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CONTRIBUTION OF INTERLEUKIN 6 TRANS SIGNALING IN PULMONARY FIBROSIS

Thuy T. Le

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By

THUY THANH LE, B.A.

APPROVED:

Michael R. Blackburn, Ph.D.

Supervisory Professor

Russell Broaddus, M.D., Ph.D.

Yang Xia, M.D., Ph.D.

Sandeep Agarwal, M.D., Ph.D.

Richard Johnston, Ph.D.

APPROVED:

Michael R. Blackburn, Ph.D.   Michelle Craig Barton, Ph.D.

Deans, The University of Texas

Graduate School of Biomedical Sciences at Houston

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A

DISSERTATION

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DOCTOR OF PHILOSOPHY

By

THUY THANH LE, B.A.

Houston, Texas

May 2014

DEDICATION

This thesis is dedicated in loving gratitude to…

The best parents I could have ever asked for –

My father Mr. MINH THANH LE

and

My mother Mrs. THACH KIM LE

ACKNOWLEDGEMENTS

Finally, I have been extremely loved and spoiled by my family. My older sister Thao and my brother-in-law Nghi have been like a second set of parents to me. Thank you for always thinking about my needs, for your constant advice and for providing the financial means for me to further my education and pursue my dreams. Bao, what an amazing man you've grown up to be! You make me aspire to be more like you, which is highly unusual since you're younger than me. Truc, my favorite little sister and best pal, what can I say? So much beauty, intelligence and

love wrapped up in that tiny little body of yours. You could always cheer me up, even on the worst days. I owe you for life and you know why! Vinh Nguyen, love of my life, light of my life, thank you for all the joy you bring into my life. I look forward to being loved and spoiled even more by you in the coming years and to whatever life brings with you by my side.

Saving the best for last, though there are few words that could fully describe my gratitude to my parents, I will try. Dad, the strongest man I know, your strength of character, perseverance through hardships and steadfast faith have inspired me to push through all obstacles and reach for the stars. Mom, your love and tender care have supported me throughout my whole life. You are the epitomy of a model wife and mother, and I hope I can be just half as wonderful as you.

Mom and Dad, you are exemplars of faith, fidelity and unconditional love. You are the reasons I am here today. I could never thank you enough or repay all you have done for me, but I hope I have, in some small way, made you proud and shown you that the love and sacrifices you've made for me were not in vain.

CONTRIBUTION OF INTERLEUKIN 6 *TRANS* SIGNALING

IN PULMONARY FIBROSIS

THUY THANH LE, B.A.

Supervisory Professor: MICHAEL R. BLACKBURN, Ph.D.

Idiopathic Pulmonary Fibrosis (IPF) is a lethal lung disease with progressive fibrosis and death within 2-3 years of diagnosis. IPF incidence and prevalence rates are increasing annually, and because the pathogenesis is unknown, there are no effective treatments available. Inhibition of interleukin 6 (IL-6) results in the attenuation of pulmonary fibrosis in mice. It is unclear whether this is due to blockade of classical signaling, mediated by membrane-bound IL-6 receptor alpha (mIL-6Rα), or trans signaling, mediated by soluble IL-6Rα (sIL-6Rα). Our study assessed the role of sL-6Rα in IPF. We demonstrated elevations of sIL-6Rα in IPF patients and in mice during the onset and progression of fibrosis and showed that protease-mediated cleavage was important in production of sL-6Rα in fibrotic lungs. *In vivo* neutralization of sIL-6Rα, and resulting antagonism of IL-6 trans signaling, attenuated pulmonary fibrosis in mice. Decreases in sIL-6Rα were associated with reductions in myofibroblasts, fibronectin and collagen. *In vitro* activation of IL-6 trans signaling enhanced fibroblast proliferation and extracellular matrix protein production, effects relevant in the progression of pulmonary fibrosis. These findings suggest that IL-6 trans signaling influences events crucial in pulmonary fibrosis *in vivo*.

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LIST OF ABBREVIATIONS

IPF	Idiopathic Pulmonary Fibrosis
IL-6	interleukin 6
mIL-6Rα	membrane interleukin-6 receptor alpha
sIL-6Rα	soluble interleukin-6 receptor alpha
JAK	Janus kinase
STAT3	signal transducer and activator of transcription 3
IPB	intraperitoneal bleomycin
ADA	adenosine deaminase
ADAM17	a disintegrin and metalloprotease 17
TGF-β	transforming growth factor beta

CHAPTER 1

Introduction

IDIOPATHIC PULMONARY FIBROSIS

Definition and Epidemiology

Idiopathic Pulmonary Fibrosis (IPF) is a lethal lung disease of unknown etiology and defined by chronic, progressive, irreversible interstitial fibrosis limited to the lungs(1, 2). It is the most common form of idiopathic interstitial pneumonia (IIP), affecting older adults, with age at diagnosis ranging from 55-75 years-old, median age of 66 years-old(2). Incidence increases with increasing age, and a diagnosis in adults less than 50-years old is uncommon. IPF is more prevalent in males than females (1.5-1.7:1)(1, 2). Recent estimates place the number of affected individuals around 128,000 (with some reports of prevalence as high as 200,000) in the USA and around 8 million worldwide. It is expected that 50,000 new IPF cases will be diagnosed each year, with that number rising annually(1-3). Over the last two decades, there has been an upward trend in incidence rates(3), emphasizing the increasing impact of this disease on the general population.

Presentation & Diagnosis:

Patients commonly present clinically with progressive dyspnea on exertion and a chronic, non-productive cough(1, 2). Other symptoms can include digit clubbing and bibasilar inspiratory crackles. Diagnosis is made based on presenting symptoms, evidence of a usual interstitial pneumonia (UIP) pattern on histology and imaging, and exclusion of other interstitial lung diseases(1, 2). UIP pattern on histology includes: a spatial and temporal heterogeneity in parenchymal involvement, appearance of fibrosis and honeycombing that is mainly subpleural and paraseptal, presence of fibroblastic foci, and absence of features suggesting other conditions(1, 2). UIP pattern on imaging includes: subpleural and basal involvement,

honeycombing with or without traction bronchiectasis, and reticular abnormalities(1, 2). Honeycombing refers to presence of airspaces that are cystic in appearance, lined with bronchiolar epithelium and may contain inflammatory cells and mucin.

IPF can be broken up into sporadic and familial forms. Familial IPF, which accounts for <5% (0.5-3.7%) of IPF cases, is indistinguishable clinically and histologically from sporadic IPF, though they may have different genetic patterns and can present at a younger age(1, 2). The genetic mode of transmission of familial IPF is most likely autosomal dominant with variable or reduced penetrance(1, 2).

Cause(s) and Risk Factors:

As its name indicates, the cause or causes of IPF remain unidentified, though potential risk factors include genetics, lifestyle choices, environmental or occupational exposures and co-morbid diseases.[2-3]

Genetic factors: Studies have suggested that the gene ELMOD2 (located on chromosome 4q31), which has unknown functions, may confer susceptibility to familial IPF(1). Mutations in human telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR), leading to telomere shortening and cellular apoptosis, have been linked to familial and sporadic IPF cases(1, 4). Mutations in surfactant protein C and A2 (SFTPC and SFTPA2) have been associated with familial IPF(1, 4). Mutations in SFTPC have not been found in sporadic IPF. SFTPA2 mutations are also associated with lung cancer(1).

Quite a number of genetic polymorphisms has been reported to be increased in sporadic IPF and suggested to be involved in disease progression. The polymorphisms occurred in genes encoding enzymes (α_1-antitrypsin, angiotensin converting enzyme), cytokines (interleukins 1α, 4,

6, 8, 10 and 12, tumor necrosis factor α, lymphotoxin α), fibrotic mediators (transforming growth factor $β_1$), immune modulators (complement receptor 1, NOD2/CARD15), coagulation pathway elements (plasminogen activator inhibitors 1 & 2), matrix metalloproteinase 1 (MMP-1), and surfactant proteins A and B (SFTPA, SFTPB)(1). Associations have also been made between human leukocyte antigen class I and II (HLA-I, HLA-II) haplotypes and MHC class I chain-related gene A (MICA) and IPF(1). A polymorphism in the promoter of the mucin gene MUC5B has been associated with familial and sporadic IPF(5).

Lifestyle choices: Cigarette smoking is a significant risk factor in both familial and sporadic IPF. There is a strong association between moking history of >20 pack-years and development of IPF(1).

Environmental factors: Exposures to wood dust, like pine, and metal dusts, like lead, steel and brass, significantly elevates risk of IPF(1). Hairdressers, farmers, and stone cutters also experience increased risk, as does anyone with exposure to birds, livestock, animal dust and vegetable dust(1).

Co-morbid conditions: Investigations into the involvement of viral infections as risk factors revealed associations between IPF and Epstein-Barr virus, hepatitis C, and a few herpes viruses(1). There were positive and negative associations studies reported with Epstein-Barr virus (EBV), cytomegalovirus (CMV), and human herpesvirus 7 and 8 (HHV-7 & HHV-8). Hepatitis C had variable associations, and elevated CMV antibodies have been reported in IPF(1).

Gastroesophageal reflux (GER) abnormalities is common in IPF patients and has long been thought to be a risk factor. Erosive esophagitis from GER was associated with development of pulmonary fibrosis(1). Diabetes mellitus, obesity, obstructive sleep apnea, and coronary artery

disease may play a role in IPF risk but that still remains to be determined(1, 2). There are many confounding factors involved in reports of risk factors, so interpretation must be taken cautiously(1).

Natural history & Clinical Phenotypes in IPF

The natural history of IPF is highly variable and unpredictable(1, 2). IPF was once thought to simply be a slowly progressive disease, but over the years, there is increasing awareness of the heterogeneity of the disease and discussion has revolved around possible distinct IPF phenotypes that have may have genotypic associations and clinical features that affect prognosis in terms of disease progression and mortality risks(2, 6).

Some patients with IPF have stable disease. Some have rapidly progressive disease. Others exhibit gradual decline in pulmonary function over time(1, 2, 6). Some have co-morbid conditions, including pulmonary hypertension and emphysema. All are at risk of possible episodes of acute respiratory worsening, known as acute exacerbations(1, 2).

Subclinical IPF: Ley et al have defined various categories of disease in IPF, including subclinical IPF, which refers to the period before diagnosis(7). Patients with IPF will typically have symptoms prior to diagnosis for a median of 1-2 years. Prior to symptoms, however, there may also be radiographic evidence of disease(7). These patients can progress from asymptomatic to symptomatic disease over years or decades. This period of subclinical disease is not well understood(7). Asymptomatic pulmonary fibrosis is now better reported because it has been identified in family members of IPF patients, particularly individuals with smoking history. The lungs of these individuals show histologic evidence of various subtypes of interstitial lung disease (ILD)(7). High-resolution CT (HRCT) is the best modality to identify asymptomatic,

subclinical IPF, though it is not yet known how to manage subclinical individuals(7). They may be at higher risk for developing acute exacerbations after procedures(7).

Stable or slowly progressive IPF: Once diagnosed, some IPF patients will have stable or slowly progressing disease(1, 2). These patients have usually presented with progressive dyspnea on exertion and cough associated with decreased lung capacities and volumes(1, 2). The rate of decline in these patients is 0.13-0.21 L in forced vital capacity (FVC)(2, 7).

Rapidly progressive IPF: IPF that is rapidly progressing typically occurs in males who are smokers(2, 6, 8). At diagnosis, these patients have similar lung capacities and volumes, and similar imaging and histological findings to stable or slowly progressive IPF patients(2). Despite the similarities, rapidly progressive IPF differ in clinical course and transcriptional profile. This accelerated version of IPF is associated with shortened survival(2, 6, 8). There is evidence of transcriptional upregulation in pathways that affect alveolar epithelial and mesenchymal functions(2). Boon et al reported upregulation in genes involved in the MAPK-EGR1-HSP70 pathway, a pathway known to regulate inflammation induced by smoking(9). Selman et al reported overexpression of genes involved in migration/proliferation, oxidative stress, morphogenesis, and fibroblast and smooth muscle cell genes along(8).

Acute Exacerbations: Acute exacerbations (AE) are defined by several measures. There must be an abrupt decline in respiratory function, as evident by worsening dyspnea (in a matter of days or weeks and less than 1 month) and worsening hypoxemia (decrease in arterial blood P_aO_2)(1, 2). This rapid deterioration is associated with new radiologic findings of diffuse alveolar damage (bilateral alveolar infiltrates and ground-glass opacities) on top of previous parenchymal involved(1, 2). Importantly, there is an absence of identifiable precipitating triggers. Other observed symptoms include fever, increased cough and increased production of sputum(1).

Acute exacerbations occur in about 5-20% of patients(1, 2). Prognosis is grim and mortality exceeds 60% with hospitalization(2). Survivors of AE have a greater than 90% mortality risk within 6 months. In about 27% of IPF AE cases, torque teno virus was detected. Pathogenesis of AE episodes are not known but excessive epithelial apoptosis and increased circulating fibrocytes have been reported(2). It is unclear whether these episodes of acute exacerbations had precipitating causes that were missed in diagnosis or if it is a reflection of progression in the inherent IPF disease processes(1).

Combined pulmonary fibrosis and hypertension: Pulmonary hypertension (PH) is a recognized complication of IPF(1). Though PH is typically seen in severe IPF, it is observed in some patients with earlier stages of IPF(6). It is defined as mean pulmonary artery pressure (mPAP) ≥25 mmHg at rest and ≥30 mmHg with exertion(6, 10, 11). Retrospective studies show prevalence of IPF patients with PH to be between 31-85%(6, 12-14). The wide range may be due to the fact that occurrence and severity of PH increases with time(15), so the number varies depending on the timing of analysis and the patient population.

A number of studies have shown associations between PH and increased mortality risk in IPF patients. Lettieri et al reported a correlation between PH (indicated by mPAP) and mortality risk(12). Higher mPAP was associated with increased mortality risk(12). Hamada et al indicated specifically that mPAP above 17 mmHg best indicates increased 5-year mortality rates(16). Nadrous et al reported an inverse correlation between PH and diffusing capacity of carbon monoxide (DL_{CO}) in IPF patients(13). They also showed that estimated systolic pulmonary artery pressure (sPAP) had a negative impact on survival, especially when higher than 50 mgHg(13). Mortality rates increase in more in the presence of pulmonary fibrosis, emphysema and pulmonary hypertension(6, 17).

Combined pulmonary fibrosis and emphysema: Recent retrospective studies place the prevalence of co-existing pulmonary fibrosis and emphysema in IPF patients around 28-55%, depending on the study population(6, 17, 18). Cottin et al first coined the name Combined Pulmonary Fibrosis and Emphysema (CPFE) in 2005 when they formally described clinical characteristics comprising this syndrome(18). Those characteristics included findings of dyspnea on exertion, basal crackles, finger clubbing, upper lobe emphysema and lower lobe pulmonary fibrosis on CT, abnormal spirometry, severe gas exchange impairment, and high prevalence of pulmonary hypertension(18).

Mejia et al also reported an association between CPFE and pulmonary arterial hypertension(17). In fact, they stratified pulmonary hypertension and further showed a positive correlation between PH and extent of emphysema in their study population(17). The presence of both pulmonary fibrosis and emphysema carries a poorer prognosis than IPF alone and mortality is even worse if pulmonary hypertension is also present(6, 17, 18). The prognosis is dismal with the combination of CPFE, severe pulmonary hypertension and severe pulmonary restriction(17). Severe PH was defined as estimated systolic pulmonary artery pressure (eSPAP) greater than 75 mmHg. Severe pulmonary restriction was defined as forced vital capacity (FVC) below 50%.

Mejia et al reported that CPFE is associated with male gender and smoking(17). Though the pathogenesis of CPFE is unclear, smoking appears to be a trigger(6, 18). Exposure to agrochemical compounds has also been suggested to be environmental risk factors(6, 19). As for genetic risk factors, it is not known if susceptibility to IPF and COPD may lead to CPFE or if CPFE may have its own distinct genetic variations(6).

Treatment of IPF

The latest published global recommendations regarding management of IPF was a joint statement released by the American, European, Japanese, and Latin American thoracic and respiratory societies in 2011. At the time, there was little evidence of effective pharmacologic therapies for IPF(1). The statement did report strong recommendations against use of: corticosteroids alone and in combination with immune modulators, colchicine, cyclosporine A, interferon γ 1b, bosentan, and etanercept(1). Therapies that received weak recommendations against (meaning it may be suitable in some patients) include: triple therapy (N-acetylcysteine, azathioprine, and prednisone), N-acetylecysteine (NAC) alone, anticoagulants and pirfenidone. Other recommendations included interventions to relieve symptoms in management of acute exacerbations and chronic disease, with treatment of pulmonary hypertension, use of long-term oxygen therapy and referral for lung transplantation in appropriate patients(1).

Pharmacologic options: Since the release of the joint statement, published outcomes from various clinical trials have raised a need to modify treatment recommendations(20, 21). Recommendations regarding the use of N-acetylcysteine monotherapy are expected to be updated in 2014, pending report of results from the PANTHER-IPF randomized controlled trial (RCT). NAC monotherapy was initially studied in the IPHIGENIA RCT. Results of IPHIGENIA, released in 2005, demonstrated a significant reduction in decline of forced vital capacity (FVC) and diffusion capacity with high-dose NAC(20, 21). The trial did not show survival benefit and was criticized for various reasons, so NAC monotherapy was further studied in the PANTHER-IPF RCT. The trial has concluded and final report is expected in 2014. In the meantime, because NAC therapy is relatively well tolerated, it is widely used for IPF(20, 21).

The PANTHER-IPF trial also tested efficacy of triple therapy (NAC, azathioprine, and prednisone) in IPF as one of the treatment groups. In October 2011, the trial terminated its triple therapy arm early when it became evident that triple therapy increased mortality, hospitalizations and adverse effects. Reports of the detrimental effects have since shifted IPF treatment emphasis away from immunosuppressants(20, 21). Another treatment option that also led to harmful effects in IPF patients is warfarin. The ACE-IPF RCT, looking at warfarin therapy in IPF, was terminated prematurely due to increased mortality. Most of the deaths in the warfarin group was attributed to disease progression and acute exacerbations, not to complications of warfarin, like bleeding(20). Ambrisentan, a type A endothelin receptor antagonist, was tested in the phase III RCT ARTEMIS. ARTEMIS was terminated prematurely due to increased disease progression and increased hospitalizations(21). Phase II testing of the efficacy of everolimus (MTOR inhibitor) was discontinued due to increased disease progression and increased acute exacerbations(21).

Therapies that have yielded no benefits in phase II RCT were imatinib mesylate (inhibitor of PDGF receptors, c-kit and c-ABL tyrosine kinases), macitentan (dual endothelin receptor antagonist) and tumor necrosis factor-α (TNF-α) antagonists(20, 21).

The good news, on the other hand, is that the pyridone analogue pirfenidone has been gaining recognition as a novel, effective therapy for IPF. Pirfenidone is thought to have anti-inflammatory, anti-fibrotic, and antioxidant effects(20-22). It is the first agent approved for treatment of mild to moderate IPF, currently available in Japan, Europe, Canada and India(20-22). It has not yet been approved in the USA despite a number of clinical trials showing efficacy and improvement in IPF patients. The first phase II RCT of pirfenidone was conducted in Japan and reported in 2005 by Azuma et al(20-22). The study was stopped early due to high numbers

of acute exacerbations in the placebo group, but they did show significant reduction in decline of FVC. Since then, three phase III RCT evaluating efficacy of pirfenidone have been completed and two were able to show significant reduction in FVC decline(20-22). The first phase III RCT, conducted in Japan, showed significant reduction in FVC decline at 52 weeks and improvement in progression-free survival with high-dose pirfenidone therapy(20-22). This led to approval of pirfenidone for use in Japan in 2008.

After that, two other phase III RCT trials, CAPACITY 1 and 2, were carried out concurrently in North America, Europe and Australia. CAPACITY 1 met its primary end-point of reduction in FVC decline at 72 weeks while CAPACITY 2 did not(20-22). However, CAPACITY 2 did show improvement in 6MWT decline as a secondary endpoint. When the data from both CAPACITY trials were pooled, there were significant differences in primary and secondary endpoints. Pirfenidone therapy resulted in significant reduction in FVC decline, mean decline in 6-minute walk test distance, and progression-free survival(20-22). When all four pirfenidone clinical trials were analyzed by Cochrane meta-analysis, it was evident that pirfenidone reduced disease progression risk by 30% in comparison to placebo(20-22). Based on that evidence, in 2011, Europe approved the use of pirfenidone for mild to moderate IPF.

In the USA, however, the FDA decided in 2010 to withhold approval of pirfenidone until more clinical trial data was obtained. They cited failure of CAPACITY 2 to replicate the improvement in FVC decline seen in CAPACITY 1 as the reason. ASCEND, another phase III RCT designed to test efficacy of pirfenidone in IPF, was initiated in 2011 and is currently in progress in North America, South America and Australia. The primary endpoint for ASCEND is FVC decline at 52 weeks(20, 21). Reassessment of FDA approval in the USA is expected to come in the future, pending results of the ASCEND trial. In the meantime, investigations of

high-dose pirfenidone side effects indicate long-term therapy is generally safe and well tolerated(20-22). Pirfenidone represents a promising therapeutic option for IPF.

Sildenafil (phosphodiesterase inhibitor 5) was tested in phase III trial STEP-IPF. No significant difference was observed in primary endpoint of ≥20% increase in 6MWT distance at 12 weeks, but secondary endpoints (quality of life and carbon monoxide diffusing capacity) showed significant improvements(21).

An option that is currently in phase III RCT testing is treatment with a nintedanib or BIBF 1120 (triple inhibitor of tyrosine kinases receptors). Nintedanib has been evaluated in the phase II RCT TOMORROW, which showed improvement in FVC decline and fewer acute exacerbation events(20). The targets of nintedanib are platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) receptors. The results from TOMORROW led to two phase III RCT currently in progress(20, 21).

Current ongoing phase II clinical trials involve targeting: interleukin 13 with lebrikizumab (QAX576, human anti-IL-13 monoclonal antibody), chemokine (C-C motif) ligand 2 or monocyte chemoattractant-1 with CNTO 888 (monoclonal antibody against CCL2), connective tissue growth factor with FG-3019 (monoclonal antibody to CTGF), transforming growth factor beta with GC1008 (antibody against TGF-β)(20, 21).

Other ideas currently in phase I testing include targeting lysyl oxidase and lysyl oxidase-like enzymes with simtuzumab (AB0024, monoclonal antibody against LOXL2) and administration of mesenchymal stem cells for treatment of IPF(20, 21).

Lung Transplantation: Lung transplantation is effective at reducing risk of death and prolonging survival in advanced IPF disease(1, 2). However, 5-year survival post-transplant is still only about 44-56%(1, 2). It is unclear whether single versus double lung transplantation

confers different survival benefits. Discussion of transplantation is appropriate at diagnosis and referral of patients for transplant evaluation is recommended at first sign of deterioration(1, 2).

Treatment of Co-Existing PH and Emphysema: Regarding the treatment of pulmonary hypertension in IPF, the joint committee gave a weak recommendation for a trial of vasodilator therapy in IPF patients with moderate to severe PH that has been confirmed by right heart catheterization(1, 2). Sildenafil, a phosphodiesterase 5 inhibitor, has been shown to improve quality of life and dyspnea in cases of IPF patients with advanced PH(1, 2).

Treatment of combined pulmonary fibrosis and emphysema has not been clearly defined. Cessation of smoking is advised and supplemental oxygen should be provided as needed for hypoxemia(6). The use of sildenafil to treat pulmonary hypertension in this group has not been evaluated(6).

Prognosis & Mortality

IPF prognosis is grim. Mortality in IPF patients is expected within 2-3 years of diagnosis(1, 2). Mortality rate is about 40,000 deaths annually(1-3). Of increasing concern is the upward trend in mortality rates for IPF over the past two decades(3). Particularly, from 1992 to 2003, the number of deaths secondary to IPF in the USA increased by 50%, a mortality rate similar to and surpassing those of many cancers, emphasizing the devastating nature of this disease(23, 24).

Pathogenesis of IPF

The dismal prognosis and paucity of effective treatment modalities in IPF is secondary to inadequate knowledge of the pathways and factors that govern the development and progression

of this complex disease. IPF was originally considered to be an inflammatory condition(1, 2). However, when anti-inflammatory therapies failed to achieve an effect in IPF patients, the focus shifted away from inflammation and towards an epithelium-centered disease model(1, 2).

Evidence to date indicates that epithelial injury and activation plays an important role in IPF pathogenesis. Involvement of local mesenchymal cells and circulating fibrocytes, in this process, leads to expansion of the fibroblast and myofibroblast populations that are responsible for the exaggerated deposition of extracellular matrix and resulting destruction of pulmonary architecture evident in IPF(2). Thus, of particular importance are efforts to understand the contribution of factors generated during tissue injury and how those factors impact development of fibrosis.

ADENOSINE IN CHRONIC LUNG DISEASES

One of the factors that has emerged as being central to injury response in the lung is the signaling nucleoside adenosine. During times of tissue stress and damage, cells will release adenosine triphosphate (ATP) into the extracellular space(25). There, ATP is rapidly dephosphorylated and converted to AMP and then adenosine(25). Adenosine can then engage its cell surface G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors) to exert various effects on inflammation, repair and remodeling processes(26), producing either tissue-protective or tissue-destructive results(25, 27).

Adenosine has been shown, in humans and mouse models, to be elevated in and contribute to chronic lung disorders, including asthma, COPD, and pulmonary fibrosis(25). Recent publications have reported, specifically, that it is the activation of the A_{2B} receptor by adenosine that mediates production of pro-fibrotic molecules, like interleukin-6, leading to

chronic lung diseases(25, 28-31). Adenosine regulates production and release of IL-6 from various cell types, including from lung epithelial cells(32), macrophages(33), smooth muscle cells(34) and fibroblasts(35).

INTERLEUKIN 6 (IL-6)

Interleukin 6 (IL-6) is a pleiotropic cytokine first identified as a secretory product of fibroblasts(36) and later recognized for the ability to stimulate the differentiation of activated B cells into immunoglobulin-producing plasma cells(37). Since then, studies have reported extensive effects in various systems, particularly the immune and hematopoietic systems. Aside from mediating the differentiation of activated B cells, IL-6 induces growth and activation of T lymphocytes and regulation of cytotoxic T cell differentiation(38, 39). With regards to hematopoiesis, IL-6 mediates the expansion of hematopoietic progenitors(40), promotes proliferation and differentiation of megakaryocytes, and regulates macrophage and neutrophil differentiation(38). IL-6 acts in the liver as a hepatocyte stimulatory factor, regulating the production of acute phase proteins like C-reactive protein (CRP) and fibrinogen, driving the acute phase response(38, 41). In the musculoskeletal system, IL-6 is important for bone health and remodeling. It acts to promote osteoclast differentiation, leading to bone resorption(42, 43). IL-6 also mediates neural cell differentiation and homeostatic activities related to fatigue, mood and pain through poorly understood mechanisms(43, 44).

INTERLEUKIN 6 SIGNALING PATHWAYS

IL-6 signals through a receptor complex consisting of two distinct glycoprotein chains – the ligand-binding interleukin 6 receptor alpha (CD126, IL-6Rα) and the non-ligand binding,

signal-transducing receptor gp130 (CD130)(38, 43, 44). While gp130 is expressed constitutively as a membrane-bound receptor on most cells in the body, expression of IL-6Rα is more limited(38, 43, 44). Membrane-bound IL-6Rα is present only on certain cells in the body, primarily hepatocytes and leukocyte subpopulations – B and T cells, monocytes, and neutrophils(45, 46). However, the presence of a natural, soluble form of the IL-6Rα (produced through alternative splicing and proteolytic cleavage) drastically widens the range of cellular targets and allows IL-6 to signal via two pathways(38, 43, 44, 47).

The first pathway is known as classical signaling and occurs on cells that co-express membrane-bound IL-6Rα and gp130. Here, IL-6 binds a membrane-bound IL-6Rα, and this IL-6/mIL-6Rα complex then triggers the association with two molecules of gp130 to initate the signaling cascade (Figure 1.1A). In the alternative pathway, known as *trans* signaling, IL-6 complexes with a soluble form of IL-6Rα in order to associate with gp130 and initiate signaling (Figure 1.1B)(38, 43, 44). In both pathways, binding of IL-6 to IL-6Rα causes association with two gp130(48). This dimerization of gp130 in turn activates associated Janus tyrosine kinases (JAK), leading to phosphorylation of gp130(49, 50). Activated gp130 then facilitates the phosphorylation of signal transducer and activator of transcription 3 (STAT3)(51). Phospho-STAT3 dimerizes and translocates to the nucleus, where it acts as a transcription factor to regulate target genes(52).

GENERATION OF SOLUBLE IL-6R ALPHA

Soluble receptors for cytokines can be generated in two ways: 1) shedding (where limited proteolysis of the membrane-bound receptor produces a soluble form of the receptor) and 2) alternative splicing (where soluble receptors are produced via translation of differentially spliced

mRNAs exhibiting deletions of transmembrane and cytoplasmic domains). Many soluble receptors retain their ligand-binding capacity and have been characterized in biological fluids(53).
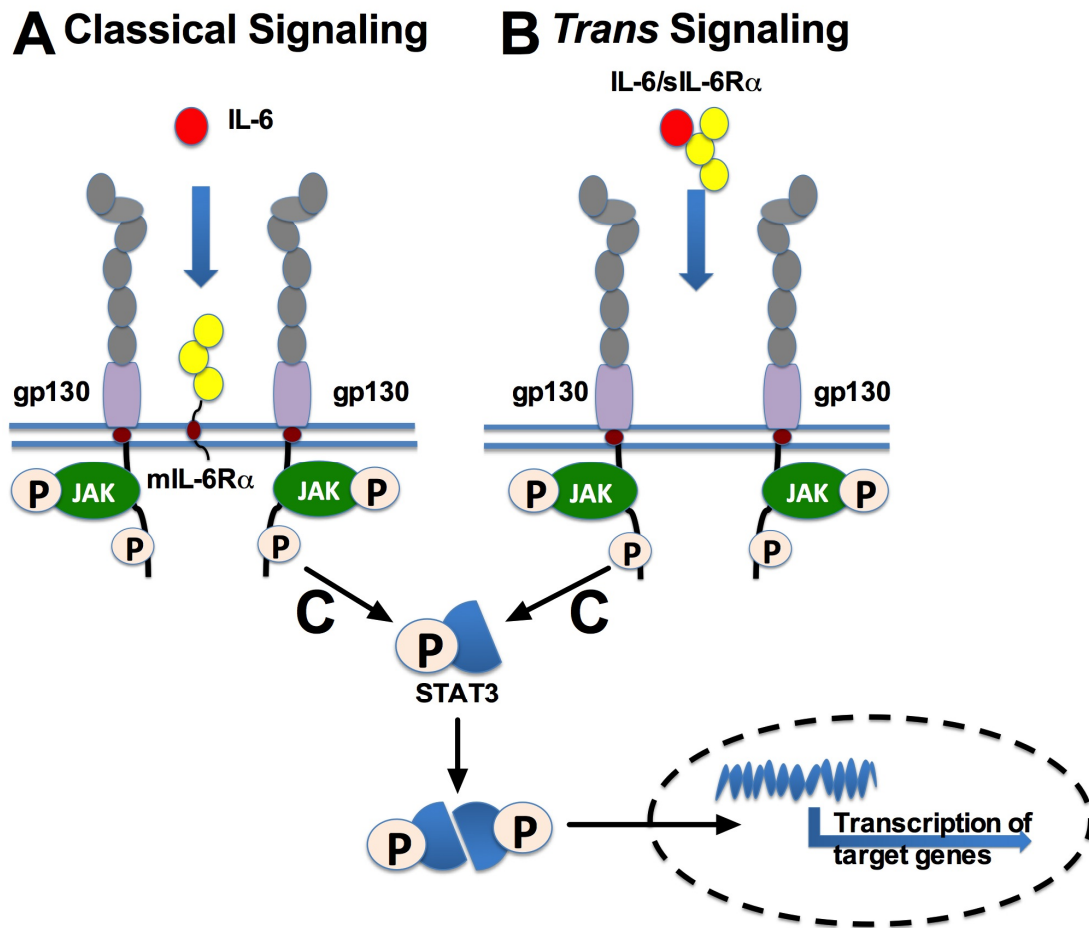
Figure 1.1. Interleukin 6 (IL-6) classical and *trans* signaling pathways. (A) Classical signaling occurs on cells that co-express membrane-bound gp130 and membrane-bound IL-6Rα (mIL-6Rα). There, IL-6 binds mIL-6Rα to form the IL-6/mIL-6Rα complex to initiate signaling. **(B)** Cells that express membrane-bound gp130 but lack mIL-6Rα can still be activated in the presence of the soluble IL-6Rα. Activation in this manner is known as *trans* signaling. There, IL-6 binds a soluble version of IL-6Rα to form the IL-6/mIL-6Rα complex. **(C)** In both pathways, binding of IL-6 to either mIL-6Rα or sIL-6Rα leads to association with 2 membrane-bound

gp130 molecules, which activates Janus kinases (JAK), which in turn phosphorylates gp130, leading to phosphorylation of Signal Transducer and Activator of Transcription 3 (STAT3). Phospho-STAT3 dimerizes and translocates to the nucleus, where it acts as a transcription factor to facilitate transcription of target genes.
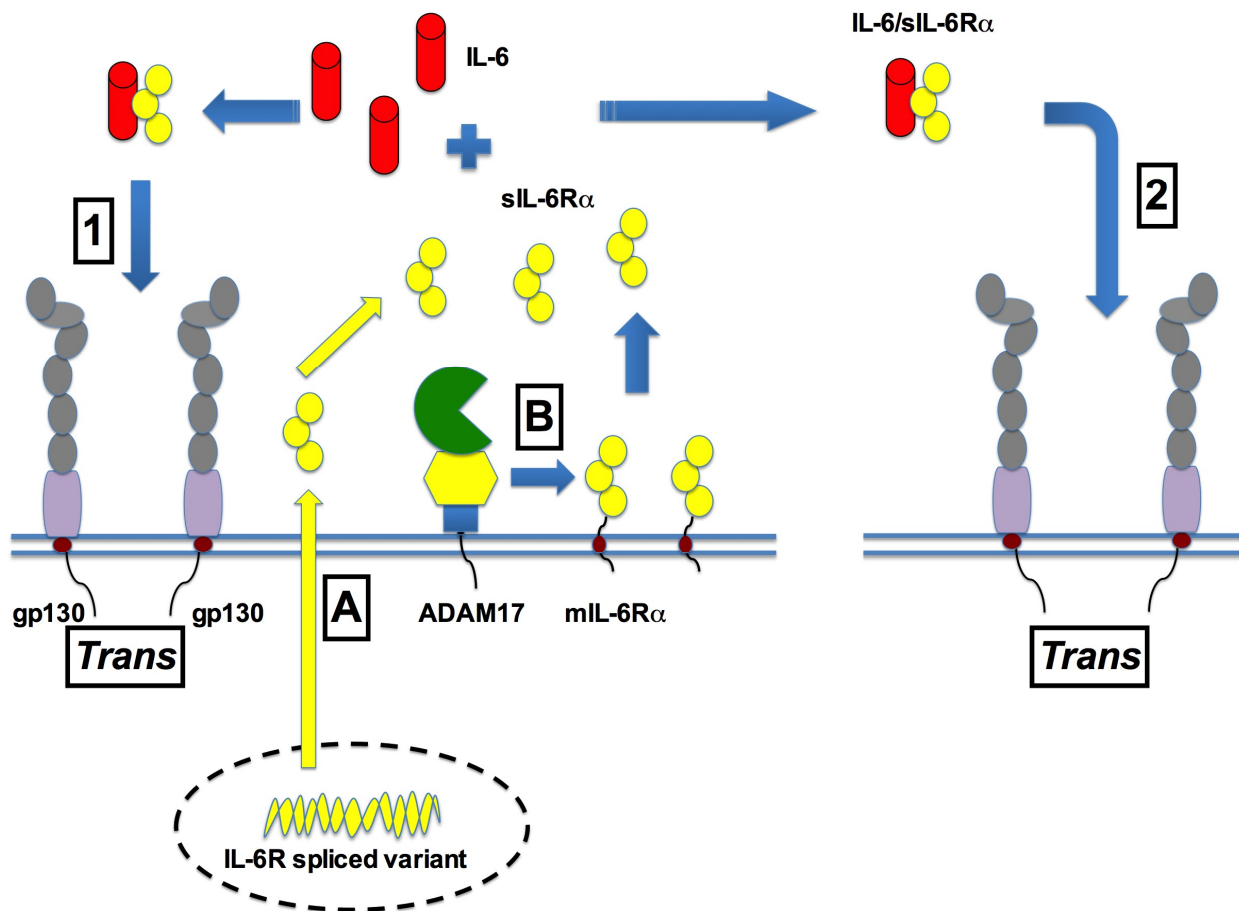
Figure 1.2. Mechanisms of generation of soluble IL-6Rα (sIL-6Rα). Soluble IL-6Rα (sIL-6Rα) can be generated via **(A)** translation of alternatively spliced mRNA or **(B)** by proteolytic cleavage of the membrane-bound receptor by metalloproteases such as ADAM17. Soluble IL-6Rα retains its ability to bind IL-6 and has a binding affinity for IL-6 similar to the membrane-bound version. The complex of IL-6/sIL-6Rα can activate cells in an autocrine **(1)** or paracrine **(2)** manner.

Soluble IL-6Rα can be generated via translation of alternatively spliced mRNA but mainly through proteolytic cleavage(44). Different studies have identified IL-6Rα mRNA isoforms that, secondary to alternative splicing and resulting reading frame shift, encodes a receptor that lacks the transmembrane and cytosolic domains and possesses a novel carboxy-terminal protein sequence(54-56). Most of the focus, however, has been on unraveling the mechanism of protease-mediated cleavage of membrane-bound IL-6Rα to produce a soluble receptor. Mullberg et al demonstrated that a soluble form of the IL-6Rα is produced via shedding of membrane-bound receptor from human peripheral blood monocytes(57). Protein kinase C activity regulates this shedding process and the shed receptor retains IL-6 binding capacity(58). Studies in subsequent years supported a role for the metalloprotease ADAM17 in mediating IL-6Rα shedding in response to various stimuli, including apoptosis(59), Ca^{2+} mobilization(60), cellular cholesterol depletion(61), leptin induction(62), TCR activation of CD4 T cells(63), and bacterial activation of bronchial epithelial cells(64).

A DISINTEGRIN AND METALOPROTEASE 17 (ADAM17)

A Disintegrin and Metalloproteinase 17 (ADAM17) (previously known as TACE – tumor necrosis factor-alpha converting enzyme) is a membrane-bound metalloprotease belonging to the ADAM (a disintegrin and metalloproteinase) family that is responsible for cleaving cell surface proteins. The list of substrates for ADAM17 includes a number of cytokines and growth factors

(e.g. TNFα, TGFα, HB-EGF), cytokine receptors (e.g. IL-6Rα, TNFα-RI and RII), and adhesion molecules (e.g. L-selectin, ICAM-1)(65), among others.

ADAM17 has been implicated as the main protease responsible for shedding IL-6Rα from cell membrane surfaces of hepatocytes, peripheral monocytes, neutrophils and T cells(38, 59, 61-63). Increased levels of this protease have been found in association with diseases that have reported increased sIL-6Rα levels(66-69). A related protease, ADAM10, has been implicated in constitutive shedding of IL-6Rα, while ADAM17 is better known for rapid, inducible shedding(38, 61).

INTERLEUKIN 6 *TRANS* SIGNALING AND DISEASE

IL-6 has comparable binding affinity to soluble IL-6Rα as it does for membrane-bound IL-6Rα (mIL-6Rα). By binding IL-6 to form the IL-6/sIL-6Rα complex, soluble IL-6Rα prolongs the plasma half-life of IL-6(70). At the same time, IL-6/IL-6Rα complex acts agonistically, interacting with gp130 to initiate signaling, allowing for activation of cells not responsive to IL-6 alone. Thus, soluble IL-6Rα provides an alternative mechanism of gp130 activation, which serves to widen the spectrum of IL-6-responsive cells and amplify IL-6 effects, leading to important roles in chronic pathological states(44, 71).

IL-6 *trans* signaling has been implicated in the pathogenesis of a number of chronic diseases, including myocardial fibrosis(72), rheumatoid arthritis(73), asthma(74), inflammatory bowel disease (colitis) and colitis-associated cancer(38). IL-6 and sIL-6Rα levels are elevated in association with these diseases and reported to contribute to their pathogenesis. Effects in suppressing the generation of regulatory T cells (T_{reg}) and promoting differentiation of T helper 2 (T_h2) and T helper 17 (T_h17) cells are important in chronic inflammatory and autoimmune

diseases. By promoting osteoclast differentiation and angiogenesis via stimulation of synovial fibroblast cells to produce receptor activator of NF-κB ligand (RANKL) and vascular endothelial growth factor (VEGF), IL-6 is implicated in the pathogenesis of arthritic conditions, most prominently in rheumatoid arthritis. Its induction of cellular proliferation, like in lymphoid and epithelial cells, contributes to tumor growth and development and has significant ramifications on inflammation-associated cancer, multiple myeloma, and lymphomas(71, 75). Given this knowledge, studies have also been conducted to specifically block IL-6 *trans* signaling *in vivo*, with resulting amelioration of disease, in colitis, arthritis, sepsis, asthma, and cancer(76-80).

INTERLEUKIN 6 SIGNALING AND FIBROSIS

In terms of its role in fibrosis, levels of IL-6 and soluble IL-6Rα are elevated in chronic hepatitis and liver cirrhosis and correlate with disease severity, suggesting involvement of IL-6 *trans* signaling(81). In the kidneys, adenosine A_{2B} receptor-mediated induction of IL-6 leads to excessive pro-collagen production, resulting in renal fibrosis. Interleukin 6 is also said to stimulate mesangial cell proliferation(75, 82). In the heart, IL-6 mediates the differentiation of cardiac fibroblasts to myofibroblasts (the main effector cell of fibrosis) and contributes to cardiac hypertrophy and myocardial fibrosis(72). IL-6 has been reported to promote collagen production in dermal fibroblasts(75). Increased IL-6 levels correlated with extent of skin fibrosis in systemic sclerosis(83), and levels of sIL-6Rα are increased in cases of systemic sclerosis patients with pulmonary fibrosis(84).

In the lungs, IL-6 is important in airway remodeling in asthma(85), induces the conversion of human lung fibroblasts to myofibroblasts(35), and promotes pancreatitis-associated lung injury(86). However, the role of IL-6 in pulmonary fibrosis was not defined until

O'Donoghue et al. demonstrated that IL-6 ablation attenuated fibrosis in a bleomycin-induced murine model(87). Pedroza et al previously demonstrated that IL-6 contributes to pulmonary fibrosis when they reported that genetic or pharmacologic removal of IL-6 resulted in attenuation of fibrosis(29). What is not known from those studies, though, is the role of IL-6 *trans* signaling in pulmonary fibrosis.

It is with the intention of mending this deficit in knowledge that we have designed a project to examine the role of IL-6 *trans* signaling in pulmonary fibrosis. We will be using IPF human samples obtained from Methodist Hospital as well as two murine models of pulmonary fibrosis to evaluate the role of this pathway in pulmonary fibrosis.

INTRAPERITONEAL BLEOMYCIN (IPB) MODEL OF PULMONARY FIBROSIS

The general consensus at this time is that there is no adequate model for IPF, though previous models have employed the use of bleomycin, silica, fluorescein isothiocyanate (FITC), irradiation, or other agents. The most frequently used model is one that involves exposure to bleomycin. Bleomycin is a chemo-therapeutic agent known to cause pulmonary fibrosis in a portion of patients exposed to it. This is secondary to the lower level of the enzyme bleomycin hydrolase (responsible for inactivating bleomycin) in the lungs(88). Models of repetitive bleomycin exposure have been demonstrated to better recapitulate features of IPF(89). Our lab currently uses the repetitive intraperitoneal bleomycin (IPB) model of pulmonary fibrosis (Figure 1.3). In this model, mice are given intraperitoneal injections of bleomycin twice weekly for four weeks, which lead to progressive development of pulmonary fibrosis(33, 90, 91). This model of chronic bleomycin exposure has been demonstrated to recapitulate features of IPF, such as the

presence of hyperplastic airway epithelial cells and fibrosis that radiates inward from the pleural

surfaces and is progressive, irreversible and lethal(92, 93).

Intraperitoneal Bleomycin (IPB) Model
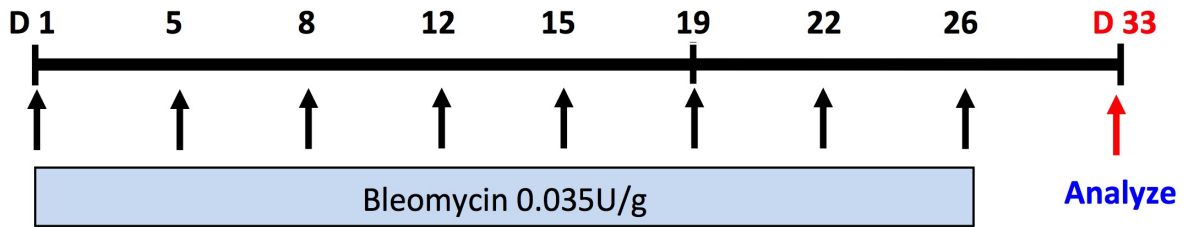
Figure 1.3. Intraperitoneal Bleomycin (IPB) mouse model of pulmonary fibrosis. Wildtype

C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4

weeks, resulting in progressive development of pulmonary fibrosis. Mice were sacrificed and

samples collected on day 33.

Adenosine Deaminase (ADA)-Deficient Model
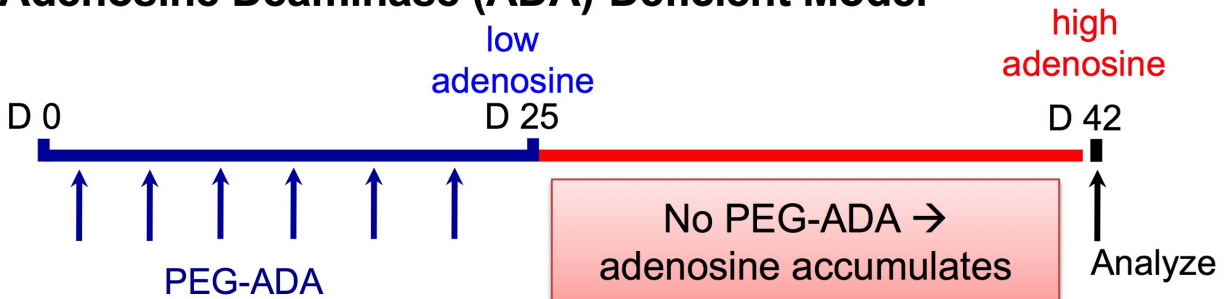
Figure 1.4. Adenosine deaminase (ADA)-deficient mouse model of pulmonary disease.

ADA-deficient (ADA$^{-/-}$) mice were identified at birth and maintained on PEG-ADA enzyme

replacement therapy until postnatal day 25. Therapy is then discontinued and endogenous

adenosine allowed to accumulate in the lung. Mice were sacrificed and samples collected for analysis on postnatal day 42.

ADENOSINE DEAMINASE (ADA)-DEFICIENT MODEL OF PULMONARY FIBROSIS

Another mouse model of pulmonary disease typically used by our laboratory is the Adenosine Deaminase (ADA)-deficient model (Figure 1.4). The benefit of this model is that it allows for evaluation of the role of adenosine in lung disease that consists of pulmonary fibrosis and emphysema(28).

Blackburn et al first reported the generation of a viable mouse with complete genetic deficiency in the enzyme ADA, an enzyme necessary to metabolize adenosine. ADA-deficient mice exhibited severe metabolic disturbances and pulmonary insufficiency resulting in mortality in the postnatal period(94). Specifically, ADA-deficient mice exhibited abnormal alveogenesis, pulmonary inflammation and airway obstruction(95). The use of ADA enzyme supplementation in these mice resulted in reduction of metabolic disturbances and survival of the animals(96).

The model now employs the use of enzyme replacement therapy from postnatal day 1 to 25 to allow normal pulmonary development in ADA-deficient mice. Discontinuation of therapy allows endogenous adenosine levels to rise, leading to increasing pulmonary inflammation, emphysematous distal airway enlargement and progressive development of pulmonary fibrosis(29, 97). These mice exhibited features similar to IPF, such as fibroblastic foci and hyperplastic airway epithelial cells (AEC)(29, 30).

Use of the ADA-deficient model allowed for exploration of adenosine in regulating events pertinent in lung injury and fibrosis. Chunn et al revealed that chronic elevation of endogenous adenosine in ADA-deficient mice resulted in pulmonary inflammation, expression

of profibrotic mediators and collagen deposition, and that these effects were all reversible with therapeutic administration of the enzyme ADA to lower adenosine(28). Blackburn et al reported that the effects of IL-13 on pulmonary inflammation, remodeling and fibrosis are mediated through augmentation of adenosine and its receptors(98). The adenosine A_1 receptor (A_1AR) serves an anti-inflammatory, protective role in adenosine-mediated lung injury(99). ADA-deficient mice lacking adenosine 2A receptor ($A_{2A}AR$) experienced enhanced pulmonary inflammation, angiogenesis and mucin production(100). Antagonism of the adenosine 2B receptor ($A_{2B}AR$) resulted in a reduction in pulmonary inflammation, fibrosis and alveolar airspace enlargement(97). Genetic removal of $A_{2B}AR$ in ADA-deficient mice, however, resulted in loss of barrier function and neutrophilia with resulting enhancement of inflammation and remodeling(101). This suggests a protective role for the $A_{2B}AR$ in acute stages. The adenosine 3 receptor (A_3AR) is important in regulating neutrophilia and mucus production in the lungs(102). Using this model, Schneider et al supported a role for osteopontin in mediating neutrophilia and alveolar airspace enlargement(30). Pedroza et al supported a role for interleukin 6 in promoting pulmonary inflammation and remodeling(29).

DISSERTION OVERVIEW

Idiopathic pulmonary fibrosis is a deadly lung disease that causes chronic, progressive, irreversible fibrosis. IPF has a highly variable and unpredictable natural history. Most patients experience respiratory decline with resulting respiratory failure and death within 2-3 years from diagnosis. There are no known causes for IPF, no cure, and at this time, no effective therapies available. The mortality rate for IPF is even worse than that of many malignancies. Of even greater concern is the fact that within the last two decades, there has been a trend for increasing

prevalence and incidence rates for IPF. There is an urgent need to better understand the pathways and events that govern the onset and progression of pulmonary fibrosis in order to remedy the lack of effective therapies. Production of the signaling nucleoside adenosine in response to lung injury is an important factor in the regulation of fibrosis. Adenosine, through activation of its adenosine 2B receptor ($A_{2B}R$), initiates production and release of the cytokine interleukin 6 (IL-6) in mice and humans with pulmonary fibrosis. Inhibition of IL-6, via pharmacologic blockade or genetic removal, resulted in attenuation of pulmonary fibrosis in mice. IL-6 can mediate tissue-protective effects via its membrane-bound receptor (mIL-6Rα) in a process termed classical signaling. However, when IL-6 signals through an alternative trans pathway, via the soluble form of its receptor (sIL-6Rα), the cytokine appears to exert a pro-fibrotic role. Preliminary data reveals increased levels of sIL-6Rα in the lungs of mice and patients with pulmonary fibrosis, suggesting involvement of IL-6 *trans* signaling. However, the role of IL-6 *trans* signaling in pulmonary fibrosis has not been examined. Given the findings in our lab, along with published literature implicating IL-6 *trans* signaling in the pathogenesis of other chronic inflammatory and fibrotic disorders, we developed the following specific aims to test the **HYPOTHESIS** that: **interleukin-6 *trans* signaling is critical for the development of pulmonary fibrosis and *in vivo* neutralization of this pathway will attenuate fibrosis.**

Specific Aim 1: **To quantify levels of soluble IL-6Rα and characterize classical and *trans* signaling components during the development and progression of pulmonary fibrosis.**

We quantified soluble IL-6Rα levels in plasma and bronchoalveolar lavage fluid during disease progression in the intraperitoneal bleomycin model. We characterized and monitor changes in expression of membrane-bound IL-6Rα and soluble IL-6Rα over time. Utilizing dual

staining protocols, we identified the pulmonary cell types that are responsive to IL-6 and delineate those that exhibit classical signaling versus those responding to *trans* signaling. The findings were validated in explanted human IPF lung samples, and the results from this aim are presented in Chapter 3.

Specific Aim 2: **Assess the role of protease-mediated shedding in the production of soluble IL-6Rα during the development and progression of pulmonary fibrosis.**

We characterized ADAM17 expression during development of pulmonary fibrosis in the IPB model. To establish the role of ADAM17 as the main protease responsible for the generation of sIL-6Rα in pulmonary fibrosis, we conducted *in vitro* stimulation and neutralization experiments using bone marrow derived macrophages as well as primary alveolar macrophages. We conducted experiments to directly stimulate shedding of IL-6Rα from cultured macrophages via activation of ADAM 17 and to prevent IL-6Rα shedding using a pharmacologic inhibitor of ADAM17 and ADAM17 siRNA. The results from this aim are presented in Chapter 4.

Specific Aim 3: **Evaluate the therapeutic benefit of blocking IL-6 *trans* signaling in pulmonary fibrosis.**

In order to assess the impact of IL-6 *trans* signaling on the development of pulmonary fibrosis, we conducted *in vivo* neutralization experiments using the IPB model. We subjected mice to bleomycin exposure while also co-injecting them with either PBS alone (vehicle control) or mouse recombinant gp130Fc (to selectively inhibit *trans* signaling). We assessed pulmonary phenotypes, inflammation, various indexes of pulmonary fibrosis, and downstream mediators of IL-6 signaling to determine the contribution of *trans* signaling. We stimulated IL-6 *trans*

signaling in normal and IPF fibroblasts *in vitro* and assess changes to proliferation and extracellular matrix protein production. The results from this aim are presented in Chapter 5.

Specific Aim 4: **To characterize IL-6 *trans* signaling in a murine model of pulmonary fibrosis and emphysema, the ADA-deficient model, and assess the impact of *trans* signaling by neutralizing it in this model.**

We attempted to verify results obtained in the IPB model by repeating experiments in the above three aims but this time using the ADA-deficient model. We characterized changes in IL-6 classical and *trans* signaling as well as ADAM17 expression during onset and progression of disease in the ADA-deficient model and conducted *in vivo* neutralization of IL-6 *trans* signaling in ADA-deficient mice and assess changes to pulmonary inflammation and pulmonary fibrosis. The results from this aim are presented in Chapter 6.

By accomplishing the above aims, we sought to gain novel insights about the pathogenesis of IPF that will translate into more effective therapies for patients.

CHAPTER 2

Experimental

Procedures

HUMAN IPF SAMPLES

Deidentified explanted lung samples from IPF patients were obtained from the Methodist Hospital J.C. Walter Jr. Transplant Center. Collection and use of these tissues for research were in accordance with the guidelines approved by the Methodist Hospital Institutional Review Board. Lung samples taken from unaffected lobes of IPF patients were used as controls.

ANIMALS

Mice used were ordered from Harlan Laboratories. They were inbred, male C57Bl/6NHsd mice, 4-5 weeks old. Once they arrive at our institution, mice were allowed a week to acclimate to their environment before the start of experiments. Maintenance and care of animals were in accordance with NIH guidelines and those set by the Animal Welfare Committee at the University of Texas Health Science Center at Houston. Mice were housed in ventilated cages equipped with microisolator lids and maintained under strict containment protocols. Housing conditions consisted of ambient temperature of 22°C and 12-hour light/dark cycles, and mice were provided with food and water ad libium. All study designs were reviewed and approved by the Animal Welfare Committee.

INTRAPERITONEAL BLEOMYCIN (IPB) MODEL

Male C57Bl/6 mice, 4-5 weeks old, were treated with intraperitoneal injections of saline or bleomycin (Teva Pharmaceutical, Petach Tikva, Israel) (0.035U/g) diluted in sterile 1X Phosphate Buffered Saline (PBS). A total of 8 injections were given twice a week for 4 weeks. Mice were sacrificed at the end of the model, on day 33, and samples were collected to assess pulmonary phenotype (Figure 1.3).

For the time course experiment, to evaluate pulmonary phenotype during development and progression of disease in the model, mice were sacrificed at various points in the model, on days 5, 10, 15, 20, 25, and 33. Day 0 mice are wildtype mice, who have not received any injections, saline or bleomycin.

ADENOSINE DEAMINASE (ADA) DEFICIENT MODEL

Adenosine deaminase heterozygous (ADA$^{+/-}$) and homozygous (ADA$^{-/-}$) mice were generated (on a C57Blk/6J background) and genotyped as described previously(96). Mice homozygous for the ADA-null allele were identified at birth and supported with polyethylene glycol modified ADA (PEG-ADA) enzyme therapy from postnatal day 1 to day 25 (Figure 1.4). Intramuscular injections were given on days 1, 5, 9, 13 and 17 (0.625, 1.25, 2.5, 2.5 and 2.5 units respectively). Intraperitoneal injections were given on days 21 and 25 (5 units each). Analysis of pulmonary phenotypes was performed on postnatal day 42. All comparisons were done among littermates of ADA-competent (ADA$^{+/-}$) and ADA-deficient (ADA$^{-/-}$) mice.

SOLUBLE GP130 INHIBITION OF INTERLEUKIN-6 *TRANS* SIGNALING

In vivo neutralization of interleukin 6 *trans* signaling was performed using recombinant mouse gp130Fc chimera (R&D Systems) (Figure 5.1A). In the IPB model, male C57Bl/6 mice, 4-5 weeks old, were treated with bleomycin or vehicle (saline) intraperitoneally twice a week for 4 weeks. Beginning on day 19 and continuing daily until day 32, mice were treated with saline (200μl 1X sterile PBS) or gp130Fc (250ng-2μg/mouse reconstituted in 200μl sterile PBS) (Figure 5.1B). On treatment days that coincide with bleomycin injections, mice received gp130Fc injections 1-2 hours before bleomycin. On day 32, arterial oxygen saturation levels

were measured. On day 33, animals were sacrificed and samples collected to assess changes to pulmonary phenotype.

In the ADA-deficient model, starting on postnatal day 30, mice were treated with saline (200μl 1X sterile PBS) or gp130Fc (1μg/mouse reconstituted in 200μl sterile PBS) until day 41 (Figure 6.4A). On day 42, animals were sacrificed and samples collected to assess changes to pulmonary phenotype.

The dosage and schedule of administration of soluble gp130 are adapted from previous published studies that have shown effectiveness of this reagent *in vivo(76-80, 103)*. The decision of when to begin treatment with soluble gp130 was based on preliminary data regarding the temporal course of disease development in both models (Figure 3.3 & 6.1).

ASSESSMENT OF ARTERIAL OXYGEN SATURATION

Assessment of arterial oxygen saturation was performed on conscious mice on day 32 using the pulse MouseOx software analysis (STARR Life Sciences Corp, Oakmont, PA). Mice were shaved on day 31 to remove the hair around the neck to allow proper measurements by collar clip light sensor. The MouseOx utilizes pulse oximetry measurements of light absorption from the red and infrared LEDs (Light Emitting Diodes) to determine real-time percent oxygen saturation of functional arterial hemoglobin.

MEASUREMENT OF RIGHT VENTRICULAR SYSTOLIC PRESSURE (RVSP)

Protocol for measurement of right ventricular systolic pressure (RVSP) is as previously published(91). Briefly, mice were anesthetized with avertin and placed on heated pad (Deltaphase Isothermal model 39; Braintree Scientific, Braintree, MA). Tracheostomy was

performed and mice were connected to a ventilator (MiniVent, Hugo-Sachs Elektronik, March-Hugstetten, Germany) via a 19-gauge blunt needle (Brico, Dayton, NJ). Mice were maintained on volume of 250μl and rate of 100/minute. Using a surgical microscope (SMZ-2B, Nikon, Tokyo, Japan), the abdomen was accessed through an incision made below the xiphoid process. A retractor (ALM-112, Braintree Scientific) was used to visualize abdominal contents. The heart was exposed through an incision in the diaphragm and pericardium was removed. The right ventricle (RV) was punctured with 27-gauge needle and catheterized with a 1-french pressure catheter (SPR-1000, Millar Instruments, Houston, TX). RVSP was recorded using Chart5.3 software (AD Instruments).

COLLECTION OF BLOOD AND ISOLATION OF PLASMA

Mice were anesthetized with avertin and blood was collected via needle aspiration of the left ventricle. Plasma was isolated via centrifugation at 10,000xG for 10 minutes at 4°C and flash frozen for storage at -80°C for later use in ELISA analyses.

BRONCHOALVEOLAR LAVAGE (BAL) FLUID COLLECTION, CELL COUNT AND DIFFERENTIALS

After collection of blood, the mouse trachea was exposed and cannulated to perform bronchoalveolar lavage. Lungs were lavaged 4 times with 0.3 ml sterile PBS each; approximately 1ml of pooled lavage fluid per mouse was recovered. An aliquot was sampled to determine total BAL cells using a hemocytometer. The rest of the BAL fluid was spun at 1200 rpm for 5 minutes at 4°C. The supernatant was removed, aliquoted and flash frozen. Cell pellets were resuspended in 100μl sterile PBS and aliquots were spun onto microscope slides (50μl for

PBS samples, 10-20μl for bleomycin samples). The slides were stained with Diff-Quick (Dade Behring, Deerfield, IL) and cellular differentials were performed. Remaining cells were pelleted once again at 10,000xG for 5 minutes at 4°C and supernatant was removed. Cell pellets were flash frozen. BAL fluid supernatant aliquots and cell pellets were stored at -80°C for later use in protein quantification, ELISA and western blot analyses.

HISTOLOGY

After lavage was performed, mouse lungs were inflated with 10% phosphate buffered formalin at 25 cm of pressure and fixed at 4°C overnight. Formalin-fixed lungs were dehydrated through an ethanol gradient and paraffin-embedded. 5μm sections were cut and collected on microscope slides for use in fibrosis assessment and immunohistochemistry staining.

IMMUNOSTAINING OF PARAFFIN-EMBEDDED LUNG SECTIONS

Lung sections were rehydrated through an ethanol gradient to water. Rehydrated sections were incubated in 10% hydrogen peroxide to quench endogenous peroxidases. Antigen retrieval (Dako Corp.) was performed at 95°C for 20 minutes and slides were allowed to cool to room temperature for 20 minutes. Endogenous avidin and biotin were blocked using the Biotin Blocking System (Dako Corp., Carpinteria, CA). Slides were blocked in 3% blocking solution (goat or rabbit serum in PBS) for 1 hour at room temperature then incubated at 4°C overnight in: anti-STAT3 (phospho S727) antibody (rabbit polyclonal, 1:100 dilution, Abcam, Cambridge, MA), anti-prosurfactant protein C (proSP-C) (rabbit polyclonal, 1:1000 dilution, EMD Millipore, Billerica, MA), anti-human S100A4 (rabbit polyclonal, 1:200 dilution, Dako Corp.), arginase 1 antibody (H-52) (rabbit polyclonal, 1:100 dilution, Santa Cruz Biotechnology, Dallas, TX), anti-

ADAM17 (rabbit polyclonal, 1:100 dilution at 37°C for 1 hour, EMD Millipore). For alpha-SMA staining, slides were processed with a Mouse on Mouse kit per manufacturer protocols (Vector Laboratories, Burlingame, CA). Slides were then incubated in anti-actin, α smooth muscle antibody (1:1000 dilution, 4°C overnight, mouse monoclonal clone 1A4, Sigma-Aldrich, St. Louis, MO).

After incubation with primary antibodies, all sections were incubated at room temperature for 1 hour in appropriate biotinylated secondary antibodies. Vectastain ABC peroxidase rabbit IgG kit (Vector Laboratories) and 3,3'-diaminobenzidine (Sigma-Aldrich) were used to develop stains for P-STAT3 and ADAM17. Vectastain ABC Alkaline Phosphatase Standard Kit (Vector Laboratories) and Vector Alkaline Phosphatase substrate kit (Vector Laboratories) were used to develop stains for alpha-SMA, pro-SPC, S100A4, and arginase 1. All sections were counterstained with methyl green. Slides were mounted with cover slip and Vectashield (Vector Laboratories) mounting medium.

IMMUNOFLUORESCENCE OF BAL CELLS

Aliquots of resuspended BAL cell pellets were cytospun onto microscope slides, allowed to air-dry, then fixed in 3.7% paraformaldehyde in PBS for 10 minutes and permeabilized in cold methanol for 10 minutes. Slides were blocked in 1% bovine serum albumin in PBS for 1 hour at room temperature and then incubated in: anti-human CD126 (IL-6R) antibody (1:50 dilution, 4°C overnight, mouse monoclonal, ABD Serotec, Raleigh, NC). Incubation in secondary antibody, Alexa Fluor 488 rabbit anti-mouse IgG (1:1000 dilution, Life Technologies, Grand Island, NY) occurred for 1 hour at room temperature, protected from light. Slides were mounted

with coverslip and Prolong Gold antifade medium with DAPI (Life Technologies) and let air dry, protected from light.

DUAL IMMUNOFLUORESCENCE OF LUNG SECTIONS FOR P-STAT3 AND α-SMA

Rehydrated sections were processed as in above section on immunostaining with regards to hydrogen peroxide quench, antigen retrieval, avidin/biotin block, and serum block. Slides were incubated in anti-STAT3 (phospho S727) antibody (1:100 dilution, O/N, 4°C) then in appropriate secondary antibody for one hour at room temperature. ABC Alkaline Phosphatase Standard Kit and Vector Alkaline Phosphatase Substrate Kit were used to develop P-STAT3 as a red fluorescence. From this point forward, all steps were performed in the dark and slides were protected from light. Slides were washed in PBS to remove excess substrate and then processed using the Mouse on Mouse Kit. Sections were incubated in blocking reagent for 1 hour at room temperature then reagent diluent for 5 minutes at room temperature and then incubated in anti-actin, α smooth muscle antibody (1:500 dilution, 4°C overnight). Secondary antibody incubation in Alexa Fluor 488 rabbit anti-mouse IgG (1:1000 dilution) was performed for 1 hour to allow detection of alpha-SMA as a green fluorescence. Slides were mounted with coverslip and Prolong Gold antifade medium with DAPI and let air dry, protected from light.

FIBROSIS ASSESSMENT

Rehydrated lung sections were stained using a Masson's trichrome protocol. Fibrosis was quantified using the Ashcroft method of scoring. Scoring was performed blinded with regards to animal treatment. All areas of the lung were scored and an overall average score was given per lung section.

The Sircol assay (Biocolor Ltd., Carrick, UK) was used to measure soluble collagen content in BAL fluid. Transcript levels of collagen isoforms were quantitated using whole lung mRNA. Fibronectin expression was determined using western blot analysis of lung lysates.

WESTERN BLOT ANALYSES OF PROTEIN LYSATES AND BAL FLUID

Protein lysates were prepared by homogenizing frozen lung tissue in cold protein lysis buffer (1M Tris pH 7.4, 1M NaCl, 1% Triton-X 100) containing 1X protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Lysates were centrifuged at 14,000 rpm for 10 minutes at 4°C, supernatant was removed and aliquoted for use in western blot analysis. Protein concentrations were determined using the Bradford assay (Bio-rad, Hercules, CA). Equal amounts of total protein per sample (25-50μg) were prepared in SDS-Sample Buffer (6X, reducing, Boston Bioproducts, Ashland, MA) and incubated at 95°C for 5 minutes. Electrophoresis was performed on 10-12% SDS-PAGE gels (Bio-rad). Transfer onto Immobilon-P PVDF membranes (EMD Millipore) was conducted at 100V for 2 hours at 4°C. Membranes were allowed to air dry, were trimmed and blocked with 5% (w/v) non-fat, dehydrated milk in Tris-buffered saline-tween 20 (TBST) for 1 hour at room temperature. Membranes were then incubated with shaking at 4°C overnight in: anti-human CD126 (IL-6R (1:500 dilution, mouse monoclonal, ABD Serotec), anti-STAT3 (phospho S727) antibody (1:500 dilution, rabbit polyclonal, Abcam), anti-ADAM17 (1:2000, rabbit polyclonal, Millipore), anti-fibronectin, cellular antibody (1:2000, mouse monoclonal clone FN 3E2, Sigma-Aldrich) and anti-β-Actin antibody (1:5000, mouse monoclonal clone AC-74, Sigma-Aldrich). Membranes were rinsed in TBST and incubated with appropriate horseradish-conjugated secondary antibodies for 1 hour at room temperature with shaking. Secondary antibodies used were: anti-rabbit IgG HRP-linked

antibody (1:1000-4000 dilution, Cell Signaling, Danvers, MA) anti-mouse IgG HRP-linked antibody (1:1000-10,000 dilutions, Cell Signaling). Signal was developed using Thermo Scientific Chemiluminescent Substrate.

For BAL fluid samples, equal volumes of samples were prepared in 6X SDS sample buffer and processed as detailed above.

ANALYSIS OF WHOLE LUNG RNA

Total RNA was isolated from frozen lung tissue using TRIzol reagent (Invitrogen) per manufacturer protocol. Samples were treated with RNAse-free DNAse 1 (Invitrogen) and used for quantitative real-time RT-PCR analysis. cDNA was made using Superscript II reverse transcriptase (Invitrogen). Equal amounts of cDNA were analyzed for transcript levels of COL1A2 and MCP-1 with normalization to beta actin and/or peptidylprolyl isomerase A (PPIA). Primer sets used were: COL1A2 – forward 5'-AAGGGTGCTACTGGACTCCC-3' and reverse 5'-TTGTTACCGGATTCTCCTTTGG-3'. Primers for MCP-1 were: forward 5'-AGCATCCACGTGTTGGCTC-3' and reverse 5'-TGGGATCATCTTGCTGGTG-3'. Data presented as mean normalized transcript levels using the comparative C_t method.

ELISA ANALYSIS OF SOLUBLE INTERLEUKIN 6 RECEPTOR ALPHA (sIL-6Rα) IN PROTEIN LYSATES, BAL FLUID AND PLASMA

Soluble IL-6Rα was quantified in protein lysates made from COPD & IPF lungs using the Human IL-6R Alpha Duoset ELISA kit (R&D Systems). 10μl of lysate was diluted in 90μl reagent diluent and subjected to ELISA analysis per manufacturer protocol. Murine BAL fluid and plasma samples were processed using the Mouse IL-6R Alpha Duoset ELISA kit (R&D

Systems). For BAL fluid samples, 100μl was used. For plasma samples, 10μl of plasma was diluted in 90μl reagent diluent.

BONE MARROW MACROPHAGE ISOLATION AND CULTURE

Male C57Bl/6 mice, 4-5 weeks old, were euthanized via cervical dislocation technique. Both femurs were isolated, removed and cleaned of surrounding muscles and tissues. The ends of each femur were carefully cut off and a 5ml syringe with 21-22G needle was used to flush the bone marrow cavity of the femur with complete macrophage medium, which is Dulbecco's Modification of Eagle's Medium (Fisher Scientific) supplemented with 10% fetal bovine serum and 20% L929 media supplement (see below for protocol for growing L929 cells and making L929 media supplement). Femur cavity was flushed with 2-5ml of media, until bone appears white. Flushed media was collected in a sterile 50ml conical tube and centrifuged at 500 X g for 10 minutes at room temperature to pellet cells. Cells were resuspended in macrophage media, counted and plated in 100mm bacterial dishes (not tissue culture treated dishes). Plate cells to have a total of $3\text{-}5\text{x}10^6$ cells in 7.5ml media per dish. A yield of approximately 10-15 dishes is typical per mouse. Cells were incubated at 37°C, 5% CO_2 for 4 days, supplemented with an extra 5ml of media per dish and cultured until day 7-8, by which time most adherent cells are macrophages. On day 7-8, culture media was discarded, fresh media was added (5ml per dish) and a cell scraper was used to dislodge adherent cells. Detached cells were collected in 50ml conical tube and centrifuged at 400 X g for 10 minutes at 4°C. Cells were then resuspended in macrophage media and seeded in tissue culture plates for experiments.

To make L929 media supplement, L929 cells were obtained from our collaborator, Dr. Sandeep Agarwal (Baylor College of Medicine, Department of Internal Medicine). Cells were

plated at a density of 5×10^5 cells per T75 flasks in 55ml of media consisting of DMEM with 1% HEPES, 1% penicillin-streptamycin, 1% L-glutamine and 10% fetal bovine serum. Cells were cultured for 7 days, then media was harvested and sterile filtered for addition to macrophage culture media.

DIFFERENTIATION OF MACROPHAGES WITH IL-4 AND IL-13

Day 7-8 bone-marrow-derived macrophages were plated in 6-well plates at a density of $1.5-2 \times 10^6$ cells/well and allowed to adhere 2-3 hours at 37°C, 5% CO_2. Culture media and non-adherent cells were removed after 3 hours. Cells were then incubated in macrophage media containing IL-4 (20 ng/ml) and IL-13 (10 ng/ml) (Peprotech, Rocky Hill, NJ) for 72 hours. Fresh reagents were added daily. After 72 hours, macrophages were washed with sterile PBS and used in TAPI-1 experiments.

TAPI-1 INHIBITION OF ADAM17 IN MACROPHAGES

The reagent phorbol 12-myristate 13-acetate (PMA) is known to activate ADAM17 and induce receptor and substrate shedding. IL-4/IL-13-stimulated macrophages were incubated in serum-free media containing PMA (10μg/ml, Enzo Life Sciences, Farmingdale, NY) for 2-3 hours at 37°C, 5% CO_2, to activate ADAM17 and induce membrane IL-6Rα shedding. Inhibition of ADAM17 activity was carried out by pre-incubation of macrophages with TNFα protease inhibitor 1 (TAPI-1) (20μM) for 1 hour followed by PMA stimulation in the presence of TAPI-1. After 2-3 hours exposure to media alone, media and TAPI-1, PMA alone, or PMA and TAPI-1, culture media was collected and soluble IL-6Rα was quantified using ELISA. Macrophages were washed with PBS and lysed in RIPA lysis buffer containing protease inhibitors. 5-10μg of

protein per sample were subjected to western blot analysis to determine expression of arginase 1 (rabbit polyclonal antibody, 1:200 dilution, O/N incubation, 4°C, Santa Cruz), IL-6R (anti-human CD126 (IL-6R) antibody, mouse monoclonal, 1:500 dilution, 4°C overnight, ABD Serotec, 1:500 dilution, O/N, 4°C), and ADAM17 (rabbit polyclonal antibody, 1:500 dilution, O/N incubation, 4°C, EMD Millipore).

ADAM17 SIRNA SILENCING IN MACROPHAGES

For siRNA silencing of ADAM17, day 7-8 bone-marrow-derived macrophages were resuspended in antibiotics-free macrophage media and seeded in 6-well plates and allowed to adhere for 2-3 hours. Cells were then incubated in antibiotics-free media containing IL-4, IL-13, Optimem (Invitrogen) with Lipofectamine RNAiMax (Invitrogen) and ADAM17 siRNA or scrambled control siRNA (Sigma-Aldrich) for 24 hours. Final concentration of siRNA was 100nM. After 24 hours, media was changed and a second transfection was performed with fresh reagents. Macrophages were incubated in the second transfection media for 48 hours. Fresh IL-4 and IL-13 were added to culture media daily. After 72 hours of stimulation and transfection, non-transfection macrophages, macrophages transfected with control siRNA, and macrophages transfected with ADAM17 siRNA were stimulated with PMA in serum-free conditions for 2 hours. Culture media were collected and soluble IL-6Rα levels were quantified using ELISA. Cells were washed with PBS and lysed in RIPA lysis buffer containing protease inhibitors. 5-10μg of protein per sample were subjected to western blot analysis to determine expression of arginase 1 (rabbit polyclonal antibody, 1:200 dilution, O/N incubation, 4°C, Santa Cruz), IL-6R (anti-human CD126 (IL-6R) antibody, mouse monoclonal, 1:500 dilution, 4°C overnight, ABD

Serotec, 1:500 dilution, O/N, 4°C), and ADAM17 (rabbit polyclonal antibody, 1:500 dilution, O/N incubation, 4°C, EMD Millipore).

SHEDDING OF MEMBRANE IL-6R ALPHA FROM PRIMARY LUNG MACROPHAGES

Bronchoalveolar lavage fluid was collected from Day 33 male C57Bl/6 mice treated with PBS or bleomycin. Lungs were lavaged 10 times with 0.5 ml sterile PBS each; approximately 5ml of pooled lavage fluid per mouse was recovered. BAL fluid from all PBS mice or all bleomycin mice were pooled and spun at 1200 rpm for 5 minutes at 4°C. The supernatant was removed and cell pellets were resuspended in complete macrophage media (RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin). Cells were counted by hemocytometer, plated in 6-well plates at $1x10^6$ cells/well and allowed to adhere 4 hours at 37°C, 5% CO_2. Culture media and non-adherent cells were removed after 4 hours by washing with serum-free RPMI. Cells were then incubated in macrophage media containing PMA (10μg/ml, Enzo Life Sciences, Farmingdale, NY) for 2 hours at 37°C, 5% CO_2, to activate ADAM17 and induce membrane IL-6Rα shedding. Inhibition of ADAM17 activity was carried out by pre-incubation of macrophages with TNFα protease inhibitor 1 (TAPI-1) (20μM) for 1 hour followed by PMA stimulation in the presence of TAPI-1. After 2 hours exposure to media alone, PMA alone, or PMA and TAPI-1, culture media was collected and soluble IL-6Rα was quantified using ELISA.

CONTROL AND IPF FIBROBLAST CELL LINES

CCD8Lu and LL97A (AlMy) are control and IPF fibroblast cell lines, respectively, purchased from American Type Culture Collection (ATCC, Manassas, VA). CCD8Lu was

cultured in ATCC-formulated Eagle's Minimum Essential Medium (ATCC), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-amphotericin B mix. LL97A was cultured in Ham's F12K medium (ATCC) supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin-amphotericin B mix. Both cell lines were maintained in T75 flask with 10ml medium, at 5% carbon dioxide atmosphere in a 37°C humidified incubator. Medium was changed every 3 days and cells were passaged using trypsin. For stimulation experiments, cells were used between passages 4-8.

IN VITRO STIMULATION OF INTERLEUKIN 6 TRANS SIGNALING IN CCD8LU AND LL97A

To assess the effects of IL-6 trans signaling on fibroblast proliferation, CCD8Lu and LL97A cells were plated in 96-well plates at a density of $3-5 \times 10^3$ cells/well. They were cultured until they reached 70-80% confluency, then washed with sterile PBS and serum starved for 24 hours. After 24 hours in their respective serum-free media, cells were maintained in serum-free conditions and stimulated for 48 hours with: recombinant human TGF beta 1 (10ng/ml), recombinant human IL-6 (50ng/ml), or IL-6 (50ng/ml) + recombinant human IL-6R alpha (100ng/ml) (R&D Systems). Fresh reagents were added to culture media every 24 hours. Total volume per well was 100µl after 48 hours. Cells were then subjected to proliferation analysis using the Apo Tox-Glo Triplex Assay kit protocol (Promega, Madison, WI). Briefly, viability reagent was added per well and fluorescence was measured after 30 minutes incubation at 37°C.

To assess effects of IL-6 trans signaling on extracellular matrix protein production, CCD8Lu and LL97A cells were seeded in 6-well plates at a density of 1×10^5 cells/well. They were allowed to reach 70-80% confluency, then washed with sterile PBS and serum starved and

stimulated as mentioned above. After 48 hours of stimulation, cells were washed with PBS and lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail. Protein lysates were subjected to western blot analysis (as detailed above) to detect extracellular matrix protein production. Typically 5-10μg of protein per sample was loaded. Incubation was performed in primary antibodies for collagen 1 (rabbit polyclonal, Abcam, 1:500 dilution, 48 hours, 4°C) and fibronectin EDA (mouse monoclonal clone FN 3E2, Sigma-Aldrich, 1:4000 dilution, O/N, 4°C).

STATISTICAL ANALYSIS:

Experimental results were reported as mean ± standard error of the mean. One-way ANOVA was used for comparisons among groups, and comparisons between groups were completed with two-tailed Student's t-test. Statistical significance for all comparisons were presented as p-values. A p value less than 0.05 was considered to be significant.

CHAPTER 3

Interleukin-6 (IL-6)

Classical and *Trans* Signaling in

Idiopathic Pulmonary Fibrosis (IPF) and

a Chronic Bleomycin Mouse Model

of Pulmonary Fibrosis

EXPERIMENTAL RATIONALE AND KEY QUESTIONS

Idiopathic pulmonary fibrosis (IPF) is a deadly lung disease that causes chronic, progressive, and irreversible fibrosis. IPF is characterized by abnormal wound healing processes related to epithelial cell injury and turnover, fibroblast proliferation and differentiation, and matrix deposition(2, 104). Most patients experience respiratory decline with resulting respiratory failure and death within 2-3 years of diagnosis. There are no known causes for IPF and no cure. At this time, there is no consensus on effective therapies though the use of an antifibrotic pirfenidone is gaining support(1). The mortality rate for IPF currently surpasses that of many malignancies, and over the last 2 decades, prevalence and incidence rates have continued to increase(3, 23, 24). Thus, there is an urgent need to better understand the onset and progression of pulmonary fibrosis in order to develop effective therapies against IPF.

The cytokine interleukin 6 (IL-6) is elevated in mice and humans with pulmonary fibrosis(29, 31). IL-6 signals through two pathways, classical and *trans* (Figure 1.1), via a receptor complex consisting of the ligand-binding interleukin 6 receptor alpha (IL-6Rα) and the non-ligand binding, signal-transducing receptor gp130. While gp130 is expressed constitutively as a membrane-bound receptor, membrane-bound IL-6Rα (mIL-6Rα) is expressed predominantly on hepatocytes and leukocytes(38, 43-46). IL-6 classical signaling occurs on cells that co-express mIL-6Rα and gp130, where IL-6 binds mIL-6Rα and associates with two molecules of gp130 to initiate the intracellular signaling cascade. In the alternative pathway, known as *trans* signaling, IL-6 complexes with a soluble form of IL-6Rα (sIL-6Rα) before it can associate with gp130 and initiate signaling(38, 43, 44, 48). Soluble IL-6Rα is generated via translation of alternatively spliced mRNA or by protease-mediated cleavage of membrane IL-6Rα to form the soluble receptor(44, 56-61, 63, 70). In both IL-6 classical and *trans* signaling, binding of IL-6/IL-6Rα

with gp130 activates associated Janus kinases and leads to phosphorylation of signal transducer and activator of transcription 3 (STAT3), which dimerizes and translocates to the nucleus where it acts as a transcription factor to regulate target genes(49-52). Thus, the presence of phospho-STAT3 is used as an indication of IL-6-mediated cellular activation.

Because of the limited expression of membrane IL-6Rα, the presence of soluble IL-6Rα, mediating IL-6 *trans* signaling, allows for activation of cells not inherently responsive to IL-6. Thus, soluble IL-6Rα widens the spectrum of IL-6-responsive cells and *trans* signaling amplifies IL-6 effects in the body, leading to important roles in chronic pathological states(44, 71). IL-6 *trans* signaling has been implicated in the pathogenesis of rheumatoid arthritis(73), asthma(74), inflammatory bowel disease (colitis)(76) and colitis-associated cancer(78). IL-6 and sIL-6Rα levels are elevated in association with these diseases, and *in vivo* blockade of IL-6 *trans* signaling using the natural inhibitor soluble gp130(105, 106) has resulted in amelioration of disease(73, 74, 76, 78).

Though the focus in published literature has been on the pathogenic role of IL-6 *trans* signaling, it must be noted that classical signaling can contribute to disease as well. As evident in studies in colitis, where it was demonstrated that IL-6 classical signaling was pathogenic but *trans* signaling amplified those effects and contribute to the propagation of disease(76). Using mouse models of arthritis, Lissilaa et al demonstrated that IL-6 *trans* signaling was responsible for local inflammatory reactions but that classical signaling is required to promote T-cell-mediated autoimmune responses that result in systemic disease(107). In an allergic asthma model, Doganci et al demonstrated that sIL-6Rα mediates T_h2 functions in T effector cells while mIL-6Rα regulates differentiation of naïve $CD4^+$ cells towards T_h2 phenotype and suppressing T_{reg} development(108). On the other hand, IL-6 classical signaling has been shown to play

protective roles in certain human conditions. Hoge et al reported that classical signaling is responsible for the innate immunity response to and control of *Listeria monocytogenes* infection(109). The authors concluded that IL-6 *trans* signaling does not protect against certain bacterial infections. Therefore, its blockade may be of more benefit than global blockade of IL-6 signaling in chronic inflammatory diseases.

In terms of fibrosis, what is known is that levels of IL-6 and soluble IL-6Rα are elevated in systemic sclerosis(83, 110) and liver cirrhosis(81), correlating with disease severity and suggesting involvement of IL-6 *trans* signaling. In the kidneys, heart and skin, IL-6 induction promotes collagen production(72, 75, 82). In the lungs, IL-6 is important in airway remodeling in asthma(85), induces the conversion of human lung fibroblasts to myofibroblasts(35), and promotes pancreatitis-associated lung injury(86). However, the role of IL-6 in pulmonary fibrosis was not defined until O'Donoghue et al. demonstrated that IL-6 ablation attenuated fibrosis in a bleomycin-induced murine model(87). Pedroza et al demonstrated that IL-6 contributes to pulmonary fibrosis when they reported that genetic or pharmacologic removal of IL-6 resulted in attenuation of fibrosis(29). What is unclear from those studies, however, is the differentiation of effects due to IL-6 classical signaling versus IL-6 *trans* signaling in the lungs.

Whether fibrosis in IPF patients develops due to effects of IL-6 classical signaling alone or *trans* signaling alone or a mixture of both pathways is unclear. It is important to understand this differentiation in order to develop targeted therapies for IPF since current therapies with immunosuppressants carry with them many adverse side effects(1, 111, 112). Thus, to begin to determine if IL-6 classical and/or *trans* signaling may be important in the pathogenesis of IPF, it was necessary to first assess whether these pathways are enhanced in IPF lungs and characterize them more thoroughly by answering the following questions:

3.1   *Is soluble IL-6Rα increased in patients with Idiopathic Pulmonary Fibrosis?*

3.2   *Is soluble IL-6Rα increased in bleomycin-induced pulmonary fibrosis?*

3.3   *What happens to soluble IL-6Rα levels as pulmonary fibrosis develops and progresses in this model?*

3.4   *Is membrane IL-6Rα increased in patients with Idiopathic Pulmonary Fibrosis? Which pulmonary cell types exhibit classical signaling in humans? Which ones are susceptible to trans signaling?*

3.5   *Is there enhanced activation of STAT3 in patients with Idiopathic Pulmonary Fibrosis?*

3.6   *Is membrane IL-6Rα increased in bleomycin-induced pulmonary fibrosis and what happens as fibrosis develops and progresses?*

3.7   *Is there enhanced activation of STAT3 in mouse fibrotic lungs? What pulmonary cell types exhibit classical signaling? Which ones are targets of trans signaling?*

RESULTS

3.1 - Soluble IL-6R alpha is elevated in Idiopathic Pulmonary Fibrosis.

As an initial assessment of whether sIL-6Rα is elevated in fibrotic lungs, we measured its levels in the lungs of Idiopathic Pulmonary Fibrosis (IPF) patients, first in bronchoalveolar lavage (BAL) fluid, then in parenchymal lung samples. Bronchoalveolar lavage is a procedure that involves flushing saline down the airways of the lungs to wash out what is present in the airways. It is often performed in IPF patients for diagnostic purposes(1). Parenchymal lung samples were obtained from the diseased lungs of patients undergoing lung transplantation because of end-stage COPD or IPF.

ELISA quantification of soluble IL-6Rα was performed in BAL fluid samples from different lobes of a single IPF lung. It revealed elevated sIL-6Rα in a fibrotic lobe (IPF LLL) but not a non-fibrotic lobe (IPF RML) (Figure 3.1A). Protein lysates were prepared from explanted COPD and IPF lung tissues, and ELISA measurement of sIL-6Rα in these samples revealed a significant, 4-fold increase in IPF lungs versus COPD lungs (Figure 3.1B). Secondary confirmation was achieved with western blot analysis of these samples, probing for expression of sIL-6Rα, which revealed increased sIL-6Rα in IPF lungs, visible as a 50 kDa band (Figure 3.1C). These findings demonstrate for the first time increased soluble IL-6Rα in IPF, specifically in regions of fibrosis, suggesting IL-6 *trans* signaling may play a role in this disease.
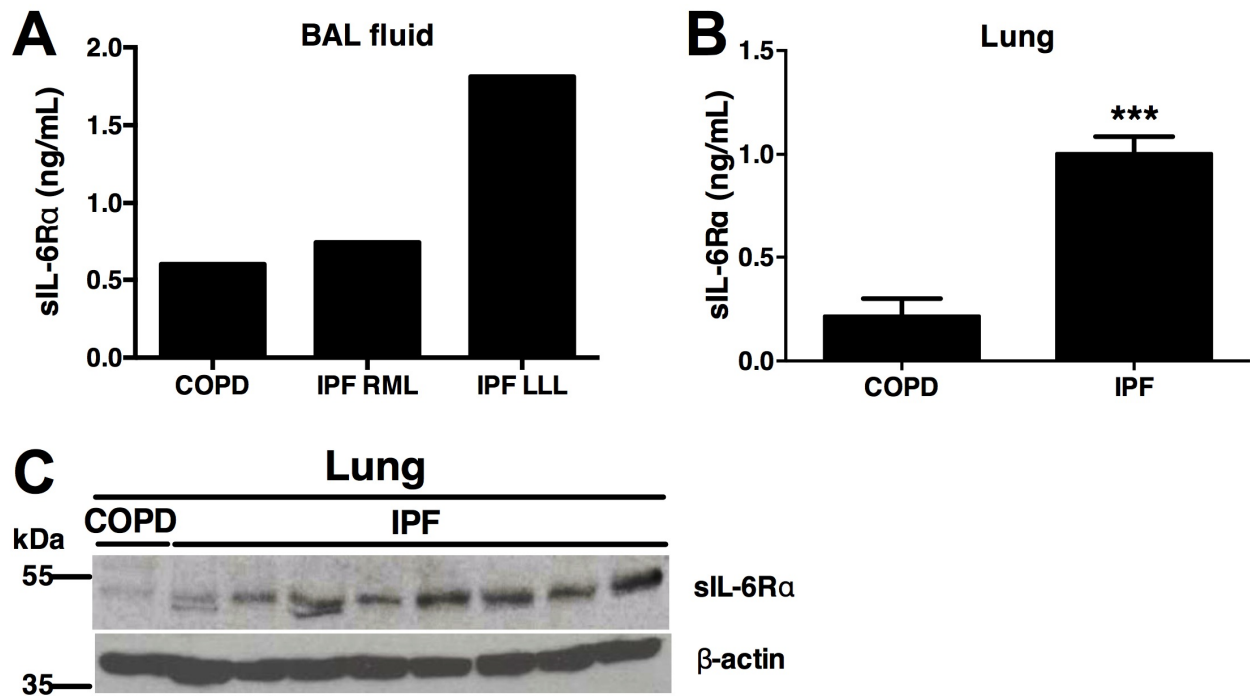
Figure 3.1. Soluble IL-6R alpha in Idiopathic Pulmonary Fibrosis (IPF). Soluble IL-6Rα expression was assessed in patients with Idiopathic Pulmonary Fibrosis. ELISA quantification of sIL-6Rα in (**A**) BAL fluid from non-fibrotic (RML) and fibrotic (LLL) lobes of an IPF patient, and (**B**) lung lysates from patients with COPD and IPF. (**C**) Western blot analysis of sIL-6Rα in lung lysates from patients with COPD and IPF. Results from A obtained by Mesias Pedroza, PhD. All data presented as mean ± SEM, n≥4 for B, n=1 for A. *significant difference from COPD; *= p<0.05, **= 0.001<p<0.01, ***= p<0.001.

To further evaluate the role of soluble IL-6Rα in IPF, we turned to a chronic model of fibrosis, the Intraperitoneal Bleomycin (IPB) model (Figure 1.3). In this model, mice are given intraperitoneal injections of bleomycin twice weekly for four weeks, which lead to progressive development of pulmonary fibrosis(33, 90, 91). This model of chronic bleomycin exposure has been demonstrated to recapitulate features of IPF, such as the presence of hyperplastic airway epithelial cells and fibrosis that radiates inward from the pleural surfaces and is progressive, irreversible and lethal(92, 93).

3.2 - Soluble IL-6R alpha is elevated in bleomycin-induced pulmonary fibrosis.

To assess expression of sIL-6Rα in this model, protein lysates were made from day 33 lungs and western blot performed. Soluble IL-6Rα was absent in PBS-injected, non-fibrotic lungs but present in bleomycin-exposed, fibrotic lungs (Figure 3.2A). Bands present at 55-65 kDa represent glycosylated versions of the soluble receptor. BAL fluid samples (which reflect the local microenvironment of the bronchoalveolar compartment in the lung) were collected and analyzed for presence of the soluble receptor. ELISA measurement of sIL-6Rα in BAL fluid demonstrated a significant, 3-fold increase in bleomycin samples in comparison to PBS samples (Figure 3.2B). Western blot analysis of sIL-6Rα in BAL fluid confirmed this increase (Figure 3.2C). ELISA measurement of sIL-6Rα in plasma (which reflects systemic conditions) from these mice demonstrated no significant difference between bleomycin and PBS samples (Figure 3.2D), supporting the hypothesis that the increase in sIL-6Rα is a local occurrence in the lungs.
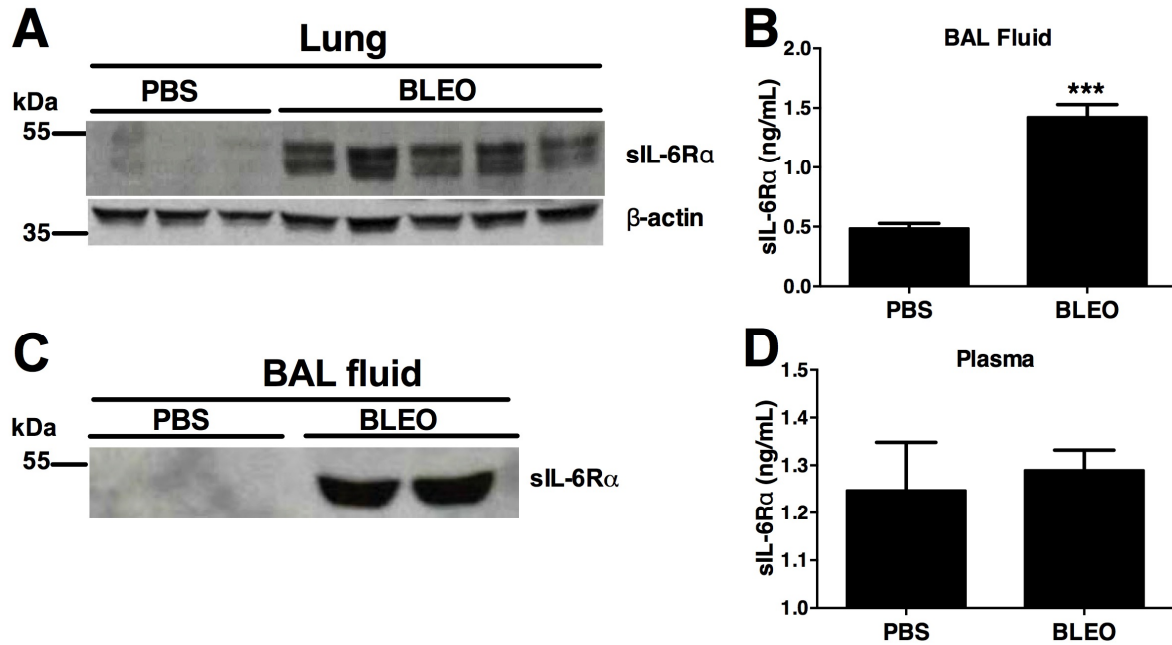
Figure 3.2. Soluble IL-6R alpha in bleomycin-induced pulmonary fibrosis. Soluble IL-6Rα expression was assessed in mice with pulmonary fibrosis. (**A**) Western blot analysis of sIL-6Rα in lung lysates, (**B** & **C**) ELISA measurement and western blot analysis of sIL-6Rα in bronchoalveolar lavage fluid (BAL fluid), and (**D**) ELISA measurement of sIL-6Rα in plasma from wildtype C57Blk/6 mice given saline or bleomycin, day 33. All data presented as mean ± SEM, n≥4. *significant difference from PBS-treated cohort; *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$.
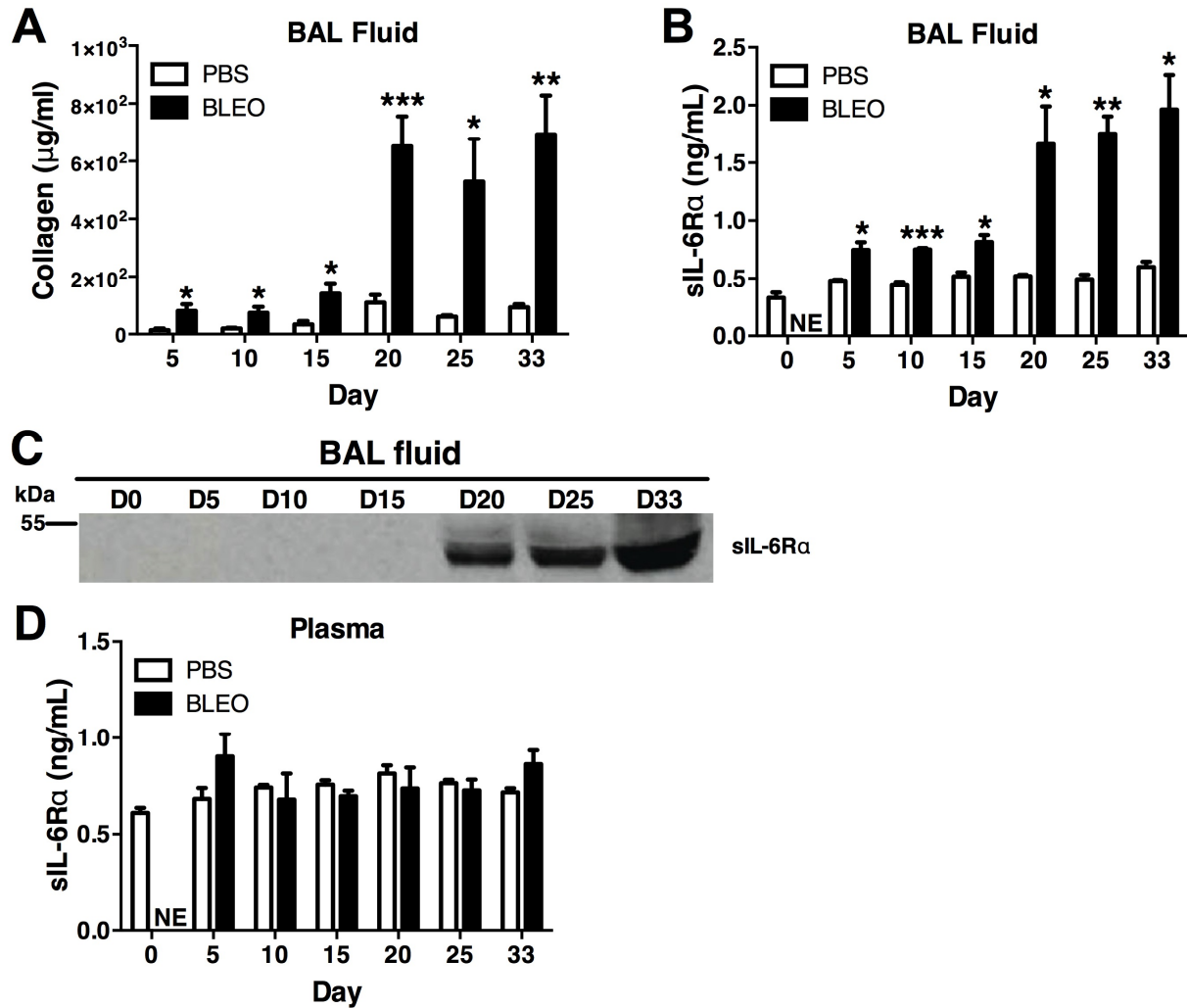
Figure 3.3. Soluble IL-6R alpha as disease develops in bleomycin-induced pulmonary fibrosis. Soluble IL-6Rα expression was assessed in mice with pulmonary fibrosis. (**A**) Sircol analysis of soluble collagen in BAL fluid over time in a chronic bleomycin mouse model. (**B**) ELISA quantification and (**C**) western blot analysis of sIL-6Rα in BAL fluid and (**D**) ELISA measurement of sIL-6Rα in plasma during development and progression of pulmonary fibrosis in the model. Results from A obtained by Ernestina Melicoff, MD. All data presented as mean ± SEM, n≥4. *significant difference from PBS-treated cohort; *= p<0.05, **= 0.001<p<0.01, ***= p<0.001.
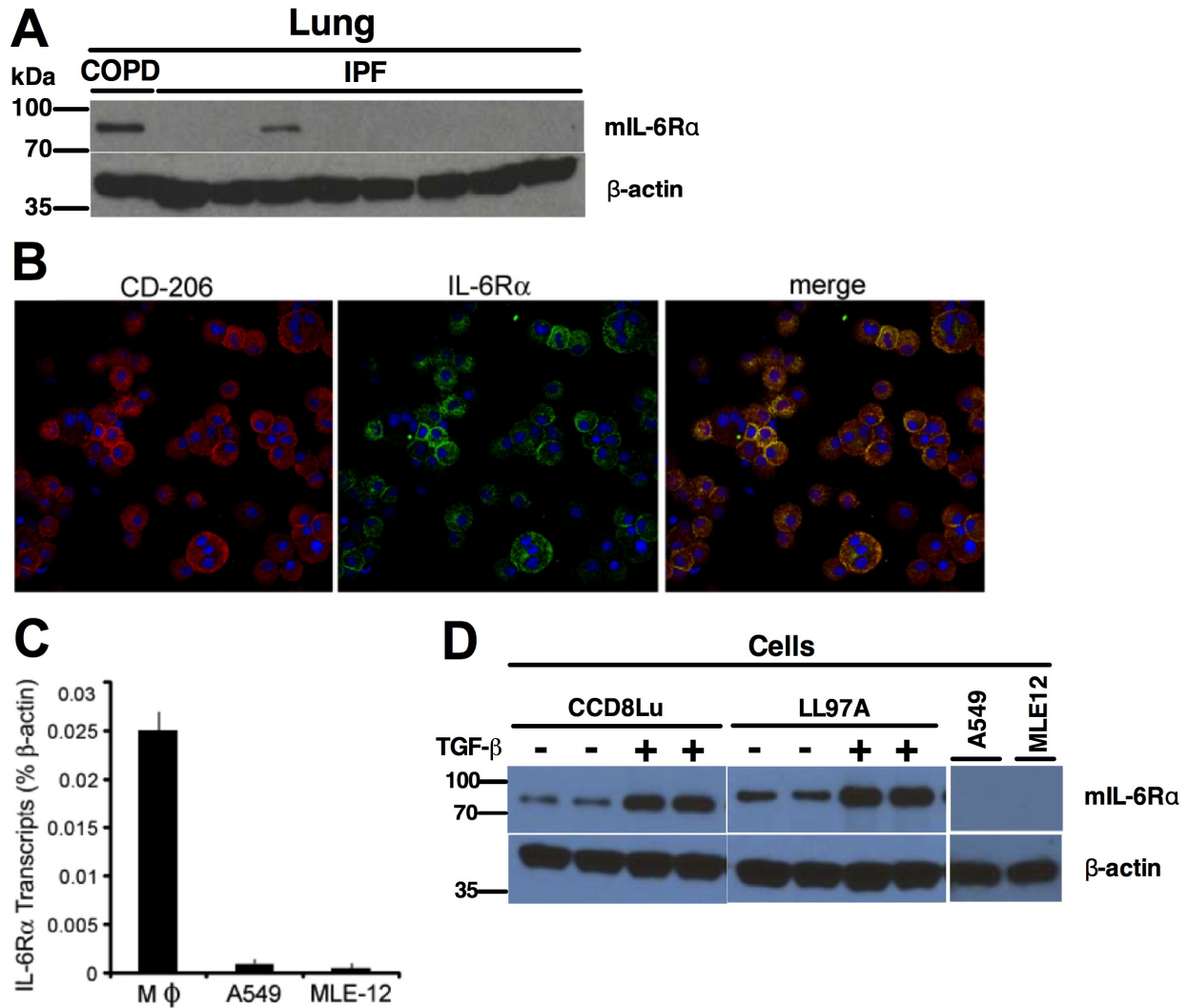
Figure 3.4. Membrane IL-6R alpha expression in human IPF lungs. (**A**) Western blot analysis of mIL-6Rα in lung lysates from explanted COPD and IPF samples. (**B**) Dual immunofluorescence to detect mIL-6Rα on macrophages isolated from IPF patients; CD-206 (red) = marker of M_2 macrophages, human IL-6Rα (green), dapi (blue). (**C**) Q-rtPCR measurement of IL-6Rα transcript in macrophages isolated from BAL fluid of IPF patients and in type II pneumocyte cell lines (A549 and MLE-12). (**D**) Western blot analysis of mIL-6Rα in protein lysates from control and IPF fibroblast cell lines (CCD8Lu and LL97A, +/- TGF-β) and type II pneumocyte cell lines. Results in B & C obtained by Yang Zhou, PhD.

3.3 - Soluble IL-6R alpha increases as pulmonary fibrosis develops and progresses.

We next asked how sIL-6Rα levels change as pulmonary fibrosis develops and progresses. BAL fluid was collected at various time points during the course of the model (days 5, 10, 15, 20, 25 and 33). Analysis of soluble collagen in BAL fluid revealed progressive development of fibrosis in the model, with significant increases in collagen already evident in bleomycin lungs by day 5 and the most prominent increase present by day 20 (Figure 3.3A). ELISA quantification of the samples showed a temporal increase in sIL-6Rα (Figure 3.3B) that mirrors the changes in collagen, which has never been shown before. This pattern was confirmed in a second manner by western blot analysis of sIL-6Rα in BAL fluid (Figure 3.3C). ELISA quantification of sIL-6Rα in plasma samples revealed no significant difference over the course of the model or between PBS and bleomycin samples (Figure 3.3D), again supporting the pattern seen in IPF samples of a local increase in sIL-6Rα in fibrotic areas. Collectively, the above findings demonstrate an association between increases in soluble IL-6Rα and increasing pulmonary fibrosis and suggest a role for the soluble receptor in disease onset and progression.

3.4 – Membrane IL-6R alpha expression is elevated in alveolar macrophages and pulmonary fibroblasts from Idiopathic Pulmonary Fibrosis lungs.

Recognizing that IL-6 classical signaling can play a pivotal role in disease pathogenesis, we asked whether the impact of this pathway is detrimental, neutral or protective in IPF. So, we proceeded to determine whether or not IL-6 classical signaling was elevated in IPF lungs.

Protein lysates from explanted COPD and IPF lung tissues were subjected to western blot analysis to determine expression of membrane IL-6Rα. Western blotting revealed decreased or absent expression of mIL-6Rα in IPF lungs in comparison to COPD lungs (Figure 3.4A). In the

absence of normal lung lysates for a control, we had to use COPD lung samples as relative controls. As such, it is unclear whether this pattern of mIL-6Rα expression in IPF lungs represents a deviation from the norm or not.

Examination of mIL-6Rα expression in specific cell types in the lung yielded more informative results. Bronchoalveolar lavage was performed on an IPF patient and alveolar macrophages were isolated from BAL fluid. These macrophages underwent immunofluorescence staining for detection of macrophage mannose receptor (CD206) and membrane IL-6Rα expression. CD206 is a marker of alternatively activated macrophages, or M_2 macrophages. M_2 macrophages are found in the lungs of IPF patients and mice with bleomycin-induced pulmonary fibrosis and are reported to drive progression of disease(31, 33, 113-117). Immunofluorescence staining for CD206 in BAL cells from an IPF patient revealed that these cells were mainly M_2 in phenotype. Immunofluorescence staining to detect membrane IL-6Rα on these cells demonstrated a significant proportion expressed membrane IL-6Rα (Figure 3.4B). Q-rtPCR analysis of the isolated macrophages was performed to assess expression of membrane IL-6Rα. IL-6Rα transcript was elevated in these macrophages (Figure 3.4C), confirming the increase in membrane protein. In comparison, type II airway epithelial cell lines, A549 and MLE-12, exhibited very low transcript levels of IL-6Rα (Figure 3.4C). Western blot analysis of mIL-6Rα expression in protein lysates from these cells revealed absence of mIL-6Rα at baseline (Figure3.4D). Evaluation of control and IPF fibroblast cell lines (CCD8Lu and LL97A) revealed that fibroblasts express mIL-6Rα at baseline (Figure 3.4D). Stimulation of these fibroblasts with transforming growth factor beta (TGF-β) induced their differentiation into myofibroblasts, which enhanced expression of mIL-6Rα.

In all, these studies allowed us to draw 2 conclusions. The first is that M_2 macrophages, fibroblasts and myofibroblasts are capable of IL-6 classical signaling while type II pneumocytes are not. All are still susceptible to *trans* signaling in the presence of the soluble receptor. The second conclusion is that M_2 macrophages and fibroblasts and myofibroblasts from fibrotic lungs are all potential cellular sources of shedding of mIL-6Rα to produce sIL-6Rα. Though soluble IL-6Rα can be generated via translation of alternatively spliced mRNA, protease-mediated cleavage of membrane IL-6Rα is largely responsible for the generation of sIL-6Rα(44, 56-61, 63, 70). Thus these potential cellular sources of sIL-6Rα may prove instrumental in the disease process.

3.5 – *STAT3 activation is increased in Idiopathic Pulmonary Fibrosis.*

Both IL-6 classical and *trans* signaling lead to phosphorylation and activation of STAT3, which acts as a transcription factor to regulate target genes(49-52). The presence of phospho-STAT3 is used as an indication of IL-6-mediated cellular activation (Figure 1.1).

As part of the attempt to further characterize IL-6 signaling in IPF, we assessed activation of STAT3 via immunostaining of lung sections from IPF patients and western blot analysis of protein lysates from explanted IPF lung samples. Both immunostaining and western blot revealed increased P-STAT3 in IPF samples (Figure 3.5A&B). Immunopositivity for P-STAT3 (brown) was seen in various cell types in IPF lungs, including what appeared morphologically to be macrophages, fibroblasts/myofibroblasts and epithelial cells (Figure 3.5A). These results suggest that, in IPF lungs, macrophages and fibroblasts/myofibroblasts could have been activated either by IL-6 classical and/or *trans* signaling while type II pneumocytes appear to have been influenced by IL-6 *trans* signaling.
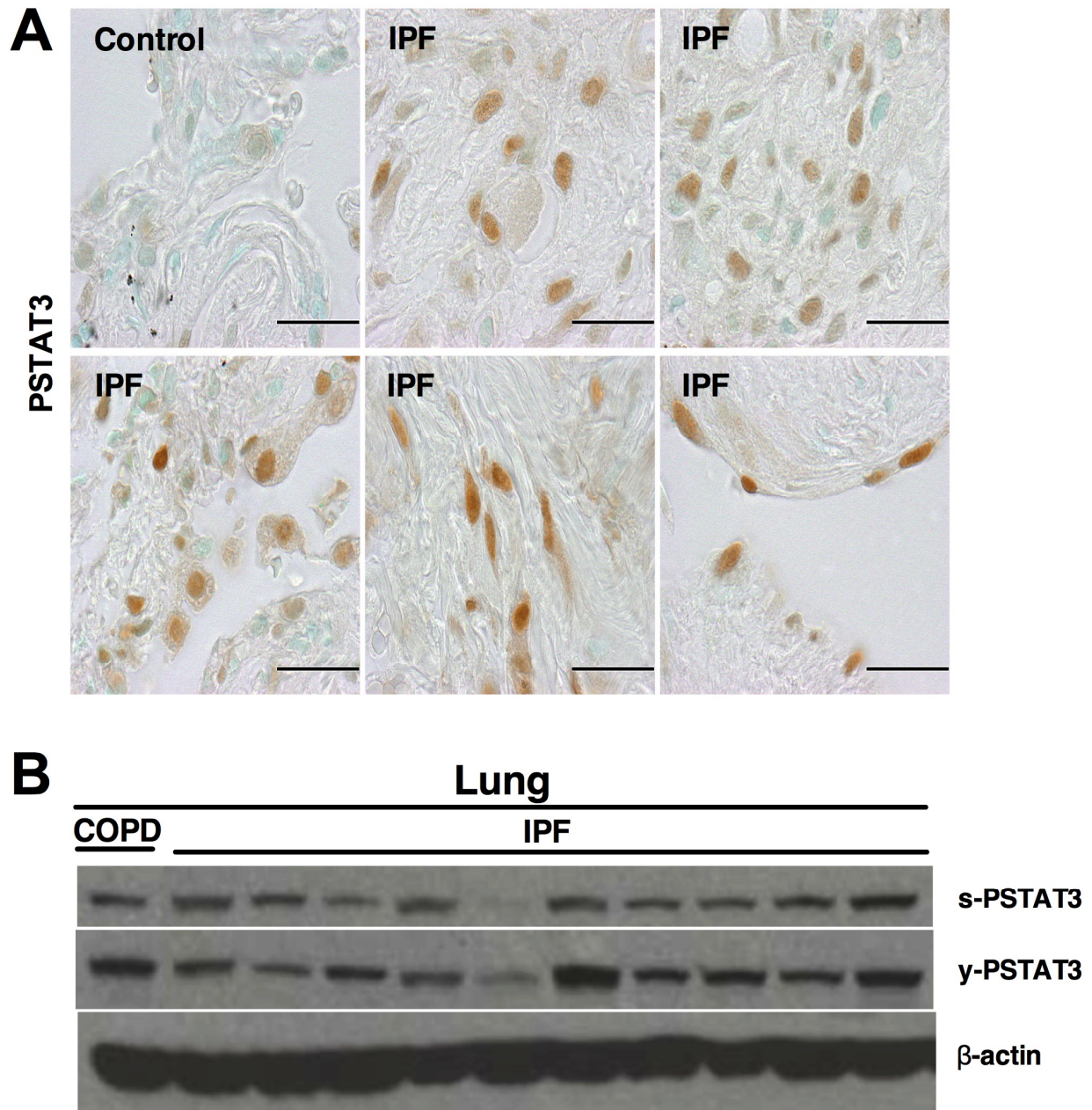
Figure 3.5. Activation of Signal Transducer and Activator of Transcription 3 (STAT3) in IPF lungs. (**A**) Immunostaining for phospho-STAT3 in lung sections from COPD and IPF patients. Scale bars: 50μm, 100x oil immersion. (**B**) Western blot analysis of phospho-STAT3 in lung lysates from explanted COPD and IPF samples.

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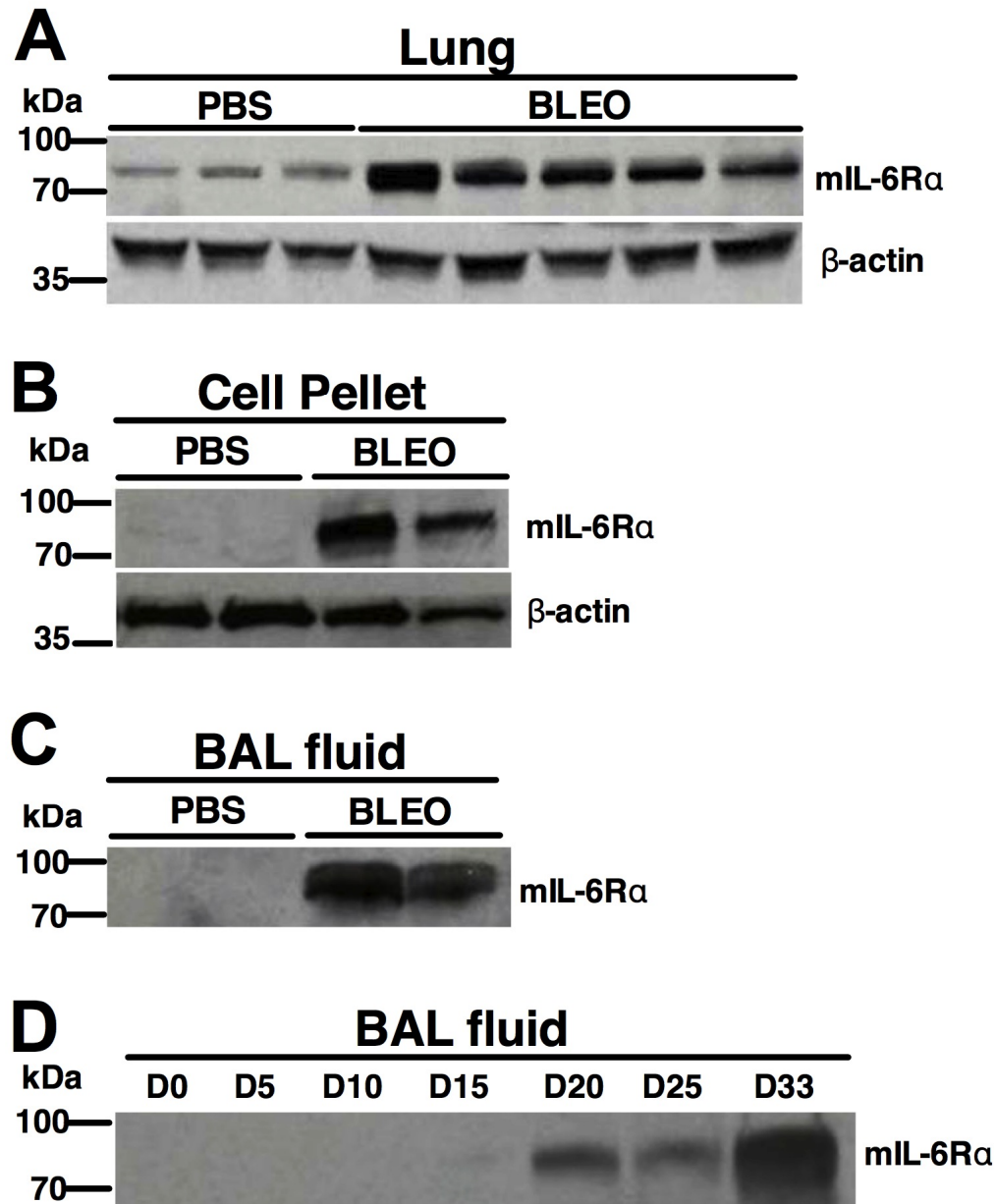
Figure 3.6. Membrane IL-6R alpha in bleomycin-induced pulmonary fibrosis. Membrane IL-6Rα expression was assessed in mice with pulmonary fibrosis. Western blot analysis of mIL-6Rα in (**A**) lung lysates, (**B**) BAL cell lysates and (**C**) BAL fluid from wildtype C57Bl/6 mice given saline or bleomycin, day 33. (**D**) Western blot analysis of mIL-6Rα in BAL fluid during development and progression of pulmonary fibrosis in a chronic bleomycin mouse model.

3.6 - Membrane IL-6R alpha is elevated in bleomycin-induced pulmonary fibrosis and increases as pulmonary fibrosis develops and progresses.

Our evaluation of membrane IL-6Rα in IPF lungs indicated variable expression in parenchymal tissue but elevation of membrane IL-6Rα was seen in M_2 alveolar macrophages. As such, we wanted to further understand classical signaling in our mouse model to better understand its role in IPF lungs.

To determine if IL-6 classical signaling was elevated in mouse fibrotic lungs, we performed western blot analysis of membrane IL-6Rα was on protein lysates from day 33 PBS and bleomycin-exposed mouse lungs. Membrane IL-6Rα expression was present in PBS-injected, non-fibrotic lungs and enhanced in bleomycin-exposed, fibrotic lungs (Figure 3.6A). Western blot analysis of mIL-6Rα in cells washed out of the lung during bronchoalveolar lavage revealed miniscule levels in PBS samples and visibly increased expression in bleomycin samples (Figure 3.6B). These cells have been shown to be primarily M_2 activated macrophages from our analysis (Figure 3.4B) and previous studies[33],[31, 113-117]. Bronchoalveolar lavage (BAL) fluid was collected and analyzed for presence of the membrane receptor in the airways and airspaces of the lung. Western blot analysis of mIL-6Rα in BAL fluid demonstrated a significant increase in day 33 bleomycin samples in comparison to PBS samples (Figure 3.6C).

To better understand how mIL-6Rα levels changed over the course of development and progression of pulmonary fibrosis, BAL fluid was collected at various time points during the course of the model (days 5, 10, 15, 20, 25 and 33). Western blot analysis of the samples showed a temporal increase in mIL-6Rα (Figure 3.6D) that mirrors the changes in collagen and sIL-6Rα. Collectively, these findings demonstrate an association between increases in membrane IL-6Rα

and increasing pulmonary fibrosis and suggest a role for the membrane receptor in disease onset and progression in our bleomycin-induced model of pulmonary fibrosis.

3.7 - STAT3 is activated in fibrotic lungs and fibroblasts, myofibroblasts and macrophages are capable of classical signaling while type II pneumocytes are not.

To determine if there was increased IL-6 activity in fibrotic, bleomycin-exposed mouse lungs, western blot analysis for expression of phospho-STAT3 was performed in lung lysates from day 33 PBS-injected and bleomycin-treated mice. Results revealed an increase in P-STAT3 in bleomycin lungs in comparison to PBS lungs (Figure 3.7A), suggesting increased IL-6 signaling.

To understand where this increased IL-6 signaling is occurring in fibrotic lungs, sections were cut from day 33 PBS and bleomycin lungs and immunostained for phospho-STAT3. Day 33 lungs were positive for P-STAT3 in what appeared morphologically to be fibroblasts/myofibroblasts, macrophages and type II airway epithelial cells (AEC) (Figure 3.7B-E). To confirm, we performed dual staining on these lung sections for co-localization of P-STAT3 and cell-specific markers, including: arginase 1 (for activated macrophages), pro-surfactant protein C (for type II pneumocytes), FSP1 (fibroblast specific protein 1 – to identify fibroblasts), and α-smooth muscle actin (marker of myofibroblasts). Dual staining revealed activation of STAT3 in fibroblasts, myofibroblasts, type II pneumocytes and activated macrophages in bleomycin lungs (Figure 3.7B-E). These findings validate what was observed in IPF samples, confirming that these cell types in fibrotic lungs are responsive to IL-6 signaling. Whether activation of STAT3 in these cells, in IPF lungs as well as bleomycin-exposed lungs, was due to IL-6 classical or *trans* signaling or both remain to be determined.
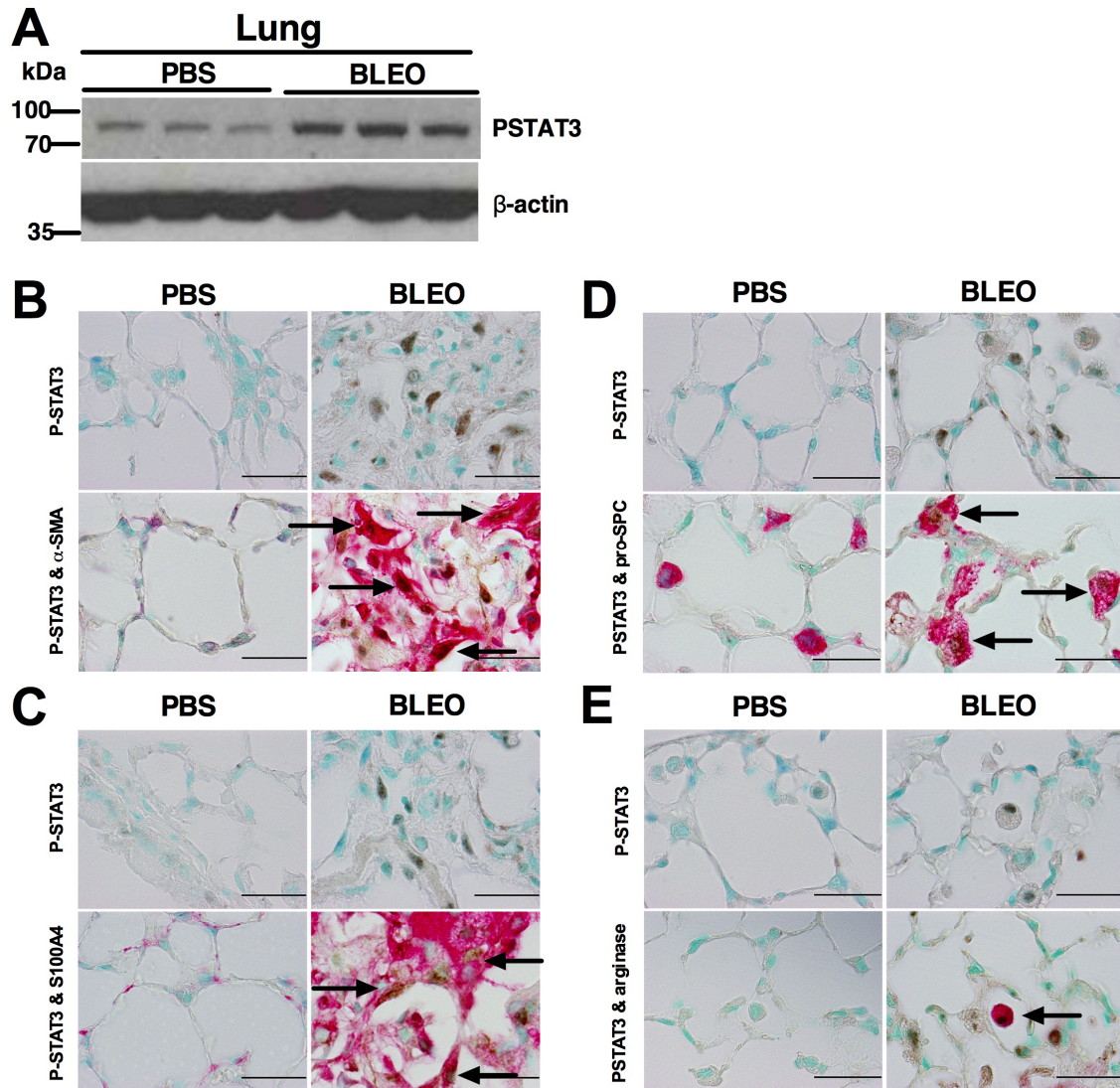
Figure 3.7. STAT3 activation and membrane IL-6R alpha expression in pulmonary cell types. (**A**) Western blot analysis of phospho-STAT3 in whole lung lysates from wildtype C57Blk/6 mice given saline or bleomycin, Day 33. (**B-E**) Day 33 lung sections from control and bleomycin-exposed mice were immunostained for P-STAT3 alone and P-STAT3 dual stains with cell-specific markers for pulmonary myofibroblasts, fibroblasts, type II pneumocytes, and macrophages. Arrows denote dual-positive cells. Images are representative of n≥4 animals from each group. Scale bars: 50μm, 100x oil immersion.

Collectively, the experiments in sections 3.5-3.7 helped delineate cell types susceptible to classical versus *trans* signaling and identified potential cellular sources of generation of soluble IL-6Rα in the fibrotic lung, key processes that regulate pulmonary fibrosis.

DISCUSSION

The experiments presented in this chapter were designed to characterize IL-6 classical and *trans* signaling pathways and their activation during onset and progression of pulmonary fibrosis in order to assess the significance of each pathway, particularly *trans* pathway.

Soluble IL-6Rα (sIL-6Rα) is a key feature of IL-6 signaling and a crucial component in the regulation of IL-6 responses. Soluble IL-6Rα acts agonistically *in vivo* to either enhance IL-6 signaling on cells already expressing membrane IL-6Rα or render cells lacking mIL-6Rα susceptible to the effects of IL-6 signaling(44, 71). Elevations of sIL-6Rα have previously been demonstrated in a number of human diseases(73, 74, 76, 78) but not in IPF.

A major observation in this study was that soluble IL-6Rα is elevated in IPF lungs and in mice with established pulmonary fibrosis (Figures 3.1&3.2), indicating its production and presence may play a role in the disease process. This is in agreement with previous studies that have demonstrated elevations in sIL-6Rα in established disease states(73, 74, 76, 78, 81, 110). Ours, however, is the first to show that sIL-6Rα levels in the lungs increased in a temporal pattern associated with the development and progression of pulmonary fibrosis in a mouse model (Figure 3.3). This suggests involvement of IL-6 *trans* signaling in onset and progression of pulmonary fibrosis. This knowledge raises the possibility of sIL-6Rα serving as a marker of staging disease severity and progression. It also supports the concept of sIL-6Rα as a potential target for therapeutic neutralization in pulmonary fibrosis. Interestingly, there is evidence that the

increase in soluble IL-6Rα in IPF is a local occurrence, only seen in a fibrotic area of the lung (Figure 3.1A). This data was supported by our mouse model findings and would suggest that sIL-6Rα could be used as a diagnostic marker, perhaps in localizing sites of active fibrosis. It may also serve prognostic values. Further investigation of soluble IL-6Rα in more IPF lavage and lung samples from fibrotic and non-fibrotic lobes is needed to validate these initial human sample findings.

To thoroughly understand the molecular mechanisms involved in regulating pulmonary fibrosis, we also characterized the presence of IL-6 classical signaling in IPF lungs and bleomycin-exposed, fibrotic mouse lungs by evaluating mIL-6Rα expression. We have seen variable mIL-6Rα expression in IPF lung samples (Figure 3.4A). Due to lack of normal lung samples for negative controls, we have had to make our comparisons of IPF findings relative to those in COPD. As such, our conclusions with regards to mIL-6Rα expression in IPF lung lysates are reported cautiously and we are aware further evaluation is necessary. In the IPB mouse model, however, IL-6 classical signaling appears enhanced as evident by elevated membrane IL-6Rα expression in whole lung protein lysates in comparison to PBS lungs (Figure 3.6). This begs the question of how much our findings about IL-6 classical signaling in the IPB model will translate to the human disease. We did, however, determine that there is increased STAT3 activation (indicative of increased IL-6 signaling) in both IPF and bleomycin-exposed lungs (Figures 3.5&3.7). Further analysis, with normal lung for negative controls, is needed in more whole lung human IPF samples to determine if mIL-6Rα is elevated or not in IPF.

Despite tentative conclusions about mIL-6Rα expression in parenchymal IPF samples, examination of classical signaling in specific cell types revealed some interesting data. Our studies clearly indicate that IL-6 classical signaling is enhanced in the bronchoalveolar

compartment of IPF lungs, specifically in M_2 alveolar macrophages. This finding is in agreement with Matsumoto et al, who reported increased mIL-6Rα expression in colonic macrophages in colitis-affected tissue (78). In our study, we were able to show augmented mIL-6Rα expression in macrophages collected via bronchoalveolar lavage of IPF lungs (Figure 3.4B). To our knowledge, this is the first study to illustrate elevations in mIL-6Rα in M_2 alveolar macrophages from IPF patients. This observation of enhanced mIL-6Rα in M_2 macrophages was confirmed in the IPB model, where membrane IL-6Rα expression was elevated in BAL cell lysates in comparison to PBS lungs (Figure 3.6). We know from the literature that M_2 alveolar macrophages are implicated in pulmonary fibrosis(31, 113-117). Thus, it is of interest to assess what role, if any, membrane IL-6Rα (and therefore IL-6 classical signaling) plays in regulating the phenotype and actions of these macrophages. The finding of increased mIL-6Rα expression in M_2 macrophages informs us that these cells are capable of classical and *trans* signaling and that they may serve as a potential source of shedding to produce the soluble IL-6Rα. Membrane IL-6Rα has been shown to be shed from cell surfaces of peripheral monocytes, bronchial epithelia cells, hepatocytes, neutrophils and T cells(38, 59, 61-64). Future experiments are needed to determine if M_2 macrophages do shed their membrane IL-6Rα to generate soluble IL-6Rα in fibrotic lungs. If they do, it would support a pathogenic role for these cells in pulmonary fibrosis.

We also evaluated mIL-6Rα expression in fibroblasts, myofibroblasts and type II pneumocytes. Current understanding of the pathogenesis of IPF identifies two cellular targets as key players in disease initiation and progression – fibroblasts and epithelial cells(118, 119). Epithelial injury and dysregulated regeneration is thought to initiate disease(118) while aberrant fibroblast activation, differentiation into myofibroblasts, and excessive collagen and fibronectin

production and deposition in the lungs propagate the problem(2). Our study provides further insight into the characterization of these cells in disease. We found that fibroblasts and myofibroblasts are capable of IL-6 classical signaling while type II pneumocytes are not. All are still susceptible to *trans* signaling in the presence of the soluble receptor. Our data indicate that fibroblasts and myofibroblasts from fibrotic lungs express more mIL-6Rα than those from normal lungs, so they are also potential cellular sources of shedding of mIL-6Rα to produce sIL-6Rα. Further examination is required to understand the impact of IL-6 classical and *trans* signaling in these individual cells.

Collectively, the findings in this aim illustrate the existence of IL-6 *trans* signaling in IPF lungs and bleomycin-induced fibrotic mouse lungs. They demonstrate that soluble IL-6Rα is present and increases in association with pulmonary fibrosis and expression of membrane IL-6Rα is elevated in fibrosis. This suggests that IL-6 classical signaling is enhanced and *trans* signaling is involved in the disease process in fibrotic lungs. Thus, there is a need to characterize the generation of soluble IL-6Rα in fibrotic lungs (Chapter 4) and to assess the role of IL-6 *trans* signaling in pulmonary fibrosis (Chapter 5). Chapter 4 will examine the role of protease-mediated generation of sIL-6Rα and Chapter 5 will evaluate the effects of *in vivo* neutralization of IL-6 *trans* signaling.

CHAPTER 4

ADAM17 Sheds Interleukin-6 Receptor Alpha (IL-6Rα) in a Chronic Bleomycin Murine Model of Pulmonary Fibrosis

EXPERIMENTAL RATIONALE AND KEY QUESTIONS

From experiments presented in the last chapter characterizing IL-6 *trans* signaling in pulmonary fibrosis, we know that soluble IL-6Rα is expressed in fibrotic lungs and increases in association with increasing fibrosis. This points to a role for sIL-6Rα in pulmonary fibrosis and emphasizes a need to evaluate the mechanism of its generation.

Soluble IL-6Rα is generated largely through protease-mediated cleavage of membrane-bound IL-6Rα(44, 58, 70) (Figure 1.2). ADAM17 (A Disintegrin and Metaolloproteinase 17) is a membrane-bound metalloprotease belonging to the ADAM (a disintegrin and metalloproteinase) family that is responsible for cleaving cell surface proteins. ADAM17 has been implicated as the main protease responsible for shedding IL-6Rα from cell membrane surfaces of hepatocytes, peripheral monocytes, neutrophils and T cells(38, 59, 61-63). Increased levels of this protease have been found in association with diseases that have reported increased sIL-6Rα levels(66-69). A related protease, ADAM10, has been implicated in constitutive shedding of IL-6Rα, while ADAM17 is better known for rapid, inducible shedding(38, 61). The roles of both ADAM17 and ADAM10 in shedding IL-6Rα in fibrotic lungs have not been examined.

In an initial attempt to determine whether or not the generation of soluble IL-6Rα in fibrotic lungs involves the actions of ADAM17 and ADAM10, transcript levels of these proteases were assessed in mice with adenosine-mediated (ADA$^{-/-}$) or bleomycin-mediated pulmonary fibrosis. Microarray analysis of lungs from these mice was conducted, and results revealed a significant increase in ADAM17 and ADAM10 transcript in fibrotic lungs (Figure 4.0). This data suggested that these proteases may mediate IL-6Rα shedding and prompted us to look more closely at this mechanism in pulmonary fibrosis.
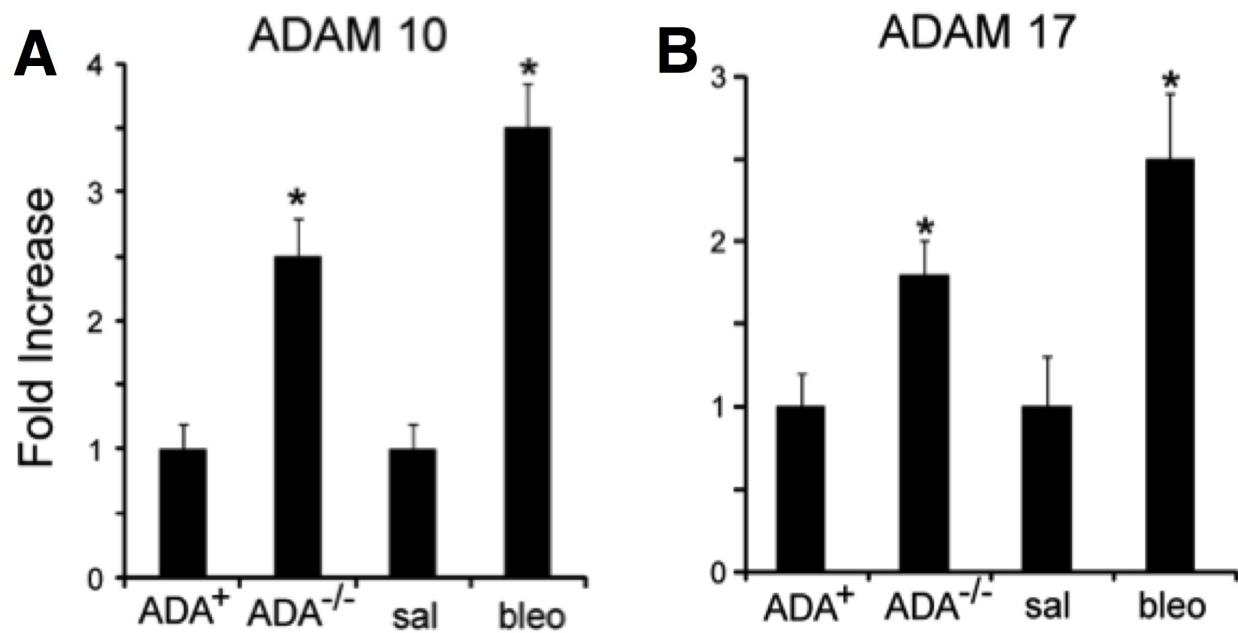
Figure 4.0. ADAM10 and ADAM17 transcripts in murine models of pulmonary fibrosis. Microarray analysis of (**A**) ADAM10 and (**B**) ADAM17 transcripts using mRNA isolated from lungs of ADA$^{+/-}$ and ADA$^{-/-}$ mice (day 42) and wild type C57Blk6 mice after exposure to saline or bleomycin (day 21). Results presented as mean fold increases ± SEM, n=3. *significant difference from ADA$^{+/-}$ or saline. Data provided by Tingting Weng, PhD.

Given the preliminary microarray results, we set out to test the hypothesis that protease-mediated shedding is the main mechanism of generation of soluble IL-6Rα in fibrotic lungs. In particular, we were interested in the role of ADAM17 in the process and wanted to answer the following questions:

4.1   ***Is ADAM17 elevated in bleomycin-induced pulmonary fibrosis?***

4.2   ***What happens to ADAM17 expression as pulmonary fibrosis develops and progresses?***

4.3-4.4   ***Can ADAM17 shed IL-6Rα from membranes of bone-marrow-derived macrophages? Can this process be blocked by pharmacologic inhibitors or small interfering RNA?***

4.5   ***Can ADAM17 shed IL-6Rα from membranes of primary alveolar macrophages from fibrotic lungs?***

RESULTS

4.1 - ADAM17 is increased in bleomycin-induced pulmonary fibrosis.

To assess the underlying mechanism of soluble IL-6Rα production in fibrotic lungs, we explored the role of the proteases ADAM 17 and ADAM 10 in cleaving membrane IL-6Rα to produce the soluble IL-6 receptor. To determine whether these proteases are elevated in pulmonary fibrosis, we evaluated their expression in the IPB model. Protein lysates were made from day 33 lungs, a stage when fibrosis was prominent, and western blotting was performed. Immature (130 kDa) and mature (93 kDa) forms of ADAM17 were increased in bleomycin-exposed, fibrotic lungs in comparison to PBS-exposed, non-fibrotic lungs (Figure 4.1A). No difference in ADAM10 expression was observed between bleomycin and PBS lungs (Figure 4.1B). Thus we focused our efforts on further characterizing ADAM17 expression.

Bronchoaveolar lavage (BAL) was performed in day 33 PBS and bleomycin-exposed mice. BAL fluid was analyzed for presence of ADAM17 in the bronchoalveolar compartment of the lung. Western blot analysis detected an increase in mature ADAM17 in bleomycin samples (Figure 4.1C). Immature ADAM17 was absent in PBS samples but present in bleomycin samples, indicating increased production of ADAM17. Immunostaining of lung sections from day 33 lungs revealed the most prominent increase of ADAM17 was in alveolar macrophages of bleomycin-exposed lungs (Figure 4.1D). This observation, in combination with results presented in the last chapter on expression of membrane and soluble IL-6Rα in IPF and bleomycin-induced fibrotic lungs, further supports the hypothesis that ADAM17 is responsible for generating the soluble IL-6 receptor in fibrotic lungs.
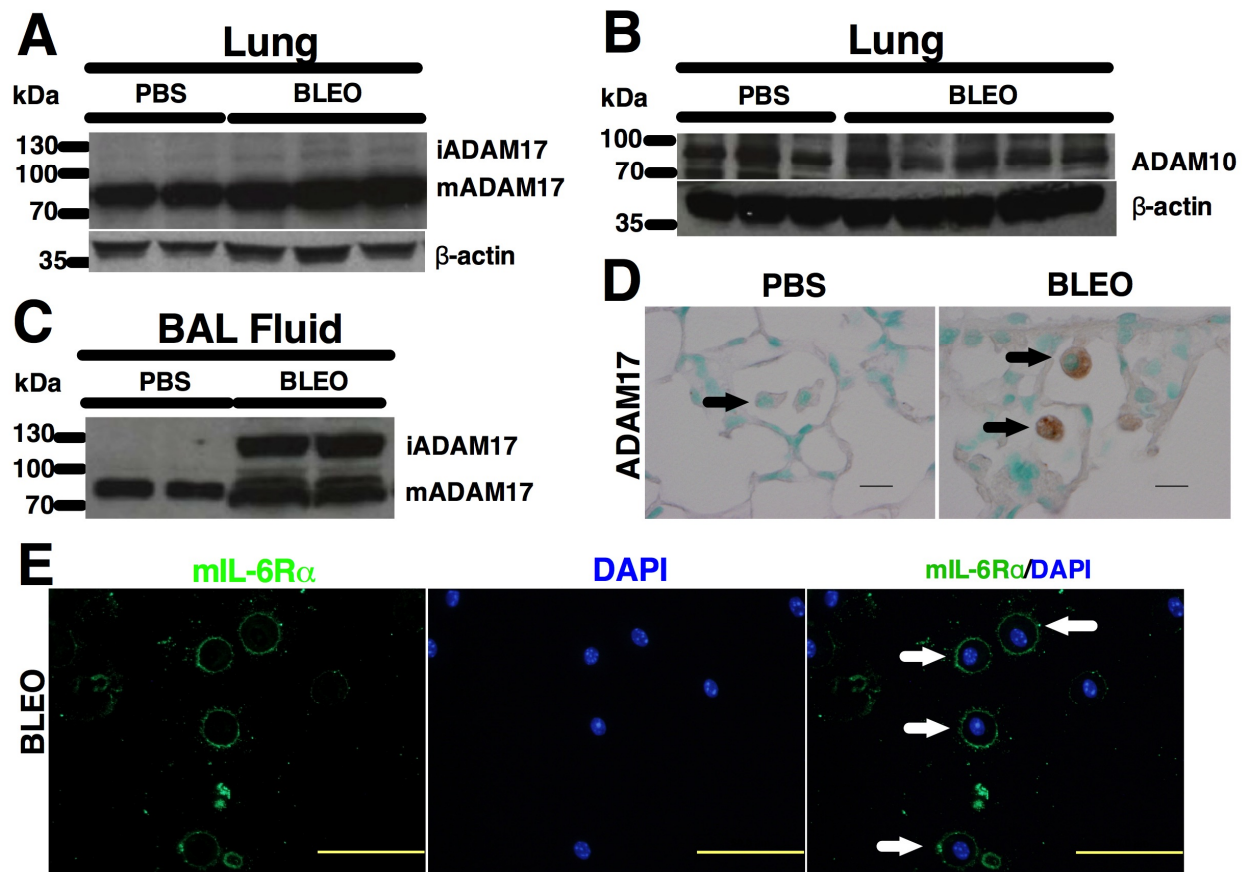
Figure 4.1. ADAM17 expression in bleomycin-induced pulmonary fibrosis. Expression of the protease ADAM17 was evaluated in mice with pulmonary fibrosis. Western blot analysis of (**A**) immature (130 kDa) and mature (89 kDa) forms of ADAM17 and (**B**) ADAM10 in lung lysates from wildtype C57Bl/6 mice given saline or bleomycin, day 33. (**C**) Western blot analysis of bronchoalveolar lavage fluid from day 33 mice. (**D**) Immunostaining for ADAM17 (brown) in the lungs of day 33 mice. Arrows denote positive cells. Images are representative of n≥4 animals from each group. Scale bars: 50μm, 100x oil immersion. (**E**) Immunofluorescence staining for membrane IL-6R alpha (green) and DAPI (blue) on BAL fluid cells. Arrows denote positive cells. Images are representative of n≥4 animals from each group, 20x magnification.

We know that macrophages isolated from IPF lungs expressed abundant mIL-6Rα (Figure 3.4B). We also saw that sIL-6Rα was increased in the bronchoalveolar compartment of fibrotic lungs (Figure 3.1 and 3.2B&C), suggesting that that might be where the soluble receptor was generated. In the last chapter, we reported increased expression of mIL-6Rα in BAL cells lavaged out of bleomycin-exposed lungs (Figure 3.6B). These cells are comprised mainly of alveolar macrophages but also include lymphocytes, neutrophils and eosinophils(33). The fact that there was an increase in ADAM17 in alveolar macrophages and these macrophages also expressed more membrane IL-6Rα than normal raise the possibility of ADAM17 acting in these cells to shed their membrane IL-6Rα to generate soluble IL-6Rα.

To provide secondary confirmation of the increase in mIL-6Rα in BAL macrophages seen on western blot analysis (Figure 3.6B), we isolated alveolar macrophages from day 33 bleomycin-exposed mouse lungs using bronchoalveolar lavage. The cells cytospun onto microscope slides and subjected to immunofluorescence staining for membrane IL-6Rα. It was evident that macrophages from day 33 bleomycin lungs had abundant expression of membrane IL-6Rα (Figure 4.1E). These findings confirm physical proximity of mature ADAM17 and membrane IL-6Rα to suggest a role for ADAM17 in shedding the membrane receptor.

4.2 - ADAM17 is increased in association with increasing soluble IL-6R alpha and alveolar macrophages in fibrotic lungs.

Further characterization of ADAM17 expression during the onset and development of fibrosis was achieved via western blot analysis of BAL fluid samples collected throughout the duration of the IPB model (days 5, 10, 15, 20, 25, and 33). Results indicated a temporal increase in ADAM17 expression (Figure 4.2A) that mirrored the increase in sIL-6Rα as pulmonary

fibrosis developed and progressed (Figure 3.3C). Specifically, just as sIL-6Rα became prominent in BAL fluid in day 20 samples, mature ADAM17 also started increasing with day 20 samples. Immature ADAM17 was absent in samples from the first 15 days of the model but appeared in BAL fluid samples from day 20. These results further strengthened our suspicions that ADAM17 is involved in generation of the soluble IL-6 receptor in fibrotic lungs.

Since our data had been pointing to the alveolar macrophage as the source of shedding, we wanted to determine if there was a temporal increase in alveolar macrophages in fibrotic lungs that would explain the temporal increase in ADAM17 in the bronchoalveolar compartment. Using BAL fluid samples collected throughout the duration of the IPB model from PBS and bleomycin mice, total BAL cell count was determined and cellular differentials performed. In comparison to total BAL cells from PBS-injected, non-fibrotic lungs, total cells from bleomycin-exposed, fibrotic lungs rose over time, starting with significant changes on day 20 (Figure 4.2B). Total BAL cells in bleomycin-exposed lungs dropped between day 25 and 33 but were still significantly different from those of PBS-exposed lungs. Analysis of cell differentials revealed that the number of BAL macrophages in these lungs also began increasing significantly on day 20, with maximum macrophage numbers in day 25 samples and a drop in numbers by day 33, though still significantly higher in comparison to controls (Figure 4.3C).

Collectively, these findings demonstrate an association between increases in alveolar macrophages and ADAM17 and increases in soluble IL-6Rα in fibrotic lungs, suggesting that ADAM17 activation in alveolar macrophages of fibrotic lungs induces shedding of membrane IL-6Rα to produce soluble IL-6Rα.
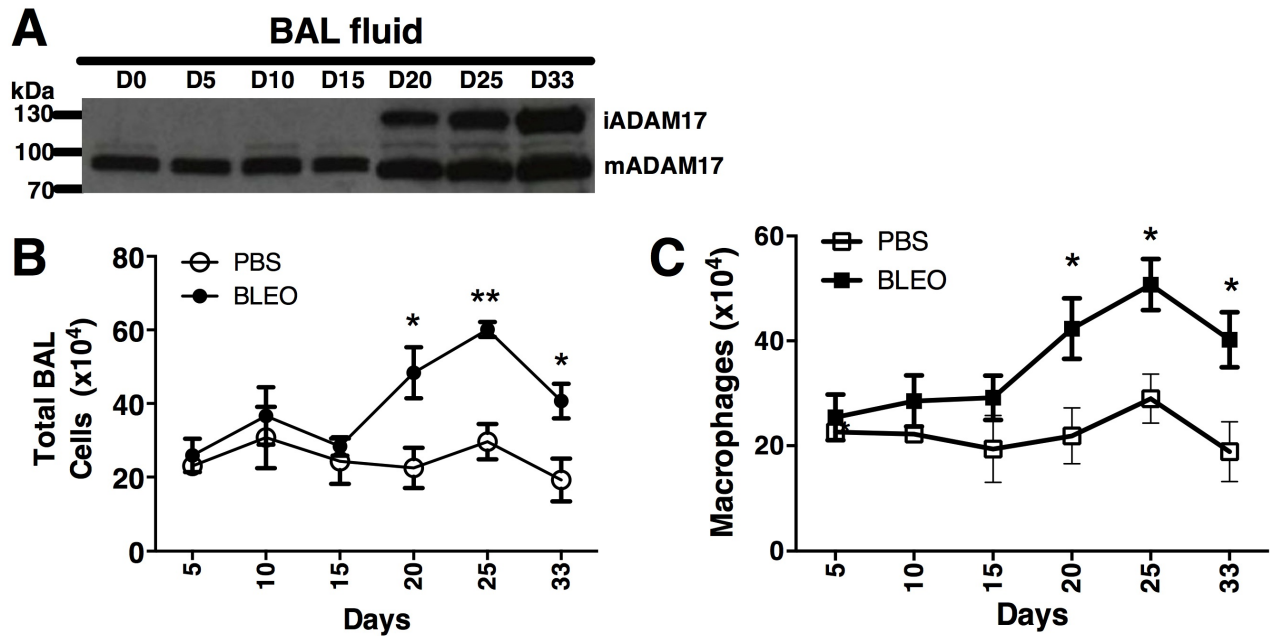
Figure 4.2. ADAM17 expression and alveolar macrophage accumulation in bleomycin-induced pulmonary fibrosis. (**A**) Western blot analysis of ADAM17 in BAL fluid samples over the course of the model, as pulmonary fibrosis develops and progresses secondary to chronic bleomycin exposure. (**B&C**) Total BAL cell and BAL macrophages in samples at various points during model. Data presented as mean ± SEM, n≥4. *significant difference from PBS-treated cohort. *= p<0.05, **= 0.001<p<0.01, ***= p<0.001. Data from B-C obtained by Tina Melicoff, MD, Tingting Weng, PhD and Harry Karmouty-Quintana, PhD.

4.3-4.5 – ADAM17 mediates shedding of IL-6R alpha from bone marrow derived M_2 activated

macrophage membranes.

Given the previous data, we suspected that the generation of sIL-6Rα revolved around the alveolar macrophages in fibrotic lungs that co-express high levels of ADAM17 and mIL-6Rα. Alveolar macrophages found in the lungs of IPF patients and mice with bleomycin-induced pulmonary fibrosis are primarily M_2 in phenotype and are reported to drive progression of disease(31, 113-117). We asked whether we could replicate the *in vivo* conditions using an *in vitro* cell system and show that activation of ADAM17 in M_2 macrophages induces shedding of membrane IL-6Rα to increase production of soluble IL-6Rα.

To generate M_2 macrophages *in vitro*, we used the protocol detailed in Figure 4.3A (courtesy of Dr. Sandeep Agarwal's laboratory). We isolated bone marrow cells from wild-type C57Blk6 mice and differentiated them into macrophages in culture. Macrophages were then stimulated with interleukin-4 (IL-4) and interleukin-13 (IL-13). These cytokines were reported to be important in the lung and their use in various studies has led to successful differentiation of macrophages to the M_2 phenotype(120-123). Arginase 1 is a marker of M_2 macrophages, and its expression after stimulation has been reported to be an indicator of successful differentiation(114, 120, 122, 123). Western blot analysis of protein lysates from IL-4/IL-13-stimulated macrophages revealed more arginase 1 expression than in unstimulated macrophages (Figure 4.3B), suggesting they are M_2 in phenotype.
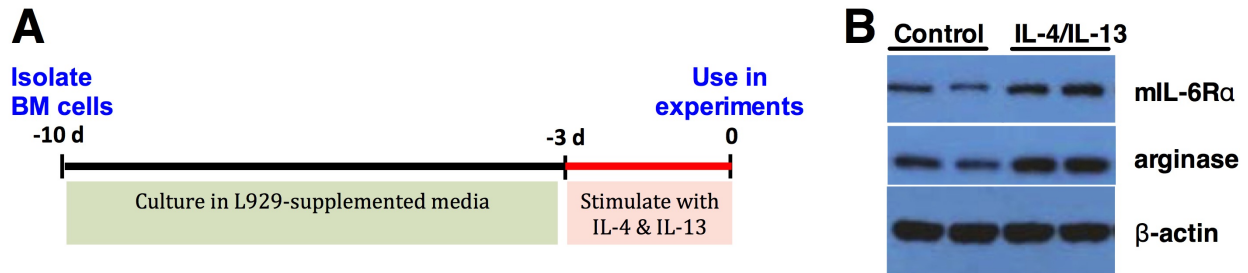
Figure 4.3. *In vitro* **culture and differentiation of bone marrow derived cells into M$_2$ macrophages.** (**A**) Experimental protocol for generating bone marrow derived M$_2$ macrophages. Bone marrow cells were isolated from the femurs of wild-type C57Blk6 mice and cultured in supplemented media for 1 week to induce macrophage development. After 1 week in culture, adherent macrophages were collected and replated then stimulated with IL-4 and IL-13 for 3 days to polarize their differentiation into M$_2$ macrophages. (**B**) Western blot analysis of membrane IL-6Rα and arginase 1 expression in bone marrow-derived macrophages stimulated with IL-4 and IL-13.
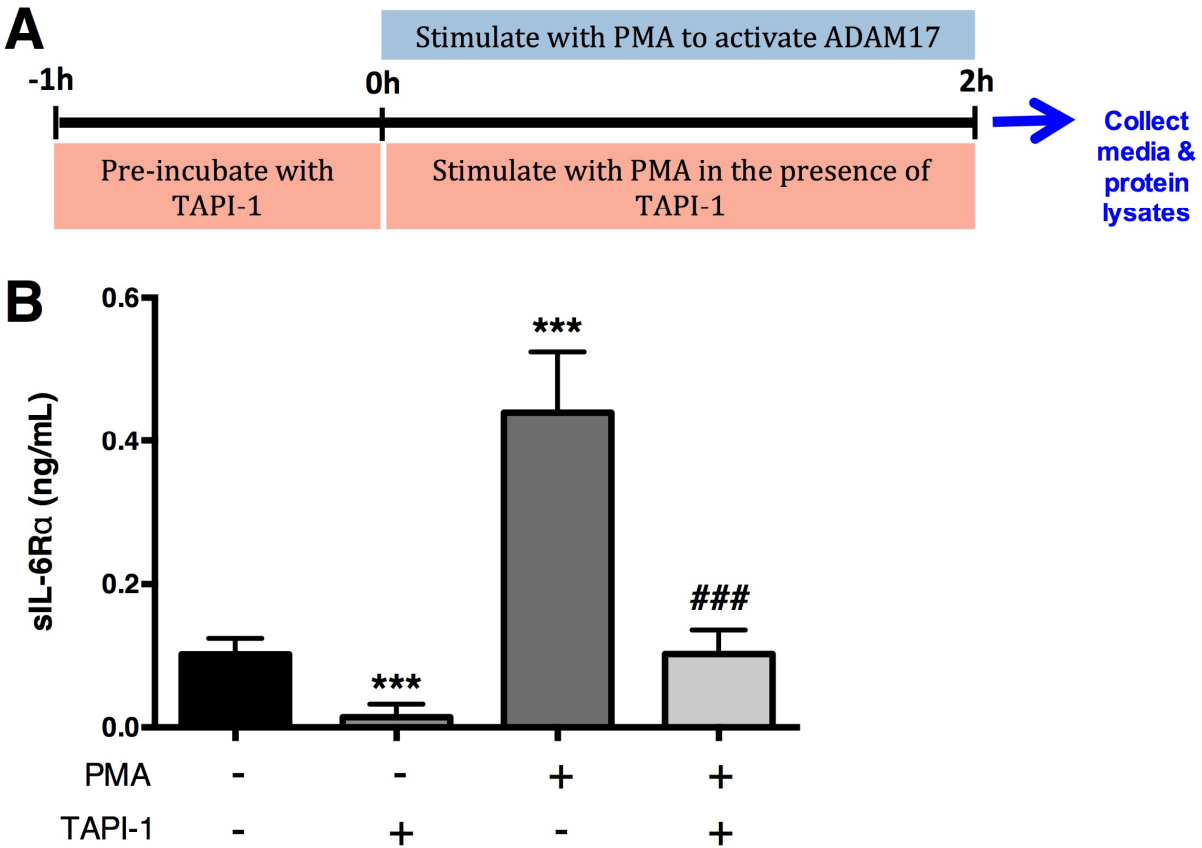
Figure 4.4. Generation of soluble IL-6Rα following pharmacologic neutralization of ADAM17 activity in bone marrow derived M$_2$ macrophages. (**A**) Bone-marrow-derived M$_2$ macrophages were either stimulated with PMA alone or pre-incubated with TAPI-1 and stimulated with PMA in the presence of TAPI-1. Culture media and cell lysates were collected after 2 hours. (**B**) ELISA measurement of soluble IL-6Rα in culture media of macrophages stimulated with PMA, with and without TAPI-1. All data presented as mean ± SEM, n≥6. *significant difference from media only cohort; #significant difference from PMA-stimulated cohort; *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$.
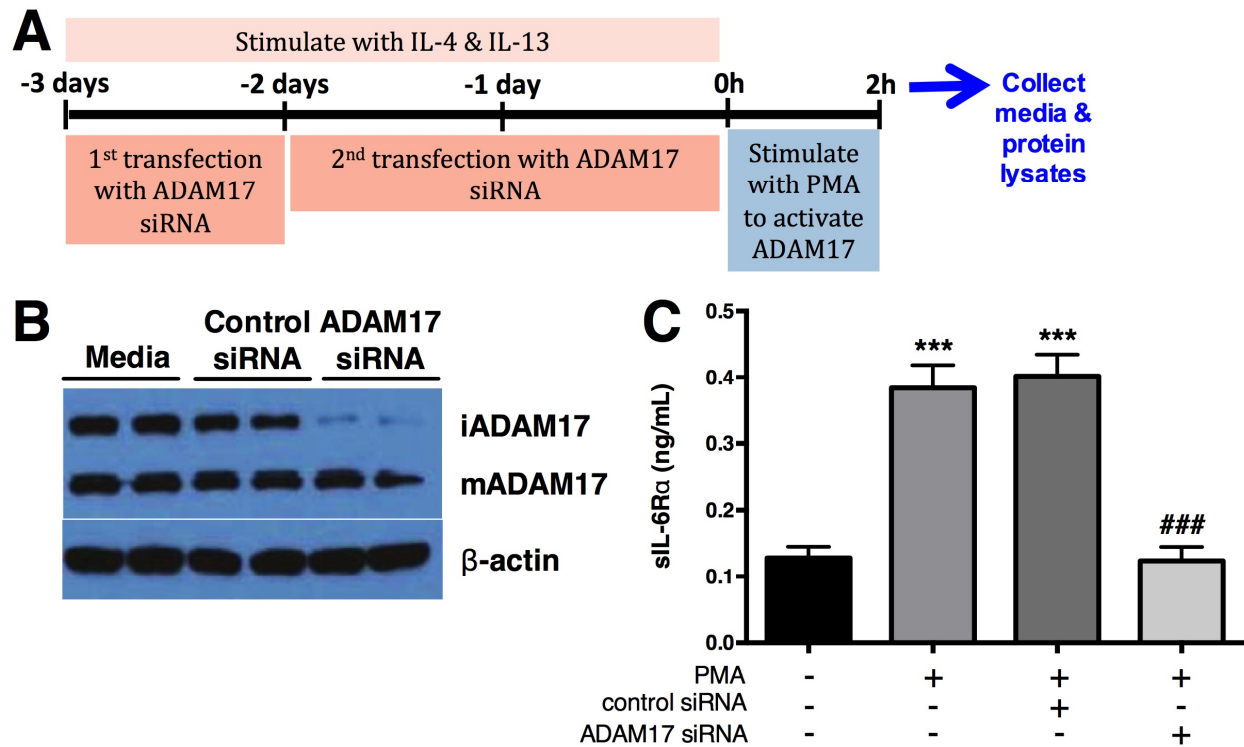
Figure 4.5. Generation of soluble IL-6Rα following siRNA-mediated silencing of ADAM17 activity in bone marrow derived macrophages. (**A**) Bone-marrow-derived macrophages were stimulated with IL-4 and IL-13 and transfected with control or ADAM17 siRNA. Macrophages are then stimulated with PMA. Culture media and cell lysates were collected after 2 hours. (**B**) Western blot analysis of ADAM17 in protein lysates of transfected macrophages. (**C**) ELISA measurement of sIL-6Rα in culture media of macrophages transfected with control or ADAM17 siRNA and then stimulated with PMA. All data presented as mean ± SEM, n≥6. *significant difference from media only cohort; #significant difference from PMA-stimulated cohort; *= p<0.05, **= 0.001<p<0.01, ***= p<0.001.

Next, we evaluated mIL-6Rα expression in the M₂ macrophages to see if the membrane receptor is present to be cleaved. Western blot analysis showed more mIL-6Rα in IL-4/IL-13 stimulated macrophages than unstimulated macrophages (Figure 4.3B). The characteristics of augmented ADAM17 and mIL-6Rα expression in our bone marrow derived M₂ macrophages mimicked those seen in alveolar macrophages isolated from IPF patients and fibrotic murine lungs (Figure 3.4B & 3.5B). Thus, we proceeded to use these cells in our shedding experiments.

The experimental protocols are illustrated in Figures 4.4A and 4.5A. The experiments involved using a phorbol ester to activate ADAM17 in bone marrow derived M₂ macrophages in order to induce shedding of IL-6R. Then, to support ADAM17's role in shedding, we blocked ADAM17 activity in the cells using a pharmacologic inhibitor or siRNA and observed changes to sIL-6Rα generation in response to phorbol ester stimulation.

The reagent phorbol 12-myrstate 13-acetate (PMA) is known to activate ADAM17-mediated shedding of membrane IL-6Rα(65, 124). IL-4/IL-13-stimulated macrophages were incubated with PMA to activate ADAM17 and induce shedding. To assess shedding efficiency, culture media was collected and sIL-6Rα was quantified using ELISA. PMA activation of ADAM17 led to a significant, 4-fold increase in soluble IL-6Rα in the culture media (Figure 4.4B), suggesting that ADAM17 is responsible for the increase in shedding.

To further support the role of ADAM17 in this process, we assessed whether blocking ADAM17 would alter the extent of shedding. We first attempted to block ADAM17 activity pharmacologically using TAPI-1, a non-selective inhibitor of ADAM proteases(59, 124) (Figure 4.4A). Addition of TAPI-1 to macrophages without PMA stimulation was able to significantly suppress baseline shedding; pre-incubation of macrophages with TAPI-1 resulted in inhibition of

sIL-6Rα release into the media with PMA stimulation (Figure 4.4B). These results demonstrated that ADAM17 mediated shedding of IL-6Rα from M_2 macrophages to generate sIL-6Rα.

We confirmed these results using a second, more specific method of neutralizing ADAM17 activity. Bone marrow derived M_2 macrophages were transfected with ADAM17 siRNA(69) to silence ADAM17 (Figure 4.5A). Successful silencing was confirmed by western blot analysis showing reduced expression of ADAM17 (Figure 4.5B). Macrophages were then stimulated with PMA. There was a 4-fold increase in release of sIL-6Rα from non-transfected macrophages and macrophages transfected with control siRNA. There was significant reduction in release of sIL-6Rα from macrophages transfected with ADAM17 siRNA (Figure 4.5B). Collectively, these findings support our hypothesis that ADAM17 activation in M_2 macrophages induces shedding of membrane IL-6Rα to increase production of sIL-6Rα in pulmonary fibrosis.

4.6 - ADAM17 promotes shedding of IL-6R alpha from alveolar macrophages collected from bleomycin-induced fibrotic lungs.

To more accurately reflect what happens in active pulmonary fibrosis, we repeated the above experiments, this time using primary alveolar macrophages isolated from day 33 mice treated with PBS or bleomycin. Lungs were thoroughly lavaged (10-12 times) to ensure evacuation of the majority of alveolar macrophages present in the bronchoalveolar compartment. There were significantly more cells collected from bleomcyin-exposed lungs than PBS lungs, consistent with our previous characterization studies, which showed higher total cell counts in BAL fluid, indicative of enhanced levels of pulmonary inflammation in bleomycin-exposed mice (Figure 4.2B). Cells collected in BAL fluid were then cultured for 4 hours in tissue culture plates to allow for adherence of alveolar macrophages. All other non-adherent cells were washed away

at the end of the incubation period. Adherent macrophages were then used in shedding experiments.

PMA-induced activation of ADAM17 in primary alveolar macrophages from PBS lungs induced an increase in soluble IL-6Rα in culture media (Figure 4.6). Pre-incubation with TAPI-1 reduced levels of released sIL-6Rα to below detection threshold. PMA stimulation of ADAM17 in macrophages from bleomycin lungs resulted in a 2-fold increase in sIL-6Rα in culture media (Figure 4.6). Pre-incubation with TAPI-1 reduced levels of released sIL-6Rα to baseline levels. This experiment validated findings from experiments with bone marrow derived M_2 macrophages and revealed that ADAM17 can shed IL-6R from primary alveolar macrophages.

Overall, the results of our shedding experiments, both in bone marrow derived M_2 macrophages and in primary alveolar macrophages from fibrotic mouse lungs have supported a role for ADAM17 in shedding membrane IL-6Rα to generate soluble IL-6Rα in fibrotic lungs.
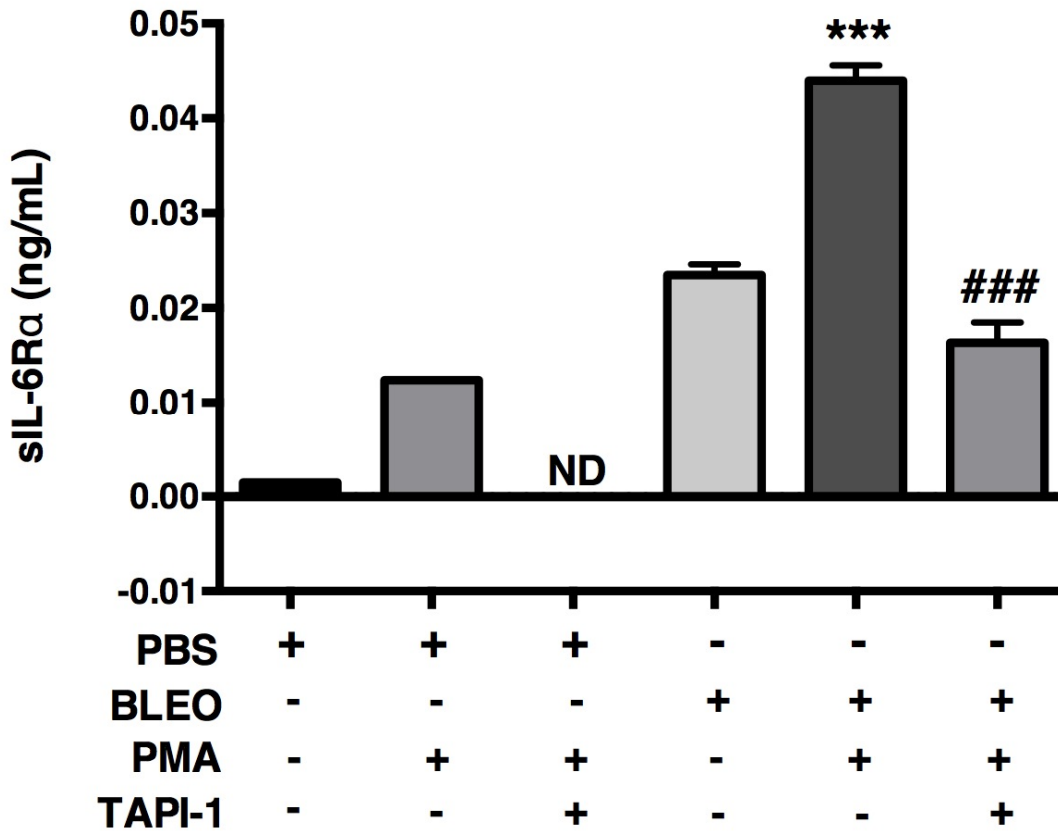
Figure 4.6. Generation of soluble IL-6Rα following pharmacologic inhibition of ADAM17 activity in primary alveolar macrophages. Primary alveolar macrophages were isolated from the lungs of day 33 PBS and bleomycin-exposed mice. Macrophages were stimulated with PMA in the presence or absence of TAPI-1. ELISA measurement of soluble IL-6Rα in culture media was performed. All data presented as mean ± SEM, n=1 for PBS and n=3 for bleomycin. *significant difference from media only cohort; [#]significant difference from PMA-stimulated cohort; *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$.

DISCUSSION

The experimental results presented in this chapter suggest that generation of sIL-6Rα in pulmonary fibrosis is due to cleavage of membrane IL-6Rα from activated pulmonary macrophages via the action of the protease ADAM17.

A major finding in this study is that ADAM17 expression was elevated in alveolar macrophages from bleomycin-induced, fibrotic mouse lungs (Figure 4.1). Lee et al have previously demonstrated increased ADAM17 expression in mice with acute lung injury in response to intratracheal bleomycin instillation(125). Our finding of increased ADAM17 in a chronic model of intraperitoneally-instilled bleomycin agrees with their results.

To our knowledge, however, this is the first study to report a temporal increase in ADAM17 expression as pulmonary fibrosis develops and progresses. This pattern of increase in ADAM17 is associated with an increasing accumulation of alveolar macrophages in the lung (Figure 4.3) and a progressively increasing level of soluble IL-6Rα (Figure 3.3). The coordinated changes in ADAM17, alveolar macrophages and sIL-6Rα all support the hypothesis that increased activation of ADAM17 in alveolar macrophages of fibrotic lungs results in shedding of IL-6Rα to increase production of sIL-6Rα. Indeed, our *in vitro* experiments with bone marrow derived M_2 macrophages and primary M_2 alveolar macrophages confirmed the role of ADAM17. Activation of ADAM17 in these macrophages led to shedding and increases generation of sIL-6Rα. Inhibition of ADAM17, both with a pharmacologic inhibitor and with ADAM17 siRNA, resulted in inhibition of shedding and a reduction in amounts of sIL-6Rα.

As mentioned in Chapter 3, fibrosis is progressive in the IPB model (Figure 3.3A). Our findings presented in this chapter suggest a mechanism for the progressive nature of fibrosis in

the model. Augmented ADAM17-mediated generation of sIL-6Rα could be the mechanism by which sIL-6Rα accumulates in the lung over time, causing progressively increasing fibrosis.

This finding is provocative in that it begs the question of ADAM17-targeted therapies in alleviating lung fibrosis. It also suggests a mechanism for the contribution of activated macrophages to pulmonary fibrosis and raises the possibility of targeting this potentially pivotal player in disease. ADAM17 could also be used as an important indicator of the progression of pulmonary fibrosis. It must be noted, however, that Garbers et al have argued that ADAM17 is the main protease responsible for cleavage of mIL-6Rα in humans, but that in mice, ADAM10 is primarily responsible(126). We do not deny that ADAM10 may be able to cleave mIL-6Rα; however, we have not been able to demonstrate an increase in ADAM10 in fibrotic murine lungs (Figure 4.1B). Our *in vitro* experiments with stimulation and neutralization of ADAM17 in macrophages have led us to conclude that ADAM17 is responsible for the generation of sIL-6Rα in our mouse model of chronic bleomycin exposure.

In summary, the experiments conducted in this chapter demonstrated a temporal pattern of increasing ADAM17 expression in association with increasing pulmonary fibrosis and increasing levels of soluble IL-6Rα. Our stimulation and neutralization experiments supported a role for ADAM17 in shedding IL-6Rα from the membrane of activated pulmonary macrophages in order to increase production of soluble IL-6Rα in fibrotic lungs. The data presented in Chapter 3 and this chapter have provided sufficient evidence and the rationale needed to propose *in vivo* neutralization of soluble IL-6Rα and resulting antagonism of IL-6 *trans* signaling in the IPB model to determine whether or not therapeutic benefits could be achieved. The results of *in vivo* neutralization of IL-6 *trans* signaling will be presented in the next chapter.

CHAPTER 5

In Vivo Neutralization of

Interleukin-6 (IL-6) *Trans* Signaling in a

Chronic Bleomycin Murine Model

of Pulmonary Fibrosis

EXPERIMENTAL RATIONALE AND KEY QUESTIONS

In the previous two chapters, we have demonstrated that soluble IL-6Rα was elevated in association with pulmonary fibrosis as a result of ADAM17-mediated cleavage of the membrane receptor. We have also provided evidence to suggest that IL-6 *trans* signaling is associated with increasing severity of disease. The role of IL-6 *trans* signaling in Idiopathic Pulmonary Fibrosis, however, is unknown.

IL-6 *trans* signaling via the soluble receptor has been implicated in the pathogenesis of rheumatoid arthritis(73), asthma(74), inflammatory bowel disease (colitis)(76) and colitis-associated cancer(78). In pulmonary fibrosis, O'Donoghue et al. and Pedroza et al demonstrated that IL-6 ablation attenuated fibrosis in bleomycin-induced murine models(29, 87). What is not known from their work is the differentiation between the contribution of IL-6 classical versus *trans* signaling with regards to the development and progression of pulmonary fibrosis. It is with the intention of filling this gap in knowledge that the experiments in this chapter were designed.

This chapter will emphasize the importance of IL-6 *trans* signaling by evaluating potential benefits of blocking this signaling pathway *in vivo* in a murine model of pulmonary fibrosis. So how is specific blockade of IL-6 *trans* signaling *in vivo* achieved? The answer lies with soluble gp130. As previously mentioned, the receptor protein gp130 is constitutively expressed in cell membranes and serves the function of signal transduction for various cytokines, including interleukin 6. Soluble forms of gp130 (sgp130) have been identified in human serum and urine(127, 128). Since the initial discovery, soluble gp130 has been shown to be a natural inhibitor of IL-6 *trans* signaling(105, 106) and a number of studies have employed its use as a mean of specifically blocking IL-6 *trans* signaling *in vivo*.

In various animal models of disease, the use of soluble gp130 has resulted in amelioration of disease states. *In vivo* neutralization of IL-6 *trans* signaling resulted in suppression of colitis in an experimental mouse model(76), lessened disease in a mouse experimental arthritis model(79), and resulted in clinical improvement in a systemic arthritis model(80). Blocking IL-6 *trans* signaling resulted in improvement in survival in a mouse sepsis model(77) and reduced colitis-associated premalignant cancer (CApC) in a mouse model(78). Specifically targeting IL-6 *trans* signaling *in vivo* reduced ascites formation and enhanced the sensitivity of ovarian tumors to chemotherapy(103).

Given the successful precedent of using soluble gp130 to neutralize IL-6 *trans* signaling *in vivo* in other disease models, we used a similar approach to assess the impact of IL-6 *trans* signaling in pulmonary fibrosis and answered the following key questions:

5.1-5.2 **Is recombinant gp130Fc able to neutralize IL-6 trans signaling in vivo?**

5.3-5.4 **Does recombinant gp130Fc treatment attenuate pulmonary inflammation?**

5.5-5.8 **Does recombinant gp130Fc treatment attenuate pulmonary fibrosis?**

5.9 **Does recombinant gp130Fc treatment improve oxygen saturation?**

5.10 **Does recombinant gp130Fc treatment reduce pulmonary hypertension?**

5.11 **Does recombinant gp130Fc treatment decrease activation of STAT3?**

5.12-18 **Does IL-6 trans signaling affect fibroblast proliferation, apoptosis and extracellular matrix protein production in vitro? If so, will recombinant gp130Fc inhibit the effects in vitro?**

5.19 **Does recombinant gp130Fc administration affect in vivo IL-6 classical signaling?**

A *In vivo* neutralization of sIL-6Rα with gp130Fc

Classical Signaling

Trans Signaling
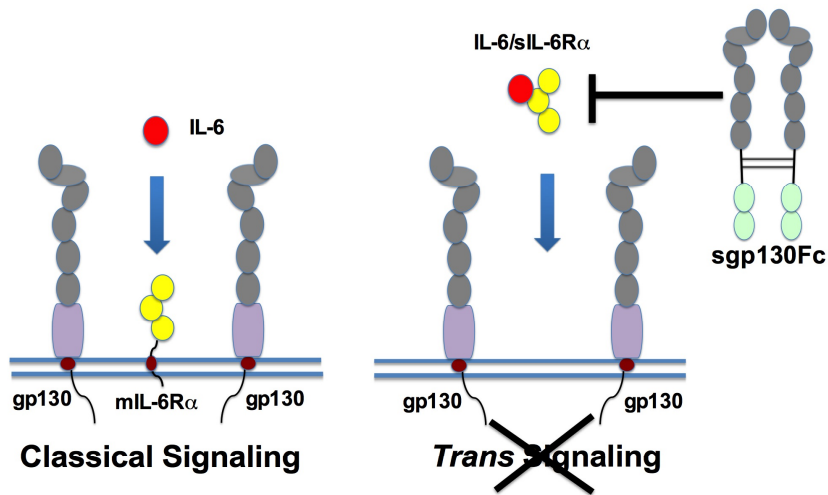
B Neutralization protocol
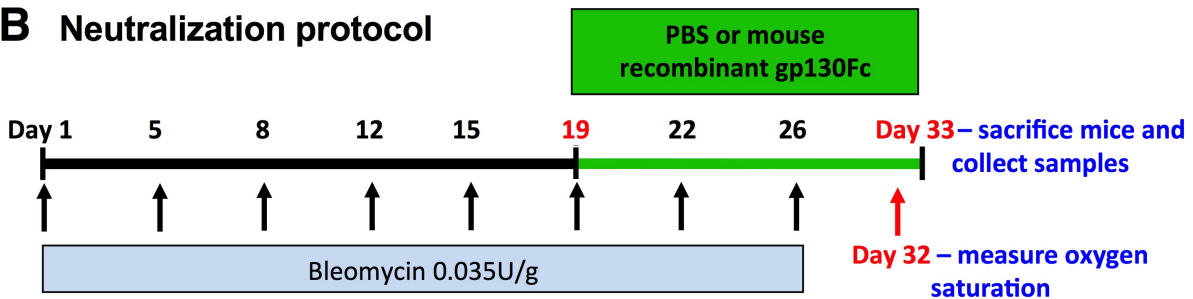
Figure 5.1. *In vivo* **neutralization of soluble IL-6Rα using mouse recombinant gp130Fc in a mouse model of chronic bleomycin exposure.** (**A**) Illustration of the mechanism of action of mouse recombinant gp130Fc on neutralization of sIL-6Rα. (**B**) Experimental setup for *in vivo* neutralization of sIL-6Rα in the Intraperitoneal Bleomycin murine model of pulmonary fibrosis. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Arterial oxygen saturation measurements were performed on live, shaved mice on day 32. Mice were sacrificed and samples collected on day 33.
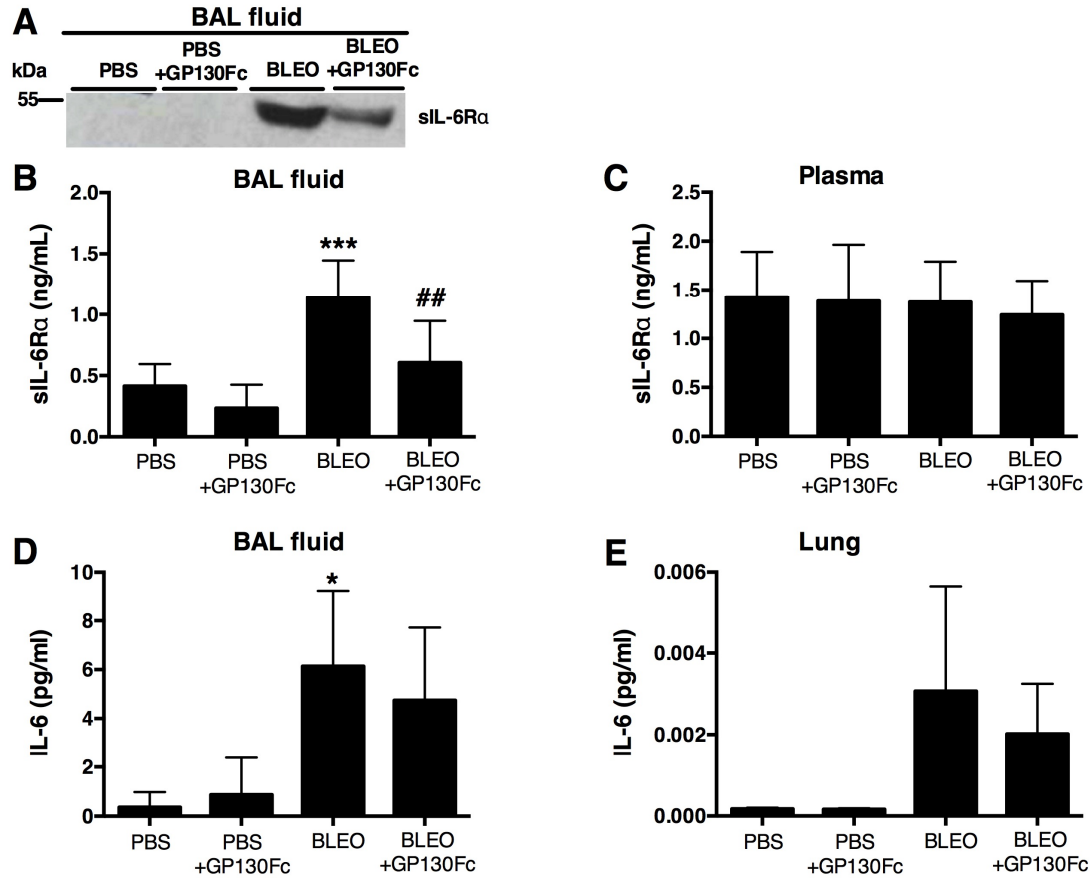
Figure 5.2. Soluble IL-6Rα and IL-6 following chronic bleomycin exposure in mice treated with recombinant gp130Fc in a mouse model of chronic bleomycin exposure. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Mice were sacrificed and samples collected on day 33. (**A**) Western blot analysis and (**B**) ELISA measurement of sIL-6Rα levels in BAL fluid from day 33 lungs. (**C**) ELISA quantification of sIL-6Rα in plasma samples. (**D**) ELISA measurement of IL-6 protein in day 33 BAL fluid and (**E**) Q-rtPCR evaluation of IL-6 transcript level in day 33 whole lung RNA. All data presented as mean ± SEM, n≥6 for B & C, n≥3 for D & E. *significant difference from PBS-treated cohort; [#]significant difference from bleomycin-exposed mice. *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$.

RESULTS

5.1 – Protocol for in vivo neutralization of IL-6 trans signaling using recombinant gp130Fc.

In vivo neutralization of sIL-6Rα was performed in the IPB model using mouse recombinant gp130Fc, a commercially available reagent shown in previous studies to be an effective and selective inhibitor of IL-6 *trans* signaling(73, 74, 76, 78). Recombinant gp130Fc binds the only the complex of IL-6/sIL-6Rα, selectively inhibiting signaling through the *trans* pathway(44), as illustrated in Figure 5.1A. It does not bind IL-6 alone or sIL-6Rα alone, mIL-6Rα, or the complex of IL-6/mIL-6Rα, thus leaving classical signaling intact.

The neutralization treatment protocol was as illustrated in Figure 5.1B. The dosage and schedule for administration of recombinant gp130Fc are adapted from previous published studies that have shown effectiveness of soluble gp130 at blocking IL-6 *trans* signaling *in vivo* in various models(76-80, 103). Treatment with gp130Fc began late in the disease process, on day 19 of the model, when pulmonary fibrosis was established. Treatment continued until day 32. Hypoxia was assessed in live mice on day 32 via measurement of arterial oxygen saturation. Mice were sacrificed on Day 33 and pulmonary phenotypes were assessed for changes to pulmonary inflammation and fibrosis.

The decision of when to begin treatment with gp130Fc was based on preliminary data regarding the temporal course of disease development in the IPB model. Data from our characterization studies informed us that though collagen levels began rising as early as 5 days after the first dose of bleomycin, levels significantly jumped between day 15 to day 20 of the model, as does levels of soluble IL-6Rα (Figure 3.3A-B). This suggests that it is around this time in the model that fibrosis becomes much more robust and *trans* signaling takes a more prominent role in the disease process. This led us to choose day 19, coinciding with the 6th injection of

bleomycin, as the best time to initiate therapeutic blockade of the *trans* signaling pathway. In this way, we were able to determine the therapeutic rather than preventative benefits of antagonizing *trans* signaling.

5.2 – Administration of recombinant gp130Fc reduced sIL-6Rα.

To determine whether our neutralization protocol was sufficient to reduce levels of the soluble receptor and inhibit *trans* signaling, Day 33 BAL fluid samples were collected and subjected to ELISA analysis of sIL-6Rα. Bleomycin-treated mice exhibited a significant elevation in sIL-6Rα in BAL fluid (Figure 5.2A&B), similar to what was demonstrated in our earlier characterization studies (Figure 3.2B&C). Treatment with gp130Fc significantly lowered levels of sIL-6Rα in BAL fluid. Interestingly, levels of sIL-6Rα in plasma were not significantly different among the treatment groups (Figure 5.2C). This suggests that recombinant gp130Fc is able to alter levels of soluble IL-6Rα and antagonize IL-6 *trans* signaling in the lung microenvironment but not the systemic circulation.

A consideration when using recombinant gp130Fc to inhibit *trans* signaling is whether it would inadvertently interact with IL-6 alone and thereby inhibit classical signaling. As previous studies have demonstrated, the inhibitory effects of gp130Fc are limited to *trans* signaling because it only binds IL-6/sIL-6Rα complexes(76-80, 103, 129). However, to confirm that gp130Fc administration in our mice did not have off-target effects, IL-6 transcript in whole lung RNA and protein levels in BAL fluid were determined. IL-6 protein and transcript levels were elevated in the lungs of bleomycin-treated mice in comparison to PBS-treated mice (Figure 5.2D&E). No significant differences were seen in protein and transcript levels between bleomycin and bleomycin + gp130Fc groups, though there was a trend for decrease. These

results tell us that gp130Fc is not directly affecting IL-6 classical signaling since IL-6 protein levels were relatively unchanged.

5.3 – Treatment with recombinant gp130Fc reduced inflammatory cells in the lungs.

Once we confirmed neutralization of *trans* signaling in our experimental setup, we began evaluating changes to pulmonary phenotype. The histopathology of IPF lungs include presence of spatial and temporal heterogeneity in fibrosis, with regions of dense scarring and honeycombing alternating with less severe fibrosis or normal pulmonary architecture(1). Regions of fibrosis are usually packed with dense collagen and scattered fibroblastic foci (areas of proliferating fibroblasts and myofibroblasts). Areas of honeycombing (cystic fibrotic airspaces) consist of airspaces that are filled with inflammatory cells and mucus and lined with bronchiolar epithelium. When inflammation is present in the interstitium, it is typically mild, with lymphocytes and plasma cells making up a patchy infiltrate(1).

To assess changes in pulmonary phenotype, we first examined the effects of recombinant gp130Fc administration on pulmonary inflammation. In the IPB model, mice develop extensive pulmonary fibrosis as well as pulmonary inflammation, consisting of an influx of inflammatory cells, including macrophages, lymphocytes and neutrophils(33, 91). Using a hemocytometer, total cell counts were performed on BAL fluid samples from day 33 mice. Mice treated with bleomycin exhibited increased inflammation, as evident by an increase in total inflammatory cells recovered in lavage fluid (Figure 5.3A). Treatment with gp130Fc was associated with decreased inflammation, as indicated by a significant reduction in total cell counts. Cell differential analysis of BAL fluid revealed a reduction in macrophages, lymphocytes, neutrophils and eosinophils (Figure 5.3B-D).
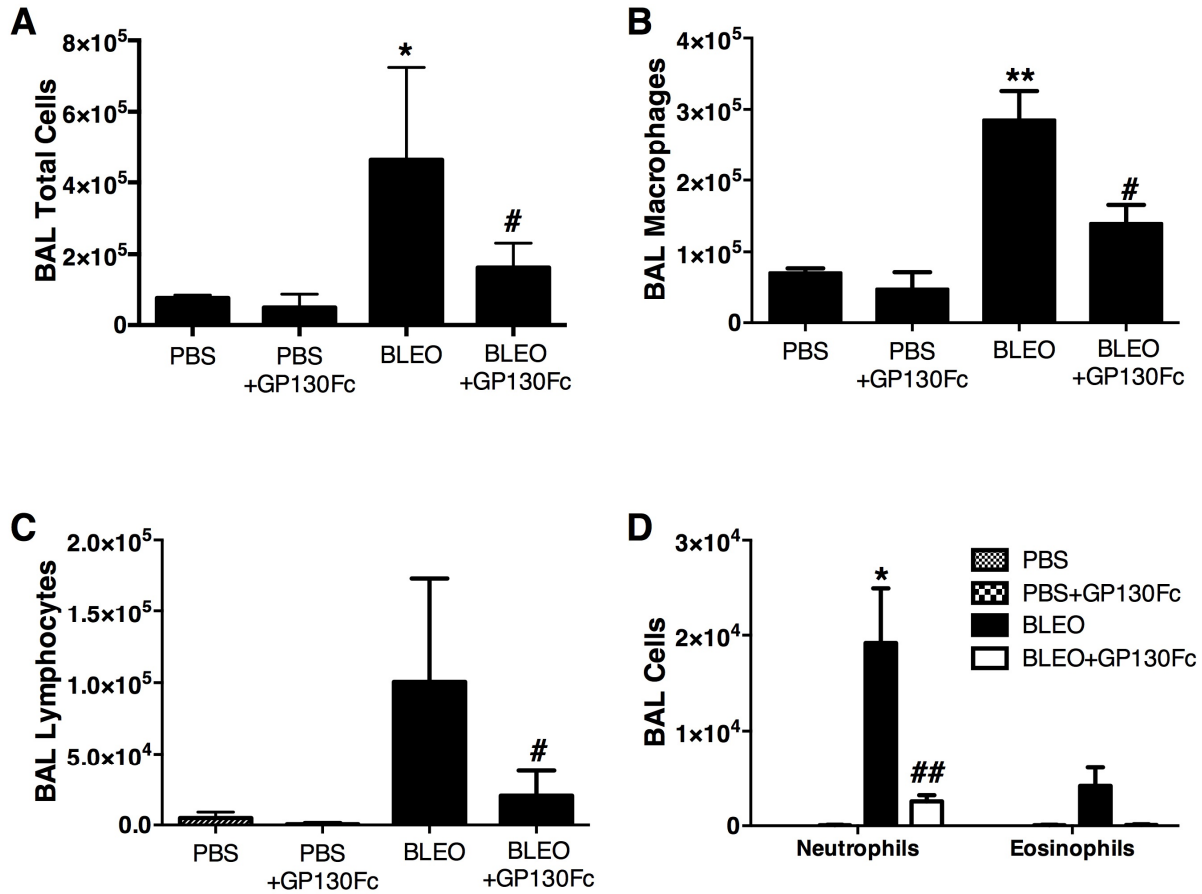
Figure 5.3. Pulmonary inflammation following chronic bleomycin exposure in mice treated with recombinant gp130Fc. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Mice were sacrificed and samples collected on day 33. (**A-D**) Total cell count and cell differential from bronchoalveolar lavage fluid (BAL fluid) of wild type C57Blk6 mice given saline or bleomycin, with and without gp130Fc. Data presented as mean ± SEM, n≥6. *significant difference from PBS-treated cohort; #significant difference from bleomycin-exposed mice. *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$.

5.4 – Treatment with recombinant gp130Fc reduced inflammatory cytokines in the lungs.

The ability of gp130Fc to dampen pulmonary inflammation led us to evaluate changes to relevant pro-inflammatory and IL-6 responsive mediators, including monocyte chemoattractant protein 1 (MCP-1), chemokine (C-X-C motif) ligand 1 (CXCL-1), interleukin 17 (IL-17), and tissue inhibitor of metalloproteinases (TIMP-1). Whole lung RNA analysis revealed a significant reduction in MCP-1, CXCL-1, IL-17 and TIMP-1 in mice treated with gp130Fc (Figure 5.4A-D). These findings demonstrate that gp130Fc-mediated neutralization of IL-6 *trans* signaling in the lungs can attenuate pulmonary inflammation and decrease pro-inflammatory and IL-6 responsive mediators in this model.

5.5 – Treatment with recombinant gp130Fc reduced collagen transcript in the lungs.

Having demonstrated attenuation of pulmonary inflammation with gp130Fc administration, we next determined the effects of gp130Fc on pulmonary fibrosis. Aberrant fibroblast activation, differentiation into myofibroblasts, and excessive collagen and fibronectin production and deposition in the lungs are hallmarks of pulmonary fibrosis(2). Therefore, we asked whether treatment with recombinant gp130Fc affected these indices.

To assess collagen production, day 33 whole lung RNA samples were analyzed for transcript expression of collagen 1 isoforms. In comparison to levels of collagen transcript in the non-fibrotic lungs of PBS-treated mice, bleomycin-treated mice exhibited increased transcript levels of collagen 1A1 and collagen 1A2 (Figure 5.5A-B), consistent with the presence of pulmonary fibrosis in those lungs. Treatment with gp130Fc resulted in significant reductions in collagen 1A2 and and a trend for reduction in collagen 1A1.
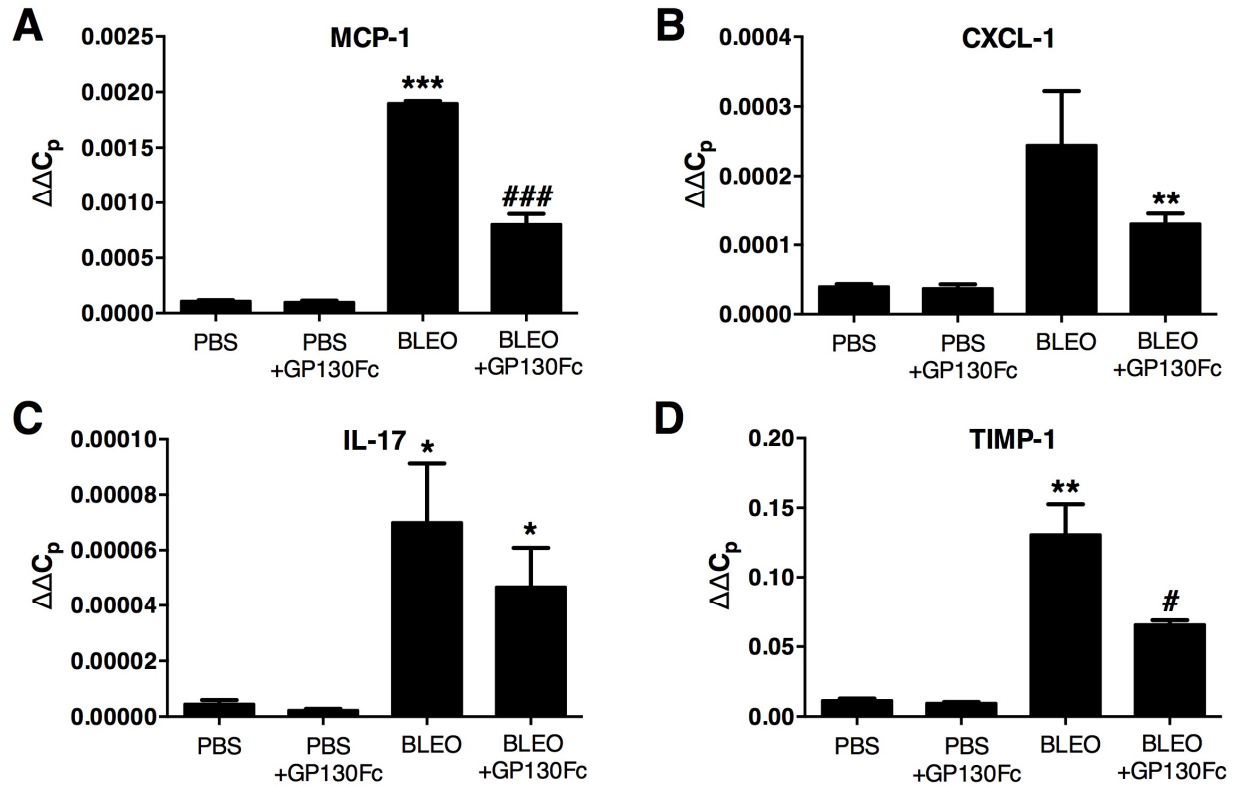
Figure 5.4. Expression of pro-inflammatory and IL-6 responsive mediators following chronic bleomycin exposure in mice treated with recombinant gp130Fc. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Mice were sacrificed and samples collected on day 33. (**A-D**) Expression of MCP-1, CXCL-1, IL-17, and TIMP-1 transcript in whole lung RNA. Data presented as mean ± SEM, n≥3. *significant difference from PBS-treated cohort; #significant difference from bleomycin-exposed mice. *= $p < 0.05$, **= $0.001 < p < 0.01$, ***= $p < 0.001$.
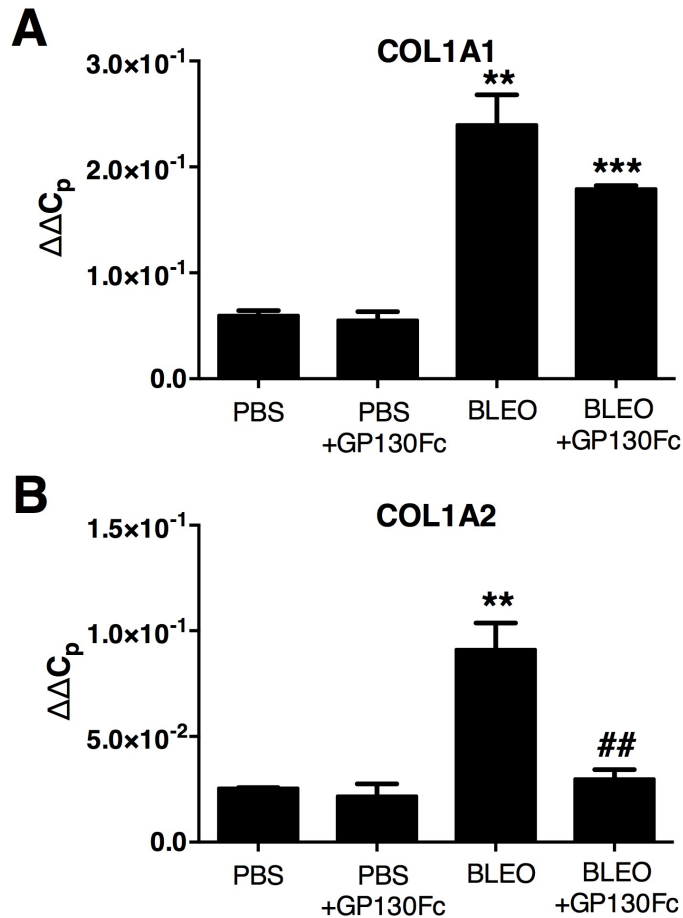
Figure 5.5. Changes in collagen transcript following chronic bleomycin exposure in mice treated with recombinant gp130Fc. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Mice were sacrificed and samples collected on day 33. (**A-B**) Whole lung RNA was prepared and subjected to q-rtPCR evaluation for analysis of transcript levels of collagen 1A1 and collagen 1A2. All data presented as mean ± SEM, n≥3. *significant difference from PBS-treated cohort; [#]significant difference from bleomycin-exposed mice. *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$.

5.6 – Recombinant gp130Fc reduced collagen protein expression in the lungs.

We validated the results of mRNA evaluation by measuring soluble collagen protein in BAL fluid using Sircol assay. Gp130Fc administration resulted in a significant reduction in collagen protein (Figure 5.6A). To see if this effect could be correlated to the decrease of soluble IL-6Rα in the lungs, we subjected matched data of soluble IL-6Rα level and soluble collagen in BAL fluid to correlation analysis. As seen in Figure 5.6B, when soluble IL-6Rα levels are displayed on the x-axis and corresponding levels of soluble collagen in each sample plotted on the y-axis, there is a significant correlation between receptor level and collagen level.

5.7 – Recombinant gp130Fc reduced collagen deposition and attenuated pulmonary fibrosis.

Changes in collagen transcripts and protein expression suggested gp130Fc mediated a reduction in collagen production, so we proceeded to evaluate whether it also led to a reduction in collagen deposition in the lungs. Day 33 lung sections were stained using Masson's trichrome protocol to visualize collagen deposition (blue). Untreated bleomycin-exposed lungs were fibrotic and had extensive collagen deposition while gp130Fc-treated lungs had diminished collagen presence (Figure 5.7A). To assess how the reduction in collagen production and deposition affected pulmonary fibrosis in our mice, we performed Ashcroft scoring on the stained sections. This is a subjective method of quantifying morphologic fibrosis that has been used extensively(130). Bleomycin-treated lungs had mean scores of about 6 on a scale of 0-8, with 8 being the highest severity of fibrosis. Gp130Fc treatment improved overall scores by almost 50% (Figure 5.7B). These findings revealed that blockade of IL-6 *trans* signaling resulted in a significant reduction in collagen production and deposition in the lungs, resulting in attenuation of pulmonary fibrosis.
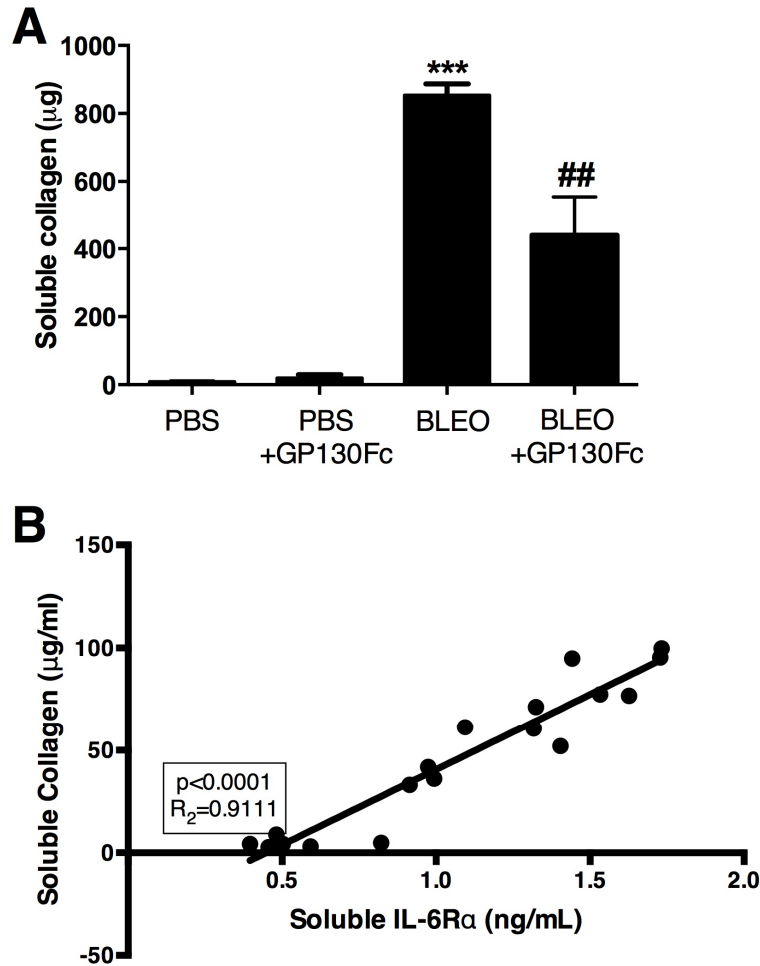
Figure 5.6. Changes in collagen protein expression following chronic bleomycin exposure in mice treated with recombinant gp130Fc. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Mice were sacrificed and samples collected on day 33. (**A**) Sircol measurement of soluble collagen in BAL fluid on day 33. (**B**) Matched results of soluble IL-6Rα level and corresponding soluble collagen level of each mouse subjected to correlation analysis; analysis representative of 2 independent experiments. All data presented as mean ± SEM, n≥6. *significant difference from PBS-treated cohort; [#]significant difference from bleomycin-exposed mice. *= p<0.05, **= 0.001<p<0.01, ***= p<0.001.
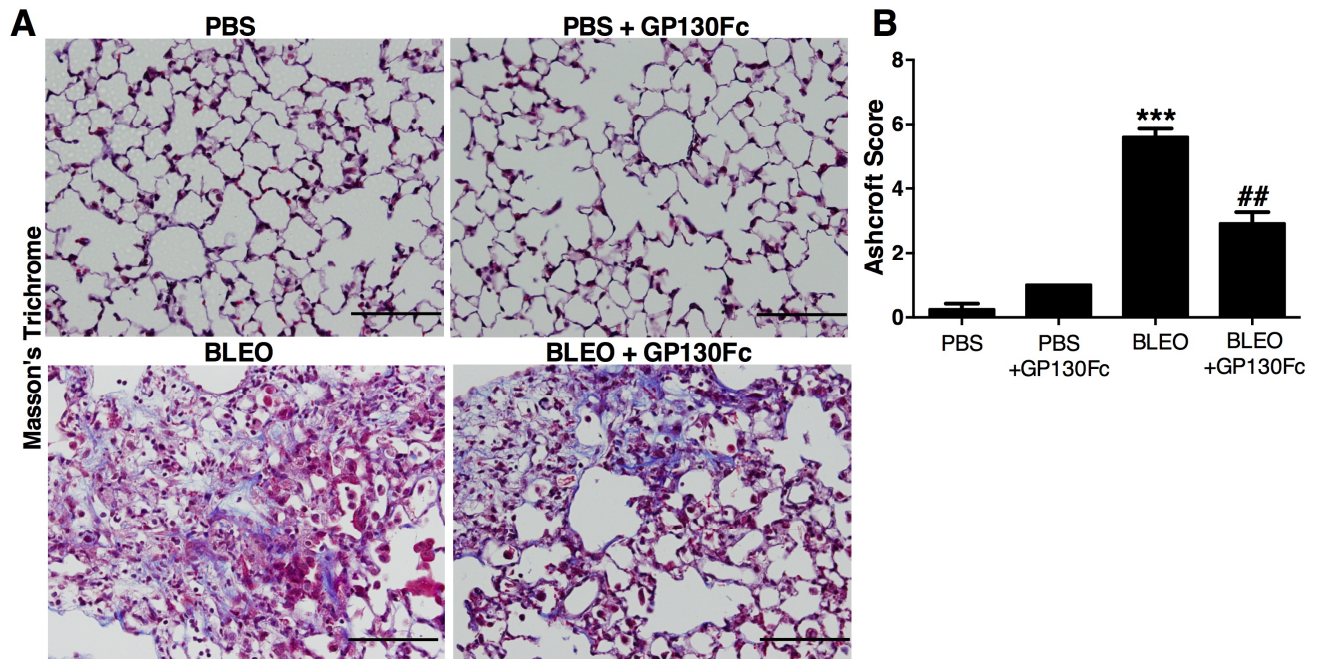
Figure 5.7. Changes in collagen deposition following chronic bleomycin exposure in mice treated with recombinant gp130Fc. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Mice were sacrificed and samples collected on day 33. (**A**) Lung sections from day 33 mice were stained with Masson's trichrome for visualization of collagen deposition (blue). Sections are representative of n≥6 mice from each group. Scale bars: 200μm. (**B**) Pulmonary fibrosis was quantified by Ashcroft method. All data presented as mean ± SEM, n≥6. *significant difference from PBS-treated cohort; #significant difference from bleomycin-exposed mice. *= p<0.05, **= 0.001<p<0.01, ***= p<0.001.

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*5.8 – Recombinant gp130Fc reduced myofibroblast accumulation and fibronectin expression.*

To better understand how *in vivo* neutralization of IL-6 *trans* signaling attenuated fibrosis, we assessed myofibroblast accumulation in gp130Fc-treated lungs. Myofibroblasts are a central cellular player in pulmonary fibrosis, the main collagen-producing culprit(131). Lung sections from day 33 mice were stained for alpha smooth muscle actin (α-SMA) for detection of myofibroblasts. Whereas fibrotic lungs exhibited prominent red α-SMA staining that is indicative of extensive myofibroblast accumulation, gp130Fc treated lungs presented with diminished myofibroblast accumulation (Figure 5.8A). Comparisons between bleomycin versus gp130Fc-treated lung sections visually emphasized the reduction in pulmonary fibrosis seen with gp130Fc treatment. We also analyzed the expression of fibronectin, an extracellular matrix protein found to be elevated in IPF lungs and is an additional indicator of fibrosis (132-135). Protein lysates were made from day 33 lungs and western blot analysis was performed to detect changes to fibronectin content. In comparison to bleomycin-exposed lungs, which have increased expression of fibronectin, lungs treated with gp130Fc exhibit less fibronectin (Figure 5.8B). PBS-injected mice treated with gp130Fc also experienced a reduction in fibronectin expression in the lungs.
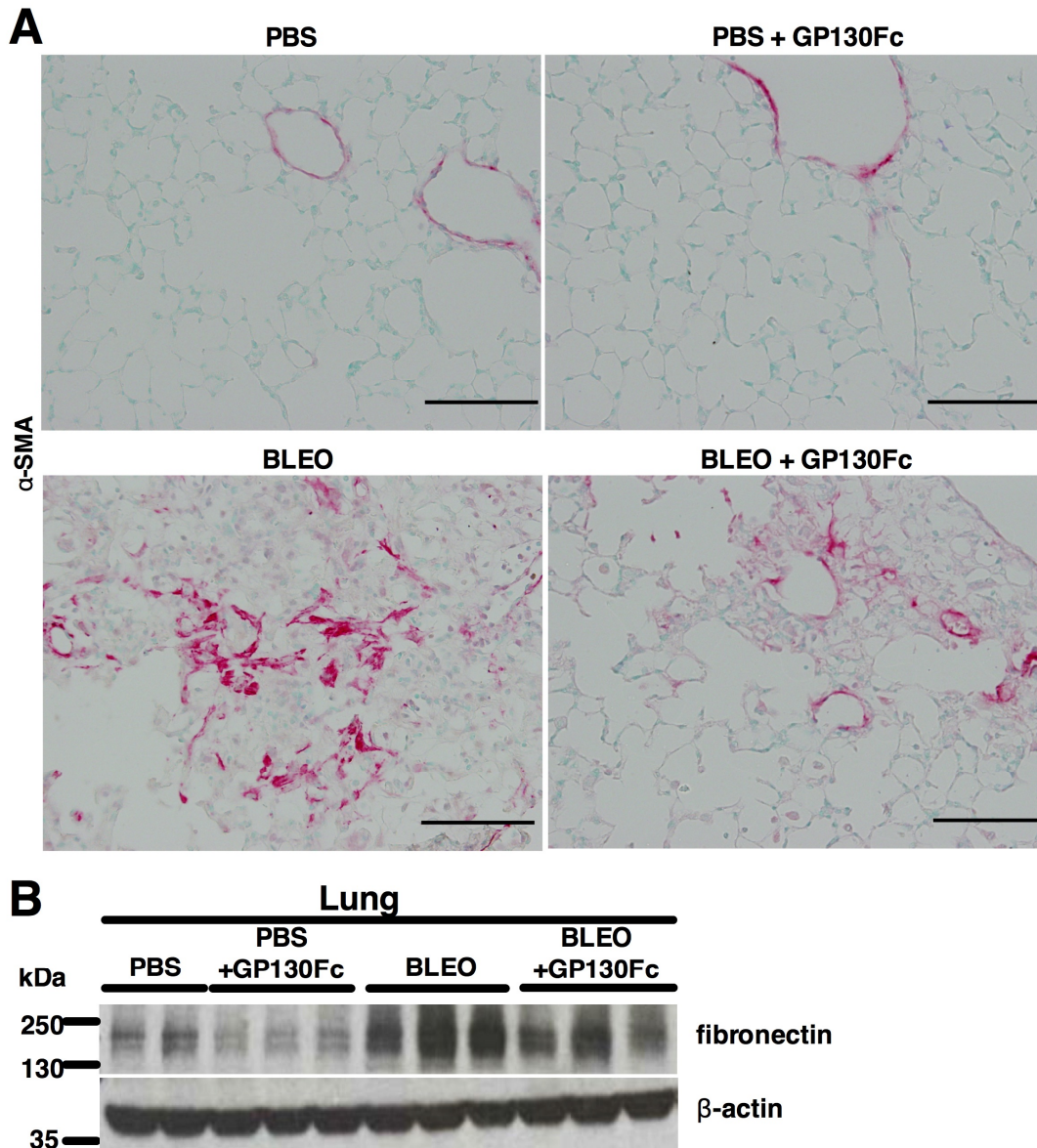
Figure 5.8. Changes in myofibroblast presence and activity following chronic bleomycin exposure in mice treated with recombinant gp130Fc. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Mice were sacrificed and samples collected on day 33. Lung sections from day 33 mice were stained for: (**A**) alpha smooth muscle actin (α-SMA) for detection of myofibroblast accumulation (red). Sections are representative of n≥6 mice from each group. Scale bars: 1mm. (**B**) Western blot analysis of fibronectin expression in whole lung lysates.

5.9 – Recombinant gp130Fc administration improved oxygen saturation.

Hypoxia is a feature of IPF and oxygen saturation measurements are often used clinically to evaluate presence and severity of hypoxia(1). We evaluated changes to hypoxia in mice treated with gp130Fc by measuring oxygen saturation. We observed a decrease in oxygen saturation in untreated, bleomycin-injected mice that was inhibited by gp130Fc treatment (Figure 5.9). Collectively, these findings demonstrate that gp130Fc treatment attenuates pulmonary fibrosis, leading to physiologic improvement in mice.

5.10 – Recombinant gp130Fc reduced pulmonary hypertension.

Pulmonary hypertension (PH, mean pulmonary artery pressure > 25 mmHg at rest) occurs in 8.1-14.9% of IPF patients, with higher numbers seen in advanced (30-50%) and end-stage (>60%) patients(136). Its presence in IPF patients is associated with increased mortality risk(1). Karmouty-Quintana et al recently presented data suggesting that adenosine receptor 2B-mediated release of IL-6 from human pulmonary artery smooth muscle cells (PASMCs) is the mechanism via which PH develops secondary to pulmonary fibrosis(91). We wanted to know whether *in vivo* neutralization of sIL-6Rα affected development of PH in our mice. Right ventricular systolic pressure (RVSP) is commonly used to indicate presence of PH. Measurement of RVSP in mice administered bleomycin revealed significantly higher RVSP values in comparison to PBS-treated mice (Figure 5.10). This is consistent with previous published results demonstrating pulmonary hypertension secondary to bleomycin-induced pulmonary fibrosis(91). Gp130Fc treatment resulted in significant reduction in RVSP, demonstrating that neutralization of *trans* signaling led to improvement in pulmonary hypertension.
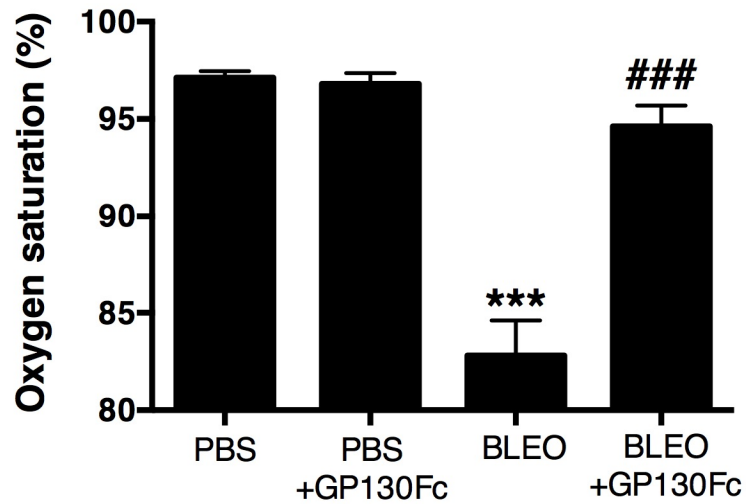
Figure 5.9. Changes in arterial oxygen saturation following chronic bleomycin exposure in mice treated with recombinant gp130Fc. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Pulse oximetry was conducted using neck collar on Day 32. All data presented as mean ± SEM, n≥4. *significant difference from PBS-treated cohort; #significant difference from bleomycin-exposed mice. *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$.
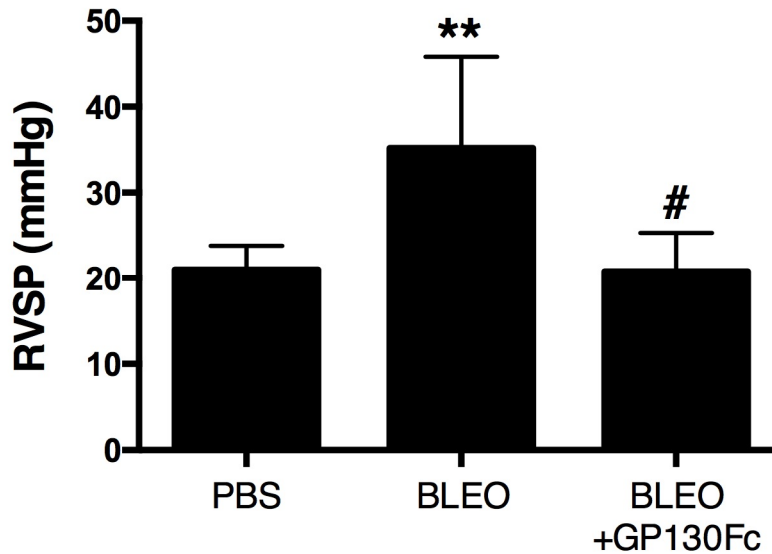
Figure 5.10. Changes in pulmonary hypertension following chronic bleomycin exposure in mice treated with recombinant gp130Fc. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Right ventricular systolic pressure (RVSP) was measured on Day 33. All data presented as mean ± SEM, n≥4. *significant difference from PBS-treated cohort; #significant difference from bleomycin-exposed mice. *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$. Data obtained by Harry Karmouty-Quintana, PhD.

5.11. – Recombinant gp130Fc decreased STAT3 activation in lungs.

In search of the mechanism behind the reduction in pulmonary fibrosis when IL-6 *trans* signaling is neutralized, we examined intracellular signaling effects of IL-6. IL-6 signaling phosphorylates and activates Signal Transducer and Activator of Transcription 3 (STAT3) (Figure 1.1), a transcription factor important in the pathogenesis of liver, skin and kidney fibrosis(137, 138). Increased STAT3 activation has been reported in IPF(87, 139). Its role in pulmonary fibrosis was evaluated by O'Donoghue et al., who demonstrated that IL-6-mediated STAT3 activation increased bleomycin-induced fibrosis in a mouse model(87). Thus, we evaluated changes to STAT3 activation in mice treated with gp130Fc. Western blot analysis of protein lysates made from day 33 lungs revealed that phospho-STAT3 was increased in bleomycin lungs compared to PBS lungs, and treatment with gp130Fc reduced STAT3 activation (Figure 5.11A). Co-staining of P-STAT3 and cell-specific markers allowed us to determine, more specifically, that there was a decrease in P-STAT3-positive myofibroblasts in gp130Fc-treated lungs (Figure 5.11B). We confirmed this finding in human samples, where we saw an abundant presence of P-STAT3-positive myofibroblasts in IPF lung sections in comparison to control sections (Figure 5.11C). These results suggest that IL-6 *trans* signaling in this population of cells could be crucial to the fibrotic process. We therefore focused our efforts on examining IL-6 *trans* signaling in fibroblast biology to understand its contribution to pulmonary fibrosis.

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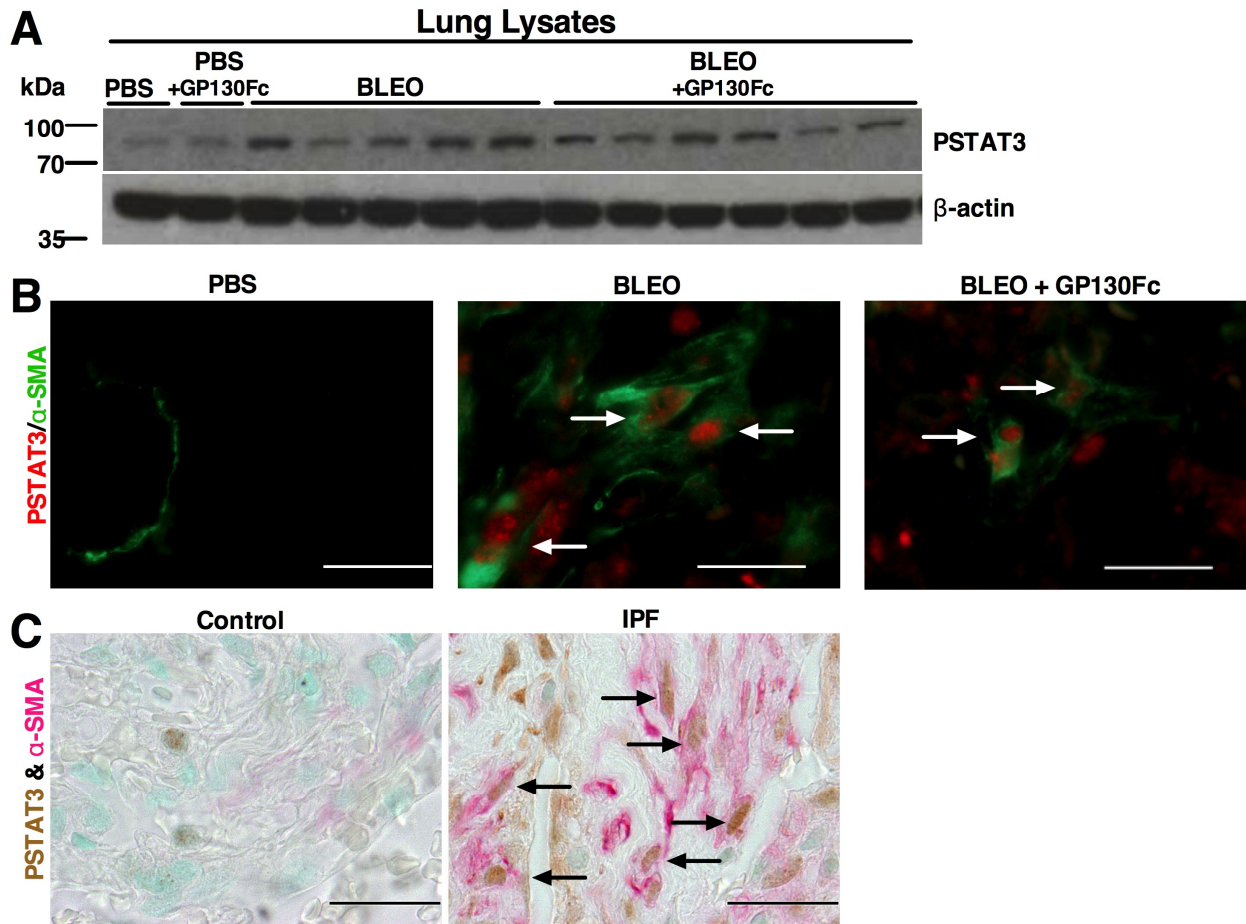
Figure 5.11. STAT3 activation following chronic bleomycin exposure in mice treated with mouse recombinant gp130Fc and in IPF lungs. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Mice were sacrificed and samples collected on day 33. (**A**) Western blot analysis of phospho-STAT3 expression in lung lysates from C57Bl6 mice given saline or bleomycin with and without gp130Fc, Day 33. (**B**) P-STAT3 immunofluorescence (red) in myofibroblasts (green) in these lungs. (**C**) P-STAT3 immunopositivity (brown) in myofibroblasts (red) in IPF lungs. Arrows denote positive cells. Images are representative of n≥4 animals from each group. Scale bars: 50μm, 100x oil immersion.

5.12-5.13 - IL-6 trans signaling promoted extracellular matrix protein production in vitro.

Aberrant fibroblast activation, differentiation into myofibroblasts, and excessive collagen and fibronectin production and deposition in the lungs are hallmarks of pulmonary fibrosis(2). In an effort to better understand the effects of IL-6 *trans* signaling on fibroblast biology, we examined these endpoints in vitro, using normal and IPF fibroblast cell lines.

To evaluate the effect of IL-6 *trans* signaling on production of extracellular matrix proteins, normal and IPF fibroblasts were serum-starved and stimulated with transforming growth factor β (TGF-β) or IL-6 and sIL-6Rα for 24, 48 and 72 hours. Protein lysates were collected from these cells and subjected to western blot analyses for detection of collagen and fibronectin. TGF-β was used as a positive control since it has been shown to be a potent inducer of extracellular matrix protein production and myofibroblast differentiation and to play a central role in pulmonary fibrosis(133, 140, 141).

In control fibroblasts, TGF-β stimulation induced expression of collagen and fibronectin and increased expression of α-SMA and membrane IL-6Rα at all three time points (Figure 5.12A-C). Stimulation of *trans* signaling via addition of IL-6 and sIL-6Rα resulted in increased collagen production visibly by 24 hours, with greater levels of protein seen at 48 hours; by 72 hours, collagen levels had begun trending down to baseline. The trend in collagen levels was associated with fibronectin levels that were unchanged at 24 and 48 hours, though mildly decreased at 72 hours. There were no changes in α-SMA at 24 and 48 hours but a decrease in α-SMA at 72 hours. Changes in expression of membrane IL-6Rα mirrored the changes in collagen production. Membrane IL-6Rα was mildly elevated by 24 hours, greatly enhanced by 48 hours, and was visibly decreased by 72 hours (Figure 5.12A-C).

A Control Fibroblasts - 24 hours

TGF-β	-	-	+	+	-	-
IL-6	-	-	-	-	+	+
sIL-6Rα	-	-	-	-	+	+

Collagen

Fibronectin

α-SMA

mIL-6Rα

β-actin

B Control Fibroblasts - 48 hours

TGF-β	-	-	+	+	-	-
IL-6	-	-	-	-	+	+
sIL-6Rα	-	-	-	-	+	+

Collagen

Fibronectin

α-SMA

mIL-6Rα

β-actin

C Control Fibroblasts - 72 hours

TGF-β	-	-	+	+	-	-
IL-6	-	-	-	-	+	+
sIL-6Rα	-	-	-	-	+	+

Collagen

Fibronectin

α-SMA

mIL-6Rα

β-actin

D IPF Fibroblasts - 24 hours

TGF-β	-	-	+	+	-	-
IL-6	-	-	-	-	+	+
sIL-6Rα	-	-	-	-	+	+

Collagen

Fibronectin

α-SMA

mIL-6Rα

β-actin

E IPF Fibroblasts - 48 hours

TGF-β	-	-	+	+	-	-
IL-6	-	-	-	-	+	+
sIL-6Rα	-	-	-	-	+	+

Collagen

Fibronectin

α-SMA

mIL-6Rα

β-actin

F IPF Fibroblasts - 72 hours

TGF-β	-	-	+	+	-	-
IL-6	-	-	-	-	+	+
sIL-6Rα	-	-	-	-	+	+

Collagen

Fibronectin

α-SMA

mIL-6Rα

β-actin

Figure 5.12. Effect of IL-6 *trans* signaling on ECM protein production in control and IPF fibroblasts over time. Control and IPF fibroblasts were serum-starved for 24 hours and then stimulated with IL-6 and sIL-6Rα. Western blot analyses were performed for expression of collagen, fibronectin, α-SMA, and membrane IL-6Rα after 24, 48 and 72 hours in (**A-C**) normal fibroblasts and (**D-F**) IPF fibroblasts. Western blot pictures are representative of n=2.

In IPF fibroblasts, TGF-β stimulation induced expression of collagen and fibronectin and increased expression of α-SMA and membrane IL-6Rα at all three time points, though to a lesser extent than in control fibroblasts (Figure 5.12D-F). Stimulation of *trans* signaling via addition of IL-6 and sIL-6Rα resulted in no difference at 24 hours and increased collagen production at 48 and 72 hours. Fibronectin levels were mildly elevated at 24 hours and experienced greater increases at 48 and 72 hours. α-SMA was mildly increased at 24 and unchanged at 48 and 72 hours. Membrane IL-6Rα was elevated at 24, 48 and 72 hours, in comparison to non-stimulated fibroblasts (Figure 5.12D-F).

These findings suggest that IL-6 *trans* signaling promote production of collagen and fibronectin in control and IPF fibroblasts, perhaps through regulation of membrane IL-6Rα expression. Since expression of collagen and fibronectin appeared to be associated with changes in membrane IL-6Rα, we asked whether IL-6 classical signaling alone would alter collagen and fibronectin levels and to what extent in comparison to *trans* signaling. So, we repeated the stimulation experiment, but this time, fibroblasts were stimulated with TGF-β, IL-6 alone, or IL-6 + sIL-6Rα. Stimulation occurred for 48 hours since the previous experiment indicated greatest changes in protein expression in 48-hour samples.
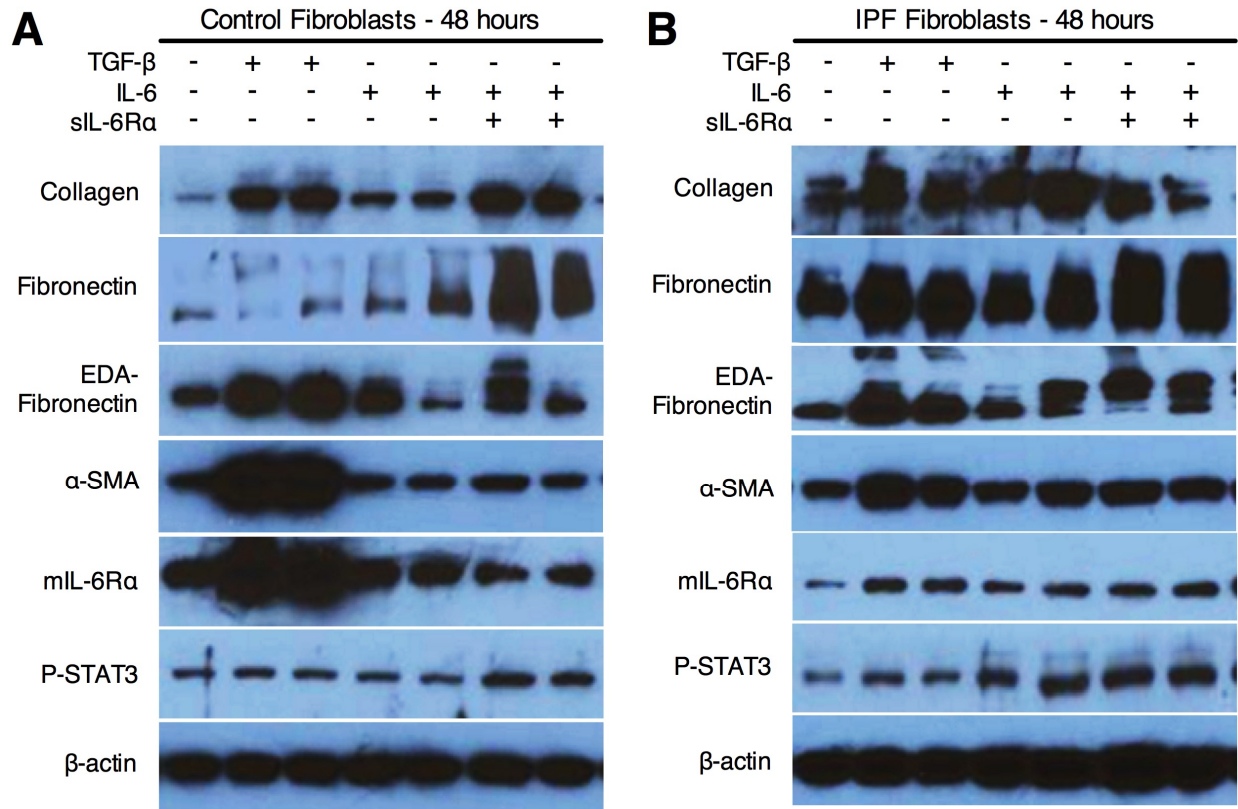
Figure 5.13. Differential effects of IL-6 classical and *trans* signaling on extracellular matrix protein production in control and IPF fibroblasts in vitro. (**A**) Control and (**B**) IPF fibroblasts were serum-starved for 24 hours and then stimulated with IL-6 alone or IL-6 and sIL-6Rα for 48 hours. Western blot analyses were performed for expression of collagen, fibronectin, EDA-fibronectin, α-SMA, membrane IL-6Rα and phospho-STAT3. Western blot pictures are representative of n=2.

In control fibroblasts, IL-6 alone increased collagen expression slightly. IL-6 + sIL-6Rα induced a pronounced increase in collagen to levels comparable to or higher than those seen with TGF-β stimulation (Figure 5.13A). In IPF fibroblasts, both IL-6 alone and IL-6 + sIL-6Rα were able to induce collagen expression to levels comparable to those of TGF-β stimulation (Figure 5.13B). In control fibroblasts, IL-6 alone increased fibronectin expression slightly. IL-6 + sIL-6Rα induced a pronounced increase in fibronectin, to levels higher than those seen with TGF-β stimulation (Figure 5.13A). IL-6 stimulation in IPF fibroblasts led to an increase in fibronectin, while IL-6 + sIL-6Rα led to an increase that was even greater than that induced by TGF-β (Figure 5.13B). Recently, Muro et al demonstrated that IPF lung fibroblasts produce more of a specific isoform of cellular fibronectin, fibronectin containing extra type III domain A (EDA-fibronectin), than normal fibroblasts(142). They also reported that mice lacking EDA-fibronectin were protected against development of pulmonary fibrosis after bleomycin challenge. Thus, we evaluated whether IL-6 *trans* signaling regulated expression of EDA-fibronectin. Results revealed variable expression of EDA-fibronectin in response to IL-6 alone or IL-6 + sIL-6Rα in control fibroblasts (Figure 5.13A). In IPF fibroblasts, however, IL-6 alone induced EDA-fibronectin expression and IL-6 + sIL-6Rα increased expression even further (Figure 5.13B).

IL-6 alone or IL-6 + sIL-6Rα did not change α-SMA and mIL-6Rα expression in control fibroblasts while inducing increased α-SMA and mIL-6Rα expression in IPF fibroblasts (Figure 5.13A&B). In control fibroblasts, IL-6 alone did not promote phosphorylation of STAT3 while IL-6 + sIL-6Rα resulted in increased P-STAT3. In IPF fibroblasts, both IL-6 alone and IL-6 + sIL-6Rα stimulated increased activation of STAT3 (Figure 5.13A&B). These results suggest that both classical and *trans* signaling can regulate collagen and fibronectin production. Although IL-6 *trans* signaling increases protein production in both normal and IPF fibroblasts, the effect on

collagen and fibronectin production is similar in normal fibroblasts, while in IPF fibroblasts, its effect on fibronectin production appear greater than on collagen production.

5.14-5.15 – IL-6 trans signaling promoted proliferation & resistance to apoptosis in IPF fibroblasts in vitro.

Having demonstrated the effect of *trans* signaling on extracellular matrix protein production, we next analyzed changes to proliferation and apoptosis subsequent to stimulation of *trans* signaling in these cells. Excessive proliferation and resistance to apoptosis are features of fibroblasts from IPF lungs that perpetuate their presence in the diseased lung and contribute to ongoing fibrotic changes(143). Moodley et al reported that IL-6 inhibited proliferation of fibroblasts isolated from normal lungs while promoting proliferation of IPF fibroblasts(144). They also demonstrated that IL-6 mediated enhanced Fas-induced apoptosis in normal fibroblasts while inhibiting apoptosis in IPF fibroblasts(145). However, evidence exists to suggest that there is much heterogeneity in fibroblast populations in IPF lungs and they respond differently to IL-6 signaling(119). Thus, we set out to determine the differential effects of IL-6 classical and *trans* signaling on normal and IPF fibroblast proliferation and apoptosis *in vitro*.

Baseline proliferation rates of IPF fibroblasts were already higher than those of normal fibroblasts (Figure 5.14A). Stimulation with IL-6 alone resulted in a significant increase in proliferation in IPF fibroblasts but not normal fibroblasts. The combination of IL-6 and soluble IL-6Rα significantly promoted proliferation in both normal and IPF fibroblasts. When proliferation rates were reported as a change from baseline rates, stimulation with IL-6 and sIL-6Rα resulted in a significant 1.2-fold difference in normal fibroblasts (Figure 5.14B). In IPF fibroblasts, IL-6 stimulation alone was sufficient to induce a significant 1.4-fold increase in

proliferation, and IL-6 in combination with sIL-6Rα resulted in a 1.5-fold increase in proliferation. The difference in response to *trans* signaling may be due to baseline expression of membrane IL-6Rα in control versus IPF fibroblasts (Figure 5.14C).

In terms of spontaneous apoptosis, IL-6 alone had no effect on normal fibroblasts while IL-6 + sIL-6Rα caused a slight decrease in apoptosis at a lower confluency (Thesis Figure 5.15A) and increased apoptosis significantly at a higher confluency (Figure 5.15B). In IPF fibroblasts, IL-6 alone enhanced spontaneous apoptosis at low and high levels of confluency (Figure 5.15C&D). IL-6 + sIL-6Rα also enhanced apoptosis at low confluency but there was no significant increase at higher confluency of fibroblasts. In all, these experiments demonstrate a role for IL-6 *trans* signaling in proliferation and apoptosis of pulmonary fibroblasts.

5.16 – *Neutralization of IL-6 trans signaling in vitro inhibited production of collagen in control and IPF fibroblasts.*

To evaluate whether blockade of IL-6 *trans* signaling affects protein production in vitro, control and IPF fibroblasts were stimulated with IL-6 and sIL-6Rα for 48 hours in the presence or absence of recombinant gp130Fc. Protein lysates were prepared and subjected to western blot analyses for detection collagen expression. Results revealed inhibition of collagen production with addition of gp130Fc in both control and IPF fibroblasts (Figure 5.16A&B).

5.17-5.18 – *Neutralization of IL-6 trans signaling in vitro inhibited fibroblast proliferation and altered spontaneous apoptosis.*

To evaluate whether blockade of IL-6 *trans* signaling affects proliferative capacity of normal and IPF fibroblasts in vitro, proliferation rates were assessed in response to in vitro

neutralization of *trans* signaling via addition of recombinant gp130Fc. In normal fibroblasts, increasing concentrations of gp130Fc resulted in significant dose-dependent inhibition of proliferation, beginning at smaller doses (Figure 5.17A&B). In IPF fibroblasts, there was a trend for increasing inhibition of proliferation, with significant inhibition occurring at a higher dose of gp130Fc (Figure 5.17C&D). The difference in response to neutralization concentrations may be due to the higher responsiveness of IPF fibroblasts to stimulation by *trans* signaling in comparison to normal fibroblasts (Figure 5.14C).

In terms of spontaneous apoptosis, in normal fibroblasts, IL-6 + sIL-6Rα caused a significant reduction in apoptosis, which was reversed with gp130Fc (Figure 5.18A). In IPF fibroblasts, IL-6 + sIL-6Rα enhanced spontaneous apoptosis, an effect that began to show evidence of reversing at the highest dose of gp130Fc (Figure 5.18B).

In all, the findings from these *in vitro* experiments support a role for IL-6 *trans* signaling in enhancing fibroblast proliferation, activation and extracellular matrix protein production that could lead to the overpopulation of the lung parenchyma and excessive protein deposition observed in pulmonary fibrosis.
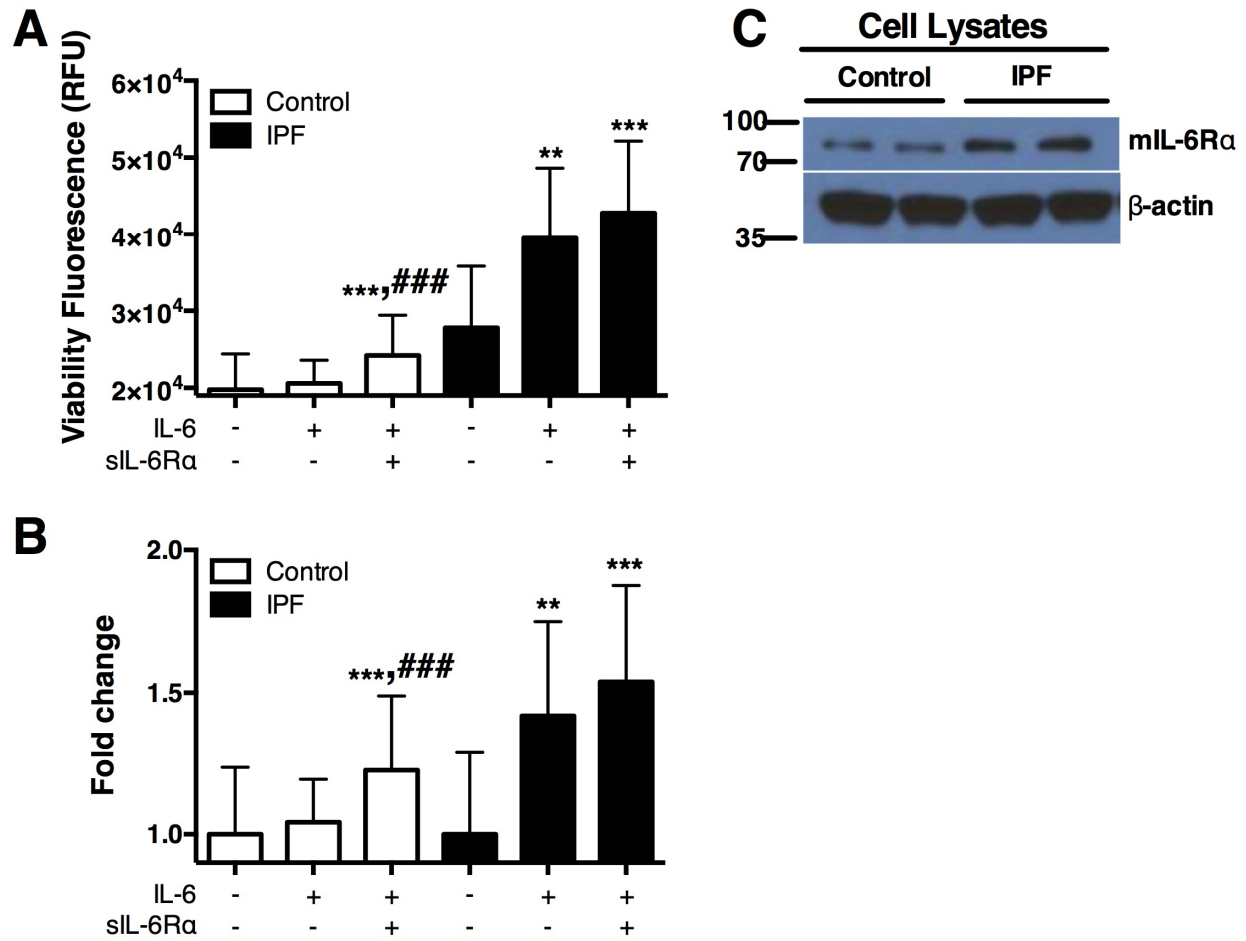
Figure 5.14. Effect of IL-6 *trans* signaling on proliferation rates in control and IPF fibroblasts in vitro. Control and IPF fibroblasts were serum-starved for 24 hours and then stimulated for 48 hours with IL-6 alone or IL-6 and sIL-6Rα. Proliferation rates were assessed in response to IL-6 and soluble IL-6Rα and expressed in raw data (**A**) and change from baseline proliferation (**B**). (**C**) Membrane IL-6Rα expression in control and IPF fibroblasts. All data presented as mean ± SEM, n≥8. *significant difference from media only cohort; #significant difference from IL-6 only group; *= p<0.05, **= 0.001<p<0.01, ***= p<0.001.
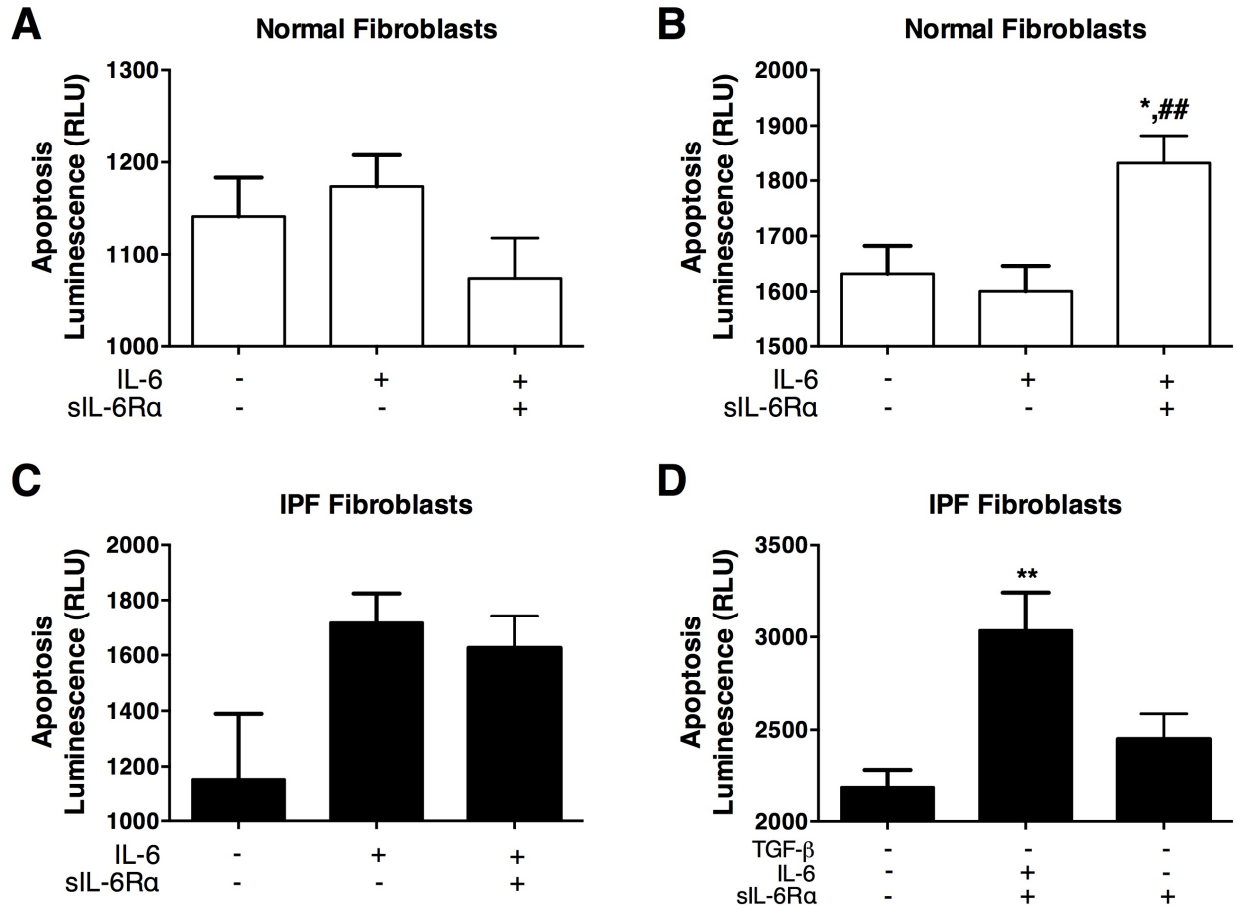
Figure 5.15. Effect of IL-6 *trans* signaling on spontaneous apoptosis in control and IPF fibroblasts in vitro. Control and IPF fibroblasts were serum-starved for 24 hours and then stimulated for 48 hours with IL-6 alone or IL-6 and sIL-6Rα. Spontaneous apoptosis at low and high confluency in (**A&B**) control fibroblasts and (**C&D**) IPF fibroblasts was assessed in response to IL-6 and soluble IL-6Rα and expressed in raw data. All data presented as mean ± SEM, n≥8. *significant difference from media only cohort; #significant difference from IL-6 only group; *= p<0.05, **= 0.001<p<0.01, ***= p<0.001.
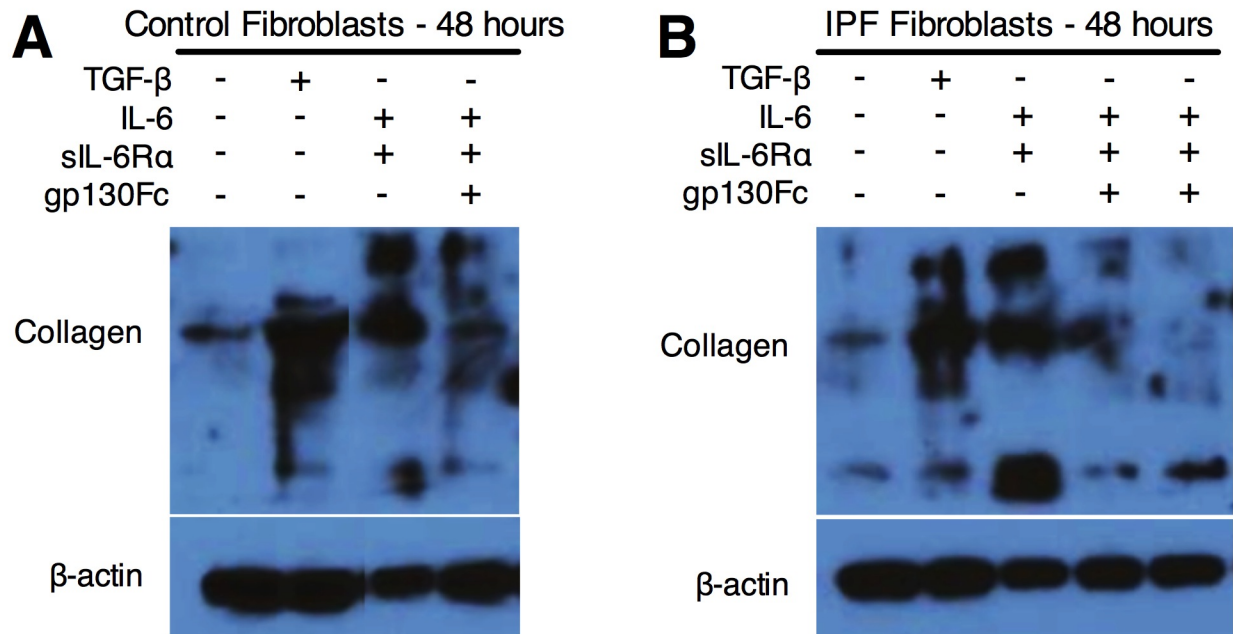
Figure 5.16. Effect of neutralizing IL-6 *trans* signaling on collagen production in control and IPF fibroblasts in vitro. Control and IPF fibroblasts were serum-starved for 24 hours and then stimulated for 48 hours with IL-6 and sIL-6Rα in the presence of absence of recombinant gp130Fc. Western blot analyses of collagen expression in (**A**) normal fibroblasts and (**B**) IPF fibroblasts.
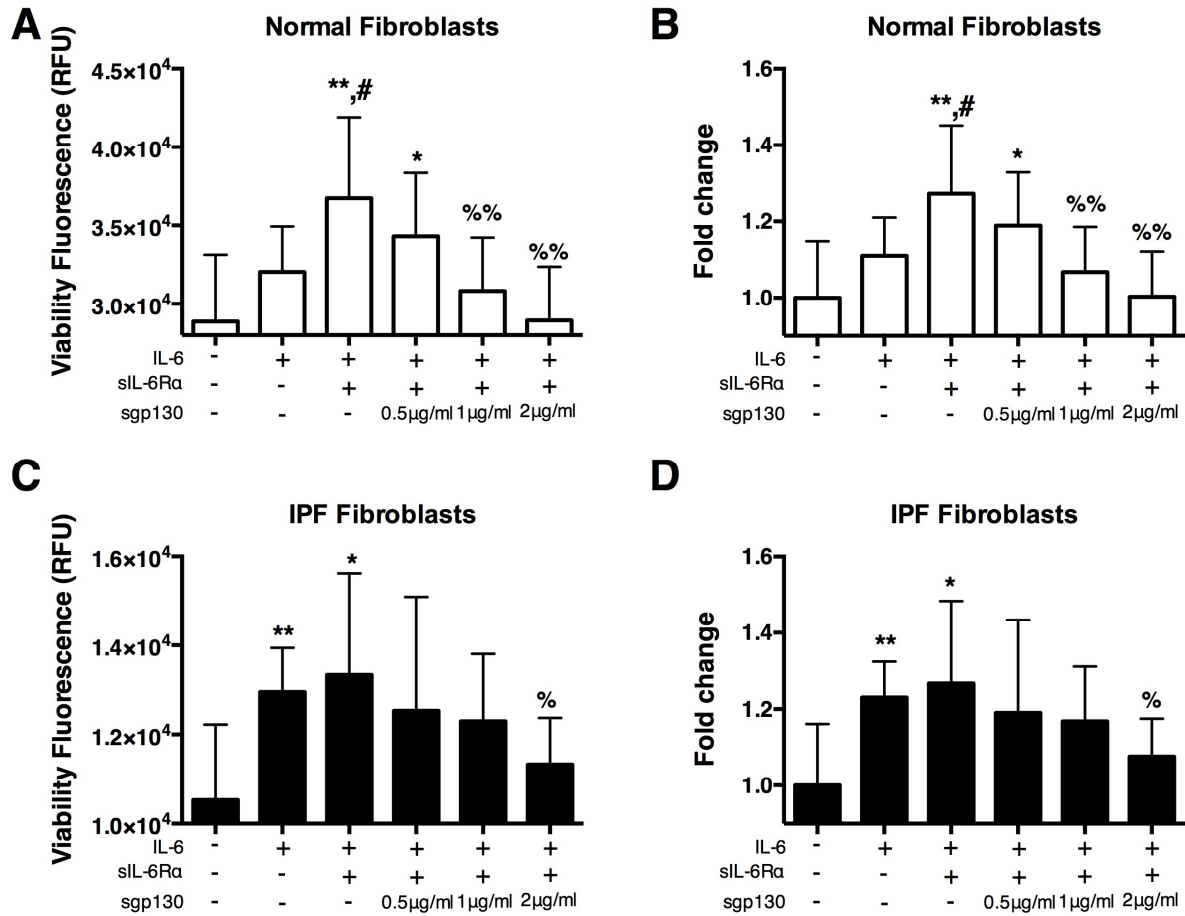
Figure 5.17. Effect of neutralizing IL-6 *trans* signaling on proliferation rates in control and IPF fibroblasts in vitro. Control and IPF fibroblasts were serum-starved for 24 hours and then stimulated for 48 hours with IL-6 alone or IL-6 and sIL-6Rα in the presence of absence of recombinant gp130Fc. (**A&B**) Changes to proliferation rate of normal fibroblasts in response to increasing concentrations of gp130Fc, expressed as raw data and changes with respect to baseline. (**C&D**) Changes to proliferation rate of IPF fibroblasts in response to increasing concentrations of gp130Fc, expressed as raw data and changes with respect to baseline All data presented as mean ± SEM, n≥6. *significant difference from media only cohort; #significant difference from IL-6 only group; %significant difference from IL-6+sIL-6Rα cohort; *= $p < 0.05$, **= $0.001 < p < 0.01$, ***= $p < 0.001$.
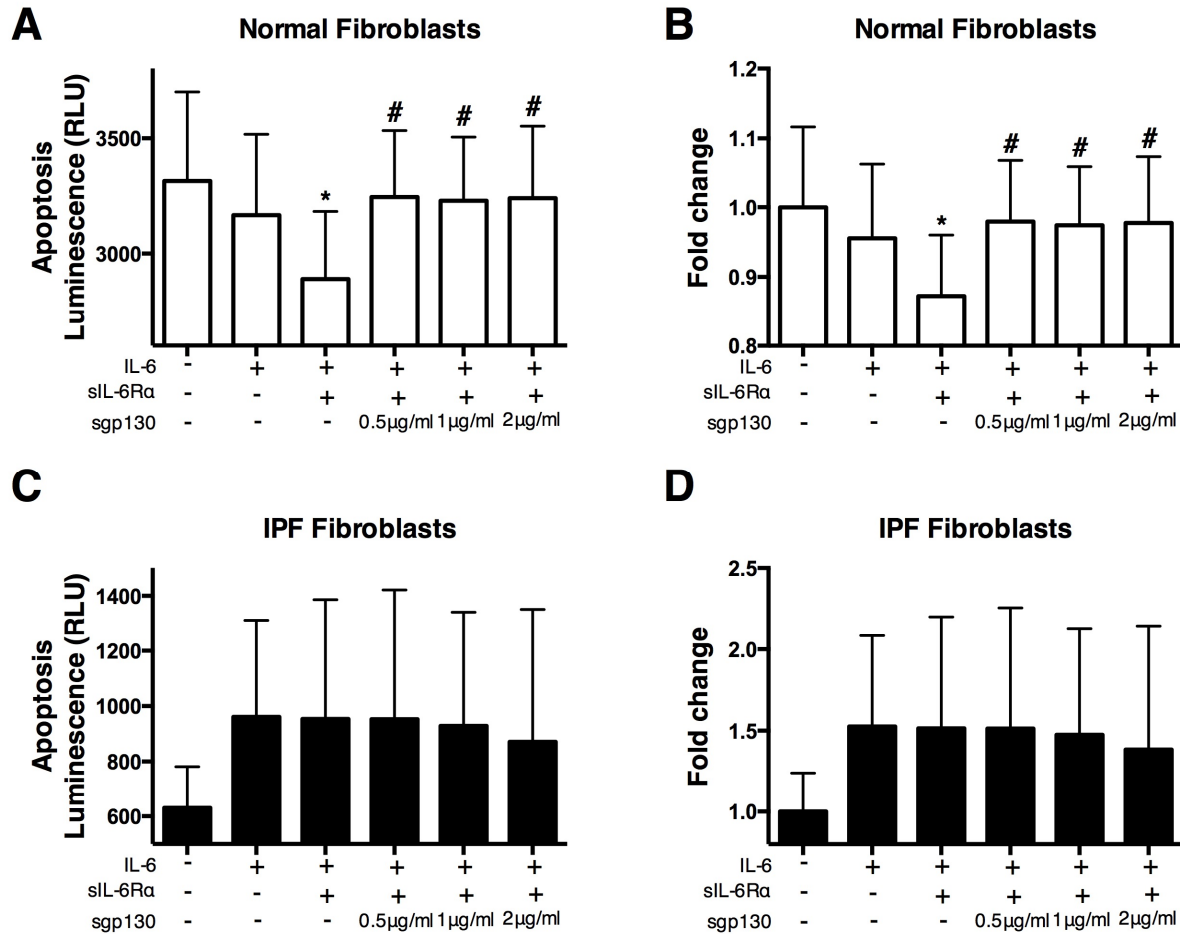
Figure 5.18. Effect of neutralizing IL-6 *trans* signaling on spontaneous apoptosis in control and IPF fibroblasts in vitro. Control and IPF fibroblasts were serum-starved for 24 hours and then stimulated for 48 hours with IL-6 alone or IL-6 and sIL-6Rα in the presence of absence of recombinant gp130Fc. (**A&B**) Changes to spontaneous apoptosis of normal fibroblasts in response to increasing concentrations of gp130Fc, expressed as raw data and changes with respect to baseline. (**C&D**) Changes to spontaneous apoptosis of IPF fibroblasts in response to increasing concentrations of gp130Fc, expressed as raw data and changes with respect to baseline All data presented as mean ± SEM, n≥6 for control and n≥4 for IPF. *significant difference from media only cohort; [#]significant difference from IL-6 only group; [%]significant difference from IL-6+sIL-6Rα cohort; *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$.

5.19 – Recombinant gp130Fc reduced mIL-6Rα protein in whole lung and BAL cells.

In our *in vitro* mechanistic studies of control and IPF fibroblasts, we saw an association between increases and decreases in membrane IL-6Rα protein expression after stimulation of *trans* signaling in control and IPF fibroblasts and resulting changes in proliferation and protein production. This association suggested a cooperative effect of IL-6 *trans* and classical signaling in development of pulmonary fibrosis. Given this knowledge, we wanted to evaluate changes to mIL-6Rα after *in vivo* blockade of *trans* signaling to see if the same association is seen *in vivo*.

Whole lung RNA and protein lysates were prepared from Day 33 bleomycin-exposed and gp130Fc-treated mice. Whole lung RNA was subjected to q-rtPCR analysis for transcript levels of mIL-6Rα. Protein lysates were subjected to western blot analysis of mIL-6Rα expression. Western blot analyses revealed elevated expression of membrane IL-6Rα protein in bleomycin-exposed lungs (Figure 5.19A), consistent with the results of our initial characterization studies (Figure 3.4A). Gp130Fc treatment resulted in a reduction in protein expression. Protein lysates were also prepared from Day 33 BAL cells and western blot performed for evaluation of mIL-6Rα. BAL cells from bleomycin-exposed lungs exhibited elevated mIL-6Rα protein expression (Figure 5.19B), once again consistent with our previous results (Figure 3.4B). Gp130Fc-treated lungs experienced a reduction in mIL-6Rα. Immunofluorescence staining of day 33 BAL cells revealed augmented expression of mIL-6Rα in bleomycin-exposed, fibrotic lungs (Figure 5.19B). BAL cells from gp130Fc-treated mice displayed less expression of mIL-6Rα. These results, in combination with our *in vitro* results, indicate that *in vivo* neutralization of IL-6 *trans* signaling using gp130Fc had effected a reduction in classical signaling as well.
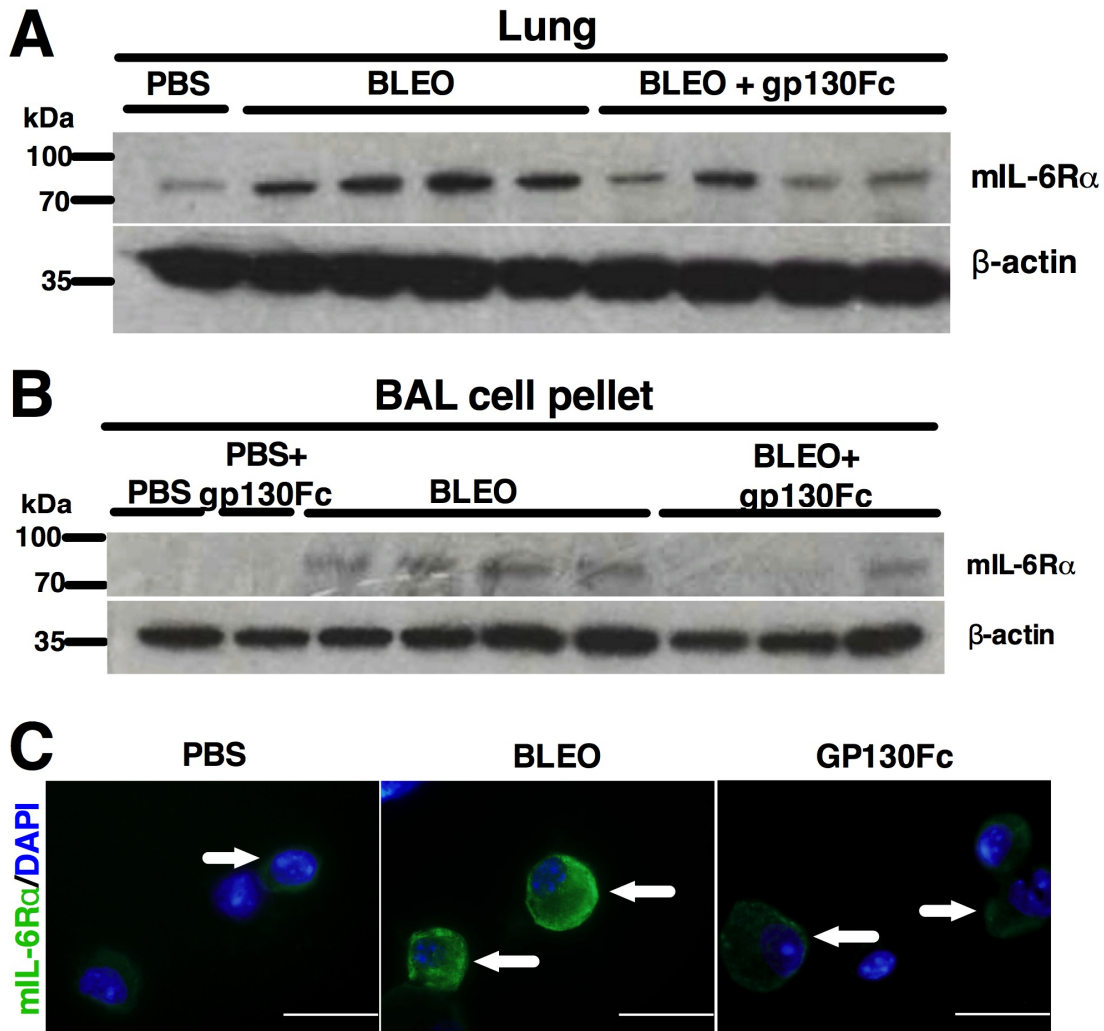
Figure 5.19. Changes in membrane IL-6R alpha following chronic bleomycin exposure in mice treated with recombinant gp130Fc. Wildtype C57Blk6 male mice were injected intraperitoneally with saline or bleomycin twice weekly for 4 weeks. Beginning on day 19, when pulmonary fibrosis has been established, daily treatment with vehicle (saline) or recombinant gp130Fc was performed. Mice were sacrificed and samples collected on day 33. Western blot analysis of mIL-6Rα expression in (**A**) whole lung and (**B**) BAL cell pellet protein lysates. (**C**) Immunofluorescence detection of mIL-6Rα on macrophages isolated from bleomycin-exposed mice treated with saline or gp130Fc; membrane IL-6Rα (green), dapi (blue). Arrows point to positive cells.

DISCUSSION

In support of a role for IL-6 *trans* signaling in pulmonary fibrosis, we have demonstrated ADAM17-mediated increases in soluble IL-6Rα in association with disease. To determine if there were therapeutic benefits to *in vivo* neutralization of sIL-6Rα and resulting antagonism of IL-6 *trans* signaling in disease, we utilized recombinant gp130Fc to neutralize sIL-6Rα and selectively block IL-6 *trans* signaling in our chronic bleomycin model and evaluated the effects on pulmonary inflammation and fibrosis.

In the IPB model, bleomycin-exposed mice developed significant pulmonary inflammation. In mice challenged with bleomycin and treated with gp130Fc, there were marked reductions in pulmonary inflammation, as evident by a reduction in the number of inflamatory cells recovered in BAL fluid, including reductions in macrophages, lymphocytes, neutrophils and eosinophils. The reduction in pulmonary inflammation with gp130Fc treatment in our model is consistent with previous studies demonstrating that inhibition of IL-6 *trans* signaling resulted in improvement in chronic inflammatory conditions like colitis(76), arthritis(73), and colitis-associated premalignant cancer(78). These results suggest that IL-6 *trans* signaling is responsible for the pro-inflammatory property of IL-6 in a model of pulmonary fibrosis. Though the role of inflammation in the pathogenesis of IPF is now controversial, there is still much interest in exploring specific cellular players in the lung and their roles in fibrosis. Of particular interest is the gp130Fc-mediated decrease in macrophages and lymphocytes that we saw in our study. This could point to mechanisms for reduced severity of disease with gp130Fc administration. Both macrophages and lymphocytes have been suggested to play roles in the development of pulmonary fibrosis (113-117, 146, 147), but their exact contribution to IPF has not been examined. Further evaluation of these cell types and their role in disease is needed.

Another point of interest is the gp130Fc-mediated reduction in IL-6-responsive mediators IL-17 and TIMP-1. Wilson et al previously reported increased IL-17A levels in bronchoalveolar lavage fluid from IPF patients and demonstrated an essential role for IL-17A in bleomycin and IL-1β-induced pulmonary fibrosis in mice(148). Conte et al demonstrated that a reduction in IL-17-producing cells and IL-17 expression protected mice from bleomycin-induced pulmonary fibrosis(149). However, other studies suggest differently and the role of IL-17 in pulmonary diseases is controversial(150). Galati et al reported a significant reduction in Th17 cells and an increased TGF-α/IL-17 ratio in IPF patients(151). Interestingly, Ge et al demonstrated that lack of IL-17 receptor A (IL-17RA), which mediates IL-17A signaling, led to decreased numbers of tissue macrophages and attenuated inflammation and fibrosis in the kidneys(152). Nuovo et al demonstrated localization of IL-17 expression to regenerating epithelial cells and alveolar macrophages in IPF lungs(147). Our study demonstrated decreased macrophage numbers in association with decreased IL-17 in mice treated with gp130Fc treatment. This would suggest that IL-6 *trans* signaling may be regulating macrophage accumulation in the lung via IL-17 and needs further study.

Our study revealed that TIMP-1 transcript was increased in bleomycin-exposed fibrotic lungs but decreased with gp130Fc treatment. This change in TIMP-1 is consistent with a previous study showing induction of TIMP-1 in human synovial fibroblasts after stimulation of IL-6 *trans* signaling, resulting in decreased collagenolytic activity(153). This suggests that blockade of IL-6 *trans* signaling by gp130Fc lifted the inhibitory effect of TIMP-1 and restored collagenolytic activity in the lung. Further study is needed to evaluate whether protein expression was reduced and whether it resulted in increased degradation of deposited collagen and resolution of fibrosis.

The compelling finding of this study was the novel observation that neutralization of IL-6 *trans* signaling resulted in attenuation of pulmonary fibrosis in our chronic bleomycin model. The hallmark features of IPF include excessive fibroblast activation, accumulation of α-sma-positive myofibroblasts and excessive production and deposition of matrix proteins, including collagen and fibronectin(2). Our study demonstrated that neutralization of IL-6 *trans* signaling resulted in a reduction in myofibroblast accumulation and extracellular matrix protein production and deposition in the lungs, which translated to a reduction in pulmonary fibrosis and improvement in pulmonary oxygenation. Previous studies have suggested a role for IL-6 *trans* signaling in liver fibrosis(154), renal fibrosis(82) and myocardial fibrosis(72), though none have demonstrated improvement in fibrosis with blockade of *trans* signaling *in vivo* using gp130Fc. This is the first study to examine the role of IL-6 *trans* signaling in pulmonary fibrosis and demonstrated a therapeutic benefit to antagonism of this pathway *in vivo*.

In search of the underlying mechanism that would explain the improvement in fibrosis seen with gp130Fc treatment, we performed *in vitro* mechanistic studies that revealed the ability of IL-6 *trans* signaling to promote proliferation and collagen and fibronectin production in control and IPF fibroblasts, events crucial to disease progression. Findings from our *in vitro* studies of IL-6 *trans* signaling in fibroblasts are consistent with previous studies that have demonstrated that *trans* signaling can induce hepatocyte proliferation and intracellular signaling(154), promote collagen production in cardiac fibroblasts(72) and induce proliferation and extracellular matrix protein production in fibroblasts from hypertrophic scars(155). What may warrant further study is the downstream effects of IL-6 classical versus *trans* signaling in fibroblasts that may explain the differences in stimulation of fibronectin versus collagen production.
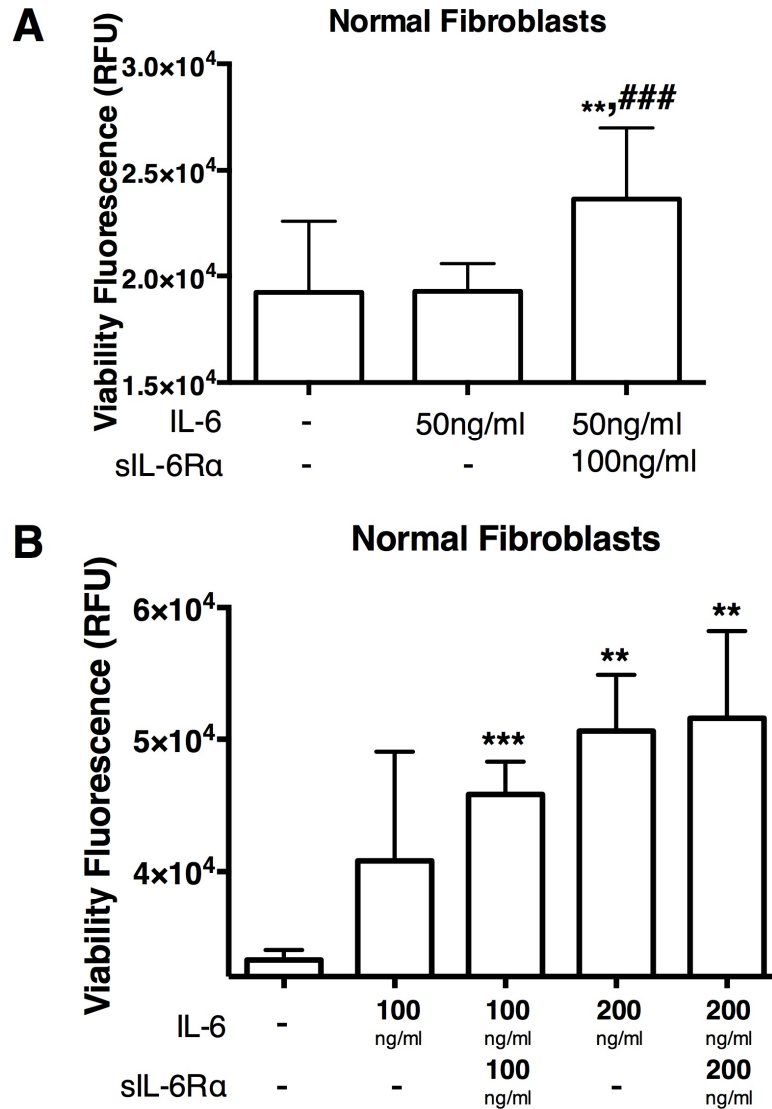
Figure 5.20. Proliferation rates in control fibroblasts *in vitro*. Control fibroblasts were serum-starved for 24 hours and then stimulated IL-6 alone or IL-6 and sIL-6Rα. (**A**) Changes to proliferation rate after 24 hours of stimulation. (**B**) Changes to proliferation rate after 48 hours of stimulation with increasing concentrations of IL-6 alone or IL-6 and sIL-6Rα. All data presented as mean ± SEM, n≥4. *significant difference from media only cohort; [#]significant difference from IL-6 only group; [%]significant difference from IL-6+sIL-6Rα cohort; *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$.

It must be noted, however, that Moodley et al examined the effect of IL-6 on fibroblast proliferation and concluded that IL-6 inhibits proliferation and enhanced Fas-induced apoptosis in normal fibroblasts but enhanced proliferation and increased resistance to apoptosis in IPF fibroblasts(144, 145) Differences between these findings may be related to the source of cells or conditions used. For example, the starvation and stimulation times were different between our studies. To confirm our results seen with 48 hours of stimulation, we evaluated proliferation in control fibroblasts after 24 hours of stimulation with IL-6 or IL-6 + sIL-6Rα. We did not show growth inhibition with IL-6 stimulation, even at 24 hours (Figure 5.20A). In the study by Moodley et al, changes in proliferation in response to IL-6 stimulation was compared to time 0 fibroblasts. In our study, changes were reported in comparison to fibroblasts that have been cultured without stimulation for the same period of time as stimulated fibroblasts, i.e. fibroblasts cultured in media only for 24 0r 48 hours. Both studies agree that stimulation of normal fibroblasts with IL-6 ≤50ng/ml does not result in inhibition of proliferation. Moodley et al reported growth inhibition with IL-6 stimulation ≥100ng/ml, while in our hands, even higher concentrations of IL-6 were not anti-proliferative in normal fibroblasts (Figure 5.20B). It is unclear the reason for this difference in observations. However, Moodley et al did not examine the effect of *trans* signaling on fibroblast proliferation, whereas our study did.

With regards to our apoptosis data, we did not examine Fas-induced apoptosis but rather only spontaneous apoptosis, so our results cannot be accurately compared to those of Moodley et al. We did not show enhanced apoptosis in normal fibroblasts stimulated with IL-6 alone, but we did see enhanced apoptosis in those stimulated with IL-6 + sIL-6Rα. In IPF fibroblasts, we saw enhanced apoptosis with stimulation of classical signaling and no change to apoptosis with stimulation of *trans* signaling. Thus, these results regarding spontaneous apoptosis, when

analyzed along with the proliferation data, suggest that classical signaling doesn't affect proliferation or spontaneous apoptosis in normal fibroblasts. *Trans* signaling in normal fibroblasts, however, increases proliferation and exert different effects on apoptosis depending on the availability of space. *Trans* signaling may act to inhibit apoptosis when there is room for growth; however, when space is limited, *trans* signaling increases apoptosis of normal fibroblasts to control expansion. On the other hand, in IPF fibroblasts, IL-6 classical signaling alone can promote proliferation and increases apoptosis in low and high confluency states. *Trans* signaling in IPF fibroblasts, promotes apoptosis at lower confluency while inhibiting apoptosis at higher confluency, failing to control growth, leading to unregulated expansion. Further work is needed to evaluate differential effects of IL-6 *trans* signaling with regards to stimulus-induced apoptosis.

The finding of improvement in pulmonary hypertension with recombinant gp130Fc treatment in our study was intriguing. As mentioned in chapter 1, PH is a recognized complication of the disease process in IPF. The presence of PH greatly increases mortality risk and decrease survival of IPF patients(2, 6, 11). Yet, there are no recommendation for therapy except in very advanced cases of PH associated with IPF. Hypoxemia (which can occur in lung disease) leads to PH and we were able to show that gp130Fc treatment caused an improvement in pulmonary hypertension. It's possible that hypoxemia affects generation of sIL-6Rα, leading to PH. The improvement in PH could be due improvement in hypoxemia or a reduction in pulmonary fibrosis. It would be interesting and fruitful to explore the role of IL-6 *trans* signaling in pulmonary hypertension. This has not been studied and needs further exploration.

Surprisingly, our analysis of changes to IL-6 classical signaling in response to neutralization of sIL-6Rα *in vivo* also generated some interesting theories. The decrease in membrane IL-6Rα mRNA in bleomycin-induced fibrotic lungs was consistent with published

results showing IL-6-mediated suppression of mIL-6Rα mRNA expression in a hepatoma cell line(156) or colitis-associated decreased mIL-6Rα mRNA(78). Yet, protein expression of membrane IL-6Rα was elevated in fibrotic lungs (in association with increased soluble receptor) and decreased with gp130Fc treatment, suggesting that the membrane receptor protein expression, and therefore IL-6 classical signaling, may be directly or indirectly regulated by *trans* signaling. Our *in vitro* studies appear to support this idea. Activation of pulmonary fibroblasts, via stimulation of *trans* signaling, can increase expression of membrane IL-6Rα (Figure 5.12). This would suggest a mechanism of action in fibrotic lungs, whereby IL-6 *trans* signaling may work to enhance classical signaling by increasing expression of the membrane receptor. The increased expression of the membrane receptor can either: 1) increase classical signaling if the membrane receptor is not cleaved or 2) increase *trans* signaling if the membrane receptor is cleaved to form the soluble receptor. In this way, there is potential for perpetuation of IL-6 signaling that leads to over-amplification of effects, resulting in progression of disease. We need to further study the effect of *trans* signaling on other pulmonary cell types as well as examine the changes to ADAM17 expression and production of sIL-6Rα from pulmonary fibroblasts after stimulation of IL-6 *trans* signaling in order to better understand the consequence of this pathway in the lung.

The results of our *in vitro* studies raises questions about the existence of a cross-talk between TGF-β and IL-6 in pulmonary fibroblasts. We know TGF-β is increased in IPF and has been shown to be a pivotal player in disease(143). There is evidence of crosstalk between these two cytokines in other diseases. Yamamoto et al reported that TGF-β potentiates IL-6 induced STAT3 activation and resulting gene expression in hepatoma cells(157). Walia et al demonstrated TGF-β1 negatively regulated IL-6 signaling in intestinal epithelial cells(158).

Yamada et al provided evidence of a crosstalk betweent TGF-β1 and IL-6 in biliary tract cancer cells resulting in conference of malignant features, including epithelial-mesenchymal transition, increased invasiveness, and resistance to chemotherapy(159). More recently, O'Reilly et al revealed that IL-6 *trans* signaling, through phosphorylation of STAT3 and downstream activation of gremlin, leads to activation of TGF-β signaling and collagen production in dermal fibroblasts from systemic sclerosis patients(160). The intricacies of interaction of IL-6 and TGF-β in IPF lungs, however, remains unclear.

Elias et al has demonstrated that TGF-β induces IL-6 production in normal human pulmonary fibroblasts(161). Gallelli et al illustrated the role of membrane IL-6 receptor alpha in mediating TGF-β-induced proliferation of normal and fibrotic lung fibroblasts by showing decreased proliferation with the use of an antagonist against the receptor(162). In this study, we showed that TGF-β stimulation of control and IPF lung fibroblasts *in vitro* resulted in enhanced expression of mIL-6Rα protein in association with increased collagen and fibronectin production and increased myofibroblast differentiation (Figure 5.12). Our results are consistent with published results demonstrating TGF-β-mediated regulation of mIL-6Rα mRNA in a hepatoma cell line(156) and mIL-6Rα protein expression in biliary tract cancer cells(159). No previous study has reported changes to mIL-6Rα protein expression in response to TGF-β stimulation of lung fibroblasts though. The change in mIL-6Rα protein expression we have shown in response to TGF-β stimulation may represent mechanisms of positively regulating TGF-β activity since IL-6 has been reported to increase TGF-β1 signaling by regulating TGF-β receptor compartmentalization and turnover in human renal tubular epithelial cells (163). It could also be a means of counteracting the effects of TGF-β. Chen et al demonstrated that IL-6 protects hepatoma cells from TGF-β-induced apoptosis(164). Exactly how IL-6 and TGF-β interact – at

what time, in what cells – in order to exert their effects and whether this crosstalk perpetuates the actions of both these cytokines in fibrotic lungs needs further analysis to fully understand.

Our *in vivo* and *in vitro* findings also led to questions regarding the role of IL-6 classical and *trans* signaling in macrophages and macrophage-fibroblast interactions. Murray et al demonstrated that TGF-β-mediated pulmonary fibrosis is dependent on macrophages of the M_2 phenotype(116). In our timecourse study, we have demonstrated an increase in macrophages in our chronic bleomycin model of pulmonary fibrosis (Figure 4.2C). In our *in vivo* neutralization study, we showed a reduction in these macrophages in gp130Fc-treated lungs associated with decreased pulmonary fibrosis (Figure 5.3B). These macrophages are known to be primarily M_2 in phenotype (Figure 2.4B) and we have reported increased mIL-6Rα protein expression on these cells as well as increased shedding of the membrane receptor from these cells to produce sIL-6Rα (Figure 4.1 & 4.2). Thus we have supported an important role for them in disease, in agreement with Murray et al. We have also suggested a role for IL-6 *trans* signaling in recruitment and activation of M_2 macrophages. Further study is needed to determine what the roles of IL-6 classical and *trans* signaling are in generation of these activated macrophages as well as in regulating their activities.

We also know from the literature that TGF-β expression has been shown to be localized to epithelial cells and alveolar macrophages after bleomycin-induced pulmonary fibrosis(165, 166). We know TGF-β transcript levels are elevated in normal alveolar macrophages and peripheral monocytes but only when the cells are activated do protein synthesis and TGF-β secretion occur(167). That knowledge, along with what we have seen in our studies, suggests a possible model of recruitment and activation of macrophages in the lungs in response to injury, which increases TGF-β secretion, which in turn can regulate mIL-6Rα expression in these

macrophages and other cells in the lung, including fibroblasts, to influence cellular activation, mediator production and induce collagen and fibronectin synthesis and secretion, all of which contributes to pulmonary fibrosis. It would also be interesting to determine if this proposed model is supported in the case of IPF patients. Of particular interest is the role of macrophage-fibroblast interactions in development of fibrosis. Ma et al reported that co-cultures of macrophages and cardiac fibroblasts led to macrophage stimulation of IL-6 production in cardiac fibroblasts, resulting in activation of TGF-β1 and promotion of cardiac fibrosis(168). The same could be true in pulmonary fibrosis and needs to be studied.

Overall, our *in vivo* and *in vitro* data suggest that IL-6 *trans* signaling mediates pro-proliferative, pro-fibrotic effects in the lungs. We demonstrated the essential role of *trans* signaling in pulmonary fibrosis and the therapeutic benefits of its neutralization, providing a novel, potentially effective treatment modality for IPF. However, the contribution of IL-6 classical signaling to disease cannot be denied as it appears that classical signaling in the lungs seem to be intimately connected with *trans* signaling. Our studies have provided a more detailed understand of IL-6 classical and *trans* signaling in IPF and our mouse model of pulmonary fibrosis, but there are many questions that remain unanswered and need further evaluation.

CHAPTER 6

Interleukin-6 (IL-6)

Classical and *Trans* Signaling in the

Adenosine-deaminase (ADA)-Deficient

Murine Model of Pulmonary Fibrosis

EXPERIMENTAL RATIONALE & KEY QUESTIONS

Having demonstrated, in chapters 3-5, the involvement and role of both IL-6 classical and *trans* signaling in IPF and in our chronic bleomycin mouse model of pulmonary fibrosis, we proceeded to characterize these pathways in another mouse model, the adenosine-deaminase (ADA)-deficient model (Figure 1.4). In the ADA-deficient murine model of pulmonary fibrosis, genetic removal of the enzyme ADA results in adenosine-mediated pulmonary inflammation, emphysematous distal airspace enlargement and pulmonary fibrosis(28, 94, 95). Studying IL-6 classical and *trans* signaling in this model will serve to confirm and enhance the results we obtained in the IPB model. At the same time, we will benefit from a second animal model that consists of the co-existence of pulmonary fibrosis and emphysema.

Based on the analyses of recent retrospective studies, the prevalence of IPF patient with features of co-existing emphysema is about 28-55% depending on the population(17, 18). Over the past decade, there has been controversy about whether the presence of pulmonary fibrosis and emphysema represents a subgroup of IPF or a distinct clinical entity in itself. Cottin et al first coined the name Combined Pulmonary Fibrosis and Emphysema (CPFE) in 2005 when they formally described clinical characteristics comprising this syndrome. Those characteristics included findings of dyspnea on exertion, basal crackles, finger clubbing, upper lobe emphysema and lower lobe pulmonary fibrosis on CT, abnormal spirometry, severe gas exchange impairment, and high prevalence of pulmonary hypertension(18). What is clear though, is that the presence of both pulmonary fibrosis and emphysema carries a poorer prognosis than IPF alone and mortality is even worse if pulmonary hypertension is also present(6, 17, 18). Thus, better comprehension of pulmonary fibrosis with co-existing emphysema is of interest to us, and the ADA-deficient mouse model provides a medium to explore these manifestations.
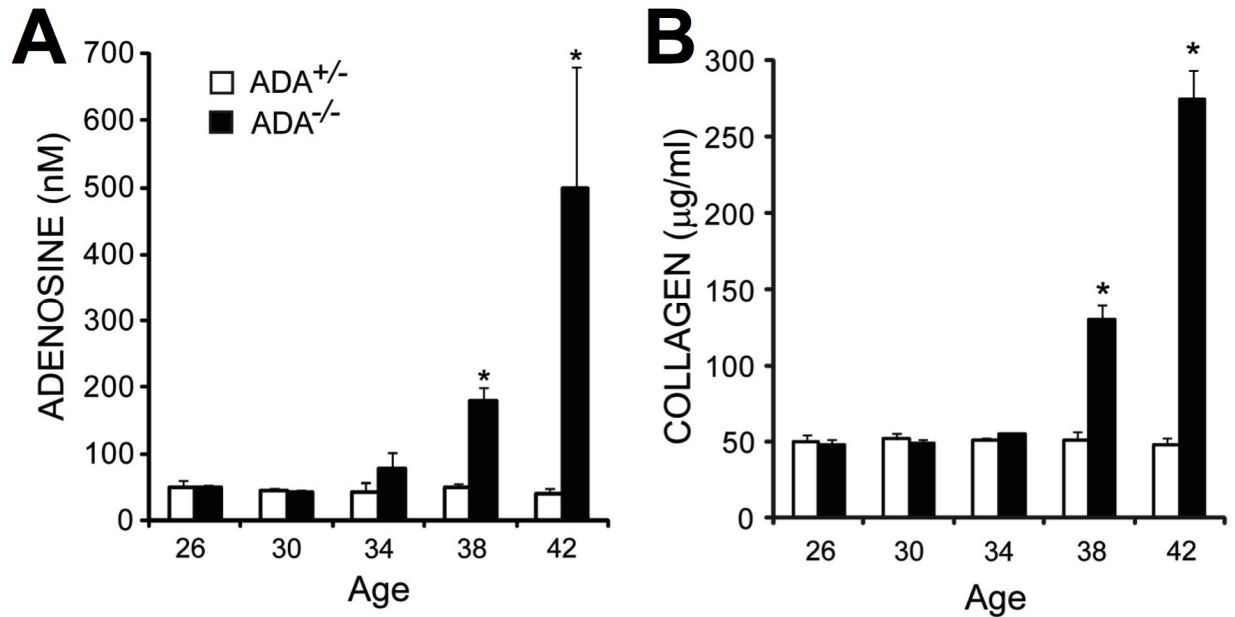
Figure 6.0. Adenosine level and disease progression in adenosine-mediated pulmonary fibrosis. ADA-deficient (ADA$^{-/-}$) mice were identified at birth and maintained on ADA enzyme replacement therapy until postnatal day 25. Therapy was then discontinued and disease allowed to develop. BAL fluid samples were collected at various timepoints during the course of disease progression. (**A**) Measurement of adenosine levels was performed in BAL fluid samples from ADA$^{-/-}$ mice and their ADA-competent (ADA$^{+/-}$) littermates. (**B**) Soluble collagen was quantified in BAL fluid samples using Sircol assay. Results from A&B obtained by Ernestina Melicoff, MD. All data presented as mean ± SEM, n≥4. *significant difference from ADA$^{+/-}$ cohort; *= p<0.05, **= 0.001<p<0.01, ***= p<0.001.

In the ADA-deficient model (Figure 1.4), ADA-deficient (ADA$^{-/-}$) mice are identified at birth and maintained on ADA enzyme replacement therapy from postnatal day 1 to 25 to allow for normal pulmonary development. Discontinuation of therapy then allows for progressive accumulation of endogenous adenosine in the lungs (Figure 6.0A), leading to chronic injury and inflammation, enlargement of distal airways and development of pulmonary fibrosis, as indicated by increasing presence of collagen in the lungs (Figure 6.0B). Elevations in adenosine have been linked to development of disease in this model and reversal of disease was seen with therapy to lower adenosine levels, thus demonstrating the influence of adenosine in lung disease(28, 95, 96). The ADA-deficient model has been used extensively by our lab as well as others to study processes involved in adenosine-mediated lung injury(28-30, 97-102, 169).

Adenosine is a signaling nucleoside generated in response to lung injury. Through activation of its G-protein-coupled receptors (A$_1$AR, A$_{2A}$AR, A$_{2B}$AR and A$_3$AR), adenosine exerts various effects on inflammation, repair and remodeling processes, and fibrosis, producing either tissue-protective or tissue-destructive results(25, 27, 170). Adenosine has been shown, in humans and mouse models, to be elevated in and contribute to chronic lung disorders, including asthma, COPD, and pulmonary fibrosis. Recent publications have reported, specifically, that it is the activation of the A$_{2B}$ receptor by adenosine that mediates production of pro-fibrotic molecules, like interleukin-6, leading to chronic lung diseases(25, 28-31, 91). Adenosine regulates production and release of IL-6 from various cell types, including from lung epithelial cells(32), macrophages(33), smooth muscle cells(34) and fibroblasts(35). More recently, Pedroza et al demonstrated the role of IL-6 in mediating pulmonary fibrosis in this model(29). To further clarify the roles of IL-6 classical versus *trans* signaling in this model, we set out to characterize the pathways and answer the following questions:

6.1 Is soluble IL-6Rα increased in adenosine-mediated pulmonary fibrosis? What happens as disease progresses?

6.2 Is membrane IL-6Rα increased in pulmonary fibrosis? What happens to levels as pulmonary fibrosis develops and progresses?

6.3 Is ADAM17 elevated in adenosine-mediated pulmonary fibrosis? What happens to expression throughout the model?

6.4-6.7 Does in vivo neutralization of IL-6 trans signaling attenuate pulmonary inflammation? What about pulmonary fibrosis?

RESULTS

6.1 – Soluble IL-6R alpha is elevated in adenosine-mediated pulmonary fibrosis.

As an initial assessment of whether sIL-6Rα is elevated in adenosine-mediated lung disease, we examined its levels in the lungs of day 42 ADA-deficient (ADA$^{-/-}$) mice and their ADA-competent (ADA$^{+/-}$) littermates. BAL fluid samples were collected and analyzed for presence of the soluble receptor in the bronchoalveolar compartment of the lung. ELISA quantification revealed a significant, more than 2-fold elevation in sIL-6Rα in ADA$^{-/-}$ samples in comparison to their ADA$^{+/-}$ littermates (Figure 6.1A). Secondary confirmation was performed using western blot analysis of these samples. It confirmed the presence of sIL-6Rα in ADA$^{-/-}$ samples (Figure 6.1B). Knowing soluble receptor levels were elevated in established adenosine-mediated lung disease, we next set out to evaluate changes to expression of sIL-6Rα during disease onset and progression in ADA-deficient lungs.

Lung protein lysates were prepared and BAL fluid samples were collected from the lungs of ADA$^{+/-}$ and ADA$^{-/-}$ mice at various stages of development between postnatal day 26 and 44 (on postnatal days 26, 30, 34, 38, and 42). Western blot analysis of lung protein lysates demonstrated increasing appearance of sIL-6Rα as adenosine accumulates and injury progresses in these lungs (Figure 6.1C). ELISA measurement and western blot analysis of sIL-6Rα in BAL fluid samples confirmed this temporal increase in sIL-6Rα in ADA$^{-/-}$ lungs (Figure 6.1D&E). There was a visible increase in soluble receptor by day 38. By day 42, there was a significantly elevated level of sIL-6Rα in ADA$^{-/-}$ lungs in comparison to ADA$^{+/-}$ lungs. These findings demonstrate an association between increases in soluble IL-6Rα and adenosine-mediated lung disease and suggest a role for IL-6 *trans* signaling in disease onset and progression.
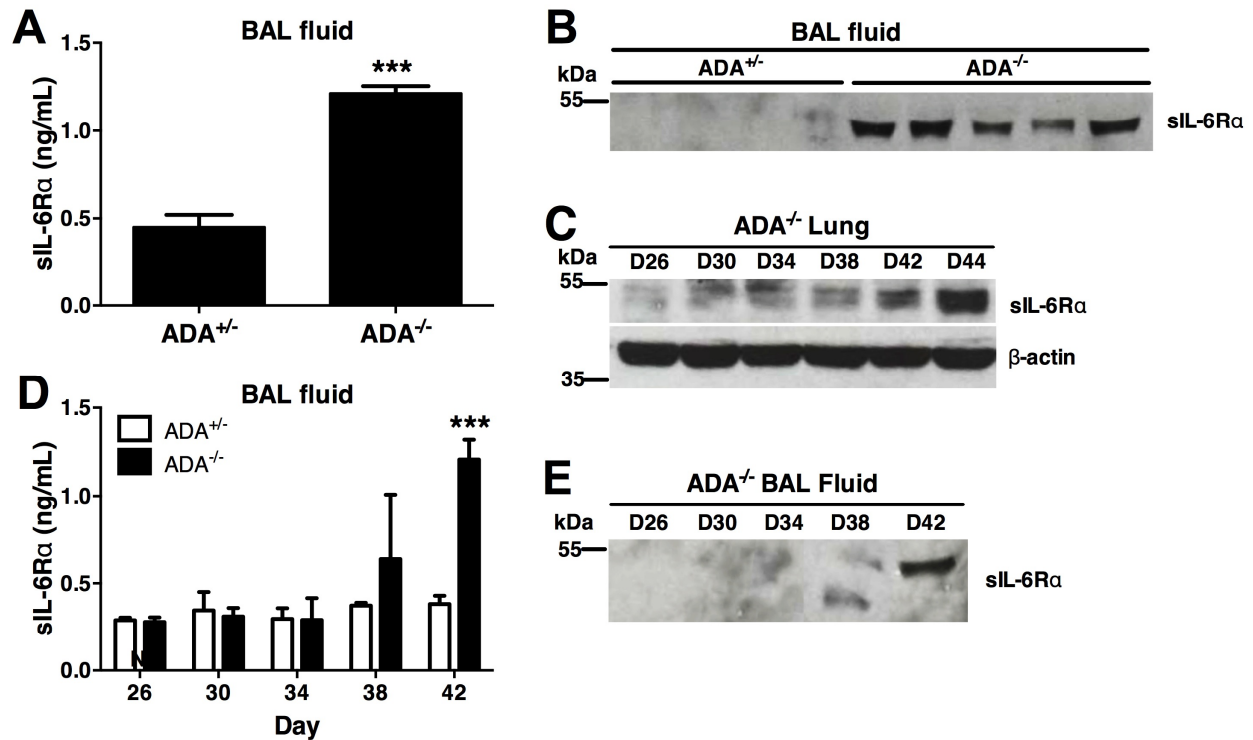
Figure 6.1. Soluble IL-6R alpha expression in an adenosine-mediated mouse model of pulmonary fibrosis. Soluble IL-6Rα expression was assessed in mice with pulmonary fibrosis. (**A** & **B**) ELISA measurement and western blot analysis of sIL-6Rα in BAL fluid from postnatal day 41-42 ADA^{+/-} and ADA^{-/-} mice. (**C**) Western blot analysis of sIL-6Rα in ADA^{-/-} lung lysates at various timepoints between postnatal day 26 and 44. (**C**) ELISA quantification of sIL-6Rα in BAL fluid samples from ADA^{+/-} and ADA^{-/-} mice at various timepoints between postnatal days 26 and 42. (**D**) Western blot confirmation of sIL-6Rα in BAL fluid samples from ADA^{-/-} mice throughout progression of disease in model. All data presented as mean ± SEM, n≥4. *significant difference from ADA^{+/-} cohort; *= $p < 0.05$, **= $0.001 < p < 0.01$, ***= $p < 0.001$.

6.2 – Membrane IL-6R alpha is elevated in adenosine-mediated pulmonary fibrosis.

It was noted from our findings in human IPF samples and the IPB model that IL-6 classical signaling also has a role in pulmonary fibrosis. There is evidence to indicate that increased IL-6 *trans* signaling may promote a feedback loop that enhances mIL-6Rα expression and enhanced mIL-6Rα expression can either increase classical signaling and/or provides more supply for production of soluble receptor. Membrane IL-6Rα was elevated in bleomycin-induced pulmonary fibrosis and neutralization of *trans* signaling *in vivo* reduced mIL-6Rα expression in association with reduction of fibrosis (Figure 5.19B&C). *Trans* signaling promoted recruitment of inflammatory cells to the lung (Figure 5.3), cells that possessed enhanced membrane IL-6Rα expression (Figure 3.4B&3.5B, 4.1E). These cells can then be stimulated to shed their receptors to produce the soluble receptor (Figure 4.5). Stimulation of *trans* signaling in fibroblasts resulted in increased mIL-6Rα, associated with increased proliferation and extracellular matrix protein production (Figure 5.12&5.14). These results support the hypothesis that, in the lungs, classical signaling and *trans* signaling interact to over-amplify the effects of IL-6, leading to fibrosis.

We wondered if the same pattern would hold in the ADA-deficient model, so we began evaluating expression of mIL-6Rα in ADA^{-/-} mice. BAL fluid samples were collected from postnatal day 41-42 ADA^{+/-} and ADA^{-/-} mice. Western blot analysis of mIL-6Rα revealed increased mIL-6Rα in ADA^{-/-} samples (Figure 6.2A). BAL cells were isolated and also examined for expression of mIL-6Rα. Immunofluorescence staining with membrane IL-6Rα (green) and DAPI (blue) demonstrated enhanced expression of mIL-6Rα in ADA^{-/-} samples (Figure 6.2B). Furthermore, to determine if mIL-6Rα levels change over time, lung protein lysates were prepared and BAL fluid samples were collected from the lungs of ADA^{-/-} mice at various stages of development between postnatal day 26 and 44 (on postnatal days 26, 30, 34, 38, and 42).

Western blot analysis of lung lysates showed no difference in mIL-6Rα expression over time (Figure 6.2C). Western blot analysis of mIL-6Rα expression in BAL fluid samples over time demonstrated a temporal increase (Figure 6.2D) resembling the pattern of increase in sIL-6Rα, adenosine and collagen. These findings suggest that there is an increase in mIL-6Rα expression in the bronchoalveolar compartment of the lungs, but perhaps not in the whole lung.

6.3 - ADAM17 is increased in adenosine-mediated pulmonary fibrosis.

Having demonstrated elevations in soluble IL-6Rα in ADA-deficient mice with pulmonary fibrosis, we next assessed the underlying mechanism of sIL-6Rα production in their lungs. From our studies in the IPB model, we know the protease ADAM17 to play a pivotal role in shedding membrane IL-6Rα from activated macrophages to produce the soluble receptor (Figure 4.5). Thus, we explored the possibility of ADAM 17 cleaving membrane IL-6Rα to produce the soluble receptor in ADA-deficient lungs.

BAL fluid was collected from postnatal day 41-42 ADA^{+/-} and ADA^{-/-} mice and analyzed for presence of ADAM17 in the airways and airspaces of the lung. Western blot analysis detected increases in immature and mature forms of ADAM17 in ADA^{-/-} samples (Figure 6.3A). We further localized the increase in ADAM17. Immunostaining of sections from postnatal day 41-42 lungs revealed the most prominent increase of ADAM17 in alveolar macrophages of ADA-deficient lungs (Figure 6.3B). These macrophages are the same ones that, when stained for membrane IL-6Rα, expressed higher levels of the receptor than in the control (Figure 6.2B).
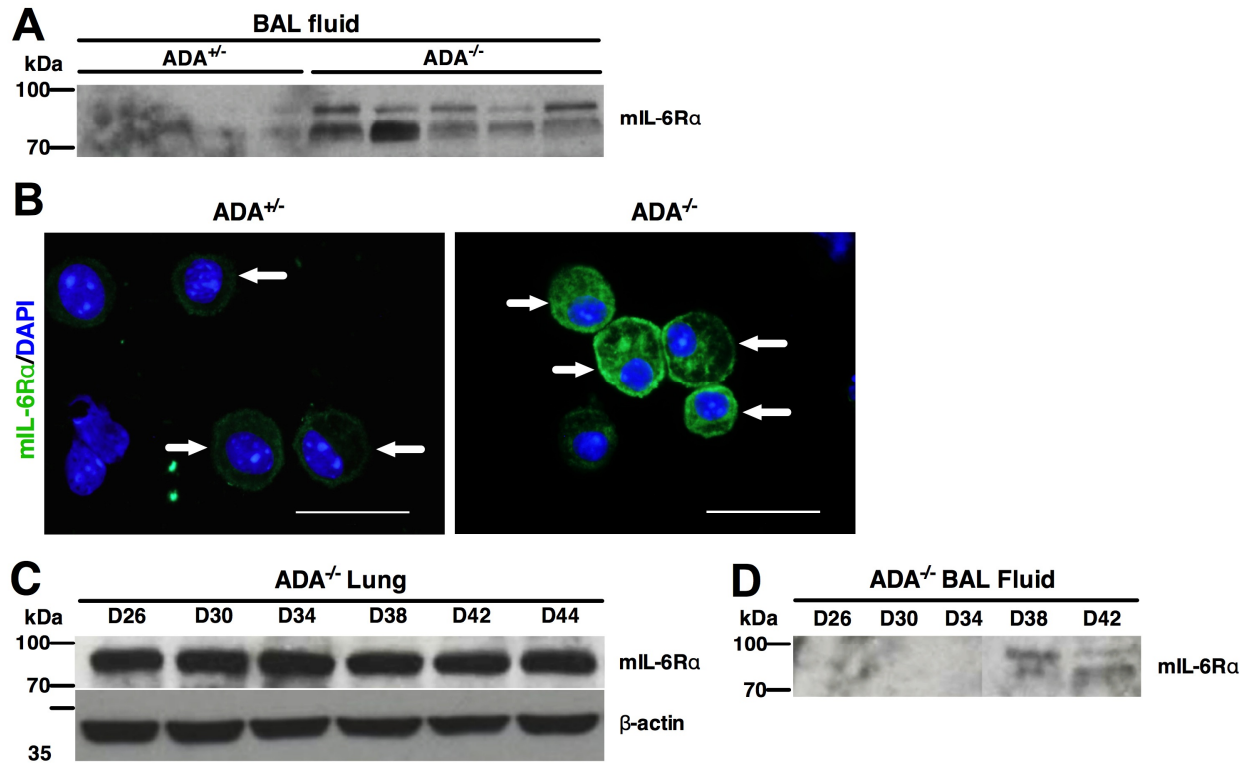
Figure 6.2. Membrane IL-6R alpha expression in a mouse model of adenosine-mediated pulmonary fibrosis. Membrane IL-6Rα expression was assessed in mice with pulmonary fibrosis. (**A**) Western blot analysis of mIL-6Rα in BAL fluid from ADA^{+/-} and ADA^{-/-} mice, postnatal day 41-44. (**B**) BAL cells were collected and immunofluorescence staining performed for detection of membrane IL-6Rα (green) and DAPI (blue). Arrows denote positive cells. Images are representative of n≥4 animals from each group. Scale bars: 50μm, 100x oil immersion. (**C**) Western blot analysis of mIL-6Rα in ADA^{-/-} lung lysates at various timepoints between postnatal day 26 and 44. (**D**) Western blot confirmation of mIL-6Rα expression in BAL fluid samples from ADA^{-/-} mice throughout progression of disease in model.
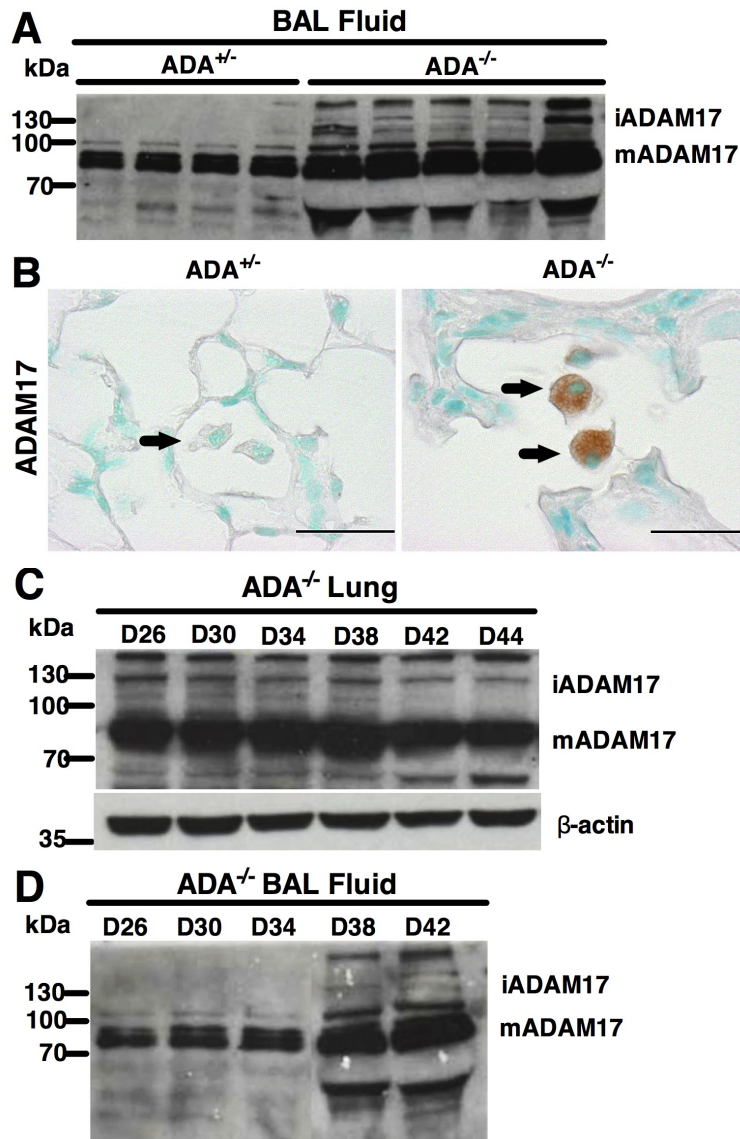
Figure 6.3. ADAM17 expression in a mouse model of adenosine-mediated pulmonary fibrosis. Expression of the protease ADAM17 was evaluated in mice with pulmonary fibrosis. (**A**) Western blot analysis of immature (130 kDa) and mature (89 kDa) forms of ADAM17 in bronchoalveolar lavage fluid from postnatal day 41-42 ADA$^{+/-}$ and ADA$^{-/-}$ mice. (**B**) Immunostaining for ADAM17 (brown) in the lungs of postnatal day 41-42 ADA$^{+/-}$ and ADA$^{-/-}$ lungs. Arrows denote positive cells. Images are representative of n≥4 animals from each group. Scale bars: 50μm, 100x oil immersion. Western blot analysis of ADAM17 in (**C**) lung protein lysates and (**D**) BAL fluid as disease develops and progresses in an adenosine-mediated model.

Further characterization of ADAM17 expression during onset and development of fibrosis was achieved via preparation of lung protein lysates and collection of BAL fluid from ADA$^{-/-}$ lungs at various stages of development between postnatal day 26 and 44 (on postnatal days 26, 30, 34, 38, and 42). Western blot analysis of lung lysates revealed no difference in ADAM17 expression, though there may be a slight reduction on days 42 and 44 in the mature form and an increase in a potential isoform found at a lower molecular weight (Figure 6.3C). Western blot analysis of mIL-6Rα in BAL fluid samples, on the other hand, indicated a temporal increase in ADAM17 expression that mirrored the increase in sIL-6Rα in this model (Figure 6.1C&E). Collectively, these findings demonstrate an association between increases in ADAM17 and increases in soluble IL-6Rα in the bronchoalveolar compartment of ADA-deficient lungs, suggesting a role for the protease in generating the soluble receptor in ADA-deficient lungs.

6.4 – In vivo neutralization of IL-6 trans signaling in the ADA-deficient model.

We have characterized increases in membrane and soluble IL-6Rα in ADA-deficient, fibrotic mouse lungs and suggested a role for ADAM17 in generation of the soluble receptor in these lungs. We saw similar patterns of expression of soluble IL-6Rα in the IPB model and were able to demonstrate therapeutic benefits of neutralizing sIL-6Rα *in vivo* in that model. We wanted to see if the same benefits would apply when we neutralize sIL-6Rα in ADA$^{-/-}$ mice.

In vivo neutralization of sIL-6Rα was performed in the ADA-deficient model using mouse recombinant gp130Fc, shown in our studies and published literature to be an effective inhibitor of IL-6 *trans* signaling(73, 74, 76, 78). The treatment protocol was as illustrated in Figure 6.4A. Treatment with gp130Fc occurred from postnatal day 30 to 41. Mice were sacrificed on postnatal day 42 and pulmonary phenotypes were assessed for pulmonary

inflammation and fibrosis. The dosage and schedule for administration of recombinant gp130Fc are adapted from previous studies that have shown effectiveness of soluble gp130 at blocking IL-6 *trans* signaling *in vivo,*(76-80, 103) including our attempts to neutralize *trans* signaling in the IPB model. The decision of when to begin treatment with gp130Fc was based on previous work by Pedroza et al, who used the ADA-deficient model to study the role of IL-6 in adenosine-mediated injury(29). They initiated pharmacologic blockade of IL-6 signaling on postnatal day 26 and demonstrated attenuation of fibrosis. Also, data from our characterization studies in this model informed us that soluble IL-6Rα was already present in the lungs as early as postnatal day 26 in ADA$^{-/-}$ mice even though significant changes in collagen levels did not occur until between day 34 and 38 (Figure 6.1C & 6.0B). This suggests that fibrosis became much more robust and *trans* signaling takes a more prominent role in the disease process between days 34 and 38. Thus, we attempted to antagonize *trans* signaling in a preventative manner by initiating recombinant gp130Fc therapy on day 30.

To determine whether our neutralization protocol was sufficient to reduce levels of the soluble receptor and inhibit *trans* signaling, Day 42 BAL fluid samples were collected and subjected to ELISA analysis of sIL-6Rα. ADA$^{-/-}$ mice exhibited an elevation in sIL-6Rα in BAL fluid (Figure 6.4B), though not significant and not to the levels observed in our characterization studies. Treatment with gp130Fc led to a trend for lower levels of sIL-6Rα in BAL fluid but not significantly different. This suggests us that the administration of recombinant gp130Fc in this experiment was unable to significantly alter levels of soluble IL-6Rα and antagonize IL-6 *trans* signaling in the lung microenvironment.
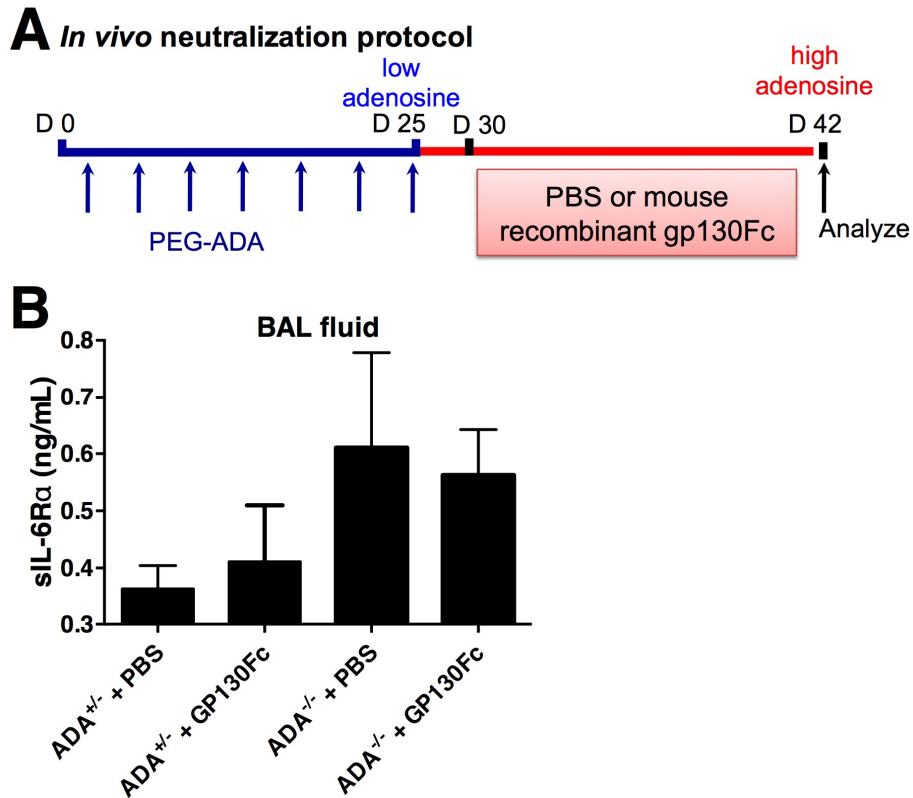
Figure 6.4. Protocol for *in vivo* neutralization of soluble IL-6Rα using mouse recombinant gp130Fc in a mouse model of adenosine-mediated pulmonary fibrosis. (**A**) Experimental setup for *in vivo* neutralization of sIL-6Rα in the adenosine deaminase (ADA)-deficient murine model of pulmonary fibrosis. ADA^{-/-} mice were identified at birth and maintained on PEG-ADA enzyme replacement therapy until postnatal day 25. Therapy is then discontinued and endogenous adenosine allowed to accumulate in the lungs. Beginning on postnatal day 30 and continuing until day 41, daily treatment with vehicle (saline) or recombinant gp130Fc was given. Mice were sacrificed and samples collected for analysis on postnatal day 42. (**A**) ELISA measurement of sIL-6Rα levels in BAL fluid from day 42 lungs. All data presented as mean ± SEM, n=2 for ADA^{+/-} + PBS, n≥4 for other cohorts. *significant difference from ADA^{+/-} cohort given PBS; [#]significant difference from ADA^{-/-} mice given PBS. $* = p<0.05$, $** = 0.001<p<0.01$, $*** = p<0.001$.

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6.5 – Effects of treatment with recombinant gp130Fc on pulmonary inflammation.

Despite not being able to show a significant reduction in sIL-6R alpha in our neutralization study, we proceeded to evaluate changes to pulmonary phenotype. The histopathology of ADA$^{-/-}$ lungs included pulmonary inflammation, emphysematous distal airway enlargement and pulmonary fibrosis.

To assess changes in pulmonary phenotype, we first examined the effects of recombinant gp130Fc administration on pulmonary inflammation. Using a hemocytometer, total cell counts were performed on BAL fluid samples from day 42 ADA$^{-/-}$ mice. Analysis of BAL fluid samples revealed a significant elevation in total BAL cells in ADA$^{-/-}$ lungs, consistent with the presence of inflammation (Figure 6.5A). Though there was a trend for decrease, there was no significant reduction in total BAL cells in ADA$^{-/-}$ mice treated with gp130Fc (Figure 6.5A). Surprisingly, cellular differential analysis revealed a significant reduction in BAL macrophages in ADA$^{-/-}$ mice treated with gp130Fc (Figure 6.5B). No significant trends could be concluded from examination of the other cell types in BAL fluid (Figure 6.5C&D).

6.6 – Effect of recombinant gp130Fc on collagen protein expression in the lungs.

We next attempted to evaluate pulmonary fibrosis in gp130Fc-treated and untreated ADA$^{-/-}$ mice. We assessed collagen production in the lungs by measuring soluble collagen protein in BAL fluid using Sircol assay. ADA$^{-/-}$ lungs exhibited significantly higher levels of soluble collagen in BAL fluid, indicative of fibrosis (Figure 6.6A), though not to the extent expected based on previous descriptive studies (Figure 6.0B). Gp130Fc administration did not result in a significant reduction in collagen protein though there was a trend for decrease. These results were confirmed in second manner by evaluation of collagen deposition in the lungs. Lung

sections from day 42 ADA$^{+/-}$ and ADA$^{-/-}$ mice, placebo and treated groups, were prepared and stained using Masson's trichrome protocol to visualize collagen deposition (blue). Untreated ADA$^{-/-}$ lungs had evidence of emphysematous distal airway enlargement and mild pulmonary fibrosis (Figure 6.6B). Gp130Fc-treated ADA$^{-/-}$ lungs appeared mildly improved, though there was not enough fibrosis in either cohorts to accurately draw conclusions about changes.

6.7 – Effect of recombinant gp130Fc on pulmonary fibrosis and myofibroblast accumulation.

We next attempted to quantify extent of pulmonary fibrosis in gp130Fc-treated and untreated ADA$^{-/-}$ mice. Ashcroft scoring was performed on Masson's trichrome-stained sections. Untreated ADA$^{-/-}$ lungs had evidence of mild pulmonary fibrosis, with Ashcroft scores of around 3 (Figure 6.7A). There was a trend for decrease in scores in gp130Fc-treated lungs, though it was not significant. Lung sections were also stained with α-SMA for detection of myofibroblasts. Neither ADA$^{-/-}$ treated nor untreated lungs displayed very much SMA staining (Figure 6.7B). In combination with the collagen data, these results support the idea that the collected ADA$^{-/-}$ + PBS samples in this experiment were not representative of what is typically expected of diseased lungs and no valid comparisons can be made between treated and untreated mice.
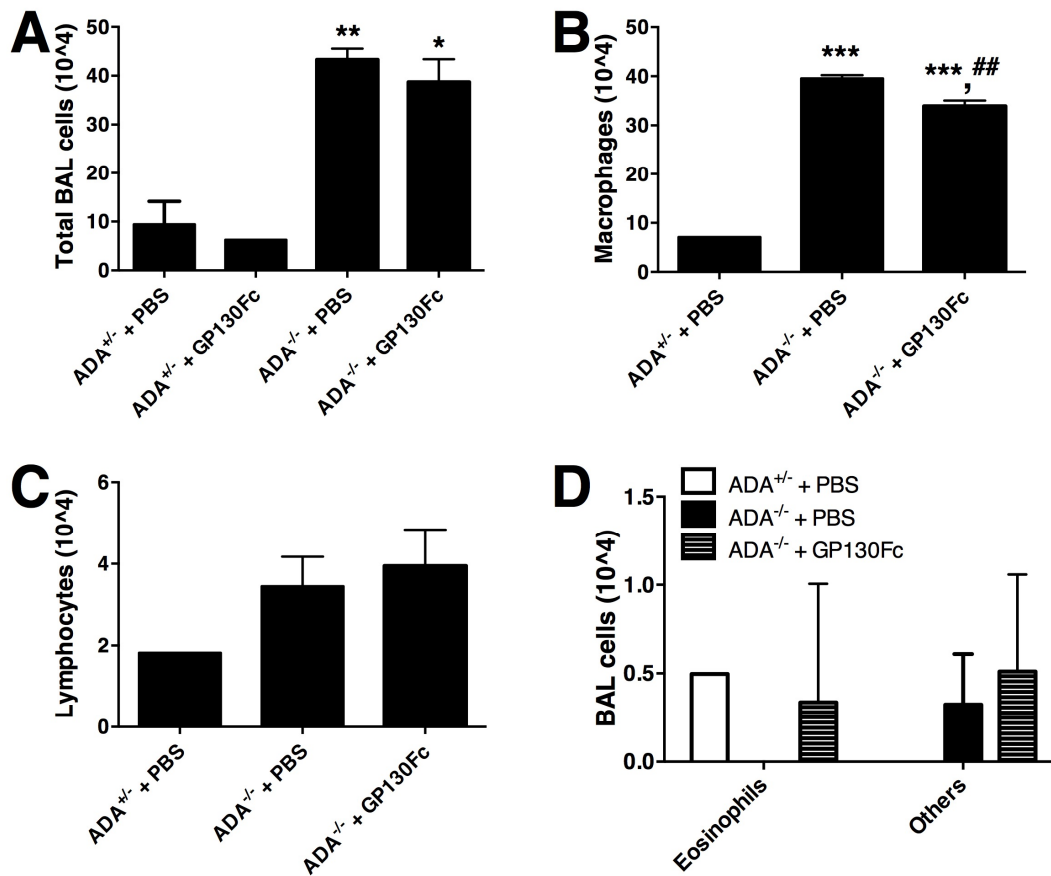
Figure 6.5. Pulmonary inflammation after recombinant gp130Fc treatment in a mouse model of adenosine-mediated pulmonary injury. ADA$^{-/-}$ mice were identified at birth and maintained on PEG-ADA enzyme replacement therapy until postnatal day 25. Therapy is then discontinued. Beginning on postnatal day 30 and continuing until day 41, daily treatment with vehicle (saline) or recombinant gp130Fc was given. Mice were sacrificed and samples collected for analysis on postnatal day 42. (**A-D**) Total BAL cells count and differentials, including macrophages, lymphocytes, eosinophils and others were determined in day 42 samples. All data presented as mean ± SEM, n=2 for ADA$^{+/-}$ groups, n≥3 for ADA$^{-/-}$ cohorts. *significant difference from ADA$^{+/-}$ cohort given PBS; #significant difference from ADA$^{-/-}$ mice given PBS. *= p<0.05, **= 0.001<p<0.01, ***= p<0.001.
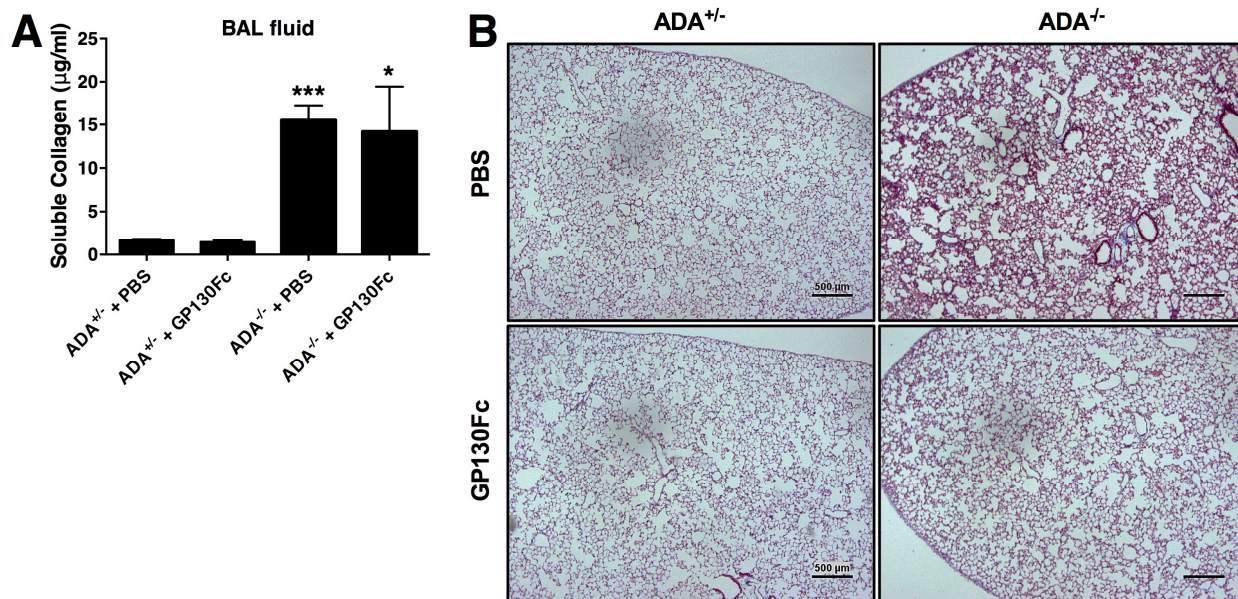
Figure 6.6. Collagen production and deposition following treatment with recombinant gp130Fc in a mouse model of adenosine-mediated pulmonary injury. ADA^{-/-} mice were identified at birth and maintained on PEG-ADA enzyme replacement therapy until postnatal day 25. Therapy is then discontinued. Beginning on postnatal day 30 and continuing until day 41, daily treatment with vehicle (saline) or recombinant gp130Fc was given. Mice were sacrificed and samples collected for analysis on postnatal day 42. (**A**) Lung sections from day 42 ADA^{+/-} and ADA^{-/-} mice, placebo and treatment groups, were immunostained using Masson's trichrome protocol for detection of collagen deposition (blue). (**B**) Sircol measurement of soluble collagen in BAL fluid samples from Day 42. All data presented as mean ± SEM, n=2 for ADA^{+/-} groups, n≥4 for ADA^{-/-} cohorts. *significant difference from ADA^{+/-} cohort given PBS; #significant difference from ADA^{-/-} mice given PBS. *= $p < 0.05$, **= $0.001 < p < 0.01$, ***= $p < 0.001$.
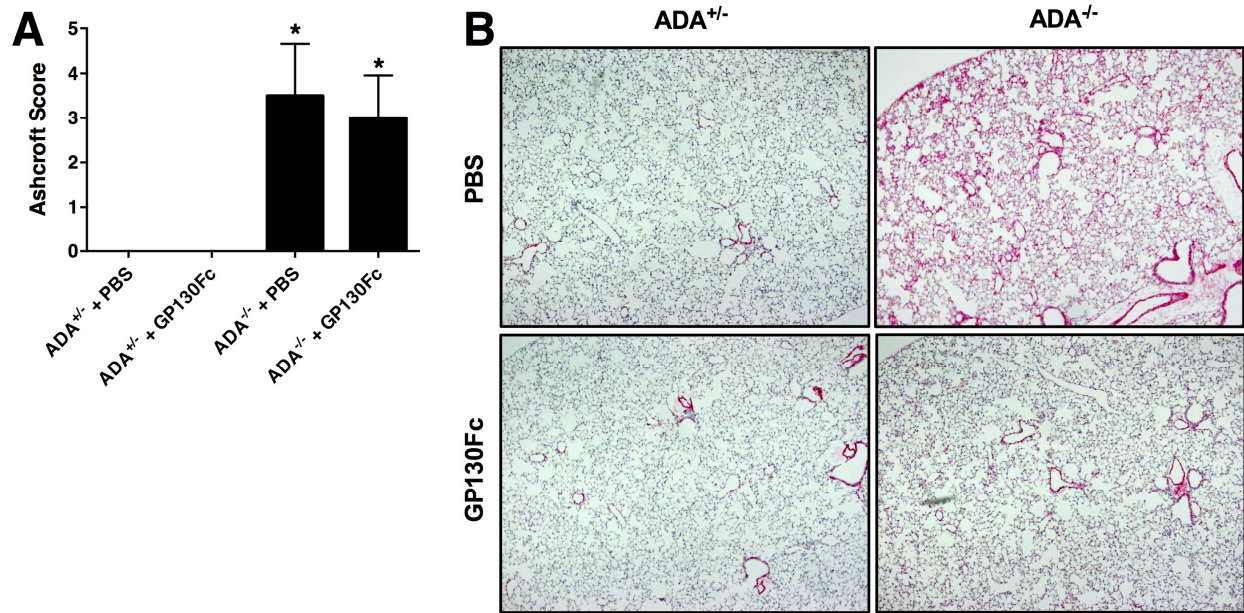
Figure 6.7. Pulmonary fibrosis following treatment with recombinant gp130Fc in a mouse model of adenosine-mediated pulmonary injury. ADA^{-/-} mice were identified at birth and maintained on PEG-ADA enzyme replacement therapy until postnatal day 25. Therapy is then discontinued. Beginning on postnatal day 30 and continuing until day 41, daily treatment with vehicle (saline) or recombinant gp130Fc was given. Mice were sacrificed and samples collected for analysis on postnatal day 42. (**A**) Ashcroft scoring was performed on Day 42 from ADA^{+/-} and ADA^{-/-} mice, placebo and treated groups. (**B**) Day 42 lung sections were immunostained with alpha-smooth muscle actin for detection of myofibroblasts (red). All data presented as mean ± SEM, n=2 for ADA^{+/-} groups, n≥4 for ADA^{-/-} cohorts. *significant difference from ADA^{+/-} cohort given PBS; [#]significant difference from ADA^{-/-} mice given PBS. *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$.

DISCUSSION

With the encouraging results found in the IPB mouse model, we approached the study of IL-6 classical and *trans* signaling in the ADA-deficient model with the hopes of secondary validation of our findings and the potential of exploring the role of these pathways in combined pulmonary fibrosis and emphysema states. We were able to characterize increases in soluble IL-6Rα, membrane IL-6Rα and ADAM17 expression in adenosine-induced pulmonary disease that were similar in most respects to the patterns seen in bleomycin-induced pulmonary fibrosis. Our characterization studies suggested: 1) a role for IL-6 classical and *trans* signaling in adenosine-mediated disease, and 2) ADAM17 in shedding IL-6Rα from alveolar macrophages. Our initial attempt to neutralize soluble IL-6Rα *in vivo* in the ADA-deficient model, however, failed to achieve significant reduction in sIL-6Rα in the lungs. Further optimization of the neutralization protocol is needed in order to more accurately determine the significance of IL-6 *trans* signaling in this model and decipher its part in the pathogenesis of adenosine-mediated pulmonary disease.

Our characterization experiments were able to support a role for IL-6 *trans* signaling in the ADA-deficient model. We saw that soluble IL-6Rα was elevated in both bronchoalveolar and parenchymal compartments of ADA-deficient lungs. This elevation was seen in established pulmonary disease, which includes pulmonary inflammation and pulmonary fibrosis. We also demonstrated that soluble IL-6Rα increased in ADA-deficient lungs in association with increasing disease, as indicated by increasing adenosine and collagen content. This pattern of increase in sIL-6Rα is in agreement with the pattern seen in the IPB model and suggests *trans* signaling could be involved in adenosine-mediated pulmonary injury in this model. This would not be surprising since adenosine regulates IL-6 expression and release in various pulmonary sources(32-35). It would be interesting to assess whether adenosine also regulates expression of

IL-6Rα. Also, more work is necessary to understand the *trans* signaling-mediated activation of intracellular pathways in the ADA-deficient model.

In contrast to the overall increase in membrane IL-6Rα expression in whole lung in the IPB model, we determined that membrane IL-6Rα is elevated only in the bronchoalveolar compartment of ADA-deficient lungs. This increase was further localized to the alveolar macrophages, similar to the observation made in the IPB model. This observation suggests that alveolar macrophages may represent the source of soluble IL-6Rα generation in this model as well. When we examined ADAM17 expression in the model, we saw increased levels over time in the bronchoalveolar compartment of ADA-deficient lungs, in association with increasing sIL-6Rα. The increase was localized to alveolar macrophages. In comparison to the pattern seen in the IPB model, the more specific localization of membrane IL-6Rα and ADAM17 to the alveolar macrophages in ADA-deficient lungs further implicate the protease and these cells in generation of sIL-6Rα. Macrophage stimulation experiments similar to those conducted in the IPB model needs to be performed to further assess the role of ADAM17 in producing sIL-6Rα.

As for our attempt at *in vivo* neutralization of sIL-6Rα in the ADA-deficient model, we were unable to demonstrate a significant reduction in sIL-6Rα in the cohort treated with mouse recombinant gp130Fc. As such, we were unable to draw solid conclusions regarding pulmonary inflammation and fibrosis since we are unsure whether or not antagonism of IL-6 *trans* signaling was successful. There could be several possibilities as to the reason for our failure to demonstrate a significant difference in sIL-6Rα levels with gp130Fc treatment. Failure could be due to inadequate dosing, treatment frequency and/or duration of therapy. Our attempt at neutralization in ADA-deficient mice was based on what was effective in the IPB model. However, every model is different, and the metabolism of recombinant gp130Fc in ADA-deficient could be

entirely different than that of bleomycin-exposed mice. Perhaps the same dose of recombinant gp130Fc is metabolized too quickly in the ADA$^{-/-}$ lungs or is being shuttled to other sites of inflammation and disease since we know ADA$^{-/-}$ mice to have metabolic disturbances in many organs, including spleen, liver and kidney(94, 95). Treatment failure could also be due to timing of therapy initiation or the duration of therapy. Perhaps gp130Fc treatment needs to be started earlier in the model.

Another valid possibility could be that we did not examine the quantity and quality of mice necessary to observe a difference with treatment. One of the difficulties of the ADA-deficient model is in timing of sample collection. Several ADA$^{-/-}$ mice treated with PBS were lost to collection due to death occurring before day 42. They were excluded from the study. Thus, the most severely ill mice were eliminated from the analysis. The ones that survived to day 42 did not appear to be sufficiently ill, given the low sIL-6Rα and soluble collagen levels measured in BAL fluid. Neither the levels of sIL-6Rα nor soluble collagen in BAL fluid samples from our untreated ADA$^{-/-}$ cohort were consistent with what was observed in our previous descriptive study (Figure 6.0B). This suggests that we weren't capturing the mice representative of the severity of disease seen in our characterization studies. As such, perhaps because we did not have adequate positive controls (i.e. sick ADA$^{-/-}$ + PBS mice), we were unable to assess changes with treatment with gp130Fc. Surprisingly, our analysis of BAL cells yielded a significant reduction in macrophages in gp130Fc-treated ADA$^{-/-}$ mice. However, in the face of unaltered levels of sIL-6Rα, we are uncertain what conclusion can be drawn from these results.

Overall, our experiments in the ADA-deficient model validated elevations in soluble sIL-6Rα in onset and progression of adenosine-mediated pulmonary disease. We confirmed elevations in mIL-6Rα and ADAM17 in alveolar macrophages of ADA-deficient lungs. Our

studies in this model have provided preliminary data to support further need for analysis of IL-6

classical and *trans* signaling as well as the role of ADAM17 in adenosine-mediated lung injury.

CHAPTER 7

Summary & Conclusions

and

Future Directions

SUMMARY OF RESULTS

The published literature suggests an important role for IL-6 *trans* signaling in diseased states, yet *trans* signaling has not been examined in IPF, a deadly form of pulmonary fibrosis and the most common interstitial lung disease(1). This project was intended to 1) characterize IL-6 classical and *trans* signaling in fibrotic lungs, 2) examine protease-mediated generation of soluble IL-6 receptor alpha, and 3) evaluate the therapeutic benefit of blocking IL-6 *trans* signaling in pulmonary fibrosis.

Characterization of IL-6 classical and *trans* signaling in IPF lungs revealed elevations of soluble IL-6Rα in parenchyma and bronchoalveolar compartments. Membrane IL-6Rα is augmented in M_2 macrophages in IPF lungs. M_2 macrophages, fibroblasts and myofibroblasts from IPF lungs are capable of responding to classical and *trans* signaling, while type II pneumocytes are only responsive to *trans* signaling. Enhanced activation of STAT3 was seen in IPF lungs in a number of different cell types. These results supported a role for IL-6 *trans* signaling in amplifying the effects of IL-6 in IPF lungs.

Evaluation of IL-6 *trans* signaling in a chronic bleomycin mouse model, the Intraperitoneal Bleomycin (IPB) model, revealed elevations in both membrane and soluble IL-6Rα levels in association with fibrosis initiation and progression. The soluble receptor was generated via protease-mediated cleavage of membrane IL-6Rα from M_2 macrophages and the protease ADAM17 was responsible. Neutralization of IL-6 *trans* signaling *in vivo* resulted in attenuation of pulmonary inflammation and fibrosis in the model, which translated to improvement in pulmonary hypertension and hypoxia. *In vitro* mechanistic studies revealed that IL-6 *trans* signaling promoted fibroblast proliferation and production of extracellular matrix

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proteins. In all, these results revealed an essential role for IL-6 *trans* signaling in development of disease in a mouse model.

Attempts to validate our findings in a second model, the Adenosine Deaminase (ADA)-deficient model, resulted in confirmation that IL-6 *trans* signaling was augmented in association with disease in ADA-deficient mice. Membrane IL-6Rα and ADAM17 were elevated in activated M_2 alveolar macrophages, same as in the IPB model. An initial attempt to antagonize IL-6 *trans* signaling in this model failed to significantly reduce levels of the soluble receptor. Further optimization of neutralization protocol is needed. Results obtained in this second model were able to validate some findings from IPF and the IPB model.
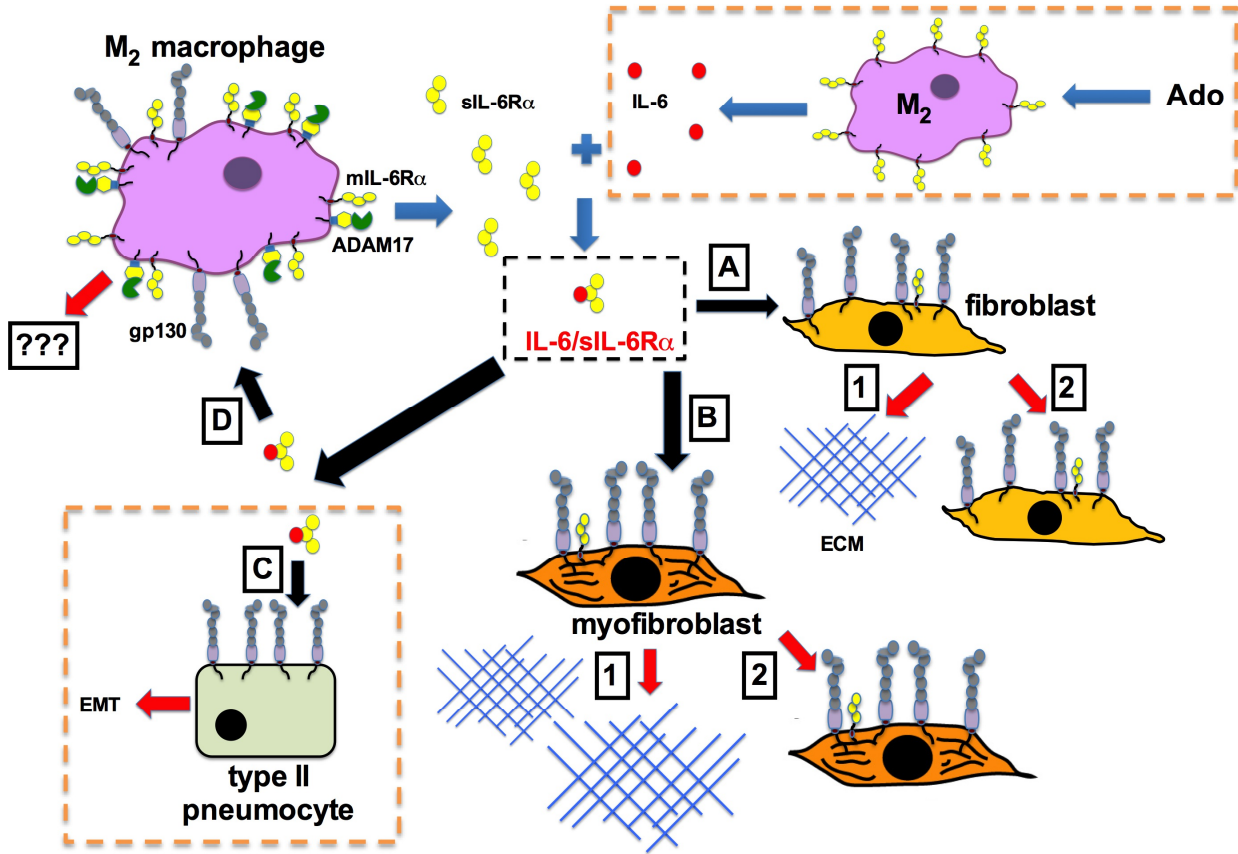
Figure 7.0. Working model of IL-6 *trans* signaling in pulmonary fibrosis. In fibrotic lungs, elevated ADAM17 expression in M_2 macrophages leads to cleavage of membrane IL-6Rα to produce soluble IL-6Rα. Soluble IL-6Rα binds IL-6, which is released by a number of cells, including alveolar macrophages, in response to adenosine stimulation (work done by Yang Zhou, PhD). The IL-6/sIL-6Rα complex can then activate various cells in the lung in a paracrine manner or further activate M_2 macrophages in an autocrine manner. (**A&B**) Stimulation of IL-6 *trans* signaling in fibroblasts results in (1) increased extracellular matrix protein production and (2) increased proliferation. (**C**) Stimulation of *trans* signaling in type II pneumocytes has been shown to induce EMT (results obtained by Mesias Pedroza, PhD). (**D**) Stimulation of IL-6 *trans* signaling in M_2 macrophages has not been examined yet. The resulting effects are unknown.

WORKING MODEL

Given what is known from published literature, the work done previously in our lab, as well as data obtained from studies in this project, we propose the following working model for IL-6 *trans* signaling in pulmonary fibrosis (Figure 7.0). As pulmonary fibrosis develops and progresses, there is increasing recruitment and accumulation of macrophages in the lung. In response to stimulatory signals present in the lung, these macrophages adopt the M_2 phenotype and express augmented levels of membrane IL-6Rα. Activation of ADAM17 in M_2 macrophages allows them to shed their membrane receptor to generate soluble IL-6Rα. Because of this, sIL-6Rα accumulates in the lung as disease progresses. Soluble IL-6Rα acts by first binding IL-6, which is released from a number of cells in fibrotic lungs, including alveolar macrophages (under stimulation by adenosine). The complex of IL-6/sIL-6Rα can then activate IL-6 *trans* signaling, amplifying the effects of IL-6. These effects include increasing recruitment of inflammatory cells, including more macrophages. IL-6/sIL-6Rα can activate IL-6 *trans* signaling in fibroblasts and myofibroblasts, promoting their proliferation and synthetic capabilities, resulting in expansion of these cells and increased production and deposition of extracellular matrix proteins like collagen and fibronectin in the parenchyma. Over time, this process results in progressive fibrosis, alterations of pulmonary architecture and impairment of gas exchange, manifested as hypoxia and respiratory dysfunction, ultimately leading to respiratory failure and death.

CONCLUSIONS

In this project, we have emphasized the significance of IL-6 *trans* signaling by showing elevations in soluble IL-6Rα in IPF lungs. We demonstrated a temporal increase in sIL-6Rα

during onset and progression of pulmonary fibrosis in two separate murine models, suggesting IL-6 *trans* signaling is involved in disease pathogenesis. We characterized IL-6 classical signaling and demonstrated a role for ADAM17 in generating soluble IL-6Rα by shedding the membrane receptor from mouse M_2 macrophages. Neutralization of sIL-6Rα and resulting antagonism of IL-6 *trans* signaling attenuated bleomycin-induced pulmonary inflammation and fibrosis in a mouse model. *In vitro* studies using human normal and IPF fibroblasts suggested activation of IL-6 *trans* signaling results in effects that are relevant to the progression of lung fibrosis. These findings are consistent with the hypothesis that IL-6 *trans* signaling is essential in mediating pro-inflammatory and pro-fibrotic effects in the lungs. Further studies are necessary to validate these observations in additional human IPF tissues and samples. However, this study presents, to our knowledge, the first *in vivo* preclinical evidence that blockade of IL-6 *trans* signaling may be of therapeutic value to the management of IPF.

FUTURE DIRECTIONS

Though we have made some progress in understanding some of the events that govern the initiation and progression of pulmonary fibrosis, much remains unclear. Our findings in this project, along with what is known from the published literature, have directed our thought process to generate the following questions that will drive our future studies and our search for more answers to this complex condition.

7.1 – Validate preliminary human findings & mouse model findings in more IPF samples

This thesis has yielded some intriguing findings regarding soluble IL-6Rα and *trans* signaling in pulmonary fibrosis. To provide even more preclinical data to support targeting this pathway in humans, we would like to validate our mouse findings in more human samples. I am most interested in the following questions.

1) Can we replicate the data showing that sIL-6Rα is increased specifically in a fibrotic lobe and not a non-fibrotic lobe (Figure 3.1A)? We would need to obtain more BAL fluid samples from different lobes of each patient and assess enough patients to see if the pattern holds. If we can show that this pattern of increased sIL-6Rα only occurs in fibrotic areas of the lungs, then it would support the idea of using sIL-6Rα as a biomarker of fibrosis in IPF.

2) Can we confirm in IPF samples the finding in both IPB and ADA-deficient models showing a temporal increase in sIL-6Rα with increasing severity of fibrosis (Figures 3.3 and 6.1)? We would need samples from patients at different stages of the disease, as representatives of varying (or increasing) severity of disease. There are a few issues with that plan. Ideally, we would recruit newly diagnosed IPF patients and follow them with periodic BALs and tissue collection. However, there are no known therapeutic justifications for subjecting patients to invasive procedures for periodic BALs and tissue collection. The other problem is that IPF is such a heterogeneous disease, with different phenotypes of IPF. How would initial classification of patients be done? What factors would be important? Imaging findings? Pulmonary function? Genotype? Duration of symptoms? As mentioned in chapter 1, rapidly progressing forms of IPF present with similar lung volumes, capacities and findings as stable or slowly progressing IPF. We would need to follow those patients to make sure they were classified correctly based on how they progress. That would take tremendous time and effort for follow-ups. However, if we are

able to gather enough samples representative of the spectrum of IPF disease and stratify them appropriately, it would provide valuable information about the disease process. If we can show the pattern of sIL-6Rα expression replicates in IPF, it would support the use of sIL-6Rα as a prognostic marker and indicator of disease severity/stage.

3) Can we validate the finding in the IPB model showing a temporal increase in M_2 macrophages and ADAM17 in association with increasing sIL-6Rα (Figure 4.2)? Can we stimulate shedding from M_2 macrophages from IPF lungs? Doing so would provide the rationale to block ADAM17 activity specifically in macrophages to prevent generation of sIL-6Rα in IPF lungs.

4) What is the expression of membrane IL-6Rα in IPF lungs? We need to determine if the preliminary results in IPF samples regarding expression of the membrane receptor is true (Figure 3.4A). We would like to evaluate mIL-6Rα expression in more IPF lung lysate samples and using a more specific antibody for the membrane receptor. Doing so would allow us to determine how much of the mouse model findings with regards to mIL-6Rα could apply to the human disease and direct our future efforts.
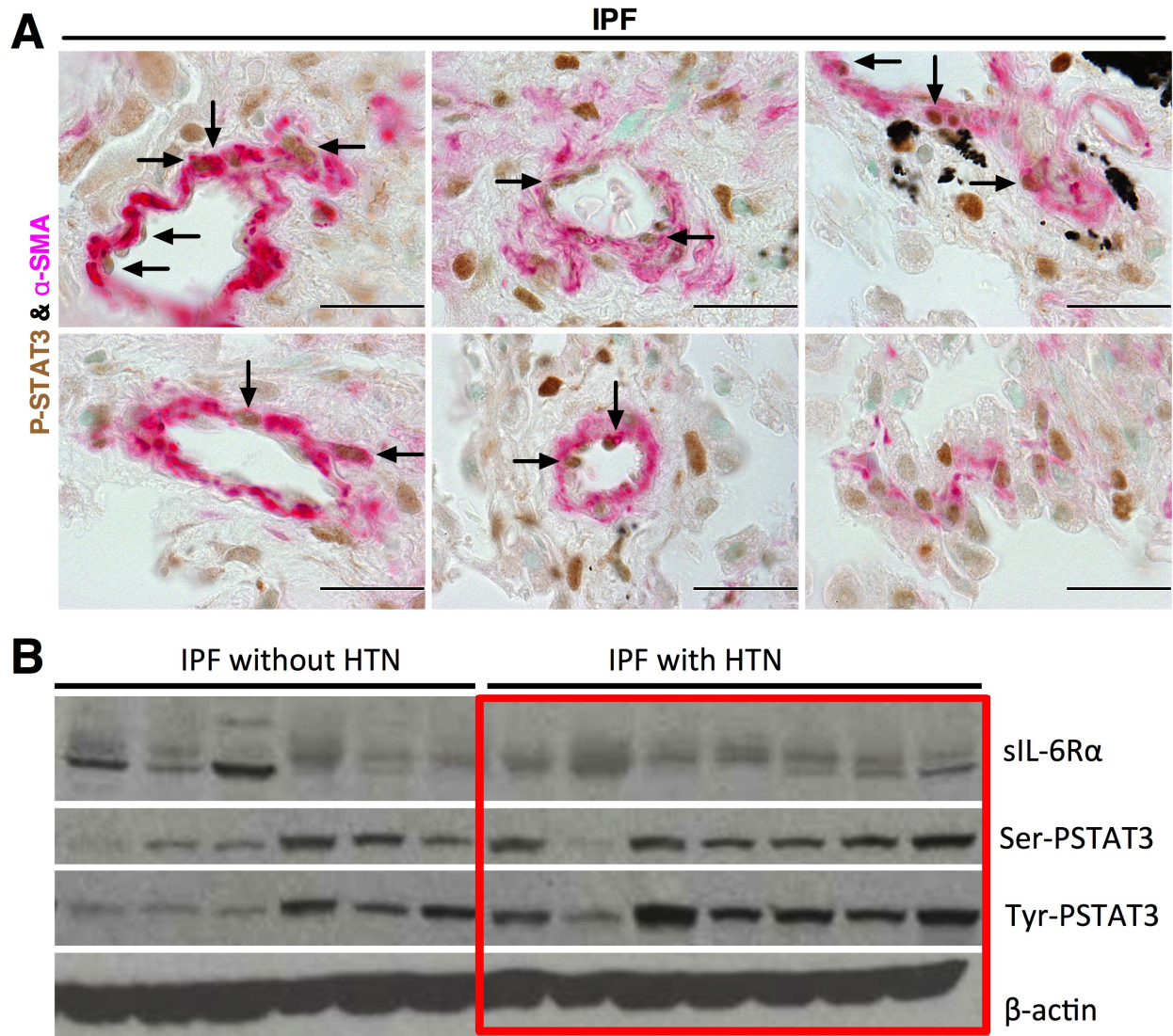
Figure 7.1. Activation of STAT3 in IPF patients with and without pulmonary hypertension. (**A**) Immunostaining for phospho-STAT3 and α-SMA around vessels in lung sections from IPF patients. Scale bars: 50μm, 100x oil immersion. (**B**) Western blot analysis of sIL-6Rα and P-STAT3 in lung lysates from explanted IPF samples from patients with and without co-existing pulmonary hypertension.

7.2 – Over-express soluble IL-6Rα to induce pulmonary fibrosis in wildtype mice

We were able to show that neutralization of IL-6 *trans* signaling using gp130Fc was therapeutic in our IPB model. To further support the role of sIL-6Rα in disease, we could over-express the IL-6/sIL-6R complex in wildtype mice to see if we can induce pulmonary fibrosis. The use of Hyper IL-6(44), a fusion protein consisting of IL-6 and sIL-6Rα, would come in handy for this purpose.

7.3 – Explore the role of IL-6 trans signaling in pulmonary hypertension associated with pulmonary fibrosis

As mentioned in chapter one, the presence of pulmonary hypertension worsens prognosis and survival. In this thesis, we showed that mice with bleomycin-induced pulmonary fibrosis also exhibit pulmonary hypertension. *In vivo* antagonism of IL-6 *trans* signaling with gp130Fc resulted in improvement in pulmonary hypertension (Figure 5.10). This could represent an effective therapy for fibrosis-associated PH.

Ammit et al reported stimulation of IL-6 *trans* signaling in airway smooth muscle cells in asthmatics promotes eotaxin and VEGF release, which may contribute to inflammation locally and vessel expansion(85). This could have significance in the vascular changes leading to development of PH. Karmouty-Quintana et al recently demonstrated the role of the adenosine 2B receptor ($A_{2B}AR$) in pulmonary hypertension associated with interstitial lung diseases (ILD)(91, 171). They provided evidence that receptor activation of vascular cells promoted IL-6 release.

Given what we have shown in our *in vivo* neutralization study as well as results from analysis of P-STAT3 in IPF lungs, there is reason to suggest involvement of IL-6 signaling in PH secondary to ILDs. Evaluation of the vessels in IPF lung sections (PH status unknown) revealed

increased activation of STAT3 in alpha-SMA positive cells (Figure 7.1A), suggesting enhanced IL-6 signaling may play a role in vascular and perivascular cells. To further assess whether trans signaling might be involved in PH associated with IPF, IPF lung lysates samples were separated by presence or absence of confirmed PH in medical history and evaluated by western blot for expression of P-STAT3 and sIL-6Rα. Western blot revealed increased phospho-STAT3 in IPF patients with PH (Figure 7.1B). Soluble IL-6Rα was present in every sample but did not show a clear pattern of increase of decrease. Further analysis is needed to evaluate whether there is a difference in sIL-6Rα or membrane IL-6Rα among IPF patients with and without PH. If not due to IL-6 signaling, then something else must explain the difference in P-STAT3 expression among IPF patients with and without PH. Other than IL-6, STAT3 is known to be activated in response to other IL-6 family cytokines, including interleukin 11, leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin 1 (CT-1) cardiotrophin-like cytokine (CLC), among others. Whatever the case, these findings need further exploration.

What was interesting in our studies was the analysis of sIL-6Rα and P-STAT3 expression in explanted samples from COPD patients, who can have pulmonary fibrosis and develop PH as a complication of their disease process. COPD patients without pulmonary hypertension expressed sIL-6Rα in 7/7 samples (Figure 7.2). COPD patients with PH did not express sIL-6Rα in 5/7 samples. Expression of P-STAT3 was variable, with 4/7 COPD with PH samples showing greater activation of STAT3 than COPD without PH. If these patterns hold up with analysis of more samples, the presence of sIL-6Rα in COPD lungs may indicate a protective effect from PH or that those COPD lungs have fibrosis and the other ones do no. It could also mean that IL-6 *trans* signaling has no role in development of PH in COPD.
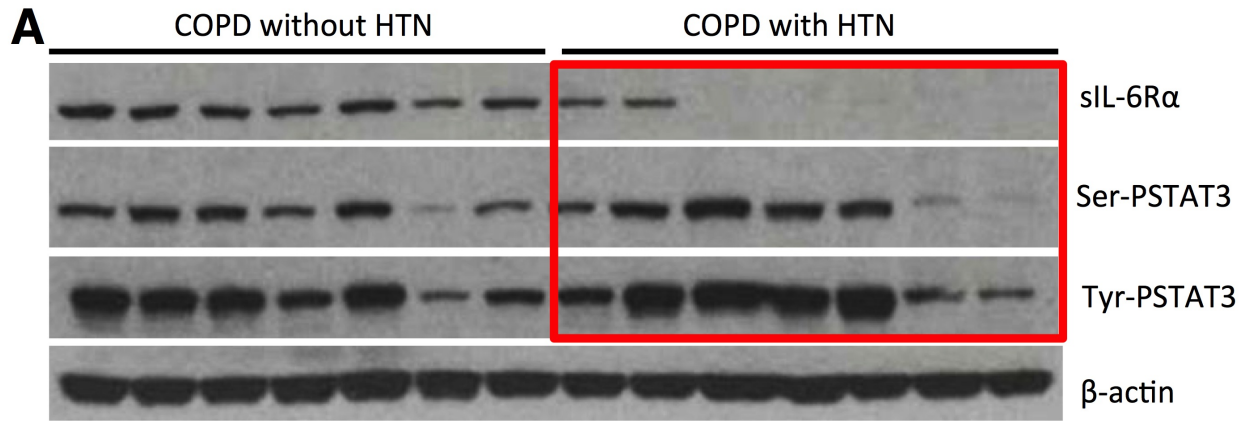
Figure 7.2. Activation of STAT3 in COPD with and without pulmonary hypertension. (**A**) Immunostaining for phospho-STAT3 and α-SMA around vessels in lung sections from IPF patients. Scale bars: 50μm, 100x oil immersion. (**B**) Western blot analysis of phospho-STAT3 in lung lysates from explanted IPF samples from patients with and without co-existing pulmonary hypertension.

7.4 – Optimize protocol for neutralization of IL-6 trans signaling in ADA-deficient model

As mentioned in the introduction and chapter 6, combined pulmonary fibrosis and emphysema represents a clinical presentation that confers bad prognosis. There are no available recommendations regarding therapy at this time due to a lack of knowledge of how these combined features developed. The ADA-deficient model provides what could be considered a potential model for this condition of combined fibrosis and emphysema since ADA activity has been shown to be significantly reduced in COPD (which consists of emphysema & chronic bronchitis) and IPF lungs and ADA-deficient mice are known to exhibit these two features of chronic lung disease secondary to chronically elevated adenosine(30, 31).

Though our first attempt at neutralizing sIL-6Rα in the ADA-deficient model did not yield significant reductions in receptor levels, we would like to make another attempt at neutralizing sIL-6Rα in this model and exploring the impact of IL-6 *trans* signaling in the disease process. We may just need to increase the dosage of gp130Fc and/or start therapy earlier to see if we can effect significant changes. The other option is to over-express IL-6/sIL-6R by injecting Hyper IL-6 in our ADA-deficient mice and see if that would worsen severity of disease or hasten disease progression.

7.5 – Evaluate role of membrane IL-6R alpha and IL-6 classical trans signaling in regulation and function of M$_2$ macrophages

Characterization studies in this thesis were able to determine that membrane IL-6Rα expression is increased in M$_2$ macrophages from IPF lungs (Figure 3.4B), bleomycin-induced fibrotic mouse lungs (Figure 4.1E), and adenosine-mediated fibrotic mouse lungs (Figure 6.2B). We provided evidence that M$_2$ macrophages from bleomycin-induced fibrotic mouse lungs shed

their membrane IL-6Rα to generate soluble IL-6Rα in those lungs (Figure 4.5). We have shown that antagonism of IL-6 *trans* signaling in bleomycin-induced pulmonary fibrosis led to a reduction in the number of macrophages in the lungs (Figure 5.3B).

These findings support an essential role for activated macrophages in the development of pulmonary fibrosis in our chronic bleomycin mouse model. Analysis of IPF human samples provided some validation. A search of the literature base also supports a role for these cells in IPF(115). We need to explore the functions of M_2 macrophages in fibrotic lungs, beyond serving as the source of IL-6R shedding. Our thesis findings have sparked the following questions.

Is the reduction in total BAL macrophages with gp130Fc treatment a reduction in M_2 macrophages? We suspect that is the case since the type of macrophage that is increased in IPF and fibrotic mouse lungs are M_2 in phenotype. However, we need to confirm that by either performing immunofluorescence staining of BAL cells from gp130Fc-treated mice for CD206, a marker of M_2 macrophages or by western blot analysis probing for expression of arginase 1, another M_2 marker.

Is enhanced expression of membrane IL-6Rα an indication of M_2 phenotype? The human and mouse data seem to suggest so. What role does IL-6 classical signaling have in M_2 macrophage function? Does it regulate release of mediators? What happens to the cells after the membrane receptor is cleaved off? Do the cells retain their M_2 phenotype?

What regulates expression of membrane IL-6Rα in these macrophages? Since adenosine can stimulate production of IL-6 from these cells(31), does it also stimulate expression of the membrane receptor? Is the A_{2B} receptor involved in the process since this receptor is also elevated in M_2 macrophages(31)?

7.6 – *Explore the effect of IL-6 trans signaling on M_2 macrophage activity*

We have shown that antagonism of IL-6 *trans* signaling in bleomycin-induced pulmonary fibrosis led to a reduction in the number of macrophages in the lungs (Figure 5.3B). We still need to confirm that this is a decrease in M_2 macrophages, though the suspicion is high that it is. As such, it would suggest that IL-6 *trans* signaling may have important effects in this cell. The effects of stimulating IL-6 *trans* signaling in M_2 alveolar macrophages have not been examined.

I am particularly interested in whether stimulation of IL-6 *trans* signaling would induce production/release of fibronectin from these cells. Rennard et al reported that alveolar macrophages from IPF patients secreted more fibronectin than those from normal lungs and that fibronectin has a chemoattractant effect on fibroblasts. Does IL-6 *trans* signaling regulate TGF-β, IL-6 or other pro-fibrotic mediator production and release from these macrophages?

7.7 – *Evaluate adenosine-mediated activation of ADAM17 in macrophages and evaluate ADAM17 neutralization specifically in macrophages on development of pulmonary fibrosis*

ADAM17 is activated by PMA and bacterial pathogens. The process of ADAM17-mediated shedding of IL-6Rα is dependent on PKC activation(58). We know adenosine can activate PKC. We know A_{2B} receptor expression is enhanced in activated alveolar macrophages(31). This begs the question of whether adenosine is involved in the regulation of ADAM17 activation in activated macrophages in order to cause shedding of IL-6Rα. Further study is necessary to elucidate the mechanism of shedding in alveolar macrophages. Further analysis is needed to support the hypothesis that adenosine activation of its $A_{2B}AR$ in M_2 macrophage leads to enhanced activation of ADAM17 in these cells, resulting in shedding of IL-6Rα.

Since the our results seem to indicate an essential role for ADAM17 in generating soluble IL-6Rα in fibrotic lungs, the next reasonable step would be to attempt neutralization of ADAM17 to see if there is any therapeutic benefit. However, because ADAM17 cleaves numerous different substrates in the body, many of which are physiologically necessary, global blockade of ADAM17 is not appropriate. In fact, ADAM17-deficiency in mice is embryonically lethal. Thus, since we demonstrate that the scene of the crime in fibrotic lungs is the activated alveolar macrophage, we wonder if it would be possible to neutralize ADAM17 specifically in macrophages to prevent shedding of IL-6Rα and determine effects on development of pulmonary fibrosis in the IPB model.

7.8 – *Explore the effect of trans signaling on senescence of airway epithelial cells*

We have demonstrated effects of stimulating IL-6 *trans* signaling in fibroblasts and myofibroblasts that are pertinent to pulmonary fibrosis. We have not examined the effects in airway epithelial cells (AEC). Given the current theory regarding IPF development, a theory that is epithelium-centered, with the thought that injury responses in alveolar airway epithelial cells is central to the initiation of a series of events that culminate in fibrosis, it would seem that we should also examine the role of IL-6 *trans* signaling in AECs. This pathway may be even more important in AECs than in other pulmonary cell types since we did not see expression of membrane IL-6Rα in AECs (Figure 3.4D), which suggests that these cells are not capable of classical signaling and only susceptible to *trans* signaling.

Pedroza et al (unpublished data) demonstrated that stimulation of IL-6 *trans* signaling in AEC induces epithelial to mesenchymal transition (EMT). We are interested in further exploring effects of *trans* signaling in AEC, particularly proliferation, senescence and apoptosis, since the

current view in the field is that there is premature senescence and excessive apoptosis of AECs in response to alveolar injury that is at the source of pulmonary fibrosis. It would be fascinating to see if IL-6 *trans* signaling has any effect on these processes.

7.9 – Clarify whether there is any change in M_1 macrophages as disease develops and progresses.

Though our focus in this thesis has been on the increase in M_2 macrophages as fibrosis progressed in our mouse model, we can't help but wonder if changes to M_1 macrophages also contribute to the pathogenesis of IPF. M_1 and M_2 macrophages appear to play different roles in renal fibrosis(172). Can the same be true in pulmonary fibrosis? We would need to characterize and monitor changes to M_1 markers in the IPB model over the course of the model to answer that question.

7.10 – Evaluate the increase in lymphocytes during disease onset and progression in IPB as another source of shedding or release of mediators

In our characterization studies, we showed a temporal increase in lymphocytes in bleomycin-induced fibrotic mouse lungs (Figure 7.3). We should determine if lymphocytes shed their membrane receptor to generate soluble receptor. We could also explore whether activation of IL-6 classical signaling in these cells alter their phenotype or activities.
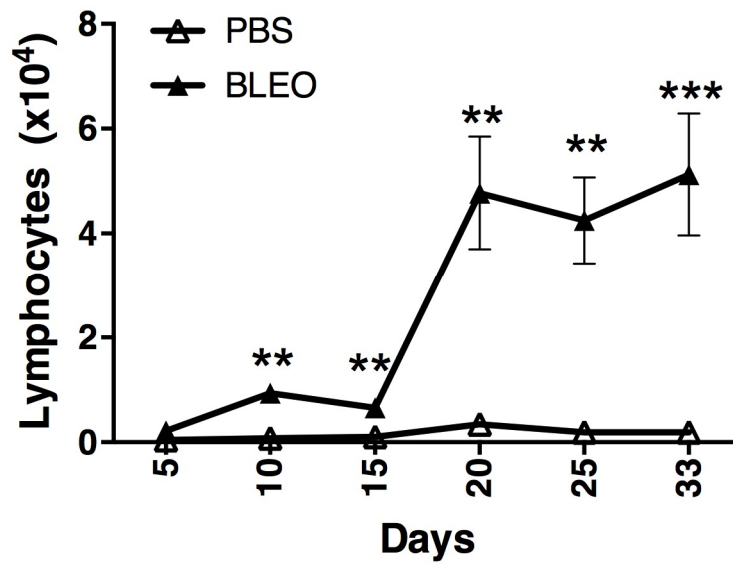
Figure 7.3. Lymphocyte accumulation in bleomycin-induced pulmonary fibrosis. Number of lymphocytes in BAL fluid samples from PBS-injected and bleomycin-exposed mice at various points throughout the model. Data presented as mean ± SEM, n≥4. *significant difference from PBS-treated cohort. *= $p<0.05$, **= $0.001<p<0.01$, ***= $p<0.001$. Data obtained by Tina Melicoff, MD, Tingting Weng, PhD and Harry Karmouty-Quintana, PhD.

7.11 – Evaluate the role of IL-6 trans signaling and ozone exposure on exacerbation of fibrosis in IPF patients

We are interested in how ozone exposures affect IPF because ozone is known to play an important role in chronic lung disorders like asthma and chronic obstructive pulmonary disease (COPD)(173) and ambient ozone has been reported to increase IL-6 production in the lungs(174, 175). However, nothing is known about the impact of ozone on pulmonary fibrosis, so much work is required in this area. I will utilize mouse models and human samples to examine the hypothesis that IL-6 *trans* signaling contributes to pulmonary fibrosis and ozone exposure exacerbates pulmonary fibrosis. First, I will characterize IL-6 classical and *trans* signaling components during the development of pulmonary fibrosis. Then I will assess the effect of block IL-6 *trans* signaling on fibrosis. Lastly, I will determine the effect of ozone exposure on the onset and progression of pulmonary fibrosis. We hope to achieve novel findings with this project that will translate into innovative therapeutics for the disease.

FINAL WORDS

As mentioned from the beginning, there is an urgent need to develop effective therapies for IPF. In this thesis, we have emphasized the significance of IL-6 *trans* signaling during the development and progression of pulmonary fibrosis and achieved novel findings that contribute to the understanding of the pathogenesis of IPF. Though the effect of recombinant gp130Fc in mice with pulmonary fibrosis was only partial, it was still significant enough to indicate this may be effective in slowing or perhaps even halting progression of disease in IPF patients. Our findings support the use of recombinant gp130Fc, and perhaps ADAM17 inhibition specifically in alveolar macrophages, for treatment of IPF patients. However, there is a need to further

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validate our mouse findings in more human samples and a need to evaluate the safety profile and effects of long-term use of gp130Fc in humans. Much work remains to better understand the complex interactions of the pulmonary micro-environment in order to develop even better therapies against this deadly disease.

BIBLIOGRAPHY

1.   Raghu, G., H. R. Collard, J. J. Egan, F. J. Martinez, J. Behr, K. K. Brown, T. V. Colby, J. F. Cordier, K. R. Flaherty, J. A. Lasky, D. A. Lynch, J. H. Ryu, J. J. Swigris, A. U. Wells, J. Ancochea, D. Bouros, C. Carvalho, U. Costabel, M. Ebina, D. M. Hansell, T. Johkoh, D. S. Kim, T. E. King, Jr., Y. Kondoh, J. Myers, N. L. Muller, A. G. Nicholson, L. Richeldi, M. Selman, R. F. Dudden, B. S. Griss, S. L. Protzko, and H. J. Schunemann. 2011. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *American journal of respiratory and critical care medicine* 183: 788-824.

2.   King, T. E., Jr., A. Pardo, and M. Selman. 2011. Idiopathic pulmonary fibrosis. *Lancet* 378: 1949-1961.

3.   Raghu, G., D. Weycker, J. Edelsberg, W. Z. Bradford, and G. Oster. 2006. Incidence and prevalence of idiopathic pulmonary fibrosis. *American journal of respiratory and critical care medicine* 174: 810-816.

4.   Kropski, J. A., W. E. Lawson, L. R. Young, and T. S. Blackwell. 2013. Genetic studies provide clues on the pathogenesis of idiopathic pulmonary fibrosis. *Disease models & mechanisms* 6: 9-17.

5.   Seibold, M. A., A. L. Wise, M. C. Speer, M. P. Steele, K. K. Brown, J. E. Loyd, T. E. Fingerlin, W. Zhang, G. Gudmundsson, S. D. Groshong, C. M. Evans, S. Garantziotis, K. B. Adler, B. F. Dickey, R. M. du Bois, I. V. Yang, A. Herron, D. Kervitsky, J. L. Talbert, C. Markin, J. Park, A. L. Crews, S. H. Slifer, S. Auerbach, M. G. Roy, J. Lin, C. E. Hennessy, M. I. Schwarz, and D. A. Schwartz. 2011. A common MUC5B promoter polymorphism and pulmonary fibrosis. *The New England journal of medicine* 364: 1503-1512.

6.   Fell, C. D. 2012. Idiopathic pulmonary fibrosis: phenotypes and comorbidities. *Clinics in chest medicine* 33: 51-57.

7.   Ley, B., H. R. Collard, and T. E. King, Jr. 2011. Clinical course and prediction of survival in idiopathic pulmonary fibrosis. *American journal of respiratory and critical care medicine* 183: 431-440.

8.   Selman, M., G. Carrillo, A. Estrada, M. Mejia, C. Becerril, J. Cisneros, M. Gaxiola, R. Perez-Padilla, C. Navarro, T. Richards, J. Dauber, T. E. King, Jr., A. Pardo, and N. Kaminski. 2007. Accelerated variant of idiopathic pulmonary fibrosis: clinical behavior and gene expression pattern. *PloS one* 2: e482.

9.   Boon, K., N. W. Bailey, J. Yang, M. P. Steel, S. Groshong, D. Kervitsky, K. K. Brown, M. I. Schwarz, and D. A. Schwartz. 2009. Molecular phenotypes distinguish patients with relatively stable from progressive idiopathic pulmonary fibrosis (IPF). *PloS one* 4: e5134.

10.   Barst, R. J., M. McGoon, A. Torbicki, O. Sitbon, M. J. Krowka, H. Olschewski, and S. Gaine. 2004. Diagnosis and differential assessment of pulmonary arterial hypertension. *Journal of the American College of Cardiology* 43: 40S-47S.

11.   Smith, J. S., D. Gorbett, J. Mueller, R. Perez, and C. J. Daniels. 2013. Pulmonary hypertension and idiopathic pulmonary fibrosis: a dastardly duo. *The American journal of the medical sciences* 346: 221-225.

12.   Lettieri, C. J., S. D. Nathan, S. D. Barnett, S. Ahmad, and A. F. Shorr. 2006. Prevalence and outcomes of pulmonary arterial hypertension in advanced idiopathic pulmonary fibrosis. *Chest* 129: 746-752.

13.   Nadrous, H. F., P. A. Pellikka, M. J. Krowka, K. L. Swanson, N. Chaowalit, P. A. Decker, and J. H. Ryu. 2005. Pulmonary hypertension in patients with idiopathic pulmonary fibrosis. *Chest* 128: 2393-2399.

14.   Shorr, A. F., J. L. Wainright, C. S. Cors, C. J. Lettieri, and S. D. Nathan. 2007. Pulmonary hypertension in patients with pulmonary fibrosis awaiting lung transplant. *The European respiratory journal* 30: 715-721.

15.   Nathan, S. D., O. A. Shlobin, S. Ahmad, J. Koch, S. D. Barnett, N. Ad, N. Burton, and K. Leslie. 2008. Serial development of pulmonary hypertension in patients with idiopathic pulmonary fibrosis. *Respiration; international review of thoracic diseases* 76: 288-294.

16.   Hamada, K., S. Nagai, S. Tanaka, T. Handa, M. Shigematsu, T. Nagao, M. Mishima, M. Kitaichi, and T. Izumi. 2007. Significance of pulmonary arterial pressure and diffusion capacity of the lung as prognosticator in patients with idiopathic pulmonary fibrosis. *Chest* 131: 650-656.

17.   Mejia, M., G. Carrillo, J. Rojas-Serrano, A. Estrada, T. Suarez, D. Alonso, E. Barrientos, M. Gaxiola, C. Navarro, and M. Selman. 2009. Idiopathic pulmonary fibrosis and emphysema: decreased survival associated with severe pulmonary arterial hypertension. *Chest* 136: 10-15.

18.   Cottin, V., H. Nunes, P. Y. Brillet, P. Delaval, G. Devouassoux, I. Tillie-Leblond, D. Israel-Biet, I. Court-Fortune, D. Valeyre, J. F. Cordier, and P. Groupe d'Etude et de Recherche sur les Maladies Orphelines. 2005. Combined pulmonary fibrosis and emphysema: a distinct underrecognised entity. *The European respiratory journal* 26: 586-593.

19.   Daniil, Z., A. Koutsokera, and K. Gourgoulianis. 2006. Combined pulmonary fibrosis and emphysema in patients exposed to agrochemical compounds. *The European respiratory journal* 27: 434.

20.   Antoniou, K. M., G. A. Margaritopoulos, and N. M. Siafakas. 2013. Pharmacological treatment of idiopathic pulmonary fibrosis: from the past to the future. *European respiratory review : an official journal of the European Respiratory Society* 22: 281-291.

21.   Woodcock, H. V., and T. M. Maher. 2014. The treatment of idiopathic pulmonary fibrosis. *F1000prime reports* 6: 16.

22.   Potts, J., and D. Yogaratnam. 2013. Pirfenidone: a novel agent for the treatment of idiopathic pulmonary fibrosis. *The Annals of pharmacotherapy* 47: 361-367.

23.   2010. SEER Cancer Statistics Review, 1975-2007. K. C. Altekruse SF, Krapcho M, Neyman N, Aminou R, Waldron W, Ruhl J, Howlader N, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Cronin K, Chen HS, Feuer EJ, Stinchcomb DG, Edwards BK (eds). , ed. National Cancer Institute, Bethesda, MD.

24.   Olson, A. L., J. J. Swigris, D. C. Lezotte, J. M. Norris, C. G. Wilson, and K. K. Brown. 2007. Mortality from pulmonary fibrosis increased in the United States from 1992 to 2003. *American journal of respiratory and critical care medicine* 176: 277-284.

25.   Zhou, Y., D. J. Schneider, and M. R. Blackburn. 2009. Adenosine signaling and the regulation of chronic lung disease. *Pharmacology & therapeutics* 123: 105-116.

26.   Fredholm, B. B. 2007. Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell death and differentiation* 14: 1315-1323.

27. Karmouty-Quintana, H., Y. Xia, and M. R. Blackburn. 2013. Adenosine signaling during acute and chronic disease states. *Journal of molecular medicine* 91: 173-181.
28. Chunn, J. L., J. G. Molina, T. Mi, Y. Xia, R. E. Kellems, and M. R. Blackburn. 2005. Adenosine-dependent pulmonary fibrosis in adenosine deaminase-deficient mice. *Journal of immunology* 175: 1937-1946.
29. Pedroza, M., D. J. Schneider, H. Karmouty-Quintana, J. Coote, S. Shaw, R. Corrigan, J. G. Molina, J. L. Alcorn, D. Galas, R. Gelinas, and M. R. Blackburn. 2011. Interleukin-6 contributes to inflammation and remodeling in a model of adenosine mediated lung injury. *PloS one* 6: e22667.
30. Schneider, D. J., J. C. Lindsay, Y. Zhou, J. G. Molina, and M. R. Blackburn. 2010. Adenosine and osteopontin contribute to the development of chronic obstructive pulmonary disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 24: 70-80.
31. Zhou, Y., J. N. Murthy, D. Zeng, L. Belardinelli, and M. R. Blackburn. 2010. Alterations in adenosine metabolism and signaling in patients with chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. *PloS one* 5: e9224.
32. Sun, Y., F. Wu, F. Sun, and P. Huang. 2008. Adenosine promotes IL-6 release in airway epithelia. *Journal of immunology* 180: 4173-4181.
33. Zhou, Y., D. J. Schneider, E. Morschl, L. Song, M. Pedroza, H. Karmouty-Quintana, T. Le, C. X. Sun, and M. R. Blackburn. 2011. Distinct roles for the A2B adenosine receptor in acute and chronic stages of bleomycin-induced lung injury. *Journal of immunology* 186: 1097-1106.
34. Zhong, H., L. Belardinelli, T. Maa, I. Feoktistov, I. Biaggioni, and D. Zeng. 2004. A(2B) adenosine receptors increase cytokine release by bronchial smooth muscle cells. *American journal of respiratory cell and molecular biology* 30: 118-125.
35. Zhong, H., L. Belardinelli, T. Maa, and D. Zeng. 2005. Synergy between A2B adenosine receptors and hypoxia in activating human lung fibroblasts. *American journal of respiratory cell and molecular biology* 32: 2-8.
36. Sehgal, P. B., and A. D. Sagar. 1980. Heterogeneity of poly(I) x poly(C)-induced human fibroblast interferon mRNA species. *Nature* 288: 95-97.
37. Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwamura, K. Nakajima, K. Koyama, A. Iwamatsu, and et al. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* 324: 73-76.
38. Chalaris, A., C. Garbers, B. Rabe, S. Rose-John, and J. Scheller. 2011. The soluble Interleukin 6 receptor: generation and role in inflammation and cancer. *Eur J Cell Biol* 90: 484-494.
39. Lotz, M., F. Jirik, P. Kabouridis, C. Tsoukas, T. Hirano, T. Kishimoto, and D. A. Carson. 1988. B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes. *The Journal of experimental medicine* 167: 1253-1258.
40. Ikebuchi, K., G. G. Wong, S. C. Clark, J. N. Ihle, Y. Hirai, and M. Ogawa. 1987. Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. *Proceedings of the National Academy of Sciences of the United States of America* 84: 9035-9039.

41. Gauldie, J., C. Richards, D. Harnish, P. Lansdorp, and H. Baumann. 1987. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proceedings of the National Academy of Sciences of the United States of America* 84: 7251-7255.

42. Kotake, S., K. Sato, K. J. Kim, N. Takahashi, N. Udagawa, I. Nakamura, A. Yamaguchi, T. Kishimoto, T. Suda, and S. Kashiwazaki. 1996. Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 11: 88-95.

43. Scheller, J., A. Chalaris, D. Schmidt-Arras, and S. Rose-John. 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta* 1813: 878-888.

44. Jones, S. A., J. Scheller, and S. Rose-John. 2011. Therapeutic strategies for the clinical blockade of IL-6/gp130 signaling. *The Journal of clinical investigation* 121: 3375-3383.

45. Bauer, J., T. M. Bauer, T. Kalb, T. Taga, G. Lengyel, T. Hirano, T. Kishimoto, G. Acs, L. Mayer, and W. Gerok. 1989. Regulation of interleukin 6 receptor expression in human monocytes and monocyte-derived macrophages. Comparison with the expression in human hepatocytes. *The Journal of experimental medicine* 170: 1537-1549.

46. Taga, T., Y. Kawanishi, R. R. Hardy, T. Hirano, and T. Kishimoto. 1987. Receptors for B cell stimulatory factor 2. Quantitation, specificity, distribution, and regulation of their expression. *The Journal of experimental medicine* 166: 967-981.

47. Rose-John, S., J. Scheller, G. Elson, and S. A. Jones. 2006. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. *Journal of leukocyte biology* 80: 227-236.

48. Taga, T., M. Hibi, Y. Hirata, K. Yamasaki, K. Yasukawa, T. Matsuda, T. Hirano, and T. Kishimoto. 1989. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* 58: 573-581.

49. Murakami, M., M. Hibi, N. Nakagawa, T. Nakagawa, K. Yasukawa, K. Yamanishi, T. Taga, and T. Kishimoto. 1993. IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. *Science* 260: 1808-1810.

50. Stahl, N., T. G. Boulton, T. Farruggella, N. Y. Ip, S. Davis, B. A. Witthuhn, F. W. Quelle, O. Silvennoinen, G. Barbieri, S. Pellegrini, and et al. 1994. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. *Science* 263: 92-95.

51. Zhong, Z., Z. Wen, and J. E. Darnell, Jr. 1994. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 264: 95-98.

52. Heinrich, P. C., I. Behrmann, G. Muller-Newen, F. Schaper, and L. Graeve. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *The Biochemical journal* 334 (Pt 2): 297-314.

53. Rose-John, S., and P. C. Heinrich. 1994. Soluble receptors for cytokines and growth factors: generation and biological function. *The Biochemical journal* 300 (Pt 2): 281-290.

54. Horiuchi, S., Y. Koyanagi, Y. Zhou, H. Miyamoto, Y. Tanaka, M. Waki, A. Matsumoto, M. Yamamoto, and N. Yamamoto. 1994. Soluble interleukin-6 receptors released from T cell or granulocyte/macrophage cell lines and human peripheral blood mononuclear cells are generated through an alternative splicing mechanism. *Eur J Immunol* 24: 1945-1948.

55. Lust, J. A., K. A. Donovan, M. P. Kline, P. R. Greipp, R. A. Kyle, and N. J. Maihle. 1992. Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor. *Cytokine* 4: 96-100.

56. Muller-Newen, G., C. Kohne, R. Keul, U. Hemmann, W. Muller-Esterl, J. Wijdenes, J. P. Brakenhoff, M. H. Hart, and P. C. Heinrich. 1996. Purification and characterization of the soluble interleukin-6 receptor from human plasma and identification of an isoform generated through alternative splicing. *Eur J Biochem* 236: 837-842.

57. Mullberg, J., H. Schooltink, T. Stoyan, M. Gunther, L. Graeve, G. Buse, A. Mackiewicz, P. C. Heinrich, and S. Rose-John. 1993. The soluble interleukin-6 receptor is generated by shedding. *Eur J Immunol* 23: 473-480.

58. Mullberg, J., H. Schooltink, T. Stoyan, P. C. Heinrich, and S. Rose-John. 1992. Protein kinase C activity is rate limiting for shedding of the interleukin-6 receptor. *Biochemical and biophysical research communications* 189: 794-800.

59. Chalaris, A., B. Rabe, K. Paliga, H. Lange, T. Laskay, C. A. Fielding, S. A. Jones, S. Rose-John, and J. Scheller. 2007. Apoptosis is a natural stimulus of IL6R shedding and contributes to the proinflammatory trans-signaling function of neutrophils. *Blood* 110: 1748-1755.

60. Jones, S. A., S. Horiuchi, D. Novick, N. Yamamoto, and G. M. Fuller. 1998. Shedding of the soluble IL-6 receptor is triggered by Ca2+ mobilization, while basal release is predominantly the product of differential mRNA splicing in THP-1 cells. *Eur J Immunol* 28: 3514-3522.

61. Matthews, V., B. Schuster, S. Schutze, I. Bussmeyer, A. Ludwig, C. Hundhausen, T. Sadowski, P. Saftig, D. Hartmann, K. J. Kallen, and S. Rose-John. 2003. Cellular cholesterol depletion triggers shedding of the human interleukin-6 receptor by ADAM10 and ADAM17 (TACE). *The Journal of biological chemistry* 278: 38829-38839.

62. Fenton, J. I., S. D. Hursting, S. N. Perkins, and N. G. Hord. 2006. Interleukin-6 production induced by leptin treatment promotes cell proliferation in an Apc (Min/+) colon epithelial cell line. *Carcinogenesis* 27: 1507-1515.

63. Briso, E. M., O. Dienz, and M. Rincon. 2008. Cutting edge: soluble IL-6R is produced by IL-6R ectodomain shedding in activated CD4 T cells. *Journal of immunology* 180: 7102-7106.

64. Gomez, M. I., S. H. Sokol, A. B. Muir, G. Soong, J. Bastien, and A. S. Prince. 2005. Bacterial induction of TNF-alpha converting enzyme expression and IL-6 receptor alpha shedding regulates airway inflammatory signaling. *Journal of immunology* 175: 1930-1936.

65. Scheller, J., A. Chalaris, C. Garbers, and S. Rose-John. 2011. ADAM17: a molecular switch to control inflammation and tissue regeneration. *Trends Immunol* 32: 380-387.

66. Booth, B. W., T. Sandifer, E. L. Martin, and L. D. Martin. 2007. IL-13-induced proliferation of airway epithelial cells: mediation by intracellular growth factor mobilization and ADAM17. *Respir Res* 8: 51.

67. Cesaro, A., A. Abakar-Mahamat, P. Brest, S. Lassalle, E. Selva, J. Filippi, X. Hebuterne, J. P. Hugot, A. Doglio, F. Galland, P. Naquet, V. Vouret-Craviari, B. Mograbi, and P. M. Hofman. 2009. Differential expression and regulation of ADAM17 and TIMP3 in acute inflamed intestinal epithelia. *Am J Physiol Gastrointest Liver Physiol* 296: G1332-1343.

68. Charbonneau, M., K. Harper, F. Grondin, M. Pelmus, P. P. McDonald, and C. M. Dubois. 2007. Hypoxia-inducible factor mediates hypoxic and tumor necrosis factor alpha-induced increases in tumor necrosis factor-alpha converting enzyme/ADAM17 expression by synovial cells. *The Journal of biological chemistry* 282: 33714-33724.

69. Franchimont, N., C. Lambert, P. Huynen, C. Ribbens, B. Relic, A. Chariot, V. Bours, J. Piette, M. P. Merville, and M. Malaise. 2005. Interleukin-6 receptor shedding is enhanced by interleukin-1beta and tumor necrosis factor alpha and is partially mediated by tumor necrosis factor alpha-converting enzyme in osteoblast-like cells. *Arthritis Rheum* 52: 84-93.

70. Peters, M., K. H. Meyer zum Buschenfelde, and S. Rose-John. 1996. The function of the soluble IL-6 receptor in vivo. *Immunology letters* 54: 177-184.

71. Neurath, M. F., and S. Finotto. 2011. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine & growth factor reviews* 22: 83-89.

72. Melendez, G. C., J. L. McLarty, S. P. Levick, Y. Du, J. S. Janicki, and G. L. Brower. 2010. Interleukin 6 mediates myocardial fibrosis, concentric hypertrophy, and diastolic dysfunction in rats. *Hypertension* 56: 225-231.

73. Cronstein, B. N. 2007. Interleukin-6--a key mediator of systemic and local symptoms in rheumatoid arthritis. *Bull NYU Hosp Jt Dis* 65 Suppl 1: S11-15.

74. Finotto, S., T. Eigenbrod, R. Karwot, I. Boross, A. Doganci, H. Ito, N. Nishimoto, K. Yoshizaki, T. Kishimoto, S. Rose-John, P. R. Galle, and M. F. Neurath. 2007. Local blockade of IL-6R signaling induces lung CD4+ T cell apoptosis in a murine model of asthma via regulatory T cells. *International immunology* 19: 685-693.

75. Tanaka, T., M. Narazaki, and T. Kishimoto. 2012. Therapeutic targeting of the interleukin-6 receptor. *Annu Rev Pharmacol Toxicol* 52: 199-219.

76. Atreya, R., J. Mudter, S. Finotto, J. Mullberg, T. Jostock, S. Wirtz, M. Schutz, B. Bartsch, M. Holtmann, C. Becker, D. Strand, J. Czaja, J. F. Schlaak, H. A. Lehr, F. Autschbach, G. Schurmann, N. Nishimoto, K. Yoshizaki, H. Ito, T. Kishimoto, P. R. Galle, S. Rose-John, and M. F. Neurath. 2000. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med* 6: 583-588.

77. Barkhausen, T., T. Tschernig, P. Rosenstiel, M. van Griensven, R. P. Vonberg, M. Dorsch, A. Mueller-Heine, A. Chalaris, J. Scheller, S. Rose-John, D. Seegert, C. Krettek, and G. H. Waetzig. 2011. Selective blockade of interleukin-6 trans-signaling improves survival in a murine polymicrobial sepsis model. *Critical care medicine* 39: 1407-1413.

78. Matsumoto, S., T. Hara, K. Mitsuyama, M. Yamamoto, O. Tsuruta, M. Sata, J. Scheller, S. Rose-John, S. Kado, and T. Takada. 2010. Essential roles of IL-6 trans-signaling in

colonic epithelial cells, induced by the IL-6/soluble-IL-6 receptor derived from lamina propria macrophages, on the development of colitis-associated premalignant cancer in a murine model. *Journal of immunology* 184: 1543-1551.

79.   Nowell, M. A., P. J. Richards, S. Horiuchi, N. Yamamoto, S. Rose-John, N. Topley, A. S. Williams, and S. A. Jones. 2003. Soluble IL-6 receptor governs IL-6 activity in experimental arthritis: blockade of arthritis severity by soluble glycoprotein 130. *Journal of immunology* 171: 3202-3209.

80.   Nowell, M. A., A. S. Williams, S. A. Carty, J. Scheller, A. J. Hayes, G. W. Jones, P. J. Richards, S. Slinn, M. Ernst, B. J. Jenkins, N. Topley, S. Rose-John, and S. A. Jones. 2009. Therapeutic targeting of IL-6 trans signaling counteracts STAT3 control of experimental inflammatory arthritis. *Journal of immunology* 182: 613-622.

81.   Migita, K., S. Abiru, Y. Maeda, M. Daikoku, K. Ohata, M. Nakamura, A. Komori, K. Yano, H. Yatsuhashi, K. Eguchi, and H. Ishibashi. 2006. Serum levels of interleukin-6 and its soluble receptors in patients with hepatitis C virus infection. *Hum Immunol* 67: 27-32.

82.   Dai, Y., W. Zhang, J. Wen, Y. Zhang, R. E. Kellems, and Y. Xia. 2011. A2B adenosine receptor-mediated induction of IL-6 promotes CKD. *Journal of the American Society of Nephrology : JASN* 22: 890-901.

83.   Sato, S., M. Hasegawa, and K. Takehara. 2001. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. *J Dermatol Sci* 27: 140-146.

84.   Hasegawa, M., S. Sato, H. Ihn, and K. Takehara. 1999. Enhanced production of interleukin-6 (IL-6), oncostatin M and soluble IL-6 receptor by cultured peripheral blood mononuclear cells from patients with systemic sclerosis. *Rheumatology* 38: 612-617.

85.   Ammit, A. J., L. M. Moir, B. G. Oliver, J. M. Hughes, H. Alkhouri, Q. Ge, J. K. Burgess, J. L. Black, and M. Roth. 2007. Effect of IL-6 trans-signaling on the pro-remodeling phenotype of airway smooth muscle. *American journal of physiology. Lung cellular and molecular physiology* 292: L199-206.

86.   Zhang, H., P. Neuhofer, L. Song, B. Rabe, M. Lesina, M. U. Kurkowski, M. Treiber, T. Wartmann, S. Regner, H. Thorlacius, D. Saur, G. Weirich, A. Yoshimura, W. Halangk, J. P. Mizgerd, R. M. Schmid, S. Rose-John, and H. Algul. 2013. IL-6 trans-signaling promotes pancreatitis-associated lung injury and lethality. *The Journal of clinical investigation* 123: 1019-1031.

87.   O'Donoghue, R. J., D. A. Knight, C. D. Richards, C. M. Prele, H. L. Lau, A. G. Jarnicki, J. Jones, S. Bozinovski, R. Vlahos, S. Thiem, B. S. McKenzie, B. Wang, P. Stumbles, G. J. Laurent, R. J. McAnulty, S. Rose-John, H. J. Zhu, G. P. Anderson, M. R. Ernst, and S. E. Mutsaers. 2012. Genetic partitioning of interleukin-6 signalling in mice dissociates Stat3 from Smad3-mediated lung fibrosis. *EMBO Mol Med* 4: 939-951.

88.   Mouratis, M. A., and V. Aidinis. 2011. Modeling pulmonary fibrosis with bleomycin. *Current opinion in pulmonary medicine* 17: 355-361.

89.   Degryse, A. L., H. Tanjore, X. C. Xu, V. V. Polosukhin, B. R. Jones, F. B. McMahon, L. A. Gleaves, T. S. Blackwell, and W. E. Lawson. 2010. Repetitive intratracheal bleomycin models several features of idiopathic pulmonary fibrosis. *American journal of physiology. Lung cellular and molecular physiology* 299: L442-452.

90.   Swiderski, R. E., J. E. Dencoff, C. S. Floerchinger, S. D. Shapiro, and G. W. Hunninghake. 1998. Differential expression of extracellular matrix remodeling genes in a murine model of bleomycin-induced pulmonary fibrosis. *The American journal of pathology* 152: 821-828.

91.   Karmouty-Quintana, H., H. Zhong, L. Acero, T. Weng, E. Melicoff, J. D. West, A. Hemnes, A. Grenz, H. K. Eltzschig, T. S. Blackwell, Y. Xia, R. A. Johnston, D. Zeng, L. Belardinelli, and M. R. Blackburn. 2012. The A2B adenosine receptor modulates pulmonary hypertension associated with interstitial lung disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 26: 2546-2557.

92.   Baran, C. P., J. M. Opalek, S. McMaken, C. A. Newland, J. M. O'Brien, Jr., M. G. Hunter, B. D. Bringardner, M. M. Monick, D. R. Brigstock, P. C. Stromberg, G. W. Hunninghake, and C. B. Marsh. 2007. Important roles for macrophage colony-stimulating factor, CC chemokine ligand 2, and mononuclear phagocytes in the pathogenesis of pulmonary fibrosis. *American journal of respiratory and critical care medicine* 176: 78-89.

93.   Chua, F., J. Gauldie, and G. J. Laurent. 2005. Pulmonary fibrosis: searching for model answers. *American journal of respiratory cell and molecular biology* 33: 9-13.

94.   Blackburn, M. R., S. K. Datta, and R. E. Kellems. 1998. Adenosine deaminase-deficient mice generated using a two-stage genetic engineering strategy exhibit a combined immunodeficiency. *The Journal of biological chemistry* 273: 5093-5100.

95.   Blackburn, M. R., J. B. Volmer, J. L. Thrasher, H. Zhong, J. R. Crosby, J. J. Lee, and R. E. Kellems. 2000. Metabolic consequences of adenosine deaminase deficiency in mice are associated with defects in alveogenesis, pulmonary inflammation, and airway obstruction. *The Journal of experimental medicine* 192: 159-170.

96.   Blackburn, M. R., M. Aldrich, J. B. Volmer, W. Chen, H. Zhong, S. Kelly, M. S. Hershfield, S. K. Datta, and R. E. Kellems. 2000. The use of enzyme therapy to regulate the metabolic and phenotypic consequences of adenosine deaminase deficiency in mice. Differential impact on pulmonary and immunologic abnormalities. *The Journal of biological chemistry* 275: 32114-32121.

97.   Sun, C. X., H. Zhong, A. Mohsenin, E. Morschl, J. L. Chunn, J. G. Molina, L. Belardinelli, D. Zeng, and M. R. Blackburn. 2006. Role of A2B adenosine receptor signaling in adenosine-dependent pulmonary inflammation and injury. *The Journal of clinical investigation* 116: 2173-2182.

98.   Blackburn, M. R., C. G. Lee, H. W. Young, Z. Zhu, J. L. Chunn, M. J. Kang, S. K. Banerjee, and J. A. Elias. 2003. Adenosine mediates IL-13-induced inflammation and remodeling in the lung and interacts in an IL-13-adenosine amplification pathway. *The Journal of clinical investigation* 112: 332-344.

99.   Sun, C. X., H. W. Young, J. G. Molina, J. B. Volmer, J. Schnermann, and M. R. Blackburn. 2005. A protective role for the A1 adenosine receptor in adenosine-dependent pulmonary injury. *The Journal of clinical investigation* 115: 35-43.

100.   Mohsenin, A., T. Mi, Y. Xia, R. E. Kellems, J. F. Chen, and M. R. Blackburn. 2007. Genetic removal of the A2A adenosine receptor enhances pulmonary inflammation, mucin production, and angiogenesis in adenosine deaminase-deficient mice. *American journal of physiology. Lung cellular and molecular physiology* 293: L753-761.

101. Zhou, Y., A. Mohsenin, E. Morschl, H. W. Young, J. G. Molina, W. Ma, C. X. Sun, H. Martinez-Valdez, and M. R. Blackburn. 2009. Enhanced airway inflammation and remodeling in adenosine deaminase-deficient mice lacking the A2B adenosine receptor. *Journal of immunology* 182: 8037-8046.

102. Young, H. W., J. G. Molina, D. Dimina, H. Zhong, M. Jacobson, L. N. Chan, T. S. Chan, J. J. Lee, and M. R. Blackburn. 2004. A3 adenosine receptor signaling contributes to airway inflammation and mucus production in adenosine deaminase-deficient mice. *Journal of immunology* 173: 1380-1389.

103. Lo, C. W., M. W. Chen, M. Hsiao, S. Wang, C. A. Chen, S. M. Hsiao, J. S. Chang, T. C. Lai, S. Rose-John, M. L. Kuo, and L. H. Wei. 2011. IL-6 trans-signaling in formation and progression of malignant ascites in ovarian cancer. *Cancer research* 71: 424-434.

104. Wynn, T. A. 2011. Integrating mechanisms of pulmonary fibrosis. *The Journal of experimental medicine* 208: 1339-1350.

105. Muller-Newen, G., A. Kuster, U. Hemmann, R. Keul, U. Horsten, A. Martens, L. Graeve, J. Wijdenes, and P. C. Heinrich. 1998. Soluble IL-6 receptor potentiates the antagonistic activity of soluble gp130 on IL-6 responses. *Journal of immunology* 161: 6347-6355.

106. Jostock, T., J. Mullberg, S. Ozbek, R. Atreya, G. Blinn, N. Voltz, M. Fischer, M. F. Neurath, and S. Rose-John. 2001. Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses. *Eur J Biochem* 268: 160-167.

107. Lissilaa, R., V. Buatois, G. Magistrelli, A. S. Williams, G. W. Jones, S. Herren, L. Shang, P. Malinge, F. Guilhot, L. Chatel, E. Hatterer, S. A. Jones, M. H. Kosco-Vilbois, and W. G. Ferlin. 2010. Although IL-6 trans-signaling is sufficient to drive local immune responses, classical IL-6 signaling is obligate for the induction of T cell-mediated autoimmunity. *Journal of immunology* 185: 5512-5521.

108. Doganci, A., T. Eigenbrod, N. Krug, G. T. De Sanctis, M. Hausding, V. J. Erpenbeck, B. Haddad el, H. A. Lehr, E. Schmitt, T. Bopp, K. J. Kallen, U. Herz, S. Schmitt, C. Luft, O. Hecht, J. M. Hohlfeld, H. Ito, N. Nishimoto, K. Yoshizaki, T. Kishimoto, S. Rose-John, H. Renz, M. F. Neurath, P. R. Galle, and S. Finotto. 2005. The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. *The Journal of clinical investigation* 115: 313-325.

109. Hoge, J., I. Yan, N. Janner, V. Schumacher, A. Chalaris, O. M. Steinmetz, D. R. Engel, J. Scheller, S. Rose-John, and H. W. Mittrucker. 2013. IL-6 controls the innate immune response against Listeria monocytogenes via classical IL-6 signaling. *Journal of immunology* 190: 703-711.

110. Hasegawa, M., S. Sato, M. Fujimoto, H. Ihn, K. Kikuchi, and K. Takehara. 1998. Serum levels of interleukin 6 (IL-6), oncostatin M, soluble IL-6 receptor, and soluble gp130 in patients with systemic sclerosis. *J Rheumatol* 25: 308-313.

111. Adamali, H. I., and T. M. Maher. 2012. Current and novel drug therapies for idiopathic pulmonary fibrosis. *Drug design, development and therapy* 6: 261-272.

112. Loomis-King, H., K. R. Flaherty, and B. B. Moore. 2013. Pathogenesis, current treatments and future directions for idiopathic pulmonary fibrosis. *Current opinion in pharmacology* 13: 377-385.

113. Murray, L. A., R. Rosada, A. P. Moreira, A. Joshi, M. S. Kramer, D. P. Hesson, R. L. Argentieri, S. Mathai, M. Gulati, E. L. Herzog, and C. M. Hogaboam. 2010. Serum

amyloid P therapeutically attenuates murine bleomycin-induced pulmonary fibrosis via its effects on macrophages. *PloS one* 5: e9683.

114. Trujillo, G., E. C. O'Connor, S. L. Kunkel, and C. M. Hogaboam. 2008. A novel mechanism for CCR4 in the regulation of macrophage activation in bleomycin-induced pulmonary fibrosis. *The American journal of pathology* 172: 1209-1221.

115. Pechkovsky, D. V., A. Prasse, F. Kollert, K. M. Engel, J. Dentler, W. Luttmann, K. Friedrich, J. Muller-Quernheim, and G. Zissel. 2010. Alternatively activated alveolar macrophages in pulmonary fibrosis-mediator production and intracellular signal transduction. *Clin Immunol* 137: 89-101.

116. Murray, L. A., Q. Chen, M. S. Kramer, D. P. Hesson, R. L. Argentieri, X. Peng, M. Gulati, R. J. Homer, T. Russell, N. van Rooijen, J. A. Elias, C. M. Hogaboam, and E. L. Herzog. 2011. TGF-beta driven lung fibrosis is macrophage dependent and blocked by Serum amyloid P. *Int J Biochem Cell Biol* 43: 154-162.

117. Collins, S. L., Y. Chan-Li, R. W. Hallowell, J. D. Powell, and M. R. Horton. 2012. Pulmonary vaccination as a novel treatment for lung fibrosis. *PloS one* 7: e31299.

118. Camelo, A., R. Dunmore, M. A. Sleeman, and D. L. Clarke. 2014. The epithelium in idiopathic pulmonary fibrosis: breaking the barrier. *Frontiers in pharmacology* 4: 173.

119. Habiel, D. M., and C. Hogaboam. 2014. Heterogeneity in fibroblast proliferation and survival in idiopathic pulmonary fibrosis. *Frontiers in pharmacology* 5: 2.

120. Modolell, M., I. M. Corraliza, F. Link, G. Soler, and K. Eichmann. 1995. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur J Immunol* 25: 1101-1104.

121. Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *The Journal of experimental medicine* 176: 287-292.

122. Mosser, D. M., and X. Zhang. 2008. Activation of murine macrophages. *Curr Protoc Immunol* Chapter 14: Unit 14 12.

123. Wynn, T. A. 2003. IL-13 effector functions. *Annu Rev Immunol* 21: 425-456.

124. Althoff, K., P. Reddy, N. Voltz, S. Rose-John, and J. Mullberg. 2000. Shedding of interleukin-6 receptor and tumor necrosis factor alpha. Contribution of the stalk sequence to the cleavage pattern of transmembrane proteins. *Eur J Biochem* 267: 2624-2631.

125. Lee, Y. J., S. H. Lee, Y. S. Youn, J. Y. Choi, K. S. Song, M. S. Cho, and J. L. Kang. 2012. Preventing cleavage of Mer promotes efferocytosis and suppresses acute lung injury in bleomycin treated mice. *Toxicology and applied pharmacology* 263: 61-72.

126. Garbers, C., N. Janner, A. Chalaris, M. L. Moss, D. M. Floss, D. Meyer, F. Koch-Nolte, S. Rose-John, and J. Scheller. 2011. Species specificity of ADAM10 and ADAM17 proteins in interleukin-6 (IL-6) trans-signaling and novel role of ADAM10 in inducible IL-6 receptor shedding. *The Journal of biological chemistry* 286: 14804-14811.

127. Narazaki, M., K. Yasukawa, T. Saito, Y. Ohsugi, H. Fukui, Y. Koishihara, G. D. Yancopoulos, T. Taga, and T. Kishimoto. 1993. Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a

potential to inhibit signals through membrane-anchored gp130. *Blood* 82: 1120-1126.

128. Zhang, J. G., Y. Zhang, C. M. Owczarek, L. D. Ward, R. L. Moritz, R. J. Simpson, K. Yasukawa, and N. A. Nicola. 1998. Identification and characterization of two distinct truncated forms of gp130 and a soluble form of leukemia inhibitory factor receptor alpha-chain in normal human urine and plasma. *The Journal of biological chemistry* 273: 10798-10805.

129. Rose-John, S. 2012. IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6. *International journal of biological sciences* 8: 1237-1247.

130. Ashcroft, T., J. M. Simpson, and V. Timbrell. 1988. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *Journal of clinical pathology* 41: 467-470.

131. Wynn, T. A. 2008. Cellular and molecular mechanisms of fibrosis. *The Journal of pathology* 214: 199-210.

132. Rennard, S. I., and R. G. Crystal. 1982. Fibronectin in human bronchopulmonary lavage fluid. Elevation in patients with interstitial lung disease. *The Journal of clinical investigation* 69: 113-122.

133. Crosby, L. M., and C. M. Waters. 2010. Epithelial repair mechanisms in the lung. *American journal of physiology. Lung cellular and molecular physiology* 298: L715-731.

134. White, E. S., F. E. Baralle, and A. F. Muro. 2008. New insights into form and function of fibronectin splice variants. *The Journal of pathology* 216: 1-14.

135. White, E. S., and A. F. Muro. 2011. Fibronectin splice variants: understanding their multiple roles in health and disease using engineered mouse models. *IUBMB Life* 63: 538-546.

136. Seeger, W., Y. Adir, J. A. Barbera, H. Champion, J. G. Coghlan, V. Cottin, T. De Marco, N. Galie, S. Ghio, S. Gibbs, F. J. Martinez, M. J. Semigran, G. Simonneau, A. U. Wells, and J. L. Vachiery. 2013. Pulmonary hypertension in chronic lung diseases. *Journal of the American College of Cardiology* 62: D109-116.

137. Knight, D., S. E. Mutsaers, and C. M. Prele. 2011. STAT3 in tissue fibrosis: is there a role in the lung? *Pulm Pharmacol Ther* 24: 193-198.

138. Prele, C. M., E. Yao, R. J. O'Donoghue, S. E. Mutsaers, and D. A. Knight. 2012. STAT3: a central mediator of pulmonary fibrosis? *Proc Am Thorac Soc* 9: 177-182.

139. Pechkovsky, D. V., C. M. Prele, J. Wong, C. M. Hogaboam, R. J. McAnulty, G. J. Laurent, S. S. Zhang, M. Selman, S. E. Mutsaers, and D. A. Knight. 2012. STAT3-mediated signaling dysregulates lung fibroblast-myofibroblast activation and differentiation in UIP/IPF. *The American journal of pathology* 180: 1398-1412.

140. Goodwin, A., and G. Jenkins. 2009. Role of integrin-mediated TGFbeta activation in the pathogenesis of pulmonary fibrosis. *Biochemical Society transactions* 37: 849-854.

141. Hetzel, M., M. Bachem, D. Anders, G. Trischler, and M. Faehling. 2005. Different effects of growth factors on proliferation and matrix production of normal and fibrotic human lung fibroblasts. *Lung* 183: 225-237.

142. Muro, A. F., F. A. Moretti, B. B. Moore, M. Yan, R. G. Atrasz, C. A. Wilke, K. R. Flaherty, F. J. Martinez, J. L. Tsui, D. Sheppard, F. E. Baralle, G. B. Toews, and E. S. White. 2008.

An essential role for fibronectin extra type III domain A in pulmonary fibrosis. *American journal of respiratory and critical care medicine* 177: 638-645.

143. Wolters, P. J., H. R. Collard, and K. D. Jones. 2014. Pathogenesis of idiopathic pulmonary fibrosis. *Annual review of pathology* 9: 157-179.

144. Moodley, Y. P., A. K. Scaffidi, N. L. Misso, C. Keerthisingam, R. J. McAnulty, G. J. Laurent, S. E. Mutsaers, P. J. Thompson, and D. A. Knight. 2003. Fibroblasts isolated from normal lungs and those with idiopathic pulmonary fibrosis differ in interleukin-6/gp130-mediated cell signaling and proliferation. *The American journal of pathology* 163: 345-354.

145. Moodley, Y. P., N. L. Misso, A. K. Scaffidi, M. Fogel-Petrovic, R. J. McAnulty, G. J. Laurent, P. J. Thompson, and D. A. Knight. 2003. Inverse effects of interleukin-6 on apoptosis of fibroblasts from pulmonary fibrosis and normal lungs. *American journal of respiratory cell and molecular biology* 29: 490-498.

146. Lo Re, S., D. Lison, and F. Huaux. 2013. CD4+ T lymphocytes in lung fibrosis: diverse subsets, diverse functions. *Journal of leukocyte biology* 93: 499-510.

147. Nuovo, G. J., J. S. Hagood, C. M. Magro, N. Chin, R. Kapil, L. Davis, C. B. Marsh, and V. A. Folcik. 2012. The distribution of immunomodulatory cells in the lungs of patients with idiopathic pulmonary fibrosis. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 25: 416-433.

148. Wilson, M. S., S. K. Madala, T. R. Ramalingam, B. R. Gochuico, I. O. Rosas, A. W. Cheever, and T. A. Wynn. 2010. Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent. *The Journal of experimental medicine* 207: 535-552.

149. Conte, E., M. Iemmolo, E. Fagone, E. Gili, M. Fruciano, T. Genovese, E. Esposito, S. Cuzzocrea, and C. Vancheri. 2014. Thymosin beta4 reduces IL-17-producing cells and IL-17 expression, and protects lungs from damage in bleomycin-treated mice. *Immunobiology*.

150. Tan, H. L., and M. Rosenthal. 2013. IL-17 in lung disease: friend or foe? *Thorax* 68: 788-790.

151. Galati, D., M. De Martino, A. Trotta, G. Rea, D. Bruzzese, G. Cicchitto, A. A. Stanziola, M. Napolitano, A. Sanduzzi, and M. Bocchino. 2014. Peripheral depletion of NK cells and imbalance of the Treg/Th17 axis in idiopathic pulmonary fibrosis patients. *Cytokine* 66: 119-126.

152. Ge, S., B. Hertel, N. Susnik, S. Rong, A. M. Dittrich, R. Schmitt, H. Haller, and S. von Vietinghoff. 2014. Interleukin 17 receptor A modulates monocyte subsets and macrophage generation in vivo. *PloS one* 9: e85461.

153. Silacci, P., J. M. Dayer, A. Desgeorges, R. Peter, C. Manueddu, and P. A. Guerne. 1998. Interleukin (IL)-6 and its soluble receptor induce TIMP-1 expression in synoviocytes and chondrocytes, and block IL-1-induced collagenolytic activity. *The Journal of biological chemistry* 273: 13625-13629.

154. Drucker, C., B. Rabe, A. Chalaris, E. Schulz, J. Scheller, and S. Rose-John. 2009. Interleukin-6 trans-signaling regulates glycogen consumption after D-galactosamine-induced liver damage. *J Interferon Cytokine Res* 29: 711-718.

155. Ray, S., X. Ju, H. Sun, C. C. Finnerty, D. N. Herndon, and A. R. Brasier. 2013. The IL-6 trans-signaling-STAT3 pathway mediates ECM and cellular proliferation in fibroblasts from hypertrophic scar. *J Invest Dermatol* 133: 1212-1220.

156. Campos, S. P., Y. Wang, A. Koj, and H. Baumann. 1993. Divergent transforming growth factor-beta effects on IL-6 regulation of acute phase plasma proteins in rat hepatoma cells. *Journal of immunology* 151: 7128-7137.

157. Yamamoto, T., T. Matsuda, A. Muraguchi, K. Miyazono, and M. Kawabata. 2001. Cross-talk between IL-6 and TGF-beta signaling in hepatoma cells. *FEBS letters* 492: 247-253.

158. Walia, B., L. Wang, D. Merlin, and S. V. Sitaraman. 2003. TGF-beta down-regulates IL-6 signaling in intestinal epithelial cells: critical role of SMAD-2. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 17: 2130-2132.

159. Yamada, D., S. Kobayashi, H. Wada, K. Kawamoto, S. Marubashi, H. Eguchi, H. Ishii, H. Nagano, Y. Doki, and M. Mori. 2013. Role of crosstalk between interleukin-6 and transforming growth factor-beta 1 in epithelial-mesenchymal transition and chemoresistance in biliary tract cancer. *European journal of cancer* 49: 1725-1740.

160. O'Reilly, S., M. Ciechomska, R. Cant, and J. M. van Laar. 2014. IL-6 trans signalling drives a STAT3 dependant pathway that leads to hyperactive TGF-beta signalling promoting SMAD3 activation and fibrosis via gremlin. *The Journal of biological chemistry*.

161. Elias, J. A., V. Lentz, and P. J. Cummings. 1991. Transforming growth factor-beta regulation of IL-6 production by unstimulated and IL-1-stimulated human fibroblasts. *Journal of immunology* 146: 3437-3443.

162. Gallelli, L., D. Falcone, G. Pelaia, T. Renda, R. Terracciano, N. Malara, A. Vatrella, A. Sanduzzi, B. D'Agostino, F. Rossi, C. Vancheri, R. Maselli, S. A. Marsico, and R. Savino. 2008. Interleukin-6 receptor superantagonist Sant7 inhibits TGF-beta-induced proliferation of human lung fibroblasts. *Cell proliferation* 41: 393-407.

163. Zhang, X. L., N. Topley, T. Ito, and A. Phillips. 2005. Interleukin-6 regulation of transforming growth factor (TGF)-beta receptor compartmentalization and turnover enhances TGF-beta1 signaling. *The Journal of biological chemistry* 280: 12239-12245.

164. Chen, R. H., M. C. Chang, Y. H. Su, Y. T. Tsai, and M. L. Kuo. 1999. Interleukin-6 inhibits transforming growth factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways. *The Journal of biological chemistry* 274: 23013-23019.

165. Khalil, N., R. N. O'Connor, H. W. Unruh, P. W. Warren, K. C. Flanders, A. Kemp, O. H. Bereznay, and A. H. Greenberg. 1991. Increased production and immunohistochemical localization of transforming growth factor-beta in idiopathic pulmonary fibrosis. *American journal of respiratory cell and molecular biology* 5: 155-162.

166. Khalil, N., O. Bereznay, M. Sporn, and A. H. Greenberg. 1989. Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation. *The Journal of experimental medicine* 170: 727-737.

167. Assoian, R. K., B. E. Fleurdelys, H. C. Stevenson, P. J. Miller, D. K. Madtes, E. W. Raines, R. Ross, and M. B. Sporn. 1987. Expression and secretion of type beta transforming growth factor by activated human macrophages. *Proceedings of the National Academy of Sciences of the United States of America* 84: 6020-6024.

168. Ma, F., Y. Li, L. Jia, Y. Han, J. Cheng, H. Li, Y. Qi, and J. Du. 2012. Macrophage-stimulated cardiac fibroblast production of IL-6 is essential for TGF beta/Smad activation and cardiac fibrosis induced by angiotensin II. *PloS one* 7: e35144.

169. Weng, T., H. Karmouty-Quintana, L. J. Garcia-Morales, J. G. Molina, M. Pedroza, R. R. Bunge, B. A. Bruckner, M. Loebe, H. Seethamraju, and M. R. Blackburn. 2013. Hypoxia-induced deoxycytidine kinase expression contributes to apoptosis in chronic lung disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 27: 2013-2026.

170. Chan, E. S., and B. N. Cronstein. 2010. Adenosine in fibrosis. *Modern rheumatology / the Japan Rheumatism Association* 20: 114-122.

171. Karmouty-Quintana, H., T. Weng, L. J. Garcia-Morales, N. Y. Chen, M. Pedroza, H. Zhong, J. G. Molina, R. Bunge, B. A. Bruckner, Y. Xia, R. A. Johnston, M. Loebe, D. Zeng, H. Seethamraju, L. Belardinelli, and M. R. Blackburn. 2013. Adenosine A2B receptor and hyaluronan modulate pulmonary hypertension associated with chronic obstructive pulmonary disease. *American journal of respiratory cell and molecular biology* 49: 1038-1047.

172. Anders, H. J., and M. Ryu. 2011. Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. *Kidney international* 80: 915-925.

173. Laumbach, R. J. 2010. Outdoor air pollutants and patient health. *American family physician* 81: 175-180.

174. Devlin, R. B., W. F. McDonnell, R. Mann, S. Becker, D. E. House, D. Schreinemachers, and H. S. Koren. 1991. Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *American journal of respiratory cell and molecular biology* 4: 72-81.

175. Koren, H. S., R. B. Devlin, S. Becker, R. Perez, and W. F. McDonnell. 1991. Time-dependent changes of markers associated with inflammation in the lungs of humans exposed to ambient levels of ozone. *Toxicologic pathology* 19: 406-411.

1. Raghu, G., H. R. Collard, J. J. Egan, F. J. Martinez, J. Behr, K. K. Brown, T. V. Colby, J. F. Cordier, K. R. Flaherty, J. A. Lasky, D. A. Lynch, J. H. Ryu, J. J. Swigris, A. U. Wells, J. Ancochea, D. Bouros, C. Carvalho, U. Costabel, M. Ebina, D. M. Hansell, T. Johkoh, D. S. Kim, T. E. King, Jr., Y. Kondoh, J. Myers, N. L. Muller, A. G. Nicholson, L. Richeldi, M. Selman, R. F. Dudden, B. S. Griss, S. L. Protzko, and H. J. Schunemann. 2011. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *American journal of respiratory and critical care medicine* 183: 788-824.

2. King, T. E., Jr., A. Pardo, and M. Selman. 2011. Idiopathic pulmonary fibrosis. *Lancet* 378: 1949-1961.

3. Raghu, G., D. Weycker, J. Edelsberg, W. Z. Bradford, and G. Oster. 2006. Incidence and prevalence of idiopathic pulmonary fibrosis. *American journal of respiratory and critical care medicine* 174: 810-816.

4. Kropski, J. A., W. E. Lawson, L. R. Young, and T. S. Blackwell. 2013. Genetic studies provide clues on the pathogenesis of idiopathic pulmonary fibrosis. *Disease models & mechanisms* 6: 9-17.

5.	Seibold, M. A., A. L. Wise, M. C. Speer, M. P. Steele, K. K. Brown, J. E. Loyd, T. E. Fingerlin, W. Zhang, G. Gudmundsson, S. D. Groshong, C. M. Evans, S. Garantziotis, K. B. Adler, B. F. Dickey, R. M. du Bois, I. V. Yang, A. Herron, D. Kervitsky, J. L. Talbert, C. Markin, J. Park, A. L. Crews, S. H. Slifer, S. Auerbach, M. G. Roy, J. Lin, C. E. Hennessy, M. I. Schwarz, and D. A. Schwartz. 2011. A common MUC5B promoter polymorphism and pulmonary fibrosis. *The New England journal of medicine* 364: 1503-1512.

6.	Fell, C. D. 2012. Idiopathic pulmonary fibrosis: phenotypes and comorbidities. *Clinics in chest medicine* 33: 51-57.

7.	Ley, B., H. R. Collard, and T. E. King, Jr. 2011. Clinical course and prediction of survival in idiopathic pulmonary fibrosis. *American journal of respiratory and critical care medicine* 183: 431-440.

8.	Selman, M., G. Carrillo, A. Estrada, M. Mejia, C. Becerril, J. Cisneros, M. Gaxiola, R. Perez-Padilla, C. Navarro, T. Richards, J. Dauber, T. E. King, Jr., A. Pardo, and N. Kaminski. 2007. Accelerated variant of idiopathic pulmonary fibrosis: clinical behavior and gene expression pattern. *PloS one* 2: e482.

9.	Boon, K., N. W. Bailey, J. Yang, M. P. Steel, S. Groshong, D. Kervitsky, K. K. Brown, M. I. Schwarz, and D. A. Schwartz. 2009. Molecular phenotypes distinguish patients with relatively stable from progressive idiopathic pulmonary fibrosis (IPF). *PloS one* 4: e5134.

10.	Barst, R. J., M. McGoon, A. Torbicki, O. Sitbon, M. J. Krowka, H. Olschewski, and S. Gaine. 2004. Diagnosis and differential assessment of pulmonary arterial hypertension. *Journal of the American College of Cardiology* 43: 40S-47S.

11.	Smith, J. S., D. Gorbett, J. Mueller, R. Perez, and C. J. Daniels. 2013. Pulmonary hypertension and idiopathic pulmonary fibrosis: a dastardly duo. *The American journal of the medical sciences* 346: 221-225.

12.	Lettieri, C. J., S. D. Nathan, S. D. Barnett, S. Ahmad, and A. F. Shorr. 2006. Prevalence and outcomes of pulmonary arterial hypertension in advanced idiopathic pulmonary fibrosis. *Chest* 129: 746-752.

13.	Nadrous, H. F., P. A. Pellikka, M. J. Krowka, K. L. Swanson, N. Chaowalit, P. A. Decker, and J. H. Ryu. 2005. Pulmonary hypertension in patients with idiopathic pulmonary fibrosis. *Chest* 128: 2393-2399.

14.	Shorr, A. F., J. L. Wainright, C. S. Cors, C. J. Lettieri, and S. D. Nathan. 2007. Pulmonary hypertension in patients with pulmonary fibrosis awaiting lung transplant. *The European respiratory journal* 30: 715-721.

15.	Nathan, S. D., O. A. Shlobin, S. Ahmad, J. Koch, S. D. Barnett, N. Ad, N. Burton, and K. Leslie. 2008. Serial development of pulmonary hypertension in patients with idiopathic pulmonary fibrosis. *Respiration; international review of thoracic diseases* 76: 288-294.

16.	Hamada, K., S. Nagai, S. Tanaka, T. Handa, M. Shigematsu, T. Nagao, M. Mishima, M. Kitaichi, and T. Izumi. 2007. Significance of pulmonary arterial pressure and diffusion capacity of the lung as prognosticator in patients with idiopathic pulmonary fibrosis. *Chest* 131: 650-656.

17.	Mejia, M., G. Carrillo, J. Rojas-Serrano, A. Estrada, T. Suarez, D. Alonso, E. Barrientos, M. Gaxiola, C. Navarro, and M. Selman. 2009. Idiopathic pulmonary fibrosis and emphysema: decreased survival associated with severe pulmonary arterial hypertension. *Chest* 136: 10-15.

18. Cottin, V., H. Nunes, P. Y. Brillet, P. Delaval, G. Devouassoux, I. Tillie-Leblond, D. Israel-Biet, I. Court-Fortune, D. Valeyre, J. F. Cordier, and P. Groupe d'Etude et de Recherche sur les Maladies Orphelines. 2005. Combined pulmonary fibrosis and emphysema: a distinct underrecognised entity. *The European respiratory journal* 26: 586-593.

19. Daniil, Z., A. Koutsokera, and K. Gourgoulianis. 2006. Combined pulmonary fibrosis and emphysema in patients exposed to agrochemical compounds. *The European respiratory journal* 27: 434.

20. Antoniou, K. M., G. A. Margaritopoulos, and N. M. Siafakas. 2013. Pharmacological treatment of idiopathic pulmonary fibrosis: from the past to the future. *European respiratory review : an official journal of the European Respiratory Society* 22: 281-291.

21. Woodcock, H. V., and T. M. Maher. 2014. The treatment of idiopathic pulmonary fibrosis. *F1000prime reports* 6: 16.

22. Potts, J., and D. Yogaratnam. 2013. Pirfenidone: a novel agent for the treatment of idiopathic pulmonary fibrosis. *The Annals of pharmacotherapy* 47: 361-367.

23. 2010. SEER Cancer Statistics Review, 1975-2007. K. C. Altekruse SF, Krapcho M, Neyman N, Aminou R, Waldron W, Ruhl J, Howlader N, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Cronin K, Chen HS, Feuer EJ, Stinchcomb DG, Edwards BK (eds). , ed. National Cancer Institute, Bethesda, MD.

24. Olson, A. L., J. J. Swigris, D. C. Lezotte, J. M. Norris, C. G. Wilson, and K. K. Brown. 2007. Mortality from pulmonary fibrosis increased in the United States from 1992 to 2003. *American journal of respiratory and critical care medicine* 176: 277-284.

25. Zhou, Y., D. J. Schneider, and M. R. Blackburn. 2009. Adenosine signaling and the regulation of chronic lung disease. *Pharmacology & therapeutics* 123: 105-116.

26. Fredholm, B. B. 2007. Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell death and differentiation* 14: 1315-1323.

27. Karmouty-Quintana, H., Y. Xia, and M. R. Blackburn. 2013. Adenosine signaling during acute and chronic disease states. *Journal of molecular medicine* 91: 173-181.

28. Chunn, J. L., J. G. Molina, T. Mi, Y. Xia, R. E. Kellems, and M. R. Blackburn. 2005. Adenosine-dependent pulmonary fibrosis in adenosine deaminase-deficient mice. *Journal of immunology* 175: 1937-1946.

29. Pedroza, M., D. J. Schneider, H. Karmouty-Quintana, J. Coote, S. Shaw, R. Corrigan, J. G. Molina, J. L. Alcorn, D. Galas, R. Gelinas, and M. R. Blackburn. 2011. Interleukin-6 contributes to inflammation and remodeling in a model of adenosine mediated lung injury. *PloS one* 6: e22667.

30. Schneider, D. J., J. C. Lindsay, Y. Zhou, J. G. Molina, and M. R. Blackburn. 2010. Adenosine and osteopontin contribute to the development of chronic obstructive pulmonary disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 24: 70-80.

31. Zhou, Y., J. N. Murthy, D. Zeng, L. Belardinelli, and M. R. Blackburn. 2010. Alterations in adenosine metabolism and signaling in patients with chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. *PloS one* 5: e9224.

32. Sun, Y., F. Wu, F. Sun, and P. Huang. 2008. Adenosine promotes IL-6 release in airway epithelia. *Journal of immunology* 180: 4173-4181.

33.   Zhou, Y., D. J. Schneider, E. Morschl, L. Song, M. Pedroza, H. Karmouty-Quintana, T. Le, C. X. Sun, and M. R. Blackburn. 2011. Distinct roles for the A2B adenosine receptor in acute and chronic stages of bleomycin-induced lung injury. *Journal of immunology* 186: 1097-1106.

34.   Zhong, H., L. Belardinelli, T. Maa, I. Feoktistov, I. Biaggioni, and D. Zeng. 2004. A(2B) adenosine receptors increase cytokine release by bronchial smooth muscle cells. *American journal of respiratory cell and molecular biology* 30: 118-125.

35.   Zhong, H., L. Belardinelli, T. Maa, and D. Zeng. 2005. Synergy between A2B adenosine receptors and hypoxia in activating human lung fibroblasts. *American journal of respiratory cell and molecular biology* 32: 2-8.

36.   Sehgal, P. B., and A. D. Sagar. 1980. Heterogeneity of poly(I) x poly(C)-induced human fibroblast interferon mRNA species. *Nature* 288: 95-97.

37.   Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwamura, K. Nakajima, K. Koyama, A. Iwamatsu, and et al. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* 324: 73-76.

38.   Chalaris, A., C. Garbers, B. Rabe, S. Rose-John, and J. Scheller. 2011. The soluble Interleukin 6 receptor: generation and role in inflammation and cancer. *Eur J Cell Biol* 90: 484-494.

39.   Lotz, M., F. Jirik, P. Kabouridis, C. Tsoukas, T. Hirano, T. Kishimoto, and D. A. Carson. 1988. B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes. *The Journal of experimental medicine* 167: 1253-1258.

40.   Ikebuchi, K., G. G. Wong, S. C. Clark, J. N. Ihle, Y. Hirai, and M. Ogawa. 1987. Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. *Proceedings of the National Academy of Sciences of the United States of America* 84: 9035-9039.

41.   Gauldie, J., C. Richards, D. Harnish, P. Lansdorp, and H. Baumann. 1987. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proceedings of the National Academy of Sciences of the United States of America* 84: 7251-7255.

42.   Kotake, S., K. Sato, K. J. Kim, N. Takahashi, N. Udagawa, I. Nakamura, A. Yamaguchi, T. Kishimoto, T. Suda, and S. Kashiwazaki. 1996. Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 11: 88-95.

43.   Scheller, J., A. Chalaris, D. Schmidt-Arras, and S. Rose-John. 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta* 1813: 878-888.

44.   Jones, S. A., J. Scheller, and S. Rose-John. 2011. Therapeutic strategies for the clinical blockade of IL-6/gp130 signaling. *The Journal of clinical investigation* 121: 3375-3383.

45.   Bauer, J., T. M. Bauer, T. Kalb, T. Taga, G. Lengyel, T. Hirano, T. Kishimoto, G. Acs, L. Mayer, and W. Gerok. 1989. Regulation of interleukin 6 receptor expression in human monocytes and monocyte-derived macrophages. Comparison with the

expression in human hepatocytes. *The Journal of experimental medicine* 170: 1537-1549.

46.   Taga, T., Y. Kawanishi, R. R. Hardy, T. Hirano, and T. Kishimoto. 1987. Receptors for B cell stimulatory factor 2. Quantitation, specificity, distribution, and regulation of their expression. *The Journal of experimental medicine* 166: 967-981.

47.   Rose-John, S., J. Scheller, G. Elson, and S. A. Jones. 2006. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. *Journal of leukocyte biology* 80: 227-236.

48.   Taga, T., M. Hibi, Y. Hirata, K. Yamasaki, K. Yasukawa, T. Matsuda, T. Hirano, and T. Kishimoto. 1989. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* 58: 573-581.

49.   Murakami, M., M. Hibi, N. Nakagawa, T. Nakagawa, K. Yasukawa, K. Yamanishi, T. Taga, and T. Kishimoto. 1993. IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. *Science* 260: 1808-1810.

50.   Stahl, N., T. G. Boulton, T. Farruggella, N. Y. Ip, S. Davis, B. A. Witthuhn, F. W. Quelle, O. Silvennoinen, G. Barbieri, S. Pellegrini, and et al. 1994. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. *Science* 263: 92-95.

51.   Zhong, Z., Z. Wen, and J. E. Darnell, Jr. 1994. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 264: 95-98.

52.   Heinrich, P. C., I. Behrmann, G. Muller-Newen, F. Schaper, and L. Graeve. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *The Biochemical journal* 334 (Pt 2): 297-314.

53.   Rose-John, S., and P. C. Heinrich. 1994. Soluble receptors for cytokines and growth factors: generation and biological function. *The Biochemical journal* 300 (Pt 2): 281-290.

54.   Horiuchi, S., Y. Koyanagi, Y. Zhou, H. Miyamoto, Y. Tanaka, M. Waki, A. Matsumoto, M. Yamamoto, and N. Yamamoto. 1994. Soluble interleukin-6 receptors released from T cell or granulocyte/macrophage cell lines and human peripheral blood mononuclear cells are generated through an alternative splicing mechanism. *Eur J Immunol* 24: 1945-1948.

55.   Lust, J. A., K. A. Donovan, M. P. Kline, P. R. Greipp, R. A. Kyle, and N. J. Maihle. 1992. Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor. *Cytokine* 4: 96-100.

56.   Muller-Newen, G., C. Kohne, R. Keul, U. Hemmann, W. Muller-Esterl, J. Wijdenes, J. P. Brakenhoff, M. H. Hart, and P. C. Heinrich. 1996. Purification and characterization of the soluble interleukin-6 receptor from human plasma and identification of an isoform generated through alternative splicing. *Eur J Biochem* 236: 837-842.

57.   Mullberg, J., H. Schooltink, T. Stoyan, M. Gunther, L. Graeve, G. Buse, A. Mackiewicz, P. C. Heinrich, and S. Rose-John. 1993. The soluble interleukin-6 receptor is generated by shedding. *Eur J Immunol* 23: 473-480.

58.   Mullberg, J., H. Schooltink, T. Stoyan, P. C. Heinrich, and S. Rose-John. 1992. Protein kinase C activity is rate limiting for shedding of the interleukin-6 receptor. *Biochemical and biophysical research communications* 189: 794-800.

59.   Chalaris, A., B. Rabe, K. Paliga, H. Lange, T. Laskay, C. A. Fielding, S. A. Jones, S. Rose-John, and J. Scheller. 2007. Apoptosis is a natural stimulus of IL6R shedding and contributes to the proinflammatory trans-signaling function of neutrophils. *Blood* 110: 1748-1755.

60.   Jones, S. A., S. Horiuchi, D. Novick, N. Yamamoto, and G. M. Fuller. 1998. Shedding of the soluble IL-6 receptor is triggered by Ca2+ mobilization, while basal release is predominantly the product of differential mRNA splicing in THP-1 cells. *Eur J Immunol* 28: 3514-3522.

61.   Matthews, V., B. Schuster, S. Schutze, I. Bussmeyer, A. Ludwig, C. Hundhausen, T. Sadowski, P. Saftig, D. Hartmann, K. J. Kallen, and S. Rose-John. 2003. Cellular cholesterol depletion triggers shedding of the human interleukin-6 receptor by ADAM10 and ADAM17 (TACE). *The Journal of biological chemistry* 278: 38829-38839.

62.   Fenton, J. I., S. D. Hursting, S. N. Perkins, and N. G. Hord. 2006. Interleukin-6 production induced by leptin treatment promotes cell proliferation in an Apc (Min/+) colon epithelial cell line. *Carcinogenesis* 27: 1507-1515.

63.   Briso, E. M., O. Dienz, and M. Rincon. 2008. Cutting edge: soluble IL-6R is produced by IL-6R ectodomain shedding in activated CD4 T cells. *Journal of immunology* 180: 7102-7106.

64.   Gomez, M. I., S. H. Sokol, A. B. Muir, G. Soong, J. Bastien, and A. S. Prince. 2005. Bacterial induction of TNF-alpha converting enzyme expression and IL-6 receptor alpha shedding regulates airway inflammatory signaling. *Journal of immunology* 175: 1930-1936.

65.   Scheller, J., A. Chalaris, C. Garbers, and S. Rose-John. 2011. ADAM17: a molecular switch to control inflammation and tissue regeneration. *Trends Immunol* 32: 380-387.

66.   Booth, B. W., T. Sandifer, E. L. Martin, and L. D. Martin. 2007. IL-13-induced proliferation of airway epithelial cells: mediation by intracellular growth factor mobilization and ADAM17. *Respir Res* 8: 51.

67.   Cesaro, A., A. Abakar-Mahamat, P. Brest, S. Lassalle, E. Selva, J. Filippi, X. Hebuterne, J. P. Hugot, A. Doglio, F. Galland, P. Naquet, V. Vouret-Craviari, B. Mograbi, and P. M. Hofman. 2009. Differential expression and regulation of ADAM17 and TIMP3 in acute inflamed intestinal epithelia. *Am J Physiol Gastrointest Liver Physiol* 296: G1332-1343.

68.   Charbonneau, M., K. Harper, F. Grondin, M. Pelmus, P. P. McDonald, and C. M. Dubois. 2007. Hypoxia-inducible factor mediates hypoxic and tumor necrosis factor alpha-induced increases in tumor necrosis factor-alpha converting enzyme/ADAM17 expression by synovial cells. *The Journal of biological chemistry* 282: 33714-33724.

69.   Franchimont, N., C. Lambert, P. Huynen, C. Ribbens, B. Relic, A. Chariot, V. Bours, J. Piette, M. P. Merville, and M. Malaise. 2005. Interleukin-6 receptor shedding is enhanced by interleukin-1beta and tumor necrosis factor alpha and is partially mediated by tumor necrosis factor alpha-converting enzyme in osteoblast-like cells. *Arthritis Rheum* 52: 84-93.

70.   Peters, M., K. H. Meyer zum Buschenfelde, and S. Rose-John. 1996. The function of the soluble IL-6 receptor in vivo. *Immunology letters* 54: 177-184.

71. Neurath, M. F., and S. Finotto. 2011. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine & growth factor reviews* 22: 83-89.
72. Melendez, G. C., J. L. McLarty, S. P. Levick, Y. Du, J. S. Janicki, and G. L. Brower. 2010. Interleukin 6 mediates myocardial fibrosis, concentric hypertrophy, and diastolic dysfunction in rats. *Hypertension* 56: 225-231.
73. Cronstein, B. N. 2007. Interleukin-6--a key mediator of systemic and local symptoms in rheumatoid arthritis. *Bull NYU Hosp Jt Dis* 65 Suppl 1: S11-15.
74. Finotto, S., T. Eigenbrod, R. Karwot, I. Boross, A. Doganci, H. Ito, N. Nishimoto, K. Yoshizaki, T. Kishimoto, S. Rose-John, P. R. Galle, and M. F. Neurath. 2007. Local blockade of IL-6R signaling induces lung CD4+ T cell apoptosis in a murine model of asthma via regulatory T cells. *International immunology* 19: 685-693.
75. Tanaka, T., M. Narazaki, and T. Kishimoto. 2012. Therapeutic targeting of the interleukin-6 receptor. *Annu Rev Pharmacol Toxicol* 52: 199-219.
76. Atreya, R., J. Mudter, S. Finotto, J. Mullberg, T. Jostock, S. Wirtz, M. Schutz, B. Bartsch, M. Holtmann, C. Becker, D. Strand, J. Czaja, J. F. Schlaak, H. A. Lehr, F. Autschbach, G. Schurmann, N. Nishimoto, K. Yoshizaki, H. Ito, T. Kishimoto, P. R. Galle, S. Rose-John, and M. F. Neurath. 2000. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med* 6: 583-588.
77. Barkhausen, T., T. Tschernig, P. Rosenstiel, M. van Griensven, R. P. Vonberg, M. Dorsch, A. Mueller-Heine, A. Chalaris, J. Scheller, S. Rose-John, D. Seegert, C. Krettek, and G. H. Waetzig. 2011. Selective blockade of interleukin-6 trans-signaling improves survival in a murine polymicrobial sepsis model. *Critical care medicine* 39: 1407-1413.
78. Matsumoto, S., T. Hara, K. Mitsuyama, M. Yamamoto, O. Tsuruta, M. Sata, J. Scheller, S. Rose-John, S. Kado, and T. Takada. 2010. Essential roles of IL-6 trans-signaling in colonic epithelial cells, induced by the IL-6/soluble-IL-6 receptor derived from lamina propria macrophages, on the development of colitis-associated premalignant cancer in a murine model. *Journal of immunology* 184: 1543-1551.
79. Nowell, M. A., P. J. Richards, S. Horiuchi, N. Yamamoto, S. Rose-John, N. Topley, A. S. Williams, and S. A. Jones. 2003. Soluble IL-6 receptor governs IL-6 activity in experimental arthritis: blockade of arthritis severity by soluble glycoprotein 130. *Journal of immunology* 171: 3202-3209.
80. Nowell, M. A., A. S. Williams, S. A. Carty, J. Scheller, A. J. Hayes, G. W. Jones, P. J. Richards, S. Slinn, M. Ernst, B. J. Jenkins, N. Topley, S. Rose-John, and S. A. Jones. 2009. Therapeutic targeting of IL-6 trans signaling counteracts STAT3 control of experimental inflammatory arthritis. *Journal of immunology* 182: 613-622.
81. Migita, K., S. Abiru, Y. Maeda, M. Daikoku, K. Ohata, M. Nakamura, A. Komori, K. Yano, H. Yatsuhashi, K. Eguchi, and H. Ishibashi. 2006. Serum levels of interleukin-6 and its soluble receptors in patients with hepatitis C virus infection. *Hum Immunol* 67: 27-32.
82. Dai, Y., W. Zhang, J. Wen, Y. Zhang, R. E. Kellems, and Y. Xia. 2011. A2B adenosine receptor-mediated induction of IL-6 promotes CKD. *Journal of the American Society of Nephrology : JASN* 22: 890-901.

83.   Sato, S., M. Hasegawa, and K. Takehara. 2001. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. *J Dermatol Sci* 27: 140-146.

84.   Hasegawa, M., S. Sato, H. Ihn, and K. Takehara. 1999. Enhanced production of interleukin-6 (IL-6), oncostatin M and soluble IL-6 receptor by cultured peripheral blood mononuclear cells from patients with systemic sclerosis. *Rheumatology* 38: 612-617.

85.   Ammit, A. J., L. M. Moir, B. G. Oliver, J. M. Hughes, H. Alkhouri, Q. Ge, J. K. Burgess, J. L. Black, and M. Roth. 2007. Effect of IL-6 trans-signaling on the pro-remodeling phenotype of airway smooth muscle. *American journal of physiology. Lung cellular and molecular physiology* 292: L199-206.

86.   Zhang, H., P. Neuhofer, L. Song, B. Rabe, M. Lesina, M. U. Kurkowski, M. Treiber, T. Wartmann, S. Regner, H. Thorlacius, D. Saur, G. Weirich, A. Yoshimura, W. Halangk, J. P. Mizgerd, R. M. Schmid, S. Rose-John, and H. Algul. 2013. IL-6 trans-signaling promotes pancreatitis-associated lung injury and lethality. *The Journal of clinical investigation* 123: 1019-1031.

87.   O'Donoghue, R. J., D. A. Knight, C. D. Richards, C. M. Prele, H. L. Lau, A. G. Jarnicki, J. Jones, S. Bozinovski, R. Vlahos, S. Thiem, B. S. McKenzie, B. Wang, P. Stumbles, G. J. Laurent, R. J. McAnulty, S. Rose-John, H. J. Zhu, G. P. Anderson, M. R. Ernst, and S. E. Mutsaers. 2012. Genetic partitioning of interleukin-6 signalling in mice dissociates Stat3 from Smad3-mediated lung fibrosis. *EMBO Mol Med* 4: 939-951.

88.   Mouratis, M. A., and V. Aidinis. 2011. Modeling pulmonary fibrosis with bleomycin. *Current opinion in pulmonary medicine* 17: 355-361.

89.   Degryse, A. L., H. Tanjore, X. C. Xu, V. V. Polosukhin, B. R. Jones, F. B. McMahon, L. A. Gleaves, T. S. Blackwell, and W. E. Lawson. 2010. Repetitive intratracheal bleomycin models several features of idiopathic pulmonary fibrosis. *American journal of physiology. Lung cellular and molecular physiology* 299: L442-452.

90.   Swiderski, R. E., J. E. Dencoff, C. S. Floerchinger, S. D. Shapiro, and G. W. Hunninghake. 1998. Differential expression of extracellular matrix remodeling genes in a murine model of bleomycin-induced pulmonary fibrosis. *The American journal of pathology* 152: 821-828.

91.   Karmouty-Quintana, H., H. Zhong, L. Acero, T. Weng, E. Melicoff, J. D. West, A. Hemnes, A. Grenz, H. K. Eltzschig, T. S. Blackwell, Y. Xia, R. A. Johnston, D. Zeng, L. Belardinelli, and M. R. Blackburn. 2012. The A2B adenosine receptor modulates pulmonary hypertension associated with interstitial lung disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 26: 2546-2557.

92.   Baran, C. P., J. M. Opalek, S. McMaken, C. A. Newland, J. M. O'Brien, Jr., M. G. Hunter, B. D. Bringardner, M. M. Monick, D. R. Brigstock, P. C. Stromberg, G. W. Hunninghake, and C. B. Marsh. 2007. Important roles for macrophage colony-stimulating factor, CC chemokine ligand 2, and mononuclear phagocytes in the pathogenesis of pulmonary fibrosis. *American journal of respiratory and critical care medicine* 176: 78-89.

93.   Chua, F., J. Gauldie, and G. J. Laurent. 2005. Pulmonary fibrosis: searching for model answers. *American journal of respiratory cell and molecular biology* 33: 9-13.

94. Blackburn, M. R., S. K. Datta, and R. E. Kellems. 1998. Adenosine deaminase-deficient mice generated using a two-stage genetic engineering strategy exhibit a combined immunodeficiency. *The Journal of biological chemistry* 273: 5093-5100.

95. Blackburn, M. R., J. B. Volmer, J. L. Thrasher, H. Zhong, J. R. Crosby, J. J. Lee, and R. E. Kellems. 2000. Metabolic consequences of adenosine deaminase deficiency in mice are associated with defects in alveogenesis, pulmonary inflammation, and airway obstruction. *The Journal of experimental medicine* 192: 159-170.

96. Blackburn, M. R., M. Aldrich, J. B. Volmer, W. Chen, H. Zhong, S. Kelly, M. S. Hershfield, S. K. Datta, and R. E. Kellems. 2000. The use of enzyme therapy to regulate the metabolic and phenotypic consequences of adenosine deaminase deficiency in mice. Differential impact on pulmonary and immunologic abnormalities. *The Journal of biological chemistry* 275: 32114-32121.

97. Sun, C. X., H. Zhong, A. Mohsenin, E. Morschl, J. L. Chunn, J. G. Molina, L. Belardinelli, D. Zeng, and M. R. Blackburn. 2006. Role of A2B adenosine receptor signaling in adenosine-dependent pulmonary inflammation and injury. *The Journal of clinical investigation* 116: 2173-2182.

98. Blackburn, M. R., C. G. Lee, H. W. Young, Z. Zhu, J. L. Chunn, M. J. Kang, S. K. Banerjee, and J. A. Elias. 2003. Adenosine mediates IL-13-induced inflammation and remodeling in the lung and interacts in an IL-13-adenosine amplification pathway. *The Journal of clinical investigation* 112: 332-344.

99. Sun, C. X., H. W. Young, J. G. Molina, J. B. Volmer, J. Schnermann, and M. R. Blackburn. 2005. A protective role for the A1 adenosine receptor in adenosine-dependent pulmonary injury. *The Journal of clinical investigation* 115: 35-43.

100. Mohsenin, A., T. Mi, Y. Xia, R. E. Kellems, J. F. Chen, and M. R. Blackburn. 2007. Genetic removal of the A2A adenosine receptor enhances pulmonary inflammation, mucin production, and angiogenesis in adenosine deaminase-deficient mice. *American journal of physiology. Lung cellular and molecular physiology* 293: L753-761.

101. Zhou, Y., A. Mohsenin, E. Morschl, H. W. Young, J. G. Molina, W. Ma, C. X. Sun, H. Martinez-Valdez, and M. R. Blackburn. 2009. Enhanced airway inflammation and remodeling in adenosine deaminase-deficient mice lacking the A2B adenosine receptor. *Journal of immunology* 182: 8037-8046.

102. Young, H. W., J. G. Molina, D. Dimina, H. Zhong, M. Jacobson, L. N. Chan, T. S. Chan, J. J. Lee, and M. R. Blackburn. 2004. A3 adenosine receptor signaling contributes to airway inflammation and mucus production in adenosine deaminase-deficient mice. *Journal of immunology* 173: 1380-1389.

103. Lo, C. W., M. W. Chen, M. Hsiao, S. Wang, C. A. Chen, S. M. Hsiao, J. S. Chang, T. C. Lai, S. Rose-John, M. L. Kuo, and L. H. Wei. 2011. IL-6 trans-signaling in formation and progression of malignant ascites in ovarian cancer. *Cancer research* 71: 424-434.

104. Wynn, T. A. 2011. Integrating mechanisms of pulmonary fibrosis. *The Journal of experimental medicine* 208: 1339-1350.

105. Muller-Newen, G., A. Kuster, U. Hemmann, R. Keul, U. Horsten, A. Martens, L. Graeve, J. Wijdenes, and P. C. Heinrich. 1998. Soluble IL-6 receptor potentiates the antagonistic activity of soluble gp130 on IL-6 responses. *Journal of immunology* 161: 6347-6355.

106. Jostock, T., J. Mullberg, S. Ozbek, R. Atreya, G. Blinn, N. Voltz, M. Fischer, M. F. Neurath, and S. Rose-John. 2001. Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses. *Eur J Biochem* 268: 160-167.

107. Lissilaa, R., V. Buatois, G. Magistrelli, A. S. Williams, G. W. Jones, S. Herren, L. Shang, P. Malinge, F. Guilhot, L. Chatel, E. Hatterer, S. A. Jones, M. H. Kosco-Vilbois, and W. G. Ferlin. 2010. Although IL-6 trans-signaling is sufficient to drive local immune responses, classical IL-6 signaling is obligate for the induction of T cell-mediated autoimmunity. *Journal of immunology* 185: 5512-5521.

108. Doganci, A., T. Eigenbrod, N. Krug, G. T. De Sanctis, M. Hausding, V. J. Erpenbeck, B. Haddad el, H. A. Lehr, E. Schmitt, T. Bopp, K. J. Kallen, U. Herz, S. Schmitt, C. Luft, O. Hecht, J. M. Hohlfeld, H. Ito, N. Nishimoto, K. Yoshizaki, T. Kishimoto, S. Rose-John, H. Renz, M. F. Neurath, P. R. Galle, and S. Finotto. 2005. The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. *The Journal of clinical investigation* 115: 313-325.

109. Hoge, J., I. Yan, N. Janner, V. Schumacher, A. Chalaris, O. M. Steinmetz, D. R. Engel, J. Scheller, S. Rose-John, and H. W. Mittrucker. 2013. IL-6 controls the innate immune response against Listeria monocytogenes via classical IL-6 signaling. *Journal of immunology* 190: 703-711.

110. Hasegawa, M., S. Sato, M. Fujimoto, H. Ihn, K. Kikuchi, and K. Takehara. 1998. Serum levels of interleukin 6 (IL-6), oncostatin M, soluble IL-6 receptor, and soluble gp130 in patients with systemic sclerosis. *J Rheumatol* 25: 308-313.

111. Adamali, H. I., and T. M. Maher. 2012. Current and novel drug therapies for idiopathic pulmonary fibrosis. *Drug design, development and therapy* 6: 261-272.

112. Loomis-King, H., K. R. Flaherty, and B. B. Moore. 2013. Pathogenesis, current treatments and future directions for idiopathic pulmonary fibrosis. *Current opinion in pharmacology* 13: 377-385.

113. Murray, L. A., R. Rosada, A. P. Moreira, A. Joshi, M. S. Kramer, D. P. Hesson, R. L. Argentieri, S. Mathai, M. Gulati, E. L. Herzog, and C. M. Hogaboam. 2010. Serum amyloid P therapeutically attenuates murine bleomycin-induced pulmonary fibrosis via its effects on macrophages. *PloS one* 5: e9683.

114. Trujillo, G., E. C. O'Connor, S. L. Kunkel, and C. M. Hogaboam. 2008. A novel mechanism for CCR4 in the regulation of macrophage activation in bleomycin-induced pulmonary fibrosis. *The American journal of pathology* 172: 1209-1221.

115. Pechkovsky, D. V., A. Prasse, F. Kollert, K. M. Engel, J. Dentler, W. Luttmann, K. Friedrich, J. Muller-Quernheim, and G. Zissel. 2010. Alternatively activated alveolar macrophages in pulmonary fibrosis-mediator production and intracellular signal transduction. *Clin Immunol* 137: 89-101.

116. Murray, L. A., Q. Chen, M. S. Kramer, D. P. Hesson, R. L. Argentieri, X. Peng, M. Gulati, R. J. Homer, T. Russell, N. van Rooijen, J. A. Elias, C. M. Hogaboam, and E. L. Herzog. 2011. TGF-beta driven lung fibrosis is macrophage dependent and blocked by Serum amyloid P. *Int J Biochem Cell Biol* 43: 154-162.

117. Collins, S. L., Y. Chan-Li, R. W. Hallowell, J. D. Powell, and M. R. Horton. 2012. Pulmonary vaccination as a novel treatment for lung fibrosis. *PloS one* 7: e31299.

118. Camelo, A., R. Dunmore, M. A. Sleeman, and D. L. Clarke. 2014. The epithelium in idiopathic pulmonary fibrosis: breaking the barrier. *Frontiers in pharmacology* 4: 173.

119. Habiel, D. M., and C. Hogaboam. 2014. Heterogeneity in fibroblast proliferation and survival in idiopathic pulmonary fibrosis. *Frontiers in pharmacology* 5: 2.

120. Modolell, M., I. M. Corraliza, F. Link, G. Soler, and K. Eichmann. 1995. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur J Immunol* 25: 1101-1104.

121. Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *The Journal of experimental medicine* 176: 287-292.

122. Mosser, D. M., and X. Zhang. 2008. Activation of murine macrophages. *Curr Protoc Immunol* Chapter 14: Unit 14 12.

123. Wynn, T. A. 2003. IL-13 effector functions. *Annu Rev Immunol* 21: 425-456.

124. Althoff, K., P. Reddy, N. Voltz, S. Rose-John, and J. Mullberg. 2000. Shedding of interleukin-6 receptor and tumor necrosis factor alpha. Contribution of the stalk sequence to the cleavage pattern of transmembrane proteins. *Eur J Biochem* 267: 2624-2631.

125. Lee, Y. J., S. H. Lee, Y. S. Youn, J. Y. Choi, K. S. Song, M. S. Cho, and J. L. Kang. 2012. Preventing cleavage of Mer promotes efferocytosis and suppresses acute lung injury in bleomycin treated mice. *Toxicology and applied pharmacology* 263: 61-72.

126. Garbers, C., N. Janner, A. Chalaris, M. L. Moss, D. M. Floss, D. Meyer, F. Koch-Nolte, S. Rose-John, and J. Scheller. 2011. Species specificity of ADAM10 and ADAM17 proteins in interleukin-6 (IL-6) trans-signaling and novel role of ADAM10 in inducible IL-6 receptor shedding. *The Journal of biological chemistry* 286: 14804-14811.

127. Narazaki, M., K. Yasukawa, T. Saito, Y. Ohsugi, H. Fukui, Y. Koishihara, G. D. Yancopoulos, T. Taga, and T. Kishimoto. 1993. Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a potential to inhibit signals through membrane-anchored gp130. *Blood* 82: 1120-1126.

128. Zhang, J. G., Y. Zhang, C. M. Owczarek, L. D. Ward, R. L. Moritz, R. J. Simpson, K. Yasukawa, and N. A. Nicola. 1998. Identification and characterization of two distinct truncated forms of gp130 and a soluble form of leukemia inhibitory factor receptor alpha-chain in normal human urine and plasma. *The Journal of biological chemistry* 273: 10798-10805.

129. Rose-John, S. 2012. IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6. *International journal of biological sciences* 8: 1237-1247.

130. Ashcroft, T., J. M. Simpson, and V. Timbrell. 1988. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *Journal of clinical pathology* 41: 467-470.

131. Wynn, T. A. 2008. Cellular and molecular mechanisms of fibrosis. *The Journal of pathology* 214: 199-210.

132. Rennard, S. I., and R. G. Crystal. 1982. Fibronectin in human bronchopulmonary lavage fluid. Elevation in patients with interstitial lung disease. *The Journal of clinical investigation* 69: 113-122.

133. Crosby, L. M., and C. M. Waters. 2010. Epithelial repair mechanisms in the lung. *American journal of physiology. Lung cellular and molecular physiology* 298: L715-731.

134. White, E. S., F. E. Baralle, and A. F. Muro. 2008. New insights into form and function of fibronectin splice variants. *The Journal of pathology* 216: 1-14.

135. White, E. S., and A. F. Muro. 2011. Fibronectin splice variants: understanding their multiple roles in health and disease using engineered mouse models. *IUBMB Life* 63: 538-546.

136. Seeger, W., Y. Adir, J. A. Barbera, H. Champion, J. G. Coghlan, V. Cottin, T. De Marco, N. Galie, S. Ghio, S. Gibbs, F. J. Martinez, M. J. Semigran, G. Simonneau, A. U. Wells, and J. L. Vachiery. 2013. Pulmonary hypertension in chronic lung diseases. *Journal of the American College of Cardiology* 62: D109-116.

137. Knight, D., S. E. Mutsaers, and C. M. Prele. 2011. STAT3 in tissue fibrosis: is there a role in the lung? *Pulm Pharmacol Ther* 24: 193-198.

138. Prele, C. M., E. Yao, R. J. O'Donoghue, S. E. Mutsaers, and D. A. Knight. 2012. STAT3: a central mediator of pulmonary fibrosis? *Proc Am Thorac Soc* 9: 177-182.

139. Pechkovsky, D. V., C. M. Prele, J. Wong, C. M. Hogaboam, R. J. McAnulty, G. J. Laurent, S. S. Zhang, M. Selman, S. E. Mutsaers, and D. A. Knight. 2012. STAT3-mediated signaling dysregulates lung fibroblast-myofibroblast activation and differentiation in UIP/IPF. *The American journal of pathology* 180: 1398-1412.

140. Goodwin, A., and G. Jenkins. 2009. Role of integrin-mediated TGFbeta activation in the pathogenesis of pulmonary fibrosis. *Biochemical Society transactions* 37: 849-854.

141. Hetzel, M., M. Bachem, D. Anders, G. Trischler, and M. Faehling. 2005. Different effects of growth factors on proliferation and matrix production of normal and fibrotic human lung fibroblasts. *Lung* 183: 225-237.

142. Muro, A. F., F. A. Moretti, B. B. Moore, M. Yan, R. G. Atrasz, C. A. Wilke, K. R. Flaherty, F. J. Martinez, J. L. Tsui, D. Sheppard, F. E. Baralle, G. B. Toews, and E. S. White. 2008. An essential role for fibronectin extra type III domain A in pulmonary fibrosis. *American journal of respiratory and critical care medicine* 177: 638-645.

143. Wolters, P. J., H. R. Collard, and K. D. Jones. 2014. Pathogenesis of idiopathic pulmonary fibrosis. *Annual review of pathology* 9: 157-179.

144. Moodley, Y. P., A. K. Scaffidi, N. L. Misso, C. Keerthisingam, R. J. McAnulty, G. J. Laurent, S. E. Mutsaers, P. J. Thompson, and D. A. Knight. 2003. Fibroblasts isolated from normal lungs and those with idiopathic pulmonary fibrosis differ in interleukin-6/gp130-mediated cell signaling and proliferation. *The American journal of pathology* 163: 345-354.

145. Moodley, Y. P., N. L. Misso, A. K. Scaffidi, M. Fogel-Petrovic, R. J. McAnulty, G. J. Laurent, P. J. Thompson, and D. A. Knight. 2003. Inverse effects of interleukin-6 on apoptosis of fibroblasts from pulmonary fibrosis and normal lungs. *American journal of respiratory cell and molecular biology* 29: 490-498.

146. Lo Re, S., D. Lison, and F. Huaux. 2013. CD4+ T lymphocytes in lung fibrosis: diverse subsets, diverse functions. *Journal of leukocyte biology* 93: 499-510.

147. Nuovo, G. J., J. S. Hagood, C. M. Magro, N. Chin, R. Kapil, L. Davis, C. B. Marsh, and V. A. Folcik. 2012. The distribution of immunomodulatory cells in the lungs of patients

with idiopathic pulmonary fibrosis. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 25: 416-433.

148. Wilson, M. S., S. K. Madala, T. R. Ramalingam, B. R. Gochuico, I. O. Rosas, A. W. Cheever, and T. A. Wynn. 2010. Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent. *The Journal of experimental medicine* 207: 535-552.

149. Conte, E., M. Iemmolo, E. Fagone, E. Gili, M. Fruciano, T. Genovese, E. Esposito, S. Cuzzocrea, and C. Vancheri. 2014. Thymosin beta4 reduces IL-17-producing cells and IL-17 expression, and protects lungs from damage in bleomycin-treated mice. *Immunobiology*.

150. Tan, H. L., and M. Rosenthal. 2013. IL-17 in lung disease: friend or foe? *Thorax* 68: 788-790.

151. Galati, D., M. De Martino, A. Trotta, G. Rea, D. Bruzzese, G. Cicchitto, A. A. Stanziola, M. Napolitano, A. Sanduzzi, and M. Bocchino. 2014. Peripheral depletion of NK cells and imbalance of the Treg/Th17 axis in idiopathic pulmonary fibrosis patients. *Cytokine* 66: 119-126.

152. Ge, S., B. Hertel, N. Susnik, S. Rong, A. M. Dittrich, R. Schmitt, H. Haller, and S. von Vietinghoff. 2014. Interleukin 17 receptor A modulates monocyte subsets and macrophage generation in vivo. *PloS one* 9: e85461.

153. Silacci, P., J. M. Dayer, A. Desgeorges, R. Peter, C. Manueddu, and P. A. Guerne. 1998. Interleukin (IL)-6 and its soluble receptor induce TIMP-1 expression in synoviocytes and chondrocytes, and block IL-1-induced collagenolytic activity. *The Journal of biological chemistry* 273: 13625-13629.

154. Drucker, C., B. Rabe, A. Chalaris, E. Schulz, J. Scheller, and S. Rose-John. 2009. Interleukin-6 trans-signaling regulates glycogen consumption after D-galactosamine-induced liver damage. *J Interferon Cytokine Res* 29: 711-718.

155. Ray, S., X. Ju, H. Sun, C. C. Finnerty, D. N. Herndon, and A. R. Brasier. 2013. The IL-6 trans-signaling-STAT3 pathway mediates ECM and cellular proliferation in fibroblasts from hypertrophic scar. *J Invest Dermatol* 133: 1212-1220.

156. Campos, S. P., Y. Wang, A. Koj, and H. Baumann. 1993. Divergent transforming growth factor-beta effects on IL-6 regulation of acute phase plasma proteins in rat hepatoma cells. *Journal of immunology* 151: 7128-7137.

157. Yamamoto, T., T. Matsuda, A. Muraguchi, K. Miyazono, and M. Kawabata. 2001. Cross-talk between IL-6 and TGF-beta signaling in hepatoma cells. *FEBS letters* 492: 247-253.

158. Walia, B., L. Wang, D. Merlin, and S. V. Sitaraman. 2003. TGF-beta down-regulates IL-6 signaling in intestinal epithelial cells: critical role of SMAD-2. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 17: 2130-2132.

159. Yamada, D., S. Kobayashi, H. Wada, K. Kawamoto, S. Marubashi, H. Eguchi, H. Ishii, H. Nagano, Y. Doki, and M. Mori. 2013. Role of crosstalk between interleukin-6 and transforming growth factor-beta 1 in epithelial-mesenchymal transition and chemoresistance in biliary tract cancer. *European journal of cancer* 49: 1725-1740.

160. O'Reilly, S., M. Ciechomska, R. Cant, and J. M. van Laar. 2014. IL-6 trans signalling drives a STAT3 dependant pathway that leads to hyperactive TGF-beta signalling promoting SMAD3 activation and fibrosis via gremlin. *The Journal of biological chemistry*.

161. Elias, J. A., V. Lentz, and P. J. Cummings. 1991. Transforming growth factor-beta regulation of IL-6 production by unstimulated and IL-1-stimulated human fibroblasts. *Journal of immunology* 146: 3437-3443.
162. Gallelli, L., D. Falcone, G. Pelaia, T. Renda, R. Terracciano, N. Malara, A. Vatrella, A. Sanduzzi, B. D'Agostino, F. Rossi, C. Vancheri, R. Maselli, S. A. Marsico, and R. Savino. 2008. Interleukin-6 receptor superantagonist Sant7 inhibits TGF-beta-induced proliferation of human lung fibroblasts. *Cell proliferation* 41: 393-407.
163. Zhang, X. L., N. Topley, T. Ito, and A. Phillips. 2005. Interleukin-6 regulation of transforming growth factor (TGF)-beta receptor compartmentalization and turnover enhances TGF-beta1 signaling. *The Journal of biological chemistry* 280: 12239-12245.
164. Chen, R. H., M. C. Chang, Y. H. Su, Y. T. Tsai, and M. L. Kuo. 1999. Interleukin-6 inhibits transforming growth factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways. *The Journal of biological chemistry* 274: 23013-23019.
165. Khalil, N., R. N. O'Connor, H. W. Unruh, P. W. Warren, K. C. Flanders, A. Kemp, O. H. Bereznay, and A. H. Greenberg. 1991. Increased production and immunohistochemical localization of transforming growth factor-beta in idiopathic pulmonary fibrosis. *American journal of respiratory cell and molecular biology* 5: 155-162.
166. Khalil, N., O. Bereznay, M. Sporn, and A. H. Greenberg. 1989. Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation. *The Journal of experimental medicine* 170: 727-737.
167. Assoian, R. K., B. E. Fleurdelys, H. C. Stevenson, P. J. Miller, D. K. Madtes, E. W. Raines, R. Ross, and M. B. Sporn. 1987. Expression and secretion of type beta transforming growth factor by activated human macrophages. *Proceedings of the National Academy of Sciences of the United States of America* 84: 6020-6024.
168. Ma, F., Y. Li, L. Jia, Y. Han, J. Cheng, H. Li, Y. Qi, and J. Du. 2012. Macrophage-stimulated cardiac fibroblast production of IL-6 is essential for TGF beta/Smad activation and cardiac fibrosis induced by angiotensin II. *PloS one* 7: e35144.
169. Weng, T., H. Karmouty-Quintana, L. J. Garcia-Morales, J. G. Molina, M. Pedroza, R. R. Bunge, B. A. Bruckner, M. Loebe, H. Seethamraju, and M. R. Blackburn. 2013. Hypoxia-induced deoxycytidine kinase expression contributes to apoptosis in chronic lung disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 27: 2013-2026.
170. Chan, E. S., and B. N. Cronstein. 2010. Adenosine in fibrosis. *Modern rheumatology / the Japan Rheumatism Association* 20: 114-122.
171. Karmouty-Quintana, H., T. Weng, L. J. Garcia-Morales, N. Y. Chen, M. Pedroza, H. Zhong, J. G. Molina, R. Bunge, B. A. Bruckner, Y. Xia, R. A. Johnston, M. Loebe, D. Zeng, H. Seethamraju, L. Belardinelli, and M. R. Blackburn. 2013. Adenosine A2B receptor and hyaluronan modulate pulmonary hypertension associated with chronic obstructive pulmonary disease. *American journal of respiratory cell and molecular biology* 49: 1038-1047.
172. Anders, H. J., and M. Ryu. 2011. Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. *Kidney international* 80: 915-925.

173. Laumbach, R. J. 2010. Outdoor air pollutants and patient health. *American family physician* 81: 175-180.
174. Devlin, R. B., W. F. McDonnell, R. Mann, S. Becker, D. E. House, D. Schreinemachers, and H. S. Koren. 1991. Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *American journal of respiratory cell and molecular biology* 4: 72-81.
175. Koren, H. S., R. B. Devlin, S. Becker, R. Perez, and W. F. McDonnell. 1991. Time-dependent changes of markers associated with inflammation in the lungs of humans exposed to ambient levels of ozone. *Toxicologic pathology* 19: 406-411.

1. Raghu, G.*, et al.* An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *American journal of respiratory and critical care medicine* **183**, 788-824 (2011).
2. King, T.E., Jr., Pardo, A. & Selman, M. Idiopathic pulmonary fibrosis. *Lancet* **378**, 1949-1961 (2011).
3. Raghu, G., Weycker, D., Edelsberg, J., Bradford, W.Z. & Oster, G. Incidence and prevalence of idiopathic pulmonary fibrosis. *American journal of respiratory and critical care medicine* **174**, 810-816 (2006).
4. Kropski, J.A., Lawson, W.E., Young, L.R. & Blackwell, T.S. Genetic studies provide clues on the pathogenesis of idiopathic pulmonary fibrosis. *Disease models & mechanisms* **6**, 9-17 (2013).
5. Seibold, M.A.*, et al.* A common MUC5B promoter polymorphism and pulmonary fibrosis. *The New England journal of medicine* **364**, 1503-1512 (2011).
6. Fell, C.D. Idiopathic pulmonary fibrosis: phenotypes and comorbidities. *Clinics in chest medicine* **33**, 51-57 (2012).
7. Ley, B., Collard, H.R. & King, T.E., Jr. Clinical course and prediction of survival in idiopathic pulmonary fibrosis. *American journal of respiratory and critical care medicine* **183**, 431-440 (2011).
8. Selman, M.*, et al.* Accelerated variant of idiopathic pulmonary fibrosis: clinical behavior and gene expression pattern. *PloS one* **2**, e482 (2007).
9. Boon, K.*, et al.* Molecular phenotypes distinguish patients with relatively stable from progressive idiopathic pulmonary fibrosis (IPF). *PloS one* **4**, e5134 (2009).
10. Barst, R.J.*, et al.* Diagnosis and differential assessment of pulmonary arterial hypertension. *Journal of the American College of Cardiology* **43**, 40S-47S (2004).
11. Smith, J.S., Gorbett, D., Mueller, J., Perez, R. & Daniels, C.J. Pulmonary hypertension and idiopathic pulmonary fibrosis: a dastardly duo. *The American journal of the medical sciences* **346**, 221-225 (2013).
12. Lettieri, C.J., Nathan, S.D., Barnett, S.D., Ahmad, S. & Shorr, A.F. Prevalence and outcomes of pulmonary arterial hypertension in advanced idiopathic pulmonary fibrosis. *Chest* **129**, 746-752 (2006).
13. Nadrous, H.F.*, et al.* Pulmonary hypertension in patients with idiopathic pulmonary fibrosis. *Chest* **128**, 2393-2399 (2005).
14. Shorr, A.F., Wainright, J.L., Cors, C.S., Lettieri, C.J. & Nathan, S.D. Pulmonary hypertension in patients with pulmonary fibrosis awaiting lung transplant. *The European respiratory journal* **30**, 715-721 (2007).

15.   Nathan, S.D.*, et al.* Serial development of pulmonary hypertension in patients with idiopathic pulmonary fibrosis. *Respiration; international review of thoracic diseases* **76**, 288-294 (2008).

16.   Hamada, K.*, et al.* Significance of pulmonary arterial pressure and diffusion capacity of the lung as prognosticator in patients with idiopathic pulmonary fibrosis. *Chest* **131**, 650-656 (2007).

17.   Mejia, M.*, et al.* Idiopathic pulmonary fibrosis and emphysema: decreased survival associated with severe pulmonary arterial hypertension. *Chest* **136**, 10-15 (2009).

18.   Cottin, V.*, et al.* Combined pulmonary fibrosis and emphysema: a distinct underrecognised entity. *The European respiratory journal* **26**, 586-593 (2005).

19.   Daniil, Z., Koutsokera, A. & Gourgoulianis, K. Combined pulmonary fibrosis and emphysema in patients exposed to agrochemical compounds. *The European respiratory journal* **27**, 434 (2006).

20.   Antoniou, K.M., Margaritopoulos, G.A. & Siafakas, N.M. Pharmacological treatment of idiopathic pulmonary fibrosis: from the past to the future. *European respiratory review : an official journal of the European Respiratory Society* **22**, 281-291 (2013).

21.   Woodcock, H.V. & Maher, T.M. The treatment of idiopathic pulmonary fibrosis. *F1000prime reports* **6**, 16 (2014).

22.   Potts, J. & Yogaratnam, D. Pirfenidone: a novel agent for the treatment of idiopathic pulmonary fibrosis. *The Annals of pharmacotherapy* **47**, 361-367 (2013).

23.   SEER Cancer Statistics Review, 1975-2007. (ed. Altekruse SF, K.C., Krapcho M, Neyman N, Aminou R, Waldron W, Ruhl J, Howlader N, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Cronin K, Chen HS, Feuer EJ, Stinchcomb DG, Edwards BK (eds).) (National Cancer Institute, Bethesda, MD, 2010).

24.   Olson, A.L.*, et al.* Mortality from pulmonary fibrosis increased in the United States from 1992 to 2003. *American journal of respiratory and critical care medicine* **176**, 277-284 (2007).

25.   Zhou, Y., Schneider, D.J. & Blackburn, M.R. Adenosine signaling and the regulation of chronic lung disease. *Pharmacology & therapeutics* **123**, 105-116 (2009).

26.   Fredholm, B.B. Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell death and differentiation* **14**, 1315-1323 (2007).

27.   Karmouty-Quintana, H., Xia, Y. & Blackburn, M.R. Adenosine signaling during acute and chronic disease states. *Journal of molecular medicine* **91**, 173-181 (2013).

28.   Chunn, J.L.*, et al.* Adenosine-dependent pulmonary fibrosis in adenosine deaminase-deficient mice. *Journal of immunology* **175**, 1937-1946 (2005).

29.   Pedroza, M.*, et al.* Interleukin-6 contributes to inflammation and remodeling in a model of adenosine mediated lung injury. *PloS one* **6**, e22667 (2011).

30.   Schneider, D.J., Lindsay, J.C., Zhou, Y., Molina, J.G. & Blackburn, M.R. Adenosine and osteopontin contribute to the development of chronic obstructive pulmonary disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **24**, 70-80 (2010).

31.   Zhou, Y., Murthy, J.N., Zeng, D., Belardinelli, L. & Blackburn, M.R. Alterations in adenosine metabolism and signaling in patients with chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. *PloS one* **5**, e9224 (2010).

32.   Sun, Y., Wu, F., Sun, F. & Huang, P. Adenosine promotes IL-6 release in airway epithelia. *Journal of immunology* **180**, 4173-4181 (2008).

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33. Zhou, Y., *et al.* Distinct roles for the A2B adenosine receptor in acute and chronic stages of bleomycin-induced lung injury. *Journal of immunology* **186**, 1097-1106 (2011).
34. Zhong, H., *et al.* A(2B) adenosine receptors increase cytokine release by bronchial smooth muscle cells. *American journal of respiratory cell and molecular biology* **30**, 118-125 (2004).
35. Zhong, H., Belardinelli, L., Maa, T. & Zeng, D. Synergy between A2B adenosine receptors and hypoxia in activating human lung fibroblasts. *American journal of respiratory cell and molecular biology* **32**, 2-8 (2005).
36. Sehgal, P.B. & Sagar, A.D. Heterogeneity of poly(I) x poly(C)-induced human fibroblast interferon mRNA species. *Nature* **288**, 95-97 (1980).
37. Hirano, T., *et al.* Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* **324**, 73-76 (1986).
38. Chalaris, A., Garbers, C., Rabe, B., Rose-John, S. & Scheller, J. The soluble Interleukin 6 receptor: generation and role in inflammation and cancer. *Eur J Cell Biol* **90**, 484-494 (2011).
39. Lotz, M., *et al.* B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes. *The Journal of experimental medicine* **167**, 1253-1258 (1988).
40. Ikebuchi, K., *et al.* Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 9035-9039 (1987).
41. Gauldie, J., Richards, C., Harnish, D., Lansdorp, P. & Baumann, H. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 7251-7255 (1987).
42. Kotake, S., *et al.* Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **11**, 88-95 (1996).
43. Scheller, J., Chalaris, A., Schmidt-Arras, D. & Rose-John, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta* **1813**, 878-888 (2011).
44. Jones, S.A., Scheller, J. & Rose-John, S. Therapeutic strategies for the clinical blockade of IL-6/gp130 signaling. *The Journal of clinical investigation* **121**, 3375-3383 (2011).
45. Bauer, J., *et al.* Regulation of interleukin 6 receptor expression in human monocytes and monocyte-derived macrophages. Comparison with the expression in human hepatocytes. *The Journal of experimental medicine* **170**, 1537-1549 (1989).
46. Taga, T., Kawanishi, Y., Hardy, R.R., Hirano, T. & Kishimoto, T. Receptors for B cell stimulatory factor 2. Quantitation, specificity, distribution, and regulation of their expression. *The Journal of experimental medicine* **166**, 967-981 (1987).
47. Rose-John, S., Scheller, J., Elson, G. & Jones, S.A. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. *Journal of leukocyte biology* **80**, 227-236 (2006).

48.   Taga, T., *et al.* Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* **58**, 573-581 (1989).
49.   Murakami, M., *et al.* IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. *Science* **260**, 1808-1810 (1993).
50.   Stahl, N., *et al.* Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. *Science* **263**, 92-95 (1994).
51.   Zhong, Z., Wen, Z. & Darnell, J.E., Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* **264**, 95-98 (1994).
52.   Heinrich, P.C., Behrmann, I., Muller-Newen, G., Schaper, F. & Graeve, L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *The Biochemical journal* **334 (Pt 2)**, 297-314 (1998).
53.   Rose-John, S. & Heinrich, P.C. Soluble receptors for cytokines and growth factors: generation and biological function. *The Biochemical journal* **300 (Pt 2)**, 281-290 (1994).
54.   Horiuchi, S., *et al.* Soluble interleukin-6 receptors released from T cell or granulocyte/macrophage cell lines and human peripheral blood mononuclear cells are generated through an alternative splicing mechanism. *Eur J Immunol* **24**, 1945-1948 (1994).
55.   Lust, J.A., *et al.* Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor. *Cytokine* **4**, 96-100 (1992).
56.   Muller-Newen, G., *et al.* Purification and characterization of the soluble interleukin-6 receptor from human plasma and identification of an isoform generated through alternative splicing. *Eur J Biochem* **236**, 837-842 (1996).
57.   Mullberg, J., *et al.* The soluble interleukin-6 receptor is generated by shedding. *Eur J Immunol* **23**, 473-480 (1993).
58.   Mullberg, J., Schooltink, H., Stoyan, T., Heinrich, P.C. & Rose-John, S. Protein kinase C activity is rate limiting for shedding of the interleukin-6 receptor. *Biochemical and biophysical research communications* **189**, 794-800 (1992).
59.   Chalaris, A., *et al.* Apoptosis is a natural stimulus of IL6R shedding and contributes to the proinflammatory trans-signaling function of neutrophils. *Blood* **110**, 1748-1755 (2007).
60.   Jones, S.A., Horiuchi, S., Novick, D., Yamamoto, N. & Fuller, G.M. Shedding of the soluble IL-6 receptor is triggered by Ca2+ mobilization, while basal release is predominantly the product of differential mRNA splicing in THP-1 cells. *Eur J Immunol* **28**, 3514-3522 (1998).
61.   Matthews, V., *et al.* Cellular cholesterol depletion triggers shedding of the human interleukin-6 receptor by ADAM10 and ADAM17 (TACE). *The Journal of biological chemistry* **278**, 38829-38839 (2003).
62.   Fenton, J.I., Hursting, S.D., Perkins, S.N. & Hord, N.G. Interleukin-6 production induced by leptin treatment promotes cell proliferation in an Apc (Min/+) colon epithelial cell line. *Carcinogenesis* **27**, 1507-1515 (2006).
63.   Briso, E.M., Dienz, O. & Rincon, M. Cutting edge: soluble IL-6R is produced by IL-6R ectodomain shedding in activated CD4 T cells. *Journal of immunology* **180**, 7102-7106 (2008).

64. Gomez, M.I.*, et al.* Bacterial induction of TNF-alpha converting enzyme expression and IL-6 receptor alpha shedding regulates airway inflammatory signaling. *Journal of immunology* **175**, 1930-1936 (2005).

65. Scheller, J., Chalaris, A., Garbers, C. & Rose-John, S. ADAM17: a molecular switch to control inflammation and tissue regeneration. *Trends Immunol* **32**, 380-387 (2011).

66. Booth, B.W., Sandifer, T., Martin, E.L. & Martin, L.D. IL-13-induced proliferation of airway epithelial cells: mediation by intracellular growth factor mobilization and ADAM17. *Respir Res* **8**, 51 (2007).

67. Cesaro, A.*, et al.* Differential expression and regulation of ADAM17 and TIMP3 in acute inflamed intestinal epithelia. *Am J Physiol Gastrointest Liver Physiol* **296**, G1332-1343 (2009).

68. Charbonneau, M.*, et al.* Hypoxia-inducible factor mediates hypoxic and tumor necrosis factor alpha-induced increases in tumor necrosis factor-alpha converting enzyme/ADAM17 expression by synovial cells. *The Journal of biological chemistry* **282**, 33714-33724 (2007).

69. Franchimont, N.*, et al.* Interleukin-6 receptor shedding is enhanced by interleukin-1beta and tumor necrosis factor alpha and is partially mediated by tumor necrosis factor alpha-converting enzyme in osteoblast-like cells. *Arthritis Rheum* **52**, 84-93 (2005).

70. Peters, M., Meyer zum Buschenfelde, K.H. & Rose-John, S. The function of the soluble IL-6 receptor in vivo. *Immunology letters* **54**, 177-184 (1996).

71. Neurath, M.F. & Finotto, S. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine & growth factor reviews* **22**, 83-89 (2011).

72. Melendez, G.C.*, et al.* Interleukin 6 mediates myocardial fibrosis, concentric hypertrophy, and diastolic dysfunction in rats. *Hypertension* **56**, 225-231 (2010).

73. Cronstein, B.N. Interleukin-6--a key mediator of systemic and local symptoms in rheumatoid arthritis. *Bull NYU Hosp Jt Dis* **65 Suppl 1**, S11-15 (2007).

74. Finotto, S.*, et al.* Local blockade of IL-6R signaling induces lung CD4+ T cell apoptosis in a murine model of asthma via regulatory T cells. *International immunology* **19**, 685-693 (2007).

75. Tanaka, T., Narazaki, M. & Kishimoto, T. Therapeutic targeting of the interleukin-6 receptor. *Annu Rev Pharmacol Toxicol* **52**, 199-219 (2012).

76. Atreya, R.*, et al.* Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med* **6**, 583-588 (2000).

77. Barkhausen, T.*, et al.* Selective blockade of interleukin-6 trans-signaling improves survival in a murine polymicrobial sepsis model. *Critical care medicine* **39**, 1407-1413 (2011).

78. Matsumoto, S.*, et al.* Essential roles of IL-6 trans-signaling in colonic epithelial cells, induced by the IL-6/soluble-IL-6 receptor derived from lamina propria macrophages, on the development of colitis-associated premalignant cancer in a murine model. *Journal of immunology* **184**, 1543-1551 (2010).

79. Nowell, M.A.*, et al.* Soluble IL-6 receptor governs IL-6 activity in experimental arthritis: blockade of arthritis severity by soluble glycoprotein 130. *Journal of immunology* **171**, 3202-3209 (2003).

80. Nowell, M.A.*, et al.* Therapeutic targeting of IL-6 trans signaling counteracts STAT3 control of experimental inflammatory arthritis. *Journal of immunology* **182**, 613-622 (2009).

81. Migita, K.*, et al.* Serum levels of interleukin-6 and its soluble receptors in patients with hepatitis C virus infection. *Hum Immunol* **67**, 27-32 (2006).

82. Dai, Y.*, et al.* A2B adenosine receptor-mediated induction of IL-6 promotes CKD. *Journal of the American Society of Nephrology : JASN* **22**, 890-901 (2011).

83. Sato, S., Hasegawa, M. & Takehara, K. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. *J Dermatol Sci* **27**, 140-146 (2001).

84. Hasegawa, M., Sato, S., Ihn, H. & Takehara, K. Enhanced production of interleukin-6 (IL-6), oncostatin M and soluble IL-6 receptor by cultured peripheral blood mononuclear cells from patients with systemic sclerosis. *Rheumatology* **38**, 612-617 (1999).

85. Ammit, A.J.*, et al.* Effect of IL-6 trans-signaling on the pro-remodeling phenotype of airway smooth muscle. *American journal of physiology. Lung cellular and molecular physiology* **292**, L199-206 (2007).

86. Zhang, H.*, et al.* IL-6 trans-signaling promotes pancreatitis-associated lung injury and lethality. *The Journal of clinical investigation* **123**, 1019-1031 (2013).

87. O'Donoghue, R.J.*, et al.* Genetic partitioning of interleukin-6 signalling in mice dissociates Stat3 from Smad3-mediated lung fibrosis. *EMBO Mol Med* **4**, 939-951 (2012).

88. Mouratis, M.A. & Aidinis, V. Modeling pulmonary fibrosis with bleomycin. *Current opinion in pulmonary medicine* **17**, 355-361 (2011).

89. Degryse, A.L.*, et al.* Repetitive intratracheal bleomycin models several features of idiopathic pulmonary fibrosis. *American journal of physiology. Lung cellular and molecular physiology* **299**, L442-452 (2010).

90. Swiderski, R.E., Dencoff, J.E., Floerchinger, C.S., Shapiro, S.D. & Hunninghake, G.W. Differential expression of extracellular matrix remodeling genes in a murine model of bleomycin-induced pulmonary fibrosis. *The American journal of pathology* **152**, 821-828 (1998).

91. Karmouty-Quintana, H.*, et al.* The A2B adenosine receptor modulates pulmonary hypertension associated with interstitial lung disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **26**, 2546-2557 (2012).

92. Baran, C.P.*, et al.* Important roles for macrophage colony-stimulating factor, CC chemokine ligand 2, and mononuclear phagocytes in the pathogenesis of pulmonary fibrosis. *American journal of respiratory and critical care medicine* **176**, 78-89 (2007).

93. Chua, F., Gauldie, J. & Laurent, G.J. Pulmonary fibrosis: searching for model answers. *American journal of respiratory cell and molecular biology* **33**, 9-13 (2005).

94. Blackburn, M.R., Datta, S.K. & Kellems, R.E. Adenosine deaminase-deficient mice generated using a two-stage genetic engineering strategy exhibit a combined immunodeficiency. *The Journal of biological chemistry* **273**, 5093-5100 (1998).

95.   Blackburn, M.R., *et al.* Metabolic consequences of adenosine deaminase deficiency in mice are associated with defects in alveogenesis, pulmonary inflammation, and airway obstruction. *The Journal of experimental medicine* **192**, 159-170 (2000).

96.   Blackburn, M.R., *et al.* The use of enzyme therapy to regulate the metabolic and phenotypic consequences of adenosine deaminase deficiency in mice. Differential impact on pulmonary and immunologic abnormalities. *The Journal of biological chemistry* **275**, 32114-32121 (2000).

97.   Sun, C.X., *et al.* Role of A2B adenosine receptor signaling in adenosine-dependent pulmonary inflammation and injury. *The Journal of clinical investigation* **116**, 2173-2182 (2006).

98.   Blackburn, M.R., *et al.* Adenosine mediates IL-13-induced inflammation and remodeling in the lung and interacts in an IL-13-adenosine amplification pathway. *The Journal of clinical investigation* **112**, 332-344 (2003).

99.   Sun, C.X., *et al.* A protective role for the A1 adenosine receptor in adenosine-dependent pulmonary injury. *The Journal of clinical investigation* **115**, 35-43 (2005).

100.   Mohsenin, A., *et al.* Genetic removal of the A2A adenosine receptor enhances pulmonary inflammation, mucin production, and angiogenesis in adenosine deaminase-deficient mice. *American journal of physiology. Lung cellular and molecular physiology* **293**, L753-761 (2007).

101.   Zhou, Y., *et al.* Enhanced airway inflammation and remodeling in adenosine deaminase-deficient mice lacking the A2B adenosine receptor. *Journal of immunology* **182**, 8037-8046 (2009).

102.   Young, H.W., *et al.* A3 adenosine receptor signaling contributes to airway inflammation and mucus production in adenosine deaminase-deficient mice. *Journal of immunology* **173**, 1380-1389 (2004).

103.   Lo, C.W., *et al.* IL-6 trans-signaling in formation and progression of malignant ascites in ovarian cancer. *Cancer research* **71**, 424-434 (2011).

104.   Wynn, T.A. Integrating mechanisms of pulmonary fibrosis. *The Journal of experimental medicine* **208**, 1339-1350 (2011).

105.   Muller-Newen, G., *et al.* Soluble IL-6 receptor potentiates the antagonistic activity of soluble gp130 on IL-6 responses. *Journal of immunology* **161**, 6347-6355 (1998).

106.   Jostock, T., *et al.* Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses. *Eur J Biochem* **268**, 160-167 (2001).

107.   Lissilaa, R., *et al.* Although IL-6 trans-signaling is sufficient to drive local immune responses, classical IL-6 signaling is obligate for the induction of T cell-mediated autoimmunity. *Journal of immunology* **185**, 5512-5521 (2010).

108.   Doganci, A., *et al.* The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. *The Journal of clinical investigation* **115**, 313-325 (2005).

109.   Hoge, J., *et al.* IL-6 controls the innate immune response against Listeria monocytogenes via classical IL-6 signaling. *Journal of immunology* **190**, 703-711 (2013).

110.   Hasegawa, M., *et al.* Serum levels of interleukin 6 (IL-6), oncostatin M, soluble IL-6 receptor, and soluble gp130 in patients with systemic sclerosis. *J Rheumatol* **25**, 308-313 (1998).

111. Adamali, H.I. & Maher, T.M. Current and novel drug therapies for idiopathic pulmonary fibrosis. *Drug design, development and therapy* **6**, 261-272 (2012).
112. Loomis-King, H., Flaherty, K.R. & Moore, B.B. Pathogenesis, current treatments and future directions for idiopathic pulmonary fibrosis. *Current opinion in pharmacology* **13**, 377-385 (2013).
113. Murray, L.A.*, et al.* Serum amyloid P therapeutically attenuates murine bleomycin-induced pulmonary fibrosis via its effects on macrophages. *PloS one* **5**, e9683 (2010).
114. Trujillo, G., O'Connor, E.C., Kunkel, S.L. & Hogaboam, C.M. A novel mechanism for CCR4 in the regulation of macrophage activation in bleomycin-induced pulmonary fibrosis. *The American journal of pathology* **172**, 1209-1221 (2008).
115. Pechkovsky, D.V.*, et al.* Alternatively activated alveolar macrophages in pulmonary fibrosis-mediator production and intracellular signal transduction. *Clin Immunol* **137**, 89-101 (2010).
116. Murray, L.A.*, et al.* TGF-beta driven lung fibrosis is macrophage dependent and blocked by Serum amyloid P. *Int J Biochem Cell Biol* **43**, 154-162 (2011).
117. Collins, S.L., Chan-Li, Y., Hallowell, R.W., Powell, J.D. & Horton, M.R. Pulmonary vaccination as a novel treatment for lung fibrosis. *PloS one* **7**, e31299 (2012).
118. Camelo, A., Dunmore, R., Sleeman, M.A. & Clarke, D.L. The epithelium in idiopathic pulmonary fibrosis: breaking the barrier. *Frontiers in pharmacology* **4**, 173 (2014).
119. Habiel, D.M. & Hogaboam, C. Heterogeneity in fibroblast proliferation and survival in idiopathic pulmonary fibrosis. *Frontiers in pharmacology* **5**, 2 (2014).
120. Modolell, M., Corraliza, I.M., Link, F., Soler, G. & Eichmann, K. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur J Immunol* **25**, 1101-1104 (1995).
121. Stein, M., Keshav, S., Harris, N. & Gordon, S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *The Journal of experimental medicine* **176**, 287-292 (1992).
122. Mosser, D.M. & Zhang, X. Activation of murine macrophages. *Curr Protoc Immunol* **Chapter 14**, Unit 14 12 (2008).
123. Wynn, T.A. IL-13 effector functions. *Annu Rev Immunol* **21**, 425-456 (2003).
124. Althoff, K., Reddy, P., Voltz, N., Rose-John, S. & Mullberg, J. Shedding of interleukin-6 receptor and tumor necrosis factor alpha. Contribution of the stalk sequence to the cleavage pattern of transmembrane proteins. *Eur J Biochem* **267**, 2624-2631 (2000).
125. Lee, Y.J.*, et al.* Preventing cleavage of Mer promotes efferocytosis and suppresses acute lung injury in bleomycin treated mice. *Toxicology and applied pharmacology* **263**, 61-72 (2012).
126. Garbers, C.*, et al.* Species specificity of ADAM10 and ADAM17 proteins in interleukin-6 (IL-6) trans-signaling and novel role of ADAM10 in inducible IL-6 receptor shedding. *The Journal of biological chemistry* **286**, 14804-14811 (2011).
127. Narazaki, M.*, et al.* Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a potential to inhibit signals through membrane-anchored gp130. *Blood* **82**, 1120-1126 (1993).
128. Zhang, J.G.*, et al.* Identification and characterization of two distinct truncated forms of gp130 and a soluble form of leukemia inhibitory factor receptor alpha-chain in

normal human urine and plasma. *The Journal of biological chemistry* **273**, 10798-10805 (1998).

129. Rose-John, S. IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6. *International journal of biological sciences* **8**, 1237-1247 (2012).

130. Ashcroft, T., Simpson, J.M. & Timbrell, V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *Journal of clinical pathology* **41**, 467-470 (1988).

131. Wynn, T.A. Cellular and molecular mechanisms of fibrosis. *The Journal of pathology* **214**, 199-210 (2008).

132. Rennard, S.I. & Crystal, R.G. Fibronectin in human bronchopulmonary lavage fluid. Elevation in patients with interstitial lung disease. *The Journal of clinical investigation* **69**, 113-122 (1982).

133. Crosby, L.M. & Waters, C.M. Epithelial repair mechanisms in the lung. *American journal of physiology. Lung cellular and molecular physiology* **298**, L715-731 (2010).

134. White, E.S., Baralle, F.E. & Muro, A.F. New insights into form and function of fibronectin splice variants. *The Journal of pathology* **216**, 1-14 (2008).

135. White, E.S. & Muro, A.F. Fibronectin splice variants: understanding their multiple roles in health and disease using engineered mouse models. *IUBMB Life* **63**, 538-546 (2011).

136. Seeger, W.*, et al.* Pulmonary hypertension in chronic lung diseases. *Journal of the American College of Cardiology* **62**, D109-116 (2013).

137. Knight, D., Mutsaers, S.E. & Prele, C.M. STAT3 in tissue fibrosis: is there a role in the lung? *Pulm Pharmacol Ther* **24**, 193-198 (2011).

138. Prele, C.M., Yao, E., O'Donoghue, R.J., Mutsaers, S.E. & Knight, D.A. STAT3: a central mediator of pulmonary fibrosis? *Proc Am Thorac Soc* **9**, 177-182 (2012).

139. Pechkovsky, D.V.*, et al.* STAT3-mediated signaling dysregulates lung fibroblast-myofibroblast activation and differentiation in UIP/IPF. *The American journal of pathology* **180**, 1398-1412 (2012).

140. Goodwin, A. & Jenkins, G. Role of integrin-mediated TGFbeta activation in the pathogenesis of pulmonary fibrosis. *Biochemical Society transactions* **37**, 849-854 (2009).

141. Hetzel, M., Bachem, M., Anders, D., Trischler, G. & Faehling, M. Different effects of growth factors on proliferation and matrix production of normal and fibrotic human lung fibroblasts. *Lung* **183**, 225-237 (2005).

142. Muro, A.F.*, et al.* An essential role for fibronectin extra type III domain A in pulmonary fibrosis. *American journal of respiratory and critical care medicine* **177**, 638-645 (2008).

143. Wolters, P.J., Collard, H.R. & Jones, K.D. Pathogenesis of idiopathic pulmonary fibrosis. *Annual review of pathology* **9**, 157-179 (2014).

144. Moodley, Y.P.*, et al.* Fibroblasts isolated from normal lungs and those with idiopathic pulmonary fibrosis differ in interleukin-6/gp130-mediated cell signaling and proliferation. *The American journal of pathology* **163**, 345-354 (2003).

145. Moodley, Y.P.*, et al.* Inverse effects of interleukin-6 on apoptosis of fibroblasts from pulmonary fibrosis and normal lungs. *American journal of respiratory cell and molecular biology* **29**, 490-498 (2003).

146. Lo Re, S., Lison, D. & Huaux, F. CD4+ T lymphocytes in lung fibrosis: diverse subsets, diverse functions. *Journal of leukocyte biology* **93**, 499-510 (2013).
147. Nuovo, G.J.*, et al.* The distribution of immunomodulatory cells in the lungs of patients with idiopathic pulmonary fibrosis. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **25**, 416-433 (2012).
148. Wilson, M.S.*, et al.* Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent. *The Journal of experimental medicine* **207**, 535-552 (2010).
149. Conte, E.*, et al.* Thymosin beta4 reduces IL-17-producing cells and IL-17 expression, and protects lungs from damage in bleomycin-treated mice. *Immunobiology* (2014).
150. Tan, H.L. & Rosenthal, M. IL-17 in lung disease: friend or foe? *Thorax* **68**, 788-790 (2013).
151. Galati, D.*, et al.* Peripheral depletion of NK cells and imbalance of the Treg/Th17 axis in idiopathic pulmonary fibrosis patients. *Cytokine* **66**, 119-126 (2014).
152. Ge, S.*, et al.* Interleukin 17 receptor A modulates monocyte subsets and macrophage generation in vivo. *PloS one* **9**, e85461 (2014).
153. Silacci, P.*, et al.* Interleukin (IL)-6 and its soluble receptor induce TIMP-1 expression in synoviocytes and chondrocytes, and block IL-1-induced collagenolytic activity. *The Journal of biological chemistry* **273**, 13625-13629 (1998).
154. Drucker, C.*, et al.* Interleukin-6 trans-signaling regulates glycogen consumption after D-galactosamine-induced liver damage. *J Interferon Cytokine Res* **29**, 711-718 (2009).
155. Ray, S.*, et al.* The IL-6 trans-signaling-STAT3 pathway mediates ECM and cellular proliferation in fibroblasts from hypertrophic scar. *J Invest Dermatol* **133**, 1212-1220 (2013).
156. Campos, S.P., Wang, Y., Koj, A. & Baumann, H. Divergent transforming growth factor-beta effects on IL-6 regulation of acute phase plasma proteins in rat hepatoma cells. *Journal of immunology* **151**, 7128-7137 (1993).
157. Yamamoto, T., Matsuda, T., Muraguchi, A., Miyazono, K. & Kawabata, M. Cross-talk between IL-6 and TGF-beta signaling in hepatoma cells. *FEBS letters* **492**, 247-253 (2001).
158. Walia, B., Wang, L., Merlin, D. & Sitaraman, S.V. TGF-beta down-regulates IL-6 signaling in intestinal epithelial cells: critical role of SMAD-2. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **17**, 2130-2132 (2003).
159. Yamada, D.*, et al.* Role of crosstalk between interleukin-6 and transforming growth factor-beta 1 in epithelial-mesenchymal transition and chemoresistance in biliary tract cancer. *European journal of cancer* **49**, 1725-1740 (2013).
160. O'Reilly, S., Ciechomska, M., Cant, R. & van Laar, J.M. IL-6 trans signalling drives a STAT3 dependant pathway that leads to hyperactive TGF-beta signalling promoting SMAD3 activation and fibrosis via gremlin. *The Journal of biological chemistry* (2014).
161. Elias, J.A., Lentz, V. & Cummings, P.J. Transforming growth factor-beta regulation of IL-6 production by unstimulated and IL-1-stimulated human fibroblasts. *Journal of immunology* **146**, 3437-3443 (1991).

162. Gallelli, L._, et al._ Interleukin-6 receptor superantagonist Sant7 inhibits TGF-beta-induced proliferation of human lung fibroblasts. _Cell proliferation_ **41**, 393-407 (2008).

163. Zhang, X.L., Topley, N., Ito, T. & Phillips, A. Interleukin-6 regulation of transforming growth factor (TGF)-beta receptor compartmentalization and turnover enhances TGF-beta1 signaling. _The Journal of biological chemistry_ **280**, 12239-12245 (2005).

164. Chen, R.H., Chang, M.C., Su, Y.H., Tsai, Y.T. & Kuo, M.L. Interleukin-6 inhibits transforming growth factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways. _The Journal of biological chemistry_ **274**, 23013-23019 (1999).

165. Khalil, N._, et al._ Increased production and immunohistochemical localization of transforming growth factor-beta in idiopathic pulmonary fibrosis. _American journal of respiratory cell and molecular biology_ **5**, 155-162 (1991).

166. Khalil, N., Bereznay, O., Sporn, M. & Greenberg, A.H. Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation. _The Journal of experimental medicine_ **170**, 727-737 (1989).

167. Assoian, R.K._, et al._ Expression and secretion of type beta transforming growth factor by activated human macrophages. _Proceedings of the National Academy of Sciences of the United States of America_ **84**, 6020-6024 (1987).

168. Ma, F._, et al._ Macrophage-stimulated cardiac fibroblast production of IL-6 is essential for TGF beta/Smad activation and cardiac fibrosis induced by angiotensin II. _PloS one_ **7**, e35144 (2012).

169. Weng, T._, et al._ Hypoxia-induced deoxycytidine kinase expression contributes to apoptosis in chronic lung disease. _FASEB journal : official publication of the Federation of American Societies for Experimental Biology_ **27**, 2013-2026 (2013).

170. Chan, E.S. & Cronstein, B.N. Adenosine in fibrosis. _Modern rheumatology / the Japan Rheumatism Association_ **20**, 114-122 (2010).

171. Karmouty-Quintana, H._, et al._ Adenosine A2B receptor and hyaluronan modulate pulmonary hypertension associated with chronic obstructive pulmonary disease. _American journal of respiratory cell and molecular biology_ **49**, 1038-1047 (2013).

172. Anders, H.J. & Ryu, M. Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. _Kidney international_ **80**, 915-925 (2011).

173. Laumbach, R.J. Outdoor air pollutants and patient health. _American family physician_ **81**, 175-180 (2010).

174. Devlin, R.B._, et al._ Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. _American journal of respiratory cell and molecular biology_ **4**, 72-81 (1991).

175. Koren, H.S., Devlin, R.B., Becker, S., Perez, R. & McDonnell, W.F. Time-dependent changes of markers associated with inflammation in the lungs of humans exposed to ambient levels of ozone. _Toxicologic pathology_ **19**, 406-411 (1991).