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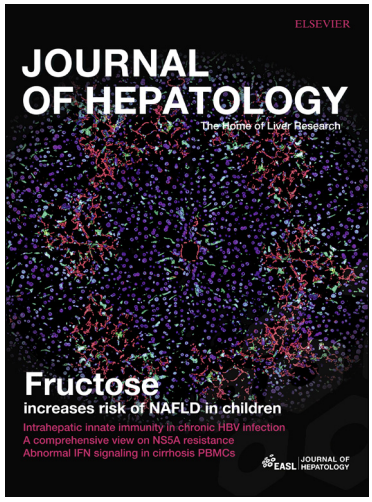

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Toll-Like Receptor 7 Agonist GS-9620 Induces Prolonged Inhibition of HBV via a Type I Interferon-Dependent Mechanism

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Key Words: TLR7; GS-9620; Hepatitis B virus; interferon-alfa; cccDNA; interferon-stimulated gene; MHC; immunoproteasome; APOBEC; Smc5/6.

Abbreviations: APOBEC3, A3; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CM, conditioned media; Ctrl, control; DEG, differentially expressed gene; FDR, false discovery rate; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IFN-α, interferon-alpha; IFNAR, IFN-α/β receptor; IPA, Ingenuity Pathway Analysis; ISG, interferon-stimulated gene; mAb, monoclonal antibody; MHC, major histocompatibility complex; ND10, nuclear domain 10; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; PHH, primary human hepatocyte; pMHC, peptide/MHC class I complex; Smc5/6, structural maintenance of chromosome 5/6 complex; TCR, T cell receptor; TLR7, toll-like receptor 7; Tx, treatment.

Conflict of interest: C. Niu, L. Li, S. Daffis, E. Salas, R. Chu, H. Ramos, C. Livingston, R. Beran, A. Garg, S. Balsitis, W. Delaney and S. Fletcher are employees of Gilead Sciences, Inc. J. Lucifora, M. Bonnin, S. Maadadi, D. Durantel and F. Zoulim have nothing to declare.

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Author's Contributions: Conceived study: S. Fletcher. Participated in research design: S. Daffis, H. Ramos, S. Fletcher, A. Garg, S. Balsitis, W. Delaney, J. Lucifora, D. Durantel, F. Zoulim. Provided supervision: S. Daffis, S. Balsitis, W. Delaney, S. Fletcher. Conducted experiments: C. Niu, S. Daffis, J. Lucifora, M. Bonnin, S. Maadadi, E. Salas, R. Chu, C. Livingston, R. Beran. Performed data analysis: L. Li, S. Daffis, J. Lucifora, D. Durantel, S. Fletcher. Wrote or contributed to the writing of the manuscript: S. Daffis, D. Durantel, J. Lucifora, S. Fletcher. All authors reviewed and approved the final manuscript.

ABSTRACT

Background & Aims: GS-9620, an oral agonist of toll-like receptor 7 (TLR7), is in clinical development for the treatment of chronic hepatitis B (CHB). GS-9620 was previously shown to induce prolonged suppression of serum viral DNA and antigens in the woodchuck and chimpanzee models of CHB. Here we investigated the molecular mechanisms that contribute to the antiviral response to GS-9620 using in vitro models of hepatitis B virus (HBV) infection. **Methods:** Cryopreserved primary human hepatocytes (PHH) and differentiated HepaRG (dHepaRG) cells were infected with HBV and treated with GS-9620, conditioned media from human peripheral blood mononuclear cells (PBMCs) treated with GS-9620 (GS-9620 conditioned media; GS-9620-CM), or other innate immune stimuli. The antiviral and transcriptional response to these agents was determined. **Results:** GS-9620 had no antiviral activity in HBV-infected PHH, consistent with low level *TLR7* mRNA expression in human hepatocytes. In contrast, GS-9620-CM induced prolonged reduction of HBV DNA, RNA, and antigen levels in PHH and dHepaRG cells via a type I interferon (IFN)-dependent mechanism. GS-9620-CM did not reduce cccDNA levels in either cell type. Transcriptional profiling demonstrated that GS-9620-CM strongly induced various HBV restriction factors — although not APOBEC3A or the Smc5/6 complex — and indicated that established HBV infection does not modulate innate immune sensing or signaling in cryopreserved PHH. GS-9620-CM also induced expression of immunoproteasome subunits and enhanced presentation of an immunodominant viral peptide in HBV-infected PHH. **Conclusions:** Type I IFN induced by GS-9620 durably suppressed HBV in human hepatocytes without reducing cccDNA levels. Moreover, HBV antigen presentation was enhanced, suggesting additional

components of the TLR7-induced immune response played a role in the antiviral response to GS-9620 in animal models of CHB.

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Lay Summary: GS-9620 is a drug in clinical trials for the treatment of chronic hepatitis B virus (HBV) infection. GS-9620 has previously been shown to suppress HBV in various animal models, but the underlying antiviral mechanisms were not completely understood. In this study, we determined that GS-9620 does not directly activate antiviral pathways in human liver cells, but can induce prolonged suppression of HBV via induction of an antiviral cytokine called interferon. However, interferon did not destroy the HBV genome, suggesting that other parts of the immune response (e.g. activation of immune cells that kill infected cells) also play an important role in the antiviral response to GS-9620.

INTRODUCTION

Approximately 240 million individuals are chronically infected with hepatitis B virus (HBV), and over 650,000 people die each year due to HBV-associated liver diseases, such as cirrhosis and hepatocellular carcinoma. Immunologic control of chronic hepatitis B (CHB), recognized as a "functional cure," is defined as sustained loss of HBV surface antigen (HBsAg) off therapy with or without seroconversion to anti-HBs antibody. Current therapies for CHB are limited to nucleos(t)ides and interferon-alpha (IFN-α). These agents reduce viral load and improve long-term outcome, they rarely lead to cure. There is therefore an urgent need for new therapies that induce durable immune control of chronic HBV infection.

GS-9620 is a potent, orally bioavailable small molecule agonist of toll-like receptor 7 (TLR7) in clinical development for the treatment of CHB [1]. TLR7 is expressed in a subset of human immune cells, primarily plasmacytoid dendritic cells (pDCs) and B cells, and recognizes single-stranded RNA as well as small molecule agonists [2]. TLR7 activation induces innate and adaptive immune responses via induction of various cytokines (including multiple IFN-α subtypes) and chemokines, direct activation of B cells, and cross-priming of cytotoxic $CD8^+$ T cells [3-5]. GS-9620 was previously shown to induce prolonged suppression of serum viral DNA and antigens in the chimpanzee and woodchuck models of CHB [6, 7]. Various immunomodulatory activities may account for the antiviral effects of GS-9620 in these animal models (e.g., induction of antiviral cytokines, activation of NK cells, $CD8^+$ T cells and B cells); however the exact mechanism remains unclear. Defining the molecular basis for response is an important

goal because mechanistic understanding of GS-9620 activity could guide rational design of novel immunotherapeutic strategies.

In the current study we investigated the molecular mechanisms responsible for the antiviral response to GS-9620 using in vitro models of HBV infection in cryopreserved primary human hepatocytes (PHH) as well as differentiated HepaRG (dHepaRG) cells.

MATERIALS AND METHODS

Ethics statement

Consent was obtained from the donor or the donor's legal next of kin for use of PHH, buffy coats from healthy human volunteers and whole blood from healthy human volunteers and CHB patients for research purposes using IRB-approved authorizations. Additional details are provided in the Supplementary Materials and the Supplementary CTAT Table.

Additional materials and methods

Chemical and reagent details are provided in the Supplementary Materials and the Supplementary CTAT Table. Methods for production and characterization of conditioned media, infection and treatment of PHH and dHepaRG cells, HBeAg and HBsAg analysis, quantitative RT-PCR (qRT-PCR), HBV DNA analysis, cell viability analysis, HBV cccDNA analysis, epifluorescence microscopy, confocal microscopy, Western blotting, pMHC complex analysis and RNA-Seq are provided in the Supplementary Materials.

Statistical analysis

Data is expressed as mean ± standard error of the mean (SEM). Statistical significance was tested using a two-tailed t-test (for two sample comparisons) or either one-way or two-way ANOVA with multiple comparison correction (for multiple comparisons). A value of $p<0.05$ was considered significant.

RESULTS

Cytokines induced by GS-9620 reduce HBV DNA, RNA and antigens, but do not alter cccDNA levels

We first determined that GS-9620 has no antiviral activity in HBV-infected PHH, consistent with low level *TLR7* mRNA expression in human hepatocytes (Supplementary Figs.1-3). These data are described in the Supplementary Materials. We next evaluated whether cytokines induced by GS-9620 in human PBMCs can suppress HBV after infection of PHH and dHepaRG cells. For these studies, PBMCs were stimulated with 10 nM GS-9620 since this concentration strongly induces IFN-α, a prototypic TLR7 cytokine with antiviral activities (Supplementary Fig. 4A). HBV-infected PHH were continuously treated (day 3-20 post-infection) with IFN-α or with conditioned media from PBMCs stimulated with GS-9620 (GS-9620-CM) or DMSO (Mock-CM). Treatment with both GS-9620-CM and 100 IU/mL IFN-α strongly reduced the levels of HBV DNA and HBeAg (>75%), as well as HBV RNA (>70%) and HBsAg (>60%), with only a very modest effect (<3% reduction) on cell viability (Figs. 1A-E, Supplementary Fig. 2B). GS-9620-CM and IFN-α also had antiviral activity in HBV-infected PHH when treatment was delayed (day 13-20 post-infection) (Figs. 1A-D). PHH viability was not affected by this shorter treatment duration (Fig. 1E, Supplementary Fig. 2B). GS-9620-CM also had antiviral activity in HBV-infected dHepaRG cells, although the HBsAg reduction was relatively modest (30%) (Fig. 1F). Importantly, Mock-CM did not have antiviral activity in either HBV-infected PHH or dHepaRG cells (Fig. 1).

In contrast to the other viral parameters, GS-9620-CM treatment did not alter cccDNA levels in HBV-infected PHH or dHepaRG cells (Fig. 2). However, even though cccDNA levels were not changed by GS-9620-CM and IFN-α, all other viral endpoints were still significantly reduced 7 days after cessation of treatment (day 3-13 post-infection) of HBV-infected PHH (Figs. 1A-D). Similarly, HBV DNA levels were significantly reduced 11 days after cessation of GS-9620-CM treatment of HBV-infected dHepaRG cells, whereas viral DNA rebounded to control levels after tenofovir treatment was stopped (Supplementary Fig. 5A). A longer duration study in HBV-infected dHepaRG cells revealed that HBV DNA and HBsAg levels were still significantly suppressed 35 days after cessation of GS-9620-CM treatment (Supplementary Fig. 6). Collectively, these data demonstrate that GS-9620-CM can induce a prolonged antiviral effect in HBV-infected PHH and dHepaRG cells without reducing cccDNA levels. In contrast to IFN-α, TNF-α did not induce prolonged suppression of viral RNA in HBV-infected PHH once treatment was stopped (Supplementary Fig. 5B). This suggests important mechanistic differences underlie the antiviral response to these cytokines.

GS-9620-induced cytokines inhibit HBV via a type I IFN-dependent mechanism

We next characterized GS-9620-CM by luminex analysis to identify the cytokine(s) responsible for the antiviral activity observed in HBV-infected PHH and dHepaRG cells. As expected, IFN-α was the predominant cytokine in GS-9620-CM, although lower amounts of other cytokines (e.g. IFN-γ, IL-1β, IL-6 and TNF-α) with antiviral activity against HBV were also present (Fig. 3A) [8]. IFN-β, like IFN-α, is a type I IFN that signals via the IFN-α/β receptor (IFNAR). In contrast to IFN-α, there were very low

levels of IFN-β in GS-9620-CM (<0.5 pg/mL). Importantly, the antiviral cytokine profile of GS-9620-CM was similar to the serum cytokine profile in chimpanzees dosed orally with GS-9620 [6]. Moreover, GS-9620 induced comparable IFN-α levels in vitro in human PBMCs from healthy donors and CHB patients (Supplementary Fig. 4B). Collectively, these data support the use of GS-9620-CM from healthy human donor PBMCs in these studies

In line with the PBMC cytokine profile, antibody blockade of the type I IFN receptor (IFNAR) or knock-down of IFNAR1 by RNA interference abrogated the antiviral activity of GS-9620-CM in HBV-infected PHH (Fig. 3B-E). The antiviral activity of GS-9620-CM in HBV-infected dHepaRG cells was also blocked by an anti-IFNAR antibody, but not by TNF-α or IFN-γ neutralizing antibodies (Supplementary Fig. 7). Taken together, these data indicate that type I IFN (most likely IFN-α) is the cytokine responsible for the HBV suppressive activity of GS-9620-CM.

Established HBV infection does not significantly modulate the transcriptional response to GS-9620-induced cytokines

A recent study suggested that HBV can inhibit IFN-α signaling in a humanized mouse model [9]. Since GS-9620-CM is principally composed of IFN-α and mediates its antiviral effect via the type I IFN receptor, we evaluated whether HBV suppresses the global transcriptional response to GS-9620-CM in PHH. The experimental set-up was designed to evaluate the potential impact of established HBV infection on the hepatocyte innate immune response, since this scenario is clinically relevant to the treatment of CHB

(Fig. 4A). High-level infection (~80-90% PHH infected) was confirmed by HBV core antigen staining (Fig. 4B). GS-9620-CM induced the greatest transcriptional response 8 hours post-treatment, with >1000 genes being differentially expressed (FDR<0.05, fold-change >2) in each PHH donor (Fig. 4C, Supplementary Table 5). Interestingly, the number of differentially expressed genes (DEGs) and the overall transcriptional profile were comparable in mock-infected and HBV-infected PHH, as well as in different PHH donors (Fig. 4C-D).

Ingenuity Pathway Analysis (IPA) and Gene Set Enrichment Analysis (GSEA) both identified IFN signaling as the top transcriptional pathway induced by GS-9620-CM in HBV-infected PHH (Fig. 5A, Supplementary Table 1). In line with the whole transcriptome analyses, GS-9620-CM induced a comparable interferon-stimulated gene (ISG) response in mock-infected and HBV-infected PHH, with no ISG being differentially modulated by GS-9620-CM in mock-infected vs. HBV-infected PHH in both donors (Figs. 5B and C, Supplementary Table 7). Taken together, these data suggest that established HBV infection does not modulate the transcriptional response to IFN-α in our cell culture system.

Established HBV infection does not significantly modulate the transcriptional response to various innate immune stimuli

In light of the GS-9620-CM transcriptome analysis, we next evaluated whether the PHH response to diverse innate immune stimuli is influenced by established HBV infection (Fig. 6A). In addition to GS-9620-CM and IFN-α, this study evaluated the ISG response

to poly(I:C) and Sendai virus (SeV), which are agonists of TLR3/MDA5 and RIG-I, respectively. The transcriptional response was measured by qRT-PCR at 8 hours post-treatment since this was the peak (or close to the peak) of the response in PHH to various innate immune stimuli in previous studies (Fig. 5B) [10]. High-level infection (~80-90% PHH infected) at the time of innate immune stimulation was confirmed by HBV core antigen staining (Fig. 6B). Consistent with the GS-9620-CM RNA-Seq data, HBV infection did not significantly reduce the ISG response to any of the innate immune stimuli tested (Fig. 6C, Supplementary Fig. 8). This was the case whether the magnitude of ISG induction was high (e.g. *RSAD2* (*viperin*), >100-fold induction) or low (e.g. *EIF2AK2* (*PKR*), <10-fold induction). Of note, although not statistically significant, there was a trend towards lower ISG induction in HBV-infected PHH for certain ISGs. However, this equated to only a 10 ± 3% (mean ± SEM) difference in fold-change for the multiple genes evaluated (n=8) in three different PHH donors with various innate immune stimuli. This lack of significant, coordinated ISG repression suggests that established HBV infection does not interfere with the hepatocyte transcriptional response to IFN-α or TLR3/MDA5 and RIG-I agonists in our cell culture system.

GS-9620-induced cytokines strongly increase the expression of various HBV restriction factors, but not APOBEC3A or the Smc5/6 complex

GS-9620-CM induced the expression of a large number of ISGs in PHH (Fig. 5B, Supplementary Table 6), many of which have antiviral effector functions [11]. The ISG expression profile in HBV-infected PHH treated with GS-9620-CM was similar to the intrahepatic ISG signature in CHB chimpanzees treated with GS-9620, although a subset

of ISGs (including several chemokines) was differentially induced in the two systems (Supplementary Fig. 9). Importantly, none of the ISGs that were strongly induced in PHH but not chimpanzee liver (i.e. *ANGPTL1*, *BCL2L14* and *CXCL9*) have been reported to possess direct antiviral effector function. This suggests that the differential induction of these genes does not impact the utility of the in vitro PHH model for investigating the molecular mechanisms responsible for the antiviral response to GS-9620 in vivo.

The HBV restriction factors *MxA* (*MX1*) and *tetherin* (*BST2*) were amongst the ISGs induced by GS-9620-CM in HBV-infected PHH (Supplementary Table 6). However, MxA and tetherin do not reduce total HBV RNA levels [12, 13], suggesting that other HBV restriction factors were also induced by GS-9620-CM. We therefore evaluated expression of APOBEC3 proteins, which have been reported to mediate cccDNA deamination and degradation [14, 15], as well as the structural maintenance of chromosome 5/6 complex (Smc5/6), which suppresses cccDNA transcription when localized to intranuclear structures known as nuclear domain 10 (ND10) [10, 16].

We determined that GS-9620-CM and IFN-α treatment induced expression of the ND10 components *PML* and *SP100*, but not Smc5/6 subunits (Supplementary Table 2, Supplementary Fig. 10A and B). Interestingly, IFN-α altered the nuclear distribution of Smc6 by significantly increasing the number of ND10 (i.e. PML foci) in uninfected PHH (Supplementary Fig. 10C and D). However, since Smc5/6 is effectively degraded in HBV-infected PHH and is not induced by IFN-α, suppression of HBV RNA by GS-9620-CM is likely independent of this restriction factor.

In contrast to Smc5/6, *APOBEC3B* (*A3B*) was modestly induced by GS-9620-CM (Supplementary Table 3). However, *A3A* expression was not increased by GS-9620-induced cytokines in these PHH donors (Supplementary Table 3). Analysis of additional PHH identified a donor in which *A3A* expression was significantly induced by GS-9620-CM as well other innate immune stimuli (Supplementary Fig. 11); however, *A3A* induction by GS-9620-CM treatment of this donor was substantially lower than observed with IFN-α treatment of freshly isolated PHH in a previous study [14]. Consistent with the antiviral response in the other PHH, GS-9620-CM did not reduce cccDNA levels in this donor (Supplementary Fig. 12, PHH donor 1). Treatment with a high concentration of IFN-α (1000 IU/mL) also did not reduce cccDNA levels in this or other PHH donors (Supplementary Fig. 12). Taken together, these data demonstrate that GS-9620-CM did not strongly increase the expression of *A3A* and *A3B* and did not induce cccDNA degradation in our cell culture system.

Cytokines induced by GS-9620 enhance presentation of an immunodominant HBV peptide

Antigen presentation by human hepatocytes is thought to be generally inefficient [17]. It was therefore interesting that transcriptional pathway analysis revealed GS-9620-CM induced antigen processing and presentation signatures in HBV-infected PHH (Fig. 5A, Supplementary Table 1). In line with the pathway analysis, GS-9620-CM induced expression of immunoproteasome subunits (*PSMB8*, *PSMB9* and *PSMB10*) as well as various MHC class I antigen presentation genes (e.g. *TAP1*, *TAP2*, *HLA* genes) and a

regulator of class I MHC expression (*NLRC5*) in PHH (Supplementary Table 4). In contrast, constitutive proteasome subunits (*PSMB5*, *PSMB6* and *PSMB7*) were not identified as GS-9620-CM DEGs (Supplementary Table 4). Consistent with the analysis of other ISGs, induction of these antigen processing and presentation genes was comparable in mock-infected and HBV-infected PHH (Supplementary Table 7).

A previous study determined that immunoproteasome subunits do not play a role in IFN-mediation inhibition of viral replication in HBV transgenic mice, but influence both the magnitude and specificity of the $CD8^+$ T cells response to HBV proteins [18]. Since presentation of the immunodominant HBsAg 183-191 (Env183-191) peptide by MHC class I is enhanced by expression of immunoproteasome subunits in transgenic mice [18], we used an antibody which recognizes the Env183-191 peptide:HLA-A0201 (pMHC) complex [19] to determine whether GS-9620-CM influences presentation of this peptide by HBV-infected PHH. This monoclonal antibody (Env183-191 mAb) was validated by demonstrating specific binding to the cognate pMHC in peptide-pulsed T2 cells (Fig. 7A and B). Using the Env183-191 mAb, we determined that GS-9620-CM significantly induced presentation of Env183-191 by HBV-infected HLA-$A0201^+$ PHH (Fig. 7C). Notably, this immunodominant viral peptide was not detected on HBV-infected PHH in the absence of IFN-γ or GS-9620-CM treatment (Fig. 7C), in line with previous data indicating that this peptide is most efficiently processed by the immunoproteasome [18]. Together, these data suggest that GS-9620-induced cytokines can modulate HBV peptide presentation in HBV-infected PHH.

DISCUSSION

The oral TLR7 agonist GS-9620 induced prolonged suppression of serum viral DNA and antigens in both the woodchuck and chimpanzee models of CHB [6, 7]. In the current study, we characterized the response of HBV-infected human hepatocytes to GS-9620 and GS-9620-induced cytokines to provide insights into the molecular mechanisms that contribute to the antiviral response in vivo. We demonstrated that GS-9620 has no direct antiviral activity in HBV-infected PHH, consistent with the low level *TLR7* mRNA expression in human hepatocytes. In contrast, type I IFN —most likely IFN-α— induced by GS-9620 in PBMCs suppressed HBV RNA, DNA and antigens in HBV-infected PHH and dHepaRG cells, although it did not reduce cccDNA levels. Importantly, this antiviral response to GS-9620-induced IFN-α is consistent with previous studies with recombinant IFN-α in PHH as well as a humanized mouse model [8, 20]. Our data are also in line with a previous study demonstrating type I IFN-dependent inhibition of HBV by a TLR7 agonist in a transgenic mouse model [21].

It has previously been reported that treatment with high dose recombinant IFN-α (1000 U/mL) triggers non-cytolytic degradation of cccDNA in human hepatocytes by APOBEC3 deaminases [14]. The current study was primarily focused on characterizing the antiviral response to lower doses of IFN-α, either in GS-9620-CM (mean: 35 pg/mL) or as recombinant IFN-α (100 IU/mL). These lower amounts of IFN-α were evaluated because they were comparable to the serum IFN-α levels (mean: 119 pg/mL) in CHB chimpanzees at the GS-9620 dose (1 mg/kg) that induced intrahepatic ISGs without modulating $CD8^+$ T and B cells responses [6, 22]. Importantly, the ISG expression profile

in HBV-infected PHH treated with GS-9620-induced IFN-α was similar to the intrahepatic ISG signature in CHB chimpanzees treated with GS-9620, indicating these cytokine levels are translationally relevant. Nonetheless, it is important to note that, in contrast to the aforementioned study, high dose recombinant IFN-α (1000 IU/mL) did not reduce cccDNA levels in our cryopreserved PHH. However, *APOBEC3A* (*A3A)* expression was not induced by GS-9620-CM in most of our PHH donors, while *A3B* expression was only modestly increased. Moreover, even in a PHH donor in which *A3A* expression was increased by GS-9620-CM treatment, the magnitude of induction was lower than observed with IFN-α treatment of freshly isolated PHH in the previous study [14]. This suggests that there may be differential effects of IFN-α on ISG expression (and hence cccDNA stability) in cryopreserved vs. freshly isolated PHH. Differences in the cccDNA qPCR quantitation protocols (e.g. DNA extraction method, T5 exonuclease treatment, primers/probes and HBV standards) may also have contributed to the discrepancy between the studies. Accordingly, once the ongoing efforts to harmonize cccDNA quantification methods have been completed, it will be important to perform additional studies (e.g. in hepatocytes freshly isolated from CHB patients) to further investigate APOBEC3-mediated cccDNA degradation.

Identification of the key molecular effectors of HBV suppression by IFN-α has been challenging. The scale of this task is illustrated by the fact that GS-9620-CM strongly induced the expression of hundreds of ISGs in HBV-infected PHH, including various well-characterized HBV restriction factors (e.g. *MxA*, *tetherin*) [12, 13]. Additional layers of complexity are that intracellular localization (and not just expression level) can be

important for the antiviral activity of HBV restriction factors [10] and that IFN-α treatment can induce epigenetic modification of cccDNA [23, 24]. Indeed, epigenetic regulation of cccDNA may underlie the prolonged effect of GS-9620-CM treatment in our cell culture system. Interestingly, the epigenetic state of cccDNA in the absence of HBx (i.e. when the viral genome is transcriptionally repressed by Smc5/6), is similar to that induced by IFN-α treatment [25]. However, the lack of Smc5/6 induction by GS-9620-CM is consistent with this complex being an intrinsic antiviral restriction factor [10] and suggests it does not play a role in type I IFN-mediated HBV suppression. However, if IFN-α reduces HBx protein levels and leads to reappearance of Smc5/6 in HBV-infected PHH, then this complex may play a role in maintaining cccDNA transcriptional suppression after cessation of treatment. Unfortunately, it is challenging to analyze HBx in HBV-infected PHH due to the lack of an antibody suitable for imaging studies.

The prolonged antiviral effect observed in vitro with GS-9620-CM may contribute to the sustained antiviral response induced by GS-9620 in animal models of CHB. However, the lack of cccDNA reduction by GS-9620-induced cytokines in our cell culture model suggests that additional components of the TLR7-induced immune response may have contributed to the antiviral response to GS-9620 in vivo. This interpretation is in line with recent transcriptomic and immunohistochemical analyses of liver tissue from the GS-9260 chimpanzee study, which suggests that cytolytic $CD8^+$ T cell effector mechanisms played a key role in the antiviral response to treatment [22]. Consequently, it is interesting that GS-9620-CM induced expression of antigen processing and presentation

genes (including immunoproteasome subunits) and enhanced presentation of an immunodominant viral peptide in HBV-infected PHH. Intrahepatic expression of many of the same genes was induced during clearance of acute HBV infection in chimpanzees [26] and during the peak of antiviral response in GS-9620-treated chimpanzees [22]. This raises the possibility that GS-9620 may modulate the $CD8^+$ T cell response to infected cells by altering presentation of HBV epitopes. However, GS-9620-CM also strongly induced *PD-L1* (*CD274*) expression in HBV-infected hepatocytes (Supplementary Table 4) and reduced viral protein levels within infected cells, both of which would be expected to influence the antiviral function of virus-specific $CD8^+$ T cells [17, 27]. GS-9620 may also impact the antiviral function of HBV-specific $CD8^+$ T cells by other mechanisms, for example by inducing immune cell migration, interactions and/or survival (as discussed in reference [22]), or by reducing HBV-mediated suppression of antiviral immunity by lowering HBV antigen levels. Further studies are required to evaluate these various hypotheses.

Another notable finding from this study is that established HBV infection does not inhibit the innate immune response of human hepatocytes in our cell culture model. Taken together with recent studies demonstrating that HBV infection does not induce a detectable innate immune response in PHH, these data suggest that HBV may passively evade innate immune detection in hepatocytes [10, 28]. These in vitro observations are in line with acute infection studies in chimpanzees, woodchucks and humans, as well as with the recently described STING deficiency of PHH [26, 29-31]. However, it is important to note that the levels of innate immune stimulation used in this study may

have been sufficient to breach modest HBV-mediated inhibition of innate immune signaling [9, 20]. In such a scenario, it is envisaged that HBV actively suppresses the (presumably low level) innate immune response induced by viral infection, but cannot counter the strong response induced by IFN-α or the innate immune activators used in this study. Additionally, our study did not examine whether HBV inhibits innate immune responses in hepatocytes early after infection, as has previously been reported [32]. These differences in experimental set-up may explain the discrepancy between our data and previous studies which have reported inhibition of hepatocyte innate immunity by HBV.

It is also possible that genetic and/or environmental factors influenced the PHH used in this study so that they did not accurately model the hepatocyte innate immune response in vivo. However, this is considered unlikely for several reasons. Firstly, the PHH were isolated from healthy donor livers and were selected based on the ability of the cells to plate and maintain high viability in culture. Mirroring the clinical situation when infected early in life, we have found that PHH from the vast majority of donors evaluated to-date can be efficiently infected when plating and viability conditions are achieved. Secondly, all PHH donors strongly responded to diverse innate immune stimuli. Thirdly, the wide age range of PHH donors used in this study (3, 8, 28 and 66 years old) would be expected to mitigate any potential role of environmental factors in the hepatocyte innate immune response. Nevertheless, it is very challenging to experimentally model chronic HBV infection in vitro. Effective in vitro modeling is further complicated by potential differences in the innate immune response and/or viral replication capacity of freshly isolated vs cryopreserved hepatocytes. Therefore, studies in hepatocytes freshly isolated

from CHB patients will be required to confirm the validity of our findings using cryopreserved PHH from healthy donor livers.

In summary, our data provide important insights into the mechanisms underlying the antiviral response to GS-9620 in animal models of CHB. In addition, this study suggests that established HBV infection does not actively inhibit innate immunity in our cell culture model. This has important implications for how HBV establishes chronicity, as well as for the therapeutic response to innate immune agonists such as GS-9620.

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FIGURE LEGENDS

Fig. 1. GS-9620-induced cytokines inhibit HBV DNA, RNA and antigen levels in HBV-infected PHH and dHepaRG cells. (A-E) HBV-infected PHH (n=3 donors) were treated with Mock-CM, GS-9620-CM or 100 IU/mL IFN-α from day 3-20, day 3-13 or day 13-20 post-infection. Plots show intracellular HBV RNA, extracellular HBV DNA, HBeAg and HBsAg, as well as cell viability at day 20 post-infection. Data are expressed as a percentage of the no treatment (Tx) control; bar height indicates the mean and the error bars represent the SEM. (F) HBV-infected dHepaRG cells were treated with Mock-CM or GS-9620-CM from day 7-17 post-infection. Plot shows intracellular HBV DNA and extracellular HBeAg and HBsAg levels, as well as cell viability at day 17 post-infection. Data are expressed as a percentage of the no treatment control; bar height indicates the mean of n=2 independent experiments and the error bars represent the SEM. Fresh media with CM or IFN-α was added every 2-4 days during the treatment phase of these studies. Statistical significance relative to the no treatment control was calculated with log-transformed values by one-way ANOVA with Dunnett's multiple comparison correction. *$p<0.05$, **$p<0.01$, ***$p<0.0001$, ns: not significant ($p>0.05$).

Fig. 2. GS-9620-induced cytokines do not alter cccDNA levels in HBV-infected PHH and dHepaRG cells. HBV-infected PHH (n=2 donors) and dHepaRG were treated with Mock-CM or GS-9620-CM from day 3-13 post-infection (PHH) or day 7-17 post-infection (dHepaRG). HBV cccDNA was measured by (A) qPCR and (B) Southern Blot at day 13 (PHH) or day 17 (dHepaRG) post-infection. HBeAg was also measured at day

13 (PHH) or day 17 (dHepaRG) post-infection. The qPCR and HBeAg data are expressed as a percentage of the no treatment (Tx) control; bar height indicates the mean and the error bars represent the SEM. Statistical significance relative to the no treatment control was calculated with log-transformed values by one-way ANOVA with Dunnett's multiple comparison correction.

Fig. 3. Antiviral activity of GS-9620-induced cytokines in HBV-infected PHH is dependent on the type I IFN receptor. (A) Cytokine levels in Mock-CM and GS-9620-CM (both at 1:100 dilution) from healthy human PBMCs (n=3 donors). Fold-induction in GS-9620-CM relative to Mock-CM is shown above each cytokine. (B-C) HBV-infected PHH (single donor, n=3 independent experiments) were treated with Mock-CM, GS-9620-CM or 100 IU/mL IFN-α from day 3-13 post-infection in the presence of IFNAR-blocking or isotype control (Ctrl) antibodies (Ab). (B) qRT-PCR data at day 13 post-infection expressed as fold-change relative to the no treatment control. (C) HBeAg levels at day 13 post-infection expressed as a percentage of the no treatment (Tx) control. (D) PHH (n=2 donors) were infected with HBV, transfected with siIFNAR1 (IFNAR) or siControl (Ctrl) 2 days post-infection and then treated with Mock-CM or GS-9620-CM from day 3-13 post-infection. qRT-PCR data at day 13 post-infection is shown; data is expressed as a percentage of Mock-CM. (E) HBeAg levels at day 13 post-infection for the study described in (D); data is expressed as a percentage of Mock-CM. For all plots, bar height indicates the mean and the error bars represent the SEM. Statistical significance relative to the no treatment control (B, C) or Mock-CM (D, E) was

calculated with log-transformed values by one-way ANOVA with Dunnett's multiple comparison correction.

Fig. 4. Established HBV infection does not significantly alter the transcriptional response to GS-9620-induced cytokines. (A) Schematic summarizing the design of the study with two independent PHH donors. d: day, h: hour. (B) Immunofluorescence staining of HBV core (red) and nuclei (blue) at day 15 post-infection with HBV (+) or mock (-). (C) Number of differentially expressed genes (DEGs) for GS-9620-CM relative to time-matched Mock-CM (red, over-expressed; blue, under-expressed). (D) Correlation analyses of the transcriptional response to GS-9620-CM by Pearson correlation. The top two plots display log_2-fold change values at 8 hours post-treatment with GS-9620-CM relative to time-matched Mock-CM for each PHH donor in HBV-infected (+HBV) and mock-infected (-HBV) cells. The bottom plot displays log_2 fold-change values at 8 hours post-treatment with GS-9620-CM relative to time-matched mock-CM for each donor in HBV-infected cells.

Fig. 5. GS-9620-induced cytokines stimulate an IFN response in PHH which is not significantly altered by HBV infection. Set-up described in Fig. 4A. (A) The top canonical pathways induced in HBV-infected PHH (both donors) at 8 hours post-treatment with GS-9620-CM relative to time-matched Mock-CM were identified by Ingenuity Pathway analysis (IPA). The –log(*p*-value) for p=0.05 and p=0.01 significance levels are indicated. The complete DEG list is provided in Supplementary Table 5. (B) Expression of ISGs significantly modulated by GS-9620-CM plotted by mean log_2 fold-

change relative to time-matched mock-CM control for each PHH donor. Rows represent individual genes; over-expression (red) and under-expression (blue) indicated by scale bars for log_2 fold change values. The ISG DEG list is provided in Supplementary Table 6. (C) RNA-Seq data for select ISGs at 8 hours post-treatment with GS-9620-CM relative to time-matched mock-CM for each donor. False discovery rate (FDR) comparing GS-9620-CM modulated expression change between mock-infected and HBV-infected PHH is displayed for each gene. Comparison data for all ISGs displayed in (B) is provided in Supplementary Table 7.

Fig. 6. Established HBV infection does not significantly modulate the transcriptional response to various innate immune stimuli. (A) Schematic summarizing the design of the study with three independent PHH donors treated with Mock-CM, GS-9620-CM, 100 IU/mL IFN-α, 10 μg/mL poly(I:C) or 10 HAU/mL Sendai virus (SeV). d: day, h: hour. (B) Immunofluorescence staining of HBV core (red) and nuclei (blue) at day 13 post-infection with HBV (+) or mock (-). (C) qRT-PCR data expressed as fold-change relative to no treatment control in mock-infected PHH for each donor. Bar height indicates mean and error bars represent the SEM. The symbols immediately above the bars denote the level of statistical significance relative to the no treatment control in mock-infected PHH: **$p<0.01$; ***$p<0.001$; ns: not significant ($p>0.05$). Statistical significance was calculated with log-transformed values by two-way ANOVA with Dunnett's multiple comparison correction. The p-values above the horizontal lines indicate the level of statistical significance between mock-infected and HBV-infected PHH for each

treatment. Statistical significance was calculated with log-transformed values by unpaired t-test.

Fig. 7. GS-9620-induced cytokines enhance presentation of an immunodominant HBsAg peptide by HBV-infected PHH. (A) Representative FACS profiles of T2 cells pulsed with 100 μg/mL of the indicated peptide and stained with the indicated antibody. (B) Representative confocal microscopy images of T2 cells pulsed with the indicated peptide and stained (red) with either the Env183-191 mAb (Env183 mAb) or an isotype control (ctrl). Nuclei were stained with DAPI (blue). The scale bar represents 10 μm. The data in (A) and (B) are representative of at least two independent experiments. (C) HBV-infected PHH (n=2 HLA-A0201$^+$ donors) were treated with Mock-CM, GS-9620-CM or 50 ng/mL IFN-γ at day 13 post-infection. Forty-eight hours later, pMHC complexes were detected by staining with the Env183-191 mAb (red). Nuclei were stained with DAPI (blue). Representative confocal microscopy images from a single PHH donor are shown. The scale bar represents 10 μm. Quantitation of the number of pMHC complexes in each PHH donor per treatment condition is displayed below the representative images. The bar height indicates the mean and error bars represent the SEM; at least 20 nuclei were analyzed per condition in each donor. The dotted lines indicate the level of background staining in uninfected PHH treated with IFN-γ. Statistical significance relative to the no treatment (Tx) control was calculated with log-transformed values by one-way ANOVA with Dunnett's multiple comparison correction.

Figure 1

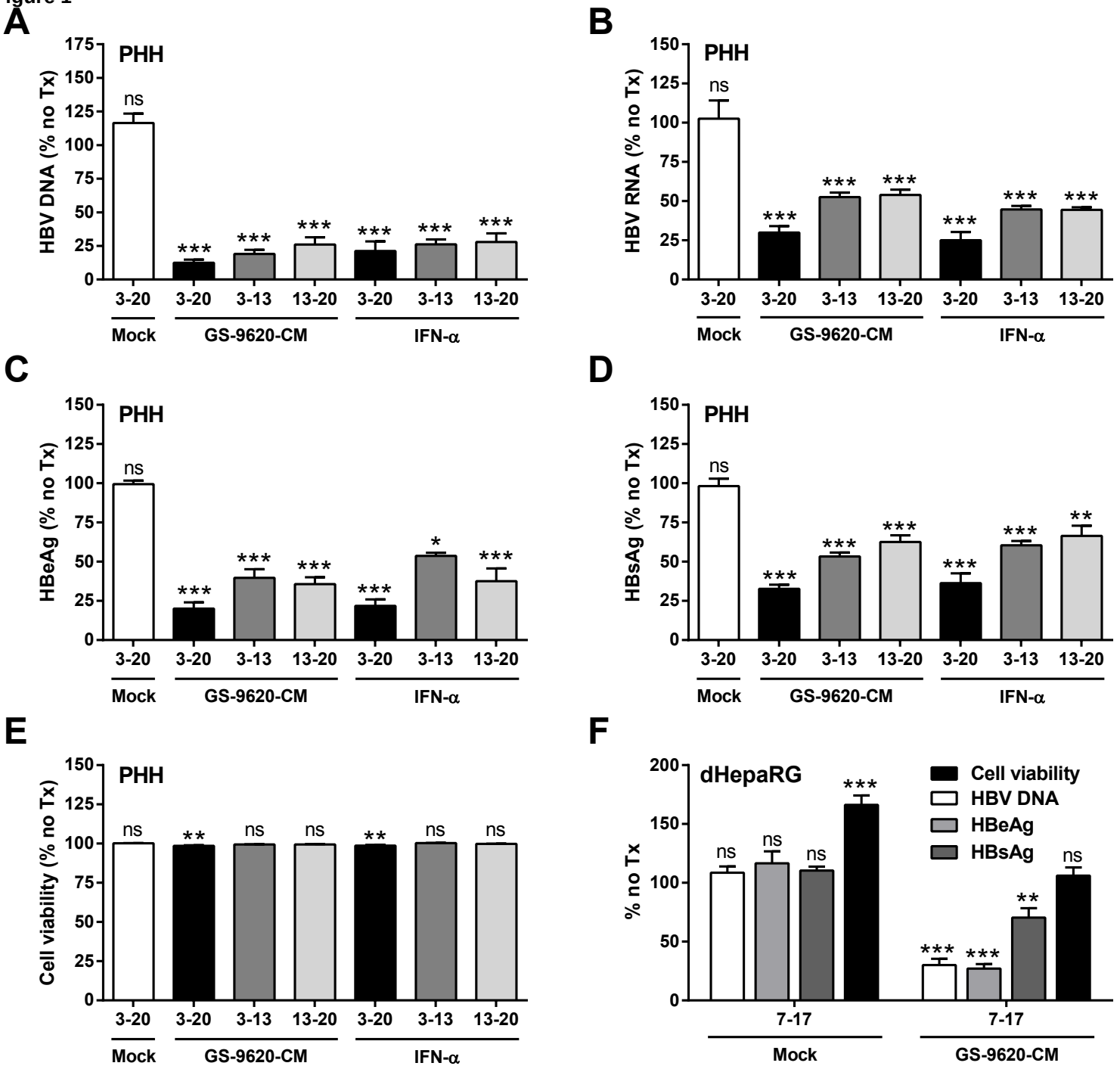
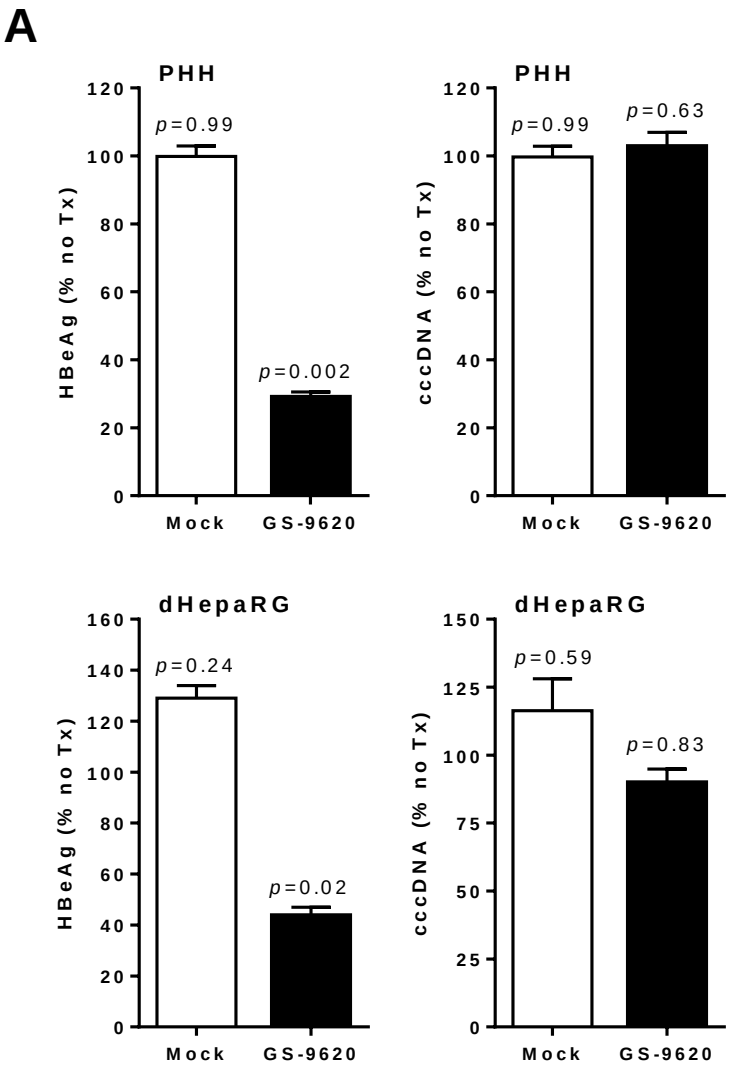

A
PHH
HBV DNA (% no Tx)
175
150
125
100
75
50
25
0
ns
***
***
***
***
***
***
3-20
3-20
3-13
13-20
3-20
3-13
13-20
Mock
GS-9620-CM
IFN-α
B
PHH
HBV RNA (% no Tx)
150
125
100
75
50
25
0
ns
***
***
***
***
***
***
3-20
3-20
3-13
13-20
3-20
3-13
13-20
Mock
GS-9620-CM
IFN-α
C
PHH
HBeAg (% no Tx)
150
125
100
75
50
25
0
ns
***
***
***
***
*
***
3-20
3-20
3-13
13-20
3-20
3-13
13-20
Mock
GS-9620-CM
IFN-α
D
PHH
HBsAg (% no Tx)
150
125
100
75
50
25
0
ns
***
***
***
***
***
**
3-20
3-20
3-13
13-20
3-20
3-13
13-20
Mock
GS-9620-CM
IFN-α
E
PHH
Cell viability (% no Tx)
150
125
100
75
50
25
0
ns
**
ns
ns
**
ns
ns
3-20
3-20
3-13
13-20
3-20
3-13
13-20
Mock
GS-9620-CM
IFN-α
F
dHepaRG
% no Tx
200
150
100
50
0
ns
ns
ns
***
***
***
**
ns
Cell viability
HBV DNA
HBeAg
HBsAg
7-17
7-17
Mock
GS-9620-CM

Figure 2


A
PHH
HBeAg (% no Tx)
p=0.99
p=0.002
Mock
GS-9620
PHH
cccDNA (% no Tx)
p=0.99
p=0.63
Mock
GS-9620
dHepaRG
HBeAg (% no Tx)
p=0.24
p=0.02
Mock
GS-9620
dHepaRG
cccDNA (% no Tx)
p=0.59
p=0.83
Mock
GS-9620


B
cccDNA
No Tx
Mock
GS-9620
Southern Blot
PHH donor 1
PHH donor 2
dHepaRG

Figure 3

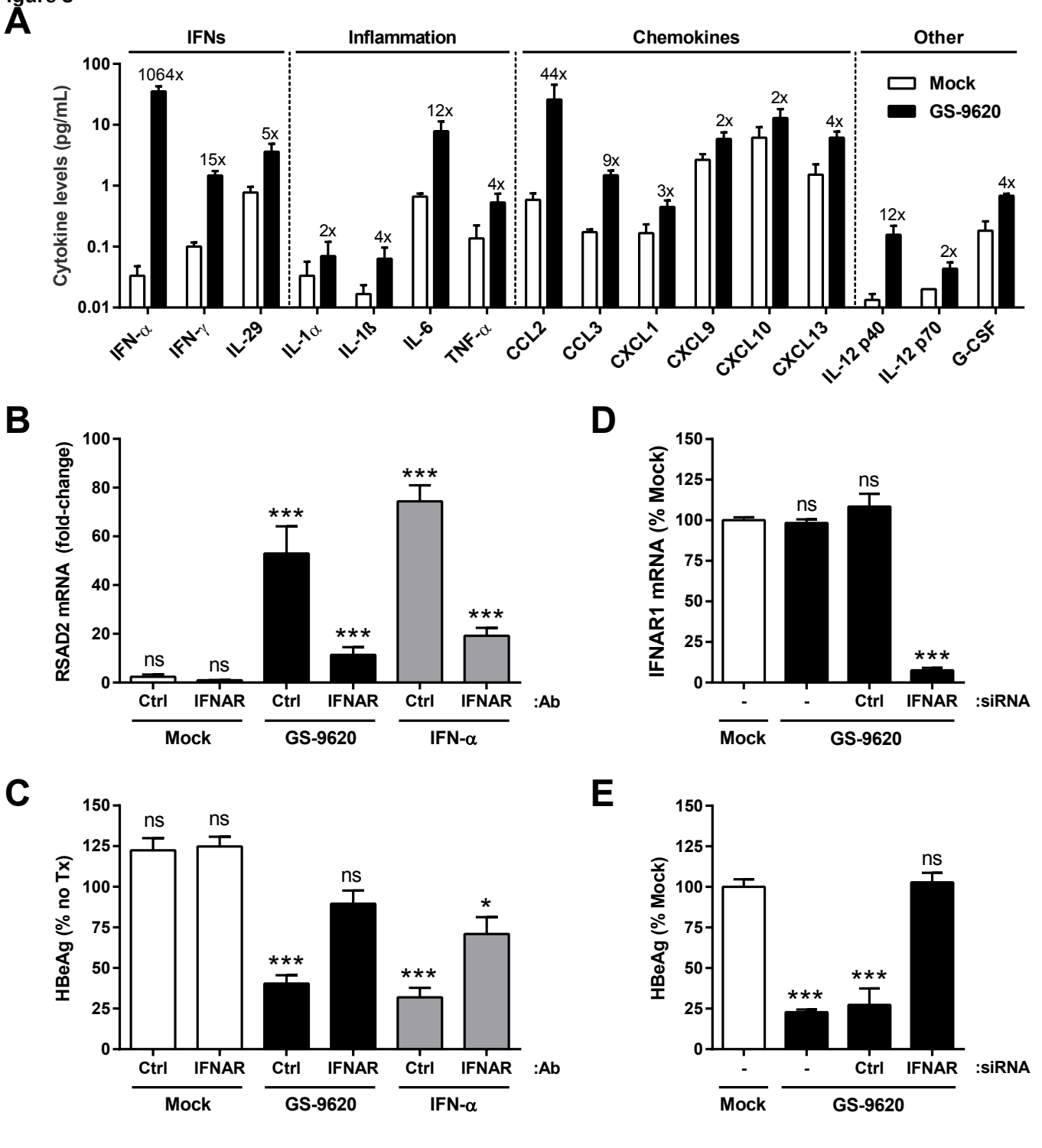

A
IFNs
Inflammation
Chemokines
Other
Mock
GS-9620
Cytokine levels (pg/mL)
100
10
1
0.1
0.01
1064x
15x
5x
2x
4x
12x
4x
44x
9x
3x
2x
2x
4x
12x
2x
4x
IFN-α
IFN-γ
IL-29
IL-1α
IL-1ß
IL-6
TNF-α
CCL2
CCL3
CXCL1
CXCL9
CXCL10
CXCL13
IL-12 p40
IL-12 p70
G-CSF
B
RSAD2 mRNA (fold-change)
100
80
60
40
20
0
ns
ns
***
***
***
***
Ctrl
IFNAR
Ctrl
IFNAR
Ctrl
IFNAR
:Ab
Mock
GS-9620
IFN-α
D
IFNAR1 mRNA (% Mock)
150
125
100
75
50
25
0
ns
ns
***
-
-
Ctrl
IFNAR
:siRNA
Mock
GS-9620
C
HBeAg (% no Tx)
150
125
100
75
50
25
0
ns
ns
ns
*
***
***
Ctrl
IFNAR
Ctrl
IFNAR
Ctrl
IFNAR
:Ab
Mock
GS-9620
IFN-α
E
HBeAg (% Mock)
150
125
100
75
50
25
0
ns
***
***
-
-
Ctrl
IFNAR
:siRNA
Mock
GS-9620

Figure 4

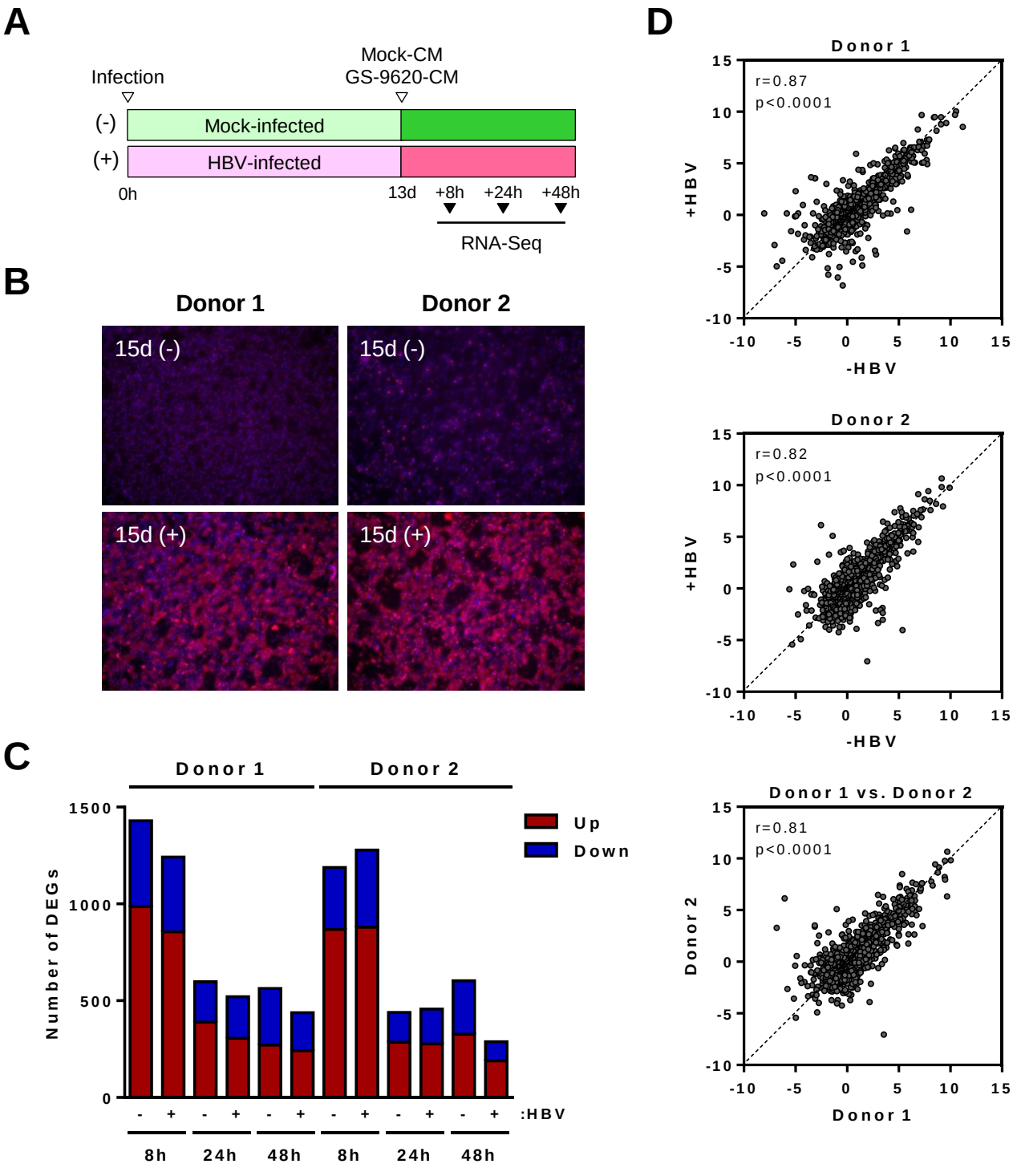

A
Infection
Mock-CM
GS-9620-CM
(-)
Mock-infected
(+)
HBV-infected
0h
13d
+8h
+24h
+48h
RNA-Seq
B
Donor 1
Donor 2
15d (-)
15d (-)
15d (+)
15d (+)
C
Donor 1
Donor 2
Number of DEGs
1500
1000
500
0
Up
Down
- + - + - + - + - + - +
:HBV
8h
24h
48h
8h
24h
48h
D
Donor 1
r=0.87
p<0.0001
+HBV
-HBV
Donor 2
r=0.82
p<0.0001
+HBV
-HBV
Donor 1 vs. Donor 2
r=0.81
p<0.0001
Donor 2
Donor 1
-10 -5 0 5 10 15

Figure 5

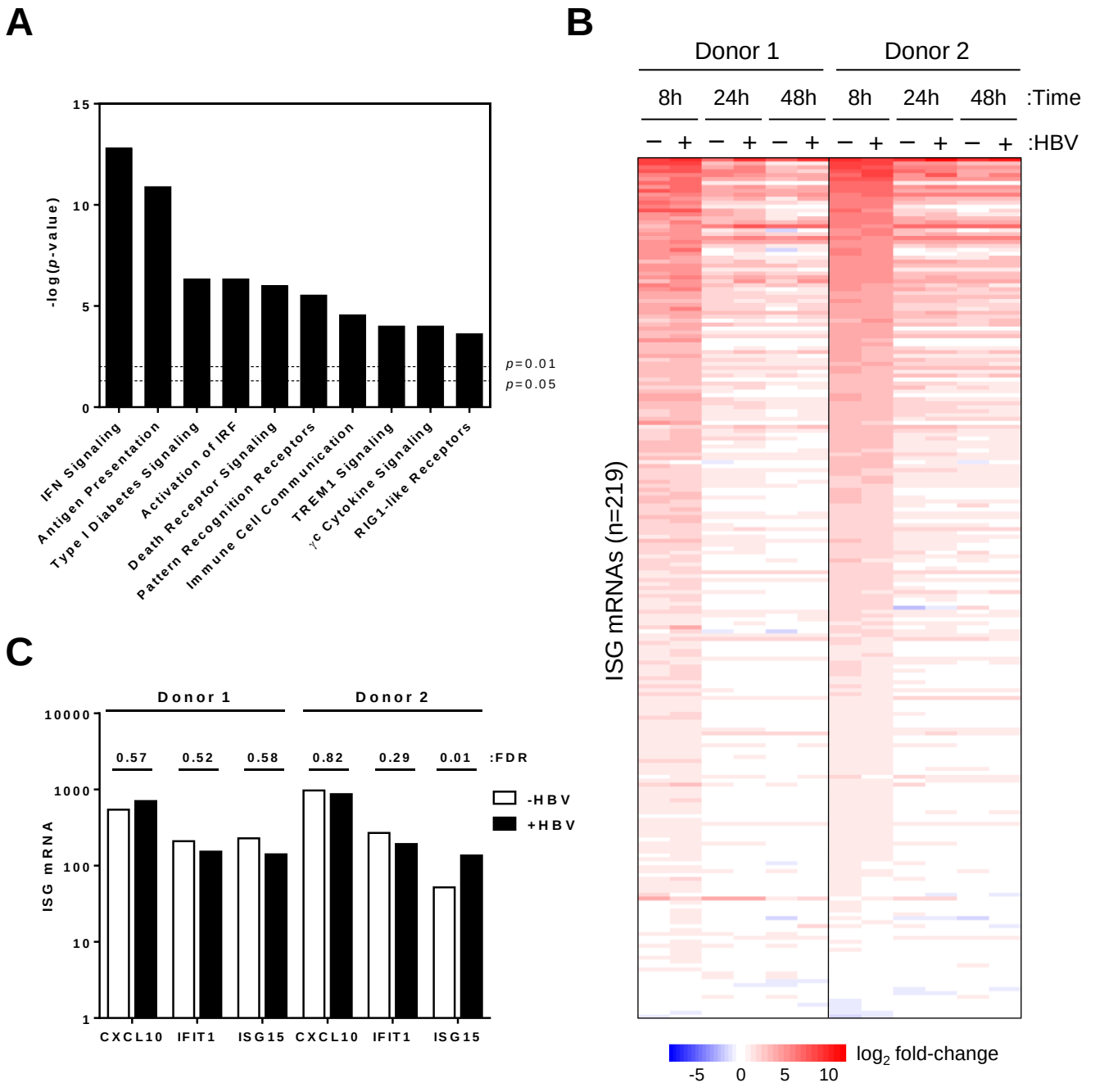

A
15
10
5
0
-log(p-value)
p=0.01
p=0.05
IFN Signaling
Antigen Presentation
Type I Diabetes Signaling
Activation of IRF
Death Receptor Signaling
Pattern Recognition Receptors
Immune Cell Communication
TREM1 Signaling
γc Cytokine Signaling
RIG1-like Receptors
B
Donor 1
Donor 2
8h
24h
48h
8h
24h
48h
:Time
− + − + − + − + − + − +
:HBV
ISG mRNAs (n=219)
log2 fold-change
-5
0
5
10
C
Donor 1
Donor 2
10000
1000
100
10
1
ISG mRNA
0.57
0.52
0.58
0.82
0.29
0.01
:FDR
-HBV
+HBV
CXCL10
IFIT1
ISG15
CXCL10
IFIT1
ISG15

Figure 6

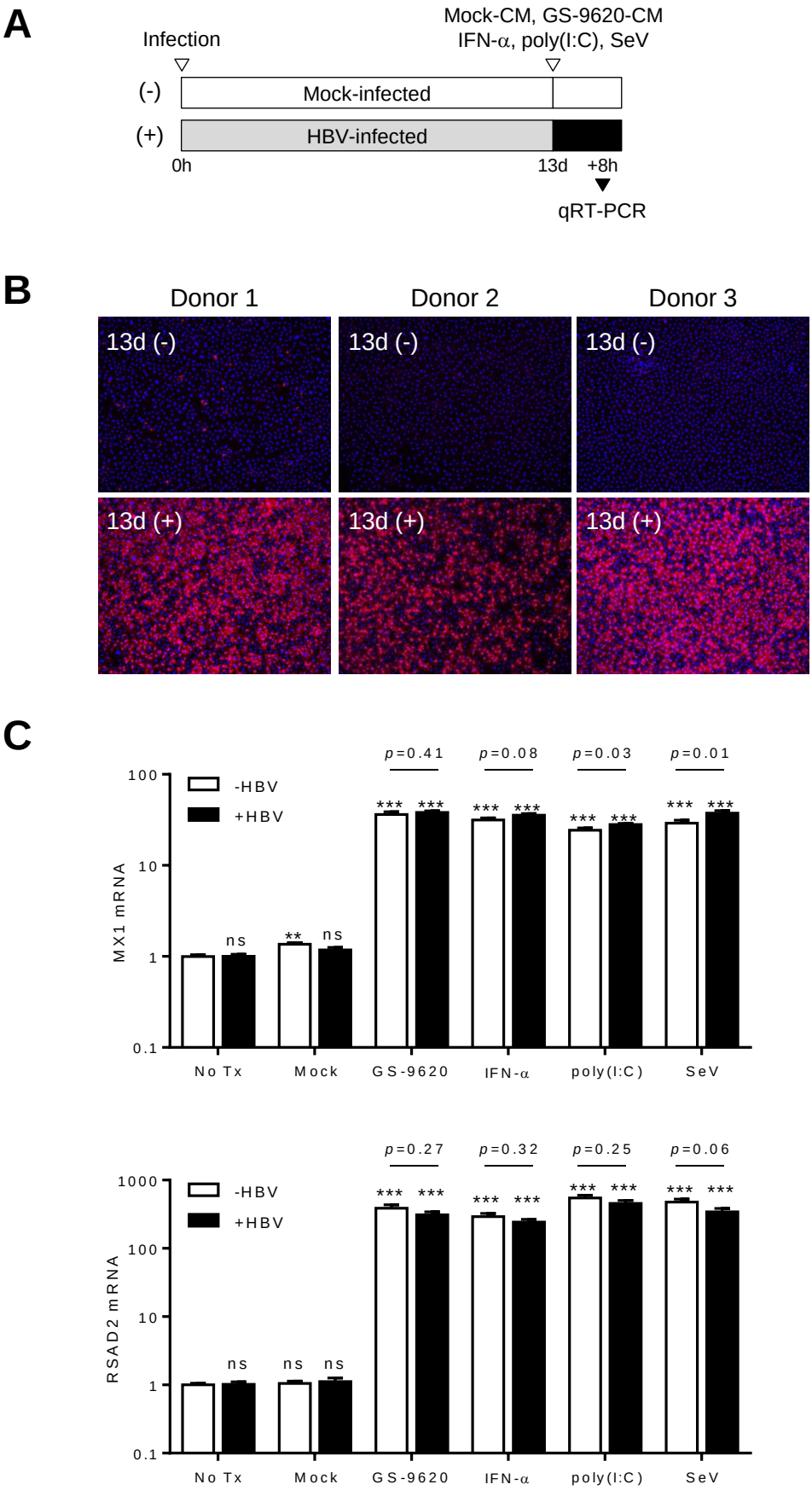
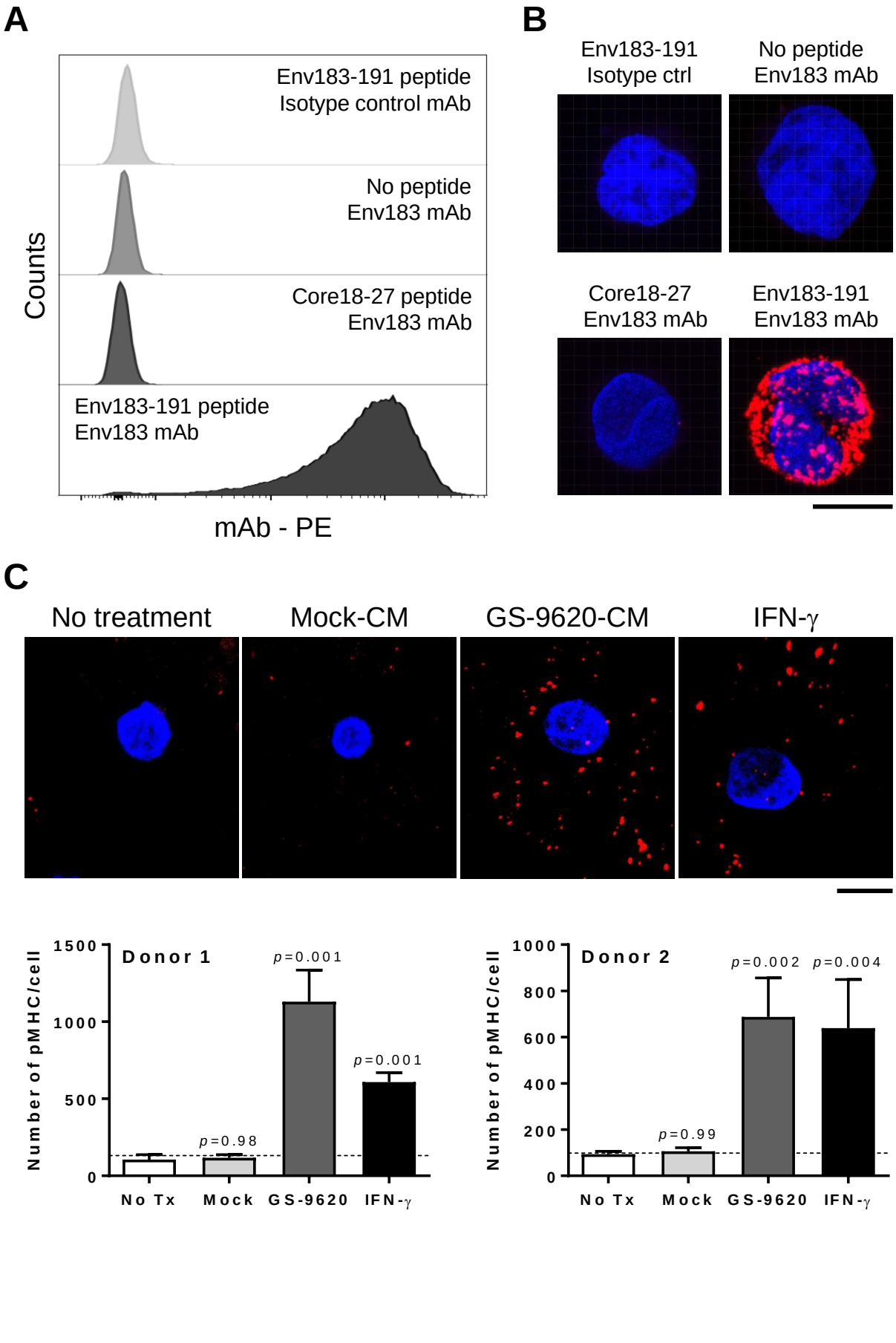

A
Infection
Mock-CM, GS-9620-CM
IFN-α, poly(I:C), SeV
(-)
Mock-infected
(+)
HBV-infected
0h
13d
+8h
qRT-PCR
B
Donor 1
Donor 2
Donor 3
13d (-)
13d (+)
C
p=0.41
p=0.08
p=0.03
p=0.01
-HBV
+HBV
MX1 mRNA
100
10
1
0.1
ns
**
***
No Tx
Mock
GS-9620
IFN-α
poly(I:C)
SeV
p=0.27
p=0.32
p=0.25
p=0.06
RSAD2 mRNA
1000
100

Figure 7


A
Env183-191 peptide
Isotype control mAb
No peptide
Env183 mAb
Core18-27 peptide
Env183 mAb
Env183-191 peptide
Env183 mAb
Counts
mAb - PE
B
Env183-191
Isotype ctrl
No peptide
Env183 mAb
Core18-27
Env183 mAb
Env183-191
Env183 mAb
C
No treatment
Mock-CM
GS-9620-CM
IFN-γ
Donor 1
Number of pMHC/cell
1500
1000
500
0
p=0.001
p=0.001
p=0.98
No Tx
Mock
GS-9620
IFN-γ
Donor 2
Number of pMHC/cell
1000
800
600
400
200
0
p=0.002
p=0.004
p=0.99
No Tx
Mock
GS-9620
IFN-γ

- GS-9620 has no direct antiviral activity against HBV
- Type I IFN induced by GS-9620 durably suppresses HBV without reducing cccDNA levels
- GS-9620-induced cytokines enhance HBV antigen presentation
- Established HBV infection does not modulate innate immune sensing or signaling

*Graphical Abstract