Supplemental Data

Fatal Hepatitis Mediated by Tumor Necrosis Factor TNFα Requires Caspase-8 and Involves the BH3-Only Proteins Bid and Bim

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T Kaufmann Fig. S1

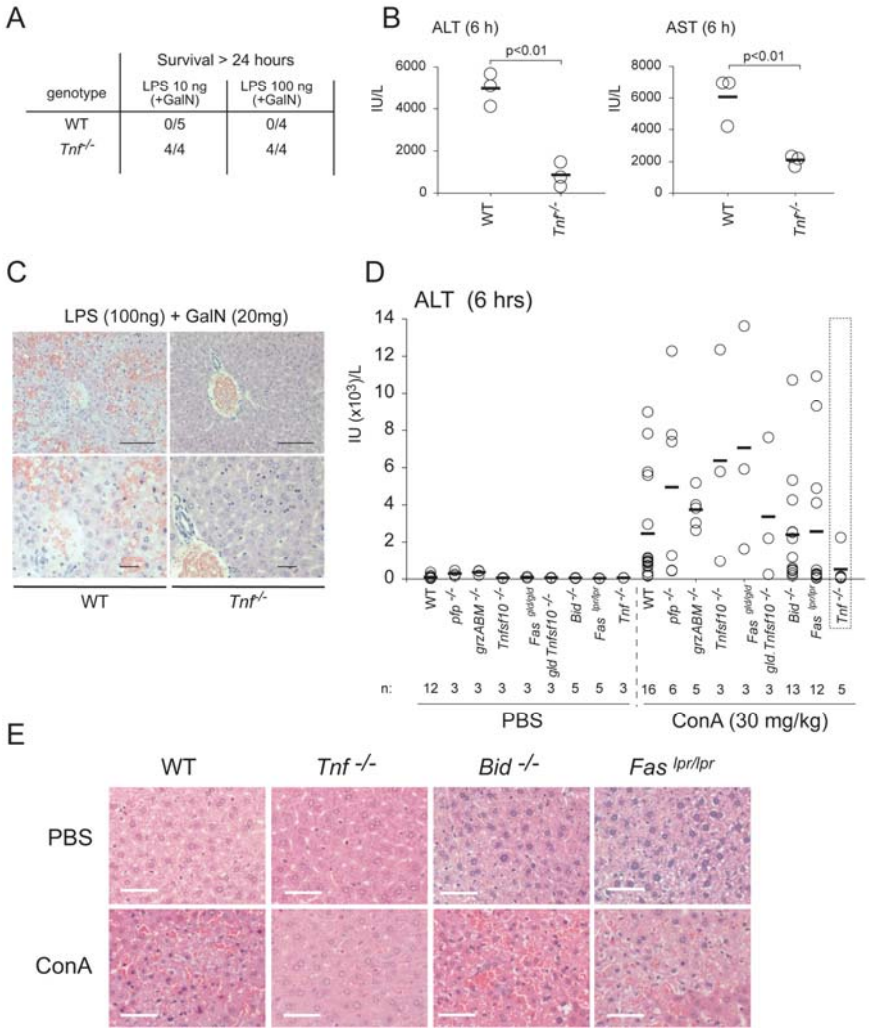

Figure S1. TNFα Is Required for LPS plus GalN- as well as for ConA-Induced Hepatocyte Destruction

(A) Table summarizing the long-term (followed up to 3 days) survival of WT or *Tnfα*$^{-/-}$ mice treated with 10 or 100 ng LPS plus 20 mg GalN. Mice that did not survive this treatment all succumbed within 24 h. After 72 h, all *Tnfα*$^{-/-}$ mice were alive and well. (B) In an independent experiment, serum levels of ALT and AST were measured after 6 h of treatment with 100 ng LPS+GalN. Data from 3 mice of each genotype are shown. Horizontal bars indicate the mean. Elevated readings of AST in 2 samples from *Tnfα*$^{-/-}$ mice are due to hemolysis (Hb above 62 mg/L). (C) Histological examination of H&E stained liver sections of *Tnfα*$^{-/-}$ and WT mice that had been treated with 100 ng LPS+GalN (low magnification: scale bars = 100 μm; high magnification: scale bar = 10 μm). Pictures shown are representative of the analysis of 3 mice for each genotype sacrificed after 6 h of treatment. (D) Mice of the indicated genotypes (*gld*: spontaneously arisen mutation in *FasL* gene; *lpr*: spontaneously arisen mutation in *Fas* gene) were injected i.v. with either PBS or a lethal dose (30 mg/kg body weight) of ConA. The mice were sacrificed after 6 h, bled and their sera analyzed for the liver enzyme ALT. Note that AST-levels could not reliably be measured in this experiment due to extensive hemolysis caused by ConA. (E) Histological examination of H&E stained liver sections of mice of the indicated genotypes, treated with PBS or ConA as described in (D). Scale bar = 50 μm. Pictures shown are representative of the analysis of at least 3 mice for each genotype sacrificed after 6 h of treatment. Data from the indicated numbers (n) of mice of the indicated genotypes are shown, Horizontal bars indicate the mean.

T Kaufmann Fig. S2

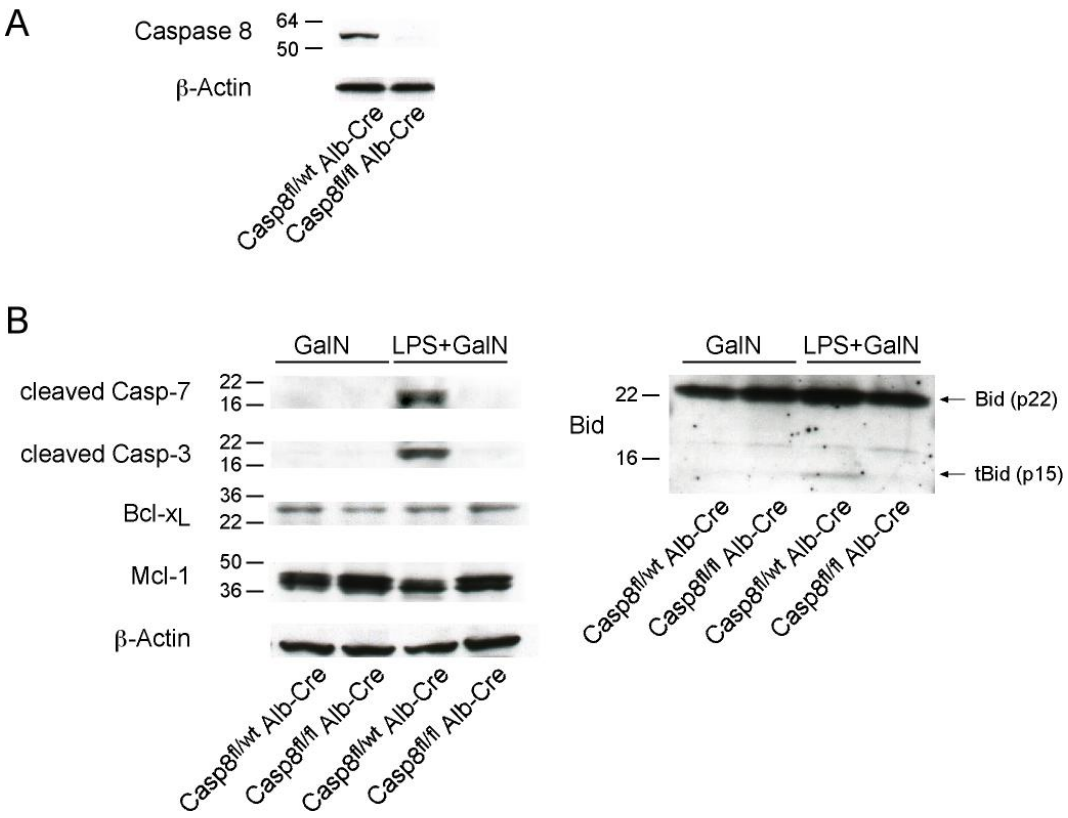

Figure S2. Absence of tBid Formation and Effector Caspase Activation in Livers of LPS plus GalN Treated Mice Lacking Caspase-8 in Hepatocytes.

(A) Western blot analysis of liver lysates from mice lacking caspase-8 in hepatocytes (*Albumin-Cre* transgenic *caspase-8*$^{fl/fl}$) and littermate controls (*Albumin-Cre* transgenic *caspase-8*$^{fl/wt}$) using antibodies to caspase-8 or β-actin (loading control). (B) Mice lacking caspase-8 in hepatocytes and littermate controls were injected with 20 mg GalN alone or with 100 ng LPS + 20 mg GalN. Mice were sacrificed after 6 h, livers excised and total protein lysates prepared. Equal amounts of protein extract were analyzed by immunoblotting using antibodies specific for effector caspase-3 and caspase-7, Bid, Bcl-x_L, Mcl-1 and β-actin (loading control).

T Kaufmann Fig. S3

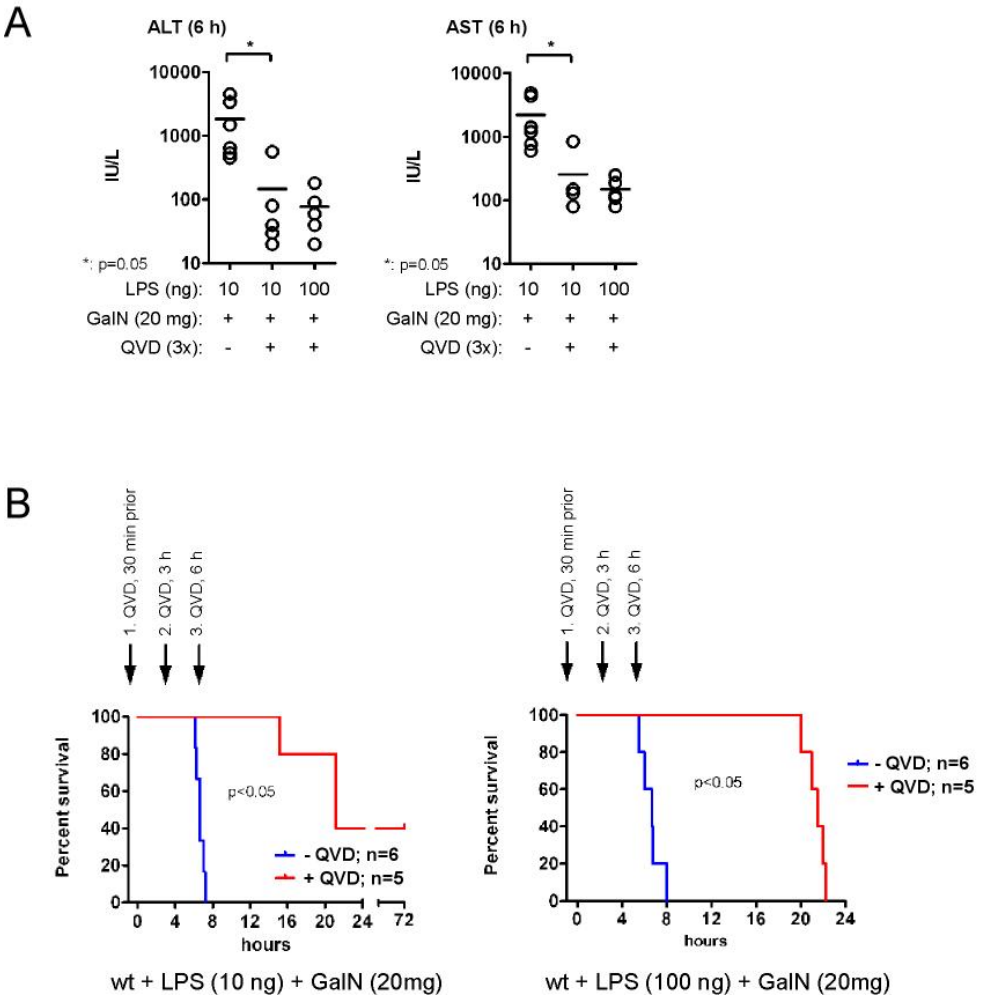

Figure S3. The Pan-Caspase-Inhibitor Q-VD-Oph Increases the Survival of Wild-type Mice Injected with LPS plus GalN

(A) WT mice were injected with LPS (10 or 100 ng) plus GalN (20 mg), with or without pre-treatment with the pan-caspase inhibitor Q-VD-oph (3x 20 mg/kg). Serum levels of the liver-specific transaminases ALT and AST were measured after 6 h. Q-VD-oph was administered 30 min prior to, and 3 and 6 h after LPS+GalN injection. N = 6 (WT + 10 ng LPS/GalN), 5 (WT + 10 ng LPS/GalN + QVD-oph), 5 (WT + 100 ng LPS/GalN + Q-VD-oph). Each circle represents measurements from an individual mouse. Horizontal bars indicate the mean. (B) Survival of WT mice injected with 10 or 100 ng LPS plus

GalN, with or without treatment with multiple injections of the pan-caspase inhibitor Q-VD-oph (3 x 20 mg/kg at the indicated time points).

T Kaufmann Fig. S4

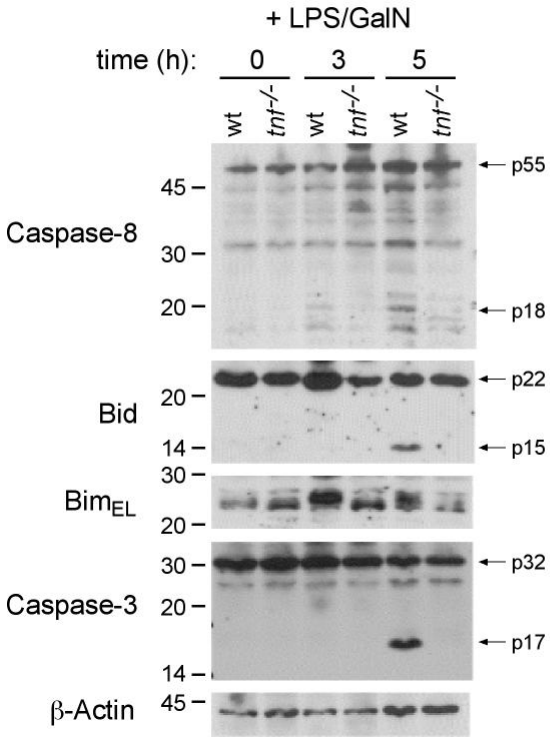

Figure S4. **TNFα Is Required for LPS plus GalN-Induced Processing of Caspase-8, Bid and Effector Caspases as well as for the Post-Translational Modification of Bim in Hepatocytes**

Mice (WT or *Tnfα*$^{-/-}$) were injected with 100 ng LPS plus 20 mg GalN and sacrificed after 6 h. Lysates from livers were analyzed by immunoblotting for processing of Bid, caspase-3 and caspase-8 and for post-translational modification of Bim. Probing with an antibody to β-actin was used as a loading control.

T Kaufmann Fig. S5

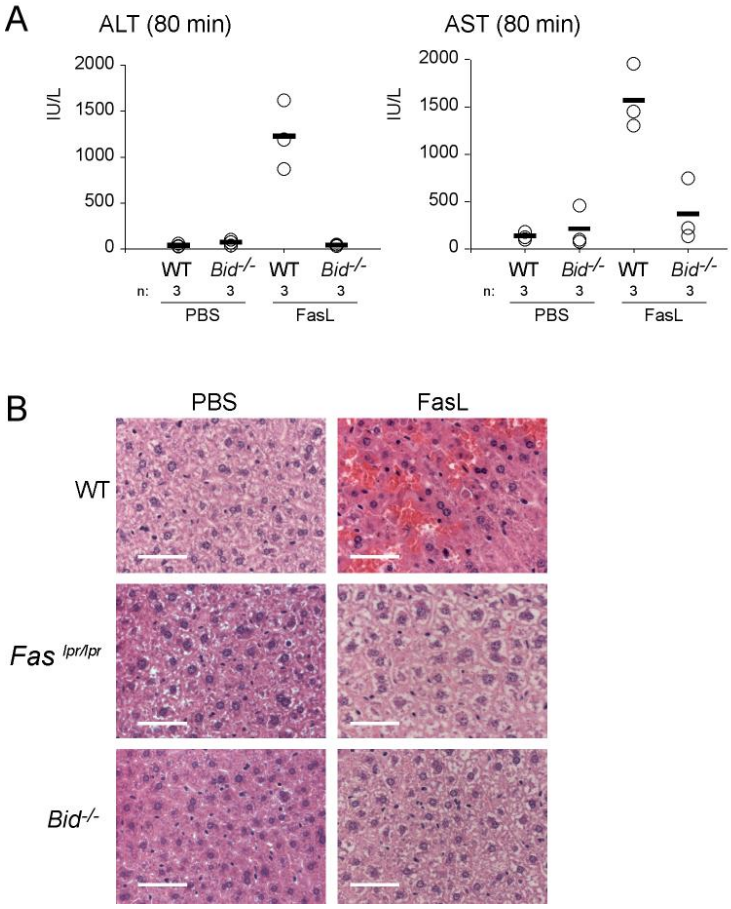

Figure S5. Bid-Deficient Mice Are Resistant to Fas Ligand (FasL)-Induced Hepatitis

(A) Bid-deficient mice and WT controls were injected i.v. with either PBS or a lethal dose (0.25 mg/kg body weight) of cross-linked recombinant FasL (soluble human FasL with a FLAG epitope plus anti-FLAG antibody for crosslinking). Mice were sacrificed after 80 min and their sera analyzed for the liver enzymes ALT and AST (*Bid-/-* vs WT: p=0.006 for ALT, p=0.012 for AST). Data from 3 mice of each genotype are shown. Horizontal bars indicate the mean. (B) Histological examination of liver sections from WT, *lpr* (Fas deficient) or *Bid-/-* mice injected 80 min earlier with FasL or carrier (PBS), respectively (bars = 50 μm). Pictures shown are representative of the analysis of at least 3 mice for each treatment and genotype.

T Kaufmann Fig. S6

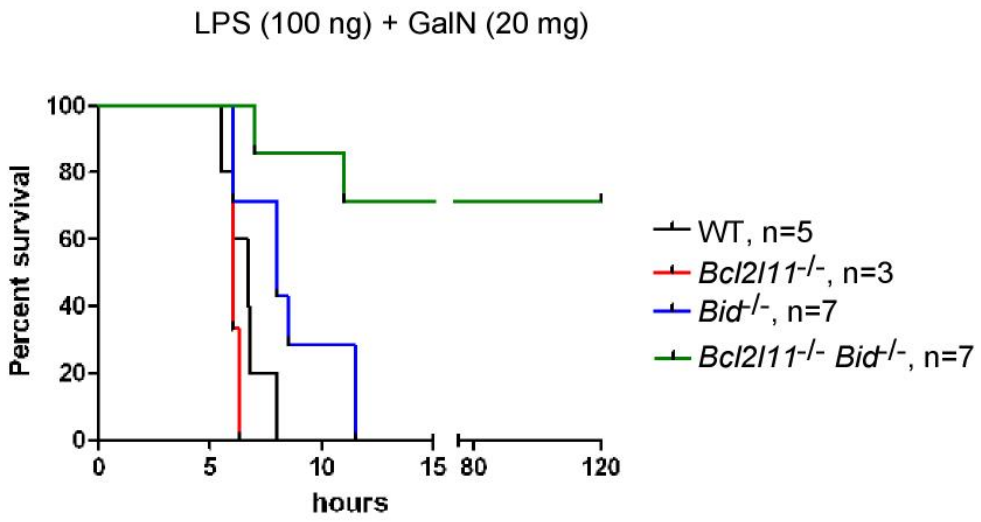

Figure S6. Survival Curve of LPS plus GalN Treated WT, *Bid*$^{-/-}$, *Bcl2l11*$^{-/-}$ and *Bcl2l11*$^{-/-}$*Bid*$^{-/-}$ Mice

Mice of the indicated genotypes were injected i.p. with 100 ng LPS plus 20 mg GalN and followed over 120 h post injection. P values: [*Bid*$^{-/-}$ vs *Bcl2l11*$^{-/-}$*Bid*$^{-/-}$] = 0.0199; [*Bid*$^{-/-}$ vs WT] = 0.1035; [WT vs *Bcl2l11*$^{-/-}$*Bid*$^{-/-}$] = 0.0017; [*Bcl2l11*$^{-/-}$ vs *Bcl2l11*$^{-/-}$*Bid*$^{-/-}$] = 0.0016. P values were calculated using a time to event analysis with a log rank test.

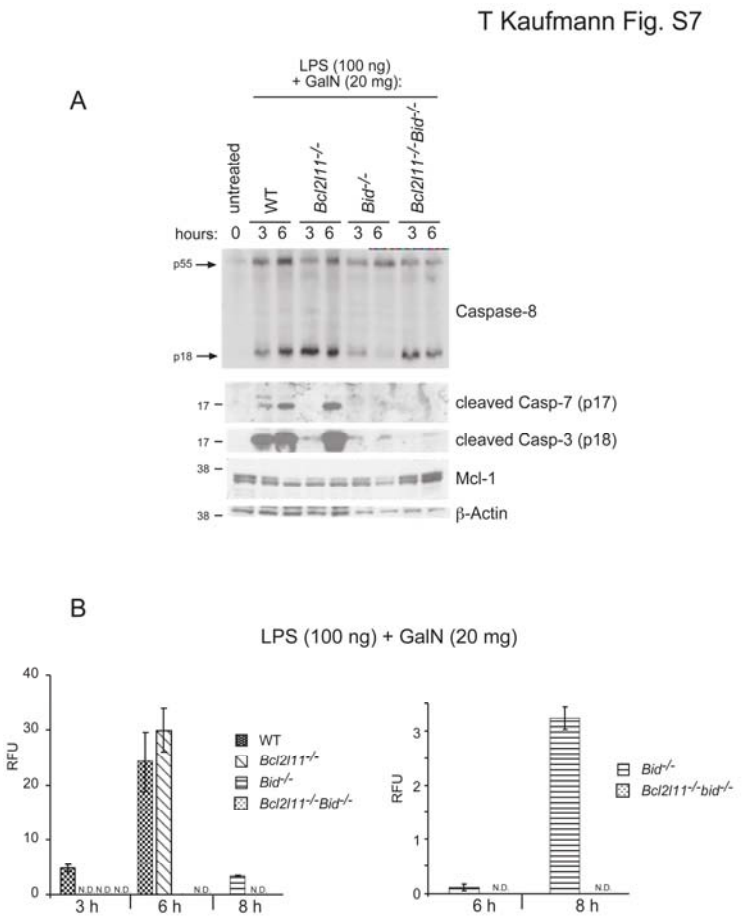

Figure S7. Lack of Both Bid and Bim Causes Profound Reduction in Effector Caspase Activation in Livers after Treatment with LPS plus GalN.

(A) Mice of the indicated genotypes were injected i.p. with 100 ng LPS plus 20 mg GalN and sacrificed after 3 or 6 h, respectively. Liver extracts were prepared and probed by immunoblotting for the active large subunits of effector caspases-3 and -7. Blots were also probed for Mcl-1 levels, the most highly expressed anti-apoptotic Bcl-2 family member found in mouse liver. Note that the levels of Mcl-1 drop, presumably as a consequence of transcriptional blockade by GalN and the relatively short half-life of Mcl-1. This drop occurs relatively late after LPS+GalN injection and we therefore believe that this drop is not a cause but rather a consequence of hepatocyte death. (B) Effector caspase activity (DEVD-AMC cleavage activity) was measured in extracts from livers of mice of the indicated genotypes that had been treated with 100 ng LPS plus 20 mg GalN for the indicated time points. N.D. indicates no detectable caspase activity. Data are derived

from four independent measurements and expressed as relative rates of cleavage of a fluorogenic caspase cleavage product (DEVD-AMC). No detectable signal was obtained from liver extracts of untreated mice. No measurements could be taken from WT and *Bcl2l11*$^{-/-}$ mice after 8 h of LPS+GalN treatment because they had all succumbed earlier. Error bars indicate mean ± SD of three individual mice.

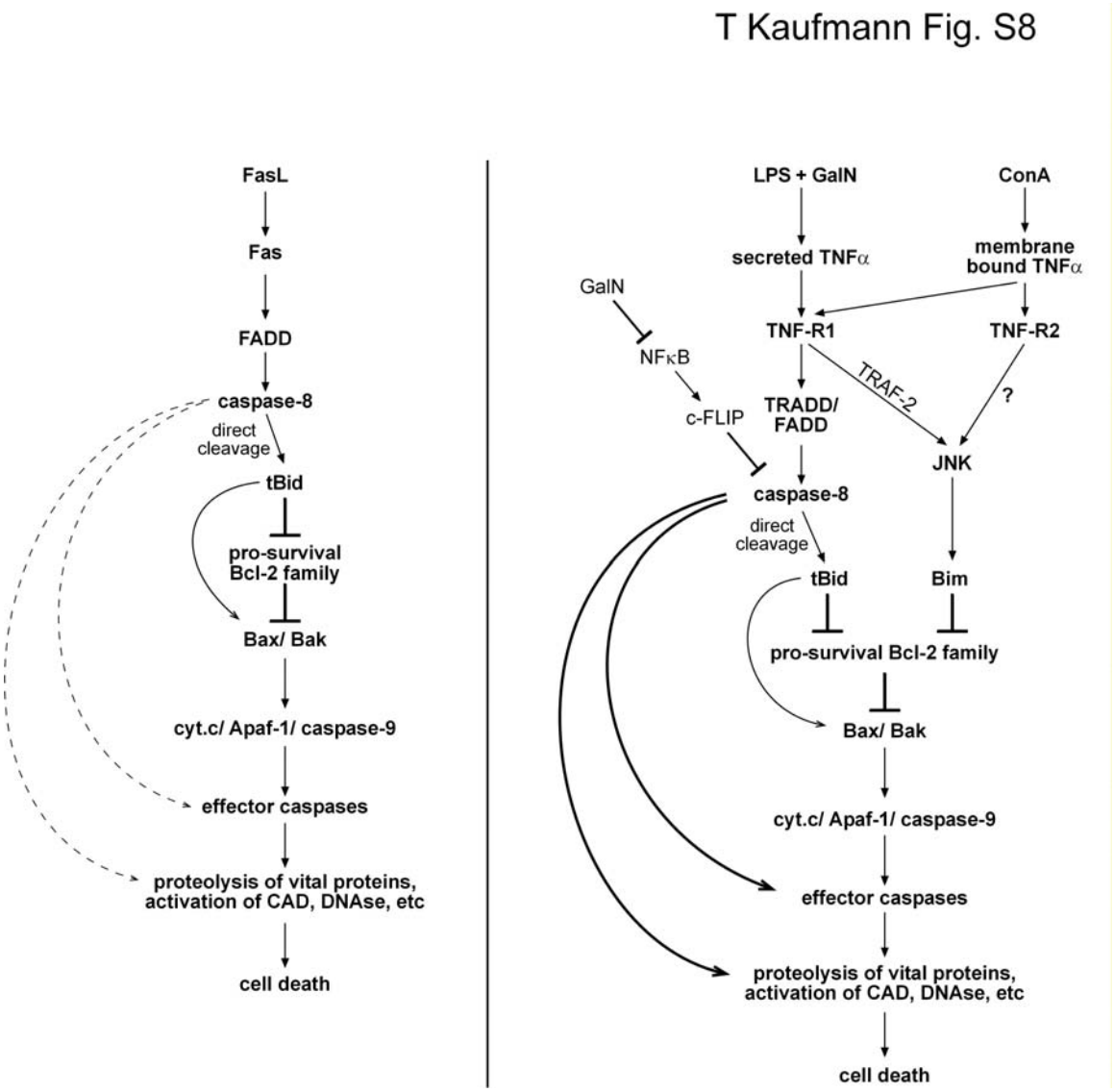

Figure S8. Speculative Models of Hepatocyte Apoptosis Induced by Treatment with FasL, LPS plus GalN or ConA

T Kaufmann Fig. S9

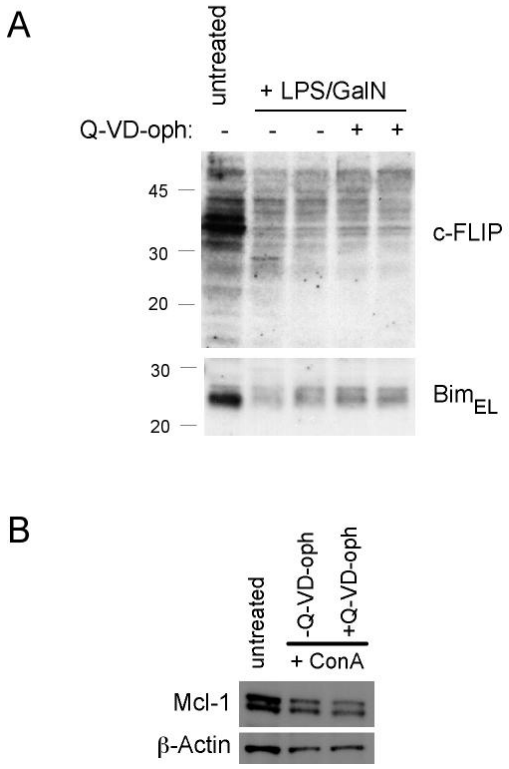

Figure S9.

(A) WT mice were injected with 100 ng LPS plus 20 mg GalN, with or without pre-treatment with the pan-caspase inhibitor Q-VD-oph (20 mg/kg, i.p. 30 min prior to LPS+GalN injection) and total protein lysates prepared from the livers after 4 h. Liver extract from an untreated mouse served as a control. Lysates were probed by immunoblotting with antibodies to FLIP or β-actin (loading control).) (B) WT mice, with or without pre-treatment with the pan-caspase inhibitor Q-VD-oph (20 mg/kg i.p. 30 min earlier), were injected with 30 mg/kg ConA and sacrificed after 5 h. Liver extracts were prepared and probed for Mcl-1 levels by immunoblotting. Liver extract from an untreated mouse served as a control.