MAHARAJ S.S. FIBROCYTES IN CHRONIC LUNG DISEASE PHD

FIBROCYTES IN CHRONIC LUNG DISEASE

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Abstract

The focus of this thesis was the role of fibrocytes in chronic lung disease. These bone marrow derived cells have been identified in the lung and the circulation in patient samples and animal models of lung injury. However, the precise mechanistic role of the fibrocyte is still to be elucidated.

Better tools are needed for assessment of lung changes in animal models of chronic lung disease. Live assessment allows for real time observation of changes and gives a readout which can be translated to humans who undergo similar tests. In this thesis, we adapted an existing model of lung injury so that it could be observed live. We delivered a discrete treatment to a single lung lobe and monitored the successful delivery. This live assessment of lung changes in an animal model represents a novel advancement to this area of work.

I also developed a robust system to examine the relationship between fibrocyte response and cytokine expression previously identified in chronic lung disease. I characterised the kinetics of fibrocytes in circulation and the lung in animal models. We found a connection between cytokine expression and fibrocyte mobilisation. Our model showed that fibrocyte mobilisation in the presence of existing lung injury does not improve and rather can worsen existing lung injury. This was a significant finding as it confirms the role of the fibrocyte as a participator or conductor in fibrogenesis. This finding suggests that this cell may be an unidentified participator in the development of chronic lung diseases where there is significant cytokine expression.

Finally, we contributed to the ongoing characterisation of the fibrocyte as a prospective biomarker. We confirmed the cell's identity by characterising it by its known markers and biological characteristics. We also identified the presence of this cell in chronic lung disease and linked its presence to disease progression.

Acknowledgements

I would like to thank my supervisors Drs Martin Kolb and Jack Gauldie for the opportunity to learn from them and for their confidence that I could accomplish this PhD project. Without their constant support and guidance, I would not have been able to complete this work. Their discipline and mentorship inspired me in a number of ways academically, professionally and personally.

I thank McMaster University for the opportunity to pursue this degree and also The Firestone Institute for Respiratory Health for their help in providing the resources I needed to perform my research. I express my sincerest gratitude to members of my supervisory committee, Drs Gerard Cox and Zhou Xing, who guided me throughout my training. I would also like to thank all of the Principal Investigators including Dr. Kjetil Ask whose critique and support was very valuable. Thanks goes out to all members of the laboratory team, especially Fuqin Duan and Jane Ann Smith, without their incredible help I would not have succeeded in my training and experimental success.

I would like to thank my family; my dear spouse Lindsay, my parents Dayanand and Taramatee and my siblings Shobha, Rameshwar, Nicole and Reggie. Without your love and support I would not have been able to accomplish this work.

I give thanks to God for the strength and guidance to do this work. The path to completing a Ph.D. was not an easy one, but as Lord Krishna told Arjuna “Without being attached to the fruits of activities, one should act as a matter of duty; for by working without attachment, one attains the supreme” (Bhagavad Gita: Chapter 3, verse 19). I dedicate the merit of this work to the patients who suffer from the diseases that we researched in this thesis and hope that our findings contribute to in some way to improving their lives

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Abbreviations

TGF-β	Transforming Growth Factor Beta
GM-CSF	Granulocyte macrophage colony stimulating factor
MIP2α	Macrophage Inflammatory Protein 2 alpha
TLC	Total lung capacity
Pfu's	Plaque forming units
3D	Three dimension
ANOVA	Analysis of Variance
CD	cluster of differentiation
CXCR4	chemokine receptor type 4
αSMA	alpha smooth muscle actin
IL-1β	Interleukin 1 Beta
TNF-α	Tumour necrosis factor alpha
IL-6	Interleukin 6
IL-10	Interleukin 10
MIP1α	Macrophage inflammatory protein 1 alpha
MIP1β	Macrophage inflammatory protein 1 beta
MCP-1	Monocyte chemoattractant protein 1
IL-8	Interleukin 8
GROα	Growth regulated protein alpha
CCR2	chemokine receptor type 2
CCR7	chemokine receptor type 7
CXCL12	CXC motif chemokine 12

IFNγ	Interferon gamma
IL-1α	Interleukin 1 alpha
IL-1	Interleukin 1
IL-12	Interleukin 12
IL-10	Interleukin 10
αvβ3	alpha v beta 3
SDF-1	stromal cell derived factor
CCL12	CC motif ligand 12
NH_4CL	Ammonium Chloride
H_2O	Water
$KHCO_3$	Potassium bicarbonate
NaEDTA	disodium edentate
RPMI	Rosswell Park Memorial Institute
IgG	Immunoglobulin G
DAPI	4',6-diamidino-2-phenylindole
Col-1	collagen 1
Col-3	collagen 3
B_2m	beta-2-microglobulin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
NaF	Sodium Flouride
Na_3VO_4	Sodium orthovanadate
µg	microgram
µl	micro-litre
FACS	Fluorescence activated cell sorting

RPMI	Dulbecco’s modified eagle’s medium
PBS	Phosphate buffered saline
FBS	Fetal Bovine Serum
DMSO	Dimethyl sulfoxide
PE	Phycoerythrin
FITC	Fluorescein isothiocyanate
PerCP	Peridinin chlorophyll protein
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonuclei acid
RNAse	Ribonuclease
cDNA	complementary Deoxyribonucleic acid
BAL	Broncho alveolar lavage
TRITC	Tetramethylrhodamine-5-(and 6)-isothiocyanate
EDTA	Ethylenediaminetetraacetic acid

Declaration of Academic Achievement

The work in this thesis was undertaken wholly by Shyam Maharaj with contributions and assistance as follows. Flexivent™ techniques were taught by Kjetil Ask and the Flexivent™ experiments were performed together with Kjetil Ask with contributions from Antje Ask for unilateral administrations and Chantal Saab for animal imaging. Contributions to Chapter 2 were made by Kjetil and Antje Ask. Fibrocyte experiments using animal models and Flow cytometry were all performed by Shyam Maharaj, technical assistance was often rendered by Fuqin Duan and Gordon Gray. Immunofluorescence experiments and biochemical assays were performed by Shyam Maharaj, training was provided by Daniela Farkas and Jane Ann Smith respectively. Collection of patient blood samples was performed by Shyam Maharaj, Tiffany Mintah, Eva Baroke and the University of Nottingham. Flow cytometry of patient samples was performed with technical assistance from Fuqin Duan. Cell culture was performed by Shyam Maharaj.

1.0 General Introduction

Overview: The topic of my thesis is to better understand the role of a newly described cell in the circulation which arises from the bone marrow and homes to sites of tissue injury, the fibrocyte. This cell may contribute to repair under normal circumstances or to chronic scarring or fibrosis under pathologic conditions, such as in Pulmonary Fibrosis. To place these studies in context, I first worked on ways to develop non-invasive methods to follow the process of fibrogenesis in an animal model of pulmonary fibrosis, eventually pursuing the circulating fibrocyte as a possible biomarker of the fibrotic process. This led to an extensive set of studies (the major portion of the thesis) with a rat experimental model of pulmonary fibrosis, based on the transgenic expression of active TGF-β in the lung through administration of an adenovirus vector. I used this model to characterize the response of the fibrocyte throughout the process of fibrosis and how the fibrocyte responds to a number of well characterized cytokines, using a similar gene transfer approach. Finally, I examined several chronic lung disorders in the human to see how the human cell behaves and to begin to determine whether the fibrocyte could be a valuable biomarker of Idiopathic Pulmonary Fibrosis. These three aspects are dealt with separately in this thesis and make up Chapters 2, 3 and 4. Chapter 5 is the Discussion and I have tried to bring the salient aspects of these different studies together.

Fibrosis: Fibrosis is a common phenomenon in biology and chronic disease, and can affect all organ systems of the body. Fibrosis is defined by the overgrowth, hardening and or scarring of various tissues and is attributed to excess deposition of extracellular matrix (ECM) components, in particular, collagen (Wynn 2008). The normal deposition of ECM is a common process in lung repair. ECM deposition is fundamental to the pathogenesis of several common conditions in the lung, such as infections and airway diseases like asthma (Mehrad and Strieter 2012). There are many proposed factors which may contribute to the switch from normal ECM deposition to excessive or aberrant deposition resulting in fibrosis, and these factors are a major area of study in fibrosis.

The work in our lab focuses on pulmonary fibrosis (PF). Pulmonary fibrosis affects an estimated 30,000 Canadians with a projected 5,000 deaths each year (Canadian Pulmonary Fibrosis Foundation). PF has several known causes including systemic disease, occupational diseases such as exposure to asbestos and silica, and also as an outcome of exposure to certain medications. In cases where there is an identifiable cause of fibrosis, addressing this cause can result in improvement of the lung fibrosis. However the most unpredictable type of PF is Idiopathic PF (IPF). Although there have been numerous epidemiological studies which have highlighted contributing factors, we still do not have a single known cause of IPF (Kottmann et al. 2009) (Gauldie, Kolb, and Sime 2002). Patients

diagnosed with IPF may experience various patterns of progressive decline in lung function (Ley, Collard, and King 2011) resulting in irreversible lung damage. This progressive disease usually results in respiratory failure 3-5 years after diagnosis (King et al. 2001).

Challenges in IPF: There are two major challenges faced in IPF: 1) The characteristic progressive fibrosis which results in death from respiratory failure (Bjoraker et al. 1998), and 2) Treatments which have been used clinically, including immunosuppressants and corticosteroids, have been shown to be either ineffective or possibly harmful in the treatment of IPF. Only lung transplantation has shown an alteration in the natural progression of IPF (Mehrad and Strieter 2012).

A recent review of the ongoing clinical trials for IPF therapies (Baroke, Maharaj, and Kolb 2011) discussed how ongoing and completed trials have enhanced our understanding of IPF and also helped identify potential targets for future trials. Importantly however, as this review also highlighted, although many of these trials have been very well designed, there has been no major breakthrough to date. In the interim, it is important that we consider other avenues which may be of benefit to patients who suffer from IPF. The heterogeneity of the patient's progression is considered to be a major confounding factor and one which has complicated the design of clinical trials (Loomis-King, Flaherty, and Moore 2013). This heterogeneity is one of the reasons why several investigators have chosen to focus on major components of the disease which can be modulated in almost all patients diagnosed with IPF.

Clinically, the progression of IPF is variable (see figure 1) and this progression presents a unique challenge. Each IPF patient may behave differently and this requires vigilance and flexibility in the design of their treatment plans.

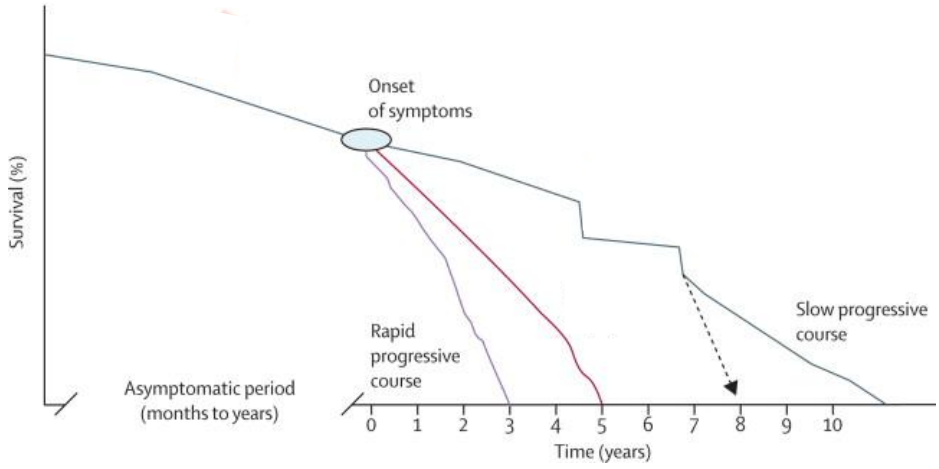


Figure 1: The variable progression course of IPF (modified from King, Pardo, and Selman 2011)

Further complicating the investigation of this disease, animal models used to examine the pathogenesis of IPF and develop therapeutic interventions are limited in their ability to faithfully reproduce clinical characteristics and reliable landmarks of disease progression. The ultimate goal in IPF research is to identify treatment strategies which can transform IPF from a fatal disease to one which can be cured or is manageable as a chronic disease. Most importantly, the goal is to halt its progressive nature. These treatments would ultimately have to be tested initially in animal models and greater clarity about disease landmarks in these models would better enable us to interpret results.

Local responses in the lung: To better characterise major components of chronic lung diseases, there is a need for a closer study of both the local responses (within the lung) and systemic responses (from the circulation and bone marrow) to lung injury. This approach may better enable us to appreciate the body's response to injurious processes that initially originated in the lung. Furthermore, an understanding of the cells which respond to lung injury and the roles which they play, may allow us to utilise these as targets for modulating disease or as

biomarkers for predicting disease progression. Looking closer at the excessive accumulation of ECM seen in pulmonary fibrosis, this change is believed to be as a result of the modulation of two homeostatic mechanisms: 1) proliferation and subsequent apoptosis of fibroblasts and 2) accumulation and subsequent breakdown of ECM. Alterations in the balance of either of these two processes can result in accumulation of ECM or increase in the number of fibroblasts and subsequent increase in the accumulation of ECM (Todd, Luzina, and Atamas 2012).

The cellular contribution to fibrosis has long been acknowledged. Early case reports and histological analysis of IPF patient biopsies led to the description of alveolitis (Scadding and Hinson 1967) (Katzenstein and Myers 1998). Inflammation and the significance of its role in the progression of IPF has been called into question within recent time, largely due to the lack of response to the anti-inflammatory effects of corticosteroids in patients diagnosed with IPF (Richeldi et al. 2003). There is however a tremendous amount of evidence, including animal studies, cell culture experiments and diagnostic/case reports in humans, which support the study of cellular responses in IPF. A recent review by Todd and colleagues (Todd, Luzina, and Atamas 2012) gives an excellent synopsis of the evidence suggesting roles for T lymphocytes, Macrophages, B lymphocytes and related cytokines, cell surface molecules and integrins in fibrosis.

As mentioned above, clinical experience and some studies have suggested that the progression of IPF is not always a slow progressive event, rather some patients may experience sharp irreversible declines in lung function (figure 1), which may be associated with an unpredictable disease course (Kim, Collard, and King 2006). The significance of these acute exacerbations on the progression and mortality seen in IPF is unclear, possibly due to differences in study design and also differences in the case definition of acute exacerbation (Collard et al. 2007). One retrospective study following a cohort of patients showed a 9.6% 2 year incidence of acute exacerbation, and the mortality for these patients was 78% (Kim et al. 2006).

The lack of validated biomarkers currently impair our ability to quickly detect impending changes in the disease status of patients who are diagnosed with IPF and those who are at risk for an acute exacerbation. The ability to better understand the contributors to changes in lung function and and also to be able to monitor a patient systemically rather than relying only on lung function changes would equip us with more time to make necessary treatment changes which may alter the prognosis for these patients.

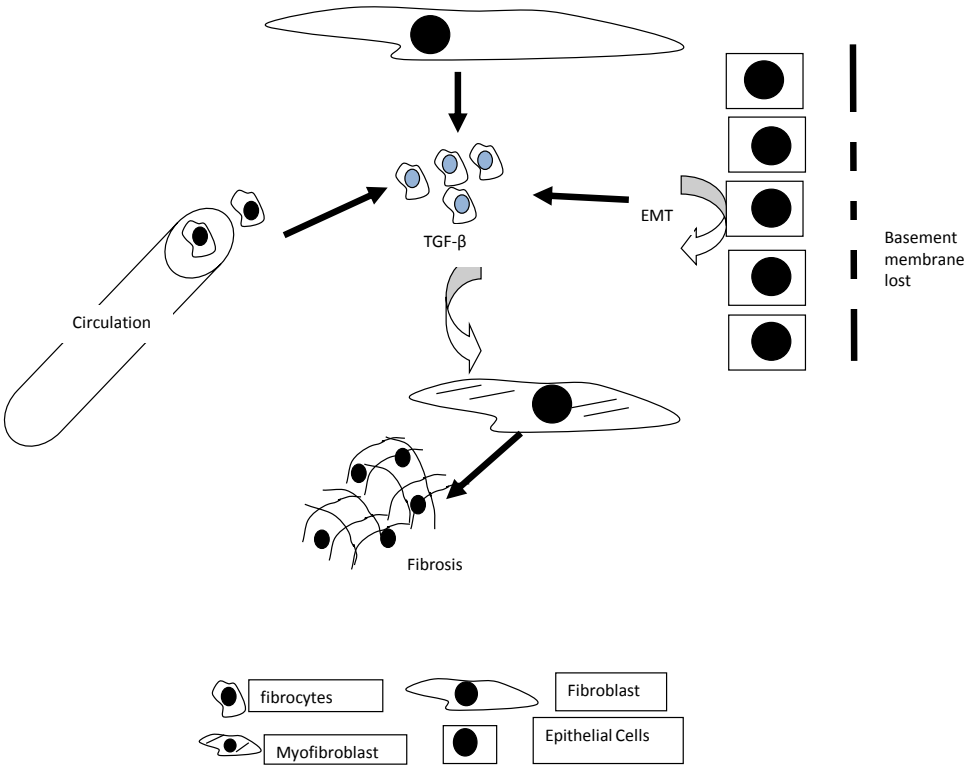


Figure 2: Proposed mechanisms of ECM accumulation (modified from Strieter and Mehrad 2009). The accumulation of ECM is proposed to arise from different cellular sources. 1) The proliferation of resident fibroblasts, 2) The recruitment of mesenchymal progenitor cells (fibrocytes) from the bone marrow (Bucala et al. 1994) and 3) Epithelial to Mesenchymal Transition (EMT) (Willis et al. 2005). All of these result in a pool of cells which, when exposed to TGF-β and other local factors, may undergo a transformation into myofibroblasts (Hinz et al. 2007) which can subsequently result in fibrosis (Strieter 2008).

Regarding the contribution of different cell types to fibrosis; of recent interest is the role of mesenchymal progenitor cells (fibrocytes) (see figure 2) and their potential contribution to the pathobiology of fibrotic lung injury. Fibrocytes were first characterised by Bucala in 1994 (Bucala et al. 1994) and have since received great interest in understanding their exact functions as well as their potential to be a biomarker of disease activity. Work by our lab (Moeller et al. 2009) and other groups (Strieter et al. 2009), (Kisseleva et al. 2006),(LaPar et al. 2011),(Abe et al. 2001),(Fujiwara et al. 2012) has added to the study of

circulating fibrocytes and their potential contribution in the prediction and or modulation of lung fibrosis. This cell's potential as a prospective biomarker was highlighted in a study where it was shown that the survival of patients with the concentration of circulating fibrocytes greater than 5% of total blood leukocytes was 7.5 months compared with 27 months for patients with fibrocyte counts less than 5% (Moeller et al. 2009).

Goals: There is, however, need for further validation with larger numbers of IPF patients and also the need to better understand the biology of the fibrocyte and the signals which recruit this cell to the lung. Furthermore, there is still a need to understand the kinetic response of fibrocytes to fibrotic lung injury. Documentation of variations of fibrocyte numbers in healthy subjects versus those with fibrotic lung injury would enable us to correlate the rise and fall in circulating fibrocyte numbers with injury, repair or an aberration of either of these two processes.

In this thesis, I will address some of the deficiencies in the knowledge of fibrocytes in IPF. Specifically, I will look at live assessment of lung function in animal models of lung injury and also the ability of these assessment methods to detect changes in disease progression. To better understand the role of the fibrocyte, I will look at the effects of modulating fibrocyte responses in animal models of lung injury. I will identify the presence of fibrocytes in patients diagnosed with chronic fibrosing lung diseases and attempt to dissect possible roles for these cells. I will conduct animal studies looking at cytokine expression and its relationship to fibrocyte response and mobilisation. I will contribute to the study of the fibrocyte as a prospective biomarker and discuss its utility as a tool in the 'live assessment' of patients diagnosed with fibrotic lung disease.

Hypothesis: A potential contributor to the characteristic fibroblast and myofibroblast accumulation in lung tissue is the fibrocyte. I propose that fibrocytes are recruited by signals generated in lung injury and that this recruitment can be modulated leading to potential changes in the resulting fibrosis

2.0 Live assessment of lung function

Despite limitations, the use of animal models to recreate human diseases is a vital component of translational research (Mouratis and Aidinis 2011). These animal models are often relied upon to reproduce complex and chronic biological processes (Ask et al. 2006). To date, there are no perfect models of pulmonary fibrosis which faithfully replicate all aspects of the disease. Many animal models are plagued by variability in the amount and approach of injury delivery. This may be driven by the need to show an injurious effect in a short period of time due to cost and other considerations. These models are often terminal models, requiring multiple animals for sequential time point determinations. Moreover, they may not be effective in faithfully replicating human disease (Esmon 2004) and/or may result in disease which is uncharacteristically heterogeneous (Chua, Gauldie, and Laurent 2005).

Over the last 30 years, numerous animal models of pulmonary fibrosis (Moore and Hogaboam 2008) have been developed to investigate pathological mechanisms implicated in these diseases and potential treatment options. There are many desired characteristics of animal models used for performing lung investigations. One of them is the ability to clearly localize the effects of an intervention in the lung tissue and another is the ability to translate the findings from an animal model, both at a single time-point, as with terminal animal studies, and in a continuous manner or via live assessment, to clinically relevant interventions.

Currently there are not many options available for localising delivery of injury to the lung of experimental animals and identifying the resultant changes. In an attempt to compensate for this, it is usual at sacrifice to submit one whole lung lobe for histology and the other lung for biochemical analysis. Histological tests are then performed on random areas and the average values, such as cell counts, seen in these random selections, are used as a measure of the overall impact. However, critics of this approach have suggested that sometimes the treatment is not equally distributed throughout all lung areas and results may be misleading. Advancements in molecular imaging have made newer techniques available for small animals. However, currently this approach is still quite costly and inconvenient for routine verification of pathogen delivery (Piwnica-Worms, Schuster, and Garbow 2004) as well as for the repeated and continuous assessment of a therapy or ongoing pathology.

There is also a need for additional methods to monitor the physiologic responses of the animal to lung injury which would help to optimise treatment and elicit the right amount of damage required for an intervention study, rather than waiting

until the end of the study to observe the pathologic outcome. With the availability of more tools for live assessment, we would also be better equipped to ensure that there is not too much injury that impairs the health of the animal to a degree that is ethically unacceptable. These additional tools would also help reduce the number of studies required prior to the actual experiment to ensure that the model is validated in terms of safety and both systemic and local effects, thereby reducing costs, labor and number of animals

Currently, there is an increased interest in delivery mechanisms to the lung that are precise and which can handle small volumes of liquid or particles in suspension as well as the need for us to better understand the role of smaller areas of injury in the pathogenesis of pulmonary fibrosis. The need for this 'zooming in' approach is driven by therapeutic advances which have shown the potential for the delivery of small volumes/particles to specifically affected areas (Azarmi, Roa, and Lobenberg 2008). We are also developing a greater understanding of the heterogeneity of human pulmonary fibrosis, which is characterised by focal points of destruction known as fibroblastic foci with increased collagen deposition occurring adjacent to normal parenchyma (Raghu et al. 2011)(King, Pardo, and Selman 2011). Furthermore, we now know that zooming into these fibroblastic foci can help our understanding of fibrogenesis.

The importance of live assessment has been highlighted by several literature reviews which have discussed the possibility that the approach of utilising new compounds in a preventive rather than therapeutic manner in experimental animals may not indicate the true efficacy of these treatments when translated to the clinic (Moeller et al. 2008)(Moore et al. 2013). Ask and colleagues (Ask et al. 2008) compared terminal methods of lung pathology assessment (histomorphometry and hydroxyproline content) to clinical measures of lung function (micro-CT and lung compliance) and the success in this study promised an option to monitor disease progress in an animal using non-invasive measures. The availability of more clinically relevant biomarkers in animal models would also make translation of these experimental findings easier.

We sought here to expand the live lung function assessments to be an approach that can be administered to an animal, given inhalation anesthesia only, and which would recover from anesthetic within 10 minutes. This would allow us to follow one single animal after multiple interventions and reduce animal mortality by removing the need for surgical anesthetic and tracheal dissection to intubate animals for Flexivent™ measurement of compliance and elastance. We also looked at the feasibility of administering agents, therapeutic or pathologic, to one individual lung and use the untreated lung as a control. This would allow us to be

able to track an individual animal's response to discrete injury as well as to reduce the number of animals needed to perform a pre-clinical trial.

I undertook this aim as a proof of principle to help me dissect the hallmarks of chronic lung disease and the usefulness of animal models in reproducing these hallmarks. I believe with this assurance, I can now further dissect the mechanisms, both local and systemic, which contribute to creating these characteristic landmarks of lung disease.

In our lab we have developed the method to deliver treatments non-surgically via the trachea of sedated rats (further discussed in methods section) and this has allowed us to reproducibly achieve a fibrotic response by the administration of Bleomycin and also TGF-β. Both of these treatments have been reported to induce fibrotic changes (Fleischman et al. 1971) (Sime et al. 1997), characterised by deposition of collagen and increased lung stiffness. Traditionally as mentioned above assessments of fibrosis are performed by dissecting the lung and performing histological quantifications. Although similar in some ways, Bleomycin and TGF-β differ in the histological pattern of fibrosis they produce as well as the time course of disease in the animal model (Izbicki et al. 2002). The use of alternate methods for the investigation of fibrotic changes would allow us to remove any bias of time-point and fibrotic distribution which can occur in these different systems. We utilised these two different systems to allow us to compare the capability of our assessment of live lung function described below. We also utilised an adenovirus vector which expresses the chemokine MIP2α. This chemokine was administered to the lung and found to result in a large increase in neutrophil influx into the lung without resulting in chronic lung injury (Foley et al. 1996). We proposed to utilise MIP2α as a potent signal which we could track in the lung to test our live assessment.

2.1 Methods

Treatments

Animals: For all experiments female Sprague-Dawley rats (Charles River) weighing 225-250 grams were used. These animals were housed under special pathogen free conditions.

Anesthesia: Rats were anesthetized with isoflurane (MTC Pharmaceuticals).

Intratracheal administration: Once fully sedated, the rat was then placed in a dorsal recumbent position and maintained this way for the entire procedure. The rat's oropharynx was visualized with a laryngoscope and intubated with a blunted

18 gauge needle. Treatments listed below suspended in a maximum volume of 300µl were then administered using a 1ml syringe.

Bleomycin group: Rats were administered Bleomycin intratracheally (Hospira, DIN#02131692) at a dose of 0.56 units

TGF-β group: Ad TGF-$\beta1^{223/225}$ an adenovirus vector that expresses biologically active porcine TGF-β1as described previously (Sime et al.1997) was administered intratracheally at a dose of $2x10^8$ pfu's

MIP2α group: AdMIP2α an adenovirus vector that expresses MIP2α, a chemotactic chemokine for neutrophils described previously (Foley et al.1996) was administered intratracheally at a dose of $1x10^8$ pfu's

Live assessment

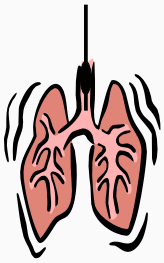
The rat was anesthetized with isoflurane. Once fully sedated the rat was then placed in a dorsal recumbent position and maintained this way for the entire procedure. The rat's oropharynx was visualized with a laryngoscope and intubated with a blunted 18 gauge needle (shown in diagram below).

The rat was then ventilated with a computer-controlled small animal ventilator. (SCIREQ, Montreal, Canada) with a mixture of oxygen and isoflurane to ensure the animal remains sedated throughout the entire procedure. Pressure volume (PV) loops were measured with FlexiVent™ (v5.1, Scireq, Montreal, PQ) allowing the rats to passively expire for 1 second against 2 cm H_2O positive end-expiratory pressure and then applying 7-step increases and decreases in volume as described by Ask and colleagues (Ask et al. 2008).

The outcomes measured were the parameter k of the Salazar-Knowles equation which reflects the curvature of the upper portion of the deflation PV curve (Ask et al. 2008). Lung stiffness was also measured by looking at data collected on Compliance and Elastance. PV loops capture the quasi-static mechanical properties of the respiratory system, the Salazar-Knowles equation can be fit to these pressure volume loops, this allows us to calculate compliance which reflects the static elastic recoil pressure of the lungs at a given lung volume. The inverse of compliance is elastance.

After all measurements were completed the animal was removed from the ventilator and assessed to ensure spontaneous breathing resumes. Once the animal is breathing regularly it is then returned to the cage and monitored for full recovery from anesthetic.

1



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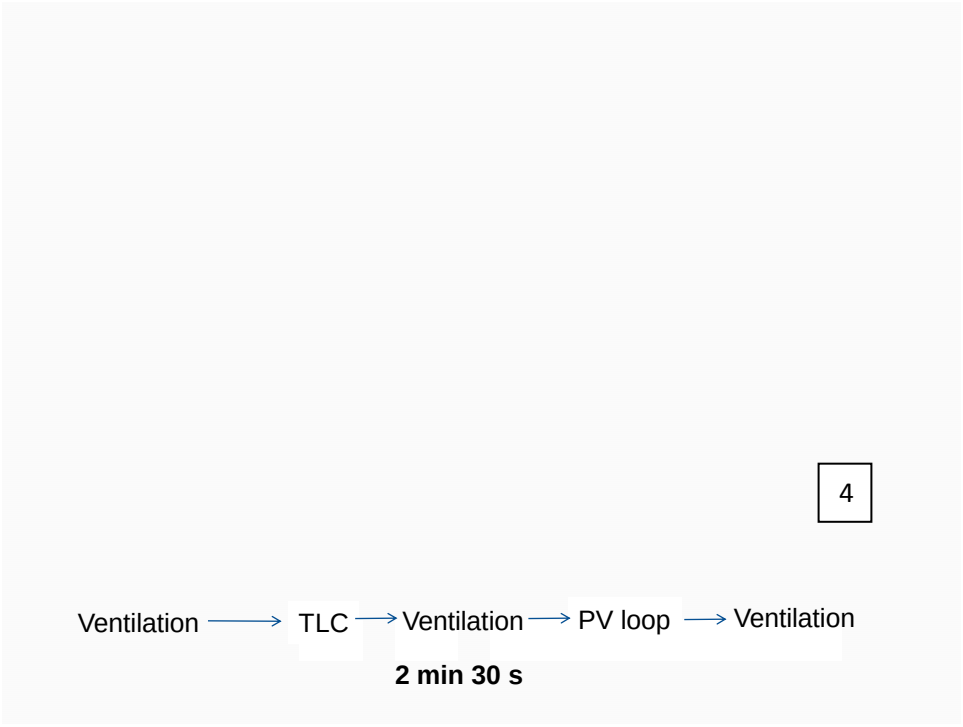


1: Blunted 18G needle use for the intubation of rats (pictured next to a penny)
2: Cartoon of approximate position of blunted needle in trachea
3: Computer-controlled small animal ventilator.
4: Overview of sequences used to run ventilator and perform measurements on sedated rat (total procedure time 2.5 minutes)

Figure 3: Outline of live lung function assessment

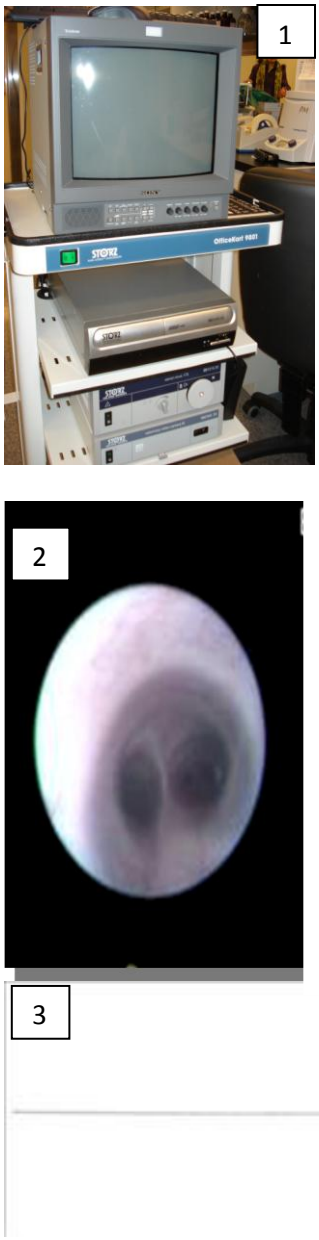
Unilateral-lobe lung instillation

In a separate set of experiments, a straight forward semi-rigid telescope (diameter 1mm, length 20cm, Karl Storz Endoscopy #11512 see figure below) was used for all procedures. Female Sprague-Dawley rats (body weight 200-250g) were anesthetized by inhalation anesthesia with isofluorane (MTC Pharmaceuticals, Cambridge, Ontario) for 3 minutes and then placed on a 45° inclined intubation board, positioned on their back. The micro-bronchoscope was first inserted into a flexible plastic tube of slightly larger diameter and both were then passed through the vocal cords of the animal. The bronchoscope unit monitor was used for further guidance to advance the instrument past the carina into the left main stem bronchus. Subsequently, the bronchoscope was withdrawn leaving the plastic tube in place which was then carefully forwarded as far as possible in the bronchi on the left distal lung.

The outer end of the tube was connected to a 1ml syringe filled with 150 µl phosphate buffered saline (PBS) containing the control vehicle or pathological agent to be instilled. AdMIP2α at a concentration of $1X10^8$pfu was administered as described above.

Imaging

Computed tomography (CT) was performed using the Micro-CT-component of an X-SPECT scanner (Gamma Medica-Ideas, Northridge, California) at different time points for longitudinal studies of radiological changes. Briefly, the rat was anesthetized by isoflurane and inserted in a Plexiglas tube with hepafilters to vehicle isofluorane/oxygen mixture. The animal was scanned over 512 angles as done previously (Ask et al. 2008).To assess neutrophilic inflammation following MIP2α administration (Foley et al. 1996) into the distal left lung, a Positron Emission Tomography (PET) scan using radio-labeled fluorine-18-fluorodeoxyglucose (18-FDG) was performed. The PET scan was performed using a µPET system (MOSIAC PET, Philips) this system uses a ring of gadolinium oxyorthosilicate (GSO) crystals coupled to photomultiplier tubes (PMTs), positioned in a ring around the subject (Counter 2013). Reconstruction of the micro-CT images was performed at 155 μm^3 voxel size, converted to Hounsfield Units (HU) selecting air as -1000 and water as 0 and filtered to reduce noise (sigma = 2 voxel Gaussian 3D filter) using Amira® visualization software (Mercury Computer Systems). Animals were monitored for weight loss and other signs of physical distress throughout the observation phase following the various interventions.



1: Bronchoscope unit monitor
2: Bifurcation of the right and left principal bronchi
3:Semi-rigid telescope

Figure 4: The equipment used in unilateral instillation

2.2 Results

Live assessment

We performed PV loops (as described above) on the treated animals and saw a change in the volume inspired and pressure required as shown below.

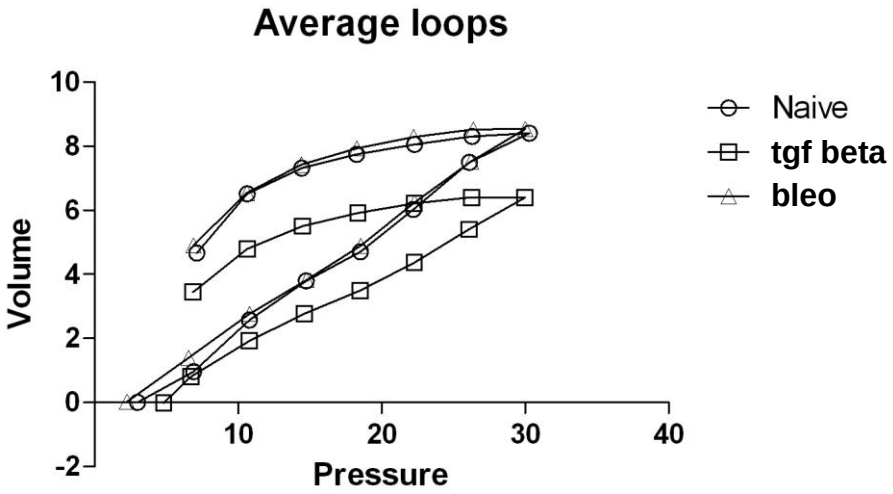
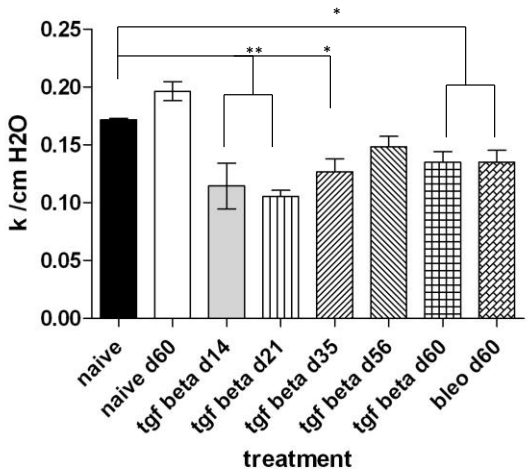


Figure 5: PV loops of animals at day 60 after Bleomycin and Ad TGF-β

tgf beta: Animal treated with Ad TGF-β intratracheally (n=6)
bleo: Animal treated with Bleomycin intratracheally (n=6)
naive: untreated animal (n=12)

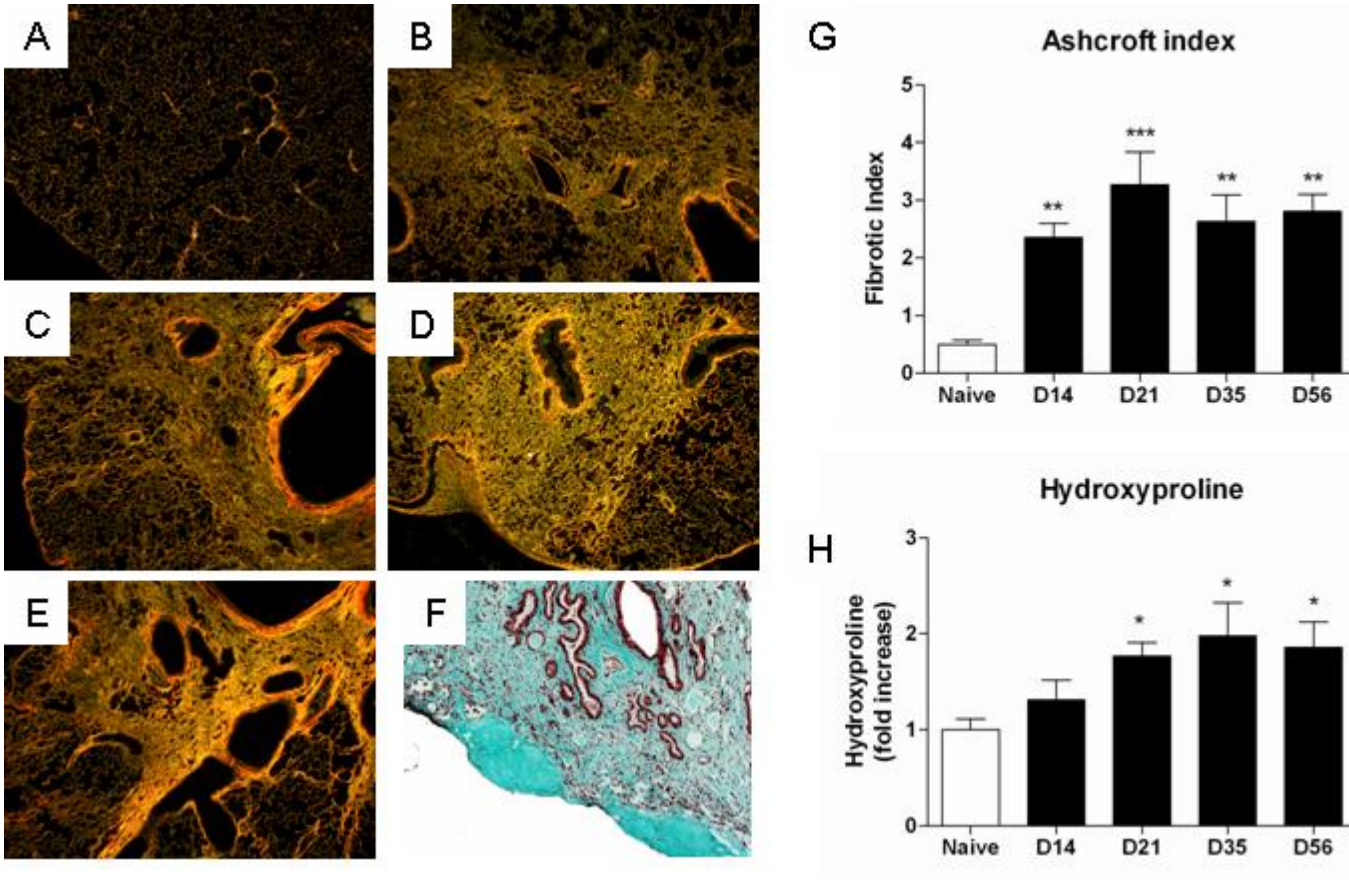
The representative pressure volume loop generated above shows the decrease in volume inspired and greater pressure required. This was seen after the administration of TGF-β. By doing repeat measurements we followed these animals for 60 days and looked at the variations seen.
One of the output measurements we recorded was the parameter k of the Salazar-Knowles equation. This parameter reflects the curvature of the upper portion of the deflation PV curve.



tgf beta: Animal received Ad TGF-β intratracheally (n=6 d14, 56 n=5 d21n=4 d35)
bleo: Animal treated with Bleomycin intratracheally (n=6)
naive: untreated animals (n=12)
naive d60: untreated animals 60 days after start of experiment (n=5)

Figure 6: k values of animals treated up to day 60 with Ad TGF- β and Bleomycin

Fibrotic lung disease causes a displacement to lower lung volume and a decrease in concavity (decrease in k), compared with normal lungs. We saw this variation in k value as we did repeated measurements on groups of rats, shown above. From our measurements, the lowest significant decrease in k value was from day 14 to day 21 after Ad TGF-β administration. This concurs with the histology seen at 21 days after administration of Ad TGF-β, as shown in the diagram below. There was also a significant difference between the naïve group and animals which were treated with Ad TGF-β at 35 and 60 days after treatment. We measured a significant difference in k value up to 60 days after administration of Ad TGF-β and Bleomycin compared to control.

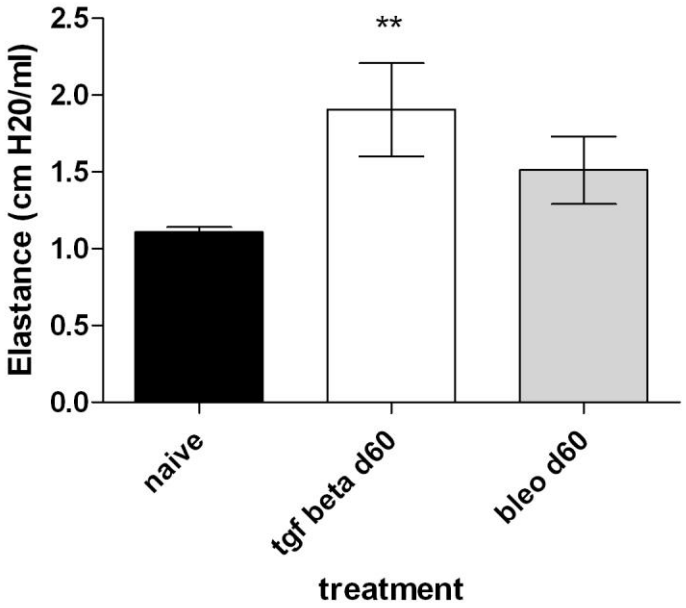


(Reproduced from *Journal of Translational Medicine* 2008, **6**:16 Ask et al. 2008)

A: Naïve lung	B: Ad TGF-β day 14	C: Ad TGF-β day 21	D: Ad TGF-β day 35
E: Ad TGF-β day 56	F: Ad TGF-β day 225	G: Ashcroft score	H: Hydroxyproline Assay

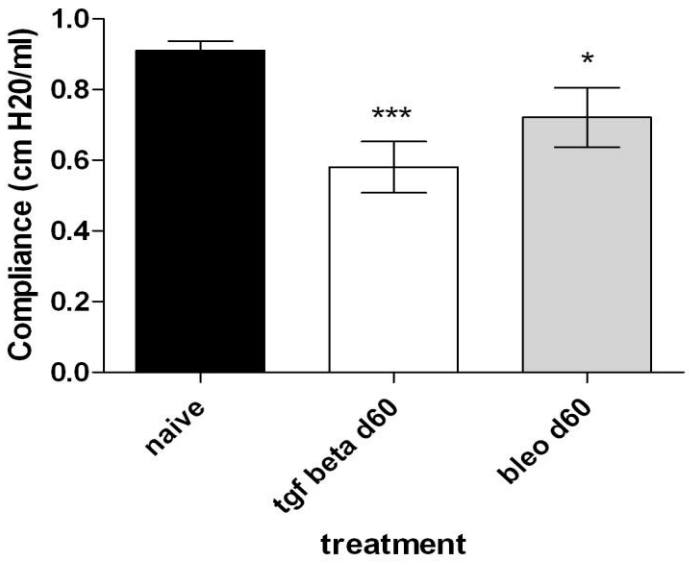
Figure 7: PicroSirus red (A-E) and Trichrome (F) staining in the lung up to day 56 after Ad TGF-β. Fibrotic tissue assessment of lung morphology from A-E are quantified by Ashcroft score (G) and biochemically by Hydroxyproline analysis (H) (x40)

We continued our measurements for up to 60 days. At the end of our observation period of 60 days we recorded increased levels of elastance and decreased levels of its inverse, compliance as shown below.



tgf beta: Animal treated with Ad TGF-β intratracheally (n=6)
bleo: Animal treated with Bleomycin intratracheally (n=6)
naive: untreated animal (n=12)

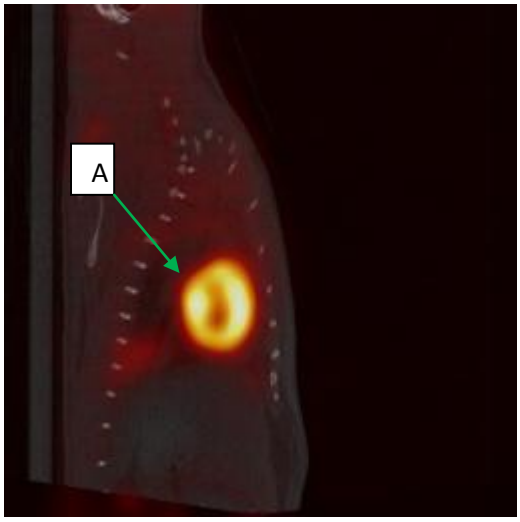
Figure 8: Elastance measured at day 60 after Bleomycin and Ad TGF-β. One way ANOVA $p<0.05$



tgf beta: Animal treated with Ad TGF-β intratracheally (n=6)
bleo: Animal treated with Bleomycin intratracheally (n=6)
naive: untreated animal (n=12)

Figure 9: Compliance measured at day 60 after Bleomycin and Ad TGF-β. One way ANOVA $p<0.05$

Unilateral Instillation



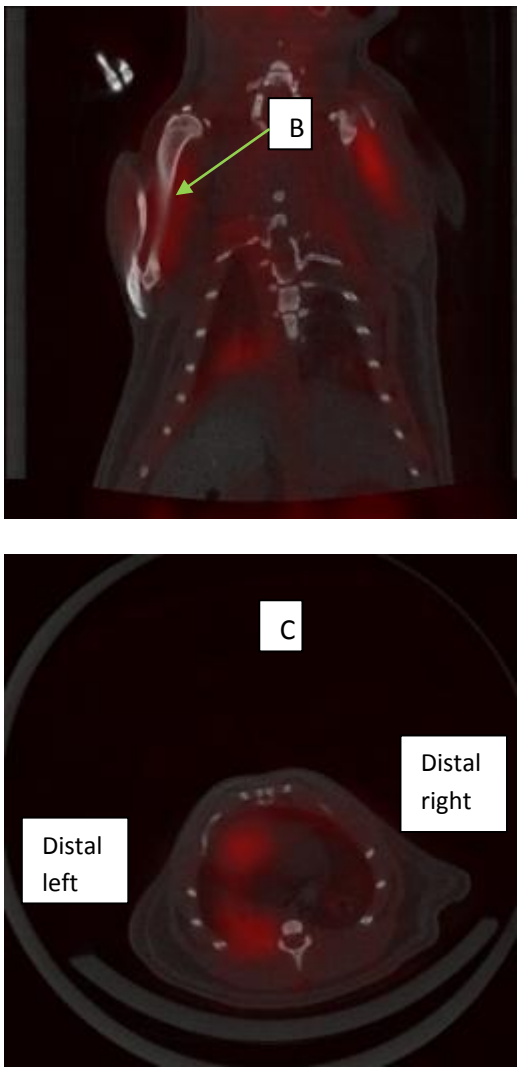


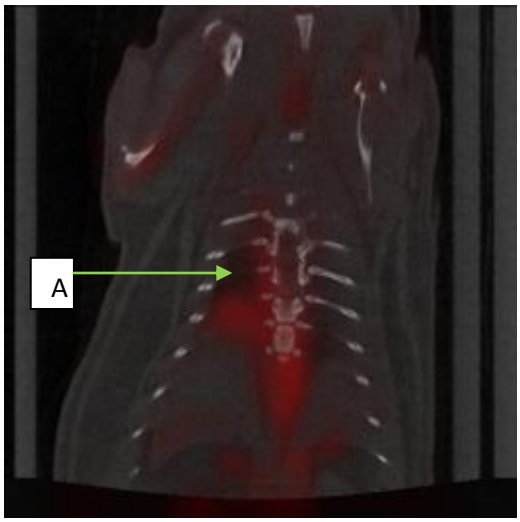
Figure 10: PET/CT image of unilateral instillation of AdMIP2α

Rat lung imaged 3 days after unilateral administration of AdMIP2α. Lung image showing uptake of fluorodeoxyglucose detected by PET scan. A: Focal point of inflammation in a single site induced by AdMIP2α and detected by FDG uptake. B: Other areas of the rodent showing absorption of FDG due to normal muscle uptake C: CT scan showing the distal lung as the focal point of inflammation.

We were able to visualise the treatment administered up to 3 days after instillation into the left lung only. The heart was excluded on PET analysis.

Figure 11: PET analysis of unilateral instillation of AdMIP2α

Uptake values showed greater absorption of the FDG on the lower left side (distal left) compared to the right. This corresponds to the location of AdMIP2α which was administered unilaterally. RU = Relative Units



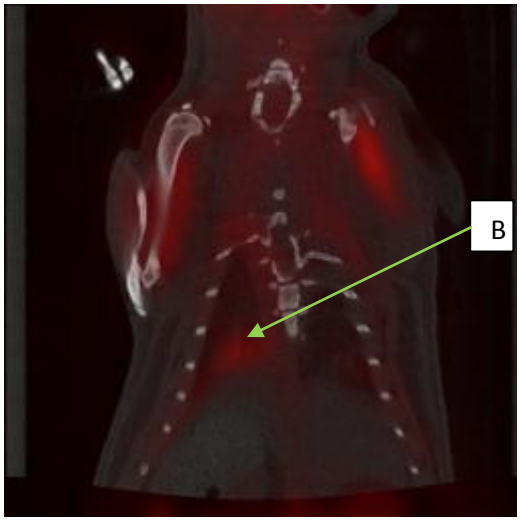


Figure 12: CT analysis of unilateral instillation of AdMIP2α

CT Scan composed of micro CT slices (A):-115 HU (B): 27 HU Having selected air as -1000 and water as 0, (B) indicates accumulation of fluid corresponding to delivery of treatment

The CT scan allows anatomical co-registration of PET signal confirming the location as the distal lung. With this approach we were able to deliver a treatment to a single lung and verify its placement and also follow the pathology or progression of the treatment using PET/CT technology

2.3 Discussion

The use of Flexivent™ is increasingly being accepted by investigators who wish to measure changes in lung compliance/elastance. We showed here a unique application of this tool to provide repeated measurements in the same animal before and after administration of treatment. This allowed us to follow these animals for up to 60 days and to see the changes in the pressure volume loop as the pathology progressed. Agreeing with the high fibrotic index from the histology seen in these animals is the low k value of the Salazar-Knowles equation. Both the TGF-β and Bleomycin systems used here confirmed that day 21 was the time-point of most severe disease after administration of TGF-β and that in both systems up until day 60 there was a significant difference in compliance and k value compared to the control. We also saw further agreement between the conventional histological methods of evaluation and Flexivent™ at day 60 where the scores for elastance was lower for Bleomycin, a reversible model of lung injury and higher for TGF-β, a progressive model of injury. This agreement of

multiple systems and detection of sensitive changes from one time point to another shows a validation of the Flexivent™ system and its utility in detecting fibrotic lung injury.

The PET/CT assessment of treatments was also a novel application of technology and we showed here a valuable proof of concept that a single treatment could be delivered successfully to the left lung of an animal. Furthermore, depending on the kinetics of the treatment, it will stay localized and can be detected up to 3 days after delivery using imaging techniques. This proof of principle demonstrates a very valuable tool for experiments designed to follow the progress of a localized treatment and one which we could apply to any number of systems. We could potentially combine this technology with the response of circulating biomarkers of lung injury to help us determine when best to sacrifice/sample blood from the animal. Also in unilateral treatments there is a potential for significant cost savings by fewer animals being required. In these unilateral treatments, the uninjured lung can be used as a control to determine, for example; whether fibrocytes, a circulating bone marrow derived cell, can be recruited to a naïve or untreated lung.

The data presented here in both arms of this study was from a small number of animals and was largely intended to demonstrate potential applications of the methods described. By completing this proof of principle we have shown that we can follow fibrotic changes in a single animal in a non-terminal manner. With the unilateral study, we can examine a highly localised treatment and uniquely, compared to other methods, we are able to use an endogenous control i.e. the unaffected lung of the treated animal.

3.0 Fibrocytes and the response to Experimental Lung Inflammation

3.1 Fibrocytes

Fibrocytes are circulating bone marrow-derived mesenchymal progenitor cells (Bucala et al. 1994) (Mori et al. 2005) (Ebihara et al. 2006) (Kisseleva et al. 2006) (Haudek et al. 2006) co-expressing haematopoietic stem cell markers, monocyte lineage markers and fibroblast cellular contents (Pilling, Tucker, and Gomer 2006) (Schmidt et al. 2003). Fibrocytes, like some dendritic cell subsets, are most likely derived from precursors in the bone marrow of the monocyte lineage, supported by the observation that they express the major histocompatibility complex, class I and class II, and the co-stimulatory molecules CD80 and CD86 (Bucala et al.1994) (Pilling, Tucker, and Gomer 2006) (Chesney et al. 1997). It is widely accepted that mature fibrocytes express both markers of haematopoietic cells (CD34, CD43, CD45, LSP-1) and the cellular content of stromal cells (collagen I and III) (Bucala et al.1994) (Abe et al. 2001) (Gomperts and Strieter 2007) (Bellini and Mattoli 2007). Fibrocytes lack lymphocyte markers such as CD3, CD4, CD8, CD19 and CD25 (Bucala et al. 1994). Although there are a panel of markers which can be used in the detection of fibrocytes (Pilling et al. 2009), Flow cytometry for CD45 and collagen 1 is routinely used for the identification of fibrocytes in circulation, as this combination seems to be practical and has been used in most studies (Maharaj et al. 2012). In the tissue fibrocytes have been mostly identified by immunohistochemistry for CD34 and pro-collagen or CXCR4 and prolyl-4-hydroxylase (Maharaj et al. 2012).

On a functional level, fibrocytes have been proposed as early responders to injury and due to their ability to function as antigen presenting cells (Chesney et al. 1997); fibrocytes have also been implicated in antigen specific immunity. There is accumulating evidence in support of fibrocytes directly contributing to the new population of fibroblasts and myofibroblasts that emerge in injured tissue during normal (Bucala et al.1994) (Mori et al. 2005) (Abe et al. 2001) or aberrant (Yang et al. 2005) wound healing in ischemic, inflammatory or fibrotic processes (Kisseleva et al. 2006) (Haudek et al. 2006) (Schmidt et al. 2003). Experimental studies in vitro showed that fibrocytes isolated from the circulating blood can differentiate into αSMA expressing cells and exhibit contractile forces after stimulation with TGF-β (Bellini and Mattoli 2007). Fibrocytes are also believed to be involved in tissue remodelling through the production of extracellular matrix proteins, including collagen I, collagen III and vimentin (Chesney et al.1998).

Fibrocytes likely participate indirectly in the process of injury and repair. Work by Chesney and colleagues showed that when stimulated in vitro by IL-1β, fibrocytes

secreted the chemokines MIP1α, MIP1β, MCP-1, IL-8, and GROα and hemopoietic growth factors, including macrophage colony stimulating factor (M-CSF), as well as interleukins, such as IL-6 and IL-10 (Chesney et al.1998). Other in vitro experiments have shown that Fibrocytes are also an important cellular source of other growth factors (Vascular endothelial growth factor (VEGF), Platelet derived growth factor A (PDGF-A), Hepatocyte growth factor (HGF), Granulocyte macrophage colony stimulating factor (GM-CSF), Basic fibroblast growth factor (b-FGF), Connective tissue growth factor (CTGF) and may contribute to autocrine and paracrine signals within the micro-environment (Hartlapp et al. 2001) (Quan et al. 2004) (Quan, Cowper, and Bucala 2006) (Wang et al. 2007).

Fibrocytes express the chemokine receptors CCR2, CCR7, and CXCR4, which may regulate their entry into inflammatory lesions (Abe et al. 2001) (Pilling et al. 2003) (Moore et al. 2005).For example, the chemokine ligand pair CXCR4-CXCL12 plays a significant role in the homing of fibrocytes to areas of tissue injury (Phillips et al. 2004). Fibrocytes have been found in rodent models of tissue repair and experimental fibrosis including wounded skin, asthma, pulmonary vascular remodeling and lung and liver fibrosis (Mori et al. 2005) (Moore et al. 2005) (Frid et al. 2006) (Postlethwaite, Shigemitsu, and Kanangat 2004) (Strieter, Gomperts, and Keane 2007). Fibrocytes can also be detected in human fibrotic disorders including idiopathic pulmonary fibrosis (IPF), chronic asthma, nephrogenic fibrosing dermopathy, scleroderma and cutaneous scars (Strieter, Gomperts, and Keane 2007) (Galan, Cowper, and Bucala 2006) (Nihlberg et al. 2006) (Iwano et al. 2002).

3.2 Cytokines in lung inflammation

The measurement and expression pattern of cytokines has long been a major area of interest in the study of fibrotic lung diseases. The vast body of knowledge about cytokines and their activities help us to understand what elevated cytokine levels might be due to or what these levels may predict. There are various cytokines which are associated with normal cell responses and which may signal an influx of these cells into areas of injury where they may perform pro-inflammatory, inflammatory and/or reparative functions. Alternately, there can also be dysregulation of cytokine activity; where over expression of cytokine(s) can result in exaggerated functions, as seen in fibrosis. It is highly likely that cytokine expression may play some role in the mobilisation and recruitment of fibrocytes. Until now the exact roles that cytokine expression plays in fibrocyte biology is still unclear. I will discuss below the relationship between several cytokines and lung inflammation making a case for the identification of the fibrocyte as one of the many cells which respond to cytokine expression.

IL-1β

Monocytes, macrophages, inflammatory and structural cells can be responsible for the production of IL-1β. This very important pro-inflammatory cytokine appears to have effects on fibrogenesis (Dinarello 1997). Due to its early release, after proteolytic cleavage, IL-1β is commonly identified in the propagation of inflammatory processes (Kolb et al. 2001). In addition to stimulation of cells, such as macrophages, by biologic substances including lipopolysaccharide (LPS), the production of IL-1β can also be stimulated by other cytokines, including TNF-α and GM-CSF. It would also appear that the pro-fibrotic effects of IL-1β may be linked to the subsequent induction of TGF-β (Kelly et al. 2003). TNF-α is another early release cytokine that plays a major role in regulating the inflammatory response and it induces the expression of GM-CSF (Bader and Nettesheim 1996). While TNF-α is chemotactic for fibroblasts (Leibovich et al.1987) and its presence has been demonstrated in IPF (Pan et al.1996), its role in fibrogenesis is controversial (Kelly et al. 2003). It is believed that the role played by TNF-α in fibrogenesis is largely mitigated through TGF-β (Liu et al. 2001).

IL-1β over expression through administration of an adenovirus vector expressing this gene (Ad IL-1) leads to transient overexpression of IL-1β in the epithelial cells of rodent lung and significant lung tissue damage and development of chronic fibrosis (Kolb et al. 2001).

Elevated concentrations of pro-inflammatory cytokines, TNF-α, IL-1β and IL-8 have been detected in the sputum of children with Cystic Fibrosis (CF) a chronic disease that usually involves the lung and has “fibrotic” changes associated with the progression of the disease. Patients with CF have been found to have significantly higher sputum concentrations of IL-1β compared to non CF children (Osika et al.1999).

This observation was confirmed in a study looking at induced sputum in patients with CF who showed an increased level of IL-1β compared to healthy patients (Eickmeier et al. 2010). Another recent study suggested that IL-1β is a clinically relevant modulator of CF lung disease by looking at single nucleotide polymorphisms in the IL-1β gene (Levy et al. 2009). The involvement of the TGF-β mediated Smad signaling pathway in CF lung changes is unknown (Shute et al. 2003), however gene modifier studies have shown possible associations with genetic variation in TGF-β and CF disease severity.

Due to the pronounced effects of IL-1β on inflammation and subsequent induction of TGF-β, the documented cellular influx into the lung after IL-1β over expression,

should be further characterised to identify a possible impact on induction of the fibrocyte.

GM-CSF

Granulocyte macrophage-colony stimulating factor (GM-CSF) is regarded as a major regulator governing the induction of mononuclear cells from the bone marrow and the function of granulocytes and macrophages at all stages of maturation (Hamilton 2002). GM-CSF is produced mainly by activated T lymphocytes, leukocytes and structural cells (Kelly et al. 2003). This cytokine has effects on quite a number of cells, with receptors being present on granulocytes, monocytes, endothelial cells and fibroblasts (Kitamura et al.1991). Many of the effects of GM-CSF are pro-inflammatory and its expression is up-regulated in a number of lung diseases including IPF (Xing et al.1996a). Rubbia-Brandt and colleagues (Rubbia-Brandt, Sappino, and Gabbiani 1991) showed that GM-CSF, when administered subcutaneously, led to the local tissue accumulation of myofibroblasts. There is some evidence which suggest that the pro-fibrotic effects seen on administration of GM-CSF by gene transfer may be mediated indirectly by an increase in TGF-β levels (Kelly et al. 2003).

GM-CSF is not detected in the circulation of healthy humans or animals (Hartung, von Aulock, and Wendell 2000) and the conditions which stimulate the release of this cytokine and its subsequent effects on other cytokines is something that has received some attention. Notably, a study by Basu and colleagues (Basu et al. 1997) showed that GM-CSF contributed to the production of IFNγ, IL-1α and IL-6 in their model of LPS mediated septic shock. Some studies have also shown that GM-CSF can also be a potent inducer of IL-1 and TNF-α, important early cytokines released in an inflammatory reaction (Hartung, von Aulock, and Wendell 2000). It is believed that due to its potential immuno-stimulatory properties, GM-CSF can be beneficial depending on the time of administration. However there are also other studies which show that systemic administration of GM-CSF can be detrimental if given before or during the pro-inflammatory phase of sepsis (Hartung et al. 1999).

Systemic GM-CSF administration into human donors increased the capacity of granulocytes to produce superoxide, increased the cytotoxicity of monocytes and gave rise to increased monocyte numbers in the blood (Wing et al. 1989). Work done by Xing and colleagues showed that intrapulmonary transfer of the gene coding for murine GM-CSF, by an adenovirus vector, induces eosinophilia, monocytosis and a limited fibrotic reaction. The transient gene transfer led to an initially high expression of GM-CSF (7 to 10 days) followed by granulation tissue

formation and an apparently irreversible limited fibrotic response starting from day 12 onward (Xing et al.1996b).

In contrast to the pro-inflammatory actions of GM-CSF which have been reported (Xing et al.1996a) (Rubbia-Brandt, Sappino, and Gabbiani 1991), some studies have shown possible anti-inflammatory activity of GM-CSF. Shindo and colleagues (Shindo et al. 1999) showed a reduction in atherosclerosis in a rabbit model after administration of GM-CSF for 7.5 months. Piguet and colleagues (Piguet, Grau, and de Kossodo 1993) showed that a continuous abdominal infusion of GM-CSF from days 7-15 prevented collagen deposition induced by the intra-tracheal administration of bleomycin on day 0. In a review by Hamilton (Hamilton 2002), he discussed the possible reasons for the conflicting information, citing the nature of the elicited inflammatory response as well as the dose of GM-CSF administered. I would also speculate that timing of the administration of GM-CSF in a model of injury may affect the underlying initial inflammatory response to the injury.

With the increased use of GM-CSF in patients, with indications for accelerating the recovery of white blood cells following chemotherapy, after bone marrow transplantation and before and/or after peripheral blood stem cell transplantation, there is a need to study the actions of this cytokine further. Furthermore, having previously identified the fibrocyte as a bone marrow derived cell, we hypothesise that GM-CSF may possibly mobilise fibrocyte release into the circulation.

TGF-β

TGF-β is a family of growth factors (5 in total of which three are present in human) which are secreted by a large number of cells and can affect the cellular functions of a variety of cell types. TGF-β1 is the most commonly secreted form and is found to be increased in fibrotic diseases (Khalil et al.1996). In the lung, there are numerous possible sources of TGF-β as it is produced by leukocytes, fibroblasts, smooth muscle cells, bronchial epithelial cells and alveolar type II pneumocytes (Kelly et al. 2003). TGF-β is expressed in an inactive or latent form as part of a complex which is comprised of a latent TGF-β binding protein and latent TGF-β. The release of the active form of TGF-β is dependent on post-translational modification which removes the latent binding protein from the active TGF-β which is then available for cell surface receptor binding (Froese 2013).There are several mechanisms involved in the activation of TGF-β in lung fibrosis including αvβ6 integrin (Tatler and Jenkins 2012) and proteolytic enzymes. TGF-β is also able to induce its own expression by positive feedback (Van Obberghen-Schilling et al.1988).

This growth factor is a potent chemoattractant for a number of cells including monocytes (Kelly et al. 2003). It can also induce the secretion of extracellular matrix (ECM) proteins and has several pro-fibrotic properties including the inhibition of myofibroblast apoptosis (Border and Noble 1994). Animal studies done by Sime and colleagues (Sime et al.1997) have used adenovirus vectors to transfer the latent and the active form of TGF-β gene to rat lung and transfer of the active form (over expressing TGF-β levels for 7 to 10 days only) resulted in severe persistent (60 days or more) and progressive fibrosis involving the lung pleura and interstitium. They also found extensive deposition of ECM and emergence of myofibroblast like cells. The TGF-β pathway has also been implicated in chronic fibrogenesis which has been demonstrated in a rodent model by Bonniaud and colleagues (Bonniaud et al. 2004) where they showed that mice lacking the Smad 3 TGF-β signaling molecule are resistant to the induction of fibrosis by adenovirus vector TGF-β gene transfer.

In a 2001 study Kolb and colleagues (Kolb et al. 2001) also found that the progressive fibrosis, seen in the rat model of fibrosis induced by adenovirus gene transfer of IL-1β to the lung, occurred in the presence of sustained induction of the profibrotic cytokine TGF-β. The interconnection between IL-1β expression and TGF-β induction gives rise to a multiple signal that can result in different patterns of cellular recruitment.

Although numerous models of TGF-β induction and/or over-expression have been examined, further study still needs to be done on the interplay of the concomitant expression of several cytokines and growth factors, as would occur in various chronic lung diseases, and examine the systemic responses to these factors including circulating responders such as the fibrocyte.

Osteopontin

Osteopontin (OPN) has been investigated by several arms of medicine at various times and from different perspectives. In 1989, a cytokine termed Eta-1 (early T cell activation 1) was sequenced from activated CD4+ cells (Patarca et al.1989). Independently OPN was discovered to be a major sialoprotein in the extracellular matrix of bone (Franzen and Heinegard 1985), and the sequence determined for osteopontin was found to be identical to the later identified Eta-1. A 1989 review (Senger, Perruzzi, and Papadopoulos 1989) also discussed some of the earliest links of the role of OPN (or 2ar, as it was initially known in cancer research) to tumour cells and host tissue matrix.

In the last two decades since the early work on OPN, there have been numerous studies looking at the ability of different cell types to secrete this cytokine. A

review in 1996 by Weber and Cantor (Weber and Cantor 1996) discussed the secretion of OPN from T lymphocytes and monocytes and its subsequent effects on phagocytes and B and T lymphocytes. Recent work in cancer biology (McAllister et al. 2008) has also highlighted the role OPN plays in the activation of Bone Marrow cells and their subsequent effects on distant tumour growth. OPN has been found to be expressed in tissues in pathological conditions and a proposed explanation has been its production by activated lymphocytes and macrophages which are known to express OPN (Patarca et al.1989).

A role for OPN was identified in type 1 cell mediated immunity by the increase of IL-12 and suppression of IL-10 production in macrophages. It was proposed that this action occurs via αvβ3 and CD44 receptors respectively (Ashkar et al. 2000). A commentary by Bateman (Bateman 2011) gives a very good perspective on the contribution of the bone marrow and its derived cells on tumour stroma growth.

OPN's link to cell mediated immunity, stimulation and activation of bone marrow cells, expression by immune cells and expression in diseased tissue all make it a very interesting target for investigation in other disease processes. There are a number of similarities in the formation of fibrotic matrix and tumour stroma, the latter in which OPN has been implicated. The association with activated lymphocytes and macrophages as well as the participation in type 1 cell mediated immunity highlights some of the conditions which may be present in fibrotic lung disease. The bone marrow stimulation in the presence of OPN most probably leads to the elevation of several cell populations in the circulation which have not yet been fully described.

We had an interest to further examine these circulating cell populations stimulated by OPN to see if the fibrocyte is present and how its levels may be affected by OPN expression. Furthermore this would also allow us to better understand a possible mechanism by which OPN may contribute to pro-fibrotic activity as proposed by other investigators (Pardo et al. 2005).

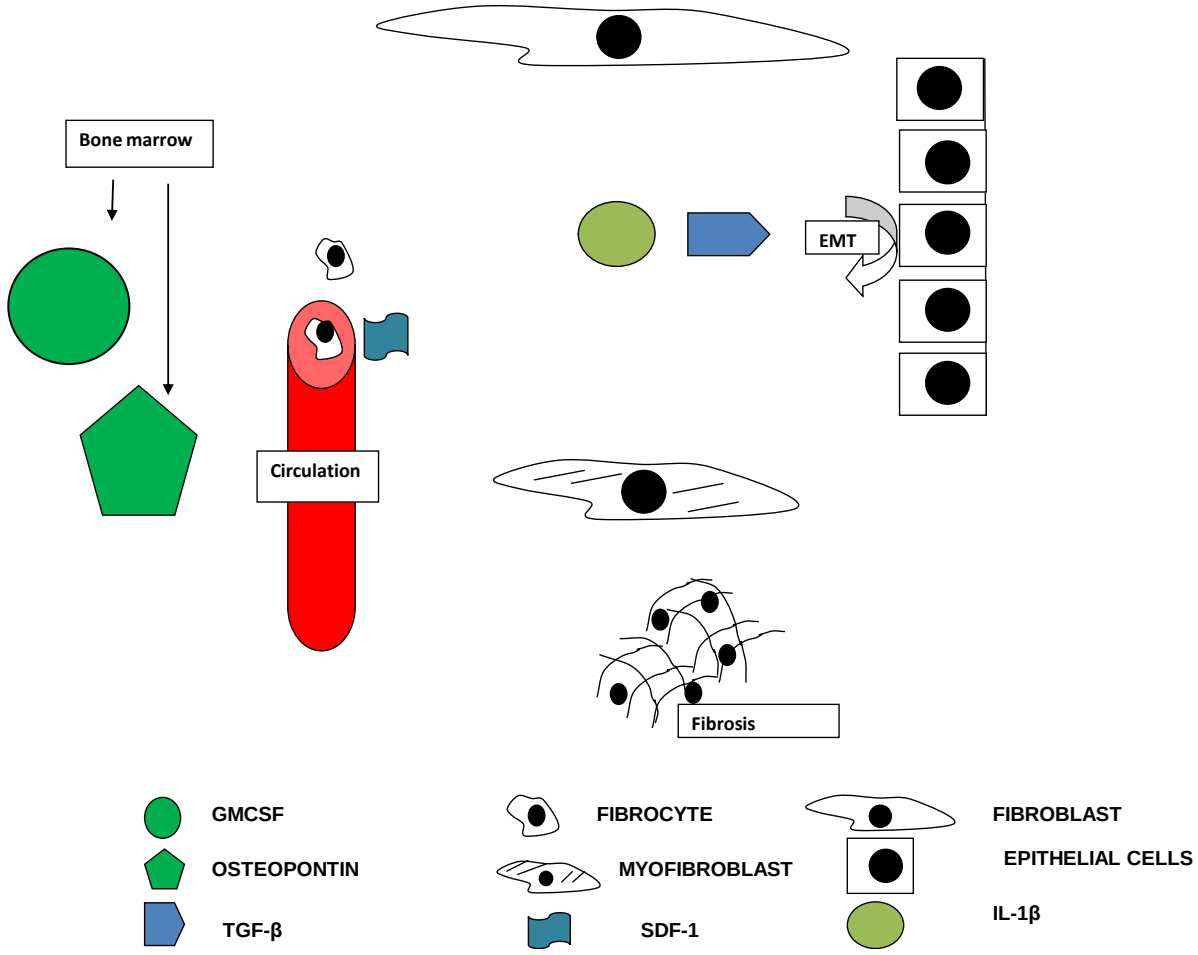


Figure 13: Proposed interaction of cytokines with cells involved in fibrosis

SDF-1

Chemokines are small chemoattractant peptides which have multiple functions; they promote immune responses, stem cell survival, chemotaxis and angiogenesis. One of their main roles is the recruitment of leukocytes to inflamed tissues (Romagnani et al. 2004). They do this by chemotaxis of cells expressing their respective receptor towards areas with higher concentrations of the chemokine. To create this gradient, chemokines are immobilised on cell or extracellular matrix surfaces where they interact with negatively charged glycosaminoglycans (Olson and Ley 2002). Chemokines can influence and also regulate the inflammatory response by the sequence of participation of inflammatory cells. Disruption of this well coordinated regulation can lead to

aberrations including chronic inflammation (Romagnani et al. 2004). In the lung, altered expression of chemokines and their receptors involved in inflammatory processes may lead to disruption of homeostatic mechanisms which regulate angiostatic and angiogenic processes which might subsequently contribute to the pathogenesis of Idiopathic Pulmonary Fibrosis (Brunetti and Pignatti 2007). There are several different chemokines and their functional diversity is maintained by their different expression patterns as well as receptor binding. There are some chemokines which have varying selectivity for receptors and others such as SDF-1 which bind only one receptor CXCR4 and CXCR4 in turn only binds to one chemokine SDF-1 (D'Apuzzo et al. 1997).

Expression of SDF-1, also referred to as CXCL12, is responsible for the recruitment of cells expressing CXCR4. The CXCR4 receptor is considered to be critical in the homing of both hematopoietic and non-hematopoietic stem cells (Murdoch 2000). The fibrocyte demonstrates high expression of CXCR4 (Pilling et al. 2009). Migration of CXCR4 positive fibrocytes to CXCL12 has been demonstrated in human studies (Phillips et al. 2004) and also in mouse studies (Phillips et al. 2004). Other pathways have been proposed for the recruitment of fibrocytes CCR2/CCL12 (Moore et al. 2006) and CCR7/SLC (Secondary lymphoid chemokine) (Abe et al. 2001). However, in human interstitial diseases, work by Mehrad and coworkers showed high plasma and lung levels of CXCL12 and they correlated this with high levels of CXCR4 expression in circulating fibrocytes (Mehrad et al. 2007). The clinical relevance of the CXCR4/CXCL12 axis in fibrocyte recruitment to the lung is further supported by additional work by Mehrad and other groups (Mehrad, Burdick, and Strieter 2009) (Andersson-Sjoland et al. 2008). There has been no work done on SDF-1 and its recruitment of fibrocytes to the lung in the rat model of lung fibrosis. To help complete our characterisation of cytokine influence on fibrocyte kinetics in the rat model, I believe that this chemokine should be examined further.

3.3 AMD3100 as a Fibrocyte Inhibitor

In addition to the relationship between cytokine expression and fibrocyte kinetics, as a proof of principle, we sought to test our ability to modulate fibrocyte response by neutralising one of its main receptors used in homing to the lung, CXCR4. By employing methods to suppress or stimulate levels of circulating fibrocytes, we can develop systems to assess this cell's potential harmful or beneficial effects by correlating its levels and presence in the lung tissue with changes seen in lung injury.

AMD 3100 is a bicyclam molecule that selectively and reversibly antagonizes the binding of stromal cell-derived factor-1 (SDF-1) to its receptor CXC motif

receptor-4 (CXCR4). AMD3100 was originally developed as a treatment for Human Immunodeficiency Virus (HIV) via its ability to block HIV entry into CD4+ T cells mediated by the HIV co-receptor CXCR4 (DeClercq 2003). Studies have looked at the administration of AMD3100 to healthy volunteers and the resultant rise in circulating CD34+ cells (Liles et al. 2003).

It is speculated that this compound liberates hematopoietic stem cells (HSC) from the bone marrow possibly by affecting their CXCR4 mediated retention to the bone marrow stroma (Lapidot and Petit 2002). This drug is currently licensed in Canada for use together with G-CSF for the liberation of HSC's in patients with lymphoma and multiple myeloma for autologous transplants.

Experimental models have shown the ability of AMD 3100 to reduce inflammation in a model of autoimmune arthritis (Matthys et al. 2001) and allergic airway inflammation (Lukacs et al. 2002). A recent study by Song and colleagues (Song et al. 2010) using a murine model of bleomycin induced fibrosis found that AMD 3100 decreased the number of fibrocytes found in the lung 3 days after bleomycin injury. The authors did not comment regarding any other cellular behavior which AMD3100 may alter.

One study looking at the effects of administration of AMD 3100 on neutrophil release found that other pathways including CXCL2 (MIP2) provided an alternate route for neutrophil trafficking (Eash et al. 2010). Another study compared AMD3100 to cyclophosphamide in administration in conjunction with Granulocyte colony stimulating factor (G-CSF) to mobilise HSC's clinically. Unlike cyclophosphamide, it was found that AMD 3100 did not inhibit osteoblasts, endosteal cytokine expression or reconstitution potential of HSC remaining in the mobilized bone marrow. Moreover, AMD3100 directly targets HSCs without altering niche function (Winkler et al. 2012). Another study looking at a rhesus macaque model found that AMD 3100 substantially mobilizes both B and T lymphocytes into the peripheral blood (Kean et al. 2011).

The literature has widely reported the expression of CXCR4 on fibrocytes and to the best of my knowledge there is no evidence to show the effects of AMD 3100 on the kinetics of circulating fibrocytes. Systemic administration of this compound may allow us to modulate a well characterised receptor on the fibrocyte without affecting the local fibrotic environment in the lung. By doing this, we can observe the subsequent effects and learn more about the role of the fibrocyte.

3.4 Experimental lung disease

Although IPF is the most common form of interstitial pneumonia found in man (ATS 2002), the absence of a clear aetiology, as in other manifestations of pulmonary fibrosis, has been a major obstacle in creating a model of this disease. However, there are several models of pulmonary fibrosis which were recently discussed by Moore and colleagues (Moore et al. 2013). The recognized pathological similarities between the fibrotic reactions seen in the lungs of animals and humans justify our continued investigation of animal models for answers to specific mechanistic questions about this disease (Chua, Gauldie, and Laurent 2005).

In choosing a model to study a disease, physiological similarity is essential in an attempt to ensure that you are able to translate your findings back to the human. The rat model of fibrosis has been discussed as being one which consistently exhibits some key features of human IPF (Gauldie and Kolb 2008). For short term experiments which address the early events, within the first month after lung injury, these animals may be a suitable candidate for study. Being larger than mice; rats are much easier to work with and allows for a more direct comparison to humans in the physiological and pathological outcomes of lung injury.

Although there have been several rat models of pulmonary fibrosis, each model is characterised by increasingly well known limitations. These limitations have not hampered further investigation into pulmonary fibrosis using animal models, as investigators have used these known characteristics to better understand the results they obtain and to ask questions which can be answered by the model they choose.

To my knowledge there has been no systematic characterisation of circulating fibrocytes. The examination of these cells has only been by random sampling in a few human studies. The characterisation of fibrocyte response in the experimental fibrosis lung, while performed in a limited number of studies, these have focused solely on the bleomycin model of lung injury in mouse and digestion of whole lungs as the primary outcome. The response of fibrocytes to cytokine over expression and also in various models of animal injury has not been fully characterised.

Having discussed above some of the most common cytokines involved in lung inflammation and fibrosis, I focus below on the specific involvement of these in animal models of fibrotic lung injury. Four models which have been shown to result in pulmonary fibrosis are; the instillation of an adenovirus vector expressing the active form of TGF-β (Ad TGF-β) (Sime et al. 1997), the instillation of an adenovirus vector expressing murine GM-CSF (AdmGM-CSF) (Xing et al. 1996b), the instillation of an adenovirus vector expressing IL-1β (Ad IL-1β) (Kolb

et al. 2001) and the instillation of Bleomycin (Fleischman et al. 1971). I will also make a case for the study of the fibrocyte in these models and hypothesise about a potential role for this cell in the propagation of lung inflammation and injury.

Bleomycin

This chemotherapeutic drug is known to possibly induce toxic side effects in patients and the incidence of these effects have been associated with dose, age and pre-existing medical conditions (Moeller et al. 2008). The lung which contains a low level of inactivating enzymes for Bleomycin is particularly susceptible to injury leading to Pulmonary Fibrosis (Sebti et al.1989). The induction of fibrosis in the lung using Bleomycin was first described by Fleischman and colleagues in 1971 (Fleischman et al.1971) and since then this has become one of the most widely used tools for the investigation of pulmonary fibrosis.

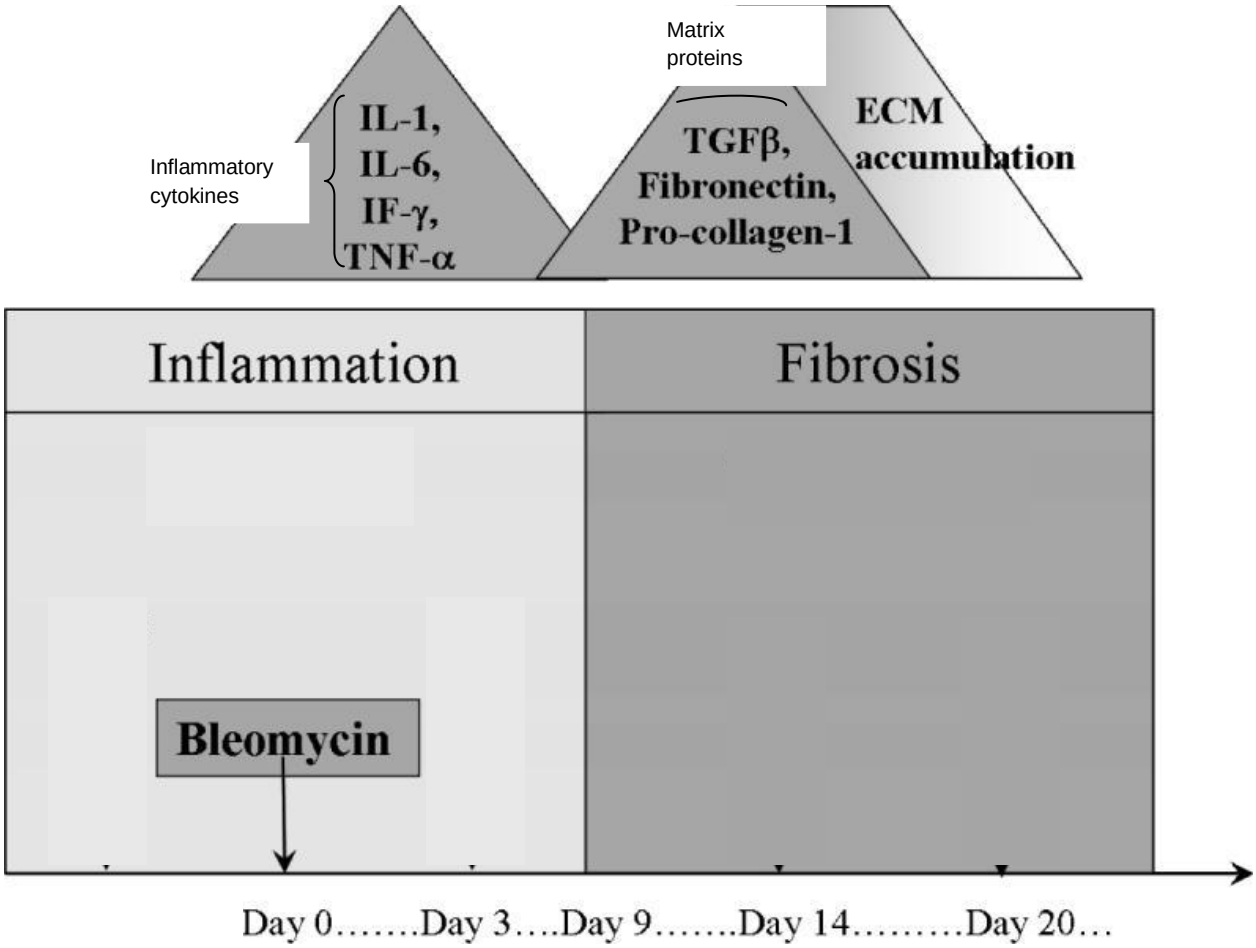


Figure 14: Time course of Bleomycin lung injury. Day 9 represents a switch from Inflammation to Fibrosis and a change in the cytokines predominantly expressed. (adapted from Moeller et al. 2008)

There have been several routes of administration of Bleomycin which have resulted in different pathologies. The intravenous administration of bleomycin twice weekly for 4-8 weeks results in a disease like that of the human with the lung endothelium being the primary target (Adamson and Bowden 1974). However, when administered intratracheally, the epithelium is the primary target of the injury (Matute-Bello, Frevert, and Martin 2008). In this model, after intratracheal instillation, there is an initial elevation of pro-inflammatory cytokines which is followed by the expression of pro-fibrotic markers (Moeller et al. 2008) (see figure 14). There is an apparent switch seen around Day 9 from inflammation to fibrosis. This switch is characterised by a decrease in

inflammatory cytokines (IL-1, IL-6, IFNγ, TNF-α) and an increase in fibrotic cytokines and matrix proteins (TGF-β, Fibronectin and Pro-collagen-1) leading to ECM accumulation. These two distinct phases of Bleomycin injury and repair, initiated by the overproduction of free radicals and activation of macrophages and neutrophils, then followed by a fibrotic phase, is somewhat reversible even without intervention (Izbicki et al. 2002).

Chaudhary and colleagues (Chaudhary, Schnapp, and Park 2006) examined the rise and fall of cytokine expression after administration of bleomycin. In their rat model, after administering a single intratracheal dose of bleomycin (2.2mg/kg), they saw an increase at Day 3 of IL-1α, IL-1β, IL-6 and IFNγ in whole lung lysates. These cytokine levels declined between day 9 and 14 after bleomycin administration.

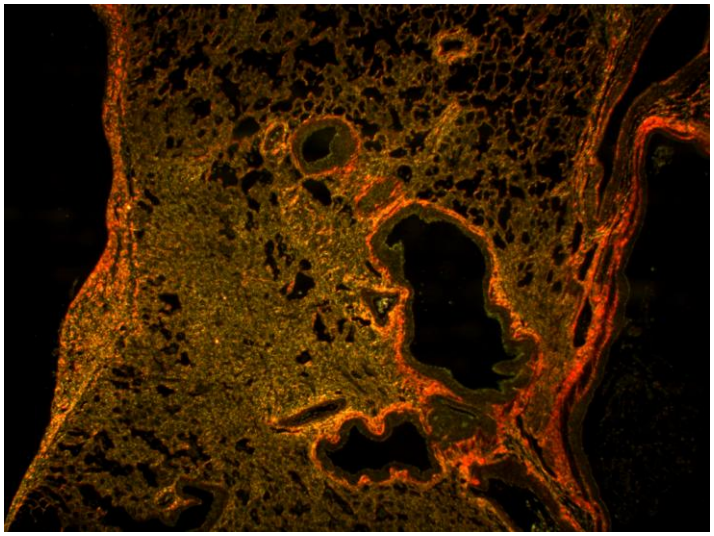
Due to the similar histology seen in animals treated with Bleomycin and that of patients with IPF, this model has gained popularity for the study of IPF. It has also been reported that this model reproduces histological features of human IPF disease, such as intra-alveolar buds, mural incorporation of collagen and obliteration of the alveolar space (Usuki and Fukuda 1995). In our lab we have developed the method to deliver treatments non-surgically via the trachea of sedated rats (further discussed in methods section) and this has allowed us to reproducibly achieve a fibrotic response in this model. The bleomycin model of fibrosis does have its limitations. As discussed by Izbicki and colleagues (Izbicki et al. 2002) this model can be partially reversible even without any intervention. The lack of the slow and irreversible progression of disease which is considered to be a major characteristic of IPF (Chua, Gauldie, and Laurent 2005) is a major limitation of this model. Furthermore, the inflammatory phase of Bleomycin injury as shown in the diagram above is characterised by the response of activated macrophages and neutrophils, thereby resembling acute lung injury. This known inflammatory phase has however enabled investigators to better target their interventions to either incorporate or avoid it.

The Bleomycin model is one of the most often utilised models of lung fibrosis and as such, some attempts have been made to identify fibrocytes and their role in the bleomycin model of injury (Hashimoto et al. 2004) (Phillips et al. 2004) (Song et al. 2010). These studies used mouse models and for the identification of fibrocytes in the circulation, pooled blood samples were used. To identify fibrocytes in the lung, whole lung digestions were performed. Our ability to translate the findings of the mouse studies to the human requires quite a bit of extrapolation. To address this disconnect in this thesis, we looked at circulating fibrocytes after Bleomycin injury in blood obtained from one single rat and also identification of fibrocytes in paraffin embedded lung samples from the same rat, both of these sampling techniques can be replicated in the human.

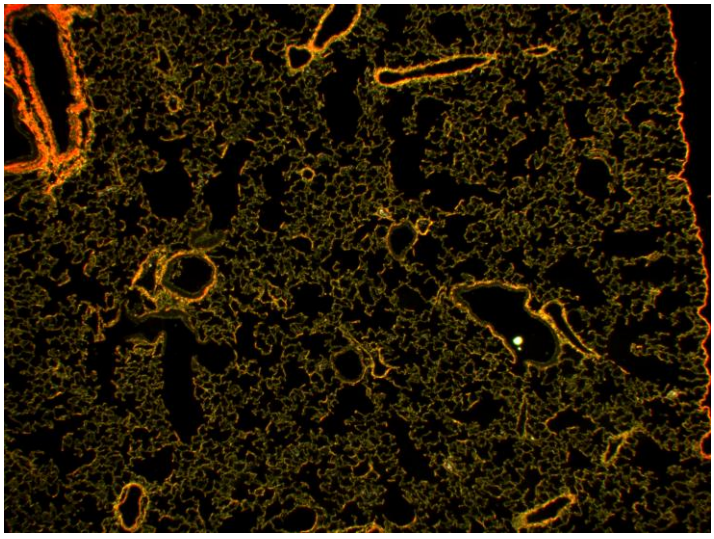
Ad TGF-β

The adenovirus vector system which was previously developed at McMaster (Graham and Prevec 1995) has given us the ability to transfer cytokine genes specifically to airway epithelium, resulting in gene expression which leads to cytokine specific changes in lung pathology (Gauldie et al. 1996). The bleomycin model has helped us to understand the roles of cytokines, growth factors and signaling pathways involved in pulmonary fibrosis. One such cytokine which has been determined to be key in the development of pulmonary fibrosis is TGF-β (Zhao et al. 2002). As noted above, Sime and colleagues (Sime et al.1997) showed that over expression of active TGF-β1 after adenoviral transfer to the lung by intratracheal administration resulted in progressive fibrosis without a major inflammatory component at any stage of the disease.

In these initial experiments, a rapid development of pulmonary fibrosis was seen around the airways, blood vessels and spreading into the interstitium up to and including the pleural surfaces. These investigators found a significant increase in the deposition of ECM proteins and also found an association between the expression of active TGF-β1 and the emergence of myofibroblasts, regarded as being one of the cells responsible for lung parenchymal changes seen in pulmonary fibrosis (Hinz 2012). In the initial studies (Sime et al. 1997), they proposed that because of the drop in TGF-β transgene expression by 14 days after administration, the fibrogenic processes seen later in this model were self perpetuating or that TGF-β was active only in lung microenvironments. Their alternate suggestion was that the normal homeostatic mechanisms of the lung were overwhelmed by the early and rapid fibrosis seen. An important difference to note in the TGF-β model compared to that of Bleomycin is the lesser degree of epithelial injury or inflammation.



Lung treated with Ad TGF-β



Control Lung

Figure 15: (A) Fibrosis in rat lung tissue (magnification x 2.5) stained with Picro Sirus Red and viewed under polarised light after treatment with Ad TGF-β versus (B) control lung

The central role played by TGF-β in fibrogenesis is now undisputed (Gauldie et al. 2007). Although the cellular contributors in this model have been characterised by several investigators, the exact relationship between TGF-β expression and fibrocyte response is still unknown.

AdmGM-CSF

Xing and colleagues (Xing et al. 1996b) in their study delivered Granulocyte macrophage colony stimulating factor (GM-CSF) into the lung of rats using an adenoviral vector (AdmGM-CSF). They found that this resulted in a transient increase in GM-CSF expression (7 to 10 days). Following the cellular response and tissue injury, these investigators saw varying degrees of persistent fibrotic reactions in the later stages after administration of this vector. Notably, in this study Xing and colleagues saw a persistent expression of TGF-β, up to 36 days after administration of GM-CSF, with a very significant level at day 7 after initial administration of GM-CSF. GM-CSF has been reported to alter the actions of cells which are normally found in lung diseases including macrophages and eosinophils (Barnes1994). Studies by Moore and colleagues (Moore et al. 2000) showed that GM-CSF knock out mice had more severe fibrosis when treated with bleomycin than wild type and subsequently showed that CCR2 deficient mice displayed less fibrosis even while showing increased levels of GM-CSF (Moore et al. 2001). It is possible that the pathogenesis of the GM-CSF fibrosis seen in the Xing study may be dependent on the recruitment or maturation of certain cell types. Furthermore based on Moore's results, it may be possible to speculate that CCR2 positive cells may play a role in the pathogenesis of this model of fibrosis including the fibrocyte.

Ad IL-1β

This cytokine has been implicated in IPF by its increased presence in fibrotic lung areas (Smith et al. 1995). A study by Piguet et al (Piguet et al.1993) showed that administering an IL-1β antagonist at the start of an animal model of fibrosis can cause an abrogation of fibrosis. This may indicate a vital need for IL-1β in the initiation of chronic inflammation leading to fibrosis. The effects of over expression of IL-1β on the rodent lung were mechanistically investigated through administration of adenovirus vectors in the rat. In this model, there was acute alveolar and parenchymal damage and marked inflammation, followed by the induction of TGF-β expression leading to progressive interstitial fibrogenesis (Kolb et al. 2001). Further work showed that expression of IL-1β alone in a murine strain that is null for the signalling molecule Smad3, a specific partner in the TGF-β signalling cascade, could not initiate the fibrotic response, thus showing the need for TGF-β signaling to proceed beyond tissue damage and inflammation (Bonniaud et al. 2004). The characterised effect of this cytokine on lung inflammation and the subsequent induction of TGF-β make this a very interesting marker for tracking ongoing lung events during fibrogenesis. The relationship between fibrocyte response and this marker would help us to better understand the potential roles of the fibrocyte in inflammation versus fibrosis.

3.5 Translation

We sought to establish here a robust platform which we can use prospectively to answer several questions about fibrocyte actions. This first required the translation of the measurement of circulating fibrocytes in humans (Moeller et al. 2009) (Strieter et al. 2009) to the rat model. The goal in this translation was to maintain the ability to look at a single subject and correlate its changes in fibrocyte kinetics to the changes in disease. With our established landmarks of disease in the animal model, the time point of fibrocyte variations can allow us to offer evidence about whether fibrocytes are a precursor to or as a result of lung injury.

3.6 Methods

Animals: For all experiments female Sprague-Dawley rats (Charles River) weighing 225-250 grams were used. These animals were housed under special pathogen free conditions.

Anesthesia: Rats were anesthetized with isoflurane (MTC Pharmaceuticals). Animals were placed in an anesthetic chamber and allowed to freely breathe isoflurane and oxygen administered by a portable anesthetic machine for 3 minutes.

Intratracheal administration (IT): Once fully sedated, the rat was then placed in a dorsal recumbent position and maintained this way for the entire procedure. The rat's oropharynx was visualized with a laryngoscope and intubated with a blunted 18 gauge needle. Treatments listed below suspended in a maximum volume of 300µl were then administered using a 1ml syringe.

Intramuscular administration (IM): Once fully sedated, the rat was then placed on its left side and the right hind leg swabbed with 70% alcohol. A 25 guage needle and a 1ml syringe were used to administer treatments listed below in a maximum volume of 100µl.

Treatments:

Reagents:

Bleomycin: Hospira, DIN#02131692: 0.56 units was administered per animal intra-tracheally.

Ad IL-1β: Ad IL-1β is an adenovirus vector that expresses human IL-1β as described previously (Kolb et al. 2001).

Ad TGF-β: Ad TGF-β1 is an adenovirus vector that expresses biologically active porcine TGF-β1as described previously (Sime et al.1997).

AdmGM-CSF: AdmGM-CSF is an adenovirus vector that expresses murine GM-CSF as described previously (Xing et al.1996b).

Ad DL70: Control vector (AdDL70) was prepared as with the other vectors but has no insert in the E1 region. All the vectors were amplified and purified by CsCl gradient centrifugation and plaque tittered on 293 cells.

Ad OPN: Ad OPN is an adenovirus vector that expresses Rat osteopontin. cDNA was amplified by PCR using pEGFP-N1 vector containing rat OPN CDNA (A kind gift from Dr J. Sodek at the University of Toronto, cloning site: EcoR I) as template.

Ad SDF-1: Ad SDF-1 is an adenovirus vector that expresses Human stromal cell-derived factor 1. cDNA was amplified by PCR using pCMV-SPORT6I vector containing human SDF-1 cDNA (ATCC® Number: 9121666, GenBank Number: BC039893) as template.

AMD 3100: Sigma Cat # A5602

Measurement of rat fibrocytes in blood

1) **To investigate fibrocyte responses in lung inflammation and fibrosis caused by pulmonary over expression of IL-1β**: A total of 2×10^8 plaque forming units (pfu) of Ad IL-1β or AdDL70 was administered to rats intratracheally in a volume of 300 µl PBS and animals were sacrificed at days 2,7,14 and 21.

2) **To investigate fibrocyte responses in lung inflammation and fibrosis caused by pulmonary over expression of TGF-β**: A total of 1 ×10^9 pfu of Ad TGF-β or AdDL70 was administered to rats intratracheally in a volume of 300 µl PBS and animals were sacrificed at days 7, 14 and 21.

3) **To investigate fibrocyte responses in lung inflammation and fibrosis caused by pulmonary over expression of GM-CSF**: A total of 2 × 10^8 pfu of AdmGM-CSF or AdDL70 was administered to rats intratracheally in a volume of 300 µl PBS and animals were sacrificed at days 3, 7 and 10.

4) **To investigate fibrocyte responses in circulation caused by <u>systemic</u> over expression of GM-CSF**: A total of 2 × 10^8 pfu of AdmGM-CSF or AdDL70 was administered to rats intramuscular (IM) in a volume of 100 µl PBS and animals were sacrificed at days 3,7,10 and 17.

5) **To investigate fibrocyte responses in circulation and accumulation in lung tissue to IT or IM over expression of GM-CSF and concurrent treatment with AMD 3100**: A total of 2 ×10^8 pfu of AdmGM-CSF or AdDL70 were administered to rats intratracheally in a volume of 300 µl PBS or IM in a volume of 100 µl PBS and the rats were treated concurrently with AMD3100 by subcutaneous injections of 1.8mg/kg daily. These animals were sacrificed at 3 and 7 days after intratracheal administration or 3 days after IM administration.

6) **To investigate fibrocyte responses in lung inflammation caused by treatment with Bleomycin alone or combined with systemic over expression of GM-CSF**: A dose of 0.56 units of Bleomycin was administered intratracheally at Day 0 alone or with the simultaneous administration of a dose total of 2 × 10^8 pfu of AdmGM-CSF administered IM in a volume of 100 µl PBS. These animals were sacrificed at days 3,6,10 and 21; or **b) combined with AMD 3100:** Animals were treated with a dose of 0.56 units of Bleomycin administered intratracheally at Day 0 and AMD3100 by subcutaneous injections of 1.8mg/kg daily then sacrificed at Day 10.

7) **To investigate fibrocyte responses in lung inflammation and fibrosis caused by treatment with Bleomycin alone or combined with a delayed systemic over expression of GM-CSF**: A dose of 0.56 units of Bleomycin was administered intratracheally at Day 0 followed by a delayed dose (Day 10) of 2 × 10^8 pfu of AdmGM-CSF administered IM in a volume of 100 µl PBS and sacrificed at days 17 or 21.

8) **To investigate fibrocyte responses in systemic circulation caused by**

systemic over expression of GM-CSF followed by pulmonary over expression of SDF-1: A total of 2×10^8 plaque forming units (pfu) of AdmGM-CSF was administered IM on day 0 in a volume of 100 µl PBS, followed by $5x10^8$ pfu of Ad SDF-1 intratracheally on Day 1. The animals were sacrificed at days 3, 8 and 10.

9) **To investigate fibrocyte responses in systemic circulation caused by systemic over expression of OPN followed by pulmonary over expression of SDF-1**:: A total of 1×10^9 pfu of Ad-OPN was administered IM on day 0 in a volume of 100 µl PBS, followed by $5x10^8$ pfus of Ad-SDF-1 intratracheally on Day 1. The animals were sacrificed at days 8 and 10.

Blood collection: After sacrifice by Carbon Dioxide inhalation, animals were exsanguinated by intracardiac puncture and the blood for each animal (5-8 mls) collected individually in heparinised tubes (BD).

Flow Cytometry

Blood samples were centrifuged and the mononuclear cell layer ("buffy coat"), found between red cells and plasma (figure16) was removed to 20ml of Ammonium Chloride (ACK) lysis solution. Plasma was stored at -80°C for later analysis by ELISA.

<u>ACK lysis solution (store at RT):</u>
8.29g NH_4Cl (0.15M)
1g $KHCO_3$ (1.0mM) } in 1000ml ddH2O
37.2mg Na_2EDTA (0.1mM)

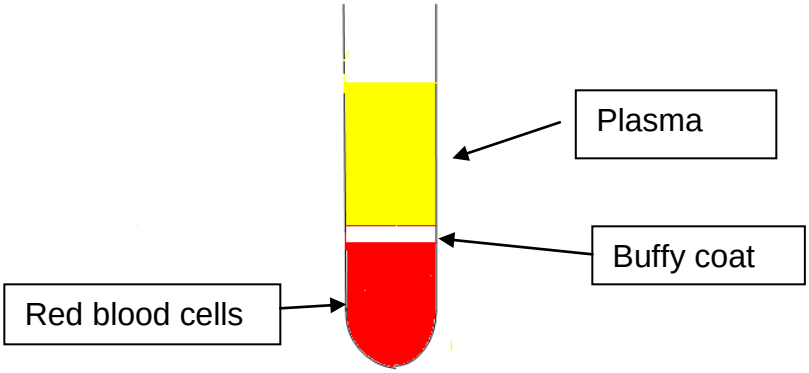


Figure 16: Isolation of buffy coat from blood samples

Leukocytes were incubated in ACK to lyse red blood cells, and then washed with 10ml Facs Analysis (FA) buffer.

FA buffer (fresh every time):
PBS + 0.1% FBS = 40ml PBS + 40µl FBS for 1 sample

Samples were centrifuged at 1200RPM, and a final wash with 20ml FA buffer and subsequent centrifugation provided a relatively pure leukocyte population. Total viable cells were quantified using Trypan blue staining and stored at 5×10^7 cells/ml in freezing solution.

Freezing solution (fresh every time):
90% FBS + 10%DMSO = 900µl FBS + 100µl DMSO for 1ml freezing solution

Cells were cooled in a Nalgene™ freezing container at -80°C for up to 3 days before transfer to a liquid nitrogen storage tank.

For flow cytometry, the procedure followed published methods (Moeller et al. 2009) (Mehrad et al. 2007). Frozen samples, prepared as described above, were allowed to thaw to room temperature (10-15 minutes), and washed with PBS. Cells were re-suspended in FA Buffer and counted. Cells were then aliquoted to

a 96-well plate at 100µl/well, for a final concentration of 1×10^6 cells/well. Cells were stained for CD45 with mouse anti-rat antibody (CD45-PE BD Cat#551402) or isotype control (0.2mg/ml), covered with foil and incubated in a fridge for 25 minutes. Cells were then washed three times with 100µl of FA buffer.

Next, cells were permeablized using the BD Cytofix/Cytoperm® kit to allow for detection of intracellular antigens. The cells were incubated with the Fixation/ Permeabilization solution for 30 min followed by 3 washes of Perm Wash solution (45ml ddH2O + 5ml perm wash stock solution). Cells were incubated in the fridge for 25 minutes with either collagen type 1 antibody (Rockland #600401103) or IgG isotype control antibody (1mg/ml). This was followed by 3 more washes and then incubation in the fridge for 25 minutes with the secondary goat anti-rabbit antibody conjugated to AlexaFluor® 488 (Invitrogen Cat # A11070), which emits in the FITC channel (2mg/ml). Finally, cells were transferred to 1% paraformaldehyde for storage up to 24 hours at 4°C.

Flow Cytometry was performed using FacsCanto. Compensation was calculated with live cells stained with each single used color antibody. For each sample a minimum of 100,000 and a max of 200,000 events were collected, depending on total cell numbers. Analysis of $CD45^+/Col\text{-}1^+$ cells was performed by identifying live cells based on forward and side scatter characteristics, gating on the $CD45^+$ cells, and then gating on the $Col\text{-}1^+$ cells within this population. Percentages of live cells coexpressing both these markers were multiplied by total viable cell count to determine the absolute number of $CD45^+/Col\text{-}1^+$ cells.

Histology

In animals receiving lung treatments, the left lung lobe was inflated with 10% buffered formalin and fixed for 24 hours. Following formalin fixation, the left lungs were paraffin embedded and stained with hematoxylin & eosin (H&E) (Core Histology McMaster University) or picrosirius red (PSR) (Core Histology Mc Master University). Immunohistochemistry stains were performed for alpha smooth muscle actin (Core Histology Mc Master University) to determine its distribution.

To count fibrocytes, Immunoflourescence staining was performed for prolyl-4-hydroxylase (Acris cat # af5110) and CXCR4 (Abcam cat # ab2074). On day 1, sections were deparaffinized and rehydrated. For antigen retrieval, slides were placed for 20min into a preheated steamer in 0.01 M citrate buffer (pH 6.0). Then, sections were blocked for 15 min with 1% normal swine serum (NSS) in PBS, followed by incubation with prolyl 4-hydroxylase (10µg/ml) in 1% NSS/PBS at 4ºC overnight. On day 2, the slides were washed with PBS and incubated with a

Dylight 594 fluorescent secondary antibody (Jackson ImmunoResearch 115-515-205) at 7µg/ml for 2 hours. The sections were then washed carefully and blocked with 10% normal mouse serum for 1 hour. After another wash, sections were incubated overnight with the primary antibody for CXCR4 (1/50 dilution) in PBS at 4ºC. On day 3, the sections were washed and then incubated in a FITC fluorescent secondary antibody for 2 hours (Sigma cat# F1262, 1/40 dilution). Slides were mounted using Vectashield with DAPI (Vector Laboratories). Fibrocytes were counted in high powered microscope fields obtained by a Axiovert microscope, Axiocam MRm and Axiovision 3.1 software (Carl Zeiss) which were used for image acquisition. Pictures were taken with the corresponding filters for DAPI, FITC and Dylight 594 and the composite images were analyzed for the presence of fibrocytes using ImageJ (National Institutes of Health) software.

RT-PCR

In animals receiving lung treatments, on sacrifice, one right lung lobe was snap frozen using liquid nitrogen. These lungs were stored at -80°C until processing. Later, while still frozen, pieces of lung about 100mg in size were quickly snapped off using a mortar and pestle cooled with liquid nitrogen to prevent the lung from thawing. These pieces were then further crushed in the cooled mortar. Crushed tissue was then transferred to a tube containing 500 µl ice cold RNAse free water (Ambion cat # AM9932). The tissue was homogenized for 40-60 seconds on ice using a tissue homogeniser.

Each homogenized sample was incubated on ice for 30 minutes with 1ml Trizol (Invitrogen) and then 300 µl of chloroform was added to each sample. These samples were then placed on a shaker for 15minutes. After shaking the samples were centrifuged at 4°C for 20 minutes at 14000 rpm. The clear supernatant was then transferred to ice-cold isopropanol and this was then incubated at -80°C for at least 2 hours.

After 2 hours, the samples were thawed and centrifuged at 14000 rpm 4°C for 30 minutes. The supernatant was discarded and the pellet washed in 500µl 70% ethanol made in nuclease free water and then centrifuged at 14000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was air dried at room temperature. The pellet was then dissolved in 20µl of RNAse-free water. The RNA concentration was measured and recorded using a NanoDrop™ spectrophotometer. Using the RNA concentration, the volume of each sample containing 1 µg of RNA was measured and combined with a variable amount of water to make up the volume to 16 µl. 4 µl of q script cDNA supermix (Quanta

biosciences cat # 95048-025) was then added to each sample. This was placed in a eppendorf Mastercycler gradient incubator and put through the incubation cycle (5 minutes at 25ºC then 30 minutes at 42 ºC then 5 minutes at 85 ºC) for 40 minutes in total and held at 4 ºC until used.

This newly formed cDNA was diluted 6 times with RNAse free water. Each cDNA sample was then used for RT-PCR analysis, 5 µl of cDNA was combined with 1 µl of gene, 6.5 µl of water and 12.5 µl of Taq Universal PCR mastermix (cat# 4304437). The genes used were Applied Biosystems: Col-1A1, CXCR4, Fibronectin, Col-3 and housekeeping genes: B2m and gapdh. This was then run in an Applied Biosystems 7500 Fast RT-PCR analyser in 96 well reaction plates using Taqman reagents and a standard run. Gene expression was expressed relative to that of naïve animals.

ELISA

For tissue: Frozen tissue 25-30mg in size was homogenised in Radioimmunoprecipitation assay buffer (RIPA) buffer approximately 10 times the volume (µl) used per mg of tissue.

RIPA working solution

1ml RIPA stock solution
10µl 0.1M Phenylmethanesulfonyl Fluoride stock
40µl 25X Roche Protease Inhibitor Cocktail stock
10 µl 1M NaF stock
5 µl 0.2M Na_3VO_4

Using a tissue homogenizer with a fine tip probe, the tissue was homogenized for 7 seconds at max disruption speed while working on ice. The probe was rinsed twice with 70% Ethanol and distilled water in between samples.

The crude lysate was then transferred into 1.5ml microcentrifuge tubes on ice and then centrifuged for 20mins at 13000prm at 4°C. The supernatants were aliquoted and transferred to a fresh chilled tube to be stored at -80°C. The supernatants were then thawed and treated and assayed as described below for plasma

For Plasma: Plasma previously stored was thawed and the protein concentration assayed spectrophotometrically using Biorad Protein assay solution (cat # 500-0006). The concentration of the protein was adjusted where necessary as

recommended by the manufacturer to meet the range of the ELISA kit. The levels of GM-CSF were measured using an ELISA kit for murine GM-CSF (R&D systems cat# MGM00) according to the manufacturer's instructions. The ELISA plate was read using a Thermo Electron Multiskan Ascent plate reader.

Hydroxyproline Assay

The hydroxyproline content in the rat lung tissue was determined using the method of Woessner (Woessner 1961). 2 mls of rat lung homogenate was placed in pre-weighed pyrex glass tubes. These samples were frozen (-70°C) then lyophilised using a Modulyod freezer dryer. These tube were then weighed and the dry lung weight calculated. 5ml of 10% trichloroacetic acid was added to each sample and these tubes were kept on ice for 1 hour. Samples were centrifuged at 3000g for 15 minutes at 4°C and the supernatants decanted. The tubes were closed with Teflon caps and secured with Teflon tape and the pellets were then hydrolysed in 2ml 6N HCL for 16 hours at 110°C in a dry water bath incubator. 100µl of methyl red, a PH indicator, was then added to each sample. Samples were then neutralised by adding NaOH and the final volume of each sample was recorded. 1ml of Chloramine T solution was added to each tube and samples were incubated at room temperature for 20 minutes. Then 1ml of 3.15N perchloric acid was added to each tube and they were incubated at room temperature for 5 minutes. Finally 1ml of Ehrlich's reagent was added to each tube and samples incubated at 65°C for 20 minutes. Absorbance of each sample was read at 557nm on the Spectrophotometer and the results calculated as µg hydroxyproline/mg dry lung weight using a hydroxyproline standard (Sigma).

BAL

BAL fluid was collected by washing the lungs of animals upon sacrifice. A small incision was made in the trachea directly after sacrifice and a canula inserted.5mls of PBS was used for the wash and the lungs were washed repeatedly 3 times with the same fluid. The final wash was termed the BAL fluid and this was kept on ice until processing. BAL fluid was centrifuged at 1500 rpm for 10 minutes at 4 °C. The supernatants were collected and stored at −80 °C for further tests. The pellet was resuspended in 1ml PBS and several dilutions done to obtain a cell smear using a Cytospin III (Thermo Scientific). Smears were stained with Wright Giemsa (Sigma, Oakville, ON, Canada) and differential cell counts were done by counting 300 cells per smear.

Statistical Analysis

A one way ANOVA followed by a Tukey's post test was performed for all groups where applicable. For comparison of two groups only the student's t test was used.

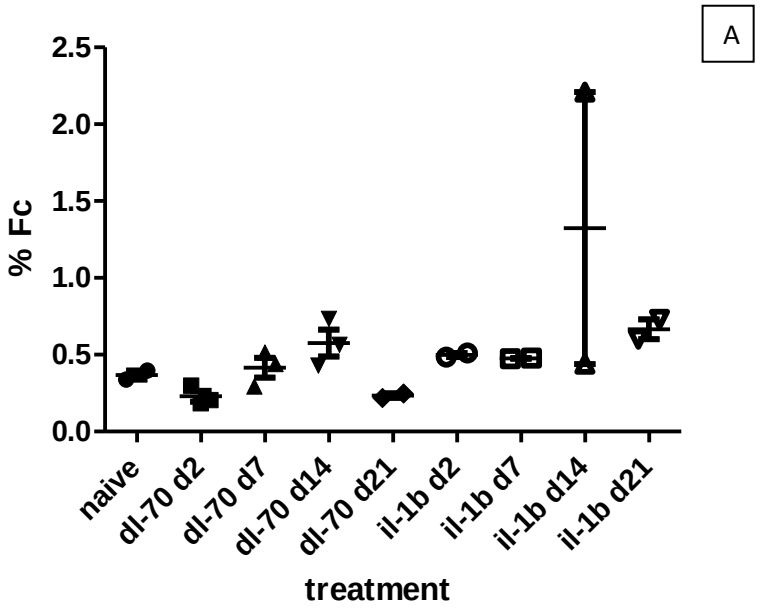
3.7 Results

Flow cytometry

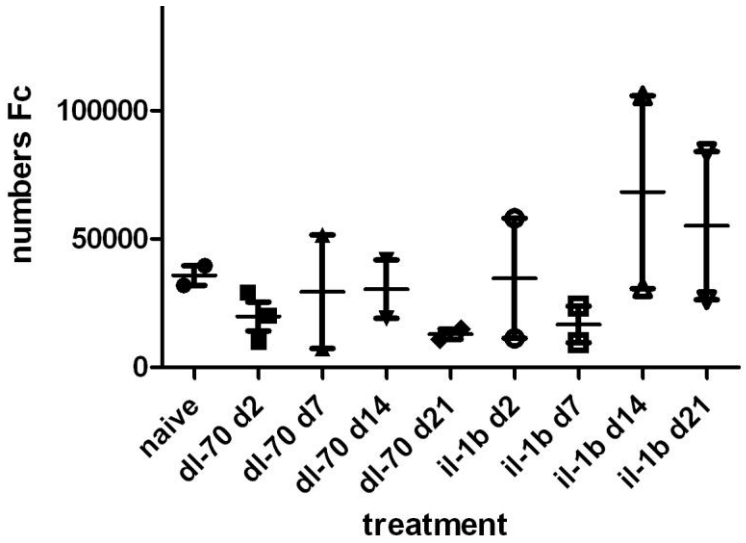
Having established a successful protocol for the isolation and measurement of fibrocytes (Fc) from the buffy coat of human patients, we reproduced this measurement in rats as described above. We sampled individual rats rather than a pooled sample and were able to get a maximum of 8 mls of blood per rat. We stained the isolated cells for CD45 and Col-1 as described above and then assayed 100,000 events by Flow cytometry. After Flow cytometry we calculated the percentage of cells which were CD45 positive by gating on the unstained cells and identifying those which were stained for PE. We then excluded the cells which were positive for CD45 only (PE positive only) and calculated the remainder as CD45/Col-1 positive (PE/FITC positive). We expressed our positive cells as percentages as described above or as number of Fc. Number of Fc was calculated from the total number of cells obtained from animal sacrifice, counted just before staining with CD45 and Col-1 antibodies.

1) To investigate fibrocyte responses in lung inflammation and fibrosis caused by pulmonary over expression of IL-1β

We administered Ad IL-1β intra-tracheally as described above and sacrificed these animals at various timepoints, days 2,7,14 and 21. We used as controls, a delete vector (DL-70) and naive animals. We saw as shown below a rise in fibrocyte numbers, particularly at day 14, followed by a decrease at day 21.



il-1b: Animals treated intra-tracheally with Ad IL-1β (n=2)
dl-70: Animals treated intra-tracheally with Ad-DL70 (n=3 d2, n=2 d7,14,21)
naïve: Animal receiving no treatment (n=2)
%Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1



il-1b: Animals treated intra-tracheally with Ad IL-1β (n=2)
dl-70: Animals treated intra-tracheally with Ad-DL70 (n=3 d2, n=2 d7,14,21)
naïve: Animal receiving no treatment (n=2)
numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1

Figure 17: (A) Percentages and (B) Absolute numbers of circulating fibrocytes after Ad IL-1β. Compared to Ad-DL 70 treated and naïve

We also detected the presence of fibrocytes, detected by a positive immunostain for CXCR4 and prolyl-4-hydroxylase, in the lungs of animals treated with IL-1β after 14 days. We speculated that these fibrocytes had 'trafficked' into the lung from the circulation, as their detection in the lung tissue corresponded with a detectable rise in circulation.

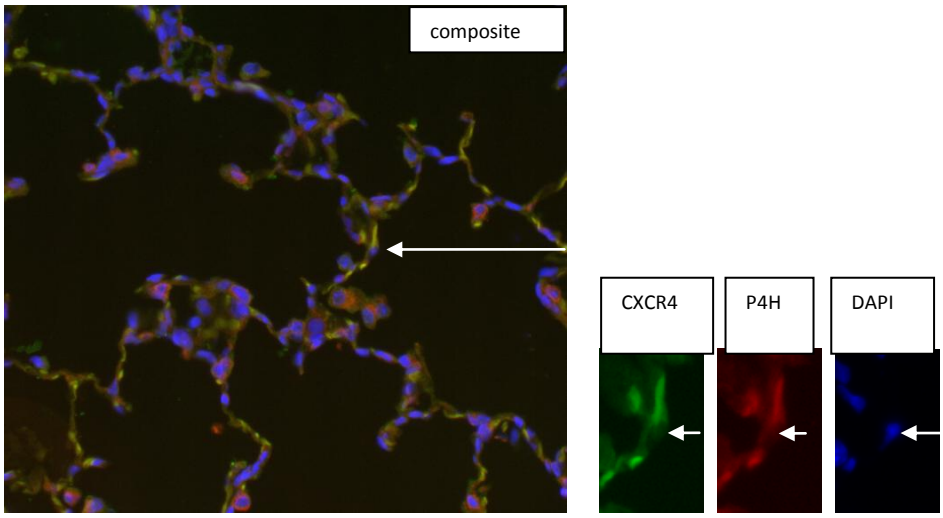
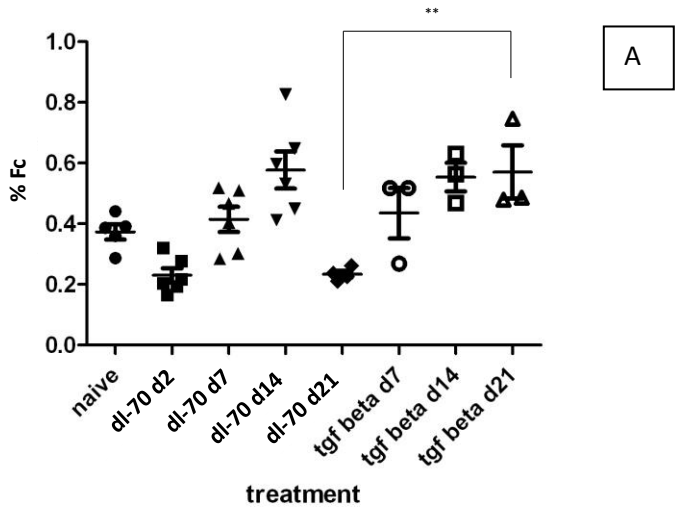


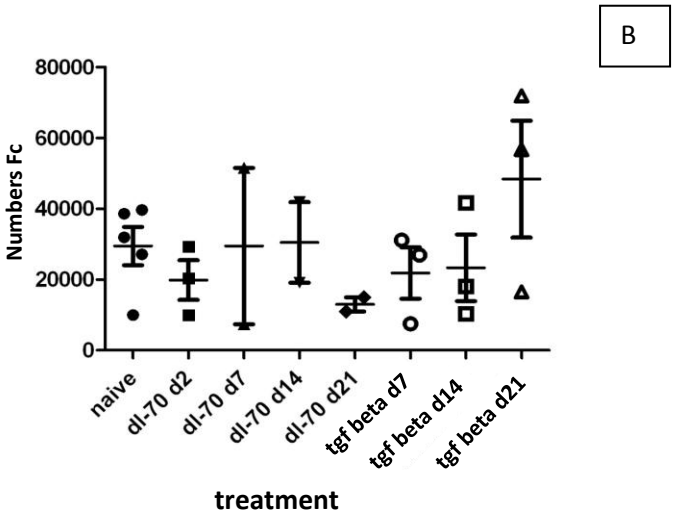
Figure18: Fibrocytes identified in the lung after IL-1β. Detected by Immunoflourescence at day 21 after treatment (x20).

DAPI indicates nuclei, CXCR4: a positive stain detected in the FITC channel and Prolyl-4-hydroxylase (P4H): a positive stain detected in the Dylight 594 channel. Arrow indicates a cell positive for CXCR4 and prolyl-4-hydroxylase.

2) To investigate fibrocyte responses in lung inflammation and fibrosis caused by pulmonary over expression of TGF-β

We administered Ad TGF-β in an attempt to characterise the fibrocyte kinetics seen in this well characterised model of lung fibrosis. We wanted to see if the levels of circulating fibrocytes changed at timepoints which have been defined in past experiments and are marked by significant changes in the rodent lung after the administration of TGF-β.

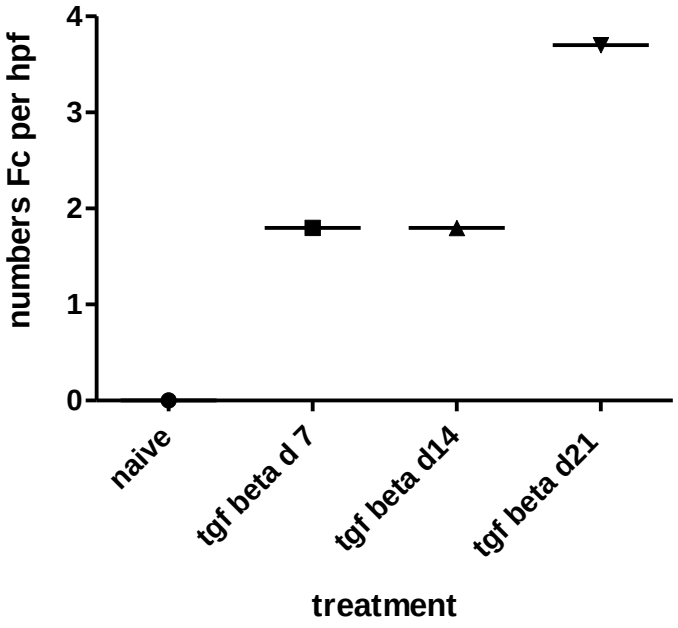




tgf beta: Animals treated intratracheally with Ad TGF-β (n=3)
dl-70: Animals treated intra-tracheally with Ad-DL70 (n=3 d2, n=2 d7,14,21)
naïve: Animal receiving no treatment (n=5)
%Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1
Numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1

Figure 19: (A) Percentages and (B) Absolute numbers of circulating fibrocytes after Ad TGF-β. One way ANOVA $p<0.05$.

We saw an increase in the number of fibrocytes from day 14 and this appears to correlate to the fibrotic response seen in these animals and there is a significant difference between the animals which received the delete vector and those that received TGF-β at day 21.



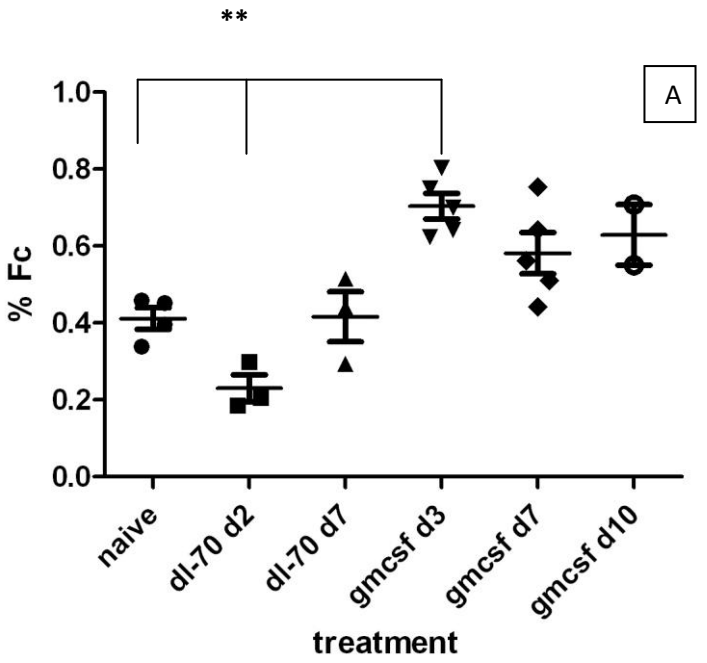
tgf beta: Animals treated intratracheally with Ad TGF-β (n=1)
numbers Fc per hpf: Average number of fibrocytes per high powered field

Figure 20: Fibrocytes identified in the lung after Ad TGF-β. Average number of fibrocytes found per high powered field in lung sections treated with TGF-β

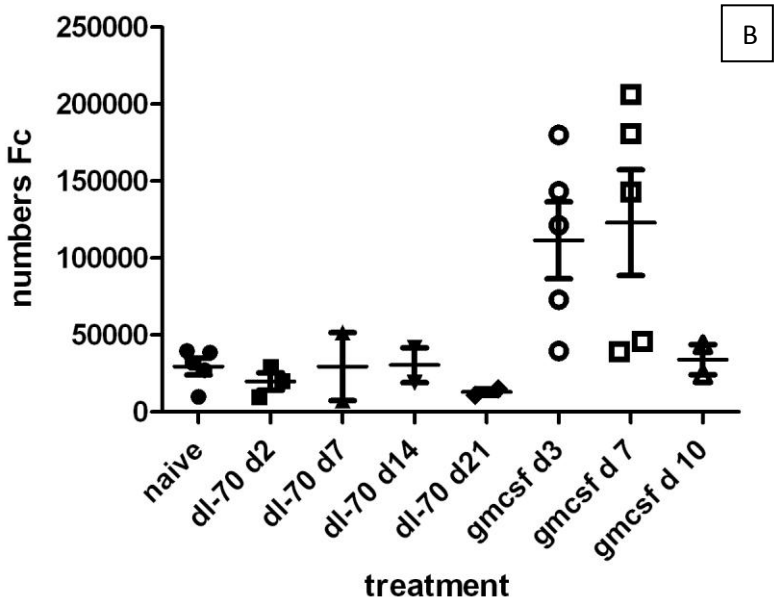
The increase in circulating number of fibrocytes concurred with that of fibrocytes we found in the lung tissue as well, further adding to the hypothesis that these cells are recruited to areas of fibrotic lung injury and may play a role in the progression the fibrosis seen.

3) To investigate fibrocyte responses in lung inflammation and fibrosis caused by pulmonary over expression of GM-CSF

We administered AdmGM-CSF intratracheally and sacrificed animals on days 3,7 and 10 and compared the levels of fibrocytes found in the circulation to that of the controls mentioned above. We found a two fold increase in the number of fibrocytes at days 3 and 7 (see below).



gmcsf: Animals treated intratracheally with AdmGM-CSF (n=5 d3,7 n=2 d10)
dl-70: Animals treated intratracheally with Ad-DL70 (n=3)
naïve: Animal receiving no treatment (n=5)
%Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1



gmcsf: Animals treated intratracheally with AdmGM-CSF (n=5 d3,7 n=2 d10)
dl-70: Animals treated intratracheally with Ad-DL70 (n=3 d2, n=2 d7,14,21)
naïve: Animal receiving no treatment (n=5)
numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1

Figure 21: (A) Percentages and (B) Absolute numbers of circulating fibrocytes after AdmGM-CSF intratracheally. One way ANOVA $p<0.05$.

We also examined the lung tissue of these animals for evidence of fibrocytes. We detected an increase in the number of fibrocytes in the lungs from day 7 as shown below

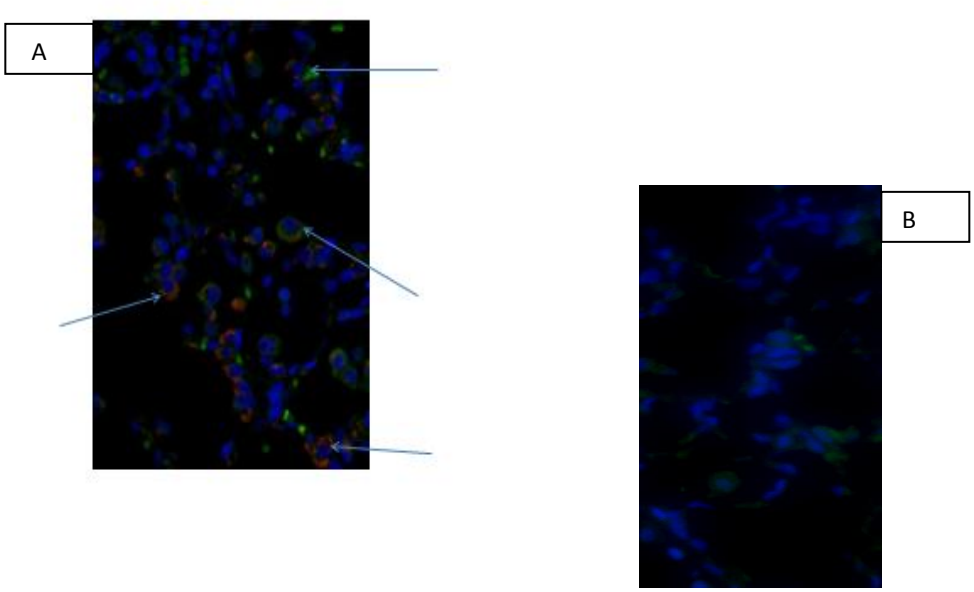
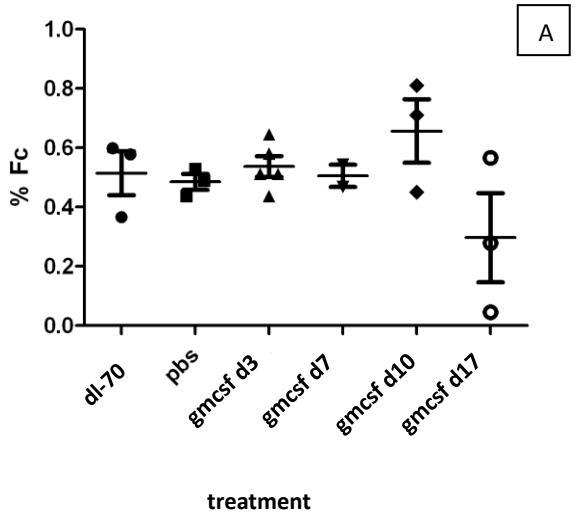


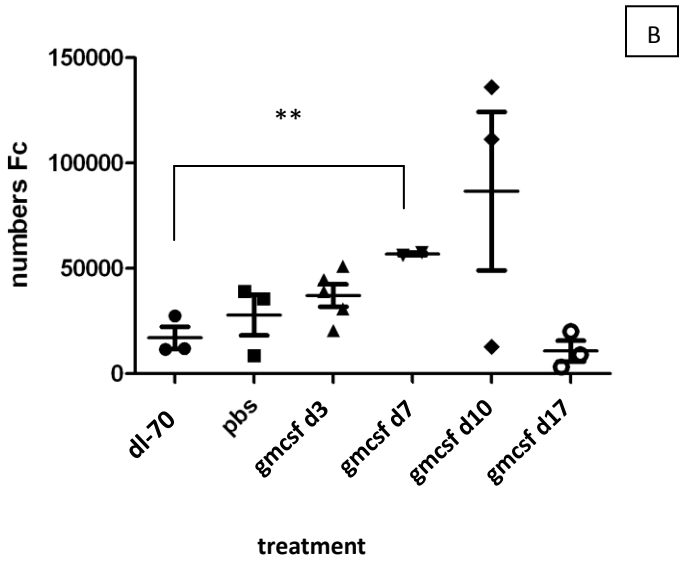
Figure 22: Fibrocytes identified in the lung after AdmGM-CSF (A) Double positive fibrocytes CXCR4 (green) and Prolyl-4-hydroxylase (red) found in AdmGM-CSF treated lungs (DAPI: blue indicates nuclei) (B) Lung of a naïve rat

4) To investigate fibrocyte responses in circulation caused by <u>systemic</u> over expression of GM-CSF

We administered AdmGM-CSF intramuscular (IM) and sacrificed animals on days 3,7,10 and 17,to see if a similar circulating fibrocyte response could be generated as when administered intratracheally. We saw a 2 fold rise in circulating fibrocytes at day 7 after administration and this remained elevated until day 10, then decreased to control levels (figure 23).



gmcsf: Animals treated intramuscular with AdmGM-CSF (n=5 d3, n=2 d7, n=3 d10)
dl-70: Animals treated intramuscular with Ad-DL70 7 days after administration (n=3)
pbs: Animal receiving pbs intramuscular (n=3)
% Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1

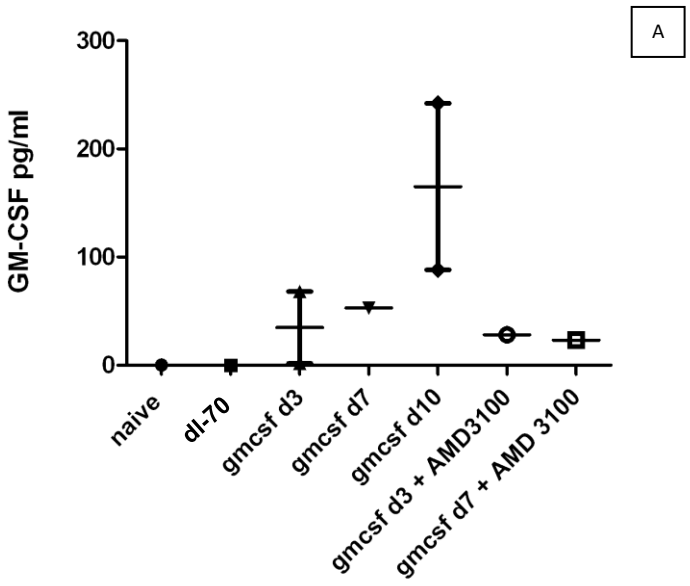


GMCSF: Animals treated intramuscular with AdmGM-CSF (n=5 d3, n=2 d7, n=3 d10)
dl-70: Animals treated intramuscular with Ad-DL70 7 days after administration (n=3)
pbs: Animal receiving pbs intramuscular (n=3)
numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1

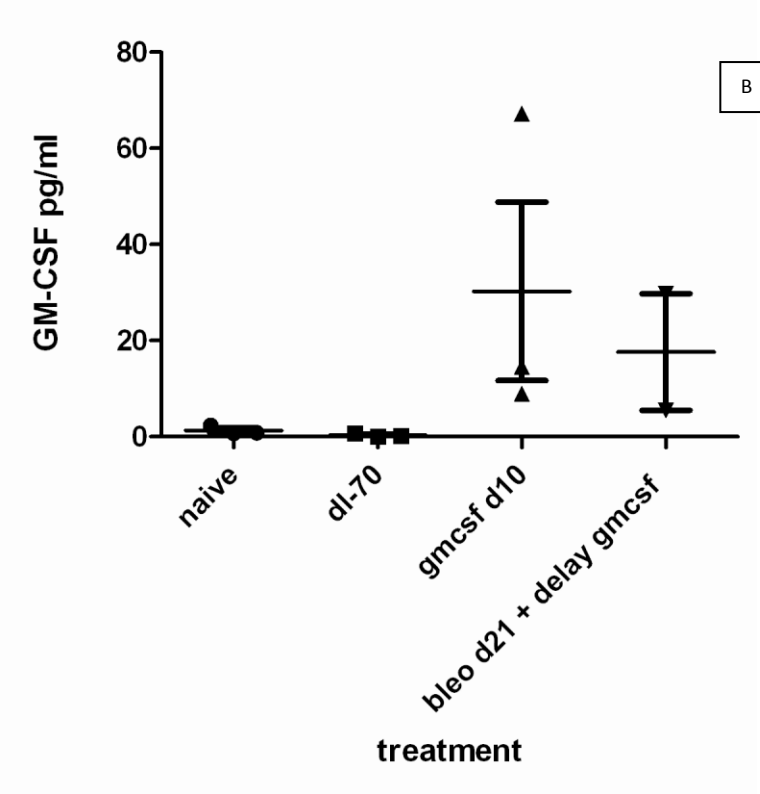
Figure 23 (A) Percentages and (B) Absolute numbers of circulating fibrocytes after AdmGM-CSF Intramuscular. One way ANOVA $p < 0.05$.

ELISA

We performed an ELISA as described above looking for the presence of GM-CSF in the treated lung tissue as well as in the serum of animals treated with AdmGM-CSF intramuscular. In both instances there was a detectable amount of GM-CSF compared to control animals (figure 24).



naive: Animal receiving no treatment (n=1)
dl-70: Animals treated intramuscular with Ad-DL70 7 days after administration (n=1)
gmcsf: Animals receiving AdmGM-CSF intra tracheally (n=2 d3,10 n=1 d7)
gmcsf + AMD 3100: Animals receiving AdmGM-CSF intra tracheally once on day 0 and subcutaneous AMD3100 daily (n=1)

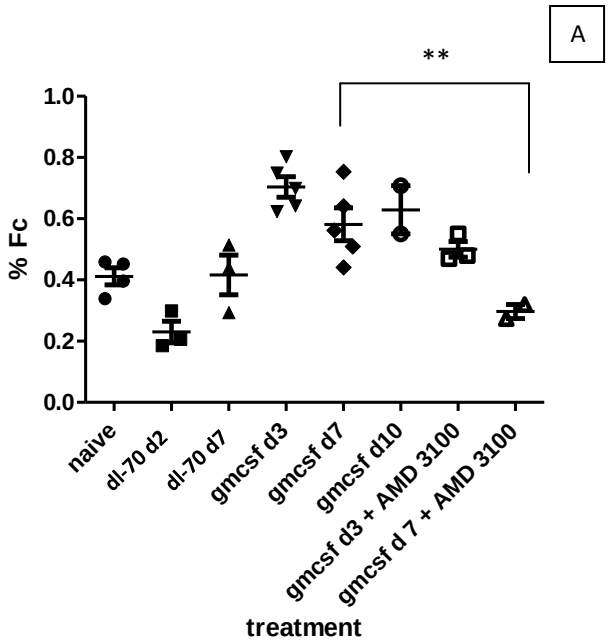


naive: Animal receiving no treatment (n=3)
dl-70: Animals treated intramuscular with Ad-DL70 9 days after administration (n=3)
gmcsf: Animals receiving AdmGM-CSF intramuscular (n=3)
bleo + delay gmcsf: Animals receiving bleomycin intra tracheally on day 0 and intramuscular AdmGM-CSF on day 10 (n=2)

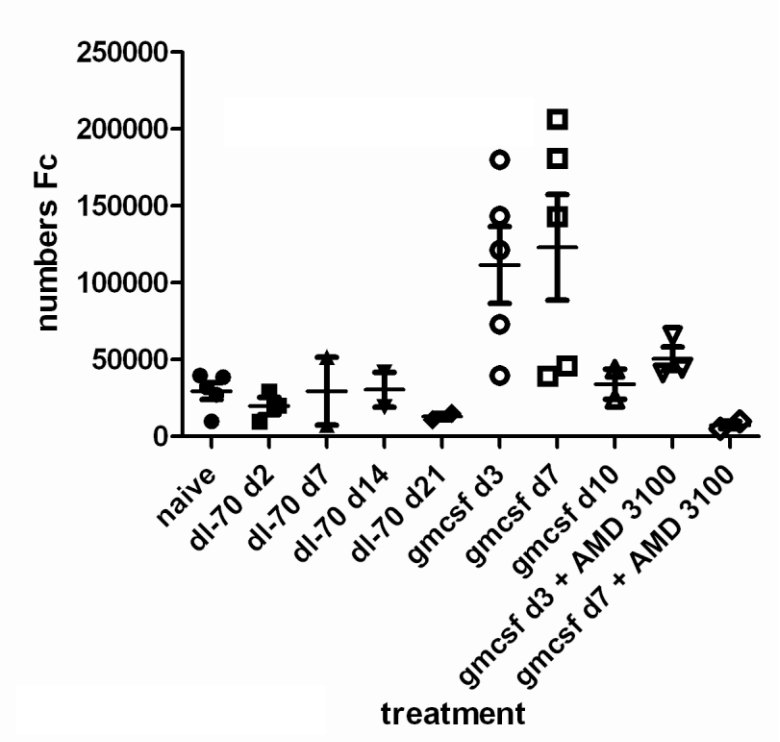
Figure 24: GM-CSF levels detected by ELISA. Using lung homogenates in (A) and serum in (B)

5) To investigate fibrocyte responses in circulation and accumulation in lung tissue to IT or IM over expression of GM-CSF and concurrent treatment with AMD 3100

We administered AdmGM-CSF intratracheally and intramuscularly followed by AMD 3100 subcutaneously (sc) daily. We conducted this procedure for 3 and 7 days (IT administration) or 3 days (IM administration). In both instances we saw a dramatic reduction in the number of fibrocytes up to 75% (figure 25 and 26).

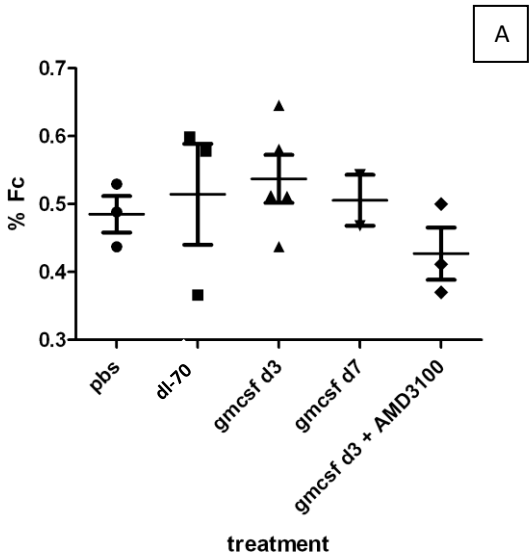


gmcsf: Animals treated intratracheally with AdmGM-CSF (n=5 d3,7 n=2 d10)
dl-70: Animals treated intratracheally with Ad-DL70 (n=3)
naive: Animal receiving no treatment (n=5)
gmcsf + AMD 3100: Animal receiving AdmGM-CSF once intratracheally on day 0 and AMD3100 subcutaneously every day (n=3 d3, n=2 d7)
% Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1

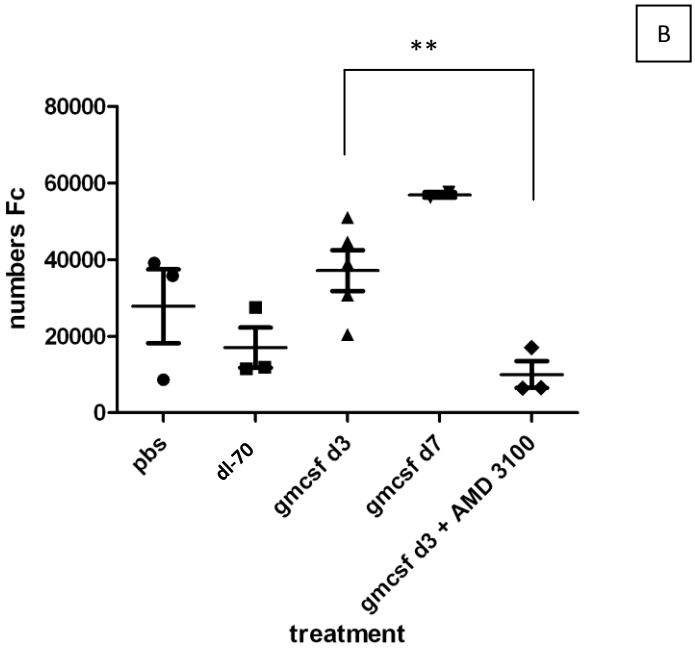


gmcsf: Animals treated intratracheally with AdmGM-CSF (n=5 d3,7 n=2 d10)
dl-70: Animals treated intratracheally with Ad-DL70 (n=3 d2, n=2 d7,14,21)
naive: Animal receiving no treatment (n=5)
gmcsf + AMD 3100: Animal receiving AdmGM-CSF once intratracheally on day 0 and AMD3100 subcutaneously every day (n=3 d3, n=2 d7)
numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1

Figure 25: (A) Percentages and (B) Absolute numbers of circulating fibrocytes after AdmGM-CSF Intratracheally and AMD 3100. AdmGM-CSF was administered intratracheally on Day 0 and animals received AMD 3100 subcutaneously daily for 3 and 7 days. One way ANOVA $p<0.05$.



gmcsf: Animals treated intramuscular with AdmGM-CSF (n=5 d3,n=2 d7)
dl-70: Animals treated intramuscular with Ad-DL70 7 days after administration (n=3)
pbs: Animal receiving pbs intramuscular (n=3)
gmcsf + AMD 3100: Animal receiving AdmGM-CSF once intramuscular on day 0 and AMD3100 subcutaneously every day (n=3)
% Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1



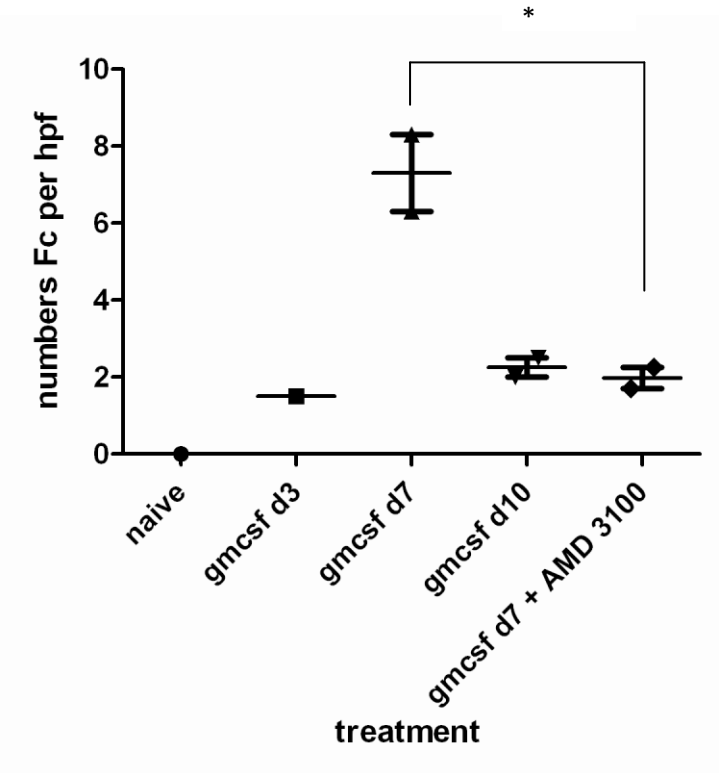
gmcsf: Animals treated intramuscular with AdmGM-CSF (n=5 d3,n=2 d7)
dl-70: Animals treated intramuscular with Ad-DL70 7 days after administration (n=3)
pbs: Animal receiving pbs intramuscular (n=3)
gmcsf + AMD 3100: Animal receiving AdmGM-CSF once intramuscular on day 0 and AMD3100 subcutaneously every day (n=3)
numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1

Figure 26: (A) Percentages and (B) Absolute numbers of circulating fibrocytes after administration of AdmGM-CSF Intramuscular and AMD 3100. AdmGM-CSF administered Intramuscularly on day 0 and administration of AMD 3100 subcutaneously daily for 3 days. One way ANOVA $p<0.05$.

Immunoflourescence staining for fibrocytes in the lung of animals which received AdmGM-CSF intratracheally and AMD 3100 subcutaneously

After staining lung sections for fibrocytes (CXCR4/prolyl-4hydroxylase double positive cells) in animals that received AdmGM-CSF intratracheally and also

treated with AMD3100, we counted the number of fibrocytes we found in 15 high powered fields. We saw an increase and then decrease in fibrocyte numbers from days 3-10 with the maximum being at day 7 (figure 27). We also saw a large decrease in the number of fibrocytes found in the lung tissue at day 7 when treated with AMD 3100. This observation supports the possible role for AMD3100 neutralising the CXCR4 receptor present on fibrocytes and preventing them from entering the lung.



naïve: animal received no treatment (n=1)
gmcsf: AdmGM-CSF administered intratracheally (n=1 d1, n=2 d7 d10)
gmcsf + AMD 3100: AdmGM-CSF administered intratracheally on Day 0 and AMD 3100 administered daily subcutaneously (n=2)
numbers Fc per hpf: average number of fibrocytes counted per animal per high powered microscope field.

Figure 27: Fibrocytes identified in the lung after AdmGM-CSF and AMD 3100. Average number of fibrocytes found per high powered field in the lung after treatment with GM-CSF alone intratracheally or with GM-CSF initially and AMD3100 daily thereafter for 7 days. Student's t test $p<0.05$.

Gene expression after administration of AdmGM-CSF

Gene expression was performed to characterise the possible function of fibrocytes which entered the lung after administration of AdmGM-CSF and furthermore to examine whether this activity could be reversed by reducing fibrocyte entry into the lung. After administration of AdmGM-CSF we detected an increase in ECM gene expression in the lung until day 10 post administration. There was a modest increase in αSMA, Collagen-1 and Fibronectin with the highest expression being detected at day 10 post administration (figure 28).

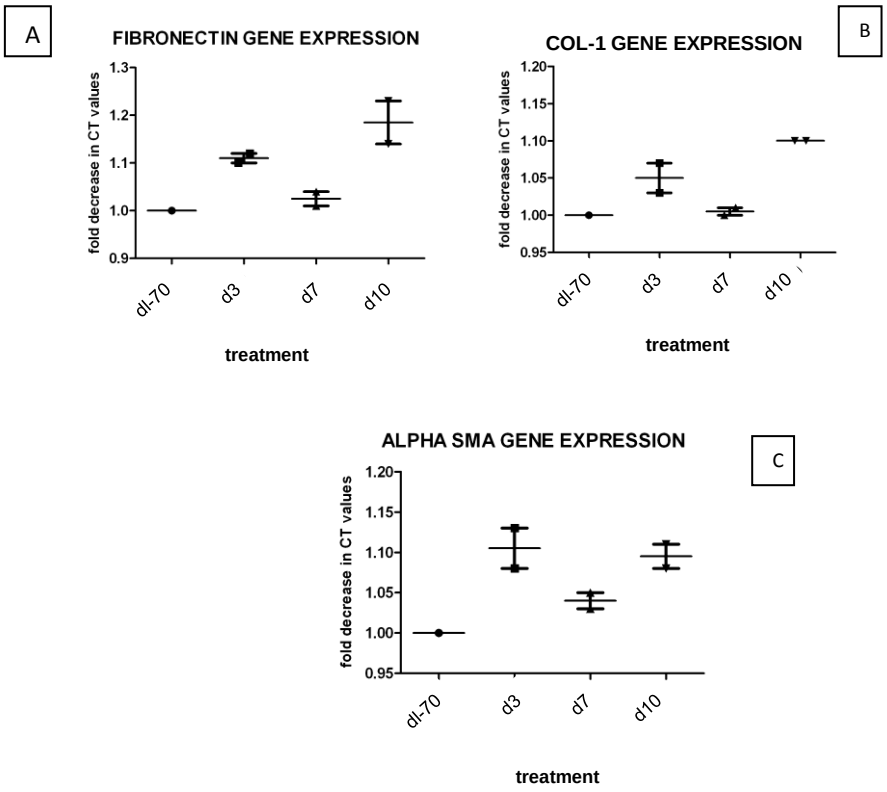
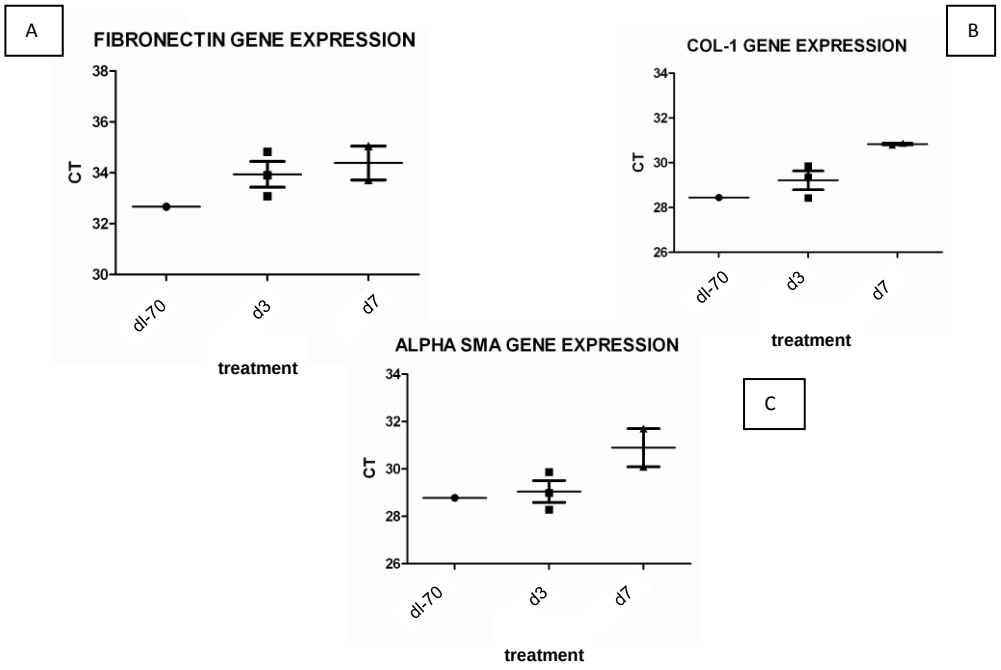


Figure 28: Gene expression in the lung after AdmGM-CSF intratracheally. (A) Fibronectin (B) Collagen-1 and (C) αSMA after the administration of AdmGM-CSF (Days 3,7 and 10 (n=2))

Fold decrease in CT values represent increase in gene expression, dL-70 represents animals which received Ad-DL70 only 7 days after administration (n=1).

Gene expression after administration of AdmGM-CSF plus AMD 3100

We examined a similar panel of ECM genes discussed above to determine if the administration of AMD3100 and its effect on reducing circulating fibrocytes had any effect on gene expression. Unlike the modest increase we saw after the administration of AdmGM-CSF alone, we saw no increases in gene expression after the administration of AdmGM-CSF and AMD 3100 (see below). There was however a possible decrease in expression of col-1 and αSMA at day 7 compared to the control. This would have to be investigated with larger numbers of controls and test subjects.



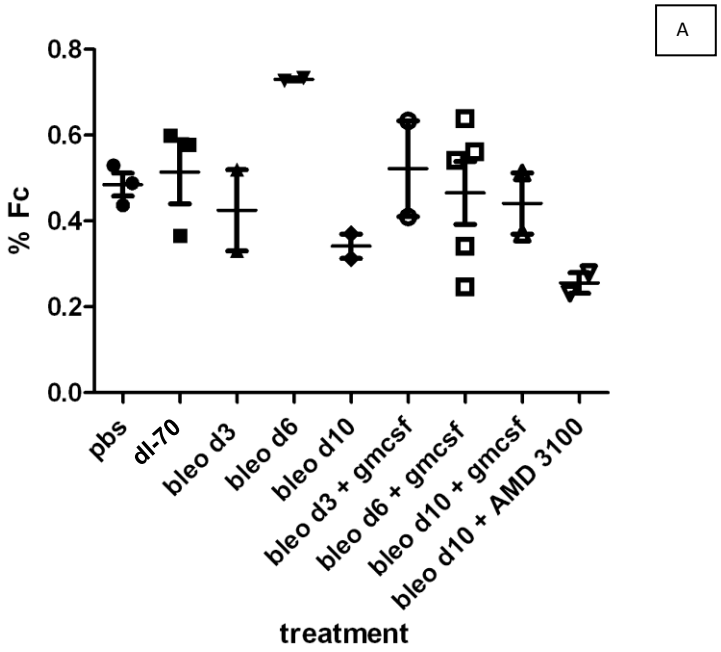
CT (cycle threshold): lower CT values represent increase in gene expression
dl-70: animals which received AdDL70 intra tracheally for 7 days (n=1)
day 3/7: animals which received AdmGM-CSF on day 0 and then AMD 3100 subcutaneously daily (n=3 d3, n=2 d7)

Figure 29: Gene expression in the lung after AdmGM-CSF intratracheally and AMD 3100. (A) Fibronectin (B) Collagen-1 and (C) αSMA after the administration of AdmGM-CSF intratracheally and AMD 3100 subcutaneously for up to 7 days.

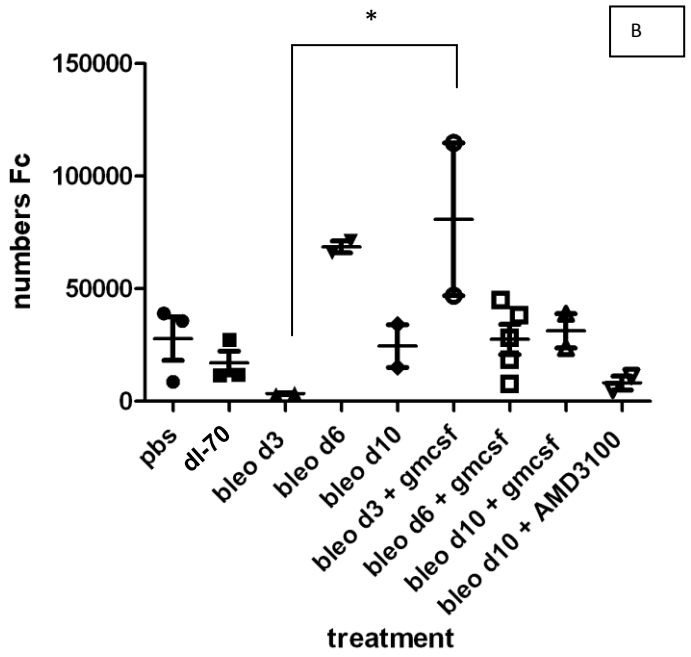
6) To investigate fibrocyte responses in lung inflammation caused by treatment with Bleomycin alone or combined with systemic over expression of GM-CSF

We combined the Bleomycin model of fibrotic lung injury with the administration of AdmGM-CSF to see if GM-CSF administration could modulate the well characterised Bleomycin injury. We administered Bleomycin intratracheally and

simultaneously administered GM-CSF IM. We sacrificed these animals at days 3,6 and 10. While we did observe some increases in fibrocyte numbers (see below) the kinetics of the fibrocyte response was not the expected smooth biological response we had observed in past testing. There was a dip in the level of circulating fibrocytes at day 6 after administration of AdmGM-CSF and bleomycin. We did however see a decrease in the circulating number of fibrocytes when AMD 3100 was administered to animals treated with Bleomycin.



pbs: Animal receiving pbs intramuscular (n=3)
dl-70: Animals treated intramuscular with Ad-DL70 7 days after administration (n=3)
bleo: Animals receiving bleomycin intratracheally (n=2)
bleo + gmcsf: Animals receiving bleomycin intratracheally and intramuscular AdmGM-CSF both on day 0 (n=2 d3,d10) (n=5 d6)
bleo + AMD 3100: Animal receiving bleomycin once intratracheally on day 0 and AMD3100 subcutaneously every day
% Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1

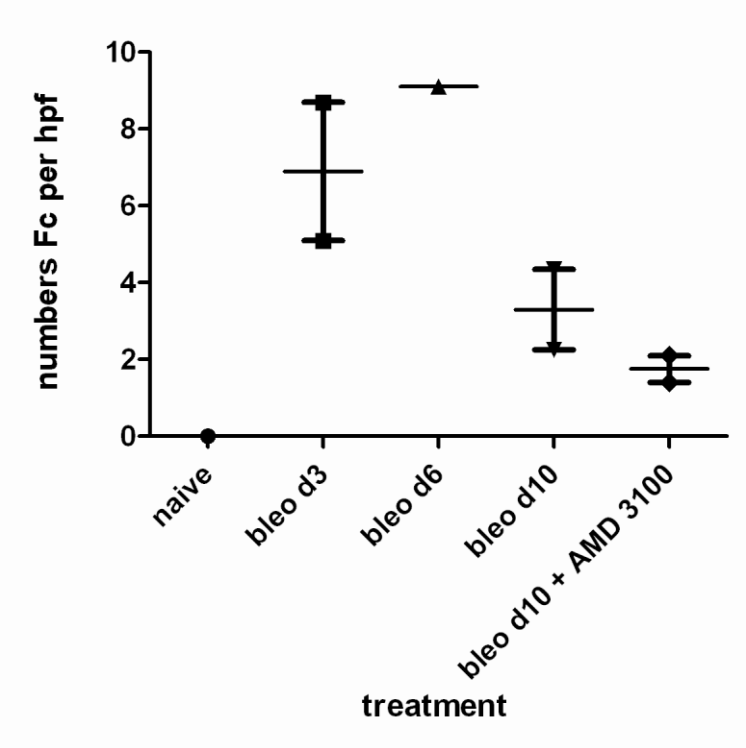


pbs: Animal receiving pbs intramuscular (n=3)
dl-70: Animals treated intramuscular with Ad-DL70 7 days after administration (n=3)
bleo: Animals receiving bleomycin intratracheally (n=2)
bleo + gmcsf: Animals receiving bleomycin intratracheally and intramuscular AdmGM-CSF both on day 0 (n=2 d3,d10) (n=5 d6)
bleo + AMD 3100: Animal receiving bleomycin once intratracheally on day 0 and AMD3100 subcutaneously every day
numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1

Figure 30: (A) Percentages and (B) Absolute numbers of circulating fibrocytes after Bleomycin intratracheally and AdmGM-CSF intramuscular. Measured by FACS after administration of 1) Bleomycin administered intratracheally alone (2) AdmGM-CSF administered concurrently with Bleomycin intratracheally 3) Bleomycin administered with AMD 3100. One way ANOVA $p<0.05$.

Immunoflourescence staining for fibrocytes in animals treated with bleomycin intra tracheally

After staining sections for fibrocytes (CXCR4/prolyl-4hydroxylase double positive cells) we counted the number of cells we found per high powered field. We saw an increase and then decrease in the numbers from days 3-10 with the maximum being at days 3-7 (see below). We also saw a decrease in the number of fibrocytes found in the lung tissue at day 10 when treated with AMD 3100. This information also supports the kinetics seen in the circulation described above.



naïve: animal received no treatment
bleo: bleomycin administered intra tracheally (n=2 d3,d10) (n=1 d6)
bleo + AMD 3100: bleomycin administered intra tracheally on Day 0 and AMD 3100 administered daily subcutaneously (n=2)
numbers Fc per hpf: average number of fibrocytes counted per animal per high powered microscope field.

Figure 31: Fibrocytes identified in the lung after Bleomycin intracheally and AMD 3100. Average number of fibrocytes found per high powered field after treatment with Bleomycin alone intratracheally or with Bleomycin initially and AMD3100 daily thereafter for 7 days

Bleomycin and AdmGM-CSF concurrent administration at day 21

To determine if the fibrocyte kinetics described above resulted from a change in the early stages of bleomycin induced fibrosis, we examined a later time point. We chose day 21, a time point which is known to be after the well characterised inflammatory phase in the development of bleomycin induced fibrosis as shown in figure 32. However, there was no significant increase in the level of circulating fibrocytes after concurrent administration of AdmGM-CSF and bleomycin compared to bleomycin alone as shown in figure 33.

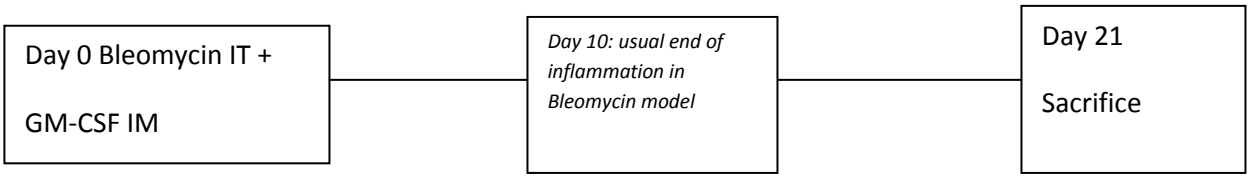
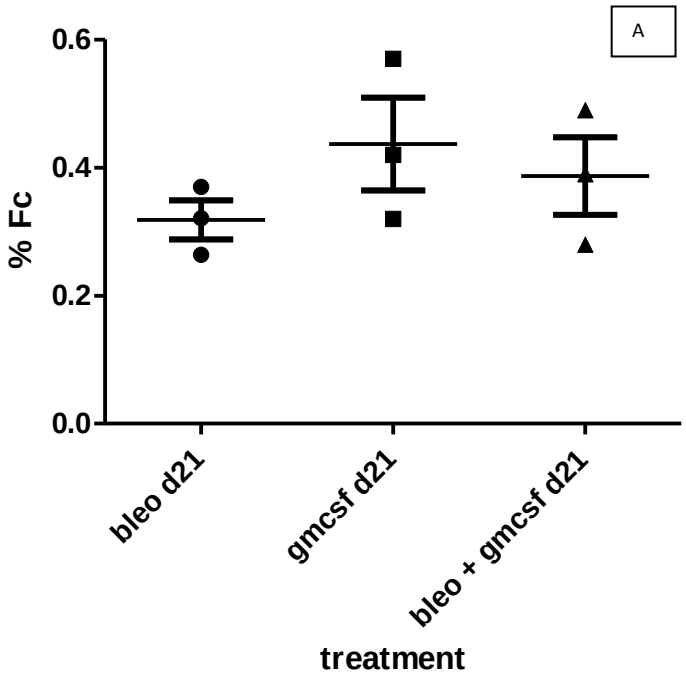
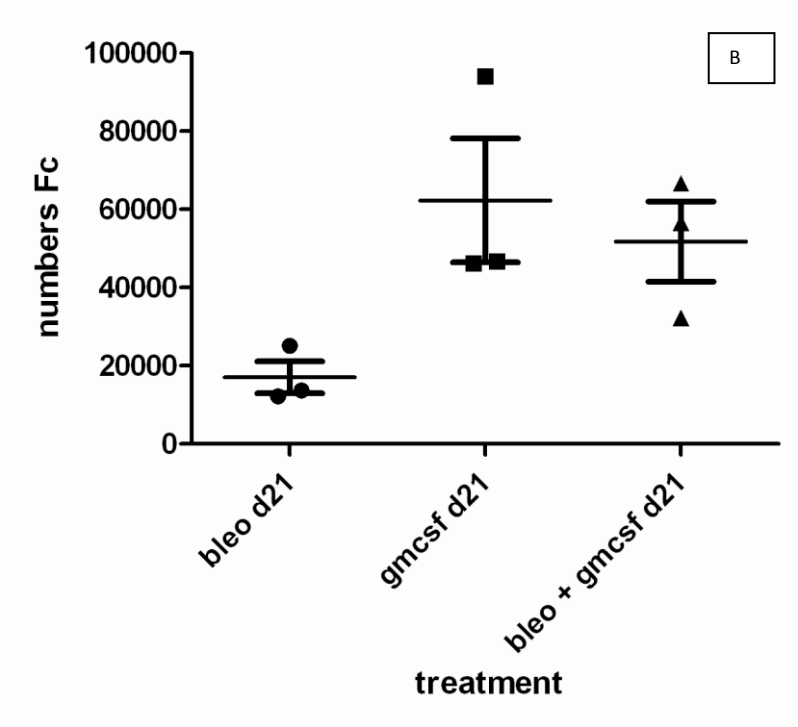


Figure 32: Concurrent administration of AdmGM-CSF and Bleomycin



bleo: Animals receiving bleomycin intratracheally (n=3)
gmcsf: Animals receiving AdmGM-CSF intramuscular (n=3)
bleo + gmcsf: Animals receiving bleomycin intratracheally and intramuscular AdmGM-CSF both on day 0 (n=3)
% Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1



bleo: Animals receiving bleomycin intratracheally (n=3)
gmcsf: Animals receiving AdmGM-CSF intramuscular (n=3)
bleo + gmcsf: Animals receiving bleomycin intratracheally and intramuscular AdmGM-CSF both on day 0 (n=3)
numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1

Figure 33: (A) Percentage and (B) Absolute numbers of circulating fibrocytes after Bleomycin intratracheally and AdmGM-CSF Intramuscular day 21.

7) To investigate fibrocyte responses in lung inflammation and fibrosis caused by treatment with Bleomycin alone or combined with a delayed systemic over expression of GM-CSF

The levels of circulating fibrocytes appeared to be dampened after the concurrent administration of AdmGM-CSF and bleomycin up until 21 days after treatment. We speculated this could be due to interference by AdmGM-CSF with the inflammatory phase of the bleomycin injury model. To test this, we delayed the treatment with AdmGM-CSF to day 10 after administration of Bleomycin as shown in figure 34 and examined the Fc response at days 17 and 21. With this sequence of treatment, we saw a significant increase in fibrocyte numbers at day 21 when we administered AdmGM-CSF 10 days after Bleomycin as show in figure 38 below.

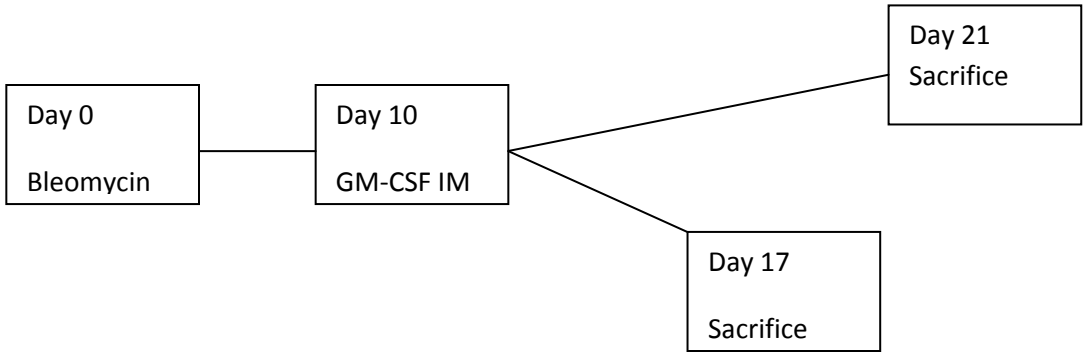
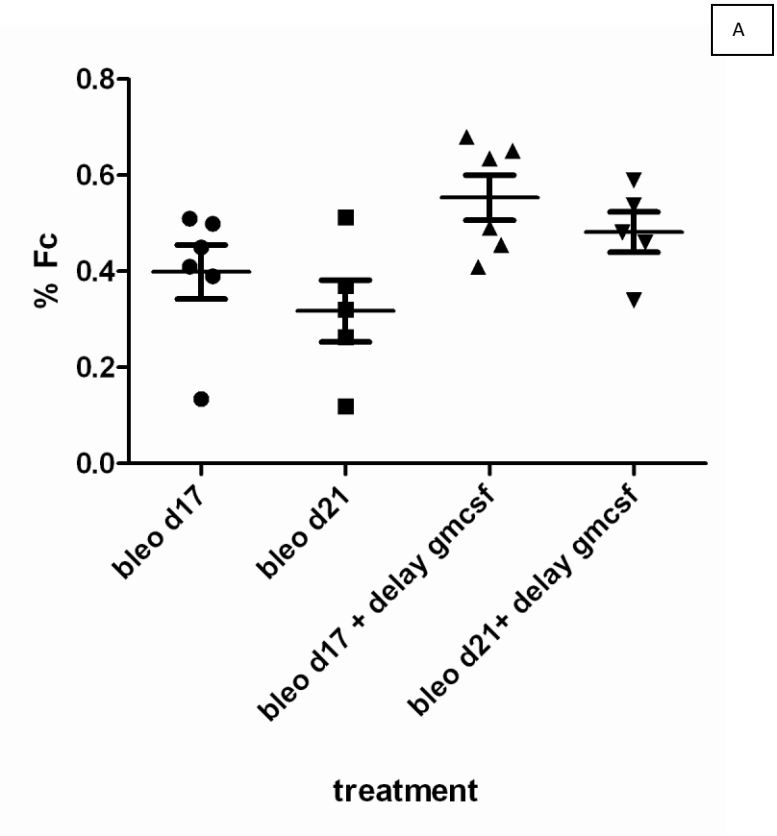
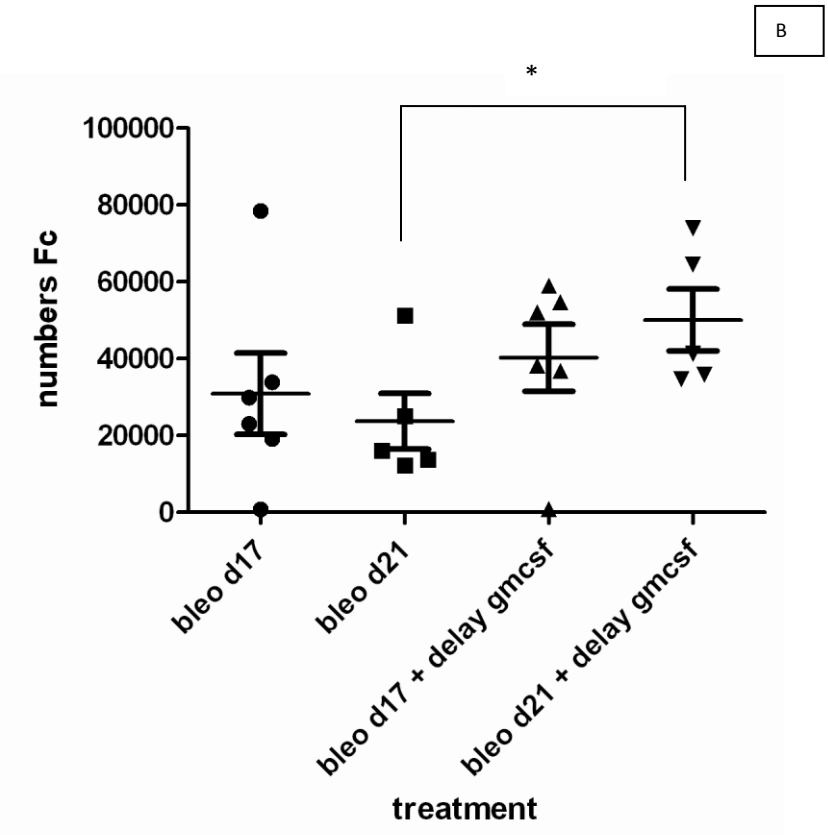


Figure 34: Delayed administration of AdmGM-CSF after Bleomycin



bleo: Animals receiving bleomycin intratracheally (d17 n=6) (d21 n=5)
bleo + delay gmcsf: Animals receiving bleomycin intratracheally on day 0 intramuscular AdmGM-CSF on day 10 (d17 n=6) (d21 n=5)
% Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1
one way ANOVA $p<0.05$



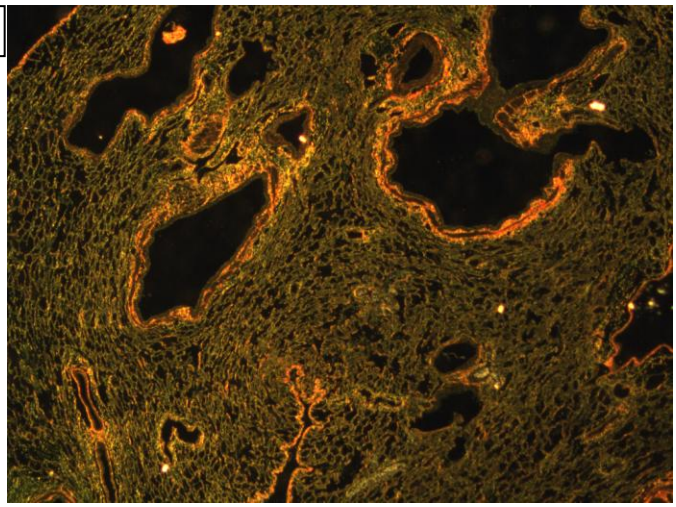
bleo: Animals receiving bleomycin intratracheally (d17 n=6) (d21 n=5)
bleo + delay gmcsf: Animals receiving bleomycin intratracheally on day 0 intramuscular AdmGM-CSF on day 10 (d17 n=6) (d21 n=5)
numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1
student t test $p<0.05$

Figure 35: (A) Percentages and (B) Absolute numbers of circulating fibrocytes after Bleomycin & delayed AdmGM-CSF. Measured in the blood of rats by FACS 17 and 21 days after administration of bleomycin followed by a delayed administration of AdmGM-CSF.

Histology after administration Bleomycin and a delayed treatment of AdmGM-CSF

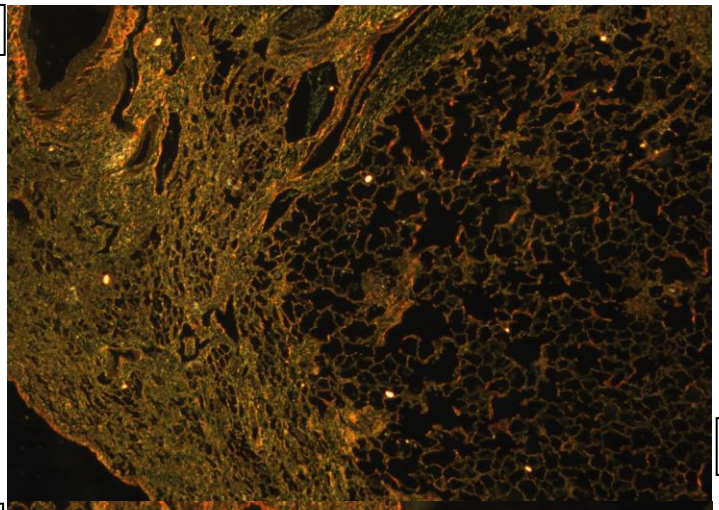
We performed Picro Sirus red staining on lung sections which had been treated with Bleomycin alone and a delayed treatment of AdmGM-CSF 10 days after bleomycin then sacrificed at either 17 or 21 days after initial treatment with Bleomycin. We examined the density of collagen in these sections to see if the increased number of fibrocytes seen was indicative of an increased or decreased amount of collagen production in the lung. In both cases we saw either the same amount of collagen or worsening by treatment with AdmGM-CSF versus treatment with Bleomycin only, which leads us to speculate that GM-CSF may be able to modulate Bleomycin pathology .

A



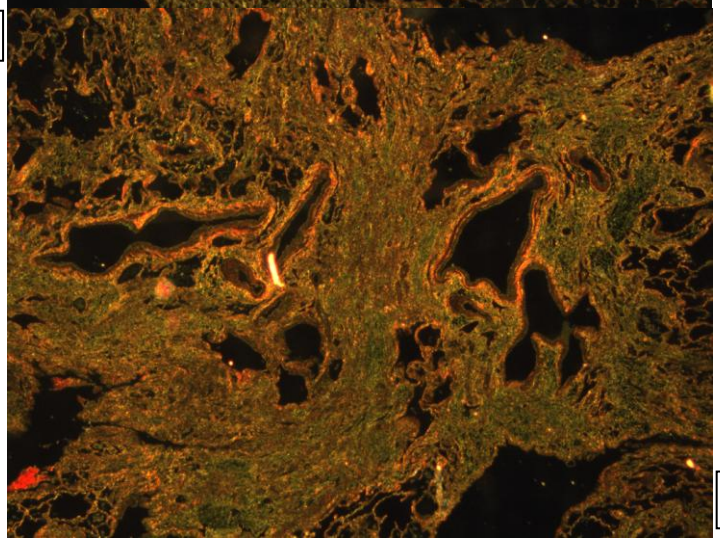
bleo d17 + delay gmcsf

B



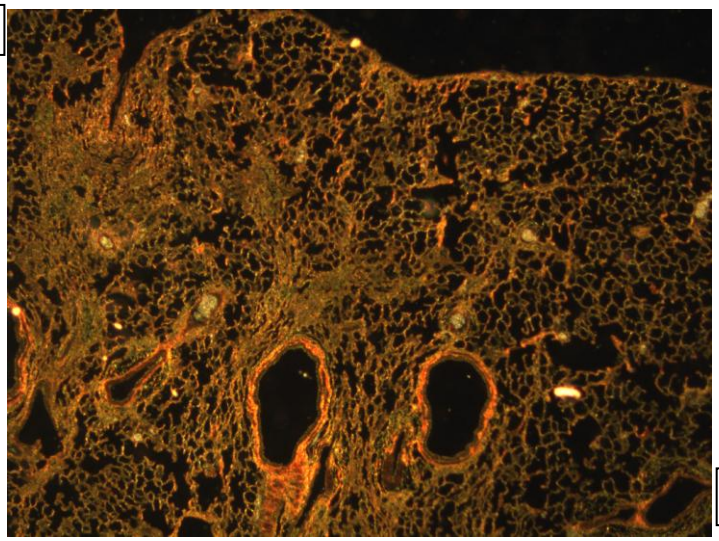
bleo d17

C



bleo d21 + delay gmcsf

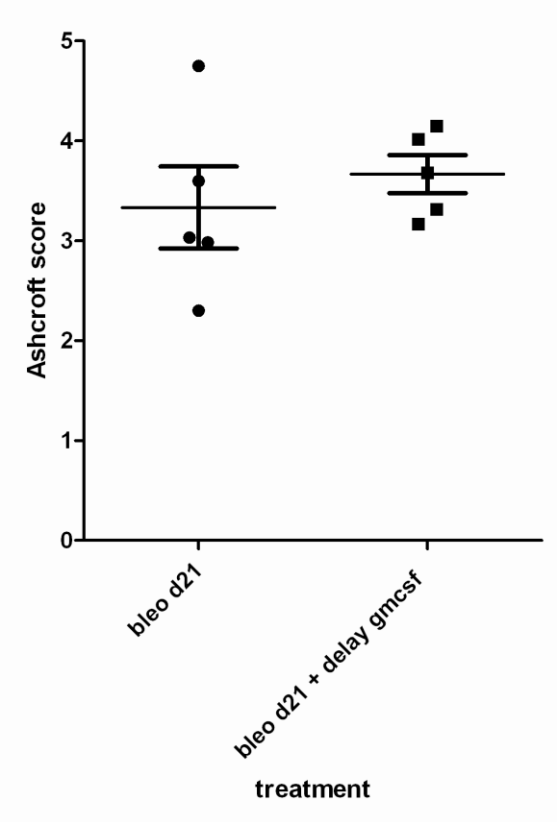
D



bleo d21

Figure 36: PicroSirus red staining of lung treated with Bleomycin intratracheally and delayed AdmGM-CSF intramuscular. Polarised light images of paraffin sections of A&C) Bleomycin at Day 0 and AdmGM-CSF at day 10. B&D) Bleomycin alone

Upon examination of PSR images above we can see some differences in the density and distribution of the fibrotic areas. When these images were analysed and ranked by Ashcroft score, we found no major increase in the intensity of collagen deposition. However, importantly we found that there was no decrease in collagen deposition in animals which reported a high number of circulating fibrocytes (figure 37).

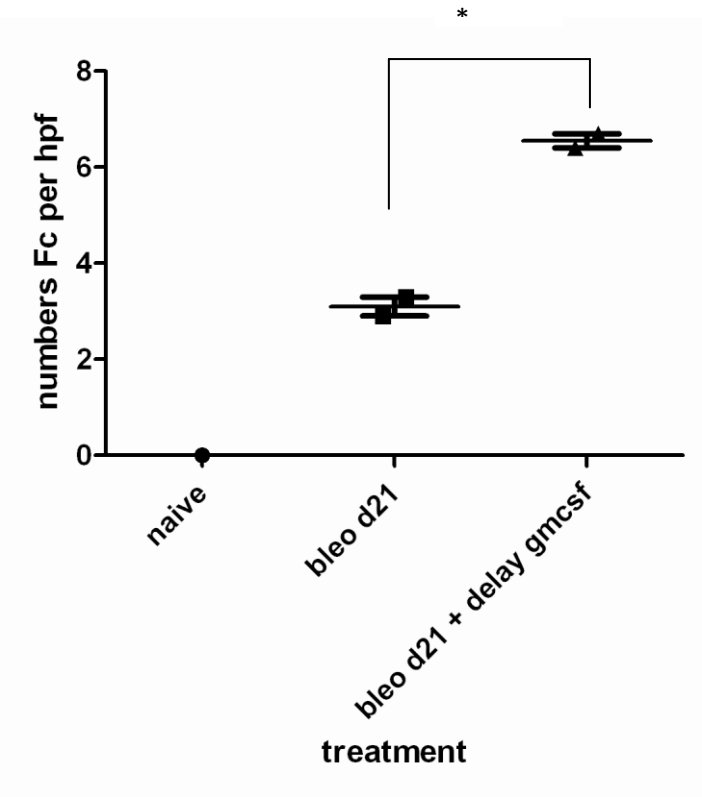


bleo: Animals receiving bleomycin intra tracheally (n=5)
bleo + delay gmcsf: Animals receiving bleomycin intra tracheally on day 0 intramuscular AdmGM-CSF on day 10 (n=5)

Figure 37: Ashcroft score of Picro Sirus Red after Bleomycin and delayed AdmGM-CSF. Lung sections stained and scored at day 21

Immunoflourescence staining for fibrocytes

After staining sections for fibrocytes (CXCR4/prolyl-4hydroxylase double positive cells) we counted the number of cells we found per high powered field. We saw an increase in the number of fibrocytes seen in the lung, when AdmGM-CSF is administered 10 days after initial treatment with Bleomycin (see below). This supports the kinetics shown above from the circulation and suggests that these cells are being recruited to the lung and may play a role in the ongoing fibrotic response seen there.

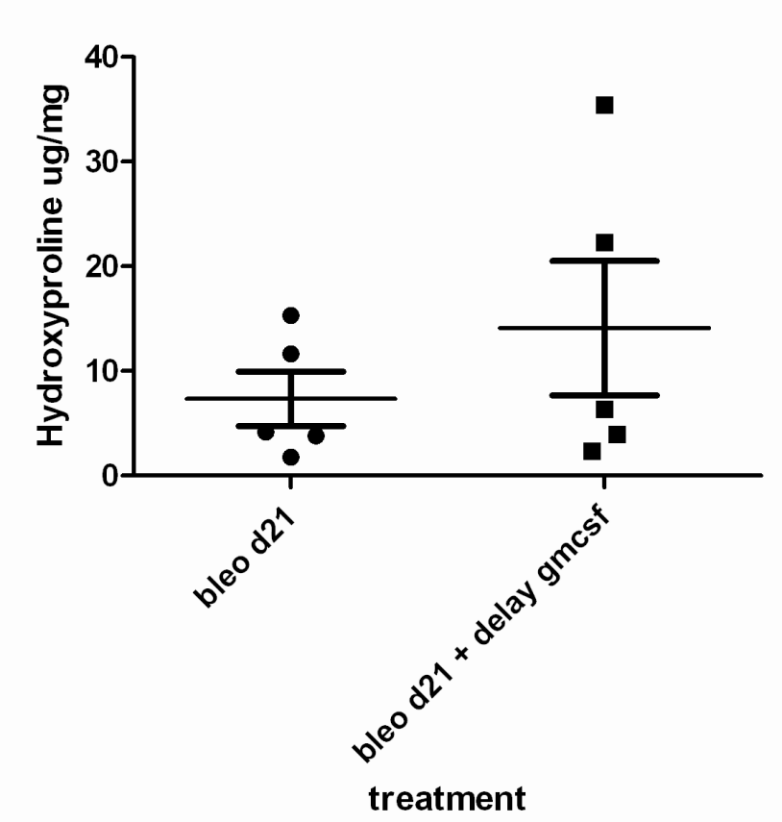


naïve: Animal received no treatment
bleo: Animals receiving bleomycin intra tracheally (n=2)
bleo + delay gmcsf: Animals receiving bleomycin intra tracheally on day 0 intramuscular AdmGM-CSF on day 10 (n=2)

Figure 38: Fibrocytes identified in the lung after Bleomycin and delayed AdmGM-CSF. Average number of fibrocytes found per 10 high powered fields after treatment with Bleomycin followed by AdmGM-CSF. Student t test $p<0.05$.

Hydroxyproline Assay

We performed the hydroxyproline assay described above on lung tissue samples from animals which were treated with Bleomycin only as well as those which received a delayed (after 10 days) treatment with AdmGM-CSF in addition to Bleomycin at day 0. We saw an increase in the hydroxyproline content in the tissue which received Bleomycin and AdmGM-CSF, this finding correlated with that of the PicroSirus red staining reported above.



bleo: Animals receiving bleomycin intra tracheally (n=5)
bleo + delay gmcsf: Animals receiving bleomycin intra tracheally on day 0 and intramuscular AdmGM-CSF on day 10 (n=5)

Figure 39: Hydroxyproline content in lung tissue after treatment with Bleomycin followed by AdmGM-CSF

BronchoAlveolarLavage (BAL)

We performed BAL counts on cell smears obtained from lung washings and counted 300 cells per smear. The figures below show the differential counts obtained in animals treated with Bleomycin alone and those treated with Bleomycin at day 0 followed by AdmGM-CSF at day 10. Both groups of animals were sacrificed 17 days after the initial treatment with Bleomycin. The animals which received both AdmGM-CSF and bleomycin showed a greater persistence of lymphocytes and monocytes.

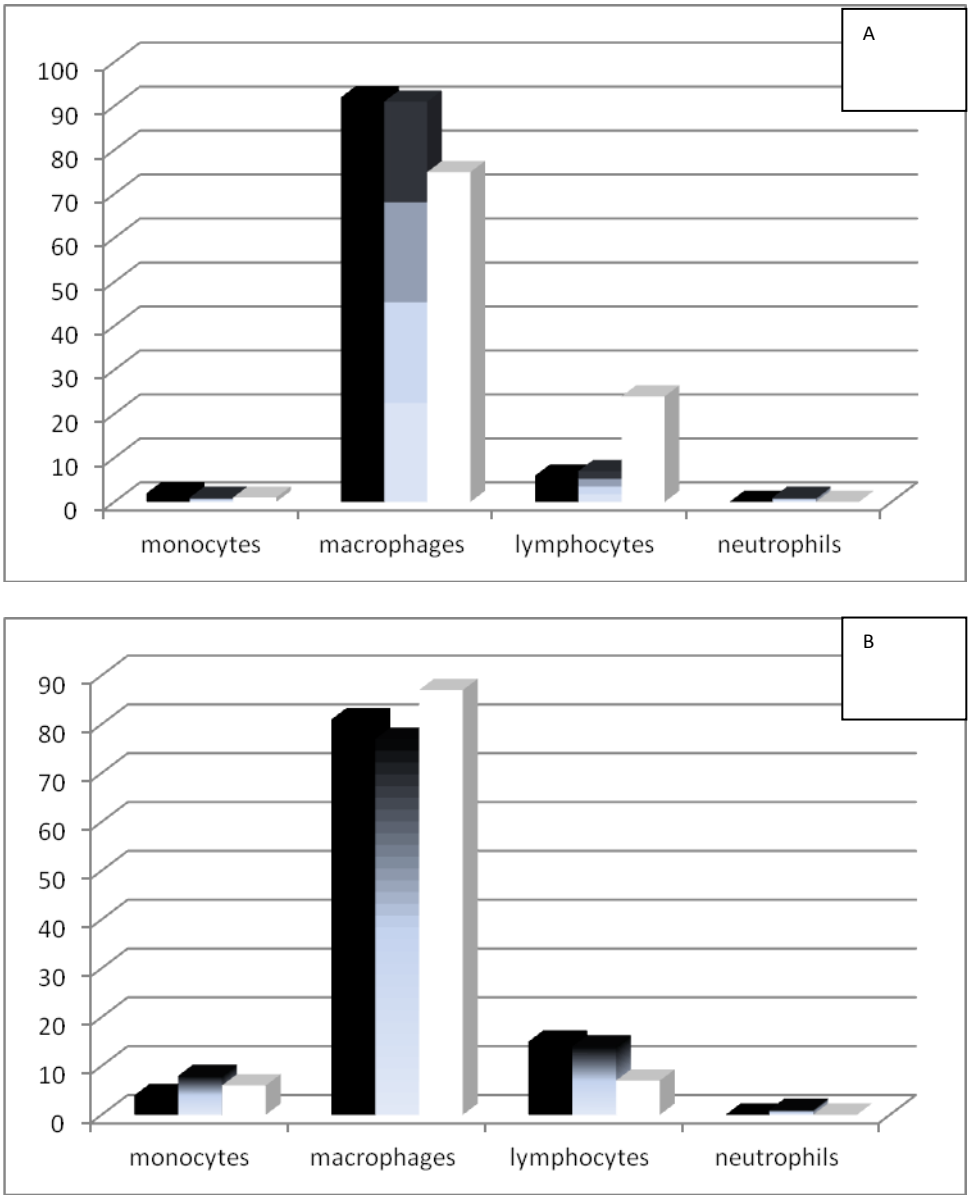
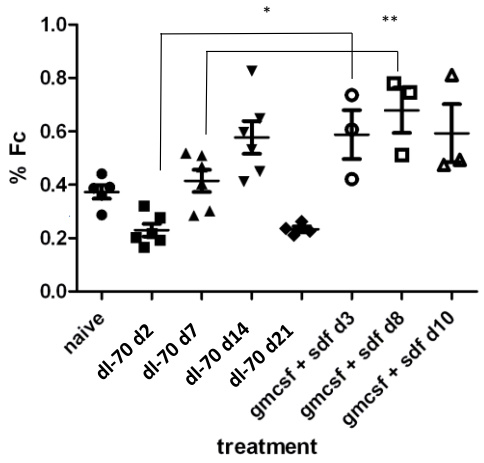
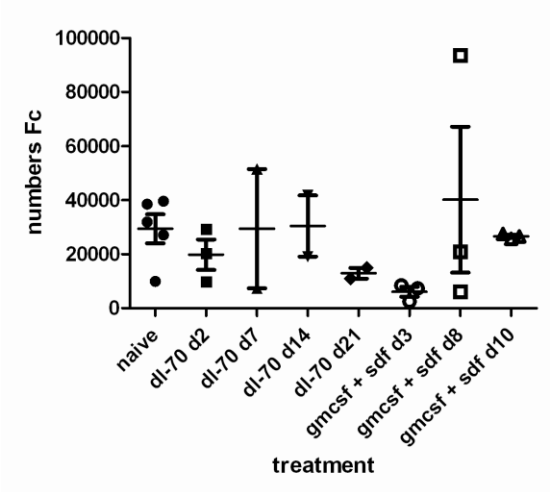


Figure 40: Percentages of cells counted in BAL Fluid Smear after treatment with Bleomycin and delayed AdmGM-CSF. Counts were done at Day 17. (A) Bleomycin Only (B) Bleomycin on Day 0 and AdmGM-CSF on Day 10. (n=3)

8) To investigate fibrocyte responses in systemic circulation caused by systemic over expression of GM-CSF followed by pulmonary over expression of SDF-1

We administered AdmGM-CSF on day 0 IM and then Ad SDF-1 on day 1 IT, to build on our previous experience of GM-CSF mobilising fibrocytes in the circulation. We wanted to see if this combination could mobilise fibrocytes from the bone marrow and traffic them to the lung.

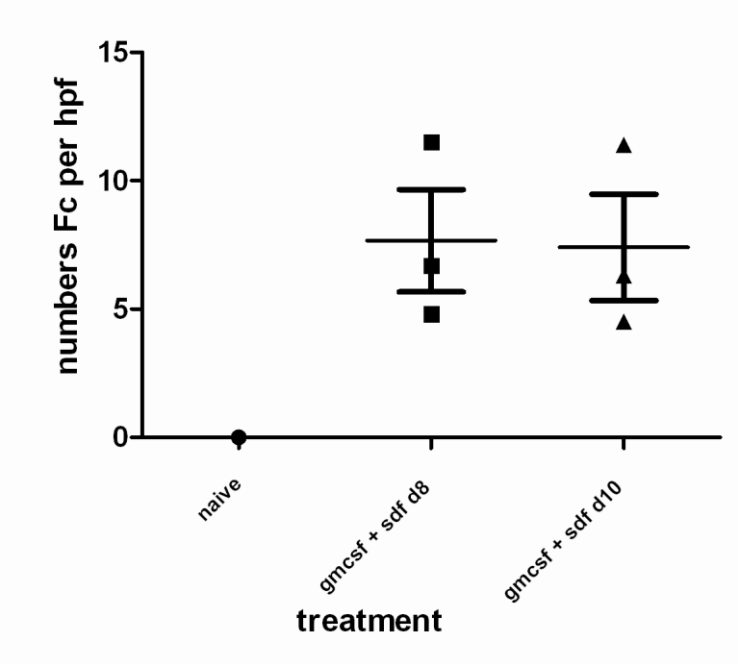




gmcsf + sdf: Animals treated intramuscular with AdmGM-CSF on day 0 and intratracheally with Ad SDF-1 on day 1 (n=3)
dl-70: Animals treated intratracheally with Ad-DL70 (n=6 *in group used for analysis)
naïve: Animal receiving no treatment (n=5)
%Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1
Numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1

Figure 41: (A) Percentages and (B) Absolute numbers of circulating fibrocytes after AdmGM-CSF and Ad SDF-1. AdmGM-CSF administered IM on Day 0 and Ad SDF-1 intratracheally on Day 1. One way ANOVA $p<0.05$

We saw an increased percentage of cells in the circulation from day 2 after the administration of Ad SDF-1 (see above) suggesting a potential relationship in the ability of SDF-1 to recruit cells and GM-CSF to mobilise cells. We also found that this combination led to the appearance of fibrocytes in the lung tissue (see below) when counted via Immunoflourescence staining for double positive fibrocytes (CXCR4 and prolyl-4-hydroxylase).

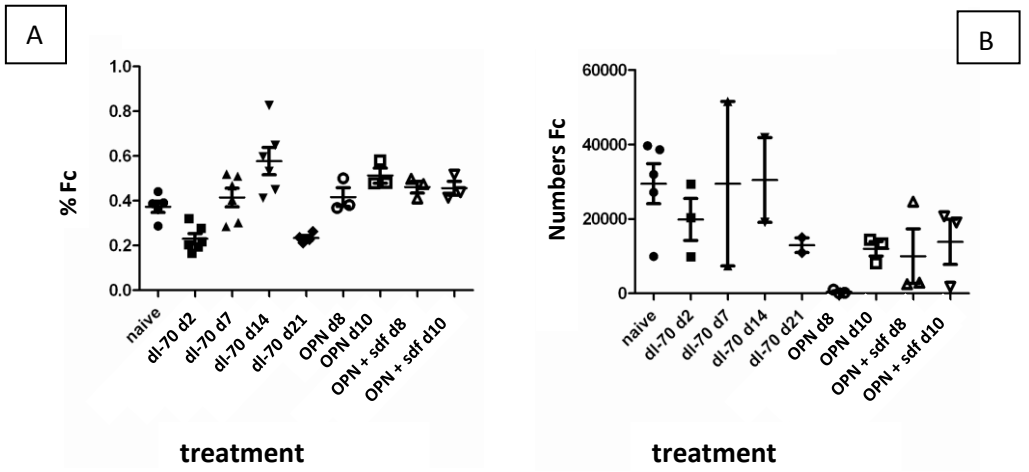


gmcsf + sdf: Animals treated intramuscular with AdmGM-CSF on day 0 and intra-tracheally with Ad SDF-1 on day 1 (n=3)
naïve: Animal receiving no treatment
numbers Fc per hpf: Average number of fibrocytes per high powered field

Figure 42: Fibrocytes identified in lung after AdmGM-CSF and Ad SDF-1. Average number of fibrocytes found per high powered field in lung sections treated with AdmGM-CSF IM Day 0 and SDF-1 intratracheally on Day 1

9) To investigate fibrocyte responses in systemic circulation caused by systemic over expression of OPN followed by pulmonary over expression of SDF-1

We completed a similar experiment as described above to test the ability of the cytokine OPN to mobilise fibrocytes and their ability in turn to respond to the expression of SDF-1 in the lung. As OPN is a completely different cytokine, our intention was to test if the fibrocyte mobilisation we have seen previously was GM-CSF specific or if these cells accompanied any cellular efflux from the bone marrow which we expected OPN to stimulate. SDF-1 was administered IT 1 day after administration of Ad-OPN IM.



OPN + sdf: Animals treated intramuscular with Ad-OPN on day 0 and intra-tracheally with Ad SDF-1 on day 1 (n=3)
OPN: Animals treated intramuscular with Ad-OPN (n=3)
dl-70: Animals treated intra-tracheally with Ad-DL70
naïve: Animal receiving no treatment (n=5)
%Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1
Numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1

Figure 43: (A) Percentages and (B) Absolute numbers of circulating fibrocytes after Ad-OPN and Ad SDF-1. OPN administered IM on Day 0 and SDF-1 intratracheally on Day 1

The ability of OPN and SDF-1 to cause any mobilisation of fibrocytes was not as noticeable as that seen with GM-CSF. Even when administered alone it appeared

that OPN elicited a different response to that of GM-CSF. The percentage of fibrocytes seen at Day 8 was no different between OPN administered IM and DL-70. The disposition of this cytokine vector when administered IM will have to be characterised further.

3.8 Discussion

Cytokine responses in lung injury have long been a focal point of investigation. There have been numerous studies involving animal models as well as patient's sputum, blood and lung samples to better understand the role of various cytokines in the onset and progression of lung fibrosis. Possibly contributing to the persistent interrogation of these systems is the hypothesis that a strong inflammatory response is one of the initiating events in fibrosis (Meneghin and Hogoboam 2007). In this chapter, we investigated the role which cytokines that have been previously implicated in lung inflammation, may play in producing a fibrocyte response. We speculated that correlating fibrocyte kinetics with known landmarks in lung injury, may help us to dissect the role of the fibrocyte. Our translation of the measurement of circulating fibrocytes from patients to rats gave us a very valuable tool with which to conduct further investigations in the animal model.

There have been numerous proposed animal models of lung fibrosis. The majority have attempted to initiate local injury by the instillation of pathological agents such as Bleomycin directly into the lung. Our group has conducted several studies on the use of Adenoviral vectors expressing bioactive cytokines, which when delivered into the lung led to the specific over expression of the respective cytokine. This has led to various animal models of fibrotic lung injury with the lead variable being the cytokine which was overexpressed. We expected to clarify possible associations between cytokine expression and the fibrocyte response. We also sought to modulate the fibrocyte response and to compare the subsequent effect of this fibrocyte modulation to that of individual cytokine over expression. We believed that the information gained from our animal models would be valuable in improving our understanding of bone marrow and other systemic contributions, including fibrocyte mobilisation, to lung injury and repair. Furthermore, by characterisation of fibrocyte kinetics in our animal models, this would equip us with a valuable tool which could be used as a single indicator of changes in lung status due to fibrotic injury.

Having established a baseline presence of circulating fibrocytes after the administration of various cytokines, we examined models of animal lung injury to better understand 1) The biological role that the fibrocyte played in lung injury

and 2) Our ability to modulate fibrocyte response and the effect that this modulation can have on lung injury. The administration of IL1-β resulted in a rise and fall of fibrocytes numbers (figure 17). Although the difference was not significant compared to the control, the kinetics was suggestive of a possible surge of circulating fibrocytes in response to injury. The peak in this case was at day 14 which was coincident with the time point that Kolb and colleagues (Kolb et al. 2001) found a sustained induction of TGF-β in this model of IL-1β mediated lung injury. The identification of fibrocytes in the lung at 21 days after administration of IL-1β (figure 18) also suggests that the decrease in circulating numbers was possibly due to a trafficking of these fibrocytes to areas of injury. We then proceeded to confirm any relationship between TGF-β and fibrocyte recruitment thus allowing us to understand any roles the fibrocyte may play in the number of processes where TGF-β has an established presence.

The administration of TGF-β resulted in an increase in the number of circulating fibrocytes which continued rising resulting in a significant difference to control at day 21 (figure 19). This finding is highly supportive of a role for this cytokine involving fibrocytes. Work on the TGF-β model showed significant fibrotic activity at 21 days after administration; the identification of fibrocytes in the lung (figure 20) as well as the circulation at day 21 helps confirm the hypothesis that these cells may be recruited into the circulation as needed for injury and repair and can migrate to areas of injury. It also appears that the signals which recruit the fibrocytes in this model might be independent of continued expression of TGF-β, as levels of expression of this cytokine are usually quite low 21 days after administration. This preliminary evidence would however need to be investigated further before any conclusions are made.

The elevation of circulating levels of fibrocytes found after the intra-tracheal administration of AdmGM-CSF (figure 21), demonstrated the ability of GM-CSF to mobilise different cell populations into the circulation including the fibrocyte. This bit of evidence fits well with known clinical applications of GM-CSF for mobilisation of bone marrow derived cells and also confirmed the fibrocyte's relationship with the bone marrow. From the kinetics of the fibrocyte response after administration of AdmGM-CSF intratracheally, we saw an early rise in circulating fibrocyte numbers, which subsequently declined around 7 days after AdmGM-CSF administration. This response concurred with the characteristic expression of this cytokine described by Xing and colleagues (Xing et al. 1996b) and suggested that the mobilisation effect on fibrocytes in this instance was dependent specifically on GM-CSF expression. This initial data provided a baseline for our other investigations; to determine if GM-CSF's fibrocyte mobilisation could be applied to other models of injury. When administered intramuscularly, AdmGM-CSF also generated a similar rise in circulating number of fibrocytes (figure 23). This finding widened the applications in which we could

use this 'mobilising agent', allowing us to administer different vectors or treatments independently by the intra-tracheal (local levels) and intra-muscular (systemic levels) routes.

We examined the effects of administering the CXCR4 blocker, AMD 3100 to animals which had been treated with AdmGM-CSF. With AMD3100, we targeted the CXCR4 receptors of fibrocytes which as we discussed earlier are implicated in the recruitment of cells to the lung via SDF-1. After administration of AMD3100 (figure 25), the dramatic reduction in circulating fibrocytes confirmed the possible neutralising effect that this drug has on CXCR4 positive cells and also the value of this intervention; should we need to alter fibrocyte kinetics. The complete effects of the administration of AMD 3100 are still not known, and we did not examine all cell types to confirm if similar to the human studies there was a liberation of other bone marrow cells. Like circulating fibrocytes, we also saw a similar reduction when we examined fibrocyte retention and subsequent detection by Immuno-flourescence in the lung (figure 27). Apart from the reduction in fibrocyte numbers, the reduction of fibrocytes in the lung seems to be related to a reduction in ECM gene expression. There was a lower expression of αSMA, collagen and fibronectin compared to treatment with AdmGM-CSF alone in the lung tissue (figure 28 & 29). Validation of this finding can be performed by the isolation of circulating fibrocytes from animals receiving AdmGMSCF with and without AMD3100. In vitro culture and fibrocyte gene expression would help us to determine if the constitutive ability of fibrocytes were changed by AMD3100 or if the reduced ECM gene expression seen in the tissue was due to the absence of fibrocytes. We sought to further apply AdmGM-CSF and AMD3100 to see if they were truly as we speculated from our findings discussed above, a fibrocyte agonist and antagonist respectively.

The Bleomycin model of lung injury is possibly one of the best characterised, both in terms of its strengths as well as its limitations. For this reason, we sought to modulate this model of fibrosis by the administration of AdmGM-CSF. From baseline measurements after administration of Bleomycin, we found there was an elevated level of circulating and lung retained fibrocytes (figure 30 & 31). These levels could be reduced by the administration of AMD3100. We did not notice any obvious difference in the lung histology with and without AMD3100 treatment after administration of Bleomycin. However notably, these experiments were performed with a dose of AMD 3100 which was selected as the median of doses reported in the literature. When AdmGM-CSF was administered concurrently with Bleomycin, there was no change in the level of fibrocytes seen (figure 33). We speculated that this might be due to the collateral effects that GM-CSF might have on other cell types involved in the initial inflammatory phase of Bleomycin lung injury. Delaying AdmGM-CSF IM administration until 10 days after initial administration of Bleomycin IT did lead to an increased level of circulating

fibrocytes, measured up until 21 days after administration of Bleomycin (11 days after AdmGM-CSF) (figure 35). Notably, even when AdmGM-CSF was administered intramuscularly in combination with Bleomycin, elevated levels of GM-CSF could be detected by ELISA up to 11 days after delivery (figure 24).

In this combined model of Bleomycin and AdmGM-CSF administration, the sustained presence of circulating fibrocytes correlated with fibrocyte numbers in the lung identified by Immunoflourescence and also appeared to be related to the degree of fibrosis seen (figure 38). Aschroft score and Hydroxyproline assay showed a maintenance and increase respectively (figures 37 & 39), after treatment with Bleomycin and AdmGM-CSF. Although the difference in the Ashcroft scores between Bleomycin only and Bleomycin plus AdmGM-CSF was non-significant, we view this as a valuable piece of data. This provides evidence to suggest that increased levels of circulating fibrocytes are not beneficial in fibrosis as previously speculated by some investigators.

The combination here of a model of injury with a mechanism for the modulation of circulating fibrocytes is a unique approach. I believe we have created a valuable approach for the continued interrogation of this model and further investigation of the preliminary evidence presented. Our findings about the fibrocyte's participation in lung injury complement that of other investigators. We have shown a kinetic response of circulating fibrocytes which supports the theory that fibrocytes are recruited from the bone marrow and can migrate to the injured lung. We also have shown here that stimulating fibrocyte release with GM-CSF can contribute to increased fibrocyte presence in the lung and possibly result in worsening injury.

In our experiments mobilising fibrocytes with GM-CSF and attempting to recruit them to the lung via local (pulmonary) expression of the chemokine, SDF-1 or CXCL12, we saw an increased number of circulating fibrocytes as well as a large number of fibrocytes being retained in the lung (figures 41 & 42). This finding fit with previous work done by Phillips and colleagues (Phillips et al. 2004) confirming the importance of the SDF-1 pathway in the recruitment of fibrocytes to the lung. Interestingly, we found with our combined administration of AdmGM-CSF/SDF-1, although fibrocytes were mobilised by GM-CSF and recruited to the lung by SDF-1, there was no injury to the lung by the increased presence of fibrocytes. This finding adds evidence to existing speculation that the presence of fibrocytes alone may not be enough to initiate injury and fibrosis, independent of pre-existing or already initiated lung injury.

We performed a similar experiment with OPN. Here we tested OPN's ability to act as a mobilising agent, and looked at the ability of the cells liberated from the bone marrow to respond to SDF-1. Although there was a small increase in the

percentage of circulating fibrocytes 10 days after the administration of OPN alone (figure 43), there was no major increase in circulating fibrocytes after the combined administration of OPN/SDF-1. To determine what this actually means we would need to thoroughly examine the presence of fibrocytes in the lung of animals treated with OPN/SDF-1 as the fibrocytes might have been liberated and recruited to the lung before our time point causing a rapid fall in circulation. We also will need to look at the expression of OPN in circulation after IM administration to ensure we have a significant signal in order to have some biological effect.

These experiments demonstrated that a relationship exists between cytokine expression and fibrocyte mobilisation. We have shown here a valuable addition to existing knowledge; fibrocytes may be mobilised by central or direct stimulation of the bone marrow by agents such as AdmGM-CSF. Also, fibrocyte release may be stimulated by lung expression of locally delivered cytokines, likely resulting in subsequent stimulation of specific chemokines such as CXCL12 resulting in fibrocyte recruitment.

4.0 Fibrocytes as prospective Biomarkers in Human Lung Disease

4.1 Introduction

Case reports and data from clinical trials have proposed several distinct trends of disease progression in patients diagnosed with IPF (Ley, Collard, and King 2011). The pattern for most patients is a slow, irreversible decline in lung function over a period of years. However, there are other patients who would experience a rapid progression of disease (Selman et al. 2007). Furthermore, some patients will experience acute exacerbations of disease, this is characterised by periods of slow decline in lung function, interrupted by rapid acute worsening in disease status (Collard et al. 2007) often leading to a permanent decrease in lung function. There are currently no validated methods of predicting these declines. If such methods were available therapeutic plans could be put in place to help ameliorate these events as well as to narrow down possible cofactors which may contribute to these exacerbations. The search continues for predictive biomarkers of disease progression in pulmonary fibrosis.

One of the key features of IPF is the presence of abundant myofibroblasts, often arranged in clusters throughout the lung parenchyma ("fibroblastic foci") (Selman et al. 2001). In fibrotic disorders, myofibroblasts are retained through, amongst other mechanisms, enhanced differentiation of local fibroblasts, trans-differentiation of epithelial cells (EMT) and inhibition of apoptosis causing continued matrix production and reduced matrix degradation. The origin of these myofibroblasts is not clear (Hinz et al. 2007). Evidence has suggested in pulmonary fibrosis that recruitment of mesenchymal cell progenitors (fibrocytes) from the circulation may be an additional contributing factor to the increased presence of myofibroblasts in fibrotic lungs (Hinz et al. 2007) (Gomperts and Strieter 2007).

The fibrocyte is a distinct cell and its cell surface features have been studied using FACS analysis, immunohistochemistry and also histomorphology. On scanning EM, fibrocytes in culture exhibit prominent cell surface projections, making them quite distinct from the appearance of other leukocytes (Bucala et al.1994). Fibrocytes are bone-marrow derived cells, they migrate to sites of tissue injury and differentiate into fibroblast-like cells (Bucala et al. 1994). It is believed that human fibrocytes in circulation probably represent an obligate intermediate stage of differentiation of one of the precursors of the monocyte lineage into mature fibroblasts and myofibroblasts at the tissue sites (Bellini and Mattoli 2007). The fibrocyte has been proposed as a predictive biomarker of lung injury but this is still to be fully validated.

The profibrotic cytokine TGF-β can stimulate fibrocytes to express α-smooth muscle actin, a typical albeit not specific myofibroblast marker, which strongly supports a potential role of these cells in myofibroblast formation (Abe et al. 2001). A typical feature of fibrocytes is that they circulate in the blood stream and are capable of producing collagen and other ECM components, and can express a variety of mesenchymal markers including collagen 1 as well as the leukocyte marker CD45 and the hematopoietic stem cell marker CD34 (Quan, Cowper, and Bucala 2006). There is sufficient evidence that at a given point in time a CD45/col-1 positive mononuclear cell is a circulating fibrocyte in some stage of differentiation, and this marker combination seems to be practical and has been used for characterization in most studies. The seemingly confusing or even controversial details about the identity of fibrocytes indicate they undergo constant changes on their way from the bone marrow to sites of injury and manifest phenotypic and functional differences. These differences may result in a heterogeneous group of cells which can then be divided into different subsets (Maharaj et al. 2012).

Using the marker combination CD45/Col-1 positivity, Bucala and colleagues estimated (Bucala et al. 1994) that fibrocytes constitute approximately 0.5% of circulating leukocytes, which has been confirmed by several other groups including our own (Moeller et al. 2009) (Strieter et al. 2009). As mentioned previously, the chemokine receptor ligand pair CXCR4-CXCL12 has been implicated to play a significant role in homing of fibrocytes to areas of tissue injury (Phillips et al. 2004). Data from animal work was supported by reports of increased levels of CXCL12 in the lung and plasma of patients with Idiopathic Pulmonary Fibrosis (IPF) (Mehrad et al. 2007) which correlated with numbers of circulating fibrocytes, an association that has previously also been found in animals (Phillips et al. 2004).

Fibrocytes have been positively identified in the lung tissue of patients with IPF by using markers for CXCR4 and Prolyl-4-hydroxylase in cells by immunohistochemistry (Andersson-Sjoland et al. 2008) and by using markers for CD34 and collagen in the airway of patients with asthma (Schmidt et al. 2003). The available data regarding circulating fibrocytes in either human or experimental studies suggests that these cells may be involved in the pathogenesis of abnormal fibrogenesis in the lungs (Strieter et al. 2009), similar to their presumed active role in liver fibrosis (Kisseleva et al. 2006). This leads us to speculate that the presence of circulating fibrocytes may be related to ongoing chronic injury and repair.

Our group has validated a FACS analysis assay in collaboration with Dr Bob Strieter (University of Virginia) to measure fibrocytes (CD45 and Col-1 positive cells) in human peripheral blood and we have shown significantly increased

levels of fibrocytes in patients with IPF and a further exacerbation during episodes of acute disease (Moeller et al. 2009). We showed in this study that fibrocyte numbers appeared to be an independent predictor of early mortality and patients with lower numbers of these cells had a longer survival. This study as well as those of other groups (LaPar et al. 2011) (Fujiwara et al. 2012) have suggested a potential biomarker role for these cells.

The Moeller 2009 study also examined circulating fibrocyte numbers in a group of ARDS patients, assuming that their acutely injured lungs are very active in creating a variety of "repair signals". However, interestingly, these patients did not show a significant elevation of fibrocyte numbers. We speculate that this suggests the presence of mesenchymal progenitor cells (the fibrocyte) may be more indicative of chronic fibrotic tissue injury or repair and not a reflection of lung injury per se.

The National Institute of Health defines a biomarker as any characteristic that can be objectively measured and act as an indicator for the different processes that occur in the human body (Biomarkers Definitions Working Group 2001). Desired properties of an ideal biomarker include that it must be objectively measured, reproducible, easily measured and inexpensive. High sensitivity and specificity combined with the ability to predict clinical changes in disease are all properties that candidate biomarkers are investigated to see if they qualify (Tunceroglu et al. 2013).

In clinical practice, pulmonary function test decline is used as one of the primary outcomes in assessing the progression of fibrotic lung disease. Limitations of pulmonary function tests and other existing endpoints include the fact that they are relatively insensitive to changes in early disease. This has energised the search for newer biomarkers of fibrotic tissue injury which meet the criteria discussed above. The availability of a biomarker may be a useful tool to detect fibrotic changes in lung disease early on and help to predict progressive functional decline. Once identified and validated, a biomarker would help to modulate therapy and clinical care in real time as opposed to waiting for a decline in FEV1 and other lung function indices in order to act.

To add to our knowledge about the potential of the fibrocyte as a biomarker we have been collaborating with the University of Nottingham on the Prospective Study of Fibrosis In the Lung Endpoints (PROFILE) study (NCT01134822), the aims of the PROFILE study are:

- Discover and validate novel biomarkers for use in subsequent intervention studies in patients with IPF

- To prospectively validate a panel of previously published biomarkers in patients with well characterized idiopathic fibrosing lung disease
- Investigate genetic associations and epigenetic modifications which affect disease severity and progression

In addition to our work in IPF we also looked at other forms of fibrotic lung injury to compare if a similar approach could be used in the prediction of disease progression. As mentioned in a previous chapter, current knowledge suggests that Idiopathic Pulmonary Fibrosis (IPF) and Cystic Fibrosis (CF) have nothing in common other than the term “fibrosis”. I believe that the comparison between IPF and CF lung disease where possible is valuable as there are some unsubstantiated associations of CF lung disease to fibrosis.

A study by our group (Bonniaud et al. 2004) showed a potential link whereby the Smad signalling pathway may be involved both in fibrosis and airspace enlargement (a feature of CF lung disease). Honeycombing and bronchiectasis usually develop in many patients with advanced IPF disease (King et al. 2001). Interestingly, bronchiectasis is also a feature in some patients with CF. In the past, basic research in CF lung disease has focused on mechanisms of tissue destruction, bronchiectasis, chronic inflammation, repetitive pulmonary infections and the biological roles of the CFTR gene. It is increasingly clear that the CF gene has far more physiologic roles than just regulating a chloride channel (Zielenski 2000). The priority for anyone involved in the care of CF patients is increasingly to identify patients who are more prone to the development of bronchiectasis, bronchial wall thickening and parenchymal changes as soon as possible. Biomarkers may be useful tools to detect these structural changes early on and help to predict progressive functional decline.

Fibrocytes can be detected in human fibrotic disorders including idiopathic pulmonary fibrosis (IPF), chronic asthma, nephrogenic fibrosing dermopathy, scleroderma and cutaneous scars (Strieter, Gomperts, and Keane 2007) (Galan, Cowper, and Bucala 2006) (Nihlberg et al. 2006) (Iwano et al. 2002). There is evidence suggesting that fibrocytes contribute to lung remodelling at some stage (Hashimoto et al. 2004) (Lama and Phan 2006). While there is no publication of a “normal” number of fibrocytes in tissue, there is some understanding of the numbers that can be expected in the circulation as discussed above. In this chapter we sought to better understand the biological function that the fibrocyte served in disease states where it was detected.

Mori and colleagues (Mori et al. 2005) looked at the cells which responded to a skin injury in a mouse model and found that they could identify fibrocytes in this wound when digested by FACS and also by in situ hybridization. Similar

digestions to identify the presence and phenotype of fibrocytes by flow cytometry have also been done by other researchers in this field (Moore et al. 2006) (Sun et al. 2011) (Tourkina et al. 2011). Because of the recognition of the major role that tissue resident fibroblasts play in contributing to fibrogenesis, it is important that we continue to examine other cells, which may be recruited to or are resident in the lung and examine their roles as well.

Once a fibrocyte is recruited to the lung, it is speculated that the environment they encounter there can largely determine the phenotype they will adopt and subsequently the activities they would participate in. This local niche and its possible effects on fibrocyte function were recently reviewed by Andersson-Sjoland and colleagues (Andersson-Sjoland et al. 2011). In this review the authors discussed the movement of fibrocytes after recruitment to the tissue and their migration to specific cytokines in the Extracellular Matrix. The presence of fibrocytes in various niche environments has not been widely reported. I believe it would be valuable to be able to identify fibrocytes in the lung and to characterise their activities in clearly defined areas of injury. Specifically, if we can correlate fibrocyte presence to the expression of cytokines of interest in fibrosis, this relationship may help us to better understand the precise role these cells play when responding to the injured lung.

Whilst identification and quantification of fibrocytes in peripheral blood is relatively straight forward, depending on the marker panel that is used, the isolation and culturing of these cells remains difficult. There are several protocols published, and most groups seem to follow the original method developed by Bucala and coworkers (Chesney et al. 1997) using RPMI- or DMEM-based culture media, supplemented with 10-20% foetal bovine serum. Modifications to this protocol have been proposed by Pilling and colleagues (Pilling, Vakil, and Gomer 2009) to change some important variables, such as blood collection methods, optimal seeding cell density, culture substrates and media which contribute to the growth rate and final cell density. Fibrocytes, grown from peripheral blood, do not easily reach confluence. This definitely makes them different from fibroblasts, but it is possible with optimization of attachment using different culture surfaces and cell numbers to have a robust growth rate. The ideal growth conditions and possible progression in the phenotype of fibrocytes, from one subset to another, still needs to be established.

Many hypotheses have been put forward about the possible mechanistic roles of fibrocytes and myofibroblasts in the pathogenesis of fibrotic reactions in the lung. The heterogeneity of lung fibrosis requires a significant number of patients to be examined in order to conclusively prove these theories. It remains difficult to unequivocally confirm that fibrocytes found in the tissue originated from the bone marrow, having migrated to the site of injury via the circulation. The literature

does not have a systematic examination of large numbers of lung samples for the presence of fibrocytes and myofibroblasts but rather cites individual cases in clinical reports.

In this chapter I looked at the circulating levels of fibrocytes in patients diagnosed with CF and IPF and the relationship between the number of cells measured and their disease status. Our goal with this aim was to determine if this method of live assessment would enable us to better understand, predict and treat drastic changes in disease status for patients diagnosed with pulmonary fibrosis. I also examined the presence of fibrocytes and myofibroblasts in lung transplant samples of patients who received lung transplant either as a result of end stage IPF or CF lung disease. I am aware that CF lung disease is an entirely different disease to IPF. However, our intention was to use CF lung disease as a comparator. The presence or absence of fibrocytes in CF lung disease may yield useful information about the role of fibrocytes in different lung diseases. Additionally, I looked at the growth kinetics of fibrocytes in vitro and repeated some experiments reported in the literature including migration to SDF-1. With the assurance that the cells I grew behaved similarly to those reported in the literature, I examined the interaction of these cells with IL-1β and TGF-β, two cytokines which have been reported to be involved in the pathogenesis of fibrosis.

As mentioned above the growth of fibrocytes in culture remains a difficult task and in this project we also encountered this problem. While primary fibrocyte cultures are usually successful for the first 21 days, secondary cultures frequently fail shortly after transferring cells. Because of the limited availability of fibrocytes and fibrocyte cultures, there was a limited number of cells to be examined, leading to most of the cell culture data being pilot or preliminary data.

4.2 Methods

Flow Cytometry

Patients were consented and recruited with McMaster University Research Ethics Board Approval from the adult CF clinic at McMaster University and IPF patients were recruited from the Chest Clinic at Firestone Institute for Respiratory Health (FIRH) St Joseph's Healthcare Hamilton. In accordance with the PROFILE study protocol, patients were recruited from the Interstitial Lung Disease clinic in Kings Mill and Nottingham, UK.

CF Patients were grouped into mild/moderate and severe disease according to FEV1 (moderate: FEV1 > 50%, severe: < 50% pred.), these patient were compared to age matched healthy volunteers as control subjects. The patients' charts were reviewed and alterations in the measurement of FEV1 were recorded over the study period.

Samples from the UK were transported to a central processing lab at Nottingham City Hospital. Mononuclear cells (MNC) were isolated as described below. MNC's were counted and stored for up to 3 months in liquid nitrogen until shipment to McMaster University.
Blood samples were centrifuged, the mononuclear cell layer ("buffy coat"), found between red cells and plasma (figure 44) was removed to 20ml of Sodium Chloride (ACK) lysis solution.

<u>ACK lysis solution (store at RT):</u>
8.29g NH_4Cl (0.15M)
1g $KHCO_3$ (1.0mM)
37.2mg Na_2EDTA (0.1mM)
} in 1000ml ddH2O

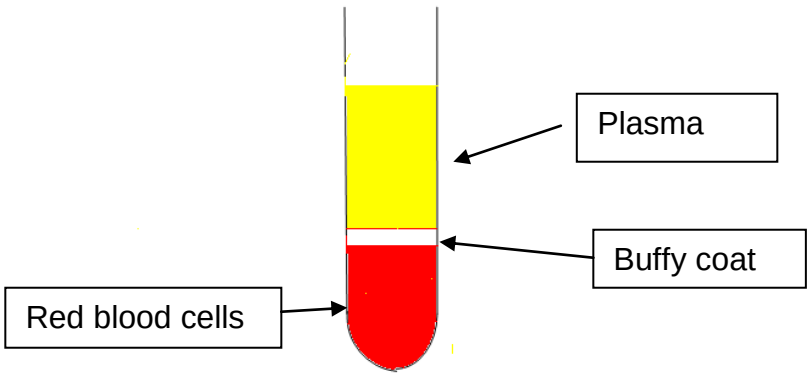


Figure 44: Isolation of buffy coat from blood samples

Leukocytes were incubated in ACK to lyse red blood cells, and then washed with 10ml FA buffer.

FA buffer (fresh every time):
PBS + 0.1% FBS = 40ml PBS + 40µl FBS for 1 sample

Samples were centrifuged at 1200RPM, and a final wash with 20ml FA buffer and subsequent centrifugation provided a relatively pure leukocyte population. Total viable cells were quantified using Trypan blue staining and stored at 5×10^7 cells/ml in freezing solution.

Freezing solution (fresh every time):
90% FBS + 10%DMSO = 900µl FBS + 100µl DMSO for 1ml freezing solution

Cells were cooled in a Nalgene™ freezing container at -80°C for up to 3 days before transfer to a liquid nitrogen storage tank.

For flow cytometry, the procedure followed published methods (Moeller et al. 2009) (Mehrad et al. 2007). Frozen samples prepared as described above were thawed to room temperature and washed. Cells were re-suspended to 1×10^7 cells/ml, and aliquoted to a 96-well plate at 100µl/well, for a final concentration of 1×10^6 cells/well. Cells were stained for CD45 with mouse anti-human antibody (CD45-PerCP BD Cat#557059) or isotype control, followed by two washes with 100µl of FA buffer.

Next, cells were permeablized using the BD Cytofix/Cytoperm® kit to allow for detection of intracellular antigens. The cells were incubated with the Fixation/ Permeabilization solution for 30 min followed by 2 washes. Cells were incubated with either specific rabbit anti-human collagen1 (Rockland #600401-103-0.1) or IgG isotype control antibody. This was followed by incubation with the secondary goat anti-rabbit antibody conjugated to AlexaFluor® 488 (Invitrogen Cat # A11070), which emits in the FITC channel. Finally, cells were transferred to 1% paraformaldehyde for storage up to 1 week at 4°C.

Flow Cytometry was performed using FacsCanto. Compensation was calculated with anti-mouse IgG κ CompBeads® stained with each single used color antibody. For each sample a min of 100,000 and a max of 200,000 events are collected, depending on total cell numbers. Analysis of CD45/Col-1+ cells was performed by identifying live cells based on forward and side scatter characteristics, gating on the CD45+ cells, and then gating on the Col-1+ cells within this population. Percentages of live cells coexpressing both these markers were multiplied by total viable cell count to determine the absolute number of CD45/Col-1 cells.

Immunoflourescence

We are fortunate to have an established collaboration with Prof Tobias Welte, Medizinische Hochschule Transplant Centre, Hannover (Germany) which gave us access to human IPF and CF tissue. The tissue collected was sampled from anatomically distinct locations (dissected into: a. macroscopically normal tissue; b. fibrotic parenchyma; c. fibrotic airways), the lungs were sampled from more than one lobe. Lung tissue was preserved in formalin and then stored in ethanol until paraffin embedded. Paraffin blocks were shipped to us and then the lung sections were cut at a thickness of 3 µm.

For fibrocyte stains: Sections were deparaffinized and rehydrated. For antigen retrieval, slides were placed for 20min into a preheated steamer in 0.01 M citrate buffer (pH 6.0). Then, sections were blocked for 15 min with 1% normal swine serum (NSS) in PBS, followed by incubation with prolyl 4-hydroxylase (Abcam cat # ab44971) in 1% NSS/PBS at 4ºC overnight. The following day, the slides were washed with PBS and incubated with a Dylight 594 fluorescent secondary antibody for 2 hours. The sections were then washed carefully and blocked with 10% normal mouse serum for 1 hour. After another wash, sections were incubated overnight with a primary antibody for CXCR4 (R&D systems cat# MAB 172) in PBS at 4ºC. The following day, the sections were washed and then incubated in a FITC fluorescent secondary antibody for 2 hours. Slides were mounted using Vectashield with DAPI (Vector Laboratories).

For myofibroblasts stains: Sections were rehydrated and antigen retrieved as described above and then incubated with primary antibody for alpha smooth muscle actin (Dako cat # M0851) overnight and then washed and incubated with a TRITC secondary antibody (Sigma cat # T2402). After blocking with Normal Swine Serum, sections were incubated in a primary antibody for fibroblast specific protein (FSP-1) (S100A4 cat # ab27957) overnight. The following day the sections were washed and incubated with a FITC secondary antibody (Sigma cat # F1262).

Axiovert microscope, Axiocam MRm and Axiovision 3.1 software (Carl Zeiss) were used for image acquisition. Pictures were taken with the corresponding filters for DAPI, FITC and Dylight 594 and the composite images were analyzed for the presence of fibrocytes using ImageJ (National Institutes of Health) software. Slides for confocal microscopy were prepared in the same manner and the images acquired using a Leica SP5 laser scanning confocal microscope.

Fibrocyte Culture

A 30ml sample of blood was collected from a patient or healthy volunteer using sodium heparinised green top collection tubes (BD vacutainer 10ml cat # 8362834). Each tube was gently inverted ten times immediately after collection to ensure thorough heparinisation. These tubes were kept at room temperature and processed immediately.

20mls of Ficoll-Paque Plus (GE Healthcare cat # 71-7167-00 AG) was added to a 50ml sterile Falcon tube and 15mls of blood was transferred from the collection tube to the top of the Ficoll solution by slow loading, taking great care not to disrupt the density gradient. For each 30 ml blood sample, two 50 ml sterile tubes were prepared as described above. These tubes were then centrifuged at 1500rpm for 30 mins.

After spinning, the top layer containing Ficoll was removed and discarded. Using a sterile disposable pipette the mononuclear layer was slowly pulled off and placed into a new sterile 50ml tube. Hanks Balanced Saline Solution (HBSS) (Cellgro cat # 20-023-cv) was added to the mononuclear layer to make the volume up to 40mls. This tube was then centrifuged at 1200 rpm for 10 mins at room temperature.

The supernatant was discarded and the pellet washed twice with 20mls of HBSS. The pellet was then gently resuspended in 3mls of DMEM cell culture media (Lonza BioWhittaker) and the number of cells was counted with a hemocytometer. A T75 Falcon cell culture flasks were then coated with fibronectin (10µg/ml) (Sigma # F4759) diluted in PBS and cells were transferred into this flask. The flask was then topped off to a total volume of 15ml with DMEM plus 20% FBS (GIBCO). Flasks were incubated at 37°C for 3-5 days and then non-adherent cells were removed and media replaced. The cells were cultured for another 7-10 days during which primary cultures of fibrocytes emerged.

Once cells reached confluence or if needed for RNA extraction, 5 mls of trypsin EDTA (GIBCO) was added to each flask and these were then placed in an incubator for 30 mins. Then 5 mls of HBSS was added and pipetted vigorously up and down and the resulting solution saved in a sterile tube. After a rinse with 10mls more of HBSS, the trypsin-edta step was repeated. This was repeated several times while verifying cell detachment under a microscope. The cells removed were centrifuged at 1200rpm for 10 mins and the supernatant discarded. The cells were again washed in 50mls HBSS and used for either 2° culture or to extract RNA as described below.

Migration assay

Migration of fibrocytes was assessed using Falcon 24 well tissue culture plates and cell culture inserts with an 8µm pore size. Fibrocytes which had grown to confluence were harvested as described above and resuspended in DMEM. The cell suspensions were added to the upper chamber $2x10^5$ cells per well. The lower chamber contained DMEM plus 20% FBS, DMEM alone or DMEM plus SDF-1 (125ng/ml R&D). These plates were incubated for 4 hours and then the filters removed, the top of the inserts were wiped of the non migrating cells and the migrating cells were viewed under a microscope. The number of cells migrated were counted and the average found per high powered field.

Secondary culture and Immuno-cytochemistry

Cells cultured and harvested as described above were grown on Nunc-LabTek™ Chamber slides (Thermo-Scientific) for characterisation by Immuno-cytochemistry stains. Cells suspended in 1.5mls DMEM plus 20% FBS was placed into chambers and incubated for 3 days. Unattached cells were removed and cells further grown for another 2 days. After cells adopted the appearance of fibrocytes, the media was removed and the chambers were removed from the slides by separating their gaskits taking care not to disrupt the cells on the remaining microscope slide.

The cells were first rinsed briefly with PBS. Then the cells were then fixed in ice cold acetone for 10 minutes and then washed 3 times with ice cold PBS. The cells were then incubated with PBS plus 4% BSA for 30 mins to block non specific antibody binding. The cells were then incubated with the primary antibody, a fibroblast antibody to detect prolyl-4-hydroxylase positive cells (abcam cat # ab44971) 1:50 diluted in 1% BSA in PBST. The cells were then washed 3 times for 5 minutes each time with TBS. The slides were drained and wiped and the secondary antibody added, a biotin conjugated antibody (Chemicon AP124B) 1:1500 in Normal Swine Serum (NSS). This was incubated for 1 hour and then washed with TBS 3 times for 5 minutes. The slides were drained and wiped and then Streptavidin/Horseradish peroxidase (HRP) (Dako P0397) was added 1:600 in 1% NSS and cells incubated for 45 minutes. The cells were then washed with TBS 3 times (5 mins each time) and then placed in a freshly prepared 3, 3′-diaminobenzidine (DAB) chromogen substrate solution (Sigma D5905) for 10 minutes. Slides were then washed in running tap water for 5 minutes and then counterstained in Mayers Haematoxylin for 20 secs. Finally slides were dehydrated and covered.

Cell stimulation

Cells in culture were stimulated by the addition of either 25ng/ml recombinant IL-1β (R&D systems cat # 201-LB-005) or 10ng/ml of TGF-β (R&D cat # 240-B-002). These cells cultures with the added cytokines were incubated for 24 hours at 37°C and the changes in the culture noted. RNA was extracted from these cells as well as described below.

RNA extraction

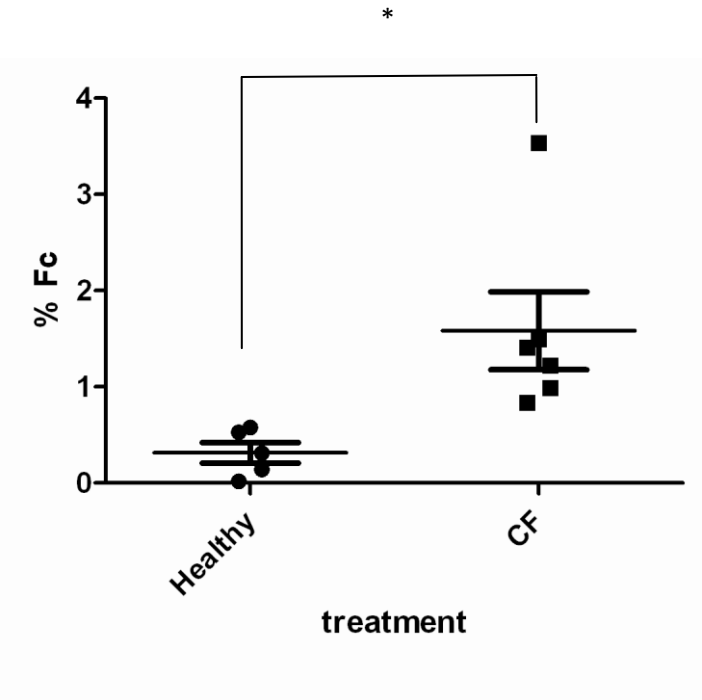
Media was removed from the flask and cells were washed with cold PBS 3 times. 3mls of Trizol (Invitrogen) was placed in the flask and left for 30 minutes on ice. Cells and Trizol was collected and aliquots were placed into eppendorf tubes on ice. 200µl of chloroform was added to each sample and then they were placed on a shaker for 15 minutes. In the meantime 800µl aliquots of isopropanol were placed in eppedorf tubes and kept on ice. After the 15 minute shake of the Trizol plus chloroform, the solution was centrifuged for 20minutes at 14000 rpm. The aqueous phase was then transferred to ice cold isopropanol and mixed 5 times. This was then frozen at -80°C for 2 hours. After 2 hours, these were thawed and centrifuged at 14000 rpm 4°C for 30 minutes. The supernatant was discarded and the pellet washed in 500µl 70% ethanol made in nuclease free water and then spun at 14000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was air dried at room temperature. The pellet was then dissolved in 20µl of RNAse-free water (Ambion cat # AM9932). The RNA concentration was measured and recorded using a NanoDrop™ spectrophotometer. This RNA was then later used for RT-PCR.

Scratch assay (modified from Liang, Park, and Guan 2007)

Fibrocytes were allowed to grow in 60-mm dishes to create a confluent monolayer. The cell monolayer was scraped in a straight line to create a scratch with a sterile p200 pipet tip. The debris was removed and the edge of the scratch smoothed by washing the cells once with DMEM and then replacing fresh DMEM plus FBS 20%. To obtain the same fields of view, marks were made on the cover of the dish with a marker. The dish was then viewed under a Nikon Eclipse TE 2000-E microscope and images taken with a Retiga 2000R QImaging camera. The dish was then placed in an incubator at 37°C for 24 hours. The dishes were removed periodically for observation and then returned to the incubator. After incubation the reference point marker on the cover of the dish was used as a guide to align the scratched area and a second image was acquired.

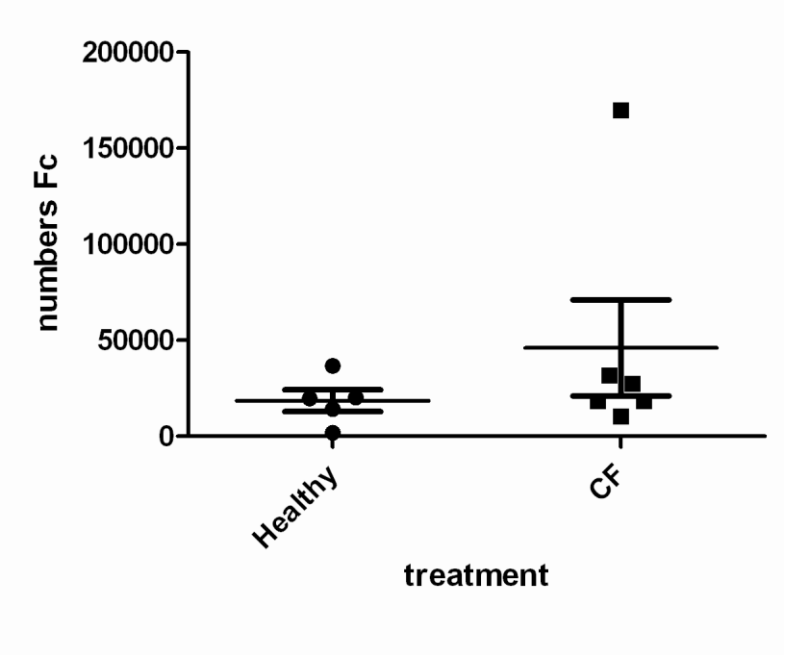
4.3 Results

We recruited patients from the adult CF clinic and measured circulating levels of fibrocytes in these patients as described above. From FACS analysis gating for double positive cells (CD45 and Col-1), we can obtain the percentage fibrocytes shown below. By using the total number of cells stained for FACS we are able to derive a corresponding number of fibrocytes. This is shown below as well, and the use of numbers corresponds to the method used by other investigators (Phillips et al. 2004 and Moore et al. 2005) to quantify fibrocytes in their studies.



Healthy: Fibrocyte levels measured in volunteers who have no known illnesses (n=5)
CF patients: Fibrocyte levels measured in patients at CF clinic (n=6)
%Fc: Percentage of fibrocytes gated positive for both CD45 and Col-1
Student t test $p<0.05$

Figure 45: Percentage of Circulating fibrocytes measured in Cystic Fibrosis outpatients



Healthy: Fibrocyte levels measured in volunteers who have no known illnesses (n=5)
CF patients: Fibrocyte levels measured in patients at CF clinic (n=6)
numbers Fc: Number of fibrocytes gated positive for both CD45 and Col-1

Figure 46: Numbers of Circulating fibrocytes measured in Cystic Fibrosis outpatients

We looked at the results obtained by using these two different analyses. We found, there were subsets of patients who we could identify that had significantly elevated levels of percentage of fibrocytes but not numbers of fibrocytes compared to healthy volunteers. The general trend however, remained the same regardless of which measure was used.

In another unmatched subset of patients we also found that there were patients in whom we could see correlations between percentages of circulating fibrocytes and decrease in lung function.

Patient	% fibrocytes in circulating blood	% of predicted fev1
Cf 20	0.743333	50
Cf 21	1.45667	61
Cf 24	0.5	67
Cf 25	1.37333	44
Cf 28	0.15667	56
Cf 29	1.773333	43

Patient: An out patient of the Cystic Fibrosis clinic diagnosed with CF lung disease
% fibrocytes in circulating blood: % of fibrocytes gated positive for both CD45 and Col-1
% of predicted fev1: 60-70% moderate obstruction, 50-60% moderately severe obstruction, 34-50% severe obstruction

Figure 47: Percentage Circulating fibrocytes and lung function of Cystic Fibrosis out patients

When we looked at a larger number of CF patients who had varying levels of disease there was no major difference between the percentage of fibrocytes seen compared to the controls. However as shown above, when we examined a subset of patients who had more severe disease we saw a significant increase in the percentage of circulating fibrocytes, suggesting that similar to what has been shown in IPF (Moeller et al. 2009) these cells may play some role in exacerbation or worsening of disease.

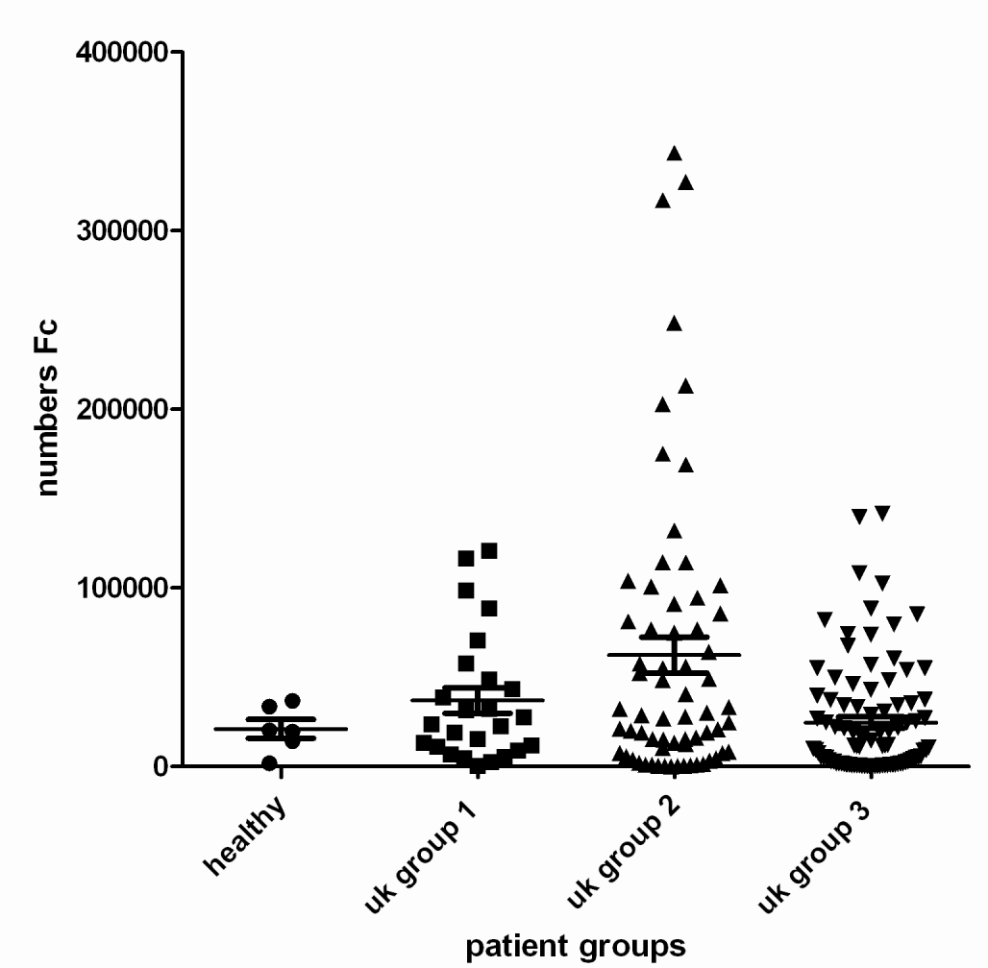


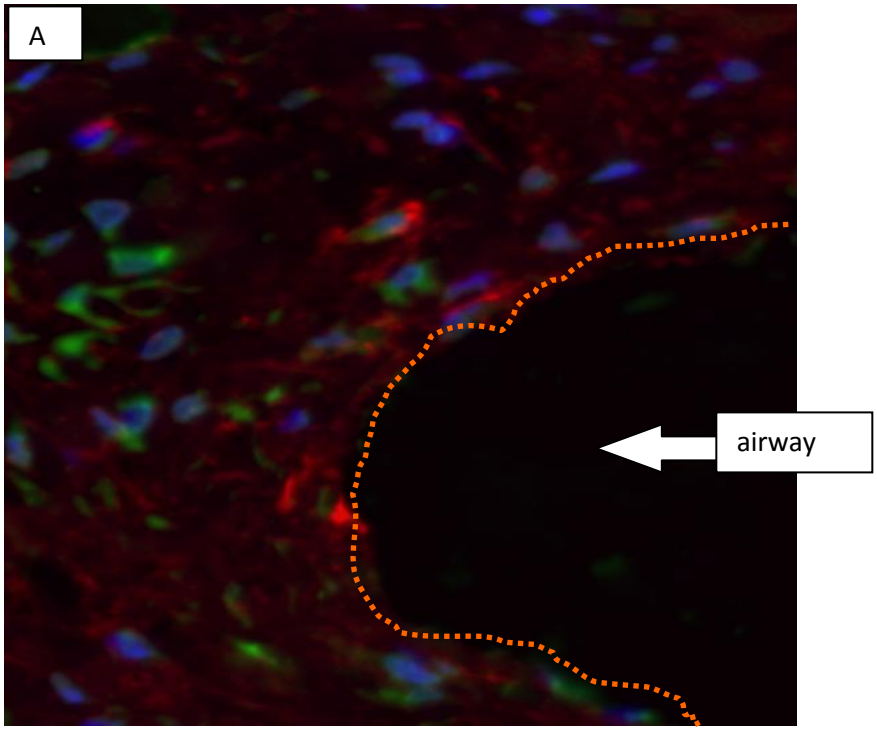
Figure 48: Fibrocyte numbers measured in patients diagnosed with IPF

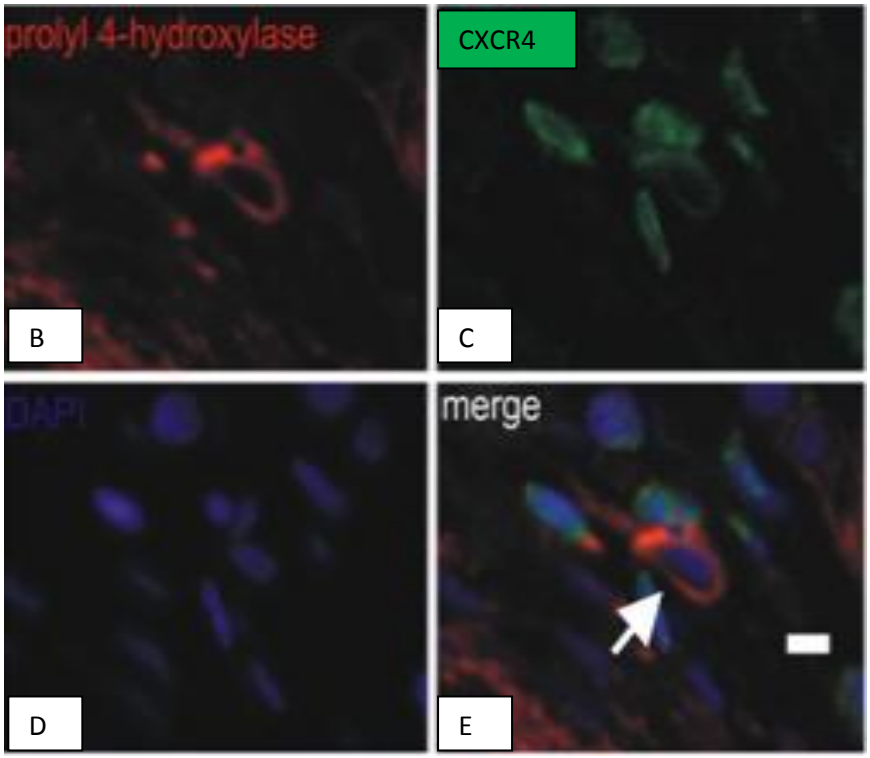
Healthy: Fibrocyte levels measured in volunteers who have no known illnesses
Numbers Fc: Absolute number of fibrocytes gated positive for both CD45 and Col-1 (n=6)
Uk group 1: First lot of IPF patients recruited into study (n=25)
Uk group 2: Second lot of IPF patients recruited into study (n=67)
Uk group 3: A combination of new IPF patients and repeat samples from lots 1 and 2 (n=94)

We have measured fibrocyte levels in several patient samples received from the University of Nottingham as part of the PROFILE study. These continued measurements have validated the robust assay we have for the measurement of fibrocytes. We expect that the fibrocyte measurements when correlated with the clinical progression of the patient as well as other biological markers will help us to better understand the utility of the measurement of fibrocytes.

Immunoflourescence: Fibrocyte detection in tissue

Images were taken of stained sections as described above. Fibrocytes were identified as double positive cells CXCR4 (green) and prolyl-4-hydroxylase (red) see figure below. These double positive fibrocytes were quantified in normal areas of lung, fibrotic areas in IPF patients and injured lung areas in CF patients, using the tissue obtained from patient lungs at transplant.





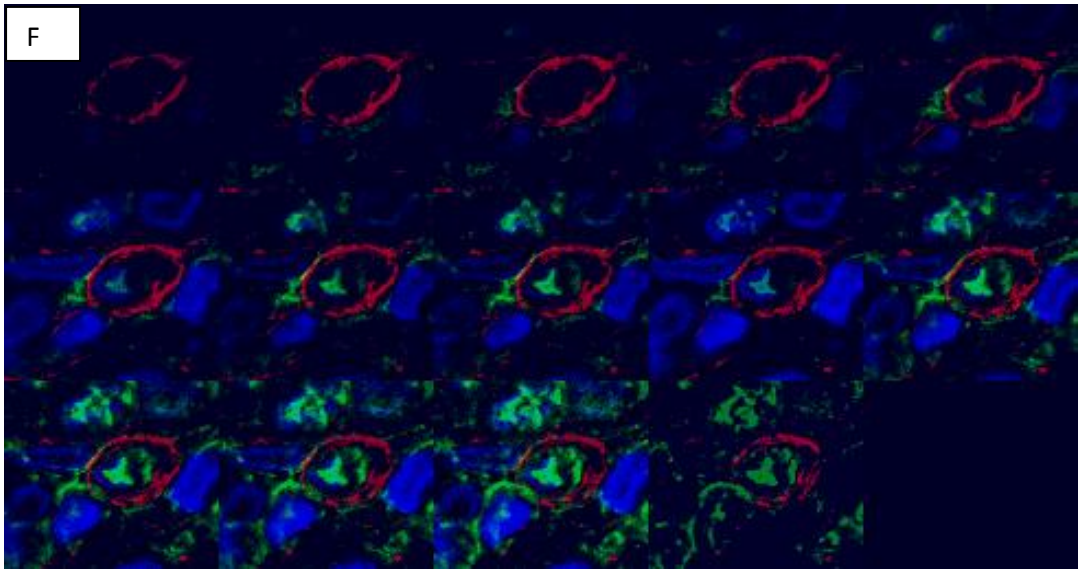


Figure 49: Fibrocytes identified in Cystic Fibrosis lung

(A) Section of a CF lung double stained for CXCR4 and prolyl-4-hydroxylase next to an airway (x20). Double positive cells identified by individual staining for (B) prolyl-4-hydroxylase (red) (C) CXCR4 (green) (D) DAPI for nuclei and then merged (E) with ImageJ software (F) Confocal montage for CXCR4 positive (green) /prolyl-4-hydroxylase (red) positive cell

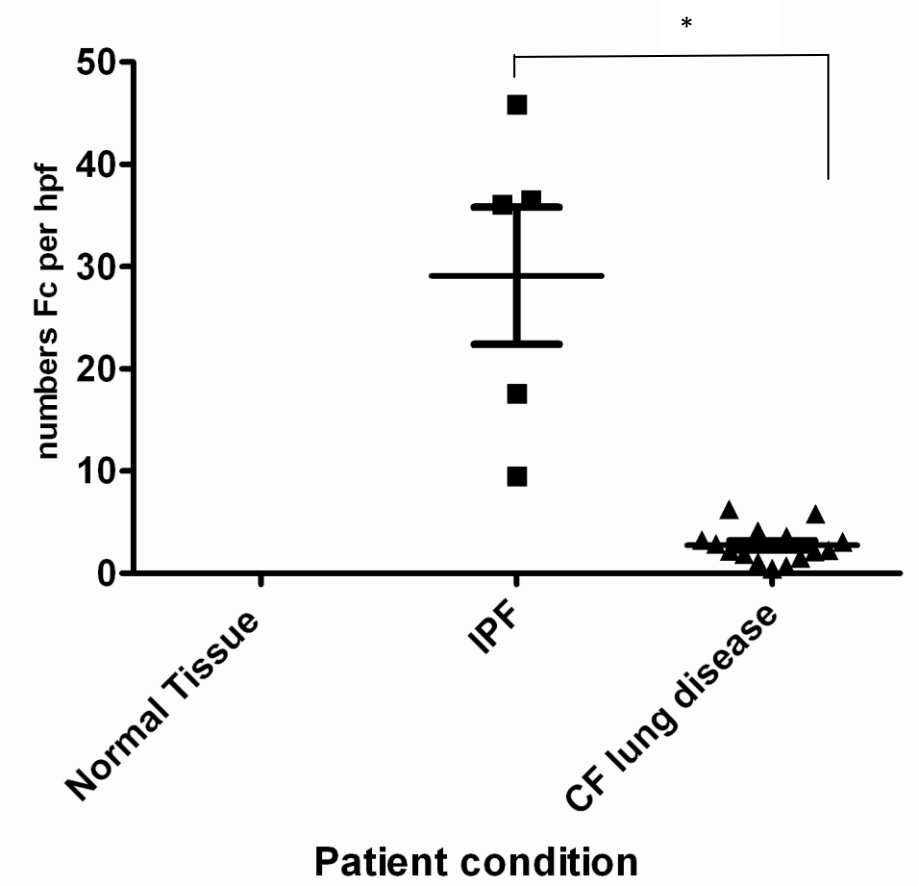


Figure 50: Fibrocytes identified in Cystic Fibrosis and Idiopathic Pulmonary Fibrosis transplant lungs

Numbers Fc per hpf: Average number of Fibrocytes counted per high powered field
Normal Tissue: Areas of unaffected tissue with normal lung structure preserved
IPF: Lung samples from patients diagnosed with Idiopathic Pulmonary fibrosis
CF lung disease: Lung samples from patients diagnosed with Cystic Fibrosis lung disease
Student's t test: $p<0.05$

These Fibrocytes were identified in lung samples obtained from patients undergoing lung transplant. There was a significantly higher number of fibrocytes in the patients diagnosed with IPF 29.08 ± 6.708 N=5 versus those with CF lung disease 2.748 ± 0.4362 N=15. From this we speculated that either fibrocytes were not being recruited at this stage in CF lung disease or not being retained in the lung matrix.

Immunoflourescence: Myofibroblast detection in CF lung tissue

We examined Myofibroblasts in CF lung to further explain the lower number of fibrocytes we found above. We examined 23 lung samples from terminal CF lungs obtained from the transplant centre. Of the 23 patients examined, we found only 5 (22%) had myofibroblasts present, the remaining 18 patients had no myofibroblasts present. The presence of some myofibroblasts does indicate the presence of some fibrosis, however taken together with the data from the previous section, the lack of abundant myofibroblast presence might be related to a scarcity of fibrocytes.

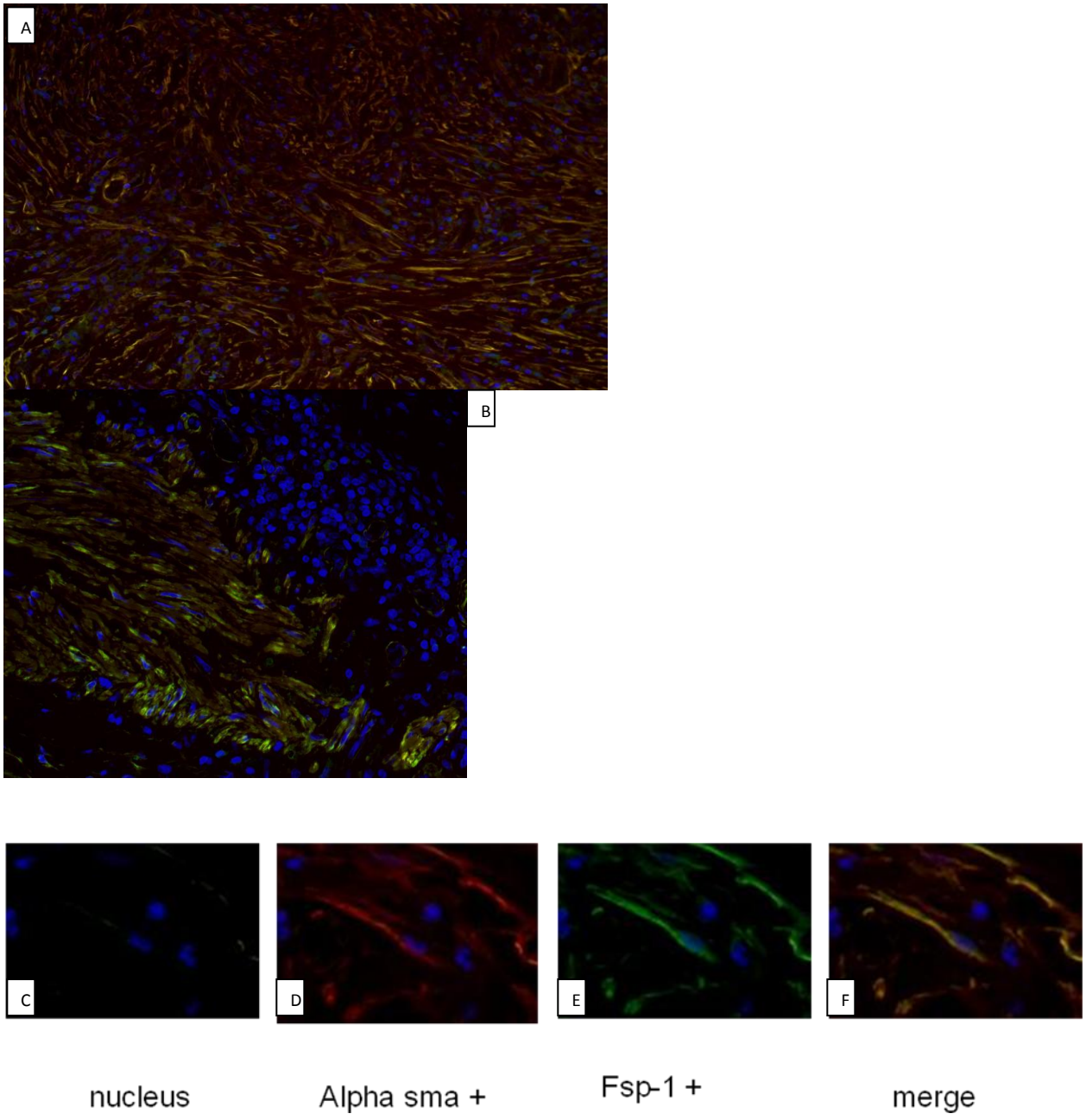


Figure 51: Myofibroblasts identified in the lungs of CF patients. Immunofluorescence staining for myofibroblasts in the lungs of CF patients. αSMA and FSP-1 (a) x5 magnification (b) x10 αSMA (red) and fsp-1(green) double positive cells. (c)DAPI staining (d) αSMA (e) FSP-1 (f) Merge (x20)

Fibrocyte cultures

We cultured fibrocytes from human peripheral blood and observed the growth kinetics of these cells as well as the characteristic change in morphology which have been reported in the literature by Bucala and others (Bucala et al. 1994, Pilling et al. 2003).

Figure 52: Primary Fibrocyte culture at day 21(x20)

We performed 30 primary cell cultures and the figure above is representative of a primary culture at day 21. These cultures were characterised by spindle shaped cells with an oval nucleus as described by Bucala and coworkers (Bucala et al.1994).

Fibrocyte Markers

Cells grown on microscope slides as described above were stained for markers which could confirm their identity. We were able to identify elongated cells with oval nuclei which were positive for prolyl-4-hydroxylase, a marker which is highly expressed in fibrocytes (Pilling et al. 2009).

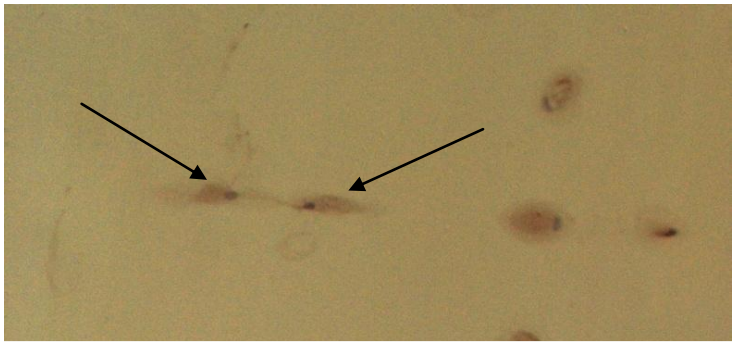
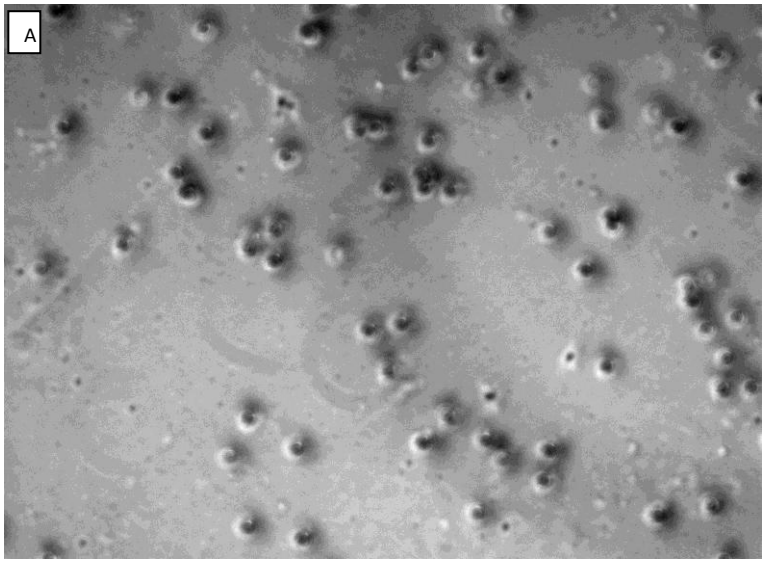
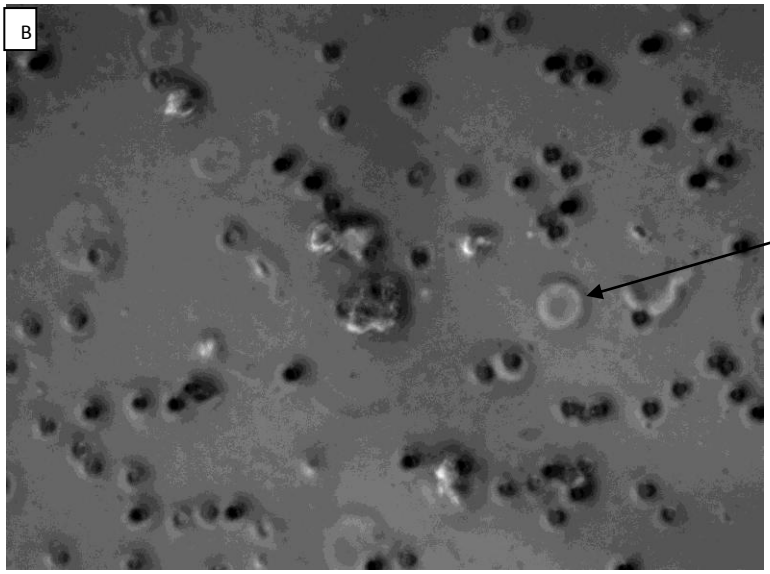


Figure 53: Fibrocyte stained positive for prolyl-4-hydroxylase. This cell was cultured from the blood of a CF patient (x20 magnification)

Migration Assay

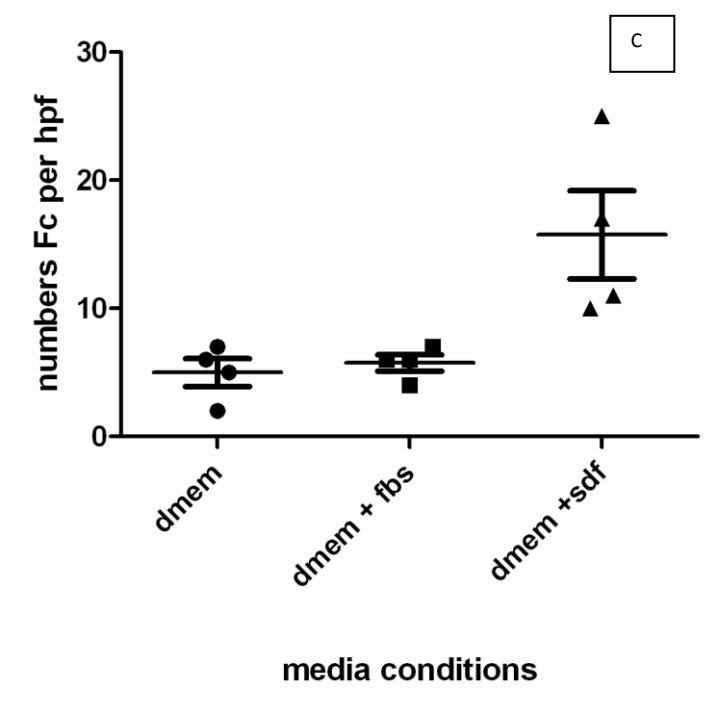


DMEM plus FBS



Cell which migrated to SDF-1

DMEM plus SDF-1



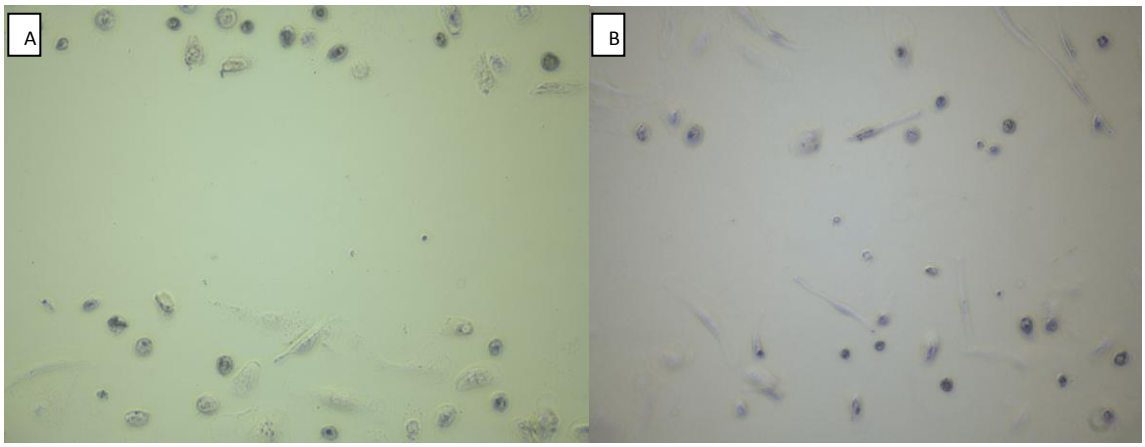
numbers Fc per hpf: Average number of Fibrocytes counted per high powered field
dmem: Media containing only DMEM
dmem + fbs: Media containing DMEM and 20% FBS
dmem + sdf : Media containing DMEM and 125 ng/ml

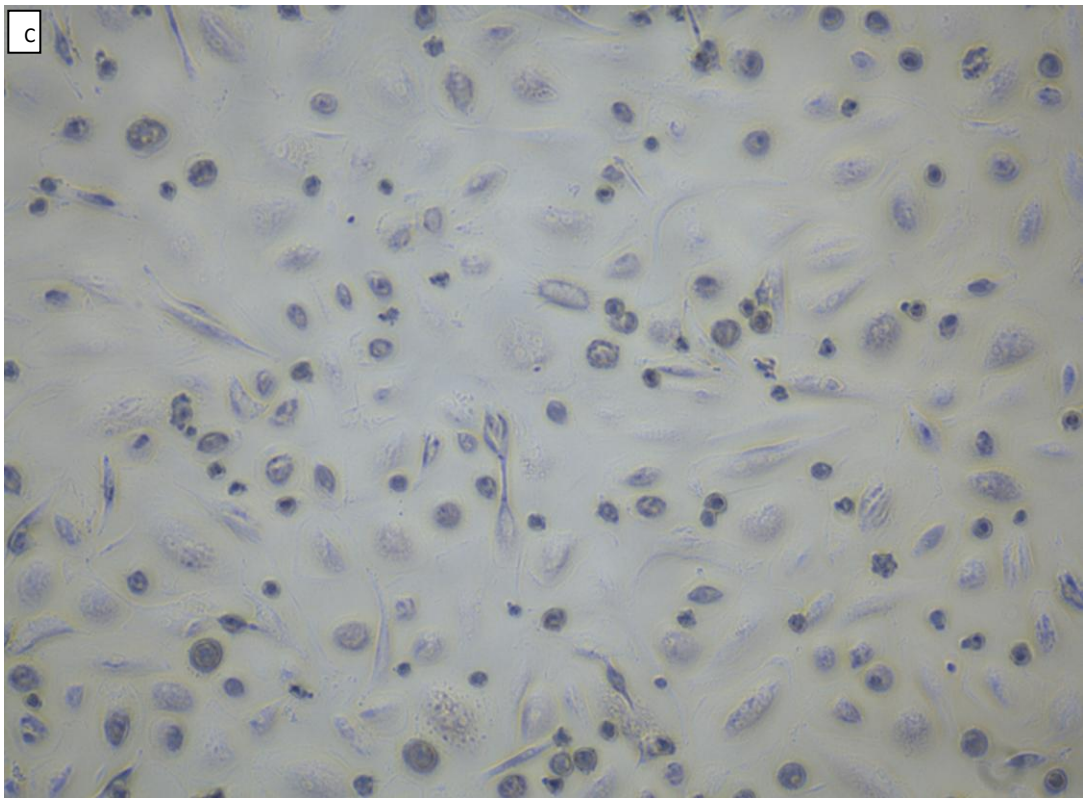
Figure 54: Migration assay of fibrocytes. (A) Cells migrating towards DMEM plus FBS (B) DMEM plus SDF-1 (x20) (C) Migrated cells counted per high powered field on filter of cell culture insert (n=4)

The results of this assay showed the cells grown in culture were indeed CXCR4 positive as they responded to the ligand SDF-1.

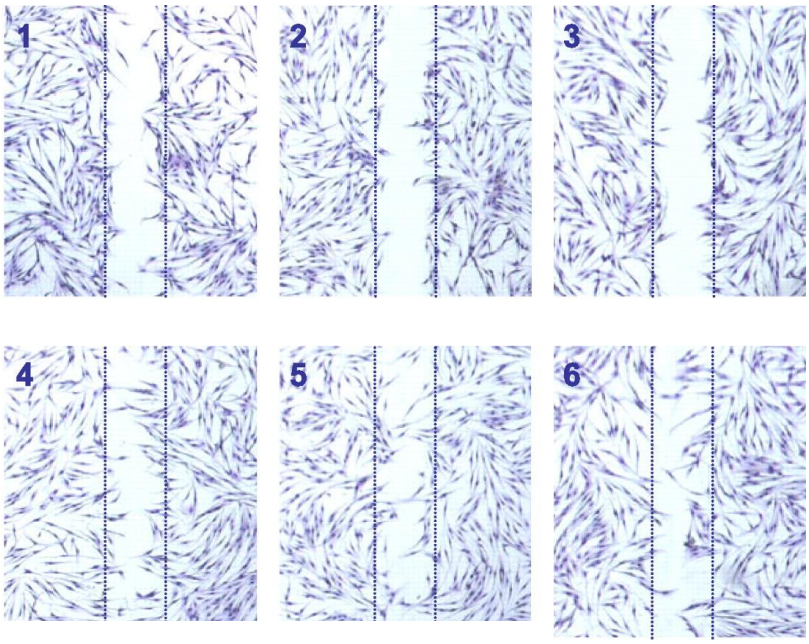
Scratch/Wound healing assay

We used a simple scratch assay to assess the ability of a primary culture of fibrocytes to heal a wound and found that after 24 hours these cells did not develop a contractile phenotype and there was a minimal amount of cell migration compared to fibroblasts.


A
B


C

This finding showed a cell phenotype which behaves differently to fibroblasts, as shown in the fibroblast cell line below which begins migration after injury and there is significant migration by 6 hours after injury.



Modified from Bogatkevich G S et al. Am J Physiol Lung Cell Mol Physiol 2008;295:L603-L611

Figure 55:Scratch wound Assay. (A) A new formed scratch in in cell culture plate (B) 24 hours later the scratch is not healed (C) A control unscratched area of the plate (x20 magnification) (D) A fibroblast cell line which shows cell migration (1-6) hours after injury in a scratch assay.

Cell Stimulation

Cells in culture were stimulated by the addition of either 25ng/ml recombinant IL-1β or 10ng/ml of TGF-β.

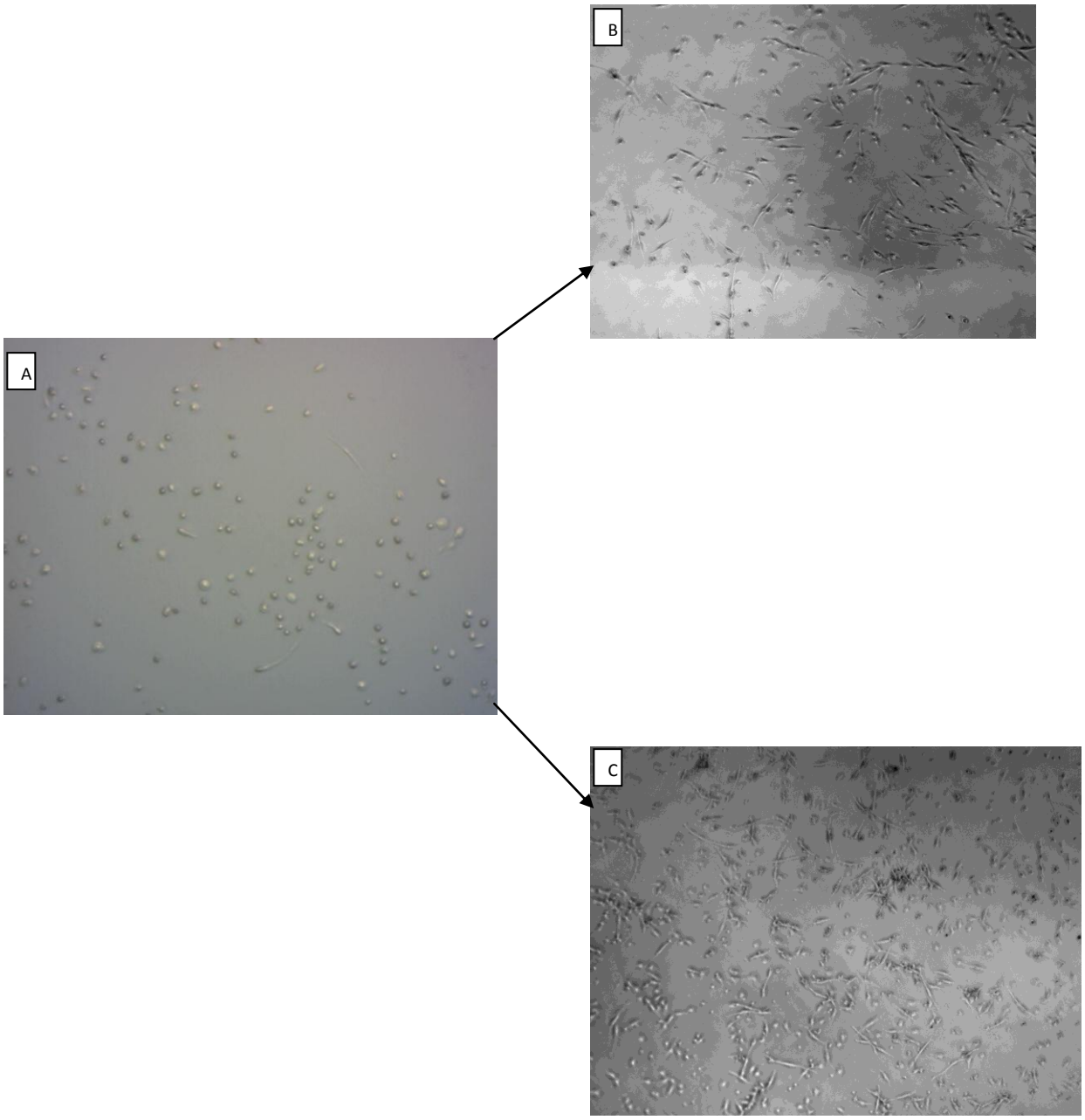
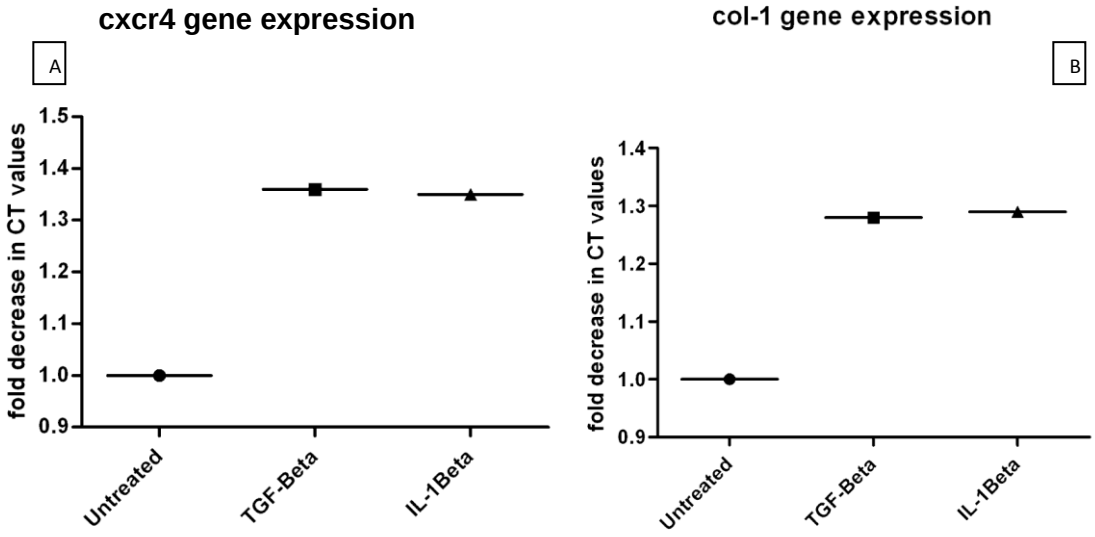


Figure 56: Fibrocyte cultures stimulated with recombinant IL-1β or TGF-β. Fibrocytes grown in culture (A) were stimulated with recombinant IL-1β (B) or

TGF-β (C) for 24 hours (x20 magnification) and the cells adopted what has been discussed in the literature (Abe et al 2001) as being a possible pro-fibrotic phenotype.

Gene expression



Fold decrease in CT (cycle threshold) values: Increase in gene expression
TGF-β: cells treated with 10ng/ml of recombinant TGF-β for 24 hours
IL-1β: cells treated with 25ng/ml of recombinant IL-1β for 24 hours

Figure 57: Gene expression of fibrocytes treated with TGF-β and IL-1β. (A) CXCR4 gene expression (B) col-1 gene expression (n=1)

In this 'activated' state, we examined the gene expression of CXCR4 and Col-1 and found a modest increase in their expression after the fibrocytes were exposed to TGF-β and IL-1β for 24 hours. These studies were performed with only cells from 1 single culture for each treatment. This limits our ability to make any definite conclusions on this data without reproducing it.

4.4 Discussion

There have been several clinical reports of fibrocytes found in association with lung injury. In many of these cases, the patients were selected based on convenience sampling, where lung biopsies were available, and not necessarily due to any careful selection. Some studies have looked at fibrocyte presence in lung sections identified by immuno-fluorescent staining and some by identification of circulating fibrocytes. In addition to our animal work discussed above, we attempted to further characterise the presence of fibrocytes in patients and to correlate the appearance of fibrocytes with the progression of fibrotic lung injury. We selected patients in whom we would be able to examine different aspects of their disease. We also looked for patients who were stable and being cared for in an outpatient setting and in addition we examined unrelated terminal lung tissue obtained from transplant patients. Our patients did have varying degrees of disease and we categorised their severity based on lung function data where available.

We find the data about circulating fibrocytes in patients diagnosed with CF or IPF to be very exciting. Our robust system is able to analyse fibrocyte numbers for patients from a 30 ml blood sample which can be routinely obtained on a clinic visit while still being able to collect sufficient blood for other necessary tests. In subsets of our CF patients, we did see a notable association between the severity of disease and increasing fibrocyte percentages, but not numbers, unfortunately, limited access to patient samples prevented us from being able to get a large enough sample size to suitably assess this. In our transplant samples, we saw a much lower retention of fibrocytes in terminal CF lung compared to terminal IPF lung tissue. Some possibilities for this observation include: 1) A defective recruitment mechanism in the CF lung with less expression of CXCL12 or periods of down regulation or 2) A lack of intact matrix in the terminal CF lung prevents the fibrocyte from its normal interaction with areas of injury.

Our measurements of circulating fibrocytes in IPF patients as part of the PROFILE trial will contribute to the much needed correlation of fibrocyte numbers with other biological markers. Most importantly we will be able to look at serial measurements and observe variations in these measurements with time and patient's disease progression. These serial measurements and their variations will better enable us to assess the merit of fibrocyte measurement as a prospective biomarker.

We attempted to grow fibrocytes in vitro as an important validation step. The biology of fibrocytes has been reported (Bucala et al. 1994) (Abe et al. 2001) (Pilling, Vakil, and Gomer 2009) in the literature and we wanted to ensure that the cells we studied had a similar profile to that discussed by others. We also

believed information about their growth characteristics and phenotype would allow us to better understand their potential biological role when identified in patient samples. The successful in vitro culture of fibrocytes remains a challenging task. We were able to establish some cultures of these cells that could grow successfully at least until a secondary culture.

We repeated in vitro experiments described in the literature (Bucala et al. 1994) (Abe et al. 2001) (Pilling, Vakil, and Gomer 2009), including the gene expression profile of these cells, their immunohistochemistry markers and their ability to migrate to SDF-1. This was done to confirm the identity of the cell population we were manipulating. We believe that further attempts to simplify the culture of these cells and also perform similar cultures in the rat are needed to help us better understand the biological properties of these cells and how they vary in the presence of concurrent disease. The literature (Andersson-Sjoland et al. 2011) has discussed that these cells appear to be very malleable in phenotype and it would be very interesting to determine if the biology of the fibrocyte in an acute disease is different to that of the cell in a chronic disease. In the animal model a valuable development would be to determine if the biology of the circulating fibrocyte in a host which has a specific cytokine overexpression, eg GM-CSF, is different to that induced by a different cytokine.

The data presented about the culture of fibrocytes is preliminary due to the small number of cultures and the small number of cells obtained. However, this data does show a cell which can be characterised by some of the markers and morphology described in the literature (Pilling et al. 2009). We also have provided preliminary data to show a cellular phenotype which can change in the presence of cytokines. This finding should be developed further and may possibly indicate a phenotype which can be switched on in the right (or wrong) conditions.

We believe that further to our results here, the results of the PROFILE and other similar trials will help us to determine the clinical utility of fibrocyte measurement. Independently however, further development is needed to reproduce the fibrocyte cell culture work of other investigators. I view this as a very important foundation step to better understanding the signature of the fibrocyte. Our evidence and that of others suggest that this cell when first isolated from the circulation has a very flexible phenotype. I speculate that only when subjected to host local conditions does the cell adopt its final role.

5.0 Conclusion

IPF is estimated to be on the rise (Nalysnyk et al. 2012) and there are still many remaining challenges in the management of these patients, despite major advances in the last decade, and the approval of the first IPF specific drug in most countries (pirfenidone) (Baroke, Maharaj, and Kolb 2011). A major question remaining is to identify factors that turn tissue repair away from normal healing towards limited, non-progressive fibrosis or even progressive fibrosis as seen in IPF. It is possible that the fundamental biological mechanisms may not be substantially different in different chronic diseases where fibrosis occurs, and the identification of unifying principles may lead to therapeutic advancements in several different diseases. To obtain these unifying principles, we hypothesised that there is a need for a closer study of both local (within the lung) and systemic responses (from the circulation and bone marrow) in fibrotic lung disease.

In this thesis, we discussed the need for improved methods of detecting changes in chronic lung disease. We examined several approaches for doing this, including; investigating new options for the assessment of ongoing lung injury in animal models, the use of models to better understand the effects of cytokines in the initiation and progression of lung injury, the use of models of lung injury to better understand the role of the fibrocyte and to modulate fibrocyte responses after lung injury, and finally the use of patient's blood samples for the assessment of the circulating fibrocyte's potential to be a prospective biomarker.

In addition to cellular markers which may indicate change in lung function and disease progression, there is a greater need for sensitive measures of structural changes in lung function. When used together, both of these can provide minimally invasive indices which can allow us to track the progress of pathological or therapeutic treatments. The availability of multiple non terminal markers to assess lung changes in animal models would be extremely useful to: 1) Persons wishing to conduct longitudinal studies to test novel compounds and 2) Investigators wishing to dissect sequential changes in lung injury after the administration of single or multiple pathological treatments. The value of these tools would be proven by the reduced number of animals that may be required for performing experiments as well as the enhanced profile of the dataset which would be obtained for models of diseases being investigated.

We provided a validation for a repeat assessment of an animal model of lung injury which could detect both early and late stage changes in lung compliance. While Flexivent™ is currently used for measuring airway resistance and other lung function indices, its use for a non terminal assessment in the rat model of lung fibrosis to the best of my knowledge, is novel. The use of PET/CT in confirming delivery of therapeutic/pathological agents in the lung discussed in

Chapter 2 was an important proof of principle. Although application of this particular approach may be potentially costly and time consuming, it would be invaluable for gaining insight into new delivery methods such as the unilateral delivery of treatments as well as the progression of lung injury without sacrificing the animal.

Until now, the exact biological role of fibrocytes in tissue repair and fibrosis has not been fully understood. Fibrocytes are believed to be involved in the pathogenesis of several fibrotic disorders, affecting lungs, liver, kidneys and other organs, and may have a role as a potential biomarker of disease activity, as they can be detected and quantified from easily available peripheral blood samples. The translation of the measurement of circulating fibrocytes from humans to the rat model was a very valuable tool generated in this thesis. The rat can offer many advantages over the sought after mouse model including easier visualisation and monitoring of ongoing changes discussed in the live assessment chapter. This model has allowed us to ask questions about the effect of cytokines implicated in fibrosis on fibrocyte kinetics. Furthermore, we were able to track the mobilisation of fibrocytes in the circulation and their movement to the lungs. The identification of fibrocytes in lungs which were injured both in human samples and animal models increased the available evidence regarding the association of fibrocytes with lung injury.

We utilised two methods for the quantification of fibrocytes in circulation, percentages (%Fc) and numbers (numbers Fc). The % Fc is the percentage of cells which gated positive for both CD45 and col-1 on analysis with FloJo software. Numbers Fc is derived from the total cell number of cells which were stained for FACS. The general trend in fibrocyte quantity remained the same whichever method was used. However when all treatments were conducted intratracheally, the %Fc appeared to be more sensitive to change. When treatments were administered intramuscularly numbers Fc appeared to be more sensitive to change. This does demonstrate that the total circulating cell count may sometimes alter the results obtained when examining small subsets of blood cell populations; thus both percentage and total cell measures should probably be examined to obtain accurate estimates of changes.

The measurements obtained were reproducible with 3-6 animals tested per time point. The fibrocyte levels in each experiment did show some variation within groups, although this is a potential threat to the robustness of the measure there was still statistical significance seen in a number of the experiments. I speculate that the variation of Fc% and number seen within groups should be expected, as with any other biological signal there will be a range within which our fibrocytes will be detected. This will however have to be validated with subsequent experiments and larger numbers of animals.

Fibrocyte retention in areas of injury at specific time points also helped us speculate on possible relationships. Interestingly in our model of TGF-β lung injury, the highest number of fibrocytes was detected in the lung at day 21, a time point of significant lung modification and suspected self perpetuating fibrogenesis. It is possible that we may be able to adapt our rat model to perform repeated measurement of fibrocytes allowing this to become a non terminal assessment increasing the utility of this approach. The induction and reduction of fibrocyte numbers by the use of GM-CSF and AMD3100 need to be examined further for any direct and indirect consequences. We showed here some direct consequences such as changes in gene expression and cellularity. However, finding a lack of a dramatic effect, such as the abrogation of a fibrotic response, may suggest compensation for the reduced/absent fibrocyte by alternate pathways. The Bleomycin model is among one of the most well characterised models of lung fibrosis and is reputed for its histological characteristics. In the combination of GM-CSF expression and this injury model, the recruitment of fibrocytes did not improve lung injury and was instead associated with some worsening of injury. I believe with a similar approach to the modalities tested in this thesis and thorough immunohistochemical analysis in a sufficient number of animals, we may be able to determine the exact impact of varying fibrocyte presence on the fibrotic outcomes in the bleomycin model of injury.

In our animal studies, cytokine overexpression did prove to be able to mobilise fibrocytes into the circulation or recruit them to the lung. The cytokines examined in this thesis are of biological significance in chronic lung disease and their ability to recruit or mobilise fibrocytes adds support to a possible role for these cells in the pathogenesis of chronic lung diseases. We have shown here a link between fibrocytes and one chronic lung disease, fibrosis. However, in the different niche environments of other lung diseases this cell may perform similar or different functions.

Our systematic approach to the measurement and characterisation of fibrocytes in both humans and the animal model has given us a great deal of information about fibrocyte behaviour in various disease states as well as in the presence of elevated levels of specific cytokines. We were able to show a presence of fibrocytes and myofibroblasts in some cases of CF lung disease, a disease which previously had a dubious link to fibrosis. The high levels of fibrocytes found in the IPF lungs, like the animal models, also suggest a definite role for these cells in fibrotic injury. The progress of studies such as the PROFILE study and hopefully others like it is a definitive approach to us being able to answer current questions about the fibrocyte's biomarker status and confirm the prognostic capabilities of this marker. A combination of our resources with those of our colleagues at the transplant centres will also allow us to look at the levels of fibrocytes pre and post

transplant allowing us to better understand the variation of fibrocytes in 1) chronic lung injury 2) end stage lung disease and 3) post transplant.

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Appendix 1 Fibrocytes in Pulmonary Fibrosis - a Brief Synopsis

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Abstract

Fibrocytes are bone marrow derived, circulating mesenchymal progenitor cells, which play a role in several fibrotic disorders, including lung fibrosis. They are attracted to injured tissue by various chemokines. It is likely that fibrocytes play a detrimental role in tissue homeostasis and promote fibrosis, although this paradigm needs further confirmation. This would make fibrocytes a possible novel treatment target for fibrotic disorders. Fibrocytes have also some potential as biomarker for IPF and other diseases, but the promising preliminary data from single center studies still require independent validation. Despite several yet unresolved issues, it has become clear that fibrocytes are more than an incidental finding in lung injury and repair and may hold great promise for the future of IPF management.

Introduction

Fibrosis is a common biological phenomenon in health and disease. It can affect all organ systems of the body. Fibrosis is defined by the overgrowth, hardening and/ or scarring of tissues and is attributed to excess deposition of extracellular matrix (ECM), including collagens, proteoglycans and glycoproteins (Wynn 2008). Controlled synthesis, deposition and degradation of ECM is a physiological process in tissue repair. In contrast, abnormal ECM accumulation is a critical part of the pathogenesis of several common conditions in the lung, including chronic infectious, airway, vascular and parenchymal diseases (Mehrad et al 2012). There are many factors that can contribute to the switch from normal, regulated ECM production and degradation to excessive or aberrant deposition in fibrosis. Many of these factors are a major area of study in fibrosis to better understand the process and develop novel strategies to manage patients with fibrotic disorders. Fibrocytes are circulating bone-marrow derived mesenchymal cell progenitors that can differentiate into fibroblasts and myofibroblasts once they enter the tissue. They are a putative source for the fibroblastic foci, which are a characteristic feature of the histopathological pattern “usual interstitial pneumonia (UIP)” found in idiopathic pulmonary fibrosis (IPF). The exact biological role of fibrocytes in tissue repair and fibrosis is not fully understood. They are believed to be involved in the disease pathogenesis of several fibrotic disorders, affecting lungs, liver, kidneys and other organs, and may become a role as a potential

biomarker of disease activity as they can be detected and quantified from easily available peripheral blood samples.

Pulmonary Fibrosis

Pulmonary fibrosis can be the result of many known diseases including systemic disease (e.g. scleroderma or mixed connective tissue disease), occupational diseases (such as asbestosis and silicosis), drug related lung disorders and others. The most progressive and unpredictable type of lung fibrosis is IPF (Collard et al 2007). This disease is a major clinical problem, particularly in the elderly population. Numerous epidemiological studies have suggested a role for certain contributing factors, but to date the initiating cause of IPF is still unknown (Kottman et al 2009). Patients with IPF suffer from worsening dyspnoe and cough and can experience various patterns of progression during the disease course, eventually resulting in irreversible lung damage and decline of lung function (Ley et al 2011). This process often leads to respiratory failure with a median survival of 3 years after diagnosis (Todd et al 2012, Antoniou et al 2013).

There are many remaining challenges in the management of IPF patients, despite major advances in the last decade and the approval of a first IPF specific drug in most countries, not yet the United States (pirfenidone) (Baroke et al 2011). The detailed understanding of the pathogenesis will help to delineate

disease mechanisms, which can be targeted by novel therapeutic compounds (Loomis-King et al 2013). Further study of disease mechanisms can also help to detect new biomarkers, which are required to measure disease activity, treatment responses and estimate prognosis in individual IPF patients. One of the key questions is to identify the factors that turn tissue repair away from normal healing towards limited, non-progressive fibrosis (such as often seen in asbestosis or idiopathic non-specific interstitial pneumonia), or even progressive fibrosis as seen in IPF. It is possible that the fundamental biological mechanisms may not be substantially different between these scenarios. Alteration of the extracellular matrix composition and microenvironment in which structural cells live in the organ tissue is an increasingly recognized problem and a potential driver of fibrogenesis in IPF (Shimbori et al 2013).

There is a need for a closer study of both local (within the lung) and systemic responses (from the circulation and bone marrow) in fibrotic lung disease. Bone marrow derived progenitor cells and systemic responses to signals that originate in damaged tissue are an important factor not only to host defense, but also in tissue repair. Better understanding of their contributions to injury might also allow to identify unifying principles which could be present in different fibrotic disorders.

Progenitor Cells and Fibrocytes in Tissue Repair and Fibrosis

There is increasing evidence suggesting that tissue resident and bone marrow derived progenitor cells contribute to injury repair in the lungs, including epithelial,

mesenchymal and endothelial cell progenitors (Beers et al 2011, Mehrad et al 2012, Moore et al 2006). Small niches of progenitor cells resident to the lungs are involved in repair and repithelialisation after injury (Warburton et al 2008). It is believed that in diseases of exaggerated repair, such as IPF, the depletion of "beneficial" progenitor cells may lead to increased recruitment of alternate sources of progenitors, leading to a switch from sucessful repair to fibrotic remodelling. Aging is an important factor in this process as well, which is consistent with the fact that IPF typcally affects individuals older than 60-65 years. Animal models have shown that old mice mobilize primarily fibrocytes from the bone marrow in response to bleomycin induced lung injury. In contrast, mesenchymal stem cells (MSC) are the predominant cell type found in the circulation of young mice following bleomycin. Consequently, young mice develop less severe fibrosis in the lungs compared to older mice (Xu et al 2009). Interestingly, MSC are more pluripotent than fibrocytes, providing a possible explanation for the difference in the wound healing process between age groups (Mattoli et al 2009).

Fibrocytes are spindle-shaped mesenchymal progenitor cells first characterised by Bucala in 1994 (Bucala et al 1994). Since then many disease models have shown that these cells respond to tissue derived signals and migrate to sites of injury where they can differentiate into fibroblast-like cells (Phillips et al 2004, Moore et al 2006). Fibrocytes circulate in the peripheral blood and are capable of

producing collagen, other ECM components, crosslinking enzymes, cytokines and growth factors. Fibrocytes express a variety of mesenchymal markers including collagen 1 as well as the leukocyte marker CD45 and the hematopoietic stem cell marker CD34 (Quan et al 2006). Different groups have reported many different marker sets as being useful in identifying fibrocytes, reviewed extensively by Pilling et al (Pilling et al 2009). However there is sufficient evidence that these cells can be reliably identified in the circulation by markers for CD45 and Col-1 (Keeley et al 2012, Moeller et al 2009, Fujiwara et al 2012, LaPar 2011).

Fibrocytes respond to the profibrotic cytokine TGF-β by expressing α-smooth muscle actin, which supports a potential role of these cells in myofibroblast formation, a prototypical feature of lung fibrosis (Abe et al 2001). In pulmonary fibrosis, the abnormal tissue remodeling is characterised by excessive accumulation of ECM, distortion of lung architecture including the vascular system (Hanumegowda et al 2012), and formation of fibroblastic foci. These foci represent the sites of active fibrogenesis and fibrocytes are a presumed source of cells that create these fibroblast clusters. Besides fibrocytes, the proliferation of resident fibroblasts, epithelial to mesenchymal transition (EMT) (Willis et al 2005) and pericytes (Hung et al 2013) are potential contributors to fibroblast foci (see Figure 1). All of these cells, when exposed to TGF-β and other local factors, may undergo transformation into myofibroblasts (Hinz et al 2007) which then drives

progressive fibrogenesis (Strieter et al 2009, Todd et al 2012). Interestingly, there is increasing evidence that not only cytokines can switch progenitor cells into active myofibroblasts, but also abnormal matrix composition and rigidity (Shimbori et al 2013). It has been shown that fibrocytes demonstrate phenotype plasticity and are affected by their niche environment (Andersson-Sjoland et al 2011, Maharaj et al 2012). Careful dissection and creation of accurate models of the fibrotic matrix are required to truly understand the functions of fibrocytes in the microenvironments of different organs.

Fibrocytes have been found in patients with various fibrotic disorders and in animal models of injury (Maharaj et al 2012, Andersson-Sjoland et al 2008, Moore et al 2006, Mori et al 2005) suggesting a relationship between mesenchymal cell progenitors and chronic injury and repair. There is evidence that these cells are involved in the pathogenesis of lung fibrosis (Strieter, Keeley et al 2009), liver fibrosis (Kisseleva et al, 2006), scleroderma (Aiba et al 1994, Tourkina et al 2011), asthma (Schmidt et al 2003, Saunders et al 2009) and others. Fibrocytes have been identified in areas of injury adjacent to newly formed scar tissue, which has raised the question whether or not these cells are good targets to improve repair and prevent fibrotic remodeling. It is also still unclear, which factors mobilize fibrocytes from the bone marrow and recruit them to the sites of tissue injury. Amongst them are human CCL2 (Moore 2006 et al), CXCL 12 (Phillips et al 2004), secondary lymphoid chemokine, SLC (Abe et al

2001) and others (see table 1). There is increasing support for the hypothesis that fibrocytes differentiate into myofibroblast as has have been shown *in vivo* (Bucala et al 2008, Mori et al 2005 and Schmidt et al 2003) and *in vitro* (Abe 2001). Serum amyloid P (SAP) seems to be an inhibitor of fibrocyte differentiation and is currently being developed for IPF treatment (Dillingh et al 2013).,More knowledge about fibrocyte biology *in vivo* is still needed before they can be confirmed as a major treatment target. However, the concept is clearly supported by studies, which have clearly shown that more fibrocytes are worse for lung injury (Moeller et al 2009, Andersson-Sjoland et al 2008).

Fibrocytes as a prospective biomarker

The clinical management of IPF remains a major challenge not only due to limited drugs available to treat the disease, but also lack of good indicators for disease progression. The identification of suitable biomarkers is an important task and would help as predictors of prognosis and possibly treatment response (Maher 2013). The National Institute of Health defines a biomarker as any characteristic that can be objectively measured and act as an indicator for the different processes that occur in the human body (Biomarkers Definitions Working Group, 2001). An ideal biomarker must be objectively measured, reproducible, easily detected and inexpensive. High sensitivity and specificity combined with the ability to predict clinical changes in disease are key properties that candidate biomarkers should fulfill (Tunceroglu et al 2013). Exciting developments in

molecular biology have opened new approaches for biomarker discovery, but circulating blood cells remain an attractive target. For fibrotic disorders, these include circulating fibrocytes.

There have been several important studies investigating the potential of fibrocytes as a biomarker of disease activity in fibrotic lung disease (table 2). Work by our group (Moeller et al 2009) and others (Strieter, Keeley et al 2009), (LaPar et al 2011),(Fujiwara et al 2012) has shown that circulating fibrocytes may have the ability to predict progression of lung fibrosis. In our study, the survival of IPF patients with circulating fibrocytes higher than 5% of total blood leukocytes was 7.5 months compared to 27 months for patients with less than 5% fibrocytes (Moeller et al 2009). Importantly, the predictive value of fibrocytes was independent of other outcomes, such as forced vital capacity (FVC) or diffusing capacity of the lungs for carbon monoxide (DLCO). This observation was recently confirmed in a smaller single center study in Japan (Fujiwara et al 2012). Another study showed correlation between numbers of circulating fibrocytes and the development of bronchiolitis obliterans syndrome after lung transplantation (LaPar et al 2011). Before these promising, yet preliminary studies can assist in the management of IPF patients or find use as outcome for clinical trials, they have to be confirmed and validated in larger multicenter trials. Some of these important studies are currently recruiting and will hopefully provide some answers to these open questions in the near future (PROFILE- Prospective Observation of

Fibrosis in the Lung Clinical Endpoints; NCT01110694and NCT01134822; Maher 2013).

Summary

Fibrocytes are bone marrow derived, circulating mesenchymal progenitor cells, which play a role in several fibrotic disorders, including IPF. They are attracted by tissue-derived chemokines, which are yet to be confirmed. It is likely that they play a detrimental role in tissue homeostasis and promote fibrosis, although even this paradigm needs further confirmation. Whether or not fibrocytes are attractive treatment targets in IPF remains to be proven. Fibrocytes may have some predictive value as biomarker for IPF and other diseases, but the promising preliminary data still requires independent and larger scale validation. Several major challenges remain in this field, which include poor understanding of fibrocyte variability in health and disease and the fact that many researchers use different panels of markers to identify fibrocytes. Some consistency would be useful to put the large amount of information in the fibrocyte literature into a common context. Despite these issues, it has become clear that mesenchymal cells and their progenitors are more than incidental finding in lung injury and repair and hold promise for the future.

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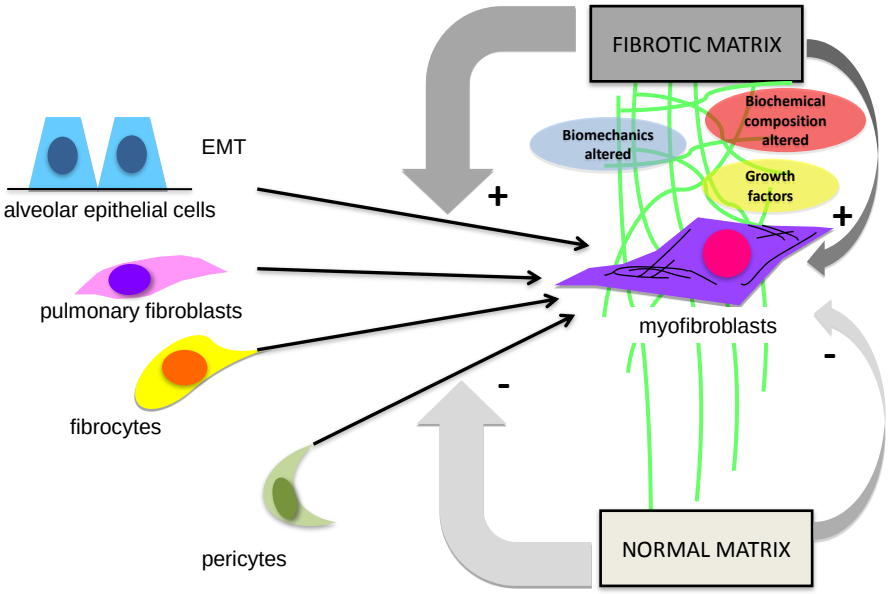

Figure 1: Proposed cellular sources for fibroblast foci and the fibrotic matrix. Several fibroblast progenitor cells, particularly fibrocytes, alveolar epithelial cell (via EMT), resident fibroblasts and pericytes can differentiate into myofibroblasts and thereby contribute to fibroblast focus formation. It is possible that the altered biochemical composition of fibrotic matrix has a profound impact on this differentiation process and facilitates the accumulation and persistence of fibrogenic cell phenotypes.

Chemokine	Location identified	Receptor	Reference
CCL 12	Lung	CCR2	Moore et al 2006
CXCL 12	Lung	CXCR4	Phillips et al 2004
SLC	Skin, Kidney	CCR7	Abe et al 2001, Sakai et al 2006

Table 1: Fibrocyte recruitment: pivotal studies on chemokines and their respective receptors which are involved in fibrocyte migration in vivo (SLC: Secondary lymphoid chemokine)

Sample	Outcome	Reference
Blood	"Quantification of circulating fibrocytes may allow prediction of early mortality in patients with IPF"	Moeller et al 2009
Blood	"Increased circulating fibrocyte levels correlate with the development of BOS after lung transplantation"	LaPar et al 2011
Blood	"Number of circulating fibrocytes was significantly increased in all patients with ILD"	Fujiwara et al 2012
Blood	"Fibrocytes are associated with the presence and extent of left ventricular hypertrophy in patients with hypertensive heart disease"	Keeley et al 2012
Blood	PROFILE study ongoing	Maher 2013
BAL	"BAL >6% provides an additive prognostic value to clinical predictors and may be useful to identify patients with acute lung injury and acute respiratory distress syndrome at highest risk of an adverse outcome"	Quesnel et al 2012
BAL	"Fibrocytes were detected in BAL fluid in about half of the patients with IPF and SSc-ILD. Their number was associated with less severe disease in IPF patients"	Borie et al 2013

Table 2: Fibrocytes as biomarker: Examples of recent studies where fibrocytes have been detected in heart and lung disease (BAL: bronchoalveolar lavage, IPF: Idiopathic Pulmonary Fibrosis, BOS: bronchiolitis obliterans, ILD: Interstitial lung disease, SSc-ILD: Systemic Sclerosis-ILD)

Appendix 2 Fibrocytes in chronic lung disease – facts and controversies (accepted Pulmonary Pharmacology and Therapeutics September 2011)

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Abstract

Fibrocytes are bone marrow-derived mesenchymal cell precursors, defined primarily by their ability to co-express markers of both hematopoietic (e.g. CD45 or CXCR4) and stromal (e.g. collagen) lineages. Fibrocytes in culture also have ultrastructural cell surface features that distinguish them from other leukocytes. Extensive efforts have helped to characterise fibrocytes phenotypically and functionally, but it is still unclear exactly how these cells contribute to tissue repair and/or pathologic fibrosis. Nevertheless, the varied levels of fibrocytes in blood have raised considerable interest as a biomarker of disease activity, such as chronic lung diseases, including pulmonary fibrosis, asthma and pulmonary hypertension. These cells also may become a novel therapeutic target for these difficult to treat disorders. This review will briefly summarize the current knowledge about fibrocytes in human lung disease and in animal disease models and highlight areas of consensus as well as issues that remain controversial to date.

Introduction

Fibrocytes are circulating mesenchymal cell precursors, derived from bone marrow and first identified by Bucala et al[1] in 1994, studying the biology of wound repair and determining which factors were contributing to the development and accumulation of fibroblasts. Since this initial discovery, additional studies have contributed to the characterization of fibrocytes, with respect to their morphology, growth characteristics in vitro, their biological roles in vivo, and their potential utility as a biomarker and/or treatment target in human disease.

Despite extensive efforts, the role and functions of this cell in tissue repair and pathobiology of chronic tissue remodeling are still unclear. In the current literature, there are discrepancies concerning the ability to culture fibrocytes, their detection in the peripheral blood and their mechanistic role in disease, most likely related to different methodologies and variable disease stages and animal models that are used to investigate the fibrocyte. In the last few years there have been a number of excellent reviews on the subject of fibrocytes highlighting the current knowledge about these cells. This review will focus on what has been consistently shown about fibrocytes in humans and some of the areas that still require further attention and scientific work.

Multiple markers are used to identify fibrocytes

Fibrocytes are circulating bone marrow-derived mesenchymal progenitor cells [2 3 4 5] co-expressing hematopoietic stem cell markers, monocyte lineage markers and fibroblast products [6 7]. Fibrocytes, like some dendritic cell subsets, are most likely derived from precursors of the monocyte lineage, supported by the observation that they express the major histocompatibility complex class I and class II and the co-stimulatory molecules CD80 and CD86 [1 6 8]. Current evidence also shows that they may be present in a subset of CD14+ CD16- monocytes that are positive for the chemokine receptor CCR2[9 10]. However, other studies debate this hypothesis and it remains to be determined whether they are truly derived from a CD14+ progenitor cell[11]. Regardless, it is widely accepted that mature fibrocytes express markers of both hematopoietic cells (CD34, CD43, CD45, LSP-1) and stromal cells (collagen I and III)[1 12 13 14]. Fibrocytes lack lymphocyte markers such as CD3, CD4, CD8, CD19 and CD25[1], and express the chemokine receptors CCR2, CCR7, and CXCR4, which may regulate their entry into inflammatory lesions [12 15 16]. The fibrocyte is a distinct cell and its cell surface features have been studied using FACS analysis, immunohistochemistry and also histomorphology. On scanning EM, fibrocytes in culture exhibit prominent cell surface projections, making them quite distinct from the appearance of other leukocytes[1].

Recent work of Pilling et al[17] highlight the rapidly evolving information about the fibrocyte. This study, observing various markers on fibrocytes in culture, questioned the completeness of the current definition of a circulating fibrocyte [18]

[19] as being a col-1 positive, CD45 positive cell. They found that at different stages in the cell lineage there appears to be a loss of CD45 positivity and speculated that by looking at only these two markers one might not get a definitive count of fibrocyte numbers. However, there is sufficient evidence that at a given point in time a CD45/col-1 positive mononuclear cell is a circulating fibrocyte in some stage of differentiation, and this marker combination seems to be practical and has been used for characterization in most studies. The seemingly confusing or even controversial details about the identity of fibrocytes indicates they undergo constant changes on their way from the bone marrow to sites of injury and manifest phenotypic and functional differences. These differences may result in a heterogeneous group of cells which can then be divided into different subsets as discussed in following sections.

Fibrocytes demonstrate phenotype plasticity

At present there is no single specific marker for fibrocytes (see figure 1). This may be due to the apparent plasticity and constant change of these cells and may explain some of the difficulties in tracking them. It is, however, believed that human fibrocytes in circulation probably represent an obligate intermediate stage of differentiation of one of the precursors of the monocyte lineage into mature fibroblasts and myofibroblasts at the tissue sites[14]. While many studies support the notion that fibrocytes differentiate into myofibroblasts[7 20 21],it has to be

acknowledged that most of this evidence is based on in vitro experiments[12] and that a firm proof of fibrocyte to myofibroblast differentiation in vivo has not been provided. The controversy of myofibroblast origins has recently been highlighted by reviewing the situation in kidney fibrosis, where fibrocytes and EMT has been studied years before lung researchers did the same in pulmonary fibrosis[22].

The combination of intracellular collagen staining and the surface expression of CD45 or LSP-1, plus either CD34 or CXCR4, has been considered as sufficient to discriminate fibrocytes from leukocytes, dendritic cells, endothelial cells and tissue-resident fibroblasts in vitro and in vivo. The presence of the hematopoietic stem cell antigen CD34 on fibrocytes isolated from circulation confirms their bone marrow origin[1]. The chemokine receptor ligand pair CXCR4-CXCL12 has been implicated to play a significant role in homing of fibrocytes to areas of tissue injury[18] and recent evidence[23] has shown that local apoptosis may be a possible contributor to the accumulation of fibrocytes. The latter finding is significant and should be explored further as it may provide critical information about the local cellular environment that attracts fibrocytes. The expression of CXCR4 and collagen is regarded as positive identification for fibrocytes when these cells are found in the tissue. It may not be wise to view fibrocytes in a static manner but rather see them as a cell in transit, whose differentiation is highly influenced by local conditions. Despite single studies which described ‘end stage’ tissue fibrocytes as CD34+ and spindle shaped[24], several groups have shown that the

progressive loss of CD34 and, eventually, also CD45 on fibrocytes appears to occur when they enter the tissue[13 14 21 25]. In early cell culture, fibrocytes are associated with expression of CD34, CD45, collagen I and vimentin; later, and especially after exposure to TGF-β or endothelin, fibrocytes seem to differentiate into myofibroblast-type cells which express α-smooth muscle actin and lose CD34 and CD45[7 18 26].

This apparent plasticity makes the fibrocyte population rather inhomogeneous, and we view a fibrocyte as a cell that is always in transition. As such it is currently difficult to identify a fibrocyte as a super-specialised cell by all its known markers in all patients at all times. We would benefit from a common agreement on which markers are reliable in what environment and at what time, to provide validity to all findings and a basis for possible division of these cells into appropriate sub-types.

Fibrocyte isolation and culture

Whilst identification and quantification of fibrocytes in peripheral blood is relatively straight forward, depending on the marker panel that is used, the isolation and culturing of these cells remains difficult. There are several protocols published, and most groups seem to follow the original method developed by Bucala et al[8 27] using RPMI- or DMEM-based culture media, supplemented with 10–20% fetal bovine serum. Modifications to this protocol have been proposed by Pilling et al[28]

to change some important variables, such as blood collection methods, optimal seeding cell density, culture substrates and media which contribute to the growth rate and final cell density. Fibrocytes grown from peripheral blood do not easily reach confluence. This definitely makes them different from fibroblasts, but it is possible with optimization of attachment using different culture surfaces and cell numbers to have a robust growth rate. The ideal growth conditions and possible progression in the phenotype of fibrocytes, from one subset to another, still need to be established.

Potential roles of fibrocytes in tissue repair

The putative roles for fibrocytes in tissue injury and repair vary between the organ systems and experimental models studied. They are found in different models of tissue repair and experimental fibrosis including wounded skin, asthma, pulmonary vascular remodelling, and lung and liver fibrosis[2 16 28 29 30 31]. Fibrocytes can be detected in human fibrotic disorders including idiopathic pulmonary fibrosis (IPF), chronic asthma, nephrogenic fibrosing dermopathy, scleroderma and cutaneous scars[31 32 33 34]. On a functional level, fibrocytes have been proposed as early responders to injury and due to their ability to function as antigen presenting cells[8], fibrocytes have also been implicated in antigen specific immunity. The following discussion will highlight their role as a mediator of tissue

repair and fibrosis and show evidence that their differentiation may be influenced by cytokines within the local micro-environment of tissue injury[35].

The pathobiology of normal wound repair and pathological tissue fibrosis are very complex with myofibroblasts playing a central role in both. In particular, in lung and airway fibrosis, there are at least three different concepts of how myofibroblasts develop in tissue. These cells could arise from: (1) Resident tissue specific precursors: through the stimulation of resident fibroblasts by local profibrotic factors[36]. This theory is supported by the fact that cultured fibroblasts *in vitro* can be induced to differentiate into myofibroblasts by treatment with cytokines, such as TGF-β and interleukin (IL)-4[37 38]. (2) Epithelial to mesenchymal transition (EMT) in response to injury: it is proposed that epithelial cells undergo major morphological changes, losing epithelial characteristics such as polarity and the expression of junctional markers, while starting to express fibroblast markers[34]. (3) Circulating bone marrow-derived progenitors: the hypothesis is that circulating fibrocytes home and extravasate into sites of tissue injury, such as the lung, differentiate into fibroblasts/myofibroblasts and contribute to the accumulation of extracellular matrix components (ECM) during the fibroproliferative process in response to injury[1 31 39 40].

At the time of tissue injury, a signal is generated that triggers the release of fibrocytes from the bone marrow into the peripheral blood, where they can migrate to inflamed sites, likely via a CCR2-mediated pathway [9] [10]. As an example, intravenously injected fibrocytes have been shown, in murine skin wounds, to exit the circulation at sites of injury and contribute to the formulation of granulomas, scars and remodeled tissue and localize to areas of ongoing ECM deposition [12]. Lung injury with subsequent fibrosis is typically studied using intratracheally administered irritants, such as bleomycin, asbestos, silica or other substances. Using one of these approaches, Moore et al[16] described the emergence of CD45+ CD13+ fibrocytes, expressing collagen I gene and protein, in cultures of minced lung tissue or bronchoalveolar lavage cells from mice treated by intratracheal instillation of an irritant, fluorescein isothiocyanate (FITC). Others have demonstrated the importance of the ligands for the known surface receptors CXCR4 (ligand CXCL12)[18] and CCR7 (ligand CCL21)[13] in the accumulation of fibrocytes in bleomycin-induced pulmonary fibrosis in mice. These findings strongly support the concept that injured tissue derived factors are able to attract circulating fibrocytes into the tissue, where they presumably participate in the repair and/or fibrotic processes. Data from animal work was supported by reports of increased levels of CXCL12 in the lung and plasma of patients with Idiopathic Pulmonary Fibrosis (IPF)[41] which correlated with numbers of circulating fibrocytes, an association that has previously also been found in animals [18].

It remains difficult to unequivocally confirm that fibrocytes found in the tissue originated from the bone marrow, having migrated to the site of injury via the circulation. However, there is accumulating evidence in support of fibrocytes directly contributing to the new population of fibroblasts and myofibroblasts that emerge in injured tissue during normal [1 2 12] or aberrant[42] wound healing, in ischemic, inflammatory or fibrotic processes [4 5 7]. Experimental studies *in vitro* showed that fibrocytes isolated from the circulating blood can differentiate into α-SMA expressing cells and exhibit contractile forces after stimulation with TGF-β [14]. Fibrocytes are also believed to be involved in remodeling by the production of extracellular matrix proteins (collagen I, collagen III and vimentin)[43].

In addition to these direct effects, fibrocytes likely participate indirectly in the process of injury and repair. Work by Hartlapp et al[44] showed that when stimulated *in vitro* by IL-1β, fibrocytes secret proinflammatory cytokines (TNF-α, IL-6, IL-8, IL-10, MIP-1 α/β) and various metalloproteinases. Fibrocytes are also an important cellular source of chemokines (MIP-1α, MIP-1ß, MCP-1, IL-8, and GROα[45]) and growth factors (VEGF, PDGF-A, M-CSF, HGF, GM-CSF, b-FGF, CNTGF[43]) and thereby contribute to autocrine and paracrine signals within the microenvironment[44 45 46 47].

Fibrocytes are present in a variety of chronic lung diseases

There is evidence suggesting that fibrocytes contribute to lung remodelling at some stage[48 49]. The presence of fibrocytes has been reported in the circulation and tissue of a variety of lung disorders in humans, e.g. in IPF, chronic asthma, and scleroderma (see table 1). While there is no publication of a "normal" number of fibrocytes in tissue, there is some understanding of the numbers that can be expected in the circulation. Using the marker combination CD45+/Col-1 positivity, Bucala estimated [1] that fibrocytes constitute approximately 0.5% of circulating leukocytes, which has been confirmed by several other groups including our own[19 35]. In disease, the numbers may be as high as 20% of circulating leucocytes[19]. We found that patients with IPF had an increased percentage of circulating CD45+/Col-1 positive fibrocytes in their blood during stable periods of the disease and even more during acute exacerbations. The percentage of fibrocytes at diagnosis was an independent predictor of subsequent mortality in patients with IPF [19]. Interestingly in this same study, patients with acute respiratory distress syndrome (ARDS) did not show an elevation of fibrocytes, indicating a specific role for these cells in the active fibrogenesis seen in IPF and not merely a reflection of tissue injury per se.

Fibrocytes (CXCR4/Prolyl-4-hydroxylase positive cells by immunohistochemistry) have been positively identified in the lung tissue of patients with IPF[20] and by

using markers for CD34 and collagen in the airway of asthma patients[7]. Preliminary work by our group have identified fibrocytes in the lungs of Cystic Fibrosis (CF) patients (unpublished data), but they occur at a much lower number than that found in IPF. This is consistent with reports of Durieu et al[50] who showed a relative lack of myofibroblasts in CF lungs and speculated that fibrogenesis in CF lung disease may even be abnormally low.

Currently there are more than 20 clinical trials ongoing for new and reformulated drug therapies in IPF. Amongst those are at least two that target processes involving fibrocytes: humanised monoclonal antibody against CCL2 and recombinant serum amyloid P, are interventions which are currently in Phase II and Phase I clinical trials respectively[51]. Whilst CCL2 is one of the putative homing factors for fibrocytes and as such a treatment target, serum amyloid P seems to inhibit the recruitment of fibrogenic cells (including fibrocytes) into the tissue and therefore is used as a drug itself.

Chronic asthma is another lung disease in which fibrocytes seem to play an important role. Spindle shaped cells co-expressing CD34 and procollagen-I mRNA (presumably fibrocytes) have been found close to areas of new extracellular matrix deposition beneath the epithelial basement membrane and their presence has been linked to the emerging population of new α-SMA positive myofibroblast-like cells[7]. Another more recent study, performed on patients with

mild asthma, found a possible link with the density of fibrocytes (CD34/CD45RO/α-SMA positive cells) in the bronchial mucosa beneath the basal membrane and the thickness of the lamina reticularis[33]. Saunders et al[52] showed increased numbers of fibrocytes (collagen-1/CD34 positive) in the blood of patients with refractory asthma compared to healthy controls; unfortunately they did not correlate this with lung function in these patients. In the same study this group also showed that *in vitro* airway smooth muscle promoted fibrocyte chemotaxis, in part by release of platelet-derived growth factor (PDGF).

To our knowledge to date, there have been no human studies examining fibrocytes in the vascular remodelling seen in Pulmonary Hypertension (PH), but it is plausible that they play some role in this process, similar to remodelling disorders of the airways (asthma) and the parenchyma (IPF). In a set of experiments using different animal models of PH (rats and bovine), investigators showed a significant accumulation of fibrocytes in the adventitia and media of remodelled pulmonary arteries[29]. In the rat model, it was further shown that sustained hypoxia leads to the development of a complex pulmonary artery specific pro-inflammatory microenvironment that promotes recruitment, retention and differentiation of circulating fibrocytes to the pulmonary vascular compartment[53]. The potential of fibrocytes as a novel drug target for PH has been elegantly discussed in a recent editorial by Stenmark and colleagues[54]. This editorial was based on a study demonstrating the involvement of fibrocytes in a

chronic hypoxia model of PH in mice[55], in which the investigators were able to modulate the recruitment of fibrocytes to the vessel wall with a stable prostacyclin analogue that is approved for the treatment of PH.

Mathai et al[56] reported that the blood of patients, with Systemic Sclerosis and associated interstitial lung disease, contained increased numbers of fibrocytes and collagen-producing monocytes when compared with healthy age-matched controls. Pilling et al[15] suggested that increased numbers of fibrocytes found in scleroderma patients may possibly be due to them having a lowered level of serum amyloid P (SAP), an inhibitor of fibrocyte differentiation. As mentioned above, there is a clinical trial underway examining the potential of SAP as antifibrotic drug for IPF.

While the presence of fibrocytes has been mostly characterised thus far in diseases of chronic inury and repair, it is still unknown what role they may play in acute lung injury. Work from our group[19] showed no difference in circulating fibrocytes between patients with ARDS and healthy persons. However, recent studies[57] suggested that the presence of fibrocytes in BAL might be associated with a poor prognosis in acute lung injury.

Summary and outlook

The study of fibrocytes is an exciting new topic in the area of injury, repair and fibrosis in many organs and tissues. The cells themselves seem to be as dynamic as the entire field of research. Sometimes the promise of a new discovery runs ahead of the actual facts and knowledge, which may be the case for fibrocytes. Some of the current "facts" are: (1) Fibrocytes are versatile and multipotent mesenchymal cell precursors found in the peripheral blood. (2) Cells that look like fibrocytes can be detected in healing wounds, scars and fibrotic tissue. (3) Fibrocytes in cell culture are able to produce a wide variety of cytokines, chemokines and matrix components. There is still some inconsistency about the exact definition of fibrocytes in the blood and the tissue, largely due to the dynamic nature of these cells, having the apparent ability to change phenotype and function throughout their migration from the bone marrow to the injured tissue. The big question *"do fibrocytes contribute to worsening or improving tissue repair?"* is still unclear, although accumulating evidence[19 20 29 452 53 56] suggests that they are detrimental to repair and contribute to fibrosis rather than being beneficial. They possibly represent "the wrong cell at the wrong time". Moving forward one must be prepared for the need to categorize fibrocytes as functionally different depending on the conditions in which they are isolated. Markers and methodologies used to perform these isolations need to be standardized for valid comparisons and characterization of these cells. Regardless of the many open questions, research on fibrocytes in injury and

repair, particularly in the lung parenchyma, airway walls and pulmonary vessels, is a very active field and holds promise to better understand fibrogenesis and develop novel biomarkers and new treatment targets.

Appendix

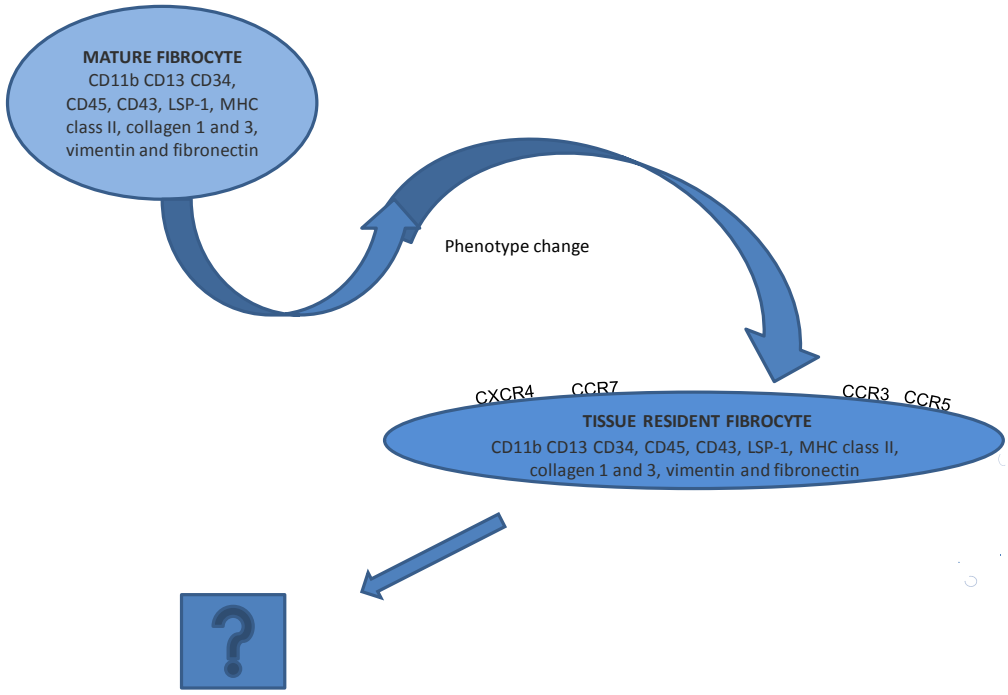

Figure 1: Phenotypical characteristics of fibrocytes isolated from humans during maturation and migration to tissue

Disease	**Location**	**Identification Markers**
Chronic Asthma	Airway	CD34 and pro-collagen 1a[7]
Idiopathic Pulmonary Fibrosis	Lung tissue, adjacent to areas of fibroblastic foci	CXCR4 and Prolyl-4-hydroxylase[20]
Nephrogenic Systemic Fibrosis	Dermis	CD34 and pro-collagen[32]
Chronic Kidney Disease	Renal Interstitium	CD34 and morphology[24]
Scleroderma	Dermis	CD34 and morphology[58]

Table 1: Human diseases in which tissue fibocytes have been implicated

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