Supporting Materials:

Cell type-dependent pro- and anti-inflammatory role of STAT3 in alcoholic liver injury

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Materials and Methods:

Materials. Anti-STAT3, anti-phospho-STAT3 (Tyr705), anti-STAT1, anti-AMPKα, and anti-phospho-AMPKα (Thr172) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-SREBP1 and anti-CYP2E1 antibodies were purchased from US Biological (Swampscott, MA) and Chemicon International (Temecula, CA), respectively.

Blood chemistry and hepatic lipid contents. Serum alanine transaminase (ALT), triglycerides, glucose, and cholesterol were determined using a clinical chemistry analyzer system (PROCHEM-V; Drew Scientific, Barrow-in-Furness, UK). Serum insulin was determined using the Ultrasensitive Mouse Insulin EIA kit (ALPCO Diagnostics, Winham, NH). Hepatic lipid content was measured as described previously[1]. Briefly, liver tissue (100 mg) was homogenized in lysis buffer. Lipids from the total liver homogenate were extracted using chloroform/methanol (2:1), evaporated, and dissolved in 2-propanol.

Histology and immunohistochemistry. Formalin-fixed liver samples were processed, and paraffin sections of 5-μm thickness were stained with hematoxylin and eosin (H&E). Inflammatory foci were counted in 10 randomly selected fields (X100 magnification).

Neutrophils were immunostained with anti-MPO antibodies (ZYMED Laboratories, San Francisco, CA).

Oil red O staining lipid content. For analysis of fat accumulation in the liver, 10-μm thick frozen sections were stained with Oil Red O (Vector Laboratories, Burlingame, CA), counterstained with Mayer's hematoxylin, and analyzed by light microscopy.

Serum cytokine levels. Serum cytokine levels were measured using cytometry bead array (BD Bioscience) according to the manufacturer's protocol.

Western blotting. Western blot analyses were performed with proteins from liver homogenates (80 μg) using anti-STAT3 (1:1000 dilution), anti-AMPKα (1:1000 dilution), and anti-phospho AMPKα (1:1000 dilution) antibodies. For Western blotting of mature SREBP1, nuclear proteins were extracted using a nuclear/cytosol fraction kit (BioVision, Mountain View, CA) according to the manufacturer's protocol, and 40 μg of proteins were loaded. Immunoreactive bands were visualized on nitrocellulose membranes using alkaline-phosphotase-linked anti-mouse or rabbit antibody and the ECF detection system with a PhosphorImager (GE Healthcare, Piscataway, NJ). The densities of bands were quantified by using an ImageQuant software (GE Healthcare). The relative density was calculated by the ratio of the interest gene density/β-actin gene density. In order to make all independent experiments comparable, we designated the ratio of the interest gene density/β-actin gene density as 1 in each experiment.

Preparation of peritoneal macrophages. To isolate peritoneal macrophages, mice were intraperitoneally injected with 2 mL of 4% thioglycollate (Sigma). Three days post injection, cells from the peritoneal exudate were isolated from the peritoneal cavity and cultured on a plastic plate for 2 h and washed three times with RPMI160. Remaining adherent cells were used as peritoneal macrophages for Western blot analyses of STAT3 protein expression.

Isolation of hepatocytes and Kupffer cells. Hepatocytes were isolated as described previously[2]. Kupffer cells were isolated by *in situ* collagenase perfusion and differential centrifugation on OptiPrep (Sigma) density gradient. Mouse livers were perfused *in situ* first with EGTA solution (5.4 mM KCl, 0.44 mM KH_2PO_4, 140 mM NaCl, 0.34 mM Na_2HPO_4, 0.5 mM EGTA, 25 mM Tricine, pH 7.2), followed by perfusion buffer (0.075% collagenase type I in GBSS buffer with 0.02% DNase I), and digestion buffer (0.009% collagenase type I in GBSS buffer with 0.02% DNase I) at 37°C for 20-30 min. To remove hepatocytes, the homogenate was filtered and centrifuged at 25 *g* for 5 min at room temperature. The supernatant was transferred to a new tube and centrifuged at 400 *g* for 10 min at 4°C, while the pellet was resuspended in 6 mL of 17% OptiPrep, and loaded carefully with 3 mL of GBSS washing buffer and centrifuged at 1600 *g* for 17 min at 4°C. The cellular fraction recovered from the GBSS and 17% OptiPrep interphase was gently aspirated, mixed with 13 mL GBSS, and centrifuged at 576 *g* for 10 min at 4°C. Pellets were then resuspended in culture medium and plated on non-collagen coated plates for 20 min. Then, cells were washed with PBS to remove debris and unattached cells. The attached Kupffer cells were detached by incubating cells with ETDA-trypsin (0.25%) for 5 min, then collected and washed with PBS once. Purity of the cultures was assessed by immunohistochemical analyses with anti-F4/80 antibody and flow cytometric analysis. More than 90% of cells were Kupffer cells with F4/80 positive staining.

Flow cytometric analysis. Kupffer cells were isolated and stimulated with IL-6 (100 ng/ml) for 30 min. The cells were then fixed and analyzed with flow cytometric analyses using anti-F4/80, anti-CD11b, and anti-pSTAT3 antibodies.

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Measurement of reactive oxidative species (ROS). Intracellular ROS was determined by DCFDA fluorescence emission (Molecular Probes), which can be hydrolyzed by cellular nonspecific esterases and oxidized to form the fluorescent product, 2′,7′-dichlorofluorescein (DCF), after reacting with ROS. Freshly isolated hepatocytes (5 x 10^4 /well) and Kupffer cells (1 x 10^5 /well) were incubated with DCFDA (5 μM) for 15 min. Fluorescence was determined at 535 nm with excitation at 485 nm (slit width: 5 nm) on a Perkin-Elmer Victor3 spectrofluorometer.

Mouse model for chronic ethanol consumption. Eight to 10-week old male mice were fed a nutritionally adequate liquid diet containing 5% ethanol or a control diet in which ethanol was substituted isocalorically with dextrin maltose (Bio Serv Inc., French town, NJ) for up to 8 weeks. Most groups were fed for 4 weeks unless specified otherwise. Both diets were dispensed in glass liquid-diet feeding tubes (Bio Serv Inc.). Ethanol was introduced gradually by increasing the content by 1% (vol/vol) every day until the mice were consuming diets containing 5% (vol/vol) ethanol for up to 8 weeks. During the entire feeding period, H-STAT3KO mice, M/N-STAT3KO mice and their WT controls were observed to consume similar volumes of ethanol diets daily and their body weight gains were also similar.

Lipopolysaccharide (LPS) stimulation of Kupffer cells and TNF-α production. Freshly isolated Kupffer cells (1 x 10^5 /well) were cultured in 96-well plates for 16 h, after which, the cell culture media were removed and replaced with 100 μL of CMRL-10% FBS with or without LPS. After stimulation with LPS for 4 h, cell culture media were removed and stored at -20°C for TNF-α assays with ELISA kits (e Bioscience)

Semiquantitive RT-PCR. Total RNA was isolated from liver tissue using TRIZOL. The cDNA was prepared using the Super Script First-Strand Synthesis System, according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Primers used are listed in Table I. The mouse ß-actin gene was also amplified and used as a control. The PCR products were separated by electrophoresis on a 1.5 to 3.0% agarose gel. RNA without reverse transcription did not yield any amplicons, indicating the absence of genomic DNA contamination. The densities of bands were quantified with a PhosphorImager (Molecular Dynamics).

Table I: Primer sequences for RT-PCR.

Name	Forward primer (5'…3')	Reverse primer (5'…3')	Size (bp)
SREBP-1c	GCAGTCTGCTTTGGAACCTC	CCACAAAGAAACGGTGACCT	447
FAS	TACCAGTGCCACAGGAGTCTCA	TAAACACCTCGTCGATTTCGTTC	158
ACC1	GTTGCACAAAAGGATTTCA	CGCATTACCATGCTCCGC	484

SCD1	CTGCCTCTTCGGGATTTTCTACT	GCCCATTCGTACACGTGATTC	161
CPT-1	TCCATGCATACCAAAGTGGA	TGGTAGGAGAGCAGCACCTT	185
PPARα	GCAGCTCGTACAGGTCATCA	ACTGCCGTTGTCTGTCACTG	327
PCK1	TGGATGTCGGAAGAGGACTT	TGCAGGCACTTGATGAACTC	236
G6PC	TCGGAGACTGGTTCAACCTC	TGGCTTTTTCTTTCCTCGAA	233
PGC1α	AAATGCAGCGGTCTTAGCACT	TTTCTGTGGGTTTGGTGTGA	207

SREBP: sterol regulatory element-binding protein; FAS: fatty acid synthase; ACC1: acetyl-CoA carboxylase-1; SCD1: stearoyl-CoA desaturase 1; CPT1: carnitine palmitoyltransferase I; PPAR-α: peroxysome proliferator-activated receptor-α; PCK-1: phosphoenolpyruvate carboxylase-1; G6PC: glucose-6 phosphatase; PGC1α: peroxisome proliferator-activated receptor-γ coactivator-1α.

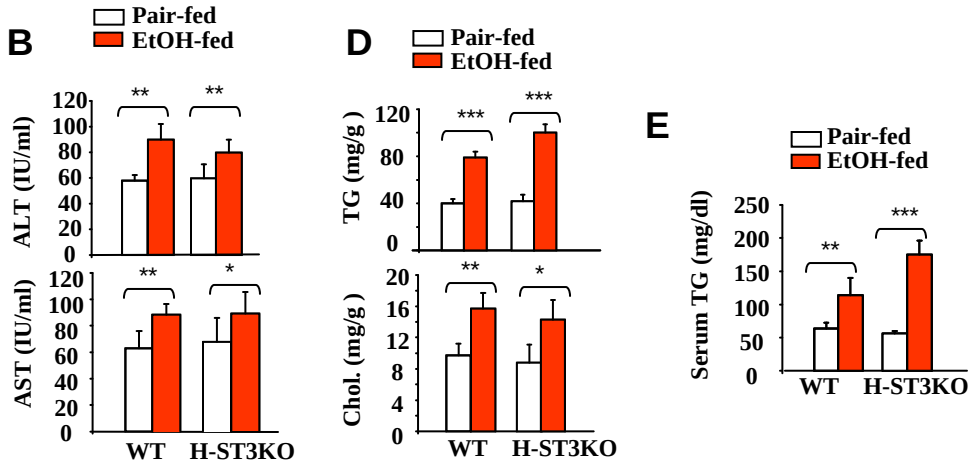
Real Time PCR. Hepatic expression of proinflammatory cytokines and chemokines was measured by real-time quantitative PCR, using a model Chromo 4 real-time PCR detection system (Bio-Rad, Hercules, CA). Primers used in real-time PCR are listed in Table II.

Table II: Primer sequences for real-time PCR.

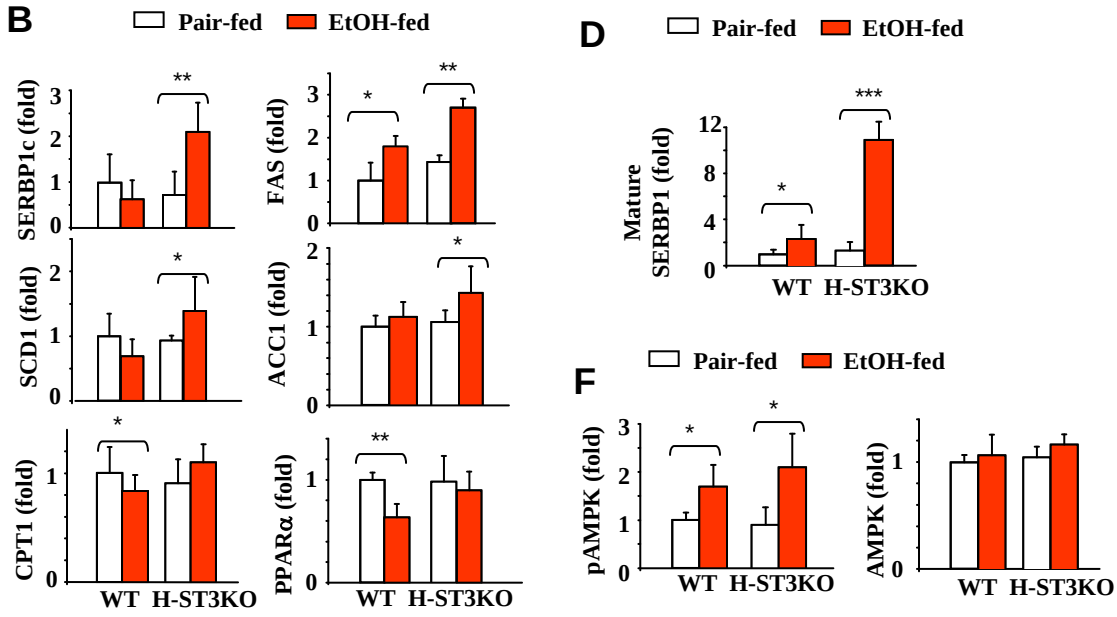
genes	Forward primer (5'…3')	Reverse primer (5'…3')
TNF-α	AAGCCTGTAGCCCACGTCGTA	AGGTACAACCCATCGGCTGG
IFN-γ	TAGCCAAGACTGTGATTGCGG	AGACATCTCCTCCCATCAGCAG
IL-1β	AAAAAAGCCTCGTGCTGTCG	GTCGTTGCTTGGTTCTCCTTG
IL-6	TCCATCCAGTTGCCTTCTTG	TTCCACGATTTCCCAGAGAAC
MCP-1	TCAGCCAGATGCAGTTAACGC	TCTGGACCCATTCCTTCTTGG
MIP1α	TGCCCTTGCTGTTCTTCTCTG	CAACGATGAATTGGCGTGG
MIP-1β	AACACCATGAAGCTCTGCGTG	TGTCTGCCTCTTTTGGTCAGG
MIP2	AGTGAACTGCGCTGTCAATGC	AGGCAAACTTTTTGACCGCC
C3	AAGCATCAACACACCCAACA	CTTGAGCTCCATTCGTGACA
C5	TGTCTTGCCACGATTCTCTG	TGTGCATCATCTGCTTCTCC
CCR2	ATGCAAGTTCAGCTGCCTGC	ATGCCGTGGATGAACTGAGG
F4/80	GGAAAGCACCATGTTAGCTGC	CCTCTGGCTGCCAAGTTAATG

MCP-1: monocyte chemoattractant protein 1; MIP: macrophage inflammatory protein; C3: complement 3; CCR2: CC chemokine receptor 2.

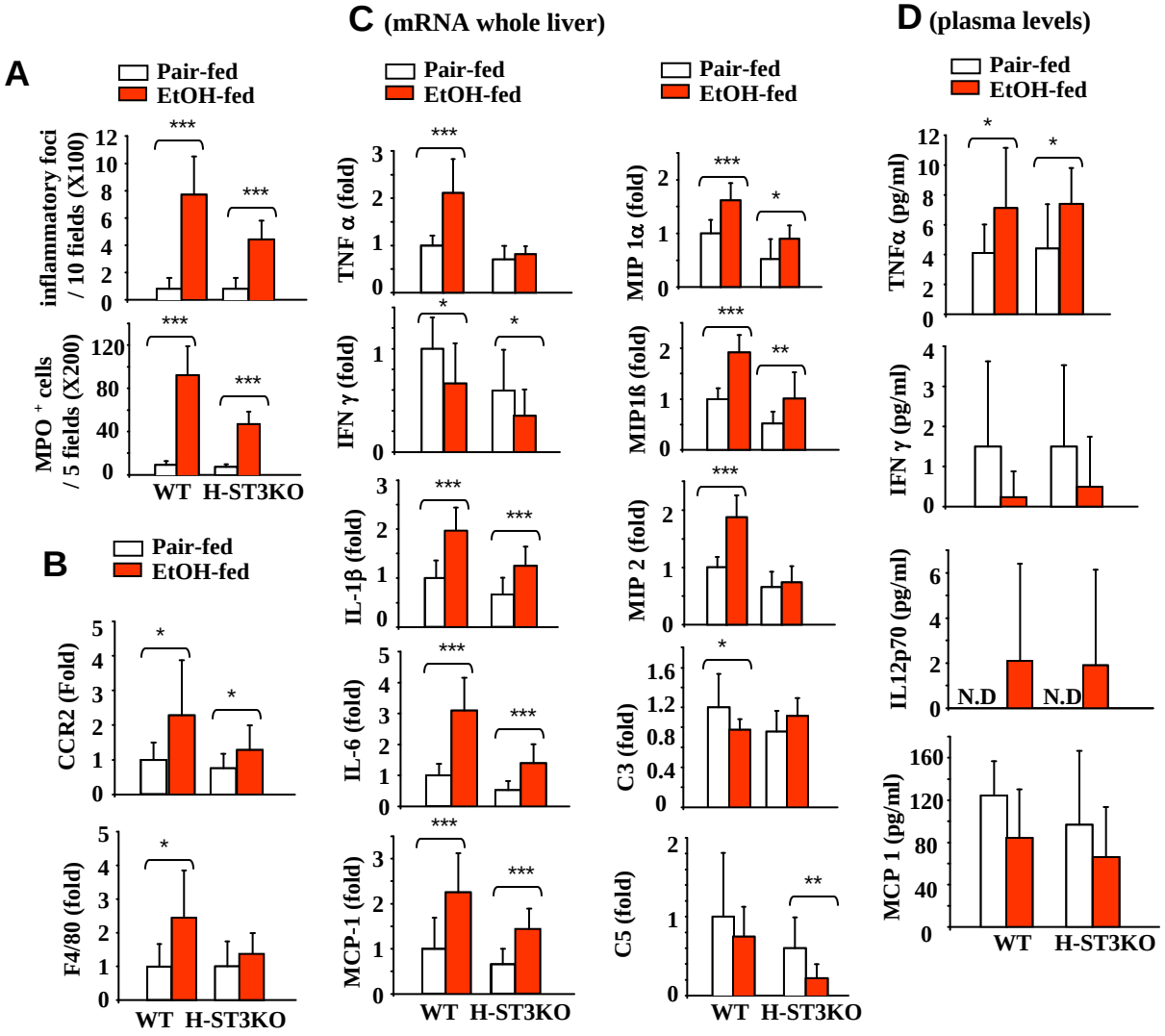
1. Hong F, Radaeva S, Pan HN, et al. Interleukin 6 alleviates hepatic steatosis and ischemia/reperfusion injury in mice with fatty liver disease. Hepatology 2004;40:933-41.
2. Sun R, Park O, Horiguchi N, et al. STAT1 contributes to dsRNA inhibition of liver regeneration after partial hepatectomy in mice. Hepatology 2006;44:955-66.



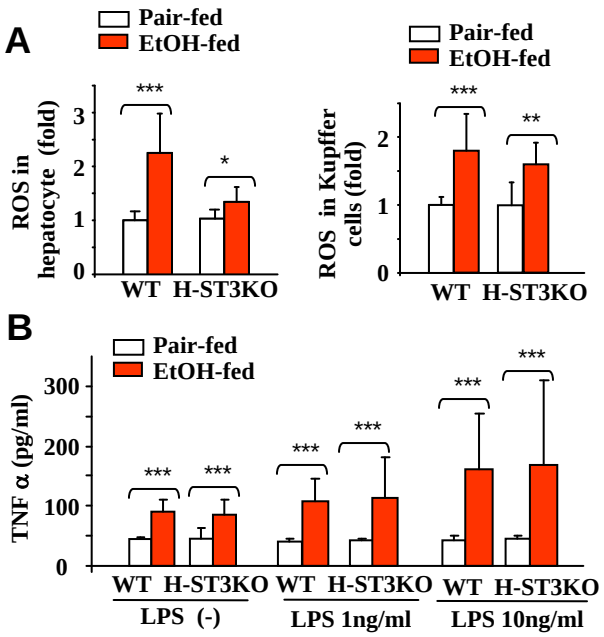
sFig. 1: Statistical comparisons between pair-fed and ethanol-fed WT mice, and between pair-fed and ethanol-fed KO mice of Fig. 1. **P*<0.05, ***P*<0.01, ****P*<0.001 denotes significant differences in comparison with corresponding pair-fed groups (white bars).



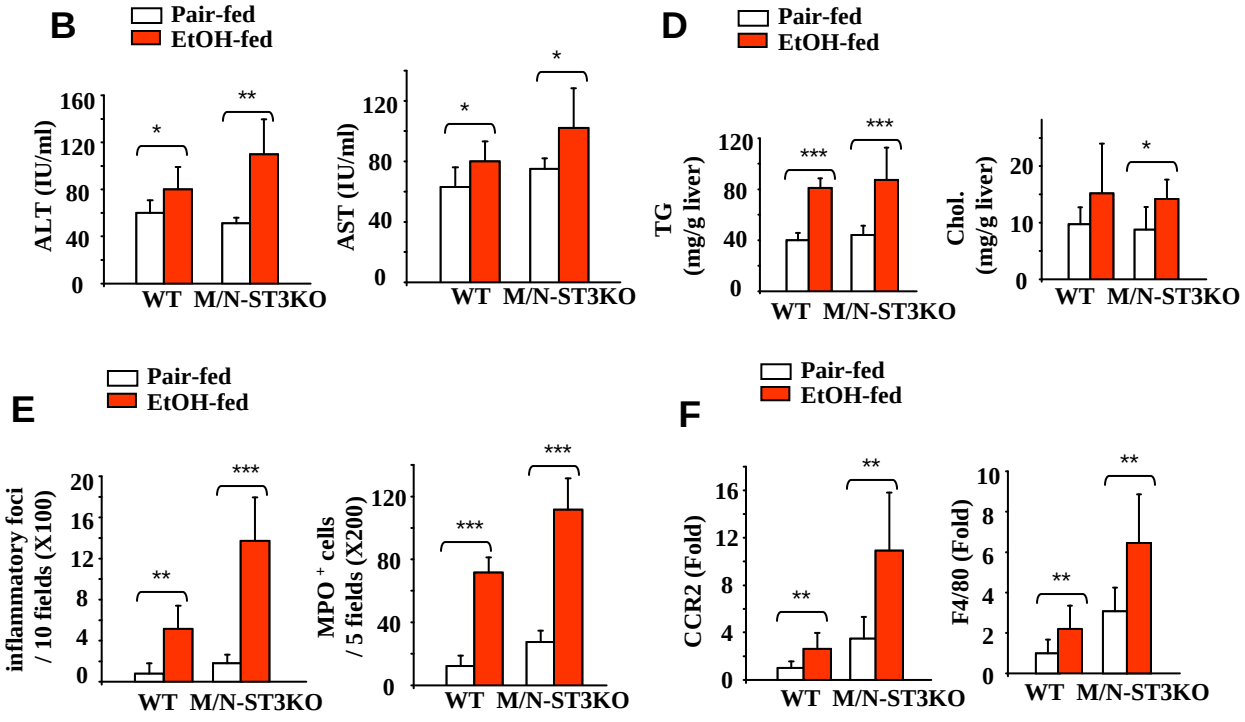
sFig. 2: Statistical comparisons between pair-fed and ethanol-fed WT mice, and between pair-fed and ethanol-fed KO mice of Fig. 2. **P*<0.05, ***P*<0.01, ****P*<0.001 denotes significant differences in comparison with corresponding pair-fed groups (white bars).



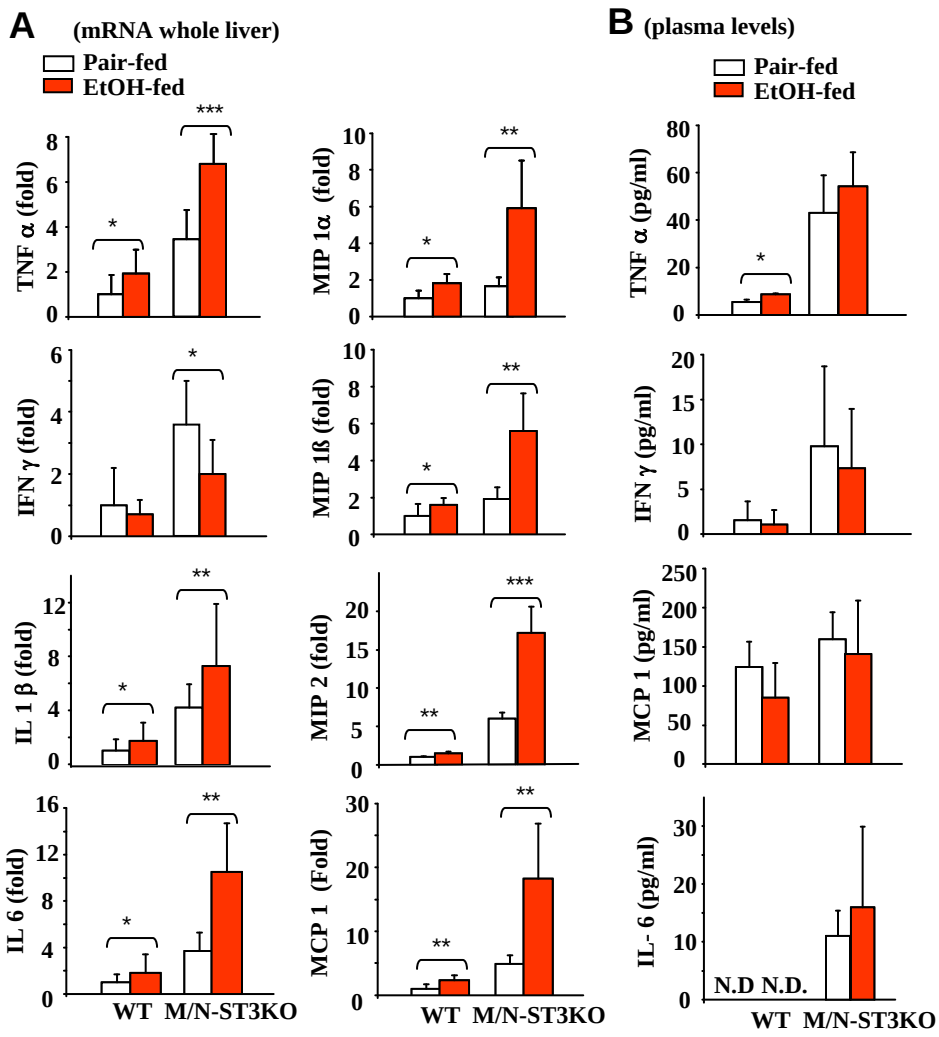
sFig. 3: Statistical comparisons between pair-fed and ethanol-fed WT mice, and between pair-fed and ethanol-fed KO mice of Fig. 3. **P*<0.05, ***P*<0.01, ****P*<0.001 denotes significant differences in comparison with corresponding pair-fed groups (white bars).



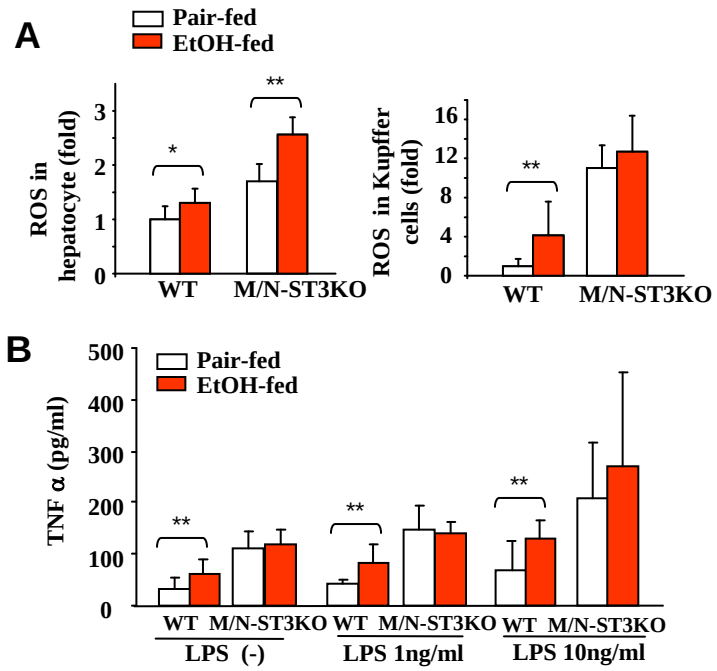
sFig. 4: Statistical comparisons between pair-fed and ethanol-fed WT mice, and between pair-fed and ethanol-fed KO mice of Fig. 4. **P*<0.05, ***P*<0.01, ****P*<0.001 denotes significant differences in comparison with corresponding pair-fed groups (white bars).



sFig. 5: Statistical comparisons between pair-fed and ethanol-fed WT mice, and between pair-fed and ethanol-fed KO mice of Fig. 5. **P*<0.05, ***P*<0.01, ****P*<0.001 denotes significant differences in comparison with corresponding pair-fed groups (white bars).



sFig. 6: Statistical comparisons between pair-fed and ethanol-fed WT mice, and between pair-fed and ethanol-fed KO mice of Fig. 6. **P*<0.05, ***P*<0.01, ****P*<0.001 denotes significant differences in comparison with corresponding pair-fed groups (white bars).



sFig. 7: Statistical comparisons between pair-fed and ethanol-fed WT mice, and between pair-fed and ethanol-fed KO mice of Fig. 7. **P*<0.05, ***P*<0.01, ****P*<0.001 denotes significant differences in comparison with corresponding pair-fed groups (white bars).

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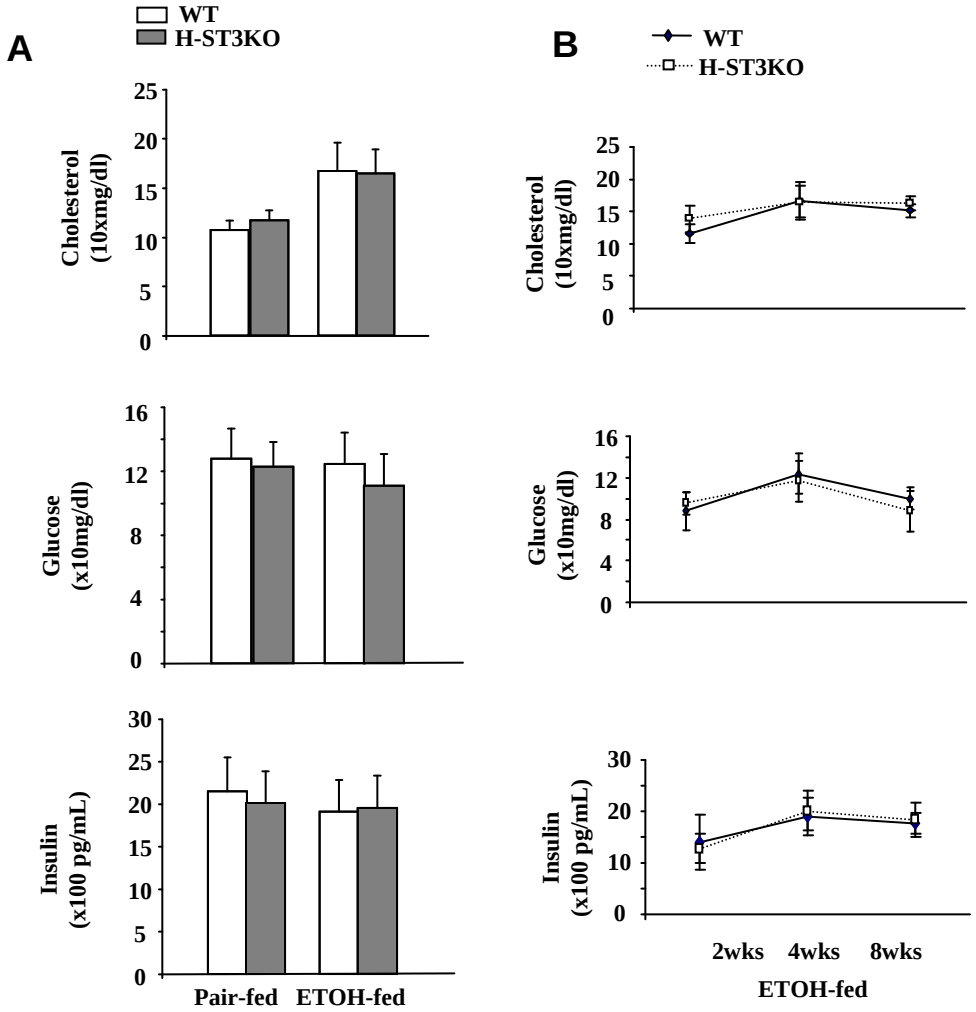
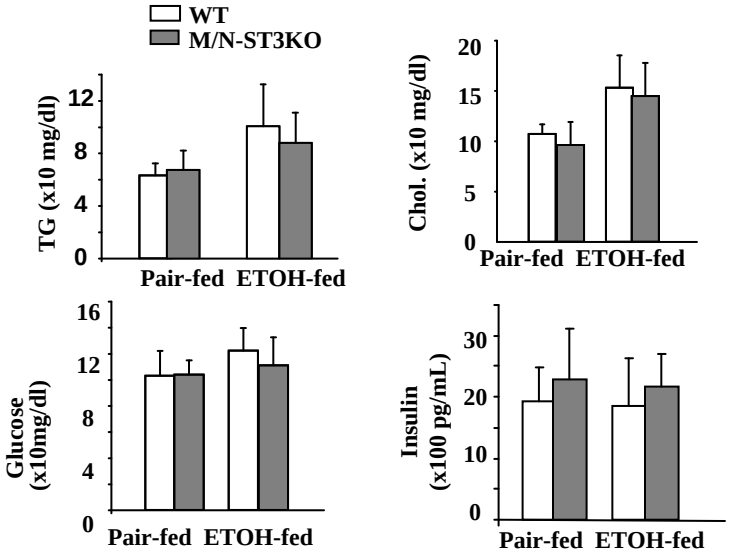
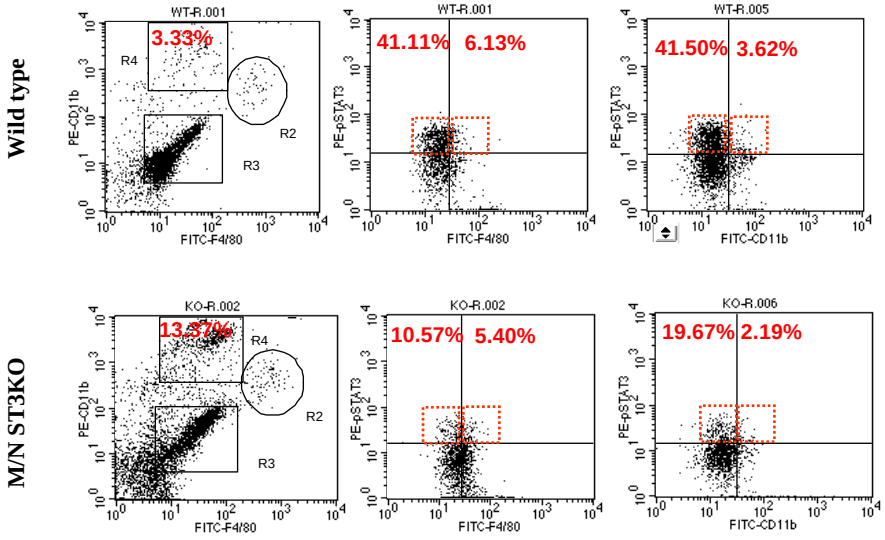


sFig. 8. Parameters in the serum. (*A*) WT and H-STAT3KO mice were given an ethanol-diet or pair-fed a control diet for 4 weeks, followed by measurement of serum levels of various factors. (*B*) An ethanol containing diet was administrated to WT and H-STAT3KO mice for 2, 4, or 8 weeks, followed by measurement of serum levels of various factors. Values represent means ± SD (2 weeks: n=8 mice/per group: 4 weeks: n=8 mice/per group in pair-fed WT and H-STAT3 KO groups; n=15 mice/per group in ethanol-fed WT and H-STAT3 KO groups; 8 weeks: n=6 mice/per group).



sFig. 9: Parameters in the serum. WT and M/N-STAT3KO mice were given an ethanol-diet or pair-fed a control diet for 4 weeks. Serum parameters were measured. Values represents means ± SD (n=6 in pair-fed group [one M/N-STAT3 KO mouse was removed from the study due to enterocolitis. n=10 in ethanol-fed group [two M/N-STAT3 KO mice were removed due to enterocolitis]).



SFig. 10: Hepatic Kupffer cells/macrophages were isolated from WT and M/NST3 KO and stimulated with IL-6 (100 ng/ml) for 30 min, then fixed and analyzed by FACS using anti-CD11b, anti-F4/80, anti-pSTAT3 antibodies. Three major populations of Kupffer cells/macrophages were detected. R4: $CD11b^{high}F4/80^{med}$, R2: $CD11b^{med}/F4/80^{high}$, R3: $CD11b^{low}/F/4/80^{med}$. The percentage of $CD11b^{high}F4/80^{med}$ was significantly increased in M/N-ST3KO mice.

The percentage of $pSTAT3^{+}$ cells was significantly reduced in M/N-ST3KO mice.