the 100% of induction. (C) dHepaRG cells were either mock or infected for 24 hr with either wild type HBV, UV-inactivated HBV, or HBx-negative HBV at two different doses (100 or 1000 vge/cell), and then super-infected with Sendai virus (10 HAU/cell). The induction of the innate response was evaluated through the induction of the relative gene expression level of IFN- β gene. Results are given as a mean \pm sem of at least 2 independent experiments (n=2-3), and differences were considered as statistically significant to the control condition when p value was ≤ 0.05 (*) or ≤ 0.005 (**).

Figure 6. Inhibition of dsRNA-induced gene expression by HBV is a very early event and is caused by HBV proteins associated with virions. (A) dHepaRG cells were mock or infected at a m.o.i. of 100 vge/cell with wild type HBV for 2, 4 or 8 hr, or wild type or UV-inactivated HBV for 24 hr, and then stimulated by poly(I:C) for 3 hr. RT-qPCR were performed to analyze the induction of indicated genes. Results are given as a mean \pm sem of 3 independent experiments and differences were considered as statistically significant to the control condition when p value was ≤ 0.05 (*). (B)

dHepaRG cells were exposed for 24 hr to a purified infectious virions (Dane) (equivalent of 100 vge/cell), HBeAg (4.4 NCU/10⁶ cells; equivalent of 100 vge/cell) or SVPs (HBsAg) (5 ng/10⁶ cells; equivalent of 100 vge/cell). Cells were then stimulated by poly(I:C) for 3 hr, and RT-qPCR performed as in panel A. (C) dHepaRG cells were exposed for 24 hr to increasing amount of HBsAg (rHBs), or HBeAg produced in yeast (rHBe) or in HepaRG cells (cHBe), then stimulated by poly(I:C) for 3 hr, and RT-qPCR performed to monitor IFN- β expression. For all panels, the mRNA expression level for each gene was normalized to housekeeping genes and relatively compared to the control mock-infected cells. The percentage of activation of each gene was then compared to the stimulated control mock-infected cells (=100%). Results are given as a mean \pm sem of at least 3 independent experiments (n=3 to 6) and differences were considered as statistically significant to the control condition when p value was ≤ 0.05 (*).

Figure 7. Entry inhibitor treatment revert the inhibitory phenotype. dHepaRG were mock-or HBV-infected (100 vge/cell) in absence or presence of the indicated amount of Myrcludex (entry inhibitor) for 24 hr, then stimulated (or not) with poly(I:C) for 3 hr. The expression of IFN- β gene was analyzed by RTqPCR. Results are given as a mean \pm sem of 3 independent experiments and differences were considered as statistically significant to the control condition when p value was ≤ 0.05 (*).

Supplementary figure legends

Supplementary Figure 1. Kinetics of HBV replication in dHepaRG cells and PHH. Cells were infected with HBV (100 vge/cell) for 2, 4, 8 and 24 hr (A-D), or infected with increasing amount of virus (1-10-100-1000 vge/ml) for 24 hr (E- F). The secretion of HBsAg and HBeAg was measured by immunoassay 3, 6 and 9 days post-infection in dHepaRG cells (A-B, E-F) and PHH (C-D). Results are given as a mean \pm SD of one representative experiment.

Supplementary Figure 2. Kinetics of Bac-HBV-mediated intracellular replication in mock or HBV-pre-exposed dHepaRG. dHepaRG cells were either mock, HBV, or UV-inactivated HBV infected (100 vge/cell) for 24 hr, then super-transduced with Bac-HBV at a m.o.i. of 25 pfu/mL. A m.o.i. of 25 was used here instead of 100 as used in other part of the work, to prevent cell toxicity. Total DNA was then extracted at indicated days post-transduction, run into a 1% agarose gel in 1x Tris/Borate/EDTA buffer, transferred into positively charged nylon membrane, and subjected to Southern blot analysis using a P^{32} radioactive probe against HBV as previously described [14].

Supplementary Figure 3. Kinetics of HBV replication after infection with UV-inactivated HBV or after inhibition with entry inhibitor myrcludex. (A) HBV/ mock inoculum was irradiated or not for 30, 60 and 90 min and dHepaRG cells were incubated for 24 hr with either the non-UV HBV or the UV-inactivated HBV (UV-HBV) inoculum (A). The mRNA level of HBV was measured by RT-qPCR 12 days post-infection to follow the effect of the UV-inactivation on the virus replication and the secretion of HBeAg and HBsAg was monitored from day 3 to day 12 post-infection in the HBV or UV- HBV infected cells and results were given as a mean \pm SD of one representative experiment. (B) dHepaRG were mock-or HBV-infected (100 vge/cell) in absence or presence of the indicated amount of Myrcludex (entry inhibitor) for 24 hr. At the end of the inoculation time cells were extensively washed with cold PBS, and HBV replication monitored over-time by ELISA (HBe and HBsAg detection) and RTqPCR (HBV RNA detection).

Supplementary Figure 4. Purification of viral components and verification of the purity of viral inocula by sucrose gradient ultracentrifugation. (A) HBV infectious particles (Dane particles), subviral particles SVPs (HBsAg) and HBeAg were purified through a sucrose cushion, then a sucrose gradient as described in the experimental procedure section. The purity of each fraction was tested by qPCR (for the Dane particles) and by immunoassay for the HBsAg and HBeAg (left axis) and the density determined by refractometry (right axis). (B) PEG precipitated HBV inocula were either mock or NP40 treated, then loaded onto a sucrose gradient (20-60%), and subjected to ultracentrifugation at 100,000g for 16h at 4°C. HBV DNA was detected in each fraction by qPCR (left axis) and the density determined by refractometry (right axis). (C) Typical HBV inoculum used for experiments was passed through a 5.6-56% iodixanol gradient to separate viral components. Twelve fractions were collected then analysed by WB to detect the core protein, by dot blot to detect viral DNA, by ELISA to detect HBs and HBe antigens, and by SDS-PAGE/Sybroruby stain to check purity of Dane containing fractions.

Supplementary Figure 5. Expression of HBeAg in HepaRG-TR-HBe cell line. A recombinant HepaRG cell line was engineered using two lentiviruses to transfer genes encoding respectively tetracycline repressor (TR) and preCore gene (HBe). The cell line was named HepaRG-TR-HBe. Another cell line was created with the core gene, named HepaRG-TR-HBc, and used as control. Both cell line were use to monitor the expression of respective proteins upon tetracycline induction. Increasing doses of tetracycline were added to cells for a period of 48 hr. Then, both cell supernatant and intracellular protein extracts were assayed in ELISA capable to detect HBe and HBc. HBe protein can be secreted, whereas HBc could not.

Figure 1

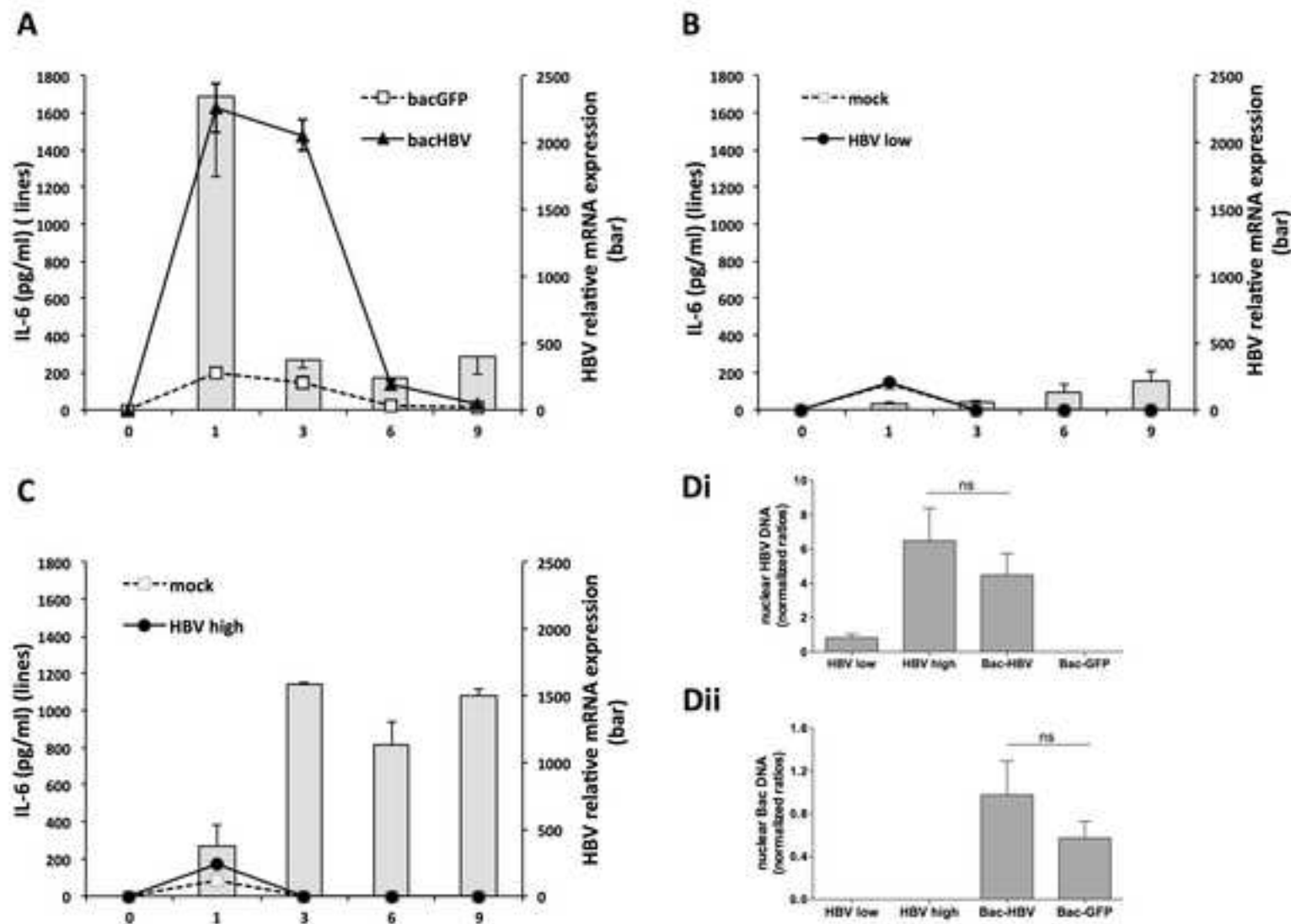


Figure 2

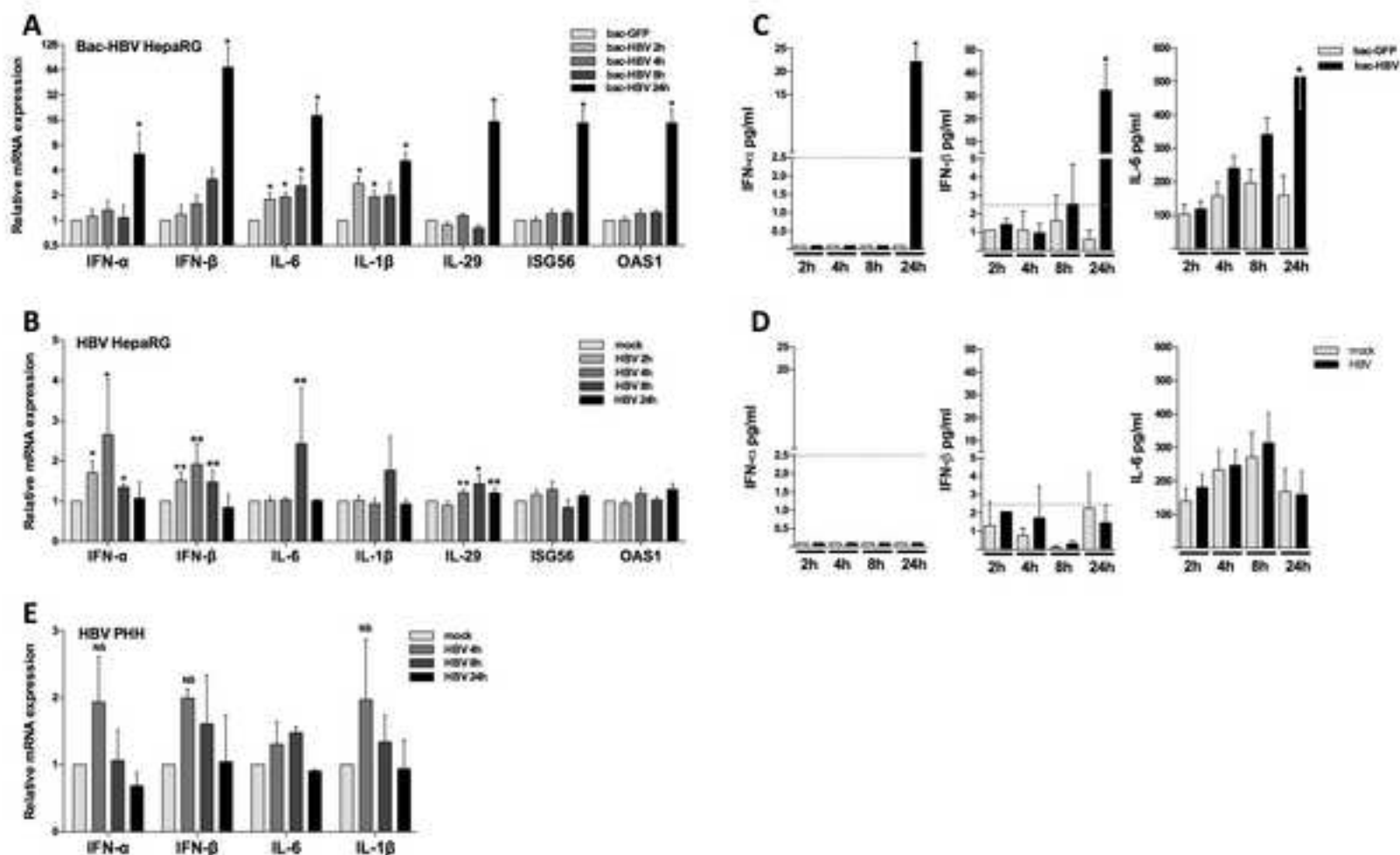


Figure 3

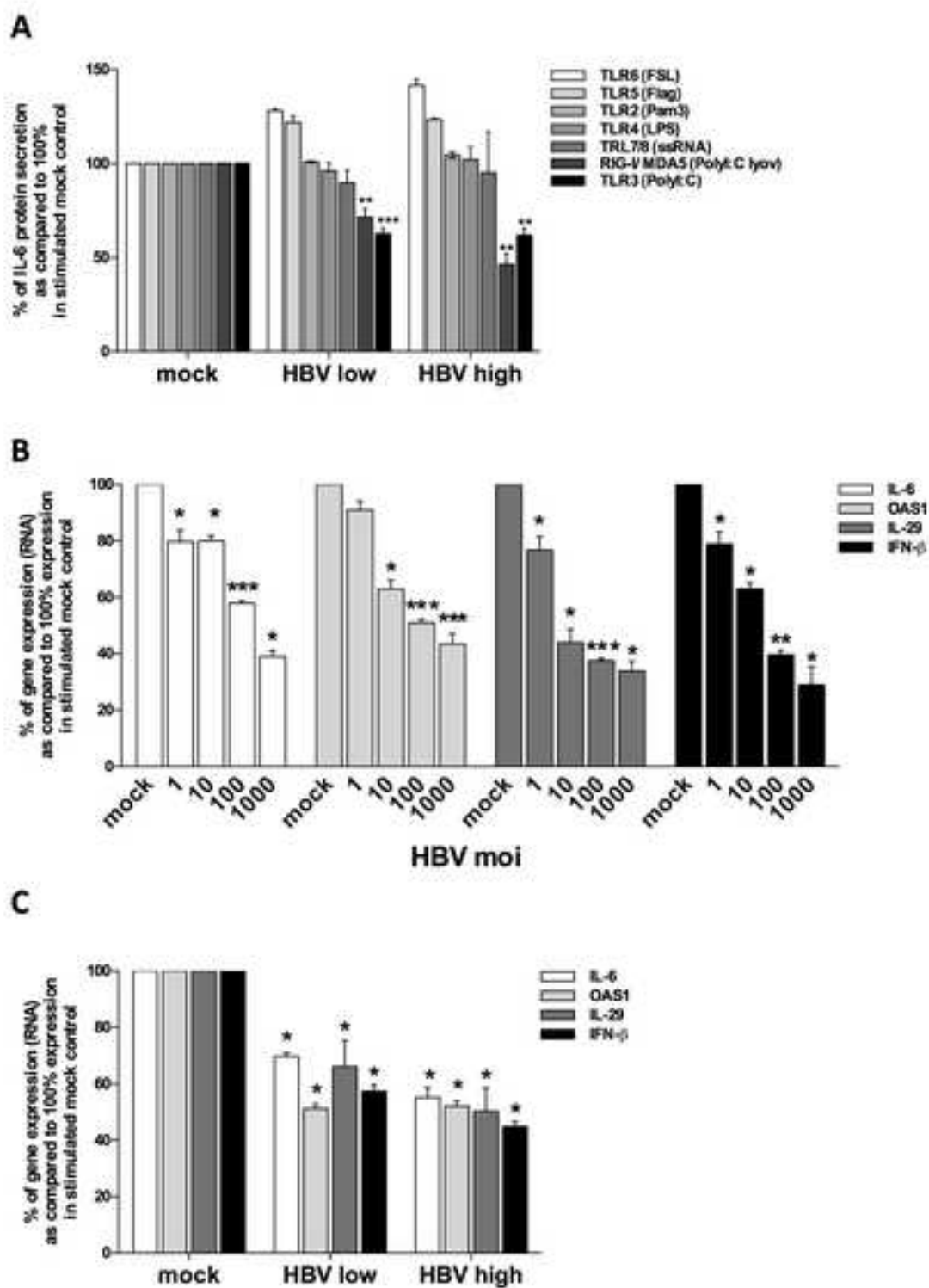


Figure 4

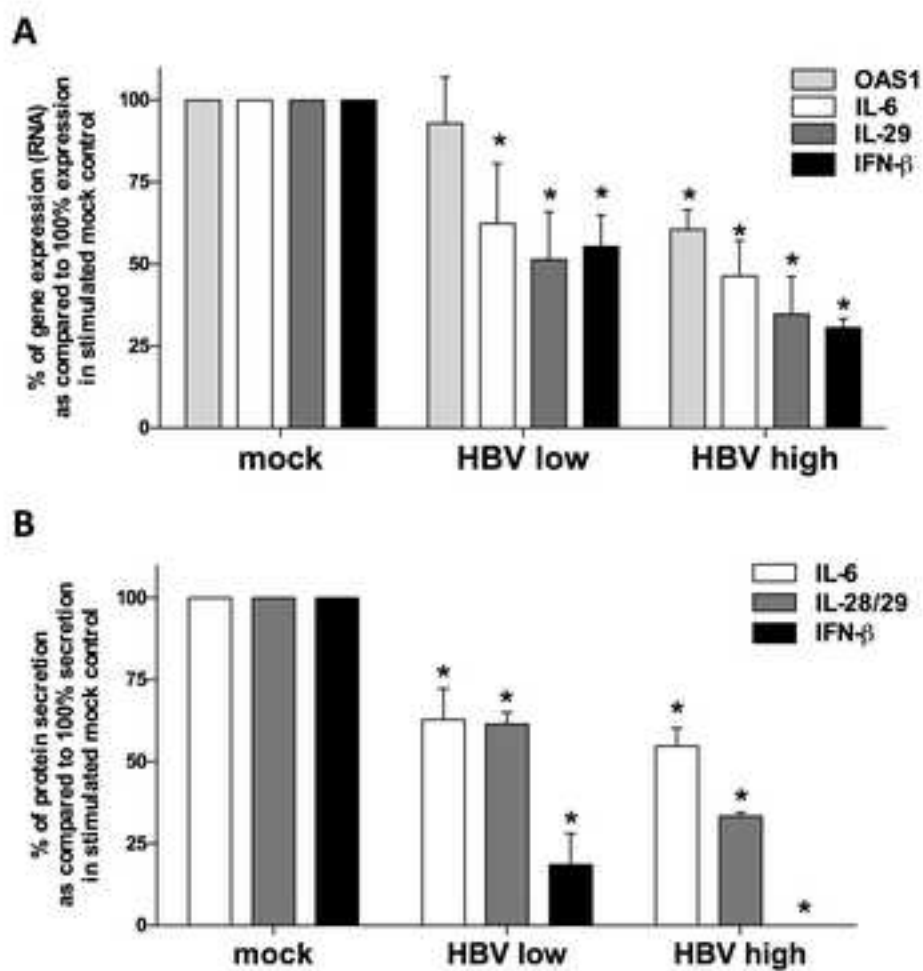


Figure 5

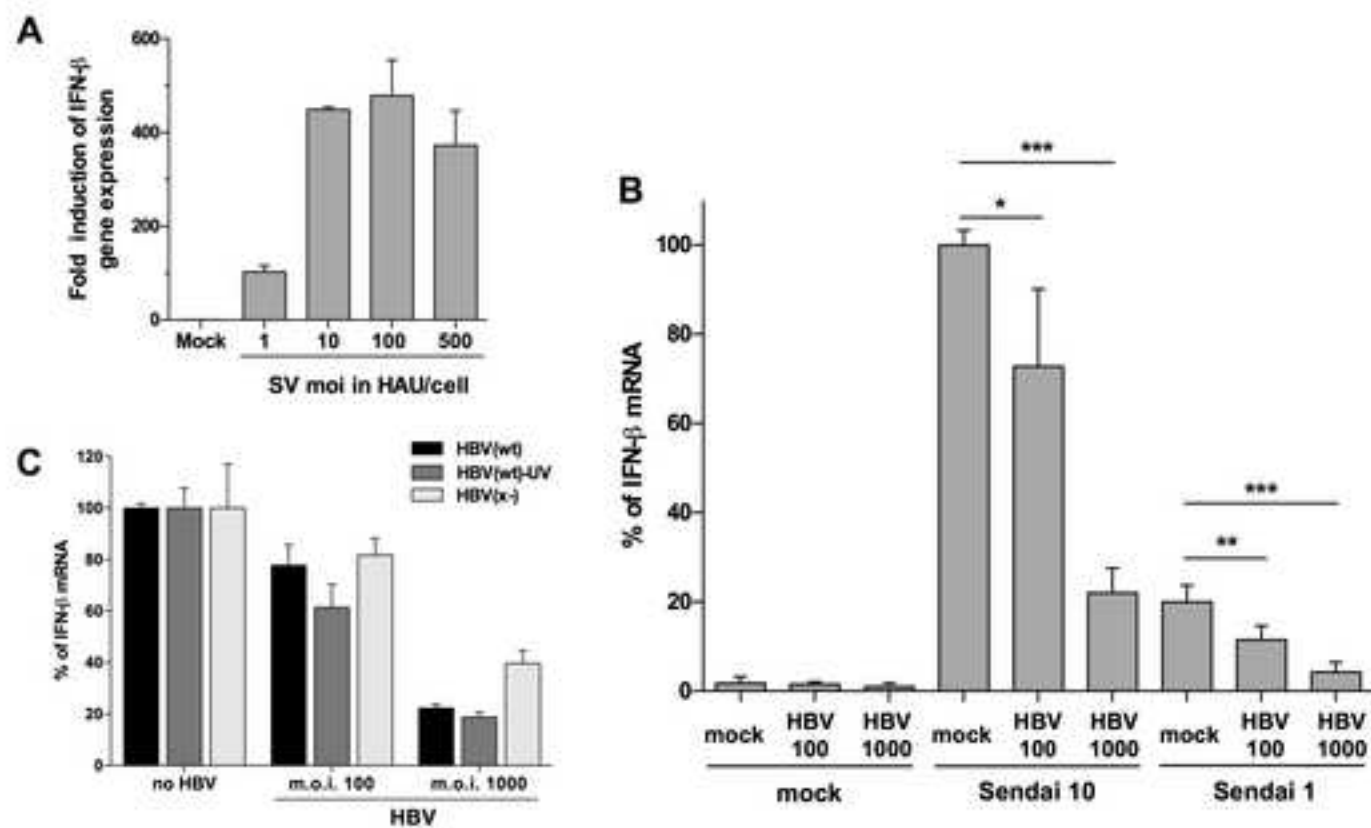


Figure 6

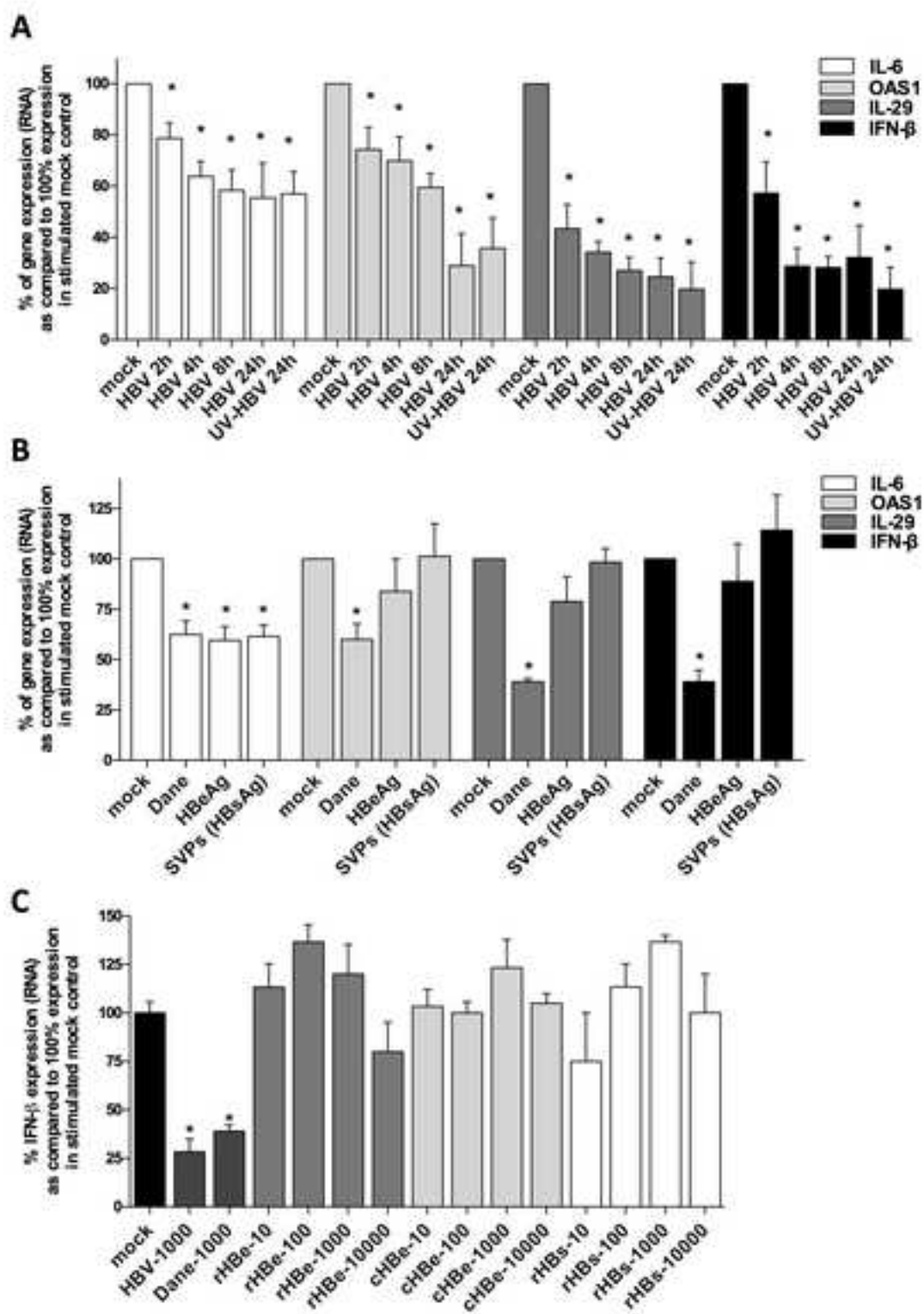


Figure 7

