Neutrophil Extracellular Traps activate hepatic stellate cells and monocytes via NLRP3 sensing in alcohol-induced acceleration of MASH fibrosis

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KEY WORDS:

NETs, MetALD, ALD, stellate cell, neutrophils, fibrosis, macrophage, inflammasome, inflammation

SHORT TITLE: NETs activate stellate cells & induce fibrosis

Supplementary materials and method sections

Animal Care and treatments

8-12-week-old male C57BL/6J mice were purchased from the Jackson Laboratory and housed in either University of Massachusetts Medical School (UMASS) or Beth Israel Deaconess Medical Center (BIDMC) in compliance with institutional guidelines. Mice were kept under controlled atmosphere in 12 hours light and dark cycles. 2 mice/cage were housed in the same facility with ad libitum supply of food and water.

All additional procedures were approved by the UMASS or BIDMC institutional animal care and use committee (IACUC, protocol #019-2019, #30-2022).

At the age of 9-10 weeks, mice (n=10/group) were randomly divided and subjected to the following conditions: 1) Standard laboratory chow fed mice (n=20) were divided into two groups and either received 5g/kg alcohol gavage or water gavage every week for 3 months. 2) MASH diet fed mice (n=20) were divided into two groups and either received 5g/kg alcohol gavage or water gavage every week for 3 months. Animals weight was recorded once a week. Mice were sacrificed after 9h of final binge. After experimental treatments, blood and liver samples were collected, processed immediately, and stored at −80°C for further analyses.

Anti-Ly6G antibody and Pulmozyme (DNase) administration

Nine to ten weeks old male C57BL/6J mice were fed on MASH diet plus 5g/kg of weekly alcohol binges for six weeks and afterwards were randomly divided into three groups and subjected to the following conditions: 1) MASH plus alcohol mice (n=15) received saline injection intraperitoneally for next six weeks, twice a week. 2) MASH diet plus alcohol mice (n=15) received 10 μg/g of anti-Ly6G antibody (Bioxcell, InVivoMAb anti-mouse Ly6G; Catalog #BE0075-1) intraperitoneally one hour after the weekly alcohol binge, for next six weeks, twice a week. 3) MASH diet plus alcohol mice (n=15) received 5mg/kg of Pulmozyme/ DNase (Genentech), intraperitoneally one hour after the weekly alcohol binge, for next six weeks, twice a week. Standard laboratory chow fed mice (n=6) was used as a control. Mice were sacrificed after 9h of final binge. After experimental treatments, blood and liver samples were collected, processed immediately, and stored at −80°C for further analyses. During the course of three months, 2-3 mice were lost from each group, therefore we use 10mice/group for further analysis. Animal experiments were reported using ARRIVE reporting guidelines.(1)

NLRP3-KO and MCC950 administration in MASH and alcohol mice

Nine to ten weeks old male C57BL/6J mice were fed on MASH diet plus 5g/kg of weekly alcohol binges for six weeks and were randomly divided into two group in which one group (n=10) received vehicle and the other group (n=10) received MCC950 via i.p. route (10mg/kg of body weight) (Cayman Chemicals, Item No. 17510) twice a week for next six weeks. Vehicle or MCC950 i.p. injection was given 2h before the alcohol gavage. Mice were sacrificed after 9h of final binge. After experimental treatments, blood and liver samples were collected, processed immediately, and stored at −80°C for further analyses. Animal experiments were reported using ARRIVE reporting guidelines.(1)

Ten to twelve weeks old NLRP3-KO male mice (n=6) were fed on MASH diet for three months in combination with 5g/kg of weekly alcohol binges.

Human Liver tissue

Human liver samples were obtained from the National Institutes of Health Liver Tissue Cell Distribution System (Minneapolis, MN; Pittsburgh, PA; Richmond, VA). Liver samples were from 6 patients with MASH with no history of alcohol use, n=5 AH patients with BMI$\leq$25 and n=7 AH patients with BMI$\geq$25. The criteria to define patients with AH were based on "Recommendation from the NIAAA Alcoholic Hepatitis Consortia."(2)

Histopathological Analysis and Immunohistochemistry

Mouse liver sections were stained with hematoxylin/eosin, Oil-red-O staining, and picrosirius red staining by BIDMC histology core. Ly6G was detected in mice livers. Briefly, deparaffinized and rehydrated sections of livers were incubated for 30 min at room temperature with 5% BSA (blocking buffer). Endogenous peroxidase activity was blocked with 3% H2O2 for 10 min. Slides were incubated with rabbit polyclonal anti-Ly6G Ab (Abcam ab238132) in a blocking buffer overnight at 4C. SignalStain® Boost IHC Detection Reagent (HRP, Rabbit or mouse) was used to detect Ly6G. Color was developed by SignalStain® DAB Substrate Kit (Cell Signaling). Sections were mounted using Mounting Medium (Abcam) and analyzed in Nikon eclipse Ni microscopy using Nikon NIS-Elements Basic Research Imaging Software.

For immunofluorescence, liver sections were deparaffinized and rehydrated, followed by permeabilizing the sections with 0.5% TritonX-100 and was later blocked with 5% Goat serum. The sections were stained with primary antibody overnight and slides were washed next day followed by addition of fluorescent conjugated-secondary antibody for 1h. Slides were mounted in ProLong™ Gold Antifade Mountant (ThermoFisher Scientific-P36930) and the slides were analysed in Zeiss LSM 880 Upright Microscope. The primary and secondary antibodies used in this study are as follows:

Vimentin (Abcam ab45939 and secondary antibody Alexa Fluor-647 Cat # A32733, ThermoFisher Scientific), α-SMA (Sigma-Aldrich, A5228 and secondary antibody-Cy™3 Jackson ImmunoResearch Inc Cat#115-165-003). Neutrophil elastase (Abcam ab68672 and secondary antibody Alexa Fluor-647 Cat # A32731, ThermoFisher Scientific), lipocalin 2 (Abcam ab216462 and secondary antibody Alexa Fluor-647 Cat # A32731, ThermoFisher Scientific) and histone (Abcam ab12079) and secondary antibody-Cy™3 Jackson ImmunoResearch Inc Cat# 705-165-003)**.**

Fasting glucose and Insulin tolerance tests

Fasting glucose was measured by starving mice overnight for 16h in clean cages with no food and feces in the bedding. Blood was collected from tail vein venipuncture, and glucose was measured using the glucometer (Nova Max, Waltham, MA).

Insulin tolerance test was performed as described previously.(3) Briefly, mice were starved for 4h in clean cages as mentioned above. Mice were administered via IP injection of 0.5 U/kg body weight of recombinant human insulin (Novolin, Novo Nordisk, Bagsvaerd, Denmark). Glucose level was measured at different time points after insulin injection.

Cell lines and isolation of stellate cells from mouse livers

For *in vitro* coculture experiments, human LX2 hepatic stellate cells and THP1 monocyte cell lines were used.

Hepatic stellate cells were harvested from mice following liver perfusions as we described previously.(4, 5) Mouse HSCs were isolated using sequential pronase-collagenase digestion. (4, 5) Livers were perfused as mentioned before with Hanks' balanced salt solution (HBSS) buffer supplemented with 0.5 mM EGTA followed by perfusing with pronase for 5 min and collagenase

for 6 min. Next, liver was digested *in vitro* for 15 min with 1% deoxyribonuclease I. HSCs were purified from remaining nonparenchymal cells and hepatocytes debris by using 9% (w/v) OptiPrep. The isolated HSCs were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Thermo Fisher Scientific) and cocultured with PA/EtOH induced NETs for 24 hours.

Neutrophil isolation

Neutrophils from healthy subjects were isolated as described previously.(6) Briefly, 40-50ml of whole blood was diluted with warm RPMI, including 1% Anti-anti to 1:1. The diluted blood, 36ml, was layered on 12ml of gradient medium Ficoll and centrifuged at 1600 rpm for 20 minutes. Next, red blood cell (RBC) lysis buffer (BD Bioscience) was added to the RBC/neutrophil fraction. After 15 minutes of incubation at room temperature, the tube was centrifuged at 200xg for 5 minutes, and the pellet was washed with PBS and harvested for neutrophils.

Neutrophils from mice were isolated from bone-marrow as described previously.(7) Briefly, bone marrow cells were collected from 8-10 weeks old WT or NLRP3-KO mice by flushing cells from both the femur and tibia by using a 25-gauge needle and 12 cc syringe cells. Following this, cells were centrifuged, and the MojoSort™ mouse neutrophil isolation kit was used, per manufacturer's instruction, to isolate neutrophils via negative selection.

Neutrophil extracellular traps (NET) formation assay

Human peripheral neutrophils were isolated as described above, and 1-1.5 million neutrophils were used per experimental condition. 50mM ethanol, 330 μM palmitic acid (PA) or 0.5mM acetaldehyde were used for 4h to test the alcohol-, PA- and acetaldehyde-induced NETs formation in neutrophils. EtOH treated neutrophils were further treated with NAC (10 mM) or Disulfiram (10 μM), 30min after treating with alcohol. Following the completion of 4h, supernatant was removed and NETs were washed with NET buffer. NETs at the bottom of the plate were treated with S7 DNase I for 15 minutes at 37°C. The cell free suspension was used to quantify NETs associated neutrophil elastase according to the manufacturer's instructions to quantify the extent of NETs formation.

Coculture experiments with NETs

Human peripheral neutrophils were treated with 50mM ethanol or 330 μM palmitic acid (PA), for 4-5 h to induce NETs formation. After an indicated time-interval, neutrophils in the supernatant were removed, and NETs were washed three times. LX2 cells (4.5-5X 10^5 cells) or THP1 cells (0.8-1X10^6) were added to the NETs for 24h, following which supernatant was collected from THP1 cells and LX2 cells for cytokine measurement. Cells were washed with PBS, and LX2 cells were lysed for western blotting.

LX2 cells and THP1 cells, were treated with either DMSO, 10μM of MCC950 (Cayman chemicals #Item No. 17510) or 10μM IL-1 receptor antagonist (Anakinra/Raleukin, MedChem express# HY-108841) before culturing with NETs for 24h.

Isolation of NETs DNA

NETs DNA was isolated as described previously.(8) Briefly, supernatants of the NETs formed after EtOH stimulation were removed, and a restriction enzyme mix containing BseRI, PacI, NdeI and AflII (New England biolabs) was added and incubated at 37°C for 60-80 min, followed by partially digested NETs treatment with Proteinase K (New England Biolabs) for 2hours. NET DNA concentration was determined using Quant-iT PicoGreen dsDNA Reagent (Thermo Fisher Scientific) and approx. 100ng of DNA along with cit-H3 was added to LX2 cells.

GST-pull down assay

Purified GST-NLRP3 protein (MedChemExpress# HY-P701027) was incubated with glutathione magnetic agarose beads (Thermo fisher scientific# 78602) for 2h at 4°C in NP-40 lysis buffer (Boston BioProducts#BP-119) comprising of 50mM Tris-Cl (pH 7.5), 150mM NaCl, 1% NP-40, and 5mM EDTA, and supplemented with 2mM DTT and 5%BSA. GST protein (Sigma Aldrich# SRP5348) was used as a control. The beads were then washed with NP-40 lysis buffer as mentioned above and supplemented with 2mM DTT and 3%BSA. Afterwards cit-H3 (Cayman Chemicals# 17926) as indicated was added to the reaction and incubated for 4-5h at 4°C. The beads were then washed three times with NP-40 lysis buffer as mentioned above with a total of 300mM NaCl, 2mM DTT and 3%BSA. Protein was eluted by adding SDS–PAGE buffer followed by boiling for 5 min. The proteins were then analyzed by western blotting.

Western blot and ELISA

Western blotting was performed on total proteins extracted from mouse livers and primary cells and cell lines. Equal amounts of total protein were resolved on a 10-15% acrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked in 5% BSA and then probed with specific antibodies for vimentin (Abcam ab92547), α-SMA (Abcam ab5694), IL-1β (GeneTex GTX74034), Cit-H3 (Abcam ab5103), GAPDH (Protein tech 60004-1-Ig), HRP anti-beta actin antibody (Abcam ab49900) as indicated.

Legend Max™ Mouse CXCL1 ELISA Kit (Biolegend#447507), Mouse Lipocalin-2/NGAL Quantikine ELISA Kit (R & D Systems# MLCN20) for serum and Mouse LCN2 (Sandwich ELISA) ELISA Kit (LifeSpan Biosciences# LS-F4586) for liver lysates. Mouse Neutrophil Elastase/ELA2 Quantikine ELISA Kit (R & D Systems# MELA20), Citrullinated Histone H3 (Clone 11D3) ELISA Kit (Cayman Chemical Company#501620), Free Fatty Acid Assay Kit (Abcam# ab65341), Mouse Total cholesterol ELISA Kit (Abcam# ab285242), Mouse IL-1 beta/IL-1F2 Quantikine HS ELISA Kit (R & D Systems# MHSLB00), Human CXCL1/GRO alpha Qunatakine ELISA kit (R & D Systems# DGR00B), Human IL-1 beta/IL-1F2 Quantikine HS ELISA Kit (R & D Systems# HSLB00D), Human TNF-alpha Quantikine ELISA Kit (R & D Systems# DTA00D). All ELISAs were performed as per the manufacturer's protocol. 30-50 ug of total liver lysates and mice serum diluted to 1:5-1:10 in the appropriate buffer was used for the ELISA.

Recombinant proteins

GST-NLRP3 was purchased from MedChemExpress (Catalog #: HY-P701027), GST protein was purchased from Sigma Aldrich (Catalog #: SRP5348), cit-H3 was purchased from Cayman Chemical Company (Catalog #: 17926) and TGF-beta Recombinant was purchased from R & D Systems (Catalog #: 7754-BH)

RNA extraction, qPCR and Nano string gene expression analysis

RNA was extracted using the RNeasy Kit (Qiagen, Germantown, MD, USA) with on-column DNase digestion. Concentration was determined using a Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA), and 1 μg RNA was used for cDNA reverse transcription (BioRad, Hercules,

CA, USA). Quantitative real-time polymerase chain reaction (qPCR) was completed using SYBR Green polymerase (BioRad), and expression was measured on a BioRad CFX96 Real-Time System. qPCR primers are listed in supplementary methods, and the expression was quantified by using the $2^{-\Delta\Delta Ct}$ method. RNA and Nanostring reactions were prepared according to the manufacturer's recommendation for the Mouse nCounter fibrosis Panel (Nanostring Technologies, Seattle, WA, USA). Data were analyzed using the nSolver Analysis Software 3.0 (Nanostring Technologies).

Flow cytometry

Liver immune cells were isolated as described previously.(4) Briefly, immune cells were incubated with a cocktail of antibodies (1:100 dilution) for 30 min on ice: APC/Cyanine7 anti-mouse CD45 Antibody (Biolegend, 157204, clone S18009F), Brilliant Violet 421™ anti-mouse F4/80 Antibody (Biolegend, 123132, clone BM8), Brilliant Violet 650™ anti-mouse CD11c Antibody (Biolegend, 117339, clone N418), Brilliant Violet 785™ anti-mouse Ly-6G Antibody (Biolegend, 127645, clone 1A8), Alexa Fluor® 700 anti-mouse Ly-6C Antibody (Biolegend, 128024, clone HK1.4), Brilliant Violet 510™ anti-mouse I-A/I-E Antibody (Biolegend, 107636, clone M5/114.15.2), Brilliant Violet 605™ anti-mouse CD86 Antibody (Biolegend, 105037, clone GL-1), FITC anti-mouse CD206 (MMR) Antibody (Biolegend, 141704, clone C068C2), Pacific Blue™ anti-mouse CD68 Antibody (Biolegend, 137028, clone FA-11), Brilliant Violet 711™ anti-mouse/human CD11b Antibody (Biolegend, 101242, clone M1/70), and Zombie NIR™ Fixable Viability Kit (Biolegend, Cat#423106). After 30 min, all samples were incubated with BD Phosflow™ Lyse/Fix Buffer (BD Biosciences, 558049) following manufacturer's protocol. Following lyse/Fix, cells were washed twice with 1× PBS containing 2% FBS, and resuspended in FACS buffer containing 2% FBS in PBS. Samples were run in Aurora Spectral Flow Cytometer (Cytek) and data analysis was done using Flowjo version 8.8.7 software.

Bioinformatic analysis

Nanostring data analyzed using the nSolver Analysis Software 3.0 was exported in csv format. Data was reorganized using the RStudio software build 732 (2021.09.1), including stringr (v1.4.0) and tidyverse (v1.3.1), and dplyr (v.1.0.8).

Volcano plot was created using the raw data including fold change and p-value. Data visualizations were performed using the RStudio software build 732 (2021.09.1), including ggplot2 (v3.3.5), tidyr (v1.2.0), dplyr (v.1.0.8), and ggrepel (v 0.9.1)

For Bubble plot we mapped all differentially expressed genes (DEGs) to terms in gene ontology (GO) database and looked for significantly enriched terms comparing to the *Mus musculus* genome background. DEGs were functionally grouped into GO networks using the Panther Classification System v17.0 online tool (http://pantherdb.org/). Panther established a web-based table list, summarizing the information of gene counts, fold enrichment and p value corresponded to each GO term. The statistical significance of the terms analyzed was calculated with fisher exact test and FDR P-value correction. The results were exported as txt format and ready to use in Rstudio. Data visualizations were performed using the RStudio software build 732 (2021.09.1), including ggplot2 (v3.3.5), tidyr (v1.2.0), and dplyr (v.1.0.8).

Supplementary Table

Supplementary Table 1: Sequences of real-time PCR primers (Mouse SYBR Green primers)

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
Mmp9	GCTGACTACGATAAGGACGGCA	TAGTGGTGCAGGCAGAGTAGGA
Mmp12	CACACTTCCCAGGAATCAAGCC	TTTGGTGACACGACGGAACAGG
Timp1	TCTTGGTTCCCTGGCGTACTCT	GTGAGTGTCACTCTCCAGTTTGC
Vim	CGGAAAGTGGAATCCTTGCAGG	AGCAGTGAGGTCAGGCTTGGAA
Nlrp3	TCACAACTCGCCCAAGGAGGAA	AAGAGACCACGGCAGAAGCTAG
Casp1	GGCACATTTCCAGGACTGACTG	GCAAGACGTGTACGAGTGGTTG
Il1b	TGGACCTTCCAGGATGAGGACA	GTTCATCTCGGAGCCTGTAGTG
Nlrc4	CTCACCACGGATGACGAACAGT	TGTCATCCAGTATGAGTCTCTCG
Aim2	AGGCTGCTACAGAAGTCTGTCC	TCAGCACCGTGACAACAAGTGG

Supplementary Figures:

Fig S1: Body weight gain and insulin resistance in mice fed on alcohol binges in combination with MASH diet. (A) Kaplan-Meier graph representing the percent survival of mice fed on MASH diet plus alcohol binges with single diet as control, for 12 weeks. **(B)** Blood alcohol level was measured from serum. **(C)** Histological scores of steatosis, inflammation, hypertrophy, and fibrosis is shown in table (n=6-8/group). **(D)** Fasting glucose was measured from the blood after 16h of starvation. **(E)** Insulin tolerance test was performed after starving mice for 4h, at different time points as indicated. *represents the significance between chow vs MASH alone group. #represents the significance between MASH vs MASH plus alcohol group.

Data is presented as mean ± SEM. Statistical significance was determined using one-way ANOVA * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.00005$

Fig S2. Western blots for α-SMA and Vimentin

Liver lysates were used to detect Vimentin (A) and α-SMA (B) by western blot (n=3-4/group). Full blots with Vimentin (A) and α-SMA (B) is shown.

Fig S3. Transcriptomic analysis shows differential expression of signaling pathways in mice fed on MASH diet and alcohol binges

(A) Volcano plot depicting DGE analysis results between MASH vs chow. **(B)** Volcano plot depicting DGE analysis results between alcohol binges vs chow. **(C-D)** Bubble plot depicting GO term analysis of the biological process in MASH diet only group and alcohol binges only.

Fig S4. *In vitro* alcohol and PA treatment induces NETs formation in human neutrophils

(A) Human neutrophils were treated with EtOH (50 mM) or acetaldehyde (0.5 mM) for 4h. In a separate group EtOH treated neutrophils were treated with NAC (10 mM) or Disulfiram (10 µM), 30min after treating with alcohol, followed which S7 DNase treatment was performed. NETs associated neutrophil elastase was measured by ELISA. **(B)** Human neutrophils were treated with PA (330 µM) or EtOH (50 mM) for 4h to induce NETs formation, followed which S7 DNase treatment was performed. NETs associated neutrophil elastase was measured by ELISA as NETosis marker. **(C)** Human neutrophils were treated with PA (330 µM) or EtOH (50 mM) for 4h to induce NETs formation, followed by lysing NETs with 1X SDS diluted in RIPA buffer.

NETs associated cit-H3 was visualized by western blot. Ponceau stained image of the membrane depicts the equal amount of protein loaded.

Fig S5. NLRP3 level in LX2 cells and GST pull down assay

(A) LX2-lysates after treating with PA/EtOH/TGF-β were used to detect NLRP3 by Western blot. The densitometry analysis is shown as bar graph (n=3). (B) Co-precipitation of cit-H3 with GST-tagged protein (NLRP3-GST or GST alone) was tested using glutathione magnetic agarose beads. Beads were first loaded with recombinant GST-tagged indicated proteins and then incubated in presence of recombinant cit-H3. Beads were washed, and eluted proteins were detected using anti-cit-H3 antibody in western blots. GST tagged proteins were detected by Anti-GST antibody as indicated.

Fig S6. mRNA level of AIM2 and NLRC4 inflammasome in combined liver injury model

Liver RNA was used to determine NLRC4 **(A)** and AIM2 **(B)** mRNA levels by qPCR. 18s was used to normalize Cq values. Liver lysates were used to detect IL-1β (C) and Cleaved Caspase-1 (D) by western blot (n=3-8/group). Full blots with pro-IL-1β (C) and Pro-caspase 1 (D) is shown.

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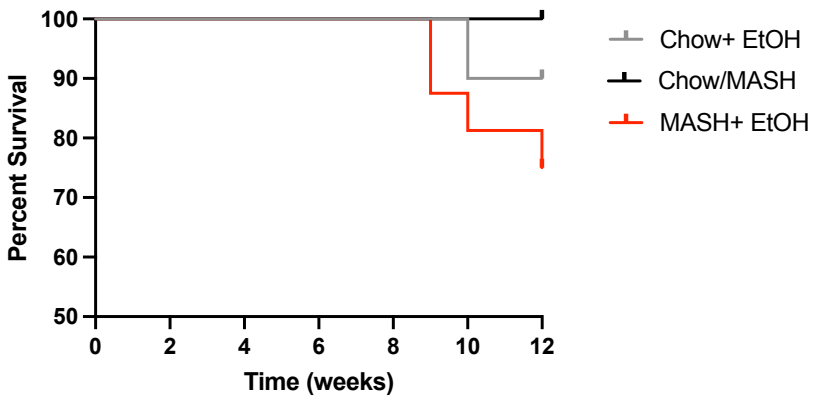
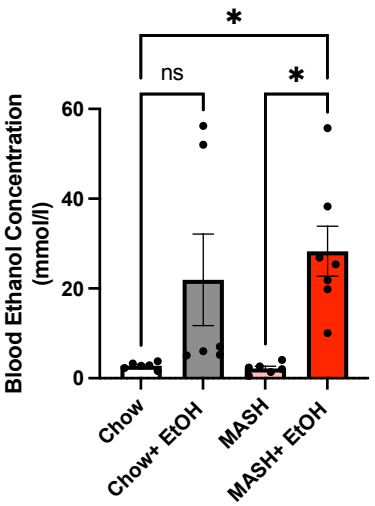
Figure S1

A



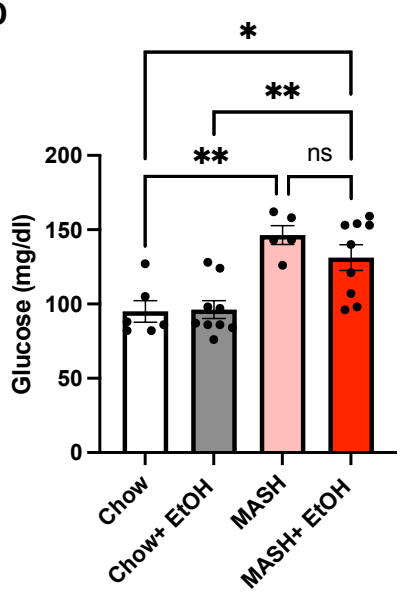
B



C

	Steatosis	Inflammation	Hypertrophy	Fibrosis
Chow (n=9)	None	None	None	None
Chow+ EtOH (n=9)	Present (n=1, positive)	Present (n=1, positive)	None	None
MASH (n=7)	Lobular variation and periportal macrovesicular	Present (n=5, positive)	Present (n=5, positive)	Focal sinusoidal fibrosis (n=6, positive)
MASH+ EtOH (n=7)	Macrovesicular steatosis and microvesicular steatosis	Present (n=4, positive)	Present (n=4, positive)	Sinusoidal, periportal and septal fibrosis (n=6, positive)

D



E

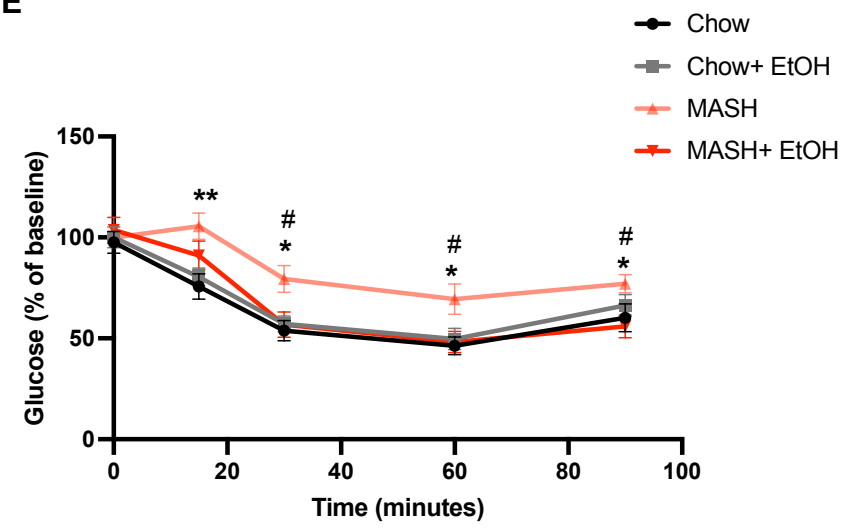
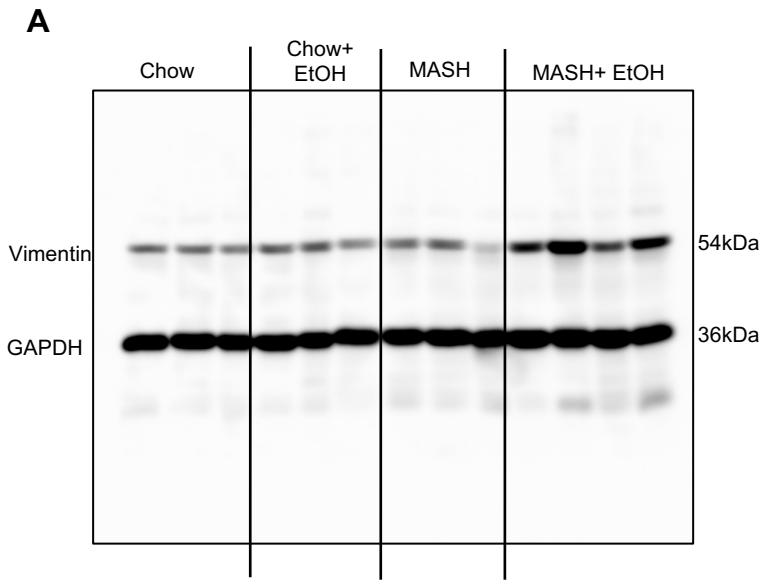


Figure S2


A
Chow
Chow+ EtOH
MASH
MASH+ EtOH
Vimentin
GAPDH
54kDa
36kDa

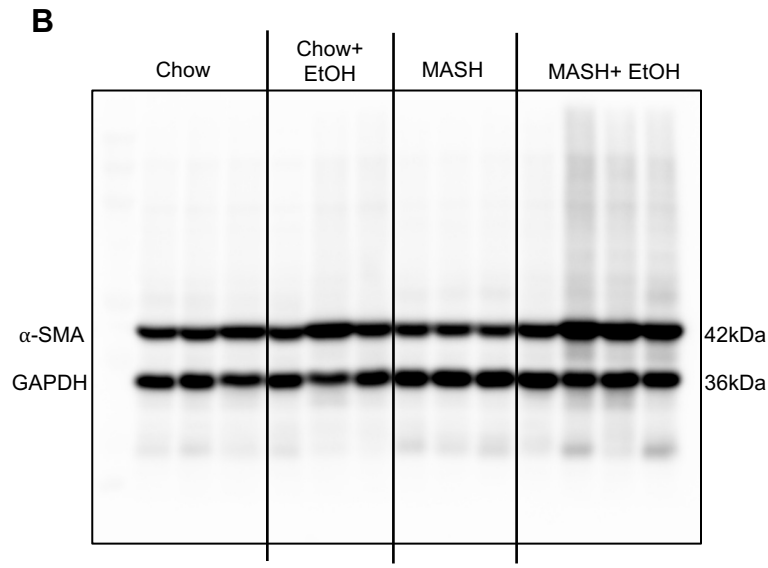

B
Chow
Chow+ EtOH
MASH
MASH+ EtOH
α-SMA
GAPDH
42kDa
36kDa

Figure S3

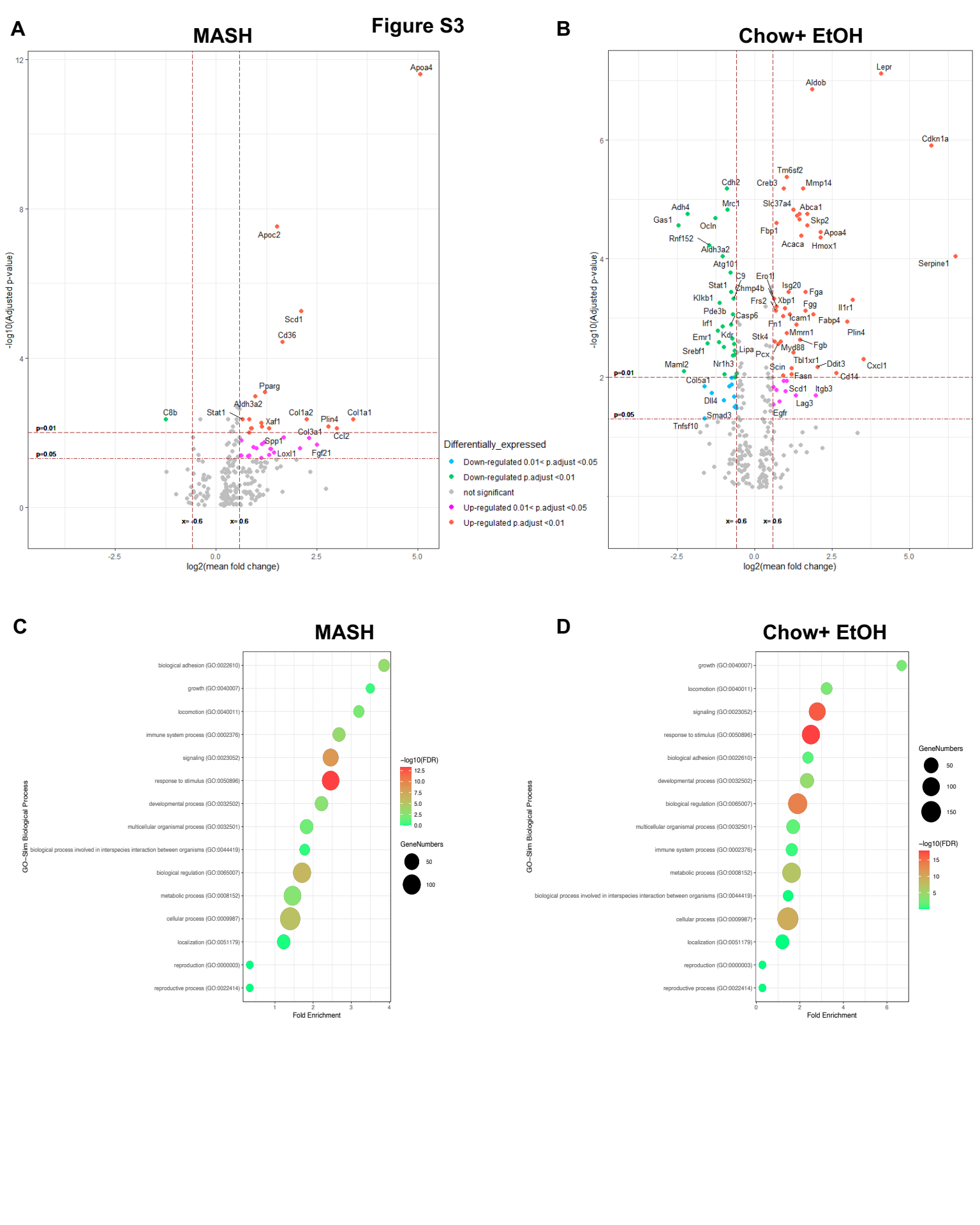

A
MASH
B
Chow+ EtOH
C
MASH
D
Chow+ EtOH
-log10(Adjusted p-value)
log2(mean fold change)
Differentially_expressed
Down-regulated 0.01< p.adjust <0.05
Down-regulated p.adjust <0.01
not significant
Up-regulated 0.01< p.adjust <0.05
Up-regulated p.adjust <0.01
p=0.01
p=0.05
x= -0.6
x= 0.6
Apoa4
Apoc2
Scd1
Cd36
Pparg
Aldh3a2
C8b
Stat1
Xaf1
Col1a2
Plin4
Col1a1
Col3a1
Ccl2
Spp1
Loxl1
Fgf21
Lepr
Aldob
Cdkn1a
Tm6sf2
Cdh2
Creb3
Mmp14
Adh4
Mrc1
Slc37a4
Abca1
Gas1
Ocln
Skp2
Fbp1
Apoa4
Rnf152
Acaca
Hmox1
Aldh3a2
Serpine1
Atg101
C9
Ero1l
Stat1
Chmp4b
Isg20
Fga
Klkb1
Frs2
Xbp1
Fgg
Il1r1
Pde3b
Casp6
Icam1
Fabp4
Irf1
Fn1
Mmrn1
Plin4
Emr1
Kdr
Stk4
Fgb
Srebf1
Lipa
Pcx
Myd88
Tbl1xr1
Ddit3
Cxcl1
Maml2
Nr1h3
Scin
Fasn
Cd14
Col5a1
Scd1
Itgb3
Dll4
Lag3
Egfr
Smad3
Tnfsf10
GO–Slim Biological Process
Fold Enrichment
–log10(FDR)
GeneNumbers
biological adhesion (GO:0022610)
growth (GO:0040007)
locomotion (GO:0040011)
immune system process (GO:0002376)
signaling (GO:0023052)
response to stimulus (GO:0050896)
developmental process (GO:0032502)
multicellular organismal process (GO:0032501)
biological process involved in interspecies interaction between organisms (GO:0044419)
biological regulation (GO:0065007)
metabolic process (GO:0008152)
cellular process (GO:0009987)
localization (GO:0051179)
reproduction (GO:0000003)
reproductive process (GO:0022414)

Figure S4

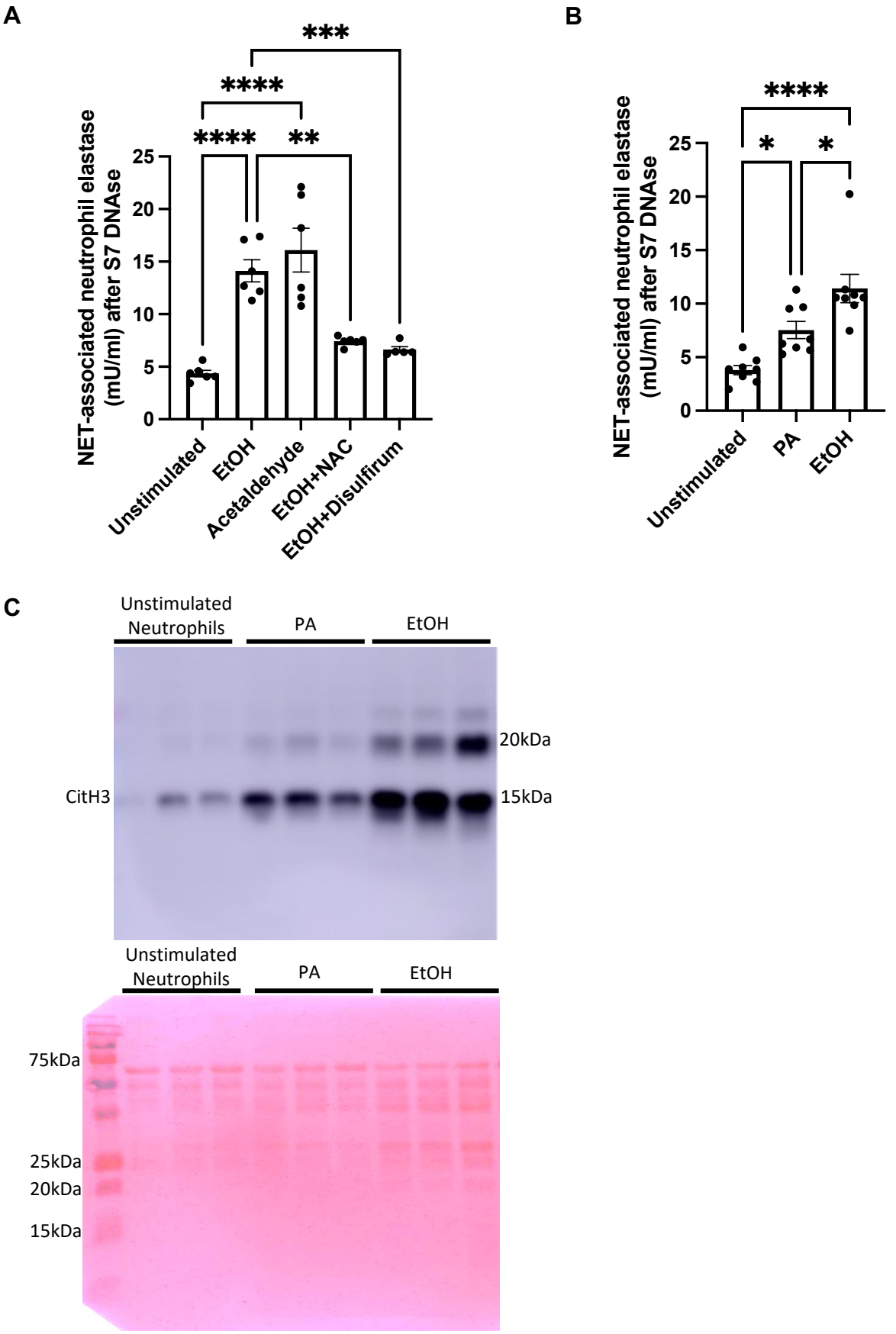
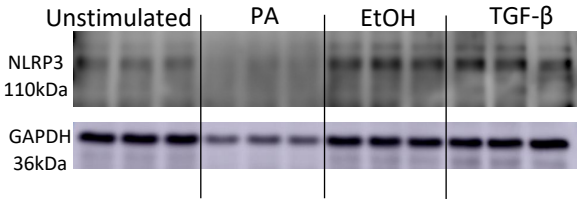
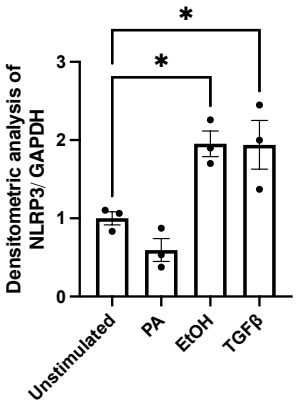

A
NET-associated neutrophil elastase (mU/ml) after S7 DNAse
***
****
****
**
25
20
15
10
5
0
Unstimulated
EtOH
Acetaldehyde
EtOH+NAC
EtOH+Disulfirum
B
NET-associated neutrophil elastase (mU/ml) after S7 DNAse
****
*
*
25
20
15
10
5
0
Unstimulated
PA
EtOH
C
Unstimulated Neutrophils
PA
EtOH
20kDa
CitH3
15kDa
Unstimulated Neutrophils
PA
EtOH
75kDa
25kDa
20kDa
15kDa

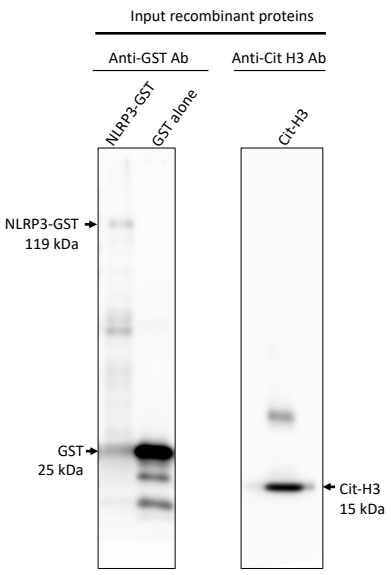
Figure S5

A


Unstimulated
PA
EtOH
TGF-β
NLRP3
110kDa
GAPDH
36kDa


Densitometric analysis of NLRP3/ GAPDH
0
1
2
3
*
*
Unstimulated
PA
EtOH
TGFβ

B


Input recombinant proteins
Anti-GST Ab
Anti-Cit H3 Ab
NLRP3-GST
GST alone
Cit-H3
NLRP3-GST
119 kDa
GST
25 kDa
Cit-H3
15 kDa

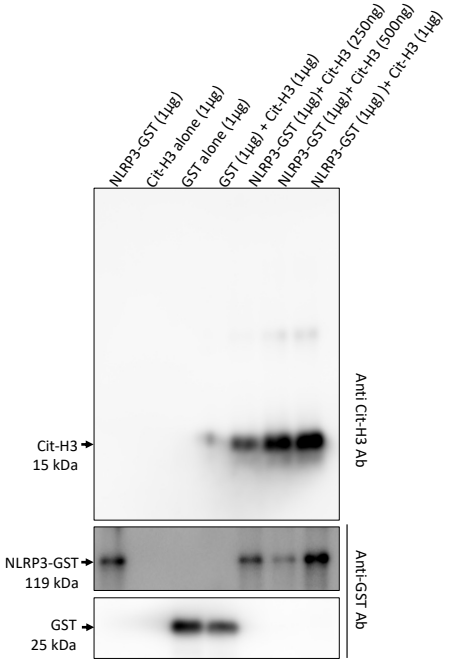
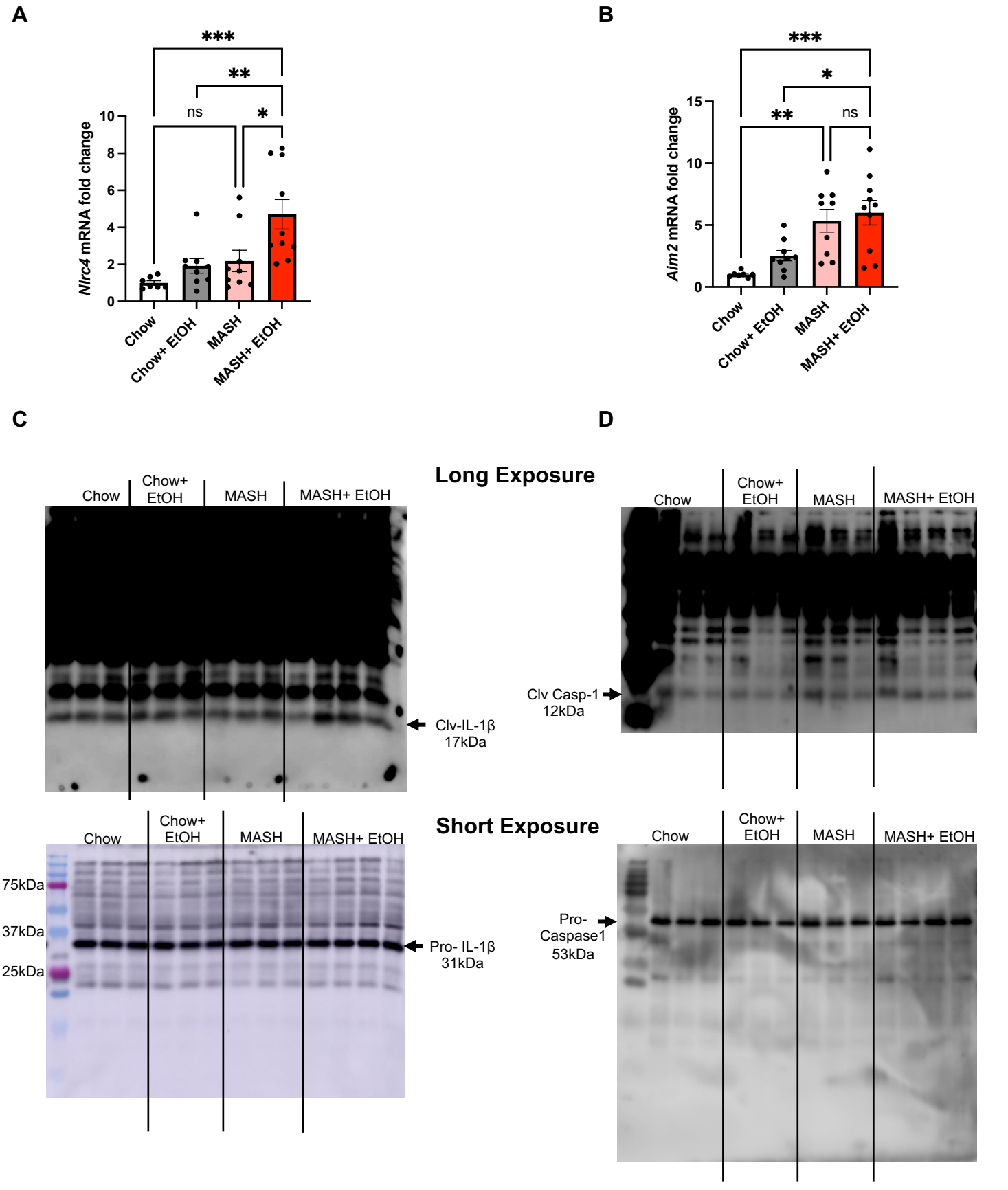

NLRP3-GST (1µg)
Cit-H3 alone (1µg)
GST alone (1µg)
GST (1µg) + Cit-H3 (1µg)
NLRP3-GST (1µg)+ Cit-H3 (250ng)
NLRP3-GST (1µg)+ Cit-H3 (500ng)
NLRP3-GST (1µg))+ Cit-H3 (1µg)
Cit-H3
15 kDa
Anti Cit-H3 Ab
NLRP3-GST
119 kDa
GST
25 kDa
Anti-GST Ab

Figure S6


A
***
**
ns
*
Nlrc4 mRNA fold change
0
2
4
6
8
10
Chow
Chow+ EtOH
MASH
MASH+ EtOH
B
***
*
**
ns
Aim2 mRNA fold change
0
5
10
15
Chow
Chow+ EtOH
MASH
MASH+ EtOH
C
Chow
Chow+ EtOH
MASH
MASH+ EtOH
Clv-IL-1β 17kDa
75kDa
37kDa
25kDa
Pro- IL-1β 31kDa
D
Long Exposure
Chow
Chow+ EtOH
MASH
MASH+ EtOH
Clv Casp-1 12kDa
Short Exposure
Chow
Chow+ EtOH
MASH
MASH+ EtOH
Pro-Caspase1 53kDa