Hepatitis B virus-induced modulation of liver macrophage function promotes hepatocyte infection
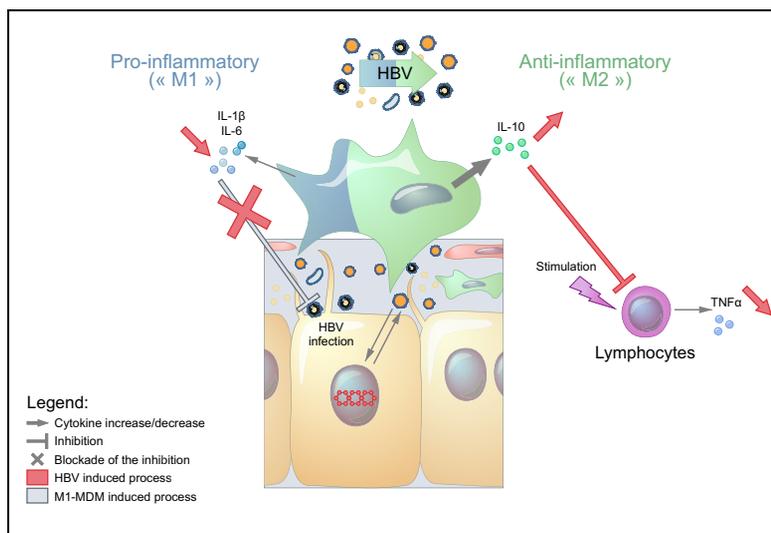
Graphical abstract

Authors

Suzanne Faure-Dupuy, Marion Delphin, Ludovic Aillot, ..., Mathias Heikenwälder, David Durantel, Julie Lucifora

Correspondence

david.durantel@inserm.fr (D. Durantel), julie.lucifora@inserm.fr (J. Lucifora)

Lay summary

Hepatitis B virus modulates liver macrophage function in order to favour the establishment and likely maintenance of infection. It impairs the production of the antiviral cytokine IL-1β, while promoting that of IL-10 in the microenvironment. This phenotype can be recapitulated in naive liver macrophages or monocyte-derived-macrophages *ex vivo* by short exposure to the virus or cells replicating the virus, thus suggesting an "easy to implement" mechanism of inhibition.

Highlights

- Hepatitis B virus proteins are observed in liver macrophages from patients.

- Hepatitis B virus impairs pro-inflammatory macrophage secretion.

- Hepatitis B virus increases anti-inflammatory macrophage secretion.

- Impairment of pro-inflammatory secretions favours the establishment of hepatitis B virus infection.

- Increase of IL-10 secretion further impairs lymphocyte activation.

Hepatitis B virus-induced modulation of liver macrophage function promotes hepatocyte infection

Suzanne Faure-Dupuy[1,2,†], Marion Delphin[1,†], Ludovic Aillot[1], Laura Dimier[1], Fanny Lebossé[1,3], Judith Fresquet[1], Romain Parent[1], Matthias Sebastian Matter[4], Michel Rivoire[5], Nathalie Bendriss-Vermare[1], Anna Salvetti[1], Danijela Heide[2], Lalo Flores[6], Klaus Klumpp[6], Angela Lam[6], Fabien Zoulim[1,3,7], Mathias Heikenwälder[2], David Durantel[1,7,*,‡], Julie Lucifora[1,*,‡]

[1]INSERM, U1052, Cancer Research Center of Lyon (CRCL), Université de Lyon (UCBL1), CNRS UMR_5286, France; [2]Division of Chronic Inflammation and Cancer, German Cancer Research Center (DKFZ), Heidelberg, Germany; [3]Department of Hepatology, Croix-Rousse Hospital, Hospices Civils de Lyon, Lyon, France; [4]University Hospital of Basel, Institute of Pathology, Basel, Switzerland; [5]INSERM U1032, Centre Léon Bérard (CLB), Lyon, France; [6]Novira Therapeutics, Part of the Janssen Pharmaceutical Companies, United States; [7]DEVweCAN Laboratory of Excellence, Lyon, France

Background & Aims: Liver macrophages can be involved in both pathogen clearance and/or pathogenesis. To get further insight on their role during chronic hepatitis B virus (HBV) infections, our aim was to phenotypically and functionally characterize *in vivo* and *ex vivo* the interplay between HBV, primary human liver macrophages (PLMs) and primary blood monocytes differentiated into pro-inflammatory or anti-inflammatory macrophages (M1-MDMs or M2-MDMs, respectively).
Methods: PLMs or primary blood monocytes, either *ex vivo* differentiated into M1-MDMs or M2-MDMs, were exposed to HBV and their activation followed by ELISA or quantitative reverse transcription PCR (RT-qPCR). Liver biopsies from HBV-infected patients were analysed by RT-qPCR or immunohistochemistry. Viral parameters in HBV-infected primary human hepatocytes and differentiated HepaRG cells were followed by ELISA, qPCR and RT-qPCR analyses.
Results: HBc protein was present within the macrophages of liver biopsies taken from HBV-infected patients. Macrophages from HBV-infected patients also expressed higher levels of anti-inflammatory macrophage markers than those from non-infected patients. *Ex vivo* exposure of naive PLMs to HBV led to reduced secretion of pro-inflammatory cytokines. Upon exposure to HBV or HBV-producing cells during differentiation and activation, M1-MDMs secreted less IL-6 and IL-1β, whereas M2-MDMs secreted more IL-10 when exposed to HBV during activation. Finally, cytokines produced by M1-MDMs, but not those produced by HBV-exposed M1-MDMs, decreased HBV infection of hepatocytes.
Conclusions: Altogether, our data strongly suggest that HBV modulates liver macrophage functions to favour the establishment of infection.

Lay summary: Hepatitis B virus modulates liver macrophage function in order to favour the establishment and likely maintenance of infection. It impairs the production of the antiviral cytokine IL-1β, while promoting that of IL-10 in the microenvironment. This phenotype can be recapitulated in naive liver macrophages or monocyte-derived-macrophages *ex vivo* by short exposure to the virus or cells replicating the virus, thus suggesting an "easy to implement" mechanism of inhibition.

Introduction

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

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

Several pro-inflammatory cytokines (IL-6, IL-1β and TNFα) and interferons (IFNα and IFNγ) were shown to induce a direct antiviral effect on HBV replication in hepatocytes,[4–7] with IL-1β

being one of the most efficient at inhibiting already established HBV infections *in vitro*.[4] IL-1β is a pro-inflammatory cytokine produced upon inflammasome activation.[8] In the liver, it is mostly produced by macrophages (MΦ) since hepatocytes do not possess functional inflammasomes.[9,10] Liver resident MΦ, named Kupffer cells (KCs), represent 80% of the MΦ count in the body at steady state.[11] They are specialised in the detection of pathogens coming from the enteric circulation, as well as in the elimination of aging blood cells, through their high phagocytic capacity.[11] Upon inflammation, monocytes from the blood circulation can be recruited in the liver and differentiate locally into MΦ that are called monocyte-derived-macrophages (MDMs).[12] KCs and MDMs have different embryonic origins and functions.[13] *In vivo*, a wide range of different phenotypes of MΦ exist, depending on their origin, activation status, localisation, and their micro-environment.[14] Inflammatory MΦ, commonly called M1, which are phenotypically described as CD40^{+} CD86^{+} HLA-DR^{+}, are characterised by their capacity to secrete pro-inflammatory cytokines/chemokines, such as IL-1β, produce various antimicrobial factors, including nitric oxide, and are therefore implicated in inflammation and elimination of pathogens.[14] Anti-inflammatory MΦ, commonly called M2, express the arginase 1, mannose receptors, and the high affinity scavenger receptor CD163, and secrete anti-inflammatory cytokines, such as IL-10 and TGFβ, as well as angiogenic factors, such as VEGF.[14] M2 MΦ are involved in the resolution of inflammation and in tolerance mechanisms.[14] They are also found abundantly in the tumour microenvironment and are called tumour-associated MΦ (TAMs).[15]

Several studies showed that HBV can influence the phenotype and biology of MΦ,[16] but few have been performed using liver resident or infiltrating primary human cells. To get further insights into the role of liver MΦ in the establishment of HBV infection in hepatocytes, we used highly relevant models such as primary human liver MΦ, pro-inflammatory/M1-like or anti-inflammatory/M2-like MΦ differentiated *in vitro* from primary blood monocytes, liver biopsies, primary human hepatocytes (PHHs), differentiated HepaRG cells (dHepaRGs), and co-culture models.

Materials and methods
Patient samples
Two different cohorts were analysed. The collection and analysis of human patient samples of the first cohort (Swiss/German cohort) was approved by the ethics committee of Northern Switzerland (EKNZ, Study ID: PB_2018-00308, 310/12), confirmed to the ethical guidelines of the 1975 Declaration of Helsinki, and was provided by the Department of Pathology, Basel, Switzerland. The biopsies were stained in Germany. Patients' characteristics are presented in Table S1. Samples from the second cohort were used under the French IRB 'CPP Sud-Est IV' approval #11/040 (2011) from the 'Biobanque INSERM CRCL Hépatologie (U1052)', France #DC2008-235 and stained in France. Written informed consent was obtained from each patient. Patient characteristics are presented in Table S2.

Immunohistochemistry
Paraffin was removed from formalin-fixed paraffin-embedded samples by successive alcohol bathes and epitopes were unmasked as previously described.[17] Endogen peroxidases were blocked by a 15 min exposition at room temperature (RT) with Dual Endogenous enzyme Block (DAKO S2003). Saturation was performed by 2 successive incubations at RT of 20 min with horse serum (VECTOR S-2012 Za0328) and 15 min with DAKO antibody diluent (DAKO S3022). Antibodies (Table S3) were incubated overnight at 4 °C in DAKO antibody diluent. Samples were incubated for 15 min at RT with secondary antibodies (Table S3). Coloration was performed with DAB substrate (DAKO K3468) or permanent red substrate (DAKO K0640), and counterstaining with haematoxylin (SIGMA MHS1). Negative controls were performed using control IgGs (Table S3) or non-infected patients, and no staining was observed (Fig. S1). Optical density (OD = log [max intensity/mean intensity]) of the DAB staining was quantified using Fiji (ImageJ) and the results are presented as 1/OD. For the CD68, HBcAg double staining, a BONDMax robotic staining machine (Leica) was used.

Primary cell purification and cell culture
Peripheral blood mononuclear cells (PBMCs) from blood donors (Etablissement Français du Sang, EFS; code 895/1052) were isolated by Ficoll gradient (Histopaque®-1077, Sigma) as previously described.[18] Lymphocytes were separated from total PBMCs by a Percoll gradient and further cultured in MΦ medium (RPMI medium supplemented with 10% of decomplemented FBS and 50 U/ml of penicillin/streptomycin). Monocytes were purified by Percoll gradient followed by a negative selection with the Monocyte Isolation Kit II (Miltenyi Biotec) and cultured in MΦ medium. Monocytes were exposed during 6 days to 50 ng/ml of GM-CSF (R&D) or 50 ng/ml of M-CSF from (Peprotech) for M1-MDM or M2-MDM differentiation, respectively. MDMs were activated by a 3 h stimulation with 10 ng/ml of lipopolysaccharide (LPS) (Invivogen). Cells were washed 3 times with PBS and cultured in fresh medium for another 3 h before a last medium exchange. Supernatants and cells were collected 24 h post stimulation (*i.e.* 18 h accumulation). Liver cells were isolated from hepatic resections obtained in collaboration with 3 surgical departments of Lyon (Centre Léon Bérard, Hôpital de la Croix Rousse and centre hospitalier Lyon-Sud) with the French ministerial authorizations (AC 2013-1871, DC 2013 – 1870, AFNOR NF 96 900 sept 2011). After a 2-step collagenase perfusion, the liver extract was filtered and centrifuged, as previously described.[19] PHHs were cultured on the collagen layer and maintained in PHH medium (Williams medium supplemented with 5% of foetal clone II serum, 50 U/ml of penicillin/streptomycin, 1× glutamax, 5 µg/ml of bovine insulin, 5×10^{-5} M of hydrocortisone, and 2% of DMSO). Liver mononuclear cells (LMNCs) or only liver MΦ were purified from the non-parenchymal cells mixture by respectively Ficoll gradients or a 2 phase iodixanol gradient.[20] Liver MΦ were isolated by negative selection using pan monocyte isolation kit (Miltenyi Biotec) and cultured in MΦ medium. HepaRG cells were cultured and differentiated as previously described.[21]

Viral infection
Differentiated HepaRG (dHepaRG) or PHHs were cultured and infected by HBV (PEG-precipitated) as previously described.[22] As PEG-concentrated HBV virions cannot be used for exposition to immune cells, because PEG activates cells, blood monocytes, LMNCs and liver MΦ were exposed to HBV inocula (or control medium), which were prepared by concentrating supernatant from HepAD38[23] (or from non-infected HepG2-NTCP respectively) by filtration/retention centrifugation using column with a cut-off retention of 100 kDa (Merk Millipore; UFC710008).

All virus inocula were tested for the absence of endotoxin (Lonza) and characterised by analyses of the fractions from a 5.6–56% iodixanol gradient and analysed by ELISA, dot blot with HBV DIG-labelled probe[24] and western-blot (DAKO, B0586) (Fig. S2). This allowed us to rule out the presence of non-enveloped nucleocapsids that may activate immune cells, as HBc was shown to be a TLR2 ligand.[25] Blood monocytes, LMNCs and liver MΦ were exposed to at least 3 different batches of HBV (full inoculum concentrated by ultrafiltration) at a multiplicity of infection of 1,000 vge/ml (if not stated otherwise). Viral titre superior to 10^{10} vge/ml were routinely obtained and uniquely used in order to allow a dilution of 100- to 1,000-fold when preparing "infection medium" at a multiplicity of infection of 1,000 vge/cell. This way, we limited the impact of "carried-away" contamination. For some experiments, virus was inactivated by a 30 min exposition to UVB. Stocks of adenovirus vector (AAV) particles were generated by the vector Core of the University of Nantes by calcium phosphate transfection of HEK-293 cells as described previously using pDG8 (as a helper plasmid). The vector particles were purified on caesium chloride gradients as previously described[26] and genome particles/ml (gp/ml) were tittered by quantitative PCR (qPCR). MΦ were exposed to AAV at a multiplicity of infection of 1,000 gp/ml.

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

Quantification of secreted proteins by ELISA
HBeAg and HBsAg secretion were quantified using chemiluminescence immunoassay kit (Autobio) following the manufacturer's instructions. Cytokine secretion was analysed using Duoset ELISA (R&D system) following manufacturer's instructions.

RNA extraction and RT-qPCR
Total mRNA from hepatocytes or MΦ were extracted with NucleoSpin® RNA II or NucleoSpin® RNA XS respectively (Macherey-Nagel). cDNA was synthesised using the SuperScript®III Reverse Transcriptase (Life technologies) according to the manufacturer's instructions. qPCR analyses were performed using "Express SYBR GreenER™ qPCR SuperMix Universal" (Invitrogen). mRNA expression was assessed by comparative cycle threshold (Ct) method ($2^{-\Delta Ct}$); GUS and RPLP0 were used as housekeeping genes for hepatocytes and MΦ respectively. Primers sequences are presented in Table S5.

DNA extraction and cccDNA quantification
Total DNA were extracted using the NucleoSpin® Tissue kit (Macherey-Nagel) following the manufacturer's instructions. Total intracellular DNA was digested for 45 min at 37 °C with T5 exonuclease (epicentre) to remove HBV rcDNA, followed by a 30 min heat inactivation. The amount of cccDNA was quantified by qPCR analyses as previously described.[1]

Cytotoxicity assay
Seven days post treatment, hepatocyte cell viability was assessed by quantification of neutral red uptake by living cells, as previously described.[4]

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

MΦ supernatant concentration
M1- or M2-MDM supernatants from at least 5 different donors were pooled and concentrated by ultrafiltration (using 10 kDa Amicon ultra-15 column; Merk-Millipore) following the manufacturer's instruction. The cytokine concentrations of the concentrated supernatants were assessed by ELISA before dilution to 1/100 in hepatocyte media (experiments from Fig. 9) or dilution to 1/2 in MΦ media (experiments from Fig. 7). Final concentrations are presented in Tables S6 and S7.

Flow cytometry
Cells were washed extensively with PBS and removed from plates with 150 µl of versene-EDTA at 37 °C for 5 min before centrifugation at 1,300 rpm for 5 min at 4 °C. Cells were incubated for 30 min at 4 °C with primary antibody (Table S3). After extensive washes, cells were incubated with secondary antibody in the dark for 30 min at 4 °C. After another wash, staining was measured by Flow Cytometry (BD FACSCALIBUR).

Statistical analysis
Results are presented as mean or median ± standard deviation. Statistical significance was analysed using Prism software. P values are indicated and non-significance is indicated by "n.s.".

Results
HBV is associated with phenotypic changes in liver MΦ in infected patients
To assess if HBV may closely interact with liver MΦ *in vivo*, levels of CD68 expression (identifying MΦ), CD163 (identifying anti-inflammatory MΦ[14]) and HBc (identifying the HBV core protein) were analysed by immunochemistry on liver biopsies from 2 distinct cohorts of patients (see Tables S1 and S2 for details on patients). As expected, signals for CD68 and CD163 were detected in the sinusoidal space (*i.e.*, in between hepatocytes where myeloid cells are), and no signals were observed with control antibodies (Fig. 1 and S1). Co-localised signals were observed for HBc and CD68 in liver samples from HBV-infected patients (Fig. 1A and zoom in Fig. 1B), demonstrating the presence of this HBV protein within liver MΦ. Of note, the proportion of MΦ co-stained with HBc varied from 1 patient to the other; approximatively 20% of all patients analysed lacked co-localisation between HBc and MΦ, 50% displayed less than 5% co-localisation, and 30% displayed between 5% to 20% co-localisation (Fig. 1A, zoom in Fig. 1B). Furthermore, quantification of the signals highlighted a 1.8-fold increase in CD163$^+$ myeloid cells in HBV-infected patients (+79%, $p = 0.0271$) compared to non-infected patients in the Swiss/German cohort and a 1.3-fold increase in the French cohort (Fig. 1C, 1D, S1B). Interestingly, a positive correlation between the level of HBc staining and the increase of CD163$^+$ cells was observed (Fig. 1E). Altogether, these data suggest that HBV interacts with liver MΦ and may affect their phenotype and functions. We therefore performed several *ex vivo* analyses to investigate the
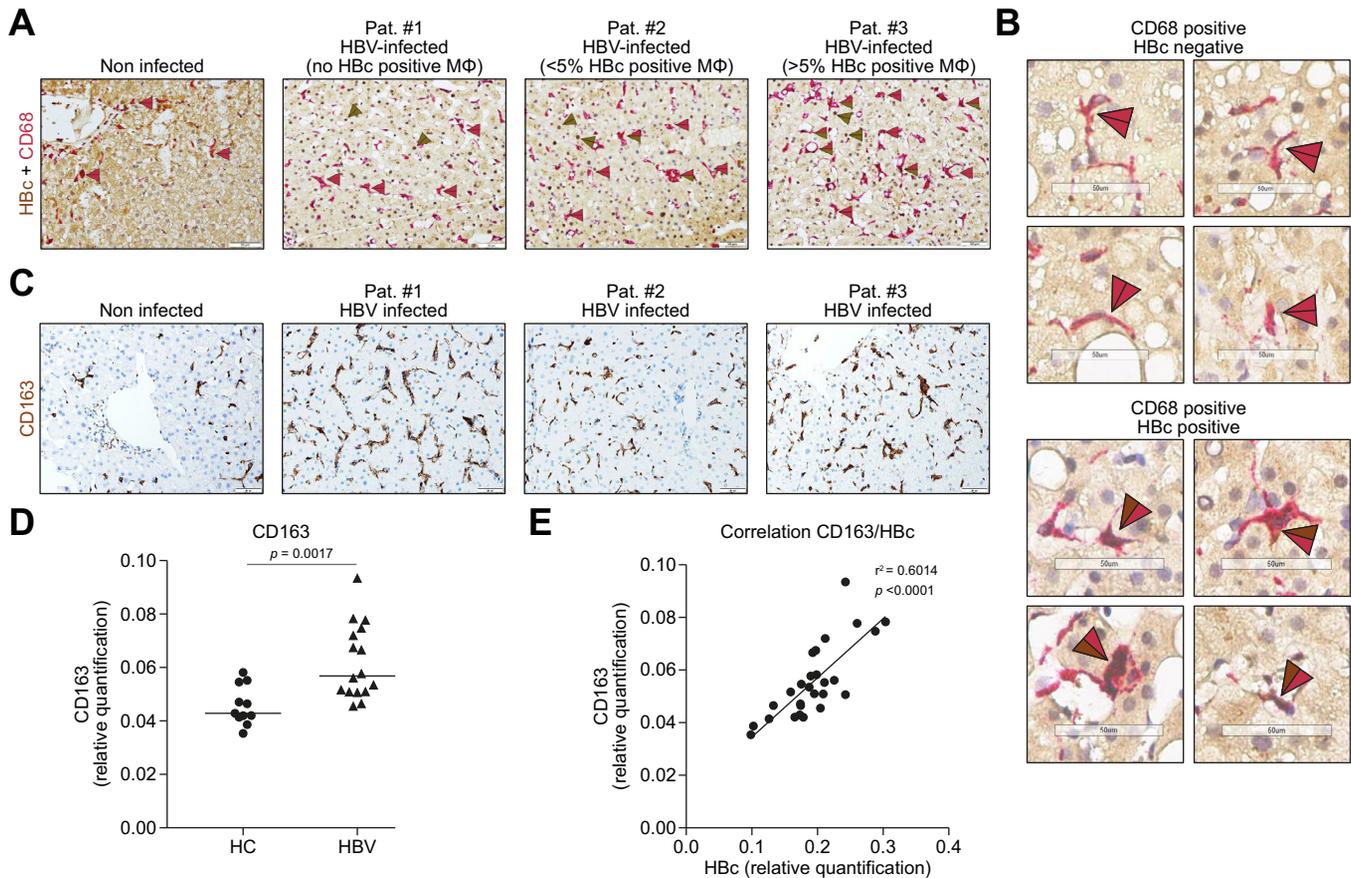
Fig. 1. An HBV protein co-localized with liver MΦ and the proportion of anti-inflammatory MΦ is higher in CHB liver biopsies. HBc (HBV capsid protein), CD68 (total MΦ) and/or CD163 (M2 MΦ), in liver biopsies from non-infected or HBV-infected patients (Table S1), were stained by IHC. (A,B) Representative photos of HBc (brown arrows) and CD68 (pink arrows) stainings. (C) Representative photos of CD163 stainings. (A,B,C) Bars represent 50 μm. (D) Quantification of CD163 stainings. Horizontal bars represent the median of values and data were submitted to Kruskal Wallis test. n.s., not significant. (E) Correlation curve between CD163 and HBc stainings. CHB, chronic hepatitis B; HBV, hepatitis B virus; IHC, immunohistochemistry.

influence of HBV on liver cells with a focus on liver MΦ, including KCs and MDMs.[9,10]

Ex vivo, HBV inhibits the activation of primary liver MΦ by direct exposure

First, we investigated the impact of HBV particles and antigens (concentrated by filtration/retention from HepAD38 cell supernatants and characterised as shown on Fig. S1) on the secretion of cytokines by non-parenchymal primary human liver mononuclear cells (LMNCs) freshly isolated from hepatic resections (5 donors) as previously described.[27] Of note, we used a virus concentrated by filtration/retention rather than PEG concentration, as PEG *per se* activates MΦ. The basal levels of 2 prototypic cytokines, namely IL-1β and IL-10, in non-stimulated LMNCs from individual donors are shown in Fig. 2A. These cells were either left untreated or exposed for 24 h to total HBV inoculum immediately after their isolation, and the same cytokines were dosed in the supernatant (Fig. 2B). The mean concentration of secreted IL-1β was 25% lower in HBV-exposed LMNC preparations compared to controls, whereas the mean IL-10 level was 64% higher (Fig. 2B).

Purified primary liver MΦ (PLMΦ) exposed to HBV or not were then stimulated with synthetic pathogen-associated molecular patterns (PAMPs); LPS was used to stimulate TLR4 and the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome,[28] whereas LPS + poly(dA:dT) was used to stimulate TLR4 and the AIM-2 (Absent In Melanoma-2) inflammasome[9] (Fig. 2C). The mean level of secreted IL-1β was 31% lower in LPS-stimulated PLMΦ exposed to HBV, compared to the unexposed condition (Fig. 2D). Similar results were obtained with HBV-exposed, LPS and poly(dA:dT)-stimulated PLMΦ, for which the mean level of secreted IL-1β was 35% lower (Fig. 2E). Interestingly, the expression of *HIF1α* mRNA, which encodes a protein tightly associated to IL-1β production and secretion in MΦ,[29] was 70% lower in LPS-stimulated-PLMΦ exposed to HBV compared to control (Fig. S3). While the mean level of secreted IL-10 was not affected by HBV with either type of stimulation in our experimental conditions (Fig. 2D and 2E), the mRNA levels of *VEGF*, an angiogenic factor expressed by anti-inflammatory cells,[30] was 56% higher in LPS-stimulated-PLMΦ exposed to HBV, compared to control cells (Fig. S3). Altogether these data suggest that HBV may interfere with the activation of PLMΦ.

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

During viral infections, a high quantity of immune cells, including monocytes, are recruited to the inflammation/injury site to mount a strong pro-inflammatory response and contribute to infection control.[31] This response is subsequently limited by an anti-inflammatory response, in order to start the scarring processes and prevent chronic inflammation.[31] Infiltrating
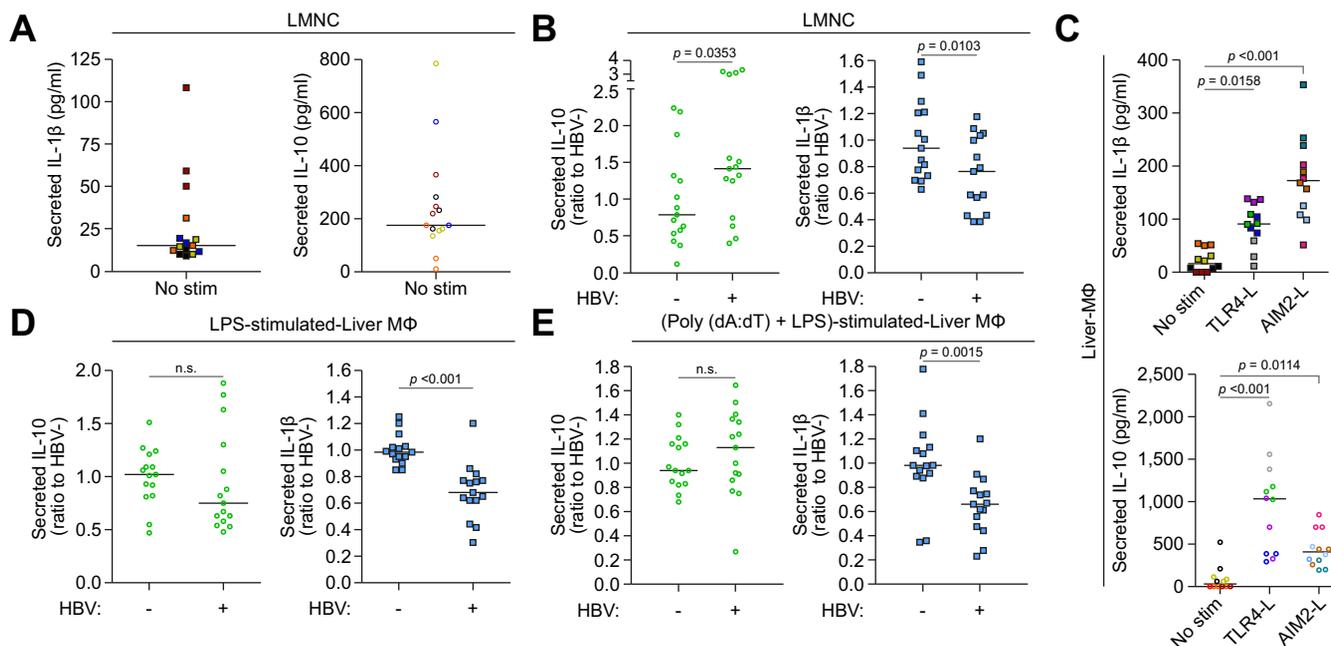
Fig. 2. Levels of pro-inflammatory markers decreased in liver MΦ exposed to HBV. (A,B) Total LMNCs were isolated from liver resection and exposed or not to HBV for 24 h. (C,D,E) Liver MΦ were isolated from liver resection, exposed or not to HBV for 24 h and stimulated 24 h later with (C,D) 100 ng/ml of LPS (TLR4-L) or (C,E) 100 ng/ml of LPS + 100 ng/ml of Poly (dA:dT) (AIM2-L) for another 24 h. (A-E) Supernatant were collected and the levels of IL-1β and IL-10 were assessed by ELISA. Data are presented either as non-normalised secretion or as ratio to non-exposed cells. Results are the median of 5 independent experiments (with 5 different donors) each performed with 3 biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test. HBV, hepatitis B virus; LMNCs, liver mononuclear cells; LPS, lipopolysaccharide; n.s., not significant. (This figure appears in colour on the web.)

monocytes differentiate into MΦ within the tissue[14] and, in the case of the human liver, cannot be fully distinguished from resident MΦ.

To assess the effect of HBV on MΦ that experimentally mimic those derived from liver-infiltrating monocytes, monocytes were purified from PBMCs and differentiated, with well-established polarizing *in vitro* conditions, into M1-like MΦ (M1-MDMs) upon GM-CSF or M2-like MΦ (M2-MDMs) upon M-CSF stimulation.[32] The differentiation process is schematically presented in Fig. 3A. M1-MDM cells derived from peripheral monocytes expressed and secreted, under a physiologic exposure to LPS (10 ng/ml), pro-inflammatory cytokines IL-6, IL-1β, and TNF-α, but not IL-10 (Fig. 3B, 3C, 3D, and 3E). In contrast, *in vitro* differentiated M2-MDM cells expressed only residual levels of IL-6, IL-1β, and TNF-α, but secreted high levels of the anti-inflammatory cytokine IL-10 in the same condition of physiologic LPS exposure (Fig. 3B, 3C, 3D, and 3E). Upon exposure to HBV during differentiation and activation (*i.e.*, 6 days exposure to HBV) (Fig. 3A), GM-CSF-treated monocytes (*i.e.*, intended to be differentiated into M1-MDMs) activated with low LPS stimulation, secreted 34% and 60% less IL-6 and IL-1β, respectively (Fig. 3F). Accordingly, a decreased level of *HIF1α* mRNA was detected (Fig. S4A). Under these conditions, no significant effect of HBV incubation was observed on the level of secreted TNF-α (Fig. 3F). M2-MDMs exposed to HBV during differentiation and activation secreted similar levels of IL-10 as unexposed cells (Fig. 3G) but expressed lower *VEGF* mRNAs levels (Fig. S4A).

We further investigated the effect of HBV on the activation of already differentiated M1-MDMs or M2-MDMs in response to physiologic LPS stimulation (Fig. 4A). No difference was

observed in the mean levels of secreted IL-6, IL-1β, or TNF-α (Fig. 4B). In contrast, lower mean mRNA level of *HIF1-α* was observed (Fig. S4B). Differentiated M2-MDMs showed a 2-fold increase of mean secreted IL-10 levels and an increase of mean *VEGF* mRNA levels in the presence of HBV (Fig. S4B). These results were confirmed with different donors and durations of exposure to HBV (Fig. S5). Moreover, increasing doses of HBV inoculum revealed an increasing effect i) on the inhibition of IL-6 secretion by M1-MDMs exposed to the virus during their differentiation and activation, and ii) on the increase of IL-10 secretion by M2-MDMs exposed to the virus during their activation (Fig. 5A and 5B).

Interestingly, the presence of cytochalasin D (Cyt D, an inhibitor of endocytosis and phagocytosis[33]), during the activation of M2-MDMs, in the presence of HBV, did not modify IL-10 secretion (Fig. 5C), suggesting that the effect of HBV on M2-MDM activation might be independent of virus productive entry within MΦ. It is also worth noting that UV-inactivated HBV has a similar effect on PLMΦ (Fig. S6A) and on M1- and M2-MDM (Fig. S6B) secretions as replication-competent HBV, and that HBV-exposed liver MΦ or MDMs did not secrete HBeAg or HBsAg (Fig. S7), highlighting that the effects of HBV on MΦ are independent of viral replication within cells. Moreover, no significant modification of cytokine secretion was observed when M1- or M2-MDMs were exposed to concentrated supernatants from non-infected HepG2-NTCP during, respectively, their differentiation and activation or activation only (Fig. S6C and S6D), suggesting that the effect is specific to HBV and not to a factor secreted by hepatocytes in the absence of HBV. Finally, AAV, either expressing GFP or the HBV genome, had no effect on M1-MDM secretion (Fig. 5D), suggesting that the
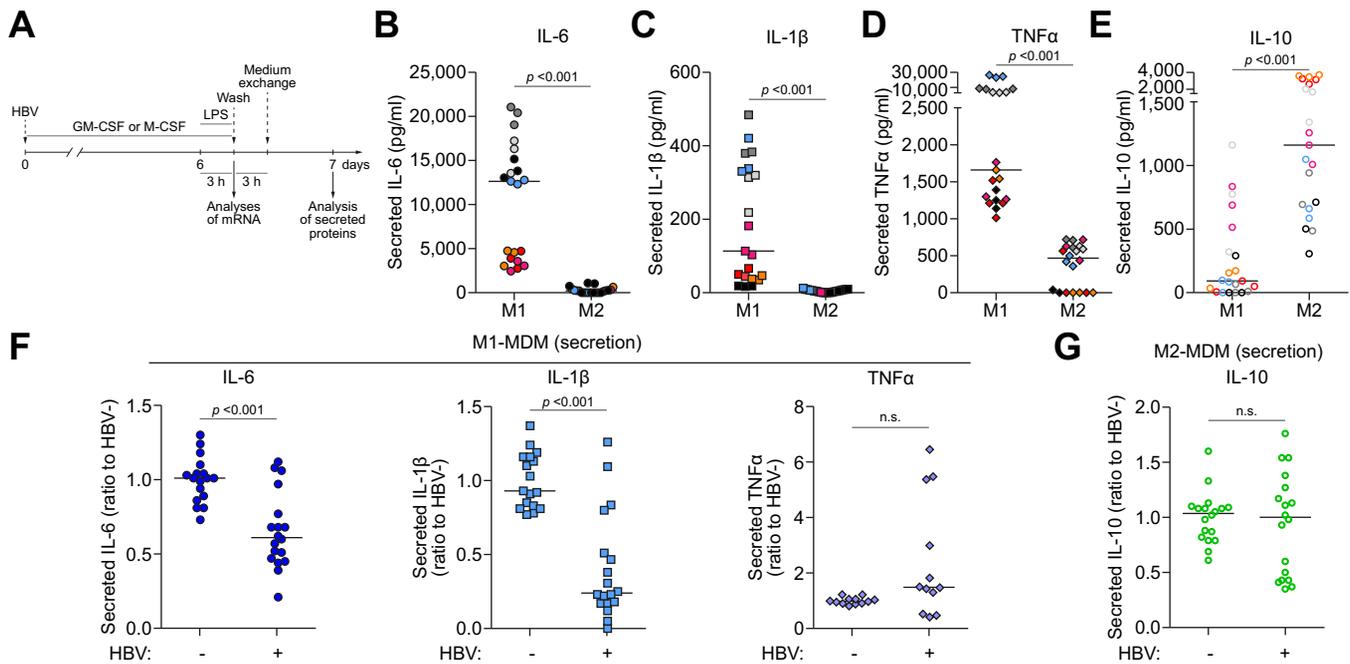
Fig. 3. HBV interferes with M1-MDM differentiation. Monocytes were purified from peripheral blood mononuclear cells, differentiated into (B-F) M1-MDM (upon GM-CSF culture) or (B-E,G) M2-MDM (upon M-CSF culture) in the presence or not of HBV before stimulation with 10 ng/ml of LPS for 3 h. (A) Schematic representation of the experiment. Medium change is indicated by dotted arrows. (B-G) Levels of the indicated secreted cytokines were assessed by ELISA and analysed either as non-normalised secretion or as ratio to non-exposed cells. Results are the mean of 6 independent experiments (with 6 different donors) each performed with 3 biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test. HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant. (This figure appears in colour on the web.)

observed effect is specific to HBV particles and/or antigens. Of note, the cells exposed to AAV-HBV did not secrete HBeAg or HBsAg (data not shown).

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

To ensure that the effect of secreted HBV components on M1-MDM differentiation and M2-MDM activation was not due to the high amount of HBV used in previously presented *ex vivo* experiments, we set up co-culture assays that allowed us to study the effect of HBV components physiologically secreted from infected hepatocytes. Monocytes, which were intended to differentiate into M1-MDMs and M2-MDMs, were co-cultured with either non-infected HepG2-NTCP cells (as HBV-negative control cells) or with HBV-producing HepAD38 cells during their respective differentiation (for M1-MDMs) or activation (for M2-MDMs) (Fig. 6A). We confirmed the results obtained with cell free-derived viruses and observed an even stronger effect of HBV since monocytes co-cultured with HBV-producing cells during their differentiation into M1-MDMs secreted between 46% and 85% lower mean levels of IL-6, IL-1β, and TNF-α (Fig. 6B). In contrast, M2-MDMs co-cultured with HBV-producing cells during their activation secreted 2.25-fold more IL-10 (Fig. 6C).

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

HBV impairs lymphocyte activation

IL-10 is well-known to impair lymphocyte activation.[34] Therefore, we assessed if an increase of IL-10 secretion induced by HBV (Fig. 4C) would further impair this activation. Total lymphocytes were purified from peripheral blood and exposed to M2-MDM-conditioned media for 2 h before their activation with PMA (phorbol 12-myristate 13-acetate) + ionomycine. Even though not significant, total lymphocytes exposed to M2-MDM supernatants secreted 17% less TNF-α than unexposed lymphocytes (Fig. 7, [no-HBV M2-MDM]-CM). Supernatants from M2-MDMs that were exposed to HBV during their activation, and contain higher levels of IL-10 (Fig. 4C and Table S6, [HBV stimulated M2-MDM]-CM), significantly decreased TNF-α secretion (30%) by total lymphocytes (Fig. 7).

Pro-inflammatory cytokines reduce the establishment of HBV infection in hepatocytes

As previously shown, pro-inflammatory cytokines can directly block HBV replication in cells persistently infected *in vitro* and IL-1β was the most efficient.[4] To investigate the effects of MΦ-derived cytokines on the establishment of HBV infection in hepatocytes, dHepaRG cells or PHHs were treated 24 h before and during the inoculation of cells with HBV with various recombinant pro-inflammatory cytokines (rhIL-1β, rhIL-6, rhTNF-α, rhIL-18), anti-inflammatory cytokines (rhIL-10, rhTGFβ), as well as with rhMCP-1, which is known to be secreted by both M1- and M2-MΦ (Table S4).[35] RhIL-1β treatment led to a 70–80% decrease of HBeAg and HBsAg secretion, HBV mRNA, and cccDNA formation, under conditions where cell viability of dHepaRG cells and PHHs were not affected (Fig. 8). Of note, a 24 h pre-treatment with rhIL-1β, followed by a

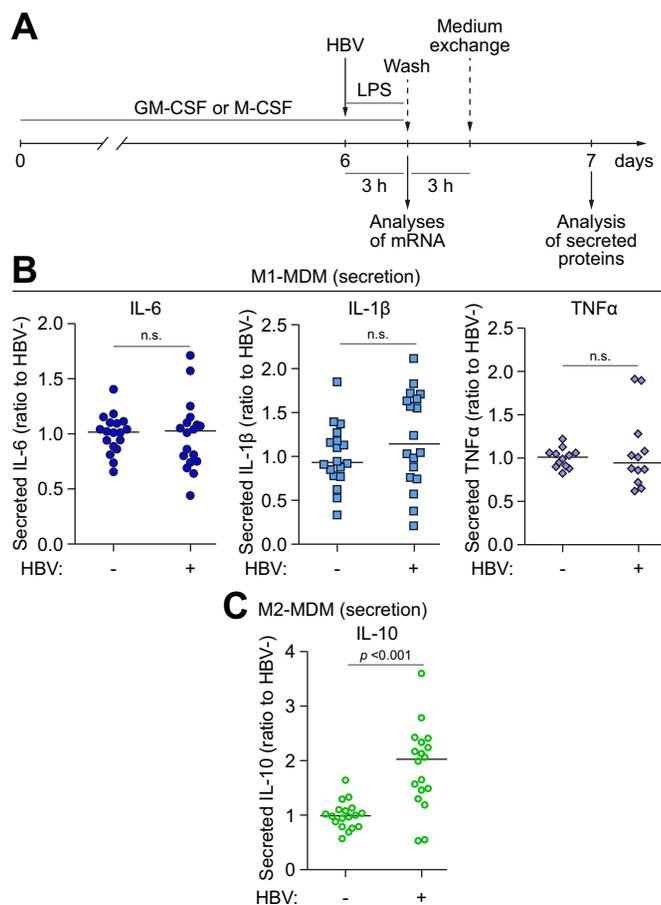**Fig. 4. HBV increases M2-MDM activation.** Monocytes were purified from peripheral blood mononuclear cells, differentiated into (B) M1-MDM (upon GM-CSF culture) or (C) M2-MDM (upon M-CSF culture) and stimulated with 10 ng/ml of LPS for 3 h in the presence or not of HBV. (A) Schematic representation of the experiment. Medium change is indicated by dotted arrows. (B-C) Levels of the indicated secreted cytokines were assessed by ELISA and analysed as ratio to non-exposed cells. Results are the mean of 6 independent experiments (with 6 different donors) each performed with 3 biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test. HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant. (This figure appears in colour on the web.)

withdrawal during HBV inoculation, was sufficient to decrease HBeAg and HBsAg secretion and HBV mRNA by 75% in dHepaRG (Fig. S8A). In addition, treatment with rhIL-6 and rhTNF-α before and during HBV inoculation resulted in an approximately 50% decrease of HBeAg, HBV RNA, and cccDNA formation. HBsAg was lowered by ~50% with rhTNF-α and by ~25% with rhIL-6 treatment (Fig. S8B). rhIL-18 had no effect on the establishment of HBV infection under these conditions (Fig. S8B). Upon rhIL-10 treatment, we observed a slight increase of mean HBeAg and HBsAg levels in the supernatant of dHepaRG cells, but not in PHHs. HBV RNA was modestly reduced in PHHs treated with IL-10 before and during infection (Fig. 8B). Other tested cytokines, TGF-β and MCP-1, had no significant effect on the establishment of HBV infection (Fig. S8B). Of note, we calculated that around 250 pg/ml of rhIL-1β and 25 ng/ml of rhTNFα are necessary to obtain a 50% decrease in the establishment of HBV infection (Fig. S9), whereas no further decrease was obtained with 20 ng/ml of rIL-6 (Fig. S9) and there was no effect with increasing doses of rhIL-10 (Fig. S9). Importantly, if the

level of NTCP (sodium-taurocholate co-transporting polypeptide), the HBV receptor, was decreased in dHepaRG after 24 h or 48 h of treatment with IL-1β (Fig. S10A), it was not affected in PHHs (Fig. S10B), indicating that the measured anti-HBV effect was not uniquely due to modulation of hNTCP expression. Collectively, these data indicate that pro-inflammatory cytokines, and IL-1β in particular, are very efficient at inhibiting the establishment of HBV infection in hepatocytes.

Next, we investigated whether cell culture supernatants from M1-MDMs exposed or not to HBV would interfere with the establishment of HBV infection in hepatocytes. Even though the final concentrations of IL-1β and IL-6 were respectively 25-fold and 10-fold lower than the concentration of recombinant cytokines used above (Fig. 8), treatment of dHepaRG cells with supernatants from differentiated M1-MDM cells (Tables S4 and S7) inhibited HBV establishment, as shown by a reduction of the mean HBeAg and HBsAg levels by ~25% (Fig. 9, [no-HBV M1-MDM]-CM). Accordingly, dHepaRG treated with supernatants from M1-MDMs exposed to HBV during activation, which contained similar amounts of pro-inflammatory cytokines to unexposed M1-MDMs (Fig. 4B and Table S7), showed a 40% decrease of secreted HBV antigens (Fig. 9, [HBV-stimulated M1-MDM]-CM). In contrast, dHepaRG cells treated with concentrated supernatants from M1-MDMs exposed to HBV during differentiation, containing less pro-inflammatory cytokines (Fig. 3F and Table S7), showed similar levels of HBV markers to those measured in non-treated dHepaRG cells (Fig. 9, [HBV diff M1-MDM]-CM).

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

Discussion

Dendritic cells and MΦ in the liver are thought to play a major role in the orchestration of adaptive responses and can also produce cytokines that may directly affect HBV infection.[36] Herein, we confirmed that cytokines secreted by pro-inflammatory MΦ (including IL-1β and IL-6) strongly inhibit the establishment of HBV infection in hepatocytes. These results are in agreement with our previously reported data showing the antiviral effects of these cytokines in already infected human hepatocytes[4] and emphasise the potential direct role of pro-inflammatory cytokines in the control of HBV. Of note, even though we observed a decrease in the level of NTCP in dHepaRG cells treated with IL-1β as previously reported,[37] NTCP levels were not changed by IL-1β treatment in PHHs under conditions that were sufficient for HBV inhibition. These results suggest that IL-1β can inhibit HBV establishment through an NTCP-independent but still ill-defined molecular mechanism.

Considering the antiviral potential of liver MΦ-derived cytokines, the main scope of this study was to investigate if HBV could interfere with such MΦ pro-inflammatory functions to facilitate the establishment of hepatocyte infection. Using human primary cells, we observed (i) that PLMΦ exposed *ex vivo* to HBV secrete less pro-inflammatory cytokines upon synthetic PAMP stimulation, thus confirming our previously published data,[8] (ii) that M1-MDMs also secrete less IL-1β and IL-6 upon activation if exposed to HBV or HBV-producing cells during differentiation and (iii) that the antiviral effect of
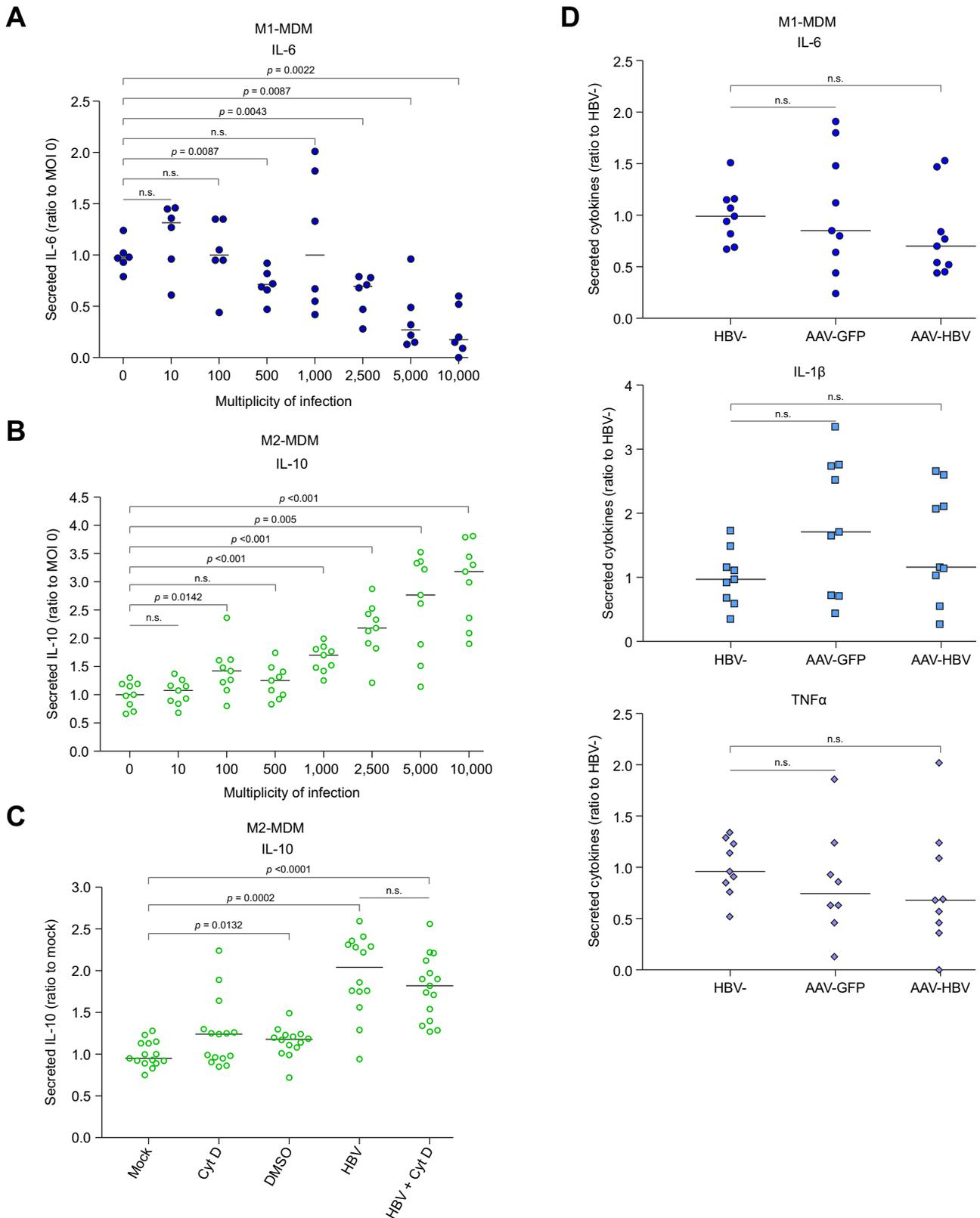
Fig. 5. HBV effect on MΦ is dependant of virus proteins quantity but independent of viral entry and replication. (A-D) Monocytes were purified from peripheral blood mononuclear cells, then differentiated into M1-MDM (upon GM-CSF culture; panels A and D) or M2-MDM (upon M-CSF culture; panels B and C) and stimulated with 10 ng/ml of LPS for 3 h. Cells were exposed or not to HBV, Cytochalasin D, AAV-GFP or AAV-HBV during their differentiation and activation (A,D), or their activation (B,C). (A-D) Levels of the indicated secreted cytokines were assessed by ELISA and analysed as ratio to non-exposed cells (A-B: MOI 0; C: Mock; D: HBV-). Results are the mean of 2 to 5 independent experiments (with 2 or 5 different donors) each performed with 3 biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test. AAV, adenovirus vector; HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant. (This figure appears in colour on the web.)
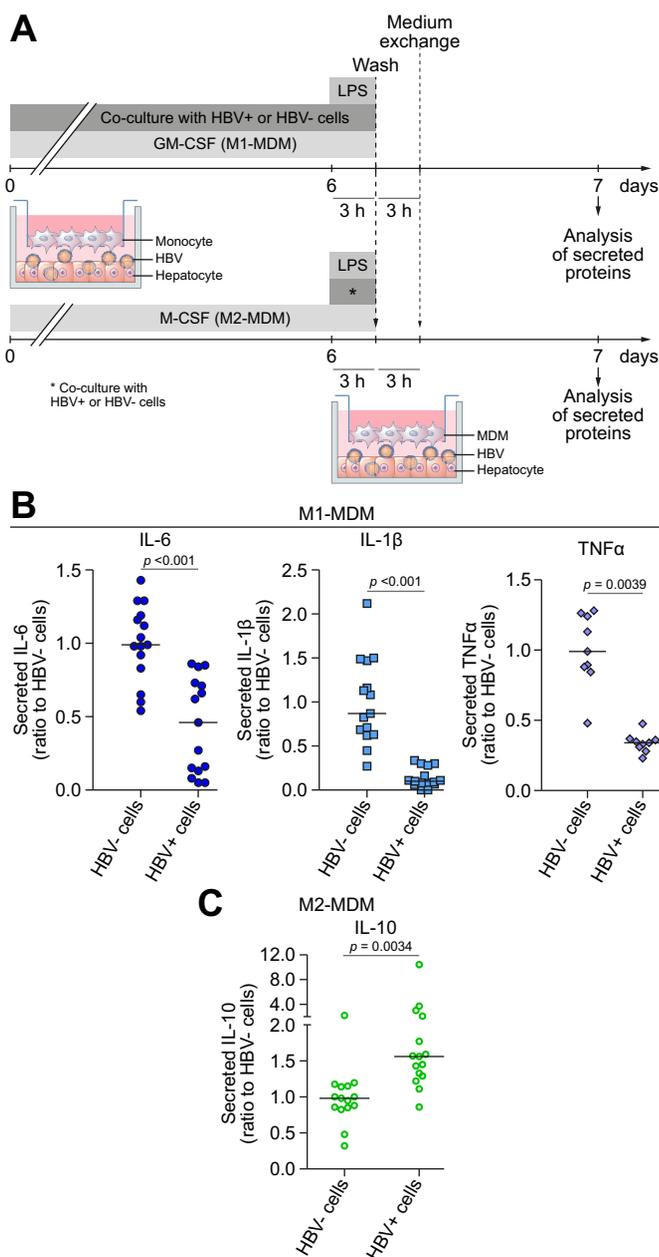
Fig. 6. HBV-producing cells impair M1-MDM differentiation and enhance M2-MDM activation. Monocytes were purified from peripheral blood mononuclear cells, differentiated into (B) M1-MDM (upon GM-CSF culture) or (C) M2-MDM (upon M-CSF culture) and stimulated with 10 ng/ml of LPS for 3 h, in the presence of non-infected HepG2-NTCP (HBV− cells) or HepAD38 (HBV+ cells) during their differentiation or activation, respectively. (A) Schematic representation of the experiment. Medium change is indicated by dotted arrows. (B, C) Levels of the indicated secreted cytokines were assessed by ELISA and analysed as ratio to cells exposed to HBV- cells. Results are the median of 5 independent experiments (with 5 different donors) each performed with 3 biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test. HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant. (This figure appears in colour on the web.)

M1-MDM-conditioned medium is lost when cells were differentiated in the presence of HBV. These results are in accordance with those from previously published studies using animal models or MΦ cell lines that reported an ability of HBV to inhibit MΦ responses,[9,38–41] and further demonstrate that HBV has
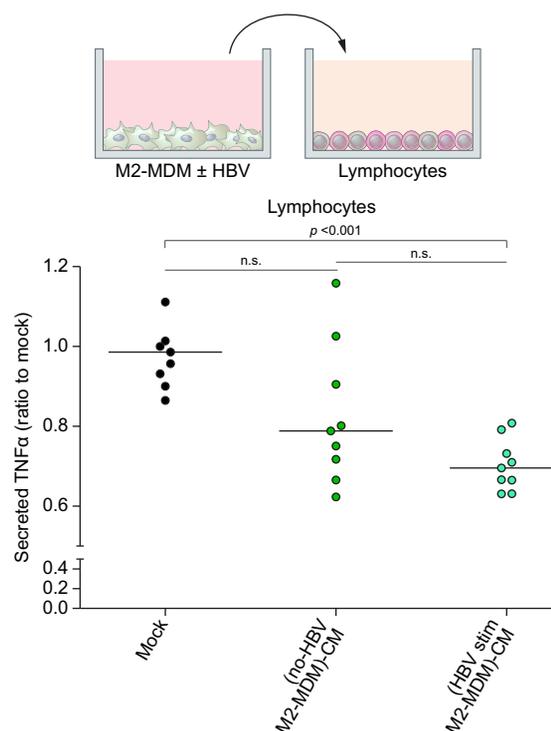

Fig. 7. HBV induces further immunosuppressive properties to M2-MDM. Lymphocytes were purified from peripheral blood mononuclear cells and exposed to media (mock) or LPS-stimulated M2-MDM in the absence (no-HBV M2-MDM) or presence (HBV stimulated M2-MDM) of HBV during their stimulation (CM; conditioned medium) for 2 h before stimulation with 20 ng/ml of phorbol 12-myristate 13-acetate and 500 ng/ml of ionomycine. Supernatants were collected 22 h later and the levels of TNF-α assessed by ELISA. Results, presented as ratio to non-treated cells (mock), are the mean ± standard deviation of 3 independent experiments (lymphocytes from 3 different donors) each performed with 3 biological replicates. Data were submitted to Wilcoxon test. HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant. (This figure appears in colour on the web.)

evolved strategies to interfere with liver myeloid cell functions, in the absence of any *bona fide* replication (*i.e.* neo-synthesis of viral genome and proteins) in these cells.

Importantly, this phenotype was similar in naive liver MΦ or MDMs following a short *ex vivo* exposure to the virus or cells replicating the virus, thus suggesting an "easy to implement" mechanism of inhibition, irrespective of a proper replication of HBV in these cells. In addition, we showed that the inhibition of pro-inflammatory cytokines and increase of anti-inflammatory cytokines is dependent on the quantity of virus. However, to be maintained, such an inhibitory phenotype would require a constant exposure to the virus and/or viral components. In this respect, our data showing increased inhibition of the secretion of pro-inflammatory cytokines by M1-MDMs upon constantly renewed exposure to HBV via co-cultures with HBV-producing cells, compared to a single exposure to concentrated virus, support the latest hypothesis. In contrast, the recent study by Suslov and colleagues convincingly showed that the stimulation with synthetic PAMPs (including TLR4 ligands) was not antagonised in *ex vivo* cultivated HBV-positive biopsies.[42] In this case, the purification procedure and *ex vivo* conditions cultures in the absence of HBV virion or viral components might have reversed the HBV-inhibitory phenotype. Moreover, additional experimental
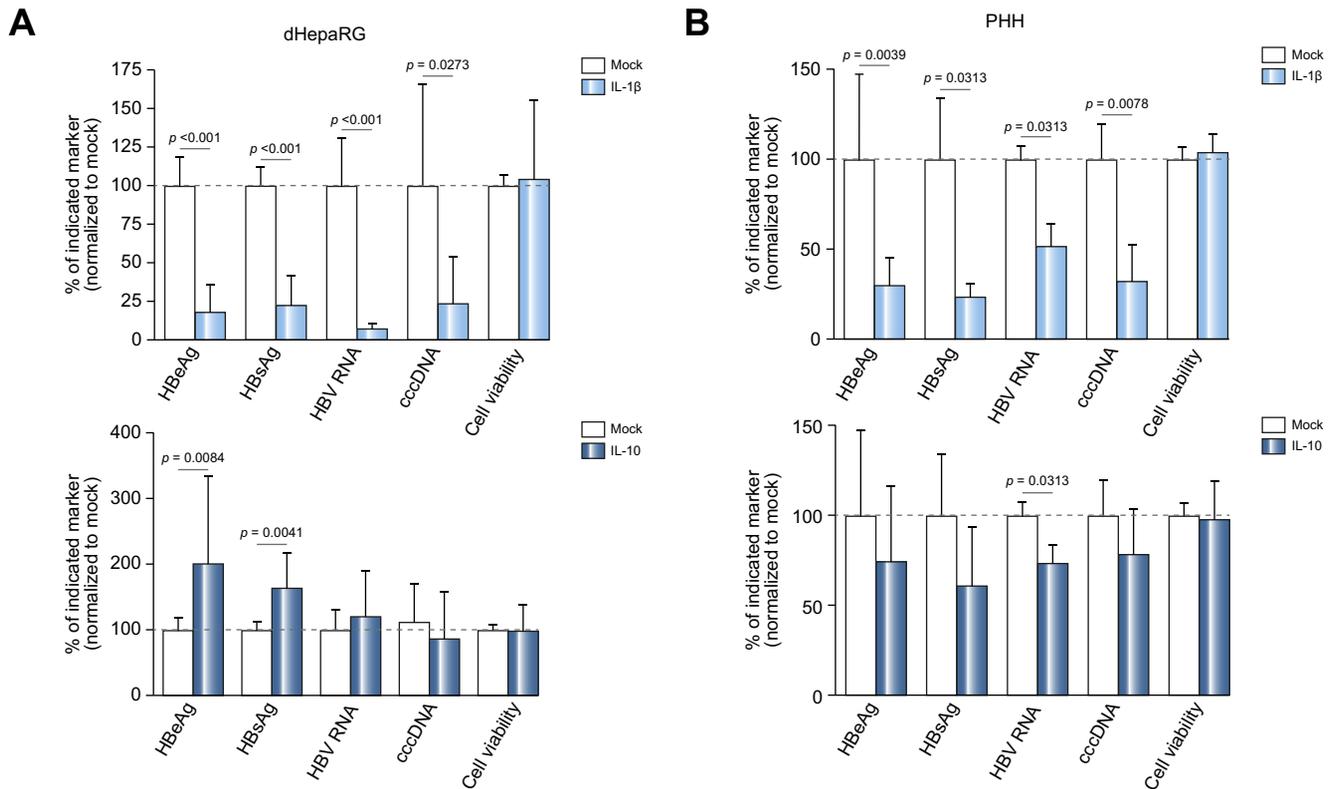
A dHepaRG

B PHH

Fig. 8. IL-1β strongly inhibits establishment of HBV infection in hepatocytes. (A) dHepaRG cells or (B) PHHs were treated with 1 ng/ml of recombinant IL-1β or 20 ng/ml of recombinant IL-10, 24 h before and during the infection with HBV. Seven days post infection, supernatants were collected and levels of HBeAg and HBsAg quantified by ELISA. Cells were harvested, total RNA or DNA extracted, and levels of HBV RNAs and cccDNA quantified by RT-qPCR or qPCR analysis. Cell viability was assessed by neutral red uptake assay. Results, presented as ratio to non-treated cells, are the mean ± standard deviation of 3 independent experiments (3 batches of dHepaRG cells and 3 donors of PHH) each performed with 3 biological replicates. Data were submitted to Wilcoxon test. cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; PHH, primary human hepatocytes; qPCR, quantitative PCR; RT-qPCR, reverse transcription quantitative PCR.

pitfalls could explain these opposite results. First, the concentration of TLR4 ligands used in Suslov *et al.*'s study was far higher than those used herein (20 μg/ml *vs.* 10 to 100 ng/ml) and probably beyond physiologic conditions. Moreover, despite the commendable effort of Suslov *et al.* to work with highly relevant biological materials, the viability of hepatocytes in these *ex vivo* maintained biopsies was limited and the viability, as well as the functionality, of other liver cells such as liver MΦ were not assessed. It would, therefore, be important to test if the addition of HBeAg/HBsAg/HBV virions in the culture medium of *ex vivo* cultured liver biopsies and the use of physiologic levels of stimulation would recapitulate the inhibitory phenotype we described here.

Importantly, we observed an increase of anti-inflammatory MΦ markers (CD68^{+} CD163^{+} cells) in liver biopsies from HBV-infected patients compared to those from non-infected patients of 2 different cohorts, as well as an increase in the levels of IL-10 secreted by M2-MDMs upon exposure to HBV during activation after *ex vivo* differentiation. These data suggest that HBV may reinforce the previously described liver immune tolerance,[16] helping to establish and/or maintain infection. This is in accordance with studies reporting an increase of anti-inflammatory cytokine secretion (especially IL-10) induced by HBV in animal models and patients[43–46] and a positive role of anti-inflammatory liver MΦ in HBV persistence.[43] Here, we observed that HBV-induced increases in IL-10 secretion by M2-MDMs might impair the activation of lymphocytes. As others had previously described,[43,47,48] the increased IL-10 expression during

HBV infection could favour a tolerogenic environment and the inhibition of functional adaptive immune responses, compromising HBV elimination. As these anti-inflammatory MΦ are also implicated in affecting distinct oncogenetic processes (through the secretion of angiogenic factors, such as VEGF, and the impairment of an anti-oncogenic responses by the secretion of regulatory mediators, such as IL-10[49]), it should be investigated if the HBV-induced modification of MΦ phenotypes may also play a role in hepatocellular carcinoma initiation and promotion.

We did not precisely determine which viral components and which HBV-infection related mechanisms influence cytokine secretion by MΦ. The secreted viral proteins (HBeAg, HBsAg) or viral proteins contained in virions (HBsAg, HBc, viral polymerase) may play a role in the modification of MΦ phenotype. Few data suggest that HBV can be internalised by MΦ (29,44). HBeAg,[10] HBsAg[9,40] and the HBV capsid protein[52] have been suggested to contribute to the inhibition of MΦ responses. In the present study, analyses of liver biopsies from HBV-infected patients revealed a co-localisation between HBc and an MΦ marker (CD68; HBcAg). How HBc is delivered to liver MΦ remains to be addressed; it could be through a specific receptor, unspecific engulfment, or by phagocytosis of dying infected hepatocytes. Moreover, it is not yet clear in which subcellular compartment (*e.g.* organelles; lysosomes) stained HBcAg is located.

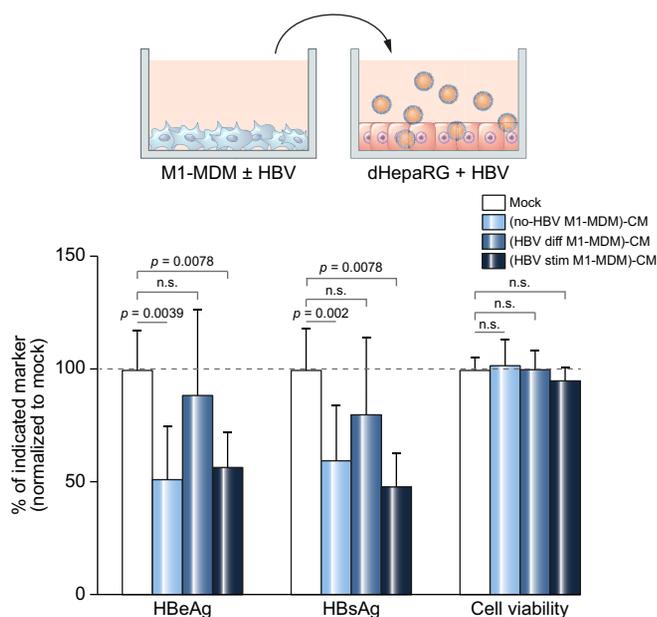Of note, the use of recombinant viral proteins to clarify the involvement of viral proteins in the modification of MΦ

Fig. 9. HBV prevents the antiviral effect of M1-MDM on HBV establishment. dHepaRG cells were treated 24 h before and during HBV infection with concentrated supernatants from LPS-stimulated M1-MDM (CM: conditioned medium pooled from at least 5 independent experiment) exposed to HBV during their differentiation or during their stimulation. Seven days post infection, supernatants were collected and levels of HBeAg and HBsAg quantified by ELISA. Cells were harvested, total DNA extracted and levels of cccDNA quantified by specific qPCR analysis. Cell viability was assessed by neutral red uptake assay. Results, presented as ratio to non-treated cells (mock), are the mean ± standard deviation of 3 independent experiments each performed with 3 biological replicates. Data were submitted to Wilcoxon test. cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant; qPCR, quantitative PCR. (This figure appears in colour on the web.)

responses may be suboptimal, as post-transcriptional modifications associated with a given protein produced in a given cell system (bacteria, yeast or mammalian cells) could be different to those found in viral proteins from infected hepatocytes and therefore have a different effect on immune cells. In addition, these modifications may vary during the different phases of HBV infection, giving the numerous functions of the viral proteins. HBV probably interferes with the activation of MΦ (and thereby cytokine secretion) at different levels. Indeed downregulation of innate sensors such as TLR[41,53,54] and inhibition of cell signalling pathways[39,40,47] have been reported. Epigenetic modulations induced by the fixation of 1 viral protein to gene promoters, as reported in hepatocytes[52] and as also reported for other viruses,[55–57] might also be implemented in MΦ even though HBV do not replicate in those cells. Importantly, the different effects observed are specific to HBV as AAVs had no impact on M1- and M2-MDM secretions. Our data suggest that HBV productive entry into cells (*i.e.* with fusion of the membrane between viral particles and cells) may not be absolutely required to induce phenotypic changes in MΦ. One of several viral proteins present in the inoculum or circulating in the blood of patients (HBsAg and HBeAg) may bind to receptor(s) of MΦ involved in modulation of inflammatory responses. As described before, the activation of specific regulatory receptors at the surface of MΦ can trigger inhibition of pro-inflammatory cytokines secretion.[58–60] It will therefore be important to determine the target(s) of HBV, on the surface of MΦ, which are responsible

for the modulations observed, in order to develop specific molecules targeting this interaction.

Importantly, in our different experimental settings, we did not detect any cytokine secretion by LMNCs, primary human liver MΦ or MDMs exposed to cell-culture-produced HBV inocula or co-cultured with HBV producing cells (data not shown). This is in sharp contrast with chronic HCV or HIV infections that induce strong host responses,[61–64] but in accordance with studies that reported an absence of measurable innate immune responses in patients, animal or *in vitro* models upon primary HBV infection,[42,45,62,65–67] leading to the definition of HBV as a stealth virus.[68] Of course, as also shown here, this stealthy character could be due to active evasion and not complete passivity. In contrast, several groups have reported an induction of innate responses following exposure to HBV.[7,25,50,69,70] The quality of the HBV inoculum used to perform the *ex vivo* analyses is probably very different between studies, since no standard inocula are available and only very few manuscripts reported the characterisation of their inocula as we did here (Fig. S2). Indeed, different HBV-producing cells, different culture conditions and concentration procedures may lead to different ratios of viral antigens, subviral particles and Dane particles, as well as contaminating non-enveloped nucleocapsids, recognised by TLR2,[25] or endotoxins, recognised by TLR4,[71] in the inocula, which may explain the discrepancies between studies.

From a therapeutic point of view, we herein confirmed that IL-1β inhibits HBV establishment and replication in hepatocytes very efficiently. However, recombinant IL-1β cannot be used systemically without risking severe side effects such as cytokine storms. New anti-HBV therapeutic options should aim at inducing the local and endogenous secretion of IL-1β to induce antiviral activity and prevent the development of pathogenesis. Different strategies may be considered to promote pro-inflammatory over anti-inflammatory phenotypes in liver resident and infiltrating MΦ. For instance, the delivery of GM-CSF in cancerous tissue has been shown to induce tumour regression[72,73] by promoting pro-inflammatory MΦ. Inversely, a monoclonal antibody against CSF-1R (colony stimulating factor 1 receptor) showed promising results in reducing the number of anti-inflammatory and tumour-associated MΦ.[15] Alternatively, inhibitors of glutaminolysis, that modify the succinate/α-keto glutarate balance in MΦ[74] or specific inflammasome and/or pathogen recognition receptor inducers could be evaluated.[75]

In summary, we showed that HBV can modulate the resident and transiting- MΦ phenotypes to favour its establishment, and likely its maintenance in the liver. HBV escapes the antiviral effect of pro-inflammatory liver resident and infiltrating MΦ by interfering with their activation/differentiation and inhibiting the production of cytokines endowed with anti-HBV activities. Besides, HBV infection promotes the activation of anti-inflammatory MΦ and the production of IL-10 in the microenvironment, likely favouring tolerance. Our data also suggest that therapeutic strategies promoting the differentiation of pro-inflammatory liver MΦ over anti-inflammatory ones or the shift from one to the other should be tested to help break immune tolerance and promote the functional cure of chronic HBV infections.

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Conflicts of interest

SFD, MD, LA, LD, JF, FL, RP, MR, NBV, MH have nothing to declare. FZ, JL, and DD received a research grant from Novira Therapeutics to perform experiments. AL, LF and KK were employees of Novira Therapeutics.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study concept and design: SFD, MD, JL, DD. Acquisition of data: SFD, MD, LA, LD, JF, DH *et al.* Analysis and interpretation of data: SFD, MD, AL, KK, JL, DD. Drafting of the manuscript: SDF, JL, DD. Critical review of the manuscript: AL, KK, NBV, FZ, MH. Statistical analyses: SFD. Material support: MR, NBV, FL, RP, MSM, FZ.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.06.032.

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