-Supplemental Data –

Sphingosine-1-phosphate lyase is an endogenous suppressor of pulmonary fibrosis: Role of S1P signaling and autophagy

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Running Title: S1P Lyase in Pulmonary Fibrosis

MATERIALS AND METHODS

Reagents and kits

Bleomycin sulfate was from Hospira Inc. (Lakeforest, IL), and the Sircol Collagen Assay Kit was from Accurate Chemical and Scientific Corp. (Westbury, NY). The neutralizing chicken anti-TGF-β1 antibody and control chicken IgG, IL6 and TGF-β1 ELISA kits were obtained from R&D systems (MN, USA). S1P, C17-S1P, a 17-carbon analog of S1P was procured from Avanti Polar Lipids (Alabaster, AL). Lysis buffer was purchased from Cell Signaling Technology Inc. (Danvers, MA). Protease inhibitor cocktail tablets (EDTA-free Complete) and phosphatase inhibitor cocktail were obtained from Roche Diagnostics (Indianapolis, IN). Recombinant human TGF-β1 was purchased from Pcpro Tech Inc (Eocky Hill, NJ). LC3-GFP plasmid construct (#11546) was obtained from Addgene [1]. Chloroquine, Rapamycin, control and human Beclin1 shRNA construct were obtained from Sigma-Aldrich (St. Louis, MO).

Murine model of bleomycin-induced experimental pulmonary fibrosis

129SV wild type mice were purchased from Charles River Laboratory (Wilmington, MA). *Sgpl1*$^{+/-}$ mice, originally provided by Dr. Philip Soriano (New York, NY). In 129SV background were bred at the University of Illinois, Chicago animal facility. For bleomycin instillation, 8 week old male mice were anesthetized with 3–5 ml/kg of a mixture of 25 mg/kg of ketamine and 2.5 ml of xylazine. The animals were challenged with either saline or bleomycin sulfate (2 U/kg of body weight in saline) by intratracheal (IT) injection in a total volume of 50 µl/animal. Animals were sacrificed on 0, 3, 7, 14 or 21 days after bleomycin challenge, BAL fluid was collected by IT injection of 0.5 ml of sterile PBS solution followed by gentle aspiration. The lavage was repeated twice with 0.5 ml of sterile PBS to recover a total volume of 0.7-0.8 ml. BAL fluid was centrifuged at 3000 X g for 10 min and the supernatant was used for total protein and cytokine measurements. Lungs were removed from mice and lobes were sectioned, embedded in paraffin, and cut into 5-µm sections. Hematoxylin and eosin (H&E) and trichrome staining were performed by the Pathology Core Facility (University of Illinois, Chicago). The studies reported here conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in

biomedical research. All animal protocols were approved by the IACUC of the University of Illinois, Chicago.

Histolopathological analysis for fibrosis

The fixed lungs were sectioned, embedded in paraffin, cut and stained with H&E for analysis of lung injury, and with Elastica-Masson trichrome stain to check for collagen deposition, an index of lung fibrosis. For the analysis of the fibrotic changes in lung tissue, the quantitative fibrotic scale (Ashcroft scale) was calculated as described previously [2-4] . Briefly, the severity of the fibrotic changes in each lung section was given a mean score from the observed microscopic fields. More than 20 fields within each lung section were observed at a magnification of 100 X, and each field was assessed individually for severity and allotted a score from 0 (normal) to 8 (total fibrosis) [5-6]. The severity was then averaged for each lung section. To avoid bias, all histologic specimens were evaluated in a blinded fashion. Each specimen was scored independently by two individuals, including a histopathologist, and the mean of their individual scores was taken as the fibrotic score.

Lipid extraction and sample preparation for LC/MS/MS

Cellular lipids were extracted by a modified Bligh and Dyer procedure with the use of 0.1N HCl for phase separation as described before [6]. C17-S1P (40 pmol) was employed as an internal standard, and was added during the initial step of lipid extraction. The extracted lipids were dissolved in methanol/chloroform (4:1, v/v), and aliquots were taken to determine the total lipid phosphorus as described previously [7]. Samples were concentrated under a stream of nitrogen, re-dissolved in methanol, transferred to auto sampler vials, and subjected to electrospray ionization tandem mass spectrometry (ESI-LC/MS/MS). The instrumentation employed was an AB Sciex 5500 QTRAP hybrid triple quadrupole linear ion-trap mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a turboionspray ionization source interfaced with an automated Agilent 1200 series liquid chromatograph and auto sampler (Agilent Technologies, Wilmington, DE, USA). S1P and dihydro (DH)-S1P were analyzed as bis-

acetylated derivatives employing reverse-phase HPLC separation, negative ion ESI, and MRM analysis [8].

Targeted Mutation of Smad3 binding sites in *SGPL1* luciferase reporter

Plasmid construct of *SGPL1* promoter luciferase reporter was purchased from GeneCopoeia, Inc (Rockville, MD). Mutation of Smad3 binding sites in *SGPL1* luciferase reporter was generated as described before [9-10], by using the site-directed mutagenesis kit (QuikChange II, Agilent Technologies, Santa Clara, CA). Mutations of Smad3 binding site in the *SGPL1* promoter area were carried out with following sequences: Mutation -143, Forward: 5'-TGGTTGAGACATTCACTGGAGGGGCGGGGTGCAGCCCGCTGCCT-3', Reverse: 5'-AGGCAGCGGGCTGCACCCTATGCCTCCAGTGAATGTCTCAACCA-3'; mutation -75, Forward: 5'-ACAGGCCGTGGGGCCCGGGGCGGGGCGTGCGCGCGGCTGG-3', Reverse: 5'-CCAGCCGCGCGCACGCCCTATGCCGGGCCCCACGGCCTGT-3'. After transfection and treatment, the luciferase reporter activity was assayed by using dual-luciferase report assay kit from Promega inc. (Madison, WI).

Infection with S1PL wild type adenoviral constructs

Infection of human lung fibroblasts with purified *SGPL1* wild type adenoviral vector was carried out as described previously [11-12]. Briefly, vector-control or *SGPL1* wild type adenoviral constructs (5 MOI) were added to primary human lung fibroblasts (~90% confluence) in complete medium containing 10% fetal bovine serum. After overnight infection, the virus-containing medium was replaced with fresh complete medium for another 24 h prior to stimulation with TGF-β (0-5 ng/ml) or S1P (0-1 μM).

Microarray profiling and analysis

The Affymetrix Human Exon 1.0 ST array (Affymetrix, Inc., Santa Clara, California) (exon array) was used to profile whole-genome expression in a cohort of 44 IPF patients. Briefly, the sample preparation, and RNA isolation were based on standard molecular biology protocols. The labeling, microarray hybridization was carried out at the University of Chicago Genomics Core Facility according to the manufacturer's

instruction. The exon array data were then normalized and summarized using the Affymetrix Power Tools. Before summarizing gene-level expression data, probes containing known polymorphisms (based on dbSNP v131) were removed as described earlier [13]. The $\log_2$ transformed gene-level (i.e., transcript clusters) expression data were then evaluated for differential expression using SAM (Significance Analysis of Microarray) [14]. Gene annotations were obtained from the Affymetrix NetAffy Analysis Center (http://www.affymetrix.com/). The microarray data have been deposited in the NCBI Gene Expression Omnibus (Accession Number: GSE38958) [15].

RNA isolation and real-time RT-PCR

Total RNA was isolated from mouse lung tissue or from cells using TRIzol reagent (Life Technology, Rockville, MD) according to the manufacturer's instructions. RNA was quantified spectrophotometrically and 1 µg of RNA was reverse transcribed using cDNA synthesis kit (Bio-Rad laboratories Inc., Hercules, CA). Real-time PCR was performed to assess expression of target genes using primers as shown in **Supplemental Table 2**. GAPDH was used as an internal control. Amplification reactions were performed in triplicate with SYBR Premix Ex Taq (Bio-Rad Laboratories, Inc., Hercules, CA), and the thermal cycling conditions were as follows: 10 seconds at 95°C, 40 cycles of 5 seconds at 95°C, and 30 seconds at 60°C [2].

Collagen content determination

The right lungs from mice were collected and homogenized in 5 ml 0.5 M acetic acid in PBS containing 0.6% pepsin. The extracts were rotated at 4°C overnight and cleared by centrifugation at 10,000 x g for 15 min. Collagen content was measured using the Sircol Collagen Assay kit according to the manufacturer's instructions. Collagen content is presented as µg acid-soluble collagen/right lungs [3].

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed as described previously [16]. Rabbit anti–p-Smad2, Smad-2, p-Smad3, Smad3, JNK, P-p38 and p38 antibodies, and mouse anti-P-Akt, Akt and P-JNK antibodies were from Cell Signaling Technology (Beverly,

MA). Horseradish peroxidase linked anti-mouse IgG and anti-rabbit IgG antibodies were from Bio-Rad Laboratories Inc. (Hercules, CA). Rabbit anti-S1PL, anti-LC3, anti-Beclin1, anti-FN, anti-GAPDH and anti-S1PL antibodies were from Santa Cruz Biotechnology Inc. Mouse anti–α-SMA and actin was from Sigma-Aldrich (St. Louis, MO).

Immunofluorescence Microscopy

Immunofluorescence microscopy to determine the expression of LC3 proteins was performed as described elsewhere [17]. Briefly, human lung fibroblasts or mouse lung fibroblasts were grown in slide chambers before infection of control or S1PL Adenovirus (5 MOI). After stimulation with TGF-β1 (0, 5 ng/ml) for 48 hours or treatment of Chloroquine (0, 10 μM) for 3 hours, cells were fixed with 3.7% paraformaldehyde in PBS for 10 min, followed by permeabilization for 4 min in Tris-buffered saline (TBS) containing 0.25% Triton X-100. Cells were then washed with PBS three times, and blocked with TBS Tween 20 (TBST) blocking buffer (containing 2% BSA) for 3 minutes at room temperature. Cells were then incubated with LC3 primary antibody (1:200 dilutions in blocking buffer) for 1 h, followed by three washings (15 min each) in TBST. Cells were then stained with Alexa Fluor secondary antibodies (1:200 dilutions in blocking buffer; Life Technologies, Grand Island, NY) for 1 h, followed by a TBST wash for 15 minutes. Slides were prepared with mounting media, examined under a Nikon Eclipse TE 2000-S fluorescence microscope (Nikon, Tokyo, Japan), and the images were recorded with a Hamamatsu digital camera (Tokyo, Japan), using a X60 oil immersion objective lens.

Transfection of plasmid constructs.

Lung fibroblast were cultured onto six-well plates or glass bottom dishes. At ~60% confluence, cells were transiently transfected with LC3-GFP plasmid (3 μg/ml), and control or Beclin1 shRNA using Fugene HD transfection reagents (Premaga, Inc, Madison, WI). Briefly, plasmid (3 μg) was condensed with Fugene HD transfection reagents according to the manufacturer's instructions. The transfection complex was diluted into 900 μl of medium and added directly to the cells. Cells were analyzed by Western blotting or confocal microscopy at 48 h after transfection.

Isolation of primary fibroblasts from murine lungs

Mouse lung fibroblasts were isolated essentially as described [2]. Briefly, mouse lungs from 8 week old mice with or without bleomycin challenge (2 U/kg, 0, 3, 7 and 14 days) were cut into small pieces, minced and subjected to collagenase type III and DNase I digestion (Worthington Biochemical, Lakewood, NJ) in Dulbecco's modified Eagle's medium with 5% FCS for 90 minutes. After filtration, cells were centrifuged, washed and cultured in T-25 flasks in DMEM medium containing 10% fetal bovine serum (FBS) for 14 days. Fibroblasts were characterized by expression of Thymocyte differentiation antigen 1 (Thy-1).

Cell culture

Primary human lung fibroblasts were obtained from Lonza (Walkersville, MD, USA). Cells were grown and maintained in 6-well dishes with FGM medium (Lonza, Walkersville, MD, USA) containing 2% FBS. Murine primary lung fibroblasts were cultured in DMEM medium containing 10% FBS. Cells (~80% confluent) were serum-starved for 24 h prior to stimulation with S1P or TGF-β1 for the indicated time. In some experiments, TGF-β neutralizing antibody was added to cover the cells 1h before stimulation with S1P or TGF-β.

Statistical analysis

Data are expressed as mean ± SEM. All results were subjected to statistical analysis using one-way ANOVA or two-tailed Student's *t* test or Spearman's correlation test. P-values < 0.05 were considered significant. Values are from three to six independent experiments. Survival analysis was performed using the Cox proportional hazards model, implemented in the *survival* library in the R Statistical Package. A log-rank test p-value <0.05 was deemed significant [18-19].

Supplemental Table 1. Characteristics of the IPF subjects.

Variable	Overall
Cases	44
Age (years)	68.7±6.8
Gender (Male/Female)	39/5
FVC % predicted	62.1±15.0
DLCO % predicted	43.2±17.4

Supplemental Table 2. cDNA primer sequences for real-time PCR.

Gene	Primer Sequences	
m SGPL1	Forward	5'-AACTCTGCCTGCTCAGGTA-3'
	Reverse	5'-CTCCTGAGGGCTTTCCCTTCT-3'
m FN	Forward	5'-TCTGGGAAATGGAAAAGGGGAATGG-3'
	Reverse	5'-CACTGAAGCAGGTTTCCTCGGTTGT-3'
m α-SMA	Forward	5'-GACGCTGAAGTATCCGATAGAACACG-3'
	Reverse	5'-CACCATCTCCAGAGTCCAGCACAAT-3'
m TGF-β1	Forward	5'-AGCGGACTACTATGCTAAAGAGGTCACCC-3'
	Reverse	5'-CCAAGGTAACGCCAGGAATTGTTGCTATA-3'
m Col1A1	Forward	5'-GGAGGGCGAGTGCTGTGCTTT-3'
	Reverse	5'-GGGACCAGGAGGACCAGGAAGT-3'
m GAPDH	Forward	5'-CGACTTCAACAGCAACTCCCACTCTTCC-3'
	Reverse	5'-TGGGTGGTCCAGGGTTTCTTACTCCTT-3'
h LC3	Forward	5'-GATGTCCGACTTATTCGAGAGC-3'
	Reverse	5'-TTGAGCTGTAAGCGCCTTCTA-3'
h beclin1	Forward	5'-CTGGTAGAAGATAAAACCCGGTG-3'
	Reverse	5'-AGGTAGAGCGTGGACTATCCG-3'
h FN	Forward	5'-GTGTTGGGAATGGTCGTGGGGAATG-3'
	Reverse	5'-CCAATGCCACGGCCATAGCAGTAGC-3'
h α-SMA	Forward	5'-CATCACCAACTGGGACGACATGGAA-3'
	Reverse	5'-GCATAGCCCTCATAGATGGGGACATTG-3'
h GAPDH	Forward	5'-GCTGGCGCTGAGTACGTCGTGGAGT-3'
	Reverse	5'-CACAGTCTTCTGGGTGGCAGTGATGG-3'
h Smad3	Forward	5'-TGGACGCAGGTTCTCCAAAC-3'
	Reverse	5'-CCGGCTCGCAGTAGGTAAC-3'
h SGPL1	Forward	5'-GATAGAGGCAGAAATTGTGAGGATA-3'
	Reverse	5'-GTATTTTGTATTTGACAGCCAGCTT-3'

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