Supplemental Information

Targeting Lactate Transporter MCT1 Modulates Macrophage Metabolism in Pulmonary Fibrosis

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Methods

Cell culture

Human monocytic (THP-1) cell lines and human fibroblast (IMR-90) cell lines were obtained from ATCC and maintained in culture at 37˚C and 5% CO_2 and grown in RPMI-1640 medium (Gibco, 11875119) with the following supplements: 10% fetal bovine serum, and penicillin/streptomycin. Primary human lung fibroblasts are isolated from lung explants of patients with idiopathic pulmonary fibrosis (IPF) undergoing transplant or failed (normal) donors and stored at UAB Lung Health Center Pulmonary Biospecimen Repository. Specimens were de-identified. Primary human lung fibroblasts were cultured in EMEM media supplemented with 10% FBS. All experiments were performed with 0.5% serum supplement. In lactate-supplemented media, neutral pH was adjusted to 7.4 by adding NaOH, using a VWR sympHony pH meter (SB70P). Experiments were done with RMPI 1640 media (Gibco, 11835), which contains ~25 mM (2 gm/L) sodium bicarbonate. No additional sodium bicarbonate or HEPES were added. The cells were cultured in 5% CO_2 incubator. Osmolality of the lactate-containing media (12.5 mM) was measured by an osmometer (Advanced Osmometer, model 3250, Advanced Instruments, Norwoord, MA) according to manufacturer's protocol. The osmolality of the 12.5 mM lactate-containing media is 303 ± 3 mOsm/kg, in comparison to the RPMI media (265 - 300 mOsm/kg).

Isolation of primary human lung fibroblasts, mouse alveolar epithelial type II cells and mouse bone marrow-derived macrophages

Primary human lung fibroblasts were isolated as previously described (61). Mouse alveolar epithelial type II cells were isolated as previously described (62). Mouse bone marrow-derived macrophages were isolated as previously described (63).

Lactate

Lactate was measured in conditioned media and BAL fluid using the Lactate Colorimetric/Fluorometric Assay Kit (BioVision) according to the manufacturer's protocol.

Conditioned Medium (CM)

Primary human normal and IPF fibroblasts were cultured in DMEM medium without serum overnight. Fractionation of the Fb-CM was achieved using Amicon Ultra centrifugal filters (3K Ultracel, Millipore-Sigma, Burlington, MA) following centrifugation at 4,000 rpm for 30 min. The < 3kDa fraction was collected and used for experiments. No differences in conditioned media pH were observed; therefore, no pH adjustment was performed.

RNA extraction and quantitative real-time PCR analysis

Total RNA was obtained using Trizol reagent (MilliporeSigma). After we performed reverse transcription using iScript reverse transcription kit (Bio-Rad Laboratories), specific gene mRNA expression was determined by quantitative real-time PCR using the SYBR Green kit (Bio-Rad Laboratories). Data were calculated by the $^{\Delta\Delta}$CT method. The mRNA measurements were normalized to ACTB or HPRT and expressed in arbitrary units.

Hydroxyproline determination

Lung tissue was dried and digested for 24 hours at 112°C with 6N hydrochloric acid. Hydroxyproline concentration was determined as previously described (64).

Indirect immunofluorescence assay

Cells were fixed with 4% formaldehyde at room temperature for 45 minutes, followed by permeabilization for 3 minutes in ice-cold buffer (0.1% sodium citrate and 0.1% Triton X-100 in distilled water). Cells were then blocked at room temperature for 1 hour in PBS with 1% BSA.

Cells were incubated with antibodies, MitoTracker Green (50 nM) (Thermo Fisher Scientific), and DAPI. All images of specific interests were quantitated using ImageJ (NIH).

Immunoblot analysis

Whole-cell lysates were obtained as previously described (65), and separated by SDS-PAGE. Immunoblot analyses were performed with the designated antibodies followed by the appropriate secondary antibodies cross-linked to horseradish peroxidase. The primary antibodies and their concentrations used are: MCT1 (AbClonal A3013, 1:1000), β-actin (Sigma A5441, 1:5000), GAPDH (Millipore MAB374, 1:1000), α-SMA (American Research Products 03-61001, 1:1000), β-tubulin (Santa Cruz sc-58884, 1:1000) and SPC (Millipore AB3786, 1:1000). Densitometry was determined using ImageJ.

Lung tissue digestion

Lung tissue was digested as previously described (19). Briefly, minced tissue was suspended in protease solution (0.25% Collagenase A, 1 U/ ml Dispase II, 2,000 U/ml Dnase I in RPMI). The suspension was incubated at 37 °C for 60 min. Cells were passed through a 70 μm cell strainer, RBCs were lysed, washed, passed through a 40 μm cell strainer, and suspended in FACS buffer containing with 1% FBS.

Flow cytometry

BAL cells were blocked with 1% BSA containing TruStain fcX (anti–mouse CD16/32) antibodies (BioLegend, 101319), followed by staining with antibodies. Antibodies used: Live/dead-eflour506 (65–0866; Invitrogen), rat anti-human CD11b-APC-Cy7 (101226; BioLegend), mouse anti-human HLA-DR-PE-Cy7 (560651; BD Biosciences), mouse anti-human CD206-FITC (551135; BD Biosciences), mouse anti-human CD169-BB700 (745772; BD Biosciences), and mouse anti-human CD163-PE (560933; BD Biosciences). rat anti-mouse CD45-FITC (Biolegend 103108),

Rat anti-mouse CD11b-APC-Cy7 (BioLegend 101226), anti-mouse CD64-PE-Cy7 (BioLegend 139314), rat anti-mouse Ly6G-AF700 (BD 561236), rat anti-mouse Siglec F-APC (BioLegend 155508), rat anti-mouse Ly6C-eflour450 (Invitrogen 48-5932-82), rat anti-mouse CD45-PE (eBiosciences 12-0451-82), rat anti-mouse CD31 (PECAM-1)-FITC (eBiosciences e11-0311-82), rat anti-mouse CD140a (PDGFRA)-PE-Cy7 (eBiosciences 25-1401-82), rat anti-mouse TER-119-FITC (BD 557915), rat anti-mouse Ep-CAM-APC (Biolegend 118214), rat anti-mouse F4/80-PE (Biolegend 123110). Hierarchical gating strategy was used to represent macrophages as lineage negative ($CD31^-$, $Ter119^-$) $F4/80^+LyG6^-$, epithelial cells as $linage^-F4/80^-EpCAM^+$, and fibroblasts as $linage^-F4/80^-EpCAM^-PDGFRA^+$. Hierarchical gating strategy (66) was used to represent the human resident alveolar macrophages as $CD11b^+HLA\text{-}DR^{++}CD206^{++}CD169^+CD163^{++}$ and monocyte-derived macrophages as $CD11b^+HLA\text{-}DR^{++}CD206^{++}CD169^+CD163^+$. Hierarchical gating strategy (30) was used to represent the murine resident alveolar macrophages as $CD45^+CD11b^{+/-}Ly6G^-CD64^+Ly6C^-SiglecF^{hi}$ and monocyte-derived macrophages as $CD45^+CD11b^{+/-}Ly6G^-CD64^+Ly6C^-SiglecF^{lo}$. Data was acquired on ARIA (BD Biosciences) using BD FACS DIVA software (version 8.0.1). Data was analyzed using FlowJo (FlowJo LLC) software (Version 10.5.0).

Albumin concentration

Albumin in BAL fluid was determined using a mouse Albumin ELISA Kit (Immunology Consultants Laboratory) according to the manufacturer's protocol.

shRNA transfection

Human MCT1 shRNA plasmids were purchased from Sigma-Aldrich. Transfection with human MCT1 shRNA and pLKO.1 puro were transfected with X-treme Gene 9 Transfection Reagent, according to the manufacturer's instructions. After 24–48 h, cells were subjected to either

treatment of specific interest or collected for downstream purposes. Two different MCT1 shRNA were tested (TCR Clone ID: 230722 and 38340).

Enzyme-Linked Immunosorbent Assay (ELISA)

Active human TGF-β1 and mouse TGF-β1, CHI3L1, PDGF-BB, IL-10, IL-5, CCL-1, CCL-2, CXCL2, TNF-α, IL-6, IL-1β levels in BAL fluid and cell media were measured by ELISA, according to the manufacturer's instructions (R&D Systems).

Arginase activity assay

Arginase activity was measured by using a QuantiChrom Arginase Assay Kit (BioAssay System), according to manufacturer's instructions.

ATP production

ATP production was quantitated by CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Quantification of mtDNA.

The ratio of mitochondrial to nuclear DNA was assessed using the Human Mitochondrial DNA Monitoring Primer Set Ratio kit (Takara Bio).

Pathway analysis

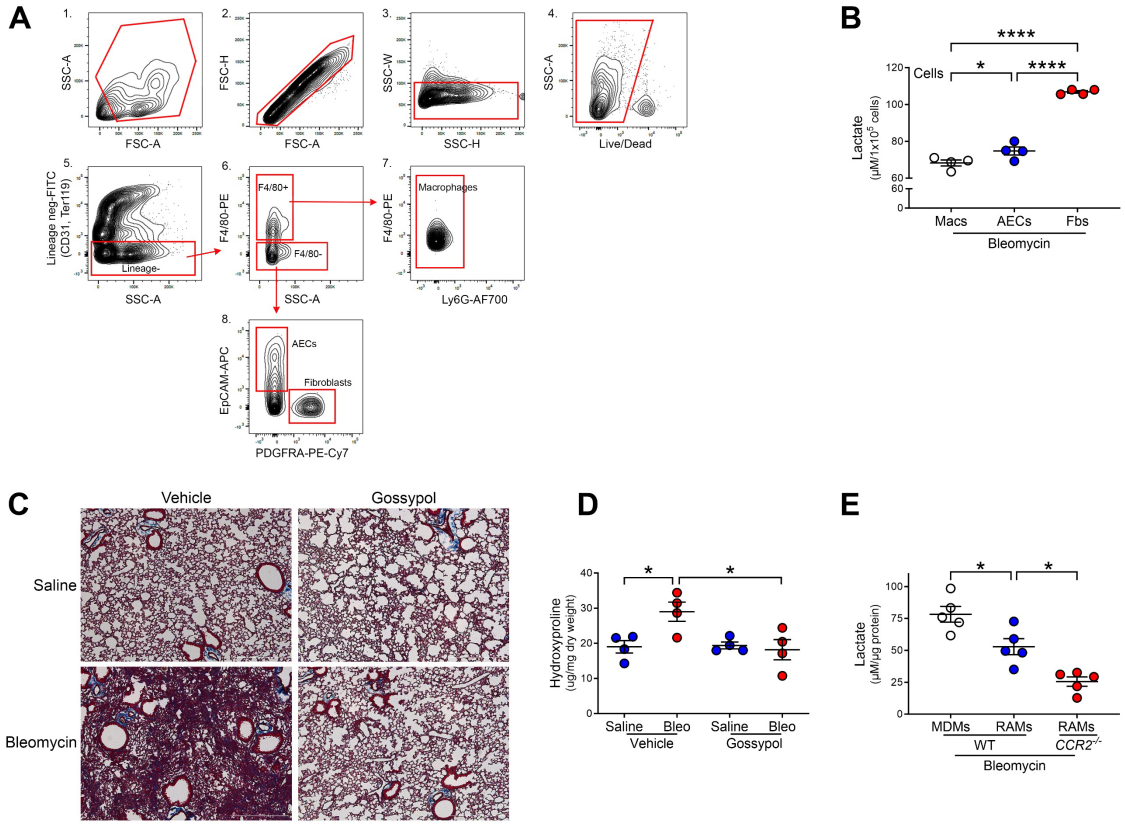
Differentiated expressed genes (DEGs) with absolute log2 fold of change $\geqslant$ 1 and *p*-value <0.05 were identified from RNA-Seq dataset (GSE70867), and subjected to pathway analysis using Ingenuity Pathway Analysis (Qiagen, Hilden, Germany) and DAVID (The Database for Annotation,

Visualization, and Integrated Discovery). Data were visualized using RStudio (2022.12.0+353) and R (version 4.3.1).

Materials availability

Slc16a1$^{fl/fl}$ mice used in this work are provided by Drs. Brett Morrison and Jeffrey Rothstein from the Johns Hopkins University, Baltimore, MD, USA.

Figure S1

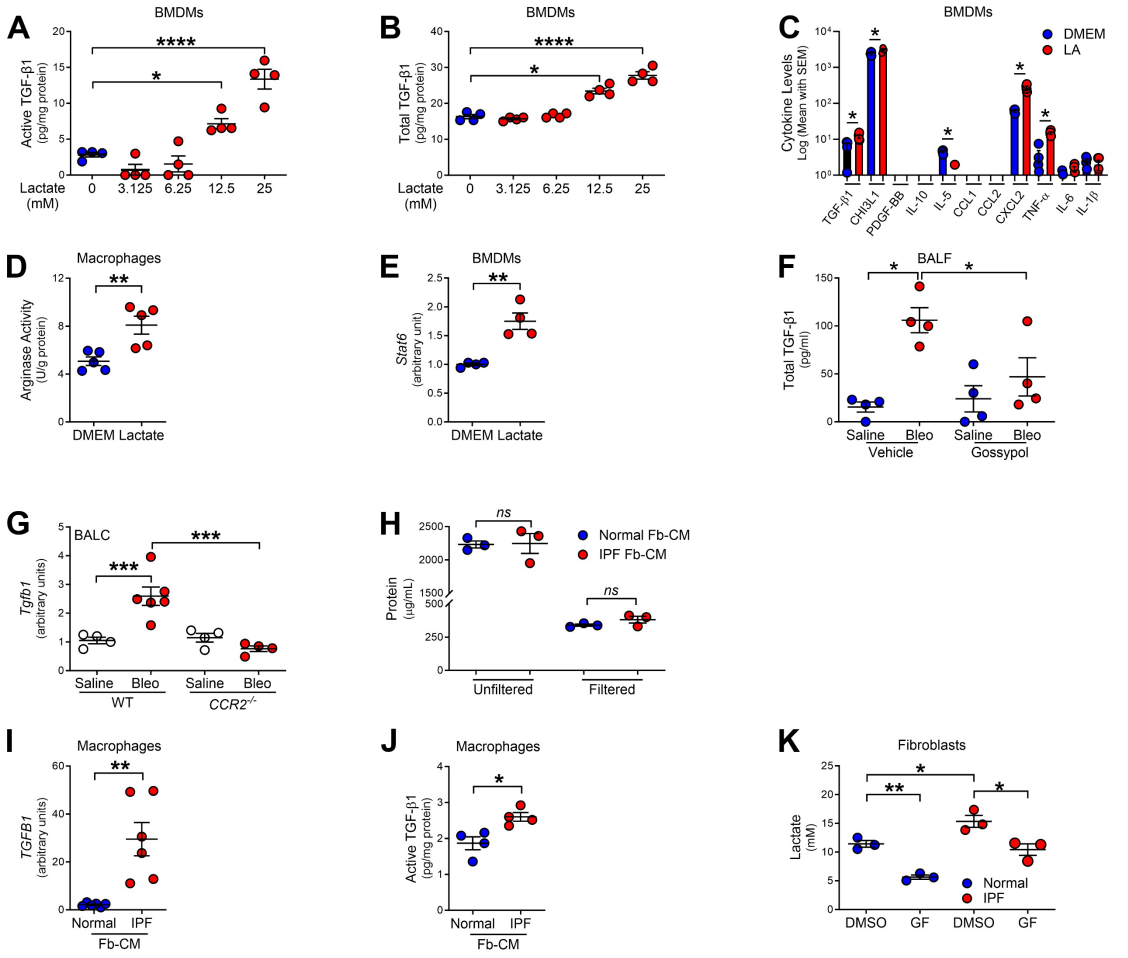


Supplemental Figure 1: Lactate contributes to pulmonary fibrosis.

(A) Gating strategy to isolated macrophages (Macs), alveolar epithelial cells (AECs), and fibroblasts (Fbs) from mouse lungs. **(B)** Intracellular lactate concentrations in isolated macrophages ($Lin^-F4/80^+LyG6^-$), AECs ($Lin^-F4/80^-EpCAM^+$), and fibroblasts ($Lin^-F4/80^-EpCAM^-PDGFRA^+$) from bleomycin-exposed mice harvested on day 21 (*n* = 4). Wildtype mice were exposed to saline or bleomycin on day 0. On day 10, gossypol-acetic acid was injected subcutaneously every day until day 21. On day 21, mice were euthanized. Lung tissues were harvested. **(C)** Representative histology of lung sections and **(D)** hydroxyproline analysis of homogenized lungs (*n* = 4). Bar indicates 400 µm. **(E)** Wildtype and *Ccr2*$^{-/-}$ mice were exposed to bleomycin and BAL was performed on day 21. RAMs ($CD45^+CD11b^{+/-}Ly6G^-CD64^+Ly6C^-Siglec\ F^{hi}$) and MDMs ($CD45^+CD11b^{+/-}Ly6G^-$

$CD64^{+}Ly6C^{-}$Siglec F^{low}) were sorted by flow cytometry, and intracellular lactate concentration was measured. $n = 5$. *, $p < 0.05$, ****, $p < 0.0001$. Values shown as mean ± S.E.M. Two-tailed *t*-test or one-way ANOVA followed by Tukey's multiple comparison test was utilized. Each dot represents one sample. All in vitro experiments were repeated independently thrice.

Figure S2

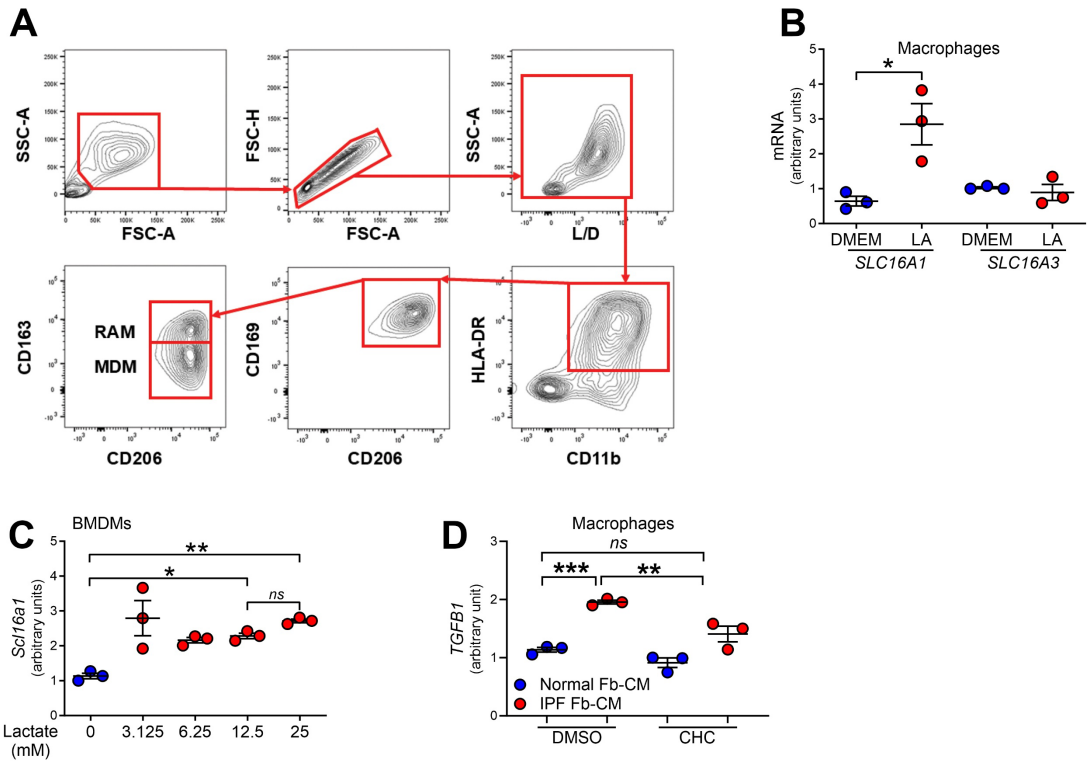


Supplemental Figure 2: Fibroblast-derived lactate induces macrophage profibrotic polarization.

Bone marrow-derived macrophages (BMDMs) cells were treated with DMEM or various concentrations of lactate. **(A)** Active and **(B)** total TGF-β1 production was measured by ELISA. *n* = 4. **(C)** Various cytokines (CHI3L1, PDGF-BB, IL-10, IL-5, CCL-1, CCL-2, CXCL2, TNF-α, IL-6, IL-1β) levels were measured by ELISA. *n* = 4. THP-1 cells were treated with DMEM or lactate (12.5 mM). (**D**) Arginase activity was measured. *n* = 5. **(E)** BMDMs were cultured in the presence or absence of lactate and total RNA was isolated. *Stat6* expression was measured by RT-PCR. *n* = 4. **(F)** Wildtype mice were exposed to saline or bleomycin on day 0. On day 10, gossypol-acetic acid was injected

subcutaneously every day until day 21. On day 21, mice were euthanized and BAL was performed. Total TGF-β1 in BAL fluid was measured by ELISA. *n* = 4. **(G)** Wildtype and *Ccr2*$^{-/-}$ mice were exposed to saline or bleomycin and BAL was performed on day 21. *Tgfb1* mRNA in BAL cells was measured by RT-PCR. *n* = 4-6. **(H)** Protein concentrations in conditioned media from primary normal and IPF human fibroblasts pre- and post-filtration. *n* = 3. **(I)** *TGFB1* mRNA expression and **(J)** active TGF-β1 production from THP-1 cells cultured in the conditioned media from primary human normal and IPF fibroblasts. *n* = 4-6. **(K)** Lactate levels in conditioned media from normal and IPF fibroblasts cultured in the presence or absence of galloflavin. *n* = 3. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$. Values shown as mean ± S.E.M. Two-tailed *t*-test or one-way ANOVA followed by Tukey's multiple comparison test was utilized. Each dot represents one sample. All in vitro experiments were repeated independently thrice. GF: galloflavin; LA: lactate.

Figure S3

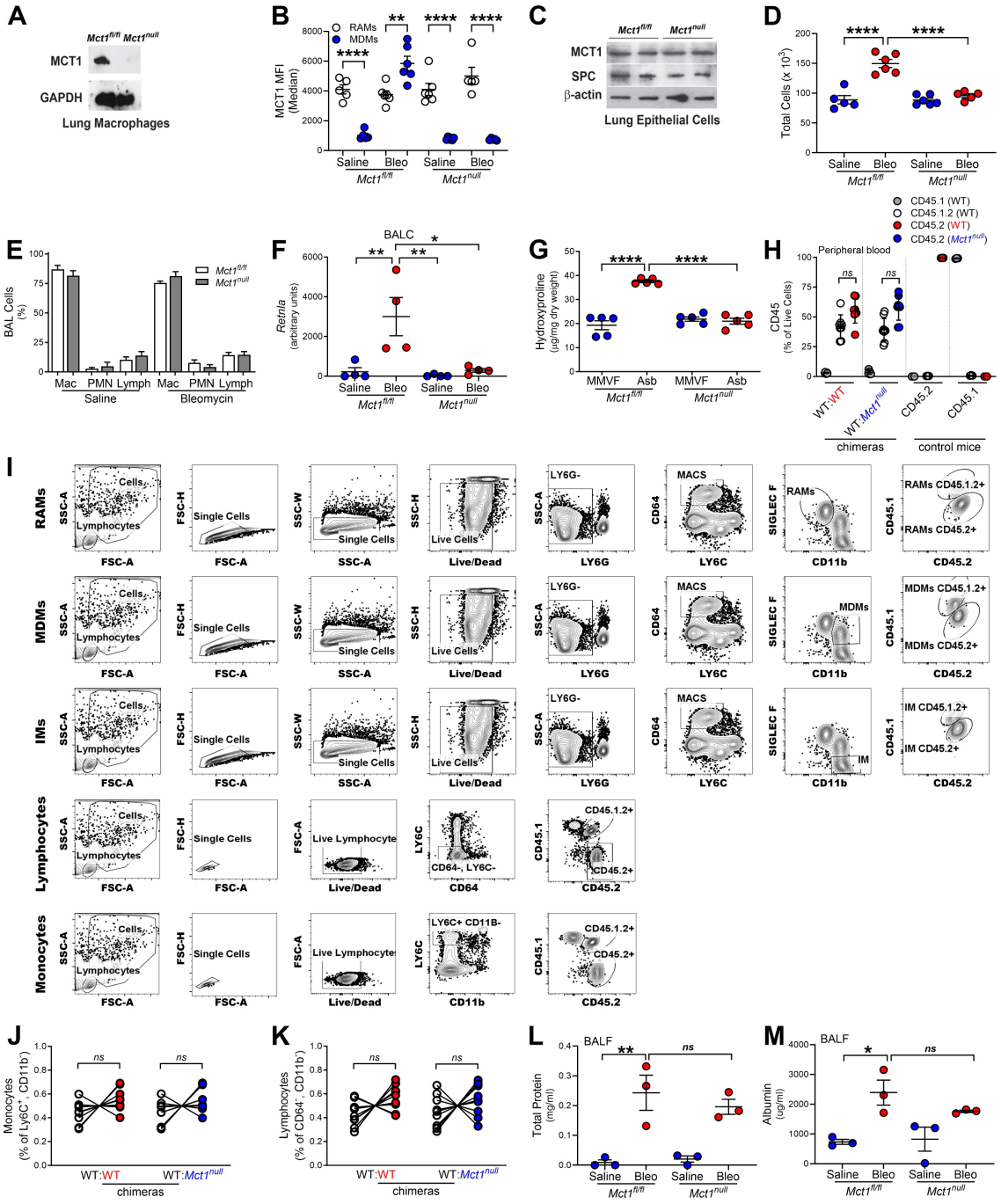


Supplemental Figure 3: MCT1 is increased in profibrotic macrophages.

(A) Gating strategy to separate monocytes-derived macrophages (MDMs, $CD11b^{+}HLADR^{++}CD206^{++}CD169^{+}CD163^{low}$) from resident alveolar macrophages (RAMs, $CD11b^{+}HLADR^{++}CD206^{++}CD169^{+}CD163^{high}$) in BAL. **(B)** THP-1 cells were treated with DMEM or lactate (12.5 mM). *SLC16A1* and *SLC16A3* expressions were measured by RT-PCR. $n = 3$. **(C)** BMDMs were treated with various concentrations of lactate, and *Slc16a1* mRNA expression was measured by RT-PCR. $n = 3$. **(D)** THP-1 cells were cultured in conditioned media from normal and IPF fibroblasts in the presence or absence of CHC (5 mM). *TGFB1* expression was measured by RT-PCR. $n = 3$. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. Values shown as mean ± S.E.M. Two-tailed t-test or one-way ANOVA followed by Tukey's multiple comparison test was utilized. Each dot represents one sample. All in vitro experiments were repeated independently thrice. CHC: α-cyano-4-

hydroxycinnamate.

Figure S4

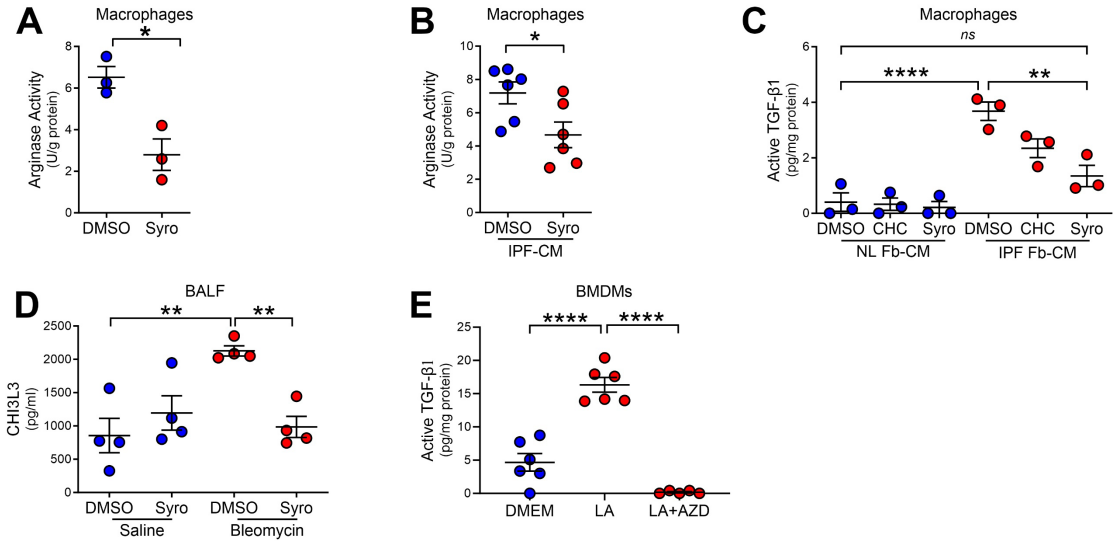


Supplemental Figure 4: Mice harboring a macrophage-specific deletion of MCT1 *Mct1*^{null} (*Slc16a1*$^{-/-}$*Lyz2-Cre*) mice have similar acute inflammation, but attenuated monocyte recruitment compared with *Mct1*$^{fl/fl}$ mice.

(A) BAL was performed in *Mct1*^{null} and *Mct1*$^{fl/fl}$ mice and BAL cells were isolated.

Immunoblot for MCT1 was performed and the representative blot is shown. **(B)** *Mct1*^{null} and *Mct1*$^{fl/fl}$ mice were exposed to saline or bleomycin. After 21 days, BAL was performed and MCT1 expression was determined by flow cytometry in RAMs and MDMs from *Mct1*^{null} and *Mct1*$^{fl/fl}$ mice. *n* = 5-6. **(C)** Epithelial cells were isolated from *Mct1*^{null} and *Mct1*$^{fl/fl}$ mice. Immunoblot for MCT1 and SPC was performed and the representative blot is shown. **(D)** *Mct1*^{null} and *Mct1*$^{fl/fl}$ mice were exposed to saline or bleomycin. After 21 days, BAL was performed and total BAL cell numbers were determined by flow cytometry. **(E)** Cell differential to identify macrophages (Mac), polymorphonuclear cells (PMN), and lymphocytes (Lymph) in BAL from *Mct1*^{null} and *Mct1*$^{fl/fl}$ mice. *n* = 4. **(F)** *Retnla* mRNA expression in BAL cells (*n* = 4) from *Mct1*$^{fl/fl}$ and *Mct1*^{null} mice exposed to either saline or bleomycin. **(G)** Hydroxyproline analysis of homogenized lungs (*n* = 5) from *Mct1*$^{fl/fl}$ and *Mct1*^{null} mice exposed to either MMVF or asbestos. **(H)** Frequency of CD45.1.CD45.2 and CD45.2 (WT:WT chimeras) or CD45.1.CD45.2 and *Mct1*^{null}.CD45.2 (WT:*Mct1*^{null} chimeras) in peripheral blood isolated 8 weeks after reconstitution. CD45.1 and CD45.2 mice are shown as controls. *n* = 8-9. **(I)** Representative gating strategy for RAMs, MDMs, IMs, lymphocytes, and monocytes. Final plots in each strategy show the frequencies of CD45.1 single positive recipient cells, CD45.1.CD45.2 double positive donor cells and CD45.2 single positive donor cells. Digested lung tissue from bleomycin exposed mice were analyzed by flow cytometry to determine the proportions of **(J)** monocytes (*n* = 10), and **(K)** lymphocytes (*n* = 10) derived from each donor. *Mct1*^{null} and *Mct1*$^{fl/fl}$ mice were exposed to saline or bleomycin. After 3 days, BAL was performed. **(L)** Total protein and **(M)** albumin concentrations in BAL fluid were measured. *n* = 3. *, $p < 0.05$; **, $p < 0.01$, ****, $p < 0.0001$. Values shown as mean ± S.E.M. Two-tailed *t*-test or one-way ANOVA followed by Tukey's multiple comparison test was utilized. Each dot represents one human subject, one animal or one sample. All in vitro experiments including western blotting were repeated independently thrice and representative blots are shown.

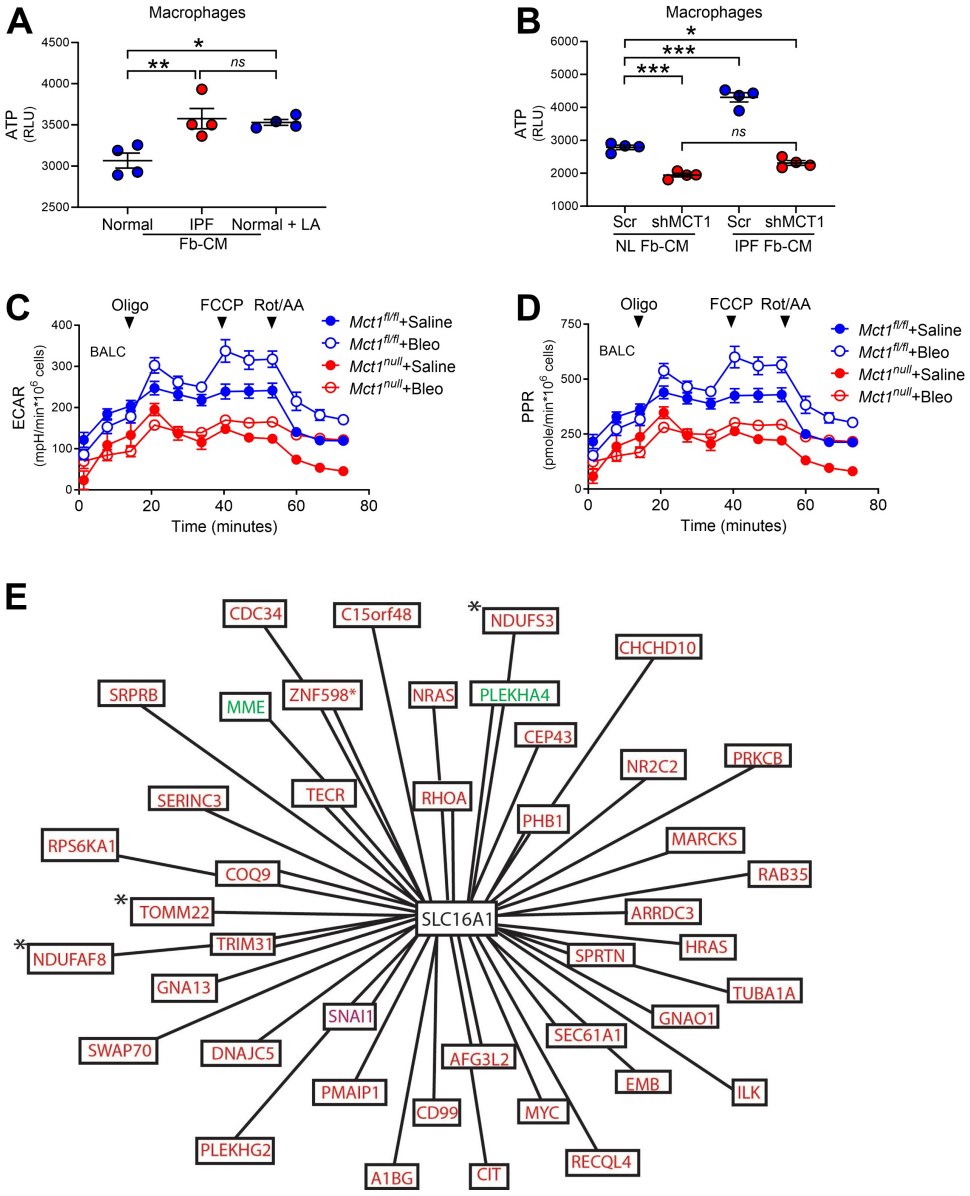
Figure S5



Supplemental Figure 5: Syrosingopine attenuates macrophage profibrotic polarization and protects mice from developing pulmonary fibrosis.

(A) THP-1 cells were treated with DMSO or syrosingopine (50 μM). Arginase activity was measured. *n* = 3. **(B)** THP-1 cells were cultured in conditioned media from IPF fibroblasts in the presence or absence of syrosingopine (50 μM). Arginase activity was measured. *n* = 6. **(C)** THP-1 cells were cultured in conditioned media from normal or IPF fibroblasts in the presence or absence of CHC or syrosingopine. Active TGF-β1 production was measured by ELISA. *n* = 3. **(D)** Wildtype mice were exposed to either saline or bleomycin on day 0. On day 10, mice were implanted with mini- osmotic pumps with either vehicle or syrosingopine (equal to 7.5 mg/kg q.o.d. i.p.). CHI3L3 level in BAL fluid was measured by ELISA. *n* = 4. **(E)** Bone marrow-derived macrophages were treated with AZD3965 (100 nM) in the presence of lactate. Active TGF-β1 production was measured by ELISA. *n* = 6. *, $p < 0.05$; **, $p < 0.01$, ****, $p < 0.0001$. Values shown as mean ± S.E.M. One-way ANOVA followed by Tukey's multiple comparison test was utilized. Each dot represents one animal or one sample.

Figure S6



Supplemental Figure 6. MCT1 regulates macrophage metabolism.

(A) THP-1 cells cultured in the conditioned media from normal or IPF fibroblasts in the presence or absence of lactate. ATP production was measured. *n* = 4. **(B)** THP-1 cells were transfected with either a scrambled shRNA or an *MCT1* shRNA and then cultured in the conditioned media from normal or IPF fibroblasts. ATP production was measured. *Mct1*$^{fl/fl}$ and *Mct1*^{null} mice exposed to either saline or bleomycin on day 0. *n* = 4. On day 21,

mice were euthanized, and BAL was performed. BAL cells were isolated and mitochondrial respiration was measured. **(C)** ECAR and **(D)** PPR were calculated. $n = 5$. **(E)** Network of the genes regulated by *SLC16A1* using Ingenuity Pathways Analysis. Genes of interest were marked with asterisks. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$. Values shown as mean ± S.E.M. One-way ANOVA followed by Tukey's multiple comparison test was utilized. Each dot represents one animal.