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 x 10\(^5\) naïve CD4\(^+\) T cells were plated in 96-well plates with 2 x 10\(^4\) of HSCs which were plated 1 day ago and then cultured in RPMI with the presence of anti-CD3\(\varepsilon\) (1 \(\mu\text{-ml}^{-1}\)), anti-CD28 (1 \(\mu\text{-g·ml}^{-1}\)), anti-IFN-\(\gamma\) (10 \(\mu\text{-g·ml}^{-1}\)) and anti-IL-4 (10 \(\mu\text{-g·ml}^{-1}\)) 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-\(\alpha\), IL-6, IL-10, IFN-\(\gamma\) and TGF-\(\beta\) were measured using an ELISA kit (Biosource International Inc.).

Staining
5 \(\mu\text{m}\) 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\textsuperscript{®}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-\(\gamma\) (5 ng·ml\(^{-1}\)) for 30, 60 and 90 minutes. Isolated Tregs were cultured with IL-6 (20 ng·ml\(^{-1}\)) (eBioscience) or IL-10 (20 ng·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, $1 \times 10^5$ HSCs from WT and CCL2^{-/-} mice were plated for 1 day in RPMI with 10% fetal bovine serum and 10% horse serum. Then, $2 \times 10^5$ 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·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, $3 \times 10^6$ 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  | GGAGTGGGGAAGGAAGTATGT | TCAACCTTGGAAGATAA | 166 |
| CCL2  | TCAGCCAGATGCAGTTAACGC | TCTGGACCCATTCCTCTTTG | 84 |
| FoxP3 | CCCAGGAAAGACAGCAACCTTTT | TTCTCACAACCAGGGCCACTTG | 88 |
| IFN-γ | AGACATCTCCTCCCATCAGCAG | TAGCCAAAGACTGTGATTGCGG | 158 |
| IL-6  | TCCATCCAGTTGCTTTTCTTG | TTCCACGATTTTCAGAGAAGAC | 166 |
| IL-10 | GGTGGCAAGCCTTTATCGGA | ACCTGTCCACTGCCTTGCT | 196 |
| Raldh1| CTCCTGCGTGGTAAACATT | CCATGGTGTCGAAACTCAAC | 244 |
| TGF-β1| TTGCTTCAGCTCCACAGAGA | TGGTTGAGGGCAAGGAC | 182 |
| β-actin| AGAGGGAATCGTGGCTGAC | CAATAGTAGCGTGCCTG | 148 |
**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·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, $1 \times 10^5$ HSCs were plated. Then, $2 \times 10^5$ 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 $\times 200$ 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 × 10⁶) 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 ×200 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 \(\times 400\) magnification. Values indicate mean ± SEM. **\(P < 0.01\) in comparison with the corresponding control.
REFERENCES

Radaeva, S., Wang, L., Radaev, S., Jeong, W.I., Park, O., and Gao, B. (2007). Retinoic acid signaling sensitizes hepatic stellate cells to NK cell killing via upregulation of NK cell activating ligand RAE1. Am J Physiol Gastrointest Liver Physiol 293, G809-816.

Suh, Y.G., Kim, J.K., Byun, J.S., Yi, H.S., Lee, Y.S., Eun, H.S., Kim, S.Y., Han, K.H., Lee, K.S., Duester, G., et al. (2012). CD11b(+) Gr1(+) bone marrow cells ameliorate liver fibrosis by producing interleukin-10 in mice. Hepatology 56, 1902-1912.

Yi, H.S., Lee, Y.S., Byun, J.S., Seo, W., Jeong, J.M., Park, O., Duester, G., Haseba, T., Kim, S.C., Park, K.G., et al. (2014). Alcohol dehydrogenase III exacerbates liver fibrosis by enhancing stellate cell activation and suppressing natural killer cells in mice. Hepatology 60, 1044-1053.