DOI:
10.1016/j.vascn.2021.107057

Document Version
Peer reviewed version

A novel murine model of pulmonary fibrosis: the role of platelets in chronic changes induced by bleomycin

Carrington R[1,2], Jordan S[2], Wong YJ[1], Pitchford SC[1], Page CP[1]

[1] Sackler Institute of Pulmonary Pharmacology, Institute of Pharmaceutical Science, King's College London, London, SE1 9NH

[2] Department of Pharmacology, Covance Laboratories Ltd, Woolley Road, Alconbury, Huntingdon, Cambs, PE28 4HS

Author for correspondence and reprint requests:
Robert Carrington

Phone: +44 1480 892 019
Fax: +44 1480 892 362
robert.carrington@covance.com

Word counts:

Abstract: 249

Main Body: 3590

Number of figures: 5

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a disease that causes scarring and destruction of lung tissue that is ultimately fatal. There is a need to develop improved treatments for IPF. One problem with identifying novel treatments of IPF is the poor predictability of current preclinical models. Few model investigate lung function changes, rather relying on histological changes which doesn't adequately reflect the complete clinical situation. The aim of this study was to establish a novel model of pulmonary fibrosis where we could investigate changes in lung function, and histology. We have also utilised this model to investigate the role of platelets in pulmonary fibrosis as platelets have been recognised as having a broader role than just facilitating haemostasis.

Lung fibrosis was induced in male C57BL6/J mice by intranasal bleomycin on Days 0, 1, 2, 5, 6 and 7. Platelets were depleted by twice-weekly administration of anti-platelet antibodies. On Day 35 mice were assessed by examining lung function, platelet infiltration into lung tissues and bronchoalveolar lavage fluid (BAL), levels of BAL Tissue growth factor (TGF)-β levels, and the degree of fibrosis evaluated histologically.

Repeated bleomycin administration caused loss of lung function associated with fibrosis assessed histologically. Platelet depletion resulted in a reduction in fibrosis and modest inhibition of lung function changes.

We have established a novel model of pulmonary fibrosis that is associated with a decline in lung function similar to the clinical setting. Furthermore, platelet depletion resulted in a less severe fibrosis suggesting that targeting platelets maybe worth further investigation.

Keywords

IPF, Platelets, Animal models

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease which is characterised by increased deposition of extra cellular matrix and scarring of lung tissue which reduces the gas exchange capacity of the lungs leading to the typical symptoms of dyspnoea, dry cough and general fatigue [1-4]. There are currently only two approved drugs for the treatment of IPF, Pirfenidone and Nintedanib, which were both shown in clinical trials to decrease the rate of progression of the disease [4-6]. However, even with the availability of these drugs, IPF is fatal with the average time from diagnosis to death being approximately 3-5 years [3,7,8]. There is therefore an urgent need to identify new drugs for the improved treatment of this debilitating condition. However, a major challenge in IPF research remains the lack of predictive preclinical models for identifying new mechanistic pathways and to investigate drugs targeting such pathways [9]Most existing models investigate the action of drugs on the effects of acute high doses of bleomycin and very few of these models attempt to investigate any physiological changes in lung function resulting from the fibrosis, rather relying on histological endpoints.

Although there are a number of risk factors identified for developing IPF, such as prolonged exposure to an environment with poor air quality, certain variations in genes such as MUC5B and TOLLIP, and a history of smoking, currently the precise pathogenesis of pulmonary fibrosis is not fully understood [7,8]. It is thought that repeated micro injuries to lung tissue may be causing the wound healing process in the lung to become overactive, leading to the characteristic increase in scar tissue and extracellular matrix associated with IPF [8]. The fact that repeated injuries are thought to lead to lung fibrosis and progressive loss of lung function suggests that the current approach in most preclinical models of administering a single intratracheal administration of a high dose of bleomycin may not be accurately modelling how the disease progresses in humans, and that a model of repeated injuries may more accurately affect the clinical situation.

It is now widely recognised that platelets can contribute to wound healing as well as playing a critical role in haemostasis, aggregating at sites of vascular injury with subsequent release of growth factors that have been suggested to be involved in remodelling of a range of tissues [10-12]. Moreover, platelets have also been increasingly implicated in a range of inflammatory disorders, particularly respiratory conditions such as asthma, acute lung injury and COPD, as well as in lung infections including SARS-CoV-2 [11-14]. However, the potential role of platelets in IPF has not yet been fully investigated and we have therefore investigated the contribution of platelets to the pulmonary fibrosis and lung function changes induced by repeated exposure to low levels of bleomycin as platelets store and release mediators such as TGF-β, Platelet derived growth factor (PDGF), and Vascular endothelial growth factor (VEGF) which have all been implicated in the fibrotic process [8, 11,13]. Furthermore, platelets are one of the first cell types to respond to injury and have been demonstrated to be able to undergo diapedesis into extravascular spaces where the release of pro-fibrotic factors could feasibly contribute to the development of fibrosis [15].

It is of interest therefore that lungs have recently been demonstrated to be a major source for platelet production, with it estimated that lungs being responsible for approximately 50% of total platelet production in mice [16]. In the present study we have therefore sought to examine whether platelets are involved in the development of pulmonary fibrosis using our novel murine model that allows the measurement of both lung function decline and the histological changes associated with repeated exposure to low levels of a fibrotic stimulus.

Methods

Animals

Animal experiments were conducted according to ARRIVE guidelines, and the Animals (Scientific Procedures) Act 1986 and 2012 amendments following local ethical approval.

Male C57BL6/J mice (obtained from Envigo RMS Ltd) were used as we have previously reported that this strain has a robust fibrotic response to bleomycin [16]. Mice were housed in a purpose-built facility with a controlled environment maintained at the following levels: temperature 21 ± 2°C, relative humidity 55 ± 15%, 12 h light and 12 h dark. Animals were given access to standard rodent diet and water ad libitum.

Bleomycin induced fibrosis

Mice were administered 6 intranasal doses of either saline or 0.25 mg/kg bleomycin A5 hydrochloride (Carbosynth Europe, Batch AB463321601) prepared in saline on Days 0, 1, 2, 5, 6 and 7. The animals were then euthanised using an intraperitoneal overdose of pentobarbital 35 days following the first administration of bleomycin.

Platelet depletion

The effects of platelet depletion on the development of fibrosis were examined by the use of twice weekly intramuscular (0.025mg/50µL per administration) anti-platelet antibodies (Emfret analytics R300) directed against mouse CD42b. Targeting of this receptor with divalent IgGs results in profound and irreversible Fc-independent platelet depletion in mice as described elsewhere [18,19].

Study design

Animals were randomized by bodyweight into 4 study groups, prior to treatment, so that the group mean weights were approximately equal. The four groups were as follows, group 1 administered saline intranasally with twice weekly administration of saline I.M. (n=10 at termination); group 2

administered saline intranasally with twice weekly administration of anti-platelet antibody I.M. (n=11 at termination); group 3 administered bleomycin intranasally with twice weekly administration of saline I.M. (n=7 at termination), and group 4 administered bleomycin intranasally with twice weekly administration of anti-platelet antibody I.M. (n=9 at termination).

Assessment of fibrosis and airways inflammation

The effect of repeated administration of low doses of bleomycin was assessed by examining lung function changes over time and at day 35, measurement of BAL fluid platelet and TGF-β levels, and the degree of fibrosis assessed histologically by Ashcroft scoring.

Lung function

Forced vital capacity (FVC) and Forced expiratory volume in 50mSec (FEV_{50}) are equivalent to parameters that are used in the clinic to assess overall pulmonary function in patients with IPF. FVC is the total volume of air that the animal is able to force out of the lungs, and FEV_{50} is the volume of air that the animal is able to force out of the lungs in 50 mSec. As scar tissue gets laid down and tissue is destroyed, the volume of air that lungs can hold will be reduced and the elasticity of the lung tissue will be reduced, reducing the speed at which air will be able to be expelled from the lung. Both FVC and FEV50 would be expected to fall as fibrosis in the lungs progresses.

Elastance and compliance are not parameters that are measured in the clinic but are parameters that are able to give valuable insight into the physical state of the lungs. Compliance is a measure of the ease with which the lung tissue can be expanded, and elastance is the inverse of this. As fibrosis in the lungs progresses and more scar tissue is laid down the stiffness of the lung tissue will increase meaning that compliance would be expected to drop and elastance increase.

Lung function was assessed in anaesthetised animals (10% 100 mg/mL ketamine, 2.5% 1 mg/mL medetomine in water for injection dosed at 10mL/kg) using a forced manoeuvres system (EMMS

152 espira) to give values for FVC and FEV_{50}, Following completion of the forced manoeuvres procedure,
153 animals were transferred to a Flexivent system. The snapshot perturbation was utilised to measure
154 for elastance and compliance.

155 Inflammatory cell infiltration and cytokines

156 At day 35 following completion of the lung function procedures, a broncho-alveolar lavage (BAL) was
157 carried out whereby the airways were washed out with 3 lots of 0.3 mL of phosphate buffered saline
158 (PBS). The lavage fluid was then centrifuged at 2000g for 10 minutes at 4°C. The resulting supernatant
159 was removed for TGF-β analysis using an R&D systems DuoSet ELISA. The remaining cell pellet was
160 resuspended in 0.5mL PBS, vortex mixed and analysed using the XT-2000i Sysmex haematology
161 analyser to analyse the number of platelets in the BAL fluid.

162 Histology

163 Following the BAL procedure, the lungs of each animal were removed and fixed in 10% neutral
164 buffered formalin. The lungs were embedded in paraffin and sectioned and stained with Masson's
165 Trichrome stain. A method adapted from Ashcroft et al, and Hübner et al [20,21] was then used to
166 histologically examine the lung sections for the extent of fibrosis. In brief, the slides were blinded and
167 then each field of view was assessed and given a score from 0-8, where 0 is normal lung tissue and 8
168 is a field of view completely obliterated by a fibrotic mass. See Figure 1 for representative images of
169 the different Ashcroft scores.

170 Immunohistochemistry

171 In order to carry out immunohistochemical staining, lung sections underwent a clearing process with
172 xylene before rehydrating with decreasing concentrations of ethanol. The lung sections were
173 consequently blocked with 4% hydrogen peroxide and heated in sodium citrate buffer (pH 6.0) at 100
174 °C for 5 minutes using a pressure cooker to unmask the antigen.

IHC staining was performed on lung sections as described in Cleary et.al [22]. Immunostaining with CD42b, anti-rabbit IgG antibodies and DAB chromogen was employed to allow visualization of infiltrated platelets in the lung tissue under light microscopy (Leica DM200).

Fixation of dried frozen lung tissue sections was carried out by immersing in 4% vol/vol paraformaldehyde in PBS for 15 minutes. Following a tap water wash, lung sections were subsequently immersed in 3% vol/vol hydrogen peroxide in ethanol to allow the blocking of endogenous peroxidase activity and bleaching of endogenous pigment. Anti-CD42b (SP219 clone, Abcam) was then incubated with the lung sections for 2 hours and washed with tap water before biotinylated anti-rabbit IgG antibodies were incubated with the lung sections for an hour. Lung sections were subsequently incubated with avidin and biotinylated horseradish peroxidase complex for another hour before the development of 1.2mM 3,3'-Diaminobenzidine (DAB) in 0.1 M Tris buffer and 0.03% hydrogen peroxidase were carried out in lung tissue for 10 minutes following a final tap water wash. Sections were counterstained with Gill's no. 2 haematoxylin and mounted with Dibutylphthalate Polystyrene Xylene (DPX).

Examination of platelets in the CD42b stained histological specimens was carried out under light microscopy using x40 objective lens. Infiltrated platelets were identified through the brown stain presented in the blue-haematoxylin treated lung parenchyma.

A digital camera connected to a light microscope with a 40x magnification, was used to randomly take images of the lung sections. The images were analyzed by ImageJ (National Institutes of Health) as described in Cleary et al [22] A colour deconvolution plugin (produced by G.Landini, University of Birmingham) was used to separate haematoxylin-DAB staining in RGB images into two component images: DAB and haematoxylin. DAB component images (brown images) were tested to set an acceptable threshold to process the DAB component images into separate greyscale images. See Figure 2 for representative images of each of the steps above.

ImageJ particle analysis was then used to determine the number and size of platelets. The size of 5 pixels^2 (equivalent to 1.5 μm^2) to infinity was chosen, in order to include all platelets (anything with 5 pixels^2 and above were counted) in the calculation of the number and size of the platelets.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Graphs were produced using GraphPad Prism 8.0.2 and for straight forward comparisons of 2 groups, data was compared using student's unpaired t-tests and for analysis of correlations, a Pearson's test was used to derive the R^2 value which was then analysed to generate a *p* value to indicate the likelihood of the magnitude of the R^2 value being generated randomly in the experimental data in the same programme.

Results

Intranasal bleomycin instillation leads to pathological features similar to IPF associated with changes in lung function, and accumulation of platelets into the lungs.

Intranasal administration of bleomycin to mice on 6 occasions over a period of seven days resulted in a significant decline in lung function at Day 35, as measured by a statistically significant decrease in FVC (saline group 0.74±0.03ml vs Bleomycin group 0.52±0.03mL, p< 0.01 Figure 3A), and a statistically significant increase in elastance (saline group 29.19±3.27 cmH_2O/ml vs Bleomycin group 55.14±5.50 cmH_20/ml, p< 0.001 Figure 3B). Furthermore, histological examination of lung tissue to measure areas of fibrosis revealed a statistically significant increase in the Ashcroft score after 35 days in mice administered bleomycin (saline group 0.16±0.02 vs Bleomycin group 4.10±0.15, p< 0.001 Figure 3C).

As platelets have been associated with lung injury, and pulmonary platelet recruitment occurs as a result of inflammation [22-25], the incidence of platelets was measured in the lavage fluid, and via histological analysis of lung sections. Thus, the induction of lung fibrosis by repeated instillation of low doses of bleomycin, was found to lead to an increase in the number of platelets recovered in the BAL fluid (saline group 29.4±2.9 million/animal vs Bleomycin group 52.3±6.0 million/animal, Fig 3D). Whilst this did not achieve statistical significance, histological analysis of lung tissue sections did reveal a statistically significant increase in the presence of platelets in the lungs of mice treated with bleomycin compared to saline treated animals (Fig 3E).

Platelet numbers in BAL fluid correlates with fibrotic biomarkers.

To investigate the relationship between platelet numbers recovered in the BAL fluid and fibrotic biomarkers the number of platelets recovered in the BAL fluid of each animal examined on Day 35 in this study was plotted against markers of lung fibrosis. As FVC decreased, representing a decline in lung function, the corresponding number of platelets in the BAL fluid increased (Fig 4A). Furthermore, as elastance increased, representing an increase in the stiffness of the lung tissue which would be expected in fibrotic lungs where there had been deposition of collagen and extracellular matrix, the number of platelets in the BAL fluid was also observed to increase (Fig 4B). Lastly, an increase in the Ashcroft score, used to determine an increase in the amount of collagen observed in the lung tissue, also corresponded to an increased number of platelets in the BAL fluid (Fig 4C). The correlation of each of these comparisons was statistically significant as determined by a Pearson test, with p values of 0.0007, 0.0024 and <0.0001 for the relationships between platelet number recovered in the BAL and FVC, elastance and Ashcroft score respectively.

Platelet depletion leads to a reversal of bleomycin-induced lung pathology and improved lung function and survival in mice.

In some experiments, on the morning of the start of bleomycin protocol (approximately 1h prior to bleomycin administration), a 98% decrease in circulating platelets was induced in animals treated with anti CD42b antibody compared to animals administered saline (Fig 5A).

Animals treated with bleomycin and previously administered anti-CD42b platelet depleting antibody showed an improved survival (platelet depleted group 75% vs platelet normal group 58%) (Fig 5B) and improved recovery from the bleomycin induced weight loss compared to bleomycin treated, saline administered animals with normal platelet numbers (Fig 5C).

There was an increase in the number of platelets recovered in the BAL fluid of bleomycin treated animals compared to the saline control animals (Fig 5D). However, there was no significant difference between the number of platelets in the BAL fluid of mice treated with anti-CD42b platelet depleting antibody compared to mice administered saline (Fig 5D). The presence of platelets in the BAL fluid of animals previously administered anti-platelet antibodies was considered to be due to a time dependent platelet recovery period as previously reported after intramuscular injection of this antibody (26), or differences in biodistribution for the antibody with respect to the extravascular location of platelets .

Studies were conducted to assess the effect of platelet depletion on lung function in mice exposed to bleomycin. At day 35, a non-significant 15% improvement in FVC (Fig 5E), and non-significant 16% improvement in elastance (Fig 5F) was observed in platelet-depleted mice compared to mice administered control IgG. Furthermore a statistically significant reduction of 16% in the Ashcroft score from 4.1 ± 0.2 in mice administered saline with normal circulating platelet levels compared to 3.4 ± 0.2 in platelet depleted animals administered antiCD42b antibody (Fig 5G) was observed. To give context to this difference a score of 3 indicates fibrotic changes in the alveolar walls (>3 x thicker than normal) and enlarged alveoli with no fibrotic masses, whereas a score of 4 indicates the presence of fibrotic masses in ≤ 10% of the field of view [21]. There was also a non-significant 40%

reduction in the levels of TGF-β detected in the BAL fluid of platelet-depleted bleomycin-treated animals compared to the platelet normal bleomycin treated animals (Fig 5H).

Discussion

Our results have shown that repeated administration of low doses of bleomycin was able to produce fibrotic changes in the lung that was associated with a significant decline in lung function, a feature observed clinically in patients with IPF [8]. These changes in lung function persisted for 35 days post the initial bleomycin administration and were associated with clear evidence of fibrotic changes in the lungs. Surprisingly most non clinical models of IPF do not assess lung function changes and most of these models often only investigate the acute effects of administering a high dose of the profibrotic agent bleomycin[9]. We believe our novel model utilising repeated exposure to low doses of bleomycin may be more akin to the clinical setting and provide an improved way of investigating both mechanisms and treatments of IPF, particularly effects of drugs on the decline in lung function. In support of this contention is the fact that FVC is a clinically relevant measurement of lung function and decline in FVC is often used as the primary endpoint examined in clinical trials for assessing IPF therapies [5,6,7]. FVC declines in IPF patients as the normally elastic lung tissue is replaced with much stiffer scar tissue, meaning that the lung is less able to expand, reducing the volume of air able to be inspired. Elastance is a measure of the pressure change that is required to induce a unit volume change i.e. the harder something is to expand the higher the elastance. As more of the lung tissue is replaced by stiffer scar tissue it will be more difficult to expand and thus the elastance will increase. This type of bleomycin protocol, where a repeated lower dose of bleomycin is utilised, is generally considered to provide a more progressive fibrosis that is more akin to the way that fibrosis develops in patients in the clinic which as we have shown in this investigation also results in progressive loss of lung function [9].

We have also shown using this novel model that platelet depletion attenuates the development of fibrotic lesions in the lungs, as demonstrated by the significantly lower Ashcroft scores measured in the platelet depleted animals. As well as the significant improvement in Ashcroft score there was also protection of the bleomycin-induced loss of lung function in the platelet-depleted mice. Our results would suggest that the changes in fibrosis may therefore contribute to the decline in lung function observed after chronic low dose bleomycin administration. Furthermore, given that TGF-β is a growth factor that has been implicated in the pathogenesis of IPF [27,28], the changes seen in lung function and Ashcroft score in platelet-depleted animals may have been in part driven by the reduction in TGF-β levels we also observed. Indeed, platelets are a major source of TGFβ [8,11,13] and the observation of platelet accumulation into lung tissue and the correlation between platelet numbers in the BAL and fibrotic markers in this model would suggest a direct contribution of platelets to the pathological response. Platelet recruitment into lungs has also been observed in models of asthma, sterile inflammation, and bacterial infection, and it has recently been reported that platelet recruitment into the lung can be independent of the recruitment of other inflammatory cells [10-16]. Furthermore, we have previously reported that platelet depletion in a murine model of chronic allergic lung inflammation was able to reduce remodelling events in the airway wall, processes that could not be inhibited by the chronic administration of dexamethasone that was otherwise effective at suppressing airways inflammation [22]. Moreover, the attenuation of the fibrosis observed in platelet-depleted animals is highly significant as it is of a similar level to that seen preclinically with the currently clinically available drugs, nintedanib and pirfenidone [29-32].

Although a number of anti-coagulant drugs have been tested (unsuccessfully) both preclinically in models of IPF and clinically in patients with IPF [9, 33], we are not aware of any studies that have evaluated the effects of anti-platelet therapy in this condition. However, given the poor prognosis associated with IPF and the continuing high level of medical unmet need with this disease, we anticipate that our results showing an important effect of platelets in contributing to both histological and physiological changes induced by repeated administration of low doses of bleomycin

to the lung, that consideration should be given to evaluating anti-platelet therapy in patients with IPF in the future.

Conclusion

We have established a novel model of pulmonary fibrosis following repeated administration of low doses of bleomycin which is associated with changes in relevant measures of lung function.

We have also shown in this study that depletion of platelets has been able to reduce bleomycin-induced fibrosis in the lungs and the associated decline in lung function. However, further research is required to elucidate the exact mechanism by which platelets are contributing to the fibrotic process and the changes in lung function. Although there was a statistically significant effect on Ashcroft score, and not lung function parameters suggesting that the platelet therapy may be a valuable adjunct therapy for IPF. Nonetheless, we hope that our findings may open up the possibility of investigating anti platelet therapy as a therapeutic strategy worth investigating further in patients with IPF.

Figure legends

Figure 1:

Representative images of Ashcroft scoring. Images captured using a x20 objective lens. The images show a healthy lung section with an Ashcroft score of 0 (A) and sections with Ashcroft scores of 2 (B), 3 (C) and 4 (D). Average scores of 2-4 are what are most commonly observed in bleomycin treated animals.

Figure 2:

Determination of standardized threshold for quantitative analysis of lung tissue sections in ImageJ. Threshold is chosen based on the criteria of minimal noise signal being presented as well as least platelets being lost in the resulting grey and grayscale image when comparing to the RGB images and DAB component images. (A) and (D) Representative CD41 immunostaining of lung tissue sections (x40 magnification) of sham and bleomycin-induced IPF mice respectively, taken under light microscopy. Platelets are immuno-stained in brown while lung tissues are counterstained in blue. (B) and (E) Representative DAB component images obtained from (A) and (D) respectively after processing with a colour deconvolution plugin. (C) and (F) Resulting representative grey and grayscale images produced after (B) and (E) underwent thresholding of 130. Black dots represents platelets that will be counted in the subsequent quantitative analysis of number and size of platelets after a suitable size filter is chosen.

Figure 3:

Lung function and histological changes confirm successful induction of IPF. A decline in lung function is seen in the bleomycin-dosed animals that is consistent with what would be expected in IPF. The decline in FVC (A) shows a decrease in the ability of the lung to draw in and expel air. This is likely due to the increase stiffness of the scarred lung tissue as demonstrated by the increase in elastance (B). The induction of IPF in the animals was confirmed histopathologically by observing a significant increase in Ashcroft score (C). An increase in the number of platelets recovered in the BAL was observed (D), this was correlated by an increase in the number of platelets observed in the lung tissue of bleomycin treated animals (E). n = 7-11 per group. Data is means± SEM. ** p<0.01, *** p< 0.001 compared to saline treated group.

Figure 4:

Platelet numbers in the BAL fluid correlate with markers of lung fibrosis. The number of platelets recovered in the BAL of all animals significantly correlated with a number of markers of lung fibrosis. As FVC decreases showing a decrease in the ability of the lung to draw in and expel air the number of platelets in the BAL increases (A) similarly as elastance increases showing an increase in the stiffness of the lung tissue the number of platelets in the BAL increases (B). As Ashcroft score increases showing an increase in the amount of collagen deposition observed in the lungs the number of platelets recovered in the BAL fluid. n=37 for each comparison, ** p<0.01, *** p< 0.001 compared to likelihood of experimental data being generated randomly.

Figure 5:

Effect of platelet depletion on development of fibrotic endpoints. The success of the platelet depletion was confirmed by measuring circulating platelet levels in n=3 animals from the saline treated platelet normal and platelet depleted animals (A). An improved survival rate was observed in platelet depleted animals compared to platelet normal animals treated with bleomycin (B). There was also an improved recovery in initial weight loss observed in platelet depleted animals compared to platelet normal animals treated with bleomycin (C). An increase in platelet numbers recovered in the BAL was observed in both the platelet normal and platelet depleted bleomycin treated animals compared to the saline treated controls. There was no difference in the number of platelets in the BAL of the platelet-normal and platelet-depleted bleomycin treated animals (D). An improvement in lung function was observed in platelet-depleted bleomycin treated animals as seen by an increase in FVC (E) and a decrease in elastance (F) compared to the platelet normal bleomycin treated animals. A significant improvement in Ashcroft score was also recorded in platelet-depleted bleomycin treated animals compared to platelet-normal bleomycin treated animals (G). A reduction in TGF-β concentration in the BAL of platelet-depleted animals compared to platelet-normal bleomycin treated animals was also observed (H).). n = 7-11 per group. Data are represented as mean± SEM. * p<0.05 ** p<0.01, *** p< 0.001 compared to saline treated group. ## p<0.01 compared to the platelet normal group. For the TGF-β analysis where 66% or greater of the group had values that were below the level of quantification (BLQ) the whole group is reported as BLQ. Where less than 66% of the group are outside the limits of quantification 0.5x the lowest level of quantification (LLOQ) has been used for the purposes of deriving a group mean. The LLOQ for this assay was 31.3 pg/mL.

Declarations

Declarations of interest: RC is an employee of Covance Laboratories ltd and CP and SP are in receipt of a grant from Covance supporting a PhD studentship for RC

The work to produce this paper did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

All animal experiments were conducted in accordance with the Animal (Scientific Procedures) Act 1986 in the UK and 2012 amendments, with the approval of local Animal Welfare and Ethics Committees at Covance Laboratories ltd.

RC, SJ, SP and CP conceived and planned the experiments, RC SJ and YW carried out the experiments. All authors contributed to the interpretation of the results. RC took the lead in writing the manuscript with input from all authors.

References:

1. Moeller A, Rodrigues-Lecompte JC, Wang L, Gauldie J, Kolb M (2006) Models of pulmonary fibrosis *Drug discovery today: disease models* **3** 243-249
2. Degryse AL, Lawson WE, (2011) Progress toward improving animal models for IPF *Am J Med Sci* **341**444-449
3. Ley B, Collard HR (2013) Epidemiology of idiopathic pulmonary fibrosis *Clin Epidemiol* **5** 483-492
4. Costabel U (2013) The changing treatment landscape in idiopathic pulmonary fibrosis *Eur Respir Rev* **24** 65-68
5. Noble PW, Albera C, Bradford WZ, Costabel U, Glassberg MK, Kardatzje D, King TE Jr, Lancaster L, Sahn SA, Szwarcberg J, Valeyre D, du Bois RM, CAPACITY study group. (2011) Pirfenidone in patients with idiopathic pulmonary fibrosis (CAPACITY): two randomised trials *Lancet* **377** 1760-9
6. Richeldi L, Costabel U, Selman M, Kim DS, Hansell DM, Nicholson AG, Brown KK, Flaherty KR, Noble PW, Raghu G, Brun M, Gupta A, Juhel N, Kluglich M, du Bois RM. (2011) Efficacy of a Tyrosine Kinase Inhibitor in Idiopathic Pulmonary Fibrosis *N Engl J Med* **365** 1079-87
7. Ley B, Collard HR, King TE (2010) Clinical Course and Prediction of Survival in Idiopathic Pulmonary Fibrosis *Am J Respir Crit Care Med* **183** 431-440
8. Wilson M.S., Wynn T.A. (2009) Pulmonary fibrosis: pathogenesis, eitology and regulation *Mucosal Immunology* **2** 103-121
9. Carrington R, Jordan S, Pitchford S.C., Page. C.P. (2018) Use of animal models in IPF research *Pulmonary Pharmacology and Therapeutics* **51** 73-78
10. Page C.P. (1988) The involvement of platelets in non-thrombotic processes *Trends Pharmacol Sci* **9** 66-71
11. Pitchford S, Cleary S, Arkless K, Amison R. (2019) Pharmacological strategies for targeting platelet activation in asthma *Current Opinion in Pharmacology* **46** 55-64
12. Petito E, Amison RT, Piselli E, Shah SA, Momi S, Pitchford SC, Gresele P, Page CP. (2018) A dichotomy in platelet activation: Evidence of different functional platelet responses to inflammatory versus haemostatic stimuli *Thrombosis research* **172** 110-118
13. Momi S, Pitchford s, Gresele P, Page CP. (2017) Platelets and Airway Diseases *Platelets in Thrombotic and Non-Thrombotic Disorders* 1149-1168
14. Zaid Y, Puhm F, Allaeys I, Naya A, Oudghiri M, Khalki L, Limami Y, Zaid N, Sadki K, El Haj RB, Mahir W, Belayachi L, Belefquih B, Benouda A, Cheikh A, Langlois MA, Cherrah Y, Flamand L, Guessous F, Boilard E (2020) Platelets can associate with SARS-CoV-2 RNA and are Hyperactivated in COVID-19 *Circulation Research* **127** 1404-1418
15. Eisinger F, Patzelt J, Langer HF (2018) The Platelet Response to Tissue Injury *Frontiers in Medicine* **5** 317
16. Lefrancais E, Ortiz-Munoz G, Caudrillier A, Mallavia B, Liu F, Sayah DM, Thornton EE, Headly MB, David T, Coughlin SR, Krummel MF, Leavitt AD, Passegue E, Looney MR (2017) The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors *Nature* **544** 105-109

17. Babin AL, Cannet C, Gérard C, Saint-Mezard P, Page CP, Sparrer H, Matsuguchi T, Beckmann N (2012) Bleomycin-induced lung injury in mice investigated by MRI: Model assessment for target analysis *Magnetic Resonance in Medicine* **67** 499-509
18. Bergmeier W, Rackebrandt K, Schroder W, Zirngibl H, Nieswandt B. (2000) Structural and functional characterization of the mouse von Willebrand factor receptor GPIb-IX with novel monoclonal antibodies. *Blood*. **95**:886-93.
19. Nieswandt B, Bergmeier W, Rackebrandt K, Gessner JE, and Zirngibl H. (2000) Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice. *Blood*. **96**:2520-27
20. Ashcroft T, Simpson JM, Timbrell V (1988) Simple method of estimating severity of pulmonary fibrosis on a numerical scale *J Clin Pathol* **41** 467-470
21. Hübner R-H, Gitter W, El Mokhtari NE, Mathiak M, Both M, Bolte H, Freitag-Wolf S, Bewig B. (2008) Standardized quantification of pulmonary fibrosis in histological samples *Bio Techniques* **44** 507-517
22. Cleary SJ, Hobbs C, Amison RT, Arnold S, O'Shaughnessy BG, Lefrançais E, Mallavia B, Looney MR, Page CP, Pitchford SC (2019) LPS-Induced Lung Platelet Recruitment Occurs Independently from Neutrophils, PSGL-1, and P-selectin. *American Journal of Respiratory Cell and Molecular Biology* **61** 232-243
23. Pitchford SC, Riffo-Vasquez Y, Sousa A, Momi S, Gresele P, Spina D, Page CP (2004) Platelets are necessary for airway wall remodelling in a murine model of chronic allergic inflammation *Blood* **103** 639-647
24. Pitchford SC, Momi S, Baglioni S, Casali L, Giannini S, Rossi R, Page CP, Gresele P (2008) Allergen induces the migration of platelets to lung tissue in allergic asthma *American Journal of Respiratory and Critical Care Medicine* **177** 604-612
25. Amison RT, O'Shaughnessy BG, Arnold S, Cleary SJ, Nandi M, Pitchford SC, Bragonzi A, Page CP (2018) Platelet depletion impairs host defense to pulmonary infection with *Pseudomonas aeruginosa* in mice *American Journal of Respiratory Cell and Molecular Biology* **58** 331-340
26. Amison, R., Cleary, S. J., Riffo-Vasquez, Y., Bajwa, M., Page, C. P., & Pitchford, S. C. (2018). Platelets Play a Central Role in Sensitization to Allergen. American Journal of Respiratory Cell and Molecular Biology, 59(1), 96-103.
27. Chambers RC, Mercer PF (2015) Mechanisms of Alveolar Epithelial Injury, Repair and Fibrosis *AnnalsATS* **12** S1 16-20
28. Sheppard D (2006) Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis *Proc Am Thorac Soc* **3** 413-417
29. Schelegle ES, Mansoor JK, Giri S, (1997) Pirfenidone attenuates bleomycin-induced changes in pulmonary functions in hamsters *Proceedings of the society for experimental biology and medicine* **216** 392-397
30. Oku H, Shimizu T, Kawabata T, Nagira M, Hikita I, Ueyama A, Matsushima S, Torii M, Arimura A (2008) Antifibrotic action of pirfenidone and prednisolone: Different effects on cytokines and growth factors in bleomycin induced murine pulmonary fibrosis *European Journal of Pharmacology* **590** 400-408
31. Wollin L, Maillet I, Quesniaux V, Holweg A, Ryffel B (2014) Antifibrotic and anti-inflammatory activity of the tyrosine kinase inhibitor Nintedanib in experimental models of lung fibrosis *J Pharmacol Exp Ther* **349** 209-220
32. Carrington R, Jordan S, Dewhurst J, Thang K, Curkovic A, Stapleton N, Meecham K (2017) *Am J Respir Crit Care Med* **195** A6787
33. Noth I, Anstrom KJ, Bristol Calvert S, de Andrade J, Flaherty KR, Glazer C, Kaner RJ, Olman MA, for the Idiopathic Pulmonary Fibrosis Clinical Research network (2012) A Placebo-Controlled Randomized Trial of Warfarin in Idiopathic Pulmonary fibrosis *Am J Respir Crit Care Med* **186** 88-95

Figure 1

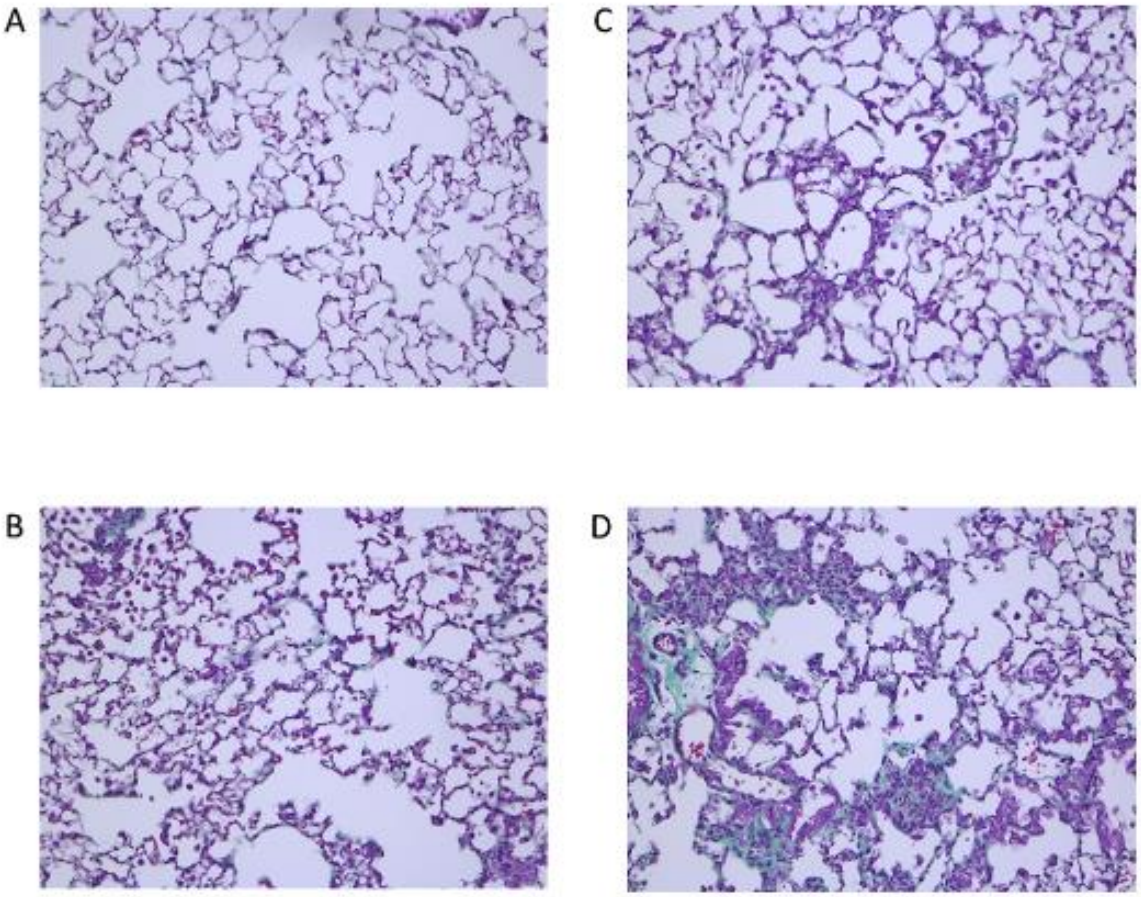


Figure 2

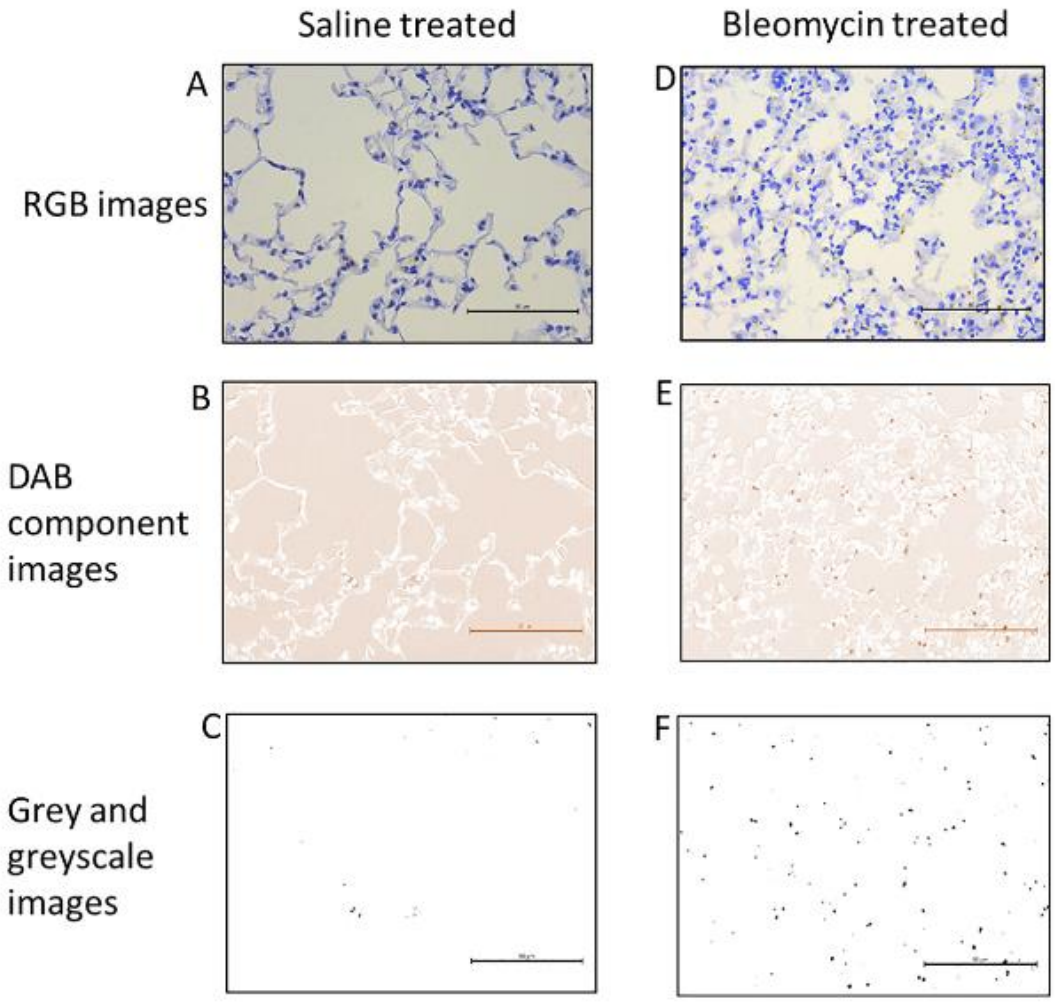

Saline treated
Bleomycin treated
A
D
RGB images
B
E
DAB component images
C
F
Grey and greyscale images

Figure 3

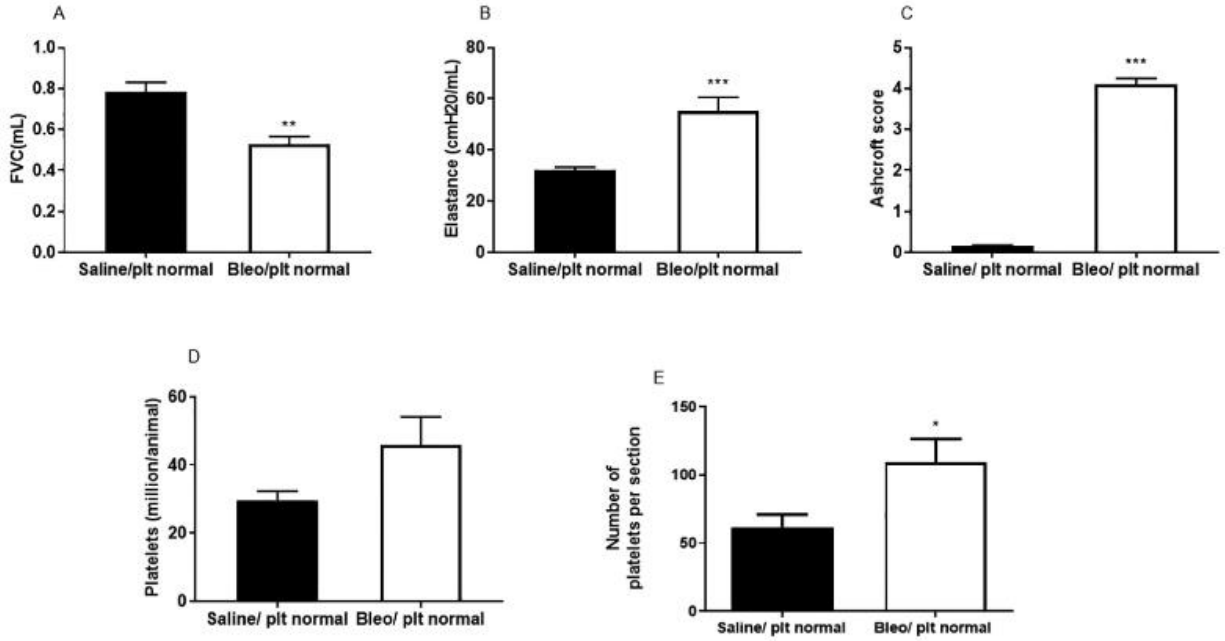

A
FVC(mL)
1.0
0.8
0.6
0.4
0.2
0.0
**
Saline/plt normal
Bleo/plt normal
B
Elastance (cmH20/mL)
80
60
40
20
0
***
Saline/plt normal
Bleo/plt normal
C
Ashcroft score
5
4
3
2
1
0
***
Saline/ plt normal
Bleo/ plt normal
D
Platelets (million/animal)
60
40
20
0
Saline/ plt normal
Bleo/ plt normal
E
Number of platelets per section
150
100
50
0
*
Saline/ plt normal
Bleo/ plt normal

Figure 4

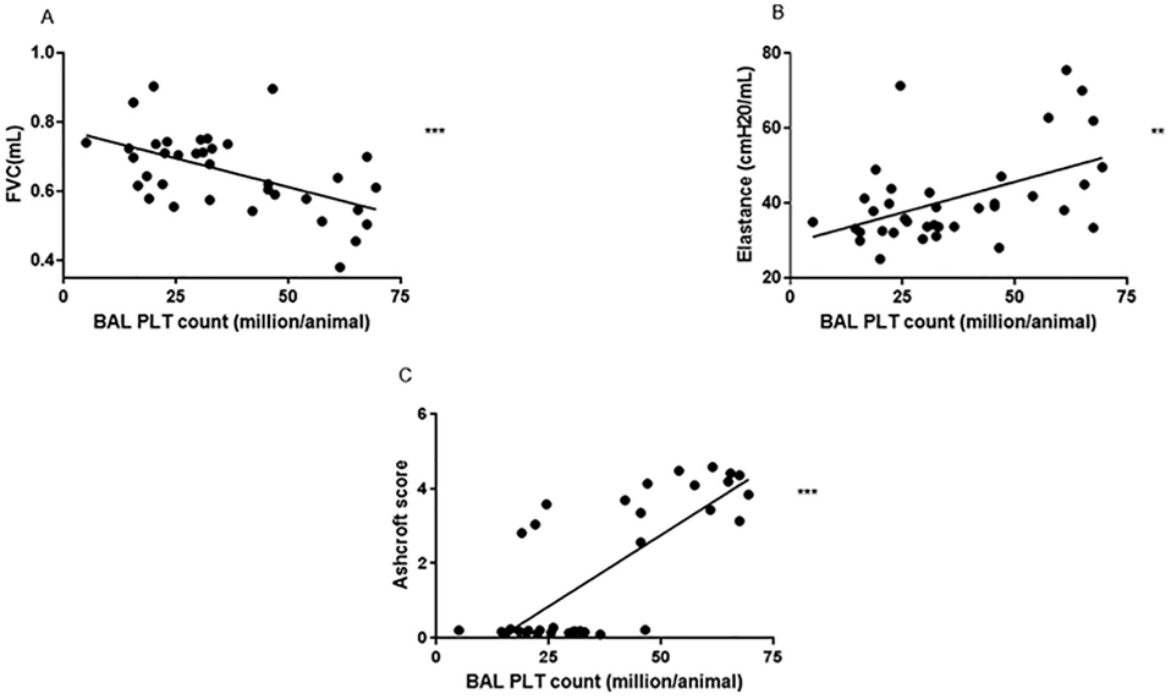
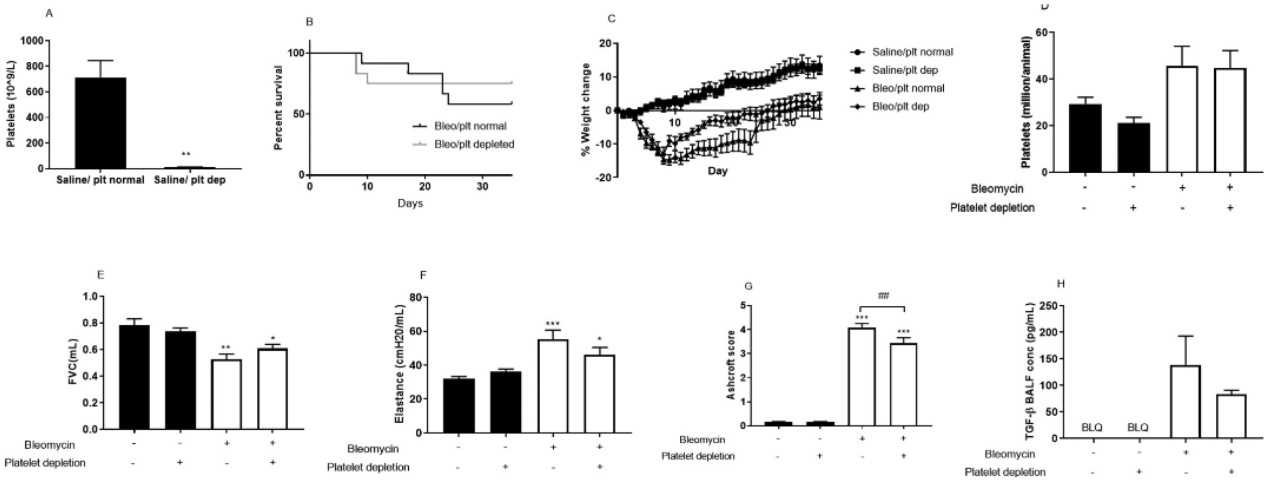

A
FVC(mL)
1.0
0.8
0.6
0.4
0
25
50
75
BAL PLT count (million/animal)
***
B
Elastance (cmH20/mL)
80
60
40
20
0
25
50
75
BAL PLT count (million/animal)
**
C
Ashcroft score
6
4
2
0
0
25
50
75
BAL PLT count (million/animal)
***

Figure 5


A
Platelets (10^9/L)
Saline/ plt normal
Saline/ plt dep
B
Percent survival
Bleo/plt normal
Bleo/plt depleted
Days
C
% Weight change
Day
Saline/plt normal
Saline/plt dep
Bleo/plt normal
Bleo/plt dep
D
Platelets (million/animal)
Bleomycin
Platelet depletion
E
FVC(mL)
F
Elastance (cmH20/mL)
G
Ashcroft score
H
TGF-β BALF conc (pg/mL)
BLQ