ROCK2 inhibition attenuates profibrogenic immune cell function to reverse thioacetamide-induced liver fibrosis

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Supplementary Methods

Immunohistochemistry

Deparaffinized liver tissues were immunolabeled as described previously[1] using 1) wide-spectrum keratin (CK-WSS) for bile duct, ductular epithelium and HPC; 2) F4/80 for monocyte/macrophage accumulation; 3) α-smooth muscle actin (SMA) for activated stellate cells and myofibroblasts and 4) Immunoglobulin (IgG) for antibody distribution. For CK-WSS (Dako, Denmark), α-SMA (Dako), and IgG (Dako EnVision anti-rabbit HRP kit, Denmark) staining, heat-induced antigen retrieval (10 mmol/L Tris Base/1 mmol/L EDTA solution, pH9) was used, whereas enzymatic digestion (Carezyme Trypsin, Biocare Medical, CA) was used for F4/80 (AbD Serotec, UK) staining. Following staining, slides were scanned using virtual microscopy (ScanScope XT Slide Scanner, Aperio, CA) and viewed in ImageScope (Aperio software version 11.2) for further analysis. For enumeration of SMA^+ and $F480^+$ cells at the 1 week timepoints, central veins were identified and immunostained cells within a 200um circumference were counted. Cells were counted at 3-5 veins and the average reported as # peri-central cells. At later time points, numbers of activated myofibroblasts were counted manually in a total of three separate areas ($5mm^2$)/section excluding the capsule and portal tracts larger than 150 mm, and reported as mean # cells/$5mm^2$. Quantification of CK-WSS staining per proportional area performed using Aperio analysis algorithm in ImageScope software.

For Immunofluorescence (IF) staining for GC in spleens, OCT-embedded frozen spleen sections were triple stained with PE-conjugated peanut agglutin (PNA, Vector laboratories) FITC-conjugated CD3 (DAKO, Denmark) and Pe-Cy7-conjugated B220

(Invitrogen, Australia) and fluorescent microscopy performed using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Australia, North Ryde, Australia).

RNA Isolation and RT-PCR Analysis

Total RNA was extracted from murine liver tissue and cultured bone marrow-derived macrophages (BMDM) using TRI Reagent according to the manufacturer's instructions (Invitrogen, Life technologies, Australia). Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA) was used to assess RNA concentration and quality. Complementary DNA was generated from 2 µg of DNAase treated RNA using SuperScript III Reverse Transcriptase with oligo dT priming according the manufacturer's instructions, including no-RT controls (Life Technologies, Australia). C*ol1a2 (*Fwd: CACCCCAGCGAAGAACTCATAC, Rev: CCCCTTCTACGTTGTATTCAAACTG, 104 nt amplicon), Il*17a (*Fwd: GCTGACCCCTAAGAAACCCC, Rev: GAAGCAGTTTGGGACCCCTT, 174 nt amplicon), T*nf (*Fwd: ATCTTCTCAAAATTCGAGTGACAA, Rev: TGGGAGTAGACAAGGTACAACCC, 174 nt amplicon), M*mp2 (*Fwd: TTGCAGGAGACAAGTTCTGGAGATA, Rev: CACGACGGCATCCAGGTTAT, 116 nt amplicon), M*mp9 (*Fwd:AGGGGCGTGTCTGGAGATTC, Rev: TCCAGGGCACACCAGAGAAC, 103 nt amplicon), M*mp12 (*Fwd: AGCTTTCCAAGTCTGGAGTGATG, Rev: GCATGGGCTAGTGTACCACCTT, 95 nt amplicon), and M*mp*13 *(*Fwd: ACAAAGATTATCCCCGCCTCAT, Rev: GGCCCATTGAAAAAGTAGATATAGCC, 99 nt amplicon) mRNA expression was measured using SYBR Select Master Mix (Life Technologies) and quantitative PCR was performed on a ViiA 7 Real-Time PCR System (Life Technologies) using standard thermal cycling conditions. qPCR primers were designed to span exon boundaries and

to detect all mRNA spliceforms and tested for specificity in silico by BLAST. For BMDM gene expression analysis each gene was normalized to the expression of the housekeeping gene, H*prt (*Fwd: CCCAGCGTCGTGATTAGCG, Rev: GCCACAATGTGATGGCCTCC, 85 nt amplicon) using the delta Ct method. For BMM gene expression analysis each gene was normalized to the expression of the housekeeping gene, H*prt (*Fwd: CCCAGCGTCGTGATTAGCG, Rev: GCCACAATGTGATGGCCTCC, 85 nt amplicon) using the delta Ct method. Expression in whole liver was calculated relative to *Hprt* and to a baseline control sample (6 wk TAA) using the delta delta Ct method. Lack of PCR amplification in no-RT controls was confirmed.

Antibodies

The following antibodies were purchased from BD Pharmingen or BioLegend: PE-conjugated F4/80 (BM8); APC-Cy7-conjugated Ly6G (1A8); AF647-conjugated Siglec-F (E50-2440); PerCpCy5.5-conjugated CD11b (M1/70); Pacific-Blue-conjugated Ly6c (HK1.4); PE-Cy7-conjugated IA/IE class 11 (M5/114.15.2); APC conjugated TNF (MP6-XT22); APC-conjugated NK1.1 (PK136); PerCpCy5.5-conjugated CD8 (53-6.7); PE-CF594-conjugated CD3 (145-2C11) and PE-conjugated CD3 (17A2). FITC-conjugated CD4 (GK1.5) was produced in-house. Phycoerythrin-labeled, α-GalCer–loaded or unloaded (control) CD1d tetramers were generously provided by Dale Godfrey (University of Melbourne, Australia).

IgG ELISA

Total IgG (H+L) concentrations in sera were determined by a sandwich ELISA. ELISA plates (Nunc) were coated with rabbit anti-mouse IgG (Southern Biotech) in 0.05 M

carbonate buffer over night at 4°C before serum samples titrated on plate along with mouse IgG (Sigma-Aldrich) as standards. Plates were then incubated with HRP-conjugated anti-mouse IgG (H+L) (Life Technologies) before the addition of substrate (Sigma-Aldrich). The absorbance was determined at 450 nm and subtract read at 620 nm as background after the addition of 1 M H_2SO_4 to stop reaction.

Western blots

Western blotting was performed using SDS-PAGE Electrophoresis System (BIO-RAD). Protein lysate (20 µg) isolated from the liver or bone marrow-derived macrophages was electrophoresed on a 10 or 15% Tris gel with Tris running buffer. The separated proteins were blotted on to a PVDF membrane. Membranes were probed with primary antibodies against pSTAT3, pSTAT5, pCofilin, Cofilin, □-Actin, and Bcl6 (Cell Signalling Technologies). HRP conjugated secondary antibodies and enhanced chemiluminescence (BIORAD) were used for detection.

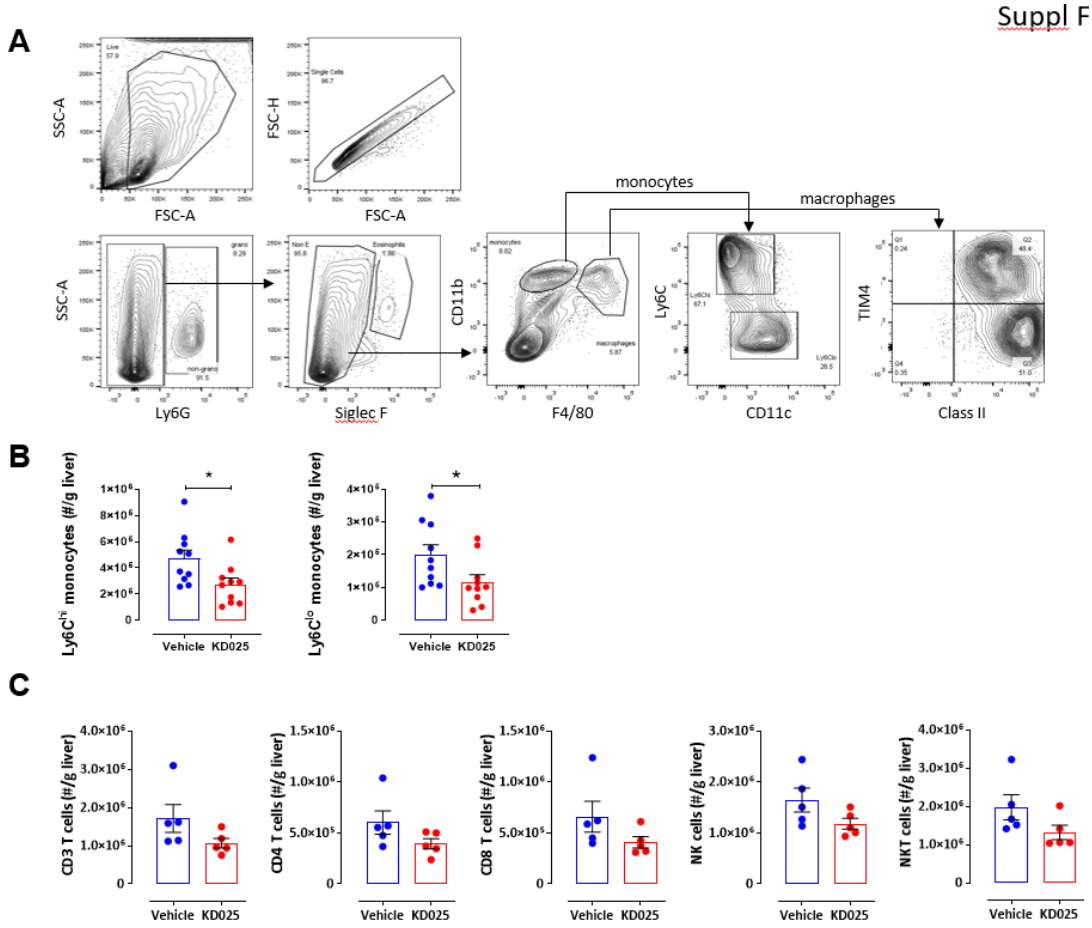

Fig. S1. Effects of KD025 administration on the liver myeloid populations. (A) FACS gating strategy for liver monocyte and macrophage analysis. Shown is a representative analysis of liver mononuclear cells following treatment with TAA for 6 weeks followed by 2 weeks of co-administration with vehicle. (B)) Absolute number of liver Ly6C^{hi} and Ly6C^{lo} monocytes and (C) lymphocyte populations in mice administered therapeutic dosing of vehicle or KD025. * p< 0.05 Mann Whitney-U test.

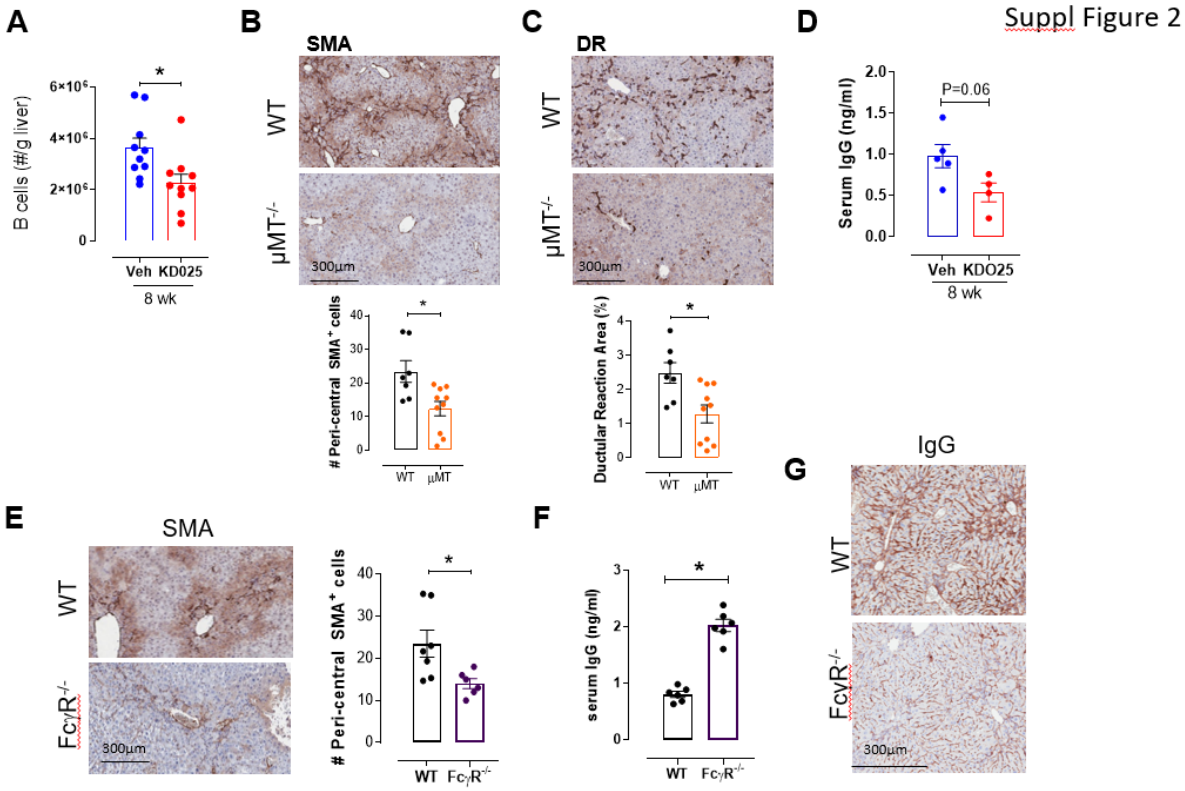

Fig. S2. Effects of B cells and IgG on hepatic fibrosis. (A) Absolute number of liver CD19⁺ B cells in mice administered therapeutic dosing of vehicle or KD025. Histochemical staining and aperio quantification of (B) Sirius red (SR) and (C) Pan-keratin (CK-WSS) staining in representative liver sections from 6-week TAA treated WT and µmt$^{-/-}$ mice (n=6-10 mice/group; combined from two independent experiments). (D) Graph illustrating serum immunoglobulin levels (IgG) in 6-week TAA treated mice, co-administered with 0.4% methylcellulose vehicle control or 100mg/kg KDO25 (n=4-5 animals/group). (E) Histochemical staining and aperio quantification of smooth muscle actin (SMA) in representative liver sections from 6-week TAA treated WT and FcγR$^{-/-}$ mice (n=6-7 animals/group). (F) Serum immunoglobulin levels (IgG) and (G) histochemical immunoglobulin staining (IgG) in 6-week TAA treated WT and FcγR$^{-/-}$ mice (n=6 animals/group). Data are presented as mean ± SEM. * p< 0.05 Mann Whitney-U test.

Suppl Figure 3

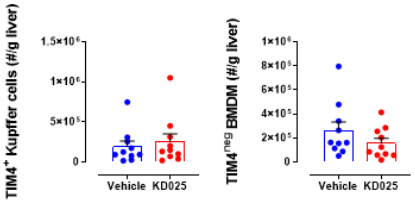

Fig. S3. Effects of KD025 administration on the liver macrophage populations. (A) Absolute numbers of Tim4^{+} Kupffer cells and BMDM following treatment with TAA for 6 weeks followed by 2 weeks of co-administration with vehicle. Gating strategy as outlined in Suppl Figure 1A.

Suppl Figure 4

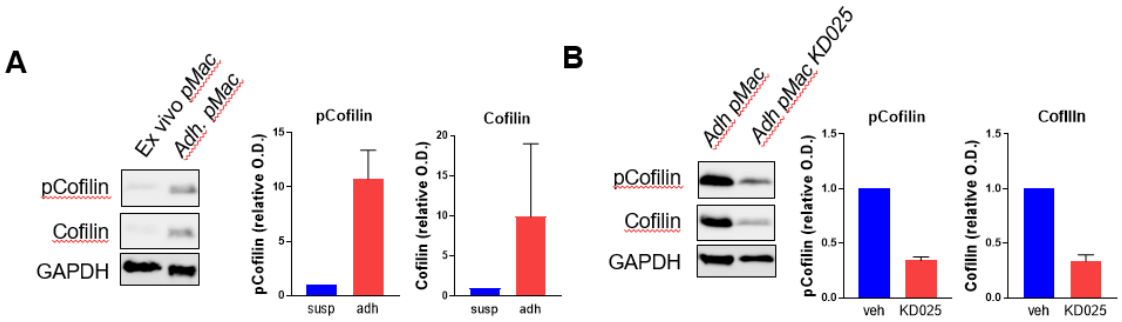

Fig. S4. KD025 inhibits cofilin in adherent macrophages. (A) pCofilin and Cofilin expression normalised to GADPH in freshly isolated peritoneal macrophages (Fresh pMac) and in cultured adherent macrophages (Adh pMac). (B) pCofilin and Cofilin expression normalised to GADPH in Fresh pMac and Adh pMac treated with KDO52/vehicle. Data are presented as mean ± SEM. (n=2 animals/group). *** p< 0.001 Mann Whitney-U test.

Reference

1. Melino M, Gadd VL, Alexander KA, Beattie L, Lineburg KE, Martinez M, et al. Spatiotemporal Characterization of the Cellular and Molecular Contributors to Liver Fibrosis in a Murine Hepatotoxic-Injury Model. *The American journal of pathology.* 2016;186(3):524-538.