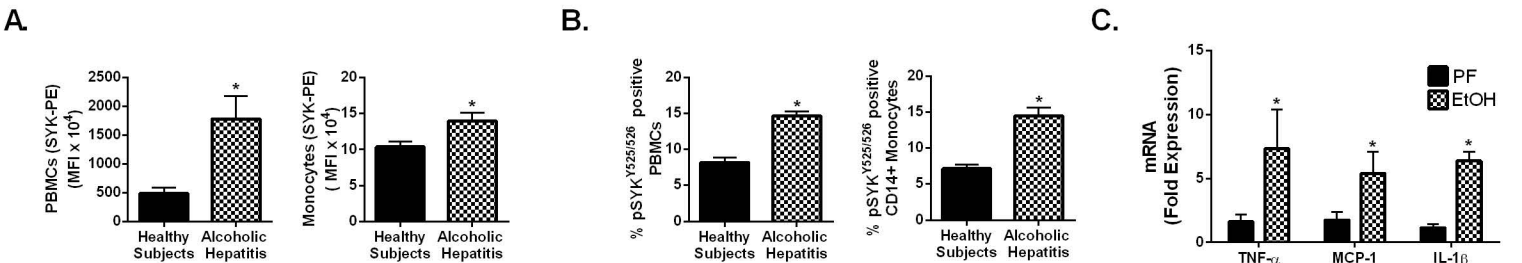
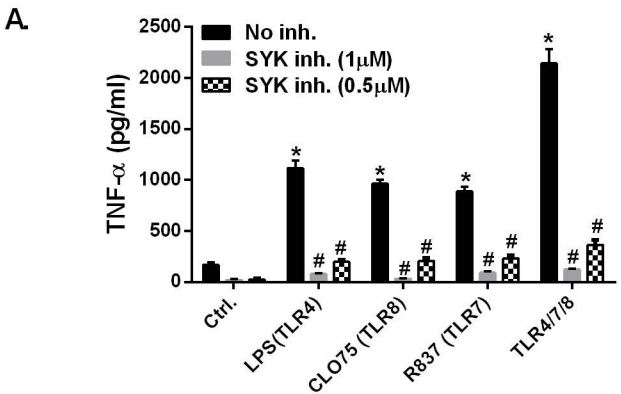
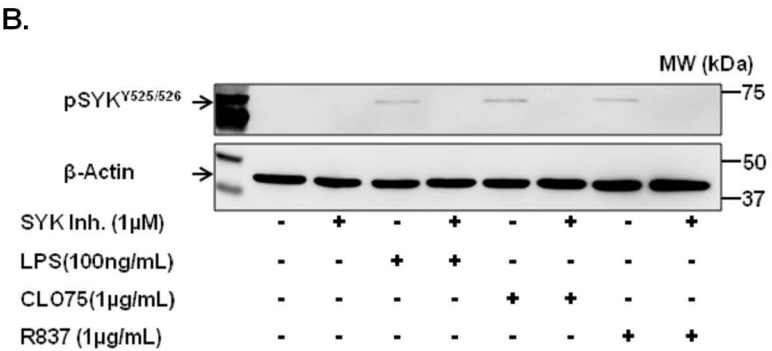
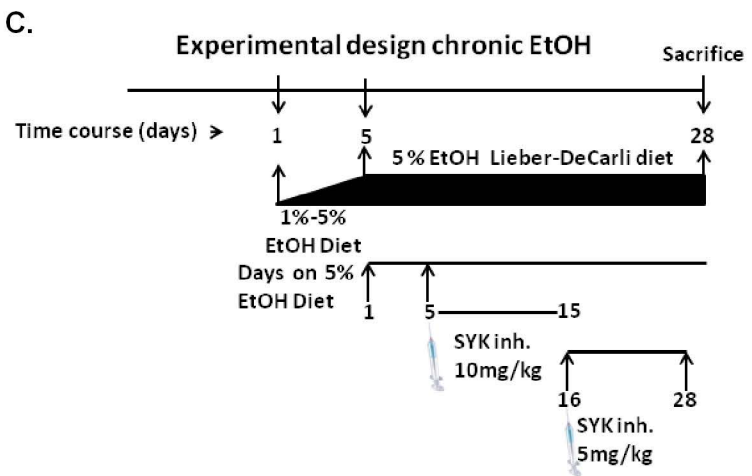
Sup. Fig. 1

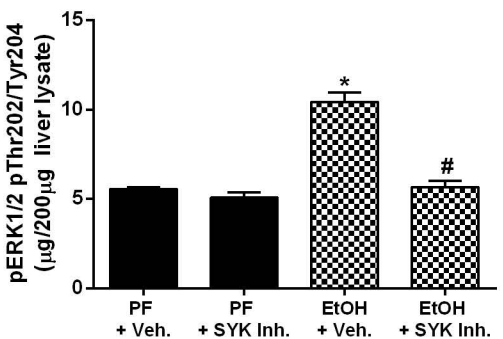

A.
PBMCs (SYK-PE) (MFI x 10^4)
2500
2000
1500
1000
500
0
*
Healthy Subjects
Alcoholic Hepatitis
Monocytes (SYK-PE) (MFI x 10^4)
20
15
10
5
0
*
Healthy Subjects
Alcoholic Hepatitis
B.
% pSYK Y525/526 positive PBMCs
20
15
10
5
0
*
Healthy Subjects
Alcoholic Hepatitis
% pSYK Y525/526 positive CD14+ Monocytes
20
15
10
5
0
*
Healthy Subjects
Alcoholic Hepatitis
C.
mRNA (Fold Expression)
15
10
5
0
PF
EtOH
*
*
*
TNF-α
MCP-1
IL-1β

Sup. Fig. 2


A.
TNF-α (pg/ml)
2500
2000
1500
1000
500
0
No inh.
SYK inh. (1μM)
SYK inh. (0.5μM)
Ctrl.
LPS(TLR4)
CLO75 (TLR8)
R837 (TLR7)
TLR4/7/8

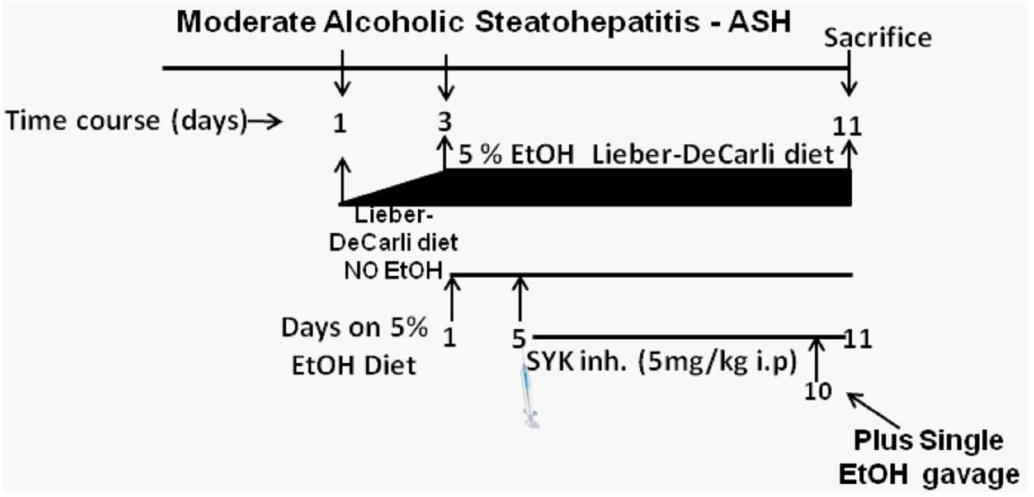

B.
MW (kDa)
pSYK Y525/526
β-Actin
75
50
37
SYK Inh. (1μM)
LPS(100ng/mL)
CLO75(1μg/mL)
R837 (1μg/mL)


C.
Experimental design chronic EtOH
Sacrifice
Time course (days)
1
5
28
5 % EtOH Lieber-DeCarli diet
1%-5%
EtOH Diet
Days on 5%
EtOH Diet
1
5
15
SYK inh.
10mg/kg
16
28
SYK inh.
5mg/kg

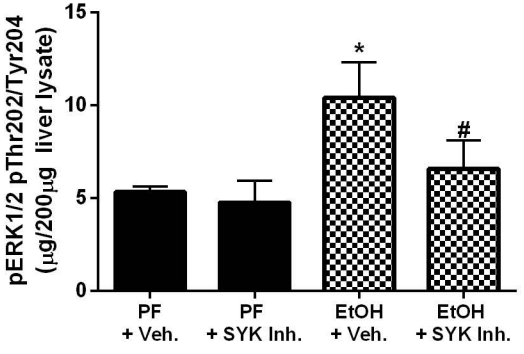

D.
pERK1/2 pThr202/Tyr204
(μg/200μg liver lysate)
15
10
5
0
PF
+ Veh.
PF
+ SYK Inh.
EtOH
+ Veh.
EtOH
+ SYK Inh.

Sup. Fig. 3

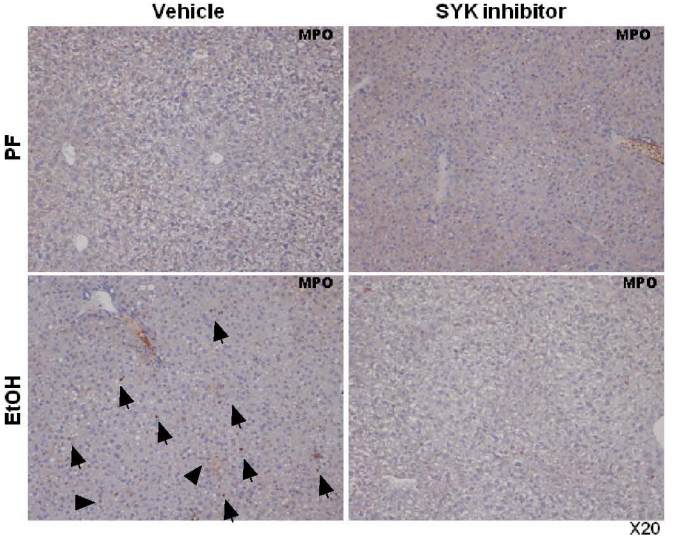
A.


Moderate Alcoholic Steatohepatitis - ASH
Sacrifice
Time course (days)→
1
3
11
5 % EtOH Lieber-DeCarli diet
Lieber-DeCarli diet NO EtOH
Days on 5% EtOH Diet
1
5
11
SYK inh. (5mg/kg i.p)
10
Plus Single EtOH gavage

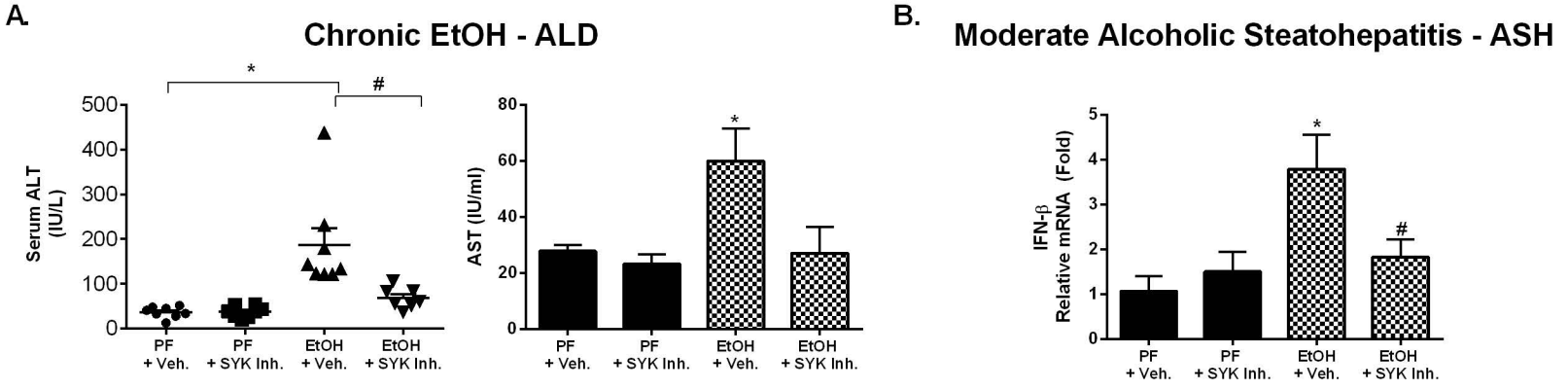
B.


pERK1/2 pThr202/Tyr204 (μg/200μg liver lysate)
15
10
5
0
*
#
PF + Veh.
PF + SYK Inh.
EtOH + Veh.
EtOH + SYK Inh.

C.

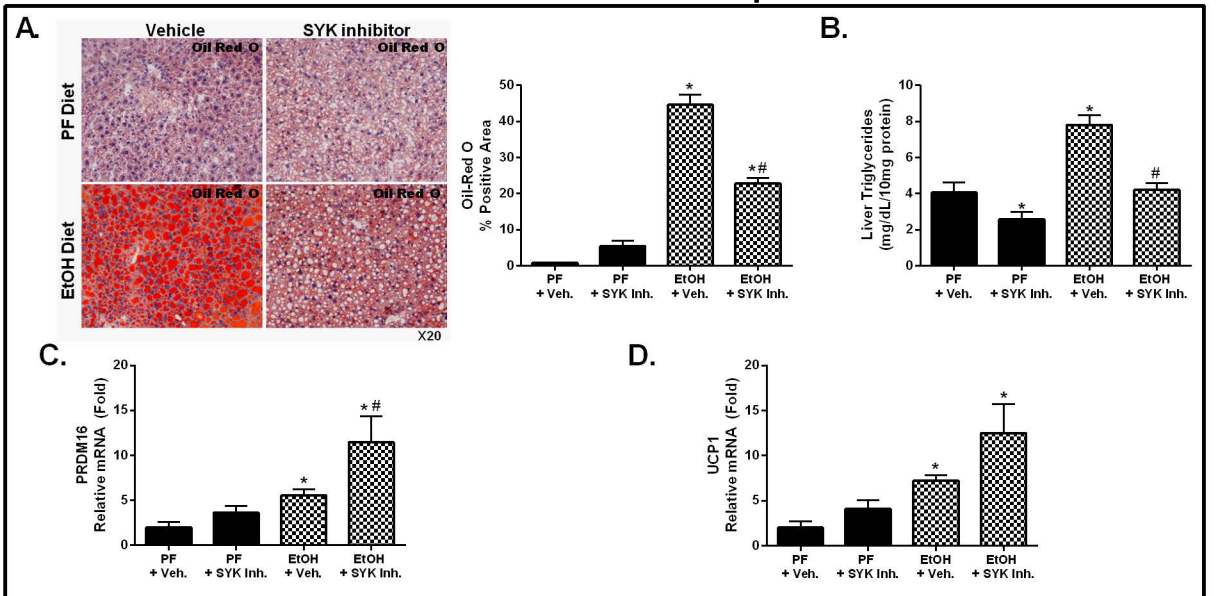

Vehicle
SYK inhibitor
PF
EtOH
MPO
X20

Sup. Fig. 4


A.
Chronic EtOH - ALD
Serum ALT (IU/L)
500
400
300
200
100
0
*
#
PF + Veh.
PF + SYK Inh.
EtOH + Veh.
EtOH + SYK Inh.
AST (IU/ml)
80
60
40
20
0
*
PF + Veh.
PF + SYK Inh.
EtOH + Veh.
EtOH + SYK Inh.
B.
Moderate Alcoholic Steatohepatitis - ASH
IFN-β Relative mRNA (Fold)
5
4
3
2
1
0
*
#
PF + Veh.
PF + SYK Inh.
EtOH + Veh.
EtOH + SYK Inh.

Sup. Fig 5

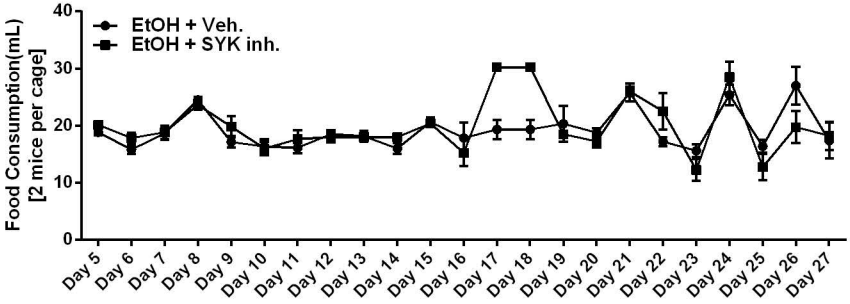
Moderate Alcoholic Steatohepatitis - ASH



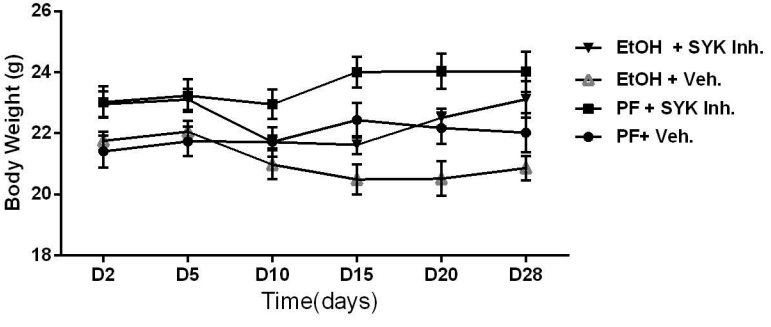
Sup. Fig. 6

Chronic model

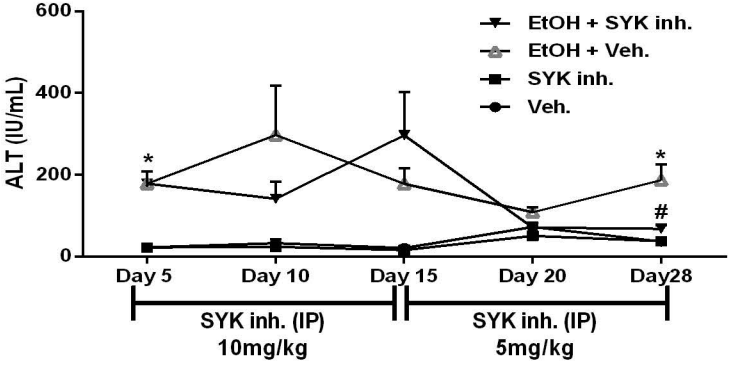
A.



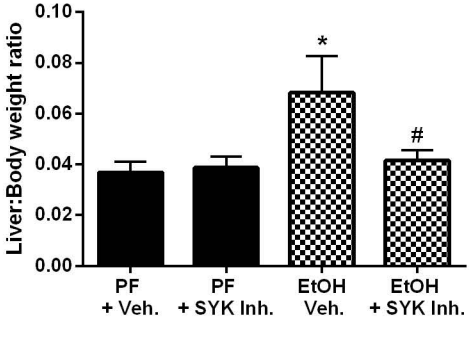
B.



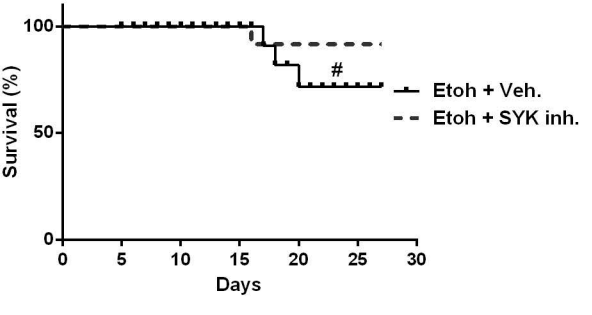
C.



D.



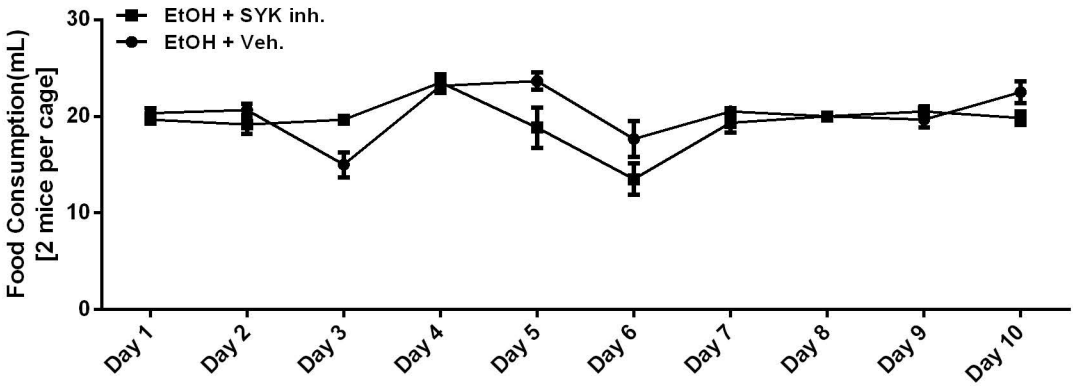
E.



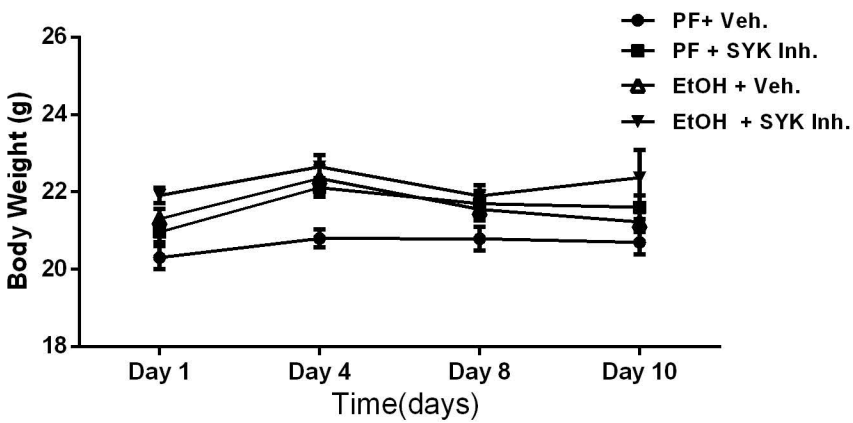
Sup. Fig. 7

Moderate Alcoholic Steatohepatitis - ASH

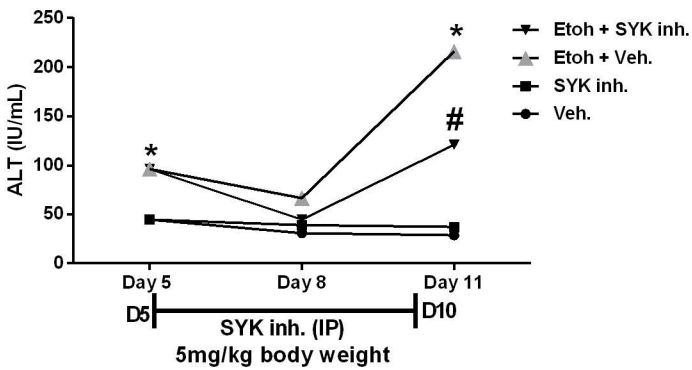
A.



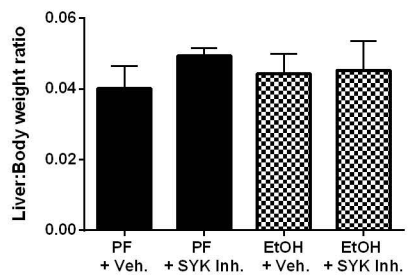
B.



C.



D.



Inhibition of spleen tyrosine kinase activation ameliorates inflammation, cell death, and steatosis in alcoholic liver disease

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Supplementary information

Methods:

SYK inhibitor administration and safety measures

For animal use, the SYK inhibitor R406 was reconstituted for in-vivo use according the manufacturers specification (Selleckchem cat # S2194). Mice on control diet or ethanol diet received daily i.p. SYK inhibitor at 5mg/kg body weight for 3-day binge and NIAAA alcohol liver disease models as indicated in the specific experimental scheme. Mice on chronic ethanol diet (sup. Fig. 2C) received daily 10mg/kg body weight of SYK inhibitor from the fifth day of ethanol diet. SYK inhibitor administered with chronic alcohol feeding was well tolerated (Sup. Fig. 6) and had no adverse effects on food consumption (Sup. Fig. 6A) and body weight gained with respect to alcohol fed vehicle group (Sup. Fig. 6B). SYK inhibitor treatment did not increase serum ALT (Fig. 6C) or liver-to-body weight at the end of experiments in the SYK treated control groups (Sup. Fig. 6D). The dose of SYK inhibitor was reduced to 5mg/kg body weight as indicated in the treatment outline (Sup. Fig. 2D) given observed increase in serum alanine aminotransferase in the SYK treated group to over 4 times upper limit of control mice on vehicle (Sup. Fig. 6C). This low dose was well tolerated with ethanol feeding leading to significantly lower mortality in the SYK treated group compared to the vehicle group (Fig.6E).

Low dose SYK inhibitor administered with moderate alcoholic steatohepatitis feeding (Sup. Fig.3A) was well tolerated and had no effect on food consumption (Sup. Fig. 7A), body weight (Sup. Fig. 7B), serum ALT (Fig. 7C) or liver-to-body weight at the end of experiments (Sup. Fig. 7D) in the control pair fed groups.

Assessment of hepatocellular damage

Serum ALT was assessed as previously described using a kinetic assay with reagents from Teco Diagnostics (USA).

Liver Histopathology

Liver specimens from mice were stained with either Hematoxylin/eosin (H&E), Oil Red O or MPO immunohistology (Antibody from Lifespan Biosciences cat # LS-B4741) using the UMASS Medical School DERC histology core facility.

Primary hepatocyte and liver mononuclear cell isolations:

Mice for this set of experiments were anesthetized with i.p. injection of ketamine/xylazine at 100-20mg/kg body weight and livers perfused by way of portal vein with saline solution followed by enzymatic digestion as previously reported(19, 46, 47). Hepatocytes and liver mononuclear cells were isolated as previously described(19, 46, 47).

Cell lines

RAW 264.7 cells were maintained in low glucose Dulbecco's minimal essential medium (DMEM) supplemented with 10% foetal bovine serum and 1% Penicillin/Streptomycin. Hepa1-6 cells for in-vitro studies were maintained in high glucose Dulbecco's minimal essential medium (DMEM) supplemented with 10% foetal bovine serum and 1% Penicillin/Streptomycin. Cells for microscopy were grown on glass cover slips in 24 well plates.

In-vitro lipid biogenesis and metabolism in Hepa1-6 cells:

Hepa1-6 cells were treated with palmitate (0.3mM) in high glucose cell culture medium with or without 1hour pretreatment of cells with SYK inhibitor (2µM) for 48h. Hepa1-6 cells were also stimulated with recombinant MCP1 (200ng/mL) with or without SYK inhibitor treatment (2µM) for 48h. At the end of experiments cells were fixed with 10% formol-saline and stained with Nile red (Life technologies cat. # N-1142) according the manufacturers specification. Stained cells were then placed on slides with mounting medium containing dapi and visualized by fluorescence microscopy.

ELISA of cell culture supernatants

Tumour Necrosis Factor alpha (TNF-α) concentrations were measured using a sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, 100µL of 1/10 diluted culture supernatants were added into flat-bottom 96-well micro titer plates pre-coated overnight with a capture antibody against TNF-α, followed by incubation for 2h at room temperature. Plates were washed three times, followed by the addition of 100 µl/well antibody against TNF-α conjugated with horseradish peroxidase. After 1 hour of incubation, plates were washed three times with wash buffer. Then 100µL substrate solution was added, and the plates were incubated for 30 min at room temperature in the dark. The reaction was terminated with acid stop solution (50µL). Optical densities of plates were read at 450 nm in a micro plate reader.

ERK1/2(pT202/Y204) was measured from liver protein lysates using the Abcam ERK1/2 (pT202/204) SimpleStep ELISA kit (cat # ab17+6640) according to the manufacturers guide. Briefly, liver tissue samples were minced and rinsed in ice cold PBS followed by homogenizing liver tissue in 1X cell extraction buffer PTR. Samples when then centrifuged at 15000rpms for 10 minutes at 4^{o}C. Supernatants were then transferred to fresh tubes and the pellet discarded. Samples were quantified for total protein using the BioRad protein assay reagent. 100µL liver protein lysate was used for ELISA according to the manufacturers guide. Optical densities of plates were read at 450nm in a micro plate reader. ERK1/2(pT202/Y204) in each sample was determined per 200µg of protein.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts from liver tissue were obtained by lysing cells with Buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% Nonidet P-40, 1 mM DTT, and 0.5 mM PMSF, followed by vortexing to shear the cytoplasmic membranes. Nuclei were pelleted by centrifugation at 3000 rpm for 5 min at 4°C in a microcentrifuge. Nuclear proteins were extracted with high-salt buffer B (20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 420 mM NaCl, 1 mM DTT, and 0.5 mM PMSF) and stored at -80°C. Total nuclear protein concentrations were determined using the Biorad protein assay. The NF-KB nuclear translocation was assessed by EMSA NF-KB consensus (Oligos ETC Inc., Wilsonville, OR) was end-labeled with T_4 polynucleotide kinase in the presence of [γ-^{32}P] ATP. Labeled probes were purified on a column (BioRad, Hercules, CA). Nuclear protein (4µg) was incubated with radioactive labeled probes for 20 min at room temperature. The mixture was electrophoresed on a polyacrylamide gel and dried gels were visualized by autoradiography. Band specificity was determined with competition experiments using a molar excess of unlabeled consensus oligonucleotide of NF-KB that were added to the nuclear extracts before the addition of radioactive labeled probes.

Quantitative Real time mRNA expression analysis

Total liver tissue RNA was purified using the RNeasy kit (Qiagen) with on-column DNASE treatment according to the manufacturer's specification. Total RNA extracted from liver tissue was quantified with Nanodrop and 1µg of total RNA used for cDNA synthesis using iScript cDNA Synthesis Kit (BIO-RAD). Real time qPCR was performed

using Sybr-Green reagent (BIO-RAD) and CFX96 Touch Real-Time PCR detection system (Bio-Rad). Comparative ΔΔCt method was used for gene expression analysis with 18s as housekeeping gene as previously described. The following primer sequences were used as detailed in table 2.

Table 2: List and sequences of primers used

Mouse Primers	Forward sequence 5' to 3'	Reverse sequence 5' to 3'
TNFα	CACCAC CATCAA GGACTC AA	AGGCAACCTGACCAC TCTCC
MCP1	CAGGTCCCT GTCATGCTTCT	TCTGGACCCATTCCTTCTTG
IL-1β	CTTTGAAGTTGACGGACCC	TGAGTGATACTGCCTGCCTG
MPO	CATCCAACCCTTCATGTTCC	CTGGCGATTCAGTTTGG
LY6G	TGCGTTGCTCTGCTGGAGATAGA	CAGAGTAGTGGGGCAGATGG
E-Selectin	ATGCCTCGCGCTTTCTCTC	GTAGTCCCGCTGACAGTATGC
PGC1α	AGACGGATTGCCCTCATTTGA	TGTAGCTGAGCTGAGTGTTGG
F4/80	TGCATCTAGCAATGGACAGC	GCCTTCTGGATCCATTTGAA
18s	GTA ACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy individuals (n=5) and alcoholic hepatitis patients (n=5). Isolated PBMCs were resuspended at a concentration of approximately 10^6 cells per 50µl in FACS staining buffer containing anti-Human BD Fc Block from BD Pharmingen (San Jose, CA) to block nonspecific binding to Fcγ receptors and incubated for 15 min at 4°C. Then we added CD14 PE-Cy7 antibody (eBioscience, San Diego, CA) for surface staining and incubated for 30 min in the dark at 4°C. For the negative control, the cells were stained with isotype-matched control antibody (eBioscience, San Diego, CA). The cells were fixed and permeabilized with BD fixation/permeabilization kit according to manufacturer's protocol (BD Pharmingen, San Jose, CA). The cells were then stained with pSYK$^{Tyr525/526}$-PE antibody (Cell signaling cat. #6485) or SYK-PE antibody or PE Mouse IgG2a isotype control (Biolegend, San Diego, CA). Cells were washed with FACS buffer and acquired on a BD-LSR II (BD Biosciences, San Diego, CA). Data obtained were acquired at the same time and analysed using FlowJo software.

Western Blot analysis and antibodies

Western blot was carried from protein extracted from liver samples using RIPA buffer (Boston Bio-products cat # BP-115) supplemented with protease inhibitor cocktail

(Roche Cat. # 11836153001) and Phospho Stop phosphatase inhibitor (Roche cat # 04906837001). Cell lysates were then centrifuged for 5minutes at 2000rpm to remove cell debris. Cell lysate supernatants containing total cell protein was quantified using the Bio-Rad protein assay to determine total soluble protein concentration following the manufacturer's protocol. Proteins were samples were boiled in Laemmli's buffer 2minutes. Proteins samples were resolved in 10% SDS-PAGE gel under reducing conditions and resolved proteins transferred onto a nitrocellulose membrane. Membranes were later probed with indicated primary antibodies followed by an appropriate secondary HRP-conjugated IgG antibody. Protein bands were developed and visualized using the Fujifilm LAS-4000 luminescent image analyzer. Antibodies used included SYK (cell Signaling cat. # 2712), phospho-SYK$^{Y525/526}$ (Cell signaling cat. # 2710), phospho-SYK$^{Y525/526}$ (Abcam Cat. # ab58575), β-Actin (Abcam cat. #ab6276), HMGB1 Caspase 3 (cat. # 9665), Cleaved caspase 3 (Cell Signaling cat. #9664), Caspase 1 (Abcam, cat. # ab108362), iNOS (Abcam cat. # ab129372), F4/80 (Abcam cat. # ab6640. Antibodies specific for phospho-IRF3, phospho-TBK1 were from Cell Signaling (Danvers, MA, USA). Antibodies against the total IRF3 was from Santa Cruz.

Statistical analysis:

All graphical representations are expressed as Mean +SEM for data analyzed, all statistical data presented in our manuscript were evaluated using two-way (Fig. 1) or one-way analysis of variance (ANOVA) with Fisher's least significant difference test for multiple comparisons with $p<0.05$ considered to be statistically significant.

Supplementary Figure Legend

Supplementary Figure 1: Alcohol induced liver inflammation is associated with increased SYK and activated SYK$^{Y525/526}$ in patient immune cells

SYK expression was assessed in healthy control individuals and patients with diagnosed alcoholic hepatitis in total PBMCs and monocytes by flow cytometry (A). Activated pSYK$^{Y525/526}$ expression determined in PBMCs and monocytes of alcoholic patients and healthy control subjects by flow cytometry (B). C57BL/6 mice received chronic EtOH feeding for 5 weeks (C). Expression of TNF-α, MCP-1 and IL-1β in the liver was analyzed by qPCR (C). *$p<0.05$ compared to baseline was considered statistically significant by ANOVA for 7-10 mice per experimental group and for 5 healthy control donors and 5-8 patients with diagnosed alcoholic hepatitis/cirrhosis.

Supplementary Figure 2: Functional SYK inhibition decreased in-vitro TLR activation and acute/chronic alcohol feeding induced inflammatory cytokine release and ERK1/2 activation

(A&B) RAW 264.7 cells were pretreated with the SYK inhibitor R406 (0.5 and 1µM) for 4h prior to TLR ligand [LPS, Clo75 and R837] activation stimulation for 6h after which culture supernatants were collected and analyzed for (A) TNF-α by ELISA and total protein extracted from cells was analysed by western blotting for (B)pSYK$^{Y525/526}$ using β-actin as loading control. Schematic illustration of alcohol feeding and SYK inhibitor treatment for chronic alcohol in mice (C). Total liver protein was analysed for phospho ERK1/2 [pThr202/Tyr204] by ELISA following acute or chronic alcohol feeding with and without SYK inhibitor treatment as indicated (D). $^{*/\#}p<0.05$ was considered statistically significant by ANOVA.

Supplementary Figure 3: SYK inhibitor treatment reduces moderate alcoholic steatohepatitis alcohol induced ERK1/2 phosphorylation, and hepatic neutrophil infiltration

C57BL/6 mice were fed alcohol or control diet using moderate alcoholic steatohepatitis ethanol model with or without SYK inhibitor or vehicle treatment as detailed (A). Total liver protein was assessed for phospho ERK1/2 by ELISA (B). Hepatic neutrophils were assessed by MPO immunohistology staining (C). $^{*/\#}p<0.05$ compared to baseline or EtOH vehicle group was considered statistically significant by ANOVA for 6-8 mice per experimental group Slide is a representation for 6-8 mice per experimental group.

Supplementary Figure 4: Acute and chronic alcohol induced hepatic cell death is reduced with SYK inhibitor treatment

Mice were fed chronic (A) or moderate alcoholic steatohepatitis alcohol (B) diet with or without SYK inhibitor treatment as indicated. Serum samples were analysed by kinetic assay for ALT and AST (A). Total liver RNA was analysed for IFN-β mRNA using 18s RNA house keeping control (B). $^{*/\#}p<0.05$ compared to baseline or EtOH vehicle group was considered statistically significant by ANOVA for 8-10 mice per experimental group.

Supplementary Figure 5: Moderate alcoholic steatohepatitis alcohol induced hepatic steatosis is ameliorated with SYK inhibitor treatment

C57BL/6 mice were fed moderate alcoholic steatohepatitis alcohol with or without SYK inhibitor treatment as indicated. Liver steatosis was evaluated by Oil-red-O staining (A) and liver triglyceride assay (B). Liver RNA was analysed for PRDM16 (C) and UCP1 (D). $^{*/\#}p<0.05$ compared to baseline or EtOH vehicle group was considered statistically significant by ANOVA for 6-8 mice per experimental group.

Supplementary Figure 6: Pharmacodynamics of the SYK inhibitor during chronic alcohol feeding in mice

C57BL/6 mice were fed chronic ethanol diet with or without SYK inhibitor or vehicle treatment as detailed in the experimental outline above. Matched pair fed, vehicle and drug control groups were assessed for all animal experiments. Food consumption for ethanol diet group with SYK inhibitor or vehicle treatment was measured daily for the entire experimental period (A). Additionally body weight (B) and serum ALT (C) was

measured weekly for all SYK inhibitor or vehicle treated groups on control and alcohol diet. Further, liver to body weight ratio (D) and mouse survival (E) for ethanol diet mice with SYK inhibitor or vehicle treatment was assessed. $^{*/\#}p<0.05$ compared to baseline or EtOH vehicle group was considered statistically significant by ANOVA for 8-10 mice per experimental group.

Supplementary Figure 7: Pharmacodynamics of the SYK inhibitor during moderate alcoholic steatohepatitis alcohol feeding in mice

C57BL/6 mice were fed moderate alcoholic steatohepatitis alcohol diet or isocaloric control diet with or without SYK inhibitor as indicated. Daily food consumption for ethanol diet group with SYK inhibitor or vehicle treatment was measured daily for the entire experimental period (A). Body weight (B) and serum ALT (C) was measured weekly for all SYK inhibitor or vehicle treated groups on control and alcohol diet. Liver to body weight ratio (D) was assessed for all groups following termination of experiments. $^{*/\#}p<0.05$ compared to baseline or EtOH vehicle group was considered statistically significant by ANOVA for 8-10 mice per experimental group.