P. Jost et al, Nature Supplementary Text

Supplementary Materials and Methods

Mice

The generation and genotyping of *Bid*$^{-/-}$ mice[1], generated on an inbred C57BL/6 background using C57BL/6-derived ES cells and *Xiap*$^{-/-}$ mice[2], generated on a mixed C57BL/6x129SV background using 129SV-derived ES cells and back-crossed onto C57BL/6 for >10 generations, have been described. The *Xiap* gene is located on the X-chromosome, however, both female (*Xiap*$^{-/-}$) and male (*Xiap*$^{y/-}$) mice used for experiments are denoted as *Xiap*$^{-/-}$ in the text and figures. Inter-crossing *Bid*$^{-/-}$ with *Xiap*$^{-/-}$ mice generated BID/XIAP double-deficient mice. All experiments with mice were performed according to the guidelines of the animal ethics committees of the Melbourne Health Research Directorate.

Anti-FAS-antibody induced hepatitis

Mice were injected intravenously (*i.v.*) with 0.25 mg/kg body weight of the agonistic hamster monoclonal anti-mouse FAS antibody (clone Jo2, no azide + low endotoxin formulation, BD Pharmingen).

FASL induced apoptosis assay of thymocytes *ex vivo*

Single cell suspensions of thymocytes were prepared from freshly excised thymi and cultured in DMEM medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/l glutamine, 10% fetal calf serum (Bovogen) at 37°C in 5% CO_2. Thymocytes were treated with 100 ng/mL recombinant soluble FASL (FLAG® tagged FASL, Alexis) that had been crosslinked with 2 μg/mL anti-FLAG® antibody (M2, SIGMA).

Lipopolysaccharide plus galactosamine (LPS+GalN) induced hepatitis

Mice were injected intra-peritoneally (*i.p.*) with 100 ng LPS (DIFCO) in the presence of 20 mg of the liver-specific transcriptional inhibitor D-(+)-galactosamine (GalN, SIGMA) per 20 g body weight of mouse.

Blockade of TNFα induced fatal hepatitis by TNFα neutralising antibody *in vivo*

Mice were injected intra-peritoneally (*i.p.*) with 250 μg TNFα neutralising antibody XT-22 or an Ig isotype-matched control mAb, GL113, per 20 g body weight of mouse 30 min prior to injection of LPS+GalN or FASL.

Immunoblotting

Liver lysates were prepared in ONYX buffer containing 20 mM Tris/HCl pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 500 μg/mL Pefabloc (AEBSF), 1 μg/mL each of Leupeptin, Aprotinin and Pepstatin, 100 μg/mL soybean trypsin inhibitor, 2 μg/mL E64 or alternatively in RIPA buffer containing 150 mM NaCl, 1% Triton X-100, 0.5 % Deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl (pH=8.0) supplemented with protease inhibitors as indicated above. Proteins in liver lysates were size-separated on pre-cast 12% or 4-20% SDS-PAGE gradient gels (Invitrogen). Membranes were probed with the following monoclonal antibodies: rat anti-BID (clone 2D1[1]), rat anti-caspase-8 (clone: 1G12, Alexis[3]), mouse anti-XIAP (clone 2F1, Abcam), rat anti-MCL-1 (clone: 19C4-15), mouse anti-β-actin (used as a loading control; clone: AC40; SIGMA), hamster anti-mouse BCL-2 (clone: 3F11), mouse anti-HSP70 (N6, gift from R. Anderson, Peter MacCallum Institute, Melbourne and W. Welch, UCSF, San Francisco) or polyclonal rabbit antibodies specific for caspase-3 (Cell Signaling), active caspase-7 (p18, Cell Signaling), caspase-9 (gift from Y. Lazebnik, Cold Spring Harbor Labs, Cold Spring Harbor, NY, US), BAK (NT, Millipore), BAX (NT, Millipore), SMAC/DIABLO (clone 7, BD Transduction Laboratories) or anti-ICAD (gift from S. Nagata, Kyoto University, Kyoto, Japan). Optical densitometry was performed on a GS-800 Calibrated Densitometer (Bio-Rad) using Quantity One 4.6.1 software (Bio-Rad).

Pull-down of active caspases and co-immunoprecipitation plus immunoblot analysis

Active caspases from liver lysates were irreversibly bound to the pan-caspase inhibitor biotin-X-VAD-fmk (Calbiochem) added at a final concentration of 2.5 μM for 30 min at

37ºC. Alternatively, in liver lysates of mice the binding of active caspase-3 to XIAP was tested by co-immunoprecipitation with an antibody to XIAP (clone 2F1, 2 μg/mL) and immunoblotting with antibodies to active caspase-3. Streptavidin-Sepharose or Protein G-Sepharose (GE Healthcare) was added as a 50% slurry in ONYX buffer and the mixture incubated for at least 2 h on a rotating wheel at 4ºC. The Sepharose beads were pelleted by centrifugation and washed 3 times in ONYX buffer prior to boiling for 5 min in reducing SDS-PAGE sample buffer. Proteins in boiled samples were separated on an SDS-PAGE gel and probed for various caspases by immunoblotting using the caspase-specific antibodies listed above.

Fluorogenic caspase activity assay

Cell lysates were prepared in RIPA buffer. Cleavage assays were set up with 8 μL of lysate plus 50 μM DEVD-AMC (Bachem, Switzerland) in a total volume of 100 μL caspase cleavage assay buffer (0.1 M HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT) and fluorescent emission measured at 37°C in a Spectra Max2-Molecular Devices Fluorimeter at 37°C for 30 min with individual readings made every 20 sec (excitation filter 360 nm; emission filter 465 nm). Data are expressed as relative rates of DEVD-AMC cleavage from four independent experiments.

Histology and TUNEL staining

Livers of treated mice were excised at indicated time points and fixed in 10% buffered formalin solution prior to preparation of histological sections and staining with hematoxylin and eosin. Tissue sections for TUNEL analysis were de-paraffinized prior to proteinase K treatment (20 μg/mL). Endogenous peroxidases were blocked by incubating the sections in 3% hydrogen peroxide in methanol for 5 min at room temperature. Sections were washed once with PBS and once with distilled water and subsequently incubated in TdT buffer for 10 min at room temperature, followed by washing the sections twice with PBS. Tissue sections were then incubated in 0.6 U/μL terminal deoxynucleotidyl transferase (Promega), 20 μM biotin-16-dUTP (Roche), 1 mM CoCl2 (Sigma) in terminal transferase buffer (Promega) for 1 h at 37ºC. After three washes,

sections were blocked with 2% FCS (Bovogen) in PBS for 10 min at room temperature. Blocking solution was removed and Vectorstain ABC (Vector Laboratories) reagents applied according to the manufacturer's instructions. Sections were then washed 3 times with PBS and once with distilled water followed by DAB staining according to manufacturer's instructions. Sections were finally washed twice with distilled water and counterstained with hematoxylin.

TNFα detection by Enzyme-Linked Immunosorbent assay (ELISA)

Measurement of TNFα levels was performed in a 96-well round-bottom plate coated overnight with 100 μl of TNFα capture antibody solution at 0.8μg/mL in PBS followed by three washes with 0.05% PBS/Tween® 20. The wells were blocked with 1% BSA/PBS for 1h at room temperature and three washes with PBS/Tween® 20. Samples (100 μL) or standard (in 1% BSA/PBS) were added per well. Plates were incubated for 2 h at room temperature, followed by three washes with PBS/Tween® 20. Detection antibodies (100 μL biotinylated goat anti-mouse TNFα antibodies at 200 ng/mL in 1% BSA/PBS) were added to each well and plates incubated for 2 h at room temperature. After three washes with PBS/Tween® 20, 100 μl Streptavidin-HRP solution (diluted 1:200 in 1% BSA/PBS) was added and the plates incubated for 20 min at room temperature. After three washes with PBS/Tween® 20, substrate solution and 20 min later stop solution were added according to the manufacturer's instructions (R&D Systems, #DY999 and R&D Systems, #DY994). The colorimetric reaction was measured at 450 nm on a Multiscan Ascent ELISA plate reader (Thermo Labsystems).

Foetal thymic organ culture

Thymic lobes were dissected from WT or *Bid*$^{-/-}$ embryos at day 15 of gestation (day of vaginal plug formation designated as day 0) and cultured as described previously[4] in RPMI medium supplemented with 15 mM HEPES and 10% (v/v) FCS at 37°C in 5% CO_2. Following 5 days of culture, lobes were transferred into "hanging drop" cultures in Terasaki plates (NUNC, USA) with medium containing 1 μg/mL FASL cross-linked with

20 μg/mL anti-FLAG® antibody and cultured for a further 4, 8 or 16 h. Single-cell suspensions of pools of 10-20 lobes were counted and sampled for preparation of cell lysates for immunoblotting and flow cytometric analysis of cell survival, using staining with propidium iodide plus antibodies to CD4 and CD8.

Pancreatic islet isolation and cell death assay

Pancreatic islets were isolated from mice using collagenase P (Roche) and histopaque-1077 density gradients (Sigma-Aldrich) as previously described[5]. Islets were washed, handpicked and cultured overnight at 37^0C and 5% CO_2 in CMRL medium-1066 (Gibco products; Invitrogen) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine, 10% fetal calf serum (JRH Biosciences). The day after isolation, 100 uniformly sized islets (excluding very large or necrotic islets) per sample were handpicked into 3.5 cm Petri dishes containing 1.1 mL complete CMRL medium and cultured for 6 days with and without 100 ng/mL FLAG-tagged FASL crosslinked with anti-FLAG antibody (2 μg/mL) and cytokines (1 U/mL recombinant murine IFNγ and 150 U/mL recombinant murine IL-1β (R&D systems). At the end of the culture period, non-attached cells and islets were transferred into polypropylene tubes and washed in PBS and dispersed (0.1 mg/mL bovine trypsin (Calbiochem) and 2 mmol/L EDTA in PBS) for 5 min at 37^0C. Cells were then washed in PBS and resuspended in 250 μL hypotonic buffer containing 50 mg/mL propidium iodide (Sigma-Aldrich), 0.1% sodium citrate, and 0.1% TritonX-100, which stains nuclear DNA. Cells were then analysed on a FACSCalibur (Becton Dickinson). Apoptotic cells were identified by their sub-diploid (<2C) DNA content as previously described[6].

***In vitro* cell culture**

Mouse fibroblastoid L929 cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine and used at 80% confluency for cell death measurement after incubation with soluble, recombinant murine MEGA-TNFα ™ (Alexis Biochemicals, Switzerland) at a

concentration of 100 ng/mL. The TNFα neutralising antibody XT-22 and an Ig isotype-control mAb, GL113, were used at a concentration of 1 μg/mL and added to L929 cells 30 min before addition of MEGA-TNFα™. Cell death was measured by FACS analysis in a FACSCalibur (Becton Dickinson) after staining with propidium iodide.

Supplementary Figure Legends

Supplementary Figure 1. Schematic diagram of FAS-induced type I and type II apoptosis signalling. Abbreviations: FASL: FAS ligand, FADD: FAS-Associated protein with Death Domain, MORT1: mediator of receptor-induced toxicity, MOMP: mitochondrial outer membrane permeabilisation, SMAC: Second mitochondria-derived activator of caspases, DIABLO: direct IAP binding protein with low pI, XIAP: X-chromosome-linked inhibitor of apoptosis protein, Cyt. C: Cytochrome C, APAF1: Apoptotic peptidase activating factor 1.

Supplementary Figure 2. Comparison of caspase activation and proteolysis of caspase substrates between FASL-treated thymocytes and hepatocytes. a, Thymocytes from WT or *Bid*$^{-/-}$ mice were treated in culture with 100 ng/mL FLAG-tagged FASL crosslinked with anti-FLAG antibody (2 μg/mL). Thymocytes were harvested at the indicated time points and total protein extracts prepared and analysed by immunoblotting using antibodies to caspase-8, BID, caspase-9, caspase-3, caspase-7 and β-actin (loading control). The percentages of dead thymocytes in culture (determined by flow cytometry) are indicated below individual lanes. **b**, WT and *Bid*$^{-/-}$ mice were injected *i.v.* with 0.25 mg/kg FLAG-tagged FASL crosslinked with anti-FLAG antibody (2 μg/μg of FASL) and sacrificed at the indicated time points. Livers were excised and total protein lysates prepared and analysed by immunoblotting as described in **a**. The percentages of dead hepatocytes (determined by TUNEL staining of liver sections) are indicated below individual lanes. **c**, E15 foetal thymic lobes from WT or *Bid*$^{-/-}$ embryos were cultured for 5 days before incubation with 1 μg/mL FLAG-tagged FASL crosslinked with anti-FLAG antibody (20 μg/mL) in "hanging-drop" cultures for 4, 8 or

16 h. Single-cell suspensions of pools of 10-20 lobes were counted and sampled for preparation of lysates and immunoblotting using antibodies to BID, caspase-3, caspase-7 and β-actin (loading control). The percentages of dead thymocytes, as determined by propidium iodide staining and flow cytometric analysis, from one experiment representative of three independent experiments are indicated below individual lanes. **d**, Thymocytes from WT or *Bid*$^{-/-}$ animals were treated with 100 ng/mL FLAG-tagged FASL crosslinked with anti-FLAG antibody (2 μg/mL) and total protein extracts prepared and analysed by immunoblotting for the levels of MCL-1, BCL-X_L, BCL-2, BAX, BAK and β-actin (loading control). **e**, WT and *Bid*$^{-/-}$ mice were injected *i.v.* with 0.25 mg/kg FLAG-tagged FASL crosslinked with anti-FLAG antibody (2 μg/μg of FASL) and sacrificed at the indicated time points. Livers were excised and total protein lysates prepared and analysed by immunoblotting as described in **d**.

Supplementary Figure 3: Analysis of the impact of inhibition of caspases or the proteasome on the levels of XIAP in FASL treated thymocytes and hepatocytes. a, Thymocytes from WT mice were treated for the indicated times in culture with 100 ng/mL FLAG-tagged FASL crosslinked with anti-FLAG antibody (2 μg/mL) with or without pre-treatment with the broad spectrum caspase inhibitor QVD-oph (25 μM, added 30 min prior to FASL administration). Cell lysates were prepared and analysed by immunoblotting using antibodies to XIAP, caspase-3 and β-actin (loading control). **b**, Thymocytes from WT mice were treated with FASL as described in **a** with or without pre-treatment with the proteasomal inhibitor MG132 (10 μM, added 30 min prior to FASL administration). Addition of MG132 had no impact on FASL-induced cell death. Cell lysates were prepared and analysed by immunoblotting using antibodies to XIAP, MCL-1 and β-actin (loading control). **c**, WT mice with or without pre-treatment with the broad spectrum caspase inhibitor QVD-oph (20 mg/kg, injected *i.p.*, 30 min prior to FASL administration) were injected *i.v.* with 0.25 mg/kg FLAG-tagged FASL crosslinked with anti-FLAG antibody (2 μg/μg of FASL) and sacrificed at the indicated time points. Livers were excised and total protein lysates prepared and analysed by immunoblotting using antibodies to XIAP, caspase-3 and β-actin (loading control). **d**,

WT and *Bid*$^{-/-}$ mice were treated as described in **c**. Livers were excised and total protein lysates prepared and analysed by immunoblotting using antibodies to XIAP, caspase-3 or β-actin (loading control). In **c** and **d**, lysates from the liver of an untreated *Xiap*$^{-/-}$ mouse served as a control for the specificity of the anti-XIAP antibody.

Supplementary Figure 4: Counting and statistical analysis of TUNEL positive cells in livers of FASL injected mice. a, WT, *Xiap*$^{-/-}$, *Bid*$^{-/-}$ and *Bid*$^{-/-}$*Xiap*$^{-/-}$ mice were injected *i.v.* with 0.25 mg/kg FLAG-tagged FASL crosslinked with anti-FLAG antibody (2 μg/μg of FASL) and sacrificed after 120 min (100 min for *Xiap*$^{-/-}$ mice) to obtain liver sections for H&E and TUNEL staining. Percentages of TUNEL positive cells in livers, with data derived by counting of three viewing fields for each section from three individual mice per genotype and treatment are shown. Error bars indicate +/- SEM. **b**, WT and *Bid*$^{-/-}$*Xiap*$^{-/-}$ mice were injected *i.v.* with FASL as described in **a**, with or without pre-treatment with the broad spectrum caspase inhibitor QVD-oph (injected *i.p.* at 20 mg/kg 30 min prior to FASL administration), and sacrificed after 120 min to obtain liver sections for H&E and TUNEL staining. Percentages of TUNEL positive cells in livers, with data derived by counting of three viewing fields for each section from three individual mice per genotype and treatment are shown. Error bars indicate +/- SEM. **c**, WT and *Bid*$^{-/-}$ mice injected *i.v.* with FASL as described in **a**, with or without pre-treatment with the SMAC/DIABLO mimetic drug BV6 (injected *i.p.* at 10 mg/kg 30 min prior to FASL administration) and with or without pre-treatment with the caspase inhibitor QVD-oph (injected *i.p.* at 20 mg/kg 30 min prior to FASL administration), were sacrificed after 180 min to obtain liver sections for H&E and TUNEL staining. Percentages of TUNEL positive cells in livers, with data derived by counting of three viewing fields for each section from at least two individual mice per genotype and treatment are shown. Error bars indicate +/- SEM. P values are indicated by asterix below individual panels.

Supplementary Figure 5. Loss of BID renders pancreatic β-cells resistant to FASL-induced apoptosis but concomitant loss of XIAP restores sensitivity. Pancreatic β-cells from WT, *Bid*$^{-/-}$, *Xiap*$^{-/-}$ and *Bid*$^{-/-}$*Xiap*$^{-/-}$ mice were cultured for 6 days with and without 100 ng/mL FLAG-tagged FASL crosslinked with anti-FLAG antibody (2 μg/mL)

and IFNγ plus IL-1β (to induce FAS expression in β-cells). Single cell suspensions of islets were prepared by trypsinisation, stained with propidium iodide in hypotonic buffer and the percentages of apoptotic cells (defined as cells containing <2C DNA content) determined by flow cytometric analysis. Data represent means +/- SEM of islet cells from 4-8 mice of each genotype from at least three independent experiments. p (WT vs *Xiap*$^{-/-}$)=0.004; p (WT vs *Bid*$^{-/-}$)<0.0001; p (WT vs *Bid*$^{-/-}$*Xiap*$^{-/-}$)= 0.0064; p (*Bid*$^{-/-}$ vs *Bid*$^{-/-}$*Xiap*$^{-/-}$)<0.0001.

Supplementary Figure 6. The SMAC/DIABLO mimetic drug BV6 enhances FASL-induced hepatocyte apoptosis in WT mice. **a**, Long-term survival of WT or *Xiap*$^{-/-}$ mice injected *i.v.* with 0.25 mg/kg FLAG-tagged FASL crosslinked with anti-FLAG antibody (2 μg/μg of FASL) with or without pre-treatment with the SMAC/DIABLO mimetic drug BV6 (10 mg/kg; injected *i.p.* 30 min prior to FASL administration) or with BV6 alone is shown. P value is indicated below graph. **b**, Serum levels of ALT and AST of mice from **a** were quantified after 45 min and 90 min of the indicated treatment. The horizontal bars indicate the mean and the error bars the SEM (n: numbers of mice analysed). P values are indicated by asterix. **c**, TNFα levels in liver extracts of WT mice prepared after 90 min of treatment as described in **a** were measured by ELISA. Data are standardised to mg of liver protein and represent means +/- SEM[7]. Sera from healthy, unstressed C57BL/6 mice were used as negative control, and as previously published[8], were found to contain undetectable levels of TNFα (n: numbers of mice analysed).

Supplementary Figure 7. TNFα blockade has no impact on FASL plus SMAC/DIABLO mimetic drug induced hepatocyte killing and fatal hepatitis in BID-deficient mice. **a**, Long-term survival of *Bid*$^{-/-}$ mice treated with FASL plus the SMAC/DIABLO mimetic drug BV6 as described in Fig. 4a, with or without pre-treatment with the TNFα-neutralising antibody XT-22 or the Ig isotype matched control mAb GL113 (each injected *i.p.* at 250 μg/mouse 30 min prior to BV6 treatment) is shown. **b**, Serum levels of ALT and AST of *Bid*$^{-/-}$ mice treated as described in **a** were quantified after 180 min of the indicated treatment. The horizontal bars represent the

mean, the error bars show the SEM and n indicates the numbers of mice analysed. p value ALT (FAS+BV6 vs FAS+BV6+XT-22)=0.49; p value AST (FAS+BV6 vs FAS+BV6+XT-22)=0.52. **c**, TNFα levels in liver extracts from *Bid*$^{-/-}$ mice prepared120 min after injection as described in **a** or injected with BV6 alone (10 mg/kg injected *i.p.*) were measured by ELISA. Data are standardised per mg of liver protein and represent the mean +/- SEM. The levels of TNFα detected in livers of healthy, untreated C57BL/6 mice were comparable to those reported in a previous study[7]. Sera from healthy,unstressed C57BL/6 mice were used as negative control and as previously published[8], were found to contain undetectable levels of TNFα (n: numbers of mice analysed).

Supplementary Figure 8. The TNFα-neutralising antibody XT-22 has no impact on FASL plus SMAC/DIABLO mimetic drug BV6 induced hepatocyte killing and fatal hepatitis although it can block TNFα-induced killing of L929 cells *in vitro* and TNFα-mediated fatal hepatitis triggered by injection of LPS plus GalN *in vivo*. a, L929 murine fibroblastoid cells were treated *in vitro* with MEGA-TNFα ™ (100 ng/mL) for the indicated time points with or without pre-treatment with the TNFα neutralising antibody XT-22 (1 μg/mL) or an Ig isotype matched control mAb, GL113 (1 μg/mL; both added 30 minutes prior to addition of MEGA-TNFα^{TM}). Cell death was measured by staining with propidium iodide and flow cytometric analysis and data represent means +/- SEM from three independent experiments. **b**, Long-term survival of *Bid*$^{-/-}$ mice injected *i.p.* with lipopolysaccharide (100 ng/mouse) plus *D*(+)-galastosamine (20 mg/mouse)**,** with or without pre-treatment with the TNFα neutralising antibody XT-22 or the Ig isotype-matched control mAb GL113 (each injected *i.p.* at 250 μg/mouse 30 min prior to administration of LPS+GalN). P value is indicated below graph. **c**, Serum levels of ALT and AST of *Bid*$^{-/-}$ mice treated as described in **b** were quantified after 6 h. The horizontal bars indicate the mean, the error bars indicate +/- SEM and n indicates the numbers of mice analysed. p value ALT (LPS+GalN vs LPS+GalN+XT22)=0.0026; p value AST (LPS+GalN vs LPS+GalN+XT-22)=0.0013; p value ALT (LPS+GalN+XT-22 vs

LPS+GalN+GL113)=0.02; p value AST (LPS+GalN+XT-22 vs LPS+GalN+GL113)=0.13.

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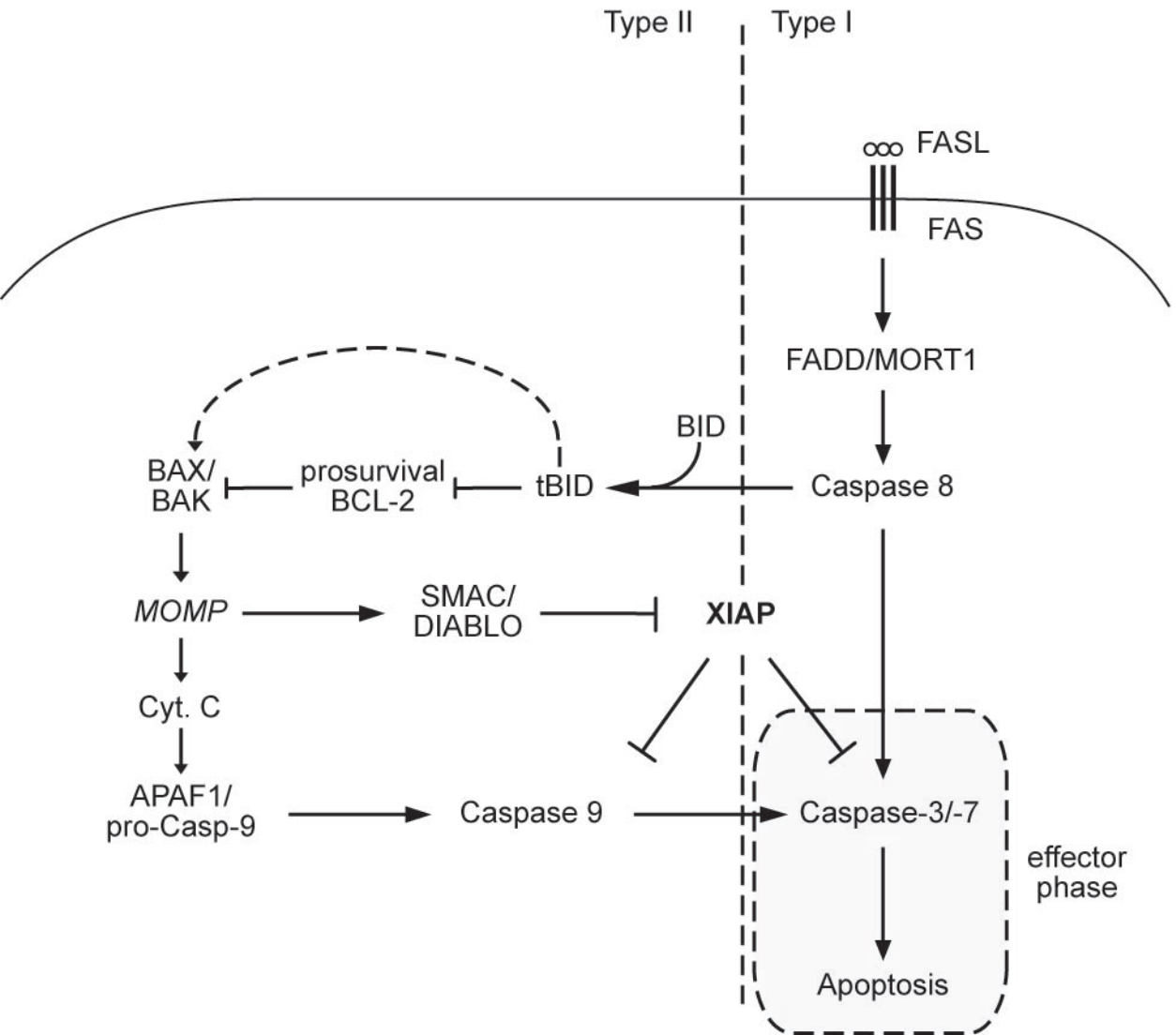
Supplementary Fig. 1



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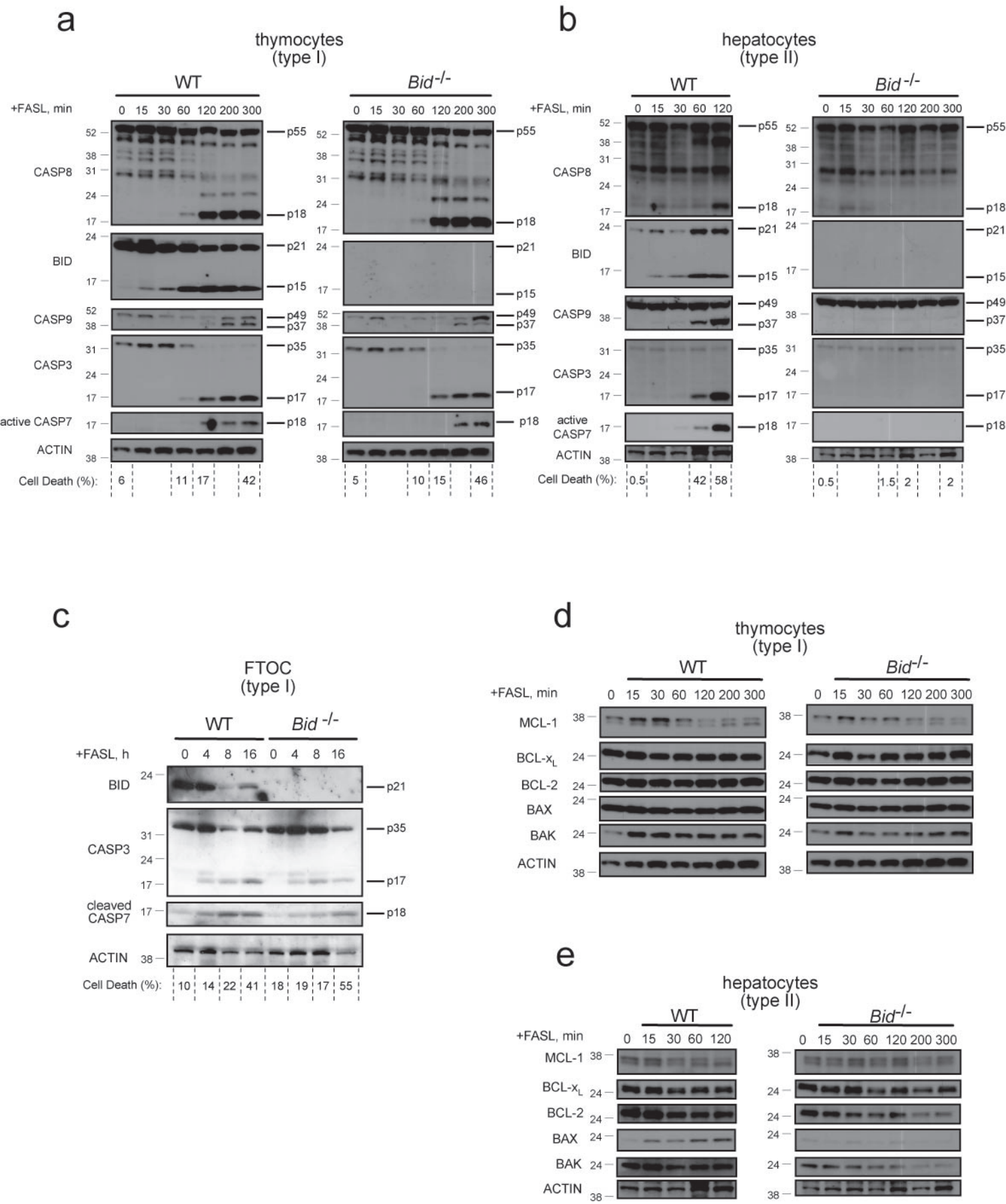

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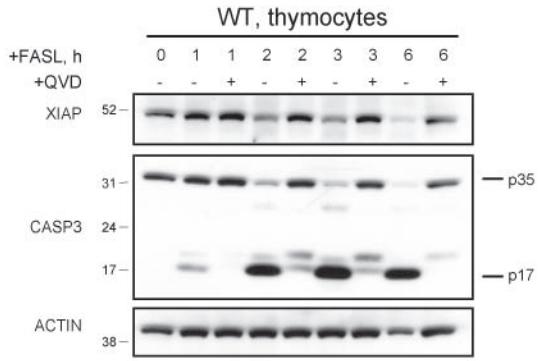
Supplementary Fig. 3

a



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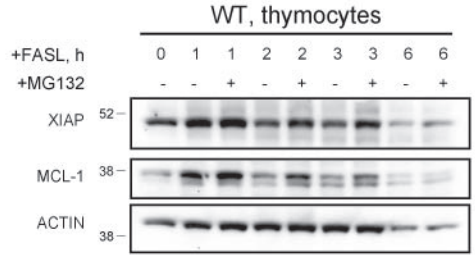

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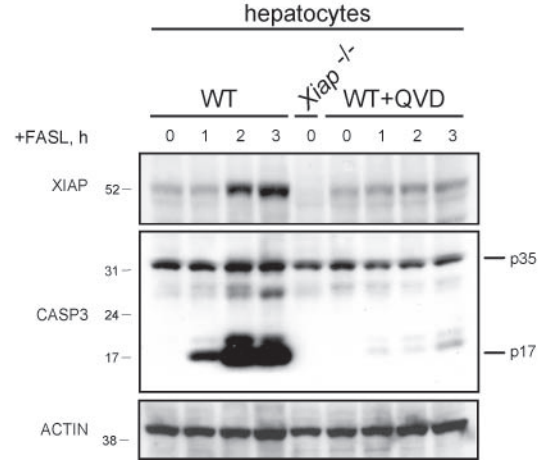

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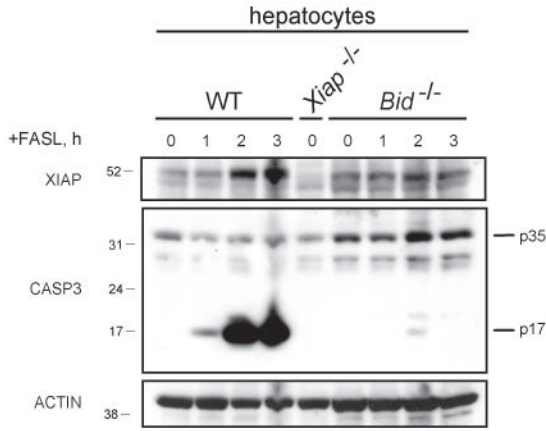

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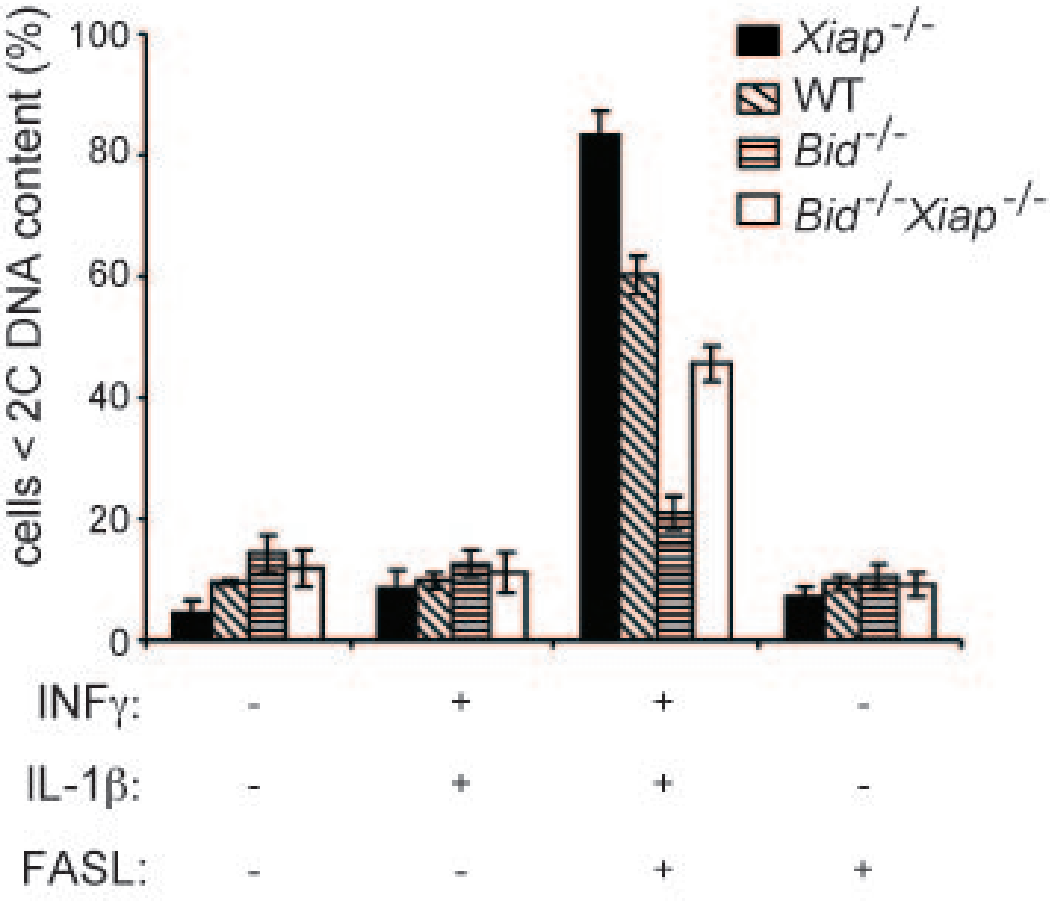
Supplementary Fig. 4



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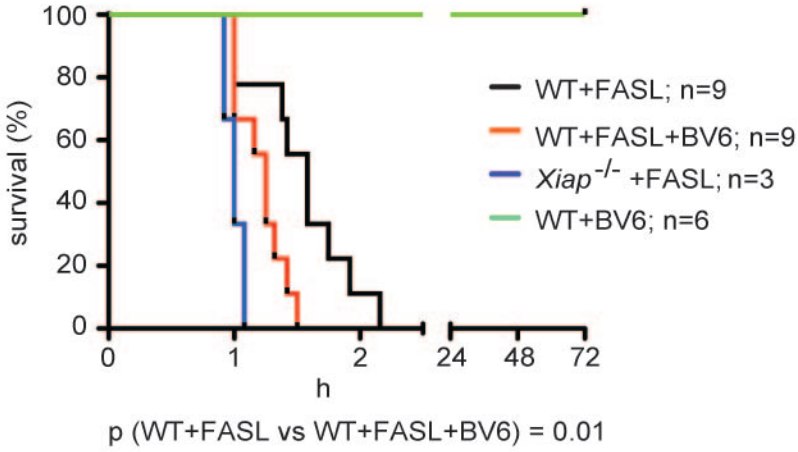
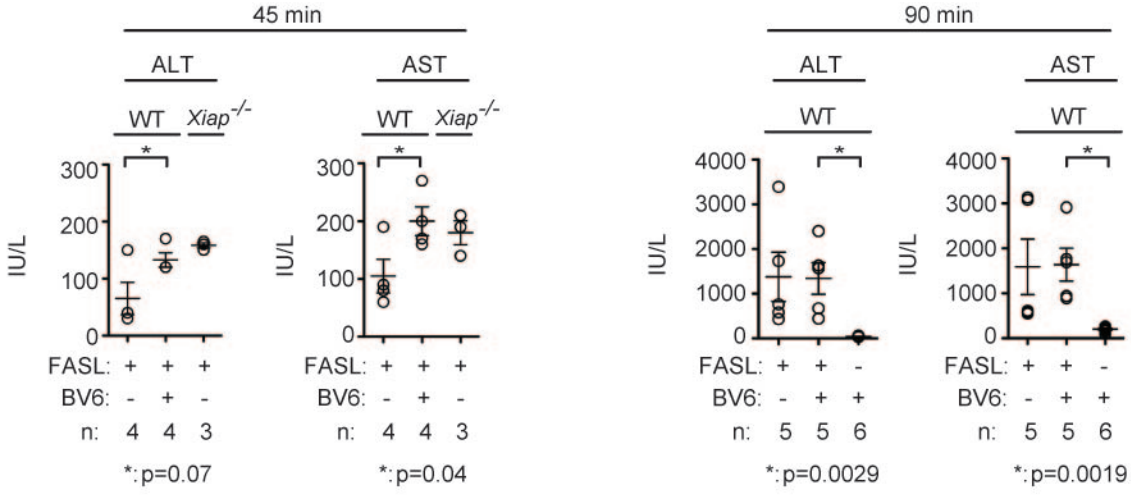
Supplementary Fig. 5

a



p (WT+FASL vs WT+FASL+BV6) = 0.01

b



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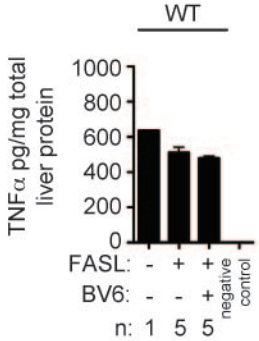

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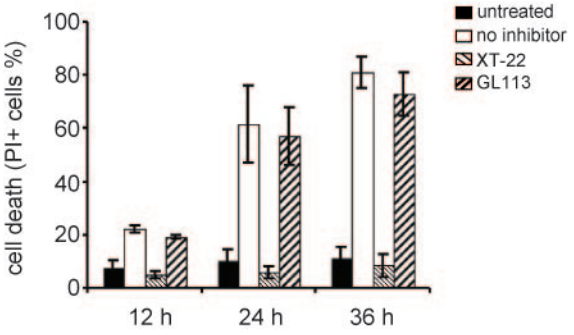
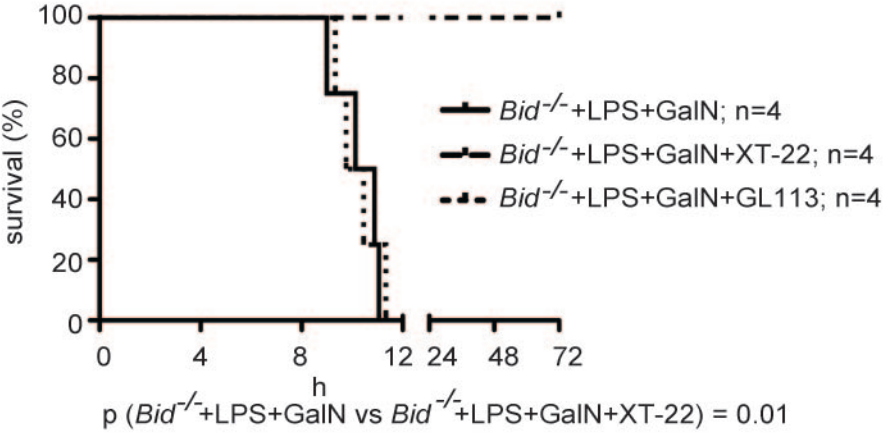
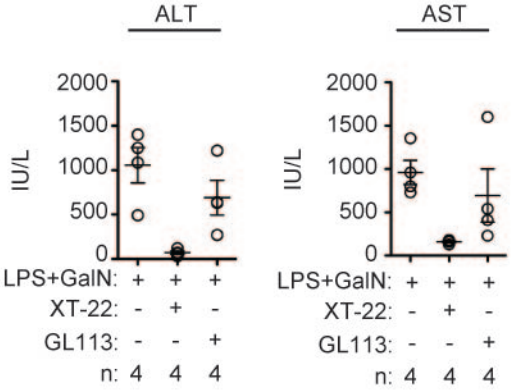

untreated
no inhibitor
XT-22
GL113
cell death (PI+ cells %)
100
80
60
40
20
0
12 h
24 h
36 h

b


survival (%)
100
80
60
40
20
0
0
4
8
12
24
48
72
h
Bid-/-+LPS+GalN; n=4
Bid-/-+LPS+GalN+XT-22; n=4
Bid-/-+LPS+GalN+GL113; n=4
p (Bid-/-+LPS+GalN vs Bid-/-+LPS+GalN+XT-22) = 0.01

c


ALT
AST
IU/L
2000
1500
1000
500
0
LPS+GalN: + + +
XT-22: - + -
GL113: - - +
n: 4 4 4

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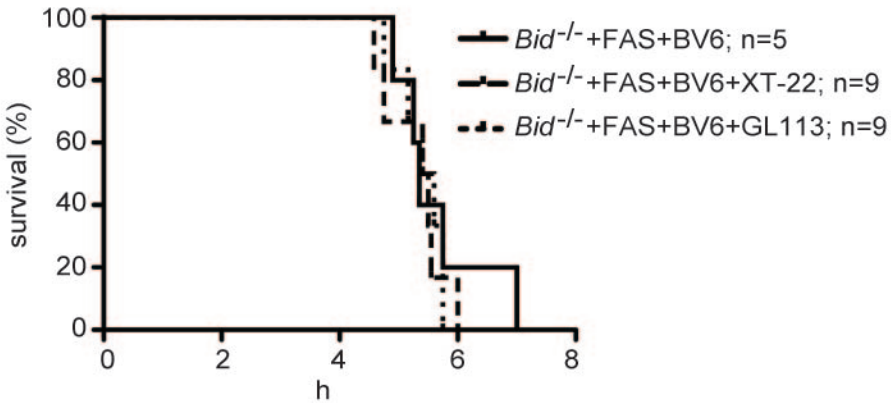
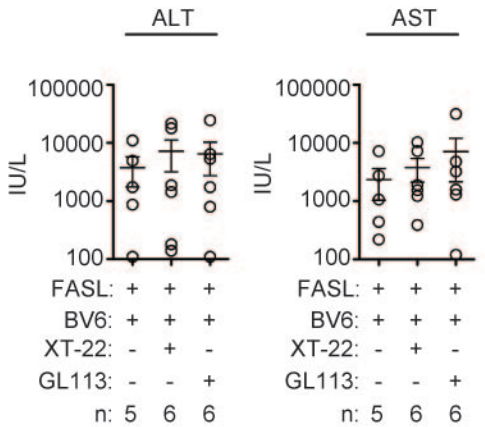
Supplementary Fig. 7

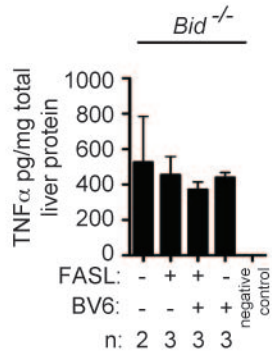
a



b



c



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Supplementary Fig. 8