Supplemental Figure 1: Hepatic expression of *MLKL* in patient cohorts of diverse liver diseases

(A) Quantification of hepatic *MLKL* mRNA in liver biopsies obtained from healthy control or liver disease patients (PBC: Primary Biliary Cirrhosis; HCV: Hepatitis C Virus; DILI: Drug-Induced Liver Injury; AIH: Autoimmune Hepatitis, n=15). **(B)** Representative image of a MLKL stained section from AIH liver (arrows demonstrate subcellular MLKL localization to plasma membranes, panel in the right represent confocal picture).

Error bars indicate +SD, gene expression levels are shown relative to *HPRT*.

Supplemental Figure 2: MLKL locates to the plasma membrane following inflammatory liver injury

(A) Quantification of hepatic *Mlkl* mRNA at different time points after ConA administration (n=3/group). $^{**}P < 0.01$, $^{***}P < 0.001$ by paired Student's *t* test. **(B)** Quantification of MLKL protein level in liver tissue lysates by Western blot, actin was used as loading control (n>3/group). $^{**}P < 0.01$ by paired Student's *t* test. **(C)** The specificity of anti-mouse MLKL mABs used in the study was analyzed by immunofluorescence and Western-blot analysis of livers of wildtype and *Mlkl*$^{-/-}$ mice subjected to saline or ConA treatment (for 7 hours). **(D)** Quantification of staining representative depicted in Fig.2D (n=5/group). **(E)** Correlations between the relative hepatic expression of *Mlkl* and circulating AST levels during the course of ConA-induced hepatitis in C57BL/6 mice (n>3/group). **(F)** Quantification of MLKL in the cytoplasm and at the plasma membrane (PM) at different time points after ConA administration (n>3/group). $^{*}P < 0.05$, $^{***}P < 0.001$ by paired Student's *t* test. **(G)** Representative images of slides of ConA challenged (for 7 hours) C57BL/6 mice stained by TUNEL assay and for MLKL.

Error bars indicate +SD, gene expression levels are shown relative to *HPRT*.

Supplemental Figure 3: MLKL is dispensable for acetaminophen induced liver injury

(A) Serum IFN-γ concentrations in control mice or *Mlkl*$^{-/-}$ mice treated with ConA (n>3/group). **(B)** The presence of STAT1 and pSTAT1^{Tyr701} in livers of unchallenged or ConA treated control or *Mlkl*$^{-/-}$ mice was analyzed by Western-blot (actin was used as loading control). **(C)** Primary hepatocytes of C57BL/6 mice were left unstimulated (mock) or were treated for 8 hours with sera of saline (B6/J_PBS) or ConA challenged control or *Mlkl*$^{-/-}$ mice. LDH release as an indicator of cell necrosis was determined by ELISA (the relative release to the B6/J_PBS group is plotted; n=6/group). **(D)** Quantification of *Mlkl* transcripts in liver lysates of saline or APAP-challenged control mice by qPCR

(n>3/group). **(E,F)** C57BL/6 and *Mlkl*$^{-/-}$ mice were i.p. injected with APAP and analyzed 24 hours later. Experiments were repeated two times with similar results. **(E)** Plasma AST/ALT concentrations (n=4/group). **(F)** Representative pictures of H&E and TUNEL assay stained tissue sections and quantification of necrotic areas in TUNEL assay stained livers (n=4/group). **(G)** Primary hepatocytes of C57BL/6 mice or *Mlkl*$^{-/-}$ mice were left unstimulated (saline) or were treated for 24 hours with APAP at 8mM. Cell death was measured by Cell death detection ELISA (relative to saline control, n=3/group).

Error bars indicate +SD, gene expression levels are shown relative to *Hprt*.

Supplemental Figure 4: Inhibition of RIPK1 kinase functions does not influence expression of hepatic *Ifng*

(A+B) B6/J mice were left untrated (0h) or challenged 1h, 3h and 6h with ConA. **(A)** Hepatic expression of Mlkl mRNA in liver biopsies (n>3/group). **(B)** Representative pictures showing double staining for RIPK1 and TUNEL in liver tissue sections. **(C)** Representative pictures of liver tissue sections from ConA treated mice stained for RIPK1 in combination with TUNEL assay. Panels on the right represent confocal images (only RIPK1 staining). **(D)** *Ifng* transcripts in unchallenged (mock) mice, mice treated with ConA alone (DMSO) or pretreated with nec1-s (nec-1s) were quantified by qPCR (n>3/group). $^{**}P < 0.01$ by paired Student's *t* test.

Error bars indicate +SD, gene expression levels are shown relative to *Hprt*.

Supplemental Figure 5: RIPK3 is expressed in F4/80^{+} kupffer cells

(A) Quantification of *Ripk3* mRNA in lysates of indicated tissues or primary mouse hepatocytes (PMH) (n=3/group, n.d. = not detectible). **(B)** Quantification of hepatic *RIPK3* transcripts in patients (n=6) or C57BL/6 mice that were control or ConA treated for 7 hours (n>3/group). **(C+D)** The specificity of anti-mouse RIPK3 mABs was analyzed by Western-blot analysis with protein lysates isolated from organs of wildtype and *Ripk3*$^{-/-}$ mice (C) and immunohistochemistry of liver cross sections from wildtype and *Ripk3*$^{-/-}$ mice (D). **(E)** Representative pictures of liver tissue sections double stained for RIPK3 and F4/80. **(F-I)** C57BL/6 and *Ripk3*$^{-/-}$ mice were i.v. injected with ConA and analyzed 7 hours later. Experiments were repeated three times with similar results. **(F)** Plasma ALT concentrations of ConA challenged control and *Ripk3*$^{-/-}$ mice (n>3/group). **(G)** Representative pictures of histological (H&E, dashed lines represent necrotic areas) and immunohistochemical (TUNEL assay) staining analysis of hepatic tissue sections. **(H)** Western Blot analysis demonstrating that endogenous MLKL

translocated from cytoplasm (C) to the plasma membrane (PM) fraction in C57BL/6 mice following ConA treatment. **(I)** RIPK1 was stained in liver cross sections obtained from ConA challenged B6/J and *Ripk3*$^{-/-}$ mice. Panel in the right represents confocal pictures.

Error bars indicate +SD, gene expression levels are shown relative to *Hprt*.

Supplemental Figure 6: Hepatocytes undergo non-apoptotic death in response to ConA

(A Quantification of TUNEL or cleaved Caspase-3/TUNEL double positive cells positive cells (n=4/group). **(B)** Representative pictures of histological (H&E, dashed lines represent necrotic areas) staining analysis of hepatic tissue sections and quantification of necrotic area in liver cross sections of ConA-challenged mice (n>3/group). **(C-E)** C57BL/6 and *Mlkl*$^{-/-}$ mice were treated with LPS/GalN and analyzed 6 hours later. Experiments were repeated two times with similar results. **(C)** Representative pictures of H&E and cleaved caspase-3 (cCasp3) stained tissue sections **(D)** Plasma ALT concentrations (n=4/group). **(E)** Quantification of cleaved caspase-3 positive area (n=4/group).

Error bars indicate +SD

Supplemental Figure 7: IFN-β-dependent STAT1 activation regulates MLKL expression

(A) IFN-γ expression constructs or empty control vectors (mock) were HD injected into C57BL/6 mice or *Stat1*$^{-/-}$ mice. 4 days later hepatic *Mlkl* mRNA was quantified by qPCR (n=3/group). **(B-D)** Plasma aminotransferase concentrations in control, *Ifng*$^{-/-}$ (B), *Stat1*$^{-/-}$ (C) or *Rag1*$^{-/-}$ (D) mice treated for 7 hours with ConA (n>3/group). ***P < 0.001 by paired Student's t test. **(E)** Quantification of *Mlkl* mRNA in liver lysates of ConA challenged control (B6/J) or *Rag1*$^{-/-}$ mice (n=4/group). ***P < 0.001 by paired Student's t test. **(F)** Relative luciferase activity in HEK293T cells after transfection with a MLKL-Luciferase promoter construct and stimulation with rhIFN-γ (relative to mock group, n=2/group). **P < 0.01 by paired Student's t test. **(G)** Hepatic expression on *Mlkl* mRNA in control, *Ifnar*$^{-/-}$ and *Il28ra*$^{-/-}$ mice treated for 7 hours with ConA. **(H)** Relative abundance of *Mlkl* mRNA in primary hepatocytes isolated from wildtype or *Stat1*$^{-/-}$ mice after stimulation with the indicated interferon factors (n=3/group). ***P < 0.001 by paired Student's t test. **(I)** The presence of STAT1^{pTyr701} in primary hepatocytes after 30 min stimulation with indicated IFNs was analyzed by Western-blot. **(J+K)** Expression of *Mlkl* mRNA in (J) livers of unchallenged (mock) or IFN-β vector injected mice (n>3/group) or (K) in BNL cells stimulated with the indicated factors (n=4/group). All experiments were performed at least two times with similar results. *P < 0.05 by paired Student's t test. **P < 0.01, ***P < 0.001 by paired Student's t test.

Error bars indicate +SD, gene expression levels are shown relative to *Hprt*.

Supplemental Figure 8: *Ripk1* gene expression is not dependent on STAT1

(A,B) *Mlkl* or *Ripk1* transcripts were quantified in primary mouse fibroblasts (Mefs, A) or BNL cells (B) stimulated *ex situ* with indicated factors (n=3/group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by paired Student's *t* test. **(C+D)** IFN-γ expression constructs or empty control vector (mock) were HD injected into C57BL/6 mice. 4 days later hepatic *Mlkl* and *Ripk1* mRNA (n=3/group) was quantified by qPCR (C) and protein amount was analyzed by Western Blot analysis (D, GAPDH was used as a loading control). ***P* < 0.01 by paired Student's *t* test. **(E)** Quantification of hepatic *Ripk1* mRNA in vehicle (mock) or ConA challenged control (BL/6) mice, *Tnfr1*$^{-/-}$ or *Ifng*$^{-/-}$ (n>3/group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by paired Student's *t* test.

Error bars indicate +SD, gene expression levels are shown relative to *Hprt*.

Supplemental Figure 9: IFN-γ is required but not sufficient for MLKL-mediated hepatocellular death

(A,B) Kaplan–Meyer survival analysis (A) and aminotransferase concentrations (B) in C57BL/6 mice injected with control vector (mock) and IFN-γ expressing minicircle DNA prior to ConA administration (n>4/group). ****P* < 0.001. **(C)** Primary hepatocytes were stimulated with IFN-γ or TNF-α for 48h. LDH release is shown as relative to mock control (n=4/group).

Error bars indicate +SD.

Supplemental Figure 10: TNF-α is required for MLKL mediated liver injury

(A-F) C57BL/6 or *Tnfr1*$^{-/-}$ mice were subjected to ConA or saline treatment (mock) and analyzed 7 hours later. Experiments were repeated three times with similar results. **(A)** AST/ALT levels (n>3/group). **(B)** Quantification of necrotic area in liver tissue of ConA challenged mice (n=3/group). ****P* < 0.001 by paired Student's *t* test. **(C)** Hepatic expression of *Mlkl* mRNA in unchallenged and ConA treated control (B6/J) and *Tnfr1*$^{-/-}$ mice was analyzed by qPCR (n>3/group). ***P* < 0.01, ****P* < 0.001 by paired Student's *t* test. **(D)** Quantification of MLKL protein level in ConA challenged mice (n=3/group, relative to ACTIN). **(E)** Representative images of liver tissue sections of ConA challenged wildtype or *Tnfr1*$^{-/-}$ mice stained for MLKL and RIPK1 alone or in combination with TUNEL by

immunohistochemistry. **(F)** Quantification of plasma membrane localized MLKL in ConA challenged mice (n=3/group).

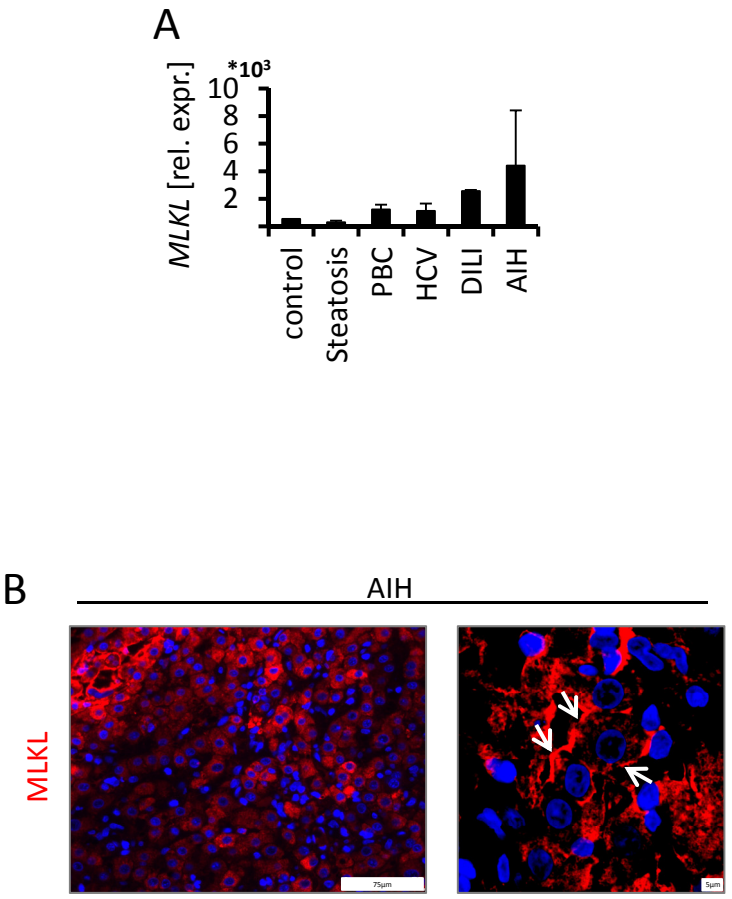
Error bars indicate +SD, gene expression levels are shown relative to *Hprt*.

Supplemental Figure 11: Model of MLKL-dependent hepatocellular death

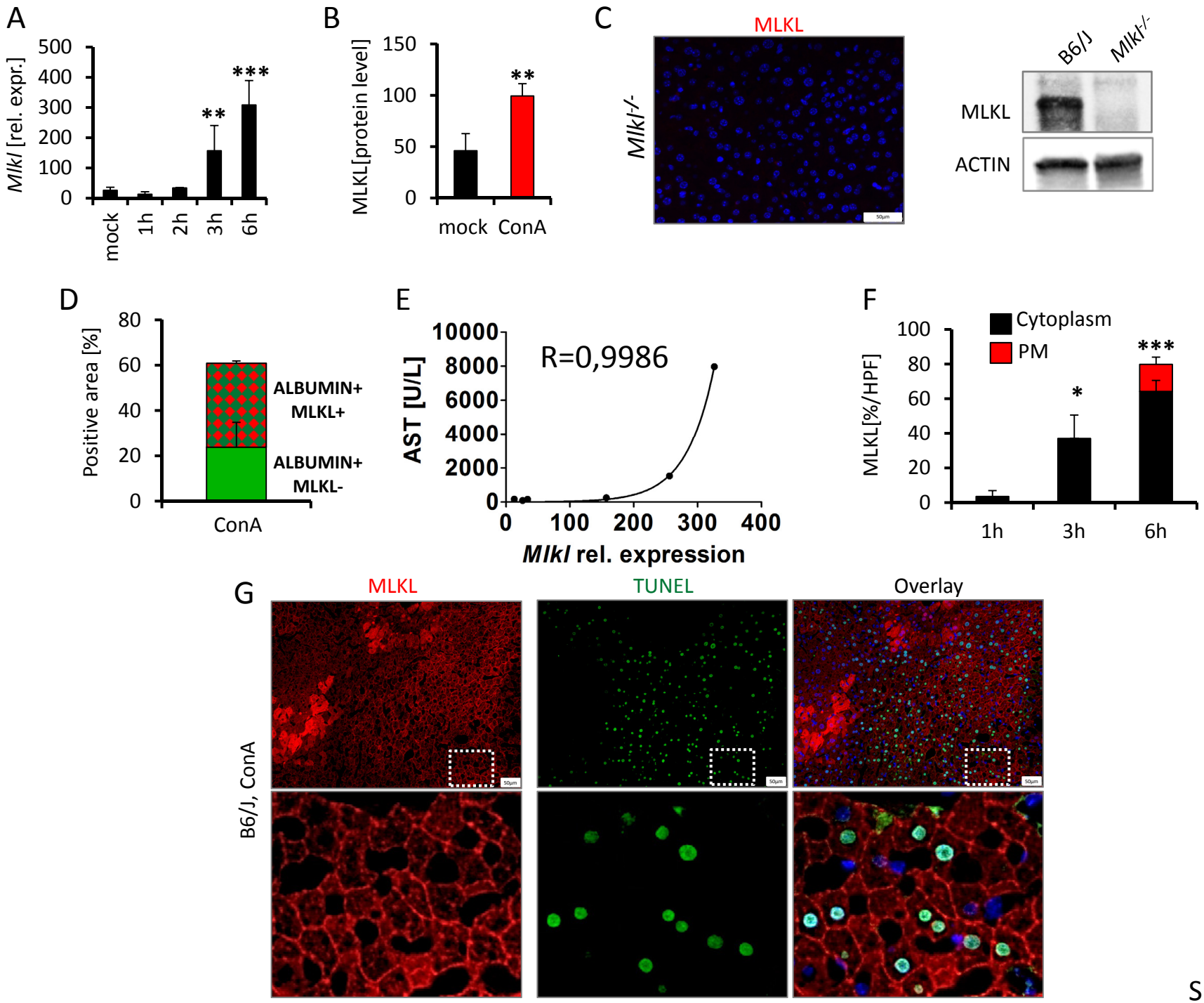
IFN-γ released by liver resident or recruited T lymphocytes, NKT cells and NK cells is strongly connected to induction of MLKL expression via activation of the transcription factor STAT1 in hepatocytes. Hepatocellular necrosis is driven by a previously unrecognized RIPK3-independent function of MLKL.

Supplemental Figure 12: MLKL dependent cell death in primary hepatocytes

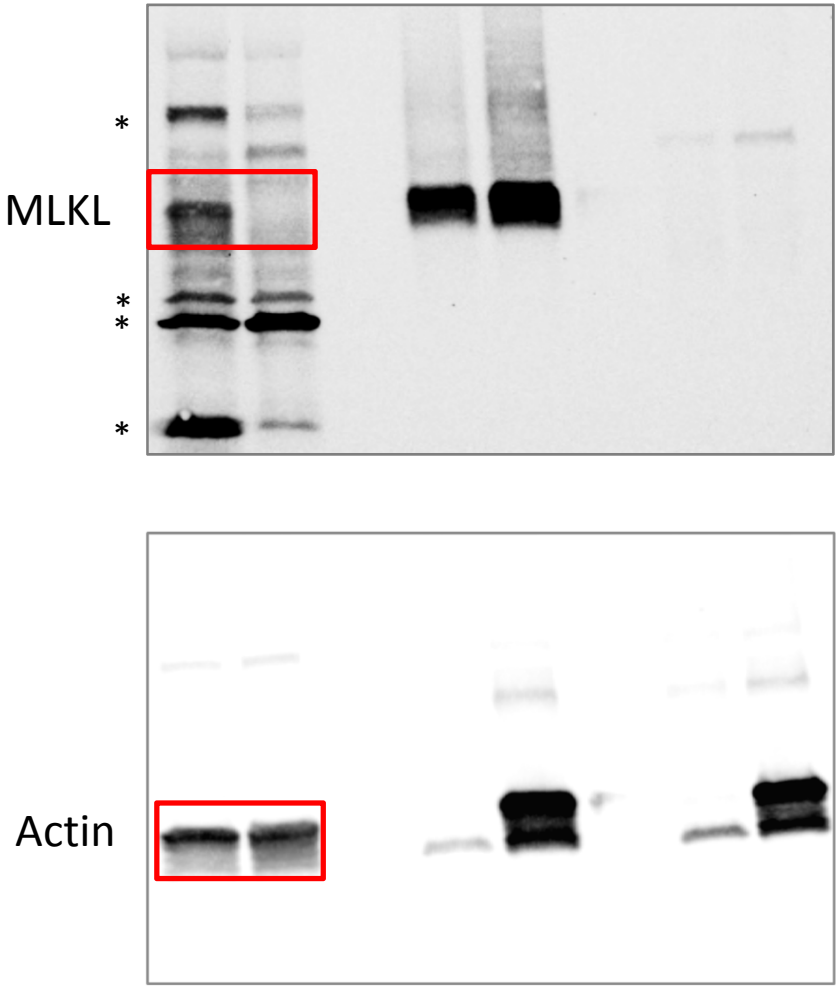
Primary hepatocytes derived from C57BL/6 mice or *Mlkl*$^{-/-}$ mice were left unstimulated (saline) or were treated for 24 hours with indicated factors after initial incubation with IFN-γ for 24 hours. Cell death was measured by Cell death detection ELISA (relative to saline control, n=4/group).



Supplemental Figure 1

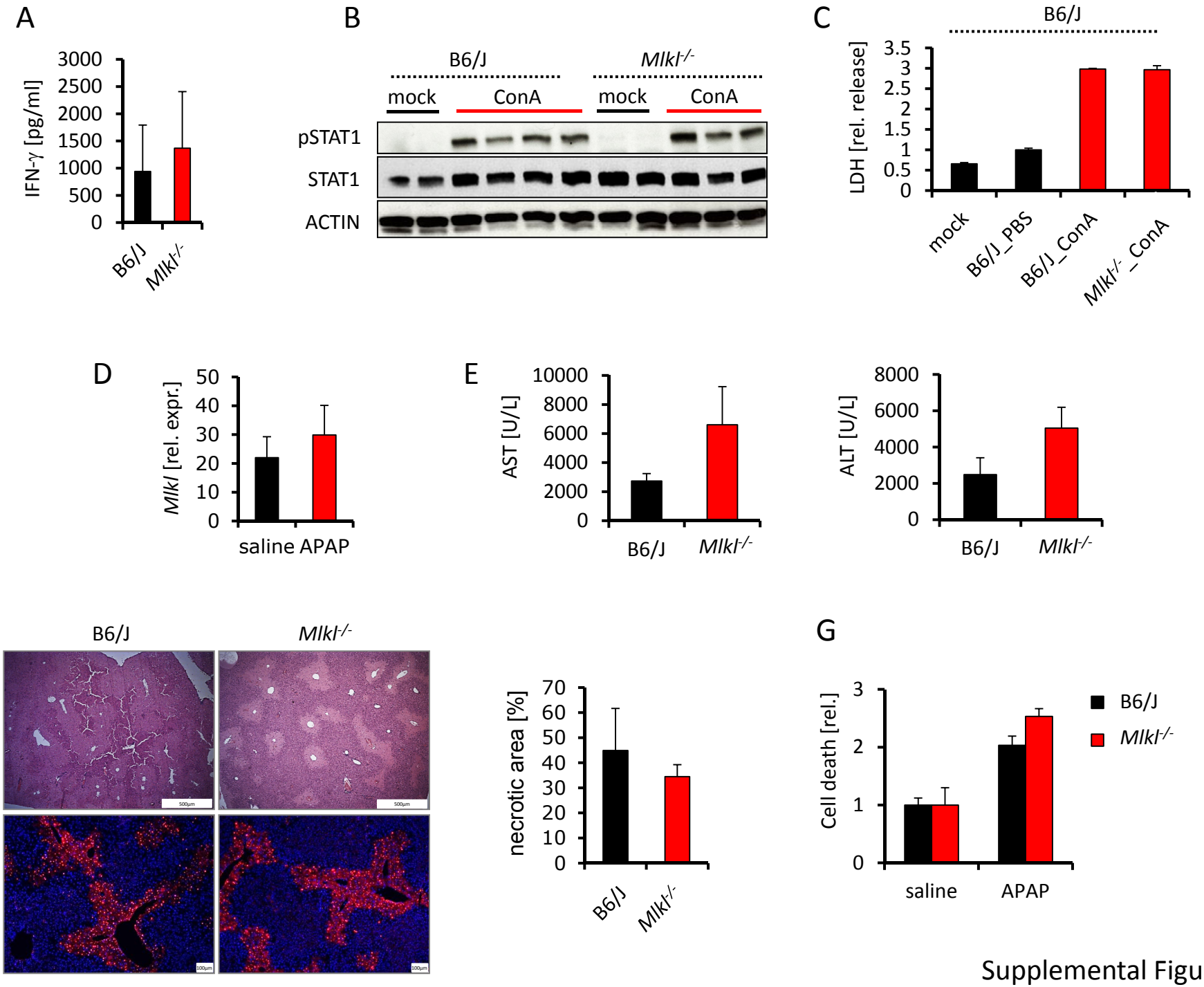

A
Mlkl [rel. expr.]
500
400
300
200
100
0
**
***
mock
1h
2h
3h
6h
B
MLKL[protein level]
150
100
50
0
**
mock
ConA
C
MLKL
Mlkl-/-
B6/J
Mlkl-/-
MLKL
ACTIN
D
Positive area [%]
80
60
40
20
0
ALBUMIN+
MLKL+
ALBUMIN+
MLKL-
ConA
E
AST [U/L]
10000
8000
6000
4000
2000
0
R=0,9986
0
100
200
300
400
Mlkl rel. expression
F
MLKL[%/HPF]
100
80
60
40
20
0
Cytoplasm
PM
*
***
1h
3h
6h
G
MLKL
TUNEL
Overlay
B6/J, ConA

Suppl. Fig.2

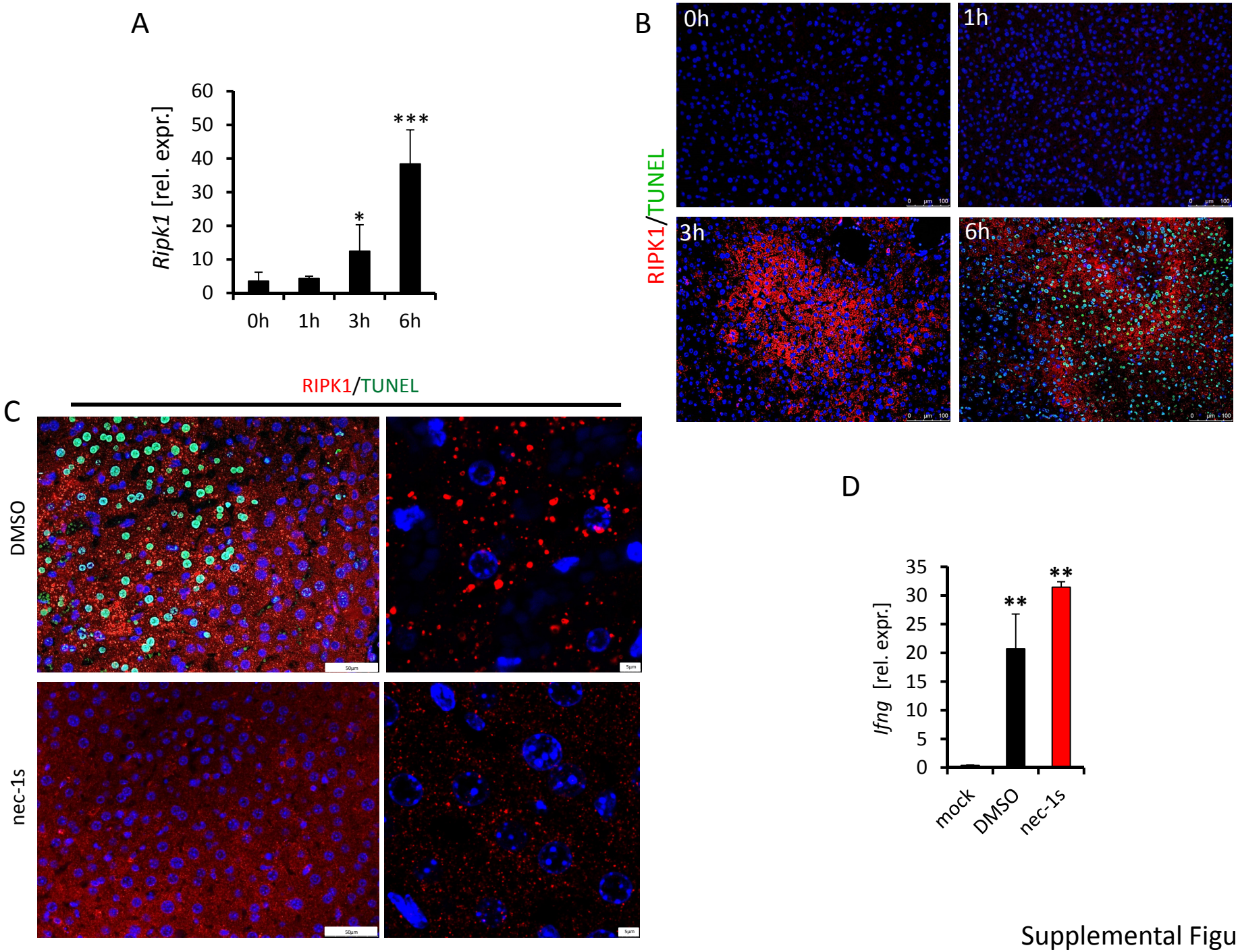


*: unspecific signal

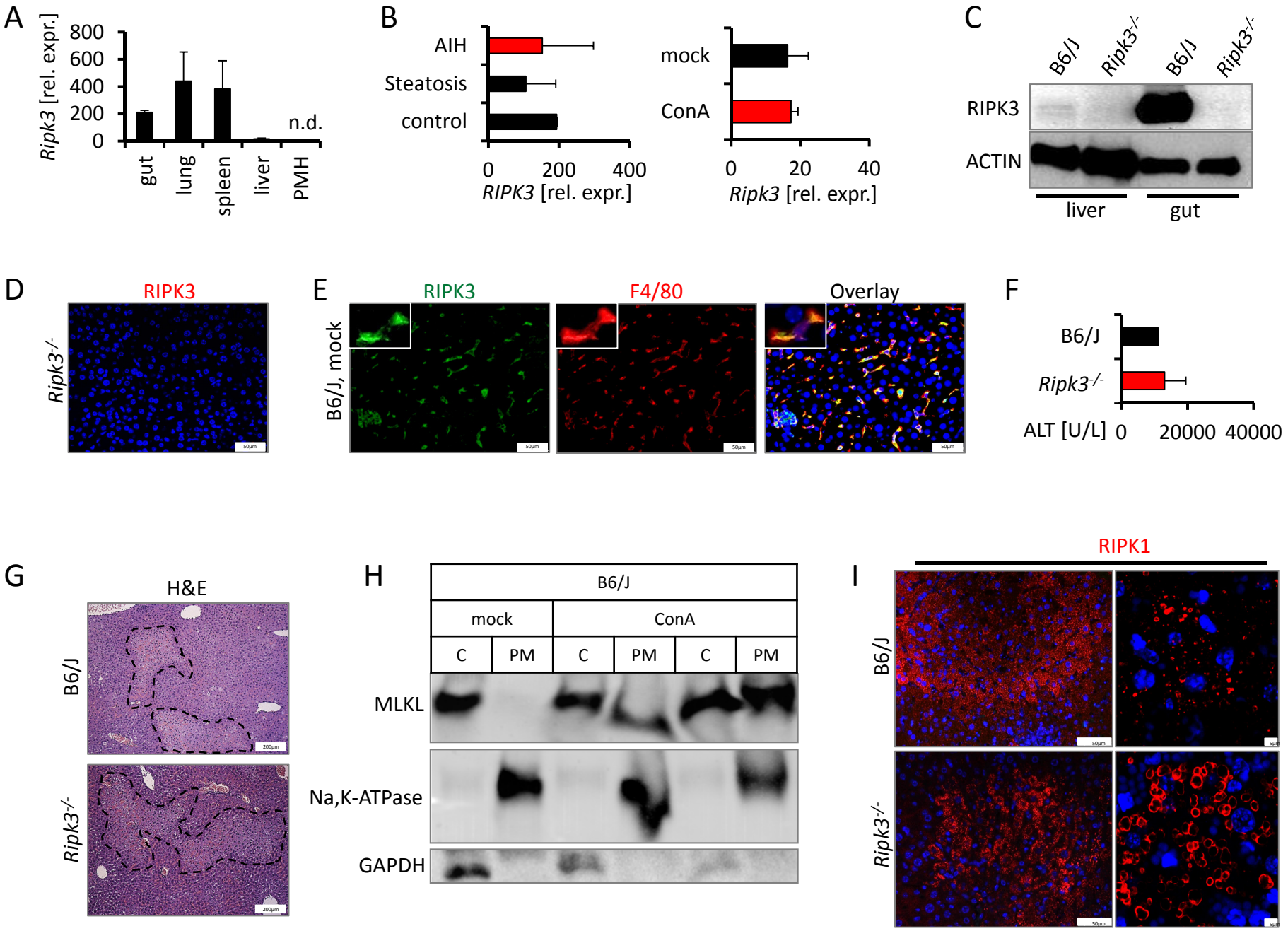
Full unedited blot for Supll. Fig. 2C



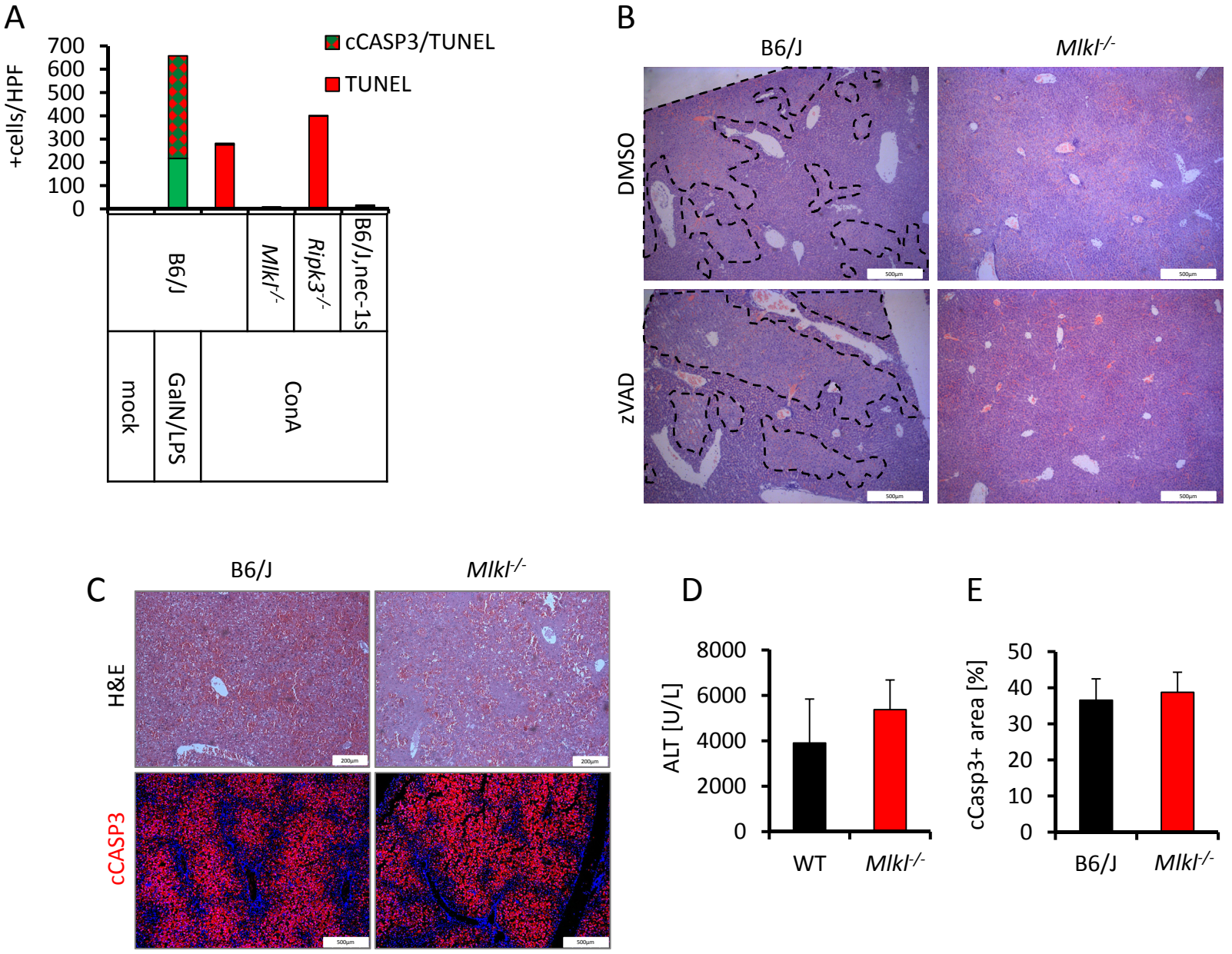
Supplemental Figure 3



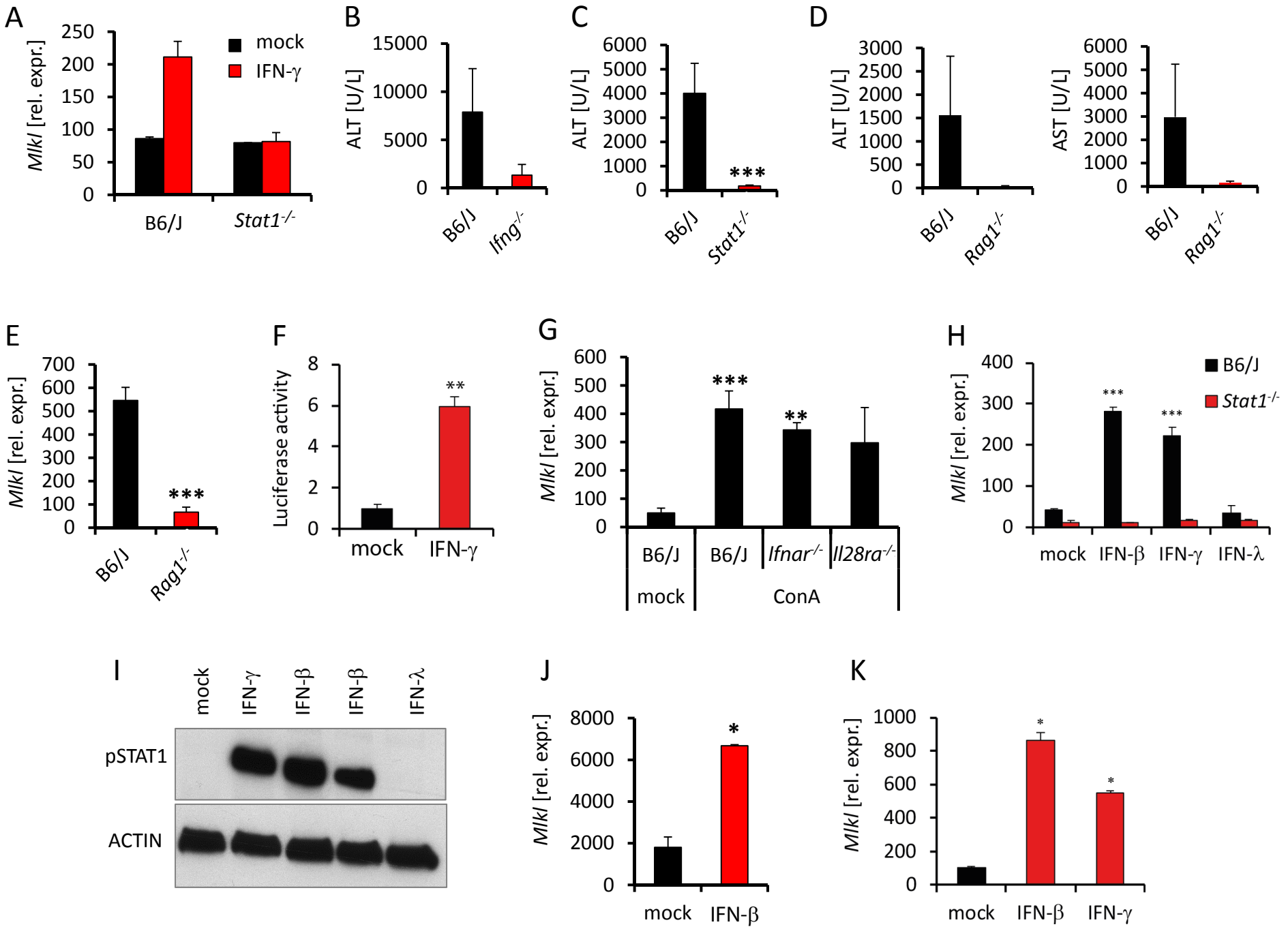
Supplemental Figure 4



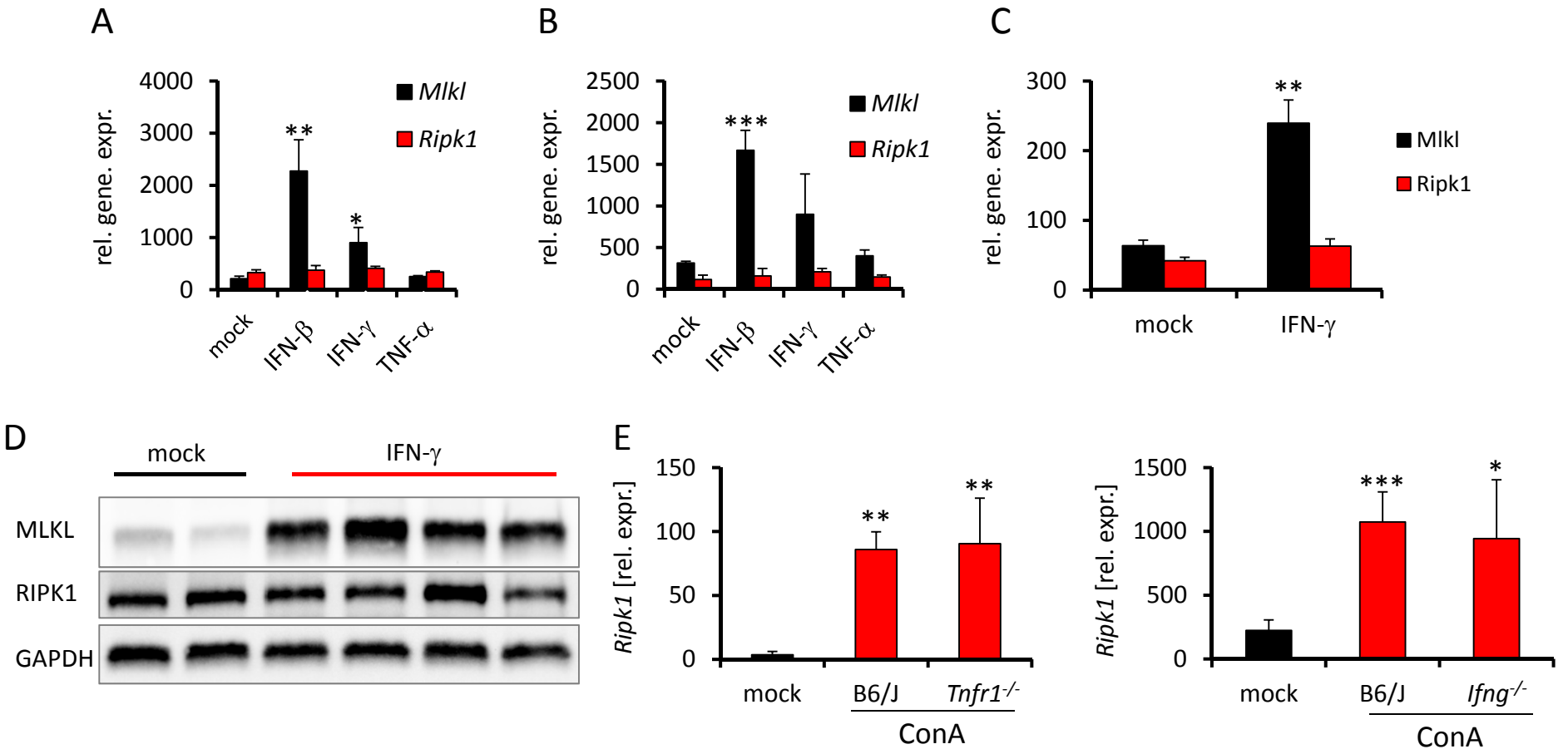
Supplemental Figure 5



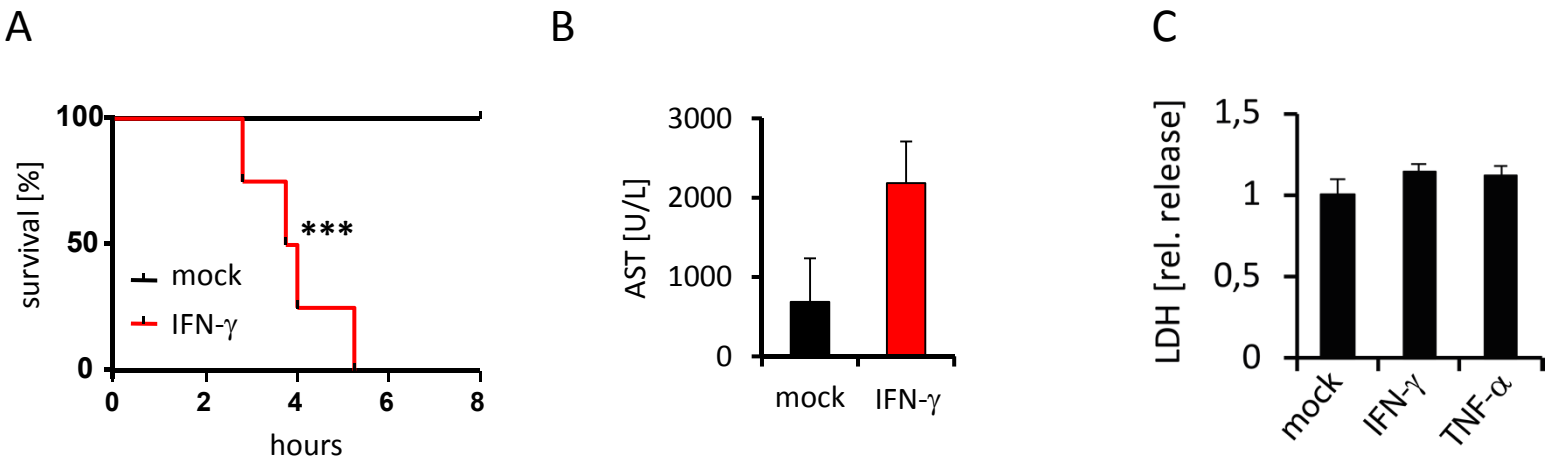
Supplemental Figure 6



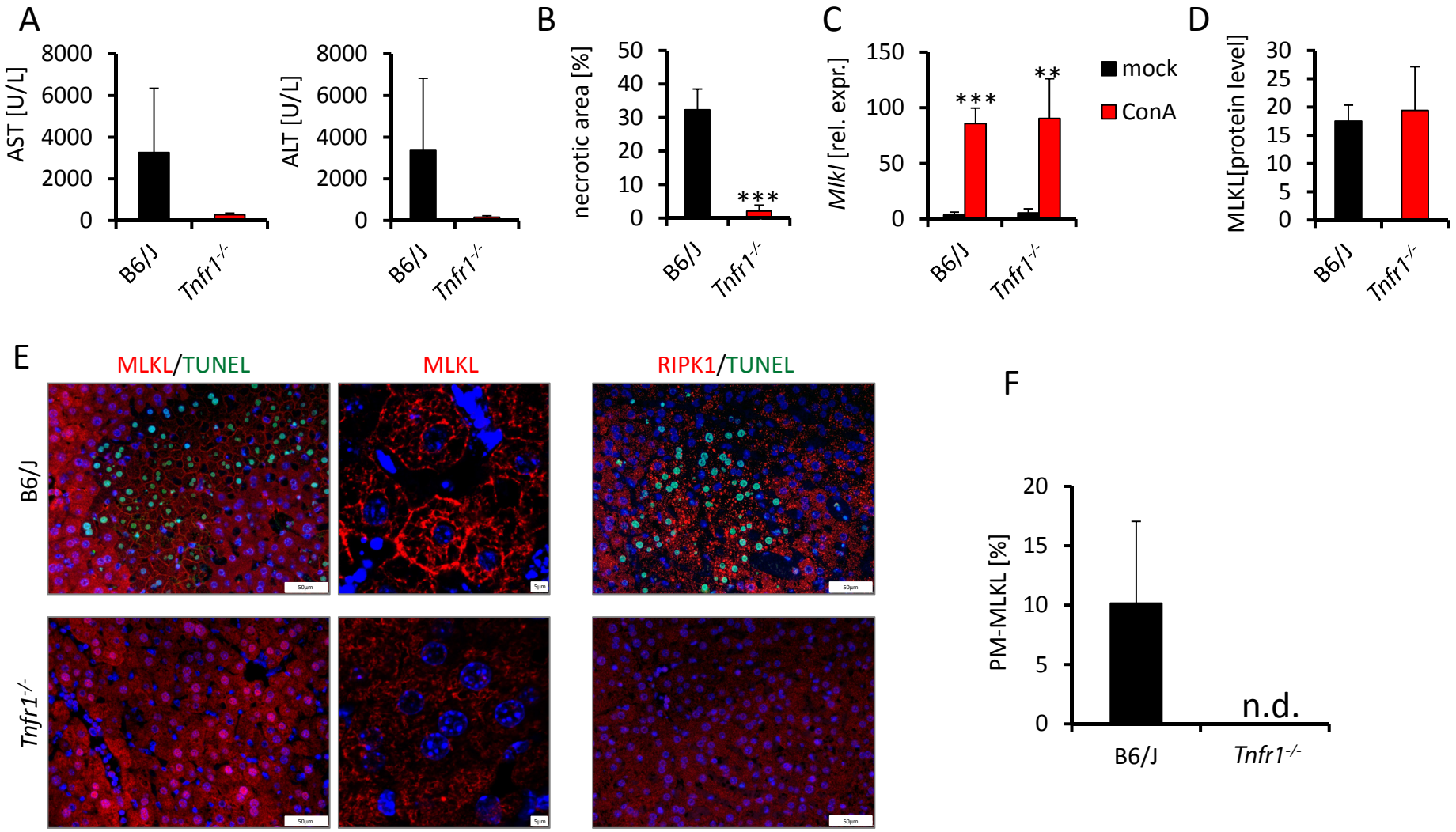
Supplemental Figure 7



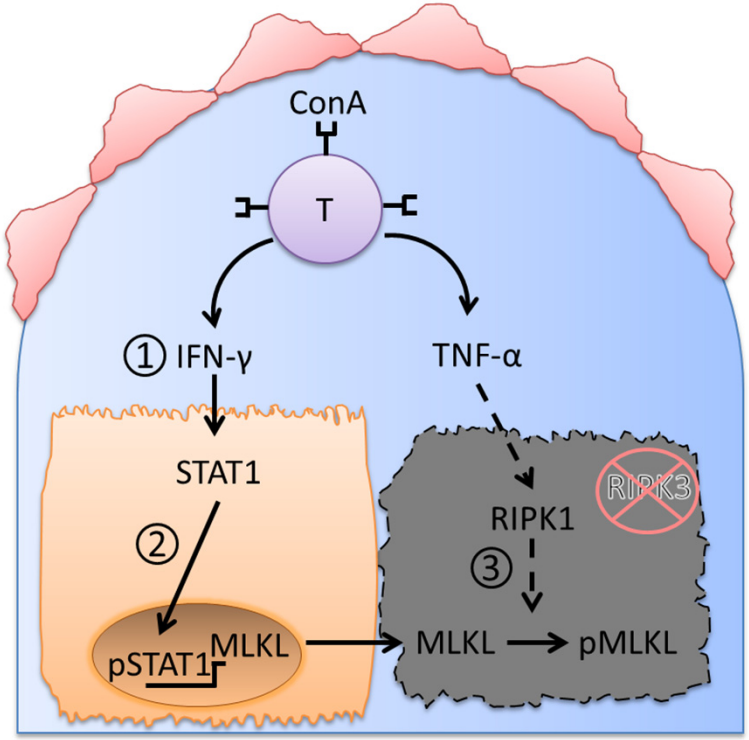
Supplemental Figure 8



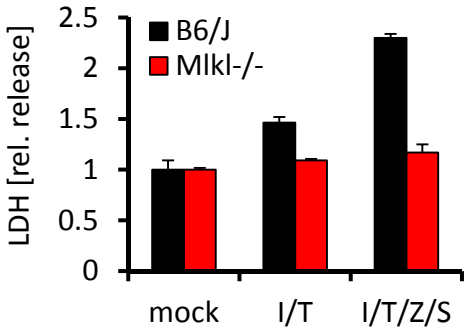
Supplemental Figure 9



Supplemental Figure 10



Supplemental Figure 11



Supplemental Figure 12