Supplemental Data

eEF2 controls TNFα translation in LPS-induced hepatitis

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Figure S1

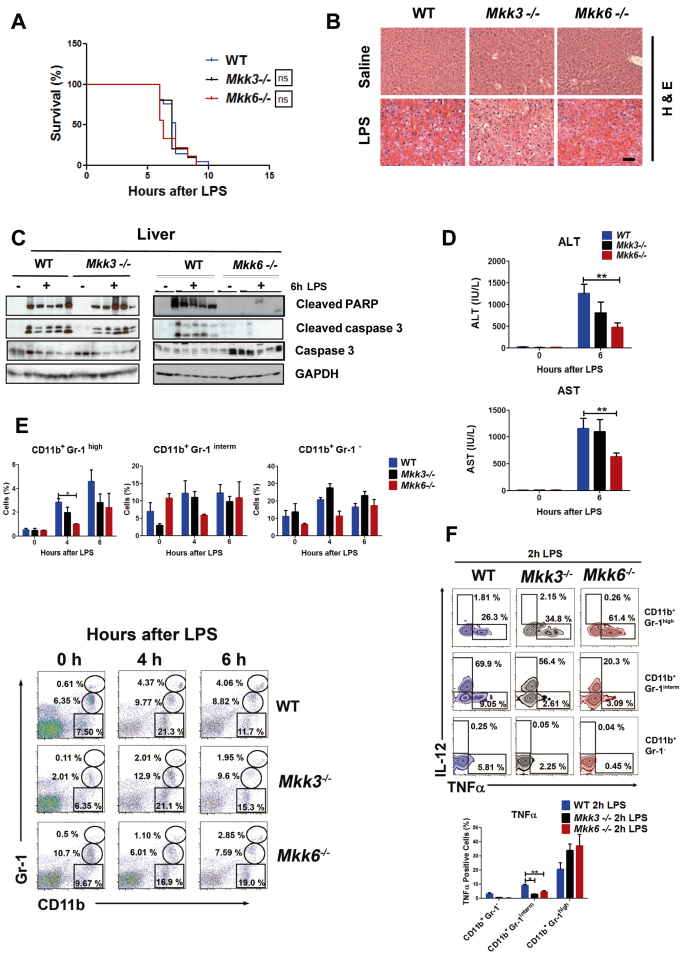

Figure S1. *Mkk3*$^{-/-}$ and *Mkk6*$^{-/-}$ mice are partially protected against LPS-induced liver damage.

Wild-type (WT), *Mkk3*$^{-/-}$ and *Mkk6*$^{-/-}$ mice were treated by intraperitoneal (i.p.) injection with 50 µg/kg LPS plus 1g/kg D-Gal (D-Gal+LPS) or saline.

(A) Survival curves after D-Gal+LPS injection (n=10). Survival curves were created with the Kaplan-Meier method and compared with Log-rank (Mantel-Cox) test.

(B) Livers were removed at 6 h post-injection. Panels show representative H&E-stained liver sections (scale bar 50µm) and livers (n= 6).

(C) Liver extracts were examined by immunoblot with antibodies to cleaved PARP, cleaved caspase 3, caspase 3 and GAPDH (n=6).

(D) Serum transaminase activity at 4 and 6 h post injection (n=6)

(E) Liver myeloid subsets (CD11b^{+} Gr-1^{high}, CD11b^{+} Gr-1^{intermediate}, CD11b^{+} Gr-1^{-}) were characterized by flow cytometry of leukocytes isolated from WT, *Mkk3*$^{-/-}$ and *Mkk6*$^{-/-}$ livers at 4 and 6 h after treatment. Bar charts show each myeloid population as a percentage of the total intra-hepatic leukocyte population; representative dot plots are shown below (n=4-7).

(F) TNFα and IL-12 production by liver myeloid populations was analyzed by intracellular staining in neutrophils (CD11b^{+} Gr-1^{high}), monocytes (CD11b^{+} Gr-1^{interm}) and CD11b^{+} Gr-1^{-} myeloid cells isolated from WT, *Mkk3*$^{-/-}$ and *Mkk6*$^{-/-}$ livers and treated in vitro with LPS (10 µg/ml) plus brefeldin A (1:1000) for 2 h. Representative dot plots are shown, and the bar charts show the percentages of TNFα-positive cells in each myeloid population. (n=4-7).

Data are means ± SD (n= 5-10). *P < 0.05; **P<0.01 (Two-way ANOVA coupled to Bonferroni post-test).

Figure S2

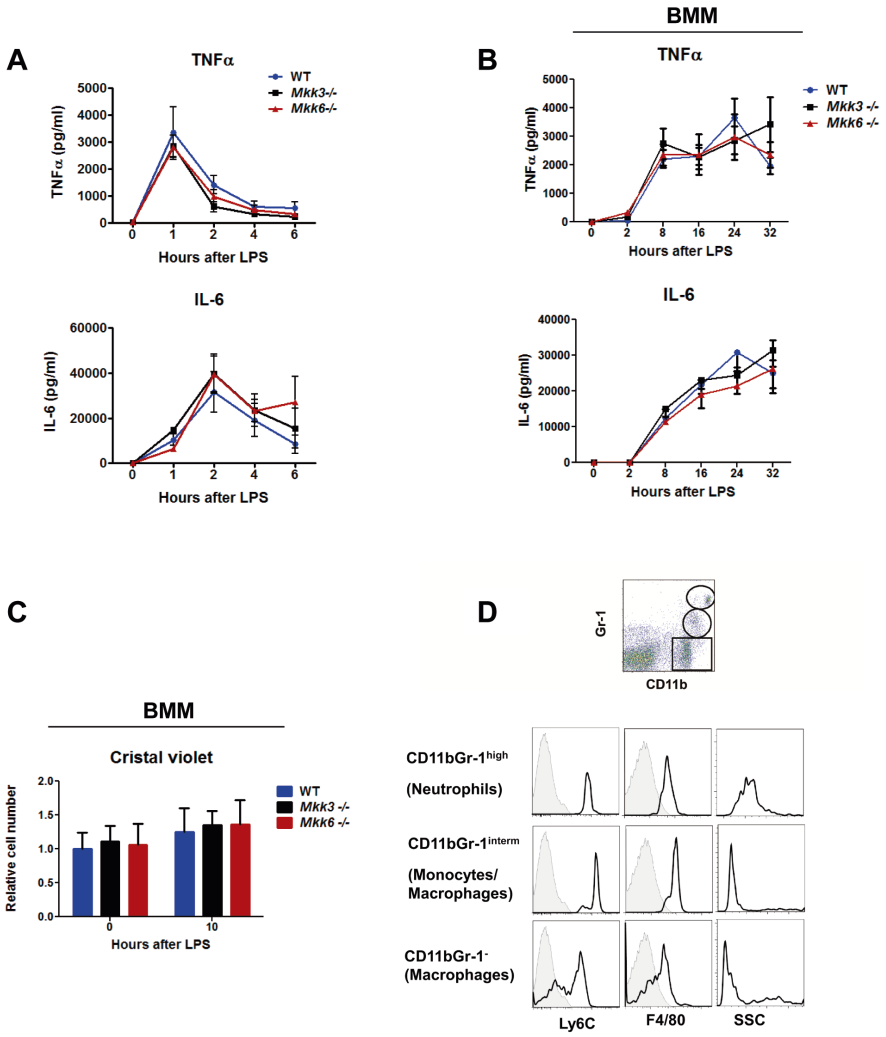

Figure S2. ***Mkk3-/-*** **and** ***Mkk6-/-*** **mice have normal levels of TNFα**

(A) Mice were treated by intraperitoneal (i.p.) injection with D-Gal+LPS (LPS/Gal), and serum cytokines (TNFα & IL-6) were measured at different times after injection by multiplexed ELISA (n = 10).

(B) Primary bone marrow macrophages from Mkk3-/-, Mkk6 -/- and WT mice were treated with LPS (10 µg/ml) in culture. The concentration of TNFα and IL-6 in the tissue culture medium was measured by ELISA (n = 4).

(C) Proliferation of Mkk3-/-, Mkk6-/- and WT bone marrow macrophages (BMM) was assayed by crystal violet staining after LPS stimulation.

(D) Representative images of flow cytometry analysis of leucocytes from WT liver based on CD11b+ and Gr-1expression levels. Three distinct populations are shown: $Cd11b^{+}$ $Gr\text{-}1^{high}$ (neutrophils), $Cd11b^{+}$ $Gr\text{-}1^{intermediate}$ (monocytes/ macrophages) and $Cd11b^{+}$ $Gr\text{-}1^{-}$ (macrophages). The $Cd11b^{+}$ subsets from WT livers were triple-stained for Cd11b, Gr-1 and indicated markers (black), and gated for SSC. Gray histograms indicate isotype control (n=7).

Data are means ± SD. No significant differences were found (Two-way ANOVA coupled to Bonferroni post-test).

Figure S3

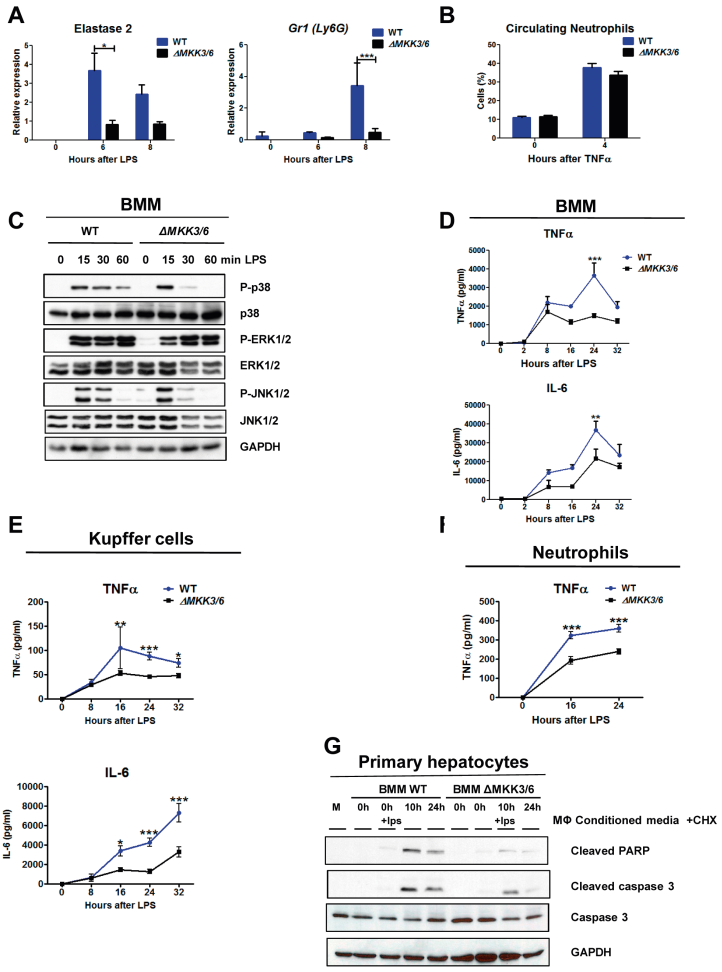

Figure S3. *ΔMKK3/6* mice show defective TNFα production and neutrophil migration

(A) WT and MKK3/6-deficient mice were injected with LPS/Gal or saline. After 6 h, liver RNA was extracted, and neutrophil marker mRNAs were determined by quantitative real-time PCR. mRNA expression was normalized to the amount of *Gapdh* mRNA (n = 8).

(B) Neutrophils were measured as the percentage of circulating leukocytes in total blood collected from WT and MKK3/6-deficient mice 4 h after TNF/Gal treatment.

(C) Primary BMM from WT and *ΔMKK3/6* mice were treated with LPS (10µg/ml) in culture for the times indicated. Cell lysates were examined by immunoblot (n= 5).

(D) ELISA analysis of the concentrations of TNFα and IL-6 in the culture medium of WT and *Mkk3*$^{-/-}$ *Mkk6*$^{-/+}$ deficient macrophages (n = 6).

(E) ELISA analysis of the concentrations of TNFα and IL-6 in the culture medium of WT and Mkk3-/- Mkk6-/+ deficient liver-isolated Kupffer cells (n = 6).

(F) ELISA analysis of the concentrations of TNFα and IL-6 in the culture medium of WT and Mkk3-/- Mkk6-/+ deficient BM- isolated neutrophils (n=6).

(G) Primary bone marrow macrophages from WT and Δ*MKK3/6* mice were treated with LPS (10µg/ml) for the times indicated. The macrophage-conditioned culture medium was collected, filtered and used to stimulate primary WT hepatocytes (12 h) in combination with cycloheximide (100µg/ml). As a control, LPS was added directly to hepatocytes treated with culture medium from untreated macrophages. Hepatocyte lysates were examined by immunoblot (n=5).

Data are means ± SD. *P < 0.05; **P<0.01; ***P<0.001 (Two-way ANOVA coupled to Bonferroni post-test).

Figure S4

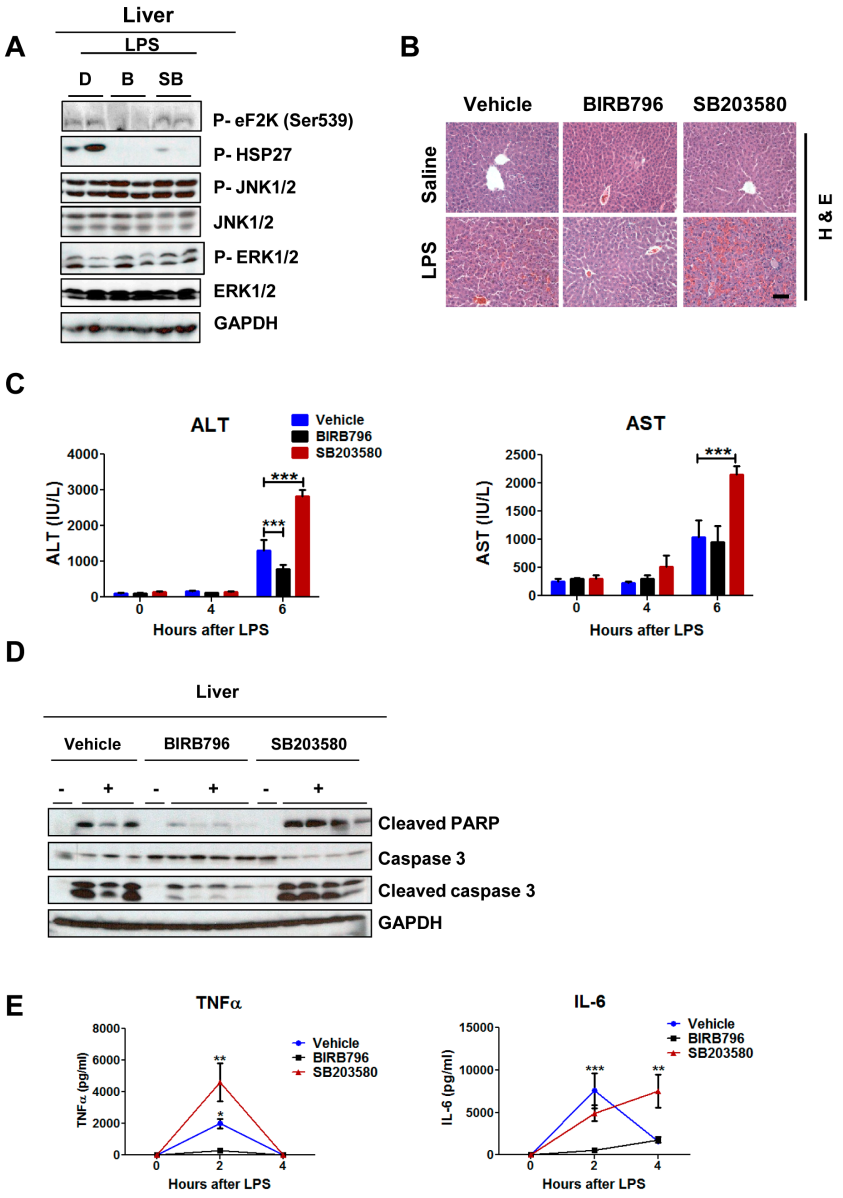

Figure S4. BIRB796 protects mice against D-Gal+LPS-induced hepatitis

Mice were i.p. injected with DMSO, BIRB796 (15mg/kg) or SB 203580 (15mg/kg). After 30min, mice were i.p. injected with D-Gal+LPS.

(A) Liver extracts were examined by immunoblot with antibodies to phospho-HSP27, phospho-EF2K (Ser359), phospho-JNK1/2, JNK1/2, phospho-ERK1/2, ERK1/2. D = DMSO; B = BIRB796; SB = SB203580, (n=8).

(B) Representative H&E-stained liver sections at 6 h after treatment. Scale bar 50µm (n=8).

(C) Serum transaminase activity at 6 h after treatment (n = 10).

(D) Liver extracts (6 h) were examined by immunoblot with antibodies to cleaved PARP, cleaved caspase 3, caspase 3 and GAPDH (n=8).

(E) Serum cytokines (TNFα, IL6) were measured at different times post-injection by multiplexed ELISA (n = 8).

Data are means ± SD. *P < 0.05; **P<0.01; ***P<0.001 (Two-way ANOVA coupled to Bonferroni post-test).

Figure S5

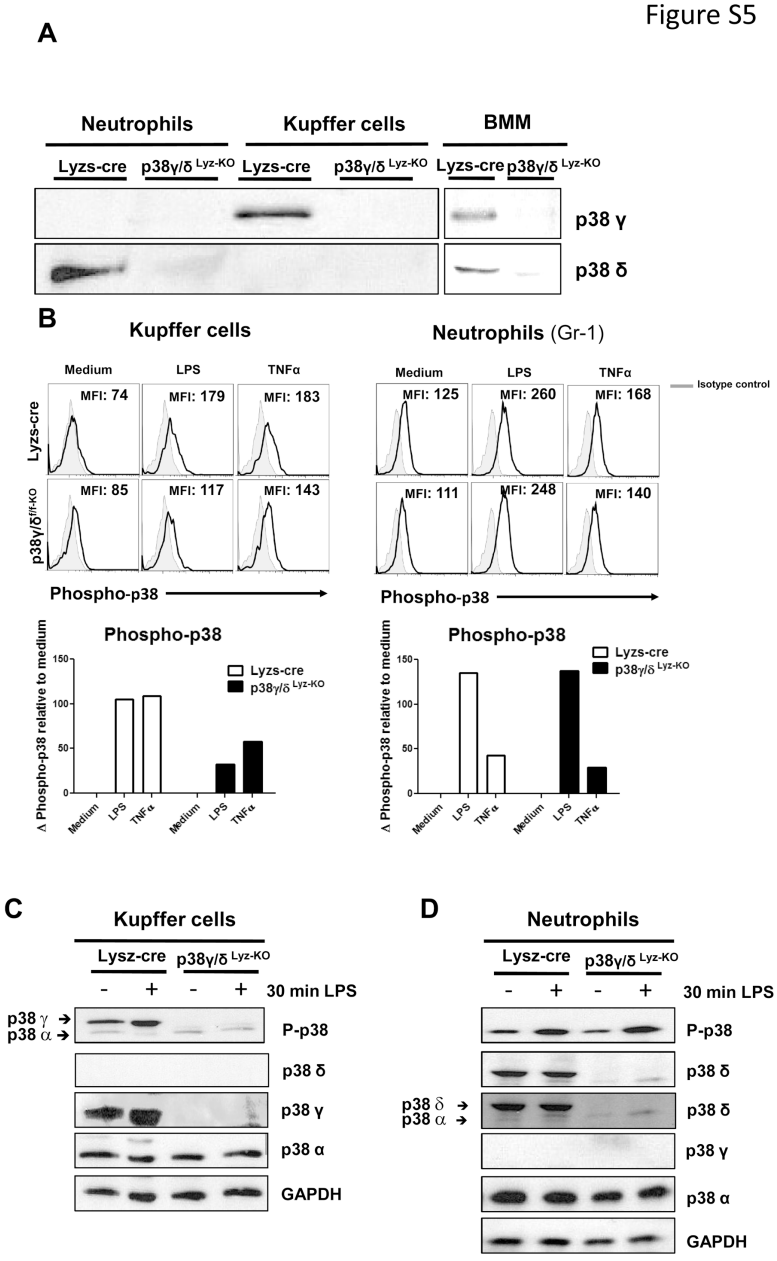

Figure S5. Myeloid expression and activation of p38γ and δ.

(A) p38γ protein expression was analyzed by immunoprecipitation of cell extracts from bone marrow-isolated neutrophils (300µg protein) or BM-derived macrophages (800µg protein), or by immunoblot of cell extracts from liver-isolated Kupffer cells (20µg protein). p38δ protein expression was analyzed by immunoprecipitation of cell extracts from bone marrow-isolated neutrophils, liver-isolated Kupffer cells (300µg protein) and BM-derived macrophages (800µg protein).

(B-C) BM-isolated neutrophils and Kupffer cells isolated from the liver of Lysz-cre mice and p38γ/$\delta^{Lyz\text{-}KO}$ transgenic mice were stimulated with LPS or TNFα for 30 min.

(B) After stimulation, cells were fixed, permeabilized, and stained for phospho-p38. Signal was detected by flow cytometry. Histograms depict intracellular phospho-p38 expression (blue histogram). Red line represents the isotype control.

(C) Cell lysates were examined by immunoblot with antibodies to phospho-p38, p38 delta, p38 gamma, p38 alpha and GAPDH.

Figure S6

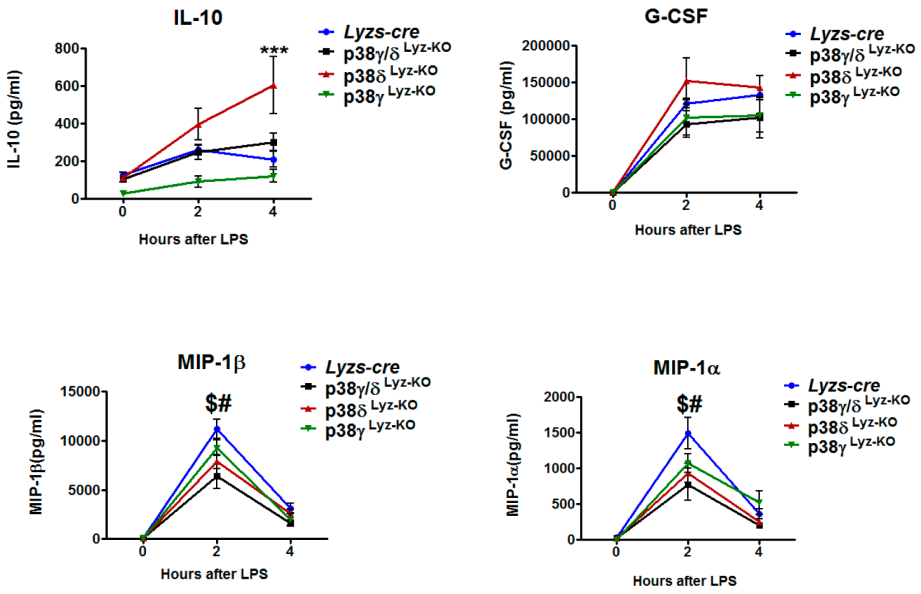

Figure S6. *p38*δ$^{Lyz\text{-}KO}$ and *p38*γ/δ$^{Lyz\text{-}KO}$ mice show defective chemokine production

*p38*γ$^{Lyz\text{-}KO}$, *p38*δ$^{Lyz\text{-}KO}$, *p38*γ/δ$^{Lyz\text{-}KO}$ and control *Lyzs-cre* transgenic mice were injected with D-Gal+LPS or vehicle.

(A) Serum cytokines and chemokines were measured at different times post-injection by multiplexed ELISA (mean ± SD; n = 10). *P < 0.05; ***P<0.001 for between-group differences in IL-10 and G-CSF. Statistically significant differences in MIP-1α and MIP-1β levels are indicated as $P < 0.001 (*Lyz-cre* versus *p38*γ/δ$^{Lyz\text{-}KO}$ mice) and #P < 0.01 (*Lyz-cre* versus *p38*δ$^{Lyz\text{-}KO}$ mice).

Figure S7

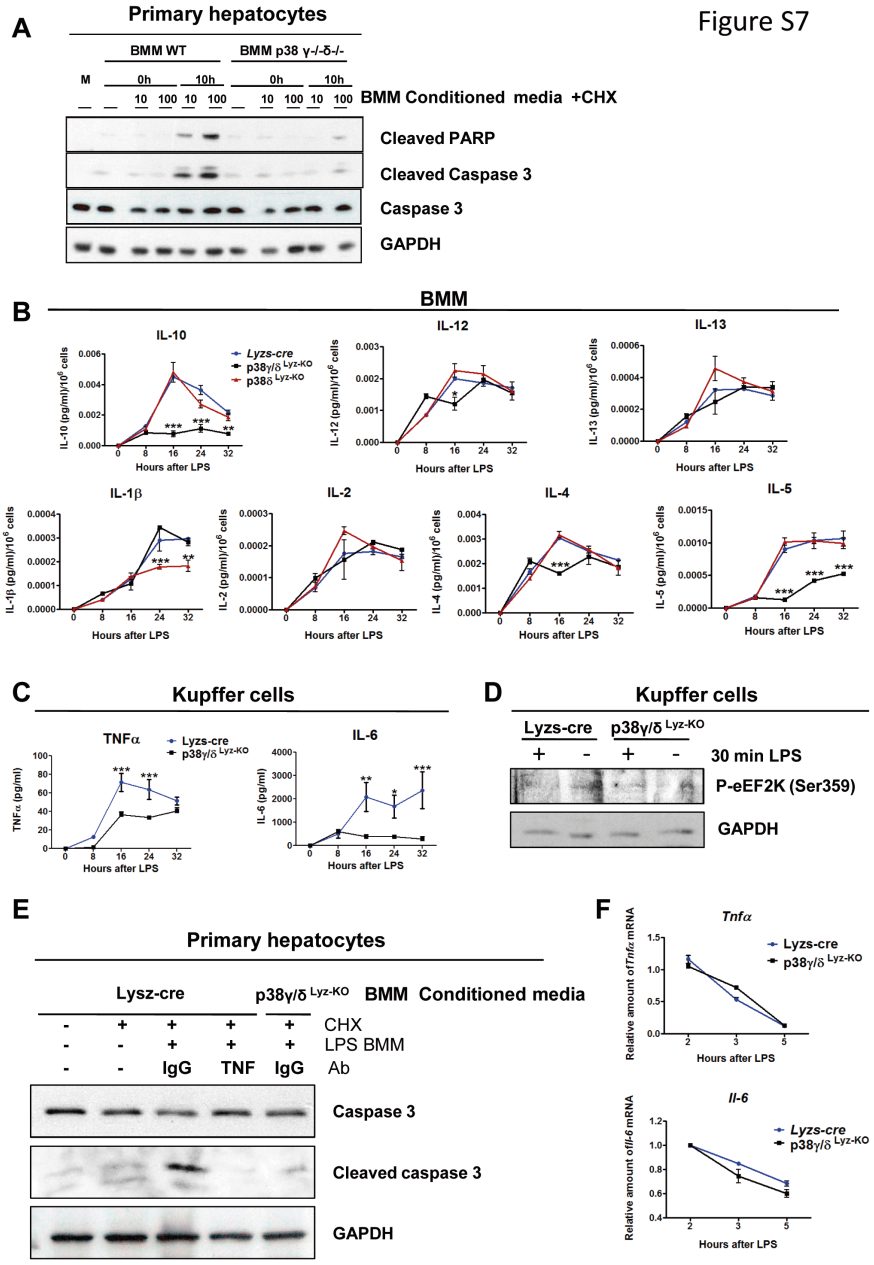

Figure sup 7. *p38*δ$^{Lyz\text{-}KO}$ and *p38*γ/δ$^{Lyz\text{-}KO}$ macrophages show defects in cytokine production

(A) *Lyzs-cre* and *p38*γ/δ deficient bone marrow macrophages were treated with LPS (10 or 100µg/ml) for 10 h. Macrophage-conditioned culture medium was collected, filtered and used to stimulate primary WT hepatocytes (12 h) in combination with cyclohexamide (100µg/ml). As a control, LPS (10 or 100µg/ml) was added directly to hepatocytes treated with culture medium from unstimulated macrophages. Hepatocyte lysates were examined by immunoblot (n=5).The concentration of cytokines in the culture medium of *Lyzs-cre*, *p38*γ/δ and *p38*δ deficient bone marrow macrophages was measured by ELISA (n=6).

(B) ELISA analysis of the concentration of cytokines in the culture medium of *Lyzs-cre*, *p38*γ/δ and *p38*δ deficient bone marrow macrophages (n = 6).

(C) Kupffer cells from the liver of Lysz-cre mice and p38γ/δ$^{Lyz\text{-}KO}$ transgenic mice were stimulated with LPS (10µg/ml) for 30 min.

(D) Cell lysates were examined by immunoblot with antibodies to phospho-eEF2K (serine 359) and GAPDH.

(E) Primary bone marrow macrophages from Lysz-cre and p38γ/δ$^{Lyz\text{-}KO}$ mice were treated with LPS (10 µg/ml) for 10 h. The macrophage-conditioned culture medium was collected, filtered and then incubated for 1 h with cycloheximide (100µg/ml) plus TNFα blocking antibodies or IgG antibodies, in the combinations indicated in the figure. The conditioned media was then used to stimulate primary WT hepatocytes for 12 h. Hepatocyte lysates were examined by immunoblot (n=5).

(F) To analyze TNFα and IL6 mRNA stability, *Lyzs-cre* and *p38*γ/δ deficient bone marrow macrophages were stimulated with LPS (10µg/ml), and actinomycin (10µg/ml) was added 1 h later. mRNA was isolated at the times indicated, and analyzed by quantitative real-time PCR. mRNA expression was normalized to the amount of *Gapdh* mRNA (n = 4).

Data are means ± SD. **P<0.01; ***P<0.001 (Two-way ANOVA coupled to Bonferroni post-test).

Figure S8

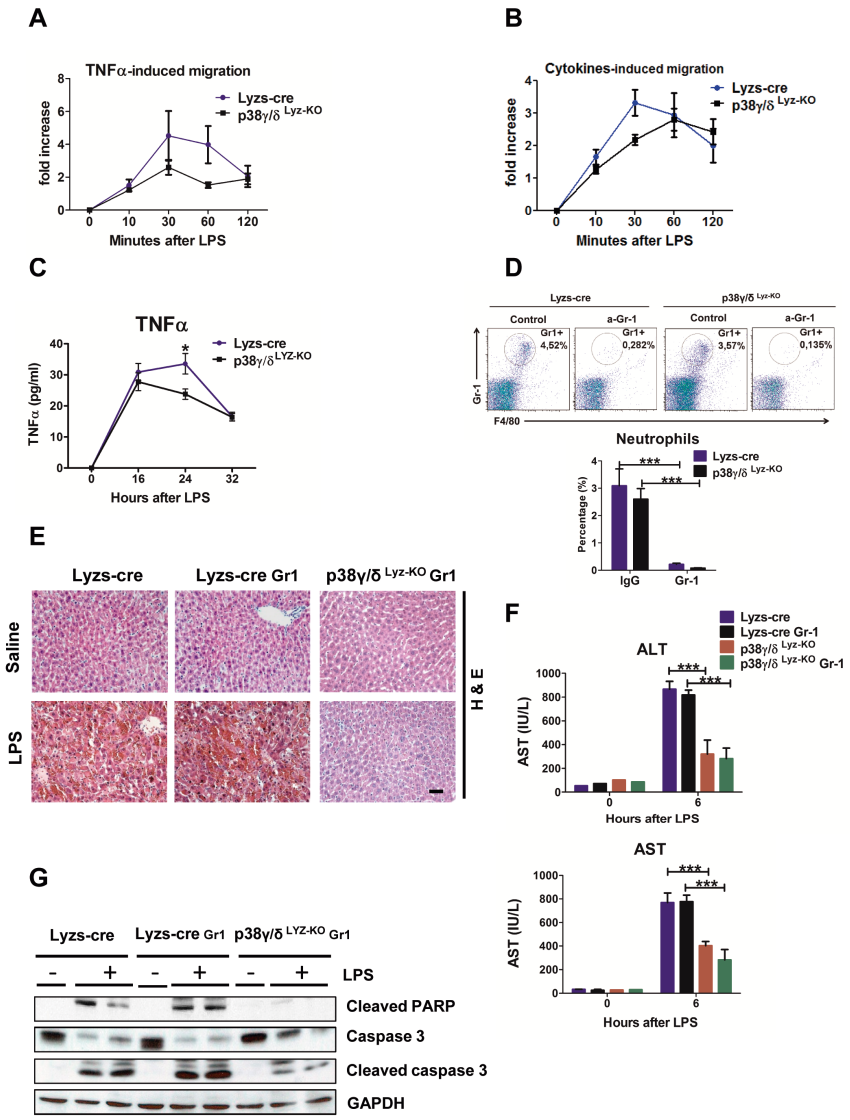

Figure S8. Role of p38γ/δ in neutrophils and LPS-induced liver injury

(A-C) Neutrophils were isolated from the bone marrow of Lysz-cre and p38γ/$\delta^{Lyz\text{-}KO}$ transgenic mice and stimulated with LPS for the indicated times. Data are means ± SD. **P<0.01; ***P<0.001 (Two-way ANOVA coupled to Bonferroni post-test).

(A) TNFα induced chemotaxis and cytokine cocktail-induced chemotaxis (B) in neutrophils. (A-B) Cells pre-labeled with 1.5 μM calcein AM were incubated with 20ng/ml TNFα or culture medium alone for 2 h using the BD FluoroBlok multiwell insert system. Fluorescence was measured at the indicated times after the addition of the chemoatractant using a Thermo Scientific Fluoroskan Ascent reader. TNFα-induced chemotaxis is expressed as the fold increases in fluorescence compared with cells treated with culture medium alone. Data are represented as mean ± SD of six replicate wells.

(C) ELISA analysis of the concentrations of TNFα and IL-6 in the culture medium of Lysz-cre and p38γ/δ deficient bone marrow-isolated neutrophils (n = 6).

(D-G) Neutrophil depletion with Gr-1 mAb does not alter LPS-induced liver injury. Lysz-cre and p38γ/$\delta^{Lyz\text{-}KO}$ mice were injected i.v. with PBS or Gr-1 mAb (250 μg) and after 24 h were injected i.p. with LPS+D-Gal or saline.

(D) The percentage of neutrophils ($Cd11b^{+}Gr\text{-}1^{high}$) in the peripheral blood of Lysz-cre mice

injected with Gr-1 mAb or saline was determined by FACS after staining with Cd11b and Gr-1 antibodies.

(E) Livers were removed at 6 h post-LPS injection. Panels show representative H&E-stained liver sections (scale bar 50μm) and livers (n= 4-8).

(F) Serum transaminase activity at 6 h after LPS treatment (n = 4-8). Data are means ± SD. ***P<0.001 (Two-way ANOVA coupled to Bonferroni post-test).

(G) Liver extracts (6 h) were examined by immunoblot with antibodies to cleaved PARP, cleaved caspase 3, caspase 3 and GAPDH (n=4-8).

Figure S9

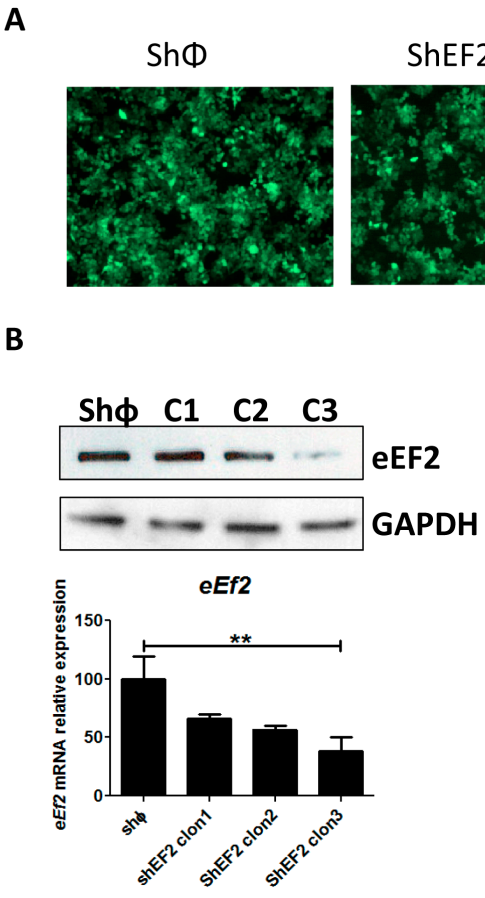

Figure S9. eEf2 silencing by lentiviral transduction of mouse BM-derived macrophage cultures.

(A) GFP expression by RAW 264.7 cells transduced with GFP^+ lentivirus expressing shRNA against eEf2 (shEF2 clon3) or with empty vector (ShΦ) by fluorescence microscopy. Magnification 10x.

(B) RAW 264.7 cells were transduced with three different lentiviruses, each one expressing a different shRNA clone against eEf2. Gene silencing efficiency was determined by immunoblot of cell extracts (top) and by qRT-PCR of eEf2 mRNA (bottom). mRNA expression was normalized to the amount of *Gapdh* mRNA. Data are means ± SD. **P<0.01 (One-way ANOVA coupled to Bonferroni post-test).

Supplemental Experimental Procedures

Tissue culture.

Conditioned medium from bone marrow-derived macrophages was obtained after stimulation of cells on day 7 of differentiation with LPS (10µg/ml) for 10 or 24 h The collected medium was sterile filtered to eliminate BM macrophages and frozen (-20ºC) until use. Primary hepatocytes were treated with the conditioned medium for 13 h.

qRT-PCR primers.

Primers were purchased from Sigma Aldrich.

Hif-1 α	Forward Primer	ACCTTCATCGGAAACTCCAAAG
	Reverse Primer	CTGTTAGGCTGGGAAAAGTTAGG
Gr-1	Forward Primer	GACTTCCTGCAACACAACTACC
	Reverse Primer	ACAGCATTACCAGTGATCTCAGT
KC	Forward Primer	CTGGGATTCACCTCAAGAACATC
	Reverse Primer	CAGGGTCAAGGCAAGCCTC
Mip-2	Forward Primer	CCAACCACCAGGCTACAGG
	Reverse Primer	GCGTCACACTCAAGCTCTG
Mcp-1	Forward Primer	TTAAAAACCTGGATCGGAACC
	Reverse Primer	GCATTAGCTTCAGATTTACGG
Icam-1	Forward Primer	GTGATGCTCAGGTATCCATCC
	Reverse Primer	CACAGTTCTCAAAGCACAGCG
Tnfα	Forward Primer	CCCTCACACTCAGATCATCTT
	Reverse Primer	GCTACGACGTGGGCTACAG
Il-6	Forward Primer	TAGTCCTTCCTACCCCAATTT
	Reverse Primer	TTGGTCCTTAGCCACTCCTTC

Statistical analysis.

Differences between groups were examined for statistical significance using Student's t-test, analysis of variance (ANOVA) coupled to the Bonferroni post-test, or the log-rank test.