Supplementary Information

Blockade of retinol metabolism protects T cell-induced hepatitis by increasing migration of regulatory T cells

Material and Methods

Isolation of liver mononuclear cells

As previously described (Radaeva et al., 2007; Yi et al., 2014), liver mononuclear cells (MNCs) were isolated. Briefly, liver was smashed and filtered through 70 μm nylon mash and suspended in cold phosphate buffered saline (PBS). Hepatocytes were removed by centrifugation at 400 rpm for 5 minutes and then supernatant was collected, washed with PBS 2 times and suspended in Percoll. The cell suspension was centrifuged at 2,400 rpm for 30 minutes. After RBC lysis, collected liver MNCs were counted and subjected to flow cytometry and real-time PCR analyses.

Preparation HSCs

After anesthesia, abdominal cavity was exposed and liver was perfused with EGTA solution and Collagenase I through portal vein which was cannulated by 23 gage-catheter. After filtering with cell strainer, hepatocytes and non-parenchymal cells were separated by centrifugation. HSCs were isolated from mice hepatic non-parenchymal cells as described previously (Suh et al., 2012; Yi et al., 2014). HSCs were isolated by density gradient using Optiprep. The isolated HSCs were cultured for 1 day in RPMI medium supplemented with 10% fetal bovine and 10% hoarse serum. The purity of HSCs was determined by flow cytometry and ranged from 90% to 95%. After Con A injection, HSCs were isolated with FACS Aria III (BD Biosciences).

Isolation of Naïve $CD4^+$ T cells and Co-Culturing with HSCs

Naïve $CD4^+$ T cells were isolated from lymph nodes using magnetic beads based on Naïve $CD4^+$ T cell Isolation Kit (Miltenyi Biotec) following the manufacture`s protocols.

To differentiate T cells from naïve $CD4^+$ cells, 1×10^5 naïve $CD4^+$ T cells were plated in 96-well plates with 2×10^4 of HSCs which were plated 1 day ago and then cultured in RPMI with the presence of anti-CD3ε (1 $\mu \cdot ml^{-1}$), anti-CD28 (1 $\mu g \cdot ml^{-1}$), anti-IFN-γ (10 $\mu \cdot ml^{-1}$) and anti-IL-4 (10 $\mu g \cdot ml^{-1}$) for 72 hours. 4-MP (0.5 mM) was treated to HSCs for 3 hours before co-culturing. After 72 hours, T cells and HSCs were collected separately and subjected to flow cytometry and real time PCR analysis, respectively.

Measurements of liver injury and cytokine Level

Serum was collected and assayed for ALT and AST using kits purchased from IDEXX Laboratories. Levels of TNF-α, IL-6, IL-10, IFN-γ and TGF-β1 were measured using an ELISA kit (Biosource International Inc.).

Staining

5 μm sections of paraffin embedded tissue blocks were stained with H&E staining. Immunofluorescence staining was performed with anti-ADH3 antibody (Abcam, UK) and anti-desmin antibody (Dako), then visualized with FITC-conjugated anti-goat IgG secondary antibody (Abcam) or Alexa Fluor®594 conjugated anti-mouse IgG secondary antibody (Invitrogen). Finally, VECTASHIELD Mounting Medium with DAPI (Vector Laboratories) as manufacturer's instructions. All of visual inspection was done using Olympus BX51 microscope (Olympus) equipped with a CCD camera and computer-assisted image analysis with DP2-BSW (Olympus) at various magnifications. For detection of apoptotic bodies, liver sections were stained with TUNEL staining kit (Roche).

Ligand treatment to HSCs and Tregs

HSCs were isolated from WT and $Raldh1^{-/-}$ mice and cultured in serum free RPMI medium with IFN-γ (5 $ng \cdot ml^{-1}$) for 30, 60 and 90 minutes. Isolated Tregs were cultured with IL-6 (20 $ng \cdot ml^{-1}$) (eBioscience) or IL-10 (20 $ng \cdot ml^{-1}$) (eBioscience) for 30, 60 and 90 minutes. After culturing, collected cells were subjected to real-time PCR analysis.

In vitro Treg migration assay

Migration assay was performed using 12-well transwell insert with 3 μm pores (Corning). In lower chamber, 1 x 10^5 HSCs from WT and $CCL2^{-/-}$ mice were plated for 1 day in RPMI with 10% fetal bovine serum and 10% hoarse serum. Then, 2 x 10^5 Tregs from WT and $CCR2^{-/-}$ mice were added to upper chamber and were incubated for 12 and 24 hours. In some plates, IFN-γ (5 $ng \cdot ml^{-1}$) and/or 4-MP (0.5mM) were added. Migrated Tregs in lower chamber were counted at 10 different areas and calculated average number.

Chimeric mice generation

Chimeric mice were generated as previously described(Yi et al., 2014). For development of chimerism, recipient mice were fed with medicated water containing antibiotics for 7 days and then treated radiation at dose of 950 Rad. After 6 hours, 3 x 10^6 BMCs from donor mice were injected via tail vein sterilely. To confirm formation of chimerism, donor mice were sacrificed at 8 weeks and subjected to flow cytometry and RT- and real-time PCR analyses. After confirmation of chimerism, we made a couple of chimeric WT (CD45.1) and $Raldh1^{-/-}$ mice transplanted with WT bone marrow. After 8 weeks, Con A was injected to chimeric mice to induce acute hepatitis and they were sacrificed at 12 hour after Con A.

Immunoblot analysis

Proteins from cells or tissues were extracted with RIPA buffer (30 mmol Tris, pH 7.5, 150 mmol sodium chloride, 1 mmol phenylmethylsulfonyl fluoride, 1 mmol sodium orthovanadate, 1% Nonidet P-40, 10% glycerol and phosphatase/protease inhibitors) after homogenization of tissues. Immunoblots were performed with 50 μg proteins from liver homogenates using antibodies for pSTAT1, STAT1 (Cell signaling) and β-actin (Sigma-Aldrich). Proteins were divided by electrophoresis through 7.5 % SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. Finally protein bands on membrane were visualized using ECL system (GE healthcare).

RNA extraction and quantitative real-time polymerase chain reaction

Isolated or collected cells were treated with TRIzol reagent (Invitrogen) to extraction of total RNA. cDNA was synthesized from RNA with amfiRivert cDNA Synthesis Master Mix (GenDEPOT) following the manufacture`s protocol. Quantitative real-time PCR was performed with SYBR Green Real-time PCR Master Mix (Toyobo) using CFX96 system (Bio-rad, Hercules). Supplementary Table 1 represented PCR primers. Each gene expression was compared with β-actin and analyzed using ΔΔCt values.

Analysis of retinoic acid with HPLC

After treatment of INF-γ at dose of 0, 10 and 50 μg ml^{-1} to HSCs for 24 hours, cells were collected and retinoids were extracted as described previously (Radaeva et al., 2007). HPLC measurements were performed using a Shimadzu SCL-10A VP HPLC system with a Shimadzu Shim-pack C18 analytical column. Peaks were detected by UV absorption at 330 nm with a diode array detector.

Supplementary Table 1. Used Primers in RT-PCR and Real-time PCR

Genes	Forward (5'-3')	Reverse (5'-3')	PCR product (base pairs)
ADH3	GTGGGAGTAGCTGCTTCAGG	GTGCATCAGATCAAAGGCTTG	215
CCR2	GGAGTGGGAAGAAGTATGT	TCAACCTTGGCAAGATAA	166
CCL2	TCAGCCAGATGCAGTTAACGC	TCTGGACCCATTCCTTCTTGG	84
FoxP3	CCCAGGAAAGACAGCAACCTTTT	TTCTCACAACCAGGCCACTTG	88
IFN-γ	AGACATCTCCTCCCATCAGCAG	TAGCCAAGACTGTGATTGCGG	158
IL-6	TCCATCCAGTTGCCTTCTTG	TTCCACGATTTCCCAGAGAAC	166
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT	196
Raldh1	CTCCTGGCGTGGTAAACATT	CCATGGTGTGCAAACTCAAC	244
TGF-β1	TTGCTTCAGCTCCACAGAGA	TGGTTGTAGAGGGCAAGGAC	182
β-actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT	148

SUPPLEMENTARY FIGURES

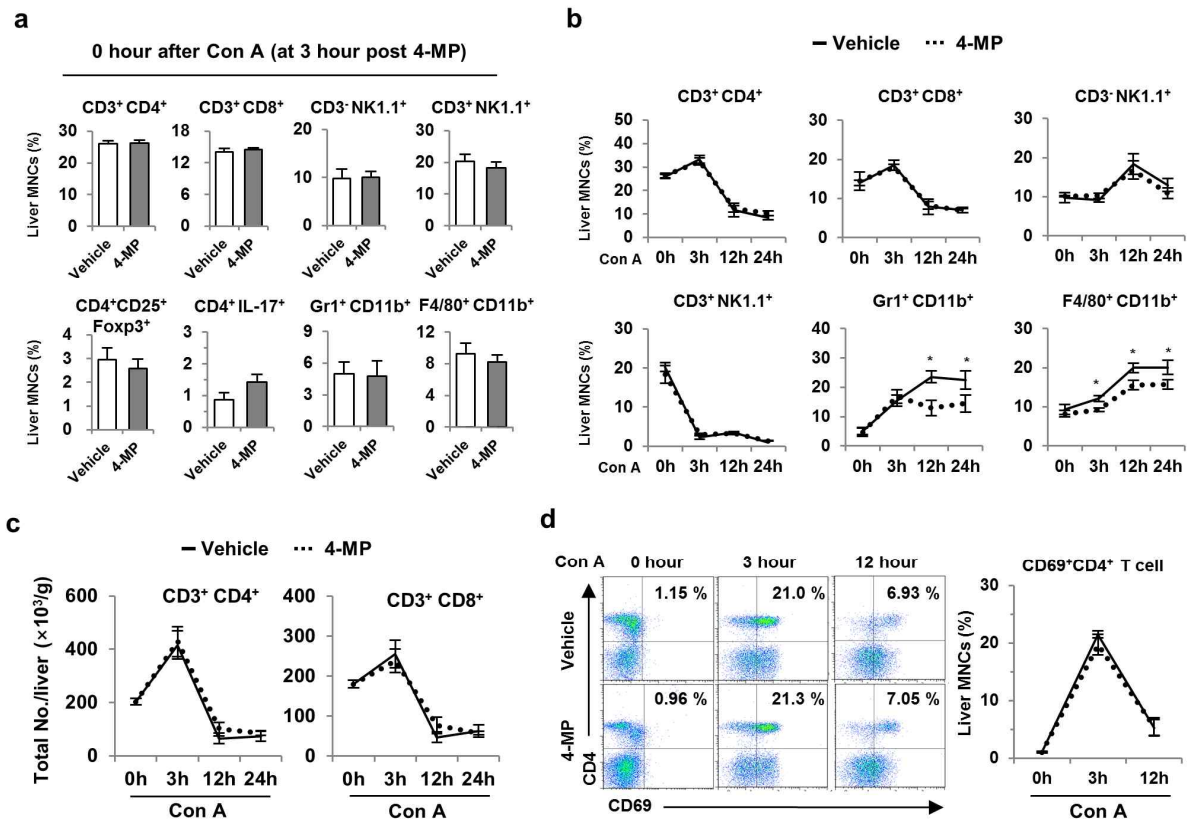

Figure S1. 4-MP treatment does not influence the population of hepatic immune cells and the Con A-mediated activation of CD4 T cells. (A) Mice (n=6/group) were sacrificed at 3 hour after 4-MP treatment (10 mg/kg) and liver MNCs were collected and analyzed by flow cytometry. Values are mean ± SEM. (B-D) After 3 hour-pretreatment of 4-MP, mice (n=8-12/group) were injected with Con A (12 $mg \cdot kg^{-1}$) and then were sacrificed at 3, 12 and 24 hours after Con A treatment. Isolated liver MNCs were stained with fluorescence conjugated-antibodies to CD3, CD4, CD8, NK1.1, Gr1, CD11b and F4/80 and then analyzed by flow cytometry. To assess early T cell activation, liver MNCs were stained with antibodies to CD4 and CD69. Representative frequencies of activated CD4 T cells were analyzed by flow cytometry. Values are mean ± SEM (n=6-12) of three independent experiments. *P < 0.05 in comparison with the corresponding control.

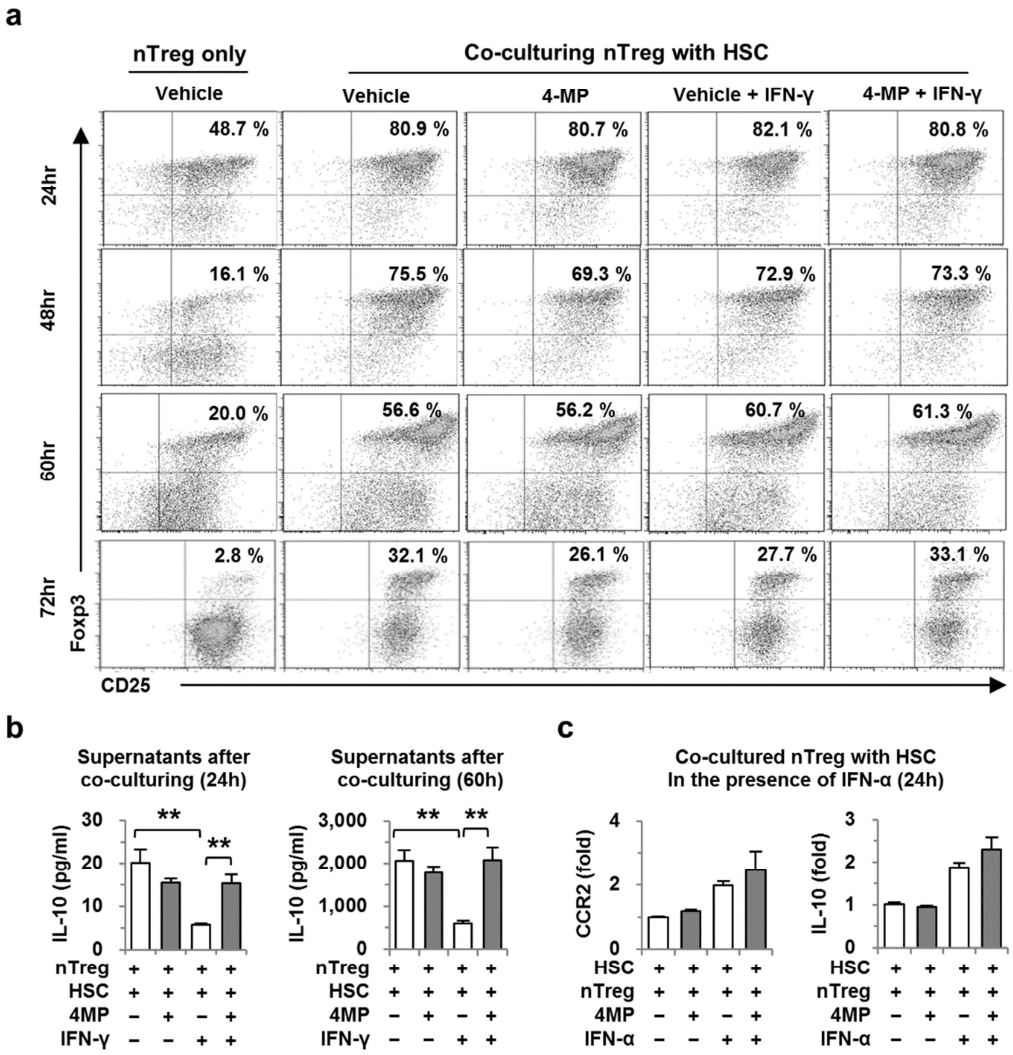

Figure S2. Increased Foxp3 expression and IL-10 production in nTregs co-cultured HSCs in the presence of IFN-γ and 4-MP. (A) Isolated nTregs were co-cultured with HSCs upto 72 hours with or without 4-MP (0.5 mM) ± IFN-γ (5 n·ml^{-1}). The Foxp3 expression was examined by flow cytometry and representative of three independent experiments. (B) Supernatant levels of IL-10 were measured at 24 and 60 hours after co-culturing. Values indicate mean ± SEM of three independent experiments. (C) The expression levels of IL-10 and CCR2 in co-cultured nTregs in the presence of IFN-α (1000 U ml^{-1}) were analyzed by quantitative PCR. Values indicate mean ± SEM of or three independent experiments. $^{**}P < 0.01$ in comparison with the corresponding control.

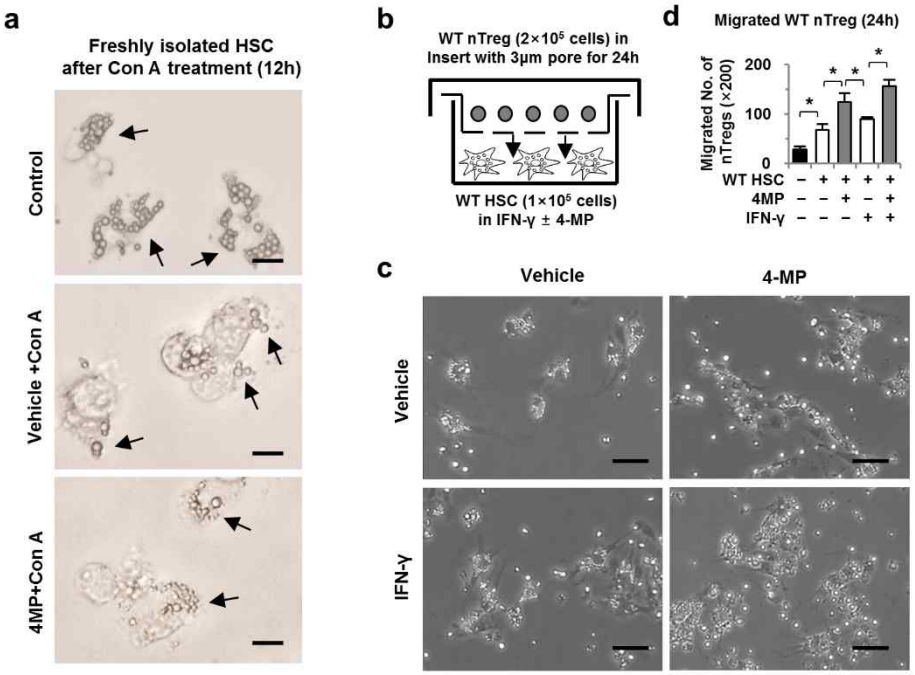

Figure S3. 4-MP treatments suppress Con A-mediated retinol metabolism in HSCs and increase Treg migration into HSCs. (A) At 12 hour after Con A and 4-MP treatment, HSCs were freshly isolated and observed under phase-contrast of light microscope. Representative HSCs of two independent experiments. Scale bars, 20 μm. (B) Migration assay was performed using 12-transwell insert with 3 μm pores. In lower chamber, 1 x 10^5 HSCs were plated. Then, 2 x 10^5 Tregs were added to upper chamber and were incubated for 24 hours. (C) Representative photos of three separate experiments. (D) Migrated nTregs were counted in 5 different areas under x200 magnification.*P < 0.05 in comparison with the corresponding control. Scale bars, 50 μm.

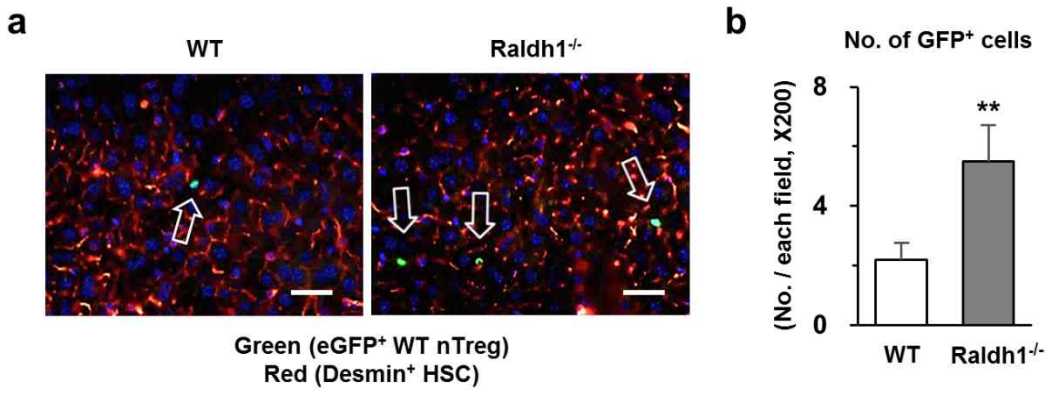

Figure S4. Raldh1 deficiency increases Treg migration. For adoptive transfer of nTregs ex vivo, isolated eGFP$^+$ nTregs (2 x 10^6) and IFN-γ (20 ng·ml^{-1}) were co-circulated for 2 hours through closed circulation system from portal vein to inferior vena cava. (A) Liver section were stained with desmin (red) for HSCs and DAPI (blue) for nuclei, manifesting in close contact between HSCs and migrated eGFP$^+$ Tregs. (B) Migrated Tregs were counted under in 5 different areas under x200 magnification. Values indicate mean ± SEM of four separate experiments. $^{**}P < 0.01$ in comparison with the corresponding control. Scale bars, 50 μm.

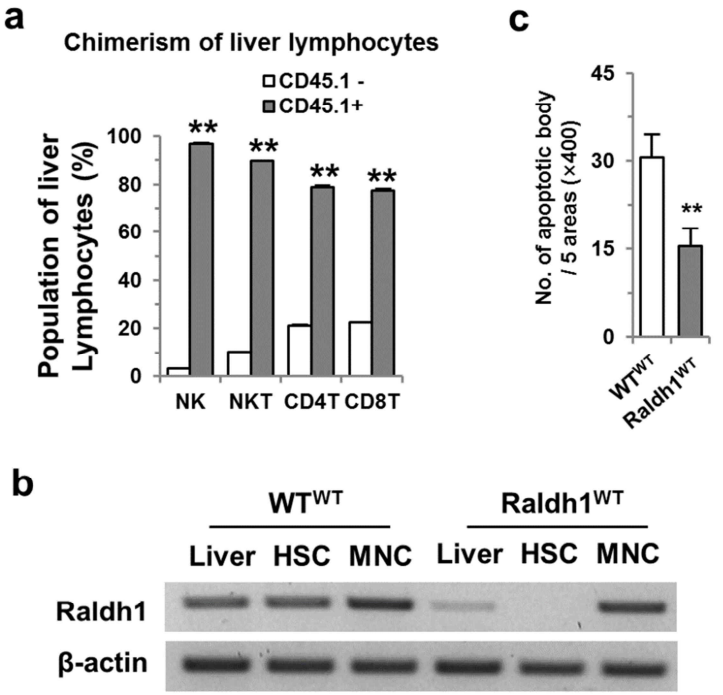

Figure S5. Raldh1-deficient chimeric mice are resistant to Con A-mediated acute liver injury. Chimeric mice were generated by transplantation of CD45.1^{+} WT bone marrow to CD45.2^{+} WT mice (WT^{WT}) and Raldh1-deficient (Raldh1$^{-/-}$) mice (Raldh1^{WT}). After 8 weeks of bone marrow transplantation, mice (n=5/group) were injected with Con A (12 mg/kg) for 12 hours. For chimerism assessment, liver MNCs were isolated and analyzed by flow cytometry and RT-PCR analyses. (A) Representative chimeric frequencies of liver MNCs were analyzed by flow cytometry. (B) The expression of Ralh1 gene in whole liver tissues, HSCs and liver MNCs of WT^{WT} and Raldh1^{WT} mice were detected by RT-PCR analyses. (C) At 12 hour after Con A injection, mice (n=5/group) were sacrificed and then liver sections were stained with TUNEL. The numbers of apoptotic bodies in liver sections were counted in 5 different areas under x400 magnification. Values indicate mean ± SEM. ***P* < 0.01 in comparison with the corresponding control.

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