Supplementary methods

Consecutive 3µm sections of the FFPE samples were stained for haematoxylin and eosin (HE) or dual immunohistochemical staining for chymase and tryptase (Ventana Discovery Ultra; Roche). Deparaffinised sections were incubated with a mouse anti-human chymase primary antibody (1ng/ml; R&D Systems), followed by incubation with an alkaline phosphatase-labelled anti-mouse IgM and IgG secondary antibody (DISCOVERY) and stained with a yellow chromogen (DISCOVERY). Next the slides were incubated with a mouse anti-human mast cell tryptase primary antibody (2ng/ml; Dako), followed by incubation with an alkaline phosphatase-labelled anti-mouse IgM and IgG secondary antibody (DISCOVERY) and stained with a red chromogen (DISCOVERY). Sections were counterstained with hematoxylin. The use of chromogens enabled visualization of chymase only positive cells in yellow, tryptase only positive cells (MC_T) in red and dual stained chymase-tryptase positive cells (MC_{TC}) in orange. Slides were digitally scanned using the Aperio Scanscope XT and analysed by OracleBio Limited (Biocity) using an image analysis algorithm software (Indica Labs HALO software) to quantify the MC density in lung tissue.

	IPF (Temple)
Age, years	66±6
Gender, male/female	8/2
Smoking status, ever/never-smokers	9/1
FVC, litres	2.3±0.7
FVC % pred, %	57.9±13.3
Steroid use yes/no	7/3

Table 1. Demographic and spirometric data of IPF (Temple) cohort. Data expressed as mean ±SD unless specified. Age is recorded at time of resection. IPF, idiopathic pulmonary fibrosis; FVC, forced vital capacity; n, number of available data.

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For microarray analysis, total RNA was extracted from tissue samples utilizing the mirVana kit (Ambion) and further purified using Agencourt RNAclean Beads following manufacturer's instructions. Total RNA was converted to cDNA using the SuperScript II kit (Life Technologies), and cRNA was generated from the cDNA using standard Affymetrix IVT labelling reagents. cRNA was fragmented for hybridization to Affymetrix HG-U133Plus2.0 microarrays. Microarray data was normalized using frozen robust multi-array analysis (fRMA) [1]. A moderated t-test as implemented in the R package LIMMA [2] was used to identify genes induced at least two fold with a Benjimini Hochberg corrected p-value <0.05 in IPF lung tissue when compared to normal tissue. Three genes encoding mast cell proteases *CPA3, TPSB2* and *TPSAB1* were among the strongest differentially expressed transcripts and comprise the mast cell protease signature. A signature score was calculated for each patient by using the median of the fRMA normalized expression value reported by the microarray of the three genes in the signature.

Mast cell culture

CD133+ cord blood cells were cultured for 8 weeks in StemSpan SFEM media (StemCell Technologies) supplemented with 100ng/ml rhSCF and 50ng/ml rhIL-6 (Peprotech) with the addition of 10ng/ml rhIL-3 (R& D Systems) for the first 3 weeks of culture only. Non-adherent cells were passaged and maintained at a density of ~5 x 10^5 cells/ml and the media replaced every 4-7 days. Cells used in these experiments were between 8 and 23 weeks of culture, and came from 4 different donors.

Preparation of mast cell conditioned media and cell lysates

Conditioned media was prepared from CBMCs that had been cultured for 7 days in StemSpan SFEM media supplemented with 100ng/ml SCF and 50ng/ml IL-6. Media alone (no cells) was included as a negative control. CBMC lysates in low serum fibroblast media was prepared by sonication of cell suspensions in a sonicating water bath followed by a freeze thaw and agitation. Supernatants were clarified by centrifugation at 3,400 x g for 5 minutes at 4°C.

Cloning, Expression and Purification of extracellular domain of human Stem cell factor

DNA encoding the human extracellular domain of the SCF gene was synthesized and optimised for expression in human cells and sub-cloned into pDESt12.2 oriP_Avi10His expression vector (Geneart, Thermo Fisher). The protein was expressed in the adherent HEK293 EBNA cell line (Thermo Fisher). The conditioned media, containing secreted protein, was harvested 94 hours after transfection, clarified by centrifugation, concentrated and diafiltered by using a 30 Kda membrane in tangential flow filtration system (Pall). The concentrated crude extract was purified by Nickel affinity column (GE Healthcare) followed by Superdex 75,16/600 size exclusion column (GE Healthcare). The protein was stored in PBS at -80°C and later used for stimulation of mast cells.

CBMC proliferation, CCL2 release and phosphorylation of ckit

CBMCs were starved of SCF overnight and the following day stimulated with recombinant SCF (4nM, MedImmune) in the presence or absence of nintedanib (300pM-1uM, LC Laboratories), DMSO vehicle (Sigma Aldrich), anti-SCF pAb (31pM- 67nM; AF-255-NA) or goat IgG isotype (both antibodies R&D Systems). Phosphorylation of ckit was measured using

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an electrochemiluminescence-based phospho-ckit assay (MesoScale Discovery) after a 10 minute stimulation and CBMC proliferation was measured by ^{3}H-thymidine incorporation after a 3 day stimulation. ^{3}H-thymidine (0.2 μCi/well) was added as a final 5 hour pulse of the 3 day stimulation and the incorporated radioactivity was assessed by liquid scintillation spectrometry. CCL2 concentrations were measured in culture supernatant harvested from the samples prior to the addition of ^{3}H-thymidine. Samples were stored at -80°C prior to analysis by MSD assay (MesoScale Discovery) according to the manufacturer's instructions.

NHLF culture

Normal human lung fibroblasts (NHLFs; (Lonza)) were cultured in Fibroblast Basal Media (FBM) supplemented with FGM-2 SingleQuot kit (Lonza). NHLFs were used in the assays at passage 4-5. IPF fibroblasts (provided by the Jenkins laboratory, University of Nottingham) were cultured in DMEM supplemented with 10% FBS, pencillin/streptomycin and fungizone.

CBMC:NHLF co-culture and flow cytometry assessment of MC and fibroblast numbers

NHLFs (10,000/well) were seeded in 96-well collagen coated plates. NHLFs were cultured at a 1:1 ratio with CBMCs that had been starved of SCF and IL-6 for 36 hours. Controls of each cell type cultured alone were included for comparison. Cells were cultured in media containing anti-SCF, goat IgG isotype control (67nM; both R&D Systems), nintedanib, (30nM; LC Laboratories), DMSO vehicle or media alone over a time-course up to 21 days. At each time-point, cells were stained with a viability dye (Blue fluorescent reactive dye, 1:1000; Life Technologies) and anti-ckit APC mIgG1 (1:1000; BD Pharmingen) and the number of ckit

positive (mast cells) and ckit negative viable cells (NHLFs) in a fixed volume was counted in a flow cytometer (LSRII Fortessa).

Fibroblast stimulation

NHLFs were seeded in 96-well plates and starved overnight by incubation in basal medium supplemented with 0.1% FBS and gentamycin only. For experiments using IPF fibroblasts, both IPF fibroblasts and NHLFs were plated in DMEM plus 10% FBS and starved in DMEM supplemented with 0.1% FBS. Cells were stimulated with recombinant human bFGF (100pM) in the presence or absence of nintedanib (1.52nM-10uM; LC Laboratories), pirfenidone (15.2nM-100uM; Selleckchem.com), or DMSO vehicle for 48 hours, or for 48-72 hours with mast cell lysates, conditioned media or mast cells at a ratio of 1:1 (see individual experiments for details). TGFβ-1 (0.33ng/ml; R&D Systems) was included as a positive control for αSMA experiments.

Proliferation of subconfluent fibroblasts was assessed by ^{3}H-thymidine incorporation. ^{3}H-thymidine (0.4 μCi/well) was added as a final 24 hour pulse of the 48 hour stimulation and the incorporated radioactivity was assessed by liquid scintillation spectrometry.

αSMA expression was assessed by immunofluorescence following a 72 hour stimulation. Cells were washed, fixed and permeabilised. After a blocking step cells were incubated with anti-αSMA Cy3 (clone 1A4; 1/500; Sigma Aldrich) together with Hoechst 33342 nuclear dye (1/5000). Plates were scanned at 20X magnification using the Cellomics ArrayScan platform

(Thermo Scientific) and a blinded count (5 fields/well) was performed to quantify high αSMA expressing cells.

Stimulation of fibroblasts with recombinant/purified mast cell mediators

NHLFs were stimulated for 48 hours with the mast cell mediators tryptase β1 or β2 (Promega), chymase (Enzo Life Sciences), histamine (Sigma) or PDGF-AA (R&D Systems). Proliferation was assessed by ^{3}H-thymidine incorporation assay.

Animals and experimental protocol

The bleomycin model of lung fibrosis was used as there is currently no suitable replacement to investigate lung fibrosis and mast cell infiltration *in vivo*. The study conforms to ARRIVE guidelines [3]. Male CD rats, 6-8 weeks of age (body weight 300-350g) and free of murine specific pathogens were obtained from Charles River UK and housed in individually ventilated cages and maintained on standard laboratory chow *ad libitum*. Rats were randomised to have similar mean body weight per group and received either 0.9U/kg bleomycin sulphate (Bleo-Kyowa) (two groups of 9) or saline (1ml/kg) (two groups of 6) intra-tracheally under isoflurane anaesthesia (study day 0). A power calculation was performed in order to reduce the number of animals used. Calculation was based on the lung hydroxyproline measurement utilised in previous studies and suggested a group size of 8 in the bleomycin treated groups was required to enable detection of a 20% change in hydroxyproline at day 14. An additional rat was included in each bleomycin group based on previous experience of loss due to exceeding the threshold of 20% loss in body weight. Nintedanib ethanesulfonate (10mg/ml in water) was dosed at 60mg/kg daily (based on a study by Wollin *et. al*; [4]) by oral gavage to one group of

bleomycin-treated and one group of saline treated animals, with the other groups receiving saline vehicle from day 0 to day 13. Soft food/gel was available to all animals during the study, as a refinement of the model to minimise weight loss following bleomycin treatment. One animal in the bleomycin plus vehicle group failed to recover post bleomycin on day 0. A second animal in the same group was culled on day five due to exceeding the 20% loss in body weight. On day 14 rats received a lethal i.p dose of Dolethal. Blood was collected by cardiac puncture and death was ensured by severance of the abdominal aorta below the heart. BAL was collected and the left lung lobe was fixed in 10% formalin and then embedded in paraffin for histological analyses. The right post caval lobe was collected and stored in RNALater for analysis of gene expression. The remaining right lung lobes were collected for analysis of hydroxyproline.

For mast cell quantification, left lung samples were fixed in 10% formalin buffer for 24 hours and then cut transversally. 3µm sections were stained using hematoxylin and eosin (HE) and Giemsa for mast cells, HE alone, and Masson's trichrome (MTC) for collagen using standard protocols (Damsgaard; Leica Autostainer). All slides were digitally scanned using the Aperio Scanscope XT and the number of positively stained cells was determined manually in the total lung area.

Image analysis of rat lung sections by Biocellvia

HE and MTC scanned images were analysed by Biocellvia using their IPF assay based on assessment of parenchymal tissue density using a proprietary software program. Biocellvia's IPF assay allows transformation of the original RGB images into digital images in which each pixel is characterised by a specific density value. Slides (5 sections per animal on a single

slide) were scanned at 20x magnification (pixel size:0.452µm). Bronchi and vessels were automatically excluded from the analysed lung sections. The high tissue density (HDFm, also referred to as percentage of foci) corresponds to the sum of the high tissue density frequency located specifically in fibrotic parenchymal tissue. The mean tissue density (Dm) corresponds to the mean density of the lung parenchyma. Quantification was carried out automatically with blinding to the group identity, with the exception that the HDFm analysis required knowledge of the control group (saline plus vehicle). Some sections were excluded from analysis if sections were too close to each other on the slide or if there was anomalously high colouration of a section.

Measurement of histamine levels

To assess histamine levels lung tissue was homogenised by bead milling (Qiagen, TissueLyser) and supernatant levels quantified by ELISA (Abnova) according to the manufacturer's instructions.

Hydroxyproline measurement in rat lung homogenates

Lung hydroxyproline levels were determined using a modified colourimetric method [5].

Gene expression analysis of rat lung homogenates

Using a bead milling procedure (QIAGEN TissueLyser), the rat post-caval lung tissue was lysed in Buffer RLT solution (Qiagen) supplemented with β-Mercaptoethanol. RNA was extracted from lung lysate using the RNeasy Fibrosis Tissue Extraction Kit (QIAGEN) and

the QIAcube automated system and quantified on a NanoDrop Spectrophotometer. Following reverse transcription, gene expression was analysed by Taqman RT-PCR using custom Taqman Rat Microfluidic Cards (Applied Biosystems). Expression was normalised against housekeeping ribosomal 18S. The relative fold change was calculated against the saline controls ($2^{-\Delta\Delta ct}$).

Supplementary results

When the number of mast cells/total area lung (no deduction of air space to account for any differences in tissue area) is compared, there is an increase in total mast cells and in each of the mast cell subtypes in the IPF lung compared to non-fibrotic lung.

	Non-fibrotic lung (n 20)	**IPF (n 17)**	**p**
Total mast cells	28.75 ± 5.318	115.8 ± 20.28	< 0.0001
Chymase only	1 (0-11)	8 (3-45)	<0.0001
Tryptase only (MC_T)	18 (0-83)	88 (2-241)	0.0011
Chymase+tryptase (MC_{TC})	0 (0-18)	2 (0-31)	0.0014

Supplementary table 1. Mast cell subtypes in IPF versus non-fibrotic lungs (number of mast cells per mm^2 area lung section). Groups were compared using an unpaired t-test (total mast cells) or Mann-Whitney test (MC subtypes).

Supplementary figure legends

Supplementary S1: Annotation of regions of lung Representative image depicting annotation of fibroblastic foci, established fibrosis and non-fibrotic areas by a registered senior pathologist on the digitally scanned HE sections. Annotations were then transferred on to the sequential serial section stained with chymase and tryptase.

Supplementary S2: MCs versus gender in healthy lung.

There is no significant difference in MC density in healthy lung between males and females, either when MC density is plotted per area lung section (total area) (A) or per area tissue (B). Median and IQR plotted and groups were compared by Mann-Whitney test.

Supplementary S3. Association of MC number/tissue area with baseline lung function

There is a trend towards decreasing baseline FVC with increasing mast cell number/area tissue (Pearson's correlation) but this does not reach statistical significance.

Supplementary S4: Mast cell mediators induce fibroblast proliferation. Proliferation was assessed by ^{3}H-thymidine incorporation assay, and the fold change compared to untreated cells is represented. (A) IPF fibroblasts were stimulated for 48 hours with MC conditioned media. The assay was repeated in four independent experiments, and NHLFs were included in two of these for comparison. (B) NHLFs were stimulated for 48 hours with the mast cell mediators tryptase β1 and β2, chymase, histamine and PDGF-AA, and the assay was repeated in at least three independent experiments. CPM, counts per minute.

Supplementary S5: Dose response of nintedanib in mast cell fibroblast co-culture. (A) Timecourse. Data from 3 separate experiments. Mean and SD plotted. (B) Day 14 data comparisons. 10nM nintedanib can partially inhibit fibroblast mediated mast cell survival.

1nM nintedanib does not have any effect. Mean and SD plotted. Groups were compared by ANOVA with Sidak's post hoc test.

Supplementary S6: Effect of nintedanib on fibroblast number in MC co-culture. Timecourse. Data from 3 separate experiments. Mean (SE).

Supplementary S7: Lung hydroxyproline and collagen and fibronectin gene expression with nintedanib in rat bleomycin model. (A) There is a trend towards increased histamine with bleomycin treatment, which is decreased with nintedanib, but this is not statistically significant. Mean and SD plotted and groups compared by ANOVA with Sidak's post hoc test. (B) There was an increase in lung collagen, measured by hydroxyproline assay in rats treated with bleomycin, but no effect of nintedanib. (C and D) There is a significant increase in col1a1 expression and a trend towards increased fn1 gene expression with bleomycin treatment but no effect of nintedanib. Data plotted as fold change relative to saline + vehicle control, normalised to 18S housekeeping gene. Median and IQR plotted for (B-D) and groups were compared by Kruskal-Wallis with Dunn's post hoc test.

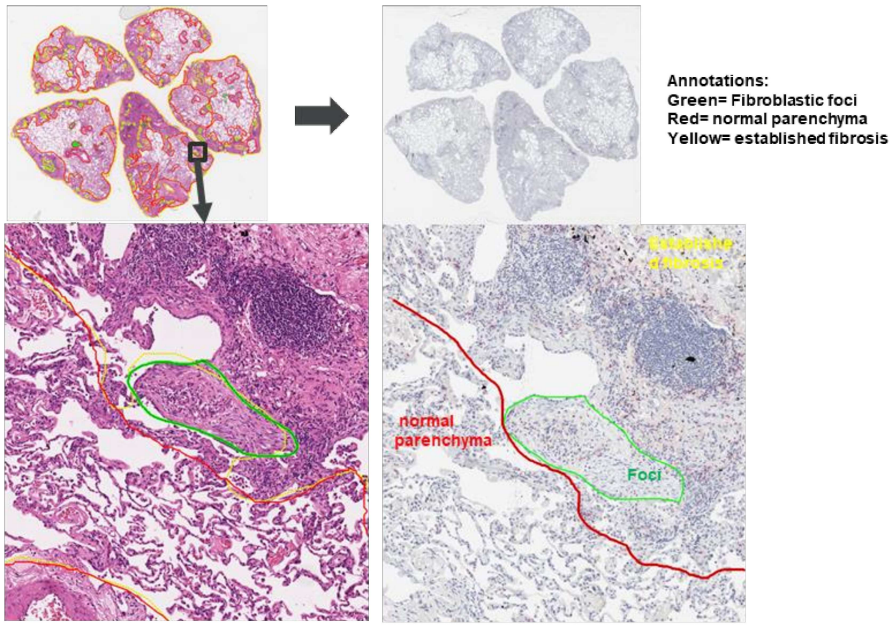
Supplementary S8: Mean tissue density in lung sections in rat bleomycin model.

There is a significant increase in the mean tissue density (Dm) in the lung parenchyma with bleomycin and this is inhibited by nintedanib treatment. Median and IQR plotted and groups were compared by Kruskal-Wallis with Dunn's multiple comparisons test.

1. McCall MN, Bolstad BM, Irizarry RA: Frozen robust multiarray analysis (fRMA)**.** *Biostatistics* 2010, 11**:**242-253.
2. Smyth GK: Linear models and empirical bayes methods for assessing differential expression in microarray experiments**.** *Stat Appl Genet Mol Biol* 2004, 3**:**Article3.
3. Kilkenny C, Browne WJ, Cuthill IC*, et al*: Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research**.** *PLoS Biol* 2010, 8**:**e1000412.
4. Wollin L, Maillet I, Quesniaux V*, et al*: Antifibrotic and anti-inflammatory activity of the tyrosine kinase inhibitor nintedanib in experimental models of lung fibrosis**.** *J Pharmacol Exp Ther* 2014, 349**:**209-220.
5. Swaisgood CM, French EL, Noga C*, et al*: The development of bleomycin-induced pulmonary fibrosis in mice deficient for components of the fibrinolytic system**.** *Am J Pathol* 2000, 157**:**177-187.

Methods:
Annotation of regions

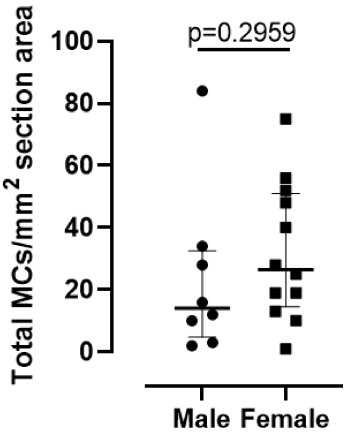
Fig S1



Annotation of regions

Supplementary S2: MCs versus gender in healthy lung

A



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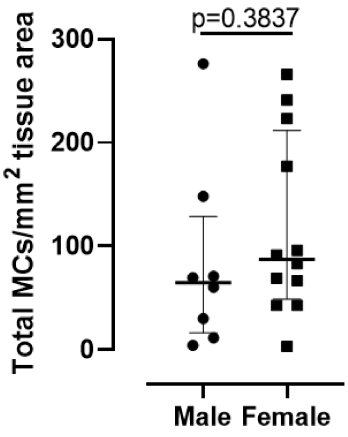
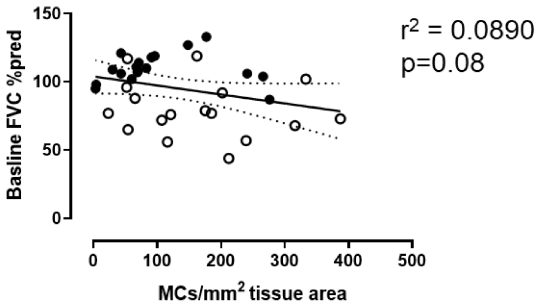
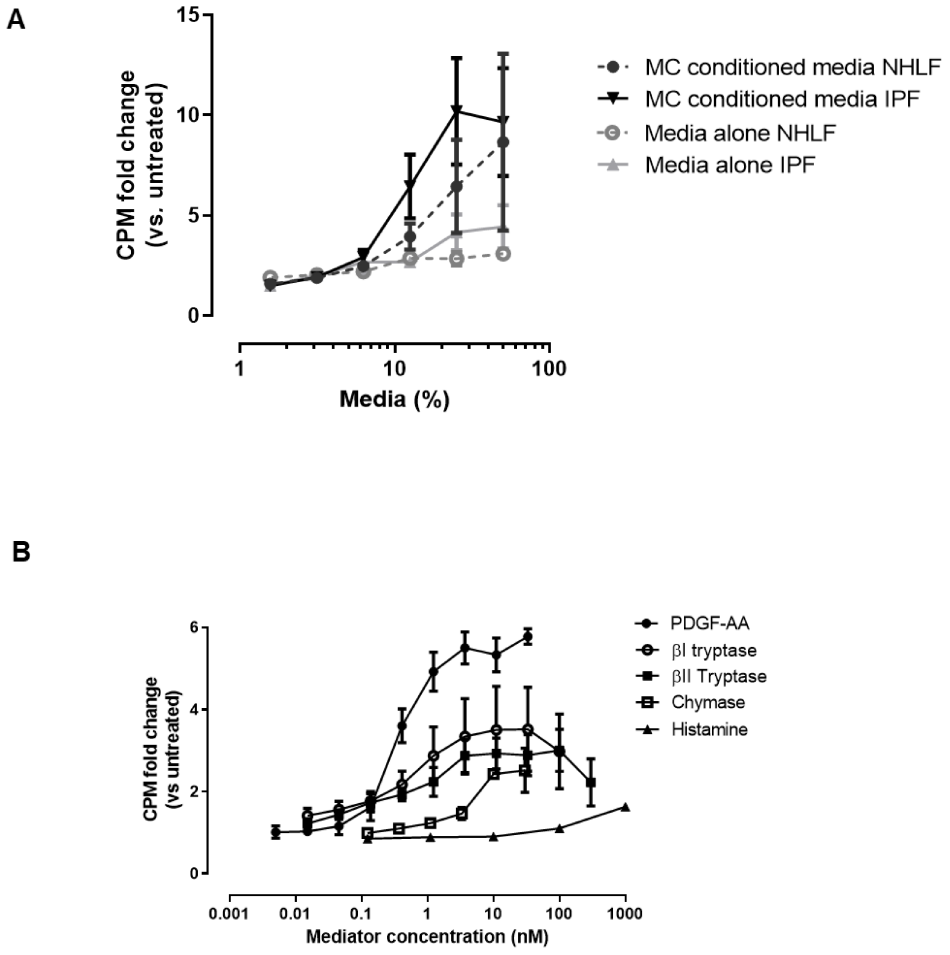


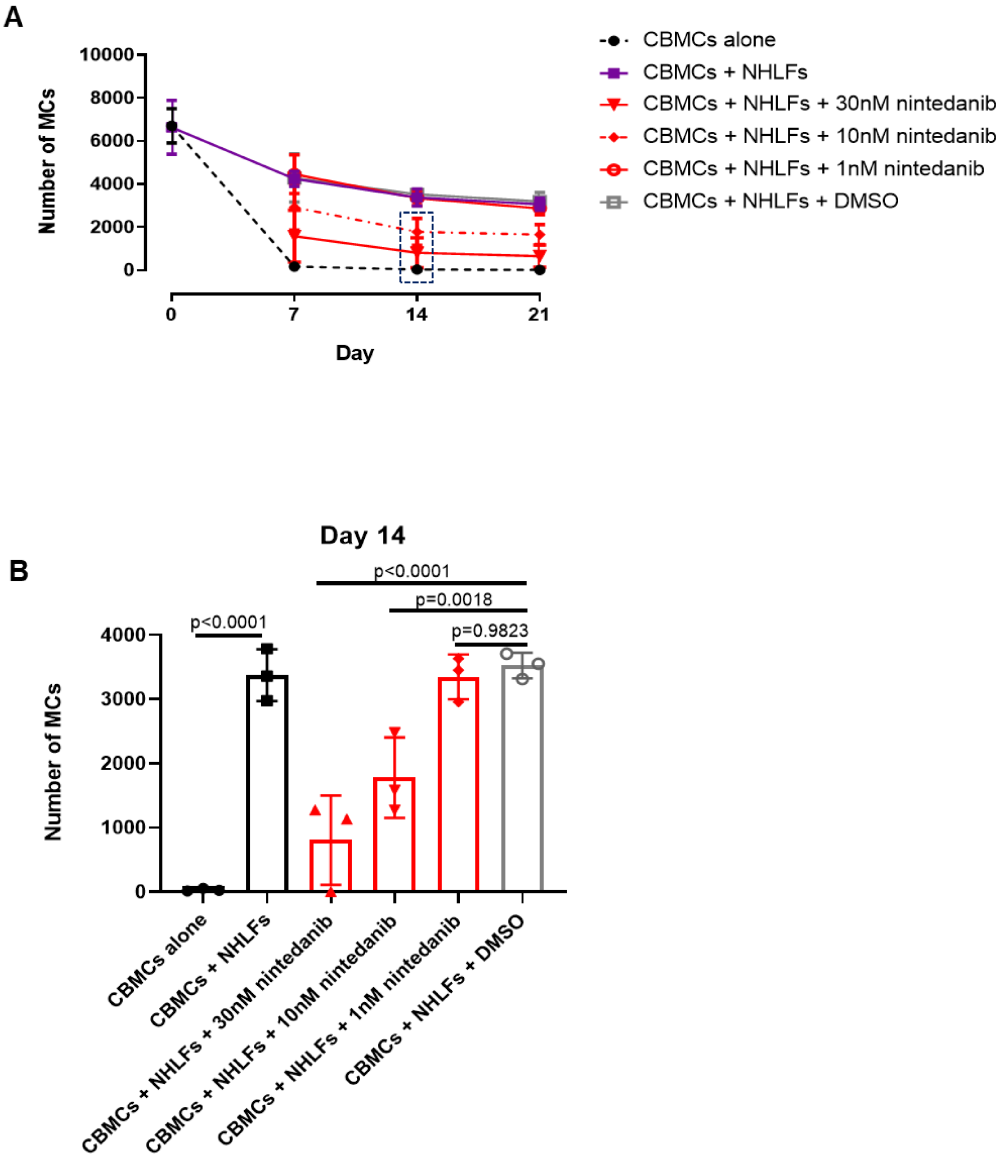
Supplementary S3. Association of MC number with baseline lung function



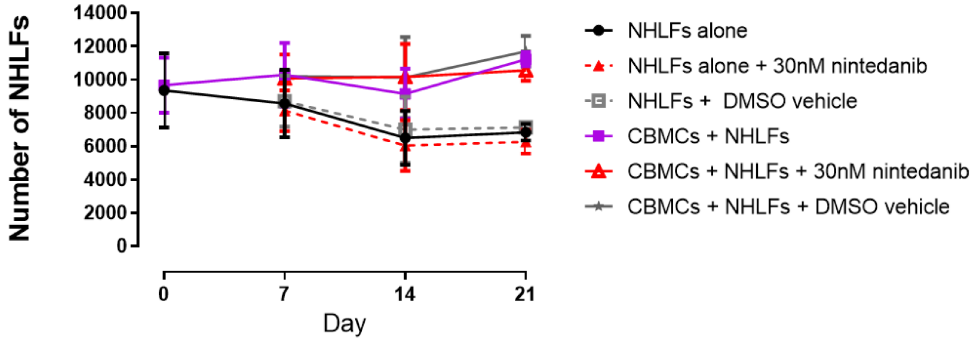
Supplementary S4:Mast cell mediators induce fibroblast proliferation



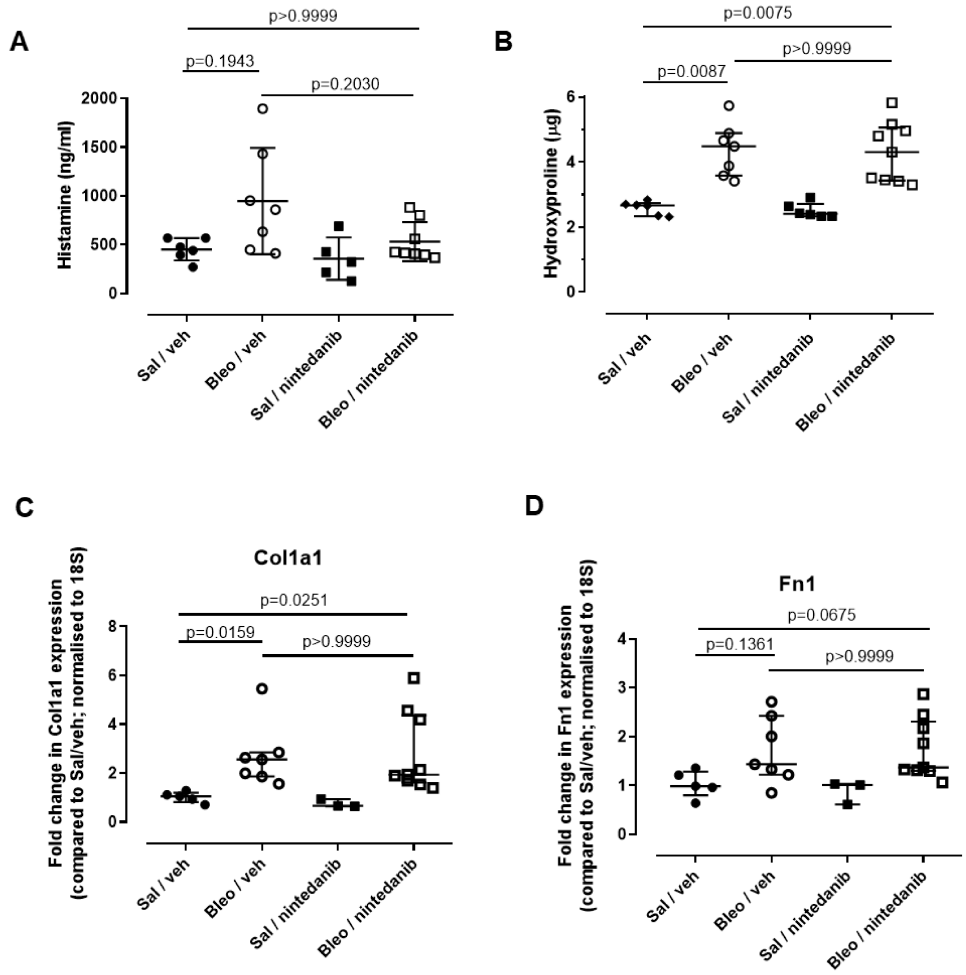
Supplementary S5 : Dose response of nintedanib on MC co-culture



Supplementary S6 : Effect of nintedanib on fibroblast number in MC co-culture



Supplementary S7: Lung histamine, hydroxyproline and collagen and fibronectin gene expression with nintedanib in rat bleomycin model



Supplementary S8: Mean tissue density