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Supplemental Data

Induction of Hepatitis by JNK-Mediated Expression of TNF-α

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Supplemental Experimental Procedures

Flow cytometry.
Peripheral blood leukocytes and splenocytes (10^6 cells) were incubated with anti-CD32/CD16 antibodies to block Fc receptors and then stained with PE-conjugated anti-CD4, APC-conjugated anti-CD8 plus FITC-conjugated anti-B220 antibody (Pharmingen) or with PE-conjugated anti-CD45.1 plus FITC-conjugated anti-CD45.2 (Pharmingen) in phosphate-buffered saline plus 2% serum. Flow cytometry was performed using a FACScan cytofluorometer (Becton Dickinson) and data were examined using FlowJo software.

Immunohistochemistry.
Livers were fixed in 4% paraformaldehyde, processed, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E) or by TUNEL assay using an *in situ* cell death kit (Roche). Hepatic damage detected in the stained liver sections was quantitated using ImagePro Plus software (Media Cybernetics).

Tissue culture.
Primary bone marrow-derived macrophages were prepared using methods described previously (Kim et al., 2004). Primary CD4+ T cells from lymph nodes and spleen were isolated by positive selection using anti-CD4 MACS beads (Miltenyi) and cultured *in vitro*. Cytokine concentration in the culture medium was measured by multiplexed ELISA using a Luminex 200 instrument (Millipore).

Taqman© probes.
Bax (Mm 00432050 _ m1), *cJun* (Mm 00495062_s1), *cFos* (Mm 00487425_m1), *Jnk1* (Mm0048915_m1), *JunB* (Mm00492781_s1), *JunD* (Mm 00495088_s1), *Il22* (Mm00444241_m1), *p53* (Mm00441964 _ g1), *p21* (Mm 00432448 _ m1), *Mdm2* (Mm00487656 _ m1), *Puma* (Mm 00519268 _ m1) and *Tnfα* (Mm_00443258_m1) probes were purchased from Applied Biosystems.

Statistical analysis.
Differences between groups were examined for statistical significance using the Student's test, analysis of variance (ANOVA) with the Fisher's test, or the log-rank test.

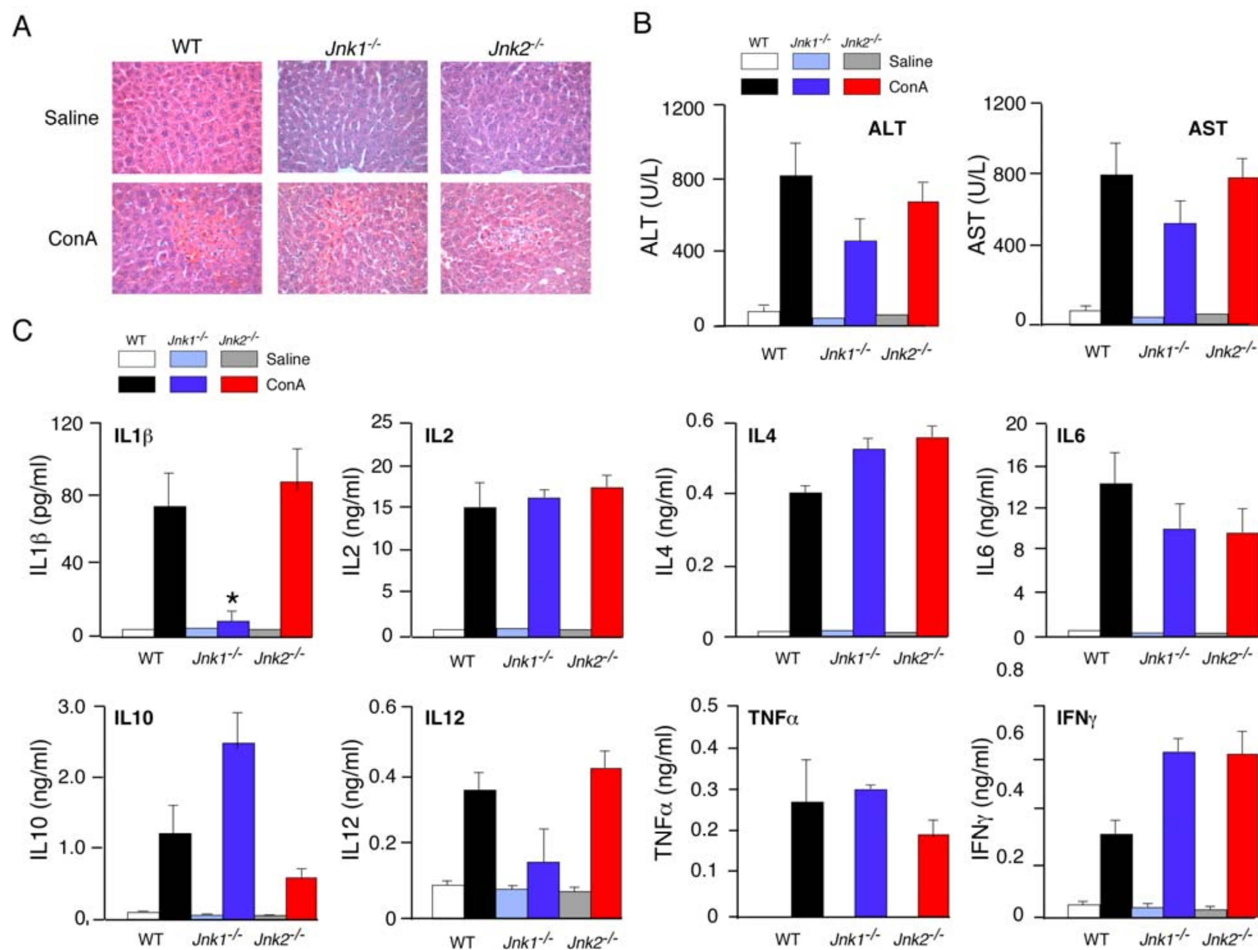

Figure S1. JNK1-deficient and JNK2-deficient mice are not protected against ConA-induced hepatitis. (**A**) Wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice were treated intravenously (8 hrs) with ConA or solvent (saline). Representative H&E-stained liver sections are presented. The amount of liver damage was quantitated (Figure S2A). (**B,C**) Serum transaminase activity (ALT and AST) in control and JNK-deficient mice after treatment (8 hrs) with ConA or solvent (saline) was measured (mean ± SD; n = 6). No statistically significant differences between wild-type and JNK-deficient mice were detected. (**D**) The amount of serum cytokines (IL1, IL2, IL4, IL6, IL10, IL12, TNFα, and IFNγ) at 8 hrs. post-treatment with ConA was measured by ELISA (mean ± SD; n = 4). Statistically significant differences between wild-type and JNK-deficient mice are indicated (*, P < 0.05).

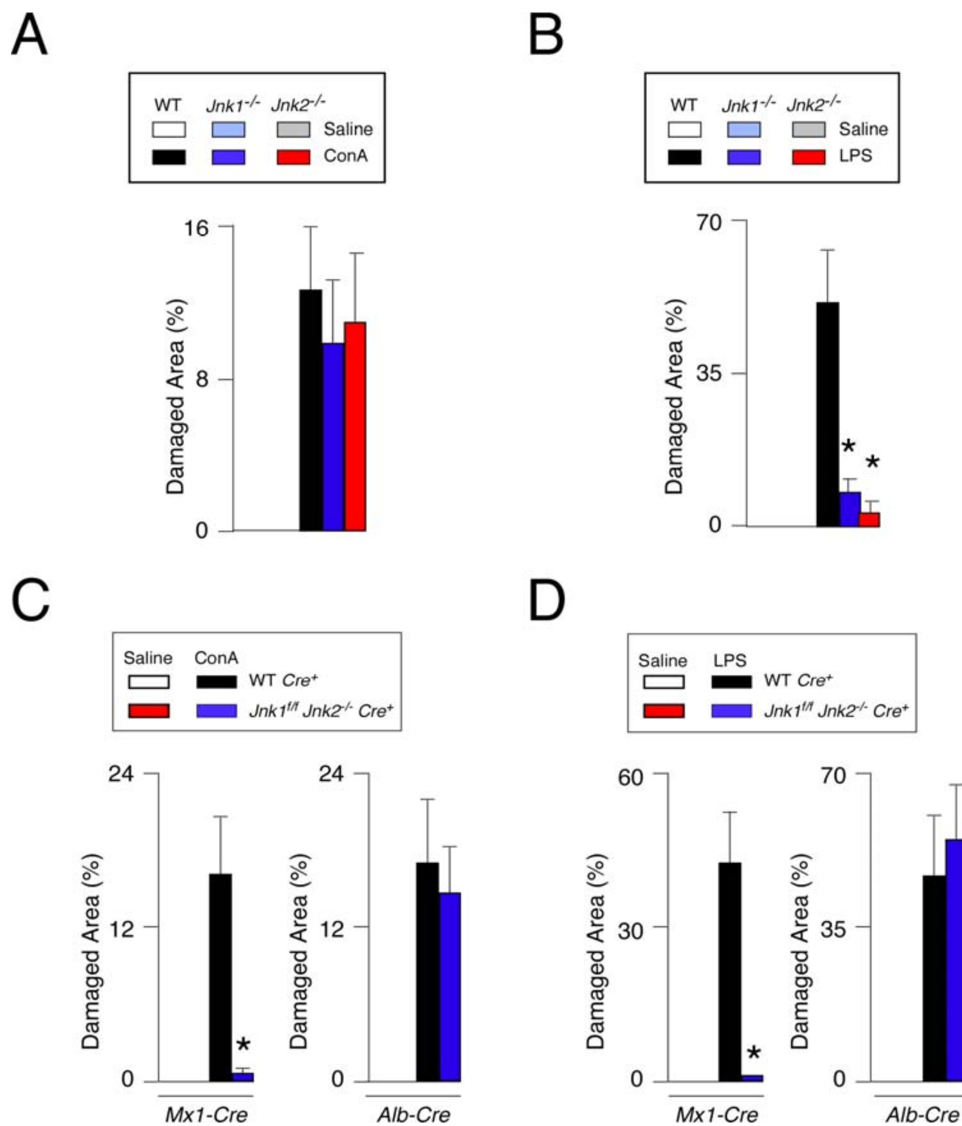

Figure S2. Quantitation of hepatic damage caused by ConA and LPS. Hepatic damage detected by staining liver sections with H&E was measured using ImagePro Plus (Media Cybernetics) software (mean % area ± SD; n= 10). Statistically significant differences between wild-type and JNK-deficient mice are indicated (*, P < 0.01).

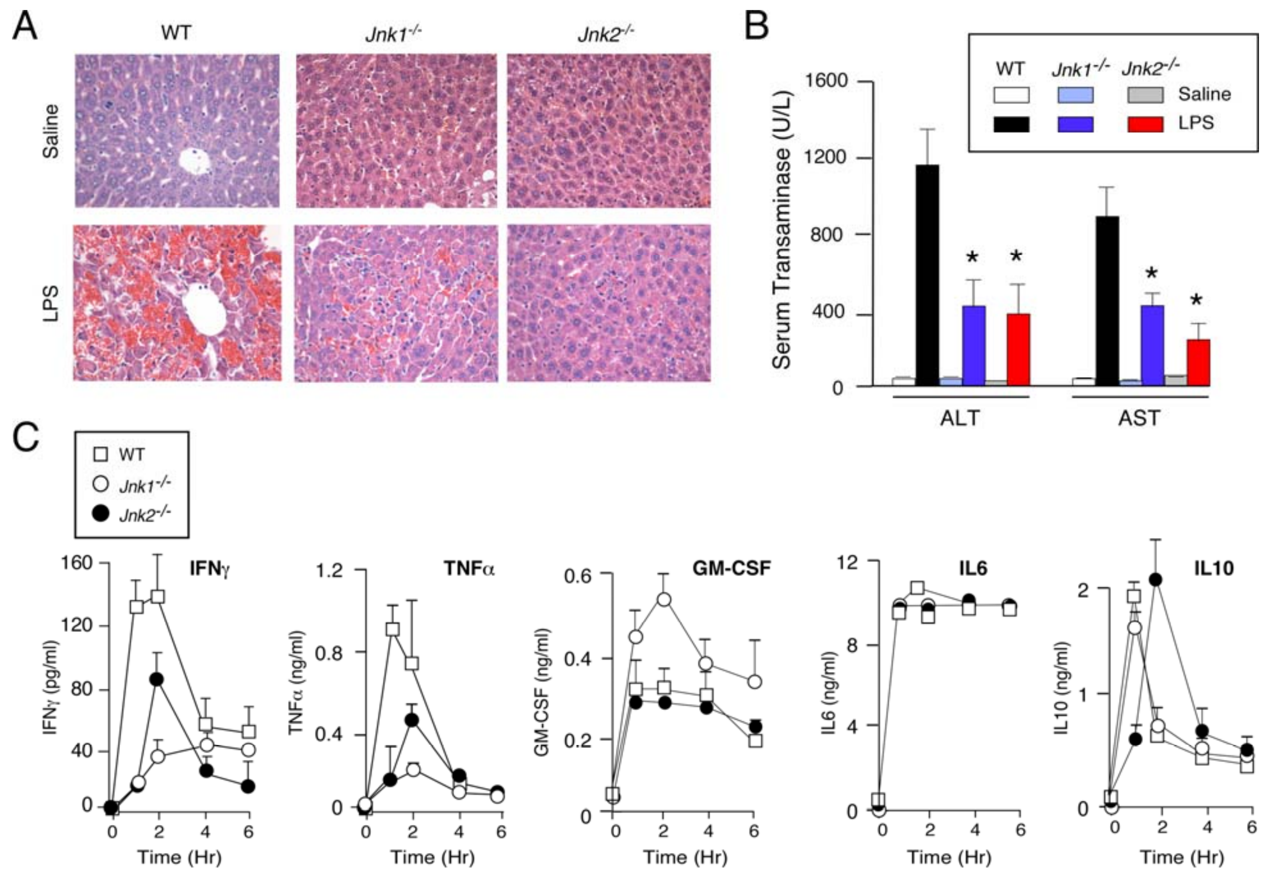

Figure S3. JNK1-deficient and JNK2-deficient mice are protected against LPS-induced hepatitis. (**A**) Wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice were treated intravenously (8 hrs) with LPS plus GalN or solvent (saline). Representative H&E-stained liver sections are presented. The amount of liver damage was quantitated (Figure S2B) (**B**) Serum transaminase activity (ALT and AST) in control and JNK-deficient mice after treatment (8 hrs) with LPS plus GalN or solvent (saline) was measured (mean ± SD; n = 6). Statistically significant differences between wild-type and JNK-deficient mice are indicated (*, P < 0.05). (**C**) The amount of serum cytokines (IFNγ, TNFα, GM-CSF, IL6, IL10) post-treatment with LPS plus GalN was measured by ELISA (mean ± SD; n = 6).

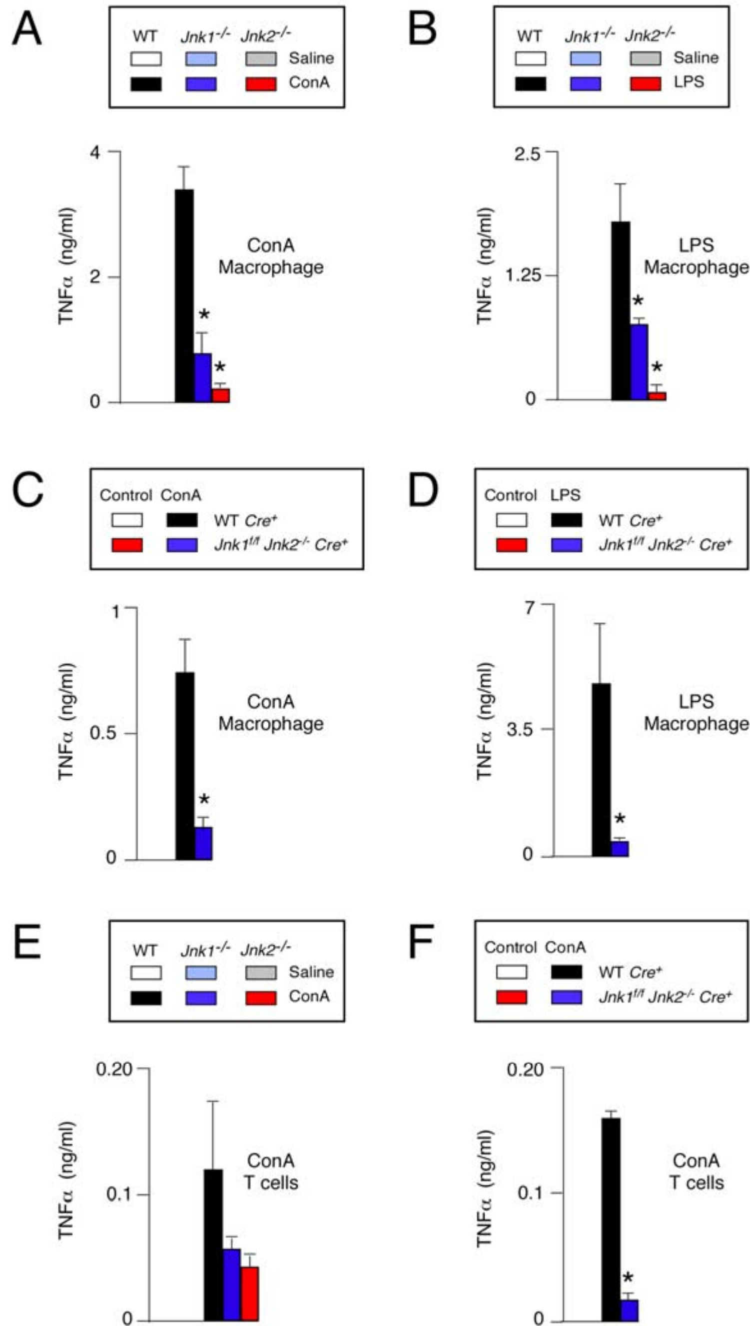

Figure S4. JNK-deficiency causes defects in the expression of TNFα by T cells and macrophages.

(**A,B**) Bone marrow-derived macrophages were isolated from wild-type, $Jnk1^{-/-}$, and $Jnk2^{-/-}$ mice. The cells (1×10^6) were cultured in 1.0 ml medium and treated without and with 2.5 µg of ConA or 1.0 µg LPS. The concentration of TNFα in the culture medium was measured by ELISA at 4 hr (ConA) or 8 hr (LPS) post-treatment. (**C,D**) Bone marrow-derived macrophages were isolated from PolyIC-treated control mice (*Mx1-Cre*$^{+}$) and JNK-deficient ($Jnk1^{f/f}$ $Jnk2^{-/-}$

Mx1-Cre^{+}) mice. The concentration of TNFα in the culture medium was measured by ELISA at 24 hr (ConA) or 8 hr (LPS) post-treatment. (**E**) CD4 T cells were isolated from wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice. The cells (5×10^5) were cultured in 0.5 ml medium and treated without and with 1.25 µg of ConA plus 0.5 µg anti-CD28 (BD-Pharmingen). The concentration of TNFα in the culture medium was measured by ELISA at 48 hr post-treatment. (**F**) CD4 T cells were isolated from PolyIC-treated control mice (*Mx1-Cre*$^{+}$) and JNK-deficient (*Jnk1*$^{f/f}$ *Jnk2*$^{-/-}$ *Mx1-Cre*$^{+}$) mice. The concentration of TNFα in the culture medium was measured by ELISA at 48 hr post-treatment with 1.25 µg of ConA plus 0.5 µg anti-CD28.

The data presented represent the mean ± SD (n = 3). Statistically significant differences between control and JNK-deficient cells are indicated (*, $P < 0.05$).

Studies of macrophages demonstrate that disruption of the *Jnk1* or *Jnk2* genes caused a significant decrease in ConA- and LPS- induced expression of TNFα (A,B). Similarly, compound deficiency of *Jnk1* plus *Jnk2* in macrophages also decreased TNFα expression in response to ConA and LPS (C,D). These data demonstrate that JNK1 and JNK2 play partially non-redundant roles in the response of macrophages to express TNFα when challenged with ConA or LPS *in vitro*. It is established that macrophages are an important target of LPS during the development of hepatitis (Dong et al., 2007). Thus, the non-redundant role of JNK1 and JNK2 in LPS-induced TNFα expression by macrophages may contribute to the observation that both *Jnk1*$^{-/-}$ mice and *Jnk2*$^{-/-}$ mice exhibit reduced LPS-induced hepatitis (Figure S3).

The sensitivity of *Jnk1*$^{-/-}$ mice and *Jnk2*$^{-/-}$ mice to ConA-induced hepatitis (Figure S1) may reflect a primary role of T cells, rather than macrophages, in the response to ConA during the development of hepatitis (Tiegs et al., 1992). We examined the response of isolated T cells to ConA *in vitro*. This analysis demonstrated that both *Jnk1*$^{-/-}$ T cells and *Jnk2*$^{-/-}$ T cells expressed reduced amounts of TNFα compared with wild-type T cells, but the difference was not statistically significant (E). In contrast, compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ T cells expressed significantly less TNFα than control T cells (F). These data indicate that JNK1 and JNK2 may have partially redundant roles in the response of T cells to express TNFα when challenged with ConA. This partial redundancy may contribute to the observation that both *Jnk1*$^{-/-}$ mice and *Jnk2*$^{-/-}$ mice exhibit only a modest reduction in ConA-induced hepatitis *in vivo* compared with wild-type mice (Figure S3).

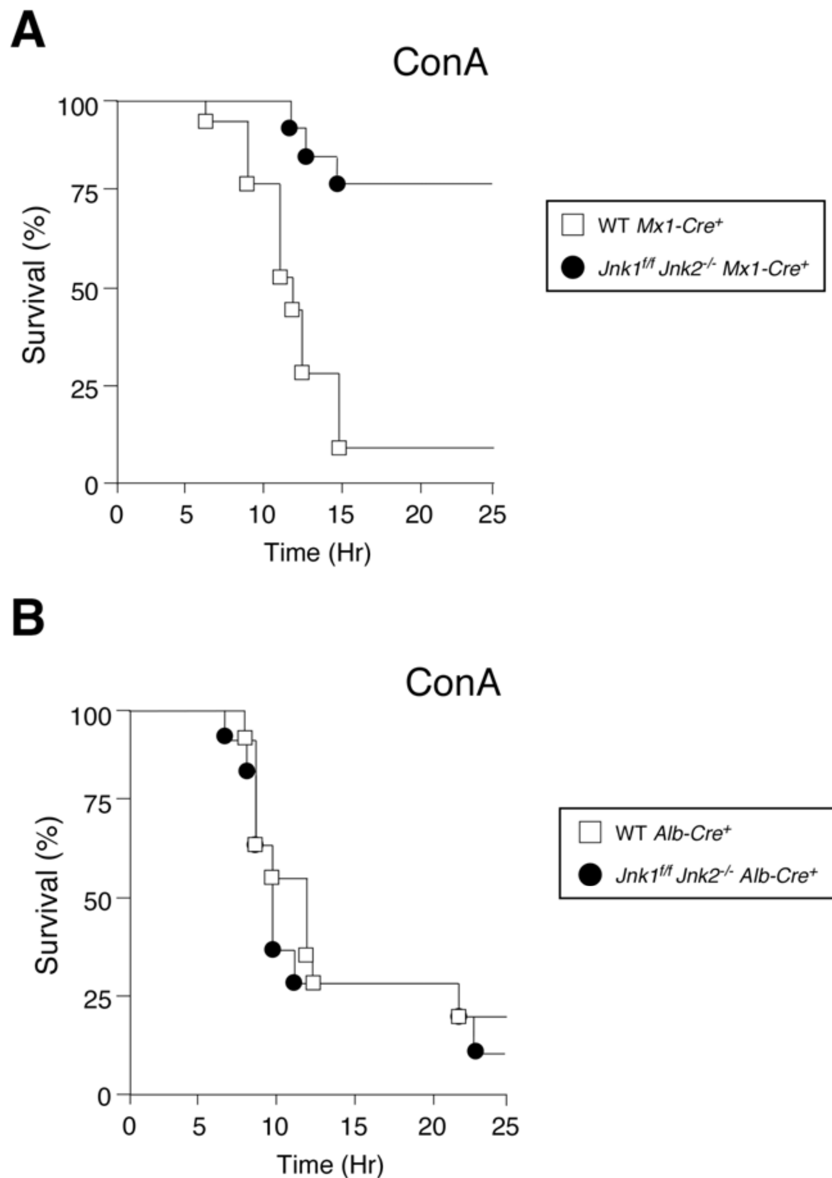

Figure S5. JNK-deficient mice exhibit reduced mortality in the ConA model of hepatitis.

(**A**) PolyIC-treated control mice (*Mx1-Cre*$^+$) and JNK-deficient (*Jnk1*$^{f/f}$ *Jnk2*$^{-/-}$ *Mx1-Cre*$^+$) mice were treated with ConA. Kaplan-Meier analysis of the survival of groups of 12 mice per genotype demonstrated that the JNK-deficient mice exhibited reduced mortality compared with control mice (log-rank test; $P < 0.005$). (**B**) Control mice (*Alb-Cre*$^+$) and JNK-deficient (*Jnk1*$^{f/f}$ *Jnk2*$^{-/-}$ *Alb-Cre*$^+$) mice were treated with ConA. Kaplan-Meier analysis of the survival of groups of 12 mice per genotype demonstrated that there was no statistically significant difference between the mortality of the control and JNK-deficient mice (log-rank test; $P > 0.05$).

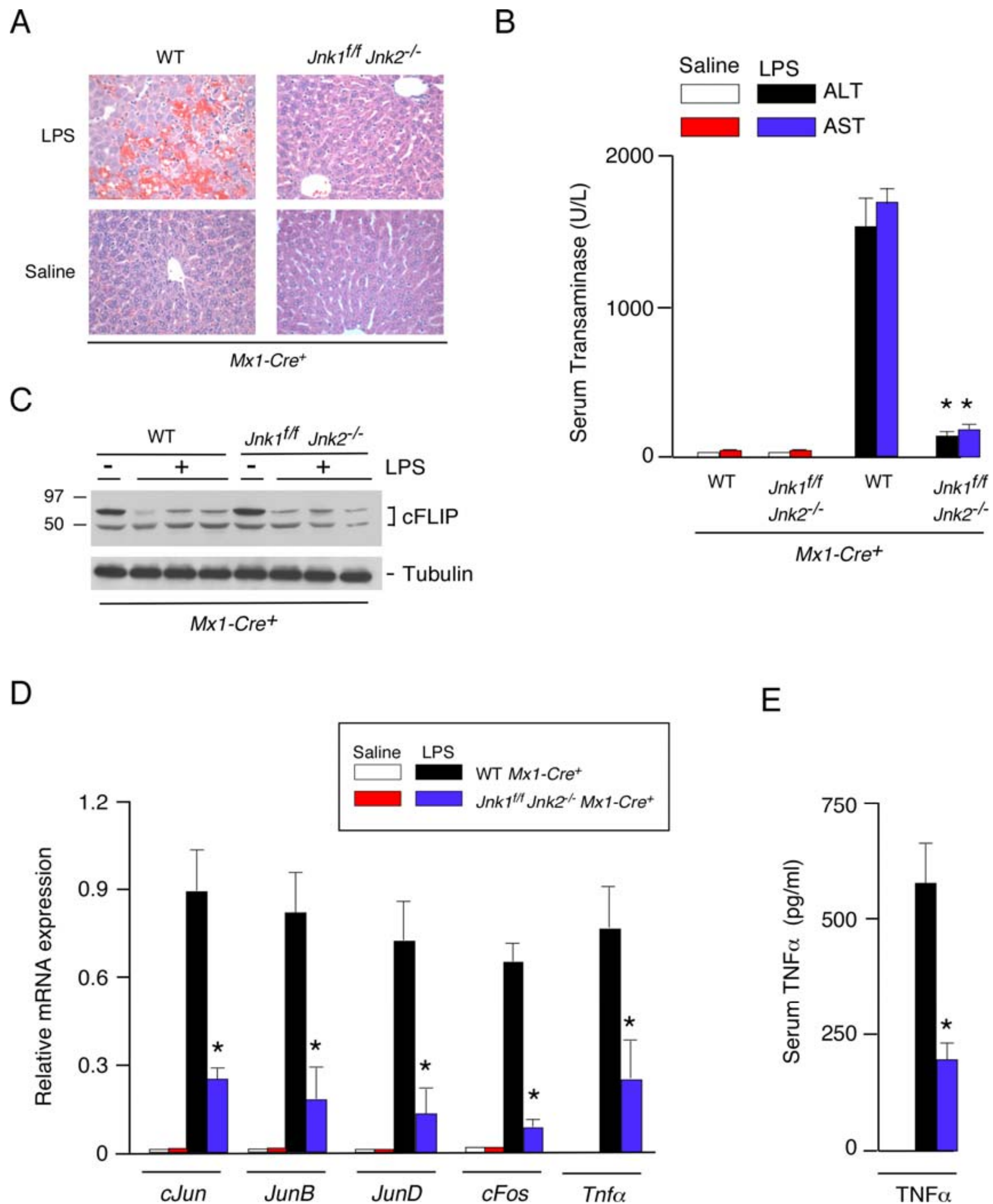

Figure S6. JNK-deficient mice are protected against LPS-induced hepatitis. (**A**) PolyIC-treated control mice (*Mx1-Cre*$^{+}$) and JNK-deficient mice (*Jnk1*$^{f/f}$ *Jnk2*$^{-/-}$ *Mx1-Cre*$^{+}$) were treated (8 hrs) with LPS plus GalN or solvent (saline). H&E-stained liver sections are presented. The amount of liver damage was quantitated (Figure S2D). (**B**) Serum transaminase activity (ALT and AST) in control and JNK-deficient mice after treatment (8 hrs) with LPS plus GalN or solvent (saline) was measured (mean ± SD; n = 6). Statistically significant differences between

wild-type and JNK1/2-deficient mice are indicated (*, $P < 0.01$). (**C**) Liver extracts were prepared from control and JNK-deficient mice at 8 hrs post-treatment with LPS plus GalN or solvent (saline) and examined by immunoblot analysis using antibodes to cFLIP, and α-Tubulin. The numbers on the left indicate the electrophoretic mobility of protein standards (kDa). (**D**) RNA was isolated from the liver of control and JNK-deficient mice after treatment (8 hrs) with LPS plus GalN or solvent (saline). The expression of *Gapdh*, *cJun*, *JunB*, *JunD*, *cFos*, and *Tnfα* mRNA was measured by quantitative RT-PCR (Taqman©) assays. The mRNA expression in each sample was normalized to the amount of *Gapdh* mRNA and presented as the mean ± SD ($n = 6$). Statistically significant differences between wild-type and JNK1/2-deficient mice are indicated (*, $P < 0.01$). (**E**) Serum TNFα was measured by ELISA after treatment (1 hr) with LPS plus GalN or saline (mean ± SD; $n = 6$). Statistically significant differences between mice transplanted with control and JNK1/2-deficient bone marrow are indicated (*, $P < 0.01$).

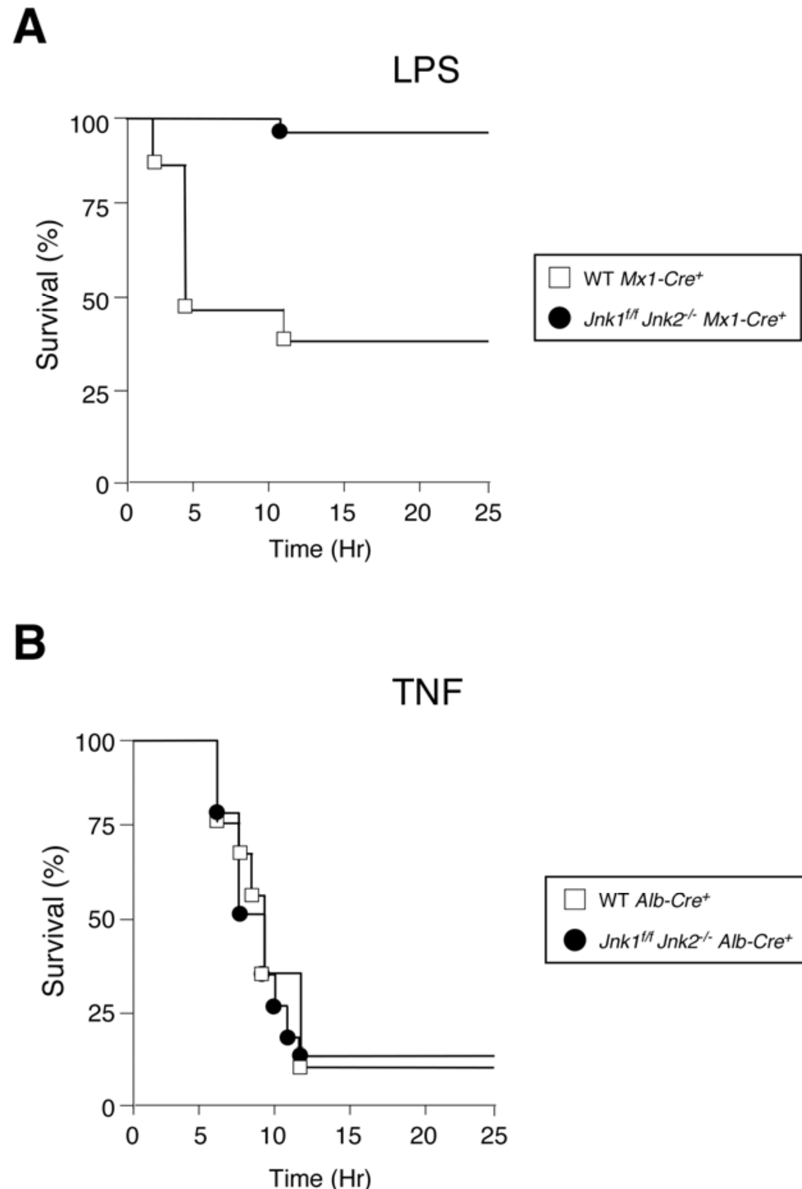

Figure S7. JNK-deficient mice exhibit reduced mortality in the LPS model of hepatitis. (**A**) PolyIC-treated control mice (*Mx1-Cre*$^{+}$) and JNK-deficient (*Jnk1*$^{f/f}$ *Jnk2*$^{-/-}$ *Mx1-Cre*$^{+}$) mice were treated with LPS plus GalN. Kaplan-Meier analysis of the survival of groups of 15 mice per genotype demonstrated that the JNK-deficient mice exhibited reduced mortality compared with control mice (log-rank test; $P < 0.01$). (**B**) Control mice (*Alb-Cre*$^{+}$) and JNK-deficient (*Jnk1*$^{f/f}$ *Jnk2*$^{-/-}$ *Alb-Cre*$^{+}$) mice were treated with TNFα plus GalN. Kaplan-Meier analysis of the survival of groups of 12 mice per genotype demonstrated that there was no statistically significant difference between the mortality of the control and JNK-deficient mice (log-rank test; $P > 0.05$).

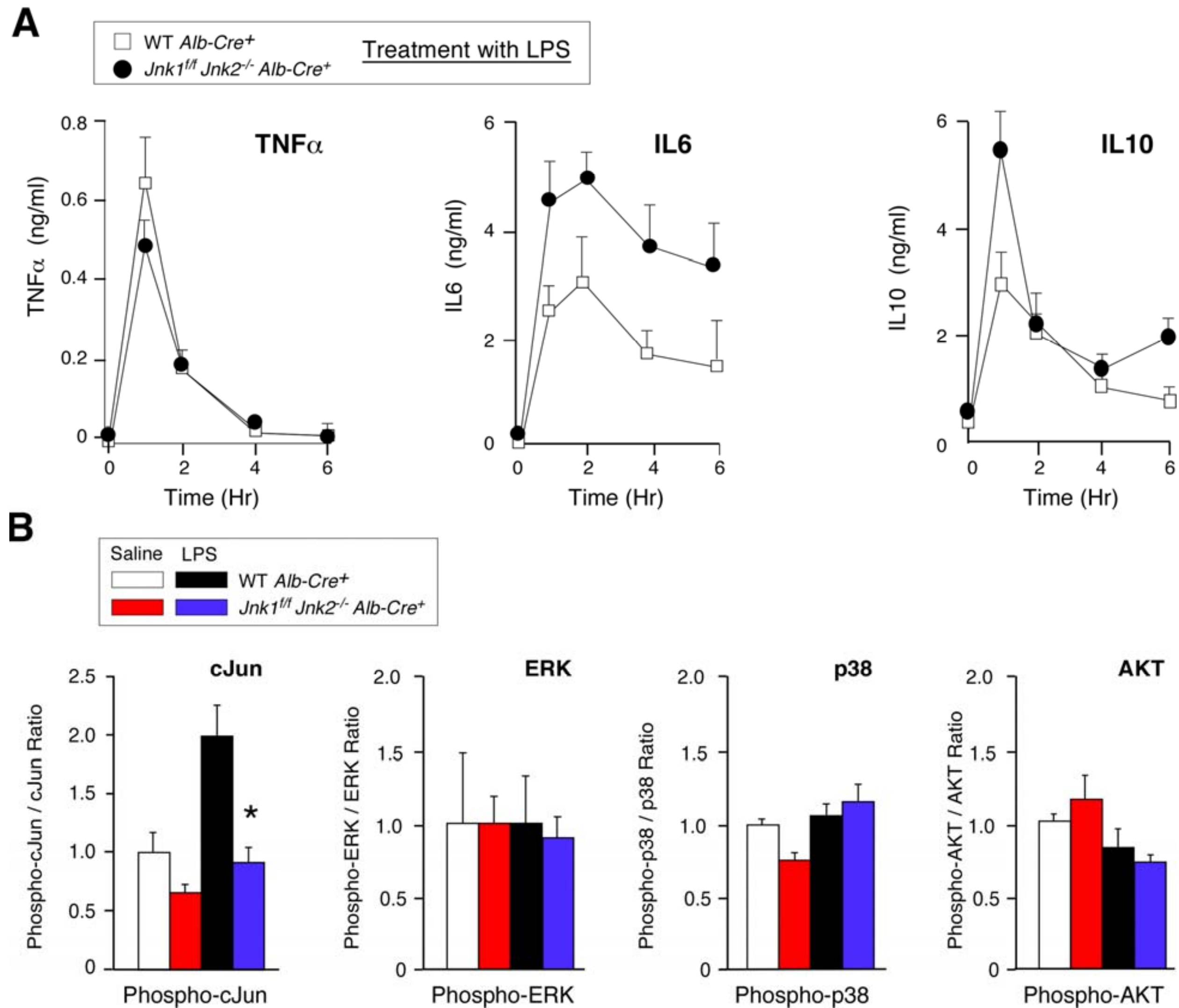

Figure S8. Effect of hepatocyte-specific JNK-deficiency on serum cytokine expression. (**A**) Control mice (*Alb-Cre*$^{+}$) and mice with hepatocyte-specific JNK-deficiency (*Jnk1*$^{f/f}$ *Jnk2*$^{-/-}$ *Alb-Cre*$^{+}$) were treated intravenously with LPS plus GalN. The amount of serum cytokines (TNFα, IL6, and IL10) post-treatment with LPS plus GalN was measured by ELISA (mean ± SD; n = 7). (**B**) The amount of total and phospho- JNK1/2, cJun, ERK1/2, p38 MAPK, and AKT in liver extracts at 8 hrs post-treatment of mice with LPS plus GalN or saline was measured by ELISA (mean ± SD; n = 5).

Statistically significant differences between wild-type and JNK1/2-deficient mice are indicated (*, $P < 0.05$).

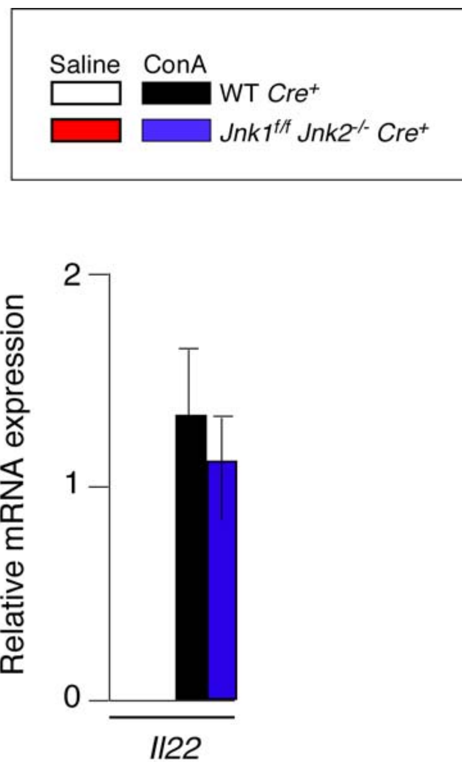

Figure S9. Effect of JNK-deficiency on the expression of IL22. Control mice (*Mx1-Cre*$^{+}$) and JNK-deficient (*Jnk1*$^{f/f}$ *Jnk2*$^{-/-}$ *Mx1-Cre*$^{+}$) were treated without and with ConA. The expression of *Il22* mRNA in the liver at 1 hr post-injection was examined by quantitative RT-PCR (Taqman©) assays. The mRNA expression in each sample was normalized to the amount of *Gapdh* mRNA and presented as the mean ± SD (n = 6). No statistically significant differences between wild-type and JNK-deficient mice were detected ($P > 0.05$).

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