Supplemental Materials and Methods

Virus production and titration

The HBV stock was produced by collecting supernatant of HepG2.2.15 cells twice a week for three weeks and HCV stock was produced by collecting supernatant daily from Huh7.5.1 infected with high titer of HCV JFH-1 strain. Mock control of HBV or HCV virus stock are prepared by collecting culture supernatant from blank HepG2 and Huh7.5.1 cultures respectively. Collected supernatant were concentrated using centrifugal filter devices (Centricon Plus-70 and Biomax 100.000; Millipore Corp., Bedford, MA). Immediately after collection, the virus or mock stocks were divided into aliquots and stored at −80°C until use. HBV stock was quantified by quantitative PCR (qPCR) and HCV stock was quantified by end-point dilution assay as previously described (1).

Extracellular viral genome quantification

Extracellular HBV DNA quantification was performed with DNA extracted from cell culture supernatant using DNeasy Blood & Tissue kit (QIAGEN, Germantown, MD, USA). SYBR Green Master (Roche, Manheim, Germany) was used for PCR with standards prepared from linearized plasmid containing 1.3X HBV genome. Primers used:
Primer 1, 5'-GGAGGGATACATAGAGGTTCCTTGA-3';
primer 2, 5'-GTTGCCCGTTTGTCCTCTAATTC-3'.

Extracellular HCV-RNA was extracted in the same way as intracellular RNA using Isolate II RNA mini kit (Bioline, London, UK). Extracted RNA was used as template in one-step RT-qPCR using QuantiTect Virus+ROX Viral Kit (QIAGEN, Germantown, MD). HCV primer probe used is the same as for intracellular HCV RNA quantification. Viremia levels in mice or in patients were quantified as previously described (2-4).

Intracellular RNA purification and RT-qPCR

Liver tissue samples from humanized chimeric mice were stored in RNAlater (QIAGEN, Germantown, MD, USA) immediately after harvest and delivered in dry iced box. Upon receipt, homogenization was done by Tissuelyser LT (QIAGEN, Germantown, MD, USA) and RNA purification was done using RNeasy Mini Kit (QIAGEN, Germantown, MD, USA). Total cellular RNA from cell cultures was purified using Isolate II RNA mini kit (Bioline, London, UK).

Complementary DNA was obtained following instruction of Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative PCR was performed using the LightCycler™ 480 system with Probe Master (Roche, Manheim, Germany). All primer probe sets for host genes are either pre-designed or self-designed and synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA) (Supplemental Table 1). Relative mRNA expression was analyzed using the second derivative maximum method that includes both normalization to the reference gene (TBP) and to primer efficiency (a dilution series of a calibrator was included in each PCR run). At least one additional reference gene ACTB or 18sRNA was also included in each run to ensure the results are not skewed due to a particular reference gene.

For chimeric tissue gene expression analysis, the specificity of human primer probes has been tested with wild type mice materials and results show no cross reactivity with murine mRNA.

RNAscope assay

In situ hybridization for CXCL10, IFNL mRNA, HCV RNA as well as HBV nucleic acid was performed with 4% PFA fixed cells using the RNAScope fluorescent multiplex detection reagent kit (#320850) according to the manufacturer's instructions (Advanced Cell Diagnostics Inc., CA, USA). Predesigned RNAscope probes Hs-CXCL10 (#311851), Hs-IFNL1 (#412341), V-HCV-GT2a-sense (#441371), V-HBV-GTD (#441351) and their alternative C2 or C3 forms were used for target binding in multiplex assay. Following signal amplification, probes conjugated to Alexa 488, Atto 550 or Atto 647 (Advanced Cell Diagnostics, Newark, CA, USA) was imaged using a Zeiss LSM 700 confocal microscope.

Image quantification

RNAscope images were quantified by counting the signal dots number for individual cells using Analyze Particles tool of Image J software. The fluorescence background of cells shows the entirety of the cells and is thus used to mark the cell border. For each experimental condition, 50-100 cells were counted for signal dots contained. Those values are presented on the dot plots (including all the cells in the view irrespective of its virus positivity). After obtaining this initial data, the dot numbers of each staining were subtracted by the highest counting from corresponding control group; all the cells with positive value were considered virus-positive, value of zero or below were taken as virus-negative. By calculating the percentage of positive cell number to total cell number, the infection efficiency was calculated and presented in the bar graph.

Supplemental References

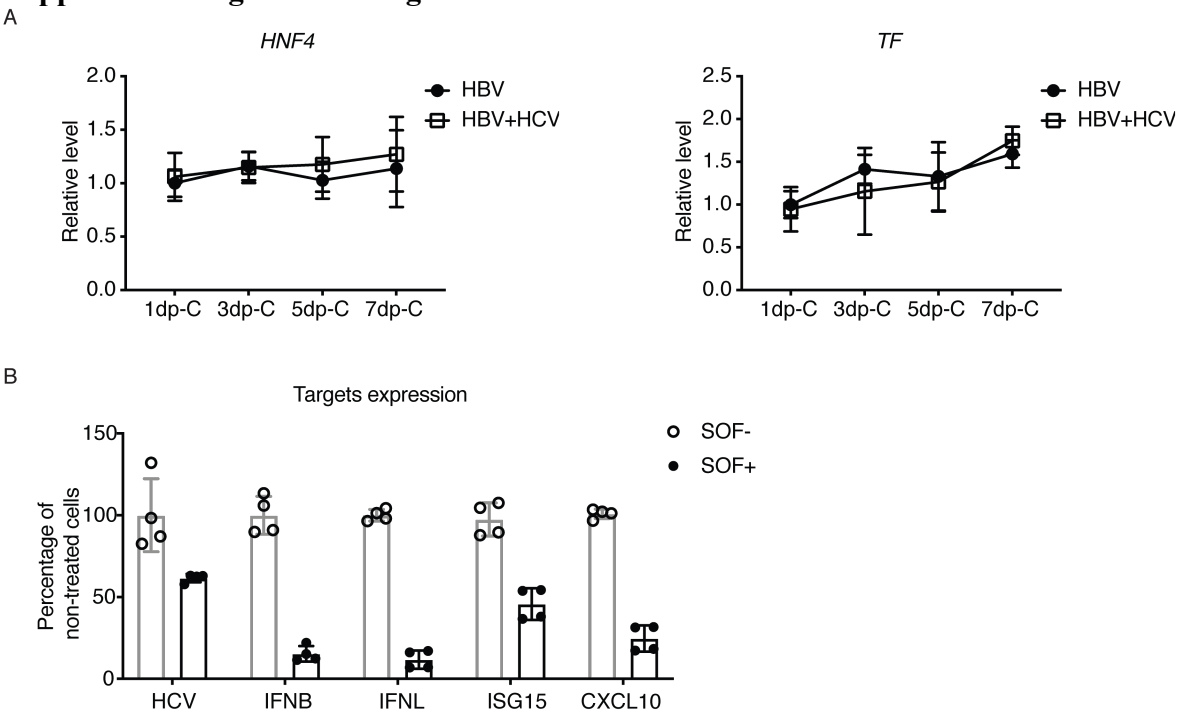
1. Li Q, Lowey B, Sodroski C, Krishnamurthy S, Alao H, Cha H, et al. Cellular microRNA networks regulate host dependency of hepatitis C virus infection. *Nature communications.* 2017;8(1):1789.

2. Hiraga N, Imamura M, Tsuge M, Noguchi C, Takahashi S, Iwao E, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. *FEBS letters.* 2007;581(10):1983-7.

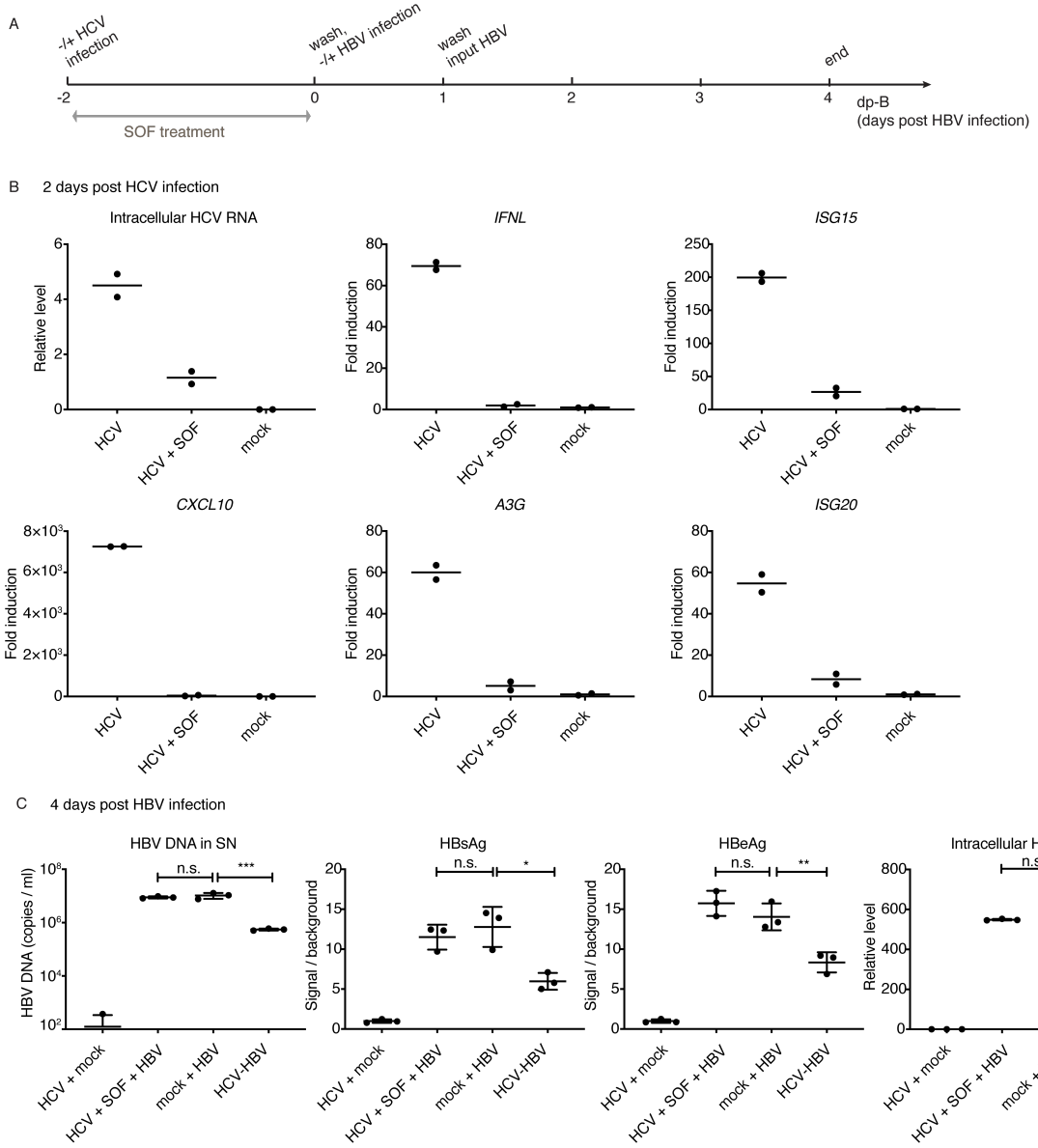
3. Tsuge M, Hiraga N, Takaishi H, Noguchi C, Oga H, Imamura M, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology.* 2005;42(5):1046-54.

4. Liu CJ, Chuang WL, Sheen IS, Wang HY, Chen CY, Tseng KC, et al. Efficacy of Ledipasvir and Sofosbuvir Treatment of HCV Infection in Patients Coinfected With HBV. *Gastroenterology.* 2018;154(4):989-97.

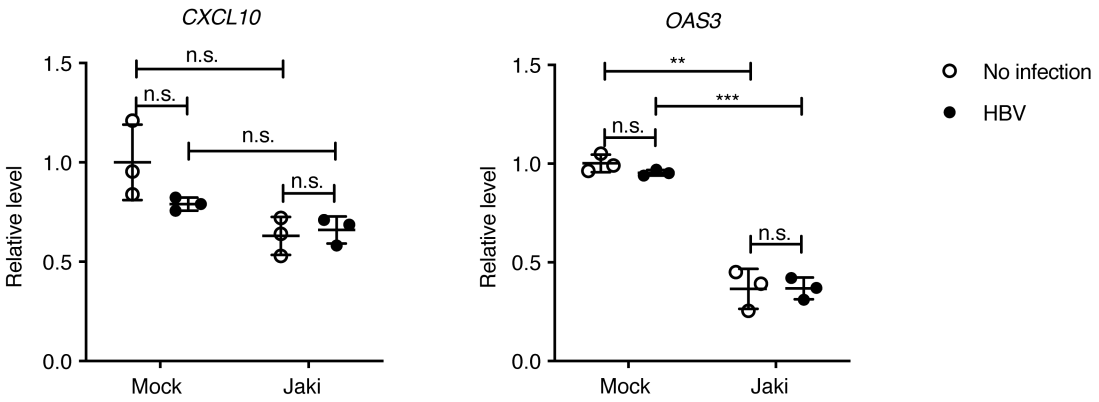
Supplemental Figures and Legends



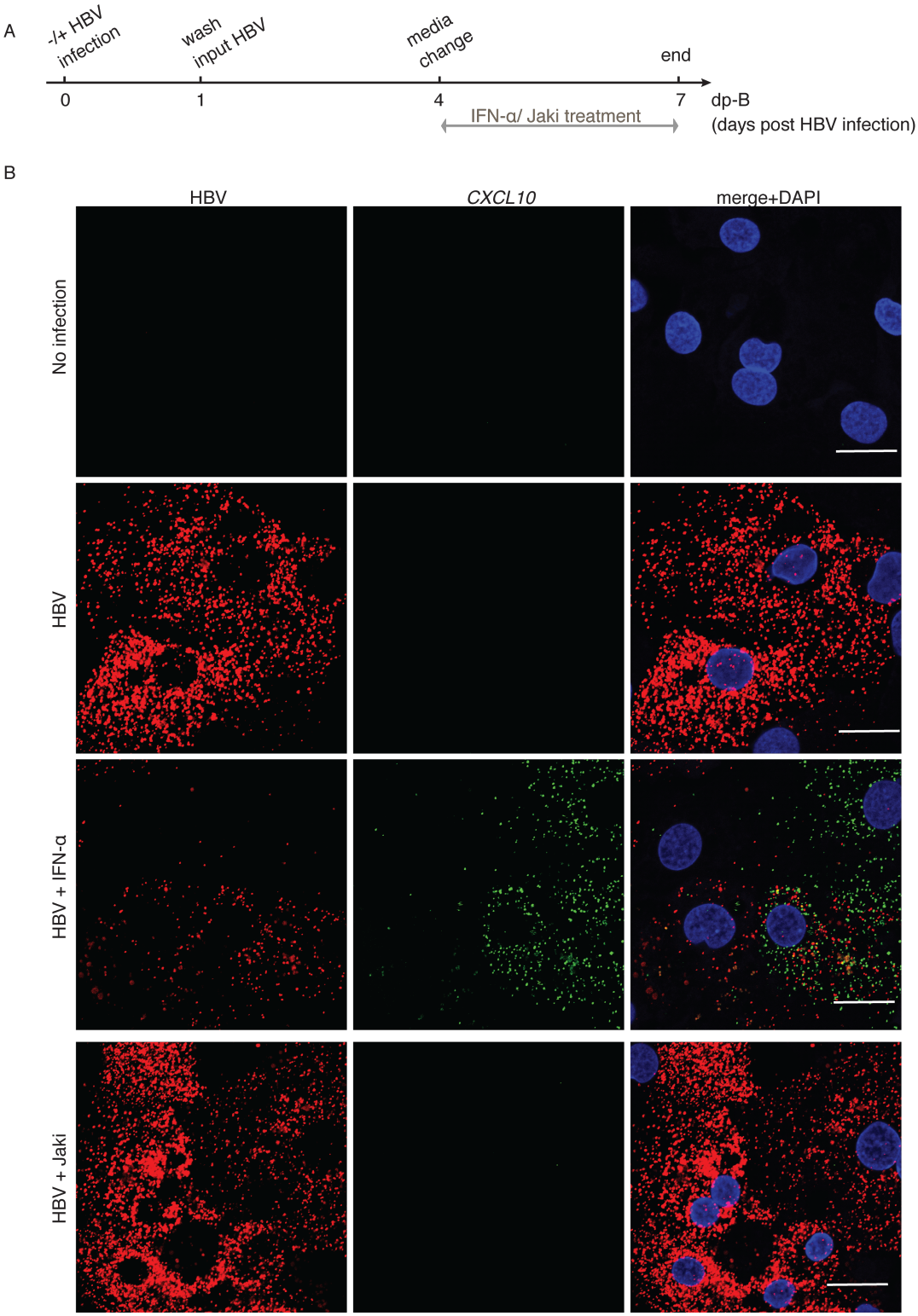
Supplemental Figure 1. Confirmation of unaltered hepatocyte function in coinfected PHH and successful HCV infection in PHH. (A) Target gene expression from the same experiment of Figure 1 were measured by RT-qPCR . Normalized gene expressions relative to those in the HBV mono-infected cells on 1 dp-C (set as 1) were shown as relative levels for *HNF4* and *TF*. (B) PHHs (lot Hu1832) were infected with HCV at MOI=1 $TCID_{50}$/cell overnight with addition of 10 μM SOF. Cells were lysed after 3 times of washing with PBS to remove the input virus. RNA was extracted for gene expression analysis by RT-qPCR. Results are shown as percentages of infected but non-treated cells. Means ± SD are shown. The results are representative of three separate experiments.



Supplemental Figure 2. HBV super-infection in HCV infected PHHs. (A) Schematic representation of the experimental setting. PHHs (lot Hu1832) were mock treated or HCV infected (MOI=1 $TCID_{50}$/mL) for 2 days with the addition of 10 µM SOF as indicated. Cells were then super-infected with HBV or mock infected. Medium was changed daily until day 4 post HBV infection. (B) 2 days after HCV infection, a set of cells were lysed to check HCV RNA and host *IFNL1*, *ISG15*, *CXCL10*, *A3G* and *ISG20* expression by RT-qPCR. Fold induction was calculated by comparing to mock. Duplicate wells were used for each group and means were ploted. (C) 4 days after HBV infection, cells (n=3) were analyzed for HBV infection markers including extracellular HBV DNA by qPCR, extracellular HBsAg and HBeAg by ELISA, and intracellular HBV RNA by RT-qPCR. Comparisons were made by unpaired t-test . Means ± SD are shown. **P*< 0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. The results are representative of three separate experiments.

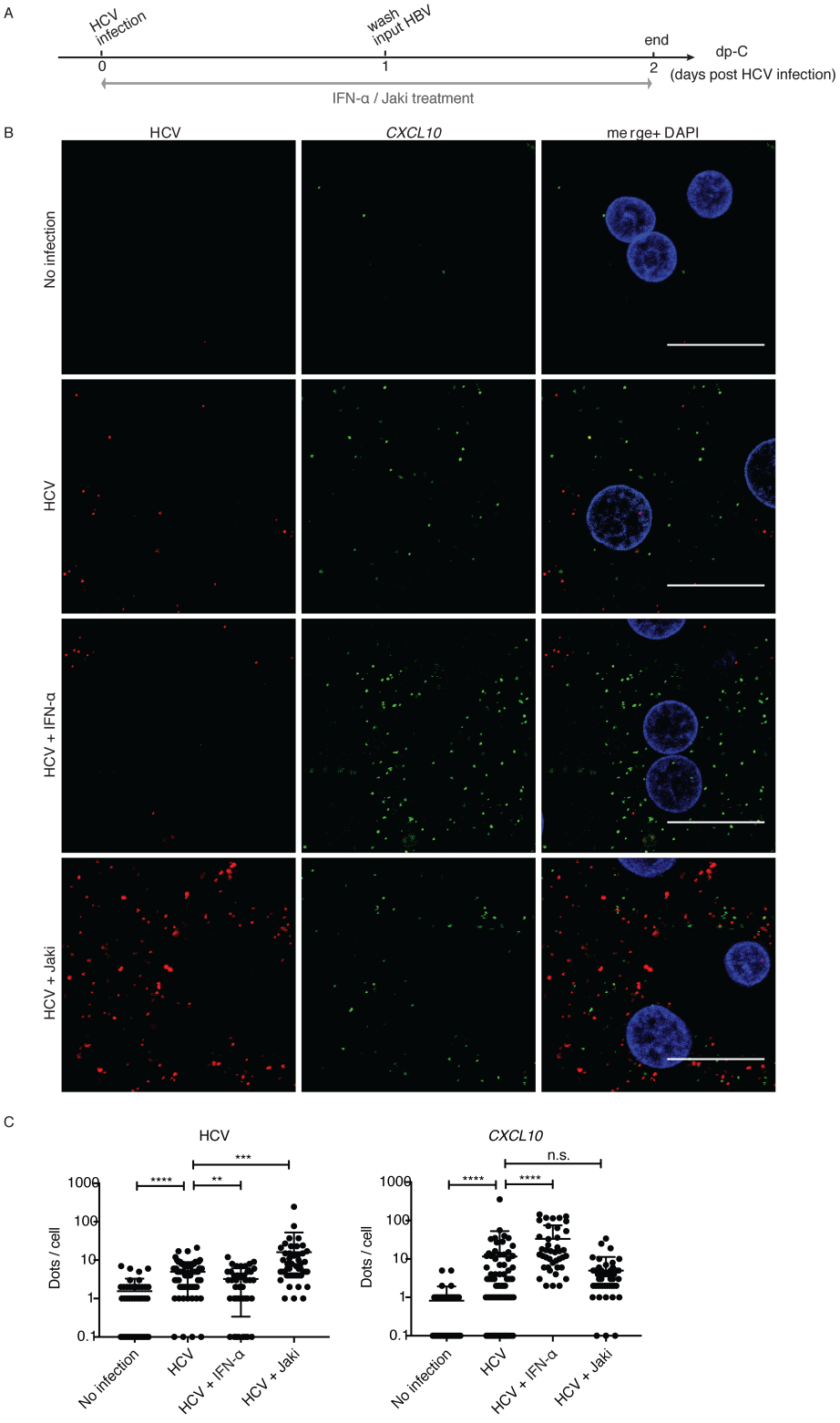


Supplemental Figure 3. Effect of Jaki treatment on basal expression of *CXCL10* and *OAS3* in PHHs. Standard culture of Hu1832 and its HBV-infected counterpart were treated with either veichle control or 1 μM Jaki for 7 days. Treatment was renewed every 3 days after virus inoculation. mRNA expression were quantified using RT-qPCR. Relative levels were calculated by comparing to mock treated non-infected cells. The comparison between two groups were made by unpaired t-test and further corrected by Hochberg procedure for multiple comparisions. ***P*<0.01, ****P*<0.001. Triplicate wells were used for each group.



Supplemental Figure 4. Specificity validation of in situ detection using HBV mono-infected PHHs. (A) Schematic representation of the experiment setting. PHHs (lot Hu1663) were infected with HBV for 7 days or only mock treated. 3 days before the end of experiment, infected cells

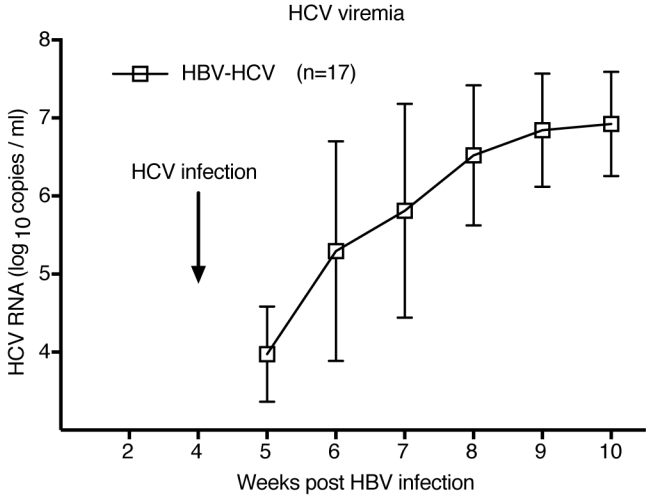
were treated with 1000 IU/ml IFN-α or 5 μM Jaki. (B) Fixed cells were hybridized with probe sets targeting RNA expression of HBV and *CXCL10*. Cell nuclei were counterstained by DAPI. Final signals are shown in red for HBV, green for *CXCL10* and blue for nuclei. Scale bar = 20 μm. The results are representative of three separate experiments.



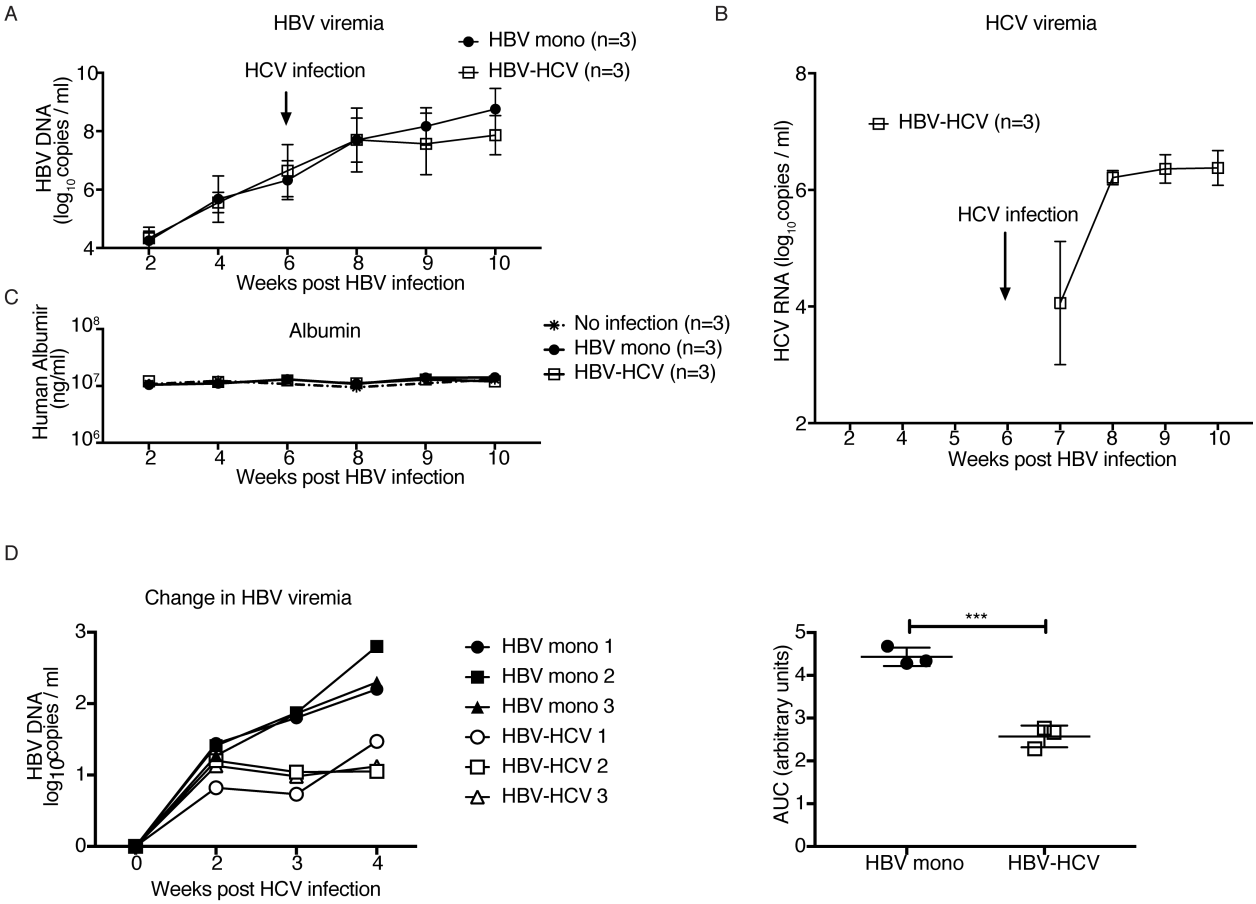
Supplemental Figure 5. Specificity validation of in situ detection using HCV mono-infected PHHs. (A) Schematic representation of the experimental setting. PHHs (lot Hu8196) were infected with HCV at MOI=0.5 $TCID_{50}$/cell overnight with addition of 1000 IU/mL IFN-α or 5 µM Jaki.

Input virus was washed away the next day and the treatment was renewed following medium change. (B) Fixed cells were hybridized with probe sets targeting HCV RNA and mRNA of *CXCL10*. Cell nuclei were counterstained by DAPI. Final signals are shown in red for HCV, green for *CXCL10* and blue for nuclei. Scale bar = 20 μm. (C) Target dots number were quantified using image J as described in Supplemental material. Comparisons were made by unpaired t-test. Means ± SD are shown. n.s.=not significant, $**P<0.01$, $***P<0.001$, $****P<0.0001$. The results are representative of three separate experiments.

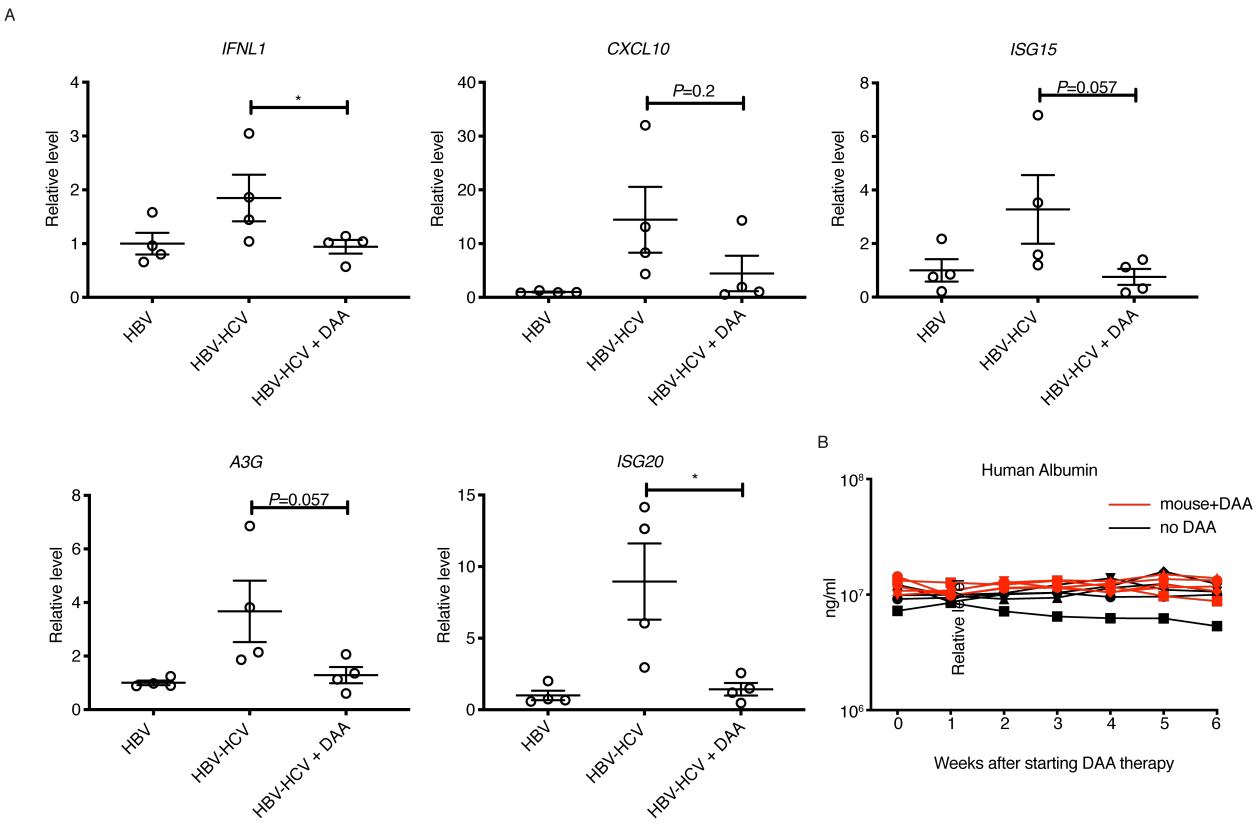
A



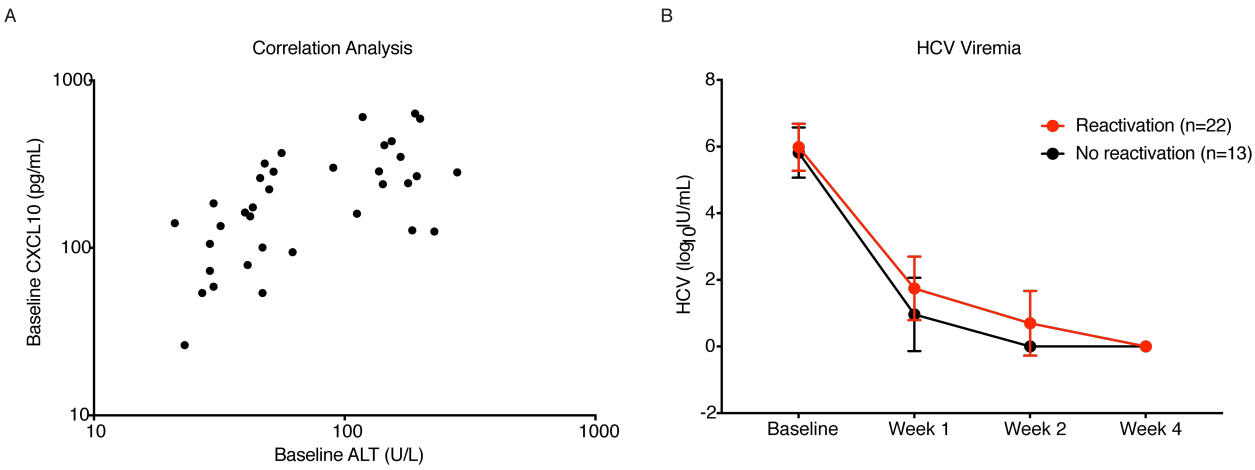
Supplemental Figure 6. HCV viremia of HBV-HCV coinfected mice. The experiment is described in Figure 5 A and B. Means ± SD are shown.



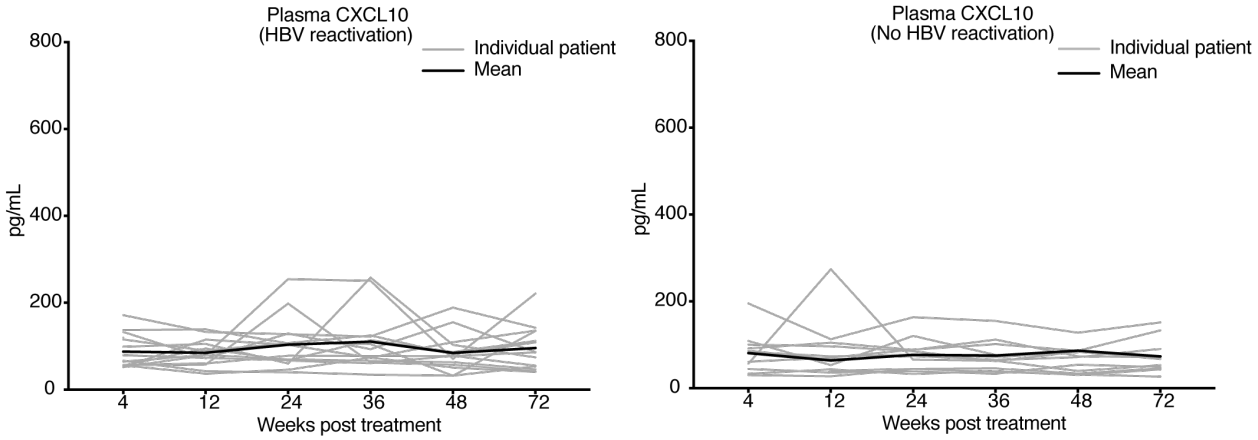
Supplemental Figure 7. Virologic profiles and human albumin concentrations of HBV-HCV coinfected mice that were sacrificed for hepatic gene expression analysis. In relation to Figure 5C&D. Serum HBV-DNA in HBV infected mice (A), serum HCV RNA in coinfected mice (B) and human albumin concentration (C) in all the mice were measured at indicated times. Changes of HBV viremia (D, left) after HCV infection were calculated by comparing the HBV DNA load at indicated time to the level before HCV inoculation. Area under the curve (AUC; D, right) was generated by Prism software for individual mice to represent net total virus production after HCV inoculation. Means ± SD are shown. Comparisons between groups were made by unpaired t-test. ***P<0.001.



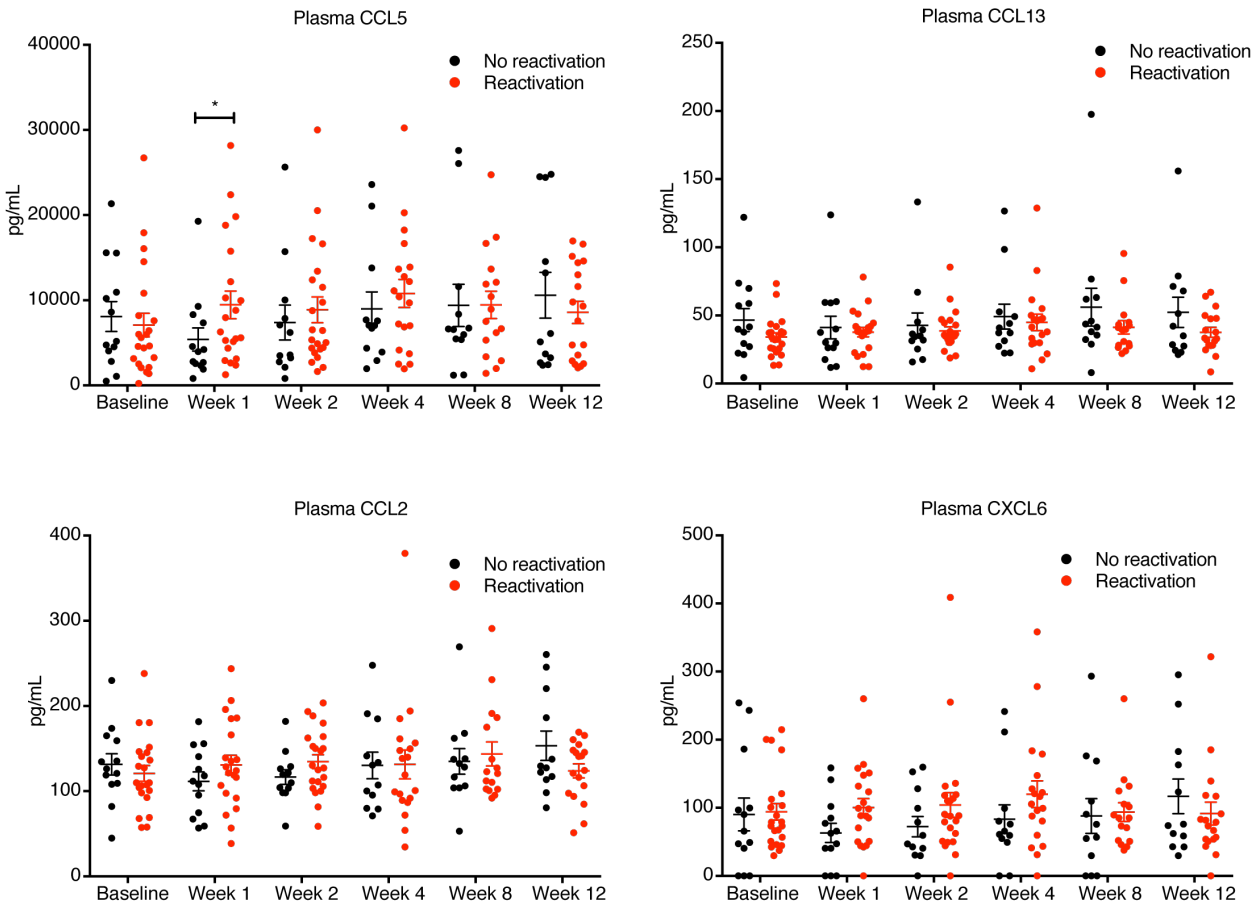
Supplemental Figure 8. Hepatic ISGs expression in coinfected mice after HCV clearance and human albumin levels in mouse serum. (A) As described in Figure 5E, DAA treated (n=4) and matched control group (n=4) coinfected mice were sacrificed 4 weeks after the end of treatment. Mice infected with HBV only for 17 weeks (n=4) were served as negative control for IFN response. Mouse liver tissues were used for RNA extraction and RT-qPCR for human *IFNL1* and ISGs transcriptional expression analysis. Relative levels were determined by normalizing to HBV mono-infected mice samples. Means ± SEM are shown. Comparisons between groups were made by unpaired t-test. **P*<0.05. (B) Human albumin levels at indicated times were shown.



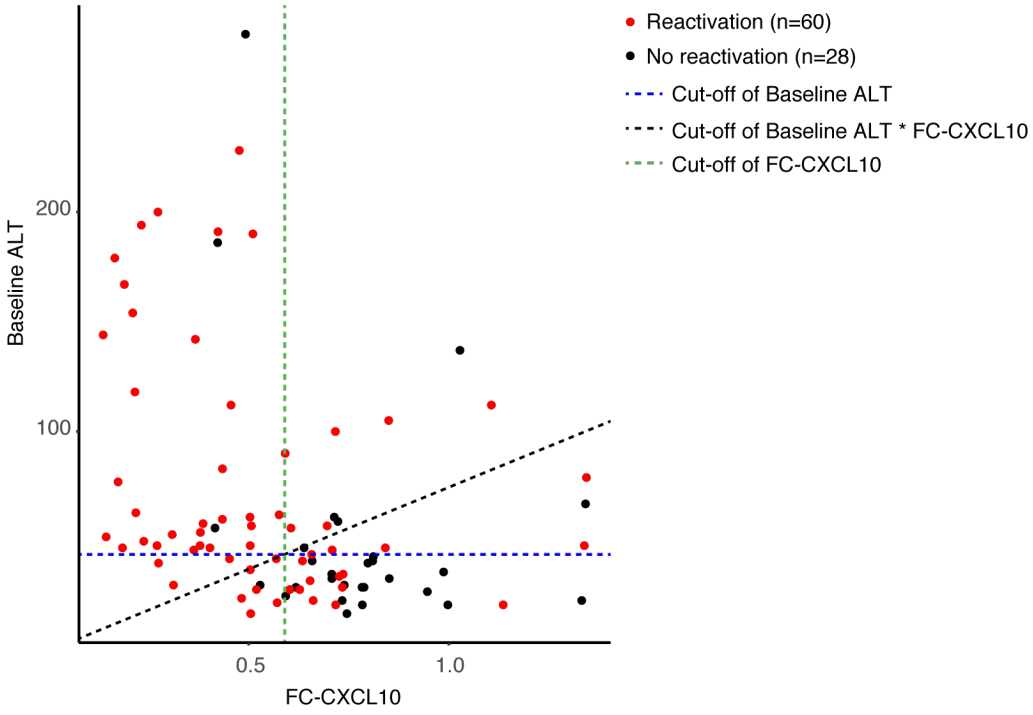
Supplemental Figure 9. Correlation of baseline CXCL10 and ALT levels in 35 patients, and baseline and longitudinal changes of HCV viremia in coinfected patients undergoing DAA regimen. (A) Non-parametric Spearman correlation analysis was performed. r=0.6231, *P*<0.0001. (B) HCV RNA level was input as 0 when below the lower limit of quantification (15 IU/mL). Means ± SD are shown.



Supplemental Figure 10. Plasma CXCL10 levels from post-treatment week 4 to post-treatment week 72 of HBV-HCV coinfected patients. Patients are grouped based on having HBV reactivation (left) or not (right). CXCL10 levels of individual patient (gray line) as well as group mean (black line) at indicated times are shown.



Supplemental Figure 11. Baseline and longitudinal changes of immune related cytokines in coinfected patients undergoing DAA regimen. Plasma samples of coinfected patients from indicated times were measured by multiplex assay for CCL5, CCL13, CCL2 and CXCL6. Means ± SEM are shown. Comparisons between groups were made by unpaired t-test.*P<0.05



Supplemental Figure 12. Scatter plot of baseline ALT and fold-change of CXCL10 of combined group of 88 patients. Baseline ALT and FC-CXCL10 values of individual patients (patients experience HBV reactivation, red dots; patients without HBV reactivation, black dots) were plotted. Youden index of the three prediction models were used as best cut-off values to separate patient groups are shown (baseline ALT, blue line; baseline ALT*FC-CXCL10, black line; FC-CXCL10, green line).

Supplemental Tables

Supplemental Table 1. Primers and Probes for Taqman RT-qPCR Assay.

Pre-designed Assay		
Name	**Gene Symbol**	**IDT Assay ID**
IFNL1	*IFNL1*	Hs.PT.56a.21113836.g
CXCL10	*CXCL10*	Hs.PT.58.3790956.g
ISG15	*ISG15*	Hs.PT.58.39185901.g
ISG20	*ISG20*	Hs.PT.58.20870590
A3G	*APOBEC3G*	Hs.PT.58.27074917
OAS3	*OAS3*	Hs.PT.58.4561974
TBP	*TBP*	Hs.PT.58v.39858774
18sRNA	*RNA18S5*	Hs.PT.39a.22214856.g
ACTB	*ACTB*	Hs.PT.39a.22214847

Self-designed Assay		
Name	**Gene Symbol**	**Sequence**
HBV		F-primer: 5’-GTTGCCCGTTTGTCCTCTAATTC-3’ R-primer: 5’-GGAGGGATACATAGAGGTTCCTTGA-3’ Probe: 5’-/56-FAM/ACCATGCCG/ZEN/GACCTGCATGACTACTG/3IABkFQ/-3’
HCV		F-primer: 5’-GCTAGCCGAGTAGCGTTGGGT-3’ R-primer: 5’-TGCTCATGGTGCACGGTCTAC-3’ Probe: 5’-/56-FAM/TACTGCCTG/Zen/ATAGGGCGCTTGCGAGTG/3IABkFQ/-3’

Supplemental Table 2. Disease characteristics and serum markers of 53 coinfected patients.

Characteristic	Reactivation (n=38)[b]	No reactivation (n=15)
Baseline HBV DNA, log_{10} IU/mL		
Mean (range)	1.9 (1.3-4.1)	2.37 (1.7-5.8)
≥LLOQ[a], n (%)	26 (68)	15 (100)
Positive HBsAg, n (%)	38 (100)	15 (100)
Baseline HCV RNA, log_{10} IU/mL		
Mean (range)	5.7 (4.2-6.9)	6.3 (5.6-6.9)
Baseline ALT, U/L		
Mean (range)	53 (17-190)	36.7 (17-67)
FC-ALT[c]		
Mean (range)	0.6 (0.28-0.94)	0.8 (0.46-1.6)
Baseline CXCL10, pg/mL[d]		
Mean (range)	72.6 (20.9-245.8)	40.8 (18.4-63.9)
FC-CXCL10		
Mean (range)	0.61 (0.17-1.34)	0.77 (0.55-1.34)
Week1 CCL5, pg/mL[d]		
Mean (range)	24383 (1338-52910)	24078 (5201-40812)
FC-CCL5		
Mean (range)	1.2 (0.3-4.1)	1.0 (0.6-1.5)

Abbreviations: HBV, hepatitis B virus; LLOQ, lower limit of quantification; HCV, hepatitis C virus; ALT, alanine aminotransferase; DAA, direct-acting antiviral; FC, fold-change

[a] LLOQ = 1.3 Log_{10} IU/mL, ≤LLOQ is considered as 1.3 in the range.

[b] 16 patients had HBV reactivation during DAA treatment and 21 patients had HBV reactivation post DAA treatment.

[c] FC = Week 1/baseline

[d] CXCL10 and CCL5 levels were measured with a different assay from that of the pilot cohort of 35 patients in Table 1, and thus cannot be directly compared to the values from the pilot cohort.

Supplemental Table 3. Summary of prediction models for HBV reactivation.

Models	Accuracy	AUC	95% Confidencial Interval	Sensitivity	Specificity
FC-CCL5	0.68	0.63	0.50-0.75	0.92	0.32
Baseline ALT	0.68	0.68	0.56-0.8	0.68	0.71
FC-CXCL10	0.7	0.81	0.71-0.90	0.67	0.86
Baseline ALT * FC-CXCL10	0.74	0.81	0.71-0.90	0.68	0.86
Baseline ALT * FC-CXCL10 + FC-CCL5	0.72	0.82	0.73-0.90	0.68	0.93

Abbreviations: AUC, area under curve; FC, fold-change

Supplemental Table 4. Characteristics of an independent cohort of HBV-HCV coinfected patients from Japan and Germany.

Characteristic	Japan (n=10)	Germany (n=6)	Total (n=16)
Age, mean (range)	59 (36-80)	45 (28-63)	53 (28-80)
Male, n (%)	8 (80%)	6 (100%)	14 (88%)
Race, n (%)	Asian, 10 (100)	Middle East, 1 (17) African, 1 (17) Caucasian, 4 (67)	Asian, 10 (63) Middle east, 1 (6) African, 1 (6) Caucasian, 4 (25)
Baseline ALT, U/L			
Mean (range)	69 (19-153)	73 (20-94)	70 (19-53)
Baseline HBV DNA, log_{10} IU/mL			
Mean (range)	2.3 (2.0-3.8)	3.5 (2.0-8.2)	2.8 (2.1-8.2)
>LLOQ[a], n (%)	2 (20)	5 (83)	7 (44)
Positive HBsAg, n (%)	10 (100)	6 (100)	16 (100)
HBV reactivation, n (%)	5 (50)	4 (67)	9 (56)
Time of HBV reactivation			
During DAA treatment, n	3	3	6
Post DAA treatment, n	2	1	3
HBV DNA level at reactivation, log_{10} IU/mL	2.8 (2.2-3.5)	3.6 (1.9-5.4)	3.2 (1.9-5.4)
ETV treatment needed?			
No, n	3	3	6
Yes, n	2	1	3
Baseline HCV RNA, log_{10} IU/mL			
Mean (range)	5.9 (4.2-7.0)	6.0 (5.4-6.5)	6.0 (4.2-7)
DAA treatment type (n)			
	SOF+RBV (6) SOF+LDV (2) DCV+ASV (2)	SOF+RBV (2) SOF+RBV+DCV (1) SOF+LDV (1) SOF+DCV(1) GLE+PIB (1)	
Treatment duration, weeks (n)			
	12 (8) 24 (2)	8 (2) 12 (2) 24 (2)	
SVR24, n (%)	10 (100)	6 (100)	16 (100)
Previous HBV treatment, n (%)			
No	10 (100)	6 (100)	16 (100)
Yes	0	0	
Previous HCV treatment, n(%)			
No	9 (90)	3 (50)	12 (75)
Yes	1 (10)	3 (50)	4 (25)

Abbreviations: HBV, hepatitis B virus; LLOQ, lower limit of quantification; HCV, hepatitis C virus; ALT, alanine aminotransferase; DAA, direct-acting antiviral; SOF, sofosbuvir; RBV, ribavirin; DCV, daclatasvir; LDV, ledipasvir; ASV, asunaprevir; GLE, glecaprevir; PIB, pibrentasvir

[a] LLOQ =2.0 Log10 IU/mL, ≤LLOQ is considered as 2.0 in the mean determination.