A dual role for hepatocyte-intrinsic canonical NF-κB signaling in virus control

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Supplementary materials and methods

Clodronate treatment

Macrophages were depleted by injecting 200 µl (~1 mg) of Clodronate-encapsulated liposomes (LIPOSOMA research, Cat no: CP-005-005 – 5 mg/ml formulation) through the i.v. route in accordance with manufacturer's directions for administration (0.1 ml/10 g body mass). 200 µl of PBS-encapsulated liposomes were used as control. On day 2 post clodronate treatments, infections were carried out using LCMV (1).

CD169 immunofluorescence

Histological analyses were performed on snap-frozen tissue as described previously (2). In brief, slides were fixed with Acetone for 10 min, incubated for 15 min in PBS and blocked with 2% FCS for 15 min. Sections were stained with rat anti-LCMV-NP antibody (clone VL4) and developed with FITC-anti-Rat-IgG1/2a (BD Pharmingen Clone: G28-5, Cat: 553881). Additionally, CD169-PE (R&D Systems, 5610P) and F4/80-APC (eBiosciences, 17-4801) were used to visualize macrophage subsets. Pictures of slides were taken with Keyence BZ-9000.

Clec4F immunofluorescence

Liver tissue samples were fixed in 4% paraformaldehyde for 5 days at room temperature and embedded in paraffin and ~2 µm tissue sections were made using a microtome. The sections were later de-paraffinized, rehydrated and boiled at 100° C with EDTA to facilitate antigen retrieval. Antigen retrieval was performed with 96°C sodium citrate buffer. Slides were washed with TBS-Tween. Primary antibody, purchased from R&D systems (1:2000), and secondary antibody, purchased from Jackson ImmunoResearch (1:250), were incubated sequentially for 1 h at RT in a

humid chambers. Slides were mounted in DAPI Fluoromount-G® and kept in the dark at 4°C. Slides were imaged with a confocal Zeiss LSM 710 ConfoCor 3.

RNA isolation and quantitative PCR analysis

Total RNA was isolated from approximately 20 mg of liver tissue lysed in RLT buffer from an RNeasy mini kit (Qiagen) using a gentleMACS™ Dissociator (Miltenyi Biotec). Spectrophotometric measurement of the quality and quantity of isolated RNA was done using Nanodrop (ThermoFisher). 1 µg of RNA was reverse transcribed to cDNA using Quantitect Reverse Transcription Kit (Qiagen) following the instructions from the manufacturer. Relative mRNA expression was analyzed in duplicates on 384-well PCR plates (ThermoFisher) using FastStart Universal SYBR Green Master (Rox) and the qPCR was run on 7900 HT qRT-PCR system (Applied Biosystems). Relative mRNA levels were calculated through ΔΔCT relative quantification method and the obtained values were normalized to housekeeping genes albumin (for liver), β-actin and GAPDH. For HBV- or HDV-infected HepaRGs, total RNA and total DNA were respectively extracted from cells with the NucleoSpin RNA II kit and Nucleospin® tissue kit according to the manufacturer's (Macherey-Nagel) instructions. RNA reverse transcription was performed using the Superscript III RT (Life Technologies). Quantitative PCR were performed specific primers and normalized to PRP housekeeping gene as previously described (3, 4). TNFα- and IFN-α-treated HepaRGs, were washed once with PBS, lysed in RLT buffer (Qiagen), passed through a QiaShredder column (Qiagen), and RNA was extracted using the RNeasy Mini Kit (Qiagen). Reverse transcription was performed with the Quantitect Reverse Transcription Kit using 500 ng total RNA and subsequent qPCR analysis was done by using the FastStart Universal SYBR Green Master (Rox) (Roche).

Immunohistological stainings

Liver tissue samples were fixed in 4% paraformaldehyde for 5 days at room temperature and embedded in paraffin and ~2 μm tissue sections were made using a microtome. The sections were later de-paraffinized, rehydrated and boiled at 100°C with EDTA to facilitate antigen retrieval. IHC staining was performed with a Leica automated BOND-MAX staining platform using Bond Polymer Refine Detection kit (Leica, Catalog #DS9800). Staining was performed using antibodies purchased from Cell Signaling for pSTAT1-pY701 (1:100 dilution), and pSTAT3 (1:100 dilution), from ACRIS for STAT2 (1:500 dilution), from Novus Biologicals for MHC-II (1:500 dilution), from Linaris for F4/80 (1:120 dilution), and from abcam for CD68 (1:300 dilution).

pSTAT1, HNF4α and F4/80 sequential staining quantification

Liver tissue samples were cut sequentially and treated and stained as described above. pSTAT1 positive cells were manually counted in three different view fields per samples. Based on cells morphology and overlaying with HNF4α positive staining, pSTAT1 positive cells were divided into two groups: pSTAT1 positive hepatocytes or pSTAT1 positive non-parenchymal cells (i.e. all other positive cells except hepatocytes).

DC maturation flow cytometry

Livers and spleens were harvested, perfused with DMEM (Gibco) in the presence of 167 μg/ml of Liberase TM Research Grade (Roche) and 200 μg/ml of DNAse I (Roche), and incubated 30 min at 37°C. Digestion was stopped by adding DMEM containing 10% FCS (Gibco). Samples were smashed through 70 μm filter and centrifuged for 5

min at 1500 rpm. Cells were resuspended and incubated for 10 min at 4°C in FACS buffer (PBS + 1% FCS + 5 mM EDTA) in presence of 1:50 FC block (CD16/CD32 monoclonal antibody, Ebioscience). The following antibody panel (ebioscience) was incubated 30 min at 4 °C: CD8α-PerCP-eFluor 710, CD11c-PE-Cy7, CD80-FITC, CD86-PE, CD40-APC, MHCII-APC-eFluor 780. Cells were washed with FACS buffer and centrifuged for 5 min at 1500 rpm. Cells were resupended in FACS buffer containing DAPI and analyzed in a BD LSRFortessa™ cytometer. Analysis was then performed on Flow-Jo™.

CXCL10 ELISA

Approximatively 30 mg of liver was disrupted using 2 mm beads in a protein buffer (100 mM TRIS pH7.4, 150 nM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X100, 0,5% Sodium deoxycholate, 1 mM PMSF, 1X PIC). Proteins were quantified and equal concentration of total protein lysate (5 µg/ml) was used to perform the CXCL10 ELISA following manufacture instructions (R&D).

Intracellular cytokine staining

Single-cell suspensions from the liver tissues were obtained by tissue dissection and grating through a 70 µm cell strainer (Falcon). RBC lysis was performed using BD Pharm Lyse Buffer (BD Biosciences). ~ $4x10^6$ cells were used for stimulation with LCMV gp33 peptide or a non-specific peptide at a concentration of 1 µg/ml of peptide along with Brefeldin A (Golgi-plug; 1 mg/ml). Plates were later incubated for 5 h at 37^0C. Surface staining was done using CD8α and cells were fixed and permeabilized (Cytofix/Cytoperm and Perm/Wash Buffer; BD Biosciences), followed by staining with mAbs to mouse IFN-γ (eBiosciences) and CD8α (eBiosciences). Cells were fixed on

ice in 2% formaldehyde (diluted from Histofix 4%; Carl Roth, Karlsruhe, Germany) for 1 h followed by fluorocytometric analysis.

HepaRG cell culture and treatments

HepaRG cells were cultured, differentiated, and infected by HBV (MOI 100) and/or HDV (MOI 10) as previously described (4, 5). HBV inoculum was prepared from HepAD38 (6) supernatants by polyethylene-glycol-MW-8000 (PEG8000, SIGMA) precipitation (8% final) as previously described (7). Viral stocks with titers of superior to $1x10^{10}$ vge/ml were tested to be endotoxin free. HDV inoculum was prepared from transfected HuH7 cells as previously described (4). Viral stocks were tested to be endotoxin free. rhPEG-IFN-α (Roferon) was purchased Roche and used at 500 UI/ml. TPCA-1 and ML120B were purchased from SIGMA-Aldrich and used as IKKβ inhibitors in Figs. 6 B and D, Figs. S5 D and E at 1 μM and 10 μM, respectively. HBeAg and HBsAg were detected in the supernatant of HBV-infected cells using the Autobio kit according to the manufacturer (Autobio Diagnostics Co.).

Evaluation of deletion efficiency and specificity in LysM-Cre $IFNAR^{fl/fl}$ and Alb-Cre $IFNAR^{fl/fl}$ mice

KCs and hepatocytes were isolated as previously described (8, 9). Following a proteinase K digestion, genomic DNA of KCs, splenocytes and hepatocytes were precipitated O/N at 4°C using 0,3M Na-acetate and ethanol as anti-solvent, washed twice with ice-cold 70% ethanol and reconstituted in nuclease-free Water. Cre-mediated excision of the loxP flanked genomic region of IFNAR exon 10 was analyzed by PCR in LysM-Cre $IFNAR^{fl/fl}$ and Alb-Cre$IFNAR^{fl/fl}$ and $IFNAR^{fl/fl}$ mice. The PCR reaction was designed to detect the WT allele at 1264 bp, floxed allele at 1200 bp and

the recombined allele at 430 bp with the following primers – dEx10For (5’-GGT TAA GCT CCT TGC TGC TAT CTG G-3’) and dEx10Rev (5’TTG GAG ATG CAA TCT CGT ACT CAG C-3’).

Single nucleotide polymorphism genotyping

Subjects were genotyped using TaqMan SNP Genotyping Assays with TaqMan Universal Master Mix and QuantStudio™ 6 Flex Real-Time PCR System (ThermoFisher Scientific). Differences in contingency were assessed Chi-squared using Prism software (Graphpad Prism version 5.0a). Associations were examined by the Fisher-exact test implemented in R (version 3.1.1). Allele and genotype frequencies were analyzed and tested for consistency with Hardy–Weinberg equilibrium using a software package designed by Strom and Wienker-TUM (http://ihg.gsf.de/ihg/snps.html). Genotype frequencies were compared between individuals with resolved and chronic infection using 2x2 contingency tables.

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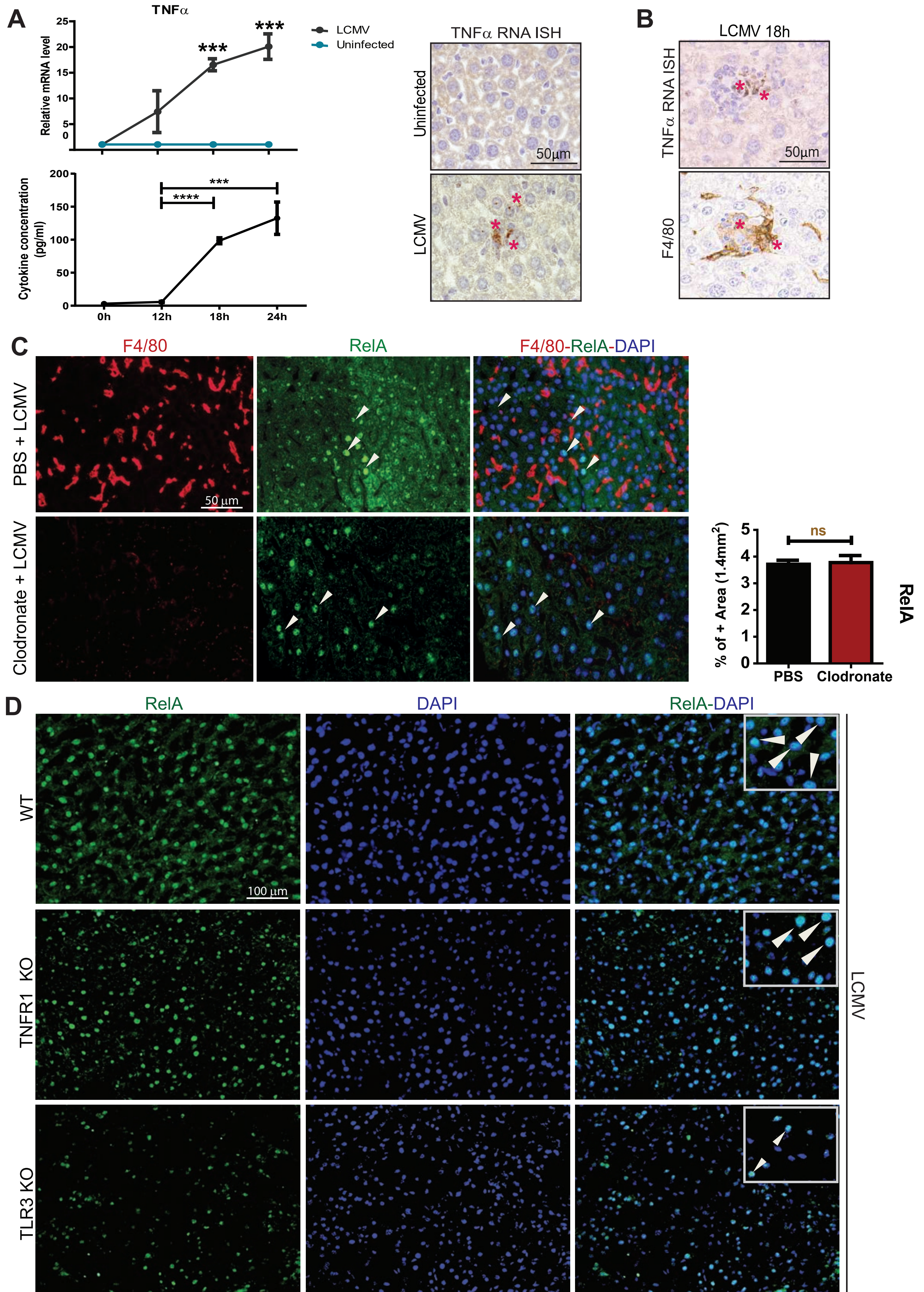
Fig. S1. LCMV infection induces TLR3, TLR7, MyD88 and MAVS mediated sensing and activation of NF-κB signaling in hepatocytes. (A) *Tnf* mRNA expression from WT livers following LCMV infection compared to uninfected WT analyzed by qRT-PCR (left upper pannel) and mRNA *in situ* hybridization (right pannel). Red asterisks: TNF mRNA positive non-parenchymal cells. TNFα was quantified in the serum of mice by ELISA and is presented as average concentration (lower left pannel). **(B)** TNFα mRNA was stained *in situ* and F4/80 was stained on consecutive slides. Red asterisks: TNF mRNA positive non-parenchymal cells. **(C)** C57BL/6 mice were injected i.v. with clodronate or with PBS liposomes on day -2. At day 0, mice were i.v. infected with $2x10^6$ LCMV-WE. At day 1 p.i., livers were isolated, and frozen sections were stained for F4/80 (red), RelA (green) and DAPI (blue) to stain nuclei (n=3-5, each). Percentage of $RelA^+$ areas was quantified densitometrically (right panel). **(D)** WT**,** $TNFR1^{-/-}$, $TLR3^{-/-}$, $TLR7^{-/-}$, $MAVS^{-/-}$, $MYD88^{-/-}$, $MAVS^{-/-}STING^{-/-}$ and $MyD88^{-/-}$-$TRIF^{-/-}$-STING mice were infected with $2x10^6$ LCMV-WE and nuclear RelA translocation in hepatocytes and other cells was analyzed in livers by IHC. White arrow heads: Nuclear RelA translocation in hepatocytes. **(E)** Percentage of $RelA^+$ areas from indicated genotypes (from Fig S1D) was quantified densitometrically (left panel). Number of RelA positive parenchymal hepatocytes and non-parenchymal cells from WT, TLR3 KO and TLR7 KO mice were identified based on morphology and quantified (right panel). **(F)** WT and $TNFR1^{-/-}$ mice were infected with $2x10^6$ LCMV-WE and day 1 p.i., livers were isolated, and parrafin sections were stained for HNF4α, RelA, and F4/80 on consecutives slides (n=3). Black arrow head: RelA+ hepatocytes. Black asterix: RelA+ non-parenchymal cell.

(G) Analysis of IKKβ deletion efficiency from WT and $IKKβ^{ΔHep}$ mice - Flox efficiency was measured from total RNA isolated from $IKKβ^{ΔHep}$ and WT livers, spleens or primary hepatocytes of the latter. **(H)** C57BL/6 and $IKK^{Δ}β^{Hep}$ mice were i.v. infected with $2x10^6$ PFU of LCMV-WE and a time course analysis was performed (n=3, each time point). Livers were isolated, and parrafin sections were stained for p-cJUN. Representative staining at 18h and 24h post infection (left pannel) and densitometrically quantification of the staining (right pannel). Black arrow heads: positive nuclear staining. **(I)** Measurement of virus titers by plaque forming assay to analyze infectious virus particles in spleens of infected mice. **(J)** Frozen liver section of day 8 p.i. were stained for $CD169^+$ splenic macrophages. Representative images are shown (left pannel). Total $CD169^+$ area was densitometrically quantified for each genotype (right pannel). **(K)** C57BL/6 and $IKK^{Δ}β^{Hep}$ mice were i.v. infected with $2x10^6$ PFU of LCMV-WE and at day 3 p.i. livers and spleen were extracted and DC maturation analyzed by flow cytometry**.** Data are presented as MFI expression on gated MHCII+ CD11c+ cells. **(L)** At day 6 and 8 p.i. livers of infected mice were stained for LCMV-NP (green) and HNF4α (red) along with DAPI (blue) staining for the nuclei. Error bars indicate mean ± SEM, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; unpaired Student's t-test.



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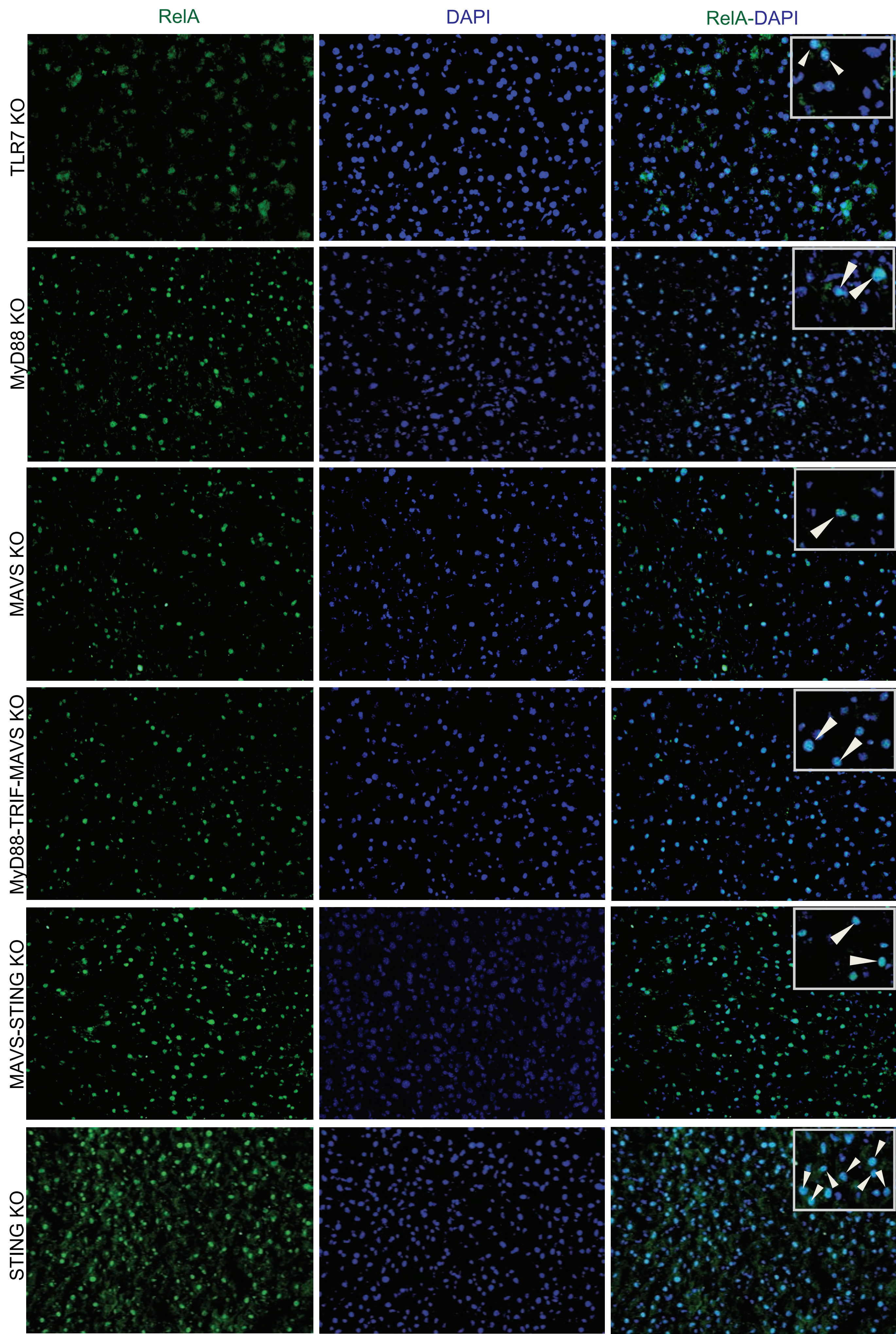

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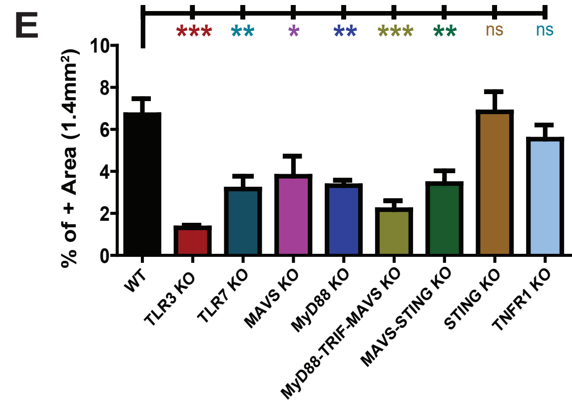

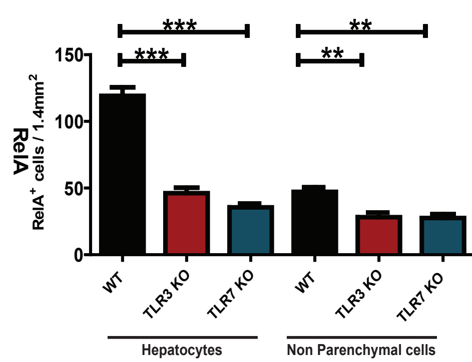

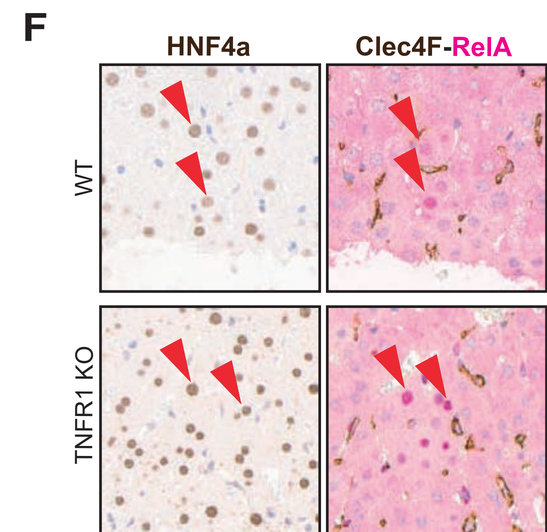

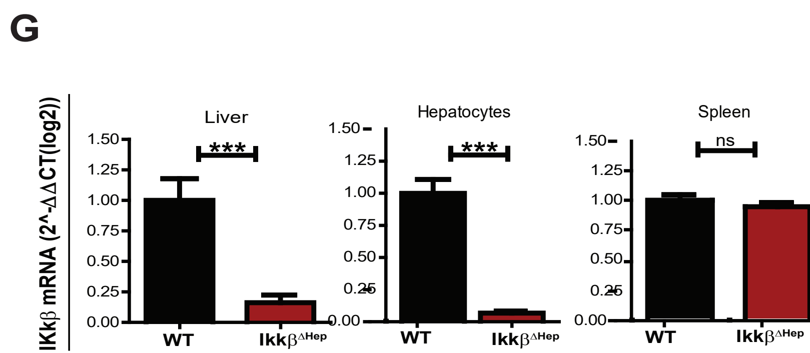

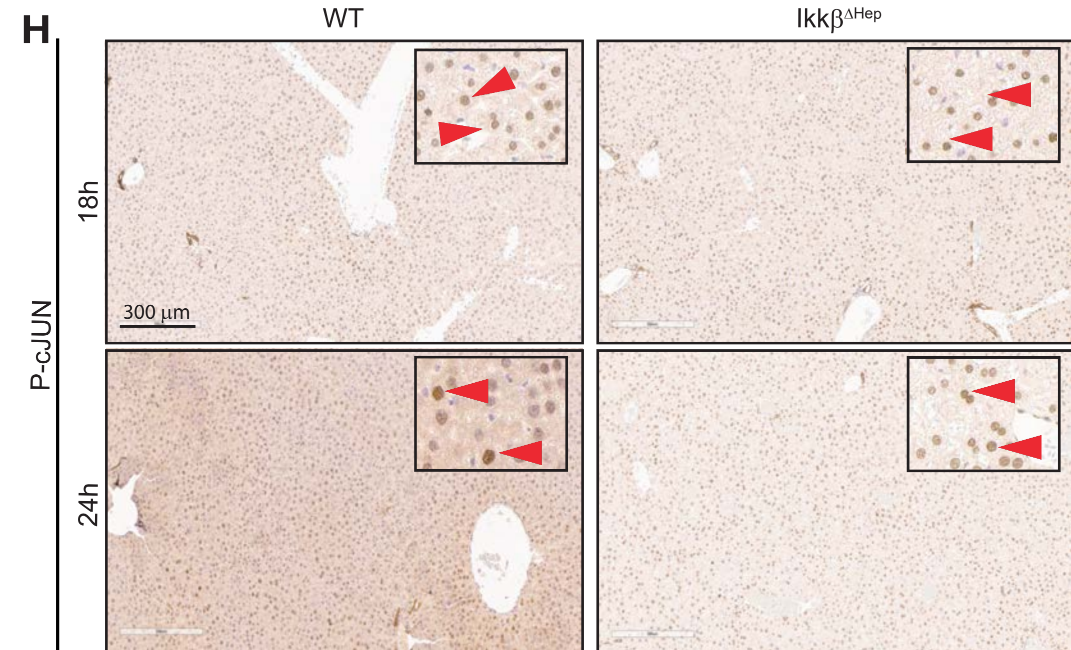

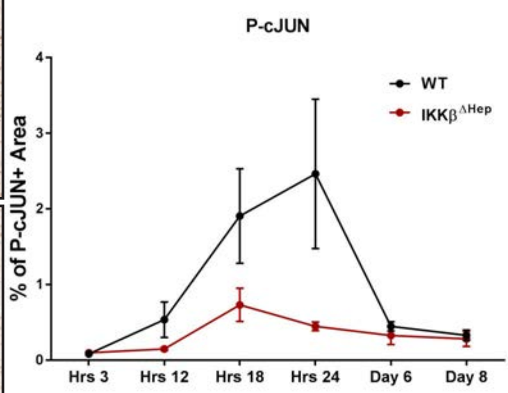

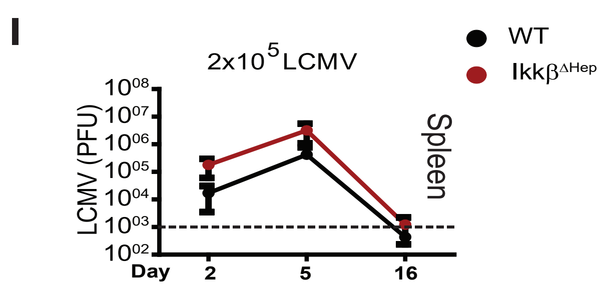

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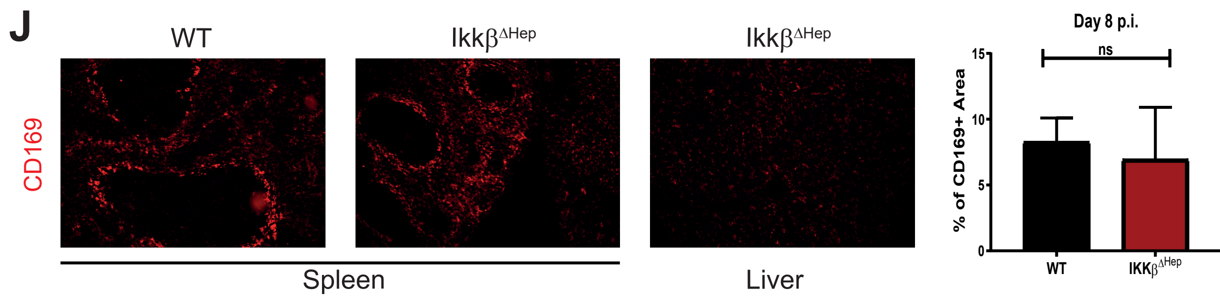
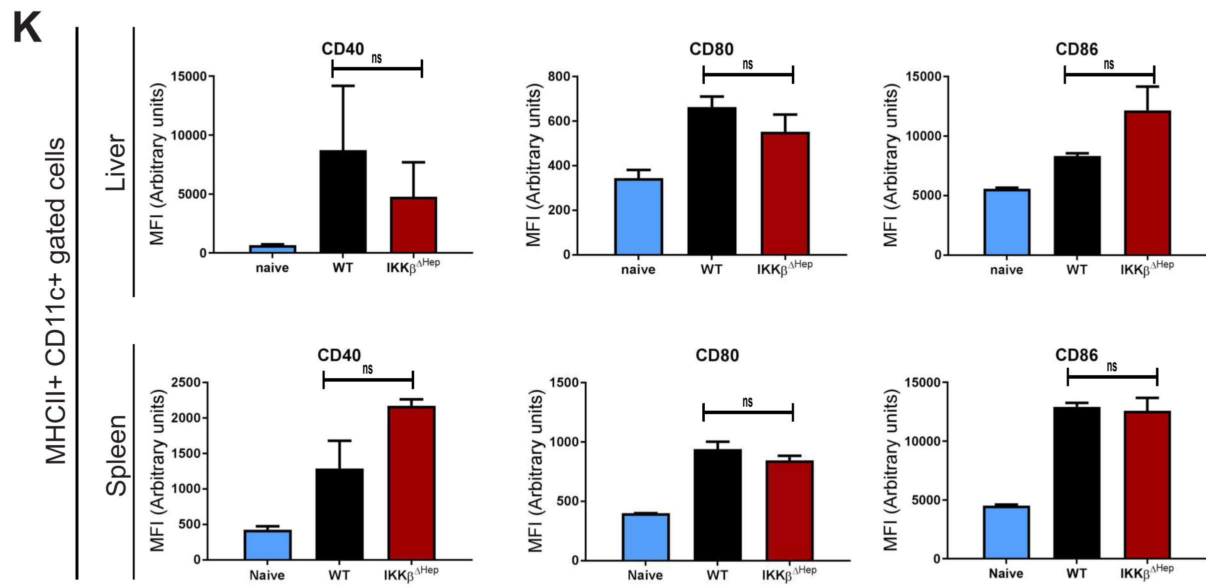
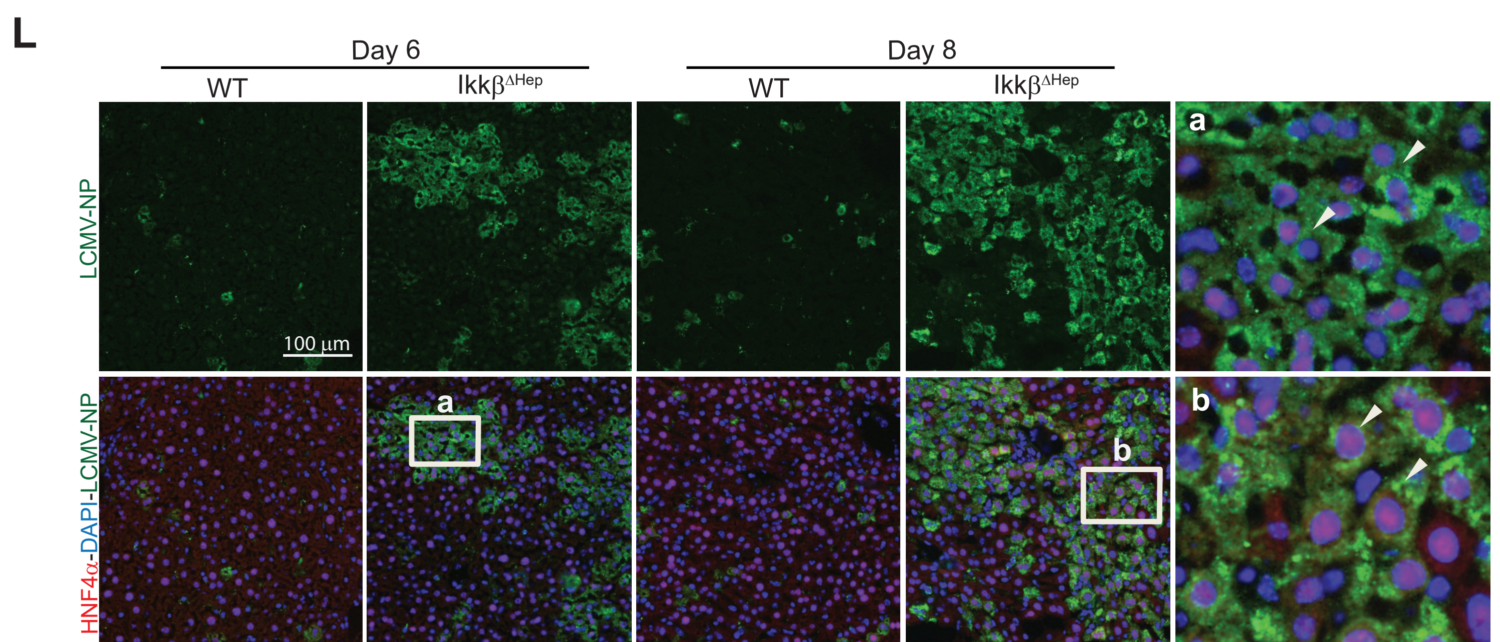






Namineni et al. Fig S1

Fig. S2. Analysis of IFN responses from WT and IKKβ$^{\Delta Hep}$ livers. C57BL/6 and IKK$^{\Delta}$β^{Hep} mice were i.v. infected with 2x10^{6} PFU of LCMV-WE and a time course analysis was performed (n=3, each time point) **(A)** Livers were analyzed for mRNA expression of IFN-α, IFN-β, and some ISGs by qRT-PCR. **(B, C, D)** Translocation of pSTAT1, pSTAT2 and pSTAT3 was analyzed by IHC. Red arrow heads: hepatocytes. Black asterisks: non-parenchymal cells. **(E)** F4/80, pSTAT1, and HNF4α stainings were performed on consecutive slides**.** pSTAT1 was quantified in hepatocytes and non parenchymal cells (NPC). Red arrow heads: pSTAT1+ hepatocytes. Black asterisks: pSTAT1+ non-parenchymal cells. **(F)** mRNA was extracted at the indicated time points from isolated hepatocytes and analyzed for mRNA expression of IFN-α and IFN-β by qRT-PCR. Error bars indicate mean ± SEM, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$; unpaired Student's t-test.

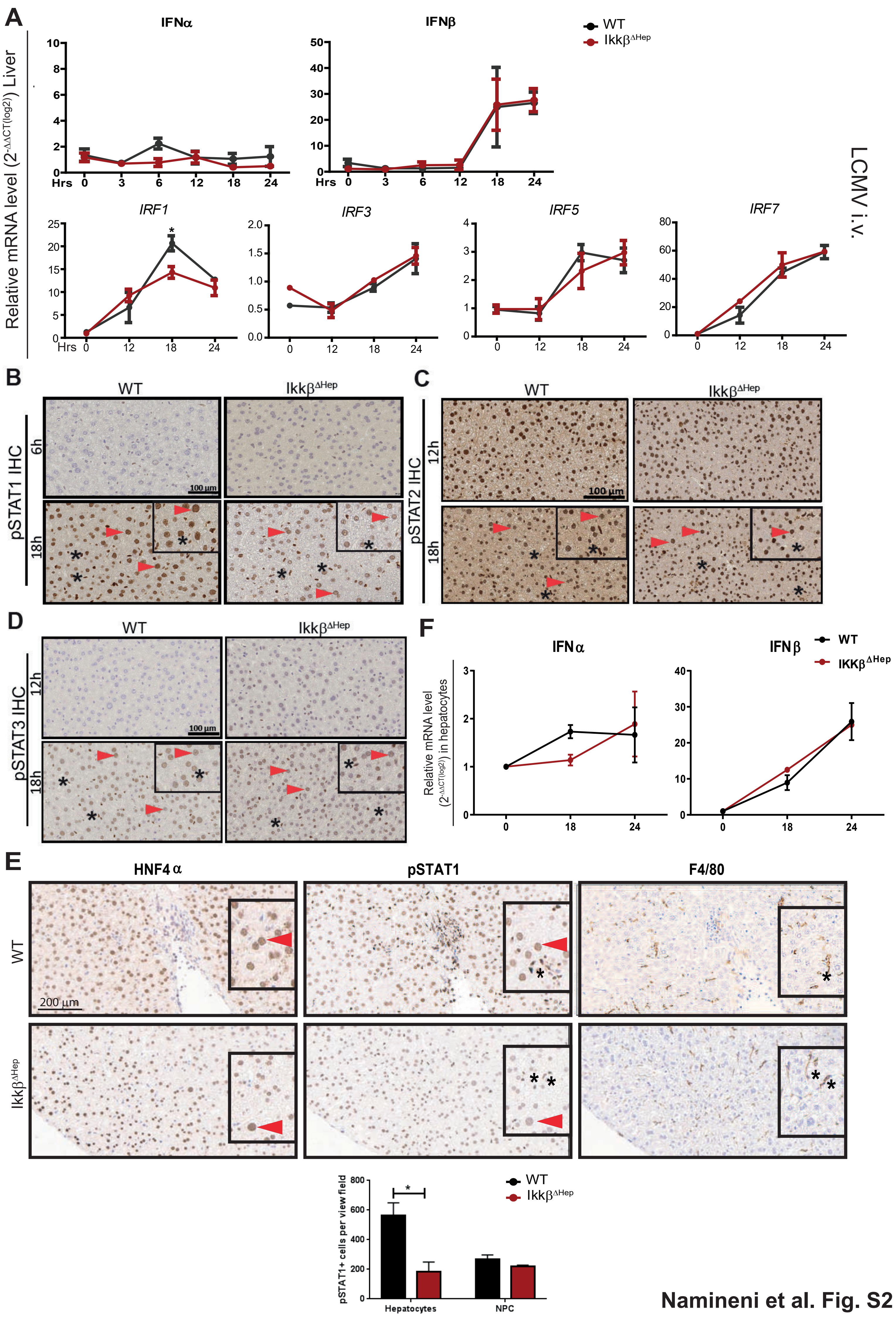
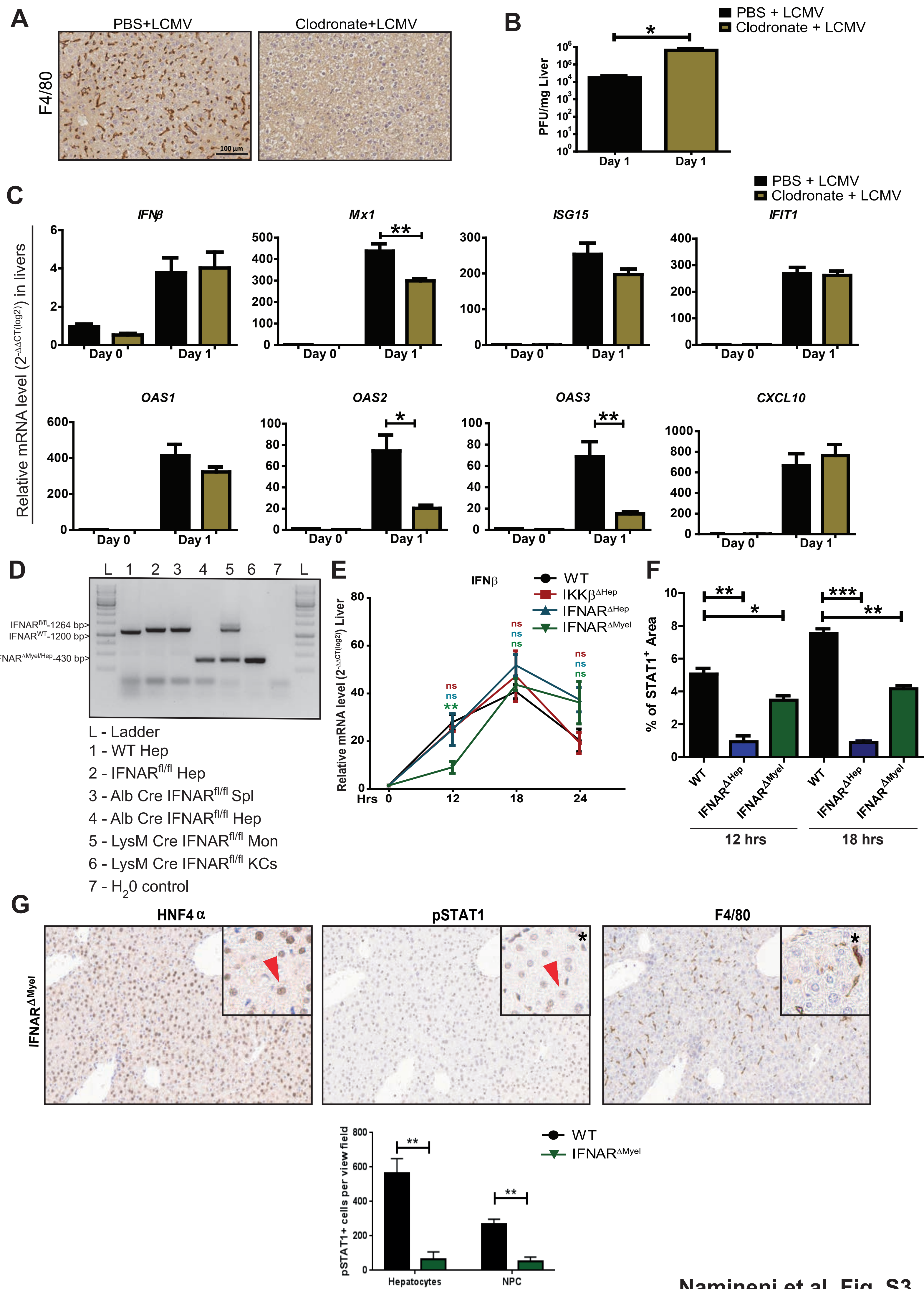
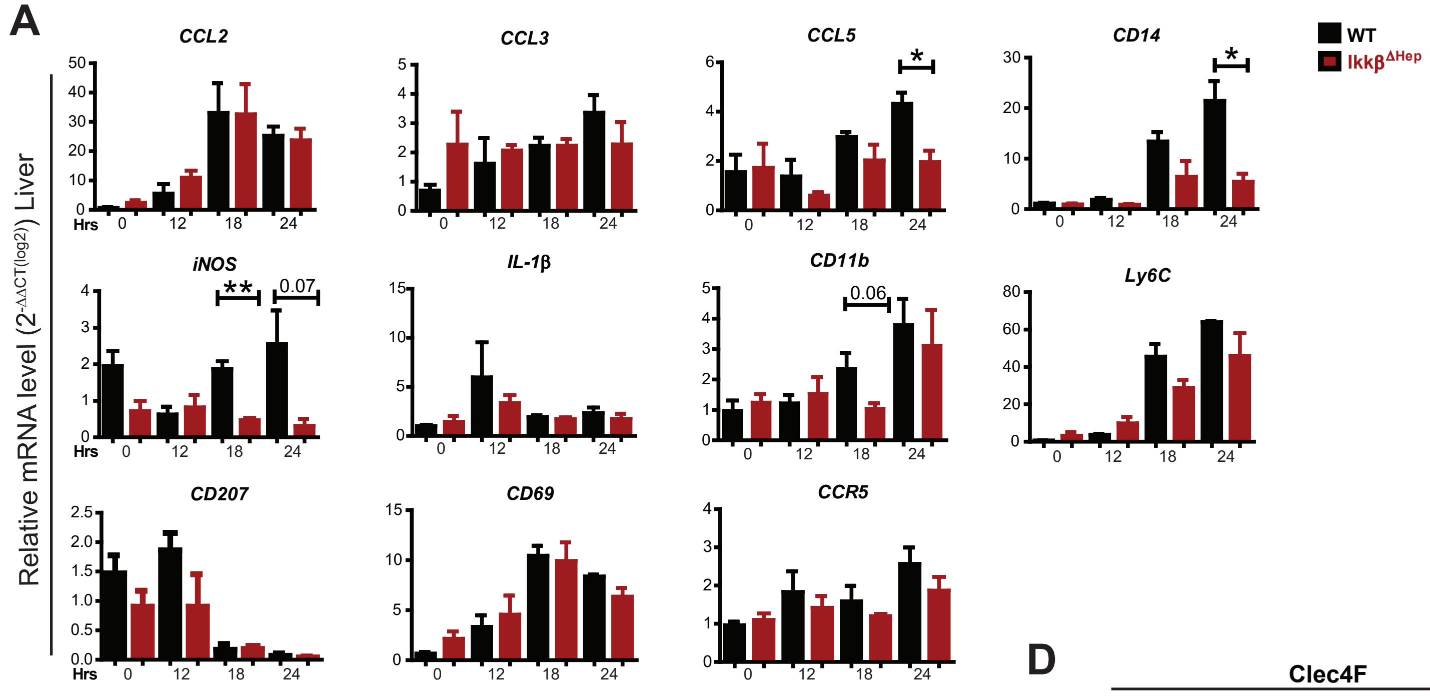

A
IFNα
IFNβ
WT
Ikkβ^{ΔHep}
Relative mRNA level ($2^{-ΔΔCT(log2)}$) Liver
Hrs
IRF1
IRF3
IRF5
IRF7
LCMV i.v.
B
WT
Ikkβ^{ΔHep}
pSTAT1 IHC
6h
18h
100 μm
C
pSTAT2 IHC
12h
D
pSTAT3 IHC
F
IFNα
IFNβ
IKKβ^{ΔHep}
Relative mRNA level ($2^{-ΔΔCT(log2)}$) in hepatocytes
E
HNF4α
pSTAT1
F4/80
200 μm
pSTAT1+ cells per view field
Hepatocytes
NPC
Namineni et al. Fig. S2

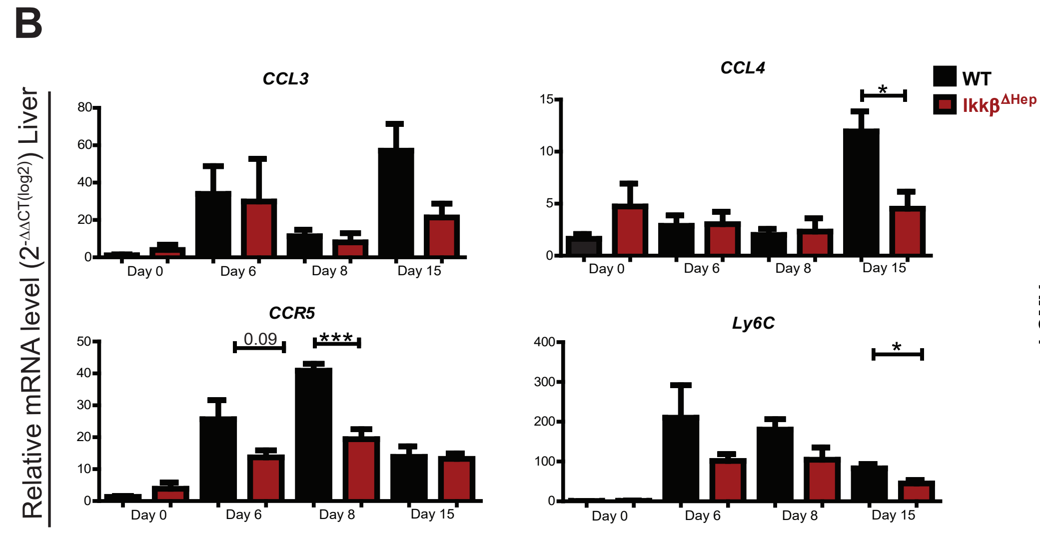
Fig. S3. Hepatocyte - Kupffer cell cross-talk at the early stage of LCMV infection. WT mice were injected i.v. with clodronate-liposomes or with PBS liposomes on day -2. At day 0, mice were i.v infected with $2x10^6$ LCMV-WE. (**A**) F4/80 staining from FFPE liver sections of clodronate-liposome and PBS-liposome treated mice to verify depletion of Kupffer cells. **(B)** Analysis of virus titers from the livers of mice through plaque forming assay (n=3-4). **(C)** Analysis of ISGs from livers of mice at indicated time points by qRT-PCR. **(D)** qRT-PCR analysis of Cre mediated excision of loxP flanked genomic region of IFNAR exon 10 in WT and IFNARKO hepatocytes. The flox allele is found at 1,264 kb in $IFNAR^{fl/fl}$ hepatocytes, the WT allele is found at 1,2kb. The recombined allele is found at 430 bp from hepatocytes (derived from $IFNAR^{\Delta Hep}$), and Kupffer cells (derived from $IFNAR^{\Delta Myel}$ mice) where as both floxed and recombined allele is seen from isolated monocytes indicating the presence of Cre^- monocytes in the isolated population. $H2_0$ control-PCR mix without gDNA template (**E**) qRT-PCR analysis of IFN-β mRNA expression in whole livers of LCMV-infected WT, $IKK\beta^{\Delta Hep}$ $IFNAR^{\Delta Hep}$, and $IFNAR^{\Delta Myel}$ mice. **(F)** Analysis of $pSTAT1^+$ signals in livers from Fig 5D. **(G)** F4/80, pSTAT1, and HNF4α stainings were performed on consecutive slides of WT, and $IFNAR^{\Delta Myel}$ mice, at 18h post LCMV infection. pSTAT1 was quantified in hepatocytes and non parenchymal cells (NPC). Red arrow heads: pSTAT1+ hepatocytes. Black asterisks: pSTAT1+ non-parenchymal cells. Error bars indicate mean ± SEM, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$; unpaired Student's t-test.

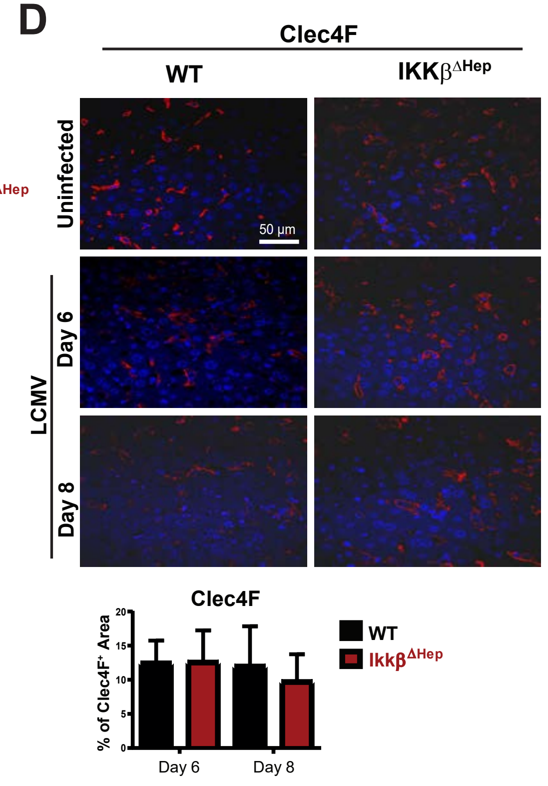


Namineni et al. Fig. S3

Fig. S4. Hepatocyte-derived IKKβ affects chemokine expression and subsequent influx of immune cells. (A, B) WT and $IKK^{\Delta}\beta^{Hep}$ mice were infected i.v. with $2x10^6$ PFU of LCMV-WE and short and long time course experiments were performed. (**A**) 0 – 24 hrs and (**B**) 0 – 15 days (n=3, each time point). Livers were isolated and analyzed for the expression of chemokines and immune cell activation markers by qRT-PCR. **(C, D)** WT and $IKK^{\Delta}\beta^{Hep}$ livers were isolated at the indicated time points, and paraffin-embedded formalin fixed liver sections were used for immunohistochemical analysis of **(C)** CD68, MHC-II, F4/80, and **(D)** immunofluorescence analysis of Clec4F. Histological analyses were quantified densitometrically. Error bars indicate mean ± SEM, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; unpaired Student's t-test.







C

CD68 — WT, IKKβ$^{\Delta Hep}$

MHC II — WT, IKKβ$^{\Delta Hep}$

F4/80 — WT, IKKβ$^{\Delta Hep}$

Uninfected

LCMV — Day 6, Day 8

100 µm

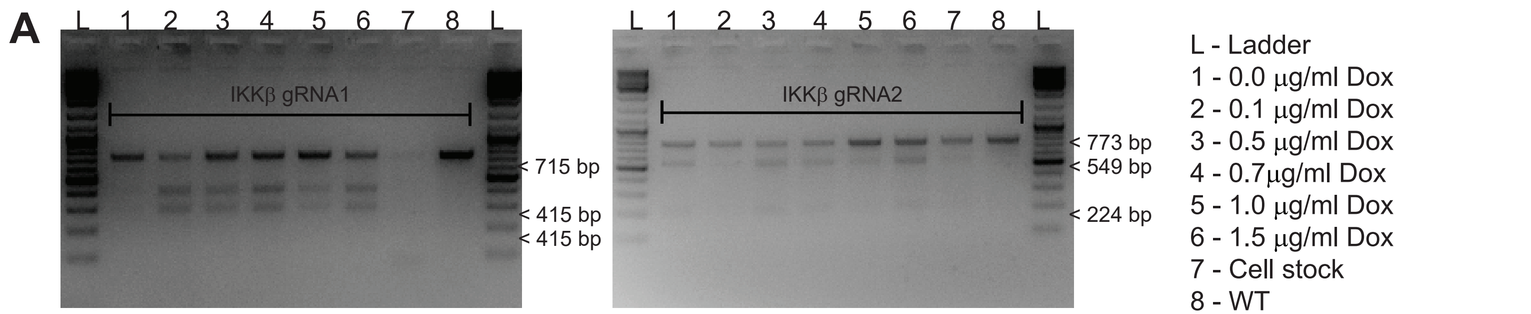
CD68: % of CD68^{+} Area — Day 6, Day 8 — WT, Ikkβ$^{\Delta Hep}$

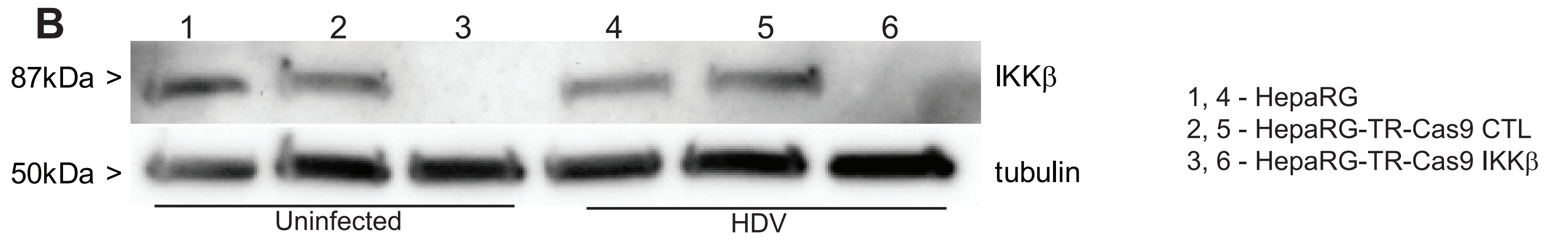
MHC II: % of MHC II^{+} Area — Day 6, Day 8 — WT, Ikkβ$^{\Delta Hep}$

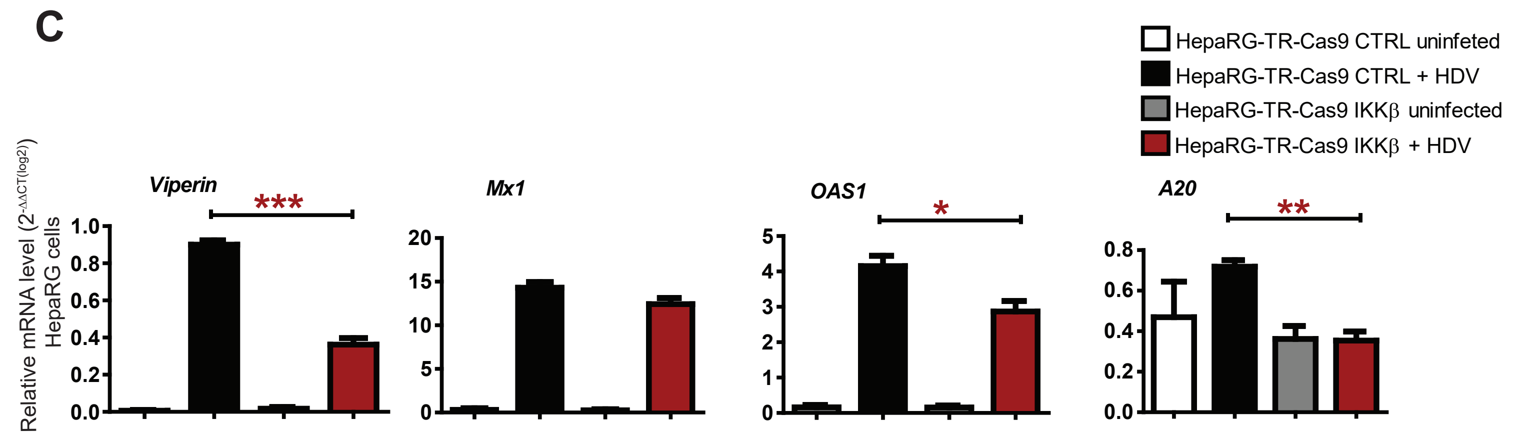
F4/80: % of F4/80^{+} Area — Day 6, Day 8 — WT, Ikkβ$^{\Delta Hep}$

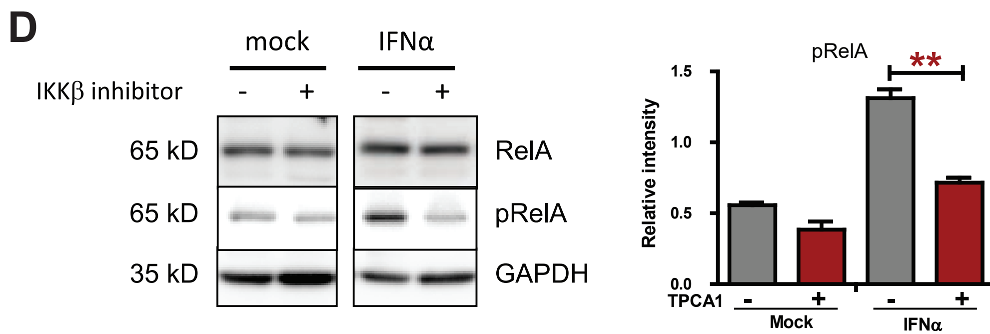
Namineni et al. Fig. S4

Fig. S5. Characterization of the HepaRG$^{\Delta IKK\beta}$ cell line. (A) Indel mutation analysis of CRISPR IKKβ Knockout Cells via T7 Assay (Surveyor Assay). For higher knockout efficiency, two gRNAs targeting IKKβ were designed and transfected into HepaRGs. The generation of indel mutations within the gene was determined performing a T7 endonuclease assay (Surveyor Assay). Both IKKβ gRNAs showed the expected, endonuclease cleaved shorter fragments (gRNA1: 415 bp, 300 bp; gRNA2: 549 bp, 224 bp) at the CRISPR cutting site, next to the whole, uncut PCR fragment (gRNA1: 715 bp; gRNA2: 773 bp) confirming the generation of indel mutations in cells of the polyclonal cell line. **(B)** Western blot analysis for the expression of IKKβ in WT HepaRG (HepaRG-TR-Cas9 CTL) or HepaRG$^{\Delta IKK\beta}$ (HepaRG-TR-Cas9 IKKβ). **(C)** IKKβ deletion in HepRG cells affects ISG expression upon HDV infection and IFN-α treatment shows reduced effectiveness in HBV clearance in HepaRG cells treated with an IKKβ inhibitor**:** WT HepaRG (HepaRG-TR-Cas9 CTRL) and HepaRG$^{\Delta IKK\beta}$ (HepaRG-TR-Cas9 IKKβ) cells were infected with HDV. qRT-PCR was performed with specific primers as indicated and normalized to PRNP (housekeeping gene). **(D)** HepaRG cells were treated for 12 hours with either DMSO or IKK2 Inhibitor IV (5 nM) and further treated for 6 hours with or without IFN-α at a concentration of 250 ng/mL. Protein lysates were blotted for RelA and pRelA, as indicated. **(E)** Analysis of HBV DNA, RNA, HBeAg and HBsAg in the context of IFN-β Inhibitor treatment. Error bars indicate mean ± SEM, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$; unpaired Student's t-test.









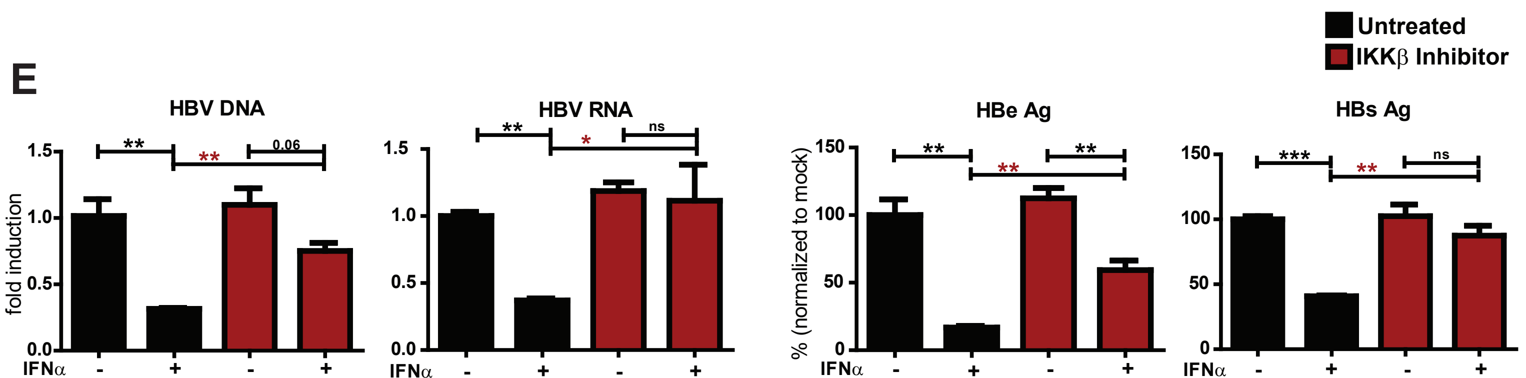


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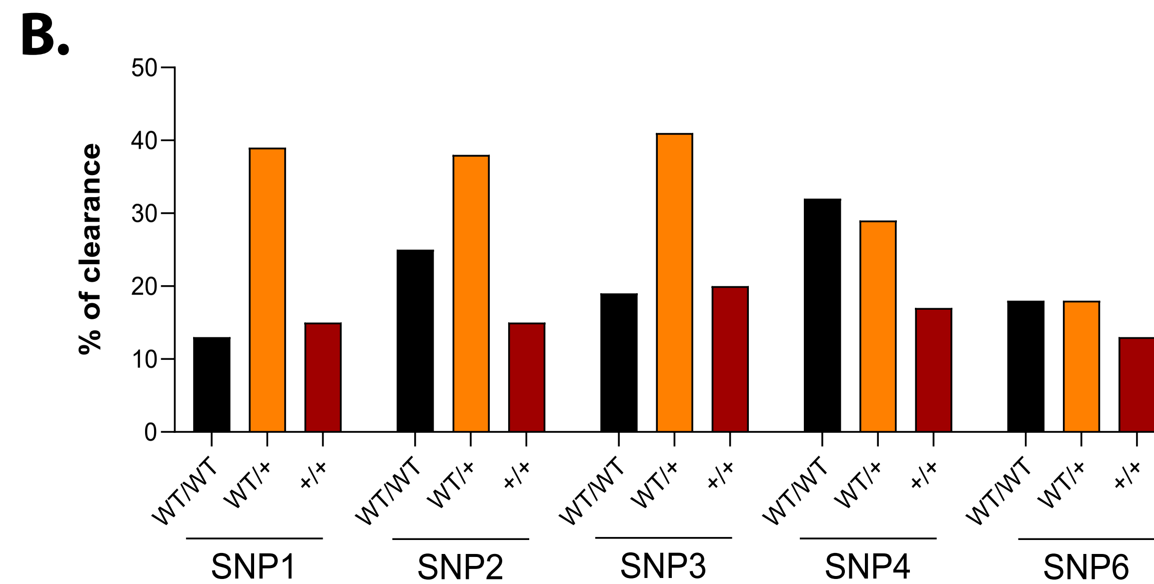
Fig. S6. NF-κB1 single nucleotide polymorphism in HCV chronically infected and resolved patients. In a cohort of 80 patients resolved (n=25) or chronically infected with HCV (n=55), blood was collected, and DNA was extracted from blood circulating cells. 6 SNPs (SNP1: rs11722146; SNP2: rs230521; SNP3: rs230542; SNP4: rs230530; SNP5: rs3774934; SNP6: rs3774963) were analyzed by Taqman SNP genotyping assay. Data are presented as percentage of resolved or chronically infected populations respectively. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$; Chi-squared test.

A.

B.



C.