

Supplementary materials and methods

Mice and transplantation of CD34⁺ human fetal liver cells

NSG mice were purchased from The Jackson Laboratory and bred in a specific pathogen free facility at the biological resource centre (BRC) in Agency for Science, Technology and Research (A*STAR), Singapore. Balb/c mice were purchased from the BRC. One to three days old NSG pups were sub-lethally irradiated at 1 Gy and transplanted with 2×10^5 CD34⁺ human fetal liver cells by intra-hepatic injections. In total, samples from 12 different donors were used to construct the HIL mice used in this study. The mice were bled at 8 weeks post-transplantation to determine the levels of human immune reconstitution and concentrations of human serum albumin. The levels of human immune cell reconstitution and human hepatocyte engraftment were similar in HIL mice that received CD34⁺ human fetal liver cells from different donors. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

HCV infection

For the laboratory strain, a cell culture-adapted HCV J6/JFH virus (known as J6/JFH-1-P47) was previously generated [1] and used throughout the experiments unless otherwise stated. Production and titration of J6/JFH-1-P47 were performed as previously described [2]. Serum samples from HCV patients (clinical strain) were obtained from National University Hospital, Singapore. Ten-week old HIL mice were infected with HCV by intravenous injection of the viruses. 1×10^6 FFU of J6/JFH-1-P47 (genotype 2a) laboratory strain or 4×10^5 virus copies of clinical strain (genotype 3a) were used.

Liver perfusion and enrichment of human hepatocyte

Liver perfusion was performed as described previously [3]. Human EGFR⁺ cells were selected using PE selection kit (18551, StemCell Technologies) according to the protocol provided by the manufacturer. The enriched cells were then used for cytospin or RNA extraction.

Immunohistochemical and immunofluorescence staining

HCV-infected/mock-infected mice were sacrificed at different time points. Mouse livers were collected, fixed with 10% formalin and embedded in paraffin for processing into liver tissue sections. Rehydrated liver tissue sections were stained with Hematoxylin & Eosin (H&E) (Thermo Scientific), Fast-green (Sigma) & Sirius red (Sigma) or were treated with heat mediated antigen retrieval with sodium citrate (pH6) or Tris-EDTA (pH9) buffer, or enzymatic antigen retrieval using 20 µg/ml proteinase K before staining with appropriate antibodies. IHC staining was performed using the SuperPicture 3rd Gen IHC Detection Kit (879673, Life Technologies) according to the protocol provided by the manufacturer. Enriched hEGFR⁺ hepatocytes were fixed in 10% formalin and stained with antibodies as indicated. Primary antibodies used in this study include anti-hCD45 (ab781, abcam), anti-αSMA (ab5694, abcam), anti-HCV core (MA1-080, Pierce), anti-human cytokeratin 18 (ab133263, abcam), anti-human tyrosine aminotransferase (ab103992, abcam), anti-human EGFR (DAK-H1-WT, Dako), and anti-hALB (ab2406, abcam) antibodies. Anti-mouse, anti-rabbit and anti-goat fluorescent secondary antibodies were obtained from Life Technologies. For immunofluorescence staining of mock-infected and HCV-infected Huh7.5 cells, sera from mock-infected or HCV-infected mice were used as the primary antibody and mouse anti-human IgG-FITC (ab99763, abcam) was used as the secondary antibody. For immunofluorescence staining of infected hepatocytes, genotype 3a HCV-infected patient serum was used as the primary antibody followed by anti-human IgG-AF 647. The patient

serum was obtained from National University Hospital in Singapore. Serum was pre-cleared by incubation with uninfected hepatocytes for few hours prior to use. For the detection of dsRNA, cytopins were fixed in ice-cold methanol for 10min and stained with a dsRNA-specific monoclonal antibodies J2 (English and Scientific Consulting, Hungary). All fluorescence images were acquired using Olympus upright confocal microscope with the Fluoview acquisition software using the 40X or 100X objective lens and immersion oil. All H&E and Sirius red/Fast green stained images were captured using MIRAX MIDI fluorescence microscope (Zeiss) using the MIRAX acquisition software.

HCV RNA quantification

Total RNA from hEGFR⁺ hepatocytes isolated by liver perfusion was extracted using the RNeasy Mini kit (Qiagen). RT-PCR was performed using the ThermoScript RT-PCR ThermoScript One-Step System (Invitrogen). Oligonucleotide primer and Taqman probe sequences as well as the reaction setups were as described [4]. 100 ng of total RNA was used in a 25 µl reaction. RNA quantities were derived from a standard curve generated using the HCV 5'UTR region. The HCV 5'UTR region was synthesized using the MEGAshortscript T7 Transcription kit (Ambion). RNA samples were analyzed in triplicate and data was expressed as the HCV RNA copies per 100 ng of total RNA.

Gene expression profiling by quantitative real-time PCR or end-point PCR

RNA extractions were performed using the RNeasy Mini kit (Qiagen) and cDNA synthesis was performed with RT kit (Qiagen), using 1 µg of total RNA. Quantitative RT-PCR (qRT-PCR) was performed on Applied Biosystems 7500 real-time PCR system (Applied Biosystems) using SYBR-green reagent (Biorad) and custom-made primers (IDT). For end-point PCR, PCR products were analyzed by agarose gel electrophoresis. The sequences of

human and mouse mRNAs were extracted from the National Center for Biotechnology Information database. Analogous genes from human and mouse were aligned using the MegAlign software (DNASTAR Lasergene 9 Core Suite) and the species-specific regions for human or mouse were identified for the design of human- or mouse-specific primers, respectively. A complete list of the oligonucleotide primer sequences used is provided in Supplementary Table 3. The relative gene expression values were normalized to species-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and derived using the $2^{-\Delta\Delta Ct}$ method.

Flow cytometry

MNCs from livers, spleens and blood were isolated as previously published [5]. The following antibodies were used for flow cytometry: anti-human CD45 (2D1) from BD Biosciences; anti-human CD14 (HCD14), CD56 (MEM-188), CD19 (HIB19), CD3 (HIT3a), CD4 (OKT4), CD8 (HIT8a), MMR (15-2) and anti-mouse CD45.1 (A20) from BioLegend. Cell suspensions of PBMCs and RBCs were stained with appropriate antibodies in 100 μ l PBS containing 0.2% BSA and 0.05% sodium azide for 30 min on ice. Flow cytometry was performed on a LSRII flow cytometer using the FACSDiva software (BD, Franklin Lakes, NJ). 10,000 events were collected per sample and analyzed using the Flowjo software 7.5.5 (Treestar).

Human cytokine antibody array

Sera from HIL mice from mock-infected and HCV-infected groups were analyzed for their cytokine profiles with a semi-quantitative human cytokine antibody array that detects 30 inflammatory cytokines in one experiment (RayBio Human Cytokine Antibody Array 3; Raybiotech, Norcross GA, USA). The arrays on glass slides were pre-treated according to the

manufacturer's instructions and incubated with 2-fold diluted serum pools at 4°C overnight. The array glass slides were washed, incubated with a biotin-conjugated mix for 2 hours, washed again, and developed for 2 hours with Cy3-conjugated streptavidin. The signals were scanned with a GenePix 4000B scanner (Axon Instruments, GenePix version 5.0) and analyzed with the Raybiotech analysis tool, a data analysis program based on Microsoft Excel technology specifically designed to analyze Raybiotech Antibody Array 3. Signals were normalized using internal, positive and negative controls included on the array.

Enzyme-linked immunosorbent assay (ELISA) and ALT

Blood samples collected from the mice were centrifuged at 3000 rpm for 10 min at 4°C, and serum samples were harvested for ELISA analysis. Human serum albumin levels were determined using the hALB ELISA kit (Bethyl Laboratories) according to the manufacturer's protocol. Human serum cytokine levels were determined using human IFN γ ELISA kit (Biolegend) and human IL6 ELISA kit (Biolegend) according to the manufacturer's protocol. For detection of virus-specific human IgG antibodies, purified HCV NS3 antigens containing either amino acids 1-180 or 400-630 were expressed as GST-fusion proteins, purified and used to coat Nunc MaxiSorp flat-bottom 96 well plates. Purified GST protein was used as negative control. Sera from mock-infected and HCV-infected HIL mice were used as the primary antibodies and anti-human IgG-HRP (Biolegend) was used as the secondary antibody. Serum alanine aminotransferase (ALT) levels were measured using a Cobas C111 Analyzer (Roche) in Comparative Medicine's (National University of Singapore) in-house veterinary diagnostic laboratory.

In vivo depletion of specific immune cells population in HIL mice

CD4⁺, CD8⁺ T cells and CD14⁺ cells were depleted from ten-week old HIL mice by intravenous injection of 50 µg of anti-human CD4 (RPA-T4) or anti-human CD8 (RPA-T8) or anti-human CD14 (M5E2) antibodies, respectively. The antibodies were purchased from Biolegend. Control mice were injected with PBS instead. Depletion of the specific immune cell population was maintained by intravenous injection of 20 µg of the respective antibodies every 3 days. Depletion efficiencies of specific immune cell population from the blood, liver and spleen of HIL mice were verified by flow cytometry.

Human IFN γ enzyme-linked immunospot (ELISPOT) assay

MNCs were isolated from fresh livers of HIL mice that were infected with HCV for 9 weeks by Percoll density gradient centrifugation and re-suspended in AIM-V medium (Invitrogen) with 2% human AB serum, supplemented with interleukin-2 at 10 IU/ml and interleukin-15 at 10 ng/ml. MNCs were seeded at 10⁶ cells/ml/well in 96-well ELISPOT plate (U-CyTech biosciences) and stimulated with and without 10 µg/ml of a pool of 16 overlapping 20-mer peptides (Genscript), covering the HCV core protein, at 37°C for 24 h. Peptides representing the 3a protein of Severe Acute Respiratory Syndrome (SARS) coronavirus were used as control peptides. A complete list of the peptide sequences is provided in Supplementary Table 2. Positive control consists of MNCs stimulated with 50 ng/ml Phorbol 12-Myristate 13-acetate (PMA) (Sigma) and 500 ng/ml ionomycin (Sigma) at 37°C for 24 h. The ELISpot assay was carried out using a commercial kit (U-CyTech biosciences) according to the manufacturer's protocol. The number of human IFN γ producing cells was determined using an ELISPOT reader (Bioreader 4000 Pro-B, Biosys) and expressed as spot-forming units (SFU) per million cells.

IFN α -2a antiviral treatment

Human recombinant IFN α -2a (PBL InterferonSource) treatment was given by intramuscular injections starting at 1 week after HCV infection until 8 weeks post-infection. The dose given is at 1000 U/g for 3 times per week.

Statistical Analysis

Either the two-tailed Student's *t* test or one-way analysis of variance was applied to evaluate the statistical significance of differences measured from the data sets. $P < 0.05$ was considered statistically significant. All data are reported as means \pm standard error of mean.

Supplementary figure legend

Fig. S1. NSG mice support the engraftment of human hepatocytes and a matching human immune system. New born NSG mice were injected with 2×10^5 fetal liver CD34⁺ cells to construct HIL mice. Eight weeks after injection, PBMCs, sera and liver paraffin sections were prepared from NSG and HIL mice (n = 50 mice per group). (A) PBMCs were stained for human CD45 (hCD45) versus mouse CD45.1 (mCD45) to determine the level of human leukocyte reconstitution [% hCD45⁺ cells / (hCD45⁺ cells + mCD45⁺ cells)]. A representative flow cytometry dot plot of hCD45 versus mCD45.1 is shown. (B) The levels of human leukocyte reconstitution between NSG and HIL mice were compared using the two-tailed Student's *t* test, and the difference was found to be statistically significant ($P < 0.05$). The values of every mouse are plotted as triangles, and the average values are plotted as solid lines. (C) Liver sections from NSG and HIL mice were stained for hALB (red) and DAPI (blue). Representative stains are shown. (D) Levels of human serum albumin between NSG and HIL mice were compared using the two-tailed Student's *t* test, and the difference was found to be statistically significant ($P < 0.05$). Values from each mouse are plotted as triangles, and the average values are plotted as solid lines. (E) The presence of human

hepatocyte markers in hEGFR⁺ cells enriched from HIL mice livers was determined by reverse transcription-PCR. Human specific primers were used for the detection of indicated genes by end-point PCR. RNA extracted from adult human hepatocytes and NSG mouse hepatocytes were used as positive and negative controls, respectively. (F) Human cytokeratin 18 (CK18) and tyrosine aminotransferase (TAT) proteins were detected in hEGFR⁺ c enriched from HIL mice livers. Shown are representative stains of hALB (in red) and CK18 (in green) or TAT (in green). The cells were counter stained with DAPI (in blue). (G) Liver sections of HIL mice were stained for hALB (green), hEGFR (red) and DAPI (blue). Representative images are shown.

Fig. S2. HCV infection in HIL mice. (A) Representative Staining of TAT (green) and HCV antigens (purple) in the hEGFR⁺ cells enriched from HIL mice infected with HCV for 2 and 5 weeks or mock-infected. (B) Representative IHC staining of hCD45 and hALB using adjacent slides from HCV-infected HIL mice at 6 weeks post-infection. Higher magnifications of the boxed areas in the upper panel are shown in the lower panel. Representative images of 3 mice are shown.

Fig. S3. No damage was observed in non-hepatic tissues of HCV-infected HIL mice and no intrahepatic human immune cell infiltration or cytokine responses were observed in Balb/c, NSG and cord blood (CB) reconstituted humanized mice after HCV infection. (A-B) Different organs were harvested from mock- and HCV-infected HIL mice at 9 weeks post-infection (n = 10 mice per group). (A) Representative image showing gross appearance of livers obtained from mock-infected mice or HCV-infected mice. (B) Representative H&E stains of lungs, kidneys, hearts and intestines that were harvested from mock-infected (top

row) or HCV-infected (bottom row) mice. (C) Balb/c, NSG and CB reconstituted humanized mice were infected with HCV (n = 5 mice per group). Livers were harvested at 9 weeks post-infection and paraffin sections were prepared. Representative H&E stains of liver sections are shown. Lower (top row) and higher (bottom row) magnifications are shown. (D-E) Sera were prepared from HCV-infected CB reconstituted humanized mice at different weeks post-infection (p.i.) and analysed for human IFN γ and IL-6 by ELISA, respectively. Data represents mean \pm SEM.

Fig. S4. Human anti-HCV antibodies were detected in HCV-infected HIL mice. (A) Sera taken from HCV-infected and mock-infected HIL mice at 12-15 weeks post-infection were used to stain HCV-infected and mock-infected Huh7.5 cells, followed by secondary antibody staining with anti-human IgG-FITC (n = 3 mice per group). Representative stains for DAPI (blue) and anti-human IgG (green) are shown. (B) Sera taken from HCV-infected and mock-infected HIL mice at 12-15 weeks post-infection were used for ELISA using plates coated with either HCV NS3-GST or GST only (n = 3 mice per group). Data represents mean \pm SEM.

Fig. S5. Depletion effects of human T cells and macrophages in HIL mice. HIL mice were treated with PBS (Ctrl), anti-human CD4, CD8 or CD14 antibody (n = 3 mice per group). MNCs were prepared from blood, spleens and livers 24 h after antibody treatments. (A-B) To prove that the antibody clones used for CD4 and CD8 depletion do not compete with the clones for FACS analysis, MNCs from blood of control HIL mice were first incubated with CD4 (clone number: RPA-T4) or CD8 (clone number: RPA-T8) antibody, followed by staining with fluorescence conjugated anti-human CD4 (clone number: OKT-4) or human CD8 (clone number: HIT-8a) antibody. Shown are representative plots of CD3 versus CD4

(OKT-4) (A) or CD8 (HIT-8a) (B). (C) MNCs from different organs of CD4 antibody (clone number: RPA-T4) treated mice were stained for human CD3 and human CD4 (clone number: OKT-4). Representative plots of CD3 versus CD4 are shown. (D) MNCs from different organs of CD8 antibody (clone number: RPA-T8) treated mice were stained for human CD3 and human CD8 (clone number: HIT-8a). Representative plots of CD3 versus CD8 are shown. (E) MNCs from different organs of CD14 antibody treated mice were stained for human macrophage mannose receptor (MMR). Representative plots of Forward-scattered light (FSC) versus MMR are shown.

Fig. S6. Liver leukocyte infiltration and fibrosis were observed in HIL mice infected with HCV clinical strain (HCV CS). Livers sections were prepared from mock-infected and HCV CS-infected HIL mice at 13 weeks post-infection (n = 5 mice per group). **(A)** Representative H&E (top row) and Sirius red/Fast green (bottom row) stains are shown. **(B)** Representative stains for human CD45 (red) and DAPI (blue) are shown.

Table S1. Mouse information and liver disease scoring

Donor Liver ID	Fetal	Human Reconstitution (%)	Immune	Human Albumin Level (ng/ml)	Virus Particles Inoculated	Weeks post-infection	Liver Disease scoring ^a
FL0915		45		20	0	0	0
FL1807		39		30	0	0	0
FL0113		66		18	0	0	0
FL0915		24		22	1e6	0	0
FL1807		51		26	1e6	0	0
FL0113		69		32	1e6	0	0
FL0915		17		27	0	3	0
FL1807		57		35	0	3	0
FL0113		44		75	0	3	0
FL0915		54		23	1e6	3	1
FL1807		18		24	1e6	3	1
FL0113		50		20	1e6	3	1
FL0915		49		30	0	5	0
FL1807		62		65	0	5	0
FL0113		67		28	0	5	0
FL0915		42		80	1e6	5	2
FL1807		31		15	1e6	5	1
FL0113		21		30	1e6	5	2
FL0915		44		25	0	9	0
FL1807		34		32	0	9	0
FL0113		36		50	0	9	0
FL0915		43		20	1e6	9	3
FL1807		25		22	1e6	9	2
FL0113		24		45	1e6	9	3

^aDetermined by H&E staining and Sirius red/Fast green staining of liver sections. 0 = no liver disease; 1 = Nodule fibrosis; 2 = Fibrosis with numerous septa; 3 = Cirrhosis

Table S2. List of SARS 3a peptides and HCV core peptides for stimulation of T cells in ELISpot.

Peptide name	Sequence
HCV core P1	MSTNPKPQRKTKRNTNRRPQ
HCV core P2	TKRNTNRRPQDVKFPGGGQI
HCV core P3	DVKFPGGGQIVGGVYLLPRR
HCV core P4	VGGVYLLPRRGPRLGVRATR
HCV core P5	GPRLGVRATRKTSERSQPRG
HCV core P6	KTSERSQPRGRRQPIKDRR
HCV core P7	RRQPIKDRRSTGKSWGKPG
HCV core P8	STGKSWGKPGYPWPLYGNEG
HCV core P9	YPWPLYGNEGLGWAGWLLSP
HCV core P10	LGWAGWLLSPRGSRPSWGPN
HCV core P11	RGSRPSWGPNDPRHRSRNVG
HCV core P12	DPRHRSRNVGKVIDTLTCGF
HCV core P13	KVIDTLTCGFADLMGYIPVV
HCV core P14	ADLMGYIPVVGAPLGGVARA
HCV core P15	GAPLGGVARALAHGVRVLED
HCV core P16	LAHGVRVLEDGVNFATGNLP
SARS 3a P1	KSKNPLLYDANYFVC
SARS 3a P2	LLYDANYFVCWHTHN
SARS 3a P3	NVFVCWHTHNYDYCI
SARS 3a P4	WHTHNYDYCIPYNSV

SARS 3a P5	YDYCIPYNSVTDITV
SARS 3a P6	PYNSVTDITIVVTEGD
SARS 3a P7	TDTIVVTEGDGISTP
SARS 3a P8	VTEGDGISTPKLKED
SARS 3a P9	GISTPKLKEDYQIGG
SARS 3a P10	CLKEDYQIGGYSEDR

Table S3. List of primers for quantitative real-time PCR and end-point PCR

Quantitative RT-PCR primers		
Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Human GAPDH	GCTTAACTCTGGTAAAGTGGATAT	ATGGAATTTGCCATGGGTGGAAT
Mouse GAPDH	CATTTGCAGTGGCAAAGTGGAGA	GTTGAATTTGCCGTGAGTGGAGT
Human Col1A1	CCAATCACCTGCGTACAGAACG	CGTCACAGATCACGTCATCGCA
Mouse Col1A1	AGCTTTGTGGACCTCCGGCTC	CTGACTTCAGGGATGTCTTCTTG
Human TIMP1	CCCACAACCGCAGCGAGGAG	GGCAGGCAAGGTGACGGGAC
Mouse TIMP1	CTGGCATCTGGCATCCTCTTGT	AGTTGCAGAAGGCTGTCTGTGG
Human AAT	GATCAACGATTACGTGGAGAAGG	CCTAAACGCTTCATCATAGGCA
Human TAT	CATTTTGGGACCCTGTACCATT	GAGTGTTGTGGTAAACTCTCCC
Human TDO2	CATAAGGATTCAGGCTAAAGAA	TTCACCTTTACTAAGGAGATGT
Human CYP7A1	GCACAGAAGCATTGACCCGATG	GGTCTTTGAGTTAGAGGAGACT
Human CK18	CTCCGTGTCCCGCTCCACCA	TCCTCACTCTGTCCAGGTAAGA
Human CK19	GTGTGGAGGTGGATTCCGCTC	TGTGGCCAGCGACCTCCCG
Human albumin	GAAAACGCCAGTAAGTGACAGA	CAGAAAGTGTGCATATATCTGCA

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