Supplemental Data

Supplemental Material and Methods

Patients recruitment for survival analysis

For the survival analysis, unselected, consecutive IPF patients from the UCLH or Exeter ILD Services were followed prospectively for mortality based on blood platelet counts. The diagnosis of IPF was made following clinico-radiological multidisciplinary team review. Survival time was taken from point of first presentation to ILD services to time of death or lung transplant. Patients with platelet counts outside the normal range, known hematological disorders or treatments known to affect platelet count were excluded. Patients were divided into 3 equally sized categories with cut-offs determined bias-free as the lower, middle and upper thirds (tertiles) as shown in the table below.

	UCLH discovery cohort	**Exeter validation cohort**
Blood platelet cut-off values for tertiles ($x10^9$/L)	**207**	**207**
	267	**256**

Cox proportional hazard modelling, with multivariate analysis for the UCLH and Exeter patient cohorts was conducted using OriginLab statistical software.

Patient BALF & plasma collection & processing

Paired blood and BALF samples were collected from patients consented for a routine bronchoscopy for diagnostic reasons. Lavages were collected bronchoscopically after saline instillation, and aliquots of BALF were frozen after centrifugation for later cytokine analysis. The remaining BAL cell pellet was resuspended in PBS before being cytocentrifuged and differentially stained with a Rapid Romanowsky Stain Kit (TCS Biosciences Ltd, Botolph Claydon, UK). Cell counts were performed using a conventional brightfield microscope. Whole blood was collected in EDTA vacutainers before centrifugation to collect the plasma for later cytokine analysis. BAL cells were stained with anti-CD61 antibody (clone VI-PL2) or isotype control (mouse IgG1, clone MOPC-21, both from BD Biosciences) and percentage of $CD61^+$

platelets in BALF was quantified on a BD FACSVerse flow cytometer (BD Biosciences, San Jose, USA) and analyzed with FlowJo Version 10 software (FlowJo LLC, Ashland, USA).

Neutrophil chemotaxis assay

Human or murine PRP were obtained by collecting whole blood in EDTA, followed by centrifugation for 8 min at 800 *g*. PRP was activated with 0.5 U/ml thrombin (Sigma-Aldrich, Gillingham, UK) for 20 min at RT prior to centrifugation at 2800 *g* for 7 min to remove the platelet pellet. Murine bone marrow-derived neutrophils were collected by flushing the femurs of transgenic mice with 3 washes of 1 ml PBS. Human neutrophils (*n*=4 healthy donors) were obtained from citrated peripheral blood collected from healthy donors after an initial 6% dextran (Sigma-Aldrich) sedimentation step to remove red blood cells. Murine bone marrow-derived neutrophils (*n*=4 littermate controls) or human neutrophils were isolated from a Percoll Plus (Sigma-Aldrich) gradient by centrifugation at 700 *g* for 30 min before labelling with 1 µM CMFDA (Invitrogen, Carlsbad, USA) in HBSS- for 30 min at 37°C. Cells were washed once in HBSS- and resuspended at $5x10^6$ cells/ml and $2.5x10^6$ cells/ml in HBSS+ for human or murine neutrophils respectively. 100 µl neutrophils were added to the top of 8 µm PET trans-wells insert (Corning, New York, USA) and chemoattractants: 100 nM fMLP (Sigma-Aldrich), 0.1-1000 pg/ml TGFβ1 (R&D systems), 10 ng/ml LTB_4 (Cambridge Biosciences, Bar Hill, UK) or pre-diluted PRP (*n*=4) added to the top or bottom of the insert. In ALK5 inhibition studies, neutrophils were pre-treated with 1 µM SB-525334 (Sigma-Aldrich) or 10 µM Galunisertib (Antibodies-online GmbH, Germany) for 30 min before migration. Neutrophils were allowed to migrate for 1 h at 37°C before collection and read on a BD FACSVerse flow cytometer and analyzed with FlowJo software. The degree of migration was expressed as the Chemotactic Index (number of cells migrating in response to the stimulus ÷ number of cells migrating in response to media control).

Cytokine analysis

To quantify active TGFβ1 in PRP, plasma or BALF samples, mink lung epithelial cells (MLEC) stably transfected with an expression construct containing the plasminogen activator inhibitor-1 (PAI-1) promoter fused to a firefly luciferase reporter gene (Abe *et al*., 1994. Anal Biochem; 216:276-284) were treated with samples or known concentrations of recombinant TGFβ1 for 14 h. Bioluminescence was

measured after cell lysis and the addition of luciferin substrate (Promega, Madison, USA). A standard curve constructed from bioluminescence readings for known concentrations of recombinant TGFβ1 was used to determine the concentration of TGFβ1 in biological samples.

Histology & Immunohistochemistry (IHC)

Murine or human lung samples were processed for histological analysis as described (Smoktunowicz *et al*., 2015. Dis Model Mech; 9:1129-1139). In brief, re-hydrated micro-CT processed murine lungs or human lung biopsies were embedded in paraffin wax blocks. 5 µm sections were stained with Hematoxylin & Eosin (H&E) or modified Martius Scarlet Blue (MSB) using an automated slide stainer (Sakura Tissue-Tek DRS 2000, Alphen aan den Rijn, The Netherlands). IHC was performed for the presence of murine CD61 (clone AB-7773, 20 μg/ml, Sigma-Aldrich) or human CD61 (clone 2f2, 550 μg/ml, Leica Biosystems, Wetzlar, Germany) under heat-induced epitope retrieval methods with citrate buffer pH 6.0. Staining was developed using HRP-conjugated secondary antibody and NovaRED Peroxide (HRP) substrate kit (all from Vector Laboratories, Burlingame, USA) for murine tissue or DAB for human tissue (Leica Biosystems) and sections counter-stained with haematoxylin. All sections were scanned on a Nanozoomer Digital Slide Scanner and images analysed using NDP.view software (both from Hamamatsu Corporation, Hamamatsu City, Japan). Quantification of collagen deposition in alveolar walls based on modified Martius Scarlet Blue (MSB) staining was performed using Orbit Image Analysis software.

LPS animal model of inflammation

15-20 week old male and female TGFβ1$^{fl/fl}$.PF4-Cre or littermate control mice were given 3.75 μg/mouse LPS (Sigma-Aldrich) in a volume of 50 µl saline or saline only via an intranasal route. Mice were monitored for disease severity and culled 6 h post instillation. BAL and lungs samples were collected for cytospins, cytokine analysis and flow cytometry as described above.

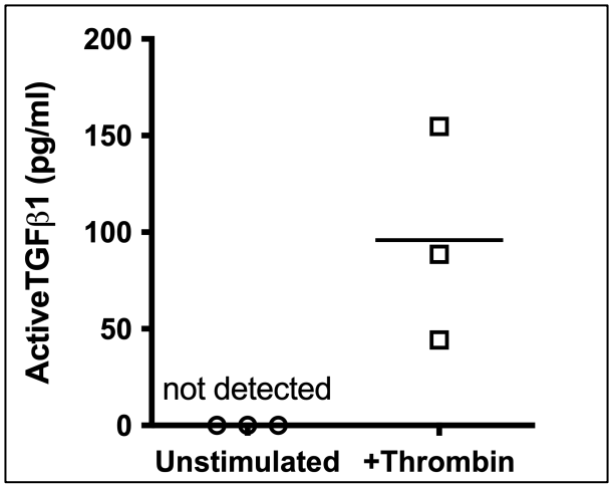
Supplemental Table 1. Summary of Non-ILD and IPF patient cohort for plasma protein analysis

Characteristics	Non-ILD cohort	IPF cohort
Blood samples recruited	10	37
Age (years ± SD)	57.5 ± 16.6	70.8 ± 10.6
% Male	50.0	73.0
Diagnosis	Haemoptysis with no lung pathology (*n*=5) Healthy control (*n*=2) Previous schwannoma (*n*=1) Asthma/COPD (*n*=1) SLE (*n*=1)	IPF (*n*=37)
Forced Vital Capacity (FVC, % predicted) (mean ± SD)	99.0 ± 47.7	76.5 ± 16.1

Supplemental Table 2. Summary of Non-ILD and IPF patient cohort for BALF protein analysis

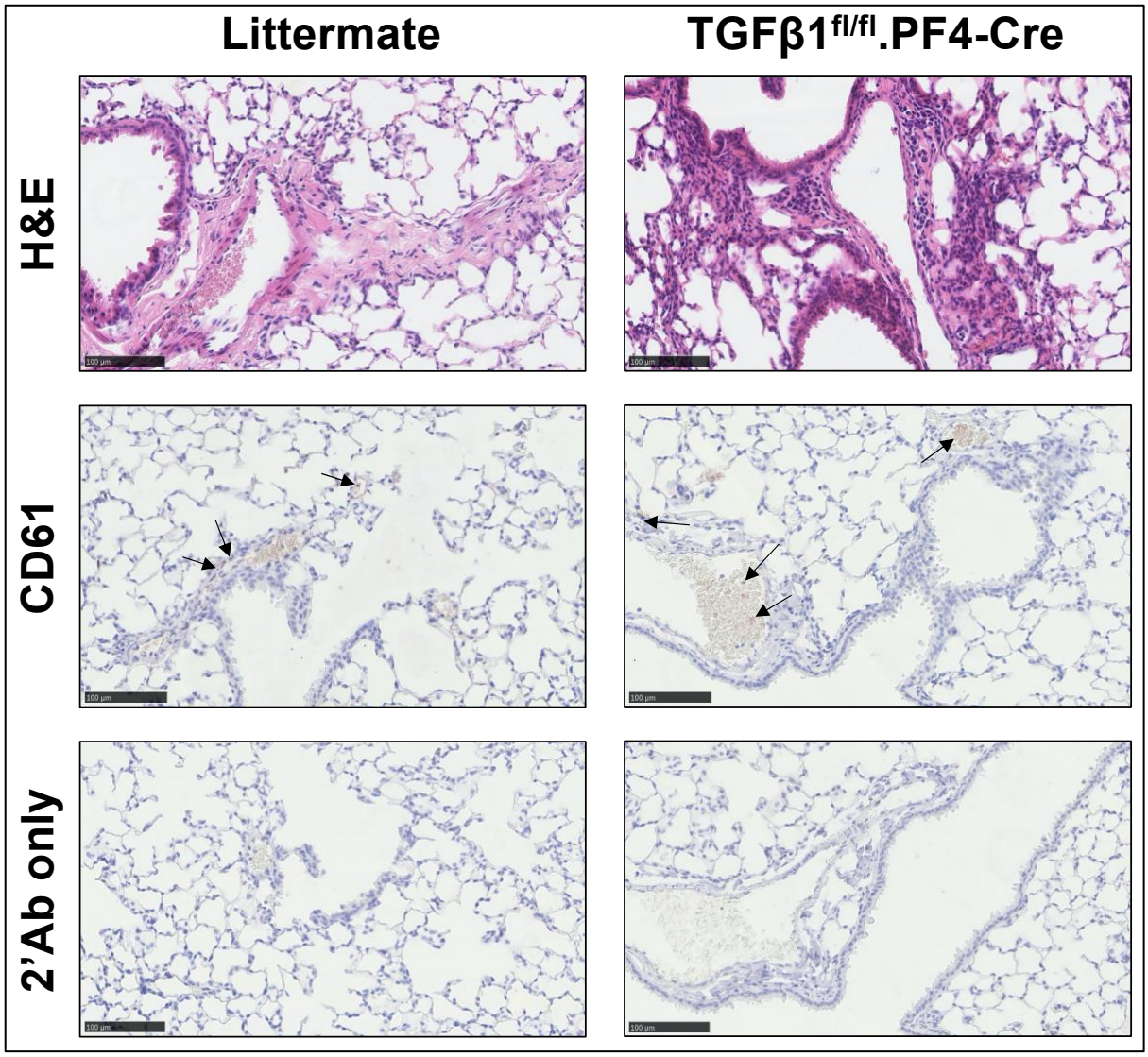
Characteristics	**Non-ILD cohort**	**IPF cohort**
Lavage samples recruited	7	16
Age (years ± SD)	42.9 ± 11.4	61.8 ± 19.7
% Male	42.9	68.8
Diagnosis	Haemoptysis with no lung pathology (*n*=5) Previous schwannoma (*n*=1) SLE (*n*=1)	IPF (*n*=16)
Forced Vital Capacity (FVC, % predicted) (mean ± SD)	104.7 ± 20.7	75.4 ± 24.4

Supplemental Figure 1. Activated human platelets are a source of active TGFβ1



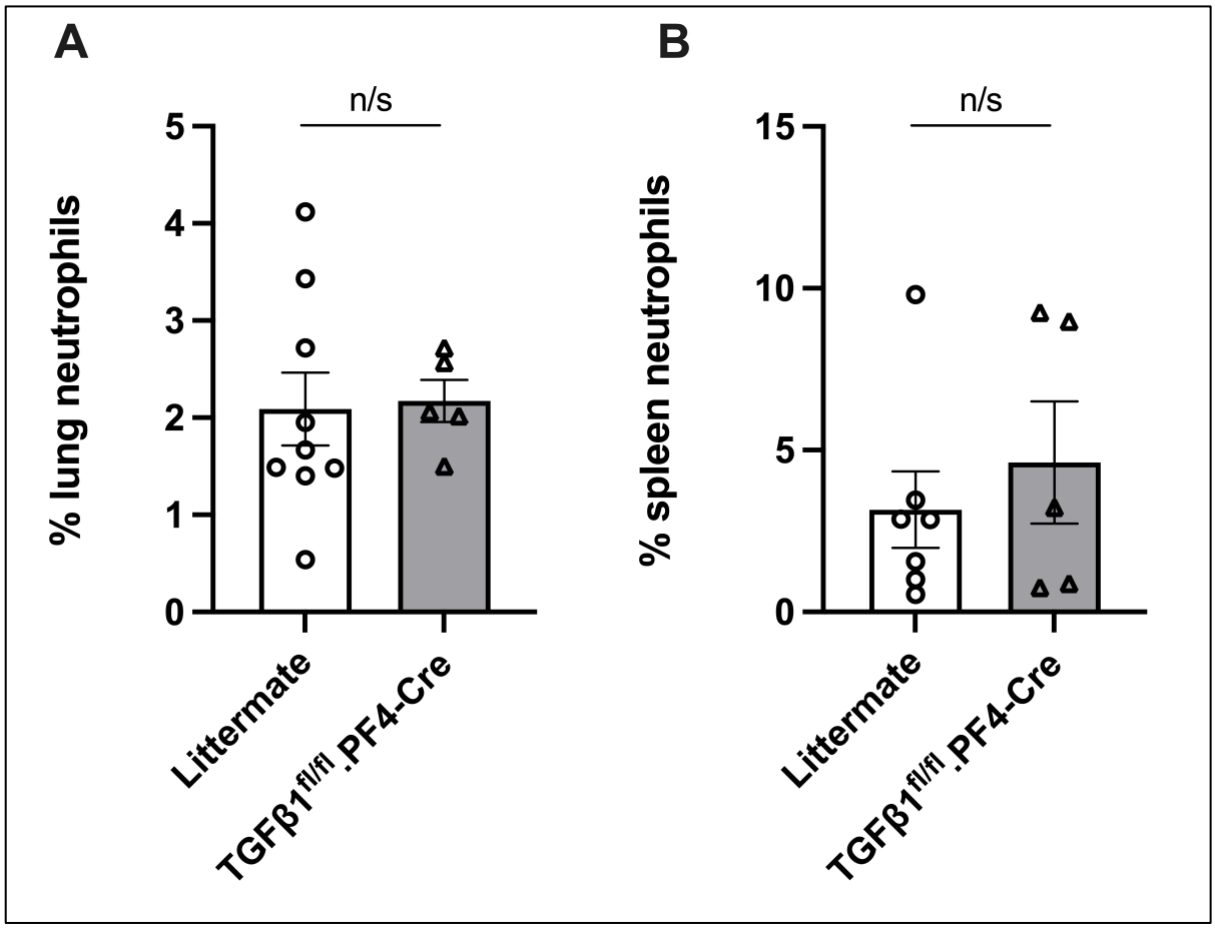
Quantification of active TGFβ1 by MLEC bioassay in unstimulated or thrombin-treated human PRP (*n*=3 healthy donors).

Supplemental Figure 2. Littermate and TGFβ1$^{fl/fl}$.PF4.Cre mice exhibit similar degrees of inflammation and platelet aggregation after *in vivo* bleomycin challenge



TGFβ1$^{fl/fl}$.PF4-Cre (*n*=10) or littermate control mice (*n*=8) were given 25 IU bleomycin via an oropharyngeal route. Lung tissue was harvested 28 days later for histological analysis. Littermate controls (left column) or TGFβ1$^{fl/fl}$.PF4-Cre lungs (right column) were processed for H&E, CD61 or secondary antibody (2'Ab) only control IHC staining (positive staining = brown, arrows denote platelets and platelet aggregates). Images are shown at 20x objective as denoted by the scale bar.

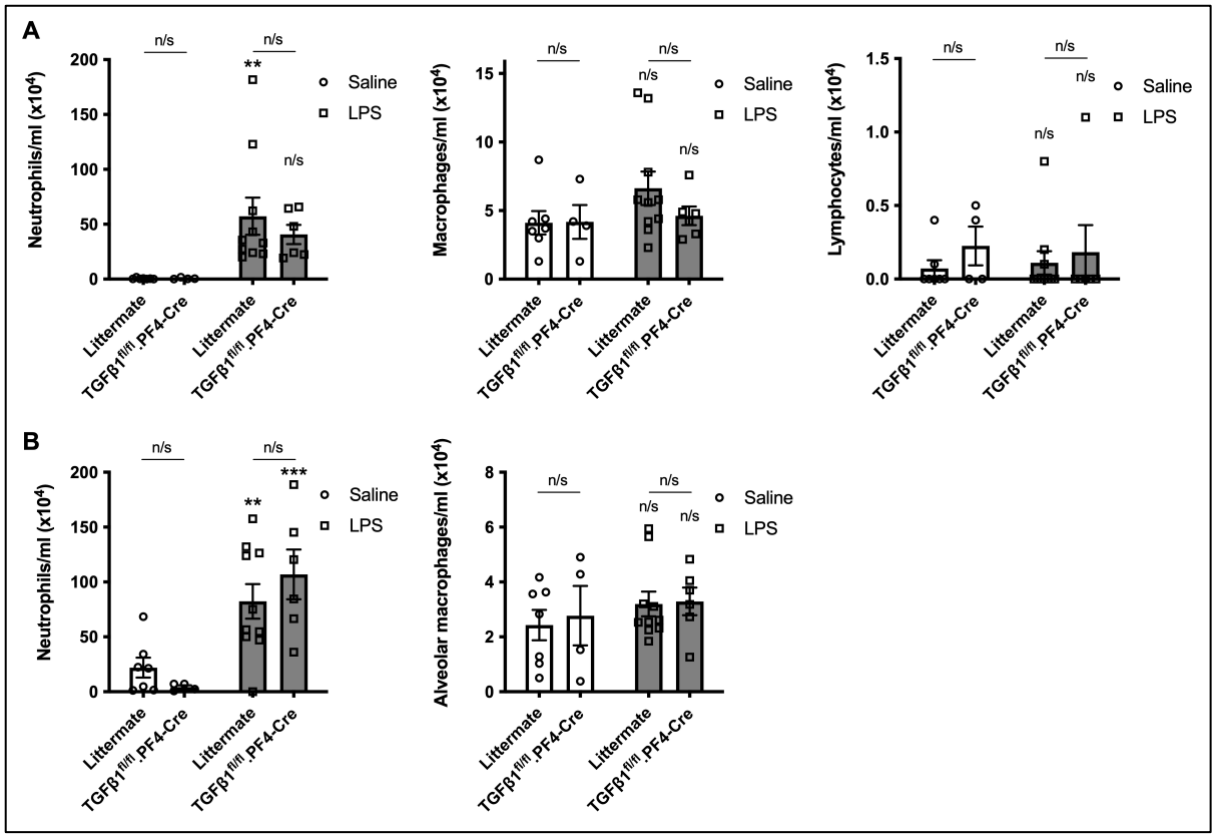
Supplemental Figure 3. Baseline neutrophils counts are unaffected in TGFβ1$^{fl/fl}$.PF4-Cre mice



Percentage of neutrophils (defined as Ly6G^{+}CD11b^{+}) in lung tissue or spleens of naïve untreated littermate controls (*n*=9) or TGFβ1$^{fl/fl}$.PF4-Cre (n=5) mice was quantified by flow cytometry.

No significant differences were calculated using the Mann Whitney U test.

Supplemental Figure 4. Platelet-derived TGFβ1 does not play a significant role in mediating inflammation after *in vivo* LPS challenge



TGFβ1$^{fl/fl}$.PF4-Cre or littermate control mice were given saline (white bars, *n*=7 littermate or *n*=4 TGFβ1$^{fl/fl}$.PF4-Cre) or 3.75 μg/mouse LPS (grey bars, *n*=10 littermate or *n*=6 TGFβ1$^{fl/fl}$.PF4-Cre) via intranasal administration. BALF and lung tissue were harvested 6 h later to determine the degree of inflammation.

A. Neutrophil, macrophage or lymphocyte cell populations were counted by cytospin from the recovered BALF.

B. Neutrophil or alveolar macrophage populations were counted by flow cytometry from the recovered lung homogenate.

Any statistical differences were determined using a 2-way ANOVA test with Holm-Sidak post-hoc testing. Asterisks above the bars represent significance between saline & LPS treatment (n/s = not significant, ** $p < 0.01$, *** $p < 0.001$).