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1           **Hepatitis B Virus-induced modulation of liver macrophage function promotes**  
2           **hepatocyte infection**

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55 - study concept and design: SFD, MD, JL, DD

56 - acquisition of data: SFD, MD, LA, LD, JF, DH, MH

57 - analysis and interpretation of data: SFD, MD, AL, KK, JL, DD

58 - drafting of the manuscript: SDF, JL, DD

59 - critical review of the manuscript: AL, KK, NBV, FZ, MH

60 - statistical analyses: SFD

61 - material support: MR, NBV, FL, RP, MSM, FZ,

62

63 **ABSTRACT**

64 **Background and aims.** Liver macrophages can be both involved in pathogen clearance  
65 and/or pathogenesis. To get further insight on their role during chronic hepatitis B virus (HBV)  
66 infections, our aim was to phenotypically and functionally characterize *in vivo* and *ex vivo* the  
67 interplay between HBV, primary human liver macrophages (PLM) and primary blood  
68 monocytes differentiated into pro-inflammatory or anti-inflammatory macrophages (M1-MDM  
69 or M2-MDM, respectively).

70 **Results.** We evidenced the presence of HBc protein within macrophages in liver biopsies from  
71 HBV-infected patients and higher levels of anti-inflammatory macrophages markers, compared  
72 to non-infected ones. *Ex vivo* exposure of naive PLM to HBV led to a reduced secretion of pro-  
73 inflammatory cytokines. Upon exposure to HBV or HBV-producing cells during differentiation  
74 and activation, M1-MDM secreted less IL-6 and IL-1 $\beta$ , whereas M2-MDM secreted more IL-10  
75 when exposed to HBV during activation. Finally, cytokines produced by M1-MDM, but not those  
76 produced by HBV-exposed M1-MDM, decreased HBV infection of hepatocytes.

77 **Conclusions.** Altogether, our data strongly suggest that HBV modulates liver macrophage  
78 functions to favour its establishment.

79

80 **Lay summary:** HBV modulates liver macrophages function in order to favour its establishment  
81 and likely its maintenance. It impairs the production of the antiviral cytokine IL-1 $\beta$  while  
82 promoting that of IL-10 in the microenvironment. This phenotype can be recapitulated in naive  
83 liver macrophages or monocytes-derived-macrophages *ex vivo* by short exposure to the virus  
84 or cells replicating the virus, thus suggesting an “easy to implement” mechanism of inhibition.

## 85 Introduction

86 Hepatitis B virus (HBV) chronically infects around 250 million people worldwide (WHO data,  
87 2016) and increases the risk of developing cirrhosis and hepatocellular carcinoma <sup>1</sup>. Current  
88 treatments, mainly based on nucleos(t)ide analogues, reduce blood viremia to undetectable  
89 levels in the majority of patients, but do not achieve virus elimination from the liver <sup>2</sup>. New  
90 treatments, including immune-therapeutic components, are therefore needed in order to  
91 progress toward a functional cure for HBV.

92 HBV is a small DNA virus that persists as a covalently-closed-circular DNA (cccDNA) within  
93 the nucleus of liver parenchymal cells (hepatocytes). Viral RNAs, including mRNAs and the  
94 pre-genomic RNA (pgRNA) are transcribed from the cccDNA. The pgRNA is encapsulated  
95 within the nucleocapsid and converted into relaxed-circular DNA (rcDNA) by an HBV  
96 polymerase-mediated reverse-transcription step. Different viral products circulate in the blood  
97 of infected patients including HBe antigens (HBeAg), Dane particles (infectious particles), HBV  
98 RNA containing particles and empty (i.e., nucleocapsid free) enveloped subviral particles  
99 (SVPs). The latter three have envelope proteins at their surface and are indistinctly detected  
100 as HBs antigens (HBsAg) <sup>2</sup>. SVPs, which are produced in large excess compared to virions,  
101 are thought to play an important role in terms of immune subversion <sup>3</sup>.

102 Several pro-inflammatory cytokines (IL-6, IL-1 $\beta$  and TNF $\alpha$ ) and interferons (IFN $\alpha$  and IFN $\gamma$ )  
103 were shown to induce a direct antiviral effect on HBV replication in hepatocytes <sup>4-7</sup>, with IL-1 $\beta$   
104 being one of the most efficient at inhibiting already established HBV infections *in vitro* <sup>4</sup>. IL-1 $\beta$   
105 is a pro-inflammatory cytokine produced upon inflammasome activation <sup>8</sup>. In the liver, it is  
106 mostly produced by macrophages (M $\Phi$ ) since hepatocytes do not possess functional  
107 inflammasomes <sup>9,10</sup>. Liver resident M $\Phi$ , named Kupffer cells (KC), represent 80% of the  
108 macrophage count in the body at steady state <sup>11</sup>. They are specialized in the detection of  
109 pathogens coming from the enteric circulations, as well as in the elimination of aging blood  
110 cells, through their high phagocytic capacity <sup>11</sup>. Upon inflammation, monocytes from the blood

111 circulation can be recruited in the liver and differentiate locally into MΦ that are called  
112 monocyte-derived-macrophages (MDM) <sup>12</sup>. KC and MDM have different embryonic origins and  
113 functions <sup>13</sup>. *In vivo*, a wide range of different phenotypes of MΦ exists depending on their  
114 origin, activation status, localization, and their micro-environment <sup>14</sup>. Inflammatory MΦ,  
115 commonly called M1, which are phenotypically described as CD40<sup>+</sup> CD86<sup>+</sup> HLA-DR<sup>+</sup>, are  
116 characterized by their capacity to secrete pro-inflammatory cytokines/chemokines, such as IL-  
117 1β, produce various antimicrobial factors, including nitric oxide (NO), and are therefore  
118 implicated in inflammation and elimination of pathogens <sup>14</sup>. Anti-inflammatory MΦ, commonly  
119 called M2, express the arginase 1, mannose receptors, and the high affinity scavenger receptor  
120 CD163, and secrete anti-inflammatory cytokines, such as IL-10 and TGFβ, as well as  
121 angiogenic factors, such as VEGF <sup>14</sup>. M2 MΦ are involved in the resolution of inflammation  
122 and in tolerance mechanisms <sup>14</sup>. They are also abundantly found in tumour microenvironment  
123 and are called tumour-associated MΦ (TAM) <sup>15</sup>.

124 Several studies showed that HBV can influence the phenotype and biology of MΦ <sup>16</sup>, but few  
125 have been performed using liver resident or infiltrating primary human cells. To get further  
126 insights into the role of liver MΦ in the establishment of HBV infection in hepatocytes, we used  
127 highly relevant model such as primary human liver MΦ, pro-inflammatory/M1-like or anti-  
128 inflammatory/M2-like MΦ differentiated *in vitro* from primary blood monocytes, liver biopsies,  
129 primary human hepatocytes (PHH), differentiated HepaRG cells (dHepaRG), and co-culture  
130 models.

131 **MATERIALS AND METHODS**

132 **Patient samples.** Two different cohorts were analysed. The collection and analysis of human  
133 patient samples of the first cohort (Swiss/German cohort) was approved by the ethics  
134 committee of Northern Switzerland (EKNZ, Study ID: PB\_2018-00308, 310/12), confirmed to  
135 the ethical guidelines of the 1975 Declaration of Helsinki, and was provided by the Department  
136 of Pathology, Basel, Switzerland. The biopsies were stained in Germany. Patients'  
137 characteristics are presented in **Table S1**. Samples from the second cohort were used under  
138 the French IRB 'CPP Sud-Est IV' approval #11/040 (2011) from the 'Biobanque INSERM CRCL  
139 Hépatologie (U1052)', France #DC2008-235 and stained in France. Written informed consent  
140 was obtained from each patient. Patients' characteristics are presented in **Table S2**.

141 **Immunohistochemistry.** Paraffin was removed from FFPE samples by successive alcohol  
142 bathes and epitopes were unmasked as previously described <sup>17</sup>. Endogen peroxidases were  
143 blocked by a 15 min exposition at room temperature with Dual Endogenous enzyme Block  
144 (DAKO S2003). Saturation was performed by two successive incubations at RT of 20 min with  
145 horse serum (VECTOR S-2012 Za0328) and 15 min with DAKO antibody diluent (DAKO  
146 S3022). Antibodies (**Table S3**) were incubated overnight at 4°C in DAKO antibody diluent.  
147 Samples were incubated for 15 min at RT with secondary antibodies (**Table S3**). Coloration  
148 was performed with DAB substrate (DAKO K3468) or permanent red substrate (DAKO K0640),  
149 and counterstaining with haematoxylin (SIGMA MHS1). Negative controls were performed  
150 using control IgGs (**Table S3**) or non-infected patients, and no staining was observed (**Figure**  
151 **S1**). Optical density ( $OD = \log(\text{max intensity}/\text{mean intensity})$ ) of the DAB staining was quantified  
152 using Fiji (ImageJ) and the results are presented as 1/OD. For the CD68, HBcAg double  
153 staining, a BONDMax robotic staining machine (Leica) was used.

154 **Primary cells purification and cells culture.** Peripheral blood mononuclear cell (PBMC) from  
155 blood donors (Etablissement Français du Sang, EFS; code 895/1052) were isolated by Ficoll  
156 gradient (Histopaque®-1077, Sigma) as previously described <sup>18</sup>. Lymphocytes were separated  
157 from total PBMC by a Percoll gradient and further cultured in macrophage medium (RPMI

158 medium supplemented with 10% of decompemented FBS and 50 U/mL of  
159 penicillin/streptomycin). Monocytes were purified by Percoll gradient followed by a negative  
160 selection with the Monocyte Isolation Kit II (Miltenyi Biotec) and cultured in macrophage  
161 medium. Monocytes were exposed during 6 days to 50 ng/mL of GM-CSF (R&D) or 50 ng/mL  
162 of M-CSF from (PeproTech) for M1-MDM or M2-MDM differentiation respectively. MDM were  
163 activated by a 3-hours stimulation with 10 ng/mL of LPS (Invivogen). Cells were washed three  
164 times with PBS and cultured in fresh medium for another 3 hours before a last medium  
165 exchange. Supernatants and cells were collected 24 h post stimulation (i.e. 18h accumulation).  
166 Liver cells were isolated from hepatic resections obtained in collaboration with three surgical  
167 departments of Lyon (Centre Léon Bérard, Hôpital de la Croix Rousse and centre hospitalier  
168 Lyon-Sud) with the French ministerial authorizations (AC 2013-1871, DC 2013 – 1870, AFNOR  
169 NF 96 900 sept 2011). After a two-step collagenase perfusion, the liver extract was filtered and  
170 centrifuged, as previously described <sup>19</sup>. Primary Human Hepatocytes (PHH) were cultured on  
171 collagen layer and maintain in PHH medium (Williams medium supplemented with 5% of fetal  
172 clone II serum, 50 U/mL of penicillin/streptomycin, 1X glutamax, 5 µg/mL of bovine insulin,  
173  $5 \times 10^{-5}$  M of hydrocortisone, and 2% of DMSO). Liver mononuclear cells (LMNC) or only liver  
174 MΦ were purified from the non-parenchymal cells mixture by respectively Ficoll gradients or a  
175 two phase iodixanol gradient <sup>20</sup>. Liver MΦ were isolated by negative selection using pan  
176 monocyte isolation kit (Miltenyi Biotec) and cultured in macrophage medium. HepaRG cells  
177 were cultured and differentiated as previously described <sup>21</sup>.

178 ***Viral infection.*** Differentiated HepaRG (dHepaRG) or PHH were cultured and infected by HBV  
179 (PEG-precipitated) as previously described <sup>22</sup>. As PEG-concentrated HBV virions cannot be  
180 used for exposition to immune cells, because PEG activates cells, blood monocytes, LMNC  
181 and liver MΦ were exposed to HBV inocula (or control medium), which were prepared by  
182 concentrating supernatant from HepAD38 <sup>23</sup> (or from non-infected HepG2-NTCP respectively)  
183 by filtration/retention centrifugation using column with a cut off retention of 100 kDa (Merk  
184 Millipore; UFC710008). All virus inocula were tested for the absence of endotoxin (Lonza) and



185 characterized by analyses of the fractions from a 5.6-56% iodixanol gradient and analysed by  
186 ELISA, dot blot with HBV DIG-labelled probe <sup>24</sup> and western-blot (DAKO, B0586) (**Figure S2**).  
187 This allowed to rule out the presence of non-enveloped nucleocapsids that may activate  
188 immune cells, as HBc was shown to be a TLR2 ligand <sup>25</sup>. Blood monocytes, LMNC and liver  
189 MΦ were exposed to at least three different batches of HBV (full inoculum concentrated by  
190 ultrafiltration) at a multiplicity of infection of 1000 vge/mL (if not stated otherwise). Viral titer  
191 superior to 10<sup>10</sup> vge/mL were routinely obtained and uniquely used in order to allow a dilution  
192 of 100 to 1000 fold when preparing “infection medium” at a multiplicity of infection of 1000  
193 vge/cell. This way, we limited the impact of “carried-away” contamination. For some  
194 experiments, virus was inactivated by a 30 min exposition to UVB. Stocks of adenoviruses  
195 (AAV) vector particles were generated by the vector Core of the University of Nantes by  
196 calcium phosphate transfection of HEK-293 cells as described previously using pDG8 (as a  
197 helper plasmid). The vector particles were purified on caesium chloride gradients as previously  
198 described <sup>26</sup> and genome particles/mL (gp/mL) were tittered by quantitative PCR (qPCR). AAV  
199 at a multiplicity of infection of 1000 gp/mL.

200 ***Cytochalasin D and cytokine treatments.*** M2-MDM were exposed to 1 µg/mL of  
201 Cytochalasin D (Sigma Aldrich; C8273-1MG) during their activation. As Cytochalasin D is  
202 diluted in DMSO, monocytes were exposed to a similar dose of DMSO as control. Cytokines  
203 references and used concentration are indicated in **Table S4**.

204 ***Quantification of secreted proteins by ELISA.*** HBeAg and HBsAg secretion were quantified  
205 using chemiluminescence immunoassay kit (Autobio) following the manufacturer’s  
206 instructions. Cytokines secretion were analysed using DuoSet ELISA (R&D system) following  
207 manufacturer’s instructions.

208 ***RNA extraction and RT-qPCR.*** Total mRNA from hepatocytes or MΦ were extracted with  
209 NucleoSpin® RNA II or NucleoSpin® RNA XS respectively (Macherey-Nagel). cDNA were  
210 synthesized using the SuperScript®III Reverse Transcriptase (Life technologies) according to  
211 the manufacturer’s instructions. qPCR analyses were performed using “Express SYBR

212 GreenER™ qPCR SuperMix Universal” (Invitrogen). mRNA expression was assessed by  
213 comparative cycle threshold (Ct) method ( $2^{-\Delta Ct}$ ); GUS and RPLP0 were used as housekeeping  
214 genes for hepatocytes and MΦ respectively. Primers sequences are presented in **Table S5**.

215 **DNA extraction and cccDNA quantification.** Total DNA were extracted using the  
216 NucleoSpin® Tissue kit (Macherey-Nagel) following the manufacturer’s instructions. Total  
217 intracellular DNA was digested for 45 min at 37°C with T5 exonuclease (epicentre) to remove  
218 rcDNA (relaxed circular HBV DNA) followed by a 30 min heat inactivation. cccDNA amount  
219 was quantified by qPCR analyses as previously described <sup>1</sup>.

220 **Cytotoxicity assay.** Seven days post treatments, hepatocytes cell viability was assessed by  
221 quantification of neutral red uptake by living cells, as previously described <sup>4</sup>.

222 **Lymphocytes activation.** Lymphocytes were cultured in different M2-MDM conditioned media  
223 at a density of 300,000 cells/cm<sup>2</sup> for 2 hours. Cells were activated by a 22h exposition to 20  
224 ng/mL of Phorbol 12-Myristate 13-Acetate (Sigma) and 500 ng/mL of Ionomycine. Activation  
225 was measured by assessment of TNF-α secretion.

226 **Macrophage supernatants concentration.** M1- or M2-MDM supernatants from at least five  
227 different donors were pooled and concentrated by ultrafiltration (using 10 kDa Amicon ultra-15  
228 column; Merk-Millipore) following the manufacturer’s instruction. Cytokines concentration of  
229 the concentrated supernatants were assessed by ELISA before dilution to 1/100 in hepatocyte  
230 media (experiments from **Figure 9**) or dilution to 1/2 in macrophages media (experiments from  
231 **Figure 7**). Final concentrations are presented in **Table S6 and S7**.

232 **Flow cytometry.** Cells were washed extensively with PBS and removed from plate with 150  
233 µl of versene-EDTA at 37°C for 5 min before centrifugation at 1300 rpm for 5 min at 4°C. Cells  
234 were incubated for 30 min at 4°C with primary antibody (**Table S3**). After extensive washes,  
235 cells were incubated with secondary antibody in the dark for 30 min at 4°C. After another wash,  
236 staining was measured by Flow Cytometry (BD FACSCALIBUR).

237 **Statistical analysis.** Results are presented as mean or median  $\pm$  standard deviation and  
238 analysed for statistical significance using Prism software. P values are indicated and non-  
239 significance is indicated by “ns”.

240 **RESULTS**

241 ***HBV is associated with phenotypic changes in liver macrophages in infected patients.***

242 To assess if HBV may closely interact with liver M $\phi$  *in vivo*, levels of CD68 expression  
243 (identifying M $\phi$ ), CD163 (identifying anti-inflammatory M $\phi$ <sup>14</sup>) and HBc (identifying the HBV  
244 core protein) were analysed by immunohistochemistry on liver biopsies from two distinct cohorts of  
245 patients (see **Table S1** and **S2** for details on patients). As expected, signals for CD68, CD163  
246 were detected in the sinusoidal space (i.e., in between hepatocytes where myeloid cells are),  
247 and no signals were observed with control antibodies (**Figure 1 and S1**). Co-localized signals  
248 were observed for HBc and CD68 in liver samples from HBV infected patients (**Figure 1A and**  
249 **zoom in Figure 1B**) evidencing the presence of this HBV protein within liver M $\phi$ . Of note, the  
250 proportion of M $\phi$  co-stained with HBc varied from one patient to the other; approximatively  
251 20% of all patients analysed lacked co-localisation between HBc and M $\phi$ , 50% displayed less  
252 than 5% co-localisation, and 30% showed between 5% to 20% co-localisation (**Figure 1A,**  
253 **zoom in Figure 1B**). Furthermore, quantification of the signals highlighted a 1.8 fold increase  
254 in CD163<sup>+</sup> myeloid cells in HBV-infected patient (+79%, p=0.0271) in the Swiss/German cohort  
255 and a 1.3 fold increase in the French cohort compared to the non-infected ones (**Figure 1C,**  
256 **1D, S1B**). Interestingly, a positive correlation between the level of HBc staining and the  
257 increase of CD163<sup>+</sup> cells was observed (**Figure 1E**). Altogether, these data suggest that HBV  
258 interact with liver M $\phi$  and may affect their phenotype and functions. We therefore performed  
259 several *ex vivo* analyses to investigate the influence of HBV on liver cells with a focus on liver  
260 M $\phi$  that include Kupffer cells (KC) and monocyte derived M $\phi$  (MDM)<sup>9,10</sup>.

261

262 ***Ex vivo, HBV inhibits the activation of primary liver M $\phi$  by direct exposure.***

263 First, we investigated the impact of HBV particles and antigens (concentrated by  
264 filtration/retention from HepAD38 cell supernatants and characterized as shown on **Figure S1**)

265 on the secretion of cytokines by non-parenchymal primary human liver mononuclear cells  
266 (LMNC) freshly isolated from hepatic resections (5 donors) as previously described <sup>27</sup>. Of note,  
267 we used a virus concentrated by filtration/retention rather than PEG concentration, as PEG per  
268 se activates macrophages. The basal levels of two prototypic cytokines, namely IL-1 $\beta$  and IL-  
269 10, in non-stimulated LMNC from individual donors are shown in **Figure 2A**. These cells were  
270 either left untreated or exposed for 24 hours to total HBV inoculum immediately after their  
271 isolation and the same cytokines were dosed in the supernatant (**Figure 2B**). The mean  
272 concentration of secreted IL-1 $\beta$  was 25% lower in HBV exposed LMNC preparations as  
273 compared to control whereas the mean IL-10 level was 64% higher (**Figure 2B**).

274 Purified primary liver M $\Phi$  (PLM $\Phi$ ) exposed to HBV or not were next stimulated with synthetic  
275 PAMPs; LPS was used to stimulate TLR4 and NLRP3 (NOD-like receptor family, pyrin domain  
276 containing 3) inflammasome <sup>28</sup>, whereas LPS + poly(dA:dT) was used to stimulate TLR4 and  
277 AIM-2 (Absent In Melanoma-2) inflammasome <sup>9</sup> (**Figure 2C**). The mean level of secreted IL-  
278 1 $\beta$  was 31% lower in LPS-stimulated PLM $\Phi$  exposed to HBV, as compared to not exposed  
279 condition (**Figure 2D**). Similar results were obtained with HBV exposed, LPS and poly(dA:dT)-  
280 stimulated PLM $\Phi$ , for which the mean level of secreted IL-1 $\beta$  was 35% lower (**Figure 2E**).  
281 Interestingly, the expression of *HIF1 $\alpha$*  mRNA, which encode a protein tightly associated to IL-  
282 1 $\beta$  production and secretion in macrophages <sup>29</sup>, was 70% lower in LPS-stimulated-PLM $\Phi$   
283 exposed to HBV as compared to control (**Figure S3**). While the mean level of secreted IL-10  
284 was not affected by HBV with both types of stimulations in our experimental conditions (**Figure**  
285 **2D and 2E**), the mRNA levels of *VEGF*, an angiogenic factor expressed by anti-inflammatory  
286 cells <sup>30</sup>, was 56% higher in LPS-stimulated-PLM $\Phi$  exposed to HBV, as compared to control  
287 cells (**Figure S3**).

288 Altogether these data suggest that HBV may interfere with the activation of PLM $\Phi$ .

289

290 ***Ex vivo, HBV interferes with blood monocyte differentiation and activation by direct***  
291 ***exposure***

292 During viral infections, a high quantity of immune cells, among which monocytes, are recruited  
293 to the inflammation/injury site to mount a strong pro-inflammatory response and contribute to  
294 infection control<sup>31</sup>. This response is subsequently limited by an anti-inflammatory response in  
295 order to start the scarring processes and prevent chronic inflammation<sup>31</sup>. Infiltrating monocytes  
296 differentiate into M $\Phi$  within the tissue<sup>14</sup> and, in the case of the human liver, cannot be fully  
297 distinguished from resident M $\Phi$ .

298 To assess the effect of HBV on M $\Phi$  that experimentally mimic those derived from liver  
299 infiltrating monocytes, monocytes were purified from peripheral blood mononuclear cells  
300 (PBMC) and differentiated, with well-established polarizing *in vitro* conditions, into M1-like M $\Phi$   
301 (M1-MDM) upon GM-CSF or M2-like M $\Phi$  (M2-MDM) upon M-CSF stimulation<sup>32</sup>. The  
302 differentiation process is schematically presented in **Figure 3A**. M1-MDM cells derived from  
303 peripheral monocytes expressed and secreted, under a physiologic exposure to LPS (10  
304 ng/mL), pro-inflammatory cytokines IL-6, IL-1 $\beta$ , and TNF- $\alpha$ , but not IL-10 (**Figure 3B, 3C, 3D,**  
305 **and 3E**). In contrast, *in vitro* differentiated M2-MDM cells expressed only residual levels of IL-  
306 6, IL-1 $\beta$ , and TNF- $\alpha$ , but secreted high level of the anti-inflammatory cytokine IL-10 in the same  
307 condition of physiologic LPS exposure (**Figure 3B, 3C, 3D, and 3E**). Upon exposure to HBV  
308 during differentiation and activation (i.e., 6 days exposure to HBV) (**Figure 3A**), GM-CSF-  
309 treated monocytes (i.e., intended to be differentiated into M1-MDM) activated with low LPS  
310 stimulation, secreted 34% and 60% less IL-6 and IL-1 $\beta$  respectively (**Figure 3F**). Accordingly,  
311 a decreased level of *HIF1 $\alpha$*  mRNA was detected (**Figure S4A**). Under these conditions, no  
312 significant effect of HBV incubation was observed on the level of secreted TNF- $\alpha$  (**Figure 3F**).  
313 M2-MDM exposed to HBV during differentiation and activation secreted similar level of IL-10  
314 than non-exposed cells (**Figure 3G**) but expressed lower *VEGF* mRNAs levels (**Figure S4A**).

315 We further investigated the effect of HBV on the activation of already differentiated M1-MDM  
316 or M2-MDM in response to physiologic LPS stimulation (**Figure 4A**). No difference was  
317 observed in the mean levels of secreted IL-6, IL-1 $\beta$ , or TNF- $\alpha$  (**Figure 4B**). In contrast, lower  
318 mean mRNA level of *HIF1- $\alpha$*  was observed (**Figure S4B**). Differentiated M2-MDM cells  
319 showed a 2-fold increase of mean secreted IL-10 levels and an increase of mean *VEGF* mRNA  
320 levels in the presence of HBV (**Figure S4B**). These results were confirmed with different  
321 donors and duration of exposure to HBV (**Figure S5**). Moreover, increasing doses of HBV  
322 inoculum revealed increasing effect i) on the inhibition of IL-6 secretion by M1-MDM, exposed  
323 to the virus during their differentiation and activation, and ii) on the increase of IL-10 secretion  
324 by M2-MDM, exposed to the virus during their activation (**Figure 5A and 5B**).

325 Interestingly, the presence of cytochalasin D (Cyt D, an inhibitor of endocytosis and  
326 phagocytosis<sup>33</sup>), during the activation of M2-MDM in the presence of HBV did not modify IL-  
327 10 secretion (**Figure 5C**), suggesting that the effect of HBV on M2-MDM activation might be  
328 independent of virus productive entry within macrophages. It is also worth noting that UV-  
329 inactivated HBV has a similar effect on PLM $\Phi$  (**Figure S6A**) and on M1- and M2-MDM (**Figure**  
330 **S6B**) secretions as compared to replication-component HBV, and that HBV-exposed liver M $\Phi$   
331 or MDMs did not secrete HBeAg or HBsAg (**Figure S7**), highlighting that the effects of HBV on  
332 M $\Phi$  is independent of viral replication within cells. Moreover, no significant modification of  
333 cytokine secretion was observed when M1- or M2-MDM were exposed to concentrated  
334 supernatants from non-infected HepG2-NTCP during, respectively, their differentiation and  
335 activation or activation only (**Figure S6C and S6D**), suggesting that the effect is specific to  
336 HBV and not to a factor secreted by hepatocytes in the absence of HBV. Finally, adenoviruses  
337 (AAV), either expressing GFP or HBV genome, had no effect on M1-MDM secretions (**Figure**  
338 **5D**), suggesting that the observed effect is specific of HBV particles and/or antigens. Of note,  
339 the cells exposed to AAV-HBV did not secrete HBeAg or HBsAg (**data not shown**).

340

341 ***HBV interferes with blood monocyte differentiation and activation in co-culture***  
342 ***conditions***

343 To exclude that the effect of secreted HBV components on M1-MDM differentiation and M2-  
344 MDM activation was due to the high amount of HBV experimentally used in previously  
345 presented *ex vivo* experiments, we set up co-cultures assays that allowed to study the effect  
346 of HBV components physiologically secreted from infected hepatocytes. Monocytes, which  
347 were intended to differentiate into M1-MDM and M2-MDM, were co-cultured with either non-  
348 infected HepG2-NTCP cells (as HBV negative control cells) or with HBV-producing HepAD38  
349 cells during their respective differentiation (for M1-MDM) or activation (for M2-MDM) (**Figure**  
350 **6A**). We confirmed the results obtained with cell free derived viruses and observed an even  
351 stronger effect of HBV since monocytes co-cultured with HBV-producing cells during their  
352 differentiation into M1-MDM, secreted between 46 and 85% lower mean levels of IL-6, IL-1 $\beta$ ,  
353 and TNF- $\alpha$  (**Figure 6B**). In contrast, M2-MDM co-cultured with HBV producing cells during  
354 their activation secreted 2.25-fold more IL-10 (**Figure 6C**).

355 Altogether these results suggest that exposure of peripheral monocytes to cell-free HBV and  
356 also cell-transmitted HBV components can interfere with their differentiation into M1-MDM, as  
357 indicated by the significantly reduced secretions of pro-inflammatory cytokines, including IL-  
358 1 $\beta$ . Moreover, an exposure to HBV during M2-MDM activation led to an increased secretion of  
359 the anti-inflammatory cytokine IL-10.

360

361 ***HBV impairs lymphocyte activation.***

362 IL-10 is well-known to impair lymphocyte activation<sup>34</sup>. Therefore, we assessed if increase of  
363 IL-10 secretion induced by HBV (**Figure 4C**) would further impair this activation. Total  
364 lymphocytes were purified from peripheral blood and exposed to M2-MDM conditioned media  
365 for 2h before their activation with Phorbol 12-Myristate 13-Acetate (PMA) + ionomycine. Even  
366 though not significant, total lymphocytes exposed to M2-MDM supernatants secreted 17% less



367 TNF- $\alpha$  than the non-exposed ones (**Figure 7, (no-HBV M2-MDM)-CM**). Supernatants from  
368 M2-MDM exposed to HBV during their activation containing higher amounts of IL-10 (**Figure**  
369 **4C and Table S6, (HBV stim M2-MDM)-CM**) significantly decreased TNF- $\alpha$  secretion (30%)  
370 by total lymphocytes (**Figure 7**).

371

### 372 ***Pro-inflammatory cytokines reduce the establishment of HBV infection in hepatocytes.***

373 As previously shown, pro-inflammatory cytokines can directly block HBV replication in cells  
374 persistently infected *in vitro* and IL-1 $\beta$  was the most efficient <sup>4</sup>. To investigate the effects of  
375 M $\Phi$ -derived cytokines on the establishment of HBV infection in hepatocytes, dHepaRG cells  
376 or primary human hepatocytes (PHH) were treated 24h before and during the inoculation of  
377 cells with HBV with various recombinant pro-inflammatory cytokines (rhIL-1 $\beta$ , rhIL-6, rhTNF- $\alpha$ ,  
378 rhIL-18), anti-inflammatory cytokines (rhIL-10, rhTGF $\beta$ ), as well as with rhMCP-1, which is  
379 known to be secreted by both M1- and M2-M $\Phi$  (**Table S4**) <sup>35</sup>. RhIL-1 $\beta$  treatment led to a 70-  
380 80% decrease of HBeAg and HBsAg secretion, HBV mRNA, and cccDNA formation, under  
381 conditions where cell viability of dHepaRG cells and PHH were not affected (**Figure 8**). Of  
382 note, a 24h pre-treatment with rhIL-1 $\beta$ , followed by a withdrawal during HBV inoculation, was  
383 sufficient to decrease HBeAg and HBsAg secretion and HBV mRNA by 75% in dHepaRG  
384 (**Figure S8A**). In addition, treatment with rhIL-6 and rhTNF- $\alpha$  before and during HBV  
385 inoculation resulted in an approximately 50% decrease of HBeAg, HBV RNA, and cccDNA  
386 formation. HBsAg was lowered by ~50% with rhTNF- $\alpha$  and by ~25% with rhIL-6 treatment  
387 (**Figure S8B**). rhIL-18 had no effect on the establishment of HBV infection under these  
388 conditions (**Figure S8B**). Upon rhIL-10 treatment, we observed a slight increase of mean  
389 HBeAg and HBsAg levels in the supernatant of dHepaRG cells, but not in PHH. HBV RNA was  
390 modestly reduced in PHH treated with IL-10 before and during infection (**Figure 8B**). Other  
391 tested cytokines, TGF- $\beta$  and MCP-1, had no significant effect on the establishment of HBV  
392 infection (**Figure S8B**). Of note, we calculated that around 250 pg/mL of rhIL-1 $\beta$  and 25 ng/mL

393 of rhTNF $\alpha$  are necessary to obtain a 50% decrease of the establishment of HBV infection  
394 (**Figure S9**) whereas no further decrease was obtained with 20 ng/mL of rIL-6 (**Figure S9**) and  
395 no still no effect with increasing doses of rhIL-10 (**Figure S9**). Importantly, if the level of NTCP  
396 (sodium-taurocholate co-transporting polypeptide), the HBV receptor, was decreased in  
397 dHepaRG after 24h or 48h of treatment with IL-1 $\beta$  (**Figure S10A**), it was not affected in PHH  
398 (**Figure S10B**), indicating that the measured anti-HBV effect was not uniquely due to hNTCP  
399 expression modulation. Collectively, these data indicate that pro-inflammatory cytokines, and  
400 IL-1 $\beta$  in particular, are very efficient to inhibit the establishment of HBV infection in hepatocytes.

401 We next investigated whether cell culture supernatants from M1-MDM cells exposed or not  
402 with HBV would interfere with the establishment of HBV infection in hepatocytes. Even though,  
403 the final concentrations of IL-1 $\beta$  and IL-6 were respectively 25 fold and 10 fold lower compared  
404 to the concentration of recombinant cytokines used above (**Figure 8**), treatment of dHepaRG  
405 cells with supernatants from differentiated M1-MDM cells (**Table 4 and 7**) inhibited HBV  
406 establishment as shown by a reduction of the mean HBeAg and HBsAg levels by ~25% (**Figure**  
407 **9, (no-HBV M1-MDM)-CM**). Accordingly, dHepaRG treated with supernatants from M1-MDM  
408 exposed to HBV during activation, that contained similar amounts of pro-inflammatory  
409 cytokines to non-exposed M1-MDM (**Figure 4B and Table S7**), showed a 40% decrease of  
410 secreted HBV antigens (**Figure 9, (HBV stim M1-MDM)-CM**). In contrast, dHepaRG treated  
411 with concentrated supernatants from M1-MDM exposed to HBV during differentiation,  
412 containing less pro-inflammatory cytokines (**Figure 3F and Table S7**), showed similar levels  
413 of HBV markers to those measured in non-treated dHepaRG (**Figure 9, (HBV diff M1-MDM)-**  
414 **CM**).

415 Altogether, these data suggest that the pro-inflammatory secretome derived from M1-MDM  
416 can inhibit the establishment of HBV infection in hepatocytes. In addition, this antiviral effect is  
417 lost when monocytes are exposed to HBV during their differentiation.

418 **DISCUSSION**

419 Dendritic cells and M $\Phi$  in the liver are thought to play a major role in the orchestration of  
420 adaptive responses and can also produce cytokines that may directly affect HBV infection <sup>36</sup>.  
421 We confirmed here that cytokines secreted by pro-inflammatory M $\Phi$  (including IL-1 $\beta$  and IL-6)  
422 strongly inhibit the establishment of HBV infection in hepatocytes. These results are in  
423 agreement with our previously reported data showing antiviral effects of these cytokines in  
424 already infected human hepatocytes <sup>4</sup> and emphasize the potential direct role of pro-  
425 inflammatory cytokines in the control of HBV. Of note, even though we observed a decrease  
426 in the level of NTCP in dHepaRG treated with IL-1 $\beta$  as previously reported <sup>37</sup>, NTCP levels  
427 were not changed by IL-1 $\beta$  treatment in primary human hepatocytes (PHH) under conditions  
428 that were sufficient for HBV inhibition. These results suggest that IL-1 $\beta$  can inhibit HBV  
429 establishment through an NTCP-independent but still ill-defined molecular mechanism.

430 Considering the antiviral potential of liver M $\Phi$  derived cytokines, the main scope of this study  
431 was to investigate if HBV could interfere with such macrophage pro-inflammatory functions to  
432 facilitate the establishment of hepatocyte infection. Using human primary cells, we observed  
433 (i) that primary liver M $\Phi$  (PLM $\Phi$ ) exposed *ex vivo* to HBV secrete less pro-inflammatory  
434 cytokines upon synthetic PAMP stimulations, thus confirming our previously published data <sup>8</sup>,  
435 (ii) that M1-MDM also secrete less IL-1 $\beta$  and IL-6 upon activation if exposed to HBV or HBV-  
436 producing cells during differentiation and (iii) that the antiviral effect of M1-MDM conditioned  
437 medium is lost when cells were differentiated in the presence of HBV. These results are in  
438 accordance with those from previously published studies using animal models or macrophage  
439 cell lines that reported an ability of HBV to inhibit macrophage responses <sup>9,38-41</sup>, and further  
440 demonstrate that HBV has evolved strategies to interfere with liver myeloid cells functions, in  
441 the absence of any *bona fide* replication (i.e. neo-synthesis of viral genome and proteins) in  
442 these cells.

443 Importantly, this phenotype was similar in naive liver macrophages or monocytes-derived-  
444 macrophages *ex vivo* by short exposure to the virus or cells replicating the virus, thus  
445 suggesting an “easy to implement” mechanism of inhibition, again irrespective of a proper  
446 replication of HBV in these cells. In addition, we showed that the inhibition of pro-inflammatory  
447 cytokines and increase of anti-inflammatory cytokines is dependent on the quantity of virus.  
448 However, to be maintained, such an inhibitory phenotype would require a constant exposure  
449 to the virus and/or viral components. In this respect, our data showing an increased inhibition  
450 of the secretion of pro-inflammatory cytokines by M1-MDM upon constantly renewed exposure  
451 to HBV via co-cultures with HBV-producing cells compared to a single exposure to  
452 concentrated virus support the latest hypothesis. In contrast, the recent study by Suslov and  
453 colleagues convincingly showed that the stimulation with synthetic PAMPS (including TLR4  
454 ligands) was not antagonized in *ex vivo* cultivated HBV-positive biopsies <sup>42</sup>. In this case, the  
455 purification procedure and *ex vivo* conditions cultures in the absence of HBV virion or viral  
456 components might have reversed the HBV-inhibitory phenotype. Moreover, additional  
457 experimental pitfalls could explain these opposite results. First, the concentration of TLR4  
458 ligands used in this study was far higher to what used here (20 µg/mL versus 10 to 100 ng/mL)  
459 and probably beyond physiologic conditions. Moreover, despite the commendable effort of this  
460 study to work with highly relevant biological materials, the viability of hepatocytes in these *ex*  
461 *vivo* maintained biopsies was limited and the viability as well as the functionality of other liver  
462 cells such as liver macrophages was not assessed. It would therefore be important to test if  
463 the addition of HBeAg/HBsAg/HBV virions in the culture medium of *ex vivo* cultured liver  
464 biopsies and the use of physiologic levels of stimulations would recapitulate the inhibitory  
465 phenotype we described here.

466 Importantly, we observed an increase of anti-inflammatory MΦ markers (CD68<sup>+</sup> CD163<sup>+</sup> cells)  
467 in liver biopsies from HBV-infected patients compared to those from non-infected patients of  
468 two different cohorts and an increase in the levels of IL-10 secreted by M2-MDM upon  
469 exposure to HBV during activation after *ex vivo* differentiation. These data suggest that HBV

470 may reinforce the already described liver immune tolerance <sup>16</sup> to establish and/or maintain its  
471 infection. This is in accordance with studies reporting an increase of anti-inflammatory cytokine  
472 secretion (especially IL-10) induced by HBV in animal models and patients <sup>43-46</sup> and a positive  
473 role of anti-inflammatory liver MΦ in HBV persistence <sup>43</sup>. Here, we observed that HBV-induced  
474 increase of IL-10 secretion by M2-MDM might impair the activation of lymphocytes. As others  
475 had previously described <sup>43,47,48</sup>, the increased IL-10 expression during HBV infection could  
476 favour a tolerogenic environment and the inhibition of functional adaptive immune response,  
477 compromising HBV elimination. As these anti-inflammatory MΦ are also implicated in affecting  
478 distinct oncogenetic processes (through the secretion of angiogenic factors, such as VEGF,  
479 and the impairment of an anti-oncogenic responses by the secretion of regulatory mediators,  
480 such as IL-10 <sup>49</sup>), it should be investigated if the HBV-induced modification of MΦ phenotypes  
481 may also play a role in HCC initiation and promotion.

482 We did not precisely determine which viral components and which HBV-infection related  
483 mechanisms influence cytokine secretion by MΦ. The secreted viral proteins (HBeAg, HBsAg)  
484 or viral proteins contained in virions (HBsAg, HBc, viral polymerase) may play a role in the  
485 modification of MΦ phenotype. Few data suggest that HBV can be internalized by MΦ (29,44).  
486 HBeAg <sup>10</sup>, HBsAg <sup>9,40</sup> and the HBV capsid protein <sup>52</sup> have been suggested to contribute to the  
487 inhibition of MΦ responses. In the present study, analyses of liver biopsies from HBV infected  
488 patient revealed a co-localisation between HBc and a macrophage marker (CD68; HBcAg).  
489 How HBc is delivered to liver macrophages remained to be addressed; it could be through a  
490 specific receptor, unspecific engulfment, or by phagocytosis of dying infected hepatocytes.  
491 Moreover, it is not clear yet in which subcellular compartment (e.g. organelles; lysosomes)  
492 stained HBcAg is located.

493 Of note, the use of recombinant viral proteins to clarify the involvement of viral proteins in the  
494 modification of MΦ responses may be suboptimal, as post-transcriptional modifications  
495 associated to a given protein produced in a given cell system (bacteria, yeast or mammalian  
496 cells) could be different as compared to those found in viral proteins from infected hepatocytes

497 and therefore have a different effect on immune cells. In addition, these modifications may vary  
498 during the different phases of HBV infection, giving the numerous functions of the viral proteins.  
499 HBV probably interferes with the activation of MΦ (and thereby cytokine secretion) at different  
500 levels. Indeed down-regulation of innate sensors such as TLR<sup>41,53,54</sup> or inhibition of cell  
501 different signaling pathways<sup>39,40,47</sup> have been reported. Epigenetic modulations induced by the  
502 fixation of one viral protein to gene promoters, as reported in hepatocytes<sup>52</sup> and as also  
503 reported for other viruses<sup>55-57</sup> might also be implemented in MΦ even though HBV do not  
504 replicate in those cells. Importantly, the different effects observed are specific of HBV as AAVs  
505 had no impact on M1- and M2-MDM secretions. Our data suggest that HBV productive entry  
506 into cells (i.e. with fusion of membrane between viral particles and cells) may not be absolutely  
507 required to induce phenotypic changes in macrophages. One of several viral proteins present  
508 in the inoculum or circulating in blood of patients (HBsAg and HBeAg) may bind to receptor(s)  
509 of macrophages involved in modulation of inflammatory responses. As described before, the  
510 activation of specific regulatory receptors at the surface of macrophages can trigger inhibition  
511 of pro-inflammatory cytokines secretion<sup>58-60</sup>. It will therefore be important to determine the  
512 target(s) of HBV at the surface of macrophages responsible for the modulations observed in  
513 order to develop specific molecules targeting this interaction.

514 Importantly, in our different experimental settings, we did not detect any cytokine secretion by  
515 LMNC, primary human liver macrophages or MDM exposed to cell-culture-produced HBV  
516 inocula or co-cultured with HBV producing cells (data not shown). This is in sharp contrast with  
517 chronic HCV or HIV infections that induces strong host responses<sup>61-64</sup> but in accordance with  
518 studies that reported an absence of measurable innate immune responses in patients, animal  
519 or *in vitro* models upon primary HBV infection<sup>42,45,62,65-67</sup> and contributed to define HBV as a  
520 stealth virus<sup>68</sup>. Of course, as also shown here, this stealthy character could be due to active  
521 evasion and not complete passivity. In contrast, several groups have reported an induction of  
522 innate responses following exposure to HBV<sup>7,25,50,69,70</sup>. The quality of the HBV inoculum used  
523 to perform the *ex vivo* analyses is probably very different from one study to the other since no

524 standard inocula are available and only very few manuscripts reported the characterization of  
525 their inocula as we did here (**Figure S2**). Indeed, different HBV producing cells, different culture  
526 conditions and concentration procedures may lead to different ratios of viral antigens, subviral  
527 particles and Dane particles as well as contaminating non-enveloped nucleocapsids,  
528 recognized by TLR2<sup>25</sup> or endotoxins, recognized by TLR4<sup>71</sup> in the inocula and may explain  
529 the discrepancies between the studies.

530 From a therapeutic point of view, we here confirmed that IL-1 $\beta$  is very efficient to inhibit HBV  
531 establishment and replication in hepatocytes. However, recombinant IL-1 $\beta$  cannot be used  
532 systemically without risking severe side effects such as cytokine storms. New anti-HBV  
533 therapeutic options should aim at inducing the local and endogenous secretion of IL-1 $\beta$  to  
534 induce an antiviral activity and prevent the development of pathogenesis. Different strategies  
535 may be considered to promote pro-inflammatory over anti-inflammatory phenotype in liver  
536 resident and infiltrating M $\Phi$ . For instance, the delivery of GM-CSF in cancerous tissue has  
537 been shown to induce tumour regression<sup>72,73</sup> by promoting pro-inflammatory macrophages.  
538 Inversely, a monoclonal antibody against CSF-1R (colony stimulating factor 1 receptor)  
539 showed promising results in reducing the number of anti-inflammatory and tumour associated  
540 macrophages<sup>15</sup>. Alternatively, inhibitors of glutaminolysis, that modify the succinate/ $\alpha$ -  
541 ketoglutarate balance in macrophages<sup>74</sup> or specific inflammasome and/or PRR inducers could  
542 be evaluated<sup>75</sup>.

543 In summary, we showed that HBV can modulate the resident and transiting-macrophage  
544 phenotypes to favour its establishment, and likely its maintenance in the liver. On the one hand,  
545 HBV escapes the antiviral effect of pro-inflammatory liver resident and infiltrating M $\Phi$  by  
546 interfering with their activation/differentiation and inhibiting the production of cytokines  
547 endowed with anti-HBV activities. Besides, HBV infection promotes the activation of anti-  
548 inflammatory M $\Phi$  and the production of IL-10 in the microenvironment to probably favour  
549 tolerance. Our data also suggest that therapeutic strategies promoting the differentiation of  
550 pro-inflammatory liver M $\Phi$  over anti-inflammatory ones or the shift from one to the other should

551 be tested to help breaking immune tolerance and promoting a functional cure of chronic HBV  
552 infections.



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- 716

717 **FIGURE LEGENDS**

718 **Figure 1: An HBV protein co-localized with liver MΦ and the proportion of anti-**  
719 **inflammatory MΦ is higher in CHB liver biopsies.** HBc (HBV capsid protein), CD68 (total  
720 macrophage) and/or CD163 (M2 macrophage), in liver biopsies from non-infected or HBV-  
721 infected patients (Table S1), were stained by immune-histo-chemistry (IHC). (A,B)  
722 Representative photos of HBc (brown arrows) and CD68 (pink arrows) stainings. (C)  
723 Representative photos of CD163 stainings. (A,B,C) Bars represent 50 μm. (D) Quantification  
724 of CD163 stainings. Horizontal bars represent the median of values and data were submitted  
725 to Kruskal Wallis test. ns = not significant. (E) Correlation curve between CD163 and HBc  
726 stainings.

727

728 **Figure 2: Levels of pro-inflammatory markers decreased in liver MΦ exposed to HBV.**  
729 (A,B) Total LMNC were isolated from liver resection and exposed or not to HBV for 24h.  
730 (C,D,E) Liver MΦ were isolated from liver resection, exposed or not to HBV for 24h and  
731 stimulated 24h later with (C,D) 100 ng/mL of LPS (TLR4-L) or (C,E) 100 ng/mL of LPS + 100  
732 ng/mL of Poly (dA:dT) (AIM2-L) for another 24h. (A-E) Supernatant were collected and the  
733 levels of IL-1β and IL-10 were assessed by ELISA. Data are presented either as non-  
734 normalised secretion or as ratio to non-exposed cells. Results are the median of five  
735 independent experiments (with five different donors) each performed with three biological  
736 replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test. ns  
737 = not significant.

738

739 **Figure 3: HBV interferes with M1-MDM differentiation.** Monocytes were purified from  
740 peripheral blood mononuclear cells, differentiated into (B-F) M1-MDM (upon GM-CSF culture)  
741 or (B-E,G) M2-MDM (upon M-CSF culture) in the presence or not of HBV before stimulation  
742 with 10 ng/mL of LPS for 3 hours. (A) Schematic representation of the experiment. Medium



743 change is indicated by dotted arrows. (B-G) Levels of the indicated secreted cytokines were  
744 assessed by ELISA and analysed either as non-normalised secretion or as ratio to non-  
745 exposed cells. Results are the mean of six independent experiments (with six different donors)  
746 each performed with three biological replicates. Horizontal bars represent the median and data  
747 were submitted to Wilcoxon test. ns = not significant.

748

749 **Figure 4: HBV increases M2-MDM activation.** Monocytes were purified from peripheral blood  
750 mononuclear cells, differentiated into (B) M1-MDM (upon GM-CSF culture) or (C) M2-MDM  
751 (upon M-CSF culture) and stimulated with 10 ng/mL of LPS for 3 hours in the presence or not  
752 of HBV. (A) Schematic representation of the experiment. Medium change is indicated by dotted  
753 arrows. (B-C) Levels of the indicated secreted cytokines were assessed by ELISA and  
754 analysed as ratio to non-exposed cells. Results are the mean of six independent experiments  
755 (with six different donors) each performed with three biological replicates. Horizontal bars  
756 represent the median and data were submitted to Wilcoxon test. ns = not significant.

757

758 **Figure 5: HBV effect on macrophages is dependant of virus proteins quantity but**  
759 **independent of viral entry and replication.** (A-D) Monocytes were purified from peripheral  
760 blood mononuclear cells, then differentiated into M1-MDM (upon GM-CSF culture; panels A  
761 and D) or M2-MDM (upon M-CSF culture; panels B and C) and stimulated with 10 ng/mL of  
762 LPS for 3 hours. Cells were exposed or not to HBV, Cytochalasin D, AAV-GFP or AAV-HBV  
763 during their differentiation and activation (A,D), or their activation (B,C). (A-D) Levels of the  
764 indicated secreted cytokines were assessed by ELISA and analysed as ratio to non-exposed  
765 cells (A-B: MOI 0; C: Mock; D: HBV-). Results are the mean of two to five independent  
766 experiments (with two or five different donors) each performed with three biological replicates.  
767 Horizontal bars represent the median and data were submitted to Wilcoxon test. ns = not  
768 significant.

769

770 **Figure 6: HBV-producing cells impair M1-MDM differentiation and enhance M2-MDM**  
771 **activation.** Monocytes were purified from peripheral blood mononuclear cells, differentiated  
772 into (B) M1-MDM (upon GM-CSF culture) or (C) M2-MDM (upon M-CSF culture) and  
773 stimulated with 10 ng/mL of LPS for 3 hours, in the presence of non-infected HepG2-NTCP  
774 (HBV- cells) or HepAD38 (HBV+ cells) during their differentiation or activation, respectively.  
775 (A) Schematic representation of the experiment. Medium change is indicated by dotted arrows.  
776 (B, C) Levels of the indicated secreted cytokines were assessed by ELISA and analysed as  
777 ratio to cells exposed to HBV- cells. Results are the median of five independent experiments  
778 (with five different donors) each performed with three biological replicates. Horizontal bars  
779 represent the median and data were submitted to Wilcoxon test. ns = not significant.

780

781 **Figure 7: HBV induces further immunosuppressive properties to M2-MDM.** Lymphocytes  
782 were purified from peripheral blood mononuclear cells and exposed to media (mock) or LPS-  
783 stimulated M2-MDM in the absence (no-HBV M2-MDM) or presence (HBV stim M2-MDM) of  
784 HBV during their stimulation (CM; conditioned medium) for 2h before stimulation with 20 ng/mL  
785 of PMA and 500 ng/mL of ionomycin. Supernatants were collected 22h later and the levels of  
786 TNF- $\alpha$  assessed by ELISA. Results, presented as ratio to non-treated cells (mock), are the  
787 mean +/- standard deviation of three independent experiments (lymphocytes from three  
788 different donors) each performed with three biological replicates. Data were submitted to  
789 Wilcoxon test. ns= not significant.

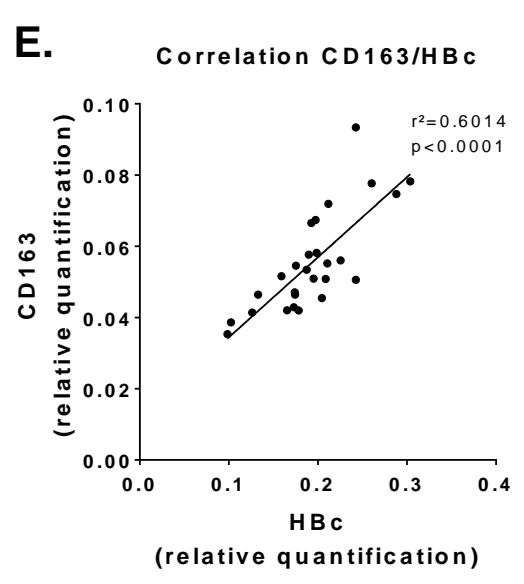
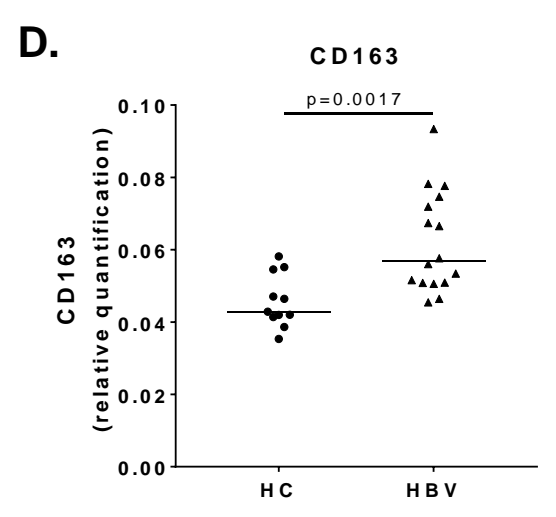
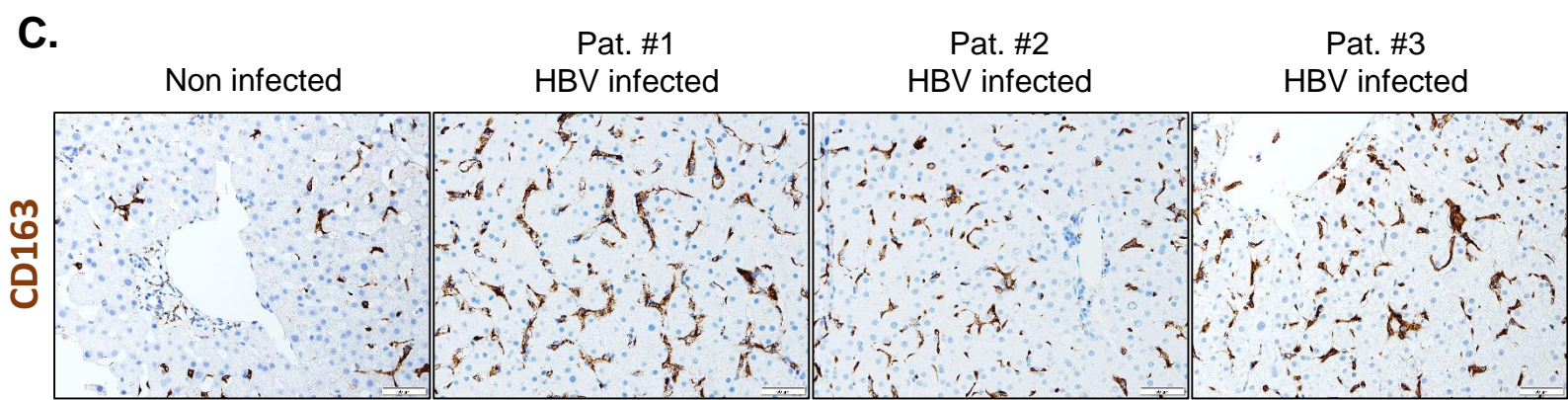
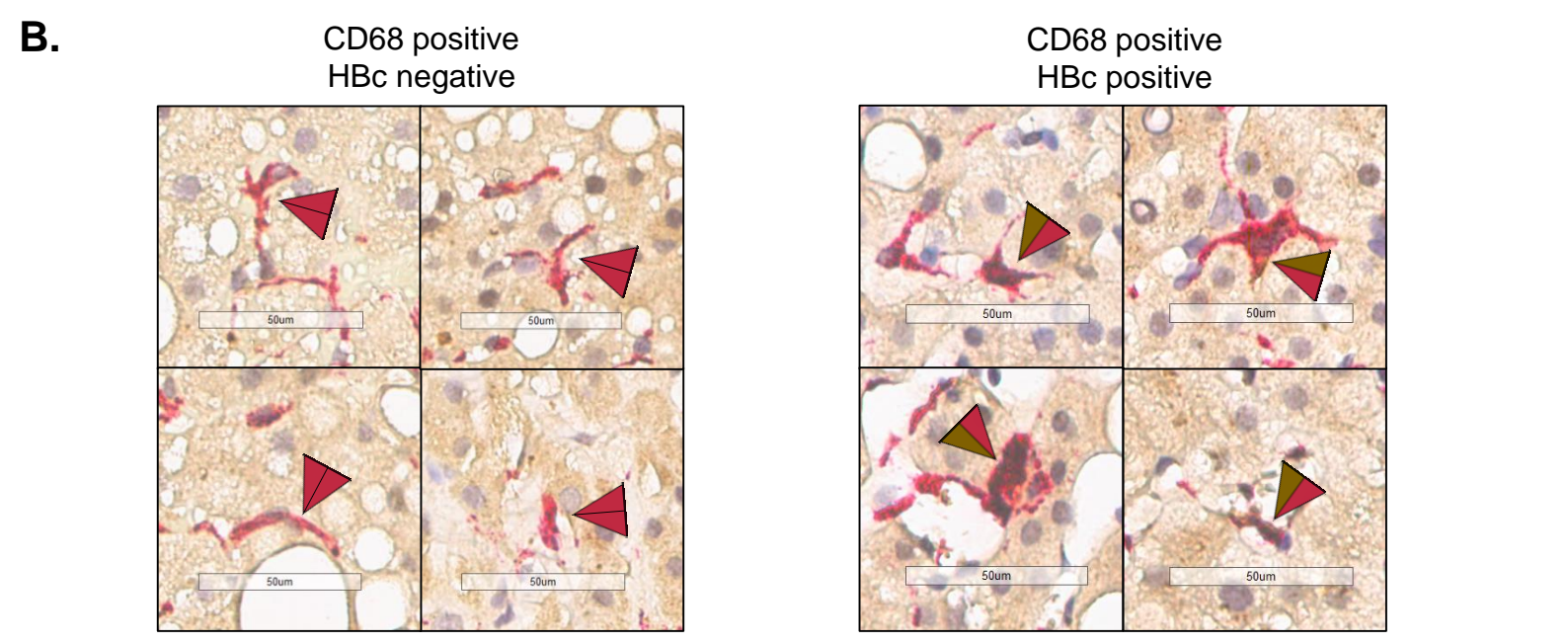
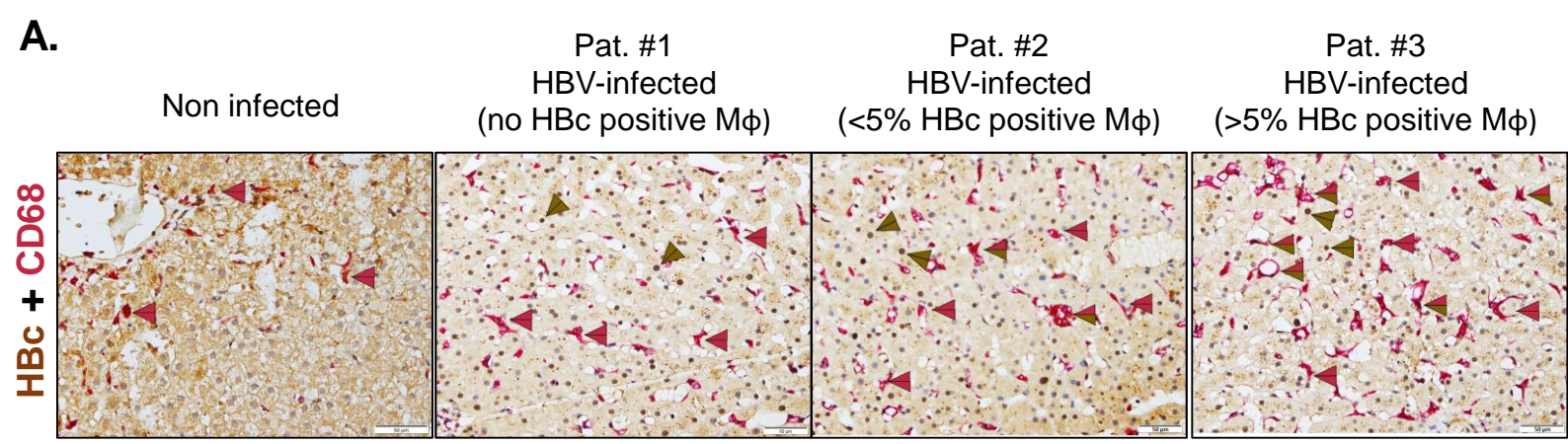
790

791 **Figure 8: IL-1 $\beta$  strongly inhibits establishment of HBV infection in hepatocytes.** (A)  
792 dHepaRG cells or (B) PHH were treated with 1 ng/mL of recombinant IL-1 $\beta$  or 20 ng/mL of  
793 recombinant IL-10, 24h before and during the infection with HBV. Seven days post infection,  
794 supernatants were collected and levels of HBeAg and HBsAg quantified by ELISA. Cells were

795 harvested, total RNA or DNA extracted, and levels of HBV RNAs and cccDNA quantified by  
796 RT-qPCR or qPCR analysis. Cell viability was assessed by neutral red uptake assay. Results,  
797 presented as ratio to non-treated cells, are the mean +/- standard deviation of three  
798 independent experiments (three batches of dHepaRG cells and three donors of PHH) each  
799 performed with three biological replicates. Data were submitted to Wilcoxon test.

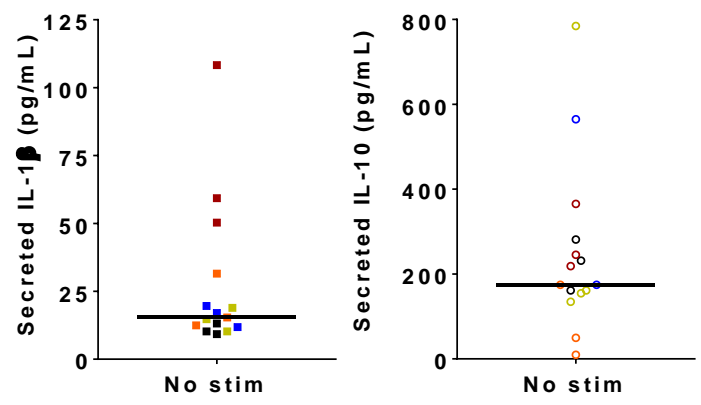
800

801 **Figure 9: HBV prevents the antiviral effect of M1-MDM on HBV establishment.** dHepaRG  
802 cells were treated 24h before and during HBV infection with concentrated supernatants from  
803 LPS-stimulated M1-MDM (CM: conditioned medium pooled from at least five independent  
804 experiment) exposed to HBV during their differentiation or during their stimulation. Seven days  
805 post infection, supernatants were collected and levels of HBeAg and HBsAg quantified by  
806 ELISA. Cells were harvested, total DNA extracted and levels of cccDNA quantified by specific  
807 qPCR analysis. Cell viability was assessed by neutral red uptake assay. Results, presented  
808 as ratio to non-treated cells (mock), are the mean +/- standard deviation of three independent  
809 experiments each performed with three biological replicates. Data were submitted to Wilcoxon  
810 test. ns= not significant.

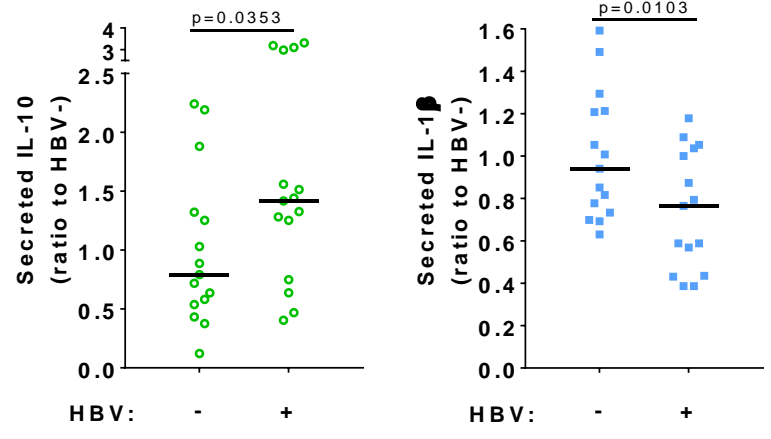


**Figure 1**

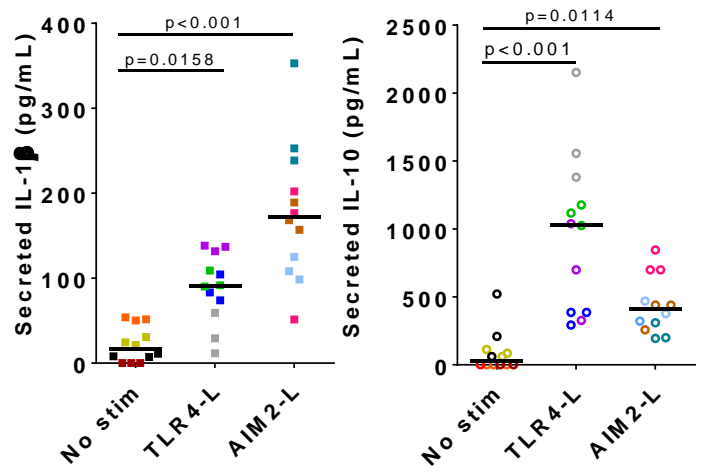
**A. LMNC**



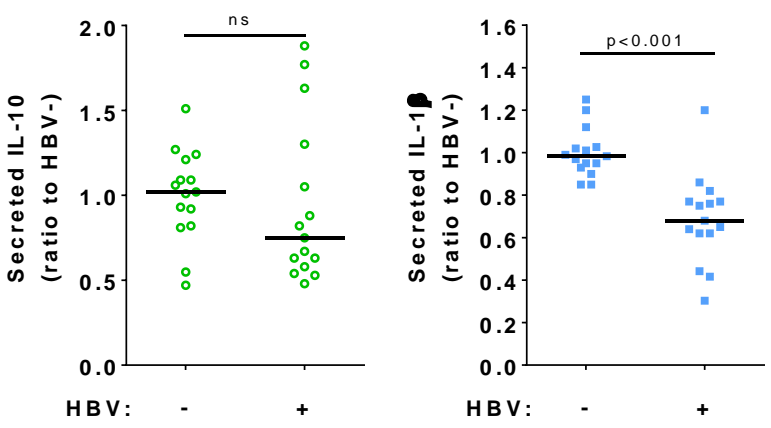
**B. LMNC**



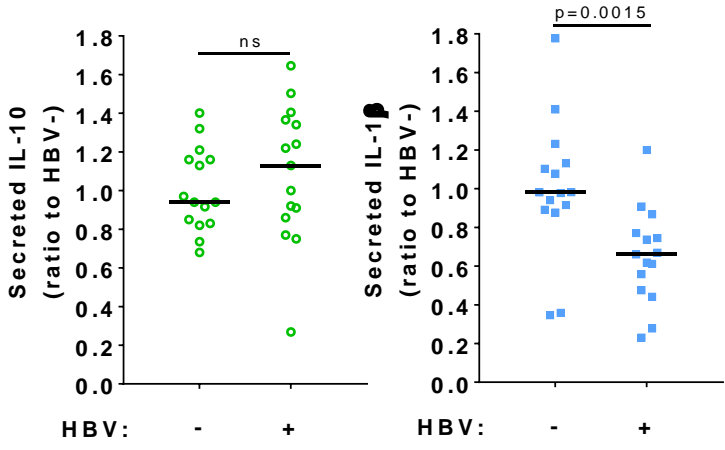
**C. Liver-MΦ**



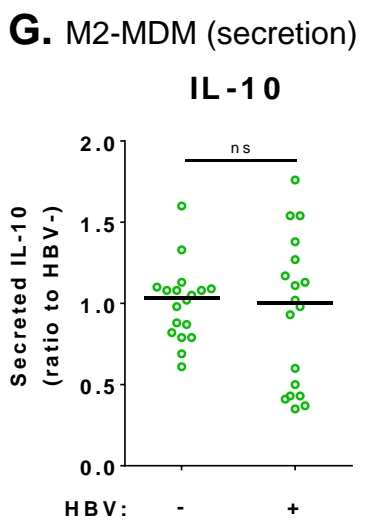
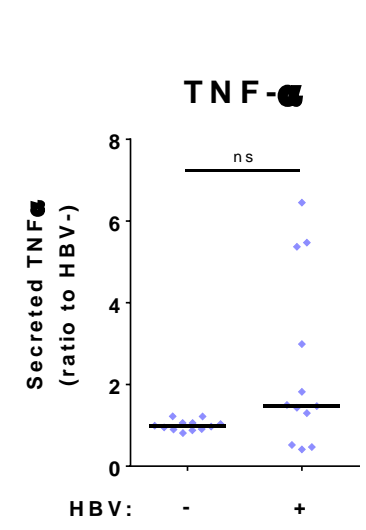
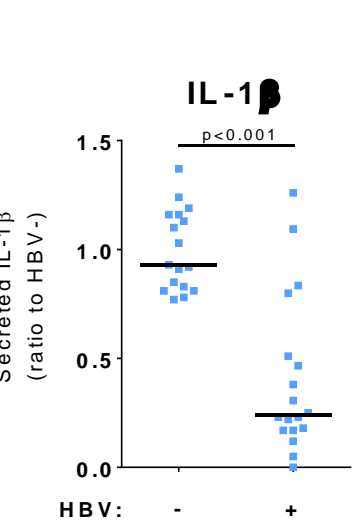
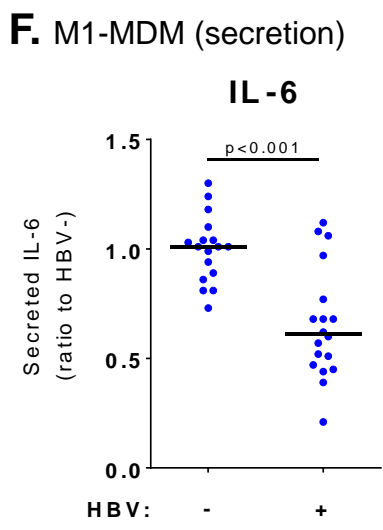
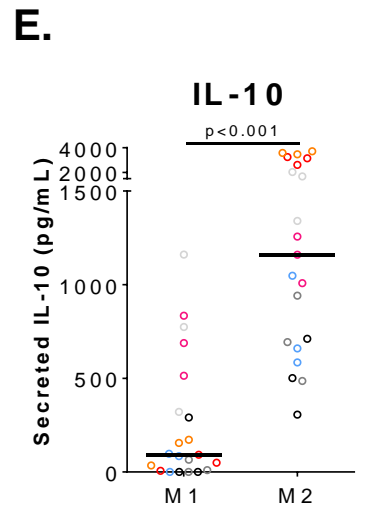
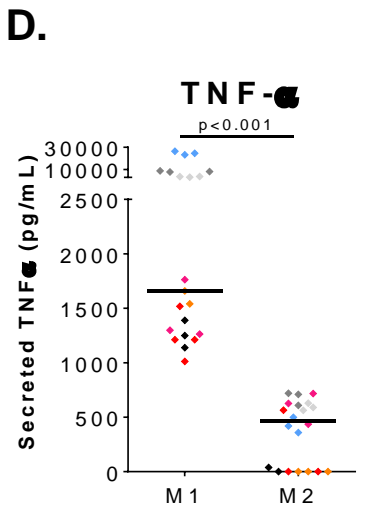
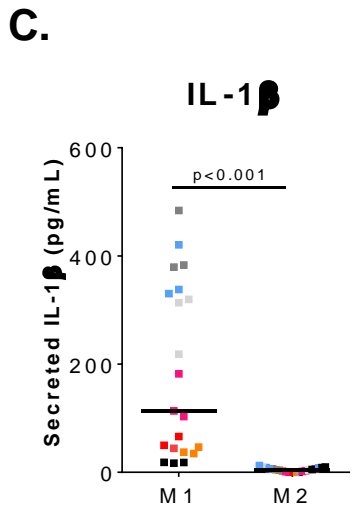
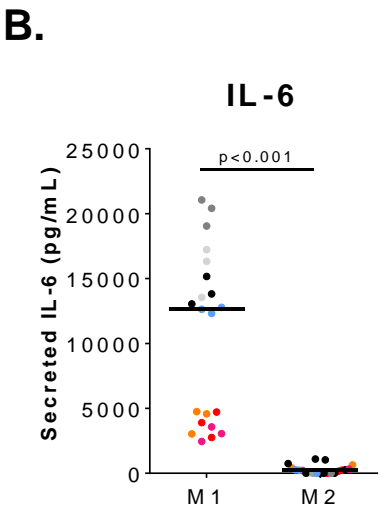
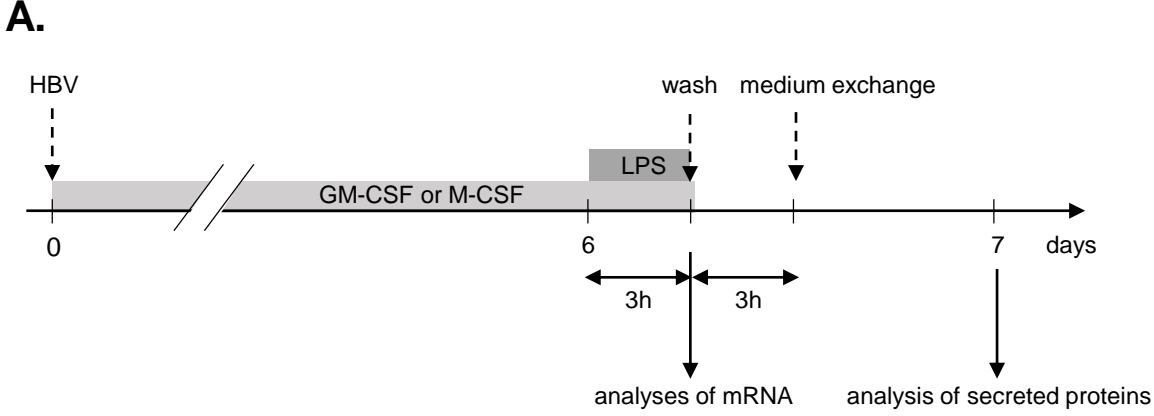
**D. LPS-stimulated-Liver MΦ**



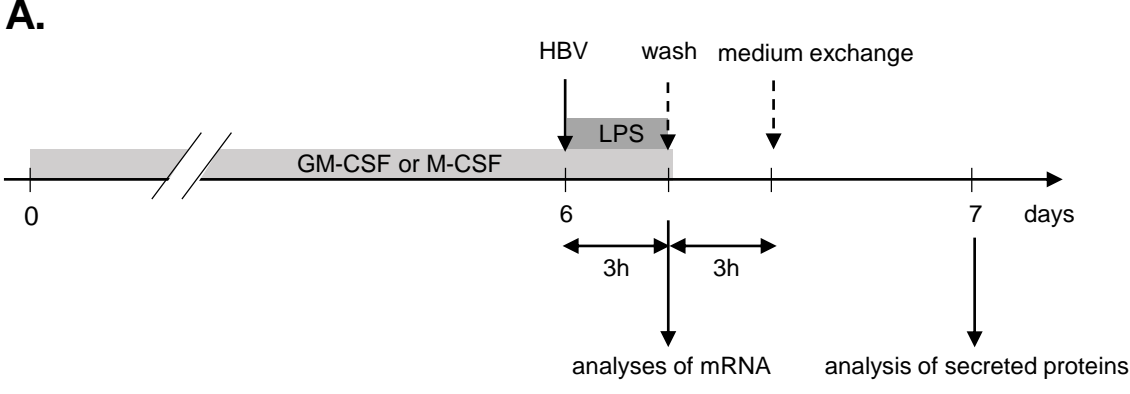
**E. (Poly (dA:dT) + LPS)-stimulated-Liver MΦ**



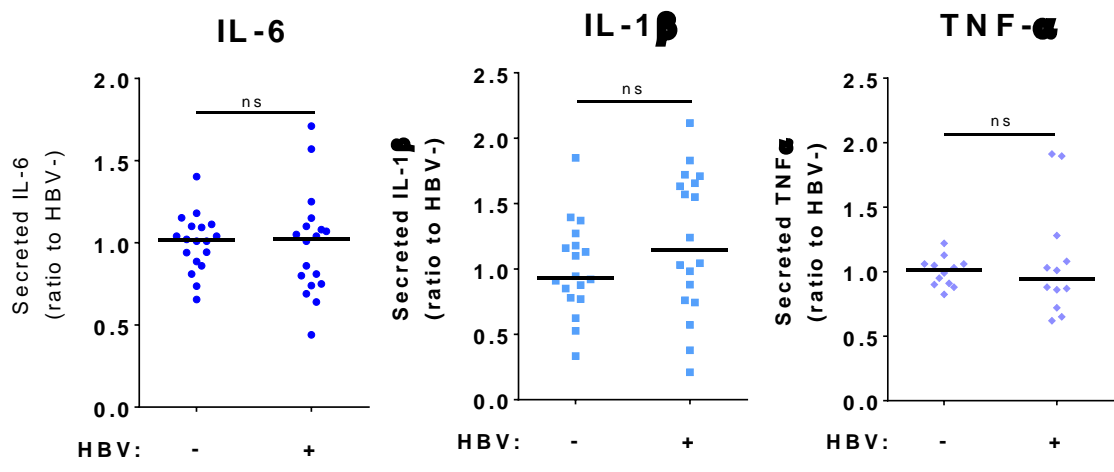
**Figure 2**



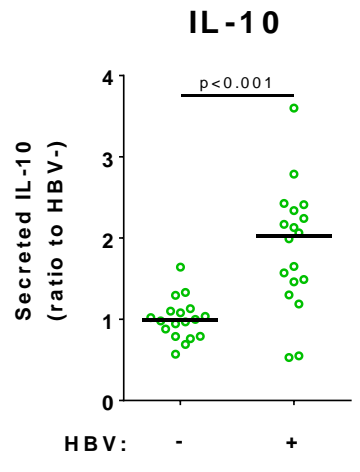
**Figure 3**



**B. M1-MDM (secretion)**

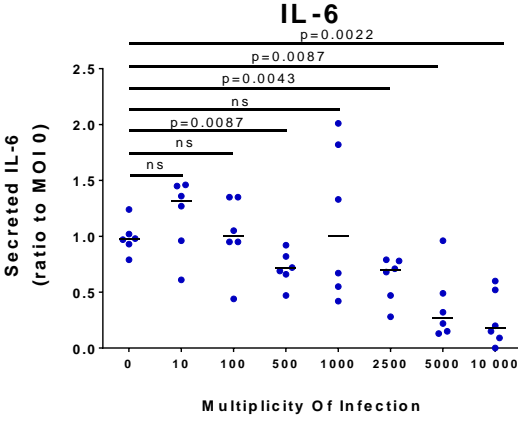


**C. M2-MDM (secretion)**

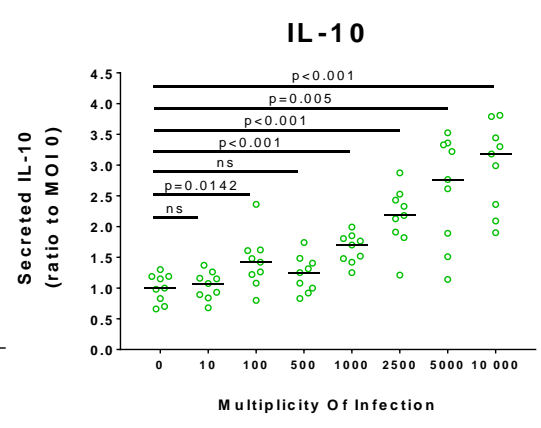


**Figure 4**

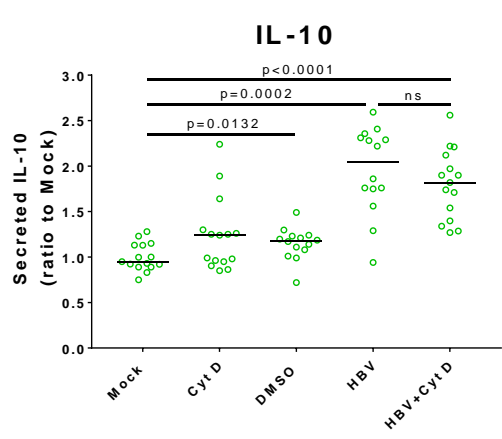
**A. M1-MDM**



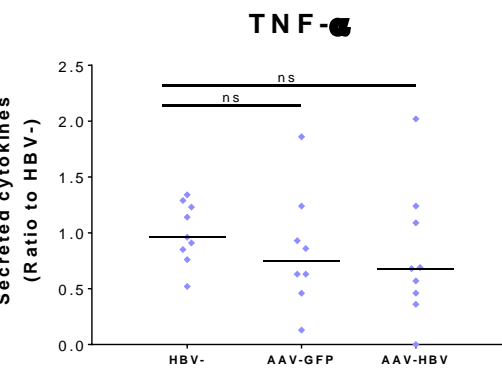
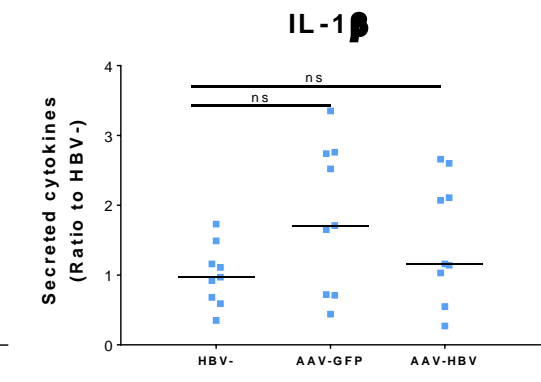
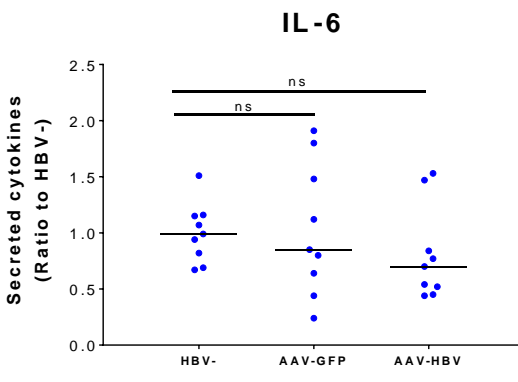
**B. M2-MDM**



**C. M2-MDM**

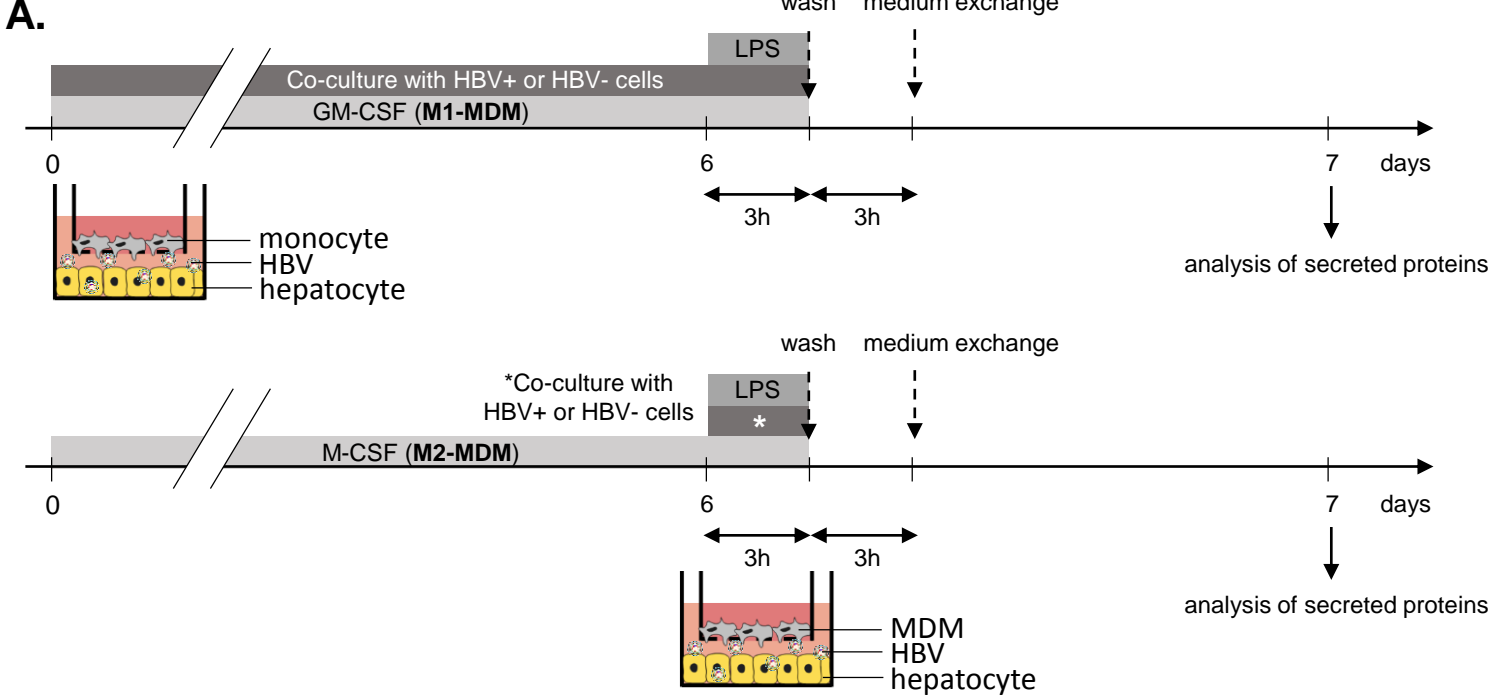


**D. M1-MDM**

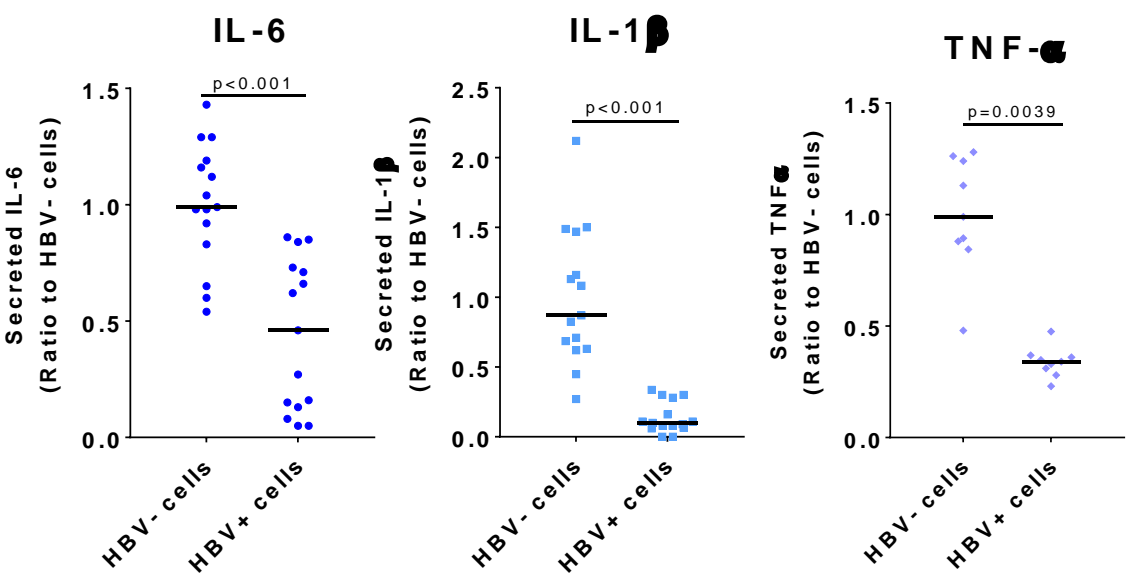


**Figure 5**

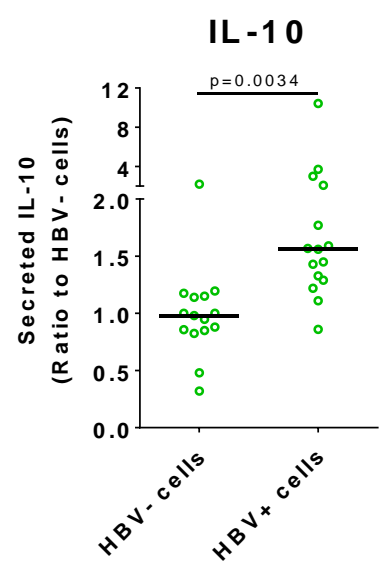




**B. M1-MDM**



**C. M2-MDM**



**Figure 6**

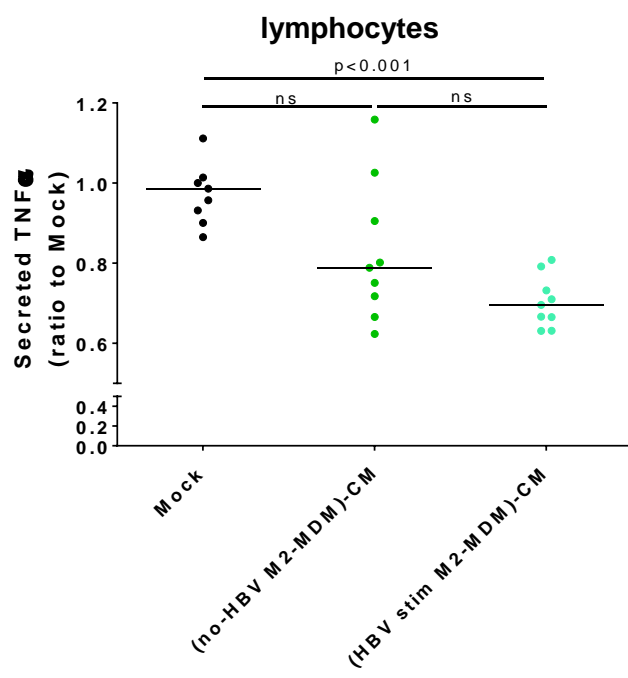
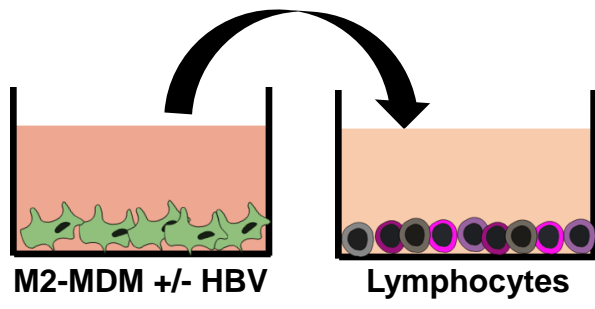
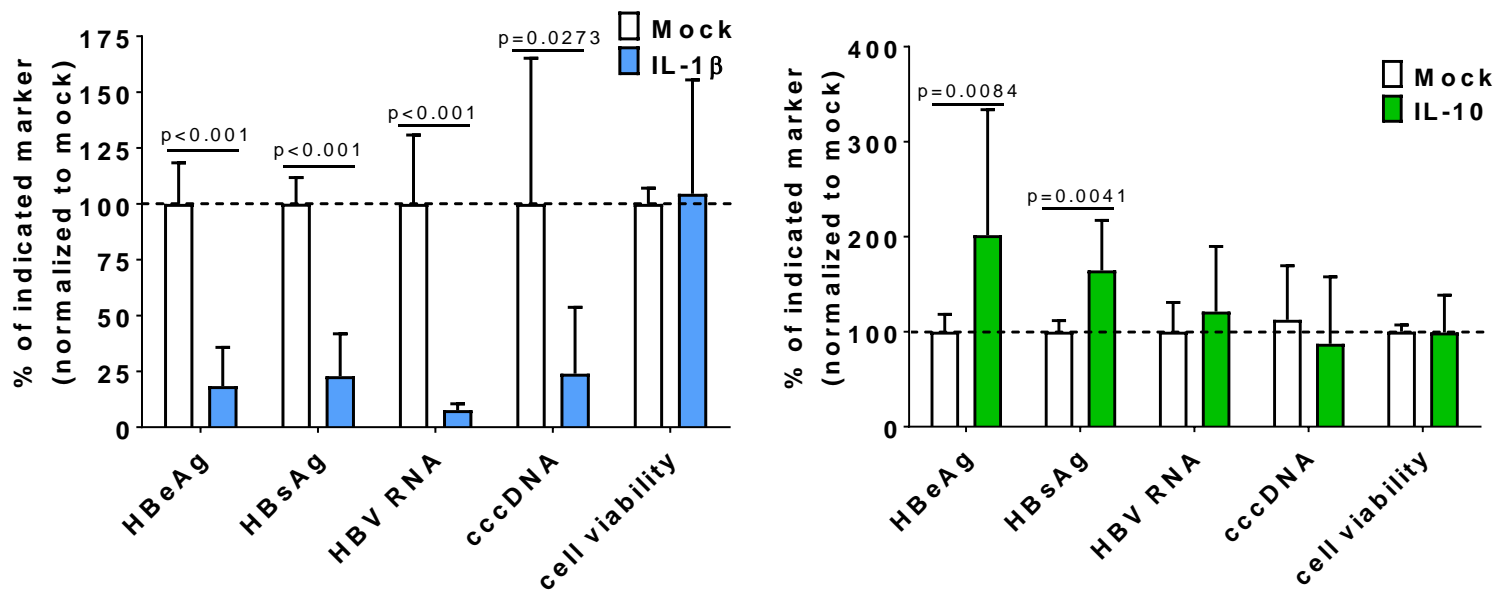
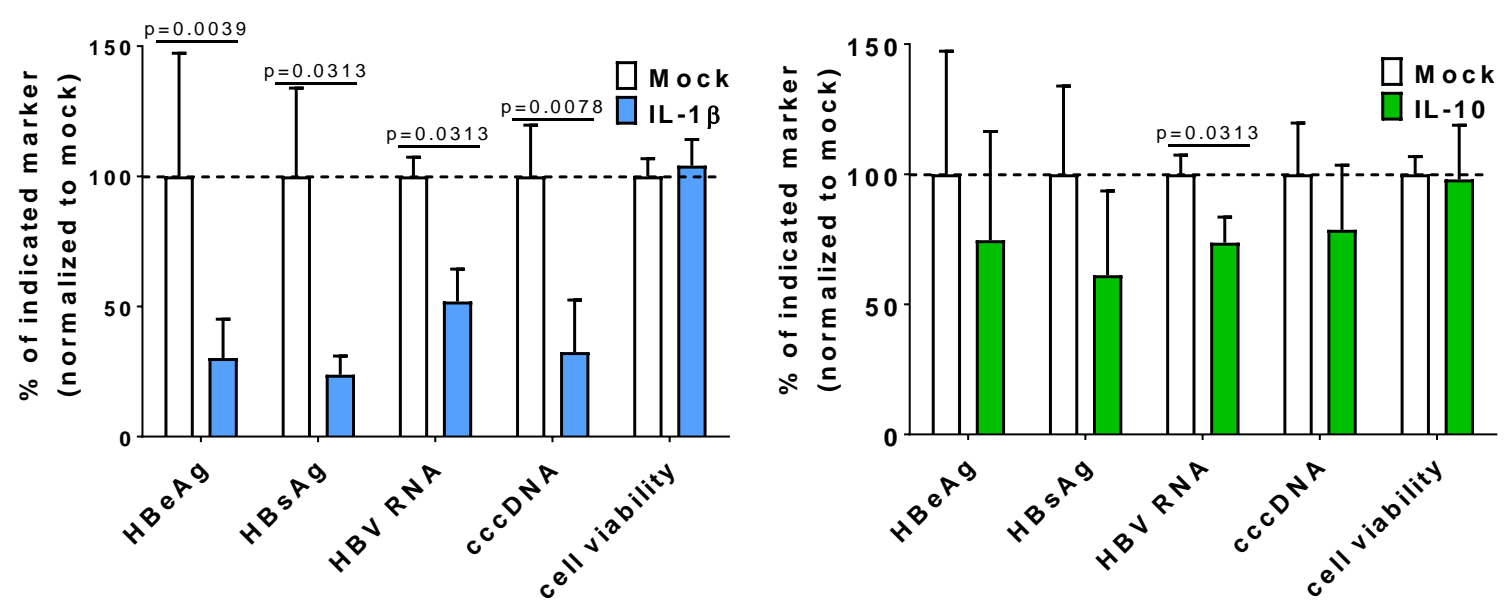


Figure 7

**A.****dHepaRG****B.****PHH****Figure 8**

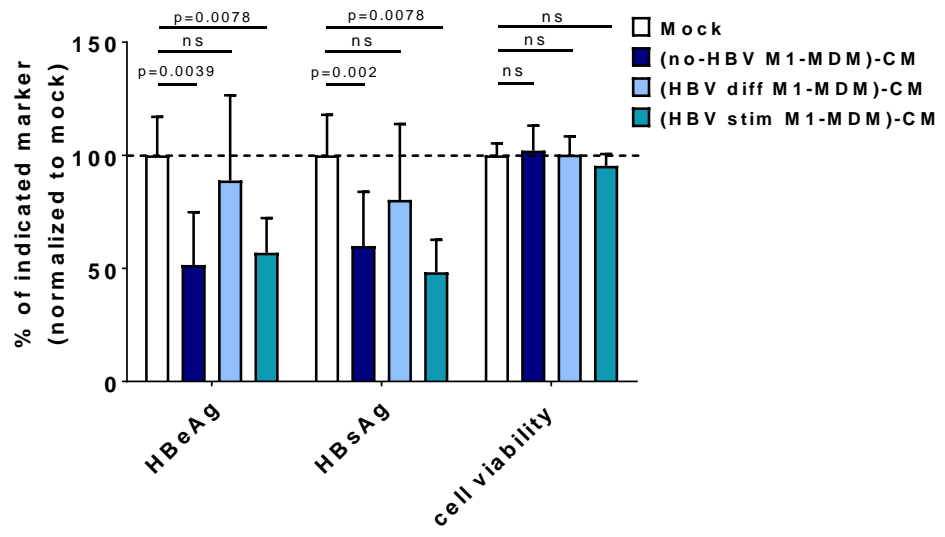
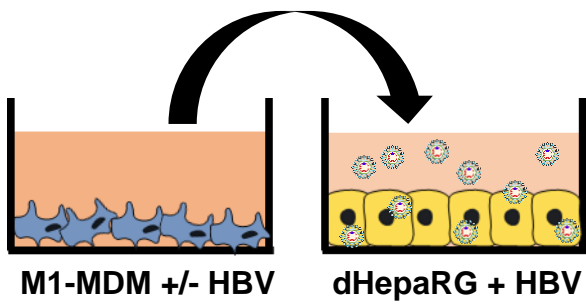
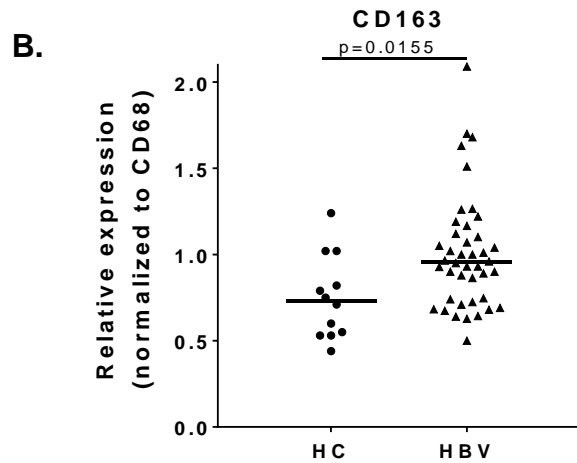
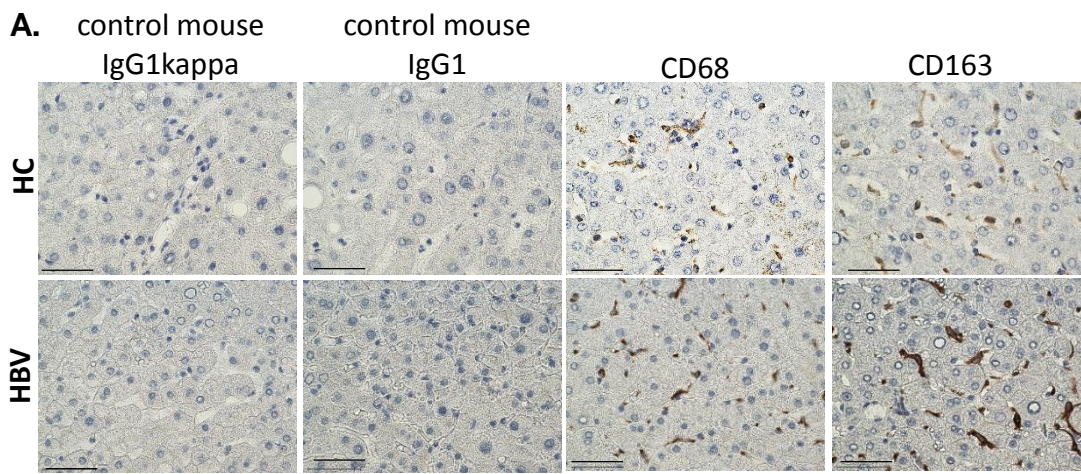
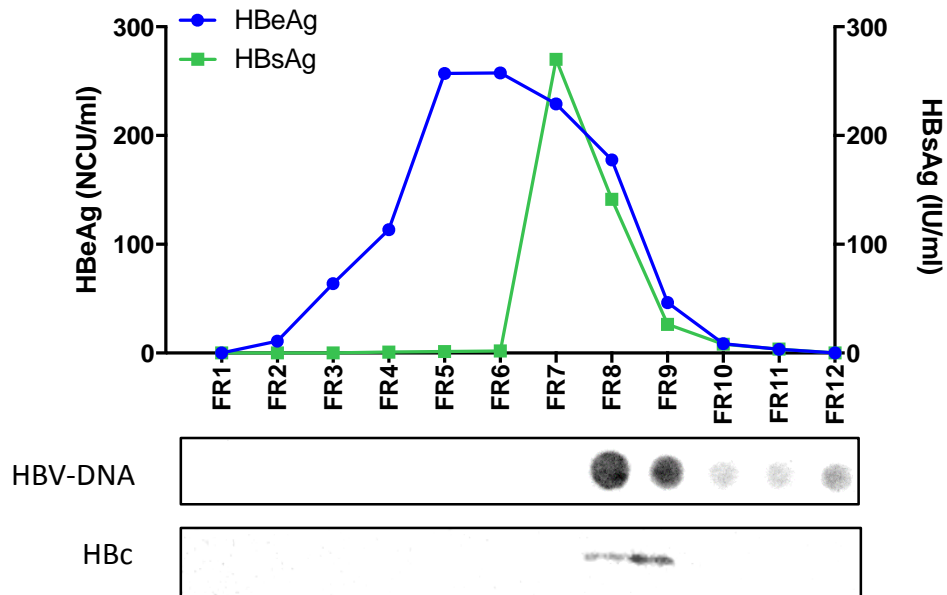


Figure 9

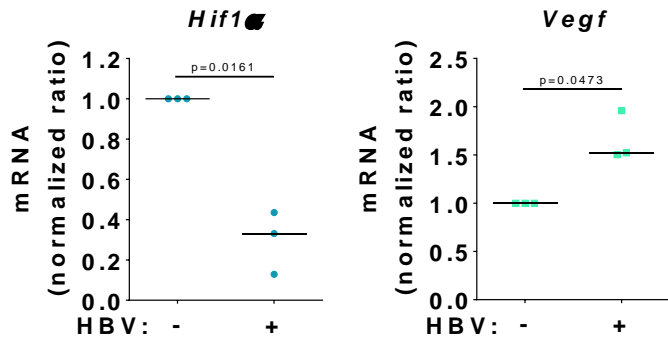


**Supplementary figure 1: The levels of anti-inflammatory macrophages are higher in liver biopsies from HBV infected patients (French cohort).** (A,B) Liver biopsies from healthy controls (HC), and HBV positive (HBV) patients were stained with control or target specific antibodies. Representative photos of the stainings are shown. The black bars represent 50  $\mu$ m. (B) The levels of positive CD163 signals were quantified compared to the levels of positive CD68 signals. Horizontal bars represent the median of values and data were submitted to Kruskal Wallis test.



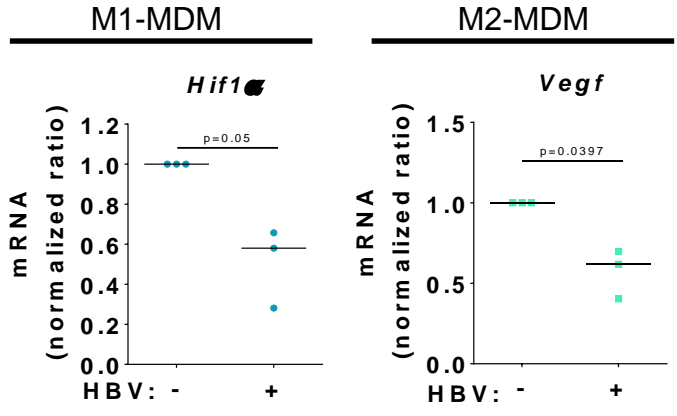
**Supplementary figure 2: Representative characterization of HBV inoculum.** Supernatants from HepAD38 were concentrated by ultracentrifugation and characterized by analysis of the fractions from a 5.6-56% iodixanol gradient. Viral parameters were assessed in each fractions by ELISA (HBeAg and HBsAg), dot blot (HBV DNA), and western-blot (HBeAg) analyses. Representative characterization of one of the three HBV inoculums used for this study.

## LPS-stimulated-Liver MΦ

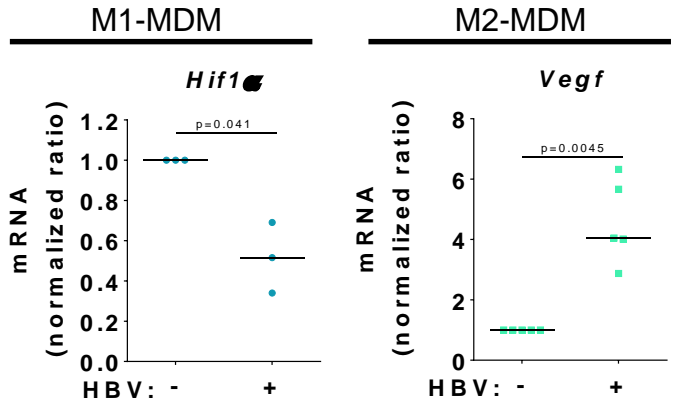


**Supplementary figure 3: Levels of *HIF1α* and *VEGF* mRNAs in liver MΦ exposed to HBV.** Liver MΦ were isolated, exposed or not to HBV for 24h before stimulation with 100 ng/ml of LPS for 3h. Cells were harvested, total RNA were extracted and the levels of the indicated mRNA were assessed by RT-qPCR. Results represent the mean of three independent experiments (three different donors). Horizontal bars represent the median and data were submitted to Mann Whitney test.

**A. MDM exposed to HBV during their differentiation**

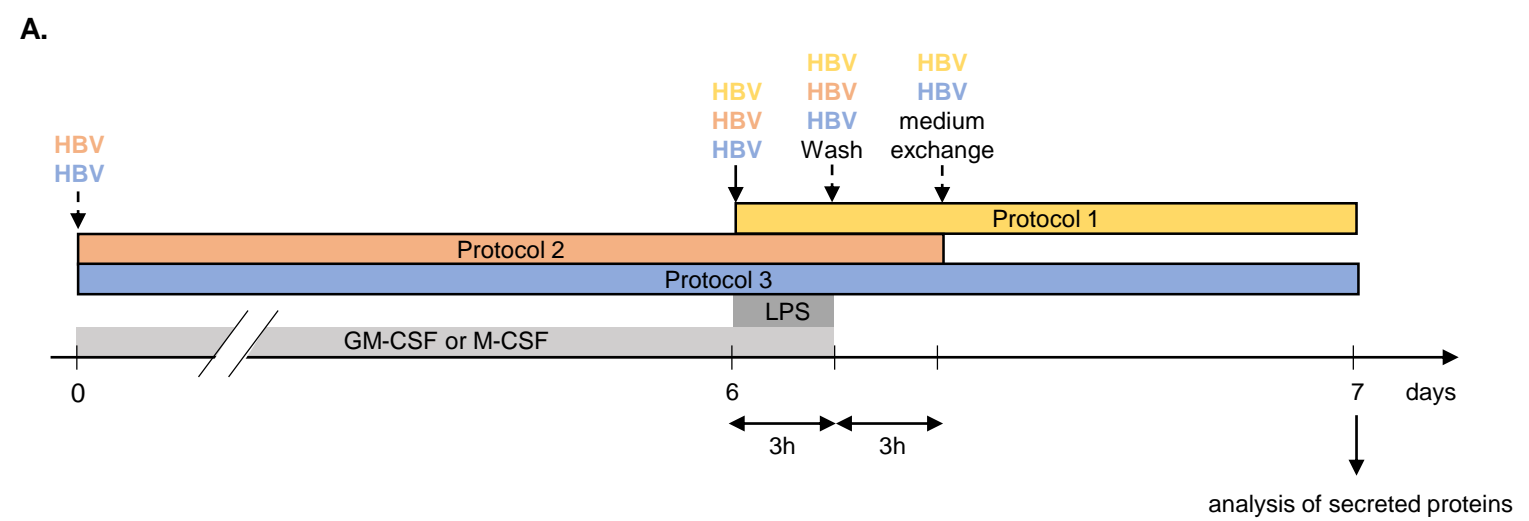


**B. MDM exposed to HBV during their activation**

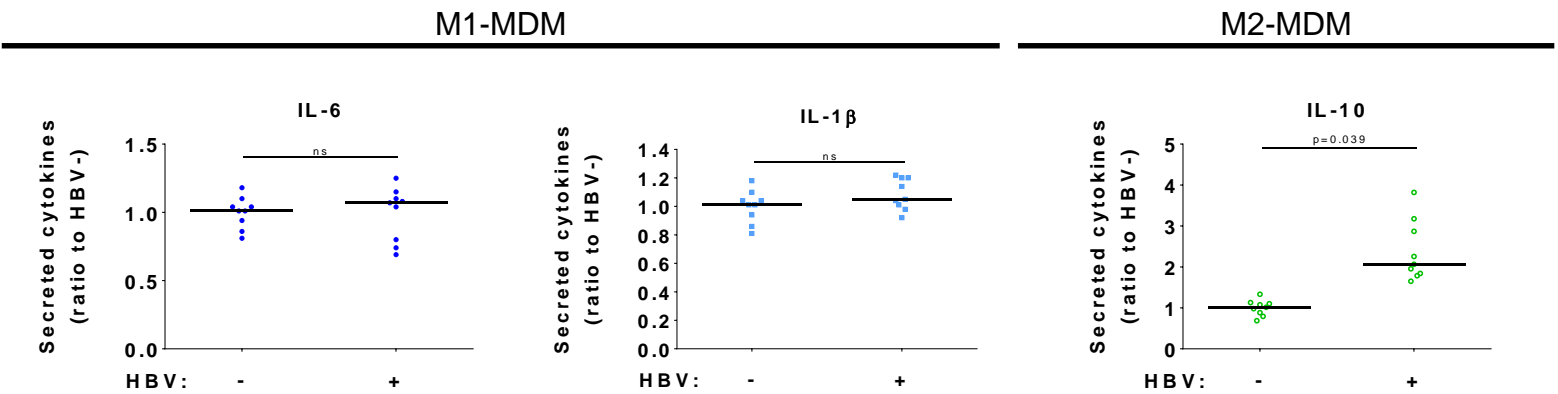


**Supplementary figure 4: Levels of *HIF1α* and *VEGF* mRNAs in MDM exposed to HBV.** (A-B) Monocytes were purified from peripheral blood mononuclear cells, differentiated into M1-MDM (upon GM-CSF culture) or M2-MDM (upon M-CSF culture) in the presence or not of HBV (A) during differentiation and activation or (B) during stimulation with 10 ng/ml of LPS for 3 hours. (A, B) Cells were harvested, total RNA were extracted and the levels of the indicated mRNA were assessed by RT-qPCR. Results represent the mean of three independent experiments (three different donors). Horizontal bars represent the median and data were submitted to Mann Whitney test.

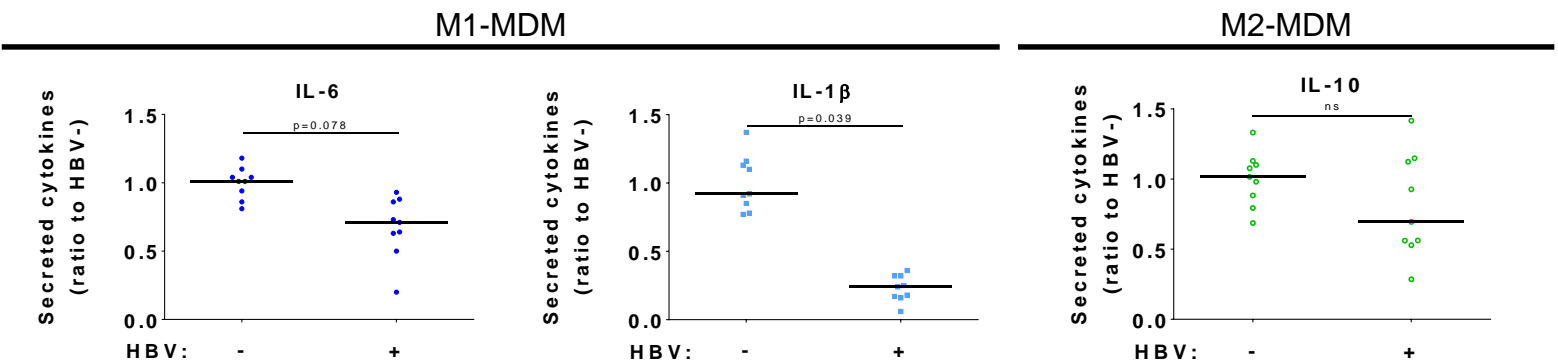




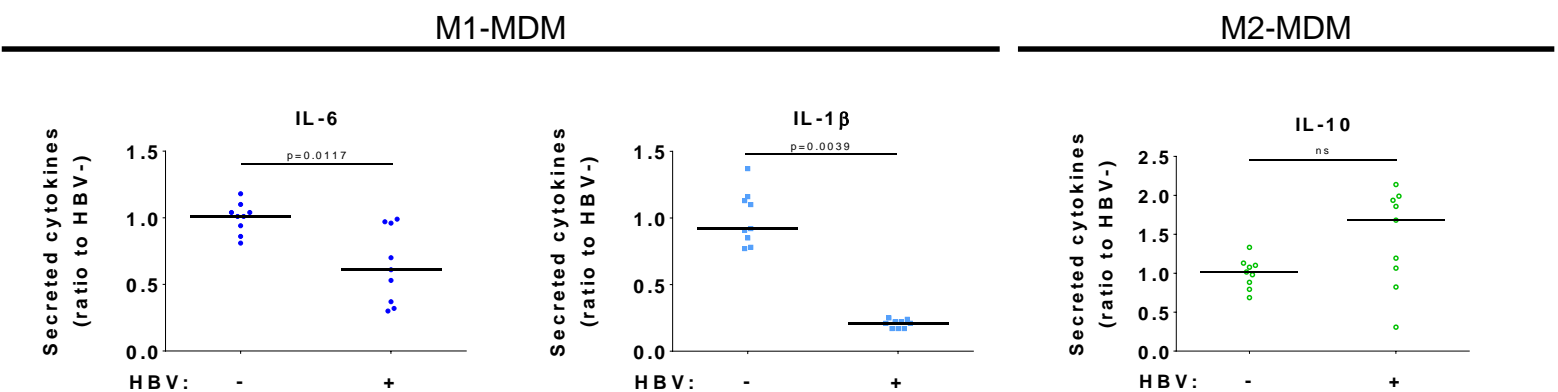
**B. M1-MDM and M2-MDM exposed to HBV according to protocol 1**



**C. M1-MDM and M2-MDM exposed to HBV according to protocol 2**

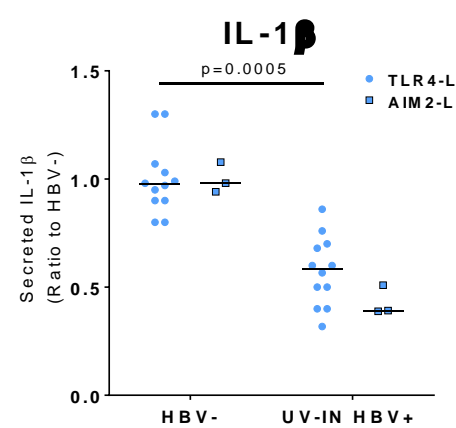
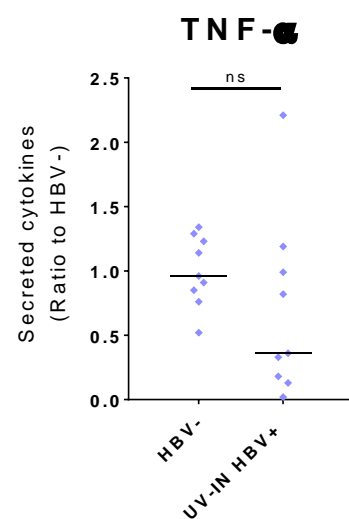
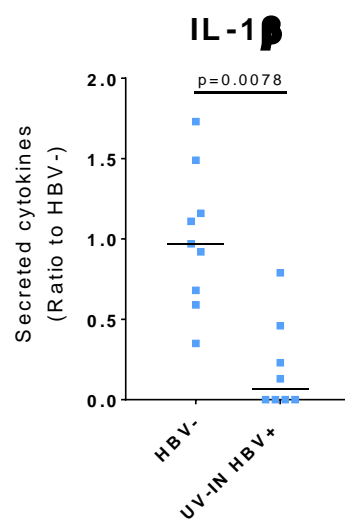
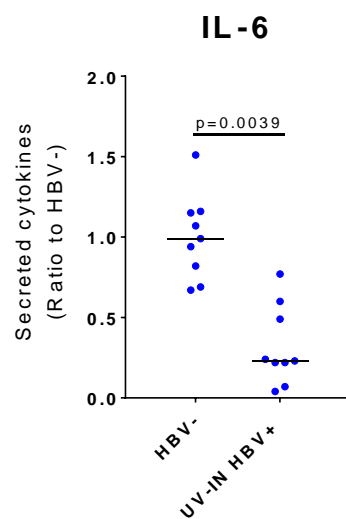
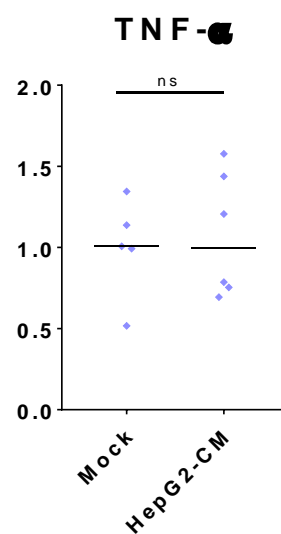
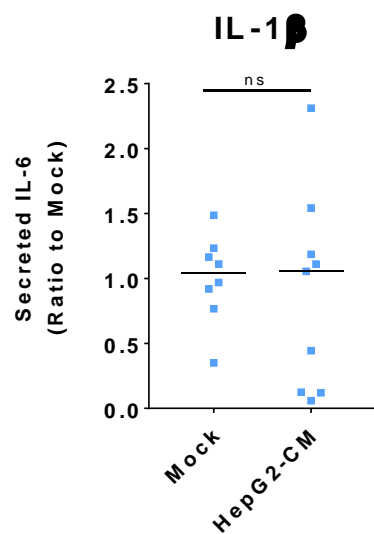
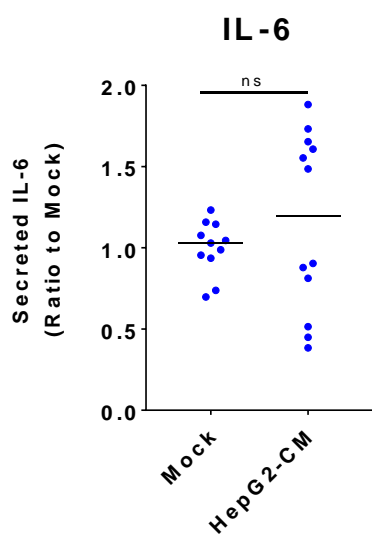
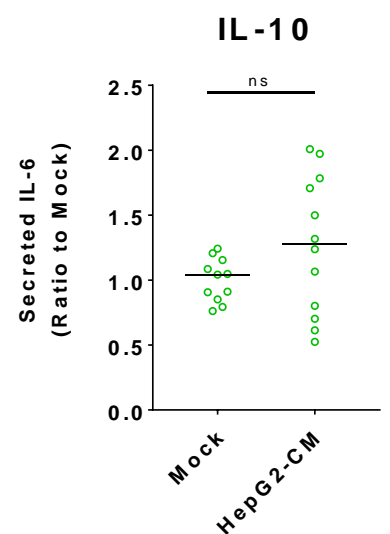


**D. M1-MDM and M2-MDM exposed to HBV according to protocol 3**

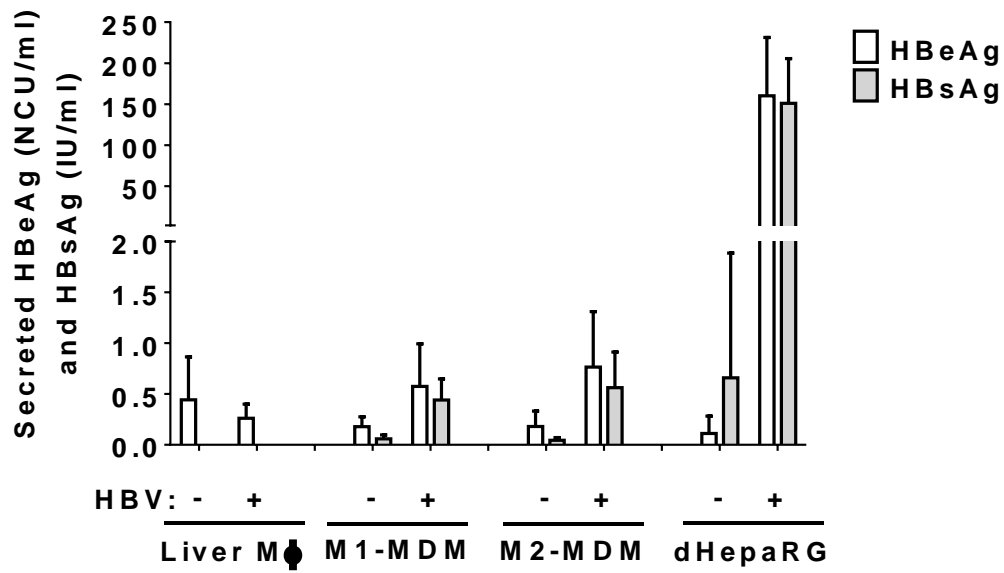


**Supplementary figure 5: HBV interfere with M1-MDM differentiation and increase M2-MDM activation.**

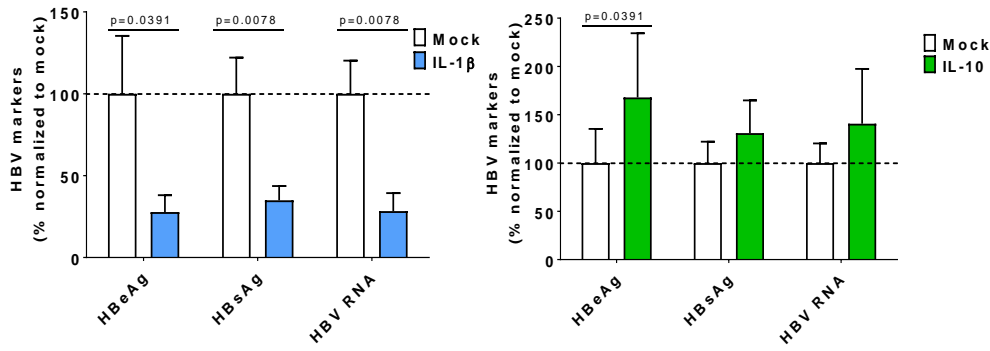
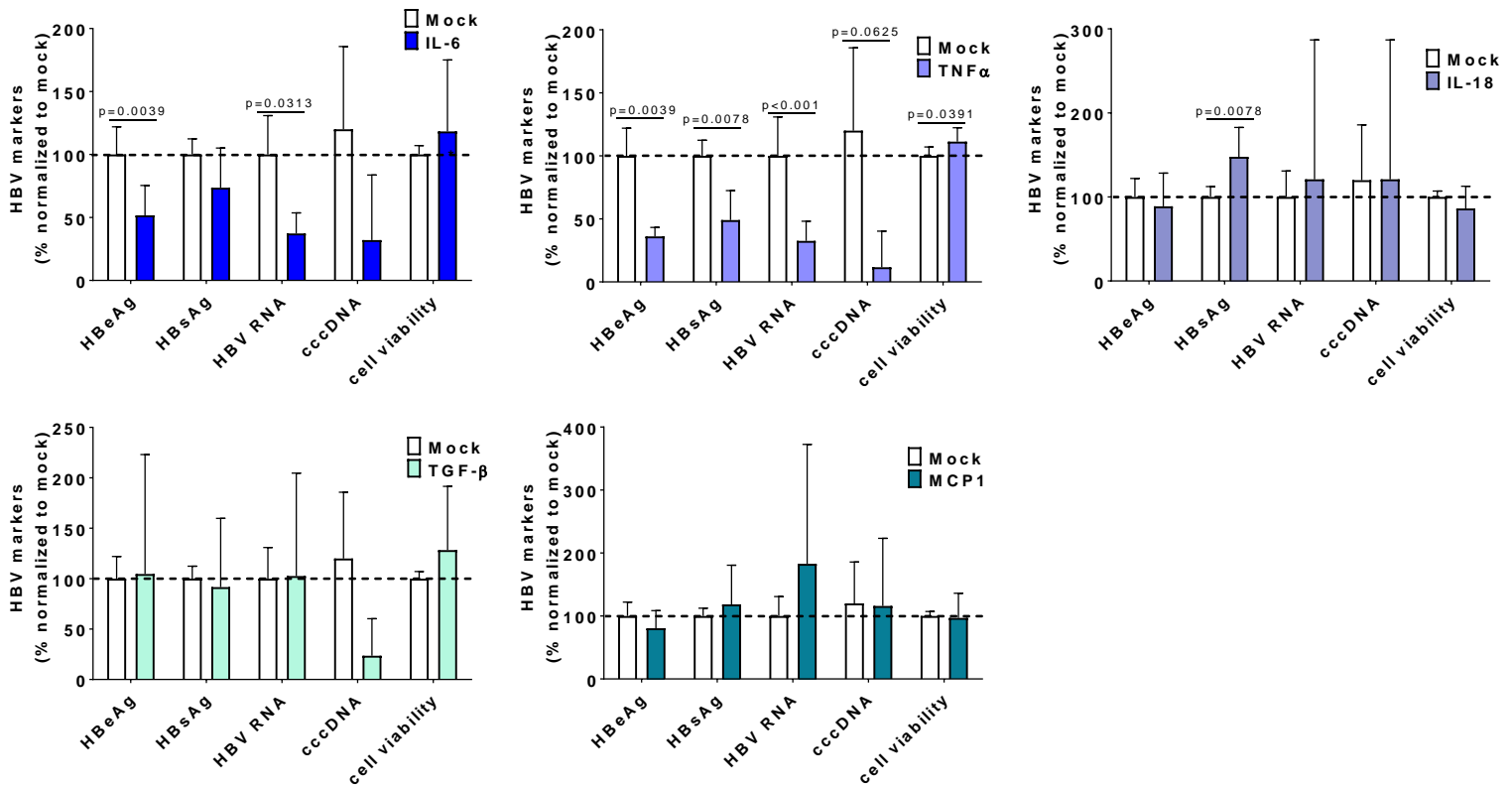
Monocytes were purified from peripheral blood mononuclear cells, differentiated into M1-MDM (upon GM-CSF culture) or M2-MDM (upon M-CSF culture) and stimulated with 10 ng/ml of LPS for 3 hours, and HBV was added at different time points. (A) Schematic representation of the experiment. Media removals are indicated by dotted arrows. (B-D) Levels of the indicated secreted cytokines were assessed by ELISA and analysed as ratio to non-exposed cells. Results are the mean of three independent experiments (with three different donors) each performed with three biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test.

**A. Liver MΦ****B. M1-MDM****C. M1-MDM****D. M2-MDM**

**Supplementary figure 6: UV-inactivated HBV has similar effect as HBV on cytokines secretion by liver MΦ and/or M1-MDM and concentrated medium from non-infected hepatocytes does not modified cytokines secretion.** (A) Liver MΦ were isolated from liver resection, exposed to medium (HBV-) or to an UV-inactivated HBV (UV-IN HBV+) for 24h, and stimulated with 100 ng/ml of LPS (TLR4-L) or 100 ng/ml of LPS + 100 ng/ml of Poly (dA:dT) (AIM2-L) for another 24h. (B,C,D) Monocytes were differentiated into M1-MDM (upon GM-CSF culture) or M2-MDM (upon M-CSF culture) in the presence or not of (B) HBV inactivated with UV light or (C,D) concentrated medium of non-infected of HepG2 (HepG2-CM), during M1-MDM differentiation and activation or during M2-MDM activation, before stimulation with 10 ng/ml of LPS for 3 hours. (A,B,C,D) Cytokine levels were assessed by ELISA and analysed as ratio to non-exposed cells (HBV- or Mock). Results are the median of one or four independent experiments (with one to four different donors) performed with three biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test.

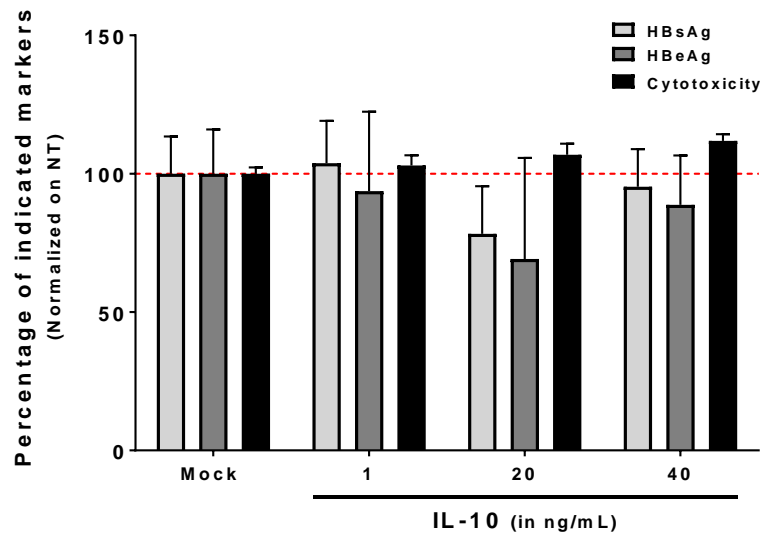
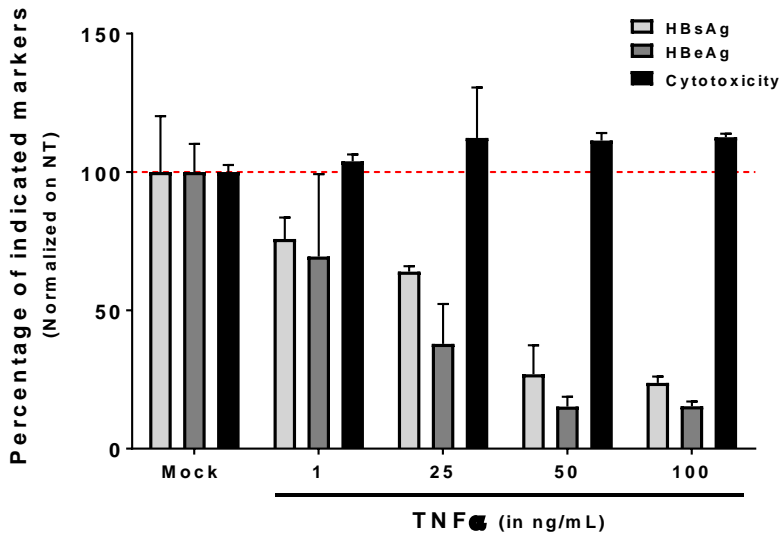
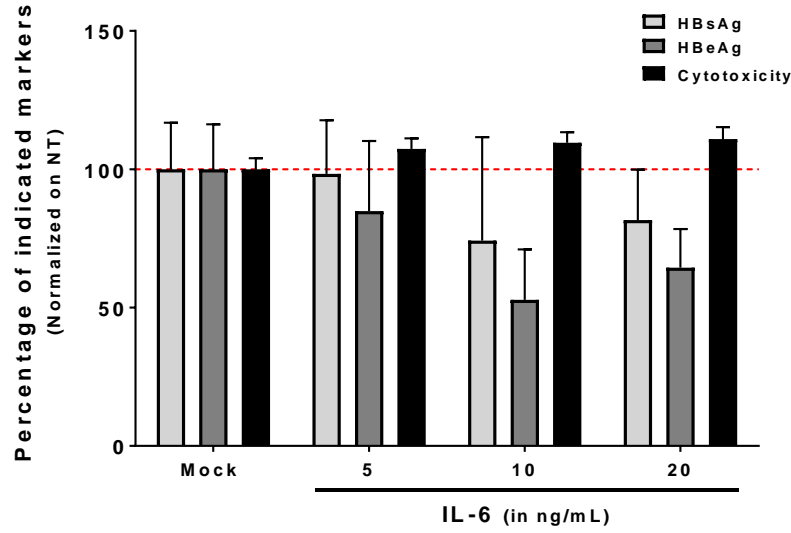
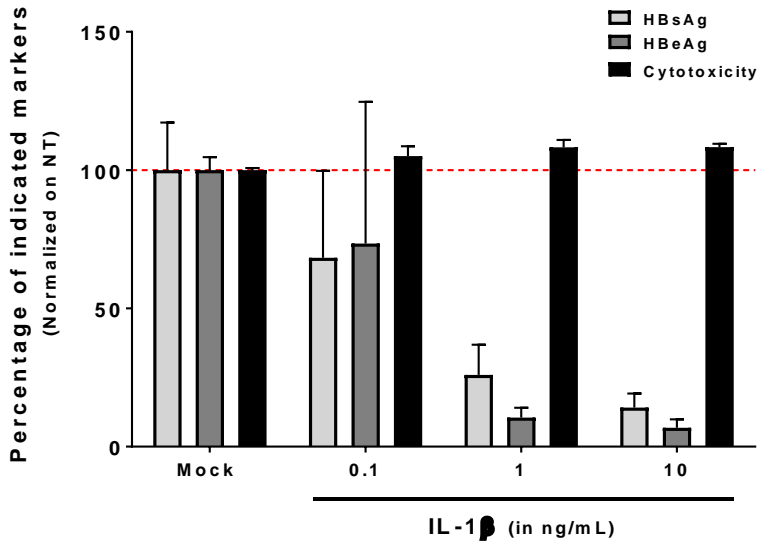


**Supplementary figure 7: HBV did not replicate in macrophages.** Liver MΦ and dHepaRG were exposed to HBV for one day and M1-MDM and M2-MDM for six days. Supernatant were collected and levels of HBeAg and HBsAg were determined by ELISA seven days post exposure. Data are presented as mean +/- standard deviation of three independent experiments (three batches of dHepaRG and three different donors) each performed in three biological replicates.

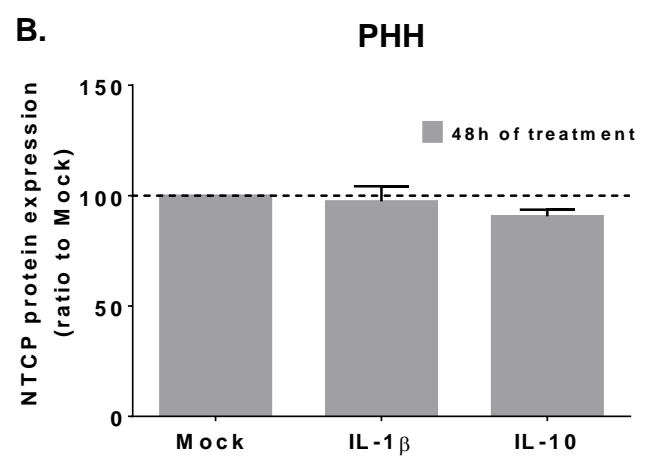
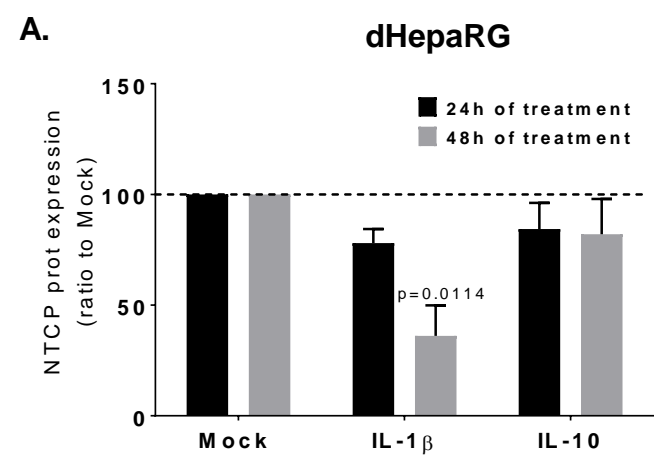
**A.****B.**

### Supplementary figure 8: Effect of recombinant cytokines on the establishment of HBV infection.

Differentiated HepaRG cells were treated with the indicated recombinant cytokines (A) 24h before or (B) 24h before and during infection with HBV. (A,B) Seven days post infection, supernatants were collected and levels of HBeAg and HBsAg were quantified by ELISA. Cells were harvest, total RNA or DNA were extracted and levels of HBV RNAs and cccDNA were quantified by RT-qPCR or qPCR analyses. Cell viability was assessed by neutral red uptake assay. Results, presented as ratio to non-treated cells, are the mean  $\pm$  standard deviation of three independent experiments each performed with three biological replicates. Data were submitted to Wilcoxon test.



**Supplementary figure 9: Dose response of recombinant cytokines on the establishment of HBV infection.** Differentiated HepaRG cells were treated with the indicated recombinant cytokines 24h before and during infection with HBV. Seven days post infection, supernatants were collected and levels of HBeAg and HBsAg were quantified by ELISA. Cell viability was assessed by neutral red uptake assay. Results, presented as ratio to non-treated cells, are the mean +/- standard deviation of two independent experiments each performed with three biological replicates.



**Supplementary figure 10: NTCP levels in dHepaRG and PHH treated with IL-1 $\beta$ .** (A) Differentiated HepaRG cells were treated with recombinant IL-1 $\beta$ , and IL-10 for 24h or 48h. (B) PHH were treated with recombinant IL-1 $\beta$ , and IL-10 for 48h. (A-B) NTCP protein expression was analysed by flow cytometry. Results are presented in percentage normalised to the non-treated cells. Data are presented as mean  $\pm$  SD of three independent experiments (three batches of dHepaRG cells and three donors of PHH). Data were submitted to Kruskal Wallis test.

**Table S1.** Patients characteristic of the Swiss/German cohort (livers biopsies analyzed by IHC). N: negative; P: positive.

ID	Group	Pathology	Grading hepatitis*	Staging hepatitis*	HBsAg (% hepatocytes)	HBcAg (N or P)	Delta-Antigen (N or P)	Fibrosis (1 to 4)	Necro-inflammatory activity
P#1	Non-infected controls (HC)	Moderate hepatitis	-	-	0	N	N	1	-
P#2		Unclear	-	-	0	N	N	-	-
P#3		Minimal steatohepatitis	-	-	0	N	N	-	-
P#4		Severe sclerosing steatohepatitis	-	-	0	N	N	-	-
P#5		Sclerosing steatohepatitis	-	-	0	N	N	-	-
P#6		Moderate to high sclerosing hepatitis	-	-	0	N	N	-	-
P#7		High sclerosing steatohepatitis	-	-	0	N	N	-	-
P#8		Moderate sclerosing steatohepatitis	-	-	0	N	N	-	-
P#9		Minimal steatohepatitis	-	-	0	N	N	-	-
P#10		Minimal sclerosing steatohepatitis	-	-	0	N	N	-	-
P#11		Moderate sclerosing steatohepatitis	-	-	0	N	N	-	-
P#12		Minimal sclerosing steatohepatitis	-	-	0	N	N	-	-
P#13		High sclerosing steatohepatitis	-	-	0	N	N	2	-
P#14	HBV-infected patients (HBV)	Chronic Hepatitis B	H-2	H-3-4	20	N	N	-	-
P#15		Chronic Hepatitis B	I-3	I-1	90	P	N	1	1
P#16		Chronic Hepatitis B	I-3	I-2	<5	N	N	-	-
P#17		Chronic Hepatitis B	I-3	I-3	<1	N	N	-	-
P#18		Chronic Hepatitis B	H-2	H-2	50	P	N	-	-
P#19		Chronic Hepatitis B	I-5	I-2	20	N	N	2	1
P#20		Chronic Hepatitis B	I-3	I-1	10	N	N	-	-
P#21		Chronic Hepatitis B	I-4	I-2	60	N	N	2	1
P#22		Chronic Hepatitis B	I-3	I-1	10	N	N	1	1
P#23		Chronic Hepatitis B	H-2	H-3-4	20	N	N	-	-
P#24		Chronic Hepatitis B	I-9	I-6	70	N	N	-	-
P#25		Chronic Hepatitis B	I-6	I-6	<1	N	N	4	1
P#26		Chronic Hepatitis B	I-3	I-1	5	N	N	2	1
P#27		Chronic Hepatitis B	I-7	I-6	>90	P	N	4	2
P#28		Chronic Hepatitis B	I-11	I-3	80	N	N	3	3
P#29		Chronic Hepatitis B	I-4	I-4	80	N	N	1	1
P#30		Chronic Hepatitis B	I-13	I-6	80	P	N	4	3
P#31		Chronic Hepatitis B	I-2	I-1	70	N	N	1	1
P#32		Chronic Hepatitis B	I-2	I-0	90	N	N	0	1
P#33		Chronic Hepatitis B	I-1	I-0	95	P	N	0	1
P#34		Chronic Hepatitis B	I-2	I-1	70	N	N	1	1
P#35		Chronic Hepatitis B	I-2	I-3	5	N	N	3	1
P#36		Chronic Hepatitis B	I-6	I-2	5	N	N	2	1
P#37		Chronic Hepatitis B	I-2	I-2	10	N	N	1	1
P#38		Chronic Hepatitis B	I-5	I-5	5	N	N	2	2
P#39		Chronic Hepatitis B	I-10	I-4	50	N	N	3	2

\* H=Hytioglou; I=Ishak et al.

**Table S2.** Patients characteristic from the French cohort (livers biopsies analyzed by IHC). N: negative; P: positive.

ID	Group	Age	Sexe	Treatment	HBsAg (IU/mL)	HBeAg (N or P)	HBV DNA (IU/mL)	cccDNA/cell	Total HBV DNA/cell	rcDNA/ cell	Replicative acitivity	ALAT (IU/ml)	Fibrosis (1 to 4)	Necro-inflammatory activity
P#1	Healthy control (HC)	84	F	None	-	-	-	-	-	-	-	-	-	-
P#2		66	M	None	-	-	-	-	-	-	-	-	-	-
P#3		76	M	None	-	-	-	-	-	-	-	-	-	-
P#4		49	M	None	-	-	-	-	-	-	-	-	-	-
P#5	HBV infected (HBV)	23	F	None	6511	N	31	0,02	0,74	0,72	31,59	15	1	1
P#6		36	M	None	1555	N	50	0,09	12,09	11,99	127,86	42	1	0
P#7		42	F	None	53,13	N	86	0,03	0,71	0,68	20,50	35	0	1
P#8		59	M	None	5926	N	440	0,02	1,89	1,87	89,17	27	1	0
P#9		40	M	None	10681	N	541	0,08	5,54	5,45	65,07	65	1	0
P#10		42	M	None	3250	N	25 238	0,41	3,09	2,68	6,58	78	3	1
P#11		42	M	None	1052	N	27 343	0,80	38,57	37,76	46,93	47	1	1
P#12		45	M	None	1205	N	17 263	0,18	2,33	2,15	11,74	62	1,5	0
P#13		32	M	None	26910	N	93 709	0,13	11,12	10,98	82,79	115	1	1
P#14		60	M	None	7170	N	1 511 762	0,34	3,75	3,41	10,12	45	1	1
P#15		28	M	None	8069	P	32 000 000	6,35	4005,87	3999,52	629,75	68	1	1
P#16		34	M	None	10539	P	139 181	0,18	28,61	28,43	155,61	61	3	1
P#17		32	M	None	66820	P	68 275 329	2,04	1975,58	1973,55	968,95	106	1	1
P#18		30	M	None	8051	P	15 800 000	4,47	5734,49	5730,02	1281,31	75	2	1



**Table S3.** Antibodies' information.

TARGET PROTEIN	TYPE	COMPANY	CATALOG NUMBER	NEGATIVE CONTROL ANTIBODY
<b>CD163</b>	IgG1	Abcam	ab74604	Biolegend 400143
<b>CD68</b>	IgG	Abcam	ab955	Abcam ab18448
<b>HBc</b>	Polyclonal	Diagnostic Biosystems	RP 017-05	-
<b>ANTI-RABBIT</b>	IgG/HRP	DAKO	P0448	
<b>ANTI-MOUSE</b>	IgG/HRP	DAKO	P0447	
<b>NTCP</b>	IgG	Abcam	ab175289	Secondary antibody only

**Table S4.** Recombinant cytokine references and working concentration

<b>Cytokine</b>	<b>Reference</b>	<b>Concentration</b>
TNF $\alpha$	Life Technologies PHC3016	50 ng/ml
IL-1 $\beta$	MBL International Corporation JM4128-10	1 ng/ml
IL-6	R&D Systems 206-IL/CF	10 ng/ml
IL-18	MBL International Corporation B003-5	10 ng/ml
IL-10	Biovision # 4155-10	20 ng/ml
TGF- $\beta$ 1	Biovision # 4342-5	5 ng/ml
MCP-1	R&D 279-MC-010	1 ng/ml

**Table S5.** Primers sequences

<b>GENE</b>	<b>FORWARD</b>	<b>REVERSE</b>
<b>GUS</b>	CGTGGTTGGAGAGCTCATTTGGAA	ATTCCCAGCACTCTCGTCGGT
<b>HBV</b>	GGAGGGATACATAGAGGTTCTTGA	GTTGCCCGTTTGTCTCTAATTC
<b>HBV cccDNA</b>	ATGGTGAGGTGAACAATGCT	GACTCTCTCGTCCCCTTCTC
<b>RPLP0</b>	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCCAAAGGAGAAG
<b>HIF1<math>\alpha</math></b>	CCACCTCTGGACTTGCCTTT	CTCCATGGTGAATCGGTCCC
<b>VEGF</b>	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA

**Table S6.** Supernatant from LPS-activated M2-MDM from one donor was collected and the indicated cytokines were determined by ELISA.

MDM Cytokines (ng/ml)	M2	
	HBV -	HBV + protocol 2
IL-6	0	0
IL-1 $\beta$	0,02	0,03
IL-12	0	0
TNF $\alpha$	0	0
IL-10	3,3	5,7

**Table S7.** Supernatant from LPS-activated M1-MDM from at least five different donors were pooled, concentrated by ultrafiltration and diluted in dHepaRG medium to the final concentration indicated.

<b>MDM</b>	<b>M1</b>		
	<b>HBV -</b>	<b>HBV + protocol 1</b>	<b>HBV + protocol 2</b>
<b>Cytokines (pg/ml)</b>			
<b>IL-6</b>	1063	585	828
<b>IL-1<math>\beta</math></b>	41	4	42
<b>TNF<math>\alpha</math></b>	1829	1492	1749
<b>IL-10</b>	39	26	40