Supplementary File

MicroRNA-223 ameliorates alcoholic liver injury by inhibiting the IL-6-p47^{phox}-oxidative stress pathway in neutrophils

Supplementary Methods:

Human subject cohort

Forty-five healthy controls were recruited at Roudebush Veterans Administration Medical Center, Indianapolis, Indiana. They were non-smokers without underlying medical diseases such as hypertension, diabetes mellitus, chronic kidney diseases, liver diseases, and atherosclerotic disease. Three hundred excessive drinkers were recruited from Fairbanks Drug and Alcohol Treatment Center (Indianapolis, IN). All subjects were at least 21 years of age or older. They were excluded if they had active and serious medical diseases (such as congestive heart failure, chronic obstructive pulmonary disease, cancer, uncontrolled diabetes, and chronic renal failure); had history of chronic hepatitis B/C infection, had history of any systemic infection within 4 weeks prior to the study; or had history of recent major surgeries within the past 3 months. The Time Line Follow-Back (TLFB) questionnaire, administered by trained study coordinators, was used to determine the quantity of alcohol consumption over the 30-day period before the enrollment. The TLFB offers a retrospective report of daily alcohol consumption over the past 30 days; drinks per drinking occasion, as well as pattern of drinking.[1-3] Based on the information from the TLFB, we dichotomized excessive drinkers into those with (significant drinking within 10 days before enrollment) and without (no drinking within 10 days before enrollment) recent alcohol drinking. Blood samples were obtained for hematogram and hepatic panel. The study was approved by the Indiana University Purdue University Institutional Review Board, the Research and Development Committee at Roudebush VA, and Fairbanks Drug and Alcohol Treatment Center. All participants provided written informed consent.

Biochemical assays

The levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with IDEXX Catalyst Dx analyzer (IDEXX Laboratories, Westbrook, Maine)

Complete blood count (CBC) test

The anticoagulated blood was collected from mice. CBC test was performed with Hemavet 950 FS Hematology Analyzer (Drew Scientific, Dallas, TX)

Real-time PCR

The expression levels of genes were measured with quantitative real-time PCR by using ABI7500 real-time PCR detection system (Applied Biosystems, Foster City, CA). The primers for mouse genes were shown in Supplemental Table 1. The primers for human genes include human IL-6: F: 5'-GTC AGG GGT GGT TAT TGC A-3'; R:5'-AGT GAG GAA CAA GCC AGA GC-3'. Human $p47^{phox}$: F: 5'-TCC CCC TCC ACA GCA GTG T-3'; R: 5'-CTT CCT CGA GCC CCT GGA C-3'.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated, followed by antigen retrieval with pH6.0 citrate buffer or proteinase K pretreatment. Sections were incubated in 0.3% H_2O_2, and followed by another 30 mins in 1% BSA. Sections were incubated with primary antibodies overnight at 4°C. Vectastain Elite ABC Staining Kit and DAB Peroxidase Substrate Kit (Vector Laboratories, Inc., Burlingame, CA) were used to visualize the staining according to the manufacturer's instructions. Primary antibodies used were listed below: anti-myeloperoxidase (MPO) (Biocare Medical, Concord, CA), anti-malonaldehyde (MDA) (Genox, Baltimore, MD), 4-hydroxynonenal (4-HNE) (Genox) and anti-F4/80 Ab (Novus Biologicals, Cambridge, U.K.). The numbers of neutrophils and macrophages and the areas of MDA and 4-HNE

in the liver were counted in 10 randomly chosen visual fields (magnification, ×100), and the average of 10 selected microscopic fields was calculated.

Isolation of circulating neutrophils from human subjects and mice

For human blood neutrophil isolation, blood was collected from each participant into purple top EDTA tubes. Peripheral blood neutrophils were isolated using the magnetic separation according to the manufacturer's protocol (Cat # 130-104-434, MACSxpress® Neutrophil Isolation Kit, Miltenyi Biotec, San Diego, CA).

Mouse blood neutrophil isolation

The anticoagulated blood was collected from mice, and ACK lysing buffer (BioWhittaker, Walkersville, MD) was added. After incubation for 5 minutes, the total white blood cellswere collected by centrifugation at 1600rpm for 5 mins and washed in PBS containing 2% fetal bovine serum. Total white blood cells were subjected to neutrophil isolation by using a neutrophil isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

Isolation of mouse liver neutrophils

Liver tissues were passed through a 70 μm cell strainer in phosphate-buffered saline (PBS), and the cell suspension was centrifuged at 30 g for 5 minutes to pellet the hepatocytes. The supernatant, which was enriched in non-parenchymal cells, was centrifuged at 300 g for 10 minutes. The pellet was re-suspended in 15 ml of 35% Percoll (GE Healthcare, Pittsburgh, PA) and centrifuged at 500 g for 15 minutes. The resulting leukocyte pellet was re-suspended in 2 ml of ACK lysing buffer (BioWhittaker, Walkersville, MD). After incubation for 5 minutes on ice, the cells were washed in PBS containing 2% fetal bovine serum. The leukocytes were subjected to neutrophil isolation by using neutrophil isolation kit obtained from Miltenyi Biotec (San Diego, CA) according to the manufacturer's instructions.

Isolation of hepatocytes, hepatic stellate cells (HSCs) and Kuppfer cells.

Mice weighing 20 to 25 g were anesthetized intraperitoneally with 30 mg/kg pentobarbital sodium, and the portal vein was cannulated under aseptic conditions. The liver was subsequently perfused with an EGTA solution [5.4 KCl, 0.44 KH2PO4, 140 NaCl, 0.34 Na2HPO4, 0.5 EGTA, and 25 Tricine (pH 7.2), all in mmol/L] and digested with 0.075% collagenase solution. The liver was cut into ~2 mm^3 piece and shake for 30mins at 240rpm in 37°C incubator and pushed through 70 μm cell strainer. Hepatocytes were collected after centrifugation at 400rpm for 5min. The non-parenchymal cells in the supernatant were collected after centrifugation 1600rpm for 10mins at 4°C and re-suspended in 20% OptiPrep. Four ml 11.5% OptiPrep and 3ml GBSS were gently loaded at the top of 20% OptiPrep. After centrifugation at 3000rpm for 17 min at 4°C. The cell fraction at the GBSS and 11.5% OptiPrep interface is collected as HSCs. Kupffer cells were purified by magnetic bead sorting from cell pellets (Mitenyibiotec).

MicroRNA-223 measurement and Pri-miR-223 measurement

Total RNA was isolated by TRIZOL Reagent (Life Technologies). Total RNA was reverse transcribed to cDNA by using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). MicroRNA-223 was amplified by using MicroRNA Assay Kit (Life Technologies)

For pri-miR-223 detection, total RNA was isolated from neutrophils using TRIzol reagents (Invitrogen, Carlsbad, CA), and then RNA samples were purified with TURBO DNA-*free*™ Kit (Ambion, CA, USA) according to the manufacturer's instructions. The pri-miR-223 strand cDNA was synthesized using TaqMan® MicroRNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-qPCR analysis for pri-miR-223 expression was performed by using TaqMan® Pri-miRNA Assays (Invitrogen) and TaqMan® Universal PCR Master Mix (Invitrogen) according to the manufacturer's instructions. The fold-change for miR-223 relative to 18s rRNA endogenous control (Invitrogen) was determined by the formula $2^{-\Delta\Delta Ct}$.

Injection of Pre-miR-223 lentivirus

Pre–miR-223 lentiviral vector and control lentiviral vector were purchased from Genecopoeia (Rockville, MD). Mice were fed an ethanol diet for 5 days, then received a tail vein injection of lentiviral particle solution containing either scrambled control miRNA (L/control) or pre–miR-223 (L/miR-223) as described previously.[4] After injection, mice were fed an ethanol diet for an additional 5 days and followed by binging a single dose of ethanol (5g/kg). Nine hours later, mice were euthanized, and serum and liver tissues were collected for analysis.

Bone marrow (BM) transplantation

BM from tibias and femurs was washed twice in Hanks balanced salt solution, and $5x10^6$ BM cells were injected into the tail vein of lethally irradiated (11 Gy) recipient mice. To determine whether BM transplantation was successful, PBMCs were isolated from WT mice with $p47^{phox}$ KO BM transplantation and analyzed by RT-qPCR to conform lack of $p47^{phox}$ gene expression.

Flow cytometry analyses of ROS levels in neutrophils and macrophages from peripheral blood and liver

For detection of superoxide production, after cell surface markers (CD11b, Gr-1, CSF-R1) staining, cells were then incubated in medium with 100 μM dihydrorhodamine 123 (DHR, Life Technologies) and catalase (1000 U/ml, Sigma Chemicals, St Louis, MO) in the dark at 37°C for 5 minutes. 200ng PMA was added into the medium and incubated for additional 20min at 37°C. DHR intensity were measured by flow cytometry (FACS Calibur, BD Bioscience) in FL1 channel.

F4/80 and TUNEL Co-staining

Formalin-fixed, paraffin-embedded tissue sections were de-paraffinized and re-hydrated with PBS, followed by antigen retrieval of proteinase K digestion. Next, sections were blocked in 1% BSA for 30 mins. Sections were incubated with primary Abs for 1 hr at room temperature (RT) and followed by Alexa Fluor555-labeled secondary Abs for 30 mins. TdT Reaction and Click-iT Reaction were done in the above sections by using

Click-iT® Plus TUNEL Assay for In Situ Apoptosis Detection kit (Life Technologies). Laser scanning confocal microscope (LSM710, Zeiss, Germany) was used to capture images.

BrdU staining

Brdu staining was done by using BrdU Flow Kits (BD Bioscience). Cells were labeled by intraperitoneal injection of BrdU (50mg/kg) in mice. After 6h, the neutrophils were isolated from bone marrow. Cell surface antigens (Gr-1 and CD11b) were stained. Cells were then permeabilized with Cytoperm permealization Buffer Plus, and followed by incubation with Cytofix/Cytoperm Buffer. Then, cells were treated with DNase I and incubated with fluorochrome-conjugated anti-BrdU antibody. Stained cells were analyzed by flow cytometry.

Flow cytometry analysis of neutrophil apoptosis

Apoptosis of neutrophils was detected with FITC Annexin V Apoptosis Detection kit (eBioscience, San Diego, CA). Neutrophils were isolated from bone marrow and cultured *in vitro*. Cells were washed and re-suspended in 1×Binding Buffer with 5 μl of FITC Annexin V and 5ul PI for 15 min at RT in the dark. Cells were analyzed by flow cytometry within 1hr.

Cell lines, plasmids, transfection and luciferase assays

HEK 293T cells were cultivated at 37°C, 5% CO2 in DMEM (Dulbecco's modified Eagle medium) with 10% fetal bovine serum (Gibco). The 3'UTR fragment of IL-6 (NM_031168) was polymerase chain reaction (PCR) amplified and ligated into the corresponding XbaI sites of GV272 vector (GeneChem, Shanghai, China) using the primers:

5'GATCGCCGTGTAATTCTAGAGTGCGTTATGCCTAAGCATATC 3'

5'CCGGCCGCCCCGACTCTAGAATAAAAATAATAAATATCAATCATAAATTAAC 3'

The mmu-mir-223 (Gene ID: 723814) was synthesized and ligated into the corresponding XhoI / KpnI of GV268 vector (GeneChem).

Plasmid transfections for luciferase assays in HEK293T cells were performed with 0.1μg 3'UTR of IL -6 expression plasmid and 0.4 μg miRNA223 expression plasmid in a 24-well plate using X-tremegene 9 and HP transfection reagent (Roche Life Science, Germany), as described by the manufacturer. Luciferase activity was measured 48-hr post transfection using the Dual Luciferase Reporter Assay System as described by the manufacturer (Promega).

Statistical Analysis

Basic descriptive statistics, including mean, standard error of mean (SEM), and frequencies (percentages) were used. Chi-square test was used for comparison of categorical variables. Group comparisons were performed using unpaired T-test or one-way ANOVA followed by Tukey's multiple comparison test. Linear regression was used to determine the relationship between two continuous variables. Analyses were performed using GraphPad prism Software (La Jolla, CA) or IBM SPSS Statistics (Version 23). The P value <0.05 was considered statistically significant.

References:

1. Sobell LC, Sobell MB, Riley DM, et al. The reliability of alcohol abusers' self-reports of drinking and life events that occurred in the distant past. J Stud Alcohol 1988;49:225-32.
2. Sobell LC, Agrawal S, Sobell MB, et al. Comparison of a quick drinking screen with the timeline followback for individuals with alcohol problems. J Stud Alcohol 2003;64:858-61.
3. Vakili S, Sobell LC, Sobell MB, et al. Using the Timeline Followback to determine time windows representative of annual alcohol consumption with problem drinkers. Addict Behav 2008;33:1123-30.
4. Qadir XV, Chen W, Han C, et al. miR-223 Deficiency Protects against Fas-Induced Hepatocyte Apoptosis and Liver Injury through Targeting Insulin-Like Growth Factor 1 Receptor. Am J Pathol 2015;185:3141-51.

Supplementary Tables:

Supplemental Table 1

Primer sequences of mouse genes for real-time PCR

Gene	Gene ID	Forward Primer/ Reverse Primer (5'-3')
Ly6G	546644	TGCGTTGCTCTGGAGATAGA CAGAGTAGTGGGGCAGATGG
F4/80	13733	CTTTGGCTATGGGCTTCCAGTC GCAAGGAGGACAGAGTTTATCGTG
CD68	12514	TGCGGCTCCCTGTGTGT TCTTCCTCTGTTCCTTGGGCTAT
18S	19791	ACGGAAGGGCACCACCAGGA CACCACCACCCACGGAATCG
TNF-α	21926	AGGCTGCCCCGACTACGT GACTTTCTCCTGGTATGAGATAGCAAA
IL-1β	16176	TCGCTCAGGGTCACAAGAAA / CATCAGAGGCAAGGAGGAAAAC
IL-6	16193	TCCATCCAGTTGCCTTCTTG/ TTCCACGATTTCCCAGAGAAC
MCP-1	20296	TCTGGACCCATTCCTTCTTGG/ TCAGCCAGATGCAGTTAACGC
MIP-1β	20303	AACACCATGAAGCTCTGCGT AGAAACAGCAGGAAGTGGGA
MIP-2	20310	AGTGAACTGCGCTGTCAATGC/ AGGCAAACTTTTTGACCGCC
VCAM-1	22329	TGAACCCAAACAGAGGCAGAGT/ GGTATCCCATCACTTGAGCAGG
ICAM-1	15894	CAATTTCTCATGCCGCACAG AGCTGGAAGATCGAAAGTCCG
MIP-1α	20302	TGAGAGTCTTGGAGGCAGCGA TGTGGCTACTTGGCAGCAAACA
NCF-1	17969	TCCTCTTCAACAGCAGCGTA/ CTATCTGGAGCCCCTTGACA
NCF-2	17970	TCTATCAGCTGGTTCCCACG/ TGGCCTACTTCCAGAGAGGA
NCF-4	17969	ATCGTCTGGAAGCTGCTCAA/ CCCATCCATCTGCTTTTCTG

gp91phox	13058	GACCATTGCAAGTGAACACCC/ AAATGAAGTGGACTCCACGCG
P22phox	13057	ATGGAGCGATGTGGACAGAAG/ TAGATCACACTGGCAATGGCC
NOX1	237038	TCGAACGCTACAGAAGAAGCC/ TGGCAATCACTCCAGTAAGGC
NOX3	224480	TCAATCCAGTCCGCACCTTTG/ TGGATGGCCAGACTGATGAAG
NOX4	50490	TCATTTGGCTGTCCCTAAACG/ AAGGATGAGGCTGCAGTTGAG
TLR4	21898	TGTCATCAGGGACTTTGCTG/ TGTTCTTCTCCTGCCTGACA

Supplemental Table 2: Demographic, clinical characteristics and laboratory results of the study cohort - MALE

				Total alcoholic – recent drinking status		
Variables	**Healthy/non excessive drinkers (n=30)**	**Total alcoholics with or without recent drinking (n=223)**	**P-value**	**Alcoholics without recent drinking≠ (n=118)**	**Alcoholics with recent drinking∫ (n=105)**	**p-value**
Age (yrs)	30±1.0	37.1±0.7	0.001	35.3±1.0	39.1±1.2	0.01
Race (White, %)	28 (93%)	187 (84%)	0.59	98 (83%)	89 (85%)	0.51
Height (cm)	181.3±1.4	179.2±0.4	0.14	178.5±0.67	179.9±0.67	0.13
Weight (kg)	93.2±2.5	90.3±1.0	0.34	89.5±1.5	91.1±1.4	0.47
BMI (kg/m^2)	28.3±0.5	28.2±0.3	0.92	28.1±0.5	28.2±4.8	0.90
Total drinks in the last 30 days	3.6±0.9	199.5±13.1	0.0001	92.4±9.4	320.0±19.9	0.0001
Number of days drinking out of 30 days	2.1±0.5	16.8±0.5	0.0001	10.1±0.5	24.3±0.3	0.001
Total bilirubin (mg/dl)	0.5±0.05	0.7±0.02	0.01	0.6±0.03	0.8±0.04	0.003
AST (U/L)	19.8±1.5	48.8±1.7	0.01	30.0±2.1	38.2±3.5	0.04
ALT (U/L)	34.1±1.1	48.8±1.7	0.003	47.5±2.0	50.2±2.9	0.43
Albumin (g/dl)	4.3±0.06	3.8±0.03	0.0001	4.0±0.03	3.7±0.03	0.0001
Total protein (g/dl)	7.9±0.1	7.2±0.3	0.39	7.1±0.07	7.4±0.6	0.68
Hemoglobin (g/dl)	15.2±0.17	14.4±0.09	0.003	14.5±0.12	14.3±0.13	0.41
Platelet counts (x10^3/µl)	234±10.2	226.8±4.9	0.57	230.4±7.1	222.6±6.7	0.43
White blood cell counts (WBC, x10^3/µl)	6.7±0.3	7.1±0.14	0.30	6.7±0.15	7.5±0.23	0.006
Percentage of neutrophils to WBC	59.9±1.4	72.1±0.88	0.0001	66.2±1.18	78.6±0.9	0.0001
Percentage of Monocytes to WBC	6.7±2.0	9.2±0.18	0.0001	9.0±0.28	9.8±0.3	0.05
Percentage of lymphocytes to WBC	30.0±1.25	16.1±0.88	0.0001	21.9±1.2	9.5±0.9	0.0001
Neutrophil counts (x10^3/µl)	3.9±0.2	5.2 ± 0.13	0.001	4.5±0.1	5.9±0.2	0.0001
Monocyte counts (x10^3/µl)	0.4±0.03	0.6±0.02	0.0001	0.6±0.02	0.7±0.03	0.0001
Lymphocyte counts (x10^3/µl)	2.0±0.14	1.28±0.06	0.0001	1.8±0.06	0.38±0.06	0.0001

≠ Excessive drinkers with last drink ≥ 10 days before the enrollment

∫ Excessive drinkers with last drinks within 10 days before the enrollment

Data presented as mean ± S.E

Supplemental Table 3: Demographic, clinical characteristics and laboratory results of the study cohort - FEMALE

				Total alcoholic – recent drinking status		
Variables	**Healthy/non excessive drinkers (n=15)**	**Total alcoholics with or without recent drinking (n=77)**	**P-value**	**Alcoholics without recent drinking≠ (n=42)**	**Alcoholics with recent drinking∫ (n=35)**	**p-value**
Age (yrs)	34±2.4	38.8±1.1	0.10	37.0±1.7	41.1±1.6	0.08
Race (White, %)	11 (73%)	62 (81%)	0.51	33 (78%)	29 (83%)	0.22
Height (cm)	164.9±0.9	166.5±0.8	0.39	166.3±1.0	166.8±1.3	0.76
Weight (kg)	75.6±3.9	76.9±2.2	0.80	78.0±3.6	75.6±2.3	0.57
BMI (kg/m^2)	27.8±1.4	27.7±0.8	0.98	28.0±1.2	27.3±0.9	0.65
Total drinks in the last 30 days	4.8±1.4	188.7±15.9	0.0001	99.5±10.6	295.9±21.5	0.0001
Number of days drinking out of 30 days	3.5±1.1	16.7±0.9	0.0001	10.8±0.89	23.8±0.48	0.0001
Total bilirubin (mg/dl)	0.5±0.06	0.6±0.03	0.08	0.54±0.04	0.71±0.05	0.02
AST (U/L)	15.3±0.4	26.3±2.2	0.03	23.7±2.1	29.3±4.1	0.23
ALT (U/L)	32.2±0.92	40.3±2.3	0.002	37.2±2.9	44.0±3.7	0.16
Albumin (g/dl)	4.0±0.09	3.6±0.05	0.003	3.7±0.06	3.5±0.08	0.14
Total protein (g/dl)	7.7±0.13	6.5±0.09	0.0001	6.7±0.14	6.3±0.11	0.02
Hemoglobin (g/dl)	12.7±0.29	12.5±0.17	0.57	12.7±0.22	12.3±0.26	0.23
Platelet counts (x10^3/µl)	242±20.9	243±8.4	0.98	259.2±10.5	224.2±12.9	0.04
White blood cell counts (WBC, x10^3/µl)	6.5±0.37	6.6±0.22	0.87	6.7±0.29	6.5±0.35	0.63
Percentage of neutrophils to WBC	62.1±8.2	74.4±1.6	0.002	68.5±2.5	81.5±1.36	0.0001
Percentage of Monocytes to WBC	6.1±0.7	8.8±0.34	0.001	8.5±0.51	9.2±2.4	0.26
Percentage of lymphocytes to WBC	28.8±1.9	12.8±1.2	0.0001	17.2±1.7	7.5±1.3	0.0001
Neutrophil counts (x10^3/µl)	4.0±0.2	4.8 ± 0.18	0.02	4.5±0.2	5.3±0.3	0.03
Monocyte counts (x10^3/µl)	0.38±0.03	0.58±0.02	0.0001	0.58±0.03	0.58±0.03	0.96
Lymphocyte counts (x10^3/µl)	1.8±0.11	1.14±0.09	0.0001	1.6±0.09	0.52±0.10	0.0001

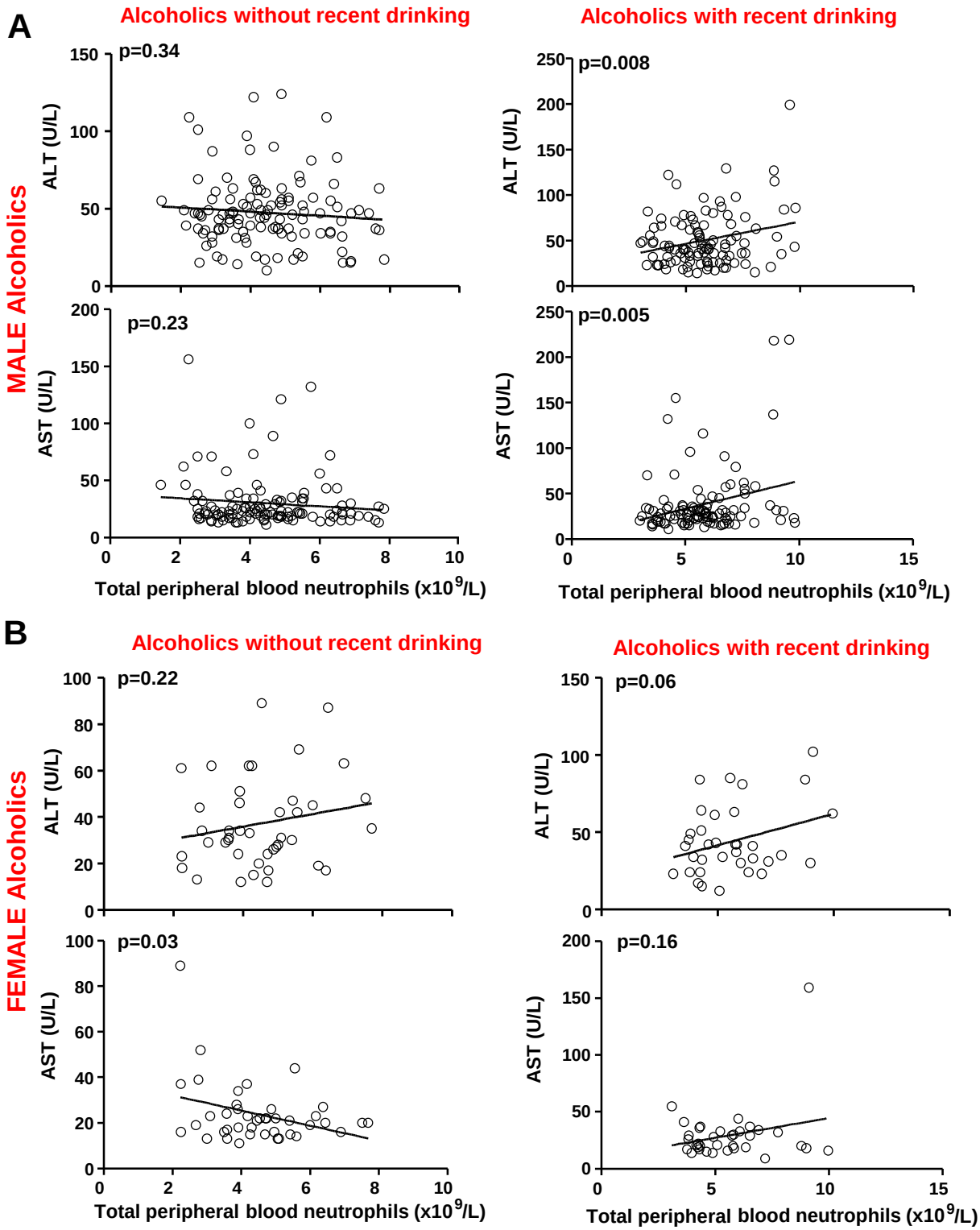
≠ Excessive drinkers with last drink ≥ 10 days before the enrollment

∫ Excessive drinkers with last drinks within 10 days before the enrollment

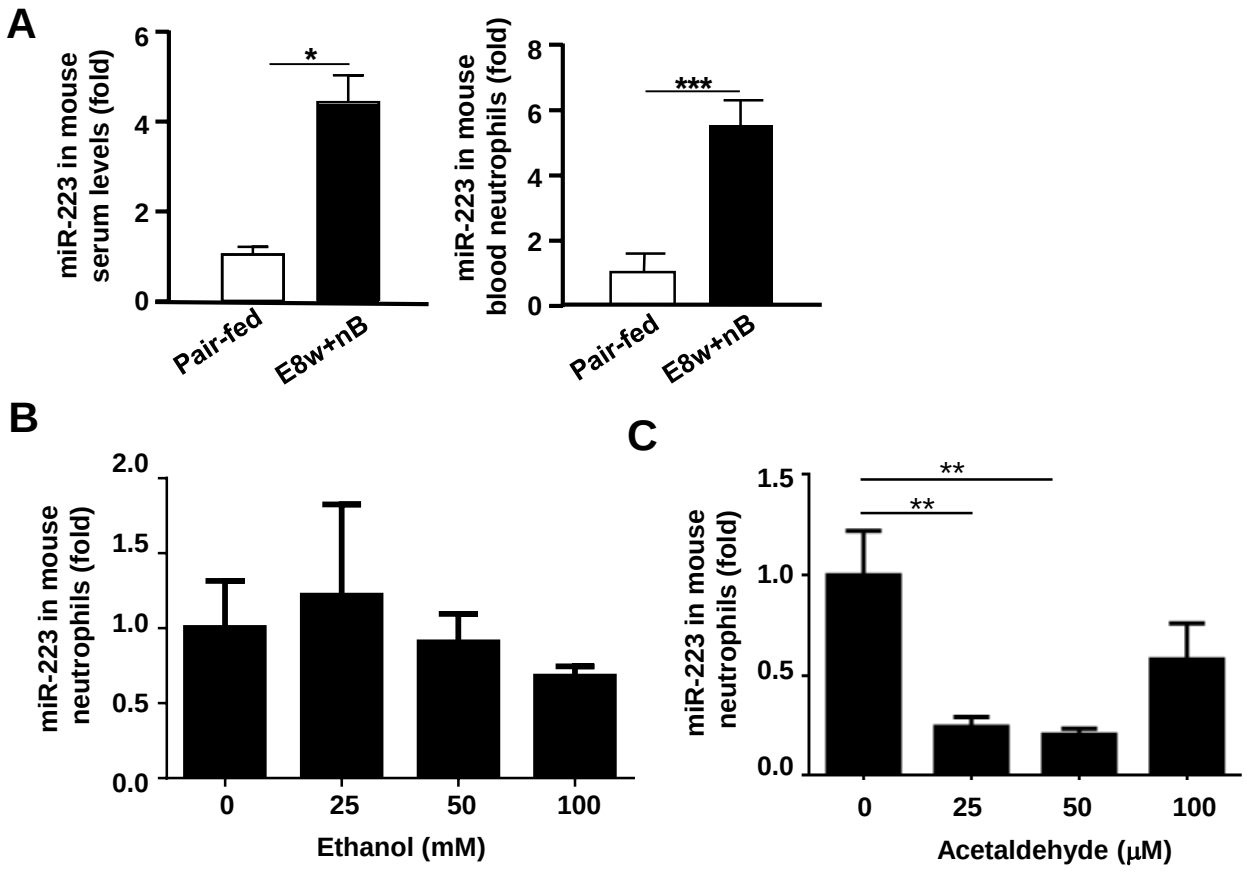
Data presented as mean ± S.E

Supplemental Table 4: Demographic, clinical characteristics and laboratory results of the study cohort, stratified by genders

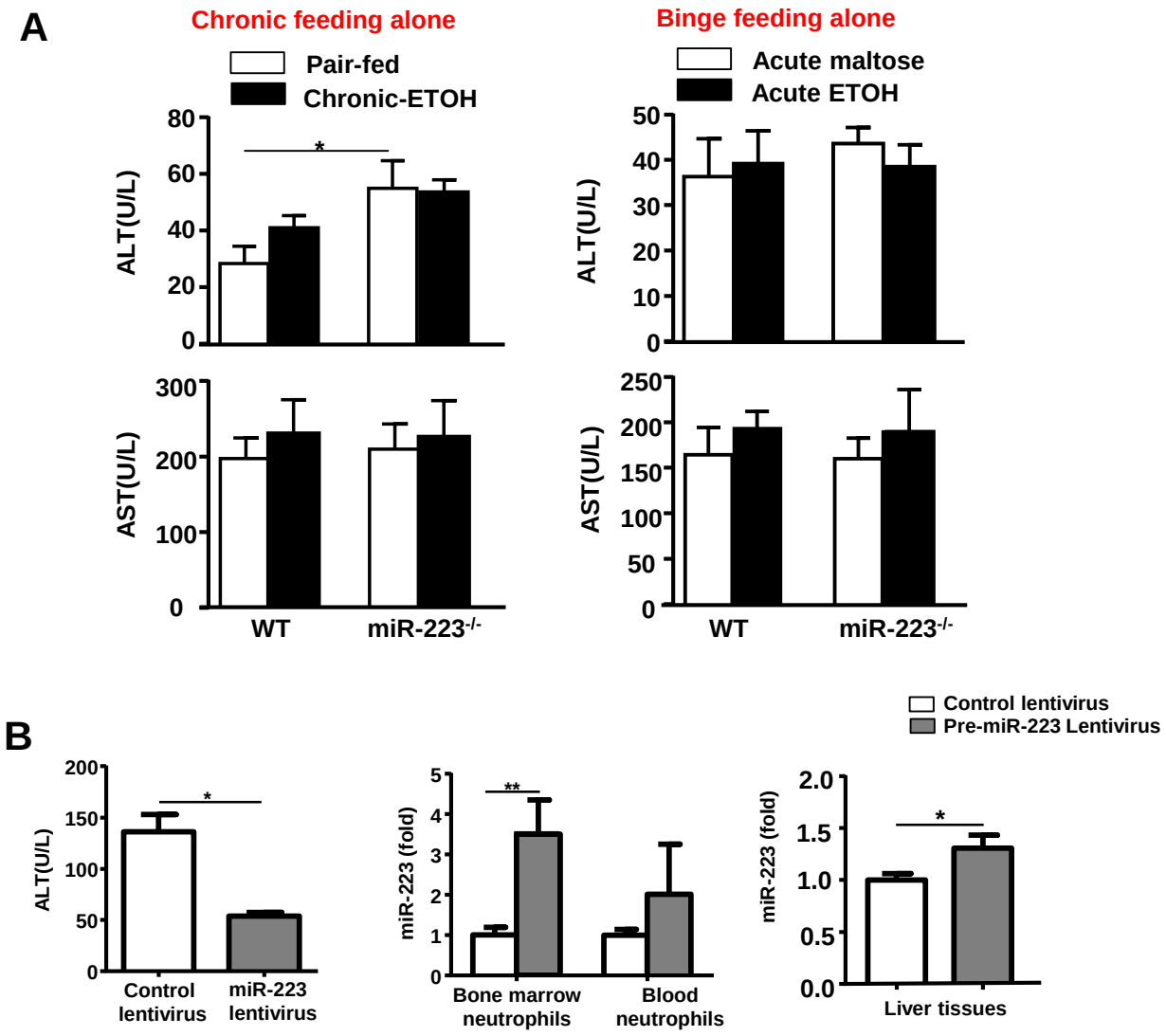
	Total alcoholics (N=300)			Alcoholics without recent drinking (N=160)			Alcoholics with recent drinking (N=140)		
Variables	**Male (N=223)**	**Female (N=77)**	**P-value**	**Male (N=118)**	**Female (N=42)**	**P-value**	**Male (N=105)**	**Female (35)**	**P-value**
Age (yrs)	37.1±0.78	38.9±1.2	0.22	35.3±1.0	37.0±1.7	0.40	39.1±1.2	41.1±1.6	0.32
Race (White, %)	28 (93%)	187 (84%)	0.59	98 (83%)	33 (78%)	0.51	89 (85%)	29 (83%)	0.51
Height (cm)	179.2±0.47	166.5±0.8	0.0001	178.5±0.67	166.3±1.0	0.0001	179.9±0.67	166.8±1.3	0.0001
Weight (kg)	90.3±1.05	76.9±2.2	0.0001	89.5±1.5	78.0±3.6	0.001	91.1±1.4	75.6±2.3	0.0001
BMI (kg/m^2)	28.2±0.3	27.7±0.7	0.60	28.1±0.5	28.0±1.2	0.94	28.2±4.8	27.3±0.9	0.43
Total drinks in the last 30 days	199.5±13.0	188.7±15.9	0.65	92.4±9.4	99.5±10.6	0.67	320.0±19.9	295.9±21.5	0.41
Number of days drinking out of 30 days	16.8±0.56	16.7±0.92	0.94	10.1±0.5	10.8±0.89	0.47	24.3±0.3	23.8±0.48	0.33
Total bilirubin (mg/dl)	0.74±0.02	0.62±0.03	0.02	0.6±0.03	0.54±0.04	0.02	0.8±0.04	0.71±0.05	0.11
AST (U/L)	33.9±2.02	26.3±2.2	0.01	30.0±2.1	23.7±2.1	0.04	38.2±3.5	29.3±4.1	0.11
ALT (U/L)	48.8±1.7	40.3±2.4	0.01	47.5±2.0	37.2±2.9	0.006	50.2±2.9	44.0±3.7	0.19
Albumin (g/dl)	3.8±0.02	3.6±0.05	0.0001	4.0±0.03	3.7±0.06	0.001	3.7±0.03	3.5±0.08	0.02
Total protein (g/dl)	7.2±0.29	6.5±0.09	0.03	7.1±0.07	6.7±0.14	0.03	7.4±0.6	6.3±0.11	0.10
Hemoglobin (g/dl)	14.4±0.09	12.5±0.17	0.001	14.5±0.12	12.7±0.22	0.001	14.3±0.13	12.3±0.26	0.0001
Platelet counts (x10^3/µl)	226±4.9	243.3±8.4	0.09	230.4±7.1	259.2±10.5	0.03	222.6±6.7	224.2±12.9	0.91
White blood cell counts (WBC, x10^3/µl)	7.1±0.14	6.6±0.22	0.08	6.7±0.15	6.7±0.29	0.95	7.5±0.23	6.5±0.35	0.02
Percentage of neutrophils to WBC	72.2±0.88	74.4±1.66	0.19	66.2±1.18	68.5±2.5	0.41	78.6±0.9	81.5±1.36	0.09
Percentage of Monocytes to WBC	9.4±0.21	8.8±0.34	0.18	9.0±0.28	8.5±0.51	0.40	9.8±0.3	9.2±2.4	0.28
Percentage of lymphocytes to WBC	16.1±0.88	12.8±1.23	0.03	21.9±1.2	17.2±1.7	0.03	9.5±0.9	7.5±1.3	0.22
Neutrophil counts (x10^3/µl)	5.1±0.13	4.8 ± 0.18	0.19	4.5±0.1	4.5±0.2	0.95	5.9±0.2	5.3±0.3	0.08
Monocyte counts (x10^3/µl)	0.66±0.01	0.58±0.02	0.02	0.6±0.02	0.58±0.03	0.64	0.7±0.03	0.58±0.03	0.002
Lymphocyte counts (x10^3/µl)	1.28±0.06	1.14±0.09	0.23	35.3±1.0	1.6±0.09	0.18	0.38±0.06	0.52±0.10	0.18



Supplemental Figure 1. Correlation between peripheral neutrophil counts and serum ALT or AST levels. (A) There is a positive correlation between peripheral neutrophil counts and serum ALT or AST levels in male alcoholics with recent drinking but not in those without recent drinking. (B) In female alcoholics, there is a trend in a positive correlation between peripheral neutrophil counts and serum ALT levels in alcoholics with recent drinking but not in those without recent drinking.



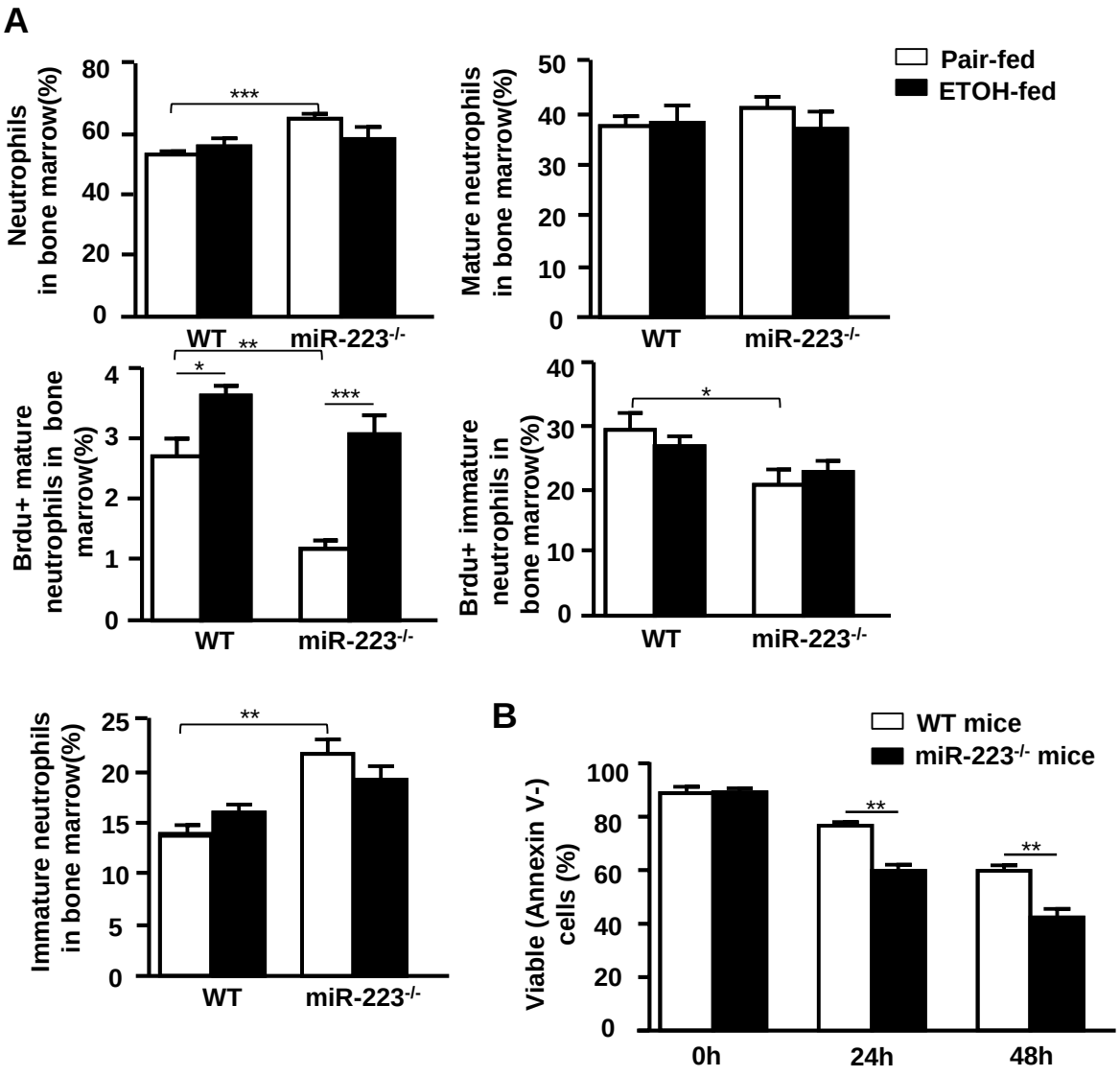
Supplemental Figure 2. A. miR-223 levels in serum and neutrophils from E8w+1B ethanol-fed mice. C57BL/6J mice were pair-fed or fed an ethanol-containing diet for 8 weeks plus multiple binges (E8w+nB). Mice were euthanized 9 h post gavage. Serum and blood neutrophils were collected for miR-223 measurement. **B. C. Effects of ethanol and acetaldehyde on miR-223 expression in mouse neutrophils.** Mouse peripheral neutrophils were isolated from C57BL/6 mice and incubated with various concentrations of ethanol or acetaldehyde for 4 hours. Expression of miR-223 was measured. Values represent means ± SEM (n=6-12). **P*<0.05; ***P*<0.01; *P*<0.001.



Supplemental Figure 3A. Deletion of the miR-223 does not affect chronic or acute ethanol-induced liver injury. WT and miR-223$^{-/-}$ mice were subjected to chronic ethanol feeding for four weeks or acute gavage alone. Sera were collected at four-week chronic ethanol feeding or 9 hours post acute gavage. Serum ALT and AST levels were measured.

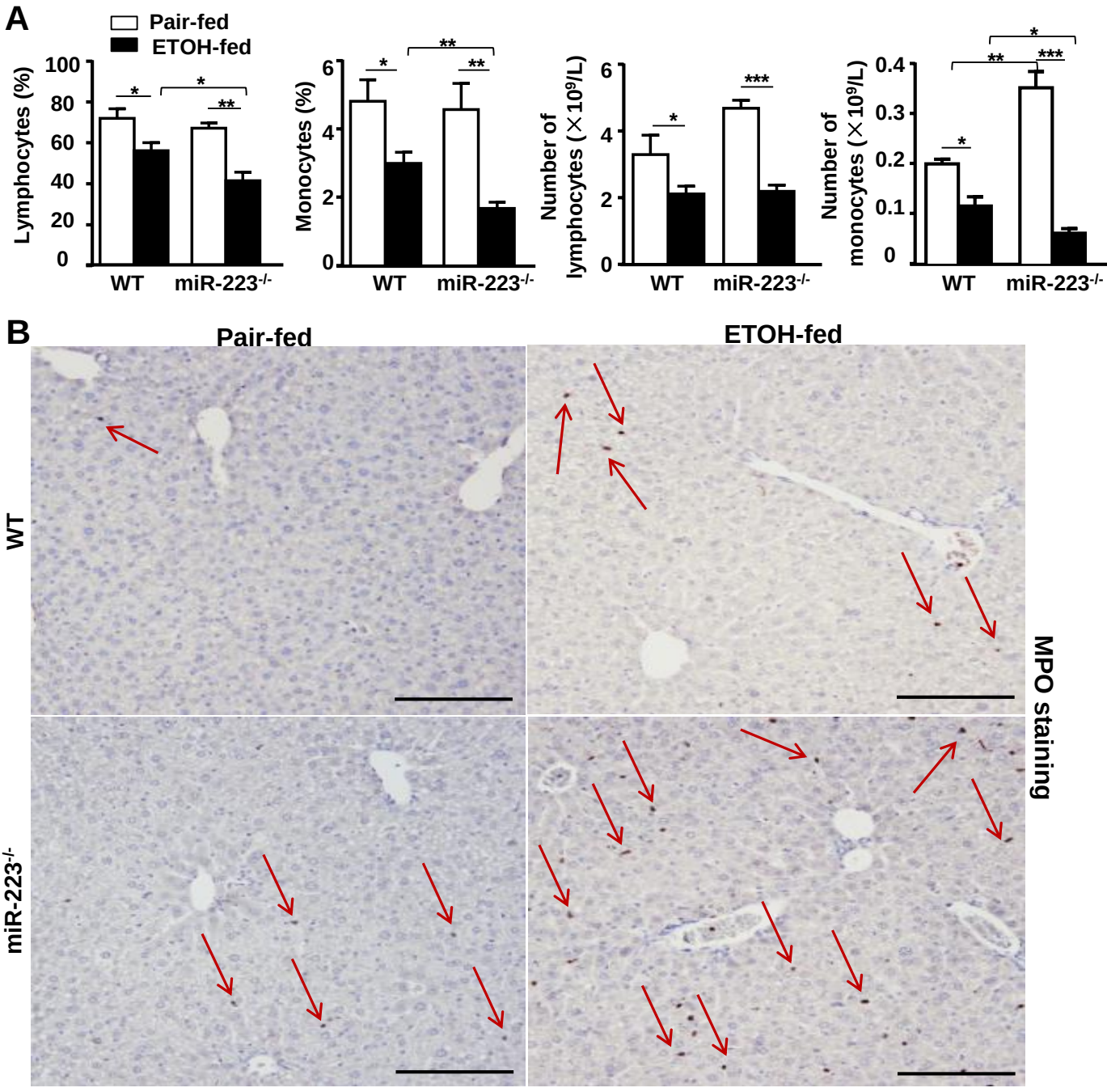
Supplemental Figure 3B. Injection of pri-miR-223 lentivirus ameliorates chronic-plus-binge-induced liver injury. Mice were subjected to chronic-plus-binge ethanol feeding or control feeding. Mice were euthanized 9 hours post gavage. Pri-miR-223 was injected 5 days before euthanization. Serum was collected for ALT measurement. Bone marrow and blood neutrophils, and liver tissues were collected for miR-223 measurement.

Values represent means ± SEM (n=4-10). *P<0.05; **P<0.01.



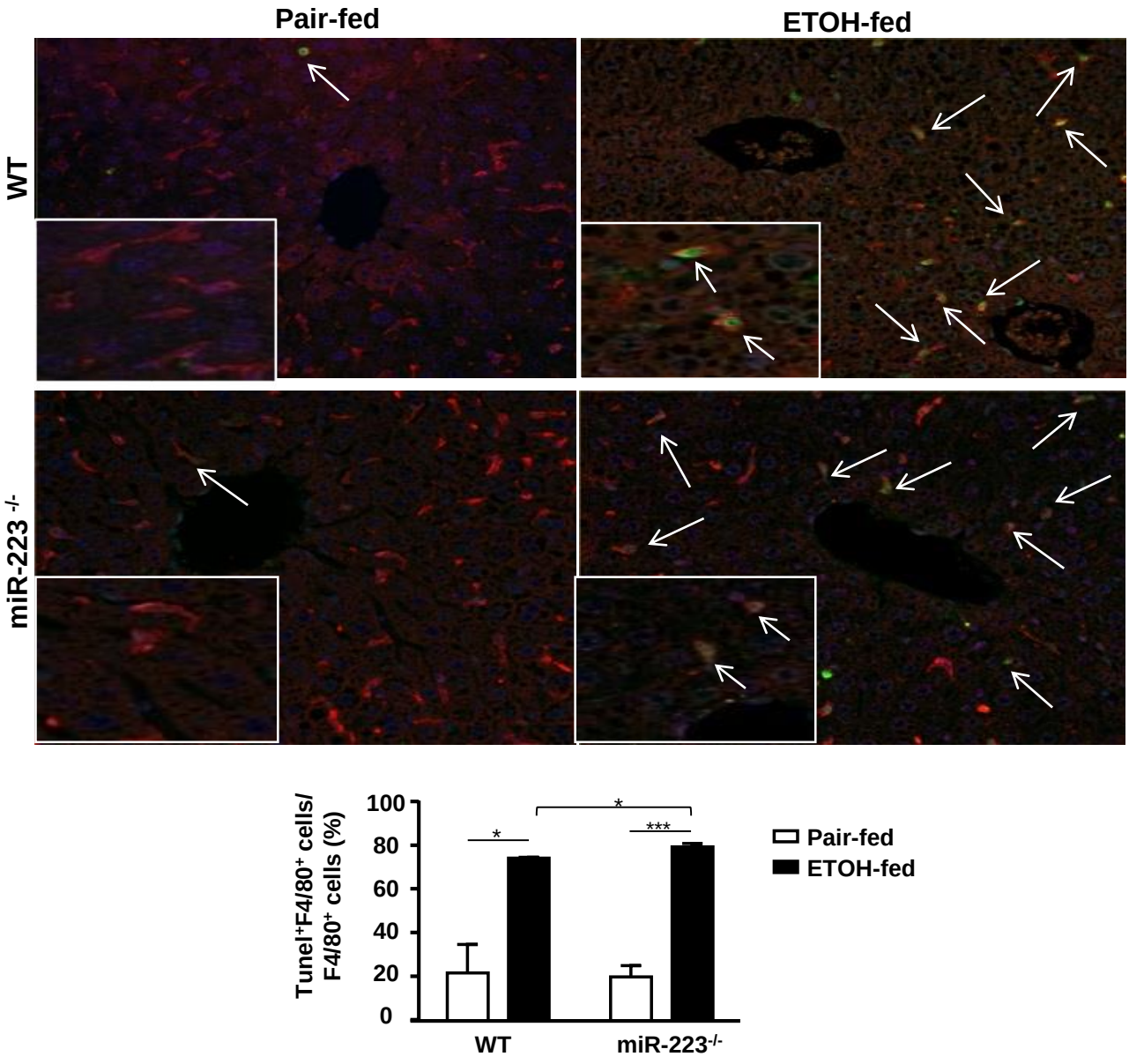
Supplemental Figure 4. Neutrophil proliferation and apoptosis in WT and miR-223$^{-/-}$ mice with or without ethanol feeding. (A) WT and miR223$^{-/-}$ mice were fed a pair food or ethanol for 10 days, and maltose or ethanol was gavaged, respectively, 9 hours before euthanization. Mice were intraperitoneally injected with Brdu and sacrificed 4h later. Neutrophils from bone Marrow were isolated. The reproduction of neutrophils was analyzed by FACS. (B) Murine neutrophils were isolated from bone marrow, and cultured for the indicated times. Spontaneous death of neutrophils was analyzed by FACS. Values represent means ± SEM (n=3-6). *P<0.05; **P<0.01; ***P<0.001.

Note: the percentage of immature neutrophils was higher, whereas the percentage of Brdu^{+} immature and mature neutrophils from bone marrow in miR-223$^{-/-}$ mice was lower than those in WT mice (supplemental Fig. 4A). Furthermore, the percentage of viable neutrophils from bone marrow in miR-223$^{-/-}$ mice was lower than that in WT mice 24h and 48h post culture (supplemental Fig. 4B). These results suggest that higher levels of neutrophils in ethanol-fed miR-223$^{-/-}$ mice compared to WT mice are likely secondary to the increase in neutrophil release from the bone marrow, rather than increased proliferation or decreased neutrophil apoptosis.

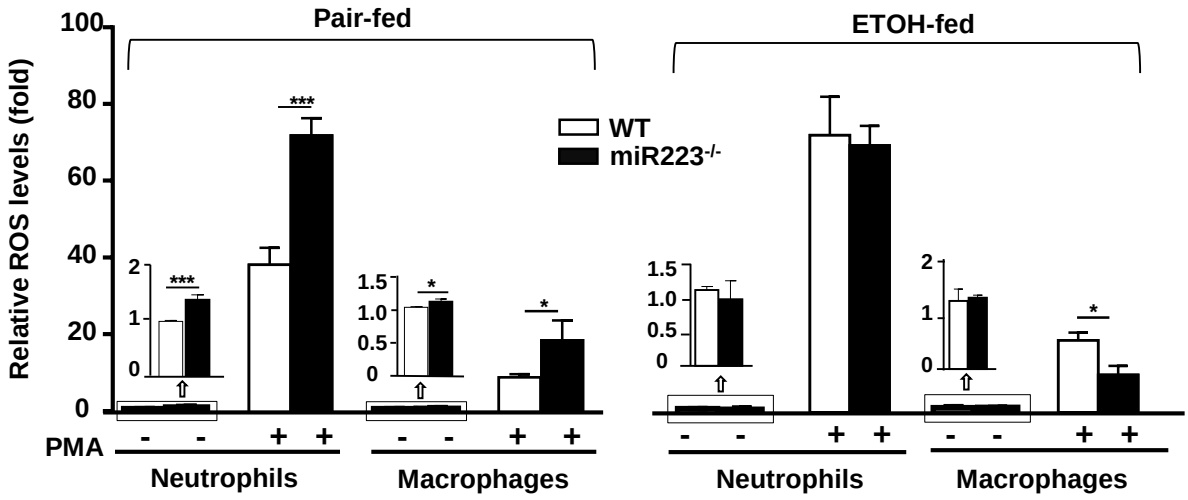


Supplemental Fig. 5A. miR-223$^{-/-}$ mice have reduced number of lymphocytes and monocytes in peripheral blood than WT mice post chronic-plus-binge ethanol feeding. Mice were subjected to chronic-plus-binge ethanol feeding or control feeding. Mice were euthanatized 9 hours post gavage. Blood leukocytes were collected, analyzed, and counted. Values represent means ± SEM (n=4-10). *P<0.05; **P<0.01; ***P<0.001.

Supplemental Fig. 5B. Representative immunostaining of MPO from ethanol-fed WT and miR-223$^{-/-}$ mice. WT and miR223$^{-/-}$ mice were subjected to pair or E10d+1B feeding. Mice were euthanized 9h post gavage. Liver tissues were subjected to immuno-staining with an anti-MPO antibody. Representative photography is shown (scale bar: 200μm).



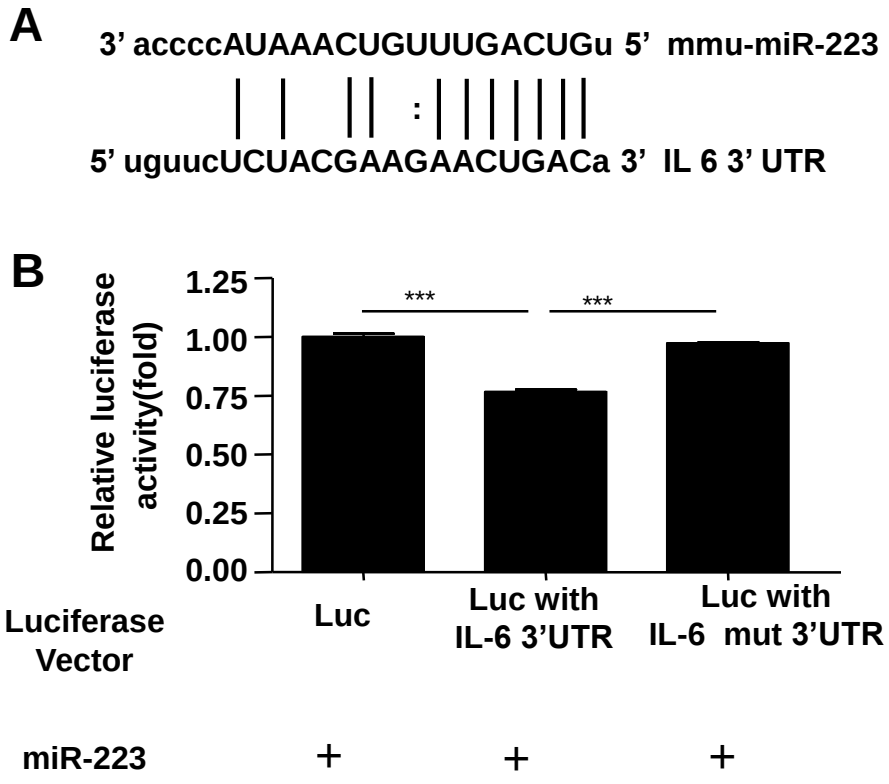
Supplemental Fig. 6. Chronic-plus-binge ethanol feeding induces kupffer/macrophage apoptosis in the liver. Mice were pair-fed or fed an ethanol diet for 10 days, followed by gavage of a single dose of maltose or ethanol. Mice were euthanatized 9 hours post gavage. Liver tissues were subjected to co-staining with Tunel (green staining) and F4/80 (red staining) (200×). Representative co-staining pictures are shown. White arrows indicate $Tunel^{+}F4/80^{+}$ cells. $Tunel^{+}F4/80^{+}$cells were counted. Values represent means ± SEM (n=4). **P*<0.05; ***P*<0.01; ****P*<0.001.



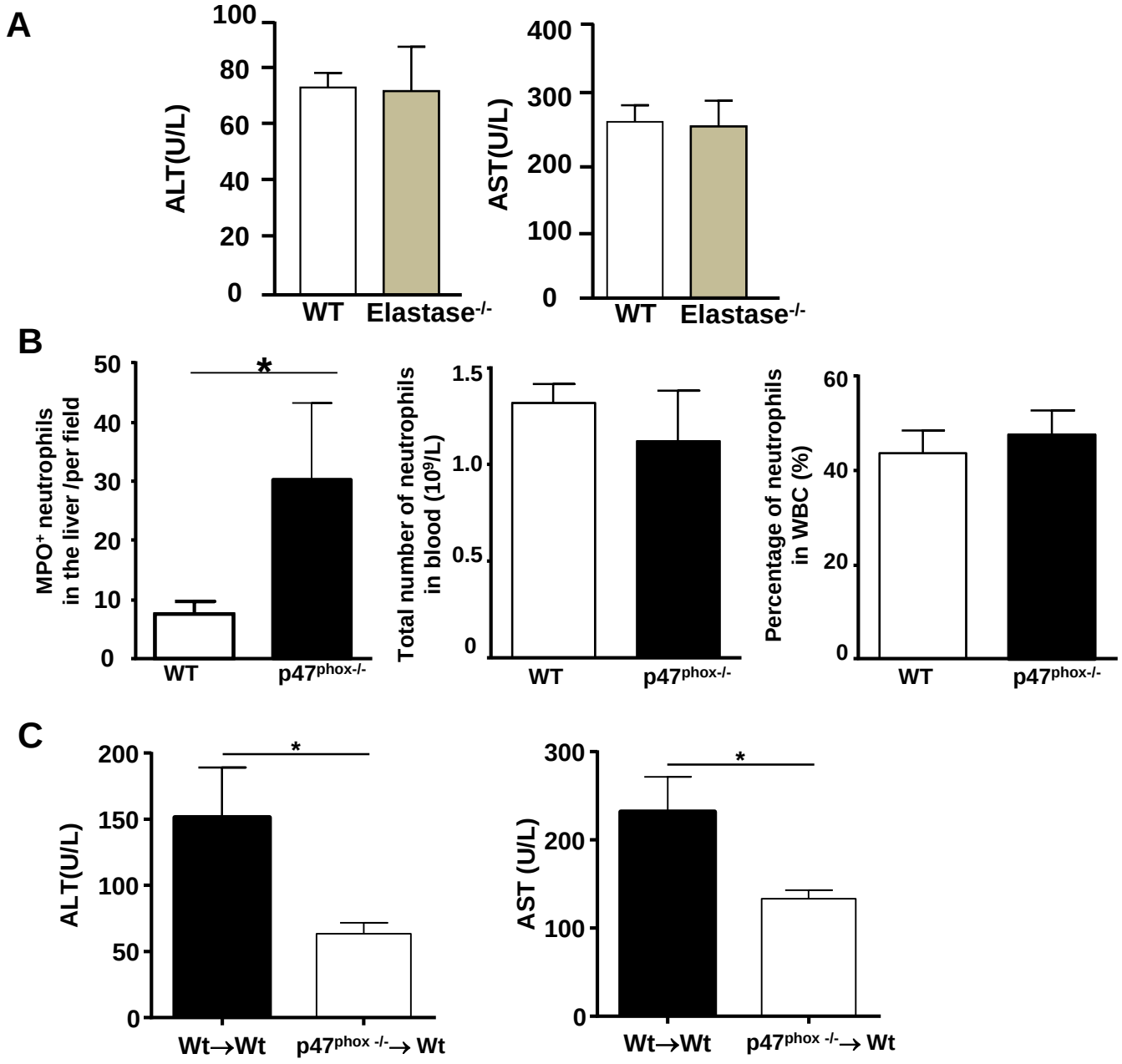
Supplemental Fig. 7. ROS production in hepatic neutrophils and macrophages from ethanol-fed and pair-fed mice. Mice were subjected to chronic-plus-binge ethanol feeding or pair-feeding. Mice were euthanized 9 hours post gavage. The neutrophils and macrophages were isolated from the livers and stimulated with PMA. The ROS levels were measured by FACS. ROS levels in macrophages were calculated. Values represent means ± SEM (n=3-6). **P*<0.05; ***P*<0.01; ****P*<0.001.

Noted: ROS data in neutrophils from Fig. 5C are included in this Figure in order to compare ROS levels between neutrophils and macrophages. As you can see, ROS levels in macrophages are five fold lower than those in neutrophils.

In pair-fed group, hepatic macrophages from miR-223$^{-/-}$ mice had higher levels of ROS than those from WT mice. In contrast, ethanol-fed miR-223$^{-/-}$ mice had lower ROS in their macrophages than WT mice (supplemental Fig. 7).



Supplemental Fig. 8. miR-223 directly targets IL-6 gene. (A), miR-223 and IL-6 3’UTR sequences. (B) Luciferase activity of 293T cells transfected with luciferase vector contain either WT or mutated 3’UTR of IL-6, or without 3-UTR, and also transfected with miR-223. ***, *P*<0.001. Data are the mean±SEM of three replicates of one representative experiment.



Supplemental Fig. 9. (A) Effects of deletion of Elastase on ethanol-induced liver injury. WT mice and Elastase$^{-/-}$ mice were subjected to E10d+1B ethanol feeding, and then mice were euthanized 9 hours post gavage. Serum ALT and AST levels were measured. **(B) Effects of p47^{phox} deletion on hepatic and peripheral neutrophils.** WT mice and p47$^{phox-/-}$ mice were fed an ethanol diet for 10 days and then ethanol was gavaged 9 hours before euthanization. Liver MPO^{+} cells were determined by immunostaining of MPO. The blood neutrophils were also counted. **(C) Role of p47^{phox} in immune cells in ethanol-induced liver injury.** WT C57BL/J mice were transplanted with WT C57BL/J or p47$^{phox-/-}$ mouse bone marrow. Two months later, mice were subjected to E10d+1B ethanol feeding, and then mice were euthanized 9 hours post gavage. Serum ALT and AST levels were measured. Values represent means ± SEM (n=6-10). * P<0.05