Macrophage Notch1 signaling modulates regulatory T cells via the TGFB axis in early MASLD

Mengya Zhang, **Kun Li**, Xiaoxing Huang, Dongqin Xu, Ruobin Zong, Qintong Hu, Xiaoyu Dong, Qinyong Zhang, Chaochen Jiang, Yue Ge, Changyong Li and Jie Ping

Materials and methods 2

Supplementary figures 11

Supplementary tables 19

References 21

Original western blot data 22

Materials and methods

Animal treatment

C57BL/6 mice were obtained from the Experimental Center of Hubei Medical Scientific Academy. Male mice at 8 weeks of age were fed with normal chow diet (NCD, 11.9% kcal fat) or high-fat diet (HFD, 60% kcal fat) (F3282, BioServ, Inc., Frenchtown, NJ) for 1, 2, 4, and 6 weeks. To generate Tregs-expansion mice, mice were injected intraperitoneally once a week with 1μg IL-2 (212-12, Preprotech, Chicago, USA) and 5 μg anti-IL-2 (16-7022-85, eBioscience, Chicago, USA) following HFD feeding. To generate Tregs-depletion mice, mice were given purified anti-CD25 antibody (200 μg) (BE0012, BioXcell, West Lebanon, NH) once a week following HFD feeding. All mice were maintained under standard conditions (25°C, 12-hour light-dark cycle) and had free access to food and water *ad libitum* at the Center for Animal Experiment of Wuhan University (Wuhan, China). All animal protocols were approved by the Committee on the Ethics of Animal Experiments at Wuhan University School of Medicine (Permit No: WP20220219). All mice received humane care in accordance with the criteria outlined in the Guide for the Care and Use of Laboratory Animals.

Human liver specimens

Human liver tissues from patients with simple steatosis and controls were collected by liver biopsy at Zhongnan Hospital of Wuhan University (Wuhan, China). Liver specimens were diagnosed as normal and simple steatosis, which is based on imaging studies and the histological diagnosis of NAFLD. Informed consent was available for each patient. This study was approved by the medical ethics committee of Zhongnan

Hospital of Wuhan University (Ethical Approval Number: 2023022). All research was conducted in accordance with both the Declarations of Helsinki and Istanbul.

Flow cytometry

Isolation of liver nonparenchymal cells of mouse liver was performed using methods previously described[1]. Subsequently, cells were stained with fluorochrome-conjugated antibodies according to the following protocols: (1) For the detection of Tregs frequency, cells were stained with surface antibodies against CD3-FITC (11-0032-82, eBioscience, San Diego, CA, USA), CD4-APC (17-0041-82, eBioscience) and CD25-PeCy7 (25-0251-81, eBioscience) for 30 min at 4°C in the dark. Then, cells were fixed with the Foxp3 Fixation/Permeabilization working solution according to the manufacturer's instruction (00-5523-00, eBioscience) and stained with Foxp3-PE (12-4771-82, eBioscience) for 30 min at RT in the dark. (2) For the detection of Notch1 in macrophages, cells were stained with surface antibody against F4/80-PeCy7 (12-4771-82, eBioscience) for 30 min at 4°C in the dark. After staining, fixation, and permeabilization, an antibody with a specific high affinity for the intracellular domain of Notch1 (12-5785-82, Biolegend, San Diego, CA, USA) was added for 30 min at RT in the dark. (3) To analyze macrophage immunophenotype, cells were stained with surface antibodies against F4/80-PeCy7, CD11c-PE (117308, Biolegend), and CD206-APC (141708, Biolegend) for 30 min at RT in the dark. (4) For the detection of TGFBR1 in $CD4^+$ T cells, cells were stained with surface antibody against CD4-FITC (11-0041-82, eBioscience) for 30 min at 4°C in the dark. After that, cells were fixed and permeabilized where further intracellular staining with TGFBR1 (ab31013, Abcam,

Cambridge, UK) and goat anti-rabbit IgG/PE (bs-0295G-PE, Bioss, Shanghai, China) antibodies were performed.

For the co-culture experiments *in vitro*, after naïve $CD4^+$ T cells were co-cultured with bone marrow-derived macrophages (BMDMs) or Exos from BMDMs, the proportion of Tregs ($Foxp3^+$) and the expression of TGFBR1 on $CD4^+$ T cells were detected by flow cytometry analysis. Cells were stained with surface antibodies against CD4-FITC or TGFBR1 and goat anti-rabbit IgG/PE for 30 min at 4°C in the dark. Subsequently, cells were fixed with the Foxp3 Fixation/Permeabilization working solution and stained with Foxp3-PE for 30 min at RT in the dark. Data were acquired at BD FACS AriaTM III flow cytometer (BD Biosciences, San Jose, USA), equipped with FACS Diva software. All data analysis was performed using FlowJo software (version 10.0.7).

Evaluation of immune cell populations

The ssGSEA algorithm was used to evaluate the immune cell populations in the GSE83452 dataset.

Isolation of Hepatic Macrophages

The hepatic macrophages were isolated as previously described[2], the purity of macrophages was determined by flow cytometry (Figure S4B).

Isolation of Hepatic T cells

Isolation of liver nonparenchymal cells of mouse liver was performed using methods previously described[1]. Subsequently, in a centrifuge tube, 70% Percoll, 40% Percoll, and the single-cell suspension were sequentially layered. The tube was then centrifuged

at 900g for 20 minutes at 4°C, with an acceleration setting of 4 and a deceleration setting of 0. The lymphocyte-enriched layer was collected from between the 40% and 70% Percoll gradients. The purity of macrophages was determined by flow cytometry (Figure S9).

Glucose and insulin tolerance testing (GTT and ITT)

For the GTT, mice were fasted overnight and then given an intraperitoneal injection of glucose (1 g/kg). For the ITT, mice were fasted 5 h, and then given an intraperitoneal injection of insulin (0.75 units/kg). Blood was collected from the tail vein before (0 min) and at 15, 30, 60, and 120 min after glucose or insulin injection to measure the glucose concentration using a glucometer (Roche, Mannheim, Germany).

Histopathological analysis

Mouse liver samples were fixed in 4% paraformaldehyde, processed for paraffin or OCT media embedding, and sectioned at 5 μm thickness. The paraffin-embedded liver sections were carried out hematoxylin and eosin (H&E) staining for histological analysis. Oil Red O staining was performed on frozen sections to determine hepatic steatosis.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-1β, TNF-α, TGF-β, and IL-10 in the liver were measured with ELISA kits (MultiSciences, ShangHai, China) according to the manufacturer's instructions.

Biochemical analysis

The levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and hepatic TC, TG, were detected by biochemical kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Isolation and characterization of Exos

For the transmission electron microscopy (TEM) analysis, the approximately 5 μL of the Exosome (Exos) suspension sample was firstly applied onto a carbon-coated copper grid, adsorbed at RT for 10 min, followed by staining with 2% phosphotungstic acid solution (pH=6.5) at RT for 2 min. After the sample was dried, the morphology of the Exos was observed by HT7700 TEM (Hitachi, Tokyo, Japan). Western blot was used for the identification of Exos membrane markers with the following primary antibodies: CD63 (ab217345, Abcam, MA, USA), CD9 (A19027, ABclonal, Wuhan, China), and TSG101 (ab125011, Abcam). To evaluate the absolute size distribution and concentration of Exos, Exos were analyzed by Flow Nanoanalyzer (NanoFCM Inc., Xiamen, China) according to the manufacturer's instructions.

Cell culture and coculture system

Primary hepatocytes were isolated as previously described[1]. $CD4^+$ $CD25^+$ Treg cells were purified from the mouse spleen with EasySep™ Mouse $CD4^+$ $CD25^+$ Regulatory T Cell Isolation Kits (Stem Cell Technologies, Vancouver, Canada).

For co-culture study, the primary hepatocytes cocultured with (1:1 ratio) or without Tregs, and then stimulated with free fatty acid (FFA) for 72 h, which was prepared at 2:1 ratio (500 μM: 250μM) of oleic acid (OA) to palmitic acid (PA). For the IL-10

neutralizing experiment, 10 μg/ml mouse IL-10 antibody (MAB417, R&D systems, Minneapolis, USA) was added to the coculture system. To assess insulin signaling, the hepatocytes were stimulated with 5 μg/mL insulin for 15 min before collection. For lipid staining, the hepatocytes were subjected to oil red O staining (C0158, Beyotime, Shanghai, China) according to the manufacturer's instructions.

BMDMs were prepared as a previous description[2]. BMDMs were treated with 100 ng/mL LPS and 250 μM PA for 12 h. The same volume of PBS was used as a control treatment. Naïve $CD4^+$ T cells were purified from the mouse spleen using EasySep™ Mouse naïve $CD4^+$ T Cell Isolation Kits (Stem Cell Technologies) (purity> 90%).

For the Treg differentiation assay, naïve $CD4^+$ T cells were cocultured with BMDMs (1:1 ratio). Cells were cultured in RPMI 1640 complete medium containing soluble anti-CD3 (1 μg/ml) (100340, Biolegend), soluble anti-CD28 (1 μg/ml) (102116, Biolegend), and recombinant murine IL-2 (5 μg/ml) (212-12, Peprotech). After 3 days, cells were harvested for Foxp3 analysis.

To verify the role of Exos, BMDMs were pretreated with 5 μM GW4869 (D1692, Sigma-Aldrich, St. Louis, USA) or DMSO for 6 h, then they were cocultured with naïve $CD4^+$ T cells for 3 days, during which GW4869 treatment was continuously performed. For the coculture of Exos and naïve $CD4^+$ T cells, after BMDMs were stimulated with LPS and PA as described above, the medium was changed, and Exos were collected as described below. Naïve $CD4^+$ T cells were prestimulated with soluble anti-CD3 (1 μg/ml), soluble anti-CD28 (1 μg/ml), and recombinant murine IL-2 (5 ng/ml) for 3 days. The Exos (30 μg/ml) were added to the system, and coculture was continued for 3 days.

Exosome-labelling for cellular uptake

The purified Exos were labeled with PKH26 membrane dye (MINI26, Sigma Aldrich) according to the manufacturer's protocol. PKH26-labeled Exos were added to naïve $CD4^+$ T cells and incubated for 8 h. Then the specific uptake of PKH26-labeled Exos by cells was visualized by the confocal microscopy (Leica Microsystems, Wetzlar, Germany).

Plasmid Constructs and Transfections.

The plasmid pEF-Flag-NICD (Notch1 intracellular domain) was constructed as follows: The NICD fragments were amplified using cDNA obtained from mouse BMDMs and subsequently inserted into the EcoRI and XbaI sites of pEF-Flag-N. The plasmid was then transferred into BMDMs with Lipo8000 transfection reagent (C0533, Beyotime) according to the manufacturer's instructions.

Transfection of miRNA mimic and miRNA inhibitor

The miR-142a-3p mimic with its miRNA negative control (NC), and miR-142a-3p inhibitor with its NC were purchased from RiboBio (Guangzhou, China). Naïve $CD4^+$ T cells were prestimulated with soluble anti-CD3 (1 μg/ml), soluble anti-CD28 (1 μg/ml), and recombinant murine IL-2 (5 ng/ml) for 3 days. After that, naïve $CD4^+$ T cells were transfected with 50 nmol/L miRNA mimic or 100 nmol/L miRNA inhibitor, and their corresponding NC using Lipo8000 transfection reagent.

Western Blot

Cells or Exos were lysed using lysis buffer and quantified by BCA assay. Equal amounts of proteins were separated on a 12% SDS-PAGE and then transferred to a

polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked with 5% skim milk. Afterward, primary antibodies were incubated overnight at 4°C, followed by HPR-conjugated secondary antibodies. Protein bands were visualized using gel imaging analyzer (Peiqing, Shanghai, China). The used primary antibodies included anti-GAPDH (PMK053M, bioprimacy, Wuhan, China), anti-β-actin (PMK083M, bioprimacy), anti-β-tubulin (PMK088M, bioprimacy), anti-Cleaved Notch1 (4147, Cell Signaling Technology), anti-Akt (4691, Cell Signaling Technology), anti-p-Akt (4060T, Cell Signaling Technology), anti-CD9, anti-CD63, anti-TSG101.

Quantitative RT-PCR Analysis

Total RNA was prepared using Trizol extraction. The cDNA was synthesized by miRNA 1st Strand cDNA Synthesis Kit (Vazyme Biotech, Nanjing, China) (for miRNA) or PrimeScript RT Reagent Kit (Takara, Tokyo, Japan) (for mRNA) according to the manufacturer's protocol. The expression of miRNA and mRNA were estimated by a real-time PCR system (Bio-Rad Laboratories, CA, USA) using SYBR qPCR Master Mix (Vazyme Biotech). β-actin or U6 were used as internal controls. Data were then analyzed using the ΔΔCt method. The primer sequences used in this study are listed in Table S1.

Immunofluorescence (IF) staining

For $CD4^+$ T cells, Foxp3 staining was performed with Foxp3 antibody (Zenbio, Chengdu, China). Human liver macrophage cleaved Notch1 intensity was assayed by co-staining sections with anti-CD68 (ab213363, Abcam) and cleaved Notch1 (4147T,

Cell Signaling Technology) antibodies. DAPI was used as nuclear staining. IF images were obtained using Olympus BX50 microscope.

Luciferase reporter assay

The wild-type (WT) and mutated (MT) sequences containing miR-142a-3p binding sites was amplified by PCR and cloned them into pmirGLO vector (Promega, Madison, WI, USA) to reconstructed luciferase reporter plasmids. Then, 100 ng WT or MT vectors were co-transfected with 50nM miR-142a-3p into HEK293T cells at 70% confluence using Lipo8000 transfection reagent. After 48 h, cells were collected and firefly luciferase and renilla activities were detected using Dual-Luciferase Reporter Assay System ((Vazyme Biotech). Data were normalized by calculating the ratio of firefly luciferase activity to Renilla luciferase activity.

Quantification and statistical analysis

Data were presented as the mean ± SD. Differences between the two groups were compared using the Student t-test, and comparisons among multiple groups were analyzed with one-way ANOVA followed by Tukey's post hoc test. All Data were analyzed using Prism 7.0 (GraphPad Software) for statistical significance. Differences were considered significant at P value <0.05.

Supplementary figures

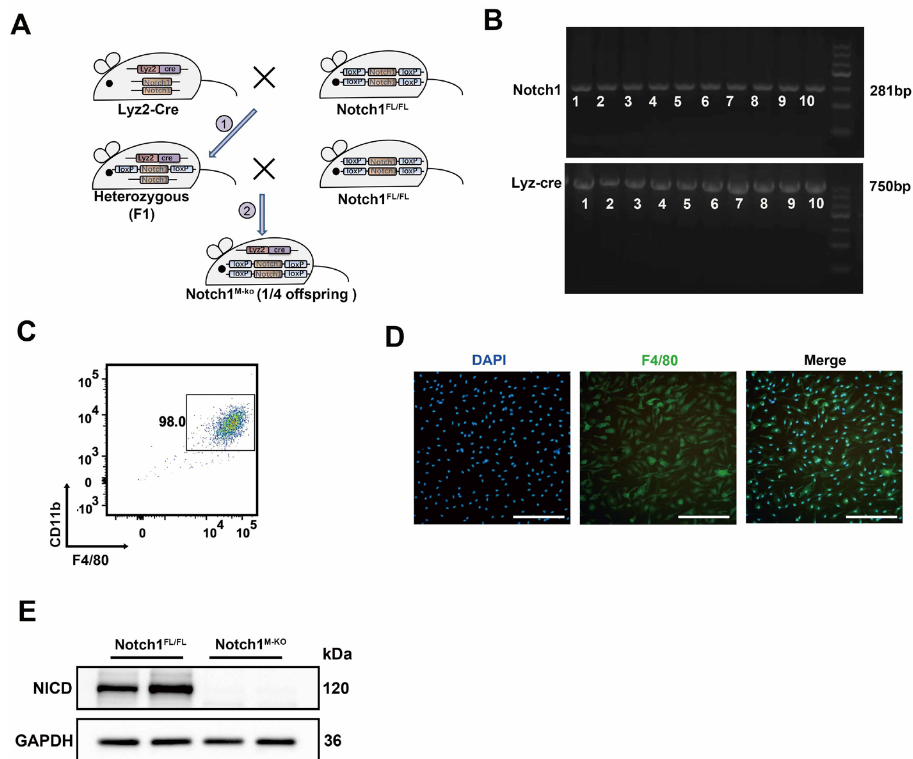


Fig. S1. Generation and characterization of Notch1$^{M\text{-}KO}$ mice. (A) Diagram of the production of Notch1$^{M\text{-}KO}$ mice. (B) PCR identified the genotypes of Notch1$^{M\text{-}KO}$ mice. (C) F4/80^{+}CD11b^{+} BMDMs from mice were detected by flow cytometry. (D) Representative pictures of immunofluorescence staining of F4/80^{+} BMDMs (scale bar: 50 μm). (E) Western blot of BMDMs from Notch1$^{FL/FL}$ and Notch1$^{M\text{-}KO}$ mice. Abbreviations: BMDMs, bone marrow-derived macrophages.

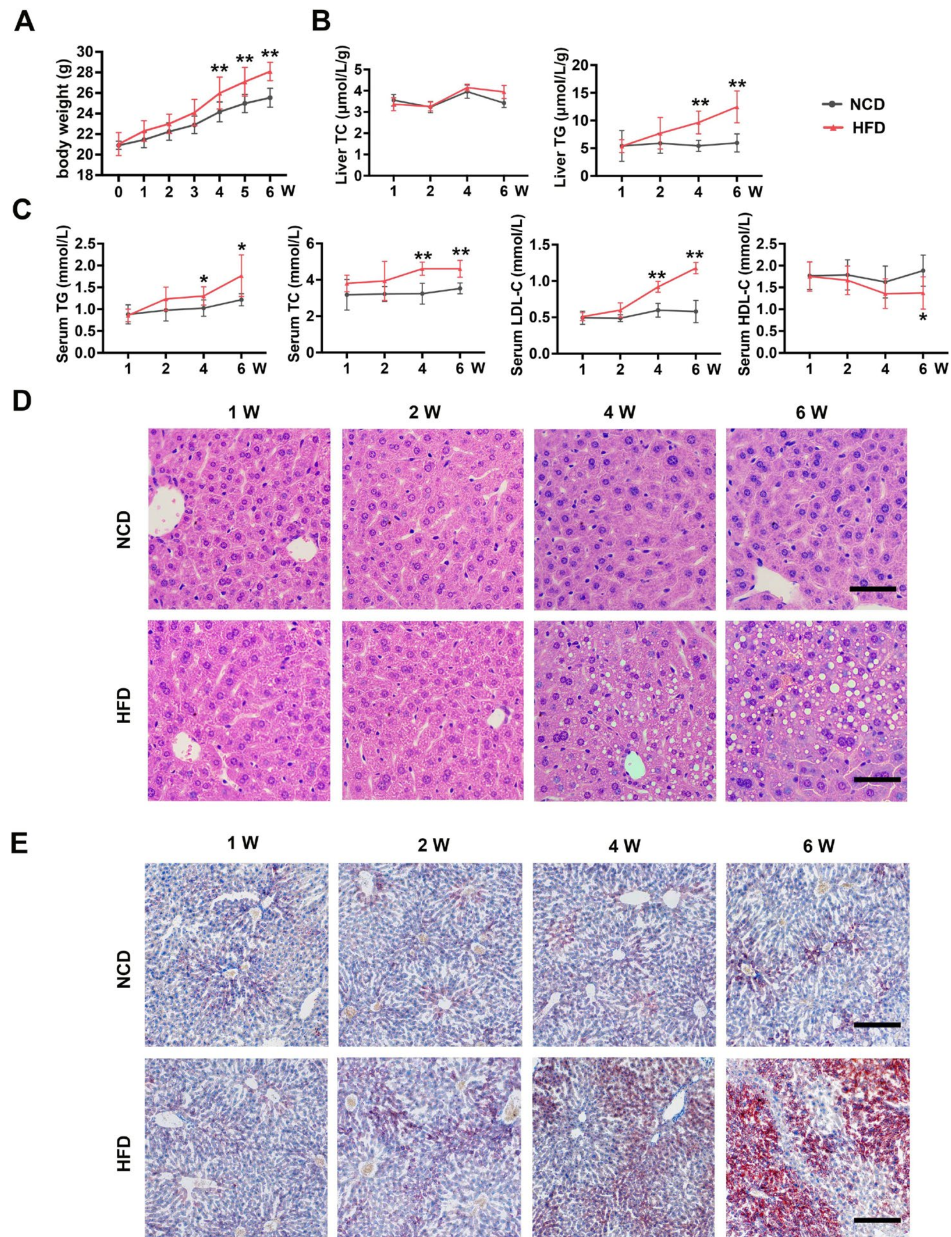


Fig. S2. Wild-type C57BL/6 mice were fed with NCD or HFD for 1, 2, 4, and 6 weeks. Dynamic changes of (A) body weight, (B) liver TG and TC levels, (C) serum TG, TC, LDL-C, HDL-C levels of NCD and HFD mice fed for 1, 2, 4, and 6 weeks (n = 8/group). (D) Representative pictures of H&E staining of liver sections (scale bar: 50 μm). (E) Representative pictures of Oil Red O staining of liver sections (scale bar: 100 μm). Values represent means ± SD. Statistical analysis was performed by two-tailed unpaired Student t-test. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: normal chow diet, NCD; HFD, high-fat diet; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; HFD, high-fat diet; LDL-C, low-density lipoprotein cholesterol; H&E, hematoxylin and eosin.

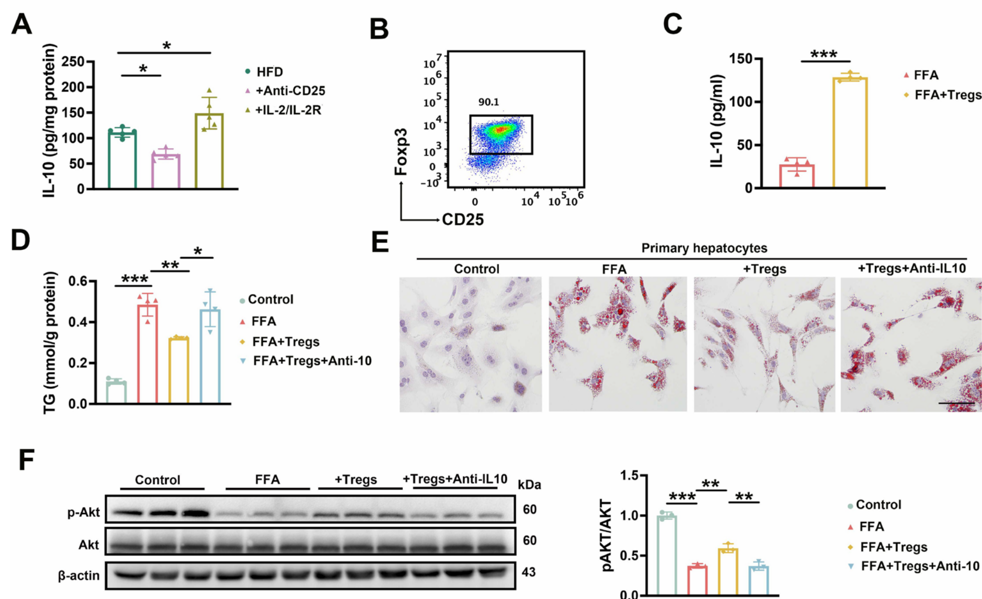


Fig. S3. (A) Wild-type C57BL/6 mice were injected intraperitoneally with anti-CD25 antibody or ldIL-2 once a week to construct Tregs-depletion mice and Tregs-expansion mice, respectively, while fed HFD for 4 weeks. The level of IL-10 in the liver was measured (n=5/group). (B) Tregs isolated from mice spleen were identified by flow cytometry. (C) The amount of IL-10 in the supernatant after primary hepatocytes co-cultured with Tregs or not were treated with FFA for 72 h (n=4/group). (D) TG content (n=4/group), (E) representative pictures of Oil Red O staining (scale bar: 100 μm), (F) immunoblot analysis of insulin-stimulated Akt and p-Akt (n=3/group) of the primary hepatocytes, or cells in cultures with the addition of Tregs and neutralizing antibody IL-10. Values represent means ± SD. Statistical analysis was performed by (A, D, F) one-way ANOVA, Tukeys test or (C) two-tailed unpaired Student t-test. **P*< 0.05, ***P* < 0.01, ****P* < 0.001. Abbreviations: ldIL-2, low-dose IL-2; Treg, regulatory T cell; HFD, high-fat diet; FFA, free fatty acid; IL-10, interleukins-10; TG, triglyceride; pAkt, phosphorylated Akt.

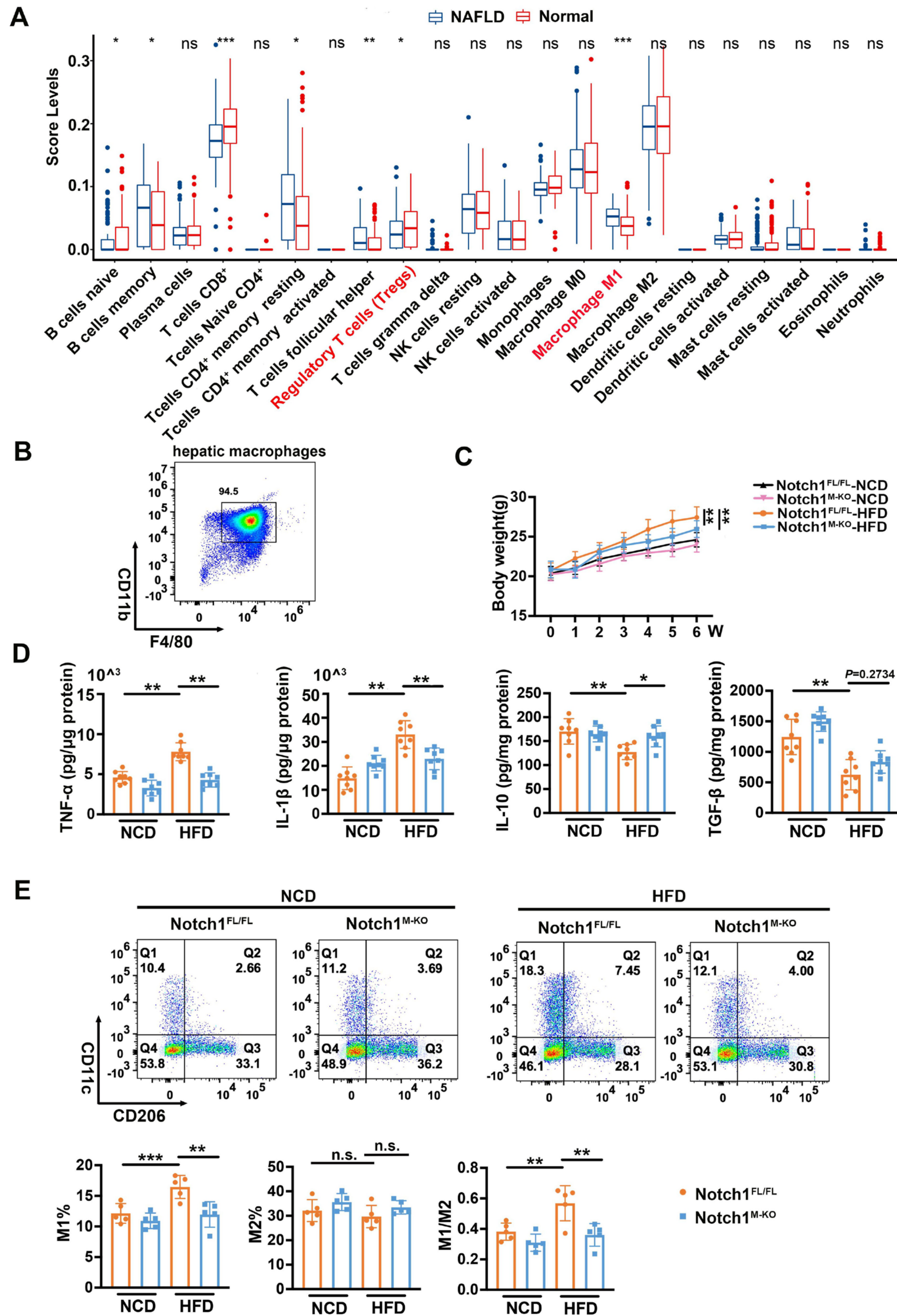


Fig. S4. (A) Bioinformatics analysis of a GEO public dataset (GEO: GSE83452) for frequency of various immune cell types in the liver of NAFLD patients. Notch1$^{FL/FL}$ mice and Notch1$^{M\text{-}KO}$ mice were fed with NCD or HFD for 6 weeks. (B) The purity of the hepatic macrophages separation. (C) The body weight was measured (n=8/group). (D) Levels of TNF-α, IL-1β, IL-10 and TGF-β in the liver (n=8/group). (E) The proportion of M1 (F4/80^{+}CD11c^{+}) and M2 (F4/80^{+}CD206^{+}) macrophages in the liver

(n=5/group). Values represent means ± SD. Statistical analysis was performed by one-way ANOVA, Tukeys test. *P< 0.05, **P < 0.01, ***P < 0.001. Abbreviations: NCD, normal chow diet; HFD, high-fat diet; TNF-α, tumor necrosis factor-α; IL-1β, interleukins-1β; IL-10, interleukins-10; TGF-β, transforming growth factor-β.

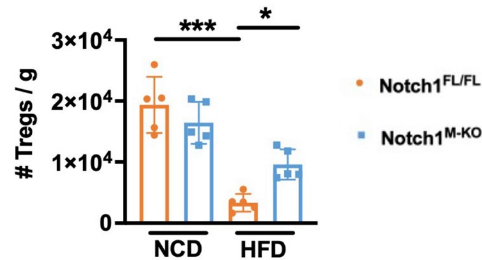


Fig. S5. Notch1$^{M\text{-}KO}$ mice and Notch1$^{FL/FL}$ mice were fed with NCD or HFD for 6 weeks, respectively. The number of Tregs in the livers of Notch1$^{M\text{-}KO}$ mice and Notch1$^{FL/FL}$ mice were analyzed by flow cytometry. Values represent means ± SD. Statistical analysis was performed by one-way ANOVA, Tukeys test. *P< 0.05, ***P < 0.001. Abbreviations: NCD, normal chow diet; HFD, high-fat diet.

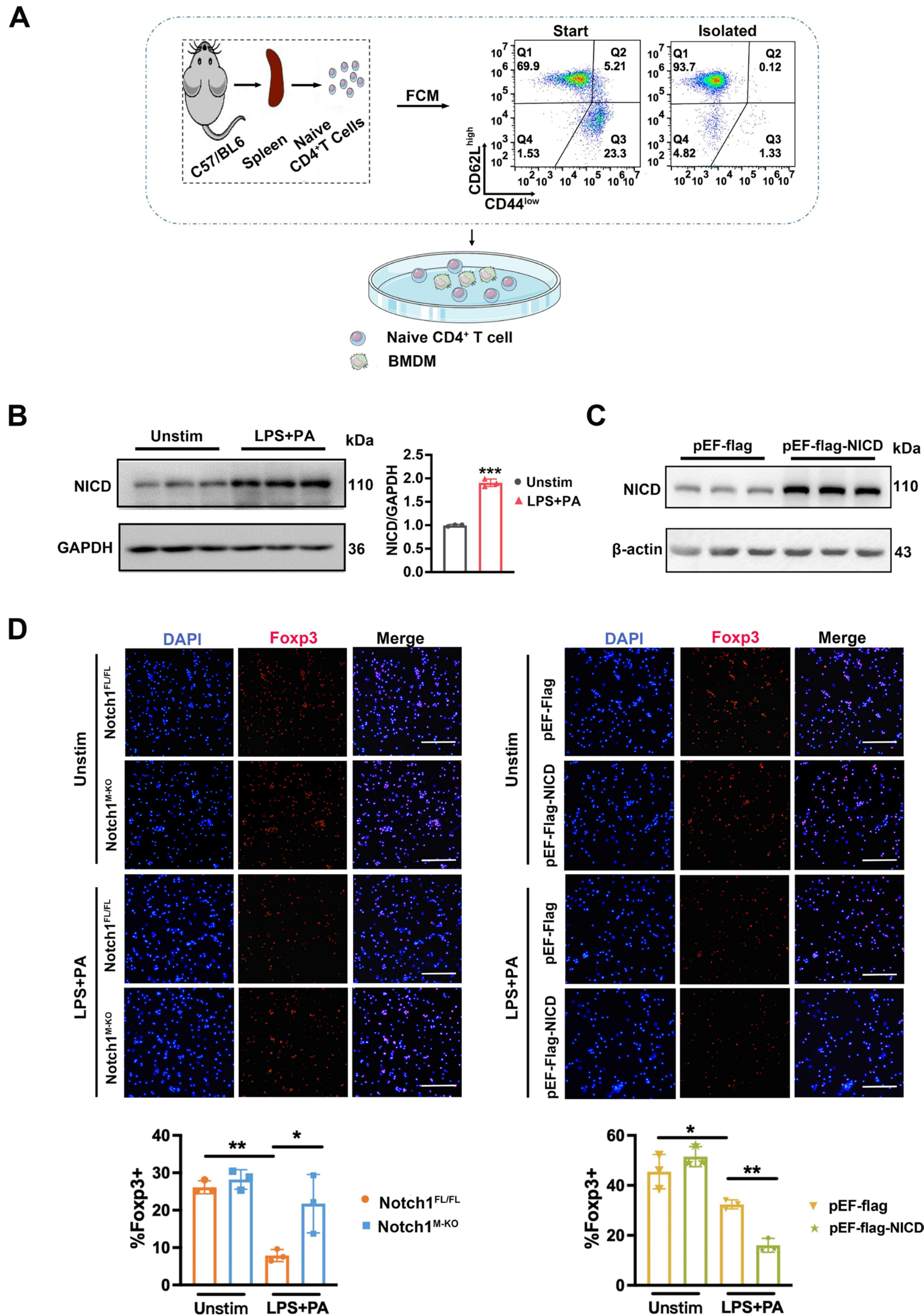


Fig. S6. (A) Schematic of naïve CD4$^+$ T cells isolated from mice spleen with purity! 90% and cocultured with BMDMs. (B) Protein expression of NICD in BMDMs after LPS (100 ng/mL) and PA (250 μM) treatment for 12 h (n=3/group). (C) The overexpression efficiency of NICD was assessed by Western Blot. (D) BMDMs from Notch1$^{FL/FL}$ and Notch1$^{M\text{-}KO}$ mice, the pEF-Flag-NICD or pEF-Flag transfected BMDMs were stimulated with LPS (100 ng/mL) and PA (250 μM) or PBS for 12 h,

respectively, and then co-cultured with naïve $CD4^+$ T cells for 3 days, the representative pictures of IF staining of T cells (scale bar: 200 μm).Values represent means ± SD. Statistical analysis was performed by two-tailed unpaired Student t-test. *P< 0.05, **P < 0.01, ***P < 0.001. Abbreviations: LPS, lipopolysaccharides; PA, palmitic acid; BMDMs, bone marrow-derived macrophages; NICD, Notch1 intracellular domain.

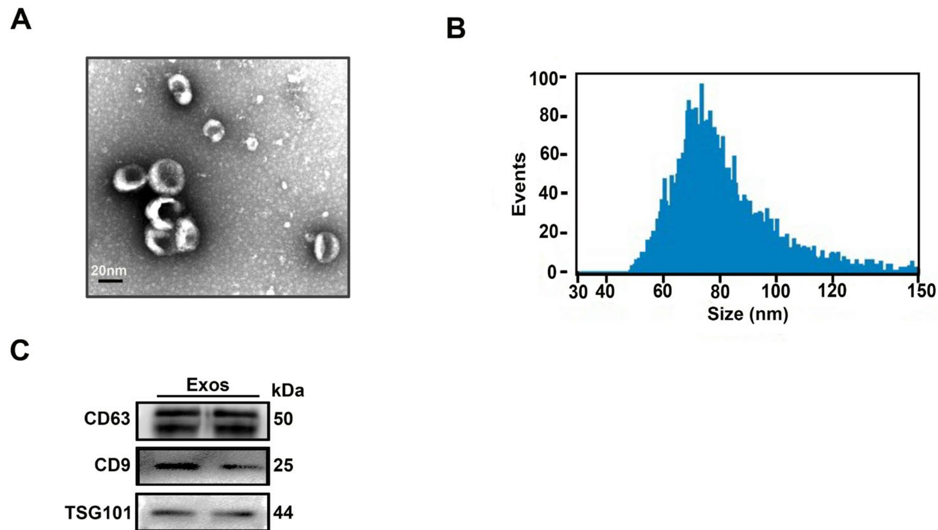


Fig. S7. Identification of Exos isolated from BMDMs. Exos isolated from BMDMs supernatants were analyzed by transmission electron microscopy (A) (scale bar: 20 nm), (B) flow nanoanalyzer, and (C) Western blot. Abbreviations: Exos, exosomes; BMDMs, bone marrow-derived macrophages.

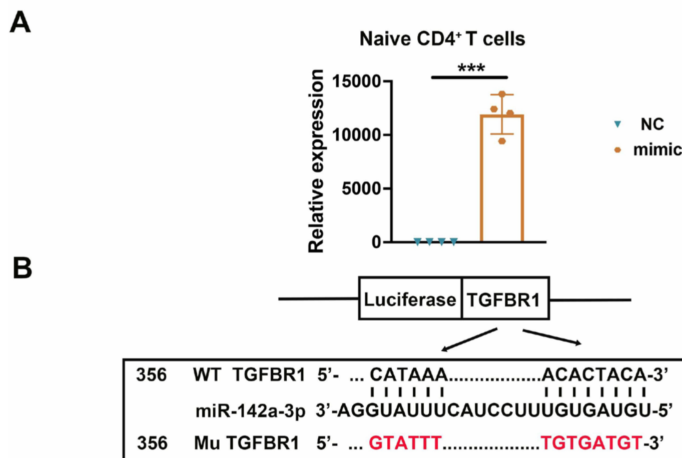


Fig. S8. (A) RT-qPCR analysis of miR-142a-3p in miRNA mimic-transfected naïve $CD4^+$ T cells after 24 h (n=4/group). (B) Schematic of TGFBR1 3'UTR WT and MT luciferase reporter vectors. Values represent means ± SD. ***P < 0.001. Abbreviations: WT, wild-type; MT, mutant; NC, negative control.

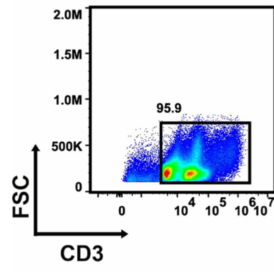


Fig. S.9. The purity assessment of isolated hepatic T cells.

Supplementary tables

Table S1. DNA sequences for mouse genotyping, RT-qPCR.

Genes	Forward (5’ to 3’)	Reverse (3’ to 5’)
Lyz2	CCCAGAAATGCCAGATTACG	CTTGGGCTGCCAGAATTTCTCTTACA
	CTTGGGCTGCCAGAATTTCTC	GTCGGCCAGGCTGAC
Notch1	TGCCCTTTCCTTAAAAGTGG	GCCTACTCCGACACCCAATA
Human-β-actin	ATGGGTCAGAAGGATTCCTATGTG	CTTCATGAGGTAGTCAGTCAGGTC
Mouse-β-actin	AGATCATTGCTCCTCCTGAGCGCA	AAACGCAGCTCAGTAACAGTCCGC
Human Foxp3	CAGCACATTCCCAGAGTTCCTC	GCGTGTGAACCAGTGGTAGATC
Mouse Foxp3	CCCAGGAAAGACAGCAACCTT	TTCTCACAACCAGGCCACTTG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
miR-142a-3p	GCGCGTGTAGTGTTTCCTACTT	AGTGCAGGGTCCGAGGTATT

Table S2. List of antibodies used in this study.

Antibodies	Company	Catalog number	Clone NO.
CD3-FITC	eBioscience	Cat#: 11-0032-82	17A2
CD4-APC	eBioscience	Cat#: 17-0041-82	GK1.5
CD25-PeCy7	eBioscience	Cat#: 25-0251-81	PC61.5
Foxp3-PE	eBioscience	Cat#: 12-4771-82	NRRF-30
F4/80-PeCy7	eBioscience	Cat#: 12-4771-82	BM8
Notch1-PE	Biolegend	Cat#: 12-5785-82	mN1A
IL-10 Antibody	R&D systems	Cat#: AB-417-NA	Polyclonal
CD11c-PE	Biolegend	Cat#: 117308	N418
CD206-APC	Biolegend	Cat#: 141708	MMR
CD4-FITC	eBioscience	Cat#: 11-0041-82	GK1.5
Anti-TGFBR1	Abcam	Cat#: ab31013	Polyclonal
Anti-CD63	Abcam	Cat#: ab217345	EPR21151
Anti-CD9	ABclonal	Cat#: A19027	ARC0330
Anti-TSG101	Abcam	Cat#: ab125011	EPR7130(B)
Anti-CD3	Biolegend	Cat#: 100340	145-2C11
Anti-CD28	Biolegend	Cat#: 102116	37.51
Anti-Notch1	Cell Signaling Technology	Cat#: 4147T	Val1744
Anti-Akt	Cell Signaling Technology	Cat#: 4691T	C67E7
Anti-p-Akt	Cell Signaling Technology	Cat#: 4060T	Ser473
Anti-Foxp3	Zenbio	Cat#: 200501-7H9	7H9
Anti-CD68	Abcam	Cat#: ab213363	EPR20545

References

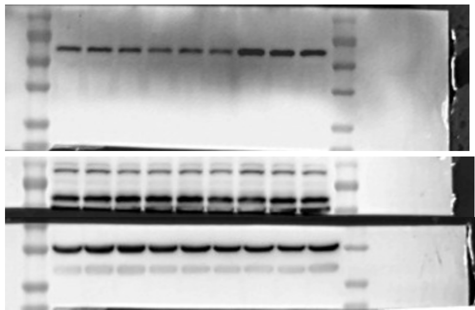
Author names in bold designate shared co-first authorship

1. **Dong X**, **Feng Y**, Xu D, et al. Targeting macrophagic 17beta-HSD7 by fenretinide for the treatment of nonalcoholic fatty liver disease. *Acta Pharm Sin B*. Jan 2023;13(1):142-156. doi:10.1016/j.apsb.2022.04.003
2. **Yang Y**, **Ni M**, Zong R, et al. Targeting Notch1-YAP Circuit Reprograms Macrophage Polarization and Alleviates Acute Liver Injury in Mice. *Cell Mol Gastroenterol Hepatol*. 2023;15(5):1085-1104. doi:10.1016/j.jcmgh.2023.01.002

Original western blot data

Figure 1

Panel H:



Panel L:

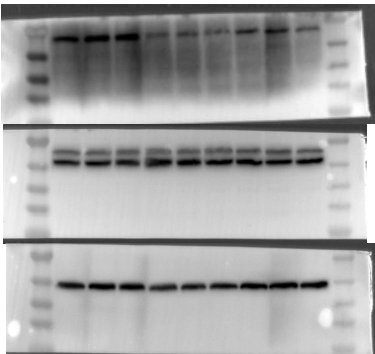


Figure 2

Panel B:

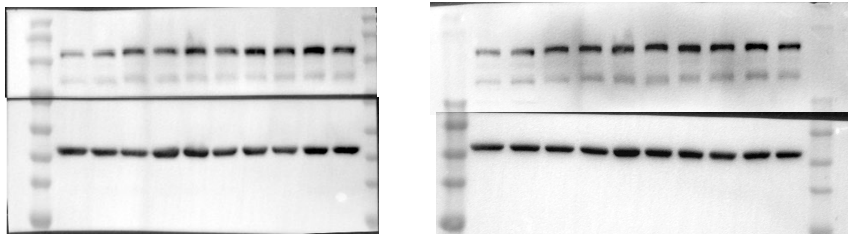


Figure 3

Panel F:

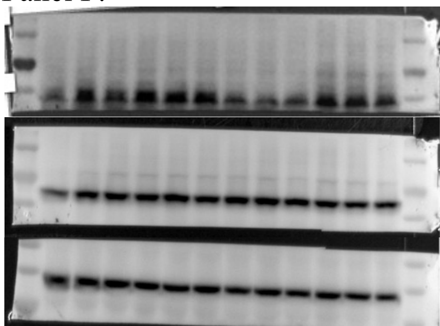


Figure 4
Panel H:

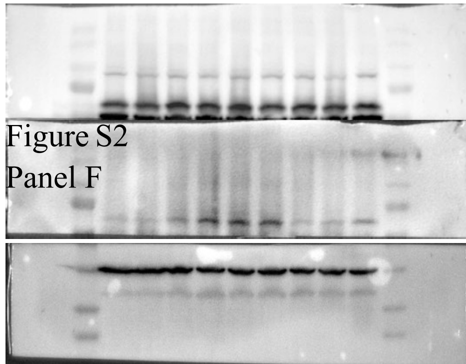


Figure S2
Panel F

Fig. S1
Panel E

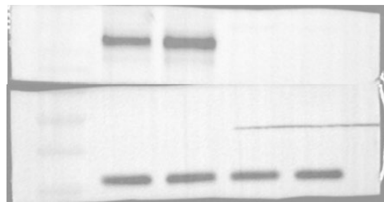


Fig. S3
Panel F

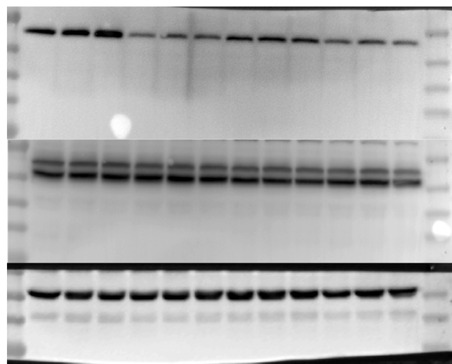


Fig. S6
Panel B

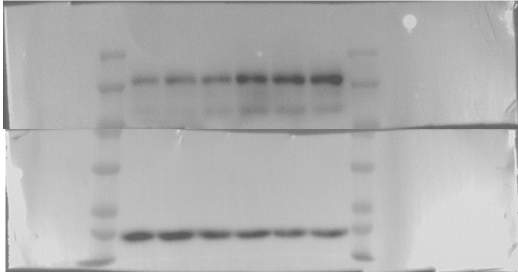


Fig. S6
Panel C

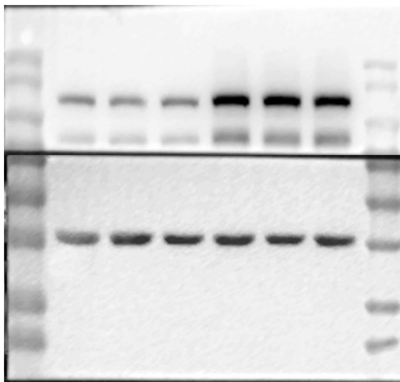
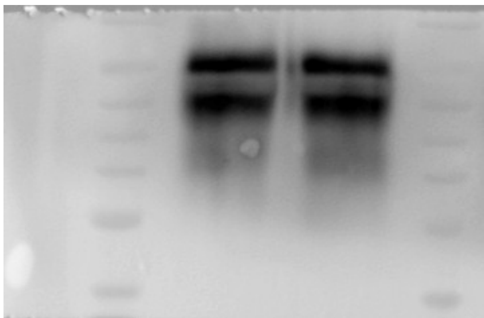
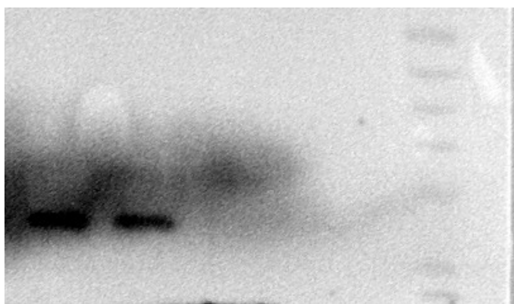


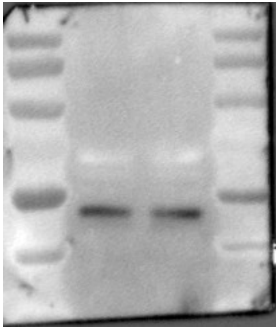
Fig. S7
Panel C
CD63



CD9

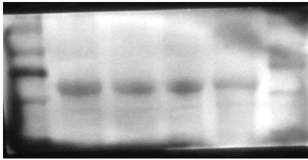


TSG101



Revision report

TGFBR1



GAPDH