

1 Supplement Materials

2

3 Methods

4 Construction of myeloid-specific FSTL1 deficient mice

5 FSTL1^{FL/FL} (carrying the a floxed allele) and Lyz2-Cre (expressing the Cre recombinase
6 specifically in myeloid cells) mice were both acquired from GemPharmatech. Co. Ltd
7 (Nanjing, China). FSTL1 conditional konckout mice were made via CRISPR/Cas9
8 system. Firstly, two sgRNAs-targeting the introns on both sides of the floxed region of
9 FSTL1 were respectively constructed and transcribed invitro. And the donor vector with
10 the loxp fragment was designed and constructed in vitro. Then Cas9 mRNA, sgRNA
11 and donor will be co-injected into zygotes. Thereafter, the zygotes were transferred into
12 the oviduct of pseudopregnant ICR females at 0.5 dpc. And F0 mice was birthed after
13 19~21 days of transplantation, all the offsprings of ICR females (F0 mice) were
14 identified by PCR and sequencing of tail DNA. And positive F0 mice were genotyped
15 by the methods. Finally, crossing F0 mice with C57BL/6J mouse to build up
16 heterozygous mice (FSTL1^{FL/WT}). A stable F1 generation mouse model was obtained
17 by mating positive F0 generation mice with C57BL/6J mice.

18 For producing myeloid-specific knockout mice, homozygous FSTL1^{FL/FL} mice were
19 bred with homozygous Lyz2-Cre mice, and their heterozygous offspring were back-
20 crossed with homozygous FSTL1^{FL/FL} mice ([supplemental figure 6](#)). Mice were
21 genotyped using primers listed in [supplemental table 5](#).

22 Protein extraction and western blot

23 Protein extraction was performed to extracted nuclear or cytoplasmic protein according
24 to manufacturer's instruction (P0027, Beyotime Biotechnology, Shanghai, China).
25 Western blot was performed as described previously ¹. Briefly, samples were lysed
26 using RIPA solution. BCA were used to normalize protein concentrations between
27 samples. Thermal denatured protein samples were subjected to 12% SDS-
28 polyacrylamide gel electrophoresis and were then transferred to a PVDF membrane
29 (Bio-Rad, Hercules, CA) for antibody incubation.

30 Antibodies

31 Primary antibodies used in this study were as follows: rabbit anti-FSTL1 mAb
32 (ab223287, Abcam); rabbit anti-PKM2 mAb (4053S, Cell Signaling Technology);
33 mouse anti-PKM2 mAb (60268-1-Ig, Proteintech); rabbit anti-p-PKM2 mAb (3827,
34 Cell Signaling Technology); rabbit anti-p-STAT6 mAb (56554S, Cell Signaling
35 Technology); rabbit anti-p-STAT1 mAb (9167S, Cell Signaling Technology); rabbit
36 anti-iNOS mAb (13120S, Cell Signaling Technology); rabbit anti-TLR4 mAb (14358S,
37 Cell Signaling Technology); rabbit anti-p65 mAb (8242S, Cell Signaling Technology);
38 rabbit anti-p-NF- κ B mAb (3033S, Cell Signaling Technology); rabbit anti-p-I κ k mAb
39 (2697S, Cell Signaling Technology); rat anti-CD68 mAb (ab53444, Abcam); rat anti-
40 F4/80 mAb (ab6640, Abcam); rabbit anti α -SMA mAb (19245S, Cell Signaling
41 Technology); rabbit anti-Ly6G mAb (87048S, Cell Signaling Technology);
42 recombinant rabbit anti-GAPDH mAb (ab181602, Abcam); recombinant rabbit IgG
43 isotype control (ab172730, Abcam); rabbit IgG isotype control (3452S, Cell Signaling
44 Technology); rabbit anti-flag-Tag mAb (14793, Cell Signaling Technology); rabbit
45 anti-myc-Tag mAb (2276S, Cell Signaling Technology).

46 Secondary antibodies used in this study were as follows: HRP-conjugated goat Anti-
47 Rabbit IgG (ab205718, Abcam); HRP-conjugated goat Anti-Rat IgG (ab205720,
48 Abcam); HRP-conjugated goat Anti-Mouse IgG (ab205719, Abcam); Alexa Fluor 488-
49 conjugated goat anti-Mouse IgG (A-11001, Invitrogen); Alexa Fluor 488-conjugated
50 goat anti-Rabbit IgG (A-11008, Invitrogen); Alexa Fluor 488-conjugated goat anti-Rat
51 IgG (A-11006, Invitrogen); Alexa Fluor 594-conjugated goat anti-Mouse IgG (A-
52 11005, Invitrogen); Alexa Fluor 594-conjugated goat anti-Rabbit IgG (A-11012,
53 Invitrogen); Alexa Fluor 594-conjugated goat anti-Rat IgG (A-11007, Invitrogen).

54 **Plasmid construction and transfection**

55 Expression plasmid constructs, including pcDNA3.1(+)-HA-FSTL1, pcDNA3.1(+)-
56 HA-FSTL1-SP domain deletion, pcDNA3.1(+)-HA-FSTL1-FK domain (follistatin-like
57 and kazal-like Domain) deletion, pcDNA3.1(+)-HA-FSTL1-EF-hand domain deletion,
58 pcDNA3.1(+)-HA-FSTL1-VWFC domain deletion, pcDNA3.1(+)-Flag-PKM2,
59 pcDNA3.1(+)-Flag-PKM2-AB-domain deletion, pcDNA3.1(+)-FlagPKM2-C-domain
60 deletion, pcDNA3.1(+)-Flag-PKM2-N-domain deletion were all constructed and

61 purchased from Shanghai (GenePharma, Shanghai, China). Lipofectamine 3000
62 (Invitrogen, Carlsbad, CA) was used to transfect. Briefly, 293T cells were plated in a
63 6-well plate (3 x10⁵ cells/well). After 24h, the 293T cells were transfected with 2.5
64 mg/well plasmid, 5 ml Lipo 3000 and 5 ml P3000. Empty pcDNA3.1(+)-vector was
65 added into each well to equalize total amounts of transfected plasmids.

66 **Co-immunoprecipitation and mass spectrometry**

67 Immunoprecipitation and mass spectrometry were performed as previously described ¹.
68 Briefly, harvested cell lysates were incubated with the specific primary antibodies
69 overnight at 4°C, and conjugated with protein A/G beads (Santa Cruz Biotechnology,
70 CA) for 4-6 h. After washing, immunoprecipitants were boiled in Laemmli sample
71 buffer for 10 min. The immunoprecipitated proteins was detected by western blot or
72 reverse phase liquid chromatography/mass spectrometry (RPLC/MS)-ESI-Q-ToFQ
73 analyzer (TripleTOF 6600 MS system, Applied Biosystem, USA).

74 **Quantitative real-time PCR**

75 Total RNA was extracted from tissue samples or cells with TRIzol reagent (Invitrogen)
76 followed by isopropyl alcohol precipitation. RNA concentration and quality were
77 evaluated using Nano-drop system and was then reverse-transcribed into cDNA by
78 commercially available kit (Vazyme, Nanjing, China). SYBR-green based real-time
79 quantitative PCR kits (Vazyme, Nanjing, China) were used to perform mRNA
80 quantification. Gene expressions were normalized to β -actin expression. Primer
81 sequences are listed in [supplemental table 6](#).

82 **Biochemical analysis**

83 Secreted cytokines (IL-1 β , IL-10 and TNF- α) were measured using ELISA kits
84 (eBioscience, San Diego, CA), according to manufacturer's instruction. To measure
85 serum alanine transaminase (ALT), aspartate transaminase (AST), albumin (ALB), and
86 total bilirubin (TB) automated chemical analyzer (Olympus, Tokyo, Japan) was used.

87 **Immunohistochemistry and Immunofluorescence (IF) staining**

88 Formaldehyde-fixed, paraffin-embedded liver samples were sectioned into 4 μ m slides.
89 Immunohistochemistry staining and immunofluorescence (IF) staining were performed
90 as previously described ¹. Briefly, for tissue samples, formaldehyde-fixed, paraffin-

91 embedded slides were subjected to dewaxing, hydration and antigen retrieval, followed
92 by blocking and antibody incubation. For *in vitro* experiments, cells were seeded on
93 coverslips and were fixed with 3% paraformaldehyde, permeated with 0.1% Triton X-
94 100 followed by 10% BSA blockage. Primary antibodies were diluted as suggested,
95 added onto slides and were incubated at 4°C overnight in a moist chamber. Then slides
96 were washed with PBS and incubated with 488-/594-/HRP-conjugated secondary
97 antibodies. For IF staining, slides were further incubated with DAPI (Invitrogen) and
98 mounted in IF mounting medium (Servicebio). Bright field and fluorescence
99 microscopy were performed using an Olympus BX53 system. Quantification of the
100 mean fluorescence intensity was performed with the ImageJ software under least three
101 randomly selected fields (supplemental figure 8). Nuclei were stained with DAPI, and
102 the percentage of nuclear p65 and PKM2 positive cells was counted under least three
103 randomly selected fields (supplemental figure 8). Confocal microscopy was performed
104 using Olympus FV3000 confocal microscopy system.

105 **Primary cell isolation, culture and treatment**

106 Recombinant M-CSF (PeproTech, USA) were used to differentiate bone marrow-
107 derived macrophages (BMDMs) as previously described ¹. Briefly, femurs and tibias
108 were carefully dislodged from sacrificed 8~12 weeks-old male mice in a laminar flow
109 hood. Bone marrow cells were then flushed out using 30 G needle on a 20 mL syringe
110 filled with DMEM. After red blood lysis using LCK buffer solution (Beyotime), cells
111 were washed with PBS and seeded in DMEM medium containing 20 ng/mL M-CSF
112 and 10% fetal bovine serum (FBS). After 4-day cultivation, cells were washed with
113 PBS and changed with new medium. Cells were regarded as fully differentiated
114 BMDMs on day 7. Liver macrophages were isolated from mice by portal perfusion
115 using pre-warmed solution containing 0.05% collagenase type IV dissolved in
116 Ca²⁺/Mg²⁺ HBSS, filtered through a 40µm nylon strainer (Falcon) and were then
117 subjected to 40 g centrifugation without brake. Primary hepatocytes were separated
118 using 50% Percoll gradient by 200 g centrifugation and were seeded in DMEM medium
119 supplemented with 10% FBS. To enrich liver macrophages, NPCs
120 (nonparenchymal cells) were suspended in HBSS and layered onto a two-layer 25%-

121 50% Percoll gradient (Sigma-Aldrich, St. Louis, MO) in a 50-ml conical centrifuge tube
122 and centrifuged at 1800g at 4°C for 15 min. Liver macrophages in the middle layer
123 were collected and allowed to attach to cell culture plates in supplemented DMEM with
124 10% FBS for 20 min at 37°C. Nonadherent cells were removed by replacing the culture
125 medium. The purity of macrophages in the adherent cells was determined by
126 immunofluorescent staining with anti-F4/80. 80-90% adherent cells were F4/80
127 positive (Supplemental figure 7A). Cell viability was assessed after 0 or 24 h using a
128 Countess II FL Automated Cell Counter (Thermo Fisher, USA) with trypan blue
129 exclusion (Supplemental figure 7B). HSC-T6 cells purchased from the Cell Center of
130 Shanghai Institutes for Biological Sciences. HSC-T6 cells were cultured in DMEM
131 medium supplemented with 10% FBS. BMDMs plasmid transfection was performed
132 using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). Adenoviruses (GenePharma,
133 Shanghai, China) expressing FSTL1 and vector were used to transfect BMDMs for 48h
134 on day 0~2 of cultivation. Cells were then treated with LPS at the concentration of 100
135 ng/mL (Sigma, St. Louis, MO) for 24 h.

136 **BMDMs Co-culture with HSCs**

137 BMDMs were co-cultured with HSCs at 1:1 using DMEM with 10% FBS. The co-
138 cultures were performed in six-well plates with HSCs seeded at lower chamber.
139 BMDMs were seeded onto 0.4 µm pore-size Transwell culture inserts (BD Biosciences).
140 Co-cultures were then treated with 100ng/ml lipopolysaccharide (LPS) for 24 hours.

141 **Bioinformatics analysis using publicly available datasets**

142 All bioinformatics analysis were performed under *R* environment version 4.0.2.
143 Publicly available omics data were provided by Gene Expression Omnibus (GEO).
144 Several datasets characterizing liver gene expression using *Affymetrix*TM expression
145 microarrays were analyzed. In dataset GSE141821, mice were treated with CCL₄ (n =
146 9) or olive oil (control, n = 8) for 8 weeks². In dataset GSE35961, mice were fed with
147 high fat, methionine- and choline-deficient diet for 8 weeks (n= 4 per group)³. In
148 dataset GSE152494, mice were subjected to common bile duct ligation or sham
149 operation and samples were harvested after 6 weeks (n = 6 per group)⁴. In dataset
150 GSE49541, liver samples were obtained from 72 patients with NASH-associated

151 fibrosis (40 with fibrosis stage F0 - F1 and 32 with F3 - F4)⁵. In dataset GSE14323, 41
152 cirrhotic human liver samples and 19 control samples were analyzed⁶. R package *Affy*
153 were used to preprocess data. R package *limma* were used to identify differentially over-
154 expressed genes, with threshold defined as log₂-fold change > 2 and adjusted *P* value <
155 0.01. Pre-processed sparse matrix files were loaded using *Seurat* R package. Genes
156 expressed in fewer than three cells in a sample were excluded. Cells that 1) had fewer
157 than 300 genes, 2) mitochondrial gene content >30% of total UMI count were excluded.
158 Data were normalized and log-transformed using function *NormalizeData* with scale
159 factor = 10,000.

160 **Extracellular acidification rate monitoring**

161 Seahorse XF-24 Extracellular Flux Analyzer (Agilent) was used for real-time recording
162 of extracellular acidification rate (ECAR). Briefly, FL/FL and M-KO BMDMs
163 extracted from mice were seeded in Seahorse XF-24 microplates (1.5x10⁵ cells/well)
164 treated with/without LPS (100 ng/mL) for 6 h. Before analysis, the cells were switched
165 into ECAR media for 1 h at 37°C. After baseline measurements, 10 mM glucose, 1 μM
166 oligomycin, and 50 mM 2-DG were sequentially injected into each well at indicated
167 time points.

168 **Extracellular lactate assay**

169 Extracellular levels of lactate were determined using lactate assay kit (BioVision)
170 according to the manufacturer's instructions.

171

172

173 **References**

- 174 1. Rao, J., et al., *Nogo-B is a key mediator of hepatic ischemia and reperfusion*
175 *injury*. Redox Biol, 2020. **37**: p. 101745.
- 176 2. Lefebvre, P., et al., *Interspecies NASH disease activity whole-genome profiling*
177 *identifies a fibrogenic role of PPARalpha-regulated dermatopontin*. JCI Insight,
178 2017. **2**(13).
- 179 3. Kita, Y., et al., *Metformin prevents and reverses inflammation in a non-diabetic*
180 *mouse model of nonalcoholic steatohepatitis*. PLoS One, 2012. **7**(9): p. e43056.
- 181 4. Gijbels, E., et al., *Robustness testing and optimization of an adverse outcome*
182 *pathway on cholestatic liver injury*. Arch Toxicol, 2020. **94**(4): p. 1151-1172.
- 183 5. Moylan, C.A., et al., *Hepatic gene expression profiles differentiate*
184 *presymptomatic patients with mild versus severe nonalcoholic fatty liver disease*.
185 Hepatology, 2014. **59**(2): p. 471-82.
- 186 6. Mas, V.R., et al., *Genes involved in viral carcinogenesis and tumor initiation in*
187 *hepatitis C virus-induced hepatocellular carcinoma*. Mol Med, 2009. **15**(3-4):
188 p. 85-94.
- 189
190

191 **Supplemental figure legends**

192

193 **Supplemental figure 1. FSTL1 expression in liver fibrosis tissues. (A-B)** H&E
194 staining, Masson's staining, and mRNA expression of TIMP-1 and COL1A1 in
195 different histological stages of human liver fibrosis; **(C)** Proportion of CD68⁺FSTL1⁺
196 cells in FSTL1⁺ cells in human liver fibrosis; **(D)** Analysis of FSTL1 expression in
197 animal models of CCl₄ injection-, BDL- and MCD diet-induced fibrosis using three
198 publicly available omics datasets. Human samples: Control (N=18), Mild fibrosis
199 (N=20), Advanced fibrosis (N=13). Data were presented as mean ± SEM; **p < 0.01.

200

201 **Supplemental figure 2. FSTL1 expression is primarily increased in macrophages**
202 **in fibrotic liver tissues. (A)** Double immunofluorescence staining of FSTL1 and α-
203 SMA in human liver fibrosis. Scale bars, 50µm. **(B)** FSTL1 mRNA expression and **(C)**
204 protein expression in isolated hepatocytes, HSCs and macrophages from mice liver
205 fibrosis. Data were presented as mean ± SEM of at least three independent experiments;
206 *p < 0.05, **p < 0.01.

207

208 **Supplemental figure 3. There were no significant differences in the PKM2 mRNA**
209 **levels in the FSTL1-knockout or FSTL1-overexpressing cells.** Data were presented
210 as mean ± SEM; N=6-8/group.

211

212 **Supplemental figure 4. The expression of PKM2 was markedly increased in the**
213 **nucleus of human liver fibrosis.** N=6/group.

214

215 **Supplemental figure 5. FSTL1-deficient BMDMs markedly reduced α-SMA**
216 **expression and attenuated the activation of HSCs. (A)** Cocultured HSCs with
217 BMDMs in an LPS-induced inflammatory environment; **(B)** α-SMA expression was
218 analyzed by WB; **(C)** α-SMA expression was analyzed by immunofluorescence staining.
219 At least three independent experiments.

220

221 **Supplemental figure 6. Generation and identification of myeloid-specific knockout**
222 **mice. (A)** Generation flow chart of myeloid-specific FSTL1 deficient mice; **(B)**
223 Identification of myeloid-specific FSTL1 deficient mice.

224

225 **Supplemental figure 7. Purity and viability of isolated macrophage were deducted.**
226 **(A)** Purity of isolated macrophage by the flowcytometry (F4/80 antibody); **(B)** Viability
227 of isolated macrophage was examined after 0 or 24 h using a Countess II FL Automated
228 Cell Counter with trypan blue exclusion.

229

230 **Supplemental figure 8. All semi-quantitative analysis for the immunofluorescence**
231 **staining in this study.**