Supplemental Figure Legend

Supplemental Figure 1: The number of mast cells is increased in IPF lung.

(A) Tryptase and chymase positive mast cells were detected by immunofluorescence. Green is tryptase, Red is chymase and blue is Dapi. Tryptase and chymase double positive cells are indicated by white arrow. The lung tissues are from non-fibrotic control lung and IPF (FVC<60%) lung. Representative pictures are shown for each group with each filter and merged. (B) TGF-β1 positive cells were detected in chymase positive mast cells by immunofluorescence. Green is chymase, Red is TGF-β1 and blue is Dapi. Chymase and TGF-β1double positive cells are indicated by white arrow. The lung tissues are from non-fibrotic control lung and IPF (FVC<60%) lung. Representative pictures are shown for each group with each filter and merged. (C) TGF-β1 positive cells were detected in tryptase positive mast cells by immunofluorescence. Green is tryptase, Red is TGF-β1 and blue is Dapi. Tryptase and TGF-β1 double positive cells are indicated by white arrow. The lung tissues are from non-fibrotic control lung and IPF (FVC<60%) lung. Representative pictures are shown for each group with each filter and merged.

Supplemental Materials and Methods

Animals

Female Sprague-Dawley rats (225–250 g; Charles River, Wilmington, MA, USA) were maintained in 12-hour light, 12-hour dark cycles with free access to food and water. Rats were subjected to bronchoalveolar lavage fluid (BALF) and lung tissues were harvested. Frozen right lung lobes were crushed in liquid nitrogen and used for molecular biology analyses. Left lung lobes were formalin-fixed and paraffin-embedded and used for histology. C57/B6 mice (8-10 weeks) were used for bone marrow derived mast cell extraction. All work was conducted under guidelines from the Canadian Council on Animal Care and was approved by the Animal Research Ethics Board of McMaster University under protocol #13.12.48. In addition, we have conducted all of our animal works follows the ERS Task Force Report[1].

TGF-β1 Adenovirus vector-induced pulmonary fibrosis

TGF-β1 adenovirus (Ad-TGF-β1223/225) or control virus (Ad-DL) was prepared and treated to rats as previously described[2]. This virus expresses active TGF-β1 in the lung over a period of 7-14 days and produces extensive and progressive fibrosis in rats[3]. Rats received 5.0×10^8 plaque-forming units (PFU) virus in 300 μl sterile saline intratracheally and were culled on days 7, 14, 21 or 35 by terminal anesthesia.

Bleomycin-induced pulmonary fibrosis

Rats received saline or bleomycin intratracheally (0.56U in 300 μl of saline). Rats were culled on days 7, 14, or 21 by terminal anesthesia.

Human plasma and lung tissue

All plasma and tissue were collected with patient consent in compliance with the Research Ethics Board of St Joseph's Healthcare Hamilton. Hamilton Integrated Research Ethics Board (HIREB #00-1839) approval was obtained prior to beginning the study. Peripheral blood was collected from healthy volunteers or IPF patients, and plasma was aliquoted and stored at −80°C. Control lung tissue was collected from patients undergoing surgery for cancer. Lung fibrosis tissue was collected from patients undergoing biopsy for the diagnosis of unclear interstitial lung disease. The biopsies analysed in this study revealed a usual interstitial pneumonia (UIP) pattern on histopathology. Non-fibrotic human lungs were used as controls. Following biopsy, all tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin.

Histology and Immunohistochemistry

Paraffin sections were prepared at 4 μm and processed in the core histology facility at McMaster. Subsequent staining was performed with haematoxylin and eosin (H&E), Masson Trichrome, PSR, and Toluidine Blue. Images from stained slides were captured on Olympus

microscope (Olympus BX41, Olympus, ON, Canada) equipped with DP Controller software (Olympus, ON, Canada). PSR pictures were take using

polarized detection. Immunohistochemical staining was performed at McMaster Histology department to characterize the localization and expression of α-SMA (Dako, Mississauga, ON, Canada) for the quantitative assessment of scarring and myofibroblast accumulation. Images from α-SMA-stained slides were captured by an automatic slide scanner microscope (Olympus VS 120-L). This automatic slide scanner can digitalize whole slides at 20X magnifications

Immunofluorescence

Immunofluorescence staining of tryptase (Abcam, ON, Canada), chymase (human: Abcam, ON, Canada, rat: Santa Cruz Biotechnology, ON, Canada), and TGF-β1 (Abcam, ON, Canada) was performed on formalin-fixed rat or human lung tissue sections. In brief, following deparaffinization and antigen retrieval process with a citric acid buffer, and saturation of non-specific sites with BSA (5%, 30 min), sections were incubated with primary antibodies overnight in a humidified chamber at 4°C. Conjugated secondary antibodies were used at a dilution of 1:2000 (Abcam, ON, Canada). Slides were mounted in ProLong-Gold with DAPI (ProLong® Gold antifade regent with DAPI; Thermo Fisher Scientific, ON, Canada). Pictures were taken using an epifluorescence microscope

(Olympus IX81: Olympus, ON, Canada) with the same setting and exposure time for all pictures.

ELISA

The levels of chymase and tryptase in human plasma were measured using tryptase ELISA kit (My Biosourse, San Diego, CA) and chymase ELISA kit (My Biosourse, San Diego, CA) according to the manufacturer's recommendations. Active and total TGF-β1 levels in cell culture from decelluralization experiments were detected by mouse/rat/porcine/canine TGF-β1-specific ELISA kit (R&D Systems, Minneapolis, MN). Bath solution from mechanical stretch study histamine level was measured by histamine ELISA kit (Cayman Chemical, Ann Arbor, MI).

Western blotting

Protein from crushed lung strips was extracted with RIPA buffer using a mechanical homogenizer (Omni International, Waterbury, CT), and proceeded Western blotting as described previously[3]. Western blotting assays were used to detect pSmad2 and Smad2 (Cell Signalings Technology, Danvers, MA).

Isolation of mRNA and gene expression

Total RNA was extracted from frozen rat lung tissue with TRIzol® reagent (Invitrogen). Two micrograms of total RNA was reverse-transcribed using qScript cDNA Super Mix (Quanta Bioscience, Gaithersburg, MD, USA). The cDNA was amplified using a Fast 7500 real-time PCR system (AB Applied Biosystems) using TaqMan® Universal PCR Master Mix and predesigned primer pairs for B2M (Rn00560865_m1), Tryptase (Rn00570928_m1), Chymase (Rn01488537_g1), SCF (Rn01502851_m1), and TGF-β1 (Rn00572010_m1) (Thermo Fisher Scientific, ON, Canada).

Mechanical stretch bath solution model

We modeled the breathing in the tissue bath by setting the resting tissue tension to 15 mN and cyclically stretching, oscillating at a frequency of 2 Hz, to a length of 1.1 times the original resting lung length[4]. Briefly, the mechanical cyclic stimuli were 2 Hz with 0.1x resting length of the tissue, corresponding to the estimated tissue stretch *in vivo* during breathing. Resting tension was set using a manual actuator controlling the stage upon which the servo-controlled arm was mounted. The estimated pressure was $15mN/4mm^2=0.37$ mN/mm^2. 0.37 mN/mm^2 is equal to 37.73 cm/H^2O, which has been proposed to be in accordance with pressures in human lung of IPF patient [5]. Bath solutions before stretch and after stretch were collected and used for ELISA and TGF-β1 Reporter Cell Assay. Non-stretched and stretched lung strip was collected 6 hours after for Western blotting. Lung strips for toluidine blue staining was fixed with 10% formalin immediately after the

mechanical stretch study and submitted to toluidine blue staining.

Inhibitors of mechanical stretch study

To detect the effect of mechanical stretch on mast cell degranulation in this model, we used mast cell stabilizers, cromoglycate and doxantrazole. Tissue slices from AdTGF-β1 treated rat lung (day 21) were pre-incubated with 100uM cromoglycate, 1mM doxantrazole or vehicle 2 hour before, during, and after stretching. The concentration of cromoglycate and doxantrazole was also determined based on previous in vitro studies[6-8]. After stretch and incubation at 37°C for 6 hours without any mechanical stimuli, Western Blot was performed for Smad2/3 phosphorylation. Tissues strips were then frozen in liquid nitrogen and stored at -80°C.

We also used αv integrin small molecule inhibitor (CWHM-000012-8, a kind gift from Dr. David Griggs, Saint Louis University) with 1 mM in fibrotic lung strips (AdTGF-β1). Lung strips were soaking with inhibitors for 2 hour before, during, and 6 hours after the stretch. Tissues strips were then frozen in liquid nitrogen and stored at -80°C.

Rodent lung ventilator model

Rats were treated with adenoviral vectors as above. On day 21 when full fibrotic changes are evident, a half hour prior to sacrifice, the rats were given 1000 U/kg sodium heparin as an intraperitoneal injection and then sacrificed via CO_2 cull. The lungs were intubated and

extracted carefully to avoid puncture and the pulmonary artery (PA) catheterized and applied to lung ventilation model as previously described[4]. Briefly, the lungs were then floated in a tissue bath of PBS held at 37°C and connected to a FlexiVent rodent ventilator (SciReq, QC, Canada). The PA catheter was connected to a Krebs+BSA buffer solution held 30 cm above the lung tissue. The left lung received the "control" ventilation whereas the right lung was given the "challenge" ventilation. Under control ventilation, the inspiratory pressure for control lungs was 5 cmH_2O, and during the expiratory cycle lungs were allowed to expire to an imposed PEEP of 2 cmH_2O. The challenged lungs were ventilated by performing 3 TLC manoeuvres each minute. This involved a 6 second inflation to a pressure of 30 cmH_2O, followed by relaxation against a PEEP of 2 cmH_2O. 30 cmH_2O is a pressure that would be reached by many PF patients at lung volumes well below TLC. Lungs were then cut into 2 mm wide transverse sections and incubated in Krebs+ BSA buffer including protease inhibitors for 6 or 10 hours. Lungs strips were fixed with 10 % formalin and submitted to toluidine blue staining.

Mouse bone marrow derived mast cells

Bone marrow derived mast cells (BMMC) were cultured from male C57BL/6 mice as described previously[9]. Briefly, bone marrow cells were cultured for up to 10 weeks in enriched RPMI-1640 medium in the presence of 50 ng/ml rmSCF plus 10 ng/ml rmIL-3 (R&D Systems, Minneapolis, MN). Non-adherent cells were hemidepleted twice each week

with enriched medium. After 3 weeks, the culture contained >98% of MC, as determined by staining with toluidine blue.

Isolation of rat Peritoneal Rat Mast Cells

Peritoneal mast cells were isolated as described previously[10]. Briefly, 20 mL ice-cold HEPES-Tyrode's buffer (HTB) injected into the rat peritoneal cavity, and the abdomen was massaged for 1 min, opened, and the liquid aspirated into ice-cold polypropylene tubes. Cells were washed by centrifugation (5 min, 150 g, 4°C) and resuspended in 5 ml of HTB. Mast cells were enriched by centrifugation through a discontinuous density gradient of Percoll (>95% purity). Cell viability was >95% as determined by trypan blue exclusion.

TGF-β1 Reporter Cell Assay

The Mink lung epithelial cell assay with the PAI-1/Luciferase construct was used to determine biologically active TGF-β1 levels in bath solution from stretch study. For active TGF-β1 measurement, we used bath solution without heating. For total TGF-β1 measurement, bath solution was heated at 80°C for 5 minutes to activate all latent TGF-β1 in a sample. Recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) was used as a reference standard. TGF-β1 data is show as active/total TGF-β1 ratio (%), and active and total TGF-β1 level (pg/mL) in the bath solution.

β-hexosaminidase Assay

β-hexosaminidase was measured in the stretch bath solution as described previously[11].

Collagen assay

Soluble collagen and insoluble collagen content was measured by Sircol soluble collagen assay kit and Insoluble collagen assay kit according to the manufacturer's instructions (Biocolor Ltd, Carrickfergus, UK).

Decellurarized lung

Decellularization is performed via TritonX (1%), sodium deoxycholate (SDC) (2%) and NaCl (1M) wash sequence, followed by storage in sterile PBS supplemented with Penicillin-Streptomycin (1%). The lungs are washed 5 times with sterile H_2O between each solution to ensure there is no mixing of the wash solutions. Lungs were perfused with 10 ml of each detergent (including water washes) using a 10ml syringe through the tracheal and the pulmonary artery. Lungs are given enough time between perfusions to deflate, ensuring proper washing. The lungs are first washed with sterile H_2O and then incubated in Triton X at 4°C overnight. Day 2, the lungs are once again washed and incubated in SDC at room temperature, overnight. The last day, the lungs are incubated in NaCl solution for 1 hr, after which they are washed and stored in PBS.

Mast cell recellularization

During recellularization, all but the designated lobes are tied off, including the heart, after which the designated lobe is perfused with 10 ml in whole lung of an equal portion of low melting agarose and mast cells in RPMI ($1x10^6$ cells / ml). The tracheal is then tied off, and the agarose is left to harden in the fridge for 5 min. The lobe is then separated from the lung and sectioned into 2 to 3 cm wide horizontal pieces. Each section is placed into a 6 well plate with 3 to 4 ml of media for 5 days, after which the lung sections and culture media was harvested.

Animal treatment study

AdTGF-β1-induced pulmonary fibrosis rats were administrated cromoglycate (40 mg/kg/ day, i.p.) for 7 consecutive days, from day 14 to day 21. The cromoglycate concentration was determined based on previous studies[12,13]. The lung was harvested at day 21 and submitted to histology and collagen assay.

Statistical analysis

All data were expressed as the mean ± deviation (SD). Data were analysed with GraphPad Prism 6.0 (GraphPad Software Inc, La Jolla, CA) with either one-way analysis of variance (followed by Tukey's post hoc tests) or non-parametric analysis (Kruskal-Wallis test), followed by post-Dunn's test for multiple comparison. Statistical analysis between two

groups was performed using a nonparametric Mann Whitney test. Shapiro-Wilk normality test was used to check normal distributon. In all cases, a p-value less than 0.05 was considered significant

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