SUPPLEMENTAL MATERIALS

The non-transcriptional activity of IRF3 dynamically modulates hepatic immune cell populations in acute on chronic ethanol in mice

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Supplemental Materials and Methods

Gao-binge ethanol exposure and tissue collection in mice

Mice were housed two per micro-isolator cage and were maintained in a temperature regulated facility with a 12 hour light-dark cycle, with Nylabones provided for environmental enrichment. Mice were allowed free access to a Lieber-DeCarli liquid diet (Dyets, Bethlehem, PA; Cat#710260) containing ethanol or a pair-fed control diet that isocalorically substituted maltose dextrin for ethanol (1). Mice were weight matched and randomly distributed to ethanol or control diets. Pair-fed control and ethanol-fed animals received control diet for 5 days and then ethanol-fed animals were allowed free access to the Lieber-DeCarli diet with ethanol at 5% (v/v) or 28% of total calories for an additional 10 days. Mice were given 3 conditioning gavages of saline at random times on days 4, 8, and 12. On the final day of the experiment, pair-fed mice were gavaged with 5g/kg maltose and ethanol-fed mice were gavaged with 5g/kg ethanol in water. Mice were euthanized 6h after gavage. Three independent feeding trials were conducted to collect sufficient material for all the analyses included in this study.

Mice were anesthetized, blood samples were collected from from the posterior vena cava into non-heparinized syringes, livers excised and mice euthanized by exsanguination. Portions of each liver were then either fixed in formalin or frozen in optimal cutting temperature (OCT) compound (Sakura Finetek U.S.A., Inc., Torrance CA) for histology, frozen in RNAlater (Qiagen, Germantown, MD) or flash frozen in liquid nitrogen and stored at -80 ^{o}C until further analysis. Blood was transferred to EDTA-containing tubes for the isolation of plasma. Plasma was then stored at -80°C.

Histopathology and immunohistochemistry

Formalin-fixed tissues were paraffin-embedded, sectioned, coded and stained with hematoxylin and eosin. Formalin-fixed paraffin-embedded sections were de-paraffinized and used to assess 4-hydroxynonenal (4-HNE) (HNE11-S, Alpha Diagnostic, San Antonio, TX) and phospho-MLKL in liver (ab196436, Abcam, Cambridge, MA) (1). Frozen liver sections were stained with Oil Red O, as described before (1). Nuclei were counter-stained with hematoxylin. Tissues were coded at time of collection to assure an un-biased analysis; at least 3 images were acquired per tissue section and semi-quantification of positive staining was performed using ImagePro Plus software (Media Cybernetics, Silver Springs, MD). No specific immunostaining was seen in sections incubated with PBS rather than the primary antibody (data not shown).

Biochemical assays

Plasma samples were assayed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using a commercially available enzymatic assay kit (Sekisui Diagnostics, Lexington, MA), following the manufacturer's instructions.

Western blot analysis

Frozen liver tissue (100mg) from mice or human subjects, as well as cells, were homogenized in lysis buffer (10 ml/ g tissue) and protein concentration measured using the DC Lowry assay (2). Liver lysates were then used for Western blot analysis using antibodies against cytochrome P450 2E1 (CYP2E1) (AB1252, Millipore, Burlington, MA); caspase-3 (8G10, #9665), Capase-9 (C-9, #9508), cleaved PARP (Asp214, #9544), phospho^{Ser396}IRF3 (4D4G, #4947), CHOP (D46F1, #5554), STING (D1V5L, #50494), eIF2α (#9722) and phospho^{Ser51} eIF2α (#3597) (Cell

Signaling, Danvers, MA) and IRF3 (FL425) (sc-9082, Santa Cruz Biotechnology, Dallas, TX). HSC70 (sc-7298, Santa Cruz Biotechnology, Dallas, TX) or GAPDH (MAB374, Millipore, Burlington, MA) were used as the loading control.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was isolated from liver stored in RNAlater using RNeasy Mini kits per the manufacturer's instructions (Qiagen, Germantown, MD). 2 μg of liver RNA was reverse transcribed and analyzed with PowerSYBR qRT-PCR kits (Applied Biosystems, Foster City, CA) on QuantStudio5 RT-PCR Machine (Applied Biosystems, Foster City, CA). In explanted human liver tissue samples, RNA was isolated from frozen pieces of liver homogenized in TRIZOL (Qiagen, Germantown, MD) and separated by chloroform extraction. RNA was purified using RNeasy Mini kits and 1 μg of liver RNA was reverse transcribed and analyzed as indicated above. Relative messenger RNA (mRNA) expression was determined using gene-specific primers (See Supplemental Table 1). Statistical analyses were performed on the ΔCt values (average Ct of gene of interest – average Ct of 18S).

Cell Isolation, Flow Cytometry and Cell Sorting

Hepatic non-parenchymal cells were prepared by digesting livers in RPMI 1640/10% FBS with Type IV Collagenase (Sigma Aldrich, St. Louis, MO, Lot# 087K8630) and DNase I (Roche, Mannheim, Germany) for 45min at 37° C. Digests were pressed through a 70 μm strainer and washed with RPMI 1640/10% FBS. Cells were centrifuged at 50 g for 2 min; supernatant was then centrifuged at 300 g for 15 min. Cells were resuspended in BD Pharm Lyse (BD Biosciences, San Jose, CA) for 5 min on ice. Cells were washed with RPMI 1640/10% FBS and

centrifuged at 300 g for 7 min. To collect the NPC fraction, cells were centrifuged at 50 g for 10 min without brake in a 30% Percoll gradient. The cell layer was removed carefully and cells collected by centrifugation at 300 g for 10 min. Cells were washed with PBS and centrifuged at 300 g for 10 min. Cells were resuspended in FACS buffer (PBS, 1% BSA, 0.05% sodium azide) and aliquoted into 96-well plates at a concentration of ~$1x10^6$ cells/mL. Cells were centrifuged at 830 g for 4 min, resuspended in 50 µl FACS buffer containing 0.5 µg of Fcγ Block (clone 93, eBioscience, San Diego, CA) and incubated for 15 minutes at room temperature. After blocking, cells were stained with fluorochrome-conjugated antibodies: CD45-Pe/Cy7 (clone I3/2.3, Abcam, Cambridge, MA); Ly6C-AF647 (clone ER-Mp20, AbD Serotec, Raliegh, NC); Ly6G-eF610 (clone 1A8, Biolegend, San Diego, CA); F4/80-Pe/Cy5 and CD11b-AF488 (clone BM8 and clone M1/70, eBioscience, San Diego, CA) and/or Annexin V (Becton Dickinson Biosciences, Mountain View, CA) for 30 min at 4° C in the dark. Cells were washed twice with FACS buffer and centrifuged at 830 g for 4 min in FACS buffer. Cells were fixed overnight in 1% PFA at 4°C and collected by centrifugation 830 g for 5 min. Cells were resuspended in 300 µl FACS buffer. Data was collected on a LSRII flow cytometer (Becton Dickinson Immunocytometry systems, Mountain View, CA) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

For cell sorting experiments, isolated hepatic non-parenchymal cells were stained with CD45-FITC (clone I3/2.3, Abcam, Cambridge, MA), Ly6C-AF647 (clone ER-Mp20, AbD Serotec, Raleigh, NC) and CD11b-PE (clone M1/70, eBioscience, San Diego, CA) as described above and then sorted on a BD FACSAria II (Becton Dickinson Immunocytometry systems, Mountain

View, CA). $CD45^+$/ $CD11b^+$/$Ly6C^{high}$ and $CD45^+$/ $CD11b^+$/$Ly6C^{low}$ events were collected for RNA isolation using RNeasy Micro Kit (Qiagen, Germantown, MD).

Blood was collected in heparin-coated tubes, red blood cells (RBC) were lysed and cells were centrifuged at 300g for 15 min. Spleens were digested mashing the tissue in a 70µm strainer with RPMI 1640/10% FBS, RBC were lysed and cells were centrifuged at 300g for 15 min. Bone marrow cells were isolated by flushing the femur and tibia bones of 10 week old mice with sterile phosphate buffered saline, RBC were lysed and cells were centrifuged at 300g for 15 min. Cells were resuspended in FACS buffer (PBS, 1% BSA, 0.05% sodium azide) and aliquoted into 96-well plates at a concentration of ~$1x10^6$ cells/mL. Cells were centrifuged at 830 g for 4 min, resuspended in 50 µl FACS buffer containing 0.5 µg of Fcγ Block (clone 93, eBioscience, San Diego, CA) and incubated for 15 minutes at room temperature. After blocking, cells were stained with fluorochrome-conjugated antibodies: CD45-Pe/Cy7 (clone I3/2.3, Abcam, Cambridge, MA); Ly6C-AF647 (clone ER-Mp20, AbD Serotec, Raleigh, NC) and CD11b-PE (clone M1/70, eBioscience, San Diego, CA) for 30 min at 4° C in the dark. Cells were washed twice with FACS buffer and centrifuged at 830 g for 4 min in FACS buffer. Cells were fixed for 30 min in 1% PFA at 4°C and collected by centrifugation 830 g for 5 min. Cells were resuspended in 300 µl FACS buffer. Data was collected on a BD LSR Fortessa flow cytometer (Becton Dickinson Immunocytometry systems, Mountain View, CA) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Primary cultures of hepatic macrophages from rats and mice

Hepatic macrophages were isolated from ethanol- and pair-fed rats and placed into primary culture for 18 h, as previously described (3). To isolate murine hepatic macrophages, mice were anesthetized, livers were perfused with PBS and digested with HEPES plus 2mg/mL of collagenase D (#11088866001, Roche, Mannheim, Germany) subjected to 4 x 20s cycles in a gentle MACS dissociator (MACS Miltenyi Biotec, Auburn, CA). Digests were then incubated for a further 45 min on a rotator and then run for 24 sec on the gentle MACS dissociator. Samples were filtered through a 100 µm cell strainer (Corning, Corning, NY) and centrifuged at 300 g for 10 min at room temperature. The cell pellet was resuspended in 5 mL, applied to a 70 µm cell strainer (Corning, Corning, NY) and then washed twice in 40 mL of PBS and centrifuged at 300g for 10 min. Pellet was resuspended in 2 mL RPMI 1640 and mixed with 8 mL of 30% Histodenz (D2158, Sigma Aldrich, St. Louis, MO). Cells were centrifuged at 1500g for 20 min at 4°C without brake, washed twice as above and then resuspended in DMEM with 10% FBS and placed into primary culture.

After 18 (rat) or 24 h (mouse) in culture, hepatic macrophages were challenged with or without 25 µg/ml Poly (I:C) in fresh media, at incubation times indicated in the Figure legends. For co-localization studies, hepatic macrophages were plated onto coded slides and challenged or not with 25 µg/ml Poly (I:C). 30 min prior to the end of the incubation period, Mito-Tracker Orange CMTMRos (Invitrogen, Grand Island, NY) was added to the media. Cells were then fixed in ice and processed for confocal microscopy, as previously described (4). After blocking, slides were incubated overnight at 4°C in primary antibody against IRF3 (FL-425) (#sc-9082, Santa Cruz, Dallas, TX) and/or Bax (#55647, BD Pharmigen, Franklin Lakes, NJ). Cells were then washed

with PBS and incubated in the dark with fluorescently-labelled secondary antibodies (goat anti-mouse IgG 488 (A11029), donkey anti-rabbit IgG 488 (A21206) or goat anti-rabbit IgG 568 (A11036), Molecular Probes, Grand Island, NY) for 1 hour at room temperature. Cells were again washed with PBS and mounted with a DAPI-containing mounting media. Images were acquired using a Leica TCS SP5 II Confocal/Multi-Photon high speed upright microscope using 63X objective. For Western blots, cells were lysed in RIPA buffer with proteinase and phosphatase inhibitors and processed in Laemmli buffer.

Endotoxin assay

All glassware used for endotoxin collection and detection was rendered endotoxin-free by baking at 225°C overnight (3). Plasma was isolated from portal blood by centrifugation at 150g for 10min at 4°C and then stored at −80°C until testing. Endotoxin Sample Preparation Kit (ESP) (BioDtech (Birmingham, AK) was used according to the manufacturer's instruction and endotoxin concentration measured with Pyrogent-5000 assay (Lonza, Basel, Switzerland). As a control, endotoxin standards were spiked into ESP-treated plasma samples to confirm endotoxin recovery within the manufacturer's acceptable range (50– 200%).

Immunoprecipitation assay from cells and whole liver

HuH7 is a well-differentiated hepatocyte-derived carcinoma cell line, obtained from Japan Health Science Research Resources Bank (HSRRB, JCRB0403). RAW264.7 is an Abelson murine leukemia virus transformed-macrophage cell line (ATCC TIB-202TM). HuH7 and RAW264.7 cells were cultured as previously described (5). Mycoplasma contamination has been excluded by testing the cultures periodically using fluorescent staining (Hoechst 33258).

RAW264.7 or Huh7 cells were transfected with V5.IRF3 plasmid (3µg)(6) using Lipofectamine 2000 reagent (11668019, Thermo Fisher Scientific, Grand Island, NY). Twenty four hours post transfection, the cells were either left untreated or treated with EtOH as indicated for 24h. After treatment, the cells were harvested in alternative lysis buffer (ALB) and subjected to ubiquitination assay using previously described protocol (6). Briefly, the cell lysates were immunoprecipitated using anti-V5 conjugated agarose (A 7345, Sigma, Saint Louis, MO) and probed with anti-Ub (sc-8017, Santa Cruz, Dallas, TX) or anti-V5 (R960-25, Thermo Fisher Scientific, Grand Island, NY). Whole livers from mice were harvested in alternative lysis buffer and immunoprecipitated using anti-Ub (13-1600, Thermo Fisher Scientific, Grand Island, NY) conjugated with True Blot Anti-Mouse IgG IP Agarose Beads (00-8811-25, Rockland, Limerick, PA) and probed with anti-IRF3 (FL425) (sc-9082, Santa Cruz Biotechnology, Dallas, Texas).[2] Immunoprecipitation with C57BL/6 and *Irf3*$^{-/-}$ livers were performed as positive and negative control for IRF3 (Sup Fig. 7)

Reference List

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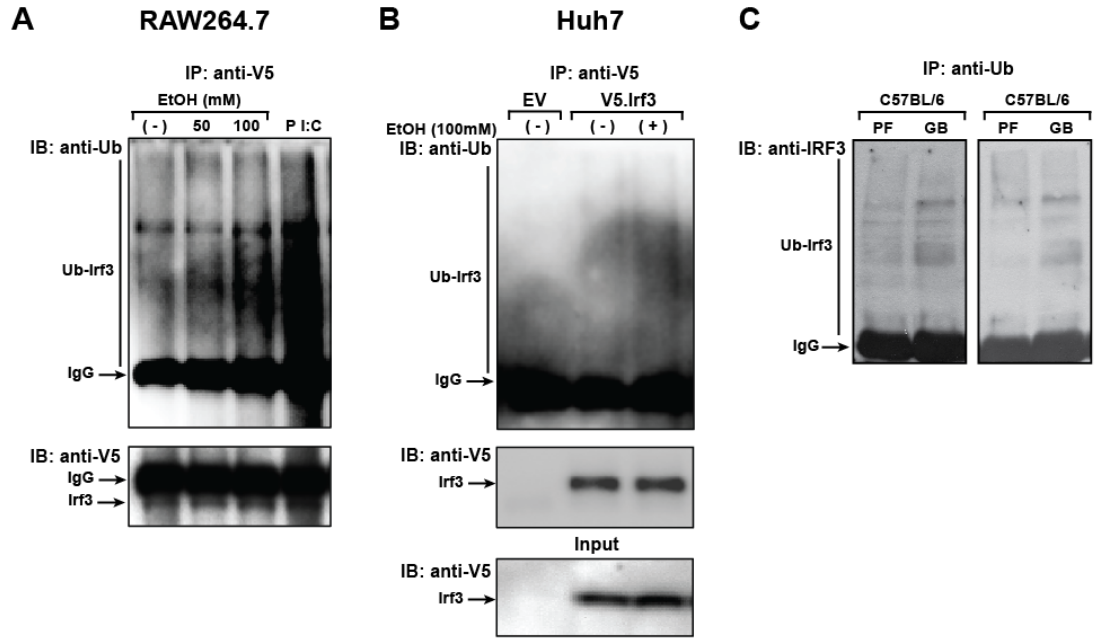
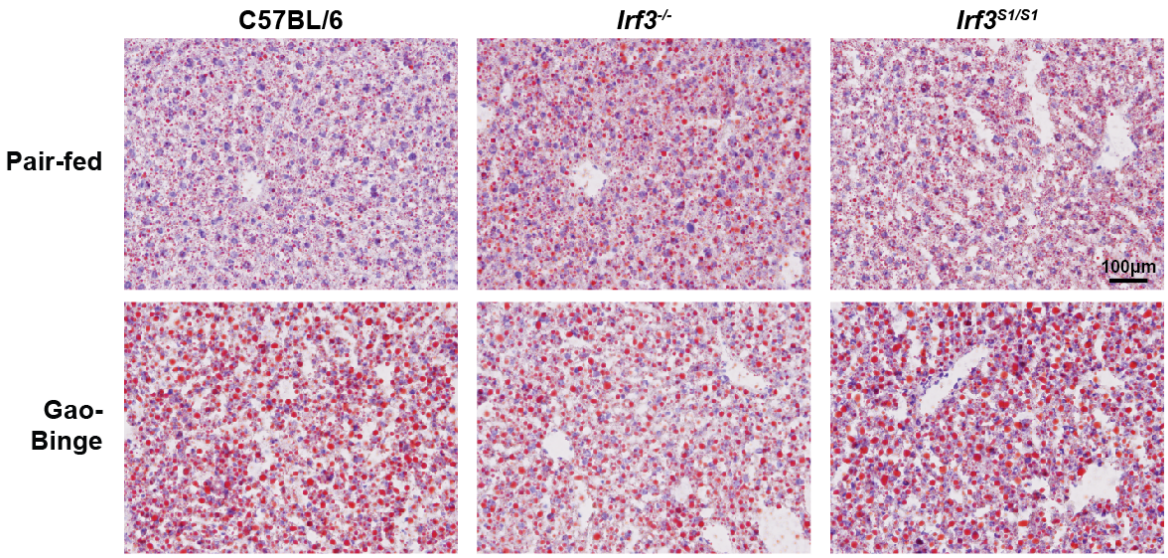
Supplemental Figure 1



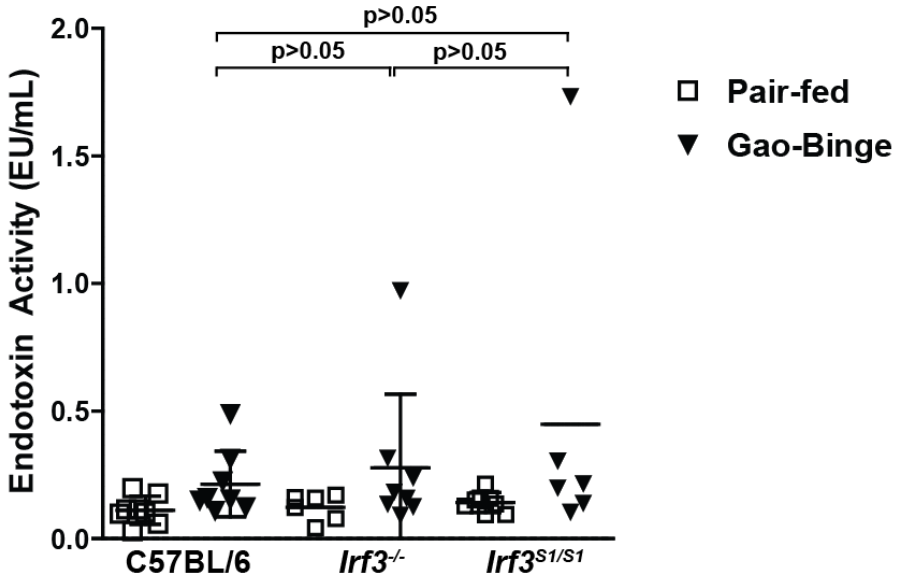
Supplemental Figure 1: Ethanol increases the ubiquitination of IRF3 in RAW264.7 macrophage and Huh7 hepatocyte cell lines. RAW264.7 cells **(A)** or Huh7 cells **(B)** were transfected with V5-IRF3 and 24h post-transfection, cells were challenged with EtOH as indicated for 24h, immunoprecipitated with anti-V5 antibody and blotted with anti-Ubiquitin or anti-V5 antibody. P I:C line: cells were transfected with Poly(I:C) as a positive control (6). Data are representative of 3 independent experiments in each cell type. **(C)** Liver lysates from C57BL/6 mice were immunoprecipitated with anti-Ubiquitin antibody and probed with anti-IRF3. (PF:Pair-fed; GB: Gao-binge ethanol). Pull-downs are representative of 4 mice per diet group.

Supplemental Figure 2



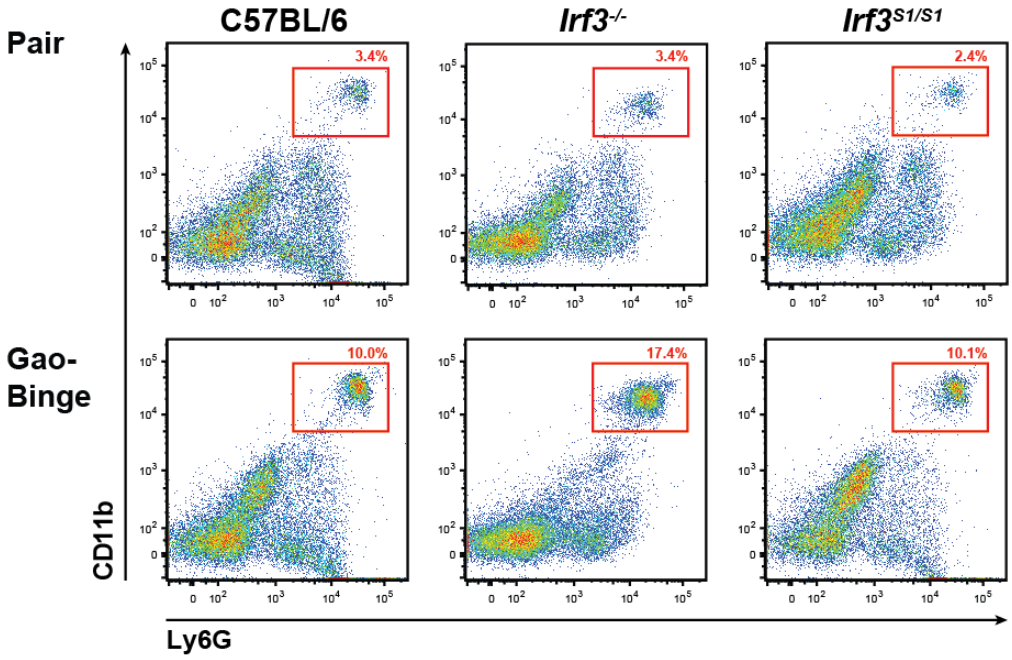
Supplemental Figure 2: The non-transcriptional activity of IRF3 contributes to ethanol-induced hepatic steatosis in the Gao-binge model of ethanol-induced liver injury. C57BL/6, *Irf3*$^{-/-}$ and *Irf3*$^{S1/S1}$ were exposed to the Gao-binge model as in Figure 1. Hepatic steatosis was assessed by Oil Red O staining. Images were collected at 20X and are representative of 4 animals per group.

Supplemental Figure 3



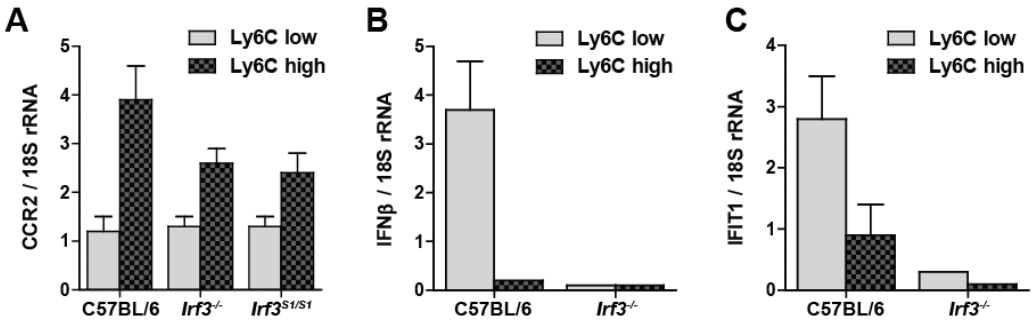
Supplemental Figure 3: Endotoxin concentrations in the portal circulation after Gao-binge ethanol exposure were not affected by genotype. C57BL/6, *Irf3*$^{-/-}$ and *Irf3*$^{S1/S1}$ mice were exposed to the Gao-binge model as in Figure 1. Plasma endotoxin was measured from portal blood. Values represent means $\pm$ SEM, n=6-8 per group. Data were not normally distributed. Therefore, the data were analyzed by ANOVA of ranked data followed by least square means testing using SAS.

Supplemental Figure 4



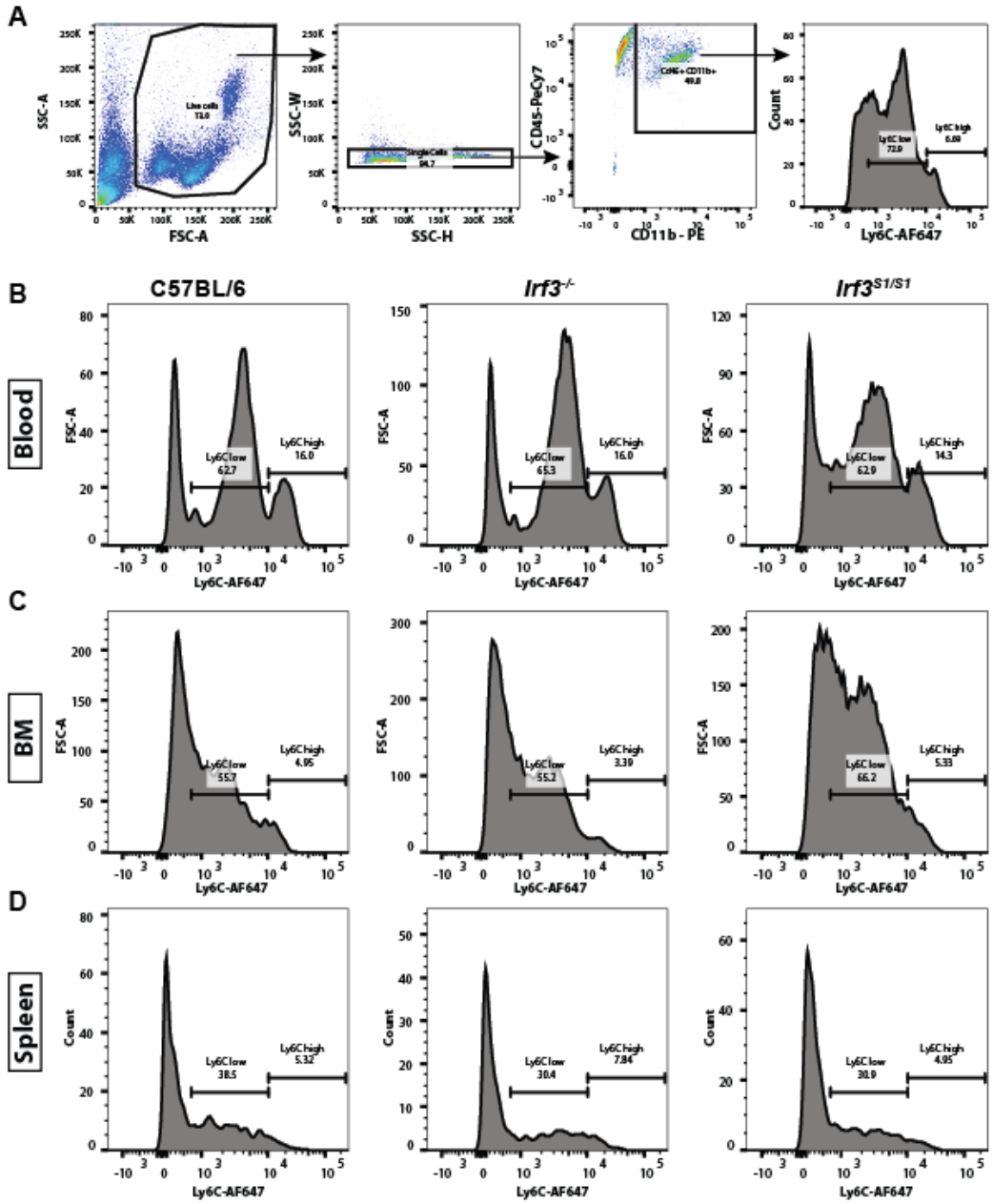
Supplemental Figure 4: Characterization of neutrophil populations from C57BL/6, *Irf3*$^{-/-}$ and *Irf3*$^{S1/S1}$ mice. C57BL/6, *Irf3*$^{-/-}$ and *Irf3*$^{S1/S1}$ mice were exposed to the Gao-binge model as in Figure 1. Total non-parenchymal cells were isolated from the liver and then labelled as described in Supplemental Methods and analyzed by flow cytometry. Representative flow diagrams for CD45^{+}/ CD11b^{+}/Ly6G^{+} are illustrated. Representative flow cytometry plots and proportions are shown from different experiments, n=4.

Supplemental Figure 5



Supplemental Figure 5: Differential gene expression from Ly6C^{hi} and Ly6C^{low} population from C57BL/6, *Irf3*$^{-/-}$ and *Irf3*$^{S1/S1}$ mice. C57BL/6, *Irf3*$^{-/-}$ and *Irf3*$^{S1/S1}$ mice were exposed to the Gao-binge model as in Figure 1. Total non-parenchymal cells were isolated from the liver and then labelled for cell sorting as described in Supplemental Methods. CD45^{+}/ CD11b^{+}/Ly6C^{high} and CD45^{+}/ CD11b^{+}/Ly6C^{low} cells were separated by cell sorting and expression of **A)** CCR2, **B)** IFNβ and **C)** IFIT1 mRNA was assessed by qRT-PCR. Values represent means ± SEM, n=4 per group.

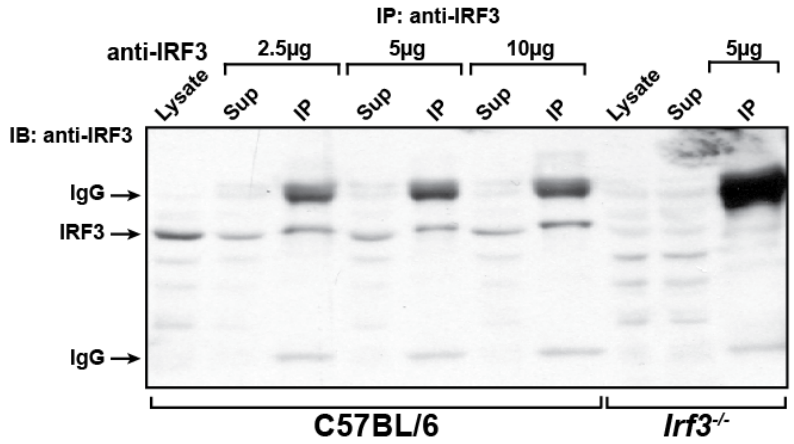
Supplemental Figure 6



Supplemental Figure 6: Distribution of Ly6C^{high} and Ly6C^{low} monocytes in blood, bone marrow and spleen in C57BL/6, *Irf3*$^{-/-}$ and *Irf3*$^{S1/S1}$ mice. C57BL/6, *Irf3*$^{-/-}$ and *Irf3*$^{S1/S1}$ mice were anesthetized and blood, spleen and tibia/femur were collected. After isolation, cells were labelled and analyzed by flow cytometry as described in Supplemental Methods. **A)** Gating strategy used to analyze the distribution of Ly6C^{high} and Ly6C^{low} monocytes in each cell

population. Live cells were gated and corrected for singlets. $CD45^+$ $CD11b^+$ cells were then gated for Ly6C. $Ly6C^{high}$ and $Ly6C^{low}$ monocytes from C57BL/6, *Irf3*-/- and $Irf3^{S1/S1}$ mice in **B)** Blood, **C)** Bone marrow (BM) and **D)** Spleen. Data are representative of three independent experiments.

Supplemental Figure 7



Supplemental Figure 7: Immunoprecipitation from C57BL/6 and *Irf3*$^{-/-}$ mice. C57BL/6 and *Irf3*$^{-/-}$ mice were exposed to the Gao-binge model as in Figure 1. Liver lysates were immunoprecipitated with different concentrations of anti-IRF3 antibody and blotted with anti-IRF3. *Irf3*$^{-/-}$ mice were used as negative controls for the immunoprecipitation. Sup: supernatant from immunoprecipitation; IP: immunoprecipitation.

Supplemental Table 1

Gene Name (Symbol)	Species	Forward	Reserve
Arginase-1 (Arg1)	mouse	CTC CAA GCC AAA GTC CTT AGA G	AGG AGC TGT CAT TAG GGA CAT C
Bak	mouse	AGA CCT CCT CTG TGT CCT GG	AAA ATG GCA TCT GGA CAA GG
C-C chemokine receptor type 2 (CCR2)	mouse	AGG AGC CAT ACC TGT AAA TGC	TAG TCA TAC GGT GTG GTG GC
Cluster of diferentation 36 (CD36)	mouse	GAA CCA CTG CTT TCA AAA ACT GG	TGC TGT TCT TTG CCA CGT CA
Death receptor 5 (Dr5)	mouse	GGT CCT CTT GAT GGG CTC TC	GTT GCT GCT TGC TGT GCT AC
ELANE	mouse	CAG AGG CGT GGA GGT CAT TT	GAA GAT CCG CTG CAC AGA GA
Endoplasmic reticulum oxidase 1 alpha (Ero1α)	mouse	CAC AGG TAC AGT CGT CCA GGT	CTT GCT CGT TGG ACT CCTG
Fatty acid-binding protein 4 (FABP4)	mouse	AAG GTG AAG AGC ATC ATA ACC C	TCA CGC CTT TCA TAA CAC ATT CC
Glucose regulated protein 78 (Grp78)	mouse	ACT TGG GGA CCA CCT ATT CCT	ATC GCC AAT CAG ACG CTC C
Glucose regulated protein 94 (Grp94)	mouse	AAA GTT CGC CTT CCA AGC TG	CTG TGA CAT GCA GCA GGT TT
Growth arrest and DNA damage-inducible 34 (Gadd34)	mouse	GGA GAT AGA AGT TGT GGG CG	TTT TGG CAA CCA GAA CCG
Interferon beta (IFNβ)	mouse	CAG CTC CAA GAA AGG ACG AAC	GGC AGT GTA ACT CTT CTG CAT
Interferon regulatory factor 3 (IRF3)	mouse	AAC CGG AAA GAA GTG TTG CG	GCA CCC AGA TGT ACG AAG TCC
Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)	mouse	CAA GGC AGG TTT CTG AGG AG	TGA AGC AGA TTC TCC ATG ACC
Interferon-induced protein with tetratricopeptide repeats 3 (IFIT3)	mouse	CCT ACA TAA AGC ACC TAG ATG GC	ATG TGA TAG TAG ATC CAG GCG T
Interleukin 1 beta (IL-1β)	mouse	ATG GCA ACT GTT CCT GAA CTC AAC T	CAG GAC AGG TAT AGA TTC TTT CCT TT
Interleukin 6 (IL-6)	mouse	TAG TCC TTC CTA CCC CAA TTT CC	TTG GTC CTT AGC CAC TCC TTC
Ly6G	mouse	TGC GTT GCT CTG GAG ATA GA	CAG AGT AGT GGG GCA GAT GG
Monocyte chemoattractant proetin 1 (MCP-1)	mouse	AGG TCC CTG TCA TGC TTC TG	TCT GGA CCC ATT CCT TCT TG
spliced X-box binding protein 1 (sXBP1)	mouse	GAG TCC GCA GCA GGT G	GTG TCA GAG TCC ATG GGA
Tumor necrosis factor alpha (TNFα)	mouse	CCC TCA CAC TAG ATC ATC TTC T	GCT ACG ACG TGG GCT ACA G
18S	mouse	ACG GAA GGG CAC CAC CAG GA	CAC CAC CAC CCA CGG AAT CG
Interferon beta (IFNβ)	human	CGC CGC ATT GAC CAT CTA	GAC ATT AGC CAG GAG GTT CT
Interferon regulatory factor 3 (IRF3)	human	GAC CTT CCA TCG TAG GCC G	GGT TGG CAG GTC TGG CTT AT

Interferon-induced protein with tetratricopeptide repeats 3 (IFIT3)	human	GAA CAT GCT GAC CAA GCA GA	GAA CAT GCT GAC CAA GCA GA
18S	human	CGG CTA CCA CAT CCA AGG AA	GCT GGA ATT ACC GCG GCT