Supporting Material and Methods

Mitochondrial double-stranded RNA in exosome promotes interleukin-17 production through toll-like receptor 3 in alcoholic liver injury

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MATERIAL AND METHODS

ANIMALS

Male C57BL/6 and TLR3 KO mice were bred or purchased from the Jackson Laboratory (Bar Harbor, ME). In detail, 8- to 10-week-old male mice were maintained in sterile, isolated, and individually ventilated cages with controlled temperature (22-24°C) and adequate light for circadian rhythm (12-hour light/12-hour dark) and ad libitum access for food and water at specific pathogen-free facility (Bio Model System Park; KAIST, Daejeon, Korea). All animals received humane care in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH) and all animal experiments were approved by KAIST Institutional Animal Care and Use Committee.

ETHANOL-INDUCED LIVER INJURY

For single binge ethanol drinking model, 4 g/kg of ethanol was orally administered to mice and the mice were euthanized and analyzed at 0, 3, 6, or 12 hour after ethanol drinking. To induce acute on chronic liver injury or simple alcoholic steatosis, mice were fed a 4.5% Lieber-DeCarli ethanol liquid diet (Dyets Inc., Bethlehem, PA) for 10 days and 4 g/kg ethanol was administered to mice by oral gavage or they were fed a 4.5% Lieber-DeCarli ethanol liquid diet (Dyets Inc., Bethlehem, PA) for 8 weeks as previously reported.[1, 2] The liquid diet was kept in angled bottles and daily supplied to prevent evaporation of ethanol.

CLODRONATE LIPOSOME TREATMENT

For depletion of Kupffer cells and resident macrophages, 10 μl/g clodronate liposomes (Haarlem, Netherlands) were injected intraperitoneally in each mouse. Depletion was confirmed at 2-day after the injection by fluorescence-activated cell sorting (FACS) analysis. The same concentration of phosphate buffered saline (PBS) liposomes was administered intraperitoneally as a control.

GENERATION OF BONE MARROW CHIMERAS

Chimeric mice were generated by reciprocal bone marrow transplantation as described previously.[3] Mice were irradiated with single dose 9 Gy and then were reconstituted with isolated bone marrow cells (2×10^6 cells per mouse) from femurs and tibias of donor wild-type (WT) mice through tail vein injection. After resting for 8 weeks, chimerism was confirmed in isolated liver mononuclear cells (MNCs), peripheral blood mononuclear cells (PBMCs), and bone marrow cells of mice by performing gel electrophoresis.

SERUM MEASUREMENTS

The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC) and triglyceride (TG) were measured by VET Test Chemistry analyzer (IDEXX Laboratories, Westbrook, ME) according to the manufacturer's instructions.

ISOLATION OF MOUSE PRIMARY HEPATIC CELLS

To isolate primary hepatocytes, hepatic stellate cells (HSCs) and Kupffer cells (KCs) from mice, liver was perfused *in situ* through portal vein puncture and flowed 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 the perfusion buffer (0.075% collagenase type I in Hanks' Balanced Salt Solution (HBSS) buffer with 0.02% DNase I) at 37 ℃ at a rate of 1.4 - 1.5 ml/min. Then, liver tissues were transferred into digestion buffer (0.009% collagenase type I in HBSS with 0.02% DNase I) and homogenized gently by transfer pipette. The cell lysate was

incubated in shaking incubator for 20 minutes at 90 rpm. After that, the cell lysate of the perfused liver was filtered through a 70 μm sized cell strainer and centrifuged at 40 *g* for 5 minutes at room temperature (RT) and pellet was resuspended to 50% Percoll gradient solution and centrifuged at 1,400 *g* for 10 minutes at 4 ℃ to isolate and purify primary hepatocytes. The supernatant was transferred to another tube and centrifuged at 510 *g* for 10 minutes at 4 ℃. The cell pellet was then resuspended in 11.5% and 20% Opti-Prep gradient (Sigman-Aldrich, St. Louis, MO, USA), and centrifuged at 1,800 *g* for 17 minutes at 4 ℃ to isolate HSCs and KCs, respectively. HSCs were collected from interfaces of 0% and 11.5% and KCs were collected from interfaces of 11.5% and 20%. Freshly isolated cells were resuspended in RPMI-1640 medium with 10% FBS and 1% antibiotic-antimycotic (Thermo Fisher Scientific, Waltham, MA, USA). Cell viability of hepatocytes, HSC, and KCs were over 90 % as assessed by Trypan blue staining and typical inverted light microscopic appearances, respectively. Primary hepatocytes were cultured in Low Glucose DMEM with 10% FBS and 1% antibiotic-antimycotic (Thermo Fisher Scientific, Waltham, MA, USA). HSCs and KCs were cultured in RPMI-1640 with 10% FBS and 1% antibiotic-antimycotic (Thermo Fisher Scientific, Waltham, MA, USA) on 6- or 12-well plates.

ISOLATION OF HUMAN PRIMARY HEPATIC CELLS

We obtained small sized-healthy liver tissues during liver resection from the department of surgery and the department of internal medicine, Chungnam National University Hospital (Daejeon, South Korea) and tried to collect human primary cells through liver perfusion. Briefly, 22-gauge catheter was inserted in a vessel and then a purse string suture was performed to fix the catheter to the tissue. After that, the liver were perfused with 300 ml of 1× EGTA solution at a rate of 30 ml per minute, and it was digested twice with 50 ml of type I collagenase solution (2 mg/ml) in closed circulation system.[4] Human HSCs and KCs were separated by differential centrifugation on 11.5% and 15% Opti-Prep gradients (Merck) as a previously described report with a little modified method.[5] The remaining procedure was identical with the isolation method of mouse hepatic cells. Authorization for the use of these tissues for research purposes was obtained from the Institutional Review Board of Chungnam National University Hospital (IRB number: 2016-03-02-003). Informed consents were received from the entire patients who had provided the tissue. Freshly isolated cells were cultured with 10% fetal bovine serum in DMEM or RPMI 1640 medium for the future experiments.

ISOLATION METHOD OF LIVER MONONUCLEAR CELLS

Total liver MNCs were isolated from the whole liver tissue as previously described.[6] Briefly, after sacrificing the mice, liver tissues were immediately grinded and homogenized through cell strainer with 70-μm Nylon mesh filter. After the elimination of hepatocytes by centrifugation at 40 *g* for 5 minutes, the supernatant was collected, washed with sterile filtered PBS, and suspended in 40 % Percoll (GE Healthcare, Little Chalfont, UK). The cell lysate was gently re-suspended well and centrifuged at 1,400 *g* for 30 minutes at 4 ℃. Red blood cells (RBCs) were lysed using RBC lysis buffer (BioLegend, San Diego, CA, USA) and liver MNCs were washed twice in sterile PBS and counted by automated cell counter TC20^{TM} (Bio-Rad, Hercules, CA, USA)

ISOLATION METHOD OF EXTRACELLULAR VESICLES

To isolate mouse hepatocyte-derived extracellular vesicles, liver perfusion was performed on 8 to 10 week-old WT male mice and primary hepatocytes were obtained. Then, primary hepatocytes were plated onto 100 mm dish and treated with 100 mM of ethanol for 24 hours. First, to remove cell debris from the media, it was centrifuged at 300 *g* for 10 minutes. Then, the supernatant was carefully collected and re-centrifuged at 2,000 *g* for 12 minutes to remove the apoptotic bodies (pellet). After collecting the supernatant, it was further centrifuged at 15,000 *g* for 30 minutes. Microvesicles were obtained from the pellet, and exosomes were collected from the supernatant using ExoQuick-TC^{TM} (System Biosciences Inc., Mountain View, CA). After separation of microvesicles and exosomes using differential centrifugation, we confirmed them through nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and transmission electron microscopy (TEM).

BIOANALYZER ANALYSES

Total RNA was extracted by TRIzol reagent (ThermoFisher Scientific, Waltham, MA, USA) from extracellular vesicles as previously described.[3] After quantification of extracted total RNA by using NANODROP LITE spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), RNA Nano Chips was used for the analysis of RNA with the Agilent 2100 Bioanalyzer

System (Agilent Technologies) according to the manufacturer's instruction. Briefly, after spinning the gel for 10 seconds, add 1 μl of dye and vortex and centrifuge 18,000 *g* for 10 minutes at RT. Gently put 9 μl into the marked G part of the gel and slowly lower the syringe of station. Then wait 30 seconds, raise to 1 ml mark again. Add 9 μl of the gel-dye mixture, and add the markers #1-12 and 5 μl to the ladder sequentially. Add 1 μl of the following ladder, then add 1 μl of extracted RNA to #1-12. Vortex chip for 1 minute and place in the Bioanalyzer machine.

CO-CULTURE OF γδ T CELLS WITH KUPFFER CELLS

Co-culture assay was performed using 12-well Transwell with 3 μm-sized pores (Corning). Freshly isolated Kupffer cells were plated at lower chamber and treated with EtOH-EVs isolated from supernatant of primary hepatocytes by differential centrifugation for 4 hours. After washing out supernatant and media change, freshly isolated mouse γδ T cells from wild-type mice were added to upper chamber and were incubated for 12 hours. Then, cells in upper and lower chamber were collected respectively and qPCR was done. Human freshly isolated primary Kupffer cells were plated at lower chamber and incubated with EtOH-EVs isolated from human primary hepatocytes for 4 hours. The procedure after this experiment is the same as the procedure of above mouse experiment.

IMMUNOFLUORESCENCE OF dsRNA STAINING

Primary hepatocytes were plated on a cover glass in a 12-well plate and they were incubated with MitoTracker Deep Red (M22426, Invitrogen, 100 nM) for 30 minutes at 37 ℃ before fixing in 4% paraformaldehyde (PFA) in PBS. Cells were washed and added with 0.5% Triton X-100 for 15 minutes at RT. After 3 washes with 0.05% phosphate buffered saline Tween-20 (PBST), blocking was performed with goat serum at RT for 1 hour. The preparation of the primary antibody was briefly described. Primary antibody was diluted as manufacturer's description (1:250). Primary antibody of anti-dsRNA (#10010500, J2 monoclonal antibody, mouse, IgG2a, kappa chain, SCICONS) was incubated for 3 hours at 37 ℃. Cells were washed three times by 0.05% PBST and then incubated with Alexa Fluor® 488 conjugated anti-mouse IgG secondary antibody (#ab150109, Abcam, Cambridge, UK) at (1:300) concentration at RT for 1 hour. And then cells were washed and covered with DAPI included mounting solution

(#ab104139, Abcam) for nuclear staining and all images were acquired using an Olympus BX51 microscope equipped with a CCD camera (Olympus, Tokyo, Japan) and computer-assisted image analysis with DP2-BSW. For acquiring of more detailed and high resolution of immune-fluorescent images, a Zeiss LSM 780 confocal microscope equipped with argon and helium-neon lasers (Carl Zeiss, Oberkochen, Baden-Württemberg, Germany) were used.

IMMUNOFLUORESCENCE OF dsRNA STAINING OF KUPPFER CELLS TREATED WITH DiI-STAINED EXOSOMES

Primary Kupffer cells were plated on a cover glass in a 12-well plate and cells were incubated with DiI-stained exosomes for 4 hours at 37 ℃ before fixing in 4% PFA in PBS. The procedure after this experiment is the same as the immunofluorescent staining procedure of dsRNA. The method of staining exosomes with DiI is as follows. Briefly, DiI was incubated with exosomes in amber tube for 1 hour at 4 ℃. DiI-stained exosomes were transferred into Quick-Seal Polypropylene centrifuge tube (#342414, Beckman Coulter, Inc., CA) and centrifuged at 54,000 *g* for 4 hours at 4 ℃. Collected DiI-stained exosomes were incubated with primary Kupffer cells.

STAINING OF TISSUES

Five μm paraffin embedded tissue blocks were stained with hematoxylin and eosin (H & E). Stained slides were mounted using Canada balsam (Sigma-Aldrich, St. Louis, MO) and carefully observed under Olympus BX51 fluorescent microscopy (Olympus, Tokyo, Japan) equipped with DP2-BSW computer-assisted image analysis.

MEASURING OF HEPATIC TRIGLYCERIDE LEVEL

Hepatic lipids were extracted from 30-50 mg of liver tissues using chloroform/methanol mixture (2/1 ratio). Lipid extract was lyophilized by vacuum concentrators and then dissolved in 5 % fatty acid-free BSA. Resuspended lipid was used for assessments of TG level using VET Test Chemistry Analyzer according to the manufacturer’s instructions (IDEXX Laboratories, Westbrook, ME, USA).

IMMUNOFLUORESCENCE OF RNase III TREATED SAMPLES

Primary hepatocytes were plated on cover glass of 6-well plate. After 100 mM ethanol treatment to primary hepatocytes for 12 hours, RNase III (M0245S, NEB, concentration 70 U/ml) were treated and incubated for additional 6 hours at 37 ℃. Supernatant was collected for differential isolation of extracellular vesicles and primary hepatocytes were washed with filtered PBS and fixed in 4% PFA (v/v). The procedure after this experiment is the same as the immunofluorescent staining procedure of dsRNA.

FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

Isolated liver MNCs were stained with anti-CD45, andi-CD11b, anti-CD3e, anti-NK1.1, anti-CD4, anti-Gr1, anti-CD8, and anti-γδ TCR (BD bioscience, San Jose, CA). For blocking non-specific antibody reactions, cells were pre-incubated with anti-mouse CD16/32 mouse Fc blocker (BD bioscience, San Jose, CA) before the staining with antibodies. For intracellular staining, isolated liver MNCs were cultured with RPMI 1640 including 10% FBS, 50 ng/ml phorbol-myristate acetate, 500 ng/ml ionomycin (Sigma-Aldrich, St. Louis, MO) and 10 μg/ml Brefeldin A (BD Bioscience, San Jose, CA) for 5 hours and then stained with IL-17A antibody (eBioscience, San Diego, CA). The stained cells were analyzed using BD^{TM} LSR II Flow Cytometer (BD bioscience, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR).

QUANTITATIVE RT-PCR

Total RNA was isolated from liver tissues or cells with TRIzol reagent (ThermoFisher Scientific, Waltham, MA, USA) or RNeasy Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The same amount of total RNA was reverse-transcribed to complementary DNA using ReverTra AceR qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Newly synthesized complementary DNA and gene-specific primers were used. Quantitative RT-PCR was performed with the CFX96 system and the SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan). Expression of each gene was normalized and expressed as a fold-change relative to housekeeping genes (β-actin). Results were analyzed by the $^{\Delta\Delta}$Ct method. Relative values were expressed as fold change in comparing with control. Sequences of mouse and human primers were described in Supporting Table S1.

WESTERN BLOT

Total proteins were isolated from primary hepatocytes, Kupffer cells or liver tissues of mice or humans using RIPA lysis buffer (30 mM Tris, pH 7.5, 105 mM NaCl, 1 mM PMSF, 1 mM Na_3VO_4, 10% SDS, 10% glycerol), containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). Samples were separated in 10 % SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Membranes were blocked in 5 % skim milk, incubated with primary antibodies at 1:1000 in 0.05% PBST. Primary antibodies of caspase-1 (#2225S), IL-1β (#12507S), NLRP3 (#15101S) (Cell Signaling Technology, Danvers, MA, USA), β–actin (#A5441) (Sigma-Aldrich, St. Louis, MO), PNPase (#BS-7100R) (Bioss Antibodies Inc., Wobrun, MA, USA) and SUV3 (#sc-365750), and HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, CA, USA) were used for detection. Comparative amount was normalized with β-actin. Detection of immunolabeled proteins was performed using a commercial chemiluminescent assay (ECL femto; Thermo Fisher Scientific, Waltham, MA, USA). Visualization and quantitative measurements were made with a CCD camera and software of LAS-3000 (GE Healthcare, Little Chalfont, UK) for western blot image analysis.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

One hundred micro liter of collected supernatants from culturing of Kupffer cells and γδ T cells were added to a plate pre-coated with monoclonal antibody against mouse IL-17A and IL-1β (eBioscience, San Diego, CA). After incubation at 4 ℃ for 12 hours, HRP-conjugated anti-mouse IL-1β was added, and incubated for additional 1 hour at RT. The color was developed with 50 μl of 3,3’5,5’-Tetramethylbenzidine (TMB) substrate (eBioscience, San Diego, CA). The reaction terminated by 50 μl of 2N H_2SO_4 and absorbance of reaction measured at 450 nm using spectrophotometer (Bio-Rad, Hercules, CA, USA).

EXOSOME qRT-PCR METHOD

Total exosomal RNA was isolated using TRIzol according to manufacturer's instructions. cDNA was generated from 0.05 μg of RNA using random hexamers and reverse transcriptase (Maxima, Thermo Scientific). qRT-PCR was carried out using SYBR green master mix (Kapa

Biosystems), and a thermal cycler (Bio-Rad). For amplifying transcripts originated from mouse or human mitochondrial genome, the primers are designed and provided in Supporting Table S2. The relative quantities of mitochondrial RNAs were calculated using the ΔΔCt method without normalization.

EXOSOMAL RNA ANALYSIS

For high throughput RNA-sequencing, we followed standard protocol of Illumina sequencing using TruSeq Stranded Total RNA kit with Ribo-Zero (Part#15031048 Rev. E) and NovaSeq6000 S4 (150bp PE). Analyses were performed on two paired-ends samples with 3.1 Gb Raw data/2.6 Gb Trimmed data for Control sample and 3.4 Gb/2.8 Gb for EtOH-treated sample. The Q30 percentage (% of bases with quality over phred score 30) of raw and trimmed data are 93%/95% and 92%/95% respectively. Trimmed reads are mapped to reference genome (HG19 with chrM transcripts)[7] with HISAT2 resulting in 27% and 44% mapping radio. After the read mapping, Stringtie was utilized for transcript assembly. Expression profile was calculated for each sample and transcript/gene as read count and FPKM (Fragment per Kilobase of transcript per Million mapped reads). (Supporting Table S3).

STATISTICAL ANALYSIS

Data are presented as the means ± SEM. To compare values obtained from two or more groups, Student's *t* test or one-way analysis of variance was performed. A value of $P < 0.01$ or 0.05 was considered statistically significant.

References

1. Bertola A, Mathews S, Ki SH, Wang H, Gao B. Mouse model of chronic and binge ethanol feeding (the NIAAA model). Nat Protoc 2013;8:627-637.
2. Choi WM, Kim HH, Kim MH, Cinar R, Yi HS, Eun HS, et al. Glutamate Signaling in Hepatic Stellate Cells Drives Alcoholic Steatosis. Cell Metab 2019.
3. Seo W, Eun HS, Kim SY, Yi HS, Lee YS, Park SH, et al. Exosome-mediated activation of toll-like receptor 3 in stellate cells stimulates interleukin-17 production by gammadelta T cells in liver fibrosis. Hepatology 2016;64:616-631.
4. Choi WM, Eun HS, Lee YS, Kim SJ, Kim MH, Lee JH, et al. Experimental Applications of in situ Liver Perfusion Machinery for the Study of Liver Disease. Mol Cells 2019;42:45-55.
5. Werner M, Driftmann S, Kleinehr K, Kaiser GM, Mathe Z, Treckmann JW, et al. All-In-One: Advanced preparation of Human Parenchymal and Non-Parenchymal Liver Cells. PLoS One 2015;10:e0138655.
6. Kim SY, Jeong JM, Kim SJ, Seo W, Kim MH, Choi WM, et al. Pro-inflammatory hepatic macrophages generate ROS through NADPH oxidase 2 via endocytosis of monomeric TLR4-MD2 complex. Nat Commun 2017;8:2247.
7. Mercer TR, Neph S, Dinger ME, Crawford J, Smith MA, Shearwood AM, et al. The human mitochondrial transcriptome. Cell 2011;146:645-658.

Supporting Table 1. Primer sequences of mouse and human for qRT-PCR

Gene	Forward (5’-3’)	Reverse (5’-3’)	Base pair
Mouse			
Actb	GTTACCAACTGGGACGAC	CTCAAACATGATCTGGGTCA	150bp
IL1b	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG	191bp
IL23a	CAGCAGCTCTCTCGGAATCTC	TGGATACGGGGCACATTATTTTT	119bp
Ccl20	ACTGTTGCCTCTCGTACATACA	GAGGAGGTTCACAGCCCTTTT	176bp
Il17a	GCTCCAGAAGGCCCTCAGA	CTTTCCCTCCGCATTGACA	139bp
Nlrp3	ATTACCCGCCCGAGAAAGG	TCGCAGCAAAGATCCACACAG	141bp
Tlr3	AAAATCCTTGCGTTGCGAAGT	TGTTCAAGAGGAGGGCGAATAA	145bp
Aim2	GTCACGGTGCTGAAAGCTATAA	GGCCACTGTTGCATGAAATATCT	81bp
Tlr9	ACAACTCTGACTTCGTCCACC	TCTGGGCTCAATGGTCATGTG	116bp
Mt-Cox3	ACCAAGGCCACCACACTCCT	ACGCTCAGAAGAATCCTGCAAAGAA	120bp
Pnpt1	AATCGGGCACTCAGCTATTTG	CAGGTCTACAGTCACCGCTC	75bp
Supv3l1	GTGCAGCTCATGTGGACGATT	GGGTGGTATCCTCAAGTCACTG	116bp
Il6	TCCATCCAGTTGCCTTCTTG	TTCCACGATTTCCCAGAGAAC	166bp
Tnf	AAGCCTGTAGCCCACGTCGTA	AAGGTACAACCCATCGGCTGG	140bp
Rorc	TGCAAGACTCATCGACAAGG	AGGGGATTCAACATCAGTGC	176bp
Cxcl1	AACCGAAGTCATAGCCACAC	CAGACGGTGCCATCAGAG	147bp
Cxcr2	ATGCCCTCTATTCTGCCAGAT	GTGCTCCGGTTGTATAAGATGAC	152bp
Clec4f	AAAAGGCAAACGCTATGACTTCC	CCTCAACGCCTGGATCTCAG	128bp
Timd4	GTGTACTGCTGCCGTATAGAGG	TGGTGGTTGGGAGAACAGATG	196bp
Fasl	CAGCCCATGAATTACCCATG	ATTTGTGTTGTGGTCCTTCT	228bp
Human			
ACTB	AGCGAGCATCCCCCAAAGTT	GGGCACGAAGGCTCATCATT	285bp
IL1B	ATGATGGCTTATTACAGTGG	GTCGGAGATTCGTAGCTGGA	132bp
IL23A	CTCAGGGACAACAGTCAGTT	ACAGGGCTATCAGGGAGCA	119bp
CCL20	TGCTGTACCAAGAGTTTGCTC	CGCACACAGACAACTTTTTCTTT	220bp
IRF3	AGAGGCTCGTGATGGTCAAG	AGGTCCACAGTATTCTCCAGG	100bp
AIM2	AGCAAGATATTATCGGCACAGTG	GTTCAGCGGGACATTAACCTT	97bp
NLRP3	CGTGAGTCCCATTAAGATGGAGT	CCCGACAGTGGATATAGAACAGA	191bp
TLR3	GAT GAA ATG TCT GGA TTT GGA CTA	GTT AGC TGG CTA TAC CTT GTG A	297bp
IL17A	AGATTACTACAACCGATCCACCT	GGGGACAGAGTTCATGTGGTA	151bp
TLR9	CTGCCACATGACCATCGAG	GGACAGGGATATGAGGGATTTGG	121bp
PNPT1	CCTTCCCAGTTTATGCCTTTGG	AAATAGCGGTCTAATTGAACGAT	153bp
SUPV3L1	CCTGTGTTGGACTGTAAGGATG	TGCGTGATAAGTCTTTCCACTG	141bp

Supporting Table 2. Primer sequences of mouse and human for qRT-PCR of exosomes

Gene	Forward (5’-3’)	Reverse (5’-3’)
Mouse		
mt-Tf	TGTAGCTTAATAACAAAGCAAAGCA	TTATGGGATACAATTATCCATCTAAGC
mt-Rnr1	TTTGCCAGAGAACTACTAGCC	GGATATAAAGTACCGCCAAGTC
mt-Tv	GTGTAGCTTAATATTAAAGCATCTGG	GTGTTCATTGGTCATGAAATCTTC
mt-Rnr2	CGATTAAAGTCCTACGTGATCTG	ATAGATAGAAACCGACCTGGA
mt-Tl1	TGGCAGAGCCAGGAAATTG	AGAGGATTTGAACCTCTGGG
mt-Nd1	TACGCCCTTTAACAACCTC	AGTGTGAGTGATAGGGTAGG
mt-Ti	AATATGTCTGATAAAAGAATTACTTTG	TAAGAGGGCTTGAACCTC
mt-Tm	GTCAGCTAATTAAGCTATCGGG	GTACGGGAAGGATTTAAACCA
mt-Nd2	CACAATATCCAGCACCAACC	TGCTAGTAGGCTGAATTCCA
mt-Tw	GTTTAGGATATACTAGTCCGCGA	AAGTTAAACTTGTGTGTTTTCTTAGG
mt-Co1	ACTTTCTTTGATCCCGCTG	AGAATCAGAACAGATGCTGG
mt-Td	GATATTAGTAAAATCAATTACATAACT	ATAGATTATTGATCTATAATTTAACTT
mt-Co2	ATAATCCCAACAAACGACCT	CTCGGTTATCAACTTCTAGCA
mt-Tk	ATGAAGCTAAGAGCGTTAACC	CACTATGGAGATTTTAAGGTCTCTAA
mt-Atp8	ATTCCCACTGGCACCTTCAC	TCTCAAGGGGTTTTTACTTTTATGGT
mt-Atp6	CCTTCAATCCTATTCCCATCC	GTTGGAAAGAATGGAGACGG
mt-Co3	GGTATAATTCTATTCATCGTCTCGG	AGAACGCTCAGAAGAATCCT
mt-Tg	CCCTTAGTATAATTAATATAACTGAC	TCTTCTGGGTTTATTCAGAAT
mt-Nd3	AGCAAATCCATATGAATGCG	TATTGAGAATGGTAGACGTGC
mt-Tr	GGTAATTAGTTTAAAAAAAATTAATGA	TTATGAACATCATCATAATCTAAT
mt-Nd4L	TCTTCAACCTCACCATAGCC	TGAGAGCGAAATATAAGTGTCC
mt-Nd4	ATCTGCTTACGCCAAACAG	CTATGTGGCTAACTGAGGAG
mt-Th	ATAGTTTACAAAAAACATTAGACTGTG	TGAATAAGGAGGTTTATTTCCTGTT
mt-Ts2	GAAAGATTGCAAGAACTGCTAATTC	AGAAAGCCATGTTTTTAAACATGG
mt-Tl2	GGATAATAGTAATCCATTGGTCTTAGG	CTTTTATTTGGATTTGCACCAAGG
mt-Nd5	TGCCTTCTCTACATCAAGCC	TGGTTGGTTTATTCCTAGCGT
mt-Cyb	TATACACGCAAACGGAGCCT	CCTCGTCCGACATGAAGGAA
mt-Tt	GATAGTATAAACATTACTCTGGTCT	TCTTGAGAAGAGAAGATCTTCATTT
mt-Tp	GAAGTAGTTTAATTAGAATACCAGCT	GAAGAAGGAGCTACTCCCC
mt-Te	TCTGTAGTTGAATTACAACGATGA	TACACAGCATTCAACTGCGA
mt-Nd6	GATGGTTTGGGAGATTGGT	CTCCTTCCAACATAACTCCA
mt-Ts1	GAAAGACATATAGGATATGAGATTGGC	GGAAGGAATCGAACCCCC
mt-Ty	AAATGGCTGAGTAAGCATTAGACT	TAAAAAGAGGATTTAAACCTCTGTGT
mt-Tc	GGTCTTAAGGTGATATTCATGTCG	GTCTTAGTAGAGATTTCTCTACACC
mt-Tn	GAAGCCAGTAATAGGGTATTTAGC	ATTGGCAGGAATTAAACCTACG
mt-Ta	GGTCTTAGCTTAATTAAAGCAATTGAT	TGTAAGACTTCATCCTACATCTATTGA
mt-Tq	AGGTGTTTAGGTAGCACGG	GGACAATAGGAATTGAACCTACAC

Human		
MT-TF	TATGTAGCTTACCTCCTCAAAGC	GGGTGATGTGAGCCCGTC
MT-RNR1	AAGATTACACATGCAAGCATCC	TTGATCGTGGTGATTTAGAGG
MT-TV	GAGTGTAGCTTAACACAAAGCA	GGTCAAGTTAAGTTGAAATCTCCT
MT-RNR2	AACTCGGCAAATCTTACCC	AATACTGGTGATGCTAGAGGTG
MT-TL1	TAAGATGGCAGAGCCCGG	TTGAACCTCTGACTGTAAAGTTTT
MT-ND1	TCCTACTCCTCATTGTACCCA	TTTCGTTCGGTAAGCATTAGG
MT-TI	AATATGTCTGATAAAAGAGTTACTT	TAAGGGGGTTTAAGCTCC
MT-TM	GGTCAGCTAAATAAGCTATCGG	GTACGGGAAGGGTATAACCA
MT-ND2	GTAAGCCTTCTCCTCACTCTC	TTAATCCACCTCAACTGCCT
MT-TW	TTAGGTTAAATACAGACCAAGAGC	GAAATTAAGTATTGCAACTTACTGAG
MT-CO1	ATATTTCACCTCCGCTACCA	TCAGCTAAATACTTTGACGCC
MT-TD	AGAAAAACCATTTCATAACTTTGTCA	AAGATATATAGGATTTAGCCTATAATT
MT-CO2	ACGCATCCTTTACATAACAGAC	GCCAATTGATTTGATGGTAAGG
MT-TK	AGCTAACTTAGCATTAACCTTTTAAGT	ACTGTAAAGAGGTGTTGGTTCT
MT-ATP8	TAAATACTACCGTATGGCCCAC	GTGATGAGGAATAGTGTAAGGAG
MT-ATP6	TCCCTCTACACTTATCATCTTCAC	GACAGCGATTTCTAGGATAGTC
MT-CO3	CTCTCAGCCCTCCTAATGAC	GCGTTATGGAGTGGAAGTG
MT-TG	AGTATAAATAGTACCGTTAACTTCCA	ACTCTTTTTTGAATGTTGTCAAAA
MG-ND3	TTGATCTAGAAATTGCCCTCC	GGCAGGTTAGTTGTTTGTAGG
MT-TR	GTATATAGTTTAAACAAAA	GTAAATATGATTATCATAA
MT-ND4L	CTCATAACCCTCAACACCCA	AGACTAGTATGGCAATAGGCAC
MT-ND4	CCCTTCCTTGTACTATCCCT	TTTGTCGTAGGCAGATGGAG
MT-TH	AATATAGTTTAACCAAAACATCAGAT	TAAGGGGTCGTAAGCCTC
MT-TS2	AAGCTCACAAGAACTGCTAAC	AGAAAGCCATGTTGTTAGACATG
MT-TL2	TTTAAAGGATAACAGCTATCCATTGG	TTTGGAGTTGCACCAAAATTTTT
MT-ND5	TCTTAGTTACCGCTAACAACC	ATAATTCCTACGCCCTCTCAG
MT-CYB	ATCACTTTATTGACTCCTAGCC	TGGTTGTCCTCCGATTCAG
MT-TT	TTGTAGTATAAACTAATACACCAGT	TCCTTGGAAAAAGGTTTTCAT
MT-TP	ATAGTTTAAATTAGAATCTTAGCTTT	AGAGAAAAAGTCTTTAACTCCAC
MT-TE	TTGTAGTTGAAATACAACGATGGT	TCGCACGGACTACAACCA
MT-ND6	GCTTTGTATGATTATGGGCGT	CACCAACAAACAATGTTCAACC
MT-TS1	AAGTCATGGAGGCCATGG	AAGGAAGGAATCGAACCCC
MT-TY	AAATGGCTGAGTGAAGCATTG	GTAAAAAGAGGCCTAACCCCT
MT-TC	CTCCGAGGTGATTTTCATATTGAA	CCCGGCAGGTTTGAAGCT
MT-TN	AGATTGAAGCCAGTTGATTAGG	GGACTTAAACCCACAAACAC
MT-TA	GGGCTTAGCTTAATTAAAGTGGC	TGCAAAACCCCACTCTGC
MT-TQ	GATGGGGTGTGATAGGTGG	TGAGAATCGAACCCATCCC

Supporting Table 3. Exosome Sequencing data of human (Excel file attached)

Supporting Figure Legends

sFig. 1. Kupffer cells are critical for increased IL-17A production in γδ T cells in acute alcoholic liver injury. WT mice were sacrificed at 6 hour after binge drinking with or without pretreatment of clodronate (10 μl/g) for 2 days (*n* = 5/group). (A) IL-17A expression was assessed in liver MNCs, Kupffer cells (KCs) or hepatic stellate cells (HSCs). (B) Liver MNCs were analyzed by flow cytometry to check removal of F4/80^{high}CD11b^{+} Kupffer cells. After clodronate treatment, F4/80^{high}CD11b^{+} Kupffer cells were disappeared but F4/80^{+}CD11b^{+} macrophages were still observed. (C) mRNA expression of *Clec4f* and *Tim4* was assessed in freshly isolated F4/80^{high}CD11b^{+} Kupffer cells and F4/80^{+}CD11b^{+} macrophages after binge ethanol consumption. (D) Serum levels of ALT and AST were measured. (E) IL-17A producing cells were counted. Values represent the results from three experimental replicates. Data are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 in comparison with the corresponding controls, by unpaired *t*-test between two groups and one way ANOVA with Dunnett's test for multiple comparison vs control.

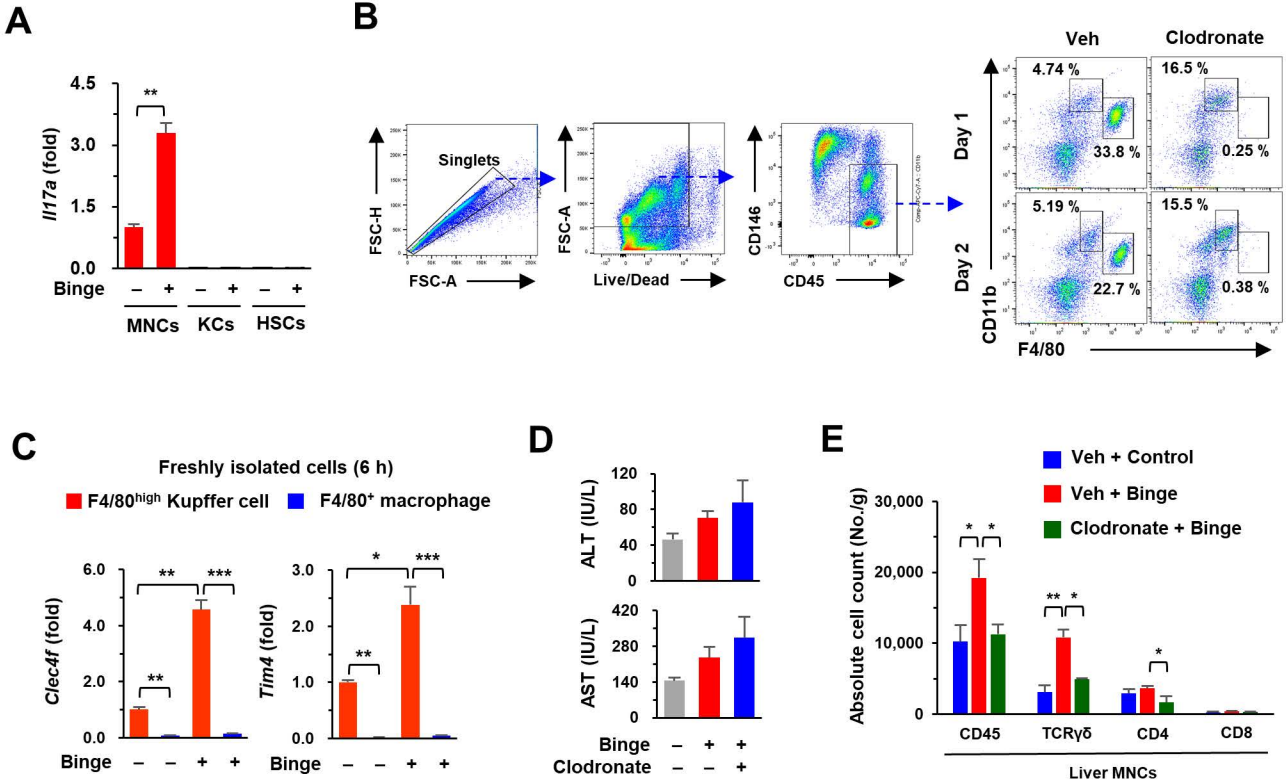
sFig. 2. Ethanol treatment increases release of extracellular vesicles from hepatocytes. (A) Medium AST levels were measured after 100 mM ethanol treatment to hepatocytes for 24 hours. (B) The shapes and numbers of twinkling exosomes (arrowhead) and microvesicles (open arrowhead) were confirmed by Nanoparticle Tracking Analysis after ethanol treatment to hepatocytes. (C) *Il23a* mRNA expression was assessed in WT Kupffer cells after co-culturing with γδ T cells in the presence of EVs. (D) Isolated WT γδ T cells were treated with poly I:C (50 μg/ml) or combination of IL-1β (10 ng/ml) and IL-23 (50 ng/ml). Then, expression and production of IL-17A were assessed and measured in WT γδ T cells and their supernatants, respectively. Values and images represent the results from three experimental replicates. Data are presented as mean ± SEM. **P* < 0.05, ****P* < 0.01 in comparison with the corresponding controls, by unpaired *t*-test between two groups.

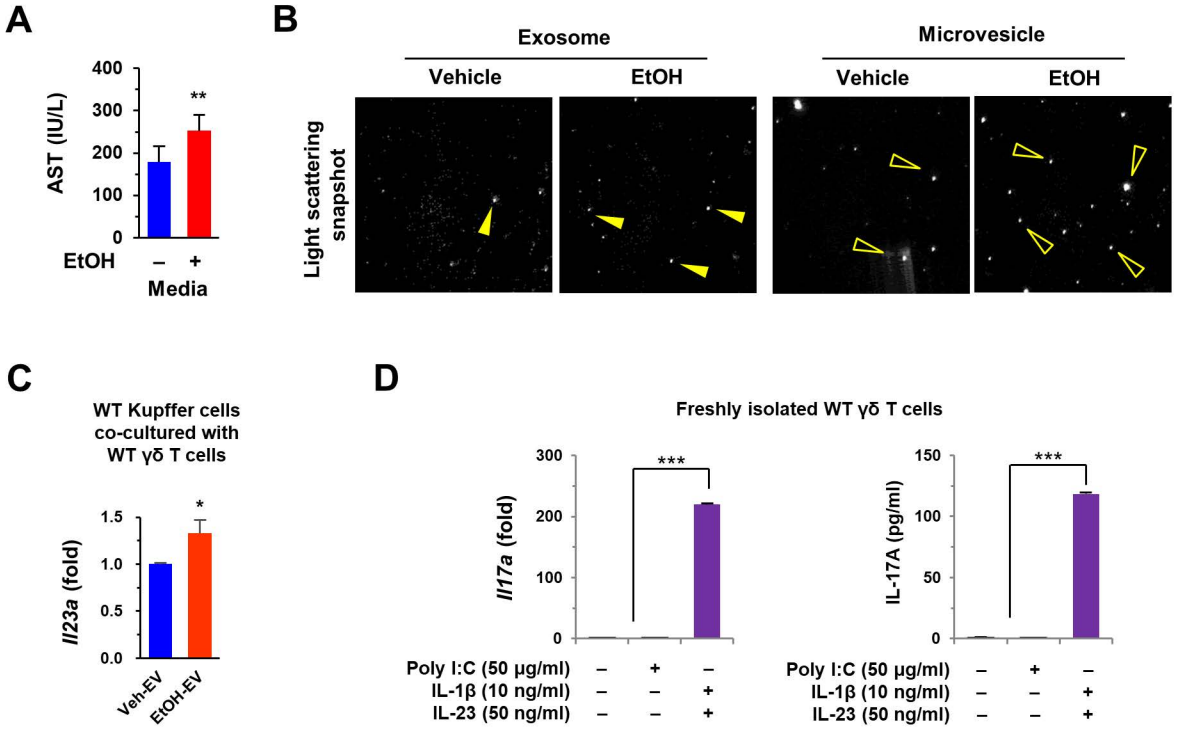
sFig. 3. TLR3-dependent IL-17A expression in acute alcoholic liver injury. Wild type (WT) and toll-like receptor 3 (TLR3) knockout (KO) mice were fed with 4 g/kg of ethanol (binge

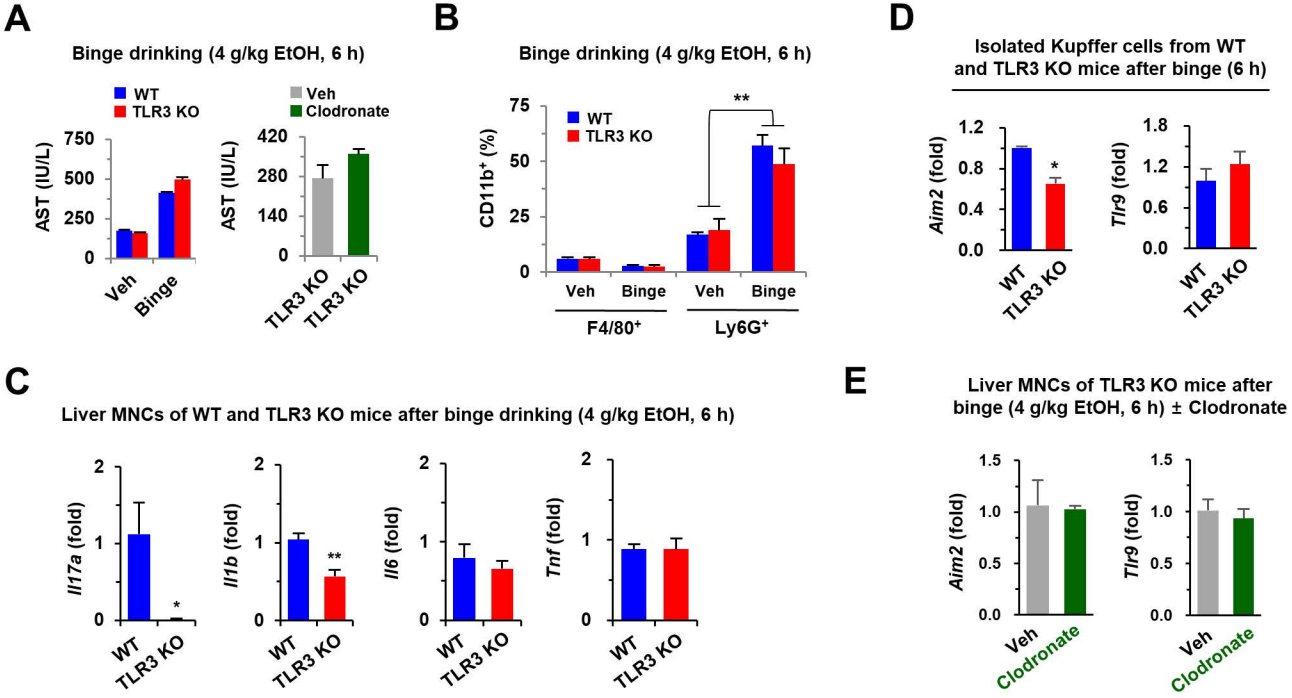
drinking) for 6 hours with or without 2-day pretreatment of clodronate (n = 5/group). (A) After binge drinking, serum AST levels were measured in WT and TLR3 KO mice or TLR3 KO mice with or without clodronate. (B) The population of $CD11b^+F4/80^+$ macrophages and $CD11b^+Ly6G^+$ neutrophils were analyzed by flow cytometry in liver mononuclear cells (MNCs) of WT and TLR3 KO mice. (C,D) Gene expression in Kupffer cells and liver MNCs of WT and TLR3 KO mice was analyzed by qRT-PCR after binge drinking (6 h). (E) Gene expressions were analyzed in liver MNCs of TLR3 KO mice with or without clodronate treatment (3 replicates). Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 in comparison with the corresponding control by unpaired t-test between two groups and one-way ANOVA with Dunnett's test for multiple comparison vs control.

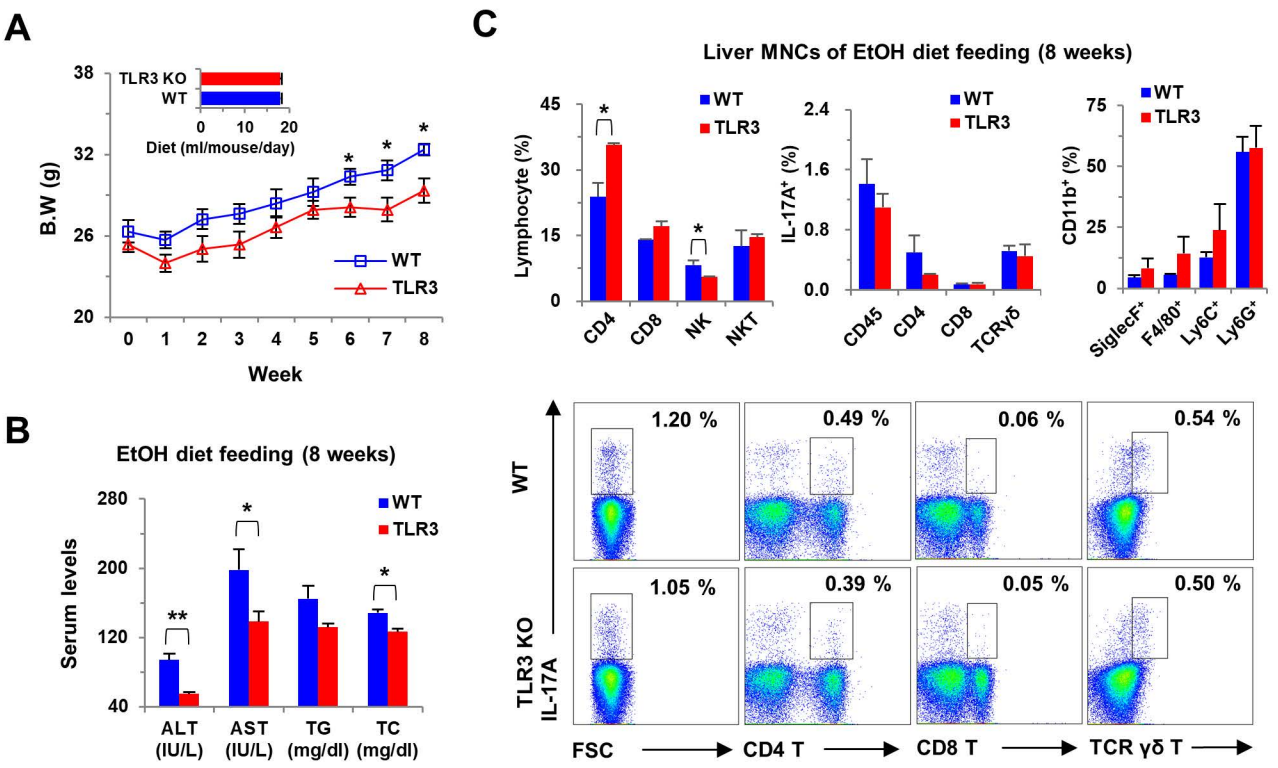
sFig. 4. TLR3 deficiency does not attenuate IL-17A production of γδ T cells in alcoholic steatosis model. Wild type (WT) and toll-like receptor 3 (TLR3) knockout (KO) mice were fed liquid ethanol diet for 8 weeks (n = 5/group). (A) Changes of body weight and diet intake were recorded. (B) Serum levels of ALT, AST, TG and TC were measured in WT and TLR3 KO mice. (C) Liver MNCs of WT and TLR3 KO mice were analyzed by flow cytometry. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 in comparison with the corresponding control by unpaired t-test between two groups and one-way ANOVA with Dunnett's test for multiple comparison vs control.

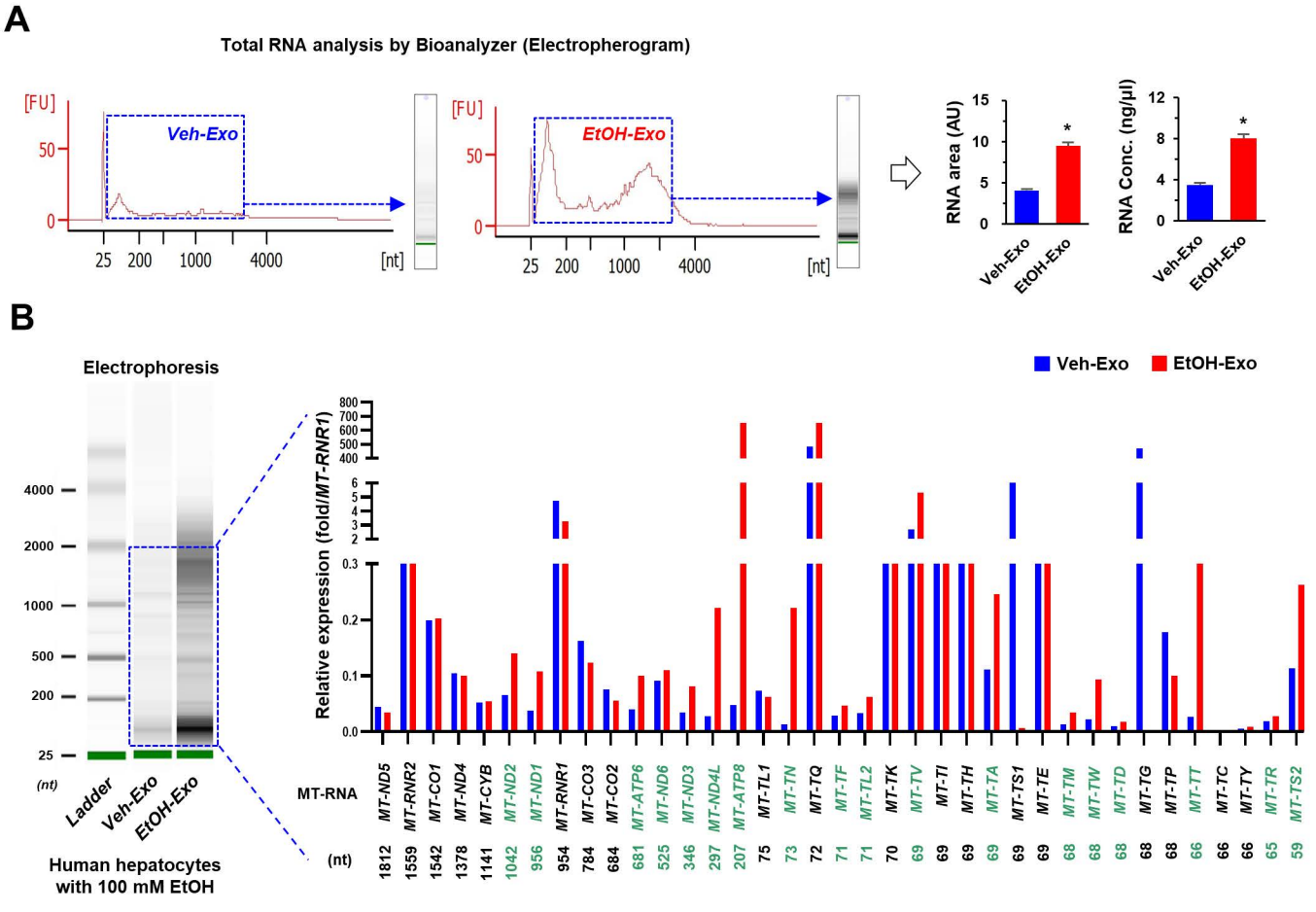
sFig. 5. RNA sequencing reveals increased mitochondrial RNA contents in exosomes of ethanol-treated human hepatocytes. Human hepatocytes were treated with or without 100 mM ethanol for 24 hours and then hepatic exosomes were isolated from media. (A) Size distribution and contents of total RNA isolated from hepatic exosomes were assessed by Bioanalyzer and Electropherogram. (B) Size distribution and contents of total RNA isolated from hepatic exosomes were shown by Electrophoresis, and relative mRNA expressions of 37 mitochondrial genes were analyzed by qRT-PCR in isolated Veh-Exo and EtOH-Exo from human hepatocytes. Green colored genes were up-regulated genes in EtOH-Exo compared to those of Veh-Exo. Values and images represent the results from three experimental replicates. Data are expressed as the mean ± SEM. *P < 0.05 in comparison with the corresponding control by unpaired t-test between two groups.


A
Il17a (fold)
4.5
3.0
1.5
0.0
**
Binge
– + – + – +
MNCs KCs HSCs
B
Singlets
FSC-H
FSC-A
FSC-A
Live/Dead
CD146
CD45
Veh
Clodronate
Day 1
Day 2
4.74 %
33.8 %
16.5 %
0.25 %
5.19 %
22.7 %
15.5 %
0.38 %
CD11b
F4/80
C
Freshly isolated cells (6 h)
F4/80high Kupffer cell
F4/80+ macrophage
Clec4f (fold)
Tim4 (fold)
Binge
– – + +
D
ALT (IU/L)
AST (IU/L)
Binge – + +
Clodronate – – +
E
Veh + Control
Veh + Binge
Clodronate + Binge
Absolute cell count (No./g)
30,000
20,000
10,000
0
CD45
TCRγδ
CD4
CD8
Liver MNCs


A
AST (IU/L)
400
300
200
100
0
**
EtOH – +
Media
B
Exosome
Microvesicle
Vehicle
EtOH
Vehicle
EtOH
Light scattering snapshot
C
WT Kupffer cells co-cultured with WT γδ T cells
Il23a (fold)
1.5
1.0
0.5
0.0
*
Veh-EV
EtOH-EV
D
Freshly isolated WT γδ T cells
Il17a (fold)
300
200
100
0
***
Poly I:C (50 μg/ml) – + –
IL-1β (10 ng/ml) – – +
IL-23 (50 ng/ml) – – +
IL-17A (pg/ml)
150
100
50
0
***
Poly I:C (50 μg/ml) – + –
IL-1β (10 ng/ml) – – +
IL-23 (50 ng/ml) – – +


A
Binge drinking (4 g/kg EtOH, 6 h)
WT
TLR3 KO
AST (IU/L)
750
500
250
0
Veh
Binge
Veh
Clodronate
AST (IU/L)
420
280
140
0
TLR3 KO
TLR3 KO
B
Binge drinking (4 g/kg EtOH, 6 h)
WT
TLR3 KO
CD11b+ (%)
75
50
25
0
**
Veh
Binge
Veh
Binge
F4/80+
Ly6G+
D
Isolated Kupffer cells from WT and TLR3 KO mice after binge (6 h)
Aim2 (fold)
1.2
0.8
0.4
0.0
*
WT
TLR3 KO
Tlr9 (fold)
1.8
1.2
0.6
0.0
WT
TLR3 KO
C
Liver MNCs of WT and TLR3 KO mice after binge drinking (4 g/kg EtOH, 6 h)
Il17a (fold)
2
1
0
*
WT
TLR3 KO
Il1b (fold)
2
1
0
**
WT
TLR3 KO
Il6 (fold)
2
1
0
WT
TLR3 KO
Tnf (fold)
2
1
0
WT
TLR3 KO
E
Liver MNCs of TLR3 KO mice after binge (4 g/kg EtOH, 6 h) ± Clodronate
Aim2 (fold)
1.5
1.0
0.5
0.0
Veh
Clodronate
Tlr9 (fold)
1.5
1.0
0.5
0.0
Veh
Clodronate


A
B.W (g)
Week
TLR3 KO
WT
Diet (ml/mouse/day)
WT
TLR3
B
EtOH diet feeding (8 weeks)
Serum levels
ALT (IU/L)
AST (IU/L)
TG (mg/dl)
TC (mg/dl)
C
Liver MNCs of EtOH diet feeding (8 weeks)
Lymphocyte (%)
CD4
CD8
NK
NKT
IL-17A+ (%)
CD45
CD4
CD8
TCRγδ
CD11b+ (%)
SiglecF+
F4/80+
Ly6C+
Ly6G+
WT
TLR3 KO
IL-17A
1.20 %
0.49 %
0.06 %
0.54 %
1.05 %
0.39 %
0.05 %
0.50 %
FSC
CD4 T
CD8 T
TCR γδ T


A
Total RNA analysis by Bioanalyzer (Electropherogram)
[FU]
Veh-Exo
EtOH-Exo
[nt]
RNA area (AU)
RNA Conc. (ng/μl)
B
Electrophoresis
Ladder
Veh-Exo
EtOH-Exo
Human hepatocytes with 100 mM EtOH
Relative expression (fold/MT-RNR1)
MT-RNA
(nt)