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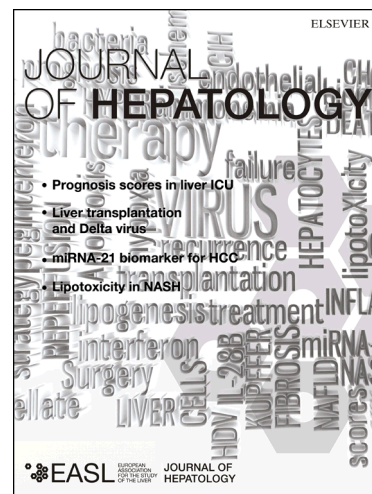
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1 Early Inhibition of Hepatocyte Innate Responses by Hepatitis B Virus

2
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44 **Key Words:**45 Hepatitis B virus; hepatocytes; early host/virus interaction; IFN response; dsRNA mediated innate
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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

57 **Abstract**

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

61 **Methods:** We used the HepaRG cell line, as well as primary human hepatocytes to analyze, within
62 24 hours of exposure to HBV, either delivered by a physiologic route or baculovirus vector (Bac-
63 HBV), the early modulation of the expression of selected antiviral/pro-inflammatory cytokines and
64 interferon-stimulated-genes. Experiments were also performed in presence or absence of innate
65 receptor agonists to investigate early HBV-induced blockade of innate responses.

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

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

78

79 **Introduction**

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

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

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

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

123

124 **Materials and methods**

125 *HBV and Sendai viral inocula*

126 HBV inoculum was either concentrated from filtered HepG2.2.15 (wild type virus) or K6 (HBx
127 negative virus) [25] supernatants by PEG precipitation as previously described [22], or partially
128 purified by heparin chromatography [26], then concentrated using centrifugal filters devices
129 (Amicon Ultracel 100K, Millipore). A mock “HBV-negative” inoculum (mock control) was
130 generated by depletion of Dane particles, HBsAg and HBeAg using centrifugal filters devices
131 (Amicon Ultracel 10K, Millipore). After DNA extraction (QIAmp Ultrasens Virus kit, Qiagen),
132 HBV inoculum was titrated by qPCR with forward 5’-GCTGACGCAACCCCACT-3’ and reverse
133 5’-AGGAGTTCCGCAGTATGG-3’ probes using a standard curve from a quantified HBV encoded
134 plasmid. All preparations were tested for the absence of endotoxin (Lonza Verviers, Belgium).
135 Sendai virus (Cantell strain; titer: 4000 HAU/mL) was obtained from Charles River Laboratories
136 (Bois des Oncins, France) and used according to recommendations.

137 *HBV virion and viral protein purification*

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

147 *Human hepatocyte culture and HBV infection*

148 The human liver progenitor HepaRG cells were cultured and infected as previously described
149 [21, 22]. Primary human hepatocytes were prepared from surgical liver resections as previously
150 described [27]. They were infected similarly to differentiated HepaRG.

151 *Baculovirus vectors and cell transduction*

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

155 *Cell stimulation*

156 Cells (10^6 per well) were stimulated with TLR agonists (Invivogen) and harvested after 6 hr
157 (except for RIGI/MDA5 after 24 hr) for the analysis of IL-6 protein production by ELISA : TLR1/2
158 (pam3CSK4, 0.8 μ g/ml), TLR3 (poly(I:C), 10 μ g/ml), TLR4 (LPS 0.4 μ g/ml), TLR5 (flagellin,
159 0.1 μ g/ml), TLR6 (FSL-1, 0.1 μ g/ml), TLR7/8 (ssRNA, 10 μ g/ml), RIGI/MDA5 (transfection of
160 poly(I:C) with the reagent lyovec, 0.2 μ g/ml). For the cytokine gene expression analysis following
161 poly(I:C) stimulation, cells were harvested after 3 hr of stimulation.

162 *Nucleic acid purification, RTqPCR, and qPCR*

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

173 *Analysis of cytokine production*

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

177 *UV inactivation*

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

182 *Statistical analysis*

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

186

187 **Results**188 *IL-6 production during vector-mediated or physiologic HBV infection of differentiated HepaRG*
189 *(dHepaRG) cells*

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

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

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

223

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

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

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

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

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

259
260 ***HBV actively represses dsRNA-mediated innate responses in hepatocytes shortly after the onset***
261 ***of infection***

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

265 measure the induction of the innate response. While stimulation of TLR5 or TLR6 receptors led to a
266 stronger secretion of IL-6 in HBV-infected cells in comparison to controls (production of IL-6 in
267 control mock-infected cells represented 100%), stimulations of TLR2, TLR4 or TLR7/8 led to
268 similar IL-6 protein secretion. Interestingly, stimulation of dsRNA sensors (TLR3 and RIG-
269 I/MDA5) with either poly(I:C) or transfected poly(I:C), led to significantly less IL-6 protein
270 secretion by HBV infected cells (**Fig. 3A**). Moreover, analysis of the induction of selected host
271 innate gene expression in poly(I:C)-stimulated cells also showed a strong inhibition reaching 60-
272 70% at the highest multiplicity of infection for IL-6 gene expression, but also for type I and type III
273 IFNs (IFN- β , IL-29) and OAS1 (i.e. used as a prototypic ISG) in HBV-infected hepatocytes (**Fig.**
274 **3B**). Although this HBV-mediated inhibition of TLR3, RIG-I/MDA5 pathways by poly(I:C)
275 seemed to be dependent on the amount of virus used to initially inoculate dHepaRG cells, it seemed
276 that a very low quantity of HBV virus (i.e. 1 vge/cell) was sufficient to significantly repress the
277 expression of IFN- β , IL-29 and IL-6 in infected hepatocytes (**Fig. 3B**). Similar observations were
278 also made in HBV-infected PHHs at both RNA (IFN- β , IL-29, IL6, OAS1) and, more importantly,
279 at level of secreted proteins (IL-6, IFN- λ and IFN- β) following poly(I:C) stimulation (**Fig. 4A and**
280 **4B**). The latter suggests that an inhibition observed at RNA level translates into a stronger
281 inhibitory phenotype at the level of secreted cytokines, thus strengthening the relevance of the
282 results. To functionally confirm this inhibition, we used two types of assays. First, we could observe
283 a significant inhibition of IFN- β gene 24 hr following Sendai virus (SV) super-infection in
284 dHepaRG cells previously infected with HBV, compared to cells not infected with HBV but “super-
285 infected” by SV in a similar manner (**Fig. 5A and B**). Second we extended Bac-HBV mediated
286 intracellular replication in super-infection experiments (**Fig. Sup 2**); indeed we had previously
287 shown that HBV intracellular replication launched by Bac-HBV delivery was abortive due to a
288 strong IFN response [30]. Pre-infection of cells with HBV was capable to block IFN response and
289 therefore extend Bac-HBV-mediated replication. Both assays suggested that HBV could suppress a
290 strong induction of the IFN response, in a context of super-infection. Finally, the repressive effect

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

295

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

298 So far the inhibition of the dsRNA-mediated innate response in hepatocytes was measured after
299 24 hr of inoculation with the virus. To determine whether this inhibition could be set even earlier,
300 we shortened the inoculation time down to 2 hr. Cells were inoculated with HBV for either 2, 4, 8,
301 or 24 hr, then stimulated for 3 additional hour with poly(I:C) after removal of the viral inoculum. In
302 this setting, the HBV-mediated inhibition of IFN- β and IL-29 gene expression and to a lesser extent
303 of OAS1 and IL-6 gene expression seemed to occur very rapidly after the virus addition; in fact, as
304 early as 2 hr after virus inoculation. Interestingly, the inhibition of expression of IFN- β and IL-29
305 upon poly(I:C) stimulation reached its maximum after only 4 hr of viral exposure (**Fig. 6A**), which
306 suggests that no viral proteins were required for this very early inhibition.

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

317 To identify which viral determinants could be responsible for the suppressive effect on the innate
318 response, we purified each viral component of the HBV inoculum (i.e. infectious viral Dane
319 particles, subviral particles (HBsAg), and HBeAg) (**Fig. Sup 4A**). Differentiated HepaRG cells
320 were exposed with either a full inoculum free of non-enveloped capsids (called HBV) (**Fig. Sup**
321 **4B**), or to the various purified viral components including infectious Dane particles purified by a
322 double cushion/gradient methodology to insure purity (**Fig. Sup 4C**), subviral particles SVPs (i.e.
323 HBsAg), and HBeAg for 24 hr. The concentration of infectious particles, SVPs and HBeAg was
324 harmonized to that of full inoculum as follow: HBsAg at 5 ng/10⁶ cells, HBeAg at 4.4 NCU/10⁶
325 cells, and HBV DNA at 100 vge/cell. After 24 hr of exposure to HBV or various viral components,
326 the impact on IL-6, IFN- β , IL-29, and OAS1 gene expression following poly(I:C) stimulation was
327 evaluated. While IL-6 expression was significantly inhibited by each viral component, a strong
328 inhibition of type I/III IFNs and OAS1 gene expression was only observed with the HBV full
329 inoculum and purified infectious Dane particles (**Fig. 6B**). To further exclude the implication of
330 HBeAg and HBsAg in this phenotype, we also used recombinant antigens produced in *Pichia*
331 *Pastoris* (HBeAg and HBsAg from commercial source) or produced in HepaRG cells (**Fig. Sup 5**)
332 and performed similar experiments to find that neither HBeAg nor HBsAg, yet used at a 10x higher
333 equivalent MOI as compared to full HBV inoculum or purified Dane particles, were capable to
334 inhibit the induction of IFN- β expression after poly(I:C) stimulation (**Fig. 6C**). To further
335 demonstrate that the very early inhibitory phenotype was associated with the entry of infectious
336 particles themselves, we treated cells with Myrcludex[®], a peptide capable to block in a specific
337 manner HBV entry into cells [31]. Indeed, we found that a concentration of as low as 100nM of
338 Myrcludex[®] could prevent viral entry and replication (**Fig. Sup 3B**), and revert HBV-mediated
339 inhibition of IFN- β gene induction (**Fig. 7**). This latter data further demonstrate that a UV-resistant
340 viral component within HBV infectious virions, is responsible for the early repression of IFN
341 responses.

342

343 **Discussion**

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

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

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

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

372 While it is not yet established which sensors are involved in the detection of HBV virions and
373 intracellular intermediates of replication, our data clearly demonstrate the particular ability of the
374 virus to efficiently and promptly inhibit, within 2 hours of viral exposure and in the absence of *de*
375 *novo* viral protein synthesis, the TLR3 and RIG-I/MDA5-mediated innate response. While HBeAg
376 and SVPs containing HBsAg alone seemed sufficient to down-regulate the IL-6 response at a
377 comparable level to infectious Dane particles and the whole HBV inoculum, neither HBeAg nor
378 HBsAg could repress the type I/III IFNs and ISGs response when cells were exposed to a complete
379 HBV inoculum, which contained the same viral protein concentration. Only viral components of the
380 infectious Dane particles (i.e. HBcAg, viral polymerase, HBV genome, or host-associated proteins)
381 [32] seemed to exert a specific inhibition on IFN response. Further studies will be needed to
382 determine which of these viral or host components composing the Dane particle could rapidly
383 impact on the IFN response and by which mechanisms. Recently, Wu and coworkers [14] showed,
384 in primary murine hepatocytes, a significant suppression of NFkB activity and to a lesser extent of
385 IFN- β secretion in TLR3 stimulated primary murine hepatocytes, LSEC and Kupffer cells exposed
386 to human HBV or secreted HBV antigens (e.g. HBeAg, HBsAg) that had been produced by an
387 immortalized hepatocyte cell line derived from primary mouse hepatocyte cultures (HBV-met cells)
388 [33]. However, in this setting, the consequences on HBV infection could not be studied, as murine
389 hepatocytes are not susceptible to HBV. In addition, the authors observed the inhibition of the IL-6
390 response in the HBV-met cells following TLR4, TLR7 and TLR9 stimulation, which implies that
391 human HBV could suppress the murine hepatocyte innate responses [14]. In our model of human
392 hepatocyte infection by HBV, none of these sensors was modulated by HBV, at least early after the
393 onset of the infection. The mechanism described here may be hepatocyte specific, while HBV may

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

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

409

410

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422

ACCEPTED MANUSCRIPT

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514 alpha production in plasmacytoid dendritic cells. *Mol Immunol* 2009;46:2640-2646.

515

516

517

518 **Table**519 **Table 1. Sequences of Human primer pairs used for RT-qPCR**

520

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

521

522

523

524 Figure legends

525

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

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

535

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

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

548

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

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

559 were considered as statistically significant to the control condition when the p value was ≤ 0.05 (*),
560 ≤ 0.005 (**), or ≤ 0.0005 (***)).

561

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

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

586 **Figure 6. Inhibition of dsRNA-induced gene expression by HBV is a very early event and is**
587 **caused by HBV proteins associated with virions.** (A) dHepaRG cells were mock or infected at a
588 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
589 24 hr, and then stimulated by poly(I:C) for 3 hr. RT-qPCR were performed to analyze the induction
590 of indicated genes. Results are given as a mean \pm sem of 3 independent experiments and differences
591 were considered as statistically significant to the control condition when p value was ≤ 0.05 (*). (B)

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

603

604 **Figure 7. Entry inhibitor treatment revert the inhibitory phenotype.** dHepaRG were mock-or
605 HBV-infected (100 vge/cell) in absence or presence of the indicated amount of Myrcludex (entry
606 inhibitor) for 24 hr, then stimulated (or not) with poly(I:C) for 3 hr. The expression of IFN- β gene
607 was analyzed by RTqPCR. Results are given as a mean \pm sem of 3 independent experiments and
608 differences were considered as statistically significant to the control condition when p value was
609 ≤ 0.05 (*).

610

611

612 Supplementary figure legends

613

614

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

620

621 **Supplementary Figure 2. Kinetics of Bac-HBV-mediated intracellular replication in mock or**
622 **HBV-pre-exposed dHepaRG.** dHepaRG cells were either mock, HBV, or UV-inactivated HBV
623 infected (100 vge/cell) for 24 hr, then super-transduced with Bac-HBV at a m.o.i. of 25 pfu/mL. A
624 m.o.i. of 25 was used here instead of 100 as used in other part of the work, to prevent cell toxicity.
625 Total DNA was then extracted at indicated days post-transduction, run into a 1% agarose gel in 1x
626 Tris/Borate/EDTA buffer, transferred into positively charged nylon membrane, and subjected to
627 Southern blot analysis using a P³² radioactive probe against HBV as previously described [14].

628

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

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

653

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

662

Figure 1

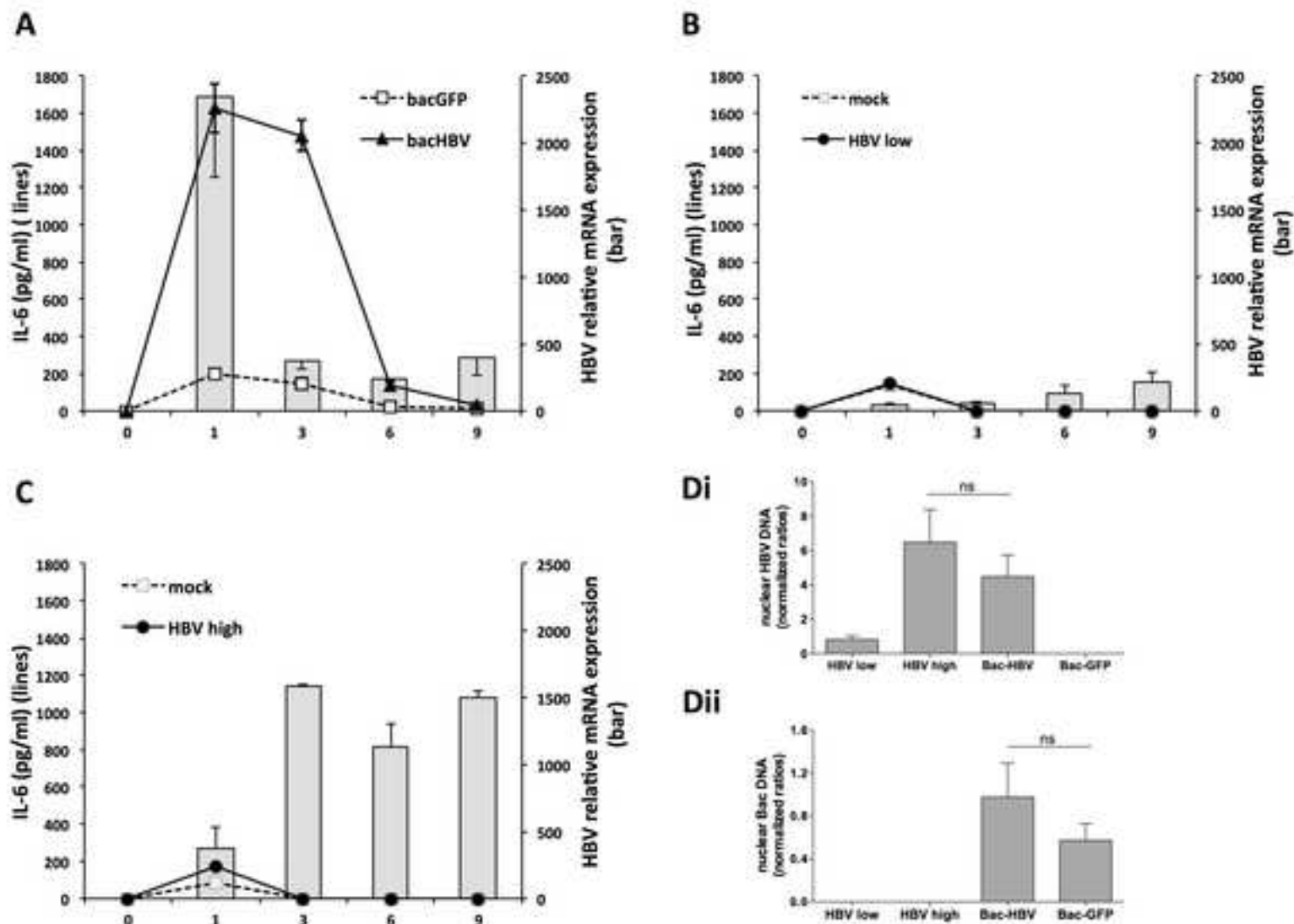


Figure 2

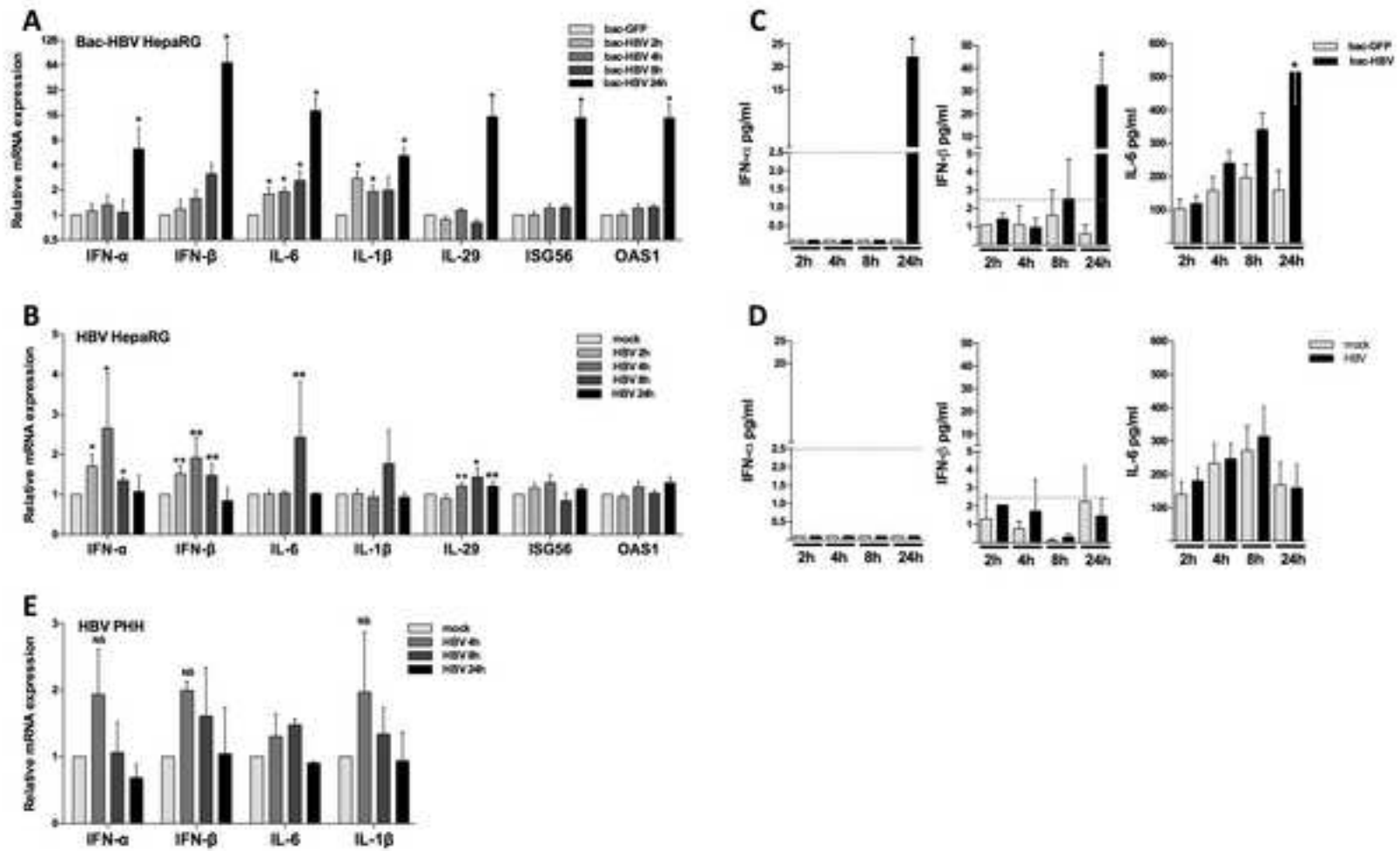


Figure 3

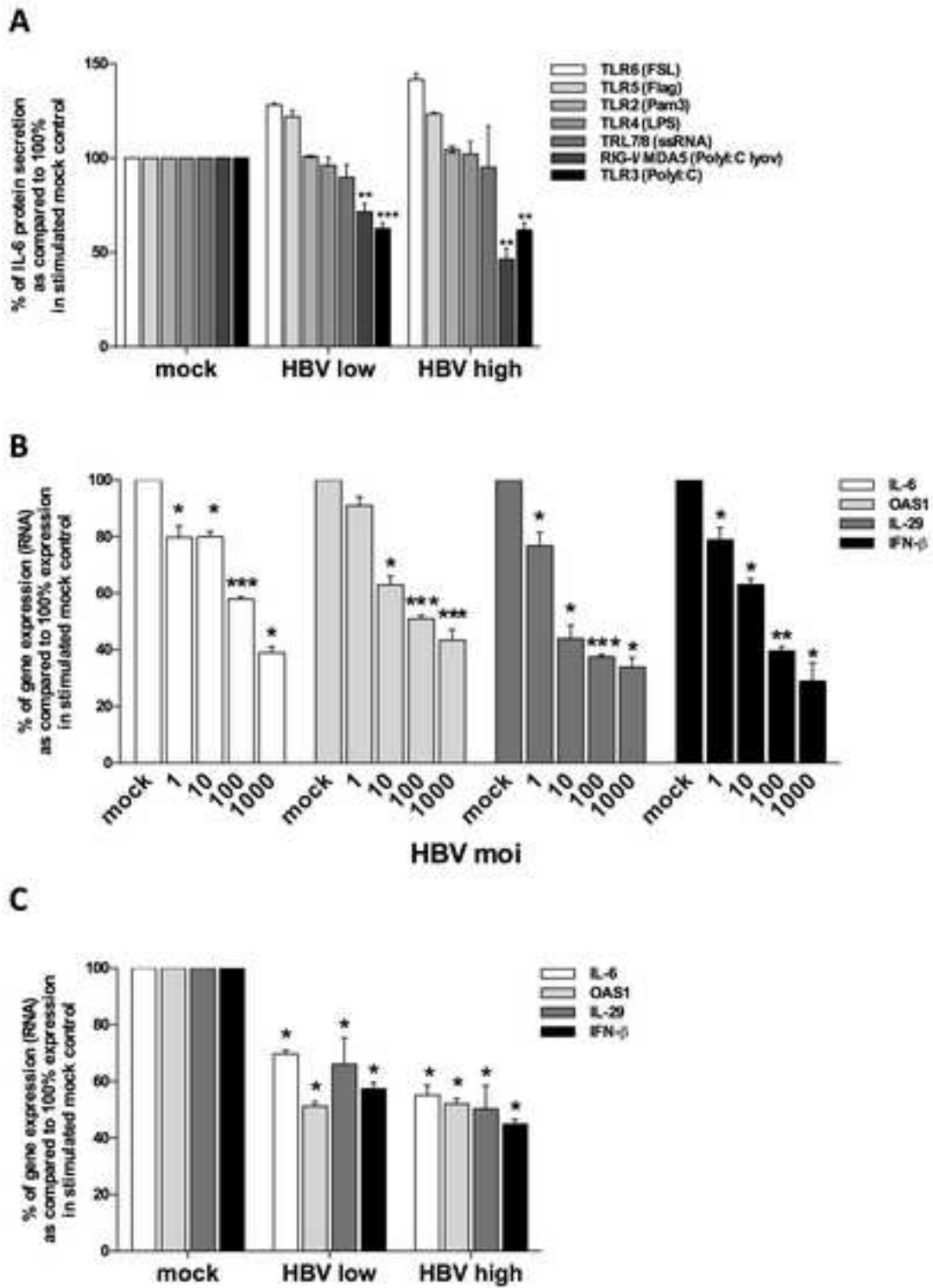


Figure 4

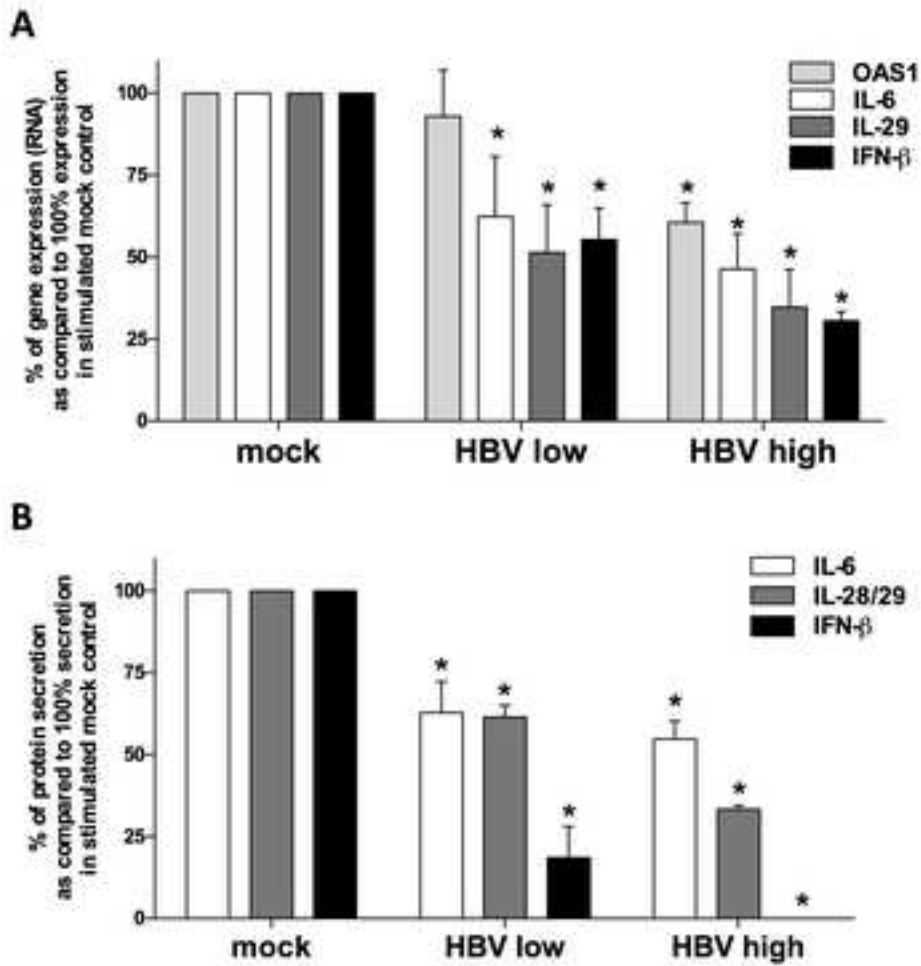


Figure 5

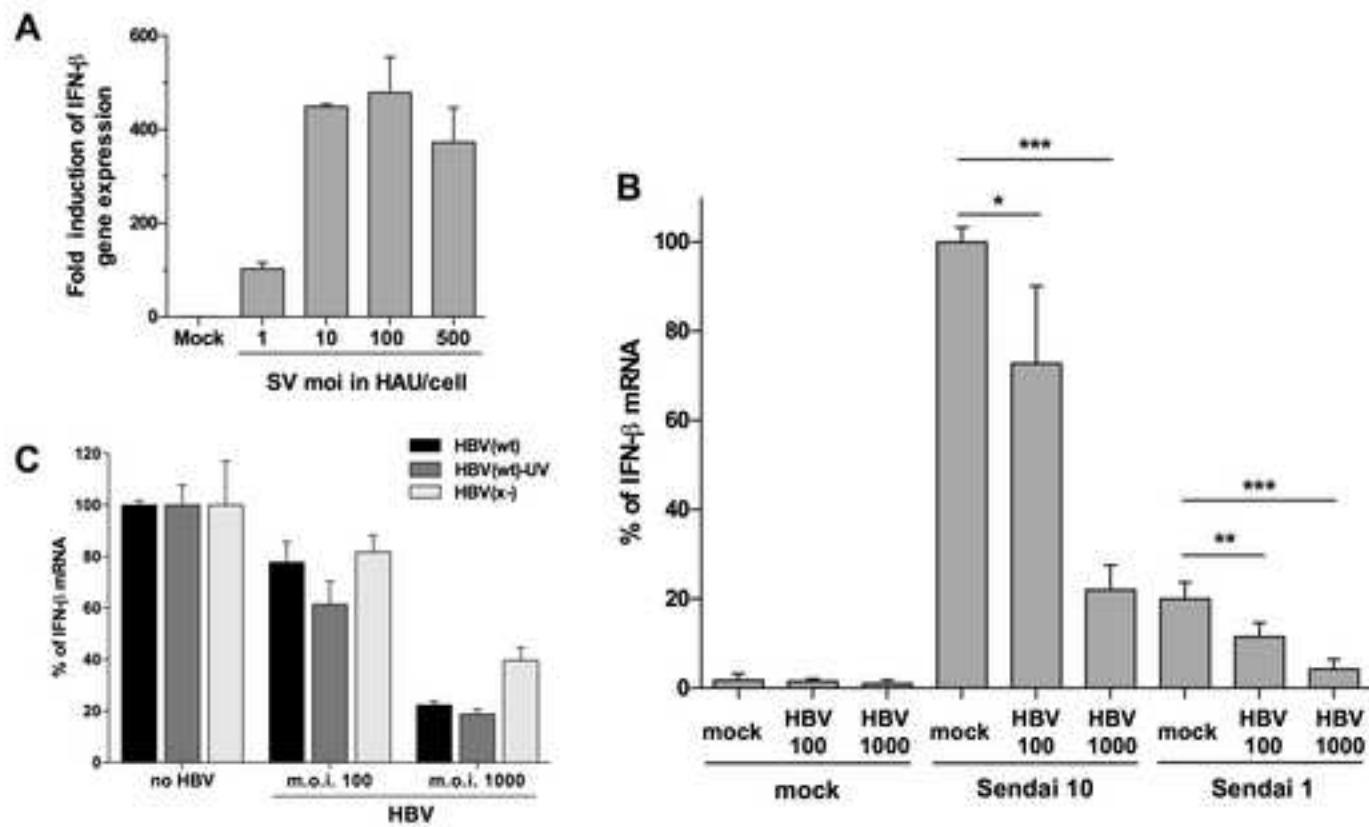


Figure 6

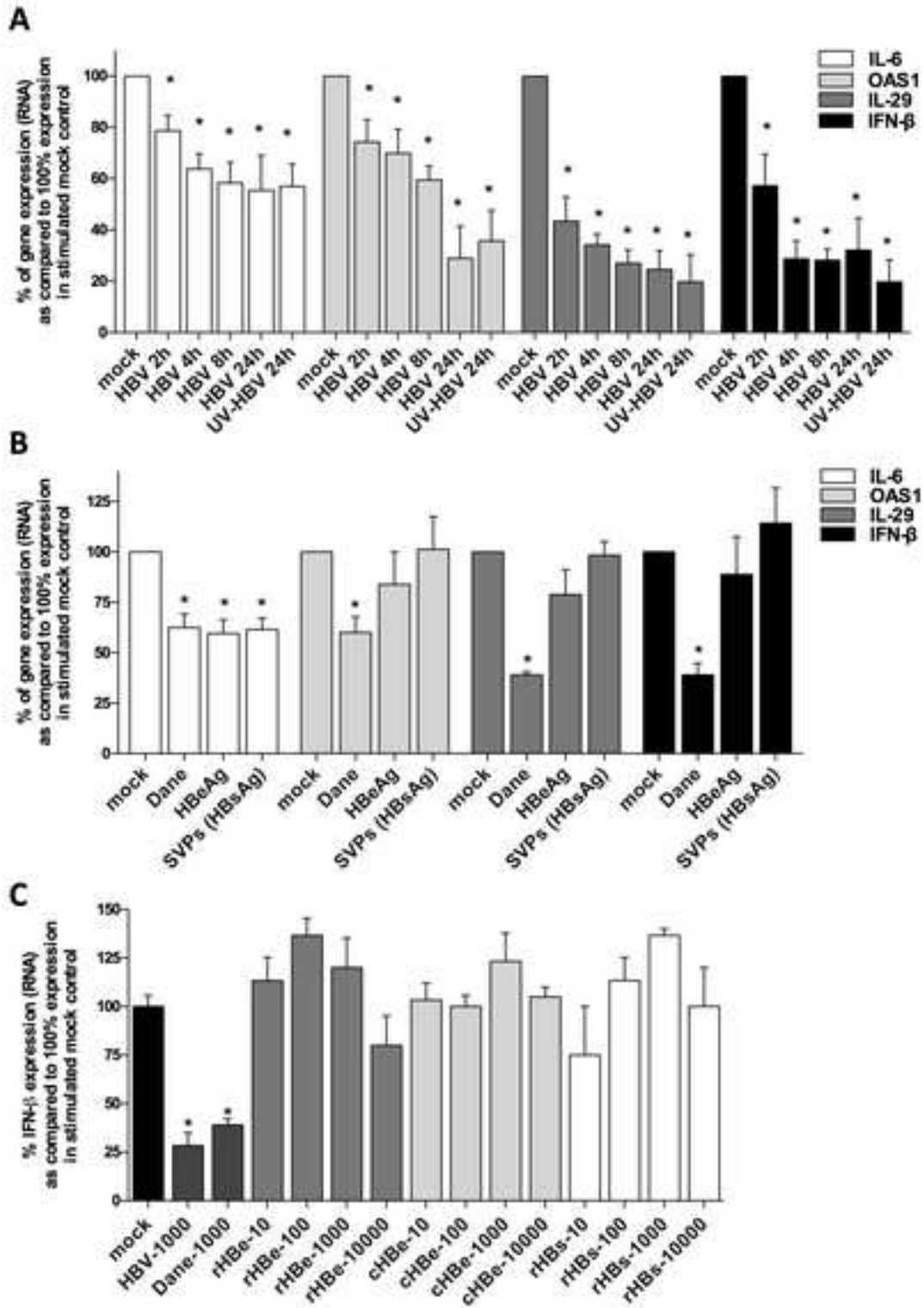


Figure 7

