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The Hypoxic Adenosine Response Modulates Macrophage Differentiation and Contributes to Lung Disease

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THE HYPOXIC ADENOSINE RESPONSE MODULATES MACROPHAGE DIFFERENTIATION AND CONTRIBUTES TO LUNG DISEASE

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A

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THE HYPOXIC ADENOSINE RESPONSE MODULATES MACROPHAGE DIFFERENTIATION AND CONTRIBUTES TO LUNG DISEASE

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Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease which affects 5 - 8 million individuals worldwide and 200,000 individuals in the United States alone. Although prevalent, we do not know what causes IPF and no effective curative treatment exists for this disease. Our laboratory has shown that extracellular accumulation of adenosine and subsequent activation of the adenosine 2B (ADORA2B) receptor promotes immune cell invasion, airspace destruction, and fibrosis in chronic lung disease. Additionally, alternatively activated alveolar macrophages (AAMs) expressing ADORA2B, have been implicated in mediating adenosine's pro-fibrotic effects in IPF. However, the exact role of AAMs in the hypoxic lungs of IPF patients is not known. Our results reveal myeloid-specific ADORA2B deletion, antagonism of ADORA2B on AAMs, and inhibition or genetic silencing of hypoxia inducible factor 1α (HIF1A) as a means to attenuate pro-fibrotic mediator production and pulmonary fibrosis in bone marrow derived macrophages (BMDMs) and in vivo models of bleomycin-induced pulmonary fibrosis. These players will be valuable as potential clinical targets to halt differentiation of macrophages into the reparative AAM subtype and attenuate their subsequent pro-fibrotic role. Ultimately, these investigations will lead to a better understanding of adenosine's role in IPF and lead to identification of targets for novel therapeutics that can prevent disease progression and possibly reverse lung fibrosis.

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IPF	Idiopathic Pulmonary Fibrosis
HIF1A	Hypoxia-inducible factor 1-alpha
IL-6	Interleukin-6
BLM	Bleomycin
PBS	Phosphate-buffered saline
AAM	Alternatively-activated macrophage

CHAPTER ONE

INTRODUCTION

IDIOPATHIC PULMONARY FIBROSIS

Definition and Epidemiology

Idiopathic pulmonary fibrosis (IPF) is a devastating lung disease of unknown cause which leads to chronic, progressive interstitial pulmonary fibrosis(1). It is a disease which typically affects older males between the ages of 55 and 75(2). IPF is believed to affect up to 200,000 alone in the United States and around 8 million individuals worldwide (2-4). Nalysnyk et al. estimate the prevalence in the United States to be between 42.7-63 cases per 100,000 based on broad case definitions of IPF(5). Approximately 14,000 – 34,000 patients receive the new diagnosis of IPF each year(3). Ultimately, prevalence and incidence of IPF has been shown to increase with age and is continuing to rise emphasizing the need for investigation into the pathogenesis of this disease and development of novel therapies to halt its increasing incidence (1, 5).

Presentation and Diagnosis

Patients with IPF typically present with a chronic, non-productive cough and progressive dyspnea, or shortness of breath, on exertion which severely limits their quality of life(1, 2, 6). Findings on physical examination will include digital clubbing or bibasilar inspiratory crackles(2). Diagnosing IPF requires excluding other known causes of interstitial lung disease, the presence of a usual interstitial pneumonia (UIP) on high resolution computed tomography (HRCT) in patients without surgical lung

biopsy, or findings of possible UIP on HRCT with surgical lung biopsy findings showing definite UIP, probable/possible UIP, or non-classifiable fibrosis in conjunction with multidisciplinary discussion(1). Findings of definite UIP on histopathology include marked fibrosis, including patchy parenchymal involvement, fibroblastic foci, and honeycombing in a subpleural or septal distribution in the absence of any features suggestive of a different diagnosis (1). Honeycombing refers to clustered cystic airspaces with thick, fibrous walls and findings of collapsed alveoli and dilated alveolar lumen on pathology (7). HRCT findings suggestive of definite UIP include subpleural or basal predominance of reticular abnormalities, honeycombing with or without traction bronchiectasis, provided there is no evidence of features inconsistent with UIP such as micronodules, cysts, or consolidation(1).

Risk Factors

Although there is no known cause for IPF, risk factors such as environmental exposures, genetic factors, or medical comorbidities may contribute to the development of disease(2). A smoking history greater than 20 pack-years has strong associations with familial and sporadic IPF(1). Environmental exposures to metal, wood, vegetable, or animal dust is associated with significantly increased risk of IPF putting those who work in farming, hair dressing, or livestock at risk(1).

Co-morbid conditions including obesity, obstructive sleep apnea (OSA), diabetes, and gastro-esophageal reflux disease (GERD) have been associated with IPF(2). 37-94% of patients with IPF are affected by GERD(8). It's postulated that fibrosis changes compliance of the lung, contributing to increased negative

intrathoracic pressure and the symptoms of acid reflux; another hypothesis that repeated microaspirations contribute to alveolar epithelial cell damage which contributes to the repair, remodeling, and abnormal extracellular matrix deposition which leads to IPF (1, 8). Diabetes mellitus is found in 10-32.7% of patients with IPF(9) however the mechanism by which it may contribute to the pathogenesis of IPF is unclear. Some studies have suggested an association with Epstein-Barr virus (EBV) and hepatitis C infection in association with IPF however these studies are limited due to negative associations in some and significant confounding variables including immunosuppressive treatment(1).

Genetic transmission between families is believed to only affect up to 3.7% of patients with IPF (2). ELMOD2, a gene found on chromosome 4q31, in addition to mutations in surfactant protein C (spc) and the rare surfactant protein A2 (SFTPA2) have been found to be strongly associated with familial cases of IPF, where two or more members of the primary biological generation are affected (1). Mutations in human telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR) have been found in 15% of familial IPF cases supporting the hypothesis that progressive telomere shortening contributes to alveolar epithelial cell apoptosis and the initiation of an abnormal wound repair response which leads to the development of IPF(1, 2). Sporadic cases of IPF have been associated with genetic polymorphisms in genes coding for cytokines (interleukin-1α, 4, 8, 10, and 12, and tumor necrosis factor alpha (TNFα)), enzymes (α1-antitrypsin, angiotensin converting enzyme), pro-fibrotic mediators including transforming growth factor β1 (TGFβ1), and matrix metalloproteinase (MMP)-1(1, 10). Recent work by Yang et al. suggest genetic polymorphisms in the promoter of mucin 5B (MUC5B) has a strong association with

the development of IPF and its increased expression in terminal bronchi suggest increased mucous production contributes to perturbed mucociliary clearance and the development of pulmonary fibrosis(11).

Natural History of IPF

A large majority of patients with IPF follow a slow, gradual progression of disease while some remain stable, show a rapid decline, or demonstrate repeated acute exacerbations (AE)(1). Risk factors that may influence the disease progression are not quite understood but emphysema and pulmonary hypertension are believed to have a role(1). Patients with a stable or *slowly progressive* course of disease typically do not present to physicians until years (typically >24 months) after the symptoms begin although mortality is typically within few years of diagnosis (2, 12). Their annual decline in forced vital capacity typically ranges from 0.13 to 0.21L(12).

Patients are diagnosed with the *rapidly progressive* IPF subtype when they present to their physician within six months of symptoms beginning(12). Although their pulmonary function and findings on histology or imaging at the time of diagnosis are similar to patients with the slowly progressive subtype, patients with accelerated IPF are typically male, smokers, and have been found to have greatly differing transcriptional profiles(2). Smoking has been shown to induce inflammation and contribute to upregulation of the MAPK-ERG1-HSP70 pathway(13). Microarray data from patients with rapidly versus slowly progressive IPF found 437 differentially expressed genes including overexpression of genes related to morphogenesis, oxidative stress, migration and proliferation of fibroblasts and smooth muscle

cells(14). Immunohistochemistry of lung sections from rapid progressors also showed increased staining of ADORA2B in alveolar epithelial cells(14).

Five to ten percent of patients will experience repeated acute exacerbations (AE) or periods of respiratory decline within days to weeks(1). Acute exacerbations may be secondary to infection or cardiac failure(1). If in the absence of a known cause but associated with worsening symptoms of one month, lung function, bilateral ground-glass opacities and reticular consolidation on HRCT, is then referred to as acute exacerbations of IPF(1, 12). Mortality amongst these patients exceeds 60% during hospital admissions and increases to 90% within 6 months after discharge(2). Although there are no established risk factors for AE of IPF, it has been associated with post-operative thoracic surgery, bronchoalveolar lavage fluid (BAL) collection, and Torque teno virus in 27% of affected patients(2, 12). It is unclear however if AE of IPF represent a separate disease process from IPF or results from acute pulmonary distress leading to exacerbations of fibrotic changes in IPF(15). Examination of BAL typically reveals neutrophilia and histology shows diffuse alveolar damage (DAD) indicative of alveolar epithelial cell injury and remodeling as a mechanism of injury in AEs of IPF(15).

Treatment

In the past, as IPF was believed to largely be a disease secondary to aggressive inflammation alone, treatment had been primarily limited to corticosteroid treatment with no significant clinical improvement, little survival benefits and significant long-term comorbidities(1, 15).

Pharmacologic therapies which have failed placebo-controlled trials include Etanercept, a recombinant soluble human TNF receptor which binds and neutralizes TNFα, an inflammatory cytokine found to be elevated in patients with IPF(15). Bosentan, an endothelin-1 (ET-1) receptor antagonist, was predicted to inhibit ET-1-mediated collagen production, and matrix turnover; however, the BUILD-1 study showed no significant difference over placebo in 6 minute walking distance (6MWD) among treated patients(15). Similarly, macitentan, a nonselective ET receptor antagonist, was shown to have little effect on forced vital capacity against placebo in a phase 2 trial(16). The phase 3, randomized, double-blind, placebo-controlled anticoagulant effectiveness in idiopathic pulmonary fibrosis (ACE-IPF) trial was suspended due to ineffectiveness of warfarin treatment in patients with IPF over placebo(2).

Sildenafil, a phosphodiesterase 5 inhibitor, decreases pulmonary vascular constriction, reduces resistance, and has been shown to improve gas exchange and dyspnea in patients with severe IPF although no differences in 6MWD, the primary outcome was observed (2, 17).

The PANTHER-IPF study evaluated the change in baseline FVC after 60 weeks among patients with mild to moderate IPF who received either treatment with placebo, N-acetylcysteine (NAC) monotherapy, or prednisone and azathioprine and NAC therapy(18). No significant difference was observed in the rate of FVC decline or frequency of AEs(18). There were frequent hospital admissions and eight deaths in the three-drug group which prompted the Data and Safety Monitoring Board to stop the study and discontinue the use of the triple-drug therapy(18).

Pirfenidone is believed to inhibit fibroblast proliferation and collagen production through its inhibition of TGFβ and TNFα. Initial Phase II trials showed no difference in pirfenidone treatment as compared to placebo with respect to changes in arterial oxygen saturation after 6MWD however vital capacity (VC) was significantly improved(19). A phase III randomized control trial in Japan revealed significant differences in VC decline between placebo and high or low dose treatment groups leading to its approval in 2008 for the treatment of IPF(20). The multinational phase II CAPACITY 1 trial showed significant difference in % FVC change from baseline after 72 weeks in patients with pirfenidone treatment as compared to placebo leading to its approval in European countries in 2011 for IPF treatment(21). It was not until the phase III ASCEND trial which demonstrated improvements in FVC, 6MWD, progression free survival, and a reduction in death at 1 year by 48% that the FDA approved pirfenidone for treatment of IPF patients in the United States in October 2014(22). Mild to moderate side effects include gastrointestinal, dermatologic, neurological symptoms, and elevated liver enzymes(23).

Nintedanib inhibits vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) receptors halting the progression of fibrosis, slowing the decline in FVC, and AEs in patients receiving treatment compared to placebo. Phase III trials showed twice daily administration of nintedanib for 52 weeks led to slowing of the rate of FVC decline although there was no difference with respect to time to AE. Given these results however, Nintedanib was approved by the FDA for treatment of patients with IPF in October 2014 as well(24). Diarrhea was a significant adverse effect which impacted 90% of patients(23). However, a recent network meta-analysis comparing Pirfenidone and Nintedanib

show no significant difference in either drug's ability to lower respiratory-specific or all-cause mortality or decline in percent FVC (22, 24, 25).

Lung transplant remains the only 'curative' treatment that can increase survival, and even then, it only mildly improves 5 year survival rates to 50-56%(1). Other supportive and preventative treatment approaches include pulmonary rehabilitation, smoking cessation, immunization against influenza and pneumococcus, control of GERD, and oxygen therapy to keep saturations above 90%(15). There is some recent evidence however which suggests that oxygen therapy may not have any additional benefit over air in reducing exertional dyspnea among IPF patients without hypoxemia at rest(6).

Recent compounds in Phase 2 clinical trials include Fresolimumab, which targets TGFβ, FG-3019, a monoclonal antibody against connective tissue growth factor, Rituximab which targets Anti-CD20, and Sirolimus which inhibits mTOR(23). Several compounds have been developed against IL-13 including Lebrikizumab, OAX-576, Tralokinumab, and SAR156597 which actually targets IL-13 and IL-4(23).

Prognosis

Median survival for patients with IPF is between 2-3 years secondary to ineffective therapies (2, 26). There were approximately 175, 000 deaths due to pulmonary fibrosis from 1992 to 2003 which is about 51 deaths per 1,000,000 people when adjusted by age and sex. In fact, mortality during that time period increased by 28.4% in men and 41.3% in women(27). Five year survival rate among patients with IPF is only 43%(28). Poor prognostic factors include age greater than 70 years old, smoking history greater than 20 pack-year, and low body mass index (2). Other factors

of poor prognosis at baseline include diffusion limited capacity less than 40% of predicted, desaturations in arterial oxygen saturation to less than 88% during 6MWT, reductions in FVC by more than 10%, extensive honeycombing on HRCT, and pulmonary hypertension(1, 23). 9.8-38% of IPF patients may go on to develop malignancies including bronchogenic carcinoma(2).

Pathogenesis of IPF

Despite its significant prevalence, little is known about the mechanisms that lead to the development and progression of IPF and contributes to its poor prognosis (15). The failure of anti-inflammatory therapies for IPF including corticosteroids has largely refuted the hypothesis that IPF is primarily an inflammatory condition (2, 15). Epithelial cell injury and activation by genetic and environmental factors discussed above including smoke exposure, chronic microaspirations, or infection are instead believed to be the main pathway contributing to the development of fibrosis(15). Alveolar epithelial cells have been shown to secrete mediators which promote recruitment, proliferation, and differentiation of mesenchymal cells, fibrocyte infiltration; in fact, transitions of epithelial cells to mesenchymal cells (termed EMT or epithelial to mesenchymal transition) is believed to account for 33% of fibroblasts in mouse models of lung fibrosis(2). Hung et al. have also demonstrated the presence of pericytes, or cells derived from Foxd1-expressing progenitors, which expand after bleomycin-induced injury to myofibroblasts and fibroblasts and contribute to the deposition of extracellular matrix and fibrosis(29).

Proteins critical to embryogenesis have also been found to be abnormal in IPF. Wnt ligands have found to be overexpressed in alveolar epithelium and fibroblasts from IPF patients in addition to downregulation of phosphatase and tensin homologue (PTEN) in myofibroblasts, contributing to their resistance to apoptosis(2). Myofibroblasts are essentially 'activated' fibroblasts with greater contractility and pro-fibrotic potential(2). Increased gremlin expression, a bone morphogenetic protein antagonist, has also been observed in IPF fibroblasts (2). Moreover, much remains to be understood regarding the pathogenesis of lung tissue injury, remodeling, and fibrosis development emphasizing the significance of work presented in this dissertation.

ACUTE LUNG INJURY

Definition and Epidemiology

Acute lung injury (ALI) is a devastating lung disease of acute onset which often progresses into acute respiratory distress syndrome (ARDS), respiratory failure, and death(30). It is characterized by pulmonary edema due to breakdown and increased permeability of the alveolar capillary barrier which contributes to impaired arterial oxygen saturation in affected patients (31). It is estimated that the incidence in the United States is 190,600 individuals per year(30, 32). Although incidence of hospital-acquired ARDS is declining since changes in standard of practice with respect to lung-protective mechanical ventilation, the incidence of community-acquired ARDS remains the same(32). Moreover, there still remains a large clinical need to identify

mechanisms behind the development of inflammation, pulmonary edema, and hypoxemia in order to develop targeted therapeutics that can reduce its incidence.

Presentation and Diagnosis

Patient typically present with tachypnea, or increased respiratory rate, and hypoxemia(30). Criteria for diagnosis of ALI include acute onset within 1 week of a known insult or worsening symptoms, the presence of diffuse pulmonary infiltrates, $PaO_2/FiO_2 \leq 300$, and the absence of symptoms that suggest cardiac failure (31, 32). Early phases of disease show bilateral, ground-glass opacities which develop secondary to interstitial edema and hyaline membrane deposition(30).

Epithelial and endothelial barrier dysfunction contribute to diffuse alveolar damage (DAD) which can be classified into three phases on histology. In the acute or exudative phase, hyaline membranes are observed lining the alveolar space in addition to edema with contributing to the eventual development of granulation tissue; alveolar hemorrhage may be present and type II epithelial cells become hyperplastic(30). In the proliferative phase, granulation tissue incorporates into the alveolar septa leading to squamous metaplasia(30). Finally, the fibrotic phase is characterized by collagen deposition and alveolar hyalinization(30).

Risk Factors

Generally, risk factors for ALI can be divided into predisposing medical conditions, including shock, sepsis, aspiration, pneumonia, and surgery, or risk

modifiers such as obesity, alcohol abuse, tachypnea, and oxygen supplementation(32). However, infection including pneumonia or sepsis are the greatest factors shown to lead to ALI development at 46% and 33%, respectively(32). Additionally triggers which directly affect the lung including microaspiration, ingestants, inhalants, hyperoxia, ventilator induced injury, and bleomycin can lead to the development of ALI (30, 31). Systemic inflammatory response syndrome (SIRS) in response to pancreatitis or transfusions can also contribute to ALI(31). Patients with other medical comorbidities including chronic lung disease or alcohol abuse are also found to have increased incidence of ALI(32). Interestingly, both Type 1 and Type 2 diabetes mellitus has been found to have a protective effect in septic and nonseptic patients with ALI(32).

Treatment

Treatment is primarily aimed at addressing the underlying cause in addition to supportive care which may include mechanical ventilation and corticosteroid therapy. Phase 3 trials demonstrated significant reductions in mortality and greater ventilator-free days when low tidal volumes are used (6cc/kg of predicted body weight)(33). Implementation of changes in mechanical ventilation based on these trials has contributed to the reduced incidence by almost 40% of ALI developing into hospital-acquired ARDS(32).

The Lung Injury Prevention study with Aspirin (LIPS-A) is a recent phase 2 trial which evaluated the impact of prophylactic aspirin administration versus placebo on reducing ALI incidence in patients already on invasive mechanical ventilation;

however no reductions in ARDS incidence was observed so a phase 3 trial was not initiated(34). The recent phase 2 clinical trial evaluating aerosolized budesonide, an inhaled corticosteroid, and formoterol, a beta-agonist, treatment against placebo shows promise however in that combined treatment demonstrated improvements in oxygenation, PaO_2/FiO_2 ratio, decreased incidence of ARDS, and reductions in hospital stay(35).

Prognosis

Typically, patients with ALI will develop ARDS within 2-5 days of hospitalization(30). Factors which influence mortality include ALI severity whether it be mild ($200 < PaO_2/FiO_2 \leq 300$), moderate ($100 < PaO_2/FiO_2 \leq 200$), or severe ($PaO_2/FiO_2 \leq 100$) associated with increasing mortality rates of 27%, 32%, or 45% respectively(32). Males and African Americans affected by ALI also exhibit increased mortality however rates generally declines as time from admission increases(30, 32). Since 2010, the rate of in-hospital mortality is approximately 45%, ICU-associated, 38%, 30d after discharge, 30%, and 32% at 60 days from discharge(36). Overall however, there has been no significant improvement in mortality over the past two decades (30, 31).

Pathogenesis of ALI

There is extensive evidence suggesting the role of inflammation in ALI. After the initial insult, neutrophils are often the first cell type to infiltrate the lung releasing chemokines that attract macrophages and other mononuclear cells(37). Activated

neutrophils release proteases, reactive oxygen species (ROS), and peptides that contribute to the apoptotic and necrotic cell death of endothelium and epithelium(37). This breakdown of the pulmonary barrier contributes to increased permeability, pulmonary edema, and further inflammation (37, 38). Therapeutic inhibition of neutrophil-dependent CXCR2 (the chemokine receptor for IL-8, a known neutrophil chemoattractant) in a bleomycin-induced model of ALI was shown to be protective in addition to bleomycin exposure in IL-1$\beta^{-/-}$ and IL-18$^{-/-}$ mice(31). These studies highlight the importance of neutrophil migration and activation in contributing to acute lung injury. Recruited anti-inflammatory macrophages also play a key role in resolving inflammation. Janssen et al demonstrated the importance of Fas-mediated apoptosis in bone-marrow recruited macrophages (CD11b^{high}) in resolving inflammation in a H1N1-model of acute lung injury while populations of resident macrophages (CD11c^{high}) were stable (39).

Chemokines including macrophage inflammatory protein-1alpha (MIP-1α), a monocyte and leukocyte chemoattractant contributes to acute inflammation in acute lung injury models through TNFα and IL-6 release (38). Monocyte chemotactic protein-1 (MCP-1) is a chemokine produced by mononuclear cells including lung macrophages and contributes to increased inflammation in the development of lung injury(38). BALF levels of chemokines including CXCL1 and CXCL8 are elevated in patients with ALI(40). As such, some investigators have focused on antibody blockade of chemokines and or their receptors to attenuate ALI in animal models of disease however these findings have yet to be translated to trials in humans with ALI/ARDS.

Pathogen associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) typically serve to initiate responses by the innate immune

system including TNFα, IL1β, and IL-8 release(30). One key DAMP includes hyaluronan, an extracellular matrix glycosaminoglycan, produced in the setting of lung injury binds Toll-like receptors (TLR) 4 and 2 inducing inflammation(30). In fact, overexpression of high-molecular weight hyaluronan has been shown to be protective against lung injury in animal models by NF-kappaB activation protecting from further epithelial cell apoptosis(41).

Biomarkers to date which have been found to be elevated in plasma and BAL amongst patients with ALI include TNFα, IL-1β, IL-6, IL-8, and IL-18(30). IL-8 is interesting of note due to its ability to activate and attract neutrophils by binding with anti-IL-8 autoantibodies which inhibit neutrophil apoptosis(42, 43). Neutrophils are one of the first cells to respond and infiltrate the lung after ALI(31). Additionally, concentration of these IL-8-anti-IL-8 autoantibodies along with IL-18 levels is a direct indicator of ALI disease severity and mortality (44, 45). Despite the advances in understanding the pathophysiology which lead to the development of acute lung injury, there are few molecular-based therapies which have been shown to reduce mortality and improve quality of life for the patients who survive ALI and ARDS; as such, findings presented in this thesis on the impact of hypoxia and HIF1A stabilization are valuable to driving development of novel treatments for ALI.

ADENOSINE SIGNALING IN LUNG DISEASE

Adenosine Metabolism and Signaling

Adenosine is a potent signaling molecule that exerts its effects by acting on four G-protein coupled receptors (GPCRs), ADORA1, ADORA2A, ADORA2B, AND

ADORA3(46, 47). These receptors lead to changes in intracellular cAMP and calcium which ultimately, regulate homeostatic functions within the cell(48). ADORA1 and ADORA3 are G_i coupled so activation leads to reduced cAMP levels while ADORA2A and ADORA2B are G_s coupled stimulating adenyl cyclase, increasing cAMP and protein kinase A activation(48). ADORA2B can also couple to G_q stimulating phospholipase C (PLC) contributing to calcium release and protein kinase C (PKC) activation(48). Generally, the ADORA1 and ADORA2A receptor have higher affinity for adenosine than the low-adenosine affinity ADORA2B and ADORA3A receptors(49). Physiologic levels of adenosine in the extracellular fluid range from 40-600nM and its accumulation in the extracellular space is secondary to passive transfer from the intracellular space (50). Two bi-directional carriers responsible for passive diffusion of adenosine are equilibrative nucleoside transporter (ENT)-1 and ENT-2(49). Adenosine can be deaminated to inosine by adenosine deaminase (ADA) or phosphorylated by adenosine kinase to ATP(49).

In settings of cellular injury induced by hypoxia, trauma, or chronic inflammation, ATP is released from affected cells and subsequently dephosphorylated by the membrane bound enzyme ectonucleoside triphosphate diphosphohydrolase CD39 to AMP followed by dephosphorylation to adenosine by ecto-5'-nucleotidase CD73 to produce elevations in adenosine in the extracellular space(51, 52). Adenosine concentrations have been found to be as elevated as 100μM in asthma and COPD(50). Activation of the different adenosine receptors have been found to have differing effects depending on the injury model and cell-type. For example, antagonism of ADORA1 and ADORA3 have been associated with reduced inflammation in models of cecal ligation (50). ADORA2A has been shown to maintain

integrity in cardiac and renal models of ischemic reperfusion injury while ADORA2A provides systemic organ protection (50).

Adenosine Signaling in Chronic Lung Disease

Previous work suggests that pulmonary fibrosis results from an abnormal tissue reparative response(53). We have demonstrated how the nucleoside adenosine plays a critical role in the regulation of this response in the lung and contributes to fibrosis(50, 54-56). Adenosine levels are elevated in chronic lung diseases including asthma, COPD, and IPF(55, 57, 58). Mouse models of lung injury show increased adenosine levels in bronchoalveolar lavage fluid associated with increased inflammation and fibrosis(56).

These elevations in adenosine have been found to have differing tissue protective versus tissue destructive effects depending on the adenosine receptor activated and cell type. One mouse model that has been used to understand the effects of adenosine-mediated lung injury is the Adenosine Deaminase (ADA) deficient model ($ADA^{-/-}$) in which adenosine accumulates in the extracellular space due to the lack of the ADA enzyme which catalyzes its breakdown(59). $ADA^{-/-}$ mice exhibit abnormalities in alveologenesis, airway enlargement and obstruction, along with pulmonary inflammation, that contributes to the development of pulmonary fibrosis which is attenuated with introduction of ADA enzyme supplementation (60, 61). This model has ultimately allowed evaluation of adenosine-mediated lung injury. Adenosine stimulation itself promotes release of IL-8, IL-4, and IL-13 from mast cells. Overall, ADORA1 and ADORA2A have been found to have a tissue-protective role in

adenosine-mediated lung injury as ADA$^{-/-}$/ADORA1$^{-/-}$ and ADA$^{-/-}$/ADORA2A$^{-/-}$ mice showed exacerbations in inflammation and mucous cell metaplasia(62, 63). Meanwhile, activation of ADORA2B and ADORA3 are associated with tissue-destructive effects enhancing inflammation and fibrosis, or mast cell degranulation and mucous metaplasia, respectively (50).

The ADORA2B receptor is unique in several ways including its low affinity for adenosine and promotion of anti-inflammatory and anti-fibrotic features in acute lung injury(54). However, our lab has previously shown that chronic extracellular adenosine accumulation and activation of ADORA2B receptor can lead to features of chronic lung disease including inflammation, fibrosis, and tissue remodeling (56). Evaluating lung sections from IPF patients who are rapid progressors has also shown increased immunohistochemical staining for ADORA2B in association with increases in matrix metalloproteinases (MMPs) in IPF BALF promoting increased fibroblast migration in comparison with slow progressors (14).

ADORA2B is found throughout many cell types in the lung including epithelial cells, macrophages, fibroblasts, and smooth muscle cells (64). However, ADORA2B-mediated release of the pro-inflammatory and pro-fibrotic cytokine, IL-6, from macrophages has proven to be of unique interest. IL-6 itself is known to target inflammatory mediators including CXCL1, MCP-1, osteopontin (OPN), and IL-17 (65). CXCL1 itself, the murine analog of human neutrophil chemoattractant, IL-8, was found to be elevated in lungs of ADA$^{-/-}$ mice and neutralization of CXCL1 and its receptor, CXCR2, was found to inhibit adenosine-mediated angiogenic activity in the lung(66).

Zhou et al. demonstrated ADORA2B expression on CD206 positive macrophages in macrophages from BALF of IPF patients and revealed a role for ADORA2B antagonism in decreasing IL-6 production from macrophages isolated from IPF patients (55). Supporting a role for ADORA2B in mediating IL-6 production, ADORA2B$^{-/-}$ mice were observed to have reduced IL-6 expression in BALF on day 33 after bleomycin exposure in association with reductions in IL-6 immunohistochemical staining localized to lung macrophages(56). Lung macrophages isolated from ADA$^{-/-}$ mice, known to have exacerbated inflammation, alveolar destruction and fibrosis secondary to adenosine elevations, showed elevations in IL-6 transcripts which were further increased with treatment by the adenosine receptor agonist, NECA, and reversed upon addition of ADORA2B-specific antagonist, MRS 1754 (65).

Adenosine Signaling in Acute Lung Injury

As discussed above, ALI is characterized by initial influx of neutrophils into the lungs and a Th1 cytokine rich environment including increases in IL-12, IFNγ, and TNFα(50). Models of LPS-induced ALI have demonstrated exacerbations neutrophilia and pulmonary barrier dysfunction in CD39 and CD73 deficient mice associated with reductions in adenosine thereby suggesting adenosine itself plays an anti-inflammatory role in ALI(67). ADORA2A and ADORA3 agonists have also been utilized in transplantation lung reperfusion injury models and cardiopulmonary bypass-induced lung injury models to attenuate inflammation and preserve pulmonary function(50). ADORA2B antagonist treatment in a ventilator-induced lung injury model demonstrated increases in pulmonary inflammation, edema, decreased gas

exchange, and reductions in survival time (68). Similarly, we have previously demonstrated a tissue-protective role for ADORA2B as exacerbations in pulmonary edema, inflammation, and barrier dysfunction were observed in ADORA2B$^{-/-}$ mice exposed to bleomycin intratracheally(56). Bleomycin treatment of ADORA3$^{-/-}$ mice also showed enhanced inflammation and eosinophilia suggesting ADORA3 also serves an anti-inflammatory role in ALI (69).

Evidence in animal models of acute and chronic lung disease and patient samples reveal that adenosine receptors have differing anti-inflammatory, tissue-protective or pro-inflammatory, tissue-destructive roles depending on the stage of disease or cell-type involved(50, 52). Although it is not clearly understood what contributes to these differences depending on disease stage, it may likely be a feature of differences in the cytokine environment with acute injury being primarily Th1 and chronic stages of disease, Th2 (50). This dissertation will focus on the role of ADORA2B on myeloid cells in acute lung injury and chronic lung disease in a bleomycin-induced model of lung injury along with how hypoxia can regulate these responses.

HYPOXIA-INDUCIBLE FACTOR 1A (HIF1A) IN LUNG DISEASE

Hypoxia inducible factor-1A (HIF1A) Signaling

Hypoxia inducible factor (HIF) is a transcription factor responsible for regulating a variety of target genes including those involved in angiogenesis, matrix metabolism, apoptosis, and glycolysis(70, 71). It is composed of two subunits: the inducible alpha (HIF1A) and constitutively expressed beta unit (HIF1B)(70). In normoxic conditions,

von hippel Lindau (VHL) protein targets the HIF1A subunit for proteasomal-mediated degradation followed by prolyl hydroxylation of the oxygen dependent domain (ODD) by three prolyl hydroxylases (PHD 1,2 and 3)(70). Another player which can disrupt HIF transcription is Factor inhibiting HIF1, or FIH. FIH hydroxylates asparagine residues within HIF1A or HIF2A, preventing the interaction of HIF with its transcriptional co-activator p300.(70).

In settings of hypoxia, PHD enzyme activity decreases resulting in decreased hydroxylation of HIF1A promoting its stabilization and accumulation in the cytoplasm(72). Next, it translocates to the nucleus where it binds to the constitutively expressed HIF1B and the heterodimer bind to hypoxia response elements (HRE) on the promoter region of target genes, recruits coactivator proteins, and initiates transcription of the target gene (71).

HIF1A can also be stabilized in normoxic conditions such as bacterial lipopolysaccharide (LPS) treatment, iron uptake by bacteria during infection, or mechanical stretch which increases ROS inhibiting PHDs(73, 74). NF-kb activation of the innate immune system, PI3K/Akt pathway activation in response to insulin-like growth factor (I-LGF) can also stabilize HIF1A in the absence of hypoxia(74, 75). Elevations in succinate have also been shown to directly inhibit PHD activity in macrophages leading to stabilization of HIF1A(76, 77). Additionally, increased succinate production can contribute to increased ROS production which can oxidize PHD cofactors ultimately resulting in the stabilization of HIF1A(77, 78).

Agents or means which have been demonstrated to stabilize HIF1A include siRNA against FIH and $CoCl_2$, which acts as PHD inhibitor(70, 79). Pharmacologic inhibitors

of HIF1A include 17-DMAG, an inhibitor of heat shock protein 90 (hsp90), leading to improper folding of HIF1A, its destabilization and subsequent degradation(80).

HIF1A in Chronic Lung Disease

On history, physical and laboratory examination, IPF patients often demonstrate features of chronic hypoxia including shortness of breath, reduced diffusion capacity of the lung and 6 minute walk distance, and decreased partial pressure of oxygen in arterial blood(2, 81). Increased immunohistochemical staining for HIF1A on histological evaluation of lungs from IPF patients also supports the presence of hypoxia(82). Similarly, animal models of bleomycin-induced chronic pulmonary fibrosis show decreased arterial oxygen saturation and increased HIF1A in the lungs(83). Weng et al. demonstrated in vitro and in vivo how hypoxia-induced stabilization of HIF1A contributes to induction of deoxycytidine kinase (DCK) and subsequent alveolar epithelial cell proliferation contributing to the development of severe lung fibrosis(84). In vivo inhibition of HIF1A in a house dust mine (HDM) model of chronic asthma led to decreases in eosinophilia, inflammation, reductions in VEGF and CXCL1 in association with fewer endothelial progenitor cells and peribronchial angiogenesis(85). Together these studies illustrate HIF1A contributes to inflammation and fibrosis in chronic lung disease.

HIF1A in ALI

A role for HIF1A in acute lung injury is only recently being elucidated and is still unclear depending on the model of lung injury and cell-type involved. HIF1A up-regulation in a hypoxia/ischemia reperfusion model led to an increase in VEGF and

disruptions of the vascular barrier suggesting a tissue-destructive role for HIF1A in acute injury(72). Similarly, stabilization of HIF1A in neutrophils was revealed to promote NF-κβ activation, preventing their apoptosis after in vitro hypoxia exposure suggesting a pro-inflammatory role for HIF1A(86). However, HIF1A-dependent reduction in adenosine kinase led to an increase in adenosine levels that protected the vascular barrier(72, 87). Also demonstrating an anti-inflammatory role for HIF1A in acute injury, a normoxic model of ventilator-induced lung injury (VILI) by mechanical stretch led to HIF1A stabilization which was associated with reductions in pulmonary edema and inflammation(88). Alveolar-epithelial cell specific HIF1A deletion however led to exacerbations of acute lung injury in association with suboptimal carbohydrate metabolism after VILI emphasizing an anti-inflammatory role for HIF1A in alveolar epithelial cells(89).

HIF1A and Adenosine Signaling

HIF1A is known to regulate elements of the adenosine pathway. Specifically, HIF1A has been shown to regulate expression of the ADORA2B receptor, by binding to the HRE of its promoter, CD73, and equilibrative nucleotide transporters (ENTs) which allow adenosine transport across the cell membrane (88, 90, 91). This regulation ultimately leads to elevation of extracellular adenosine levels and enhancement of ADORA2B signaling.

The promoter of CD73 has also been found to have a HIF1 binding site and in vivo studies of a mouse model of hypoxia-induced intestinal injury showed CD73 inhibition increased barrier disruption, supporting a protective role for CD73 in hypoxia-mediated acute injury(92). Cyclic mechanical stretch of pulmonary epithelia

and in vivo model of VILI has been shown to promote HIF1A stabilization and significant increases in ADORA2B which were reduced with HIF1A inhibition in vitro and epithelial-cell specific HIF1A deletion (88). These studies suggest a role for HIF1A in modulating players in the adenosine signaling pathway that contribute to tissue protection in acute lung injury.

Most recently, we demonstrated up-regulation of HIF1A along with ADORA2B, CD73, and ENT-1 in tissue samples from patients with Group 3 pulmonary hypertension (PH), or PH associated with chronic lung disease, in association with increased succinate levels, a known HIF1A stabilizer as discussed above(93). As HIF1A has been shown to regulate ADORA2B expression and ADORA2B has been found to be elevated and localized to alternatively-activated macrophages in IPF patients, this dissertation will further investigate the role HIF1A plays on modulating ADORA2B expression on myeloid cells and macrophage differentiation.

ALTERNATIVELY-ACTIVATED MACROPHAGES (AAMs)

Macrophage Subtypes

Monocytes circulate in the vasculature prior to entering tissues where they become macrophages, either long-living tissue-specific as residents, or are recruited to phagocytose and kill infectious agents, scavenge toxins, or act as antigen presenting cells(94, 95). Therefore, mononuclear cells play a key role in the inflammatory response and wound repair response in a variety of tissues (48, 94-96).

Macrophages can generally be classified into two larger subtypes: classically activated and alternatively-activated. Classically-activated macrophages (CAMs) are activated by Th1 cytokines including TNFα, IFNγ, granulocyte macrophage-colony

stimulating factor (GM-CSF), PAMPs (i.e. LPS), or DAMPs(48, 74, 96). CAMs produce inducible nitric oxide synthase (iNOS), TNFα, IL-1, and IL-12(96). Their primary function is pro-inflammatory to contain microbes and tumoricidal and anaerobic glycolysis predominantes (74, 96).

Alternatively-activated macrophages (AAMs) are polarized by activation with Th2 cytokines including IL-4 (binds IL-4Rα activating Signal transduction and activator of transcription (STAT)6), IL-13 (shares STAT6 signaling), IL-6, IL-10 (activates STAT3), M-CSF, and MCP-1(74, 96-98). A novel pharmacologic inhibitor of STAT6 signaling is AS1517499 which disrupts IL-4 and IL-13-mediated differentiation of macs into AAMs(97, 99-101). Mauer et al have demonstrated in a diet-induced obesity mouse model that IL-6 expression induced IL-4R on macrophages, increasing their responsiveness to IL-4 and subsequent AAM activation(102).

AAMs predominantly express Arginase-1 and mannose receptor C, or CD206, along with found in inflammatory zone-1 (FIZZ-1), and chitinase3-like-3 (Ym1). Arginase-1 is an enzyme which metabolizes arginine to urea and ornithine, which is subsequently utilized for proline and collagen synthesis thereby contributing to extracellular matrix (ECM) deposition(48, 96). In fact, Arginase-1 competes with iNOS, produced by CAMs, for the arginine substrate thereby reducing nitric oxide production (96). AAMs also utilize oxidative glycolysis and fatty acid oxidation as opposed to anaerobic pathways(74). CD206 is also a key glycoprotein important to AAM phagocytosis function by increasing efferocytosis of pathogens and apoptotic cells(103).

Ultimately, AAMs regulate wound healing through release of matrix degrading enzymes and growth factors, and lead to fibrosis by stimulating myofibroblast

activation, proliferation, and survival(94, 95, 98). Flow cytometry analysis of lung macrophages after a bleomycin-model of lung injury reveal during initial stages or at rest, markers which predominate are $CD11c^{+}CD11b^{-}$ indicative of resident macrophages however in later stages of injury after fibrosis development, a majority of macrophages are recruited macrophages, or $CD11c^{+}CD11b^{+}$(104). Additional functions of AAMs including protection from parasites and suppression of the Th1 response(74). TNFα treatment of bleomycin exposed mice led to reductions in fibrosis and AAM expression supporting a role for AAMs in the development of lung fibrosis and the potential of encouraging macrophage switch to CAMs as a possible treatment(105). AAMs have been found to be a regulatory effector cell type involved in remodeling in many diseases including asthma, COPD, CF, and liver fibrosis(95).

Adenosine and Macrophages

There is evidence that the key effector cell type influenced by ADORA2B receptor signaling in pulmonary fibrosis is the lung macrophage. Lung macrophages increase over time in animal models of fibrosis(106). Previous work by our lab has shown that IPF patients have increased ADORA2B receptor positive alternatively activated macrophages (AAM) and we have also shown that the presence of the ADORA2B receptor on these macrophages is critical to the production of pro-fibrotic cytokines such as interleukin-6 (IL-6)(55, 56). In support of a pro-fibrotic and AAM promoting role for ADORA2B in another chronic lung disease, global and myeloid-specifc ADORA2B deletion in a cockroach allergen mouse model of asthma led to reductions in inflammatory cell infiltration, airway mucin production, and decreased secretion of pro-inflamamtory cytokines including TNFα, tmγ, and IL-17(107). Th2

cytokines IL-4, IL-5, and IL-13 were also reduced demonstrating ADORA2B on myeloid cells as a regulator of a Th2 rich environment in the development of chronic inflammation in asthma (107). Additionally, Csoka et al have indicated a role for the ADORA2B receptor, and to a lesser extent, ADORA2A, in mediating differentiation of macrophages into the AAM phenotype (108).

Like ADORA2B, the other adenosine receptors have also been found to affect macrophage differentiation and function. Adenosine itself has been shown to stimulate IL-10-induced STAT3 signaling and subsequent activation of M2c macrophages(109). Activation of ADORA1 on monocytes leads to differentiation of multinucleated giant cells(48). Both ADORA2A and ADORA2B have been found to be pivotal to macrophage metabolism as they can increase Specificity protein (Sp1) expression promoting increase in glycolytic flux, a metabolic shift believed to play a role in shifting CAMs to AAMs(48). Moreover, there is significant evidence that ADORA2B drives differentiation of AAM, however this dissertation seeks to delineate the disease signals which drive this response whether it be Th2-cytokine-STAT6 mediated or hypoxia through HIF1A.

HIF1A and Macrophages

Hypoxia in the setting of inflammation has been observed to cause significant changes in functional plasticity of macrophages, which account for 95% of the recruited cells(74). Changes observed in gene expression include up-regulation of VEGF, MMP7, MMP9, and M2 markers such as Arginase-1(96, 110). Cramer et al demonstrate that HIF1A deletion on myeloid cells leads to depletion of the ATP pool which is associated with poor myeloid cell invasion, motility, and bactericidal abilities

suggesting that HIF1A can directly impact macrophage metabolism and subsequent survival and function in the setting of inflammation(110). HIF1A$^{-/-}$ macrophages have reductions in bactericidal activity, angiogenesis, worsened systemic infection, and reduced TNFα and IL-6 responses(111). Hypoxia through HIF1A in T lymphocytes has been shown to promote the shift of cells from T helper 1 (Th1) to T helper 2 (Th2); this shift in the T helper cell environment can directly influence cytokines released into the extracellular environment and mediate macrophage differentiation as discussed above(112). Limited studies so far suggest that HIF1A in myeloid cells affects macrophage metabolism, function, and gene expression suggestive of an AAM subtype, however the mechanisms by which HIF1A mediates changes in macrophage differentiation and subsequent fibrosis development remain to be understood and will be investigated in this dissertation.

DISSERTATION OVERVIEW

IPF is a common, chronic disease which significantly impacts the quality of life of millions of individuals worldwide(2). Median survival after diagnosis is typically 3-5 years due to the absence of any effective therapies outside of lung transplantation(26). The incidence and prevalence of IPF continues to rise hastening the need to better understand the pathways which mediate the development and progression of pulmonary fibrosis(5). We have previously demonstrated a role for the signaling molecule adenosine in the setting of acute or chronic lung injury and demonstrated its elevation in patients with IPF and animal models of bleomycin-induced lung injury(56). Activation of ADORA2B by extracellular accumulation of adenosine can lead to features of chronic lung disease including inflammation, fibrosis, and tissue

remodeling(56). We have shown previously the increase in expression of ADORA2B and AAMs which are correlated with increased pro-fibrotic mediator production(55, 65). The stabilization of HIF1A has been found to up-regulate ADORA2B however its role as a direct modulator of macrophage phenotype remains to be understood (88). Additionally, factors which regulate adenosine receptor expression on alternatively activated macrophages and their role in IPF are unknown. An understanding of AAMs and previous work demonstrating a pro-fibrotic role for ADORA2B receptor led to the development of my proposed project which investigates the hypothesis that both **hypoxia and lung macrophage differentiation contribute to the progression of pulmonary fibrosis through up-regulation of ADORA2B; and myeloid-specific ADORA2B deletion or HIF1A inhibition in late stages of disease will attenuate fibrosis.**

Specific Aim 1: Evaluate the expression and regulation of ADORA2B on AAMs and determine the role of ADORA2B on AAMs in pulmonary fibrosis.

Conditional knockout mice lacking ADORA2B receptor on myeloid cells ($ADORA2B^{f/f}LysM^{Cre}$) were exposed to the intraperitoneal bleomycin injections over 33 days. $ADORA2B^{f/f}LysM^{Cre}$ after bleomycin treatment showed evidenced of improved arterial oxygen saturation and decreased fibrosis consistent with the presence of fewer AAMs as compared to wild type mice after bleomycin exposure (113). To validate these findings in vitro, BMDMs were isolated from C57Bl6 and $ADORA2B^{-/-}$ mice, treated with Th2 cytokines and hypoxia in addition to pan-adenosine receptor agonist, with and without ADORA2B antagonist, to evaluate the

effect on AAM and CAM expression along with pro-fibrotic molecule production. BMDMs were also utilized to determine the effect of Th1 in comparison to Th2 cytokine treatment on adenosine receptor expression. Results of this aim are presented in Chapter 3.

Specific Aim 2: Evaluate the expression of HIF1A in AAMs and determine the effect of HIF1A inhibition in pulmonary fibrosis.

HIF1A stabilization and localization to AAMs in addition to ADORA2B expression was characterized in lung sections from patients with IPF. To demonstrate HIF1A stabilization in AAMs in a mouse lung sections, Hif1α-luciferase reporter mice were then exposed to bleomycin. A HIF1A pharmacologic inhibitor, 17-DMAG, was then delivered in late stages of bleomycin-induced lung injury in C57BL/6 mice to evaluate the role of HIF1A inhibition on ADORA2B expression, macrophage differentiation, and the development of fibrosis. Finally, BMDMs from control mice were cultured in the presence of a HIF1A stabilizer, inhibitor, STAT6 inhibitor, along with genetic silencing to establish the role of HIF1A as a driver of ADORA2B expression on macrophages, mediating their differentiation into the AAM subtype, and promoting production of pro-fibrotic molecules and the subsequent development of pulmonary fibrosis. Chapter 4 discusses the results of this aim.

Specific Aim 3: Evaluate the role of HIF1A on myeloid cells in lung injury.

Myeloid-specific HIF1A knockout mice ($HIF1A^{f/f}LysM^{Cre}$) were exposed to bleomycin-induced lung injury to evaluate the role of HIF1A in acute and chronic

stages of disease. A time course experiment of varying degrees of bleomycin exposure reveal an anti-inflammatory role for HIF1A in acute lung injury. Evaluation of fibrotic markers in later stages of disease reveal exacerbations in fibrosis despite HIF1A-mediated reductions in ADORA2B and AAM expression. BMDMs from $HIF1A^{f/f}LysM^{Cre}$ mice were stimulated in culture with Th2 cytokines, hypoxia, and adenosine receptor agonist to establish the effect HIF1A deletion on myeloid cells places in regulation of ADORA2B and macrophage differentiation.

Insight into the mechanisms contributing to the development and progression of fibrosis in IPF will lead to direct development of novel therapies which target key pathways and cells included in this project such as ADORA2B antagonists or HIF1A inhibitors which modulate macrophage phenotype, pro-fibrotic mediator production, and the development of lung fibrosis.

CHAPTER TWO

EXPERIMENTAL PROCEDURES

Human Subjects

Explanted lung tissue samples from healthy, normal (n=6) and IPF patients (n=19) were de-identified and obtained from the International Institute for the Advancement of Medicine (IIAM, Edison, NJ) and the Methodist Hospital J.C. Walter Jr. Transplant Center (Houston, TX), respectively (Table I). These lung tissue are those normal or affected lungs that come from lung transplants or lungs gathered but not used for transplants. IIAM and Methodist Hospital Institutional Review Board guidelines were strictly adhered to for tissue collection and use.

Table I. Distribution of demographic and clinical variables for patient population.

	Normal (n=6)	IPF (n=19)	p-value
Gender, n (%)			
Males	1 (16.67)	12 (63.2)	**0.00410**
Females	5 (83.33)	7 (36.8)	0.04362
Age (Mean ± SD)			
Overall	43.6 ± 17.07	63 ± 9.22	0.06314
Males	63	67 ± 7	0.08801
Female	38.75 ± 15.22	56 ± 9.45	0.09926
BMI (Mean ± SD)	29.46 ± 5.50	28.69 ± 6.24	0.79276
P_aO_2, mmHg (Mean ± SD)	138.68 ± 60.44	72.23 ± 29.11	0.07121
P_aCO_2, mmHg (Mean ± SD)	37.1 ± 9.20	40.29 ± 6.22	0.49692
mPAP , mmHg (Mean ± SD)		18.95 ± 6.47	
Systolic PAP, mmHg (Mean ± SD)		30.63 ± 10.36	
Diastolic PAP, mmHg (Mean ± SD)		13.16 ± 5.12	
6MWD, feet (Mean ± SD)		795.24 ± 458.77	
FVC, % (Mean ± SD)		54 ± 24	
DLCO, % (Mean ± SD)		29.06 ± 10.94	
FEV_1, % (Mean ± SD)		7.99 ± 3.80	

Abbreviations: mPAP = mean pulmonary arterial pressure; PAP = pulmonary arterial pressure; 6MWD = six-minute walk distance; BMI = body mass index; FVC = forced vital capacity; DLCO = diffusing capacity of the lung for carbon monoxide; TLC = total lung capacity; FEV_1 = forced expiratory volume in one second; P_aO_2 = = partial pressure of oxygen in arterial blood; P_aCO_2 = partial pressure of carbon dioxide in arterial blood.

Generation and Genotyping of Mouse Lines

All inbred, male, 5-6 week old C57BL/6 and LysM^{Cre} mice were obtained from Harlan (Envigo) Laboratories (Houston, TX). ADORA2B$^{f/f}$LysM^{Cre}, HIF1A$^{f/f}$LysM^{Cre}, and Hif1α::LUC reporter mice (FVB.129S6-*Gt(ROSA)26Sor*$^{tm2(HIF1AA/luc)Kael}$/J) were obtained from Holger K. Eltzschig(89). DNA was extracted from tail samples to confirm genotypes with PCR. Animals were maintained and cared for in agreement with McGovern Medical School at University of Texas Health Sciences Center Animal Welfare Committee (AWC) guidelines. Study designs were approved by AWC.

Intraperitoneal Bleomycin Mouse Model

Bleomycin is a glycopeptide chemotherapeutic antibiotic used to treat different types of cancer including testicular cancer and Hodgkin's lymphoma (114). 10% of patients treated develop pulmonary toxicity secondary to reduced levels of the enzyme bleomycin hydrolase, responsible for its inactivation (114). Thus, repeated bleomycin exposure contributes to inflammation and the subsequent development of pulmonary fibrosis(114). Ultimately, bleomycin leads to breaks in the DNA strand contributing to the production of ROS(115). C57BL/6 mice are more susceptible to bleomycin-induced injury and demonstrate increased inflammation in the first 14 to 21 days followed by the development of fibrosis by day 20 onwards in an intraperitoneal model (56, 115). This model however is limited in that the fibrosis that develops is reversible once systemic bleomycin administration is stopped (115). Despite this limitation, the bleomycin-induced model of lung injury is more frequently used than exposure to

silica, fluorescein isothiocyanate (FITC), or irradiation as it better models features observed in patients with IPF including the presence of hyperplastic epithelial cells, greater concentration of pleural fibrotic patches, and the fibrosis development is progressive (49, 116).

Utilizing this model, male, 5-6 weeks old, C57BL/6, ADORA2B$^{f/f}$LysM^{Cre}, HIF1A$^{f/f}$LysM^{Cre}, LysM^{Cre}, and Hif1α::LUC reporter mice were treated with 8 intraperitoneal bleomycin (0.035U/g, Teva Pharmaceutical, Irvine, CA) or saline injections, twice a week over a four week period as described previously (Figure 2.1A,B,C) (83, 113). HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice were sacrificed and samples collected on day 3, 7, 14, 20, and 33. ADORA2B$^{f/f}$LysM^{Cre}, LysM^{Cre}, and C57BL/6 were sacrificed and samples collected at the following time points after bleomycin or saline exposure: 14, 17, 20, 21, 25, and 33. Samples from Hif1α::LUC reporter mice were collected on day 33.

In vivo Inhibition of HIF1A

In vivo inhibition of HIF1A was carried out using 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG, InvivoGen, San Diego, CA). Male, 5-6 week old C57BL/6 mice were treated with intraperitoneal bleomycin or vehicle control (saline) twice a week for 33 days. Beginning on day 20 and every other day until day 33, mice were treated with saline (100μL 1X sterile PBS) or 25mg/kg 17-DMAG(117). Mice received 17-DMAG injections 3-4 hours before bleomycin on treatment days were both were to be given. Arterial oxygen saturation levels were measured on day 33 prior to animal sacrifice and sample collection.

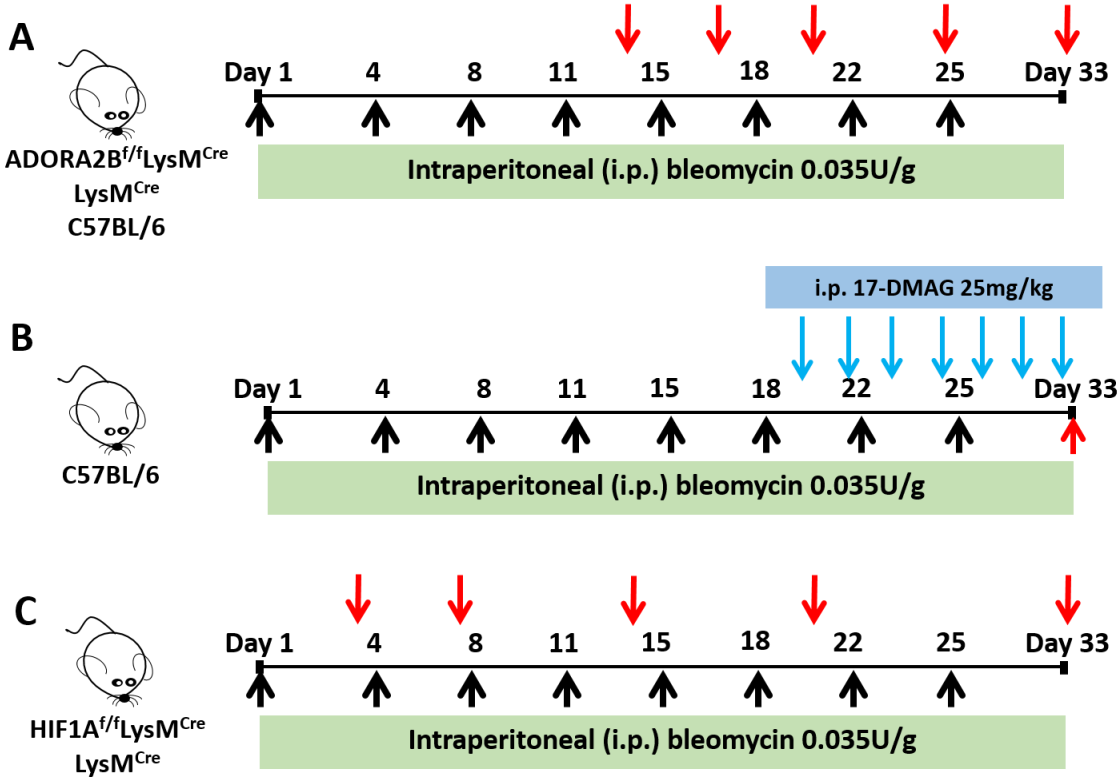

Figure 2.1. Intraperitoneal (i.p.) bleomycin model of pulmonary fibrosis. 8 intraperitoneal bleomycin (0.035U/g, Teva Pharmaceutical, Irvine, CA) or saline injections are administered twice a week over a four week period in the i.p. bleomycin model. (A) In Chapter 3, samples were collected at day 14, 17, 21, 25, and 33 from ADORA2B$^{f/f}$LysM^{Cre}, LysM^{Cre}, and C57BL/6 mice exposed to bleomycin or PBS. (B) 17-DMAG, an inhibitor of HIF1A, was administered every other day from day 20 onwards to evaluate the impact of HIF1A inhibition on fibrosis development in Chapter 4. (C) HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice were sacrificed and samples collected on day 3, 7, 14, 20, and 33 in Chapter 5. (Red arrows indicate sacrifice and sample collection days).

Assessment of Arterial Oxygen Saturation

Mice were shaved along the neck and awake for arterial oxygen saturation measurement on day 33 using the Pulse MouseOx software (STARR Life Sciences Corp, Oakmount, PA).

Plasma, Bronchoalveolar lavage fluid (BALF), Cellular differentials, and Histology

Once mice were anesthetized with Avertin, blood was collected and centrifuged to collect plasma. Lungs were lavaged four times with 0.3mL PBS to collect BALF. A hemocytometer was used to determine total cell counts. Aliquots of BAL cells spun onto microscope slides and stained with Diff-Quick (Dade Behring, Deerfield, IL) were used to assess cellular differentials. BAL fluid remaining was centrifuged and cell pellet and supernatant were stored for further analysis. Once BALF was collected, lungs were pressure inflated, fixed in 10% formalin, and embedded in paraffin. 5μm lung sections were used for immunostaining and further histological analysis.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed on 5μm sections from formalin-fixed, paraffin-embedded lungs. Antigen retrieval was performed on rehydrated sections using Tris-based Antigen Unmasking Solution (Vector Laboratories) followed by blocking of endogenous avidin and biotin using the Biotin-Blocking system (Vector Laboratories, Burlingame, CA), Bloxall (Vector Laboratories) for 30 minutes, and

normal horse serum (Vector Laboratories) for 1 hour at room temperature. Nuclear permeabilization with 1% Triton-X 100 was also carried out for 10 minutes after antigen retrieval and prior to blocking for anti-HIF1A and anti-firefly luciferase staining. Slides for immunohistochemistry were incubated with anti-αSMA (1:1000 mouse monoclonal; Sigma-Aldrich), anti-CD206 (1:100 rabbit polyclonal; Bioss) , anti-iNOS (1:100 rabbit polyclonal; Abcam Inc.), anti-IL-6 (1:100 rabbit polyclonal; Abcam Inc) , or anti-Ly6.b (1:500 rat anti-mouse; AbD SeroTec) overnight at 4°C and developed the following day with the ImmPRESS™-AP Anti-Rabbit IgG (Alkaline Phosphatase) Polymer Detection Kit (Vector Laboratories) prior to counterstaining with haematoxylin, dehydration, and mounting with coverslip.

Dual immunofluorescence staining of F4/80 and CD206 began with primary antibody incubation for CD206 (1:100 rabbit polyclonal; Bioss) overnight at 4°C, development with Vector Red, followed by incubation with F4/80 (1:500 rat polyclonal; Acris Antibodies) and development with goat anti-rat Alexa Fluor-488 (1:500; Life Technologies). For immunofluorescence staining of alpha smooth muscle actin (αSMA), slides were incubated with Cy3-conjugated mouse monoclonal anti-αSMA (1:1000; Sigma-Aldrich) overnight at 4°C, prior to mounting with with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories). For dual immunofluorescence of HIF1A and CD206, slides were incubated in anti-HIF1A (1:200 rabbit polyclonal; Bethyl Laboratories Inc.) overnight at 4°C and then developed using the ImmPRESS™-AP Anti-Rabbit IgG (Alkaline Phosphatase) Polymer Detection Kit described above (Vector Laboratories). Slides were next incubated in anti-CD206 (1:100 rabbit polyclonal; Bioss) overnight at 4°C prior to secondary incubation with Alexa 633 goat anti-rabbit (1:500; Life Technologies) and

mounting with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories). Dual immunofluorescence staining of Firefly Luciferase and CD206 began with primary antibody incubation with Cy3-conjugated anti-firefly luciferase (1:1000 rabbit polyclonal; abcam) overnight at 4°C, followed by blocking and incubation with anti-CD206 (1:100 rabbit polyclonal; Bioss) overnight at 4°C. Secondary incubation with Alexa 633 goat anti-rabbit (1:500; Life Technologies) was carried out at room temperature the next day followed by incubation with sudan black for 10 minutes, copper sulfate for 10 minutes, and Levisamole (Vector Laboratories) for 5 minutes to quench any autofluorescence and mounting with DAPI (Vector Laboratories).

Immunofluorescence of BAL cells

Cytospun BAL cells from Hif1α::LUC reporter mice after saline or bleomycin exposure, described above, were air-dried, fixed with 3.7% paraformaldehyde for 10 minutes, followed by permeabilization in cold methanol for 10 minutes, and nuclear permeabilization with 1% Triton-X 100 for 10 minutes. Endogenous avidin and biotin were blocked with the Biotin-blocking kit (Vector Laboratories), followed incubation with Bloxall (Vector Laboratories) for 10 minutes, and normal horse serum (Vector Laboratories) for 20 minutes at room temperature. Slides were then incubated over night at 4°C with Cy3-conjugated anti-firefly luciferase (1:500, rabbit polyclonal, Bioss). After washing with TBS-T, slides were blocked again with Bloxall and normal horse serum (Vector Laboratories) before incubation with anti-CD206 (1:500, rabbit polyclonal, Bioss) overnight at 4°C. The following day, slides were incubated with secondary antibody, Alexa Fluor 633 goat anti-rabbit (1:500; Life Technologies) for

one hour at room temperature in the dark. Slides were mounted with coverslip and Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories).

Assessment of Fibrosis

Masson's trichrome collagen staining was performed according to the manufacturer's instructions on rehydrated lung sections (EM Science, Gibbstown, NJ). A modified Ashcroft scoring method was used to quantify fibrosis among stained slides (118). Slides were blinded to reviewers with respect to treatment group. Soluble collagen content in BALF was measured using the Sircol assay (Biocolor Ltd., Carrick, UK). Whole lung RNA was used to quantify collagen transcripts and whole lung protein lysates utilized for determining fibronectin expression.

Western Blot Analysis

Mouse lung tissues were pulverized or cells from bone marrow-derived macrophages in culture were lysed in RIPA lysis buffer (50mM Tris-HCL pH 7.4, 150 mM NaCl, 1% NP-40) containing a protease inhibitor cocktail to extract protein (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein were separated on SDS-PAGE and transferred to PVDF membranes (GE Healthcare, Life Sciences, Pittsburgh, PA). Membranes were blocked for 1 hour at room temperature with 5% nonfat milk and then washed with Tris-buffered saline-Tween-20 and incubated with primary HIF1A (1:500 rabbit polyclonal; LifeSpan Biosciences), ADORA2B (1:1000 rabbit polyclonal; LifeSpan Biosciences), CD206 (1:250 rabbit polyclonal; Bioss), fibronectin (1:1000 rabbit polyclonal; Sigma-Aldrich), collagen I (1:1000 rabbit

polyclonal; abcam), β-actin (1:1000 rabbit polyclonal) overnight at 4°C. Washed membranes were incubated with corresponding secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology, Danvers, MA, USA) for 1 hour at room temperature and developed with Amersham ECL Prime Western Blotting Direction Agent (GE Healthcare, Life Sciences) or Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

Analysis of whole lung and BAL cell pellet RNA

RNA was isolated from frozen lung tissue, bronchoalveolar lavage cell pellet, or macrophage cell lysate using TRIzol (Invitrogen) as per manufacturer's instructions. cDNA was synthesized using the iScript™ cDNA synthesis kit (BioRad) and equal amounts were analyzed for transcript levels of COL1A1, COL1A2, ARG-1, ADORA2B, NOS2, IL-6, and CXCL1 with normalization to beta actin or 18s rRNA. Primers for COL1A1 were forward 5'-GCTCCTCTTAGGGGCCACT-3' and reverse 5'-CCACGTCTCACCATTGGGG-3'. Primers for COL1A2 were forward 5'-AAGGGTGCTACTGGACTCCC-3' and reverse 5'-TTGTTACCGGATTCTCCTTTGG-3'. Primers for ARG-1 were forward 5'-CAGAAGAATGGAAGAGTCAG-3' and reverse 5'-CAGATATGCAGGGAGTCACC-3'. Primers for ADORA2B were forward 5'-GCGTCCCGCTCAGGTATAAAG-3' and reverse 5'-CGGAGTCAATCCAATGCCAAAG-3'. Primers for NOS2 were forward 5'-GTTCTCAGCCCAACAATACAAGA-3' and reverse 5'-GTGGACGGGTCGATGTCA-3'. Primers for IL-6 were forward 5'-TAGTCCTTCCTACCCCAATTTCC-3' and reverse 5'-TTGGTCCTTAGCCACTCCTTC-3'. Primers for CXCL1 were forward 5'-CTGCACCCAAACCGAAGTC-3' and reverse 5'-AGCTTCAGGGTCAAGGCAAG-3'.

Primers for MCP-1 were: forward 5'-TCAGCCAGATGCAGTTAACGC-3' and reverse 5'-TGATCCTCTTGTAGCTCTCCAGC-3'. Next, the comparative C_t method was used to present data as mean normalized transcript levels.

Quantification of Protein in Bronchoalveolar Lavage Fluid

IL-6 levels in BALF from C57BL/6 and ADORA2B$^{f/f}$LysM^{Cre} mice after bleomycin or PBS treatment was quantified using the murine IL-6 ultrasensitive kit (Meso Scale Discovery) and read on the Sector Imager 2400 (Meso Scale Discovery) per manufacturer's protocol. Collagen levels were assessed in BALF from C57BL/6 mice after PBS or BLM exposure ± 17-DMAG on day 33 utilizing the BioColor kit per manufacturer's instructions. The Mouse ELISA Kit from Immunology Consultants Laboratory was used to quantify albumin levels and Hycult Biotech ELISA Kit to assess MPO levels in BALF from HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice after PBS or BLM exposure on day 3 and 7.

Dual-luciferase Assay

Whole lung lysate and BALF from Hif1α::LUC reporter mice after saline or bleomycin treatment was prepared for luciferase activity measurement per manufacturers protocol (Dual-Luciferase Reporter Assay System, Promega Corporation, Madison, WI). Briefly, samples were diluted in Passive Lysis Buffer (PLB, Promega), protein concentration measured using the Pierce^{TM} BCA Protein Assay Kit (ThermoFisher Scientific), and samples were diluted to 0.5μg/uL of PLB in a 96-well plate. Equal volumes of Luciferase Assay Reagent II (LAR, Promega) were added to

each well prior to measurement of Firefly luciferase activity, followed by addition of equal volumes of Stop & Glo Reagent (Promega) to each well and Renilla luciferase activity. Data are presented as relative luciferase activity, or Firefly luciferase to Renilla luciferase activity.

Bone Marrow Macrophage Isolation and Differentiation

Bilateral femurs were isolated from male C57BL/6 mice (5-6 wk old, inbred, Envigo Laboratories). Complete macrophage medium, DMEM (Fisher Scientific) supplemented with 10% FBS and 20% L929 media supplement was next used to flush the marrow cavity. L929 media supplement was made by seeding L929 cells at a density of $5x10^5$ cells per T75 flask in 55mL media of DMEM with 1% HEPES, 1% penicillin-streptomycin, 1% L-glutamine, and 10% FBS. L929 cells were then cultured for 7 days and harvested media was sterile filtered for addition to macrophage culture media. Bone marrow cells were pelleted, resuspended in complete macrophage media and plated in 100mm bacterial dishes at a density of $5x10^6$ cells in 10mL media per dish. Cells were incubated at 37°C, 5% CO_2 for 4 days and then supplemented with 5mL of media. On day 7, adherent bone marrow-derived macrophages were detached, collected, pelleted, and resuspended in macrophage media for seeding in six-well tissue culture plates at a density of $1.5x10^6$ cells/mL and allowed to adhere for 2-3h. Adherent cells were then incubated in macrophage media with and without 20ng/mL Interleukin 4 (IL-4) and 10ng/mL Interleukin-13 (IL-13) (Peprotech, Rocky Hill, NJ) for three days with addition of fresh cytokines daily or 100ng/mL LPS (Sigma) and 30ng/mL IFNγ (PeproTech) for 24 hours. Macrophages were washed and used

for ADORA2A, ADORA2B, STAT-6, and HIF1A stimulation and antagonism experiments (Figure 2.2).

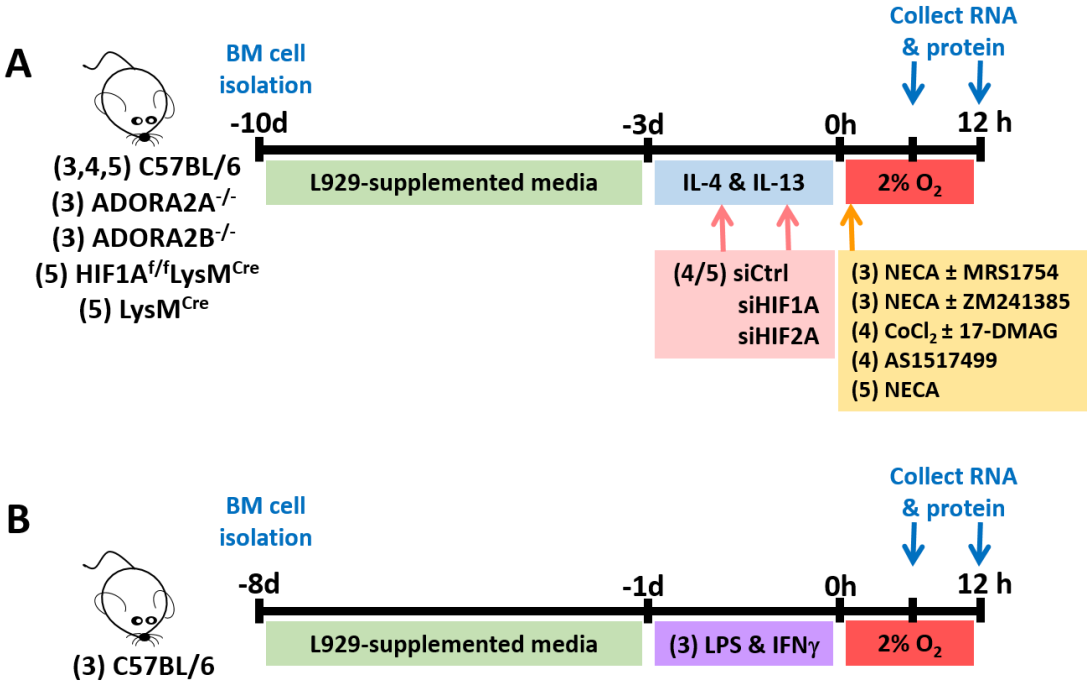

Figure 2.2 Bone marrow derived macrophages isolation, culture, and treatment. Bone marrow (BM) cells were isolated from femurs of C57BL/6 (A,B), (A) ADORA2A$^{-/-}$, ADORA2B$^{-/-}$, HIF1A$^{f/f}$LysM^{Cre}, and LysM^{Cre} mice and cultured with L929-supplemented media for 7 days. In Chapter 3 (denoted (3)), these macrophages were then treated with (A) 10ng/mL IL-4 and 10ng/mL IL-13 for 72 hours or (B) 100ng/mL LPS and 30ng/mL IFNγ for 24 hours followed by 12 hour 2% O_2 exposure alongside treatment with NECA ± MRS1754 and NECA ± ZM241385. In Chapter 4 (4), C57BL/6 BMDMs were treated with $CoCl_2$ ± 17-DMAG at time 0hr or double-transfected with control or siRNA against HIF1A on -2d and -1d prior to hypoxia exposure. In Chapter 5 (5), HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} BMDMs were treated with NECA and C57BL/6 BMDMs were double transfected with control siRNA, siRNA against HIF1A and HIF2A on -2d and -1d. Cells were lysed and RNA and protein collected at 12hours (12h).

Primary Lung Macrophage and Isolation

Mouse lungs from male C57BL/6 mice exposed to bleomycin over 33 days, as described above, were isolated and treated with 1 to 2mL Dispase (BD Bioscience, San Jose, CA, USA) and immediately stored on ice. The lungs were then incubated in 2mL Dispase at room temperature for 45 minutes, diced into 1mm cubes and filtered through 100μm, 40μm, and a 25μm nylon mesh. Filtered cells were incubated in an IgG plate for 45 minutes at 37°C in lung macrophage media (RPMI 1640 (Fisher Scientific), 10% FBS, and 1% penicillin/streptomycin) to select for macrophages. Cells were washed in fresh macrophage media to remove detached cells or other cell-types and allow for macrophages to settle prior to treatment with $CoCl_2$ and 17-DMAG.

Stimulation and Antagonism of Adenosine Receptors, STAT-6, AND HIF1A in Macrophages

Bone marrow-derived macrophages treated with and without IL-4/IL-13 for 72 hours, were next incubated in serum-free media for up to 12 hours. Macrophages were pre-incubated with Adenosine deaminase (PEG-ADA; Roche) to inhibit endogenous adenosine activity prior to 12 hour hypoxia exposure, 2% O_2, and treatment with 10μm 1-(6-Amino-9*H*-purin-9-yl)-1-deoxy-*N*-ethyl-β-D-ribofuranuronamide (NECA, Tocris Bioscience, Minneapolis, MN) or 1μm Bay 60-6085 (Tocris Bioscience, Minneapolis, MN) with and without 1μm N-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-9-yl)phenoxy]-acetamide (MRS 1754, Tocris Bioscience, Minneapolis, MN) to stimulate and antagonize ADORA2B; 10μM ZM241385 (Tocris Bioscience) to stimulate and antagonize ADORA2A; 100nM AS 1517499 (axon

MEDCHEM, Netherlands) to inhibit STAT-6; 3μm 17-DMAG with and without 100μm $CoCl_2$ (Sigma-Aldrich) to stabilize and antagonize HIF1A. After 12 hours exposure with the above treatments, macrophages were lysed in RIPA lysis buffer (20μg of protein per sample) or TRIzol (Invitrogen) for western blot and RNA analysis, respectively.

HIF1A and HIF2A Small Interfering RNA Silencing in Macrophages

Bone marrow derived macrophages from day 7-8 were resuspended in antibiotic-free macrophage media, seeded in 6-well plates, and allowed to adhere for 2-3 hours. Macrophages were then incubated in media containing IL-4, IL-13, Optimem (Invitrogen) with Lipofectamine RNAiMax (Invitrogen), HIF1A siRNA and or HIF2A siRNA, or scrambled control siRNA (Sigma-Aldrich) for 24 hours. Final concentration of siRNA was 100nM. 24 hours later, media was changed and the transfection was repeated with fresh reagents for another 24 hours prior to hypoxia exposure (2% O_2) for 12 hours. Cells were lysed in RIPA lysis buffer with protease inhibitors for western blot analysis (20μg of protein per sample) or TRIzol reagent (Invitrogen) and processed for RNA analysis as described above.

Statistical Analysis

Experiments were repeated at least two times with biological and technical duplicates at minimum. One-way ANOVA was used for comparisons among multiple groups. Unpaired, two-tailed Student's t-test was used for comparisons between two

groups. A p-value of less than or equal to 0.05 was considered statistically significant. All statistical analyses were performed with GraphPad Prism 6.0 software (La Jolla, CA).

CHAPTER THREE

ADORA2B ON MYELOID CELLS CONTRIBUTES TO THE DEVELOPMENT OF PULMONARY FIBROSIS

INTRODUCTION

Note: This chapter is based upon: Karmouty-Quintana H., K. Philip, L.F. Acero, N.Y. Chen, T. Weng, J.G. Molina, F. Luo, J.Davies, N.B.Le, I. Bunge, K.A. Volcik, T.T. Le, R.A. Johnson, Y.Xia, H.K. Eltzschig, and M.R. Blackburn. 2015. Deletion of ADORA2B from myeloid cells dampens lung fibrosis and pulmonary hypertension. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* 29: 50-60. http://www.fasebj.org/content/29/1/50.full Copyright permission granted 06 Feb 2017 for FJ 14-260182.

Adenosine Signaling on AAMs in IPF

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease which affects up to 8 million individuals worldwide (2-4). Despite its significant prevalence, little is known about the mechanisms that lead to the development and progression of IPF(15). There are few effective treatments for IPF and the five year survival rate among patients with IPF is only 43%(28). Prognosis in IPF patients is poor secondary to few effective long-term therapies. As such, there is a significant need to better understand the pathogenesis of IPF in order to develop novel pharmaceutical therapies to prevent and reverse this disease.

Previous work suggests that pulmonary fibrosis results from an abnormal tissue reparative response(53). Evidence suggests that the nucleoside adenosine plays a critical role in the regulation of this response in the lung and contributes to fibrosis (52, 55, 119). Adenosine levels are elevated in patients with IPF and mouse models of lung injury in association with increased inflammation and fibrosis(55, 56). Chronic adenosine accumulation and subsequent activation of ADORA2B can lead to features

of chronic lung disease including inflammation, fibrosis, and tissue remodeling (56). There is evidence that the key effector cell type influenced by ADORA2B receptor signaling in pulmonary fibrosis is the pro-fibrotic AAM subtype, typically activated by Th2 cytokines, IL-4 and IL-13 (94, 113). Recent work also suggests ADORA2B mediates differentiation of macrophages into the AAM phenotype (48, 108).

Experimental Rationale

Previous work by our lab has shown that IPF patients have increased ADORA2B receptor positive alternatively activated macrophages (AAM) and we have also shown that the presence of the ADORA2B receptor on these macrophages is critical to the production of pro-fibrotic cytokines such as interleukin-6 (IL-6)(55, 56). In this chapter, a myeloid-specific ADORA2B knockout mice is exposed to bleomycin along with bone marrow-derived macrophages to investigate the hypothesis that ADORA2B on myeloid cells mediates differentiation of macrophages into the AAM subtype, increases pro-fibrotic mediator production, and the subsequent development and progression of pulmonary fibrosis.

RESULTS

Deletion of ADORA2B on myeloid cells attenuates pulmonary fibrosis in association with improved arterial oxygen saturation

We have previously demonstrated an increase in ADORA2B-expressing AAMs in patients with IPF, however the role of this receptor on myeloid-cells in contributing to pulmonary fibrosis remains to be understood (55). As such, we utilized the well-

established intraperitoneal bleomycin model of chronic lung disease to evaluate how genetic deletion of ADORA2B on myeloid cells would impact the development of fibrosis. C57BL/6, LysM^{Cre}, and ADORA2B$^{f/f}$LysM^{Cre} mice were treated with intra-peritoneal bleomycin or saline for 14, 17, 21, 25, and 33 days. On day 33, whole lung lysate and lung sections were isolated to assess fibrotic markers. Fibronectin protein expression (Figure 3.1 A,B) were increased in C57BL/6 exposed to bleomycin but reduced in myeloid-specific ADORA2B knockout mice after bleomycin exposure. Collagen 1A2 transcript levels, another marker of fibrosis, was increased after bleomycin exposure in control mice but significantly reduced in mice lacking ADORA2B on myeloid cells treated with bleomycin (Figure 3.1C). Immunohistochemistry of lung sections for alpha-smooth muscle actin (αSMA), a marker of myofibroblasts, demonstrated an increase in myofibroblasts after bleomycin exposure which was attenuated in ADORA2B$^{f/f}$LysM^{Cre} after bleomycin treatment (Figure 3.1D). Masson's Trichrome staining for collagen in whole lung sections was also decreased in ADORA2B$^{f/f}$LysM^{Cre} mice after bleomycin exposure as compared to wild-type control mice, C57BL/6, after 33 days of bleomycin treatment; the Ashcroft scoring method was used to quantify these histologic findings over multiple lung sections from each treatment group (Figure 3.2A,B).

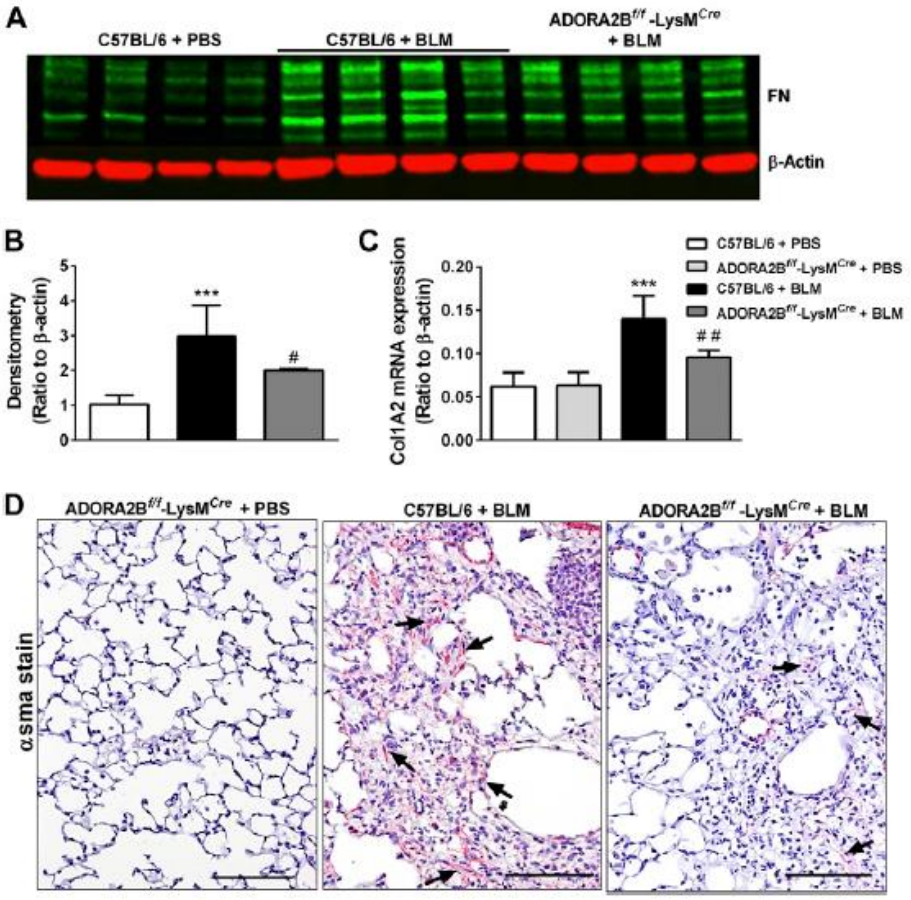

Figure 3.1. Myeloid-specific ADORA2B deletion attenuates fibrotic markers on day 33 after BLM or PBS exposure. [Adapted from Figure 1 of (113)]**.** (A) Immunoblot for fibronectin and β-actin in whole-lung lysates from C57BL/6 mice treated with PBS or BLM and ADORA2B$^{f/f}$LysM^{Cre} mice treated with BLM with densitometry analysis (B). (C) Col1A2 mRNA expression levels from C57BL/6 mice treated with PBS or BLM or ADORA2B$^{f/f}$LysM^{Cre} mice exposed to PBS or BLM. D) Immunohistochemistry for alpha-smooth muscle actin (αSMA) with Gill's hematoxylin counterstain from formalin-fixed paraffin-embedded (FFPE) lung sections from ADORA2B$^{f/f}$LysM^{Cre} mice exposed to PBS (left), C57BL/6 mice exposed to BLM (middle),or ADORA2B$^{f/f}$LysM^{Cre} mice treated with BLM (right). (Positive staining for αSMA is red/pink; Black arrows point at fibroblasts positive for αSMA.) (A-C) Used

with permission from Ning-Yuan Chen and Harry Karmouty-Quintana who performed immunoblot, PCR, and assembled data, respectively. $^{***}P \leq 0.001$, ANOVA comparisons between C57BL/6 + PBS and C57BL/6 + BLM treatment groups. $^{\#}P \leq 0.05$ and $^{\#\#}0.001$, $P \leq 0.01$, ANOVA comparisons between C57BL/6-BLM and ADORA2B$^{f/f}$LysM^{Cre} + BLM treatment groups. Collagen 1a2 mRNA expression assessed using real-time PCR. Data presented as %β-actin. Images are representative of n ≥ 4 animals from each group. Scale bar, 200 mm.

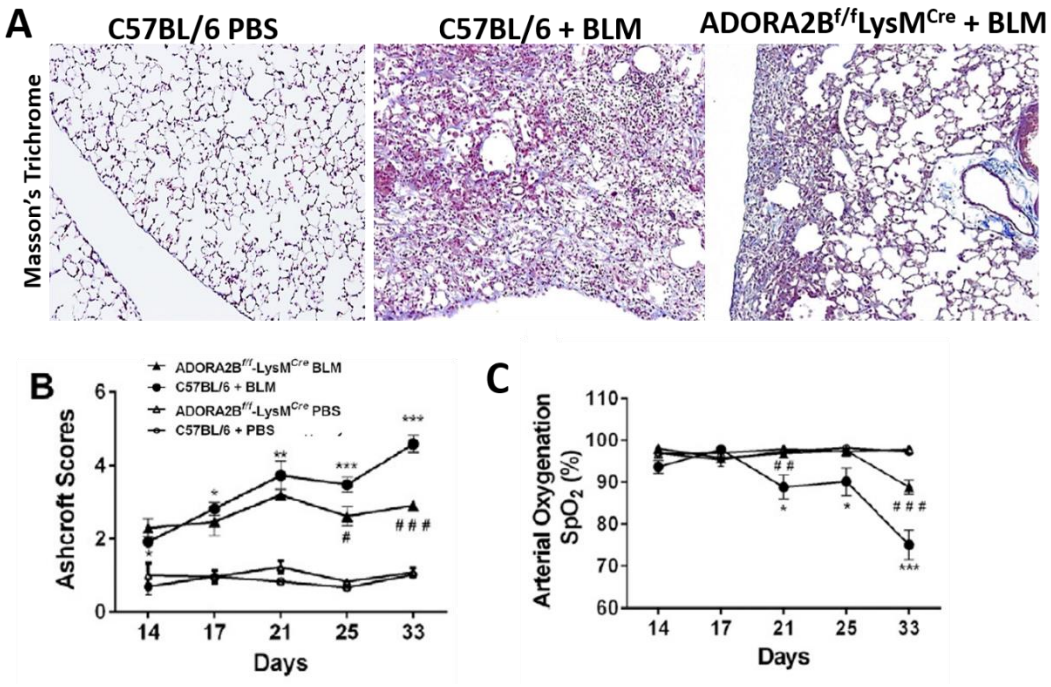

Figure 3.2. Myeloid-specific ADORA2B deletion attenuates development of pulmonary fibrosis after BLM treatment [Adapted from Fig.2 and Fig 3. of (113)]**.** (A) Masson's Trichrome collagen staining in whole lung sections with quantitative, fibrotic histologic scores as given by (B) Ashcroft scores in C57BL/6 and ADORA2B$^{f/f}$LysM^{Cre} mice after 33-day i.p. PBS or Bleomycin. (C) Arterial oxygen saturation in C57BL/6 and ADORA2B$^{f/f}$LysM^{Cre} mice after PBS or BLM treatment. (A-C) Used with permission from Ning-Yuan Chen who performed Trichrome staining and Harry Karmouty-Quintana who performed pulse oximetry measurements and assembled data. Results are presented as mean ± SEM, n = 5-7. $^{***}P < 0.001$, $^{**}0.001 < P < 0.01$, and $^{*}P < 0.05$, comparisons between C57BL/6 + PBS and C57BL/6 + BLM treatment groups. $^{\#\#\#}P < 0.001$ and $^{\#}P < 0.05$, ANOVA comparisons between C57BL/6 + BLM and ADORA2B$^{f/f}$LysM^{Cre} + BLM groups. Images are representative of n ≥ 4 animals from each group. Scale bar: 200μm (4x).

This reduction in fibrotic endpoints in mice lacking ADORA2B on myeloid cells after bleomycin treatment were in association with improved arterial oxygen saturation as compared to bleomycin treatment of C57BL/6 mice (Figure 3.2C). These findings ultimately indicate myeloid-specific deletion of ADORA2B in a bleomycin-induced model of lung fibrosis can attenuate pulmonary fibrosis, suggesting a role for ADORA2B on myeloid cells as a therapeutic target.

BLM exposure of myeloid-specific ADORA2B knockout mice yields reductions in AAMs and subsequent pro-fibrotic mediator production

To distinguish the changes in macrophage cell type after bleomycin exposure in ADORA2B$^{f/f}$LysM^{Cre} mice, lung sections were evaluated for CD206 expression, a marker of AAMs. Immunostaining for CD206 and semi-quantification of CD206+ macrophages over time showed a decrease in AAMs in bleomycin-treated ADORA2B$^{f/f}$LysM^{Cre} mice as compared to C57BL/6 mice after bleomycin exposure (Figure 3.3A,C). Co-immunofluorescence for pan-macrophage murine marker, F4/80, and CD206 also revealed a decrease in F4/80^{+}CD206^{+} macrophages (yellow signal) in myeloid-specific ADORA2B knockout mice after bleomycin exposure as compared to C57BL/6 mice (Figure 3.3B). No significant difference was observed however in iNOS^{+} macrophages, a marker for the classically-activated macrophage (Figure 3.4A,B). These data reveal the attenuation in fibrosis is due to the absence of ADORA2B on myeloid cells reducing AAM expression with no effect on CAMs.

Interleukin-6 (IL-6) is a key pro-fibrotic mediator known to be released by macrophages after activation of ADORA2B(55). IL-6 levels were found to be significantly increased as early as 14 days after bleomycin exposure and continued to

increase by day 33 in bronchoalveolar lavage fluid (BALF) from C57BL/6 mice. However, these levels were significantly reduced in ADORA2B$^{f/f}$LysM^{Cre} mice after bleomycin treatment on days 25 and 33 (Figure 3.5A). Immunohistochemistry for IL-6 shows fewer IL-6 positive macrophages on day 33 in ADORA2B$^{f/f}$LysM^{Cre} mice after bleomycin treatment as compared to control mice, supporting the observed changes in IL-6 levels in BALF (Figure 3.5B).

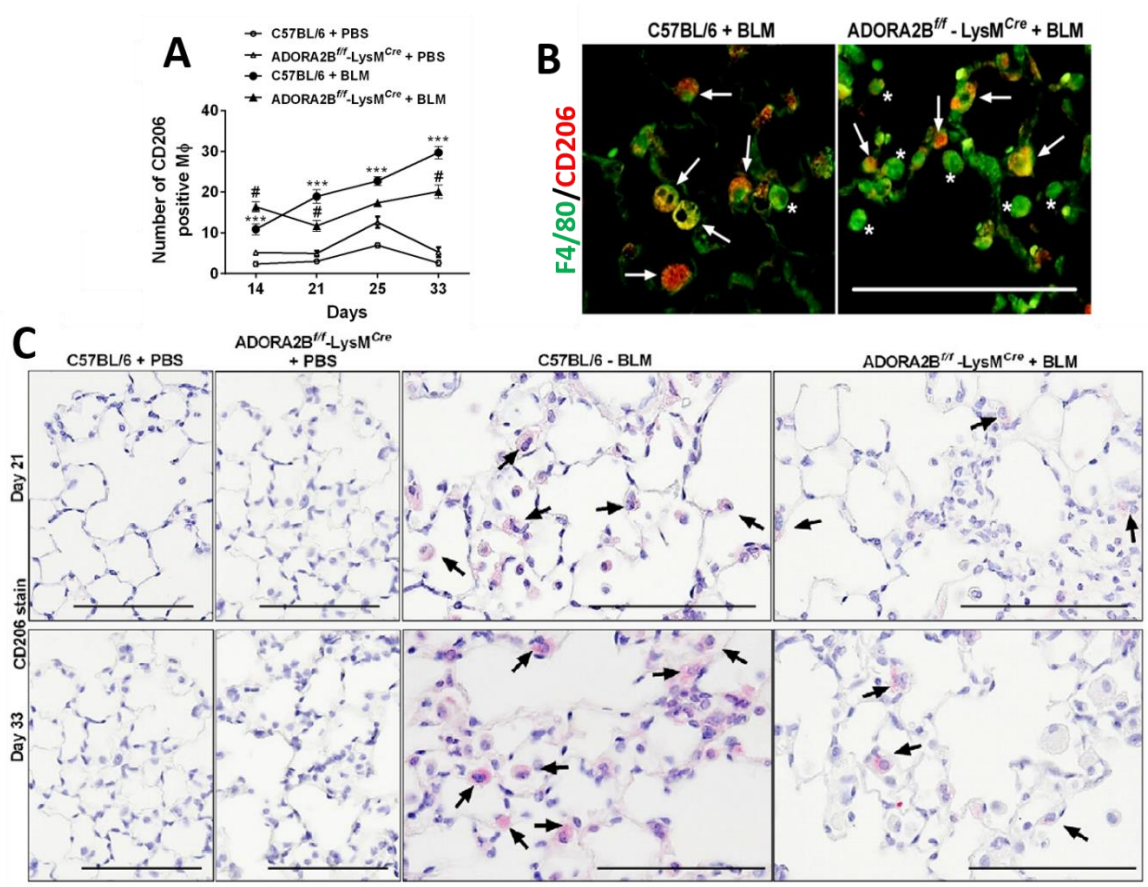

Figure 3.3 Fewer AAMs are observed on day 33 after treatment with BLM or PBS in myeloid-specific ADORA2B knockout mice [Adapted from Figure 7 and Supplemental Figure 2 of (113)]**.** (A) Quantification of CD206+ macrophages identified morphologically from CD206-stained sections from C57BL/6 + BLM, C57BL/6 + PBS, and ADORA2B$^{f/f}$LysM^{Cre} + BLM and ADORA2B$^{f/f}$LysM^{Cre} + PBS groups at 14, 21, 25,

and 33 d of treatment. (B) Double immunofluorescence for F/480 (green signals) or CD206 (red signals) from C57BL/6 mice exposed to BLM (left) and LysM^{Cre} mice exposed to BLM (right) on day 33. White arrows indicate cells that express both F4/80 and CD206 (cells that co-localize F4/80 and CD206 appear yellow). White asterisks represent cells that only express F4/80. (C) Immunohistochemistry for CD206 from formalin-fixed paraffin-embedded (FFPE) lung sections from C57BL/6 + PBS (left) ADORA2B$^{f/f}$LysM^{Cre} + PBS (left middle), C57BL/6 + BLM mice (right middle), or ADORA2B$^{f/f}$LysM^{Cre} + BLM (right) at day 21 (upper row) or day 33 (bottom row). Positive staining for CD206 is red/pink; sections were counterstained with Gill's hematoxylin. Black arrows point at macrophages positive for CD206. $^{***}P < 0.001$ and $^{**}0.001 < P < 0.01$, ANOVA comparisons between C57BL/6 + PBS and C57BL/6 + BLM treatment groups. $^{\#}P < 0.01$, ANOVA comparisons between C57BL/6 + BLM and ADORA2B$^{f/f}$LysM^{Cre} + BLM treatment groups. Images are representative of $n \geq 4$ animals from each group. Scale bar, 200 μm.

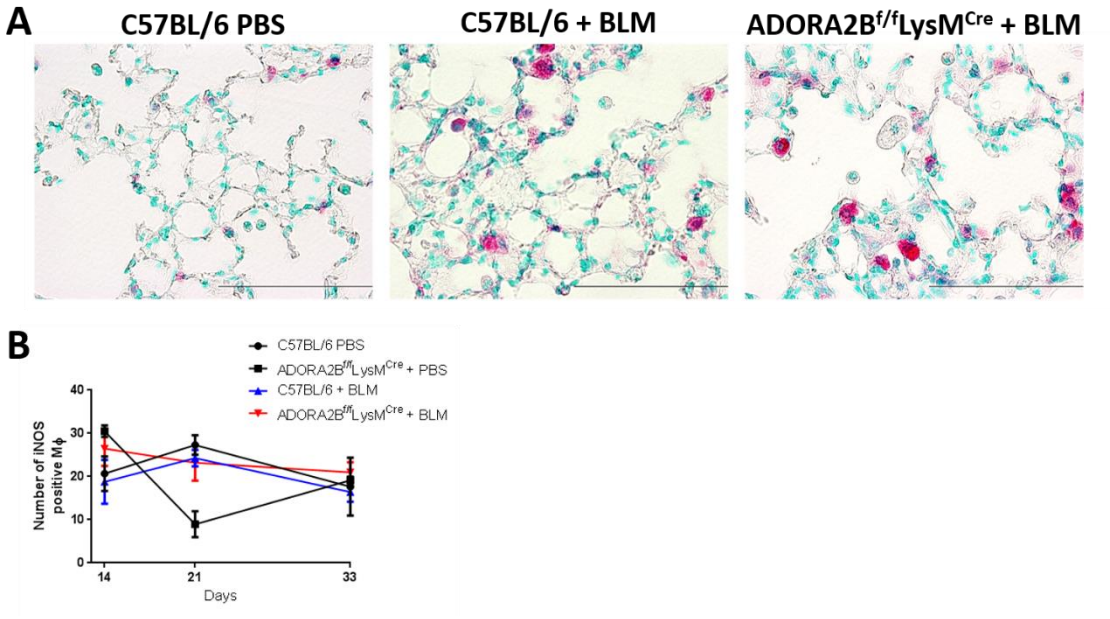

Figure 3.4. Classically-activated macrophages 33 days after BLM or PBS exposure in myeloid-specific ADORA2B knockout mice. (A) Immunohistochemistry for iNOS from formalin-fixed paraffin-embedded (FFPE) lung sections from C57BL/6 + PBS (left), C57BL/6 + BLM mice (middle), or ADORA2B$^{f/f}$LysM^{Cre} + BLM (right) on day 33. Positive staining for iNOS is red/pink; sections were counterstained with methylgreen. (B) Quantification of iNOS+ macrophages identified morphologically from iNOS-stained sections from C57BL/6 + BLM, C57BL/6 + PBS, and ADORA2B$^{f/f}$LysM^{Cre} + BLM and ADORA2B$^{f/f}$LysM^{Cre} + PBS groups at 14, 21, 25, and 33 d of treatment. Images are representative of n ≥ 4 animals from each group. Scale bar, 200 μm.

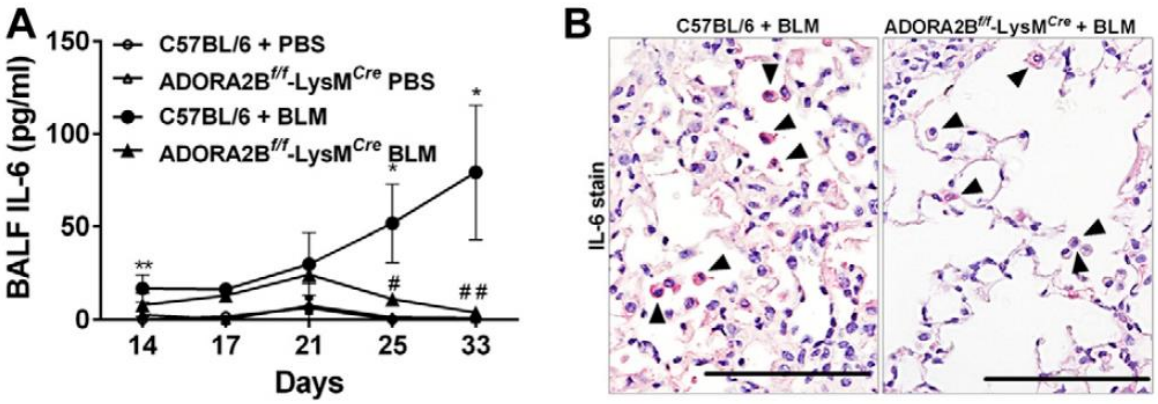

Figure 3.5. IL-6 levels are reduced in ADORA2B$^{f/f}$LysM^{Cre} mice after BLM exposure [Adapted from Fig.8 of (113)]**.** (A) IL-6 levels in BALF from C57BL/6 mice exposed to BLM or PBS and ADORA2B$^{f/f}$LysM^{Cre} mice treated with BLM or PBS at 14, 17, 21, 25, and 33 d after treatment. Immunohistochemistry for IL-6 (B) showing positive staining for IL-6 (red/pink signals) in macrophages (arrowheads) from a C57BL/6 mouse (left) or an ADORA2B$^{f/f}$LysM^{Cre} mouse (right) treated with BLM. (A) Used with permission from Ning-Yuan Chen who performed ELISA for IL-6. Results are presented as mean ± SEM. $^{*}P < 0.05$ and $^{**}0.001 < P < 0.01$, comparisons between C57BL/6 + PBS and C57BL/6 + BLM treatment groups. $^{\#}P < 0.05$ and $^{\#\#}0.001 < P < 0.01$, ANOVA comparisons between C57BL/6 + BLM and ADORA2B$^{f/f}$LysM^{Cre} + BLM groups.

ADORA2B mediates hypoxia-induced macrophage differentiation and production of pro-fibrotic mediators in BMDMs

The data above demonstrate that the absence of ADORA2B on myeloid cells in bleomycin exposure, reduces AAM expression, and subsequent pro-fibrotic mediator production. To further clarify the role of ADORA2B in mediating macrophage differentiation in a robust, in vitro BMDM system, BMDMs were isolated from wild-type (C57BL/6) and mice with global ADORA2B deletion ($ADORA2B^{-/-}$) prior to IL-4/IL-13 co-treatment with hypoxia exposure. $ADORA2B^{-/-}$ BMDMs no longer showed robust increases in Arginase-1 expression or subsequent pro-fibrotic mediator, IL-6, expression supporting a role for ADORA2B in mediating differentiation of macrophages into the pro-fibrotic alternatively-activated subtype as described previously (Figure 3.6A, 3.7C)(48, 108). Similarly, treatment with a pan-adenosine receptor agonist, 10μM NECA, led to significant increases in Arginase-1 expression and subsequent pro-fibrotic mediators, IL-6 and CXCL1, which were reduced in the presence of an ADORA2B-specific antagonist, 1μM MRS 1754 (Figure 3.6B, 3.7A, B). However, genetic deletion of ADORA2B in the presence of Th1 cytokines, lipopolysaccharide (LPS) and interferon-gamma (IFNγ), showed no significant difference in classically-activated macrophage (CAM) marker, iNOS, expression (Figure 3.6C) highlighting the importance and supporting in vivo data above of ADORA2B in mediating macrophage differentiation into AAMs but not CAMs. These data reveal the importance of ADORA2B on macrophages in mediating their differentiation into the pro-fibrotic AAM after hypoxia exposure, and further highlight a role for ADORA2B antagonists in treatment of pulmonary fibrosis.

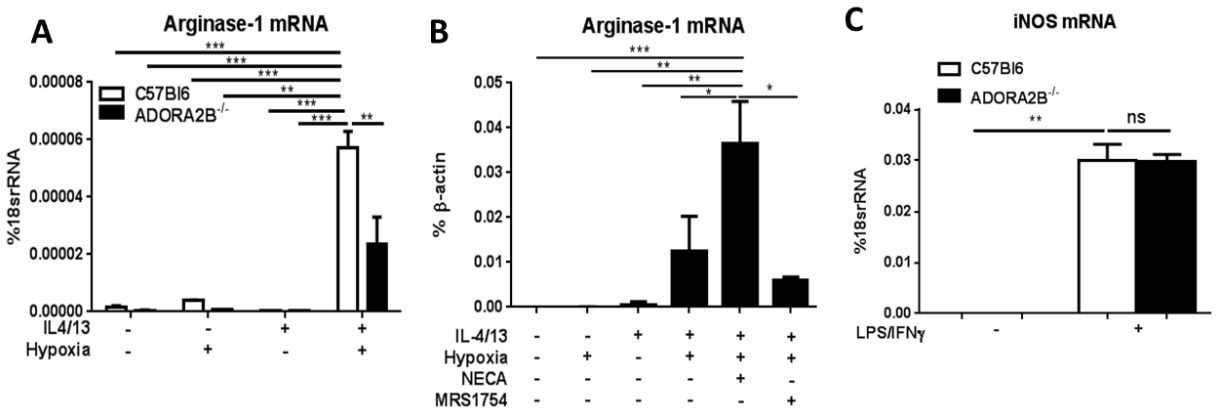

Figure 3.6. Absence or antagonism of ADORA2B disrupts macrophage differentiation into AAMs. C57Bl6 and (A,C) ADORA2B$^{-/-}$ BMDMs were polarized with IL-4 and IL-13 for 72 hours and then incubated in hypoxia (2% O_2) for 12 hours in the presence of (B) 10μM NECA, 1μM MRS1754, or (C) 100ng/mL lipopolysaccharide (LPS) with 30ng/mL interferon-gamma (IFNγ) treatment. (A,B) Arginase-1 and (C) iNOS, mRNA expression was assessed using real-time PCR. Data presented as %β-actin. $^{***}p < 0.001$, $^{**}0.001 < p < 0.01$, and $^{*}p < 0.05$, ANOVA comparisons between treatment groups.

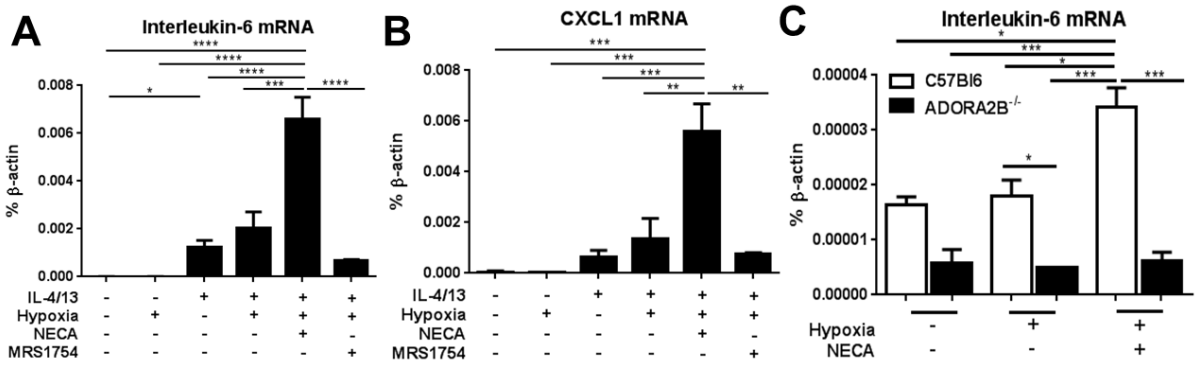

Figure 3.7. Absence or antagonism of ADORA2B disrupts pro-fibrotic mediator production. (A-C) C57Bl6 and (C) ADORA2B$^{-/-}$ BMDMs were polarized with IL-4 and IL-13 for 72 hours and then incubated in hypoxia (2% O_2) for 12 hours in the presence of (A-C) 10μM NECA or (A,B) 1μM MRS1754. (A, C) Interleukin-6 and (B) CXCL1 mRNA expression was assessed using real-time PCR. Data presented as %β-actin. $^{***}p < 0.001$ and $^{**}0.001 < p < 0.01$, ANOVA comparison between IL-4/13 + hypoxia ± NECA treatment vs NECA ± MRS treatment.

DISCUSSION

ADORA2B has been shown extensively to mediate pro-fibrotic effects in pulmonary fibrosis (56). Despite its expression throughout various cell types in the lung as demonstrated through in-situ hybridization previously, work by Zhou et al. have illustrated an elevation in ADORA2B-expressing, CD206+ macrophages in IPF patients and animal models of bleomycin exposure(55, 64). Our studies demonstrate myeloid-specific ADORA2B deletion followed by bleomycin exposure yields reductions in pulmonary fibrosis in association with reduced AAM expression and improved arterial oxygen saturation. A role for ADORA2B in mediating macrophage differentiation into the pro-fibrotic AAM and subsequent pro-fibrotic mediator production was illustrated in vitro using ADORA2B genetic deletion and pharmacologic agonism and antagonism in BMDMs. In accord with previously published work, ADORA2B deletion in vivo after bleomycin treatment and in vitro BMDM work revealed ADORA2B does not mediate differentiation of macrophages into the classically activated pro-inflammatory phenotype. Together, these data suggest a clinically significant role for myeloid-specific ADORA2B antagonists in treating patients with IPF.

This work emphasizes the role of ADORA2B on myeloid cells in mediating the development of pulmonary fibrosis through a mouse model of bleomycin-induced lung fibrosis and in vitro bone marrow derived macrophage system. Bleomycin treatment of mice lacking ADORA2B on myeloid cells resulted in decreased pulmonary fibrosis as evidenced by reductions in fibrotic extracellular matrix proteins including fibronectin and collagen. Decreases in lung fibrosis were associated with fewer CD206+ AAMs in late stages of disease as assessed in lung sections along with significant

improvements in arterial oxygen saturation. These data further support a key role for AAMs in mediating fibrosis and remodeling in the lung. Although fibrosis was attenuated with myeloid-specific ADORA2B deletion, it was not completely eradicated pointing to the importance of other cell-types which may also express ADORA2B in mediating the development and progression of pulmonary fibrosis. As such, further work includes delineating the contribution of other cell types in the lung by exposing conditional knock-out mice for type 1 or type 2 epithelial cells to bleomycin, separately or in combination with myeloid-specific deletion.

A key finding of this work includes demonstrating the role of ADORA2B on mediating differentiation of macrophages into the AAM subtype. AAM marker expression was increased in control BMDMs after hypoxia and Th2 cytokine exposure but significantly decreased in those BMDMs from ADORA2B-deficient mice. Pharmacologic antagonism of ADORA2B in BMDMs also led to disruption of AAM expression as well, in addition to a significant reduction in production of pro-fibrotic mediators, including IL-6 and CXCL1. Of note however, genetic deletion of ADORA2B on BMDMs did not inhibit differentiation of macrophages into the classically-activated macrophage subtype in the presence of Th1 cytokines, IFNγ and LPS. In fact, Th1 cytokine treatment and hypoxia exposure of BMDMS instead leads to robust increase in the ADORA2A receptor (Figure 3.8A) while Th2 cytokine treatment contributes to statistically significant increases in ADORA2B mRNA transcripts as compared to ADORA2B (Figure 3.8B,C). ADORA3 is known to mediate anti-inflammatory effects and predominantly expressed in peripheral mononuclear cells and as such, the increased expression in BMDMs as compared to other adenosine receptors is expected (Figure 3.8B)(120). In fact, we have previously shown that bleomycin

exposure in a global ADORA3$^{-/-}$ leads to no significant differences in pulmonary fibrosis as compared to control ADORA3$^{+/+}$ mice despite initial increases in inflammatory markers indicating a role for ADORA3 in regulating anti-inflammatory features of acute lung injury(69).

In the presence of IL-4 and IL-13 treatment however, both ADORA2B and to a lesser extent, ADORA2A, mediate differentiation of macrophages to AAMs and inhibition of these receptors reduce pro-fibrotic mediator production as evidenced by reductions in Arginase-1 and IL-6 expression with global deletion of ADORA2A or ADORA2B from BMDMs (Figure 3.9A,B) or pharmacologic antagonism in control BMDMs (Figure 3.9C,D).

These findings support work by Hasko et al. which illustrate a role for ADORA2B activation in augmenting the AAM phenotype with production matrix remodeling and anti-inflammatory mediators in addition to ADORA2A activation as a means to promote the pro-inflammatory CAM population by suppressing production of tumor necrosis factor-α (TNFα), interleukin-12, and nitric oxide(48, 108). Further work includes validating the observations above in lung macrophages isolated from bleomycin-treated mice in addition to lung macrophages from patients with and without IPF.

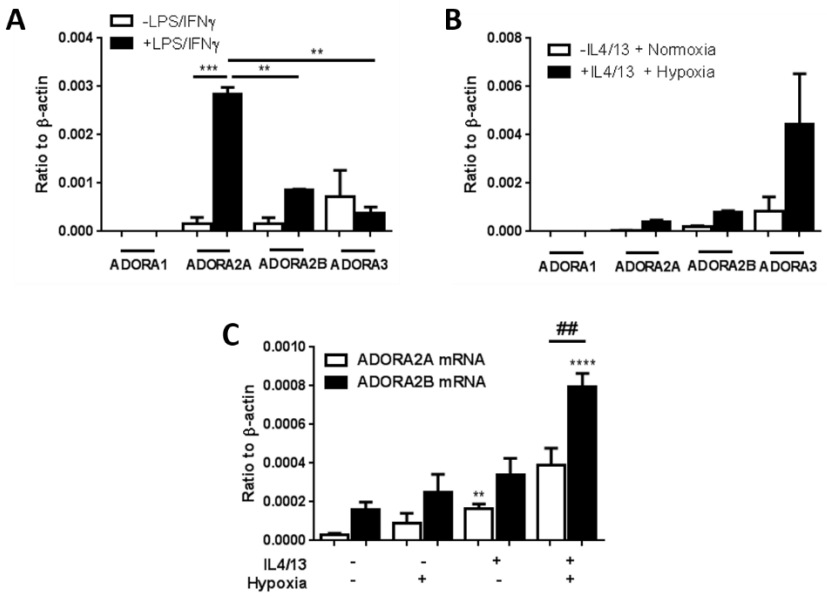

Figure 3.8. T-helper cell cytokine treatment and hypoxia exposure alters adenosine receptor expression. BMDMs were isolated from C57Bl6 mice. On day 7, macrophages were polarized with (A) 100ng/mL LPS and 30ng/mL IFNγ for 24 hours or (B,C) 10ng/mL IL4 and 10ng/ml IL-13 for 72hrs followed by serum starvation for 12hrs. PEG-ADA added 30min prior to incubation at 37°C in 21% O_2 or 2% O_2 for 12 hours. Results represent pooled data from biological duplicate experiments. ADORA1, ADORA2A, ADORA2B, and ADORA3 mRNA expression assessed using real-time PCR. Data presented as %β-actin.

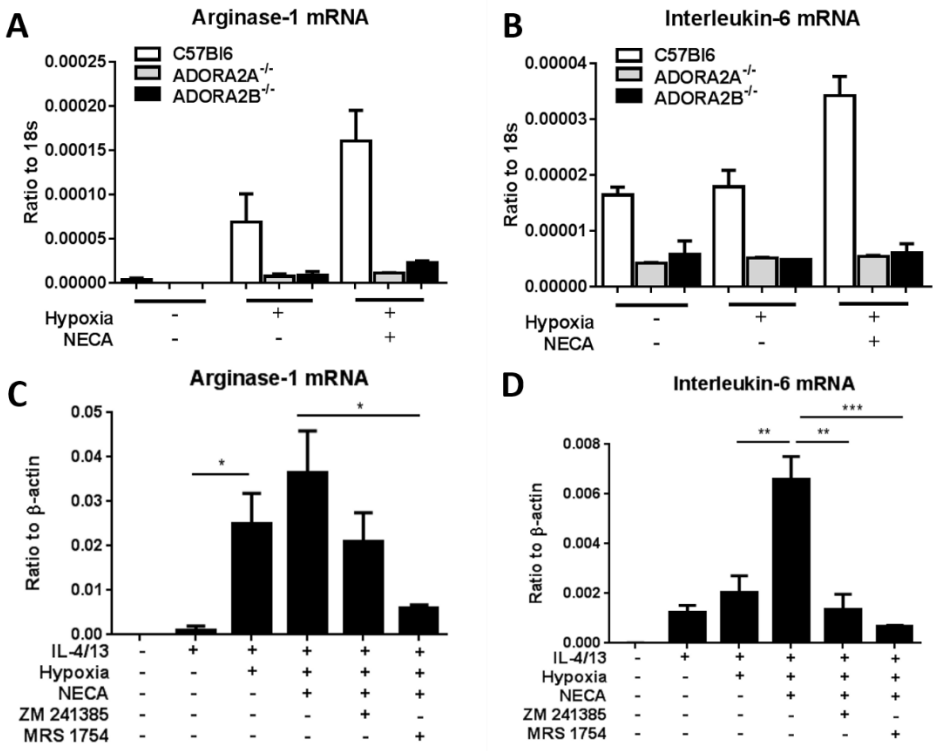

Figure 3.9. ADORA2A and ADORA2B mediate macrophage differentiation and pro-fibrotic mediator production. BMDMs from C57BL/6, ADORA2A-/-, and ADORA2B-/- mice were polarized with 10ng/mL IL4 and 10ng/ml IL-13 for 72hrs followed by serum starvation for 12hrs. PEG-ADA added 30min prior to treatment with 10μM NECA, 10μM ZM 241385, or 1μM MRS 1754. PEG-ADA added 30min prior to incubation at 37°C in 21% O_2 or 2% O_2 for 12 hours. Results represent pooled data from biological duplicate experiments. Arginase-1 and Interleukin-6 mRNA expression assessed using real-time PCR. Data presented as %β-actin.

Together, these data demonstrate the role of ADORA2B on myeloid cells in a bleomycin-induced model of pulmonary fibrosis. They demonstrate that bleomycin treatment of mice lacking ADORA2B on myeloid cells leads to reductions in AAMs in association with improved pulmonary function. These results are supported by in vitro data in BMDMs which reveal the importance of ADORA2B itself in mediating differentiation of macrophages to the AAM phenotype in the presence of both hypoxia and a Th2 cytokine rich environment. Chapter 4 assesses the contribution of hypoxia itself through the hypoxia inducible factor 1A (HIF1A) transcription factor in regulating ADORA2B expression, macrophage differentiation, and the development of pulmonary fibrosis utilizing pharmacologic inhibition in an in vivo model along with BMDMs. Chapter 5 examines the effects of myeloid-specific HIF1A deletion on pulmonary fibrosis development revealing a role for HIF1A in acute stages of lung injury.

CHAPTER FOUR

HIF1A UPREGULATES THE ADORA2B RECEPTOR ON ALTERNATIVELY ACTIVATED MACROPHAGES AND CONTRIBUTES TO PULMONARY FIBROSIS

INTRODUCTION

Note: This chapter is based upon: Philip K, Mills T, Davies J, Chen NY, Karmouty-Quintana H, Luo F, Molina JG, Amione-Guerra J, Sinha N, Guha A, Eltzschig HK, and Blackburn MR. HIF1A Up-regulates the ADORA2B receptor on Alternatively Activated Macrophages and Contributes to Pulmonary Fibrosis. *Under Review.*

In the preceding chapter, we provide evidence that activation of ADORA2B on myeloid cells contributes to the development of lung fibrosis by promoting macrophage differentiation into the pro-fibrotic AAM phenotype in a bleomycin-induced model of chronic lung disease(113). In support of these findings, antagonism of ADORA2B on BMDMs yields reduced AAM expression and subsequent reductions in pro-fibrotic mediators including interleukin-6 and CXCL1. However, the disease signals leading to this are unknown, thereby limiting the development of targeting therapies for IPF.

Hypoxia and Adenosine Signaling

We hypothesized that hypoxia may be the driver of changes in ADORA2B and AAMs in IPF. Hypoxia, or low oxygen supply, is common in IPF patients and is known to regulate elements of the adenosine pathway. On history, physical and laboratory

examination, IPF patients often demonstrate features of chronic hypoxia including shortness of breath, reduced diffusion capacity of the lung and 6 minute walk distance (6MWD), and decreased partial pressure of oxygen in arterial blood(2, 81). Increased immunohistochemical staining for hypoxia inducible factor 1α (HIF1A) on histological evaluation of lungs from IPF patients also supports the presence of hypoxia(82). Similarly, animal models of bleomycin-induced chronic pulmonary fibrosis show decreased arterial oxygen saturation and increased HIF1A in the lungs(83). Hypoxia stabilizes the transcription factor HIF1A which binds to hypoxia-response elements within the promoters of target genes involved in the growth of new blood vessels, programmed cell-death, and glucose metabolism(71). Specifically, HIF1A has been shown to regulate expression of the ADORA2B receptor, CD73, and equilibrative nucleotide transporters (ENTs) which allow adenosine transport across the cell membrane(90, 91). This regulation ultimately leads to elevation of extracellular adenosine levels and enhancement of ADORA2B signaling.

Experimental Rationale

Although hypoxic conditions are present in IPF and animal models of pulmonary fibrosis, the role of hypoxia as a direct modulator of macrophage phenotype remains to be understood. Additionally, factors which regulate adenosine receptor expression on AAMs and their role in IPF are unknown. An understanding of AAMs and previous work demonstrating a pro-fibrotic role for ADORA2B receptor led to hypothesis that both hypoxia through HIF1A and alveolar macrophage differentiation contribute to the progression of pulmonary fibrosis through up-regulation of ADORA2B(55, 56). To address this, I examined lung tissues from

patients with and without IPF along with luciferase reporter mice during the progression of pulmonary fibrosis to show activation and stabilization of HIF1A in alternatively activated macrophages. Next, to understand the role of HIF1A in regulating ADORA2B expression and the development of pulmonary fibrosis, we inhibited HIF1A pharmacologically in late stages of disease in a mouse model and observed attenuated pulmonary fibrosis in association with reductions in ADORA2B and AAM expression and improved respiratory function. Confirming these in vivo findings, in vitro studies in BMDMs support a role for ADORA2B in mediating differentiation of macrophages into AAMs, and further demonstrate that antagonism or silencing of HIF1A leads to reductions in ADORA2B expression and ADORA2B-mediated pro-fibrotic mediator production. These studies shed light on the importance of HIF1A in altering the hypoxic adenosine response in pulmonary fibrosis and presents a novel means to halting the progression of IPF.

RESULTS

AAMs show HIF1A stabilization in patients with IPF

To characterize ADORA2B expression in IPF, whole lung lysate from patients with and without IPF was isolated for mRNA and protein analysis. Both ADORA2B transcript levels and protein expression were increased in patients with IPF as compared to normal controls along with increased HIF1A protein expression (Figure 4.1A, B). Co-immunofluorescence for the AAM marker, CD206, DAPI nuclear signal, and HIF1A were carried out in human lung sections from patients with and without IPF to demonstrate HIF1A stabilization. CD206+ macrophages in patients with IPF show nuclear localization, or co-localization of DAPI signal and HIF1A, suggesting HIF1A

stabilization in comparison to normal lungs (Figure 4.1C). These findings support previous work that patients with IPF show evidence of tissue hypoxemia, leading to stabilization of HIF1A, and further highlight the importance of alternatively activated macrophages as the key effector cell type involved in activation and stabilization HIF1A-mediated pathways.

BLM exposure yields AAMs which exhibit HIF1A stabilization

After demonstrating HIF1A stabilization in AAM's from patients with IPF, we next examined HIF1A stabilization in an experimental model of lung fibrosis using intraperitoneal bleomycin exposure(113). HIF1A luciferase (Hif1α::LUC) reporter mice were injected with bleomycin intra-peritoneally over a four week period to assess HIF1A stabilization and localization in vivo. Luciferase activity, as measured by relative Firefly-luciferase to Renilla-luciferase activity, was increased in whole lung lysate and bronchoalveolar lavage fluid (BALF) from Hif1α::LUC reporter mice after 33-day bleomycin-treatment as compared to PBS treatment (Figure 4.2A, B). Cellular differential of BALF cells demonstrate monocytes and macrophages were the predominant cell-type which exhibited statistically significant increases in luciferase activity after bleomycin treatment (Figure 4.2C).

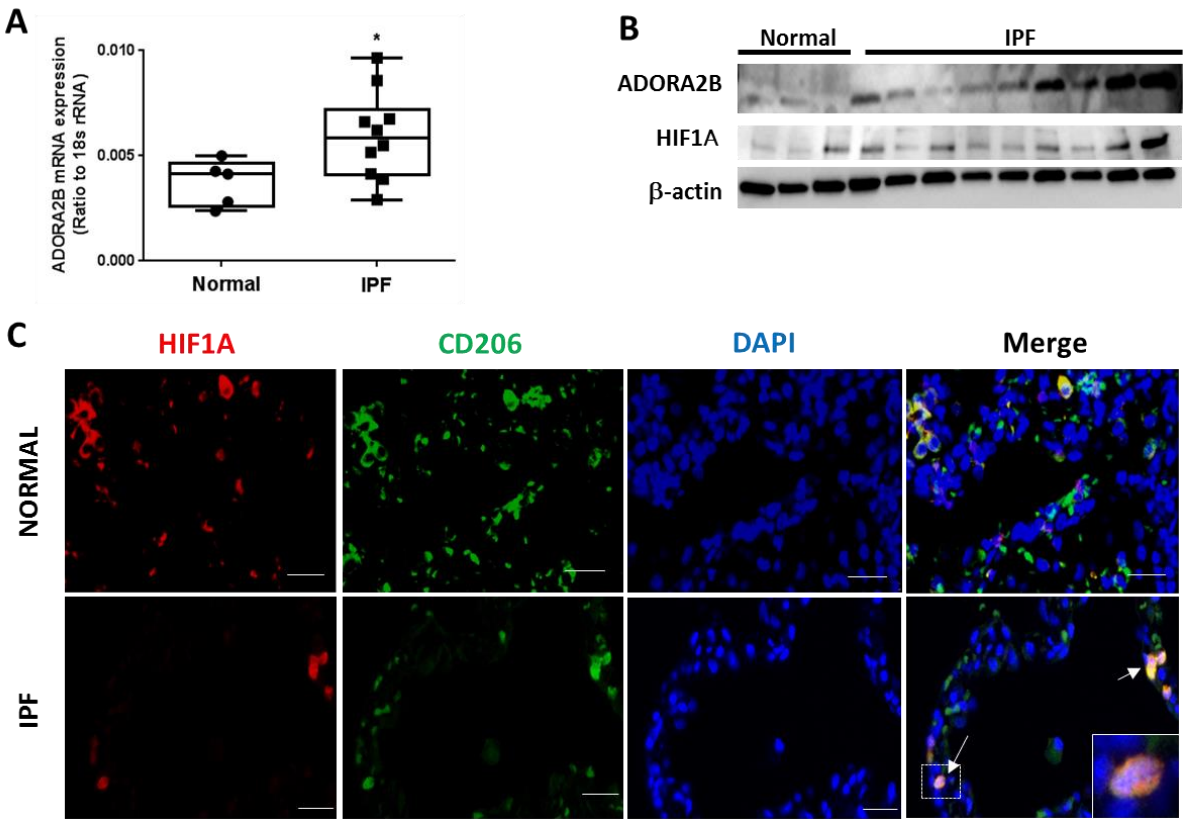

Figure 4.1 CD206+ macrophages exhibit HIF1A stabilization in idiopathic pulmonary fibrosis. (A) ADORA2B mRNA expression in whole lung lysate from patients without (n=5) and with IPF (n=10). ADORA2B expression was assessed using real-time PCR. Data presented as %18s rRNA. (B) Immunoblot for ADORA2B, HIF1A, and β-actin in whole lung lysate from patients without (n=3) and with IPF (n=9). (C) Immunofluorescence for HIF1A (red), CD206 (green), and DAPI (blue) from lung sections of human patients with and without IPF. (White arrow: CD206+ cells that localize red HIF1A signal in the nucleus with DAPI blue-signal, appear to have purple nuclei). Results are presented as mean ± SEM. *$p < 0.05$, Normal healthy lungs vs IPF patients). Scale bar: 25μm (40x).

To better understand localization of HIF1A stabilization after bleomycin exposure, immunofluorescence for Firefly-luciferase, CD206, and DAPI were carried out in lung sections and bronchoalveolar lavage cells. Bleomycin exposure yields increased Firefly-luciferase expression, or HIF1A stabilization, in CD206+ AAMs in whole lung sections and BALF cells as evidenced by greater intensity yellow signal due to co-localization of red Firefly-luciferase and green CD206 signal in comparison to Hif1α::LUC mice treated with PBS (Figure 4.3A, B). These findings support a role for hypoxia-dependent HIF1A stabilization in AAMs in mediating fibrosis in a bleomycin-induced mouse model of pulmonary fibrosis.

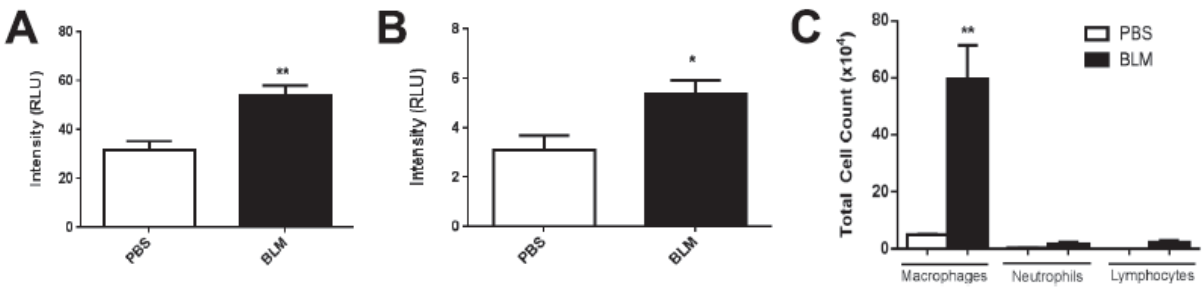

Figure 4.2. Macrophages exhibit HIF1A stabilization in pulmonary fibrosis. Luciferase activity as measured by relative Firefly-luciferase to Renilla-luciferase activity in whole lung lysate (A) and bronchoalveolar lavage cells (B) of Hif1α::LUC reporter mice after treatment with PBS (n = 2) or BLM (n = 4) for 33 days. (C) Cellular differential in bronchoalveolar lavage fluid (BALF) from Hif1α::LUC reporter mice after exposure to BLM or PBS for 33 days. Results are presented as mean ± SEM, n = 2-4. $^{**}0.001 < p < 0.01$, and $^{*}p < 0.05$, PBS vs BLM treatment.

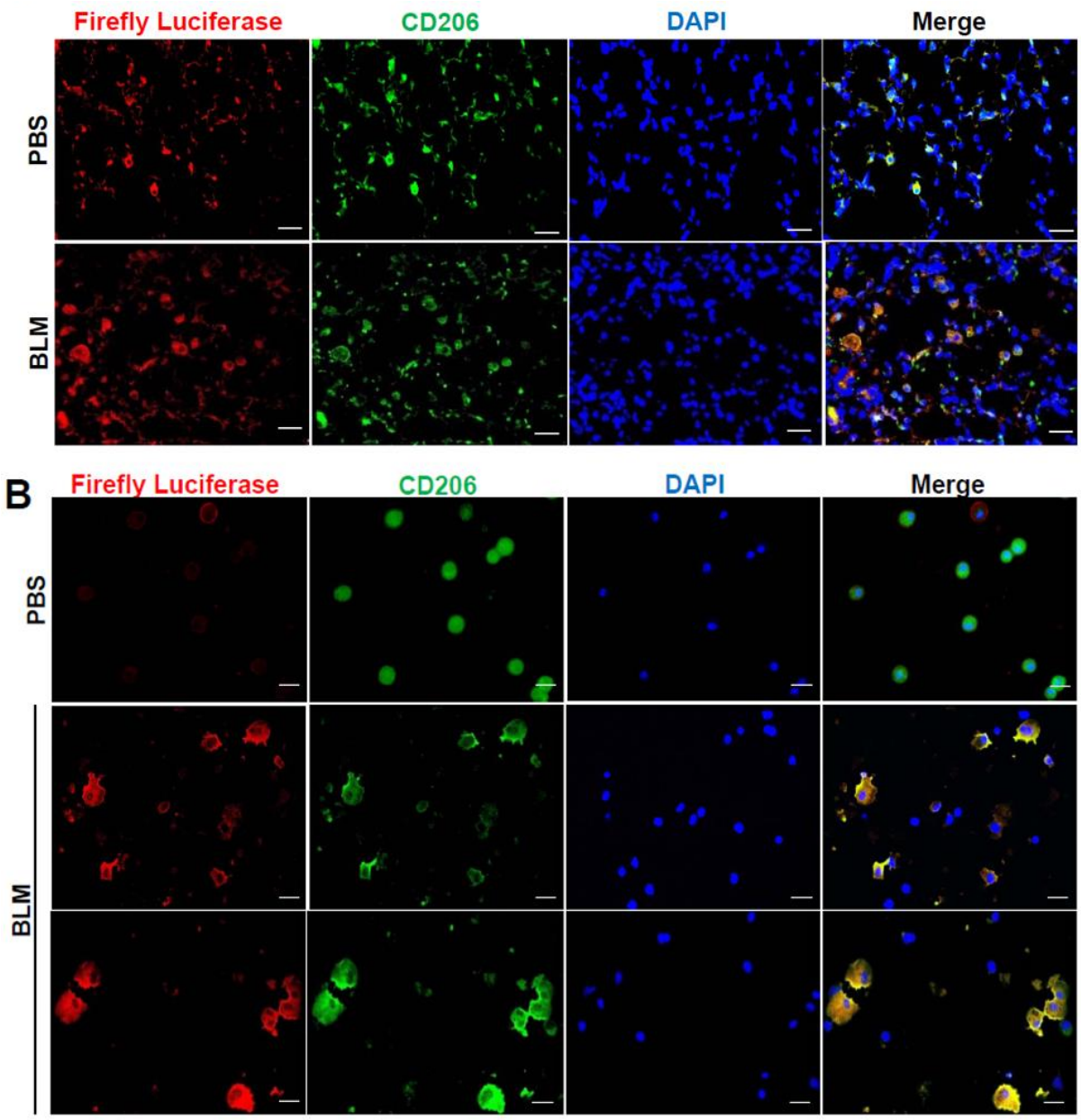

Figure 4.3. CD206^{+} macrophages exhibit HIF1A stabilization in pulmonary fibrosis after BLM exposure. Immunofluorescence for Firefly-luciferase (red), CD206 (green), and DAPI (blue) in (A) formalin-fixed paraffin-embedded (FFPE) lung sections and (B) bronchoalveolar lavage cells in Hif1α::LUC reporter mice after treatment with PBS or BLM for 33 days. Scale bar: 25μm (40x).

A HIF1A inhibitor attenuates pulmonary fibrosis in association with improved arterial oxygen saturation

Our results suggest bleomycin exposure in a mouse model of lung fibrosis and in patients with IPF have increased expression and stabilization of the transcription factor, HIF1A, in AAM's and may mediate the development of lung fibrosis through activation of hypoxia-responsive genes. These findings in concert with previous studies suggesting a role for hypoxia-induced HIF1A in pulmonary fibrosis led us to investigate whether pharmacologic inhibition of HIF1A would attenuate pulmonary fibrosis(88, 121). C57BL/6 mice were exposed to intra-peritoneal bleomycin and a subset were treated with the HIF1A inhibitor, 17-DMAG, beginning on day 20, a time point where lung fibrosis is already evident(56). 33 days after bleomycin or PBS exposure, whole lung lysate and lung sections were isolated to assess markers of fibrosis. HIF1A protein expression in whole lung lysate was reduced in mice treated with bleomycin and 17-DMAG as compared to bleomycin alone demonstrating the efficacy of the pharmacologic antagonist in HIF1A knockdown (Figure 4.4A). Collagen 1A1 transcript levels and Collagen 1 protein expression were increased in C57BL/6 mice after bleomycin treatment as compared to PBS but reduced in the presence of combined bleomycin and 17-DMAG treatment (Figure 4.4B, C, D). Similarly, another marker of fibrosis, fibronectin protein expression, was increased in the cohort exposed to bleomycin as compared to PBS but decreased with bleomycin and 17-DMAG treatment (Figure 4.4E, F). Immunofluorescence for α-smooth muscle actin (αSMA) was next carried out to identify myofibroblasts in lung sections after bleomycin exposure. Myofibroblasts were increased in fibrotic lesions of the lung in mice after

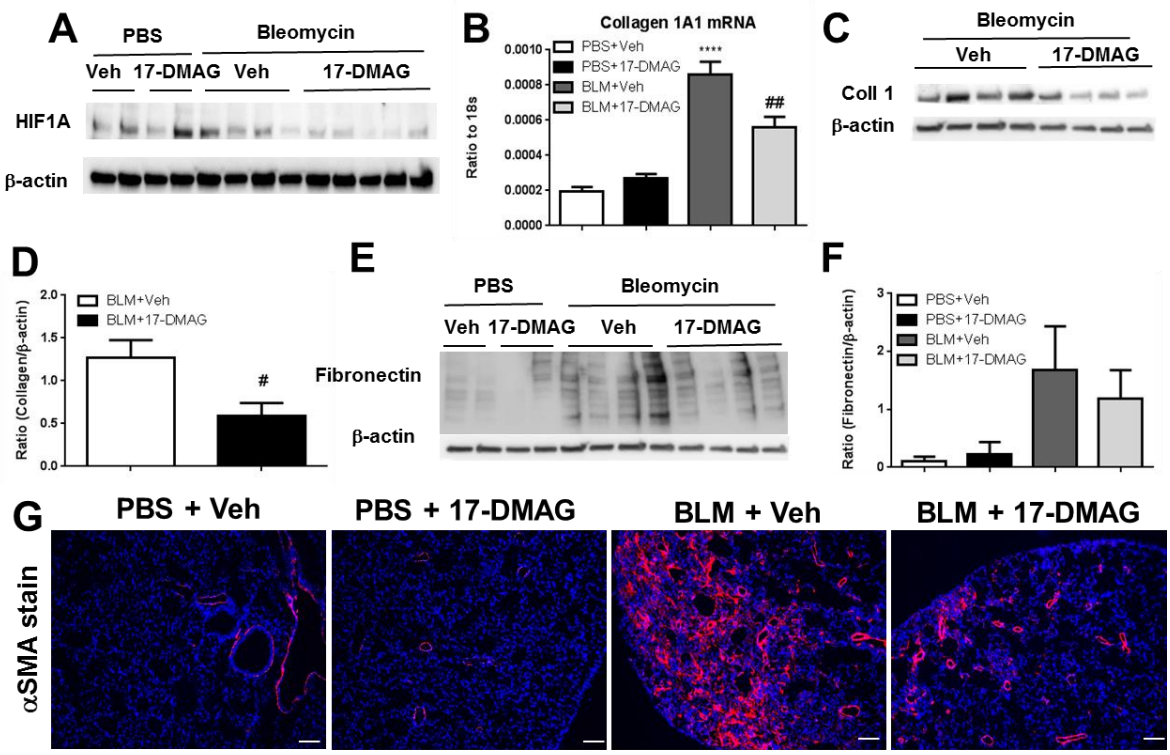

Figure 4.4. HIF1A inhibition attenuates fibrotic markers on day 33 after BLM treatment. Immunoblot for (A) HIF1A, (C) Collagen, and (E) Fibronectin and (A,C,E) β-actin in whole lung lysate from in C57BL/6 mice after 33-day i.p. PBS or Bleomycin treatment with vehicle or 25mg/kg 17-DMAG. (B) Collagen 1a1 mRNA expression in whole lung lysate and densitometry analysis from immunoblots for (D) collagen and (F) fibronectin from C57BL/6 mice after treatment with PBS or BLM ± 17-DMAG. (G) Immunofluorescence for α-smooth muscle actin staining from formalin-fixed paraffin-embedded (FFPE) lung sections from C57BL/6 mice after PBS or BLM ± 17-DMAG treatment. Results are presented as mean ± SEM, n = 5-7. $p < 0.001$ PBS-Veh vs Bleomycin-veh, $^{\#\#}0.001 < p < 0.01$ and $^{\#}p < 0.05$, C57BL/6 Bleomycin-Veh vs C57BL/6 Bleomycin + 17-DMAG. Collagen 1a1 mRNA expression assessed using real-time PCR. Data presented as % 18s rRNA. Images are representative of $n \geq 4$ animals from each group. Scale bar: 100μm (10x).

bleomycin treatment which was attenuated after 17-DMAG treatment of bleomycin-treated mice (Figure 4.4G). Masson's Trichrome collagen staining in whole lung sections was reduced in mice after bleomycin and 17-DMAG exposure as compared to bleomycin treatment alone; these histological findings were quantified over multiple lung sections by Ashcroft scores (Figure 4.5A, B). Collagen levels in bronchoalveolar lavage fluid as measured by Sircol assay were also reduced in bleomycin-exposed mice after 17-DMAG treatment in association with improved arterial oxygen saturation as compared to bleomycin treatment alone (Figure 4.5C, D). These findings reveal treatment with 17-DMAG, an antagonist to HIF1A, during later stages of disease (day 20 onwards) in a bleomycin-induced model of pulmonary fibrosis can attenuate lung fibrosis, thereby suggesting a role for HIF1A antagonists in treatment of pulmonary fibrosis.

HIF1A inhibition attenuates ADORA2B expression and the number of AAMs

Mice treated with intra-peritoneal bleomycin over 33 days showed increased total, macrophage, lymphocyte, and neutrophil cell counts for which 17-DMAG treatment led to statistically significant reductions in total and macrophage cell counts; lymphocytic cells also appeared to be reduced with bleomycin and 17-DMAG treatment although not statistically significant (Figure 5A, B, C, and D).To elucidate these changes in macrophage cell count and type, we next evaluated whole lung lysate and lung sections for CD206 expression, a marker of AAMs. CD206 protein expression was increased with bleomycin exposure yet reduced with 17-DMAG co-treatment (Figure 4.7A, B).

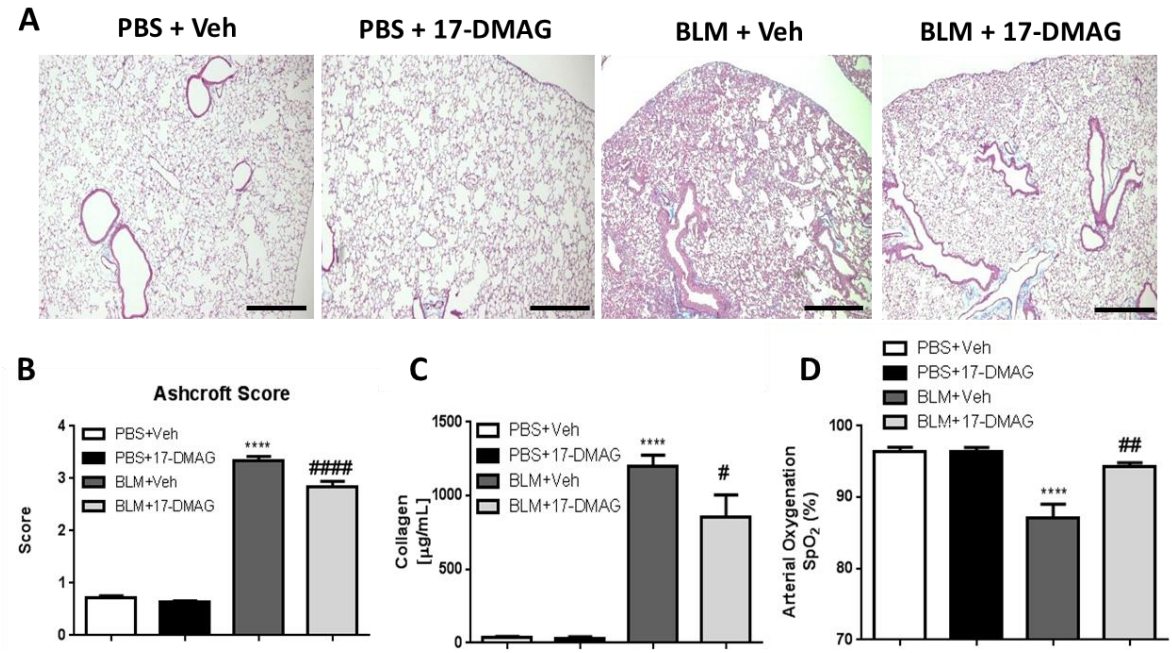

Figure 4.5. HIF1A inhibition attenuates development of pulmonary fibrosis after BLM treatment. (A) Masson's Trichrome collagen staining in whole lung sections with quantitative, fibrotic histologic scores as given by (B) Ashcroft scores in C57BL/6 mice after 33-day i.p. PBS or Bleomycin treatment with vehicle or 25mg/kg 17-DMAG. (C) Sircol Collagen assay in BALF and (D) Arterial oxygen saturation in C57BL/6 mice after PBS or BLM treatment ± 17-DMAG. Results are presented as mean ± SEM, n = 5-7. $^{****}p < 0.001$ PBS-Veh vs Bleomycin-veh, $^{####}p < 0.001$, $^{##}0.001 < p < 0.01$, and $^{#}p < 0.05$, ANOVA comparison between C57BL/6 Bleomycin-Veh vs C57BL/6 Bleomycin + 17-DMAG. Images are representative of n ≥ 4 animals from each group. Scale bar: 200µm (4x).

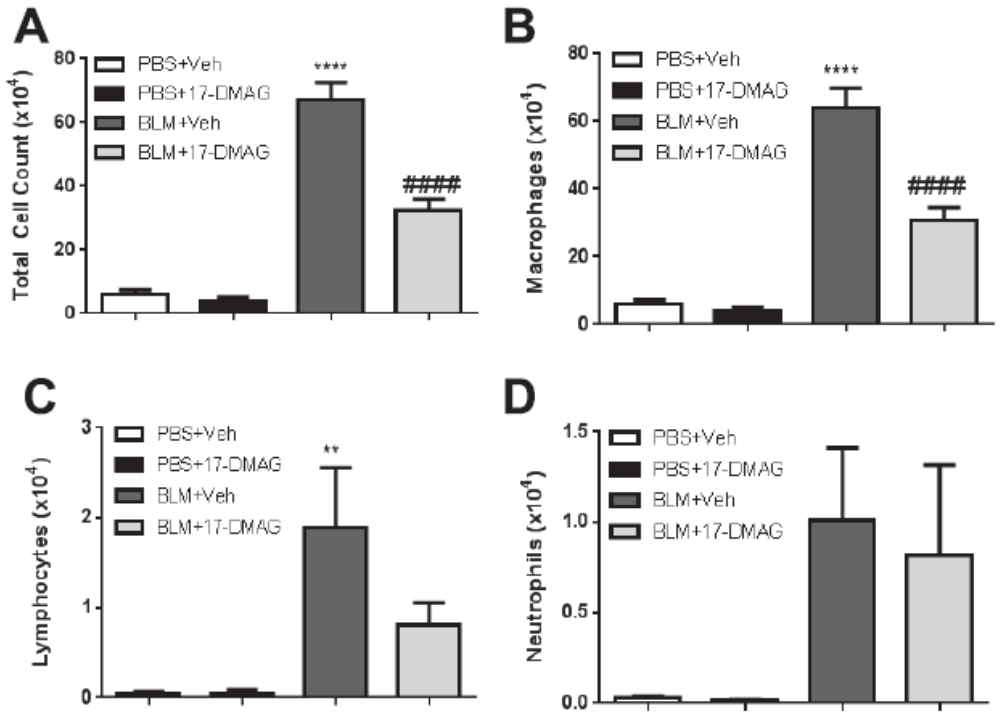

Figure 4.6. Cellular infiltration after 17-DMAG treatment in a BLM-induced pulmonary fibrosis model. (A) Total cell counts, (B) macrophages, (C) lymphocytes, and (D) neutrophils present in bronchoalveolar lavage fluid (BALF) from C57BL/6 mice after 33-day i.p. PBS or Bleomycin treatment with vehicle or 25mg/kg 17-DMAG. Results are presented as mean ± SEM, n = 5-7. $^{****}p < 0.001$ and $^{**}0.001 < p < 0.01$ PBS-Veh vs Bleomycin-veh, $^{####}p < 0.001$ C57BL/6 Bleomycin-Veh vs C57BL/6 Bleomycin + 17-DMAG.

Immunostaining of lung sections for CD206 and semi-quantification of CD206+ macrophages showed increased AAM in bleomycin-treated mice as compared to mice treated with bleomycin and 17-DMAG (Figure 4.7C, D). Previously, we demonstrated a role for ADORA2B expression in myeloid-cells mediating pulmonary fibrosis through up-regulation of pro-fibrotic AAMs and subsequent production of pro-fibrotic mediators including IL-6(113). To assess the role of HIF1A in mediating ADORA2B expression and subsequent pulmonary fibrosis, ADORA2B transcripts were measured in whole lung lysate and BALF cells after bleomycin and 17-DMAG treatment. Results revealed increases in ADORA2B transcript in whole lung lysate and BALF cells which were significantly reduced after treatment with 17-DMAG among BALF cells (Figure 4.7E, F), shown earlier to be predominantly macrophages. These findings demonstrate 17-DMAG treatment attenuates AAMs and ADORA2B expression in lungs of mice after bleomycin exposure.

Inhibition of HIF1A in bone marrow-derived macrophages lowers ADORA2B and Arginase-1 expression

In vivo inhibition of HIF1A after bleomycin exposure demonstrated reduced pulmonary fibrosis consistent with evidence of decreased ADORA2B and AAM expression and improved arterial oxygen saturation. Next, an in-vitro system of bone marrow derived macrophages (BMDMs) was utilized to understand the role of hypoxia through HIF1A in regulating ADORA2B expression and macrophage differentiation into the AAM subtype. BMDMs cultured in the presence of Th2 cytokines, Interleukin-4 (IL-4) and Interleukin-13 (IL-13), as previously described, and exposure to hypoxia (2% O_2) for 6 and 12 hours led to robust increases in AAM marker, Arginase-1

expression, in comparison to BMDMs treated with IL-4 and IL-13 alone (Figure 4.8A, B).

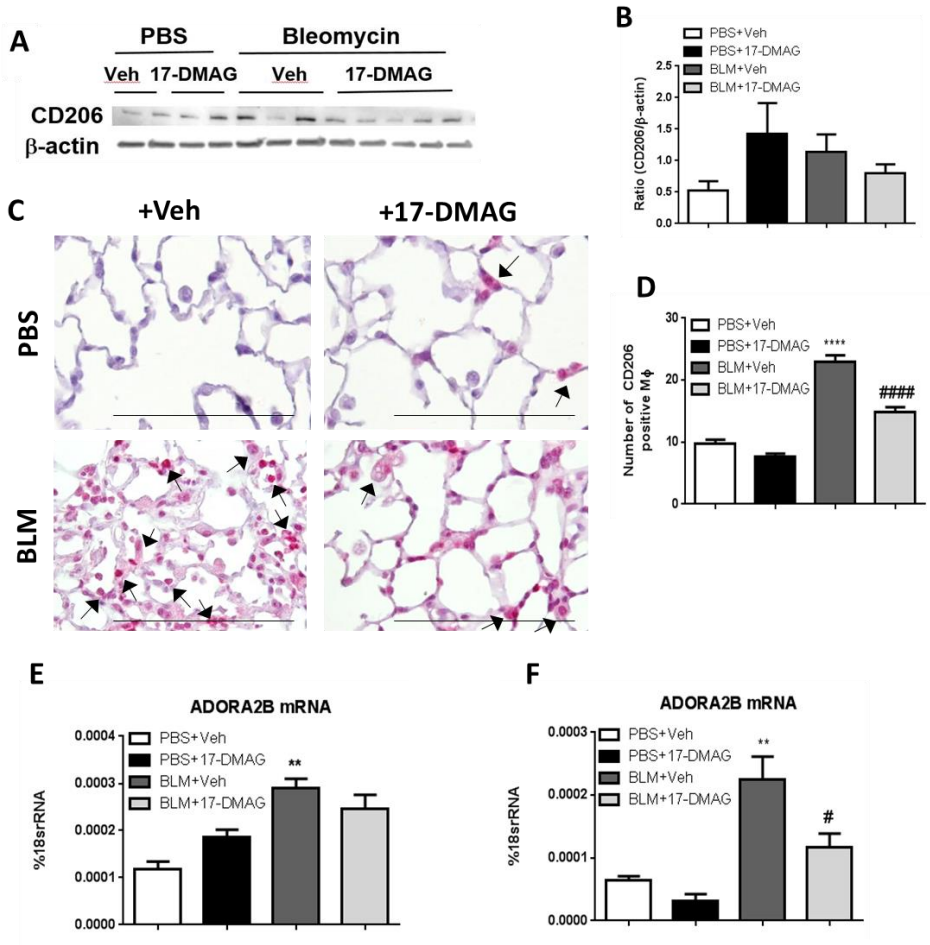

Figure 4.7. HIF1A inhibition reduces ADORA2B expression in BALF cells and subsequent markers of AAMs. (A) Immunoblot for AAM marker, CD206, and β-actin in whole lung lysate from C57BL/6 mice after 33-day i.p. PBS or BLM treatment with vehicle or 25mg/kg 17-DMAG. (B) Densitometry analysis for CD206 immunoblot in C57BL/6 mice after BLM 33 day treatment with 17-DMAG. (C) Immunohistochemistry for CD206 in FFPE lung sections from C57BL/6 mice after PBS or BLM treatment with or without 17-DMAG. Positive staining for CD206 is red/pink; sections were counterstained with Gill's hematoxylin. Black arrows represent macrophages positive for CD206. Scale bar: 200 μm (40x). (D) Quantification of CD206+ macrophages

identified morphologically from CD206-stained lung sections from C57BL/6 mice with PBS or BLM ± 17-DMAG treatment. (E) ADORA2B mRNA expression in whole lung lysate and (F) BALF cells. ADORA2B (D) expression assessed using real-time PCR. Data presented as %18s rRNA. (*p≤0.05 PBS-Veh vs Bleomycin-veh, #p≤0.05 C57BL/6 Bleomycin-Veh vs C57BL/6 Bleomycin + 17-DMAG).

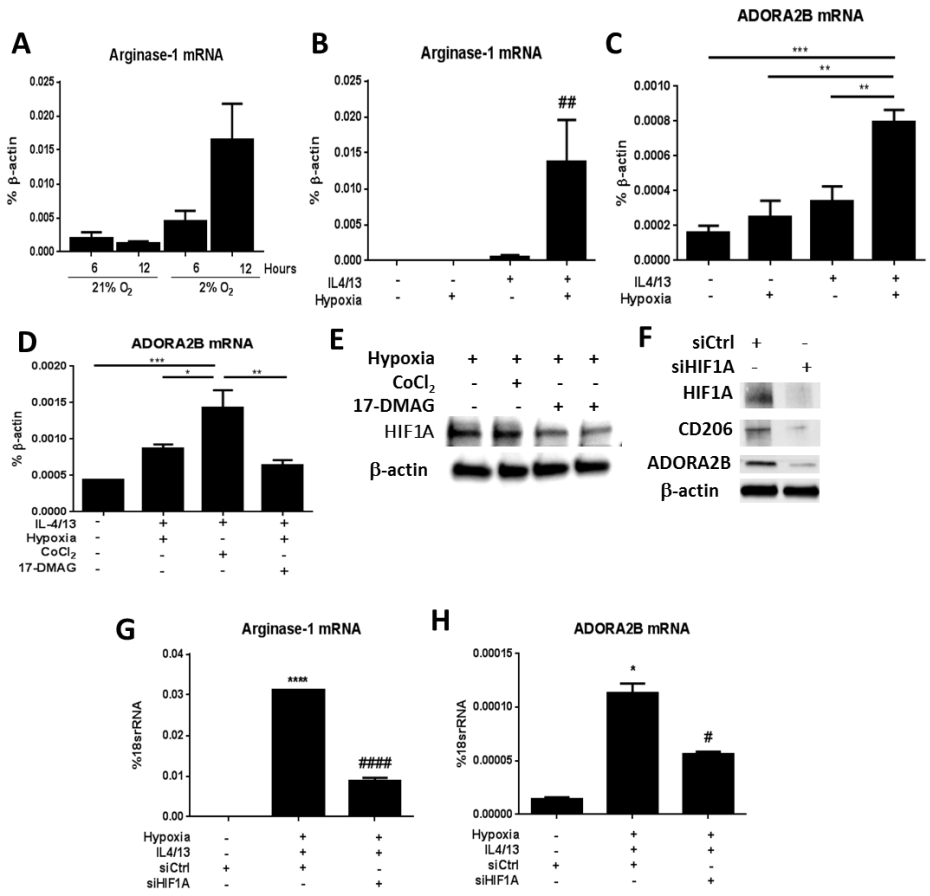

Figure 4.8. Hypoxia exposure yields robust increases in AAMs and regulates ADORA2B and AAM marker expression in vitro through HIF1A. Bone marrow derived macrophages from C57Bl6 mice were treated with 10ng/mL IL-4 and 10ng/mL IL-13 for 72 hours and then incubated in 2% O_2 hypoxia for 6 (A) or 12 (A-I) hours in the presence of (D,E) 100μM $CoCl_2$ or 3μM 17-DMAG, and (F-H) control siRNA and siRNA against HIF1A. Immunoblot for HIF1A (E, F), (F) AAM marker, CD206, ADORA2B, and β-actin after (E) $CoCl_2$ or 17-DMAG and (F) control siRNA and siRNA against HIF1A in BMDMs after 72 hour Th2 cytokine and 12 hour hypoxia treatment . Arginase-1 (A,B,G) and ADORA2B (C, D,H) expression was assessed using real-time PCR. Data presented as %β-actin.

Similarly, ADORA2B expression was robustly increased in the presence of combined IL-4/IL-13 and hypoxia treatment as compared to either IL-4/IL-13 or hypoxia exposure alone (Figure 4.8C). To test the hypothesis that hypoxia through HIF1A is largely responsible for mediating ADORA2B expression on Th2-treated BMDMs, a HIF1A stabilizer, $CoCl_2$, was used in addition to 17-DMAG. Treatment with $CoCl_2$ led to increased ADORA2B expression which was reversed in the presence of 17-DMAG in association with reductions in HIF1A protein expression (Figure 4.8D, E).

Signal Transduction and Activator of Transcription-6 (STAT-6) is known to be the predominant signaling pathway through which IL-4 and IL-13 activate differentiation of macrophages into AAMs (97, 99, 100, 122). To understand the role for STAT-6 as a regulator of ADORA2B expression on AAMs in culture, BMDMs were exposed to IL-4/IL-13 and hypoxia in the presence of a STAT-6 inhibitor, AS 1517499 (100nM)(101). STAT-6 inhibition of Arginase-1 expressing BMDMs led to no significant difference in ADORA2B expression (Figure 4.9A,B).

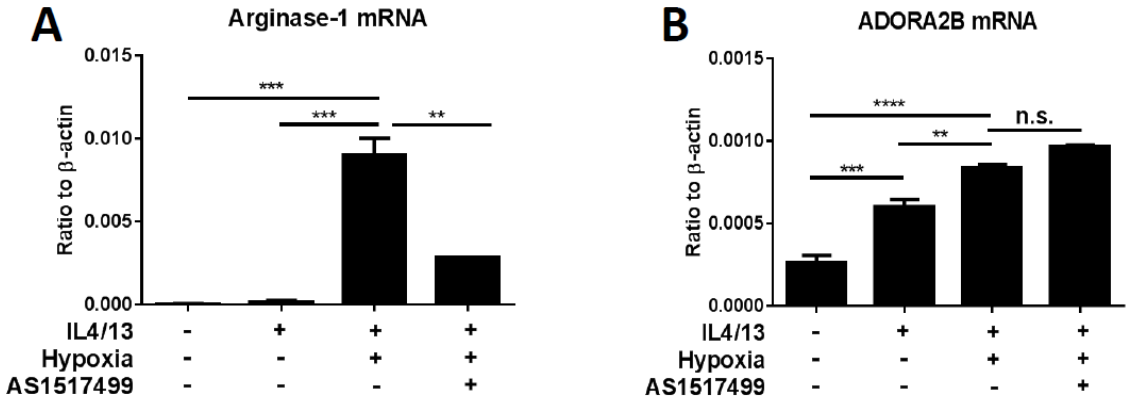

Figure 4.9. ADORA2B expression on AAMs is STAT6-independent. Bone marrow derived macrophages from C57Bl6 mice were treated with 10ng/mL IL-4 and 10ng/mL IL-13 for 72 hours and then incubated in 2% O_2 hypoxia for 12 hours in the presence

of 200nm AS1517599, a STAT-6 inhibitor. Arginase-1 (A) and ADORA2B (B) expression was assessed using real-time PCR. Data presented as %β-actin.

Confirming the results of HIF1A stabilization and inhibition from above with $CoCl_2$ and 17-DMAG, genetic knockdown of HIF1A through siRNA silencing in IL-4/IL-13-treated BMDMs exposed to hypoxia led to reduced HIF1A protein expression and subsequent ADORA2B mRNA and protein expression in conjunction with reduced AAMs, as evidenced by decreased CD206 protein and Arginase-1 mRNA expression (Figure 4.8G, H, and I). Together, these data highlight the role of hypoxia through HIF1A as the predominant driver of ADORA2B expression on Arginase-1 expressing BMDMs.

HIF1A antagonism in lung macrophages after bleomycin exposure reduces ADORA2B and pro-fibrotic mediator expression

To validate the findings above in macrophages after bleomycin-exposure, lung macrophages were isolated from whole lung after intra-peritoneal bleomycin treatment and treated with $CoCl_2$ and 17-DMAG. Treatment with HIF1A stabilizer, $CoCl_2$, led to increases in ADORA2B, IL-6, and CXCL1 expression which were reduced in the presence of the HIF1A inhibitor, 17-DMAG (Figure 4.10A, B, and C). These data suggest a role for antagonism of HIF1A in treatment of pulmonary fibrosis by mediating reductions in ADORA2B expression on AAMs, differentiation of macrophages further into AAMs, and subsequent pro-fibrotic mediator production.

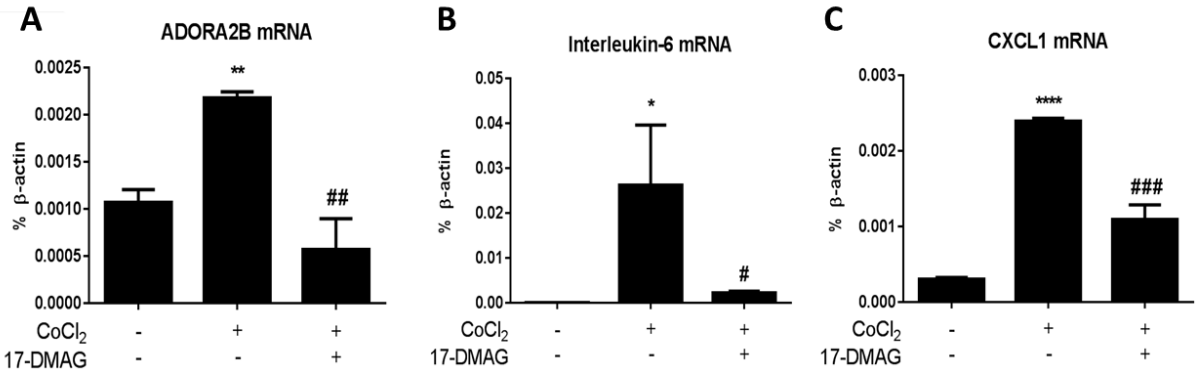

Figure 4.10. Antagonism of HIF1A reduces ADORA2B expression and subsequent pro-fibrotic mediator production in lung macrophages after BLM treatment. Macrophages were isolated from whole lung lysate of C57BL/6 mice after i.p. bleomycin treatment for 33 days. Isolated macrophages were then treated with 100μM $CoCl_2$ or 3μM 17-DMAG for 12 hours. (A) ADORA2B, (B) Interleukin-6, and (C) CXCL1 mRNA expression was assessed using real-time PCR. Results are presented as mean ± SEM, n = 2-4. Data presented as %β-actin. $^{*}p < 0.05$ $CoCl_2$ treatment vs no $CoCl_2$, $^{\#\#}$ $0.001 < p < 0.01$ and $^{\#}p < 0.05$ $CoCl_2$ vs $CoCl_2$ + 17-DMAG.

DISCUSSION

Previous work has demonstrated a role for ADORA2B-mediated pro-fibrotic effects in pulmonary fibrosis(56). Although ADORA2B is expressed throughout the lung, Zhou et al. demonstrated that ADORA2B-expressing, CD206+ macrophages, a marker for AAMs, was elevated in patients with IPF and animals models of bleomycin-induced chronic lung disease(55). AAM's have been implicated in many diseases as a key effector cell type involved in remodeling including asthma and systemic scleroderma(103, 123). Although, it is known that patients with IPF demonstrate

clinical features of hypoxia and hypoxemia, the role for hypoxia as a modulator of adenosine signaling and macrophage differentiation in pulmonary fibrosis remains unknown. Our studies demonstrated the transcription factor HIF1A as a key hypoxia-dependent mediator which is stabilized in IPF patients and mice after bleomycin exposure. We illustrated a role for HIF1A in mediating ADORA2B expression and subsequent AAM differentiation and pro-fibrotic mediator production. Inhibition of HIF1A in vivo attenuated pulmonary fibrosis in association with reduced ADORA2B and AAM expression in association with improved arterial oxygen saturation. These findings were validated in lung macrophages from bleomycin-exposed mice and a robust in vitro bone marrow-derived macrophage system illustrating the involvement of hypoxia through HIF1A being a key regulator of ADORA2B expression on macrophages, their differentiation into the pro-fibrotic AAM subtype, and production of pro-fibrotic mediators including IL-6 and CXCL1. Our data suggest a clinically significant use for HIF1A antagonists in targeting the hypoxic adenosine response as a treatment for patients with IPF.

Hypoxia is known to regulate elements of the adenosine signaling pathway including down-regulation of CD73 and ENTs leading to increase concentrations of adenosine in the extracellular space(90, 91). The presence of a hypoxia response element (HRE) on the promoter of ADORA2B supports the notion that hypoxia through HIF1A will lead to up-regulation of ADORA2B as described previously(88, 90). HIF1A transcript and protein expression have been found to be elevated in patients with IPF and mouse models of bleomycin-induced lung fibrosis(82, 83). A key finding of this work however emphasizes the nuclear localization and stabilization of HIF1A specifically in CD206+ macrophages for the first time in patients with IPF and a mouse

model after bleomycin exposure. These data further support a key role for AAM's as a cell type mediating release of pro-fibrotic mediators including IL-6, which further increase extracellular matrix production and the fibrotic phenotype through IL-6 mediated trans-signaling on fibroblasts in the lung(124). Further studies in macrophages from bronchoalveolar lavage fluid from patients with and without IPF is needed to support whole lung findings of HIF1A stabilization on histology. Results from bleomycin treatment of Hif1α::LUC reporter mice support the importance of other cell-types in stabilization of HIF1A including hypoxia-mediated induction of deoxycytidine kinase which has been implicated in alveolar epithelial cell proliferation(84). Further work is needed to delineate the contribution and interplay of these various cell types in the development and progression of pulmonary fibrosis.

A major finding of this work was the demonstration of a role for HIF1A inhibition in attenuating pulmonary fibrosis in mice after bleomycin exposure. We previously showed a role for the presence of ADORA2B on myeloid cells in mediating the development of pulmonary fibrosis through regulation of AAMs(113). This finding led us to investigate the hypoxia pathways upstream of ADORA2B which may be involved and their role in regulating ADORA2B expression and macrophage differentiation. We showed that treatment with a HIF1A antagonist, 17-DMAG, decreases pulmonary fibrosis as evident by reductions in key extracellular matrix proteins including collagen I and fibronectin. This reduction in fibrosis was associated with improvements in arterial oxygen saturation and significant reductions in ADORA2B transcript levels in bronchoalveolar lavage cells, known to be comprised significantly of macrophages. To further elucidate the role of HIF1A-mediated ADORA2B expression on macrophages, we assessed expression of an AAM marker, CD206, and found

significant reductions in CD206+ macrophages after 17-DMAG treatment in whole lung lysate and histology sections. These findings suggest a critical role for antagonizing hypoxia-dependent HIF1A stabilization through treatment with an antagonist in late stages of disease as a potential therapeutic for patients with IPF. These studies are limited however in that 17-DMAG was delivered systemically through intraperitoneal injection versus localized delivery to the lung; however, western blot analysis of HIF1A in whole lung lysate suggest that despite systemic delivery of the drug, we were still able to achieve significant decreases in HIF1A expression. Recent work suggesting a protective role for HIF1A in epithelial cells in acute lung injury could lead to further experiments investigating the role of HIF1A antagonism or genetic knockdown in early stages of disease as well(89).

Patients with IPF are known to have a Th2 cytokine rich environment as evident through increased presence of IL-4 and IL-13 in bronchoalveolar lavage fluid from patient samples and immunohistochemical staining(125-128). Although patients with IPF are known to be hypoxic, the contribution of hypoxia to Th2 cytokine treatment on ADORA2B expression on macrophages and subsequent differentiation and pro-fibrotic mediator production is unknown. To address this knowledge gap, bone marrow derived macrophages were utilized and we observed a robust increase in ADORA2B expression after hypoxia and Th2 cytokine treatment; however, we further illustrated that this increase in ADORA2B expression on macrophages is predominantly mediated by hypoxia through HIF1A as demonstrated by reductions in ADORA2B expression with addition of HIF1A antagonist treatment to hypoxia as compared to no significant change in ADORA2B expression with addition of a STAT-6 inhibitor. Similar results were observed with reductions in ADORA2B expression, Arginase-1 and

CD206 expression with genetic silencing of HIF1A. These findings were validated in another macrophage system, lung macrophages isolated from mice after bleomycin treatment, wherein co-treatment of 17-DMAG with $CoCl_2$, HIF1A stabilizer, led to reductions in ADORA2B, IL-6, and CXCL1. These data support another major finding from this study that hypoxia through HIF1A regulates ADORA2B expression on macrophages, differentiation of macrophages into AAMs, along with pro-fibrotic mediator production. Antagonism of HIF1A in BMDMs led to decreases in pro-fibrotic mediator production revealing new targets for halting the progression of pulmonary fibrosis in patients with IPF. Future work will include elucidating the impact of hypoxia-independent pathways, whether stretch-induced or metabolite-mediated, on upstream HIF1A stabilization. The HIF2A subunit has also gained attention as it has been shown to play a role in the development of pulmonary hypertension and fibrosis in genetically-modified mice(121). Recent work by Cowburn et al. suggest the development of pulmonary hypertension may be due to hypoxia-induced HIF2A stabilization which increases arginase expression thereby disrupting protective effects of nitric oxide on the pulmonary vasculature(129). As such, further work may include investigating the contribution of HIF2A in the development of pulmonary fibrosis and associated pulmonary hypertension after bleomycin exposure. It may also be insightful to elucidate the possibly differing role of adenosine receptor expression, role of HIF1A stabilization, and differentiation on alveolar, interstitial, or recruited macrophages in pulmonary fibrosis alongside validation in macrophages isolated from patients with and without IPF.

In Chapter 3 of the dissertation, we reveal a role for the presence of ADORA2B on myeloid cells in promoting their differentiation into the AAM subtype, production of

pro-fibrotic mediators, and ultimately, the development of pulmonary fibrosis after bleomycin exposure. The contribution of hypoxia as a regulator of ADORA2B expression on AAMs and development of pulmonary fibrosis is explored in this chapter. I found that antagonism or genetic silencing of HIF1A can attenuate pro-fibrotic mediator production and pulmonary fibrosis in bone marrow derived macrophages (BMDMs) and in vivo models of bleomycin-induced pulmonary fibrosis. In the following chapter, I will examine the effect of myeloid-specific HIF1A deletion on the development and progression of pulmonary fibrosis and a pivotal role which HIF1A plays in acute stages of disease.

CHAPTER FIVE

MYELOID-SPECIFIC HIF1A DELETION EXACERBATES ACUTE LUNG INJURY CONTRIBUTING TO PULMONARY FIBROSIS

INTRODUCTION

Note: This chapter is based upon: Philip K, Mills T, Davies J, Chen NY, Karmouty-Quintana H, Hernandez A, Luo F, Molina JG, Eltzschig HK, and Blackburn MR. Myeloid-specific HIF1A deletion exacerbates acute lung injury. *In Preparation.*

In the previous chapter, I demonstrate a role for ADORA2B on myeloid cells in mediating macrophage differentiation and subsequent pro-fibrotic mediator production in an in vivo bleomycin-induced model of lung fibrosis and bone marrow derived macrophages(113). Chapter 4 then explores how hypoxia through HIF1A acts as a regulator of ADORA2B expression on AAMs and HIF1A inhibition in late stages of disease can be used to attenuate the development of pulmonary fibrosis in an in vivo mouse model. Additionally, bone marrow derived macrophages were used to decipher between the roles of Th2 cytokine signaling through STAT-6 and hypoxia through HIF1A in culture in mediating ADORA2B expression, AAM differentiation, and subsequent interleukin-6 production. Although these studies provide evidence for a therapeutic role of HIF1A or ADORA2B inhibition in the development and progression of pulmonary fibrosis, the role of HIF1A on myeloid cells in altering adenosine signaling and the inflammatory mileau in early stages of lung injury remains to be understood and will be discussed in this chapter.

HIF1A in Lung Disease

Numerous pulmonary diseases are known to result in hypoxia and more attention has been drawn to the interplay between hypoxia and inflammation(51). Systemic or organ-specific hypoxia has been shown to promote an inflammatory response including immunocyte recruitment, increased endothelial cell permeability and resulting vascular leakage, and the activation of proinflammatory signaling pathways, cytokines, and chemokines(130). Inflammation itself, including increases in mediators such as nitric oxide, can contribute to the development of hypoxia and subsequent HIF1A expression, ultimately disrupting epithelial cell wound repair(72, 131). Although multiple mechanisms exist to activate HIF, hypoxia-dependent PHD inhibition and subsequent HIF1A stabilization is the focus of this chapter as HIF1A is known to be stabilized in patients with IPF, mouse models of bleomycin-induced lung injury, and we have demonstrated in the preceding chapters its activation in lung macrophages from patients with IPF and animal models of disease (75, 82, 83)

Recent work by Eckle et al. observed exacerbations in pulmonary edema and inflammation after alveolar-epithelial cell specific HIF1A knockout mice were exposed to ventilator-induced lung injury suggesting a protective, anti-inflammatory role for HIF1A (89). They later showed that stabilization of HIF1A in this cyclic mechanical-stretch induced model of lung injury lead to upregulation of ADORA2B in epithelial cells and subsequent anti-inflammatory effects in agreement with previous work from our laboratory revealing an anti-inflammatory, protective role for ADORA2B itself in acute lung injury(56, 88). In support of a pro-fibrotic role for HIF1A in this same cell type, alveolar epithelial cells, our lab has shown that hypoxia-mediated HIF1A stabilization can also induce deoxycytidine kinase expression in alveolar epithelial

cells contributing to their proliferation and the development of lung fibrosis in a model of bleomycin-induced chronic lung disease (84). Myeloid-specific HIF1A knockout mice showed decreased airway hyperresponsiveness and recruitment of asthmatic inflammatory mediators, eosinophils, when exposed to ovalbumin supporting however a protective anti-inflammatory role of HIF1A in acute lung injury in another cell type (132). Together these studies demonstrate a tissue-protective, anti-inflammatory role for HIF1A in acute stages of lung injury in differing cell types. However, the role of HIF1A in myeloid cells in acute stages of a bleomycin-induced lung injury model and its subsequent effects on late stages of disease remain to be understood and will be the focus of this chapter.

Experimental Rationale

We have previously demonstrated differing roles for ADORA2B depending on the stage of disease. In an intra-tracheal model of bleomycin-induced acute lung injury, ADORA2B$^{-/-}$ mice are found to exhibit increases in pulmonary edema and acute inflammatory markers revealing a protective and anti-inflammatory role for ADORA2B in acute lung injury(54, 56). ADORA2B$^{-/-}$ mice however exposed to an intra-peritoneal model of bleomycin-induced chronic lung disease show reductions in fibrotic markers such as collagen and fibronectin, attenuated fibrosis and profibrotic mediators including macrophages expressing interleukin-6, and clinical improvement demonstrating a detrimental role for ADORA2B in chronic lung disease(56).

The previous chapter directly illustrates how antagonism of HIF1A can lead to reductions in ADORA2B, disrupted macrophage differentiation into the profibrotic

AAM subtype, and subsequently attenuate lung fibrosis; ultimately, revealing a novel therapeutic target for patients with IPF. To investigate the impact of HIF1A on lung disease in acute stages of injury, a myeloid-specific HIF1A knockout mice was generated and exposed to saline or bleomycin over the 33 day time course. Samples were collected at initial stages of injury, day 3, 7 and 14, in addition to late stages of disease, 21 and 33 to evaluate the role HIF1A may play as a regulator in mediating the differing roles of ADORA2B on myeloid cells in acute versus chronic lung injury.

RESULTS

Myeloid-specific HIF1A deletion alters temporal changes in cellular inflammation and increases neutrophil infiltration after BLM exposure

HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice were treated with intra-peritoneal bleomycin over 33 days, sacrificed, and samples collected at varying time points. Overall, mice lacking ADORA2B on myeloid cells showed similar trends but still a significant reduction in total cell count in bronchoalveolar lavage fluid when compared to control mice after bleomycin treatment by day 20 (Figure 5.1A). Upon closer evaluation of the cellular differential in BALF, it is evident that HIF1A$^{f/f}$LysM^{Cre} treated with bleomycin show significant increases in acute inflammatory mediators, including neutrophils by day 7 and lymphocytes by day 14 as compared to LysM^{Cre} mice after bleomycin exposure (Figure 5.1B,C, 5.2A). Macrophage cell counts followed an increase by day 14 and 20 after bleomycin treatment for both HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice although they were significantly reduced in mice lacking ADORA2B on myeloid cells (Figure 5.1D). To further distinguish these changes in neutrophil cell count by day 7,

lung sections were stained for Ly.6b expression, a surface marker found on murine neutrophils (Figure 5.2B) (133). Semi-quantification of Ly.6b positive cells shows increased neutrophils in bleomycin-treated HIF1A$^{f/f}$LysM^{Cre} mice as compared to LysM^{Cre} mice after bleomycin exposure (Figure 5.2C).

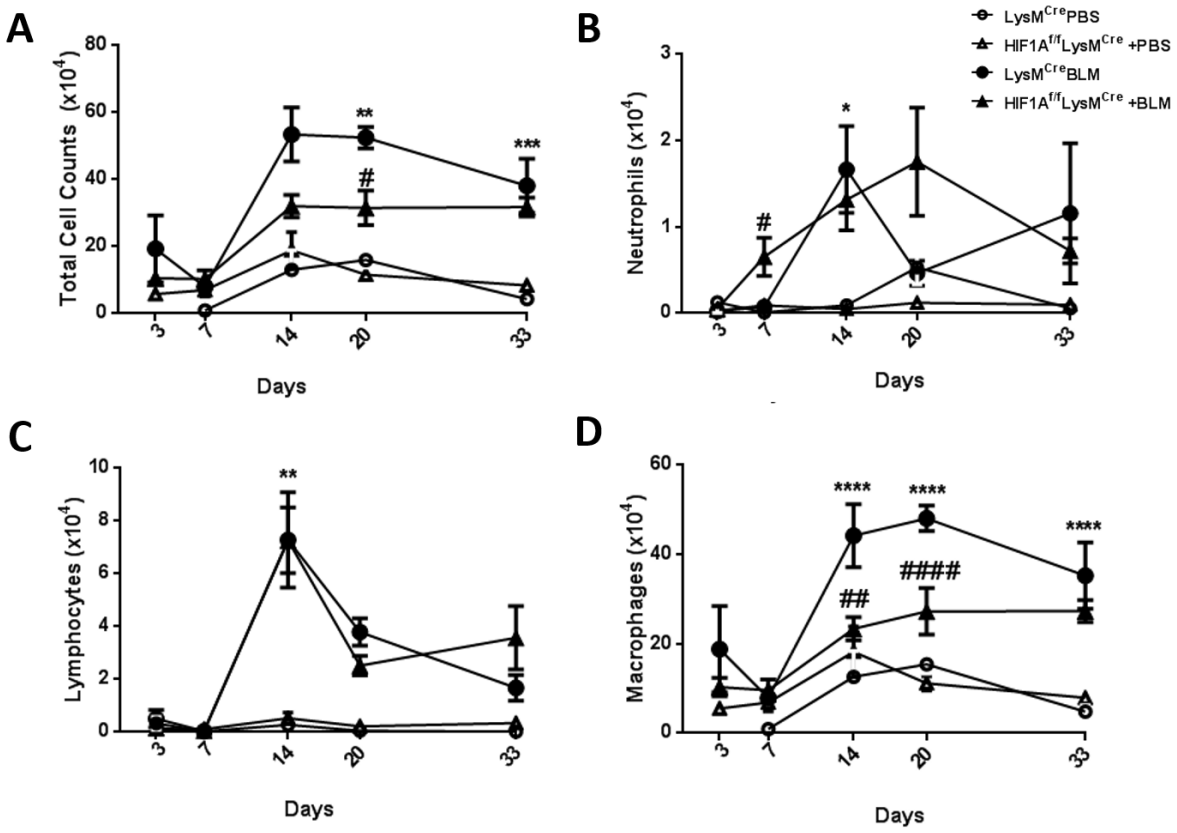

Figure 5.1. Temporal changes in cellular infiltration after BLM exposure. (A) Total cell counts, (B) neutrophils, (C) lymphocytes, and (D) macrophages present in bronchoalveolar lavage fluid (BALF) from HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice after i.p. PBS or Bleomycin treatment for 3, 7, 14, 20, or 33 days. Results are presented as mean ± SEM, n = 5-12. $^{****}p < 0.001$, $^{**}0.001 < p < 0.01$, $^{*}p < 0.05$, ANOVA comparison between LysM^{Cre} + PBS and LysM^{Cre} + Bleomycin. $^{####}p < 0.001$, $^{##}0.001 < p < 0.01$, and $^{#}p < 0.05$, ANOVA comparison between LysM^{Cre} + Bleomycin and HIF1A$^{f/f}$LysM^{Cre} + Bleomycin.

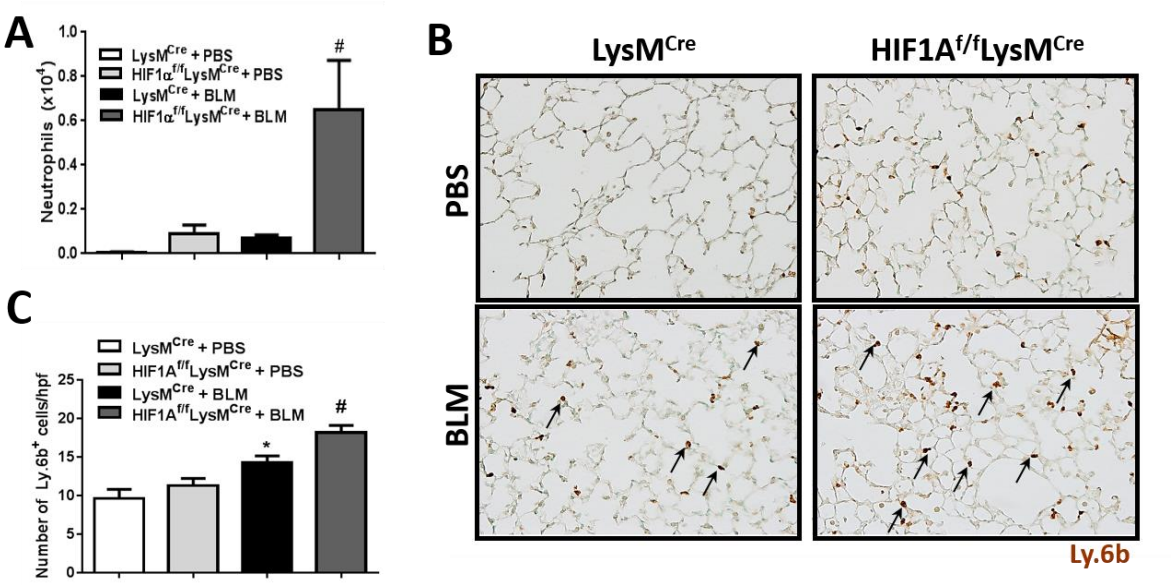

Figure 5.2 BLM exposure in myeloid-specific HIF1A knockout mice increases early neutrophil infiltration. (A) Neutrophils cell counts in bronchoalveolar lavage fluid (BALF), (B) Immunohistochemistry for Ly.6b in FFPE lung sections, and (C) semi-quanitification of Ly.6b positive cells identified morphologically in stained sections from HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice after i.p. PBS or Bleomycin treatment for 7 days. Positive staining for Ly.6b is brown; sections counterstained with methylgreen. Black arrows represent Ly.6b positive cells, or neutrophils. Results are presented as mean ± SEM, n = 5-12. *$p < 0.05$, ANOVA comparison between LysM^{Cre} + PBS and LysM^{Cre} + Bleomycin. $^{\#}p < 0.05$, ANOVA comparison between LysM^{Cre} + Bleomycin and HIF1A$^{f/f}$LysM^{Cre} + Bleomycin. Images are representative of n ≥ 4 animals from each group.

HIF1A deletion on myeloid cells exacerbates acute lung injury after BLM exposure

Results above suggest bleomycin exposure in mice lacking HIF1A on myeloid cells lead to alterations in acute inflammatory mediators including neutrophils and lymphocytes in comparison to control mice after bleomycin treatment. These observations lead us to further investigate other markers of acute lung injury. Next, to assess loss of the pulmonary barrier function and the development of pulmonary edema, we evaluated albumin levels in the BALF on day 7 and found significant increases in HIF1A$^{f/f}$LysM^{Cre} mice after bleomycin exposure as compared to control mice with bleomycin treatment (Figure 5.3A). To further evaluate chemokines involved in neutrophil recruitment in the setting of acute lung injury, we evaluated mRNA levels of monocyte chemotactic peptide-1 (MCP-1) and found increases in control bleomycin-treated LysM^{Cre} mice which were robustly increased after bleomycin treatment in mice lacking HIF1A on myeloid cells (Figure 5.3B) (31, 37, 38). Similarly, another indicator of acute lung injury, Myeloperoxidase (MPO), a major component of neutrophil cytoplasmic granules revealing neutrophil presence, was found to be significantly increased in BALF on day 7 after bleomycin exposure in HIF1A$^{f/f}$LysM^{Cre} mice (Figure 5.3C)(134). These findings reveal myeloid-specific HIF1A deletion followed by acute lung injury through bleomycin exposure will not only increase neutrophil count, but contribute to worsening pulmonary edema, greater neutrophil recruitment, and ultimately, increased inflammation, thereby suggesting a protective role for HIF1A in acute lung injury.

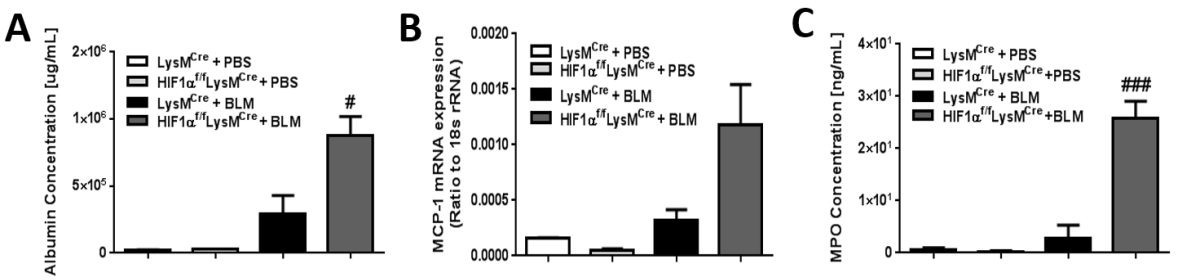

Figure 5.3 Deletion of HIF1A on myeloid cells exacerbates ALI markers in BLM-induced lung fibrosis. (A) Albumin concentration in bronchoalveolar lavage fluid, (B) MCP-1 mRNA expression in whole lung lysate, and (C) myeloperoxidase activity in bronchoalveolar lavage fluid after bleomycin exposure at day 7 in HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice. Results are presented as mean ± SEM, n = 5-12. $^{###}p < 0.001$, $^{#}p < 0.05$, ANOVA comparison between LysM^{Cre} + Bleomycin and HIF1A$^{f/f}$LysM^{Cre} + Bleomycin. MCP-1 mRNA expression assessed using real-time PCR. Data presented as % 18s rRNA.

HIF1A deletion on myeloid cells increases pulmonary fibrosis in association with worsened arterial oxygen saturation

Thus far, data reveal an anti-inflammatory role for HIF1A on myeloid cells in acute stages of bleomycin exposure as evidenced by increases in acute inflammatory mediators, neutrophil recruitment, count, MPO, and loss of barrier function in bleomycin-treated myeloid specific HIF1 knockout mice. This worsening of acute lung injury due to loss of HIF1A on myeloid cells led us to investigate whether pulmonary fibrosis would also be exacerbated as has been shown previously(135). LysM^{Cre} control mice and HIF1A$^{f/f}$LysM^{Cre} mice were treated with bleomycin intraperitoneally

over 33 days. Assessing fibrotic markers in whole lung lysate on day 33 reveal increased fibronectin protein expression and collagen 1a1 transcript levels in mice lacking ADORA2B on myeloid cells as compared to bleomycin treatment of control mice alone (Figure 5.4 A,B). Immunofluorescence for α-smooth muscle actin (αSMA), a marker of myofibroblasts in lung sections, revealed increased staining after bleomycin treatment of control mice as compared to saline which was further increased in HIF1A$^{f/f}$LysM^{Cre} mice after bleomycin treatment in association with reductions in arterial oxygen saturation (Figure 5.4C,D). Masson's Trichrome collagen staining in whole lung sections was increased in LysM^{Cre} mice after bleomycin exposure by day 14 and continued to increase by day 21 and 33 (Figure 5.5A). HIF1A$^{f/f}$LysM^{Cre} mice exposed to bleomycin shows similar increase in collagen staining by day 14 but demonstrated statistically significant increases in collagen staining as quantified by Ashcroft histological score by day 33 (Figure 5.5 A,B). Together, these data reveal myeloid specific HIF1A deletion in a bleomycin-induced model of lung fibrosis will worsen lung fibrosis, as a result of the loss of the protective anti-inflammatory effects of HIF1A in acute stages of disease.

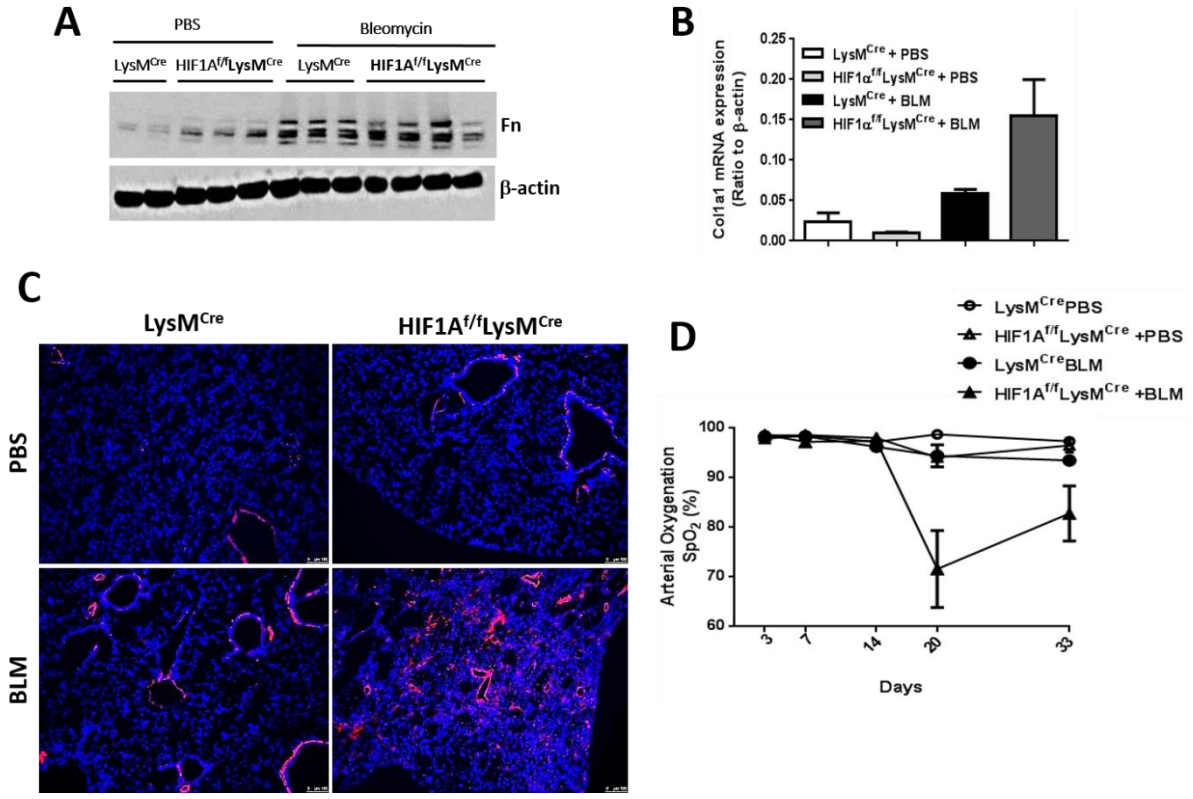

Figure 5.4 Fibrotic markers are increased on day 33 after BLM exposure in mice lacking HIF1A on myeloid cells. Immunoblot for (A) Fibronectin and (B) Collagen 1a1 mRNA expression in whole lung lysate from HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice after i.p. PBS or Bleomycin exposure on day 33. (C) Immunofluorescence for alpha-smooth muscle actin staining from formalin-fixed paraffin-embedded (FFPE) lung sections and (D) Arterial oxygen saturation in HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice after 33 days of bleomycin treatment. Images are representative of n ≥ 4 animals from each group. Scale bar: 100μm (10x).

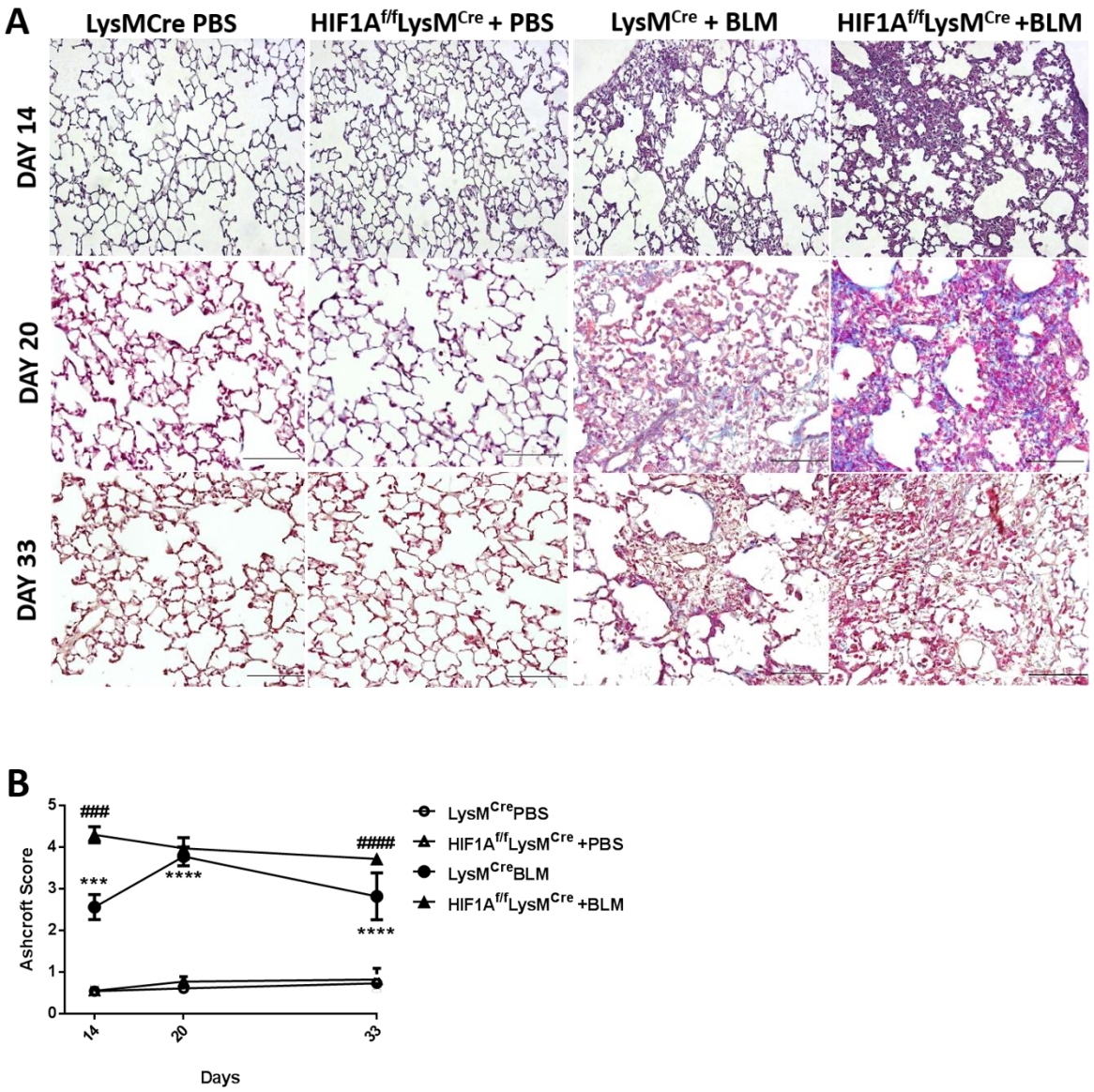

Figure 5.5 Myeloid HIF1A deletion exacerbates the development of pulmonary fibrosis after BLM treatment. (A) Masson's Trichrome collagen staining in whole lung sections with quantitative, fibrotic histologic scores as given by (B) Ashcroft scores in HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice after i.p. PBS or Bleomycin exposure on day 14, 20, and 33. Results are presented as mean ± SEM, n = 5-7. $^{****}p < 0.001$ LysM^{Cre} + PBS vs LysM^{Cre} + Bleomycin and $^{####}p < 0.001$, ANOVA comparison between LysM^{Cre} + Bleomycin and HIF1A$^{f/f}$LysM^{Cre} + Bleomycin.

Myeloid-specific HIF1A deletion attenuates ADORA2B and AAM expression after BLM exposure

HIF1A$^{f/f}$LysM^{Cre} after bleomycin treatment showed increases in macrophage cell count as observed in bleomycin-treated LysM^{Cre} however, they were significantly reduced compared to treated control mice at day 14 and 21 (Figure 5.1D). To decipher these changes in macrophage cell count, whole lung lysate was evaluated for markers of AAMs. CD206 protein expression was increased with bleomycin exposure in control LysM^{Cre} mice as compared to PBS treatment but reduced in those bleomycin-treated mice lacking ADORA2B on myeloid cells (Figure 5.6A). In accord with this, transcript levels in lung lysate of another AAM marker, Arginase-1, were increased with bleomycin exposure and reduced in myeloid-specific HIF1A knockout mouse (Figure 5.6B). In Chapter 3, I demonstrated that activation of ADORA2B on myeloid cells up-regulates pro-fibrotic AAMs, profibrotic mediators, and contributes to the development of pulmonary fibrosis(113). In chapter 4, HIF1A was found to mediate ADORA2B expression on BMDMS and in vivo attenuating lung fibrosis. In agreement with these findings, ADORA2B transcript levels were increased after bleomycin exposure in control mice yet reduced in bleomycin-treated mice lacking HIF1A on myeloid cells. Together, these results demonstrate myeloid-specific HIF1A deletion reduces AAM and ADORA2B expression after bleomycin exposure.

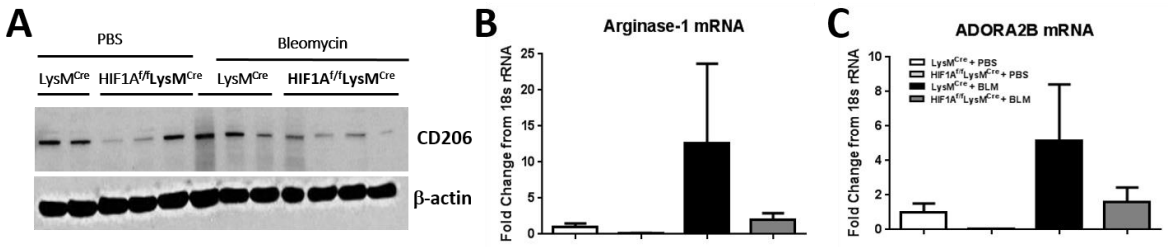

Figure 5.6. Conditional myeloid-specific HIF1A deletion yields decreased ADORA2B and AAM expression after BLM-induced pulmonary fibrosis. (A) Immunoblot for CD206 and β-actin, (B) Arginase-1, and ADORA2B transcript levels (C) in whole lung lysate from HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice exposed to bleomycin or PBS 33 days after treatment. Arginase-1 and ADORA2B expression was assessed using real-time PCR. Data presented as %18s rRNA.

***HIF1A deletion in myeloid cells in bone marrow-derived macrophages lowers Arginase-1 and* production of pro-fibrotic mediators**

In vivo bleomycin exposure in myeloid-specific HIF1A conditional knockout mice resulted in exacerbated pulmonary inflammation and subseqent increases in fibrosis despite reductions in ADORA2B and AAM expression. To confirm the role of hypoxia through HIF1A in regulating macrophage differentiation observed in chapter 4, bone marrow-derived macrophages from HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} mice were cultured in the presence of IL-4 and IL-13 and exposed to hypoxia for 12 hours. Treatment of hypoxia and Th2-cytokine treated LysM^{Cre} BMDMs with NECA, a pan-adenosine receptor agonist**,** resulted in increases in Arginase-1, Interleukin-6, and CXCL1 transcript levels which were significantly reduced in BMDMS lacking HIF1A (Figure 5.7A,B,C). This suggests HIF1A on myeloid cells is needed for ADORA2B-

mediated stimulation of macrophage differentiation into AAMs and profibrotic mediator production.

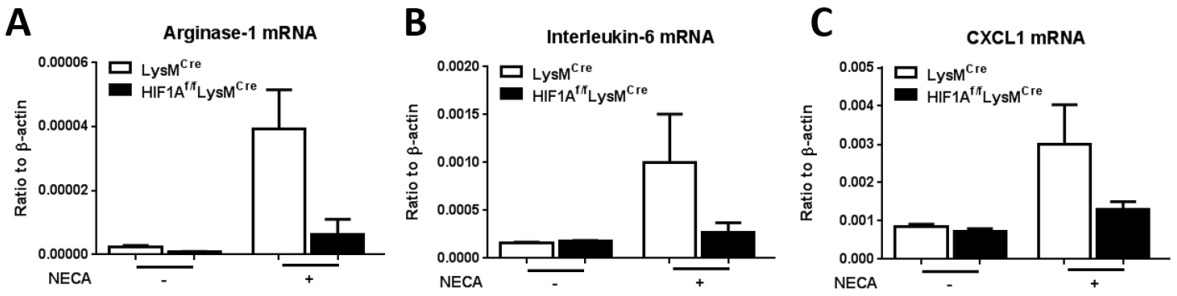

Figure 5.7. AAM and pro-fibrotic mediator expression is reduced after adenosine receptor activation in BMDMS lacking HIF1A. HIF1A$^{f/f}$LysM^{Cre} and LysM^{Cre} BMDMs were treated with IL-4 and IL-13 for 72 hours, then incubated in hypoxia (2% O_2) for 12 hours in the presence of 10μM NECA. (A) Arginase-1, (B) Interleukin-6, and (C) CXCL1 mRNA expression was assessed using real-time PCR. Data presented as %β-actin.

DISCUSSION

HIF1A has received recent attention as a transcription factor which can play a protective role in various types of acute lung injury including normoxic ventilator-induced lung injury and ovalbumin challenge models of airway hyperresponsiveness and asthma(89, 132). These findings however were specific to other cell types in the lung including alveolar epithelial cells and eosinophils. In Chapter 4, we demonstrated stabilization of HIF1A in macrophages from IPF patients and bleomycin-induced models of lung injury; in this chapter however, I utilized a myeloid-specific HIF1A

knockout mice to understand the role HIF1A plays in acute and chronic stages of a bleomycin-induced model of lung injury.

The results above demonstrate myeloid-specific HIF1A deletion followed by bleomycin exposure leads to worsening of pulmonary inflammation in acute stages and subsequent pulmonary fibrosis in association with reductions in arterial oxygen saturation. In accord with results of the previous chapter, a role for HIF1A in regulating ADORA2B expression, macrophage differentiation, and subsequent profibrotic mediator production was illustrated in chronic stages of a bleomycin-induced lung disease and BMDMs from conditionally modified mice lacking HIF1A on myeloid cells. Moreover, these studies support a role for myeloid-specific HIF1A agonists or stabilizers in treating patients with acute lung injury or myeloid-specific HIF1A antagonists or inhibitors in patients with chronic lung diseases such as IPF.

A mouse model of bleomycin-induced lung injury in acute stages of disease reveals a protective role for HIF1A on myeloid cells in reducing inflammation. Mice lacking HIF1A in myeloid cells showed exacerbations of acute lung injury including increased pulmonary edema, acute inflammatory markers, chemokines, and neutrophil recruitment. These data are novel in that they are the first to suggest HIF1A in AAMs as a key factor in reducing inflammation and protecting pulmonary barrier function in a bleomycin-induced model of lung injury. Crotty et al. recently showed inhibition of HIF1A and myeloid-specific HIF1A knockouts exposed to ovalbumin had reductions in airway hyper-responsiveness and eosinophil recruitment, features of asthma pathogenesis(132). Supporting a similar anti-inflammatory role, Eckle et al. demonstrate HIF1A stabilization in normoxia under mechanical stretch and ventilator-induced acute lung injury leads to decreases in pulmonary edema and inflammation

associated with maximal carbohydrate metabolism in vivo; these observations were exacerbated in alveolar-epithelial cell specific HIF1A knockout mice after VILI, localizing this anti-inflammatory role of HIF1A to alveolar epithelial cells(89). As such, our findings further support previous work indicating HIF1A plays an anti-inflammatory role in myeloid cells in acute lung injury, or bleomycin exposure as demonstrated here. Further work includes investigating the contribution of other cell types which exhibit HIF1A stabilization in the bleomycin lung injury model. Additionally, evaluating conditions that promote HIF1A stabilization in normoxic conditions in myeloid cells may be key to preventing inflammation and subsequent fibrosis development in patients at risk for the development of IPF or other chronic lung diseases.

This work supports the consistent finding observed in previous chapters that HIF1A drives ADORA2B expression, macrophage differentiation, and pro-fibrotic mediator production. Thirty-three day bleomycin exposure in myeloid-specific HIF1A knockout mice resulted in decreased CD206 protein, Arginase-1, and ADOR2B transcript levels in whole lung lysate. Given the reductions in ADORA2B as regulated by HIF1A, one may expect lung fibrosis to be reduced as well based on findings from the two previous chapters; however, the resultant increase in fibrosis differs from the previous studies in that lung injury due to bleomycin exposure was exacerbated acutely secondary to myeloid-specific HIF1A deletion in comparison to myeloid-specific ADORA2B deletion (Chapter 3) or HIF1A inhibition in late stages of disease (Chapter 4). We have previously observed similar increases in inflammation as evidenced by lowered adenosine levels and increasing cellular infiltration, pro-inflammatory mediators including interleukin-1β, TNFα, which contributed to increased lung fibrosis after bleomycin treatment of CD73$^{-/-}$ mice(135). CD73 is known

to have a HRE on its promoter and as such it maybe that myeloid-specific HIF1A deletion leads to decreased levels of CD73, lowering adenosine levels, and ultimately exacerbating injury in the acute phase of a bleomycin-induced model of lung injury(92). TGFβ is another mediator which was found to be elevated in lung macrophages after adenosine-dependent lung injury in which increased inflammation and subsequent fibrosis was observed in ADA-/- mice which improved with antagonism of ADORA2B (136). These elevations in TGFβ were also observed in ADORA2B$^{-/-}$ mice during acute stages of the bleomycin-induced lung injury model (56). Further work to evaluate the mechanism by which HIF1A deletion on myeloid cells exacerbates acute lung injury includes assessing adenosine levels in bronchoalveolar lavage fluid, CD73 and TGFβ expression in whole lung lysate and lung sections, in addition to evaluating pro-inflammatory and pro-fibrotic mediators.

Moreover, these data demonstrate an anti-inflammatory role for HIF1A on myeloid cells in acute lung injury. Mice lacking HIF1A on myeloid cells exhibit increased inflammation and pulmonary edema which contributes to increases in fibrotic markers including collagen and fibronectin and the development of fibrosis in later stages of disease. Chapter 3 and 4 revealed how activation of ADORA2B and HIF1A stabilization ultimately mediate macrophage differentiation into AAMs and pro-fibrotic mediator production. In agreement, this chapter illustrated a decrease in AAM and ADORA2B expression in conditional myeloid-specific HIF1A knockouts after bleomycin exposure and BMDMS after NECA treatment.

CHAPTER SIX

SUMMARY, FUTURE DIRECTIONS, AND CONCLUSION

SUMMARY

IPF is a common, chronic disease which significantly impacts the quality of life of millions of individuals worldwide. Median survival after diagnosis is typically 3-5 years due to the absence of any curative treatment(1, 128, 137). Previously published work from our lab and others have demonstrated a role for adenosine accumulation and activation of ADORA2B as a key regulator of fibrosis in vivo and in conjunction with human samples of IPF, ADORA2B expression and IL-6 production has been localized to alternatively, activated macrophages as a key effector cell-type in this process(50, 55, 56). The work in this dissertation sought to identify factors which regulated expression of ADORA2B on macrophages, elucidate the role ADORA2B in macrophage differentiation and development of fibrosis, and assess the impact of inhibiting HIF1A in late stages of disease versus myeloid-specifIc HIF1A deletion on pulmonary fibrosis.

Bleomycin exposure of myeloid-specific ADORA2B knockouts revealed reductions in markers of fibrosis including collagen, fibronectin, and myofibroblast expression in association with improvements in arterial oxygen saturation. Upon further inspection of macrophage cell counts and type, we found that the attenuation of fibrosis was also associated with reductions in AAMs in whole lung lysate and sections along with less ADORA2B-mediated pro-fibrotic cytokine IL-6 in BALF and fewer IL-6 positive macrophages. Antagonism and genetic deletion of ADORA2B followed by exposure to Th2 cytokines and hypoxia revealed a role for ADORA2B

itself in mediating macrophage differentiation into AAMs and the production of profibrotic mediators. These results supported a role for ADORA2B on myeloid cells in mediating their differentiation into AAMs and promoting production of profibrotic cytokines and chemokines that lead to the development and progression of pulmonary fibrosis.

Lung sections from patients with and without IPF along with bleomycin-induced lung fibrosis in Hif1α::LUC reporter mice for the first time demonstrated stabilization of HIF1A in AAMs. Next, inhibition of HIF1A in late stages of a bleomycin-induced lung injury model revealed reductions in ADORA2B and AAM expression in association with attenuation of pulmonary fibrosis. Inhibition and genetic silencing of HIF1A in BMDMs also showed disruption of HIF1A-mediated ADOR2B expression, subsequent AAM marker expression including Arginase-1 and CD206. These results were confirmed in lung macrophages from mice after bleomycin treatment along with exhibiting reductions in IL-6 and CXCL1.

To further characterize the role of HIF1A in regulating ADORA2B expression subsequent development of fibrosis, a myeloid-specific HIF1A knockout mouse was exposed to saline and bleomycin. Results showed exacerbations in inflammatory parameters in acute stages of disease which lead to exacerbated lung fibrosis in late stages of disease as well. However, both ADORA2B and AAM expression remained reduced on day 33 after bleomycin exposure, confirming previous findings that HIF1A does regulate ADORA2B expression on AAMs. Further work to elucidate the mechanisms by which HIF1A deletion on myeloid cells contributes to breakdown of the alveolar-epithelial cell barrier and increased inflammation leading to the development of fibrosis is needed.

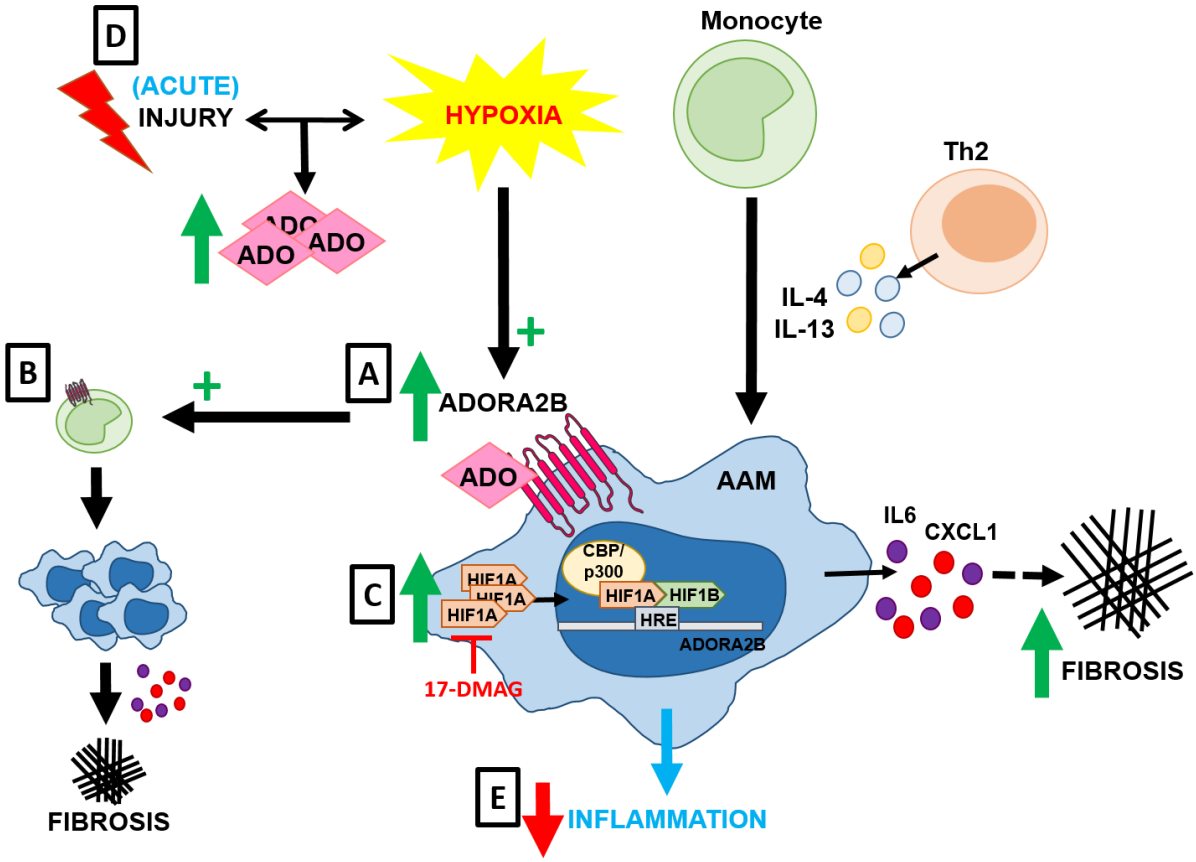

Figure 6.1. Working model of the hypoxic adenosine response on AAMs in lung disease. Patients with IPF have increased levels of adenosine and ADORA2B-expressing AAMs (work done by Yang Zhou, PhD). (A) We demonstrate a role for ADORA2B on AAMs in contributing to the development of lung fibrosis due to (B) ADORA2B-mediated increases in macrophage differentiation into AAMs and subsequent pro-fibrotic mediator production. (C) Hypoxia-induced HIF1A stabilization in AAMs contributes to increased ADORA2B expression, (B) AAM differentiation, and development of pulmonary fibrosis which can be reversed with 17-DMAG treatment (a HIF1A inhibitor). (D) In acute lung injury, myeloid-specific HIF1A plays a tissue protective and (E) anti-inflammatory role.

This dissertation address several knowledge gaps with respect to how hypoxia alters adenosine signaling in lung disease and contributes to the development of fibrosis. We demonstrate a role for ADORA2B on myeloid cells in contributing to macrophage differentiation into AAMs, pro-fibrotic mediator production, and the development of lung fibrosis(113). I later illustrated how hypoxia through HIF1A acts as a major driver of ADORA2B on macrophages in BMDMs and for the first time, demonstrated its stabilization in human lung sections from patients affected by IPF in addition to murine models of bleomycin-induced lung injury. I then illustrated how inhibition of HIF1A in late stages of disease can attenuate lung fibrosis by reducing ADORA2B expression, AAM differentiation, and pro-fibrotic mediator production in vivo with supporting findings in BMDMs through genetic silencing and pharmacologic antagonism. Lastly, through myeloid-specific HIF1A deletion followed by bleomycin exposure, an anti-inflammatory role for HIF1A in myeloid cells is revealed in acute stages of disease.

The findings from this dissertation are significant in that they emphasize the clinical importance of myeloid-specific ADORA2B antagonism or HIF1A inhibition in late stages of disease in attenuating the progression of lung fibrosis. These therapies could significantly prolong the lives of patients living with IPF by reducing symptoms or acute exacerbations, and ultimately, provide them a better quality of life. HIF1A stabilization in acute lung injury to reduce inflammation, breakdown of the pulmonary barrier, and worsened fibrosis in the long-term, also presents a novel therapy for patients affected by acute lung injury. Before translating to patient care however, the exact mechanisms behind HIF1A stabilization contributing to decreased inflammation need to be investigated further.

FUTURE DIRECTIONS

The work presented in this dissertation contributes to our understanding of how hypoxia modulates adenosine signaling and macrophage differentiation, contributing to pulmonary fibrosis. However, there are still many questions which remain to be answered and findings confirmed to delineate the mechanisms and key players in this complex disease process.

What role does ADORA2B on myeloid cells contribute to pulmonary fibrosis development in comparison to other cell-types in the lung?

Deletion of ADORA2B on myeloid cells followed by bleomycin exposure showed attenuation of fibrosis but not complete abolishment of lung fibrosis. This suggests that myeloid-specific deletion alone is not enough to halt the progression of fibrosis and that the presence of ADORA2B on other cell-types may play a role including type 1 or 2 alveolar epithelial cells. To investigate this, ADORA2B$^{f/f}$ mice crossed with mice carrying Cre recombinase under differing tissue-specific promoters, such as surfactant protein c (SPC) for type 2 epithelial cell-specific deletion or aquaporin 5 (Aqp5) for type 1 epithelial cell-specific deletion, should be exposed to bleomycin- induced lung fibrosis to delineate the role ADORA2B may play on these cell types in contributing to the development and progression of fibrosis(138).

Differing roles for adenosine receptors on myeloid cells

Bone marrow derived macrophages exposed to Th2 cytokine treatment followed by hypoxia exposure and co-treatment with either pan-adenosine receptor agonist and ADORA2A or ADORA2B antagonism showed reductions in AAM

expression and profibrotic mediator production. Similar reductions were observed after hypoxia and Th2 cytokine treatment in mice with ADORA2A and ADORA2B global deletion as compared to wild-type control mice. Although, reductions in AAM and IL6 expression were more significant with ADORA2B antagonism or deletion, reductions secondary to ADORA2A antagonism or deletion are also evident. These observations are not surprising however given the work by Csoka et al. showing a role for ADORA2B and to a lesser extent ADORA2A in mediating differentiation of macrophages after IL4 and IL13 treatment to the AAM subtype(108). As such, it would be interesting to evaluate how a mouse lacking both ADORA2A and ADORA2B on myeloid cells would respond to bleomycin treatment.

Contribution of recruited versus resident macrophages

The robust response of ADORA3 to combined Th2 cytokine and hypoxia treatment do reveal limitations of the in vitro bone marrow derived macrophage system. ADORA3 is known to be elevated in bone marrow and peripheral mononuclear cells and are more representative of the circulating monocytes which travel through the vasculature and migrate to sites of injury or tissue of interest where they mature (139{Lech, 2013 #218, 140). Resident macrophages are believed to be the key players involved in acute inflammation in the lung even differentiating to the AAM subtype to promote repair in addition to Arginase-1 positive recruited macrophages(140). This observation elicits the question of what if any differences exist in adenosine receptor expression between recruited macrophages, such as those derived from bone marrow monocytes, and resident lung macrophages. Future

work investigating the contribution of ADORA2B or HIF1A specific deletion in resident ($CD11c^+$) or recruited ($CD11b^+$) macrophages in a bleomycin-induced model of lung injury will be useful to distinguish the differences in HIF1A-mediated changes in adenosine receptor expression, macrophage differentiation, and subsequent effects on acute or chronic lung disease(141, 142). Flow cytometry can be utilized to identify lung macrophage as alveolar ($CD45^+CD68^{hi}F4/80^+CD11b^-CD11c^+Gr1^-$), interstitial ($CD45^+CD68^{low}F4/80^+CD11b^+CD11c^+Gr1^-CD14^{low}$), monocytes ($CD45^+CD68^{low}F4/80^+CD11b^+CD11c^-Gr1^{low}CD14^{hi}$), or dendritic cells ($CD45^+CD68^{hi}F4/80^-CD11c^+Gr1^-CD103^+$major histocompatibility complex (MHC) class II^{hi}) (143). Chao et al suggest that it is the alveolar macrophages when activated by hypoxia that contribute to mast cell degranulation and subsequent inflammation (144). Additionally, adenosine receptor expression may also differ between BALF macrophages of patients with and without IPF in comparison to murine lung or BAL macrophages after bleomycin exposure.

Does HIF1A stabilization in other cell-types in contributing to the development of lung fibrosis?

Although we demonstrated that HIF1A is stabilized in AAMs in patients with and without IPF patients along with bleomycin-induced models of lung injury, these findings should be validated in BALF macrophages from human IPF patients samples as well. Examination of HIF1A stabilization in bleomycin exposed Hif1α::LUC reporter mice along with work by Weng et al. suggest HIF1A activation in other key cell types,

such as type 2 alveolar epithelial cells, can contribute to epithelial cell proliferation and the development and progression of fibrosis(84).

Effect of HIF1A inhibition, systemic versus localized, or myeloid-specific deletion on the development of pulmonary hypertension

In this dissertation, HIF1A was inhibited systemically through intraperitoneal delivery of 17-DMAG in late stages of bleomycin-induced lung injury. Evaluation of HIF1A levels in whole lung lysate showed that systemic 17-DMAG delivery was still able to achieve significant HIF1A knockdown which was associated with reductions in ADORA2B, AAM expression, and reduced lung fibrosis. Further experiments however should utilize targeted delivery to the lung through intranasal or intra-tracheal delivery of antisense oligonucleotides against HIF1A in order to minimize adverse effects of systemic 17-DMAG delivery, including fatigue, anorexia, renal dysfunction, and peripheral neuropathy(145). Localized or targeted delivery is also more desirable for translating therapies into novel treatments for patients.

Pulmonary hypertension is a known co-morbidity of patients with IPF. We demonstrated that myeloid-specific ADORA2B deletion followed by bleomycin exposure will result in improved pulmonary function parameters in comparison to control mice possessing ADORA2B on myeloid cells. Investigating the impact of myeloid-specific HIF1A deletion and inhibition of HIF1A in late stages of disease on the development of pulmonary hypertension will be insightful and could be potentially used in conjunction with existing therapies to treat PH associated with lung disease and idiopathic PH.

Possible contribution of HIF2A in myeloid cells to ADORA2B expression, macrophage differentiation, and fibrosis development

HIF2A has recently been shown to contribute to the development of pulmonary hypertension and fibrosis in mouse models as well. Hickey et al. illustrated that homozygous mutation of VHL, contributes to the development of pulmonary edema, vascular remodeling, pulmonary hypertension, macrophage infiltration, and fibrosis which was reduced with the introduction of HIF2A heterozygosity(121). Hypoxia-induced HIF2A stimulation has also been found to drive miR-120 expression in fibroblasts, repressing the c-myc inhibitor, MNT, driving the proliferation of IPF fibroblasts(146). Similarly, hypoxia-dependent HIF2A stabilization was recently shown to increase Arginase-1 expression inhibiting vasodilatory effects of its competing nitric oxide, leading to the development of pulmonary hypertension(129). To date, HIF2A expression has been believed to be limited to vascular endothelium and type 2 alveolar epithelial cells(72). Preliminary work with genetic silencing of HIF2A by siRNA in BMDMs after Th2 cytokine treatment and hypoxia exposure suggest that HIF2A knockdown can be used as a means to reduce ADORA2B and AAM expression (Figure 6.2). Given these recent findings, future work will include evaluating the effects of inhibiting both HIF1A and HIF2A in myeloid cells in addition to myeloid-specific HIF2A deletion alone after bleomycin exposure on the development of fibrosis.

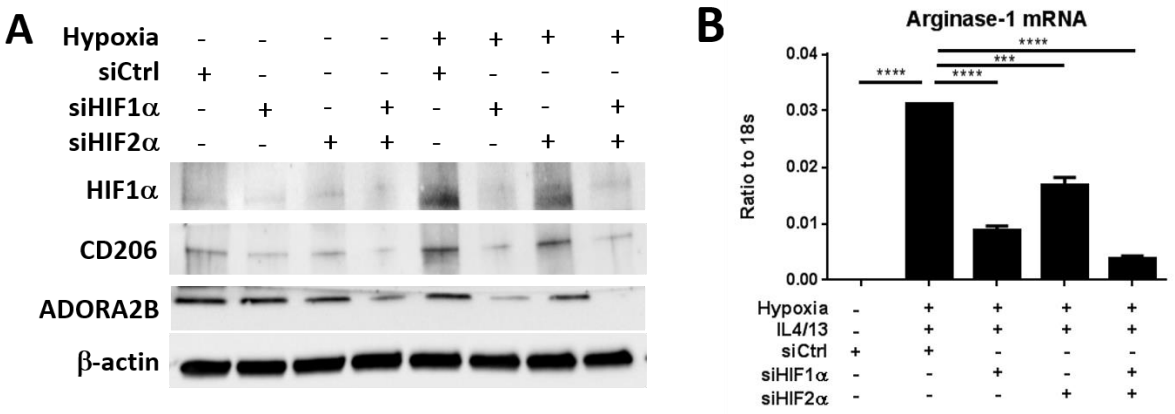

Figure 6.2. Genetic silencing of HIF1A and HIF2A alter macrophage differentiation and ADORA2B expression. Bone marrow derived macrophages from C57Bl6 mice were treated with 10ng/mL IL-4 and 10ng/mL IL-13 for 72 hours and then incubated in 2% O_2 hypoxia for 12 hours in the presence of control siRNA, siRNA against HIF1A, and siRNA against HIF2A. (A) Immunoblot for HIF1A, AAM marker, CD206, ADORA2B, and β-actin and (B) Arginase-1 expression, assessed using real-time PCR. Data presented as %β-actin.

Effect of Hypoxia-dependent versus hypoxia-independent pathways on ADORA2B and the development of pulmonary fibrosis

HIF1A has also been shown to be stabilized in the absence of hypoxia through metabolites such as succinate, mechanical stretch, NF-kb activation (73-77). There is also evidence that ADORA2A and ADORA2B can mediate changes in glycolytic flux in association with the observation that CAMs and AAMs also utilize differing metabolic pathways(48, 74). Future work will include delineating the impact of ADORA2B in myeloid cells on macrophage metabolism, HIF1A stabilization, and macrophage differentiation. Understanding these pathways and changing

metabolomics of macrophages in the setting of hypoxia-induced and normoxia-induced HIF1A stabilization in a bleomycin-induced model of lung injury can potentially be used as novel targets to stabilize HIF1A in acute lung injury or to inhibit HIF1A in chronic lung disease.

Understanding the mechanisms behind which HIF1A stabilization in myeloid cells reduces acute lung injury

We discussed in Chapter 5 the need to discriminate the mechanism by which HIF1A deletion on myeloid cells exacerbates acute lung injury. This includes assessing adenosine levels in BALF and evaluating CD73 or expression of other pro-fibrotic or pro-inflammatory cytokines including TGFβ expression in whole lung lysate and lung sections. It is possible that HIF1A deletion contributes to CD73 down-regulation, thus reducing the production of adenosine in the extracellular space and reducing adenosine-ADORA2B mediated protective effects in acute stages of disease.

Interplay of HIF1A stabilization, endoplasmic reticulum (ER) stress, and macrophage differentiation, and lung injury

Pereira et al. have demonstrated that combined insults of ER stress through activation of the unfolded protein response (UPR) and HIF1A stabilization can lead to significant elevations in hypoxia-response genes, including VEGF expression contributing to pulmonary angiogenesis and pulmonary hypertension development (147). Additionally, GRP78 is a chaperone protein which in the setting of ER stress,

recognizes exposed misfolded proteins and activates the UPR. Continued UPR activation in the setting of ER stress leads to up-regulation of C/EBP homologous protein (CHOP). $Grp^{+/-}$ mice showed protection from bleomycin-induced lung fibrosis in association with fewer lung macrophages that were indeed TUNEL, CHOP, and cleaved caspase-3 positive. $CHOP^{-/-}$ mice after bleomycin treatment however showed increase in fibrosis and arginase-1 positive lung macrophages (148).

In support of a role for ER stress in AAMs in IPF, lung sections from patients with IPF and BLM model of lung fibrosis shows increase in $CHOP^{+}CD206^{+}$ macrophages and other ER stress markers. $CHOP^{-/-}$ protects against bleomycin-induced lung injury in association with reductions in fewer Arginase-1 positive macrophages and reductions in TGFβ (149). STAT-mediated macrophage activation is through suppressor of cytokine signaling (SOCS) proteins of which IL-4 up-regulates SOCS1, inhibiting STAT1, while IFNγ up-regulates SOCS3, inhibiting STAT3(96). The authors conclude that CHOP deficiency up-regulates SOCS1 and SOCS3 leading to down-regulation of STAT6/PPARγ and subsequent disruption of AAM programing and infiltration into the lungs after bleomycin exposure, reducing fibrosis. Oh et al. have also demonstrated a role for ER stress in AAM differentiation in a mouse model of atherosclerosis (150).

These recent studies suggest ER stress as a mechanism to HIF1A stabilization, AAM differentiation, and the development of pulmonary fibrosis or hypertension in mouse models of disease. Future work to evaluate the impact of ER stress on adenosine signaling and HIF1A-mediated ADORA2B expression, macrophage differentiation, and pro-fibrotic mediator production will be insightful and potentially lead to the identification of novel targets for drug development.

Evaluate role of hyaluronan production by myeloid cells in acute lung injury

We have previously demonstrated that myeloid-specific ADORA2B mediated deletion in mice followed by bleomycin exposure is associated with reduced hyaluronan production from macrophages in association with reductions in pulmonary fibrosis and hypertension (113). ADORA2B antagonism has also been shown to attenuate pulmonary hypertension in a mouse model of COPD in association with decreases in hyaluronan and enzymes involved in its synthesis including hyaluronan synthase 2 (HAS2)(151). Interestingly, high molecular weight (HMW) hyaluronan production by epithelial cells has been found to have a protective effect in ALI (41). The role of HIF1A and adenosine signaling in mediating this protective effect on epithelial cells in addition to characterizing hyaluronan production by myeloid cells in ALI should be investigated in future work to evaluate adenosine-mediated ECM production as a target for ALI or IPF.

CONCLUSIONS

We utilize an established mouse model of chronic lung injury, lung samples from patients with IPF, along with a robust in vitro system of bone marrow derived macrophages to elucidate the effects and interactions of adenosine signaling, macrophage differentiation, and hypoxia on pulmonary fibrosis. We have illustrated a role for ADORA2B in mediating macrophage differentiation into AAMs, promoting the production of pro-fibrotic cytokines in mouse models of lung injury and in vitro culture. For the first time, we demonstrate inhibition or genetic silencing of HIF1A can

attenuate pro-fibrotic mediator production and pulmonary fibrosis in bone marrow derived macrophages (BMDMs) and in vivo models of bleomycin-induced pulmonary fibrosis. Finally, we reveal a tissue-protective and anti-inflammatory role for HIF1A in acute stages of lung injury. Further investigations are needed to validate the mechanism by which deletion of HIF1A contributes to exacerbated inflammation and subsequent fibrosis in addition to validation in human IPF tissue and macrophage samples.

There exists a great need to develop novel therapeutics to halt the development and progression of IPF. Through this work, we have found a role for HIF1A in mediating ADORA2B expression on macrophages, contributing to their differentiation into the reparative AAM subtype, and ultimately, contributing to the secretion of pro-fibrotic mediators leading to the progression of lung fibrosis. Genetic deletion of ADORA2B on myeloid cells and inhibition of HIF1A late stages of disease has illustrated significant reductions in the development of fibrosis in bleomycin-induced models of lung injury suggesting their importance as therapeutic targets for drug development; additionally, myeloid-specific HIF1A stabilization may be another approach to reduce inflammation in acute lung injury. Much work remains to validate findings in human tissue samples before moving to clinical trials in patients to understand the safety, efficacy, and long-term effects of ADORA2B antagonism or HIF1A inhibition, and HIF1A stabilization, for treatment of IPF or ALI. Ultimately, these investigations will lead to a better understanding of the role of adenosine in IPF and ALI, leading to identification of targets for novel therapeutics that can prevent disease progression and possibly reverse lung fibrosis and inflammation. Additionally, the inflammatory cell response and fibrosis observed in IPF shares features of other

common diseases including asthma and systemic scleroderma which respectively affect up to 300 million and 2.5 million individuals worldwide(152-154). Treatments developed for IPF may overlap and offer potential benefit in these diseases as well.

REFERENCES

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. Coultas, D. B., R. E. Zumwalt, W. C. Black, and R. E. Sobonya. 1994. The epidemiology of interstitial lung diseases. *American journal of respiratory and critical care medicine* 150: 967-972.
5. Nalysnyk, L., J. Cid-Ruzafa, P. Rotella, and D. Esser. 2012. Incidence and prevalence of idiopathic pulmonary fibrosis: review of the literature. *European respiratory review : an official journal of the European Respiratory Society* 21: 355-361.
6. Nishiyama, O., H. Miyajima, Y. Fukai, R. Yamazaki, R. Satoh, T. Yamagata, H. Sano, T. Iwanaga, Y. Higashimoto, H. Nakajima, H. Kume, and Y. Tohda. 2013.

Effect of ambulatory oxygen on exertional dyspnea in IPF patients without resting hypoxemia. *Respiratory medicine* 107: 1241-1246.

7. Johkoh, T., F. Sakai, S. Noma, M. Akira, K. Fujimoto, T. Watadani, and Y. Sugiyama. 2014. Honeycombing on CT; its definition, pathologic correlation, and future direction of its diagnosis. *European journal of radiology* 83: 27-31.
8. Fahim, A., M. Crooks, and S. P. Hart. 2011. Gastroesophageal reflux and idiopathic pulmonary fibrosis: a review. *Pulmonary medicine* 2011: 634613.
9. Kim, Y. J., J. W. Park, S. Y. Kyung, S. P. Lee, M. P. Chung, Y. H. Kim, J. H. Lee, Y. C. Kim, J. S. Ryu, H. L. Lee, C. S. Park, S. T. Uh, Y. C. Lee, K. H. Kim, Y. J. Chun, Y. B. Park, D. S. Kim, Y. Jegal, J. H. Lee, M. S. Park, and S. H. Jeong. 2012. Clinical Characteristics of Idiopathic Pulmonary Fibrosis Patients with Diabetes Mellitus: the National Survey in Korea from 2003 to 2007. *Journal of Korean medical science* 27: 756-760.
10. Kropski, J. A., T. S. Blackwell, and J. E. Loyd. 2015. The genetic basis of idiopathic pulmonary fibrosis. *The European respiratory journal* 45: 1717-1727.
11. Yang, I. V., T. E. Fingerlin, C. M. Evans, M. I. Schwarz, and D. A. Schwartz. 2015. MUC5B and Idiopathic Pulmonary Fibrosis. *Annals of the American Thoracic Society* 12 Suppl 2: S193-199.
12. 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.
13. 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.

14. 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.
15. Harari, S., and A. Caminati. 2010. IPF: new insight on pathogenesis and treatment. *Allergy* 65: 537-553.
16. Raghu, G., R. Million-Rousseau, A. Morganti, L. Perchenet, and J. Behr. 2013. Macitentan for the treatment of idiopathic pulmonary fibrosis: the randomised controlled MUSIC trial. *The European respiratory journal* 42: 1622-1632.
17. Zisman, D. A., M. Schwarz, K. J. Anstrom, H. R. Collard, K. R. Flaherty, and G. W. Hunninghake. 2010. A controlled trial of sildenafil in advanced idiopathic pulmonary fibrosis. *The New England journal of medicine* 363: 620-628.
18. Raghu, G., K. J. Anstrom, T. E. King, Jr., J. A. Lasky, and F. J. Martinez. 2012. Prednisone, azathioprine, and N-acetylcysteine for pulmonary fibrosis. *The New England journal of medicine* 366: 1968-1977.
19. Azuma, A., T. Nukiwa, E. Tsuboi, M. Suga, S. Abe, K. Nakata, Y. Taguchi, S. Nagai, H. Itoh, M. Ohi, A. Sato, and S. Kudoh. 2005. Double-blind, placebo-controlled trial of pirfenidone in patients with idiopathic pulmonary fibrosis. *American journal of respiratory and critical care medicine* 171: 1040-1047.
20. Taniguchi, H., M. Ebina, Y. Kondoh, T. Ogura, A. Azuma, M. Suga, Y. Taguchi, H. Takahashi, K. Nakata, A. Sato, M. Takeuchi, G. Raghu, S. Kudoh, and T.

Nukiwa. 2010. Pirfenidone in idiopathic pulmonary fibrosis. *The European respiratory journal* 35: 821-829.

21. Noble, P. W., C. Albera, W. Z. Bradford, U. Costabel, M. K. Glassberg, D. Kardatzke, T. E. King, Jr., L. Lancaster, S. A. Sahn, J. Szwarcberg, D. Valeyre, and R. M. du Bois. 2011. Pirfenidone in patients with idiopathic pulmonary fibrosis (CAPACITY): two randomised trials. *Lancet* 377: 1760-1769.

22. King, T. E., W. Z. Bradford, S. Castro-Bernardini, E. A. Fagan, I. Glaspole, M. K. Glassberg, E. Gorina, P. M. Hopkins, D. Kardatzke, L. Lancaster, D. J. Lederer, S. D. Nathan, C. A. Pereira, S. A. Sahn, R. Sussman, J. J. Swigris, and P. W. Noble. 2014. A Phase 3 Trial of Pirfenidone in Patients with Idiopathic Pulmonary Fibrosis. *New England Journal of Medicine* 370: 2083-2092.

23. Fujimoto, H., T. Kobayashi, and A. Azuma. 2015. Idiopathic Pulmonary Fibrosis: Treatment and Prognosis. *Clinical medicine insights. Circulatory, respiratory and pulmonary medicine* 9: 179-185.

24. Richeldi, L., R. M. du Bois, G. Raghu, A. Azuma, K. K. Brown, U. Costabel, V. Cottin, K. R. Flaherty, D. M. Hansell, Y. Inoue, D. S. Kim, M. Kolb, A. G. Nicholson, P. W. Noble, M. Selman, H. Taniguchi, M. Brun, F. Le Maulf, M. Girard, S. Stowasser, R. Schlenker-Herceg, B. Disse, and H. R. Collard. 2014. Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis. *The New England journal of medicine* 370: 2071-2082.

25. Canestaro, W. J., S. H. Forrester, G. Raghu, L. Ho, and B. E. Devine. 2016. Drug Treatment of Idiopathic Pulmonary Fibrosis: Systematic Review and Network Meta-Analysis. *Chest* 149: 756-766.

26. Blackwell, T. S., A. M. Tager, Z. Borok, B. B. Moore, D. A. Schwartz, K. J. Anstrom, Z. Bar-Joseph, P. Bitterman, M. R. Blackburn, W. Bradford, K. K. Brown, H. A. Chapman, H. R. Collard, G. P. Cosgrove, R. Deterding, R. Doyle, K. R. Flaherty, C. K. Garcia, J. S. Hagood, C. A. Henke, E. Herzog, C. M. Hogaboam, J. C. Horowitz, T. E. King, Jr., J. E. Loyd, W. E. Lawson, C. B. Marsh, P. W. Noble, I. Noth, D. Sheppard, J. Olsson, L. A. Ortiz, T. G. O'Riordan, T. D. Oury, G. Raghu, J. Roman, P. J. Sime, T. H. Sisson, D. Tschumperlin, S. M. Violette, T. E. Weaver, R. G. Wells, E. S. White, N. Kaminski, F. J. Martinez, T. A. Wynn, V. J. Thannickal, and J. P. Eu. 2014. Future directions in idiopathic pulmonary fibrosis research. An NHLBI workshop report. *American journal of respiratory and critical care medicine* 189: 214-222.

27. 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.

28. Kaunisto, J., E. R. Salomaa, U. Hodgson, R. Kaarteenaho, and M. Myllarniemi. 2013. Idiopathic pulmonary fibrosis--a systematic review on methodology for the collection of epidemiological data. *BMC pulmonary medicine* 13: 53.

29. Hung, C., G. Linn, Y. H. Chow, A. Kobayashi, K. Mittelsteadt, W. A. Altemeier, S. A. Gharib, L. M. Schnapp, and J. S. Duffield. 2013. Role of lung pericytes and resident fibroblasts in the pathogenesis of pulmonary fibrosis. *American journal of respiratory and critical care medicine* 188: 820-830.

30. Butt, Y., A. Kurdowska, and T. C. Allen. 2016. Acute Lung Injury: A Clinical and Molecular Review. *Archives of pathology & laboratory medicine* 140: 345-350.

31. Grommes, J., and O. Soehnlein. 2011. Contribution of neutrophils to acute lung injury. *Molecular medicine (Cambridge, Mass.)* 17: 293-307.

32. Sweatt, A. J., and J. E. Levitt. 2014. Evolving epidemiology and definitions of the acute respiratory distress syndrome and early acute lung injury. *Clinics in chest medicine* 35: 609-624.

33. 2000. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network. *The New England journal of medicine* 342: 1301-1308.

34. Kor, D. J., R. E. Carter, P. K. Park, E. Festic, V. M. Banner-Goodspeed, R. Hinds, D. Talmor, O. Gajic, L. B. Ware, and M. N. Gong. 2016. Effect of Aspirin on Development of ARDS in At-Risk Patients Presenting to the Emergency Department: The LIPS-A Randomized Clinical Trial. *JAMA : the journal of the American Medical Association* 315: 2406-2414.

35. Festic, E., E. Ortiz-Diaz, A. Lee, G. Li, D. J. Kor, A. Adebola, O. Akca, J. Hoth, J. E. Levitt, R. Carter, and O. Gajic. 2013. Prehospital use of inhaled steroids and incidence of acute lung injury among patients at risk. *Journal of critical care* 28: 985-991.

36. Maca, J., O. Jor, M. Holub, P. Sklienka, F. Bursa, M. Burda, V. Janout, and P. Sevcik. 2017. Past and Present ARDS Mortality Rates: A Systematic Review. *Respiratory care* 62: 113-122.

37. Bhatia, M., R. L. Zemans, and S. Jeyaseelan. 2012. Role of chemokines in the pathogenesis of acute lung injury. *American journal of respiratory cell and molecular biology* 46: 566-572.

38. Bless, N. M., M. Huber-Lang, R. F. Guo, R. L. Warner, H. Schmal, B. J. Czermak, T. P. Shanley, L. D. Crouch, A. B. Lentsch, V. Sarma, M. S. Mulligan, H. P. Friedl, and P. A. Ward. 2000. Role of CC chemokines (macrophage inflammatory protein-1 beta, monocyte chemoattractant protein-1, RANTES) in acute lung injury in rats. *Journal of immunology (Baltimore, Md. : 1950)* 164: 2650-2659.

39. Janssen, W. J., L. Barthel, A. Muldrow, R. E. Oberley-Deegan, M. T. Kearns, C. Jakubzick, and P. M. Henson. 2011. Fas determines differential fates of resident and recruited macrophages during resolution of acute lung injury. *American journal of respiratory and critical care medicine* 184: 547-560.

40. Goodman, R. B., R. M. Strieter, D. P. Martin, K. P. Steinberg, J. A. Milberg, R. J. Maunder, S. L. Kunkel, A. Walz, L. D. Hudson, and T. R. Martin. 1996. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. *American journal of respiratory and critical care medicine* 154: 602-611.

41. Jiang, D., J. Liang, J. Fan, S. Yu, S. Chen, Y. Luo, G. D. Prestwich, M. M. Mascarenhas, H. G. Garg, D. A. Quinn, R. J. Homer, D. R. Goldstein, R. Bucala, P. J. Lee, R. Medzhitov, and P. W. Noble. 2005. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nature medicine* 11: 1173-1179.

42. Krupa, A., H. Kato, M. A. Matthay, and A. K. Kurdowska. 2004. Proinflammatory activity of anti-IL-8 autoantibody:IL-8 complexes in alveolar edema fluid from patients with acute lung injury. *American journal of physiology. Lung cellular and molecular physiology* 286: L1105-1113.

43. Fudala, R., A. Krupa, M. A. Matthay, T. C. Allen, and A. K. Kurdowska. 2007. Anti-IL-8 autoantibody:IL-8 immune complexes suppress spontaneous apoptosis of neutrophils. *American journal of physiology. Lung cellular and molecular physiology* 293: L364-374.

44. Kurdowska, A., E. J. Miller, J. M. Noble, R. P. Baughman, M. A. Matthay, W. G. Brelsford, and A. B. Cohen. 1996. Anti-IL-8 autoantibodies in alveolar fluid from patients with the adult respiratory distress syndrome. *Journal of immunology (Baltimore, Md. : 1950)* 157: 2699-2706.

45. Dolinay, T., Y. S. Kim, J. Howrylak, G. M. Hunninghake, C. H. An, L. Fredenburgh, A. F. Massaro, A. Rogers, L. Gazourian, K. Nakahira, J. A. Haspel, R. Landazury, S. Eppanapally, J. D. Christie, N. J. Meyer, L. B. Ware, D. C. Christiani, S. W. Ryter, R. M. Baron, and A. M. Choi. 2012. Inflammasome-regulated cytokines are critical mediators of acute lung injury. *American journal of respiratory and critical care medicine* 185: 1225-1234.

46. Ohta, A., and M. Sitkovsky. 2001. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 414: 916-920.

47. Fredholm, B. B., I. J. AP, K. A. Jacobson, J. Linden, and C. E. Muller. 2011. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature

and classification of adenosine receptors--an update. *Pharmacological reviews* 63: 1-34.

48. Hasko, G., and P. Pacher. 2012. Regulation of macrophage function by adenosine. *Arteriosclerosis, thrombosis, and vascular biology* 32: 865-869.

49. Della Latta, V., M. Cabiati, S. Rocchiccioli, S. Del Ry, and M. A. Morales. 2013. The role of the adenosinergic system in lung fibrosis. *Pharmacological research* 76: 182-189.

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

51. Eltzschig, H. K., Carmeliet P. 2011. Hypoxia and inflammation. . *New Engl J Med* 364: 656-665. .

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

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

54. 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 (Baltimore, Md. : 1950)* 182: 8037-8046.

55. 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.

56. 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 (Baltimore, Md. : 1950)* 186: 1097-1106.

57. Huszar E, V. G., Vizi E, Csoma Z, Barat E, Molnar VG, Herjavecz I, Horvath I. . 2002. Adenosine in exhaled breath condensate in healthy volunteers and in patients with asthma. *The European respiratory journal* 20: 1393-1398. .

58. Esther, C. R., Jr., A. L. Lazaar, E. Bordonali, B. Qaqish, and R. C. Boucher. 2011. Elevated airway purines in COPD. *Chest* 140: 954-960.

59. 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.

60. 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.

61. 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.

62. 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.

63. 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.

64. 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.

65. 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.

66. Mohsenin, A., M. D. Burdick, J. G. Molina, M. P. Keane, and M. R. Blackburn. 2007. Enhanced CXCL1 production and angiogenesis in adenosine-mediated lung disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 21: 1026-1036.

67. Reutershan, J., I. Vollmer, S. Stark, R. Wagner, K. C. Ngamsri, and H. K. Eltzschig. 2009. Adenosine and inflammation: CD39 and CD73 are critical

mediators in LPS-induced PMN trafficking into the lungs. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 23: 473-482.

68. Eckle, T., A. Grenz, S. Laucher, and H. K. Eltzschig. 2008. A2B adenosine receptor signaling attenuates acute lung injury by enhancing alveolar fluid clearance in mice. *The Journal of clinical investigation* 118: 3301-3315.

69. Morschl, E., J. G. Molina, J. B. Volmer, A. Mohsenin, R. S. Pero, J. S. Hong, F. Kheradmand, J. J. Lee, and M. R. Blackburn. 2008. A3 adenosine receptor signaling influences pulmonary inflammation and fibrosis. *American journal of respiratory cell and molecular biology* 39: 697-705.

70. Stolze, I. P., Y. M. Tian, R. J. Appelhoff, H. Turley, C. C. Wykoff, J. M. Gleadle, and P. J. Ratcliffe. 2004. Genetic analysis of the role of the asparaginyl hydroxylase factor inhibiting hypoxia-inducible factor (FIH) in regulating hypoxia-inducible factor (HIF) transcriptional target genes [corrected]. *The Journal of biological chemistry* 279: 42719-42725.

71. Carroll, V. A., and M. Ashcroft. 2005. Targeting the molecular basis for tumour hypoxia. *Expert reviews in molecular medicine* 7: 1-16.

72. Shimoda, L. A., and G. L. Semenza. 2011. HIF and the lung: role of hypoxia-inducible factors in pulmonary development and disease. *American journal of respiratory and critical care medicine* 183: 152-156.

73. Eltzschig, H. K., and P. Carmeliet. 2011. Hypoxia and inflammation. *The New England journal of medicine* 364: 656-665.

74. Escribese, M. M., M. Casas, and A. L. Corbi. 2012. Influence of low oxygen tensions on macrophage polarization. *Immunobiology* 217: 1233-1240.

75. Frohlich, S., J. Boylan, and P. McLoughlin. 2013. Hypoxia-induced inflammation in the lung: a potential therapeutic target in acute lung injury? *American journal of respiratory cell and molecular biology* 48: 271-279.

76. Tannahill, G. M., A. M. Curtis, J. Adamik, E. M. Palsson-McDermott, A. F. McGettrick, G. Goel, C. Frezza, N. J. Bernard, B. Kelly, N. H. Foley, L. Zheng, A. Gardet, Z. Tong, S. S. Jany, S. C. Corr, M. Haneklaus, B. E. Caffrey, K. Pierce, S. Walmsley, F. C. Beasley, E. Cummins, V. Nizet, M. Whyte, C. T. Taylor, H. Lin, S. L. Masters, E. Gottlieb, V. P. Kelly, C. Clish, P. E. Auron, R. J. Xavier, and L. A. O'Neill. 2013. Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. *Nature* 496: 238-242.

77. Mills, E., and L. A. O'Neill. 2014. Succinate: a metabolic signal in inflammation. *Trends in cell biology* 24: 313-320.

78. Kietzmann, T., and A. Gorlach. 2005. Reactive oxygen species in the control of hypoxia-inducible factor-mediated gene expression. *Seminars in cell & developmental biology* 16: 474-486.

79. Epstein, A. C., J. M. Gleadle, L. A. McNeill, K. S. Hewitson, J. O'Rourke, D. R. Mole, M. Mukherji, E. Metzen, M. I. Wilson, A. Dhanda, Y. M. Tian, N. Masson, D. L. Hamilton, P. Jaakkola, R. Barstead, J. Hodgkin, P. H. Maxwell, C. W. Pugh, C. J. Schofield, and P. J. Ratcliffe. 2001. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107: 43-54.

80. Burroughs, S. K., S. Kaluz, D. Wang, K. Wang, E. G. Van Meir, and B. Wang. 2013. Hypoxia inducible factor pathway inhibitors as anticancer therapeutics. *Future medicinal chemistry* 5: 553-572.

81. Malli, F., A. Koutsokera, E. Paraskeva, E. Zakynthinos, M. Papagianni, D. Makris, I. Tsilioni, P. A. Molyvdas, K. I. Gourgoulianis, and Z. Daniil. 2013. Endothelial progenitor cells in the pathogenesis of idiopathic pulmonary fibrosis: an evolving concept. *PloS one* 8: e53658.

82. Tzouvelekis, A., V. Harokopos, T. Paparountas, N. Oikonomou, A. Chatziioannou, G. Vilaras, E. Tsiambas, A. Karameris, D. Bouros, and V. Aidinis. 2007. Comparative expression profiling in pulmonary fibrosis suggests a role of hypoxia-inducible factor-1alpha in disease pathogenesis. *American journal of respiratory and critical care medicine* 176: 1108-1119.

83. 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.

84. Weng, T., J. M. Poth, H. Karmouty-Quintana, L. J. Garcia-Morales, E. Melicoff, F. Luo, N. Y. Chen, C. M. Evans, R. R. Bunge, B. A. Bruckner, M. Loebe, K. A. Volcik, H. K. Eltzschig, and M. R. Blackburn. 2014. Hypoxia-induced deoxycytidine kinase contributes to epithelial proliferation in pulmonary fibrosis. *American journal of respiratory and critical care medicine* 190: 1402-1412.

85. Byrne, A. J., C. P. Jones, K. Gowers, S. M. Rankin, and C. M. Lloyd. 2013. Lung macrophages contribute to house dust mite driven airway remodeling via HIF-1alpha. *PloS one* 8: e69246.

86. Walmsley, S. R., C. Print, N. Farahi, C. Peyssonnaux, R. S. Johnson, T. Cramer, A. Sobolewski, A. M. Condliffe, A. S. Cowburn, N. Johnson, and E. R. Chilvers. 2005. Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. *The Journal of experimental medicine* 201: 105-115.

87. Morote-Garcia, J. C., P. Rosenberger, J. Kuhlicke, and H. K. Eltzschig. 2008. HIF-1-dependent repression of adenosine kinase attenuates hypoxia-induced vascular leak. *Blood* 111: 5571-5580.

88. Eckle, T., E. M. Kewley, K. S. Brodsky, E. Tak, S. Bonney, M. Gobel, D. Anderson, L. E. Glover, A. K. Riegel, S. P. Colgan, and H. K. Eltzschig. 2014. Identification of hypoxia-inducible factor HIF-1A as transcriptional regulator of the A2B adenosine receptor during acute lung injury. *Journal of immunology (Baltimore, Md. : 1950)* 192: 1249-1256.

89. Eckle, T., K. Brodsky, M. Bonney, T. Packard, J. Han, C. H. Borchers, T. J. Mariani, D. J. Kominsky, M. Mittelbronn, and H. K. Eltzschig. 2013. HIF1A reduces acute lung injury by optimizing carbohydrate metabolism in the alveolar epithelium. *PLoS biology* 11: e1001665.

90. Kong, T., K. A. Westerman, M. Faigle, H. K. Eltzschig, and S. P. Colgan. 2006. HIF-dependent induction of adenosine A2B receptor in hypoxia. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 20: 2242-2250.

91. Morote-Garcia, J. C., P. Rosenberger, N. M. Nivillac, I. R. Coe, and H. K. Eltzschig. 2009. Hypoxia-inducible factor-dependent repression of equilibrative

nucleoside transporter 2 attenuates mucosal inflammation during intestinal hypoxia. *Gastroenterology* 136: 607-618.

92. Synnestvedt, K., G. T. Furuta, K. M. Comerford, N. Louis, J. Karhausen, H. K. Eltzschig, K. R. Hansen, L. F. Thompson, and S. P. Colgan. 2002. Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. *The Journal of clinical investigation* 110: 993-1002.

93. Garcia-Morales, L. J., N. Y. Chen, T. Weng, F. Luo, J. Davies, K. Philip, K. A. Volcik, E. Melicoff, J. Amione-Guerra, R. R. Bunge, B. A. Bruckner, M. Loebe, H. K. Eltzschig, L. M. Pandit, M. R. Blackburn, and H. Karmouty-Quintana. 2016. Altered Hypoxic-Adenosine Axis and Metabolism in Group III Pulmonary Hypertension. *American journal of respiratory cell and molecular biology* 54: 574-583.

94. Murray, P. J., and T. A. Wynn. 2011. Protective and pathogenic functions of macrophage subsets. *Nature reviews. Immunology* 11: 723-737.

95. Mosser, D. M., and J. P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology* 8: 958-969.

96. Wang, N., H. Liang, and K. Zen. 2014. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. *Frontiers in immunology* 5: 614.

97. Takeda, K., M. Kamanaka, T. Tanaka, T. Kishimoto, and S. Akira. 1996. Impaired IL-13-mediated functions of macrophages in STAT6-deficient mice. *Journal of immunology (Baltimore, Md. : 1950)* 157: 3220-3222.

98. Wynn, T. A., and T. R. Ramalingam. 2012. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nature medicine* 18: 1028-1040.

99. Goenka, S., and M. H. Kaplan. 2011. Transcriptional regulation by STAT6. *Immunologic research* 50: 87-96.

100. Kaplan, M. H., U. Schindler, S. T. Smiley, and M. J. Grusby. 1996. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4: 313-319.

101. Chiba, Y., M. Todoroki, Y. Nishida, M. Tanabe, and M. Misawa. 2009. A novel STAT6 inhibitor AS1517499 ameliorates antigen-induced bronchial hypercontractility in mice. *American journal of respiratory cell and molecular biology* 41: 516-524.

102. Mauer, J., B. Chaurasia, J. Goldau, M. C. Vogt, J. Ruud, K. D. Nguyen, S. Theurich, A. C. Hausen, J. Schmitz, H. S. Bronneke, E. Estevez, T. L. Allen, A. Mesaros, L. Partridge, M. A. Febbraio, A. Chawla, F. T. Wunderlich, and J. C. Bruning. 2014. Signaling by IL-6 promotes alternative activation of macrophages to limit endotoxemia and obesity-associated resistance to insulin. *Nature immunology* 15: 423-430.

103. Jiang, Z., and L. Zhu. 2016. Update on the role of alternatively activated macrophages in asthma. *Journal of asthma and allergy* 9: 101-107.

104. Gibbons, M. A., A. C. MacKinnon, P. Ramachandran, K. Dhaliwal, R. Duffin, A. T. Phythian-Adams, N. van Rooijen, C. Haslett, S. E. Howie, A. J. Simpson, N. Hirani, J. Gauldie, J. P. Iredale, T. Sethi, and S. J. Forbes. 2011. Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis. *American journal of respiratory and critical care medicine* 184: 569-581.

105. Redente, E. F., R. C. Keith, W. Janssen, P. M. Henson, L. A. Ortiz, G. P. Downey, D. L. Bratton, and D. W. Riches. 2014. Tumor necrosis factor-alpha accelerates the resolution of established pulmonary fibrosis in mice by targeting profibrotic lung macrophages. *American journal of respiratory cell and molecular biology* 50: 825-837.

106. Thanh-Thuy T Le, H. K.-Q., Ernestina Melicoff, Thanh-Truc T. Le, Ning Yuan Chen, Tingting Weng, Mesias Pedroza, Anuh T. George, Luis J. Garcia-Morales, Raquel R. Bunge, Brian A. Bruckner, Matthias Loebe, Harish Seethamraju, Sandeep K. Agarwal, and Michael R. Blackburn. . 2014. Blockade of interleukin-6 trans signaling attenuates pulmonary fibrosis. .

107. Belikoff, B. G., L. J. Vaickus, M. Sitkovsky, and D. G. Remick. 2012. A2B adenosine receptor expression by myeloid cells is proinflammatory in murine allergic-airway inflammation. *Journal of immunology (Baltimore, Md. : 1950)* 189: 3707-3713.

108. Csoka, B., Z. Selmeczy, B. Koscso, Z. H. Nemeth, P. Pacher, P. J. Murray, D. Kepka-Lenhart, S. M. Morris, Jr., W. C. Gause, S. J. Leibovich, and G. Hasko. 2012. Adenosine promotes alternative macrophage activation via A2A and A2B receptors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 26: 376-386.

109. Koscso, B., B. Csoka, E. Kokai, Z. H. Nemeth, P. Pacher, L. Virag, S. J. Leibovich, and G. Hasko. 2013. Adenosine augments IL-10-induced STAT3 signaling in M2c macrophages. *Journal of leukocyte biology* 94: 1309-1315.

110. Cramer, T., Y. Yamanishi, B. E. Clausen, I. Forster, R. Pawlinski, N. Mackman, V. H. Haase, R. Jaenisch, M. Corr, V. Nizet, G. S. Firestein, H. P. Gerber, N.

Ferrara, and R. S. Johnson. 2003. HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* 112: 645-657.

111. Strehl, C., M. Fangradt, U. Fearon, T. Gaber, F. Buttgereit, and D. J. Veale. 2014. Hypoxia: how does the monocyte-macrophage system respond to changes in oxygen availability? *Journal of leukocyte biology* 95: 233-241.

112. McNamee Eó, N., D. K. Johnson, D. Homann, and E. T. Clambey. 2013. Hypoxia and hypoxia-inducible factors as regulators of T cell development, differentiation, and function. *Immunologic research* 55: 58-70.

113. Karmouty-Quintana, H., K. Philip, L. F. Acero, N. Y. Chen, T. Weng, J. G. Molina, F. Luo, J. Davies, N. B. Le, I. Bunge, K. A. Volcik, T. T. Le, R. A. Johnston, Y. Xia, H. K. Eltzschig, and M. R. Blackburn. 2015. Deletion of ADORA2B from myeloid cells dampens lung fibrosis and pulmonary hypertension. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 29: 50-60.

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

115. Moore, B. B., and C. M. Hogaboam. 2008. Murine models of pulmonary fibrosis. *American journal of physiology. Lung cellular and molecular physiology* 294: L152-160.

116. Baran, C. P., J. M. Opalek, S. McMaken, C. A. Newland, J. M. O'Brien, 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.

117. Tomcik, M., P. Zerr, J. Pitkowski, K. Palumbo-Zerr, J. Avouac, O. Distler, R. Becvar, L. Senolt, G. Schett, and J. H. Distler. 2014. Heat shock protein 90 (Hsp90) inhibition targets canonical TGF-beta signalling to prevent fibrosis. *Annals of the rheumatic diseases* 73: 1215-1222.

118. Hubner, R. H., W. Gitter, N. E. El Mokhtari, M. Mathiak, M. Both, H. Bolte, S. Freitag-Wolf, and B. Bewig. 2008. Standardized quantification of pulmonary fibrosis in histological samples. *BioTechniques* 44: 507-511, 514-507.

119. 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 (Baltimore, Md. : 1950)* 175: 1937-1946.

120. Fishman, P., S. Bar-Yehuda, B. T. Liang, and K. A. Jacobson. 2012. Pharmacological and therapeutic effects of A3 adenosine receptor agonists. *Drug discovery today* 17: 359-366.

121. Hickey, M. M., T. Richardson, T. Wang, M. Mosqueira, E. Arguiri, H. Yu, Q. C. Yu, C. C. Solomides, E. E. Morrisey, T. S. Khurana, M. Christofidou-Solomidou, and M. C. Simon. 2010. The von Hippel-Lindau Chuvash mutation promotes pulmonary hypertension and fibrosis in mice. *The Journal of clinical investigation* 120: 827-839.

122. Martinez, F. O., L. Helming, and S. Gordon. 2009. Alternative activation of macrophages: an immunologic functional perspective. *Annual review of immunology* 27: 451-483.

123. Laria, A., A. Lurati, M. Marrazza, D. Mazzocchi, K. A. Re, and M. Scarpellini. 2016. The macrophages in rheumatic diseases. *Journal of inflammation research* 9: 1-11.

124. Le, T. T., H. Karmouty-Quintana, E. Melicoff, T. T. Le, T. Weng, N. Y. Chen, M. Pedroza, Y. Zhou, J. Davies, K. Philip, J. Molina, F. Luo, A. T. George, L. J. Garcia-Morales, R. R. Bunge, B. A. Bruckner, M. Loebe, H. Seethamraju, S. K. Agarwal, and M. R. Blackburn. 2014. Blockade of IL-6 Trans Signaling Attenuates Pulmonary Fibrosis. *Journal of immunology (Baltimore, Md. : 1950)*.

125. Park, S. W., M. H. Ahn, H. K. Jang, A. S. Jang, D. J. Kim, E. S. Koh, J. S. Park, S. T. Uh, Y. H. Kim, J. S. Park, S. H. Paik, H. K. Shin, W. Youm, and C. S. Park. 2009. Interleukin-13 and its receptors in idiopathic interstitial pneumonia: clinical implications for lung function. *Journal of Korean medical science* 24: 614-620.

126. Jakubzick, C., E. S. Choi, S. L. Kunkel, H. Evanoff, F. J. Martinez, R. K. Puri, K. R. Flaherty, G. B. Toews, T. V. Colby, E. A. Kazerooni, B. H. Gross, W. D. Travis, and C. M. Hogaboam. 2004. Augmented pulmonary IL-4 and IL-13 receptor subunit expression in idiopathic interstitial pneumonia. *Journal of clinical pathology* 57: 477-486.

127. Rottoli, P., B. Magi, M. G. Perari, S. Liberatori, N. Nikiforakis, E. Bargagli, R. Cianti, L. Bini, and V. Pallini. 2005. Cytokine profile and proteome analysis in bronchoalveolar lavage of patients with sarcoidosis, pulmonary fibrosis associated with systemic sclerosis and idiopathic pulmonary fibrosis. *Proteomics* 5: 1423-1430.

128. Rafii, R., M. M. Juarez, T. E. Albertson, and A. L. Chan. 2013. A review of current and novel therapies for idiopathic pulmonary fibrosis. *Journal of thoracic disease* 5: 48-73.

129. Cowburn, A. S., A. Crosby, D. Macias, C. Branco, R. D. Colaco, M. Southwood, M. Toshner, L. E. Crotty Alexander, N. W. Morrell, E. R. Chilvers, and R. S. Johnson. 2016. HIF2alpha-arginase axis is essential for the development of pulmonary hypertension. *Proceedings of the National Academy of Sciences of the United States of America* 113: 8801-8806.

130. Eckle, T., M. Faigle, A. Grenz, S. Laucher, L. F. Thompson, and H. K. Eltzschig. 2008. A2B adenosine receptor dampens hypoxia-induced vascular leak. *Blood* 111: 2024-2035.

131. Krick, S., B. G. Eul, J. Hanze, R. Savai, F. Grimminger, W. Seeger, and F. Rose. 2005. Role of hypoxia-inducible factor-1alpha in hypoxia-induced apoptosis of primary alveolar epithelial type II cells. *American journal of respiratory cell and molecular biology* 32: 395-403.

132. Crotty Alexander, L. E., K. Akong-Moore, S. Feldstein, P. Johansson, A. Nguyen, E. K. McEachern, S. Nicatia, A. S. Cowburn, J. Olson, J. Y. Cho, H. Isaacs, Jr., R. S. Johnson, D. H. Broide, and V. Nizet. 2013. Myeloid cell HIF-1alpha regulates asthma airway resistance and eosinophil function. *Journal of molecular medicine (Berlin, Germany)* 91: 637-644.

133. Lee, P. Y., J. X. Wang, E. Parisini, C. C. Dascher, and P. A. Nigrovic. 2013. Ly6 family proteins in neutrophil biology. *Journal of leukocyte biology* 94: 585-594.

134. McCabe, A. J., M. Dowhy, B. A. Holm, and P. L. Glick. 2001. Myeloperoxidase activity as a lung injury marker in the lamb model of congenital diaphragmatic hernia. *Journal of pediatric surgery* 36: 334-337.

135. Volmer, J. B., L. F. Thompson, and M. R. Blackburn. 2006. Ecto-5'-nucleotidase (CD73)-mediated adenosine production is tissue protective in a model of bleomycin-induced lung injury. *Journal of immunology (Baltimore, Md. : 1950)* 176: 4449-4458.

136. 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.

137. Izumi, S., M. Iikura, and S. Hirano. 2012. Prednisone, azathioprine, and N-acetylcysteine for pulmonary fibrosis. *The New England journal of medicine* 367: 870; author reply 870-871.

138. Rawlins, E. L., and A. K. Perl. 2012. The a"MAZE"ing world of lung-specific transgenic mice. *American journal of respiratory cell and molecular biology* 46: 269-282.

139. Koscso, B., B. Csoka, P. Pacher, and G. Hasko. 2011. Investigational A(3) adenosine receptor targeting agents. *Expert opinion on investigational drugs* 20: 757-768.

140. Lech, M., and H. J. Anders. 2013. Macrophages and fibrosis: How resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair. *Biochimica et biophysica acta* 1832: 989-997.

141. Kirby, A. C., J. G. Raynes, and P. M. Kaye. 2006. CD11b regulates recruitment of alveolar macrophages but not pulmonary dendritic cells after pneumococcal challenge. *The Journal of infectious diseases* 193: 205-213.

142. Misharin, A. V., L. Morales-Nebreda, G. M. Mutlu, G. R. Budinger, and H. Perlman. 2013. Flow cytometric analysis of macrophages and dendritic cell subsets in the mouse lung. *American journal of respiratory cell and molecular biology* 49: 503-510.

143. Zaynagetdinov, R., T. P. Sherrill, P. L. Kendall, B. H. Segal, K. P. Weller, R. M. Tighe, and T. S. Blackwell. 2013. Identification of myeloid cell subsets in murine lungs using flow cytometry. *American journal of respiratory cell and molecular biology* 49: 180-189.

144. Chao, J., J. G. Wood, V. G. Blanco, and N. C. Gonzalez. 2009. The systemic inflammation of alveolar hypoxia is initiated by alveolar macrophage-borne mediator(s). *American journal of respiratory cell and molecular biology* 41: 573-582.

145. Kummar, S., M. E. Gutierrez, E. R. Gardner, X. Chen, W. D. Figg, M. Zajac-Kaye, M. Chen, S. M. Steinberg, C. A. Muir, M. A. Yancey, Y. R. Horneffer, L. Juwara, G. Melillo, S. P. Ivy, M. Merino, L. Neckers, P. S. Steeg, B. A. Conley, G. Giaccone, J. H. Doroshow, and A. J. Murgo. 2010. Phase I trial of 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), a heat shock protein inhibitor, administered twice weekly in patients with advanced malignancies. *European journal of cancer (Oxford, England : 1990)* 46: 340-347.

146. Bodempudi, V., P. Hergert, K. Smith, H. Xia, J. Herrera, M. Peterson, W. Khalil, J. Kahm, P. B. Bitterman, and C. A. Henke. 2014. miR-210 promotes IPF fibroblast proliferation in response to hypoxia. *American journal of physiology. Lung cellular and molecular physiology* 307: L283-294.

147. Pereira, E. R., K. Frudd, W. Awad, and L. M. Hendershot. 2014. Endoplasmic reticulum (ER) stress and hypoxia response pathways interact to potentiate hypoxia-inducible factor 1 (HIF-1) transcriptional activity on targets like vascular endothelial growth factor (VEGF). *The Journal of biological chemistry* 289: 3352-3364.

148. Ayaub, E. A., P. S. Kolb, Z. Mohammed-Ali, V. Tat, J. Murphy, P. S. Bellaye, C. Shimbori, F. J. Boivin, R. Lai, E. G. Lynn, S. Lhotak, D. Bridgewater, M. R. Kolb, M. D. Inman, J. G. Dickhout, R. C. Austin, and K. Ask. 2016. GRP78 and CHOP modulate macrophage apoptosis and the development of bleomycin-induced pulmonary fibrosis. *The Journal of pathology* 239: 411-425.

149. Yao, Y., Y. Wang, Z. Zhang, L. He, J. Zhu, M. Zhang, X. He, Z. Cheng, Q. Ao, Y. Cao, P. Yang, Y. Su, J. Zhao, S. Zhang, Q. Yu, Q. Ning, X. Xiang, W. Xiong, C. Y. Wang, and Y. Xu. 2016. Chop Deficiency Protects Mice Against Bleomycin-induced Pulmonary Fibrosis by Attenuating M2 Macrophage Production. *Molecular therapy : the journal of the American Society of Gene Therapy* 24: 915-925.

150. Oh, J., A. E. Riek, S. Weng, M. Petty, D. Kim, M. Colonna, M. Cella, and C. Bernal-Mizrachi. 2012. Endoplasmic reticulum stress controls M2 macrophage differentiation and foam cell formation. *The Journal of biological chemistry* 287: 11629-11641.

151. 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.

152. Organization., W. H. 2013. Asthma. .

153. Network, T. G. A. 2014. The Global Asthma Report 2014. Auckland, New Zealand. 16-20.

154. Barnes, J., and M. D. Mayes. 2012. Epidemiology of systemic sclerosis: incidence, prevalence, survival, risk factors, malignancy, and environmental triggers. *Current opinion in rheumatology* 24: 165-170.

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PUBLICATIONS:

Philip K, Mills T, Davies J, Chen NY, Karmouty-Quintana H, Luo F, Molina JG, Amione-Guerra J, Sinha N, Guha A, Eltzschig HK, and Blackburn MR. HIF1A Up-regulates the ADORA2B receptor on Alternatively-Activated Macrophages and Contributes to Pulmonary Fibrosis. *Submitted 24 January 2017. Under Review*.

Philip K, Mills T, Davies J, Chen NY, Karmouty-Quintana H, Hernandez A, Luo F, Molina JG, Eltzschig HK, and Blackburn MR. Myeloid-specific HIF1A deletion Exacerbates Acute Lung Injury. *In Preparation.*

Bosques G, Dauer K, and **Philip K**. Pediatric Immune-mediated Brachial Plexopathy. *PM&R Knowledge NOW*. Accepted 21 December 2016.

Karmouty-Quintana H, Molina JG, **Philip K**, Wu M, Chen NY, Collum S, Agarwal SK, Zhong H, Zeng D and Blackburn MR. ADORA2B modulates dermal fibrosis. *Under Review.*

Chen NY, Collum S, Luo F, Weng T, Le T, Hernandez A, **Philip K**, Molina JG, Garcia-Morales LJ, Cao Y, Ko TC, Amione-Guerra J, Al-Jabbari O, Bunge RR, Youker K, Bruckner BA, Hamid R, Davies J, Sinha N, and Karmouty Quintana H. Macrophage Bone Morphogenic Protein 2 (BMPR2) deletion in Idiopathic Pulmonary Fibrosis (IPF) and Group III Pulmonary Hypertension. *AJPLung.* Accepted 10 June 2016.

Luo F, Le NB, Mills T, Chen NY, Karmout-Quintana H, Molina JG, Davies J, **Philip K**, Volcik K, Liu H, Xia Y, Eltzschig HK, and Blackburn MR. Extracellular Adenosine Levels are Associated with the Progression and Exacerbation of Pulmonary Fibrosis. *FASEB*. Accepted 19 October 2015.

Garcia-Morales LJ, Chen NY, Weng T, Luo F, Davies J, **Philip K**, Volcik K, Melicoff E, Amione-Guerra J, Bunge RR, Bruckner BA, Loebe M, Eltzschig HK, Pandit LM, Blackburn MR, and Karmouty-Quintana H. Altered Hypoxic-Adenosine Axis and Metabolism in Group III Pulmonary Hypertension. *AJRCMB.* 28 September 2015.

Karmouty-Quintana H, **Philip K**, Acero LF, Chen NY, Weng T, Molina JG, Luo F, Davies J, Le N, Bunge I, Volcik K, Le T, Johnston R, Xia Y, Eltzschig HK,Blackburn MR. Deletion of ADORA2B from myeloid cells dampens lung fibrosis and pulmonary hypertension. *FASEB*. Submitted 14 May 2014. Accepted 26 August 2014.

Le TT, Karmouty-Quintana H, Melicoff E, Le TT, Weng T, Chen NY, Pedroza M, Zhou Y, Davies J, **Philip K**, Molina J, Luo F, George AT, Garcia-Morales LJ, Bunge RR, Bruckner BA, Loebe M, Seethamraju H, Agarwal SK, Blackburn MR. Blockade of IL-6 Trans Signaling Attenuates Pulmonary Fibrosis. *J Immunol*. 2014 Aug 29.