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## THE ROLE OF A2B ADENOSINE RECEPTOR SIGNALING IN ADENOSINE DEPENDENT LUNG DISEASE

Yang Zhou

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# THE ROLE OF A<sub>2B</sub> ADENOSINE RECEPTOR SIGNALING IN ADENOSINE DEPENDENT LUNG DISEASE

By

Yang Zhou, B.S.

APPROVED:

---

Michael R. Blackburn, Ph.D.  
Supervisory Professor

---

Scott Drouin, Ph.D.

---

Christopher M. Evans, Ph.D.

---

Renhao Li, Ph.D.

---

Jayasimha N. Murthy, M.D.

APPROVED:

---

George Stancel, Ph.D.  
Dean, The University of Texas  
Graduate School of Biomedical Sciences at Houston

# **THE ROLE OF A<sub>2B</sub> ADENOSINE RECEPTOR SIGNALING IN ADENOSINE DEPENDENT LUNG DISEASE**

A

## **DISSERTATION**

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M.D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment  
  
of the Requirements  
for the Degree of

## **DOCTOR OF PHILOSOPHY**

By

Yang Zhou, B.S.

Houston, Texas

May, 2010

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# **The Role of A<sub>2B</sub>R Signaling in Adenosine Dependent Lung Disease**

Publication No. \_\_\_\_\_

Yang Zhou, B.S.

Supervisory Professor: Michael R. Blackburn, Ph.D.

Chronic lung diseases and acute lung injuries are two distinctive pulmonary disorders that result in significant morbidity and mortality. Adenosine is a signaling nucleoside generated in response to injury and can serve both protective and destructive functions in tissues and cells through interaction with four G-protein coupled adenosine receptors: A<sub>1</sub>R, A<sub>2A</sub>R, A<sub>2B</sub>R, and A<sub>3</sub>R. However, the relationship between these factors is poorly understood. Recent findings suggest the A<sub>2B</sub>R has been implicated in the regulation of both chronic lung disease and acute lung injury. The work presented in this dissertation utilized the adenosine deaminase-deficient mouse model and the bleomycin-induced pulmonary injury model to determine the distinctive roles of the A<sub>2B</sub>R at different stages of the disease. Results demonstrate that the A<sub>2B</sub>R plays a protective role in attenuating vascular leakage in acute lung injuries and a detrimental role at chronic stages of the disease. In addition, tissues from patients with chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis were utilized to examine adenosine metabolism and signaling in chronic lung diseases. Results demonstrate that components of adenosine metabolism and signaling are altered in a manner that promotes adenosine production and signaling in the lungs of these patients. Furthermore, this study provides the first evidence that A<sub>2B</sub>R signaling can promote the production of inflammatory and fibrotic mediators in patients with these disorders. Taken together, these findings suggest that the A<sub>2B</sub>R may have a bi-phasic effect at different stages of lung disease. It is protective in acute injury, whereas pro-inflammatory and pro-fibrotic at the chronic stage. Patients with acute lung injury or chronic lung disease may both benefit from adenosine and A<sub>2B</sub>R-based therapeutics.

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# **CHAPTER 1**

## **INTRODUCTION**

Chronic lung diseases are featured by processes such as inflammation and tissue remodeling that contribute to a progressive pulmonary function decline. The prominent and deadly features in these disorders make them the third leading cause of death in the US following cancer and cardiovascular disease. Acute lung injury and acute respiratory distress syndrome contribute significantly to critical illness. Although chronic lung diseases and acute lung injuries exhibit distinct molecular basis and pathogenesis, they both result in significant morbidity and mortality and some overlapping features are observed in these disorders. Studies are needed to investigate the mechanisms involved in disease progression and the similarities and differences between these disorders.

### **Chronic Lung Disease and Acute Lung Injury**

Chronic lung disease describes pulmonary disorders where persistent inflammation and structural alternation contribute to progressive loss of lung function (1). Examples of chronic lung disease include asthma, chronic obstructive pulmonary disease (COPD) and interstitial lung disease. Asthma is characterized by episodic airway obstruction, airway hyperresponsiveness, cellular infiltration, mucus hypersecretion and airway remodeling (2, 3). COPD is accompanied by chronic bronchitis and emphysema (4), while interstitial lung

diseases exhibit varying degrees of airway fibrosis and inflammation (5, 6). These lung diseases afflict numerous individuals and result in hundreds of thousands of dollars in annual health costs. Currently there are no effective treatments, especially during the chronic phase of these diseases. The inflammatory response seen in these disorders involves the recruitment of multiple types of inflammatory cells and results in the release of a number of soluble mediators that drive inflammation and remodeling processes in the lung (7, 8). Although the mechanisms responsible for the genesis of inflammation and the regulation of tissue remodeling have been well established, relatively little is described about the signaling pathways that serve to regulate the progressive and chronic nature of these diseases (9).

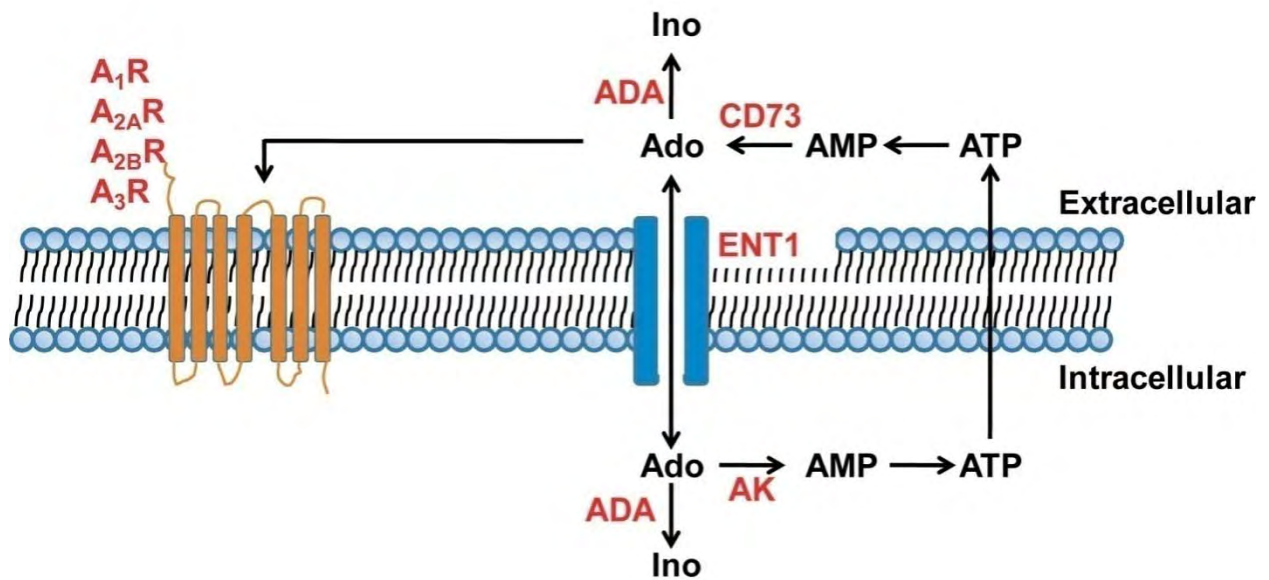
A normal wound healing response is involved in chronic lung diseases. For example, collagen deposition, epithelial cell remodeling and angiogenesis around the bronchial airways are characteristic features in asthma. Interstitial lung diseases are featured by fibroblast proliferation and extensive extracellular matrix deposition, whereas imbalances in matrix proteases and anti-proteases and destruction of the alveolar airways are often observed in patients with COPD. It has been suggested that the excessive tissue remodeling processes that are observed in chronic lung diseases result from overactive or deregulated wound healing pathways (2, 10). Efforts targeted at understanding the molecular pathogenesis of these diseases and how the diseases progress will lead to better treatment options and decreased mortality.

Acute lung injury and acute respiratory distress syndrome can develop in a number of different clinical conditions such as pneumonia, acid aspiration, major trauma, or prolonged mechanical ventilation (11). Experimental and clinical studies have demonstrated that the pathogenesis of TH1 inflammation and acute lung injury are characterized by pulmonary trafficking of polymorphonuclear leukocytes (PMNs) into the lungs (12). Activation of circulating PMNs initiates the recruitment of leukocytes to the lung (13). Transendothelial migration and transepithelial migration into the lung interstitium and into the alveolar space are initiated following PMNs adherence to the pulmonary vessel wall. Enhanced inflammation and damage is associated with enhanced permeability of microvasculature, thus pulmonary vascular barrier function serves as a protective barrier in acute stages of injury (14, 15). However, the molecular mechanisms underlying PMN trafficking in the lung is not fully understood. Better understanding the substantial mechanisms of PMN migration into the tissue in ALI and ARDS-typical symptoms, including the regulation of microvascular permeability, expression of adhesion molecules, release of cytokines and chemokines, will help prevent or treat these acute injuries (16, 17).

### **Adenosine Metabolism and Signaling**

Adenosine is a signaling molecule that is generated at sites of organ damage and tissue injury and is therefore increased during tissue hypoxia and inflammation (18). ATP is released into the extracellular space, where it is quickly





**Figure 1**

Adenosine metabolism and signaling. In response to cellular stress and damage, ATP is released into the extracellular space and is rapidly dephosphorylated by extracellular nucleotidases. CD73 catalyzes the formation of extracellular adenosine from AMP. Extracellular adenosine can interact with seven-transmembrane adenosine receptors,  $A_1R$ ,  $A_{2A}R$ ,  $A_{2B}R$ , and  $A_3R$ , which are coupled by heterotrimeric G proteins to various second messenger systems, or it can be transported into cells via facilitated nucleoside transporters, such as ENT1. Both extracellular and intracellular adenosine can be deaminated to inosine by adenosine deaminase (ADA). Intracellular adenosine can be secreted or phosphorylated back to ATP. The first step in this process is catalyzed by adenosine kinase (AK).

dephosphorylated by extracellular nucleotidases to form extracellular adenosine (19). Adenosine can enter cells via facilitated nucleoside transporters, or function on cells by interacting with G protein coupled adenosine receptors (20). Both extracellular adenosine and intracellular adenosine can be deaminated to inosine by the enzyme adenosine deaminase (ADA) (21) (Figure 1). Adenosine has potent anti-inflammatory as well as pro-inflammatory functions (22). Expression profiles of specific adenosine receptors on specific organs and cells likely mediate the distinct pro- and anti-inflammatory roles of adenosine (18). Four adenosine receptors have been identified: A<sub>1</sub>R, A<sub>2A</sub>R, A<sub>2B</sub>R and A<sub>3</sub>R. These receptors have a seven membrane-spanning structure and are coupled to heterotrimeric G proteins. Each adenosine receptor has a unique affinity and a distinct cellular and tissue distribution (20, 23). Adenosine receptor signaling can influence cellular physiology through a variety of mechanisms and access multiple intracellular signaling pathways (24). A<sub>1</sub>R and A<sub>3</sub>R are coupled to inhibitory G proteins and can downregulate adenylyl cyclase activity to decrease cAMP levels, while A<sub>2A</sub>R and A<sub>2B</sub>R are coupled to stimulatory G proteins and increase cAMP levels. In addition, adenosine receptors are coupled to other signaling molecules such as Phospholipase C, PI3K and MAPKs.

Adenosine plays distinctive roles in chronic versus acute lung injuries. In chronic lung diseases, elevations in adenosine are found in these patients (25) and inhaled adenosine and/or its precursor AMP elicit bronchoconstriction in asthmatics and COPD patients (26). Adenosine is generated following injury and it can promote processes that are critical in wound healing such as angiogenesis,

matrix production and the regulation of inflammation. An A<sub>2A</sub>R agonist can increase the rate of wound healing by increasing the production of extracellular matrix and angiogenesis (27). A<sub>2A</sub>R and A<sub>2B</sub>R activation promotes fibroblast differentiation and matrix production (28-30). It also results in VEGF production (31), endothelial cell proliferation (32), and granulation tissue formation, thus leading to angiogenesis (33). In addition, the engagement of adenosine receptors influences mast cell degranulation (34), eosinophil function (35) and stimulates mucus production by airway epithelium (36). These findings suggest that adenosine signaling can influence cellular events in chronic lung diseases and adenosine is suggested to play an important role in regulating the chronic nature of asthma and COPD. Whereas it appears that adenosine plays a largely detrimental and tissue-destructive role in the regulation of chronic lung disease, substantial evidence suggests that extracellular adenosine signaling plays important anti-inflammatory roles in acute lung injury through engaging its receptors. During acute injuries, adenosine is generated in extracellular space to regulate the acute inflammatory response. CD39 and CD73 are the enzymes that coordinate a two-step conversion of adenosine from its precursors. It has been shown that CD39 and CD73 levels are elevated in the lungs following LPS stimulation and neutrophil accumulation was enhanced in mice treated with inhibitors of these enzymes and in CD39 and CD73-deficient mice (17, 37). In addition, numerous genetic and pharmacologic antagonism studies have identified specific adenosine receptors involved in tissue protection in various acute injury models such as LPS induced injury (38), ventilator-induced lung

injury (39), hypoxia-induced injury (14), and ischemia reperfusion injury (40-44). Because of the diverse effects of adenosine and its receptors, extensive research has been dedicated to developing potential therapeutics targeting adenosine metabolism and signaling.

### **Adenosine Deaminase-deficient Mouse Model**

A mouse model of adenosine-mediated pulmonary injury has been developed in our lab and it has allowed us to examine the consequences of adenosine elevations on pulmonary inflammation and tissue remodeling. The purine catabolic enzyme, ADA, decreases the levels of adenosine as well as deoxyadenosine in tissues and cells. Genetic removal of ADA results in a significant elevation of concentrations of these substrates systemically. A severe combined immunodeficiency is observed in ADA-deficient mice because of the accumulation of deoxyadenosine in the thymus and spleen (45, 46), while adenosine accumulations result in the development of pulmonary inflammation and lung architecture remodeling, features consistent with various aspects of chronic lung disease seen in human. ADA-deficient mice spontaneously develop signs of respiratory distress and progressive pulmonary injury characterized by increases in activated alveolar macrophages, enlargement of alveolar space, mucus production in bronchial airways, and airway hyperresponsiveness. They also develop subepithelial fibrosis and extracellular matrix deposition, features consistent with interstitial lung diseases (47, 48). In addition, there is an up-regulation in levels of cytokines and pro-inflammatory mediators commonly

elevated in association with asthma and COPD including serum IgE and TH2 cytokines (47, 49), mediators of airspace enlargement such as matrix metalloproteases and cathepsins (49). These features are seen in patients with chronic lung diseases and are related to increases in lung adenosine levels.

Additional evidence suggesting that the phenotypes seen in ADA-deficient mice are directly mediated by adenosine levels is seen through the use of exogenous ADA enzyme therapy. ADA covalently linked to polyethylene glycol (Peg-ADA) was used to treat ADA-deficient mice and prevented or reversed many aspects of the pulmonary phenotype (45, 50). If ADA-deficient mice are kept on PEG-ADA enzyme therapy from birth, the phenotype is completely prevented allowing normal development and life span. In addition, ADA enzyme therapy can be used to treat ADA-deficient mice even after they have established severe pulmonary phenotypes, thus reversing pulmonary injury. These findings display a clear correlation between adenosine levels, its contribution to the overall morbidity in this model, and the presence of lung pathology. The ADA-deficient mouse model and the use of ADA enzyme therapy provide us a great system to characterize the role of adenosine in the pathology of chronic lung diseases.

### **A<sub>2B</sub> Adenosine Receptor**

The A<sub>2B</sub>R is widely expressed in tissues and cells and has the lowest affinity for adenosine (20). It has been shown that all cell types in the lung express A<sub>2B</sub>R transcripts and the levels of expression increase in inflammatory

environments. It is generally accepted that A<sub>2B</sub>R is coupled to Gs proteins to activate adenyl cyclase and increase cAMP levels (51, 52). In addition, regulatory proteins of the Gq family are thought to play a role in the coupling of A<sub>2B</sub>Rs to phospholipase C (PLC) in human mast HMC-1 cells (53). A<sub>2B</sub>R activation has both anti-inflammatory and pro-inflammatory actions and both pro- and anti-fibrotic roles (54). Numerous studies have shown that the A<sub>2B</sub>R has tissue destructive functions in that the engagement of the A<sub>2B</sub>R can promote the differentiation of pulmonary fibroblasts into myofibroblasts and hence increase the deposition of collagen and fibrosis (30). Engagement of the A<sub>2B</sub>R can mediate the direct expression of pro-inflammatory cytokines such as IL-8, IL-4 and IL-13 from human HMC-1 cells (53, 55) and mouse mast cells (56). Consistent with this, treatment of ADA-deficient mice with an A<sub>2B</sub>R antagonist resulted in diminished pulmonary inflammation and fibrosis (57). In contrast to these results, recent observations using A<sub>2B</sub>R knockout mice suggest that the ablation of the A<sub>2B</sub>R causes low-grade inflammation and enhanced leukocyte adhesion to the vasculature (58). In addition, the deletion of this Gs-coupled receptor on mouse bone marrow-derived mast cells leads to decreased levels of cAMP and enhances mast cell activation (59). Moreover, it is proposed that A<sub>2B</sub>R signaling is responsible for the phosphorylation of vasodilator-stimulated phosphoprotein, which controls the geometry of actin-filaments and mediates junctional protein distribution, thereby promoting increases in endothelial barrier functions by affecting the characteristics of the endothelial junctional complex (3). Clearly, discrepancies exist when investigating the roles of the A<sub>2B</sub>R. These

findings suggest that engagement of the  $A_{2B}R$  is complex and additional studies are needed in order to clarify the  $A_{2B}R$ 's multiple inflammatory and remodeling roles during the course of injury and repair.

## **Dissertation Overview**

The overall aim of this dissertation is to better understand the role of the  $A_{2B}$  adenosine receptor in pulmonary disease. To accomplish this goal, I have utilized two animal models to characterize  $A_{2B}R$ 's role from different aspects and at different stages of lung disease. In Chapter 3, I characterized the phenotypes in ADA/ $A_{2B}R$  double knockout mice to study the contribution of this receptor in adenosine dependent lung injury. Based on a previous receptor antagonism study shown that the  $A_{2B}R$  plays a tissue-destructive and pro-fibrotic role, I hypothesized that the genetic removal of the  $A_{2B}R$  from ADA-deficient mice would lead to enhanced pulmonary injury and damage. Unexpectedly, lack of the  $A_{2B}R$  in ADA-deficient mice resulted in loss of vascular barrier functions and ADA/ $A_{2B}R$  double knockout mice showed enhanced pulmonary phenotypes compared to ADA-deficient mice (60). I then hypothesized that the  $A_{2B}R$  may play a homeostatic and protective role in regulating vascular barrier function during the early or acute stage of pulmonary injury, while a tissue-destructive and pro-fibrotic role at the chronic stage of the disease. In Chapter 4, I utilized an animal model of pulmonary fibrosis to directly investigate the bi-phase role of  $A_{2B}R$  at different stages of lung injury. Finally, in Chapter 5, I utilized specimens from patients with chronic lung disease to further investigate the roles of the  $A_{2B}R$  in

human disease. I hypothesized that adenosine metabolism and signaling are altered in human diseases. My findings suggest that A<sub>2B</sub>R signaling pathways are enhanced in severe diseases and that these pathways play important roles in exacerbating disease status.

The findings in this dissertation raise the possibility that adenosine-based therapeutics may be beneficial in the treatment of chronic lung diseases. They also suggest that A<sub>2B</sub>R signaling has important anti-inflammatory activities during early stages of lung disease that were revealed by the global genetic removal of the A<sub>2B</sub>R, whereas A<sub>2B</sub>R engagement during active disease may contribute to disease progression. As the A<sub>2B</sub>R antagonist is under phase II clinical trials to treat chronic lung disease, these findings are particularly timely and important. Our findings suggest that the stage of disease and the degree of A<sub>2B</sub>R antagonism or agonism will be critical variables to assess when using A<sub>2B</sub>R based therapeutics in the treatment of lung diseases. Overall, these studies provide novel insight into the role of the A<sub>2B</sub>R in pulmonary disease and will aid in the development of novel therapies.



# CHAPTER 2

## EXPERIMENTAL PROCEDURES

### Generation and Genotyping of Mouse Lines

ADA-deficient mice were generated and genotyped as previously described (46). Mice homozygous for the null *Ada* allele were designated ADA-deficient ( $ADA^{-/-}$ ). Mice heterozygous for the null *Ada* allele were designated as control mice ( $ADA^{+}$ ). ADA/ $A_{2B}R$  double knockout mice were generated by mating  $ADA^{-/-}$  mice with  $A_{2B}R$ -deficient mice ( $A_{2B}R^{-/-}$ ). Mice were congenic on C57BL/6 background, and phenotypic comparisons were conducted among littermates. Animal care was in accordance with NIH guidelines and the Animal Care Committee at the University of Texas Health Science Center at Houston. All animals were housed under strict containment protocols and maintained in ventilated cages equipped with microisolator lids. Serologies on cage littermates were negative for 12 of the most commonly seen viruses in mice. No evidence of fungal, parasitic, or bacterial infection was found.

### ADA Enzyme Therapy

Polyethylene glycol modified-ADA (PEG-ADA) was generated by the covalently linking purified bovine ADA with activated polyethylene glycol (45).  $ADA^{-/-}$  mice were injected intramuscularly with Peg-ADA on postnatal days 1, 5, 9, 13, 17 (0.625, 1.25, 2.5, 2.5 and 2.5 units, respectively) and intraperitoneal

injections on postnatal day 21 (5 units). Animals were sacrificed on postnatal day 35 (14 days after the last Peg-ADA injection).

### **Bronchial Alveolar Lavage and Histology**

Avertin was used to anesthetize the animals, and airways were lavaged 4 times with 0.3 ml PBS; about 1 ml of pooled lavage fluid was able to be recovered. Total cell numbers were determined by counting on a hemocytometer. Cellular differentials were determined by cytopinning BAL aliquots onto microscope slides and staining with Diff-Quick (Dade Behring) for. After lavage, the lungs were inflated with 10% buffered formalin phosphate in PBS at 25 cm of pressure and then fixed at 4°C overnight. Formalin-fixed lungs were dehydrated in ethanol gradients, and then paraffin embedding was performed. 5 µm tissue sections were cut and collected on microscope slides and stained with H&E (Shandon-Lipshaw) according to manufacturer's instructions.

### **Immunostaining on Mouse Lung Sections**

Rehydrated slides were quenched with hydrogen peroxide (3%), followed by antigen retrieval for 30 min at 95°C (Dako Corp.), and endogenous avidin and biotin blocking was performed with a Biotin Blocking System (DAKO Corp.). Slides were incubated with Rat Anti-mouse Neutrophils Antibody (AbD SeroTec, 1:500 dilution, overnight at 4°C). ABC Streptavidin reagents and individual appropriate secondary antibodies were used to incubate lung sections, and then lung sections were developed with 3, 3'-diaminobenzidine (Sigma-Aldrich) and

methyl green counterstaining was performed. Slides were coverslipped with Vectashield (Vector Laboratories) mounting medium.

### **Airspace Size Measurement**

Mean chord lengths on H&E-stained lung sections cut from a constant-pressure infused lungs were used to determine the average alveolar airspace size (47). Representative images were captured, and overlaid with a grid consisting of 53 black lines at 10.5- $\mu$ m intervals. This line grid was subtracted from the lung images using Image-Pro Plus image analysis software (Media Cybernetics), and the mean chord length of the alveolar airspaces were determined by measuring and averaging the resultant lines. 10 non-overlapping images of each lung were used to determine the final average mean chord lengths.

### **TUNEL Analysis**

ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon) was used and end labeling of exposed 3'-OH ends of DNA fragments in paraffin-embedded tissue was undertaken under the instructions provided by the manufacturer. After staining, in each section a minimum of 300 positive-labeled cells were evaluated visually. The amount of positive-labeled cells was expressed as a percentage of total nuclei evaluated.

## **RT-PCR and Quantitative RT-PCR**

Whole lung RNA was extracted from frozen lungs using Trizol reagent (Invitrogen Corp.). RNA samples were digested with RNase-free DNase-1 (Invitrogen Corp.). A Superscript One-Step RT-PCR (Invitrogen Corp.) reaction with  $\beta$ -actin or A<sub>2B</sub>R specific primers were performed using lung RNA (1 $\mu$ g) following the manufacturer's instruction. Real-time quantitative RT-PCR was performed to quantitate transcript levels. Smart Cyclyer (Cepheid) was used to determine A<sub>2B</sub>R and  $\beta$ -actin transcripts using Taqman probes. Cytokine and chemokine transcripts were analyzed using SYBR Green method or Taqman probes on the Smart Cyclyer RT-PCR machine (Cepheid). Primer sequences were the same as previously used for the transcripts examined (47, 48, 57, 61, 62). Specific gene transcript levels were calculated either by generating a standard curve from template dilutions and the PCR amplification, or 18S ribosomal RNA was used as a normalizer and the comparative Ct method ( $2^{\Delta\Delta C_t}$ ) was used to present the data as mean normalized transcript levels (63).

## **Western Blot Analysis**

Frozen lungs samples were homogenized and lysed on ice with protein lysis buffer (1M Tris pH7.4, 1M NaCl, 1% Triton-X 100) containing 1 $\times$  protease inhibitor cocktail (Roche Diagnostics). After vortexing the lysates vigorously, lysates were cleared by centrifugation at 14,000rpm for 15 min at 4°C. Electrophoresis was performed using 50 micrograms/sample of total protein on 10% SDS-PAGE gels and transferred overnight at 4°C to Immobilon-P PVDF

membranes (Millipore), and Western blotting analysis was performed as previously described (64). The following antibodies were used: rat monoclonal anti-E-selectin antibody (R&D Systems; 1:5,000 dilutions), rabbit polyclonal anti-ICAM-1 antibody (Santa Cruz Biotechnology.; 1:200 dilution), rabbit monoclonal anti-I $\kappa$ B- $\alpha$  antibody (abcam; 1:5,000 dilution), mouse monoclonal anti- $\beta$ -actin antibody (Sigma-Aldrich; 1:5,000 dilution). Secondary antibodies are: anti-rabbit IgG-HRP (eBioscience; 1:10,000 dilution), anti-mouse IgG-HRP (eBioscience; 1:10,000 dilution), and anti-rat IgG-HRP (Sigma-Aldrich; 1:10,000 dilution). Chemiluminescence signal were detected (Pierce).

### **Cytokine and Chemokine Analysis**

The levels of the cytokines IL-1 $\alpha$ , IL-1 $\beta$  and the chemokines CXCL-1, MCP-1, LIF, RANTES and MIG were examined. Blood was collected using EDTA as an anticoagulant. The blood was centrifuged at 1,200rpm for 20 min to obtain plasma. BAL fluid was collected as described previously and was centrifuged to remove cells. Plasma and the BAL fluid supernatants were then used to quantitate the amount of these cytokines and chemokines by using the Milliplex mouse cytokine/chemokine panel (Millipore) per the manufacturer's instructions.

### **Measurement of Vascular Permeability**

BAL fluid was collected as previously described and was centrifuged to remove cells. BAL fluid supernatants were then used to determine total protein content by Bradford Assay (BioRad). To assess pulmonary edema, lungs (n = 6)

were weighed and dried. Weight ratios before and after drying were used to determine lung water content. Organ vascular permeability was quantified by intraperitoneal administration of Evans blue dye (0.2 mL of 0.5% in PBS). Circulation was perfused with PBS and the heart and lung were harvested four hours after the dye injection. After formamide extraction (55°C Overnight), organ Evans blue concentrations were quantified by measuring absorbances at 610 nm with subtraction of reference absorbance at 450 nm. The contents of Evans blue dye were determined by generating a standard curve from dye dilutions.

### **Mucus index**

Periodic acid-Schiff (PAS)-staining was performed and the amount of produced mucus in bronchial airways was calculated by determining the amount of PAS positively labeled material using Image-Pro Plus analysis software (Media Cybernetics). PAS-positive areas were identified and separated on digitized images, and the averaged intensities of pixel of each color channel (red, blue, and green) were calculated. After repeating the same procedure for each image, the average values were used to calculate the area (M) and intensity (I) of PAS-stained material in major and bronchial airways. In addition, the area (A) of the total epithelium (including both PAS-negative and positive areas) was quantitated. The equation  $(M \times I)/A$  was used to determine the mucus index. Averages of 10 pictures from each lung including both small and large bronchial airways were used to determine the final indices. All quantitative studies were performed blinded.

## **Whole Mount Immunohistochemistry for CD31 on Tracheas**

Whole mount immunohistochemistry on tracheas were performed as described previously (65). Tracheas were cut and removed from mice and then washed with cold PBS. Insect pins onto silicone polymers were used to flatten tracheas after removing surrounding tissues. PBS was used to wash tracheas for 10 min and the zinc fixative (BD Pharmingen) was used to fix tracheas at least overnight. 3 PBS washes for 5 min per wash were performed after fixation. PBS containing 1% Triton X-100 was then used to permeabilize tracheas for 300 min were. 0.6% hydrogen peroxide was used to block endogenous peroxidase activity by incubating tissues for 30 min. Then Immunohistochemistry for CD31 was conducted with an anti-rat Ig horseradish peroxidase detection kit (BD Pharmingen) according to the manufacturer's instructions. Tissues were incubated overnight with a 1:250 dilution of rat anti-mouse CD31 antibody (BD Pharmingen). Permount (Fisher) was then used to mount the tissues after dehydration.

## **Quantification of BAL Fluid Adenosine Levels**

Mice were anesthetized with avertin, and lungs were lavaged 4 times with 0.3 ml PBS containing 2 $\mu$ M dipyridamole and 5 $\mu$ M DCF; 0.95-1 ml of pooled lavage fluid was recovered. Adenosine was separated and quantified using reverse-phase HPLC.

### **Intratracheal Bleomycin Treatment**

Avertin (250 mg/kg, intraperitoneally) was used to anesthetize 10-week old female wild type or  $A_{2B}R^{-/-}$  mice, and saline alone or 2.5 U/kg bleomycin (Teva Parenteral Medicines, Inc. Irvine, CA) diluted in 50  $\mu$ l sterile saline was instilled intratracheally. Endpoints were examined at 7 and 21 days after challenge.

### **Intraperitoneal Bleomycin Treatment**

6-week old male WT or  $A_{2B}R^{-/-}$  mice were treated with 0.035 U/g bleomycin (Teva Parenteral Medicines, Inc. Irvine, CA) diluted in 150  $\mu$ l sterile PBS or PBS alone intraperitoneally. Mice were treated twice weekly for 4 weeks and were weighed and sacrificed on day 33. Endpoint at day 10 was also examined after 3 injections.

### **Fibrosis Assessment**

The Sircol assay (Biocolor Ltd., Carrick, UK) was used to quantitate soluble collagen levels in BAL fluid. Ashcroft scores were determined on H&E-stained lung sections. Twenty fields per section were used to determine the Ashcroft score blinded. 4 mice per group for controls and 6 mice per group for Bleomycin-treated mice were examined.



## **Human Subjects**

Deidentified human open lung biopsy tissue samples were obtained from the Lung Tissue Research Consortium (LTRC) (Table 2.1). Patients were classified as Stage 0 COPD, Stage 4 COPD, Mild IPF and Severe IPF according to spirometry, pathological specimen and high resolution CT scan. Stage 0 COPD and Mild IPF patients with preserved lung functions were used as controls in comparison to Stage 4 COPD and Severe IPF patients. For ex vivo culture experiments, macrophages were isolated from lavage fluid collected from Stage 4 COPD or Severe IPF patients that were lavaged as part of routine diagnostic procedures.

## **Quantitative RT-PCR for Human Samples**

Tissue RNA was isolated from frozen lung tissue using Trizol reagent (Invitrogen Corp.). RNA was purified through an RNA-purification column (Qiagen) and treated using RNase-free DNase (Invitrogen Corp.). Transcript levels were quantified using Taqman real-time quantitative RT-PCR. Primer sequences for the transcripts examined are found in Table 2.2. Specific transcript levels were determined through comparison to a standard curve generated from the PCR amplification of template dilutions, and normalized to 18S ribosomal RNA and presented as mean transcript levels %18s RNA.

**Table 2.1 Study Population**

Parameter	Stage 0 COPD <sup>1</sup>	Mild IPF <sup>2</sup>	Stage 4 COPD <sup>3</sup>	Severe IPF <sup>4</sup>
N	4	10	10	10
Age, yrs	69 (61-78)	60 (50-77)	50 (44-63)	54 (26-62)
Sex, M/F	1/3	6/4	6/4	7/3
Pack-yrs smoking	25 (25-50) <sup>a</sup>	8 (3-10) <sup>b</sup>	36 (1-120) <sup>c</sup>	20 (3-32) <sup>d</sup>
Smoking status				
Ever/Never	4/0	5/4 <sup>e</sup>	10/0	4/5 <sup>e</sup>
FEV1, % pred	86 (84-89)	92 (66-109)	20 (12-40)*	38 (30-46) <sup>#</sup>
FVC, % pred	107 (80-113)	89 (80-105)	54 (13-77)*	38 (25-43) <sup>#</sup>
FEV1/FVC, %	60 (50-80)	80 (60-90)	30 (20-60)*	90 (70-100)

Data are presented as median (interquartile range). M/F: male/female; FEV1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity. <sup>1</sup>: Stage 0 COPD is defined as FEV1, % pred > 80; <sup>2</sup>: Mild IPF is defined as FVC, % pred > 80; <sup>3</sup>: Stage 4 COPD is defined as FEV1, % pred < 50; <sup>4</sup>: Severe IPF is defined as FVC, % pred < 50; <sup>a</sup>: data available for 3/4 Stage 0 COPD patients; <sup>b</sup>: data available for 5/10 Mild IPF patients; <sup>c</sup>: data available for 9/10 Stage 4 COPD patients; <sup>d</sup>: data available for 4/10 Severe IPF patients; <sup>e</sup>: data available for 9/10 Mild or Severe IPF patients. \*: p<0.05 compared with Stage 0 COPD patients; #: p<0.05 compared with Mild IPF patients.

**Table 2.2 Primer pairs and internal probe sequences**

Gene	Accession Number	Sequences
CD73	NM_002526	1447+GACAGAGTAGTCAAATTAGATG 1511-TGAGAGGGTCATAACTGG 1471+FAM TCTTTGCACCAAGTGTCTGAGTGC
ADA	NM_000022	264+CTGCTGAACGTCATTGG 340-GCAGGCATGTAGTAGTC 281+FAM CATGGACAAGCCGCTCACCC
AK	NM_006721	1092+CCACTATGCAGCAAGCATC 1156-GGAAGTCTGGCTTCTCAGG 112+FAM TAATTAGACGGACTGGCTGCACCTT
ENT1	NM_001078177	1413+CCAGCCGTGACTGTTGAG 1489-CAGGACACAGGAATGAAGTAAC 1438+FAM CCAGCATCGCAGGCAGCAGC
A <sub>1</sub> R	NM_000647	1147+GCTGGCTGCCTTTGCAC 1215-GGATGCTGGGCTTGTGG 1165+FAM TCCTCAACTGCATCACCTCTTCTGC
A <sub>2A</sub> R	NM_000675	838+ATGCTGGGTGTCTATTTGCG 902-TGGCTCTCCATCTGCTTCAG 865+FAM CTGGCGGCGCGACGACA
A <sub>2B</sub> R	NM_000676	977+CACTGAGCTGATGGACCACTC 1040-CAGTGAATTGGCTGCATGG 1018-FAM TCCCGCTGGAGGGTGGTCCT
A <sub>3</sub> R	NM_000677	708+CCCTACAGACGGATCTTGCTG 777-TGTTGGGCATCTTGCCTTC 734+FAM CCTGTCCCTGTGGAGGTTCCCCT
IL-6	NM_000600	153+ CCCCCAGGAGAAGATTCCA 223- TCAATTCGTTCTGAAGAGGTGAG 173+FAM 223- TCAATTCGTTCTGAAGAGGTGA
IL-8	NM_000584	100+TCTTGGCAGCCTTCCTGA 182-GCACTGACATCTAAGTTCTTTAGCACT 121+FAM CTGCAGCTCTGTGTGAAGGT
OPN	NM_000582	619+GGACTGAGGTCAAAATCTAAGAAG 693-GGTGATGTCCTCGTCTGTAG 646+FAM CGCAGACCTGACATCCAGTACCCT

## **Enzyme Activity Assay**

Lungs were homogenized and lysed on ice with protein lysis buffer (50mM Tris pH7.4, 150mM NaCl, 1% Triton-X 100, 0.1%SDS, 0.5% Na Deoxycholate) freshly supplemented with 1× protease inhibitor cocktail (Roche Diagnostics). Lysates were vigorously vortexed and cleared by centrifugation at 14,000rpm for 15 min at 4°C. To quantify CD73 enzyme activity, 5 µg protein extracts were incubated with with 100 µM AMP at 37°C for 30 min in the presence of 1 µM deoxyconformycin in HEPES buffer, with or without 100 µM CD73 inhibitor adenosine- 5'- O- (α, β- methylenediphosphate) (AOPCP). To quantify ADA enzyme activity, 10 µg protein extracts were incubated with with 0.2 mM adenosine at 37°C for 60 min in HEPES buffer with or without 1 µM deoxyconformycin. Heat-inactive protein extracts were used as negative controls. Reactions were terminated at 95 degree for 5 min. Reaction mixtures were then analyzed by reversed-phase (C18) HPLC which permitted direct separation, identification, and quantitation of enzymatic products (45). Enzyme specific activity is given as nmol product formed per min per mg protein (nmol/min/mg protein).

## **Histology and Immunostaining of Human Lung Sections**

HOPE or paraformaldehyde fixed lung samples were dehydrated, and embedded in paraffin, and 5 µm sections were cut and collected on microscope slides and H&E staining was performed (Shandon-Lipshaw) according to manufacturer's instructions. For CD73 immunostaining, HOPE-fixed sections

were deparaffinized in isopropanol at 60°C and rehydrated in 70% acetone. Rehydrated slides were quenched with 1% hydrogen peroxide and endogenous avidin and biotin blocking was performed using a Biotin Blocking System (DAKO Corp.). Slides were incubated with Mouse Anti-human CD73 Antibody (Hycult Biotechnology, 1:50 dilution, overnight at 4°C). For A<sub>2B</sub>R immunostaining, rehydrated slides were quenched with 1% hydrogen peroxide, antigen retrieval performed (Dako Corp.), and endogenous avidin and biotin blocking was performed using a Biotin Blocking System (DAKO Corp.). Slides were incubated with Rabbit Anti-human A<sub>2B</sub>R Antibody (Chemicon, 1:500 dilution, 1 hour room temperature). ABC Streptavidin reagents and individual appropriate secondary antibodies were used to incubate the sections, and then sections were developed with 3, 3'-diaminobenzidine (Sigma-Aldrich) and stained with methyl green. Slides were coverslipped with Vectashield (Vector Laboratories) mounting medium.

### **Immunocolocalization**

Paraformaldehyde fixed lung samples were dehydrated, and embedded in paraffin, and 5 µm sections were cut and collected on microscope slides. For immunofluorescence on tissue sections, rehydrated slides were fixed in 1:1 acetone-methanol and treated with 1% NaBH<sub>4</sub>. Slides were blocked in 1% BSA, and incubated overnight at 4°C with the primary antibodies. For immunofluorescence on primary human macrophages, cells were cytopun and fixed in 3.7% paraformaldehyde in PBS and permeabilized in cold Methanol.

Slides were blocked with 1% rabbit serum and incubated overnight at 4°C with the primary antibodies. Primary antibodies include: Mouse Anti-human CD73 Antibody (Hycult Biotechnology, 1:50 dilution), Rabbit Anti-human A<sub>2B</sub>R Antibody (Chemicon, 1:500 dilution), Rat Anti-human MMR (CD206) Antibody (R&D Systems, 1:50 dilution). Sections and cells were incubated with the following secondary antibodies: Alexa Fluor 488 rabbit anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 568 goat anti-rat IgG (Intritrogen) then coverslipped with Vectashield with DAPI (Vector Laboratories).

### ***In vitro* Stimulation of Human Primary Alveolar Macrophages**

Primary alveolar macrophages were obtained from BAL fluid of Severe IPF and Stage 4 COPD patients. BAL fluid was spun and cell pellets were resuspended in RPMI1640 containing 10% FBS and 10,000 U/ml penicillin/streptomycin. Cells were portioned into aliquots of 2 X 10<sup>5</sup> cells/well, allowed to adhere for 4 hours at 37°C 5% CO<sub>2</sub>, and then rinsed twice with RPMI1640 without FBS. Cells were either preincubated with 100nM CVT-6883 (selective A<sub>2B</sub>R antagonist) for 30 min followed by NECA or incubated with NECA alone (in DMSO, 10µM NECA/well; Tocris Bioscience) for 12 h at 37°C 5% CO<sub>2</sub>. Tissue culture supernatants were collected and IL-8 and IL-6 levels were quantitated using Human Quantikine ELISA kits (R&D Systems).

## **Statistics**

As appropriate, groups were compared by analysis of variance; follow-up comparisons between groups were conducted using 2-tailed Student's t test. Associations between transcript levels of two genes were established by linear regression. Correlation significances were analyzed using Pearson correlation calculator software. Values are expressed as mean  $\pm$  SEM. A P value of  $\leq 0.05$  was considered to be significant.

# **CHAPTER 3**

## **ENHANCED AIRWAY INFLAMMATION AND REMODELING IN ADENOSINE DEAMINASE- DEFICIENT MICE LACKING THE A<sub>2B</sub> ADENOSINE RECEPTOR**

### **INTRODUCTION**

#### **A<sub>2B</sub>R Antagonism in ADA-Deficient Mice**

To investigate the contribution of A<sub>2B</sub>R signaling in the pulmonary inflammation and injury observed in ADA-deficient mice, ADA-deficient mice were treated with the selective A<sub>2B</sub>R antagonist CVT-6883. Following the treatment, reduced influx of neutrophils and macrophages into the lungs of ADA-deficient mice was seen and this was associated with decreased expression levels of cytokine and chemokine. Meanwhile, the A<sub>2B</sub>R antagonist treatment also resulted in decreased alveolar airspace destruction and diminished expression of matrix metalloprotease. Moreover, ADA-deficient mice treated with the antagonist showed attenuated pulmonary fibrosis, which was accompanied by decreased production of pro-inflammatory and pro-fibrotic mediators such as IL-6, TGF-β1 and osteopontin. These findings suggest that the activation of the A<sub>2B</sub>R mediates many of the pathological phenotypes seen in the lungs of ADA-deficient mice.



Treatment with the antagonist resulted in diminished pulmonary inflammation, fibrosis, and airspace enlargement, suggesting that the  $A_{2B}R$  plays a pro-inflammatory, tissue destructive role in this model (57).

### **Experimental Rationale and Goal**

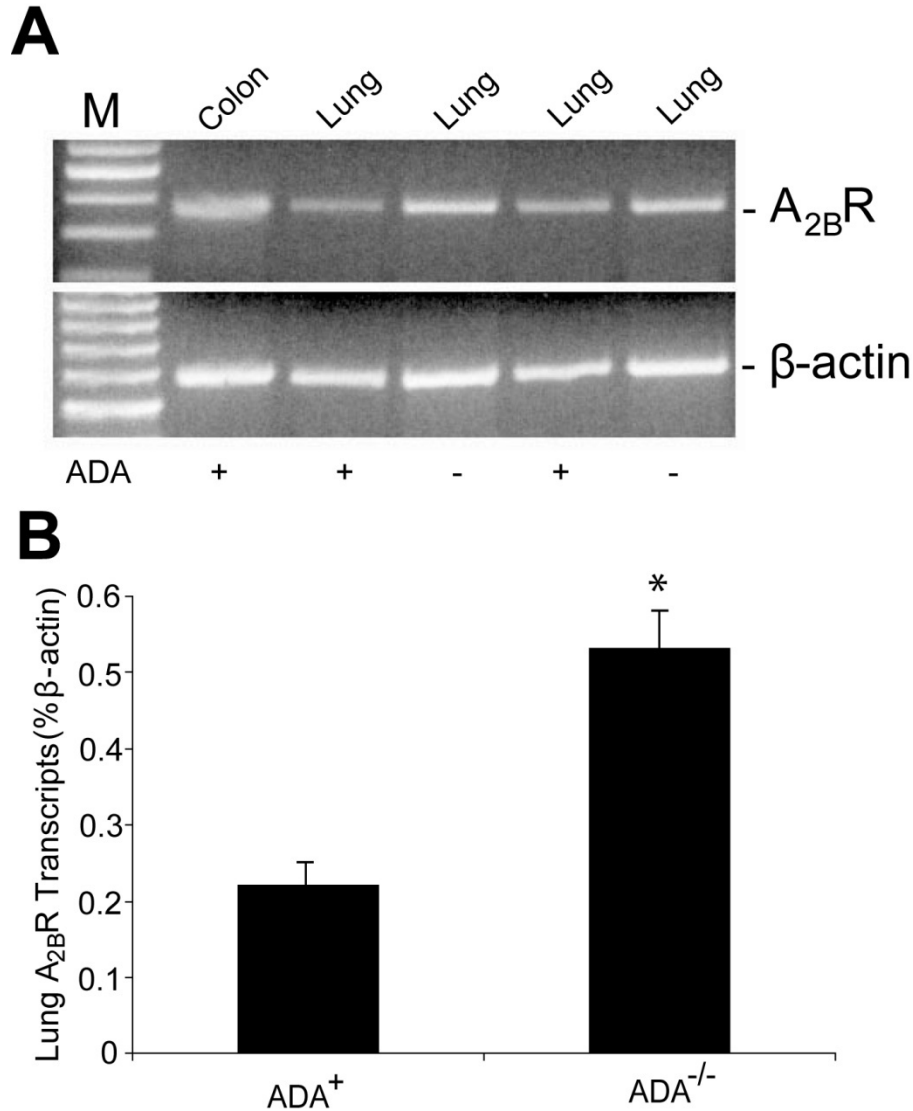
In addition to the findings in ADA deficient mice, substantial evidence has suggested a tissue destructive role of the  $A_{2B}R$ . Studies showed that engagement of the  $A_{2B}R$  promotes the differentiation of pulmonary fibroblasts into myofibroblasts and hence increases the deposition of collagen and fibrosis (30). Engagement of the  $A_{2B}R$  can mediate the direct expression of pro-inflammatory cytokines such as IL-8, IL-4 and IL-13 from human HMC-1 mast cells (53, 55). However, these findings are in contrast to recent studies in  $A_{2B}R$  knockout mice. The ablation of the  $A_{2B}R$  causes low-grade inflammation and enhanced leukocyte adhesion to the vasculature (58). In mouse models of hypoxia and ventilator-induced pulmonary injury, treatment with an  $A_{2B}R$ -selective antagonist in wild type mice resulted in enhanced pulmonary edema, inflammation, and attenuated gas exchange. Similar findings were observed in mice with genetic deletion of the  $A_{2B}R$  gene, where  $A_{2B}R^{-/-}$  mice showed increased pulmonary damage following acute injuries. Moreover,  $A_{2B}R$  agonist treatment could attenuate the pulmonary damage and reverse injury (14, 15, 39). These findings indicate that adenosine and  $A_{2B}R$  signaling play a critical role in the maintenance of pulmonary barrier function. In addition, loss of this Gs-coupled receptor on mouse bone marrow-derived mast cells results in decreased

levels of cAMP and enhanced mast cell activation (59). In order to further investigate the role of the  $A_{2B}R$ , we genetically removed the  $A_{2B}R$  from ADA-deficient mice to further characterize the function of the  $A_{2B}R$  in adenosine-mediated pulmonary injury and tissue remodeling. Results indicate that the genetic removal of  $A_{2B}R$  from ADA-deficient mice leads to the exacerbation of pulmonary disease, which suggests a critical role for this receptor in protecting pulmonary disorders where elevated adenosine levels are involved.

## **RESULTS**

### **Elevated $A_{2B}R$ Expression in the Lungs of ADA-Deficient Mice**

In order to determine if there is enhanced  $A_{2B}R$  signaling in ADA-deficient mice, levels of  $A_{2B}R$  transcripts in the lungs of ADA-deficient ( $ADA^{-/-}$ ) mice was determined by semiquantitative RT-PCR.  $A_{2B}R$  transcripts were detectable in the lungs of ADA-containing ( $ADA^{+}$ ) and  $ADA^{-/-}$  mice, which was significantly elevated in the lungs of  $ADA^{-/-}$  mice (Figure 3.1A). Quantitative RT-PCR was used to confirm these findings. A mean 2.5-fold increase of  $A_{2B}R$  transcripts in whole-lung RNA extracts was observed in ADA-deficient mice (Figure 3.1B). These findings demonstrate that  $A_{2B}R$  transcripts are elevated in the lungs of  $ADA^{-/-}$  mice.



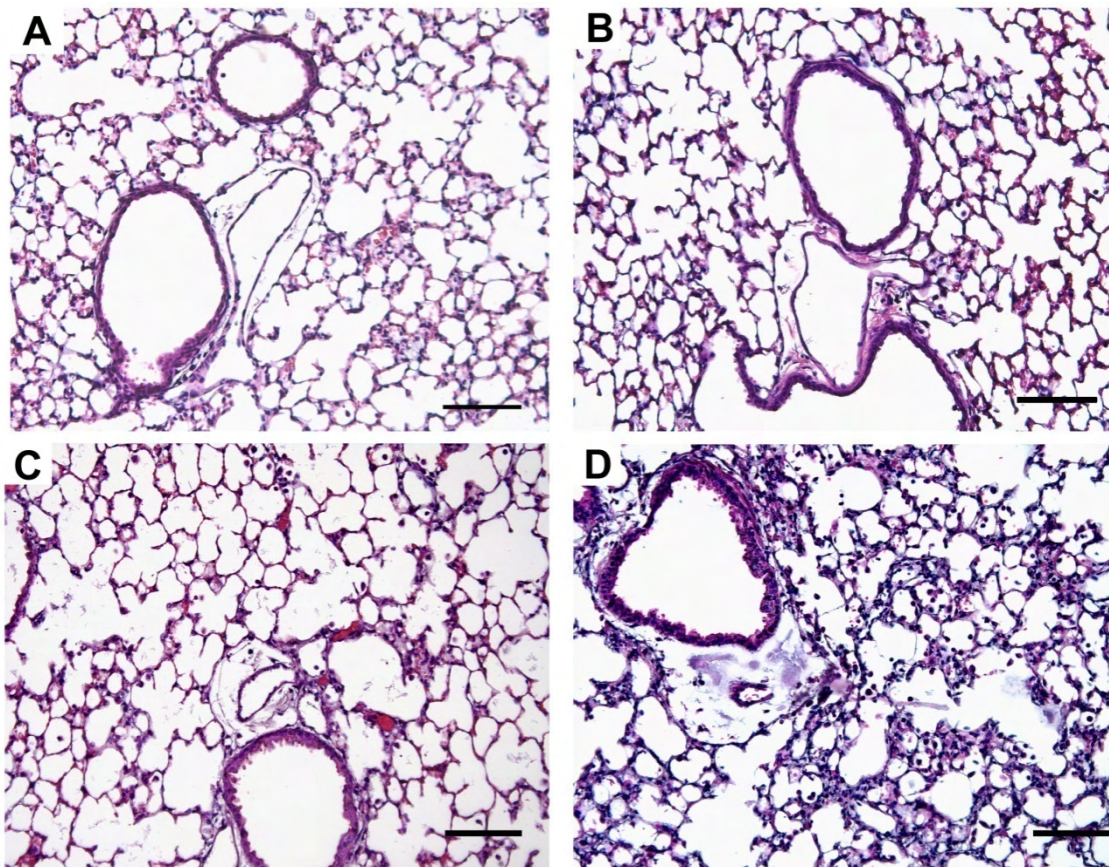
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**Figure 3.1**

The expression of A<sub>2B</sub>R in the lungs of ADA<sup>-/-</sup> mice. **(A)** Transcript levels for the A<sub>2B</sub>R were measured in whole lung mRNA extracts from ADA-containing (ADA<sup>+</sup>) and ADA-deficient (ADA<sup>-/-</sup>) mice on postnatal day 18, using semiquantitative RT-PCR. Findings from 2 different pairs of littermates are shown. RNA extracted from the colon of an ADA<sup>+</sup> mouse was used as a positive control, and  $\beta$ -actin was used as an RNA-positive control for each sample. M, DNA size ladder. **(B)** Quantitative RT-PCR was used to determine the levels of A<sub>2B</sub>R transcripts in day 18 whole-lung extracts from ADA<sup>+</sup> and ADA<sup>-/-</sup> mice. Data are presented as mean percentage of  $\beta$ -actin transcripts  $\pm$  SEM; n = 4 for each. \*p $\leq$ 0.05 compared to ADA<sup>+</sup>.

## **ADA/A<sub>2B</sub>R Double Knockout Mice Exhibit Enhanced Pulmonary Pathology and Die Precociously**

ADA<sup>-/-</sup> mice were mated with A<sub>2B</sub>R<sup>-/-</sup> mice to generate ADA/A<sub>2B</sub>R double knockout mice to assess the effect of genetically removing A<sub>2B</sub>R on the pulmonary phenotypes seen in ADA<sup>-/-</sup> mice. ADA<sup>-/-</sup> mice develop progressive pulmonary inflammation and die between postnatal day 18-21 (47). ADA/A<sub>2B</sub>R double knockout mice did not survive past postnatal day 11-12 (data not shown). These findings demonstrate that genetic removal of the A<sub>2B</sub>R from ADA<sup>-/-</sup> mice leads to precocious lung injury and death. The death of animals at such a young age made detailed assessment of pulmonary phenotypes difficult. In order to circumvent this problem, ADA<sup>-/-</sup> mice and ADA/A<sub>2B</sub>R double knockout mice were maintained on exogenous ADA enzyme therapy from birth and for the first 21 days of life and examined following the removal of ADA enzyme therapy. ADA<sup>-/-</sup> mice treated in this manner exhibit signs of respiratory distress approximately 16 days after the cessation of ADA enzyme therapy. In contrast, ADA/A<sub>2B</sub>R double knockout mice began to show signs of respiratory distress as early as day 11 after the cessation of enzyme therapy, and this labored breathing became increasingly severe up to the death of the animals around postnatal day 16. Therefore, detailed phenotype analysis was conducted 14 days after the cessation of ADA enzyme therapy. At this stage, ADA<sup>-/-</sup> mice had diffuse monocytic inflammation when compared to that seen wild type mice (Figure 3.2C). There was no evidence of inflammation in ADA<sup>+</sup>A<sub>2B</sub>R<sup>-/-</sup> mice (Figure 3.2B)



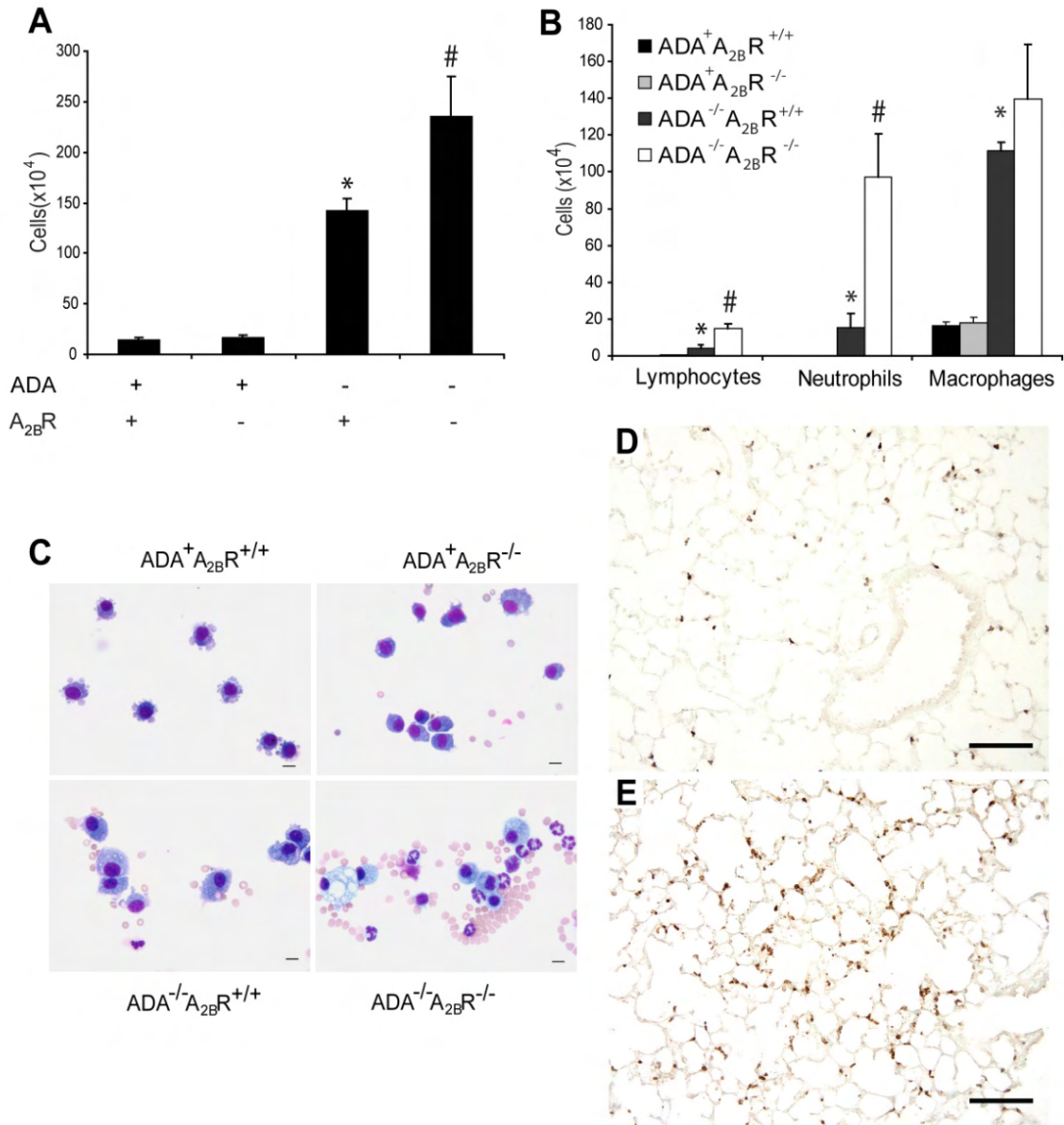
**Figure 3.2**

Histopathology of the lungs. Lungs were collected on postnatal day 35 and prepared routinely for sectioning and H&E staining. (A) Lung section from an ADA<sup>+</sup>A<sub>2</sub>B R<sup>+/+</sup> mouse. (B) Lung section from an ADA<sup>+</sup>A<sub>2</sub>B R<sup>-/-</sup> mouse. (C) Lung section from an ADA<sup>-</sup>A<sub>2</sub>B R<sup>+/+</sup> mouse. (D) Lung section from an ADA<sup>-</sup>A<sub>2</sub>B R<sup>-/-</sup> mouse. Sections are representative of 6–8 different mice from each genotype. Scale bars=100 μm.

while enhanced pulmonary inflammation and increased congestion of the alveoli were observed in ADA/A<sub>2B</sub>R double knockout mice (Figure 3.2D).

### **Enhanced Pulmonary Inflammation in ADA/A<sub>2B</sub>R Double Knockout Mice**

To quantify the level of inflammation in ADA/A<sub>2B</sub>R double knockout mice, bronchial alveolar lavage (BAL) was performed and cells recovered from BAL Fluid were counted on a hemacytometer. There was a significant increase in the number of inflammatory cells recovered from ADA/A<sub>2B</sub>R double knockout mice compared to that seen in the lungs of ADA<sup>-/-</sup> mice containing the A<sub>2B</sub>R (Figure 3.3A). Differential staining of recovered airway cells demonstrated a significant increase in neutrophils and lymphocytes as well as alveolar macrophages. Many of these macrophages were activated and enlarged to form foam cells (Figure 3.3, B and C). To better visualize the increase in lung neutrophilia, lung sections were immunostained using an antibody against mouse neutrophils. There were robust increases in tissue neutrophils in the lung parenchyma of ADA/A<sub>2B</sub>R double knockout mice compared to ADA<sup>-/-</sup> mice (Figure 3.3, D and E). Taken together, these data suggest that enhanced pulmonary inflammation is seen in the lungs of ADA<sup>-/-</sup> mice lacking the A<sub>2B</sub>R.



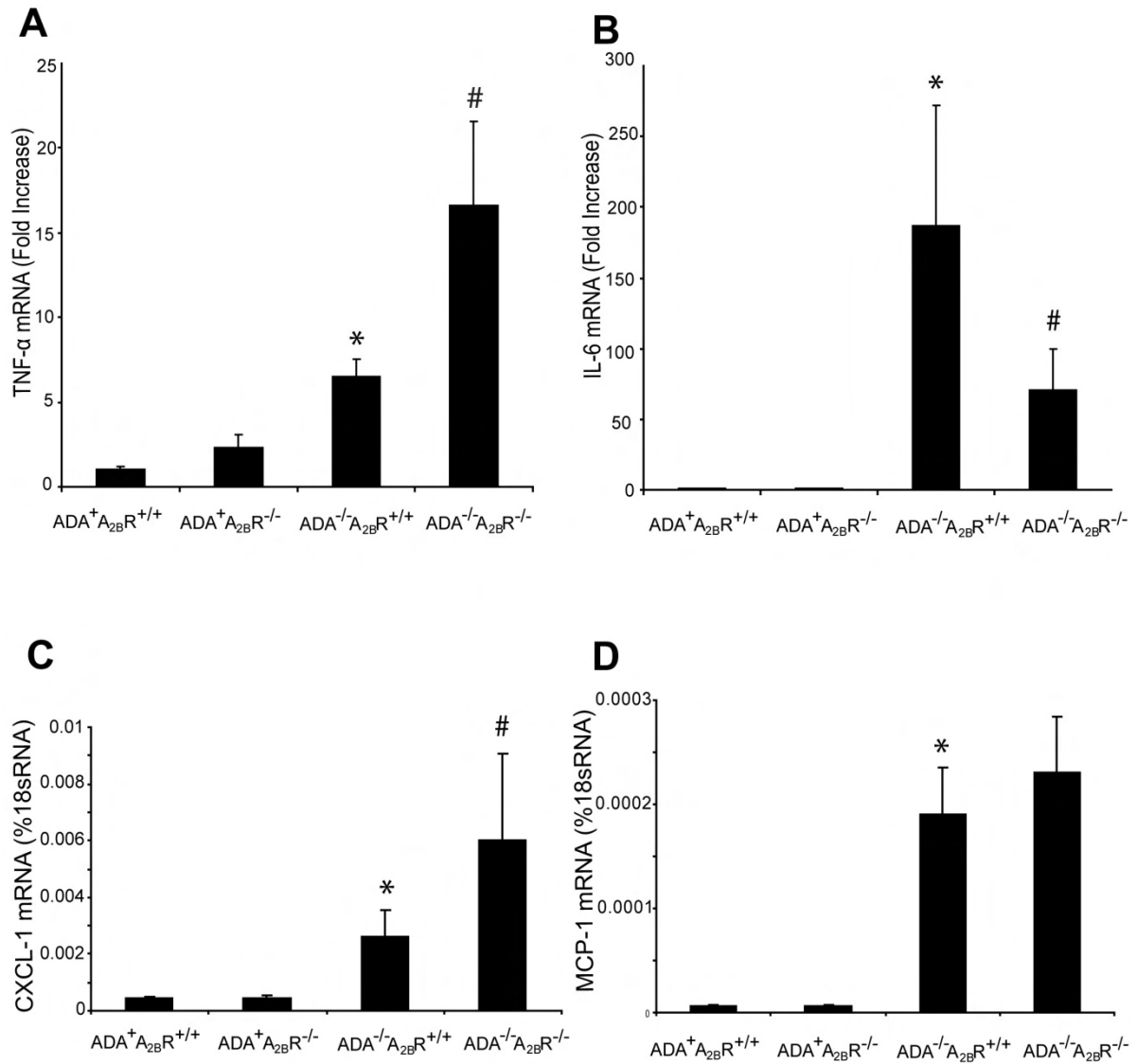
**Figure 3.3**

Pulmonary inflammation. **(A)** BAL fluid was collected on postnatal day 35 and total cell numbers were determined. **(B)** BAL cells were cytopun and stained with Diff-Quick, allowing for determination of cellular differentials. Data are mean cell counts  $\pm$  SEM. \* $p \leq 0.05$  versus ADA<sup>+</sup> mice; # $p \leq 0.05$  versus ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mice;  $n = 6$  (ADA<sup>+</sup>), 8 (ADA<sup>-/-</sup>). **(C)** Cytopun BAL cells stained with Diff-Quick. Scale bars=10  $\mu$ m. **(D)** Lung section from an ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mouse and an ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>-/-</sup> mouse **(E)** were stained with an antibody against neutrophils to visualize infiltrated tissue neutrophils (brown). Scale bars=100  $\mu$ m.

## **Inflammatory Cytokine and Chemokine Production Is Enhanced in ADA/A<sub>2B</sub>R Double Knockout Mice**

In order to better understand the mechanisms involved in the regulation of inflammatory responses in ADA/A<sub>2B</sub>R double knockout mice, transcript levels of cytokines and chemokines were monitored in whole-lung RNA extracts. TNF- $\alpha$  transcript levels were elevated in the lungs of ADA<sup>-/-</sup> mice, and they were further elevated in the absence of the A<sub>2B</sub>R (Figure 3.4A). IL-6 transcripts were elevated in the lungs of ADA<sup>-/-</sup> mice. Surprisingly, they were significantly decreased with the removal of the A<sub>2B</sub>R (Figure 3.4B). Levels of chemokines were also examined, levels of monocyte chemotactic protein-1 (MCP-1) and CXCL-1 were found to be significantly increased in the lungs of ADA<sup>-/-</sup> mice and levels of CXCL-1 were further elevated after the genetic removal of the A<sub>2B</sub>R (Figure 3.4, C and D). These data indicate that removal of the A<sub>2B</sub>R results in exaggerated transcription of TNF- $\alpha$  and CXCL-1 in the lungs of ADA<sup>-/-</sup> mice, whereas selective removal of A<sub>2B</sub>R leads to decreased IL-6. Additionally, cytokine and chemokine protein levels were determined in plasma and BAL fluid using the Milliplex mouse cytokine/chemokine panel (Table 3.1). Plasma cytokine and chemokine protein levels were measured to determine the status of systemic inflammation. CXCL-1 and MCP-1 protein levels were elevated in the plasma of ADA<sup>-/-</sup> mice, and levels were significantly elevated after the genetic removal of the A<sub>2B</sub>R. Levels for IL-1 $\alpha$  and IL-1 $\beta$  were not elevated in ADA<sup>-/-</sup> mice containing the A<sub>2B</sub>R; however, they were significantly increased in ADA/A<sub>2B</sub>R double knockout mice. Interestingly, MIG protein levels were decreased in the plasma of ADA<sup>-/-</sup> mice and were further





**Figure 3.4**

Production of proinflammatory cytokines. Transcript levels of various proinflammatory cytokines were measured in whole-lung extracts from postnatal day 35 mice using quantitative RT-PCR. Shown are levels of **(A)** TNF-α, **(B)** IL-6, **(C)** CXCL1, **(D)** MCP-1. Results are presented as mean fold increase compared to controls or mean pg transcript/μg RNA ± SEM. \*p ≤ 0.05 versus ADA<sup>+</sup> mice; #p ≤ 0.05 versus ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mice. n = 4 (ADA<sup>+</sup>), 8 (ADA<sup>-/-</sup>).

**Table 3.1 Cytokine and Chemokine Protein Levels in Plasma and BAL Fluid**

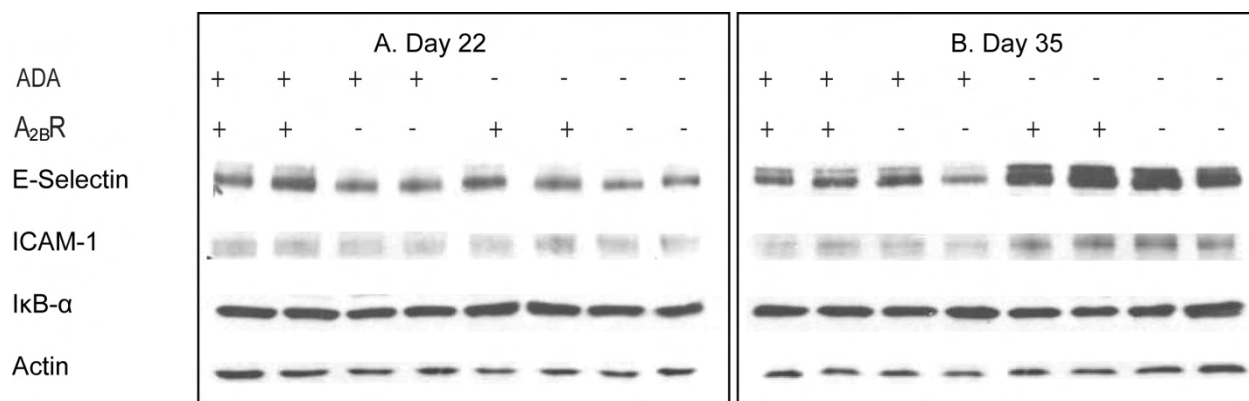
	Plasma (pg/ml)				BALF (pg/ml)			
	ADA <sup>+</sup> A <sub>2B</sub> R <sup>+/+</sup>	ADA <sup>+</sup> A <sub>2B</sub> R <sup>-/-</sup>	ADA <sup>-/-</sup> A <sub>2B</sub> R <sup>+/+</sup>	ADA <sup>-/-</sup> A <sub>2B</sub> R <sup>-/-</sup>	ADA <sup>+</sup> A <sub>2B</sub> R <sup>+/+</sup>	ADA <sup>+</sup> A <sub>2B</sub> R <sup>-/-</sup>	ADA <sup>-/-</sup> A <sub>2B</sub> R <sup>+/+</sup>	ADA <sup>-/-</sup> A <sub>2B</sub> R <sup>-/-</sup>
CXCL-1	111±10	155±18	493±121 <sup>*</sup>	1086±191 <sup>#</sup>	3±1	5±1	50±24 <sup>*</sup>	116±29 <sup>#</sup>
MCP-1	7±1	11±4	27±8 <sup>*</sup>	190±98 <sup>#</sup>	18±16	26±14	153±83 <sup>*</sup>	245±85
LIF	ND	ND	ND	ND	0.3±0.2	0.1±0.1	9±4 <sup>*</sup>	26±8 <sup>#</sup>
RANTES	18±4	25±4	18±6	20±4	0.4±0.3	0.2±0.1	0.6±0.2	2±1 <sup>#</sup>
MIG	143±21	123±8	41±14 <sup>*</sup>	19±3	ND	ND	ND	ND
IL-1α	89±19	52±6	68±37	522±218 <sup>#</sup>	4±1	5±2	2±1	3±2
IL-1β	17±4	26±4	33±7	81±29 <sup>#</sup>	5±2	4±1	5±1	5±1

Levels of inflammatory mediators in plasma and BAL fluid. Plasma and BAL fluid were collected from postnatal day 35 mice. Protein levels of inflammatory cytokines and chemokines were determined by luminex assay. Data are presented as mean ± SEM. \*p ≤ 0.05 versus ADA<sup>+</sup> mice; #p ≤ 0.05 versus ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mice. n = 4 (ADA<sup>+</sup>), 6 (ADA<sup>-/-</sup>). ND, not detected.

decreased in ADA/A<sub>2B</sub>R double knockout mice. BAL fluid protein levels were used to examine cytokines and chemokines in these mice. Levels of CXCL-1 and LIF-1 were elevated in the lungs of ADA<sup>-/-</sup> mice, and levels were significantly elevated after the genetic removal of the A<sub>2B</sub>R. RANTES protein levels were not changed in the BAL fluid of ADA<sup>-/-</sup> mice containing the A<sub>2B</sub>R; however, they were significantly elevated in ADA<sup>-/-</sup> mice lacking the A<sub>2B</sub>R. MCP-1 protein levels were not changed after the genetic removal of the A<sub>2B</sub>R though they were increased in ADA<sup>-/-</sup> mice compared to wild type mice. These data suggest that the A<sub>2B</sub>R differentially regulates the expression of cytokines and chemokines where elevated adenosine level is involved.

### **Expression of Cell Adhesion Molecules in the Absence of the A<sub>2B</sub>R**

It has been demonstrated that the removal of the A<sub>2B</sub>R is associated with the activation of NF-κB pathways as indicated by down-regulation of the NF-κB inhibitor IκB-α, and hence the up-regulation of adhesion molecules such as E-selectin, P-selectin, and ICAM-1 (58). Increased expression of cell adhesion molecules is the potential underlying mechanism for the adhesion of leukocytes to blood vessels and the migration to sites of infection in the tissue. Based on the fact that more infiltrated neutrophils were found in the lung of ADA/A<sub>2B</sub>R double knockout mice compared to that in ADA<sup>-/-</sup> mice, we examined the expression levels of adhesion molecule as well as IκB-α. Whole lung extracts from postnatal day 22 and day 35 mice were subjected to Western Blot analysis to determine



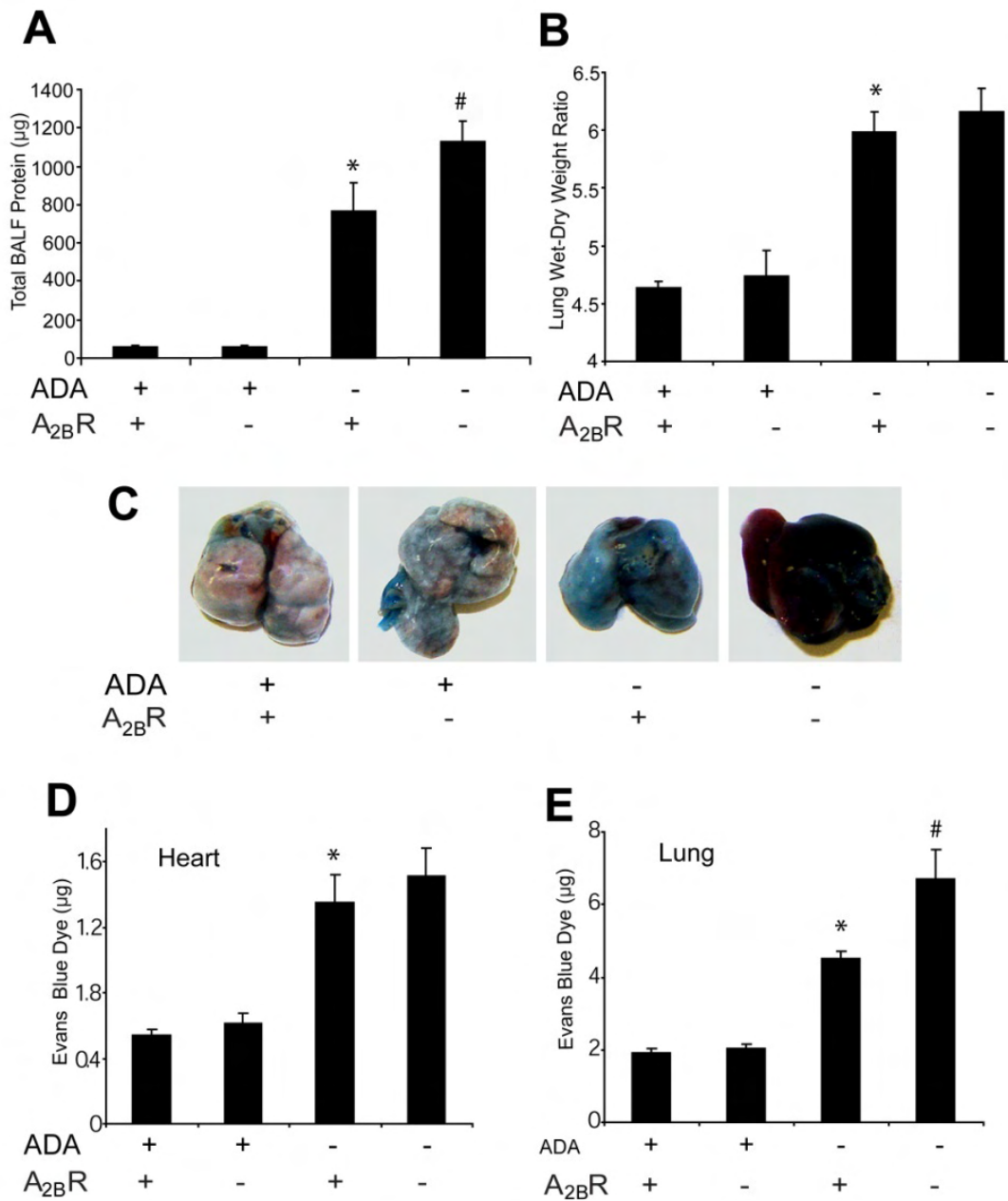
### Figure 3.5

Western blot analysis of E-selectin, ICAM-1 and IκB-α in whole lung extracts. Samples from postnatal day 22 (**A**) and postnatal day 35 (**B**) mice were subjected to Western blotting with the indicated antibodies. Probing with anti-actin was used as loading controls.

the protein levels of E-Selectin, ICAM-1 and I $\kappa$ B- $\alpha$ . Day 22 was examined because it is a stage when adenosine levels are not elevated in ADA<sup>-/-</sup> mice and it is a stage that precedes the appearance of phenotypes. The expression of E-Selectin, ICAM-1 and I $\kappa$ B- $\alpha$  were not altered in all genotypes compared to the controls on day 22 (Figure 3.5A). However, on day 36 when the disease status is established and adenosine levels are high, the expression of E-Selectin and ICAM-1 were elevated in ADA<sup>-/-</sup> mice and ADA/A<sub>2B</sub>R double knockout mice, though there was no apparent difference in the level of expression with or without the A<sub>2B</sub>R (Figure 3.5B). In addition, the expression of I $\kappa$ B- $\alpha$  was not altered in all genotypes (Figure 3.5, A and B). These data demonstrate that there is no baseline activation of NF- $\kappa$ B and increased expression of cell adhesion molecules in the lung in the absence of the A<sub>2B</sub>R. The increased expression of these adhesion molecules lead to the inflammation seen in ADA<sup>-/-</sup> mice but enhanced tissue neutrophilia in ADA/A<sub>2B</sub>R double knockout mice compared to that in ADA<sup>-/-</sup> mice is likely not due to enhanced neutrophil adhesion.

### **Enhanced Vascular Permeability in ADA/A<sub>2B</sub>R Double Knockout Mice**

Previous studies have shown a central role of the A<sub>2B</sub>R in the preservation of endothelial barrier functions during hypoxia (14). We hypothesized that A<sub>2B</sub>R deficiency would significantly influence vascular leakage in ADA<sup>-/-</sup> mice. To assess this, BAL fluid was collected from postnatal day 35 mice and total protein levels were determined. Total protein content was elevated in the lungs of ADA<sup>-/-</sup>



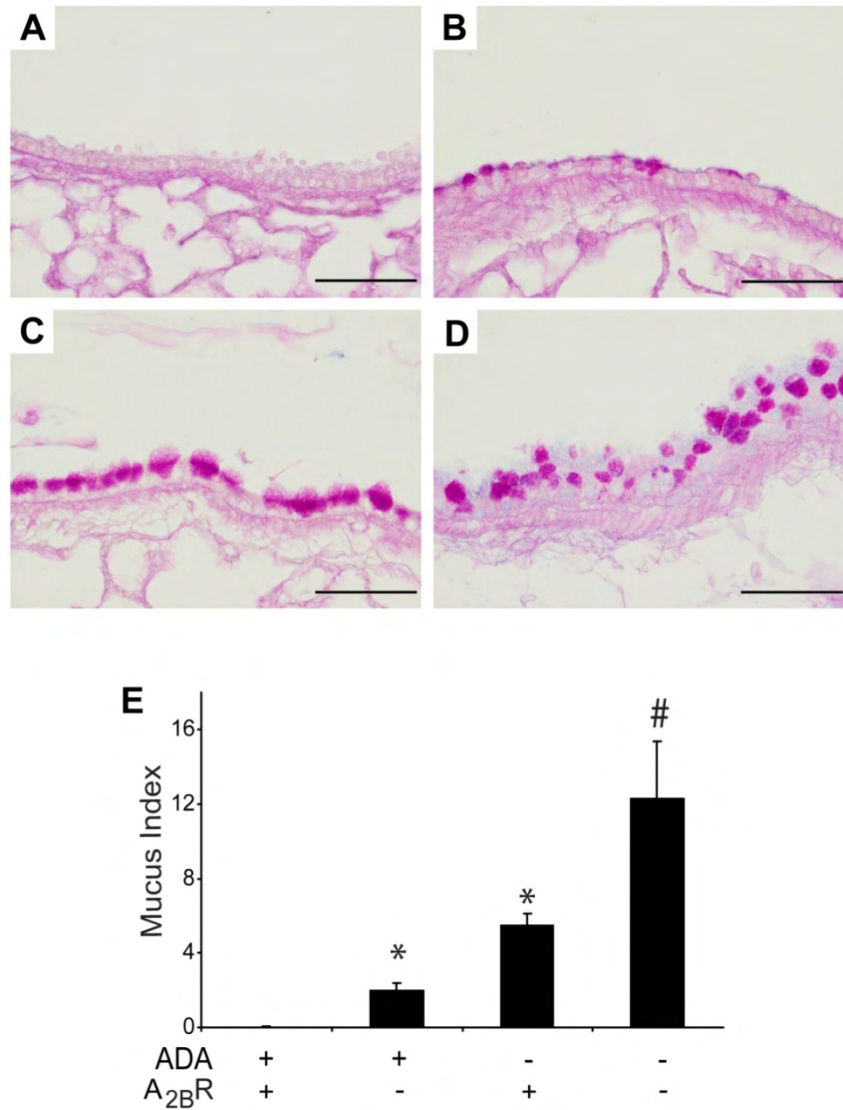
**Figure 3.6**

Vascular permeability analysis. **(A)** BAL fluid was collected from postnatal day 35 mice and total BALF protein levels were determined by Bradford assay. Data are mean total protein content  $\pm$  SEM. \* $p \leq 0.05$  versus ADA<sup>+</sup> mice; # $p \leq 0.05$  versus ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mice.  $n = 6$  (ADA<sup>+</sup>),  $8$  (ADA<sup>-/-</sup>). **(B)** Lungs from postnatal day 35 mice were collected and lung water content was determined by lung wet-dry weight ratio. Data are mean ratio  $\pm$  SEM. \* $p \leq 0.05$  versus ADA<sup>+</sup> mice; # $p \leq 0.05$  versus ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mice.  $n = 4$  (ADA<sup>+</sup>),  $6$  (ADA<sup>-/-</sup>). Mice were administered intraperitoneal Evan blue dye (0.2 mL of 0.5% in PBS) and sacrificed 4 hours later and the hearts and lungs were harvested. **(C)** Representative images of lungs. Evan blue dye concentrations were quantified in heart **(D)** and lung **(E)** as described in "Methods". Data are mean dye content  $\pm$  SEM. \* $p \leq 0.05$  versus ADA<sup>+</sup> mice; # $p \leq 0.05$  versus ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mice.  $n = 4$  (ADA<sup>+</sup>),  $6$  (ADA<sup>-/-</sup>).

mice, and increases were markedly enhanced in the absence of the A<sub>2B</sub>R (Figure 3.6A). Lung water content was determined by measuring lung wet-dry weight ratio. ADA deficiency was associated with a significant degree of lung water increase. However, this increase was not affected by the genetic removal of the A<sub>2B</sub>R (Figure 3.6B). However, comparative analysis of vascular permeability as assessed by Evans blue dye extravasation revealed that vascular leakage was significantly increased in lungs of ADA/A<sub>2B</sub>R double knockout mice compared to that in ADA<sup>-/-</sup> mice (Figure 3.6, C and E). Basal permeability in ADA<sup>+</sup> mice was not altered with or without the A<sub>2B</sub>R (Figure 3.6, D and E). These findings demonstrate that signaling through the A<sub>2B</sub>R is a critical control point for pulmonary endothelial barrier function associated with elevated levels of adenosine in ADA<sup>-/-</sup> mice.

### **Enhanced Mucous Cell Metaplasia in the Lungs of ADA/A<sub>2B</sub>R Double-knockout Mice**

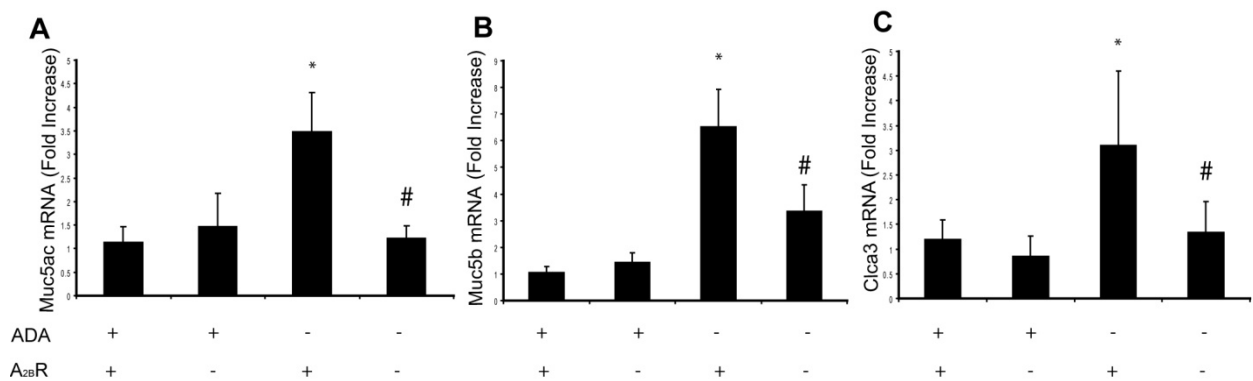
One of the prominent features seen in the bronchial airways of ADA<sup>-/-</sup> mice is mucous cell metaplasia. To determine what impact genetically removing the A<sub>2B</sub>R had on mucous cell metaplasia, tissue sections were stained with PAS, and mucous cell metaplasia was quantitated by morphometry. In the bronchial airways, A<sub>2B</sub>R<sup>-/-</sup> mice showed increased numbers of PAS-positive cells (Figure 3.7, A, B, and E). As expected, in the bronchial airways of ADA<sup>-/-</sup> mice, a large number of mucin-containing cells were observed (Figure 3.7, C and E). Moreover, in the bronchial airways of ADA/A<sub>2B</sub>R double knockout mice, there was



**Figure 3.7**

Mucus metaplasia. On postnatal day 35, lungs were collected and prepared for sectioning and PAS staining. (A), Lung section from an ADA<sup>+</sup>A<sub>2B</sub>R<sup>+/+</sup> mouse. (B), Lung section from an ADA<sup>+</sup>A<sub>2B</sub>R<sup>-/-</sup> mouse. (C), Lung section from an ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mouse. (D), Lung section from an ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>-/-</sup> mouse. Sections are representative of eight different mice from each genotype. Bars, 100 μm. (E), Mucus index was determined, and data are presented as mean mucus index ± SEM. \*,  $p \leq 0.05$  vs ADA<sup>+</sup> mice; #,  $p \leq 0.05$  vs ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mice.  $n = 4$  (ADA<sup>+</sup>A<sub>2B</sub>R<sup>+/+</sup> and ADA<sup>+</sup>A<sub>2B</sub>R<sup>-/-</sup>),  $n = 8$  (ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> and ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>-/-</sup>).





**Figure 3.8**

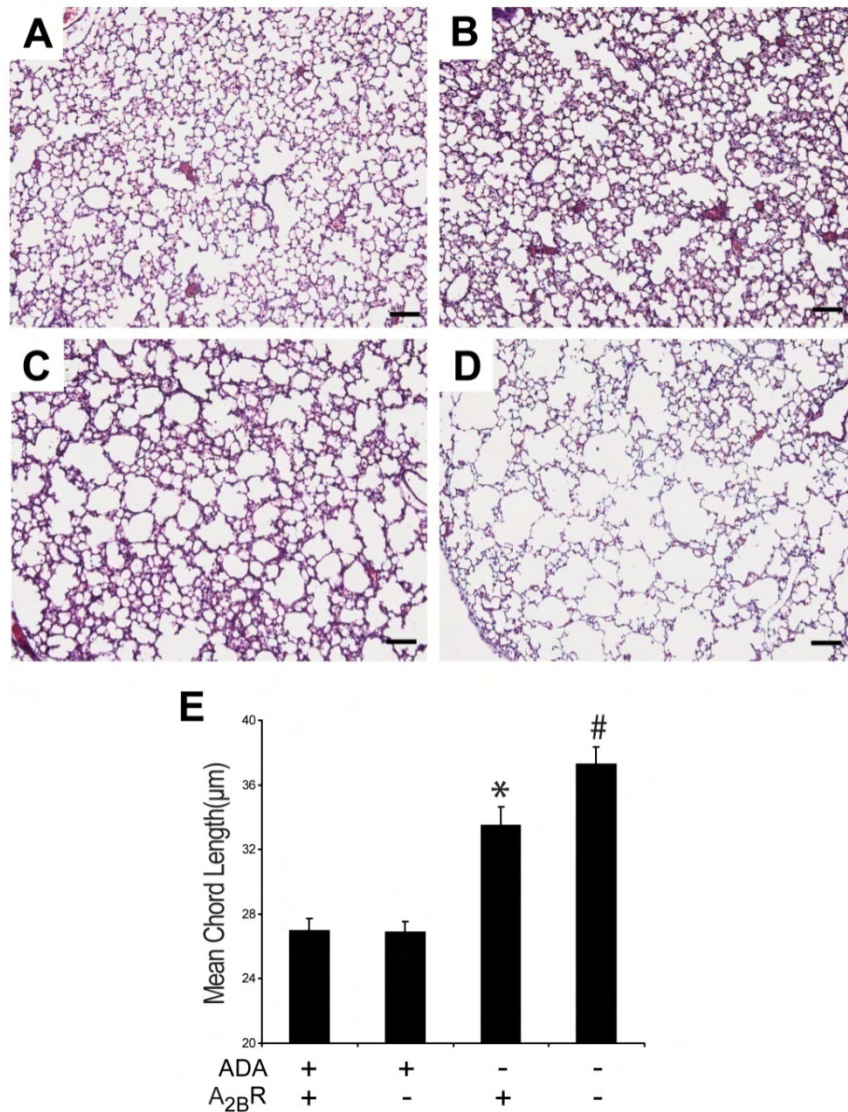
Mucin Gene Expression. Whole lung RNA extracts were analyzed using quantitative RT-PCR assays for Muc5ac (**A**), Muc5b (**B**) and Clca3 (**C**). Results are presented as mean fold increase compared to controls  $\pm$  SEM. \* $p \leq 0.05$  versus ADA<sup>+</sup> mice; # $p \leq 0.05$  versus ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mice.  $n = 4$  (ADA<sup>+</sup>), 8 (ADA<sup>-/-</sup>).

increased PAS staining (Figure 3.7, D and E). These results demonstrate that mucin accumulated in  $A_{2B}R^{-/-}$  mice at baseline and that mucus metaplasia is enhanced in ADA/ $A_{2B}R$  double knockout mice.

Major mucin gene transcripts levels were quantitated by Real-time PCR. Interestingly, although mucus metaplasia was enhanced in ADA/ $A_{2B}R$  double knockout mice, there were decreases in levels of expression of the major mucins, Muc5ac (Figure 3.8A), and Muc5b (Figure 3.8B), and the mucous metaplasia marker calcium-activated chloride channel 3 (Clca3) (Figure 3.8C). This finding suggests that the  $A_{2B}R$  may play an important role in mucin secretion. Although mucin gene expression is decreased in the double-knockout mice compared to ADA-deficient mice, the defect in mucociliary clearance lacking the  $A_{2B}R$  would still result in enhanced mucus accumulation.

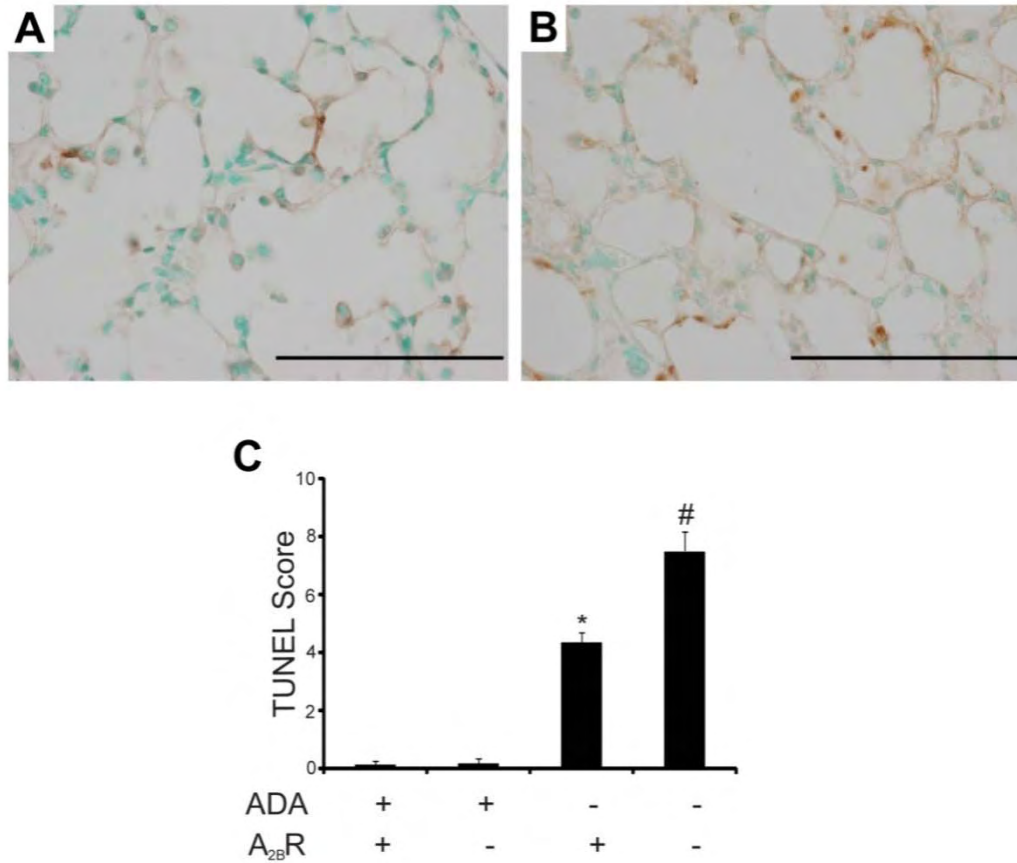
### **Enhanced Alveolar Destruction in the Lungs of ADA/ $A_{2B}R$ Double Knockout Mice**

ADA<sup>-/-</sup> mice develop characteristics of emphysema and features of alveolar airspace enlargement (47). To determine whether  $A_{2B}R$  was involved in mediating airspace enlargement, alveolar destruction was analyzed in ADA<sup>-/-</sup> mice and ADA/ $A_{2B}R$  double knockout mice 14 days after the cessation of exogenous ADA enzyme therapy. The size of alveolar airspaces in ADA<sup>-/-</sup> mice showed a significant increase (Figure 3.9, C and E). The degree of airspace



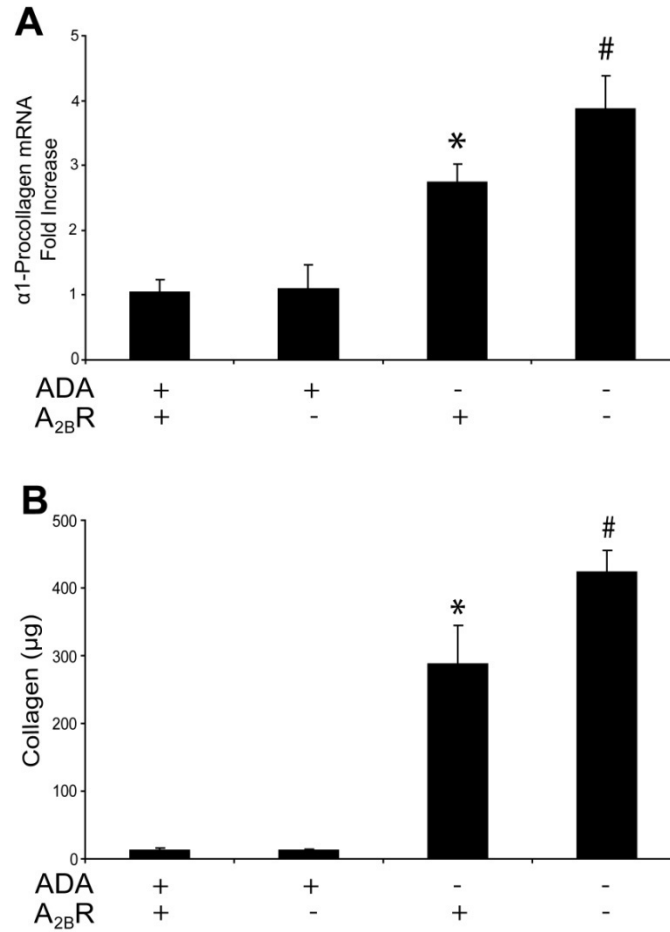
**Figure 3.9**

Alveolar airspace enlargement. Lungs from postnatal day 35 mice were infused with fixative under constant pressure (25 cm H<sub>2</sub>O) and processed for H&E staining. (A) Lung section from an ADA<sup>+</sup>A<sub>2B</sub>R<sup>+/+</sup> mouse. (B) Lung section from an ADA<sup>+</sup>A<sub>2B</sub>R<sup>-/-</sup> mouse. (C) Lung section from an ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mouse. (D) Lung section from an ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>-/-</sup> mouse. Images are representative of 6-8 animals from each genotype. Scale bars=100 μm. (E) Alveolar airspace size was calculated using ImagePro analysis software; data are mean chord length ± SEM. \*p ≤ 0.05 versus ADA<sup>+</sup> mice; #p ≤ 0.05 versus ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mice. n = 4 (ADA<sup>+</sup>), 6 (ADA<sup>-/-</sup>).



**Figure 3.10**

TUNEL Staining. (A) TUNEL-stained lung section from an ADA<sup>-/-</sup> mouse. (B) TUNEL-stained lung section from an ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>-/-</sup> mouse. Images are representative of eight animals from each genotype. (C), TUNEL-positive cells were counted, and data are presented as mean TUNEL scores ± SEM. \*,  $p \leq 0.05$  vs ADA<sup>+</sup> mice; #,  $p \leq 0.05$  vs ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mice.  $n = 4$  (ADA<sup>+</sup>A<sub>2B</sub>R<sup>+/+</sup> and ADA<sup>+</sup>A<sub>2B</sub>R<sup>-/-</sup>),  $n = 8$  (ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> and ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>-/-</sup>). Bars = 100 μm.



**Figure 3.11**

Collagen production. **(A)** Whole lung  $\alpha 1$ -pro-collagen transcript levels were measured from postnatal day 35 mice using quantitative RT-PCR, and data are presented as mean fold increase compared with controls or mean pg transcript/ $\mu$ g RNA  $\pm$  SEM. **(B)**, Soluble collagen protein levels in BAL fluid from postnatal day 35 mice were quantified using the Sircol assay. Results are presented as mean collagen levels  $\pm$  SEM. \*,  $p \leq 0.05$  vs ADA<sup>+</sup> mice; #,  $p \leq 0.05$  vs ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> mice.  $n = 4$  (ADA<sup>+</sup>A<sub>2B</sub>R<sup>+/+</sup> and ADA<sup>+</sup>A<sub>2B</sub>R<sup>-/-</sup>),  $n = 8$  (ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>+/+</sup> and ADA<sup>-/-</sup>A<sub>2B</sub>R<sup>-/-</sup>).

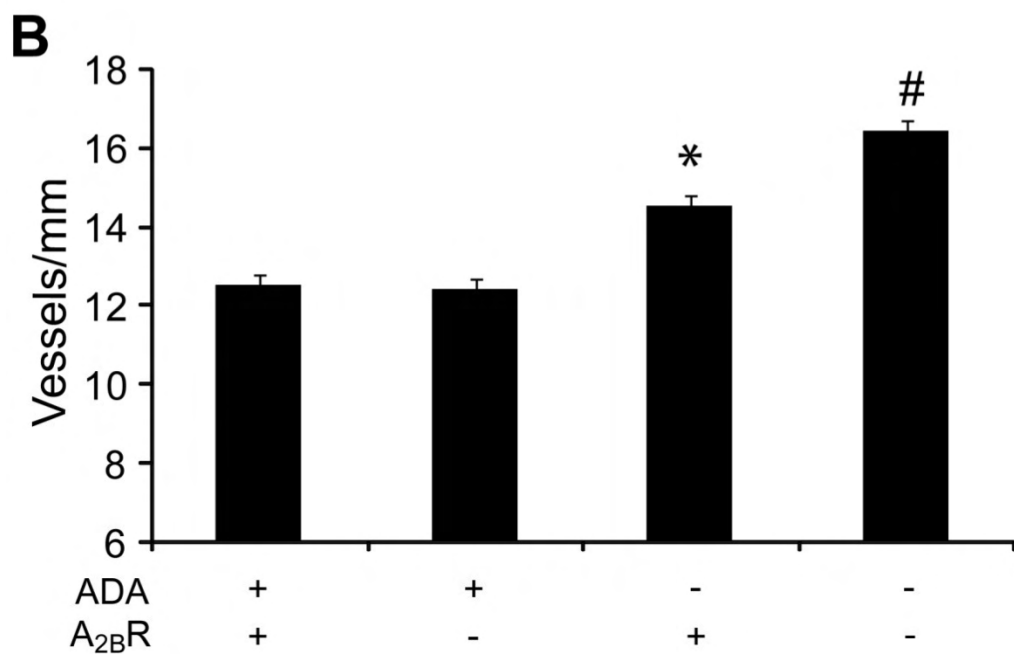
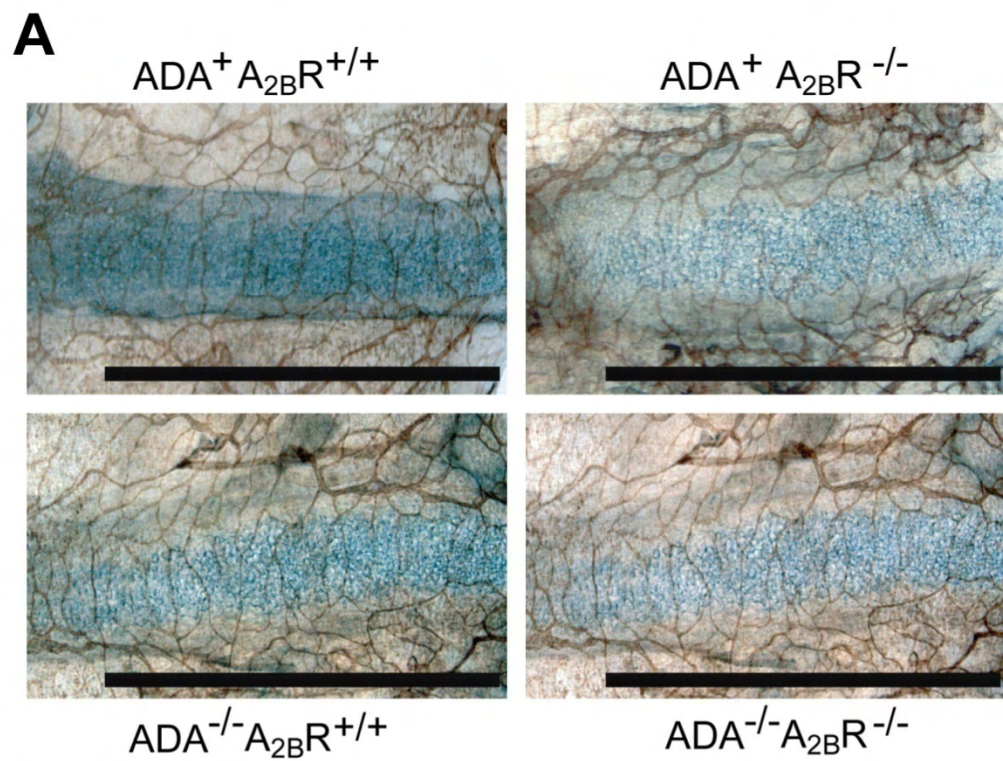
enlargement was further increased in the lungs of ADA/A<sub>2B</sub>R double knockout mice (Figure 3.9, D and E). Consistent with these findings, increased apoptosis in the alveolar airspaces of ADA/A<sub>2B</sub>R double-knockout mice were observed compared to ADA<sup>-/-</sup> mice (Figure 3.10). This finding demonstrates that there is increased alveolar destruction in the lungs of ADA/A<sub>2B</sub>R double knockout mice.

### **Enhanced Pulmonary Fibrosis in ADA/A<sub>2B</sub>R Double Knockout Mice**

Enhanced extracellular matrix deposition and collagen production are prominent features noted in the lungs of ADA<sup>-/-</sup> mice. Examination of the transcript levels of  $\alpha$ -1 procollagen (Figure 3.11A) and soluble BAL collagen levels (Figure 3.11B) revealed enhanced collagen production and signs of enhanced pulmonary fibrosis in the lungs of ADA/A<sub>2B</sub>R double-knockout mice.

### **Enhanced Tracheal Vascularity in ADA/A<sub>2B</sub>R Double Knockout Mice**

ADA<sup>-/-</sup> mice have increased tracheal vascularity in association with elevated adenosine levels (65). To determine whether A<sub>2B</sub>R plays a role in regulating tracheal vascularity, CD31 whole mount immunohistochemistry was performed on tracheas (Figure 3.12). Consistent with the previous finding, results suggest that there are increased numbers of vessel in ADA<sup>-/-</sup> mice compared to wide type mice. Interestingly, ADA/A<sub>2B</sub>R double knockout mice exhibited further increased tracheal vascularity compared with ADA<sup>-/-</sup> mice possessing the A<sub>2B</sub>R (Figure 3.12). These results indicate that the genetic removal of the A<sub>2B</sub>R leads to enhanced tracheal angiogenesis in ADA<sup>-/-</sup> mice.



Amir Mohsenin

**Figure 3.12**

Tracheal vascularity and angiogenesis. **(A)** Tracheas were removed from postnatal day 35 mice and angiogenesis was analyzed by whole mount CD31 immunostaining for the visualization of vessels. Results are representative of 4 mice from each genotype. Scale bars=100  $\mu$ m. **(B)** Tracheal vascularity was determined in the samples shown in A by counting the number of vessels intersecting a line down the length of the cartilage ring. At least 12 cartilage rings were analyzed per sample. Data are represented as mean vessels (in millimeters)  $\pm$  SE; n = 4. \*p  $\leq$  0.05 versus ADA<sup>+</sup> mice; #p  $\leq$  0.05 versus ADA<sup>-/-</sup> A<sub>2B</sub>R<sup>+/+</sup> mice.

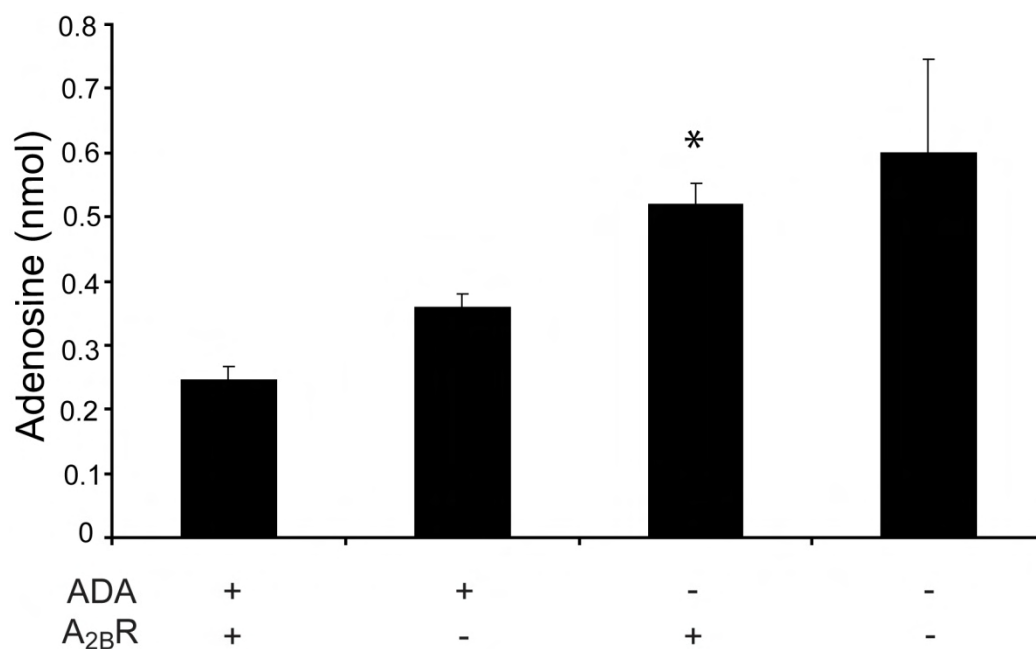
## **Adenosine Levels in ADA/A<sub>2B</sub>R Double Knockout Mice**

To determine if elevated adenosine levels in ADA/A<sub>2B</sub>R double knockout mice could contribute to the enhanced pulmonary pathology, adenosine levels in BAL fluid were quantitated using HPLC. Results suggest that there are increased adenosine levels in ADA<sup>-/-</sup> mice compared to wide type mice. Interestingly, adenosine levels were not further elevated in ADA/A<sub>2B</sub>R double knockout mice compared with ADA<sup>-/-</sup> mice possessing the A<sub>2B</sub>R (Figure 3.13).

## **DISCUSSION**

Adenosine can have both tissue-protective and tissue-destructive functions depending on endogenous adenosine concentrations generated following injury, the nature and duration of injury, and the expression profile of each adenosine receptor on specific cell types (22, 66). Adenosine's tissue-destructive role is supported by the ADA-deficient mouse model where elevated adenosine levels lead to features of chronic lung disease including inflammation, mucin overproduction, alveolar airway enlargement, fibrosis and angiogenesis (47, 65). The A<sub>2B</sub>R is widely expressed in tissues and has been indicated to play an important role in inflammation and tissue remodeling. In the current study, A<sub>2B</sub>R was genetically removed from ADA-deficient mice to generate double knockout mice to examine the contribution of the A<sub>2B</sub>R to the pulmonary





**Figure 3.13**

Adenosine levels in BAL fluid. BAL fluid was collected on postnatal day 35 and adenosine levels were measured using reversed phase HPLC. Data are presented as mean nmol adenosine  $\pm$  SEM.  $n = 3$  for each group. \*  $p \leq 0.05$  versus ADA<sup>+</sup> mice.

phenotype seen in ADA-deficient mice. Findings demonstrate that the genetic removal of the A<sub>2B</sub>R from ADA-deficient mice leads to precocious lung injury and death. The precocious pulmonary damage and death of these mice was associated with increased lung inflammation, vascular permeability, alveolar destruction and tracheal angiogenesis. These results demonstrate that the A<sub>2B</sub>R plays an important role in protecting pulmonary inflammation and damage seen in ADA-deficient mice.

One of the major observations in this study was that ADA/A<sub>2B</sub>R double knockout mice exhibited enhanced pulmonary inflammation compared to ADA-deficient mice possessing the A<sub>2B</sub>R. There were increases in lymphocytes and particularly neutrophils in the BAL fluid of ADA-deficient mice lacking the A<sub>2B</sub>R. Consistent with this, immunostaining showed that there were more neutrophils in the lungs of ADA/A<sub>2B</sub>R double knockout mice compared to ADA-deficient mice, suggesting that A<sub>2B</sub>R signaling may be protective in regulating pulmonary neutrophilia. These findings differ somewhat from our recent observations that blockade of the A<sub>2B</sub>R in ADA-deficient mice using an A<sub>2B</sub>R antagonist results in diminished pulmonary inflammation (57). The A<sub>2B</sub>R has been shown to exhibit both pro- and anti-inflammatory functions. The first in vivo evidence showing A<sub>2B</sub>R's anti-inflammatory roles comes from a study where the genetic ablation of the A<sub>2B</sub>R was associated with low-grade inflammation that was associated with the augmentation of pro-inflammatory cytokines, such as TNF- $\alpha$ , and a consequent downregulation of I $\kappa$ B- $\alpha$ , the inhibitor of NF- $\kappa$ B signaling pathway. In this study, the activation of NF- $\kappa$ B was proposed to lead to the expression of pro-

inflammatory cytokines, chemokines and cell adhesion molecules (58). We therefore examined whether activation of these pathways might account for the increased neutrophil counts seen in the lungs of ADA/A<sub>2B</sub>R double knockout mice. Interestingly, expression levels of cell adhesion molecules such as ICAM-1 and E-selectin were not altered in the lungs of ADA-deficient mice lacking the A<sub>2B</sub>R, nor was expression of I $\kappa$ B- $\alpha$  affected. Thus, activation of NF- $\kappa$ B signaling pathways and altered adhesion molecule expression do not appear to be responsible for enhanced pulmonary inflammation in ADA/A<sub>2B</sub>R double knockout mice.

However, our experiments did reveal an increase in pulmonary vascular permeability in the lungs of ADA/A<sub>2B</sub>R double knockout mice, which may account for the increased neutrophil infiltration into the lung. This is consistent with the observations that pharmacologic blockade and genetic removal of the A<sub>2B</sub>R is central to hypoxia-associated vascular leak (14). Activation of the A<sub>2B</sub>R is associated with increases in intracellular cAMP concentration following activation of PKA. PKA-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP) may result in changes in junctional protein geometry and distribution, and hence regulating the characteristics of the junctional complex, leading to barrier function increases (3, 67, 68). Hypoxia exposure and ADA deficiency both result in a prolonged action of extracellular adenosine and enhanced adenosine signaling. Adenosine plays a protective role in controlling vascular leakage that mice deficient in enzymes involved in extracellular adenosine generation showed

profound increases in vascular leakage (3, 69). Therefore, the genetic removal of the A<sub>2B</sub>R from ADA-deficient mice may lead to enhanced vascular permeability and subsequent neutrophil accumulation in the tissue.

Inflammatory cell accumulation in the lung occurs following pro-inflammatory stimuli that result in the recruitment of cells into tissue from the circulatory system. Thus, increased expression levels of inflammatory cytokines and chemokines could also contribute to neutrophil infiltration into the lungs. Transcript levels of TNF- $\alpha$  and CXCL-1 were elevated in the lungs of ADA-deficient mice, and levels were markedly increased in the absence of the A<sub>2B</sub>R. Interestingly, IL-6 levels were decreased with the removal of the A<sub>2B</sub>R, suggesting the A<sub>2B</sub>R regulates the production of IL-6 in this model. Consistent with this, engagement of the A<sub>2B</sub>R increases IL-6 release from bronchial smooth muscle cells (70), human lung fibroblasts (30), and airway epithelia (71); while adenosine inhibits TNF- $\alpha$  release from mouse peritoneal macrophages and human monocytes via the A<sub>2B</sub>R (72, 73). Additionally, cytokine and chemokine protein levels were determined from BAL fluid and plasma to monitor the inflammatory status in lung and systemically. As expected, levels of CXCL-1, LIF-1 and RANTES were elevated in BAL fluid from ADA/A<sub>2B</sub>R double knockout mice compared to that in ADA-deficient mice. Although expression levels of certain cytokines and chemokines did increase after the genetic removal of the A<sub>2B</sub>R from ADA-deficient mice, we did not see increased percentage of neutrophils in peripheral white blood cell counts (data not shown). As a result, increases in lung

neutrophil numbers appeared to depend largely on enhanced vascular permeability following the genetic removal of the A<sub>2B</sub>R.

An interesting finding in this study is that mucus metaplasia was enhanced in ADA/A<sub>2B</sub>R double-knockout mice, consistent with the other enhanced pulmonary histopathology. However, there was a decrease in degree of expression of the major mucin genes and mucous metaplasia marker Clca3. Although we cannot rule out the roles of other mucin genes that possibly take part in mucus accumulation, this finding suggests that the A<sub>2B</sub>R may play an important role in mucociliary clearance. Previous studies have shown that the A<sub>2B</sub>R is an important regulator of cystic fibrosis transmembrane conductance regulator (CFTR) functions and lacking this receptor may lead to dehydration of airway surface liquid volume and mucus stasis (74). As a result, even though there was decreased mucin gene expression in the absence of A<sub>2B</sub>R signaling, the defect in mucus clearance would still lead to mucus accumulation in the airways. Further studies are needed to clarify the role of the A<sub>2B</sub>R in mucin gene expression as well as mucociliary clearance.

Another prominent feature of ADA/A<sub>2B</sub>R double knockout mice was enhanced tissue remodeling, including destruction of alveolar airspaces, a response reminiscent of emphysema, and trachea angiogenesis (47, 61, 62). The degree of the enlargement of airspace was significantly increased in the lungs of ADA-deficient mice lacking the A<sub>2B</sub>R. It is possible that this pulmonary phenotype noted in ADA/A<sub>2B</sub>R knockout mice is attributed to the increases in pulmonary neutrophils seen. For example, neutrophils produce degradative

enzymes such as matrix metalloproteinase-9 and neutrophil elastase, which can contribute to alveolar airway enlargement and could account for the enhanced airspace enlargement seen in ADA/A<sub>2B</sub>R double knockout mice exhibiting increases in airway neutrophils. ADA-deficient mice have increased tracheal angiogenesis in association with increased CXCL-1 level (65). Similarly, the current study showed that enhanced tracheal vascularity is associated with increased CXCL-1 expression seen in the lungs of ADA/A<sub>2B</sub>R double knockout mice. These findings demonstrate that CXCL-1 level in the lung may directly regulate pathological angiogenesis and may serve as an important therapeutic target.

Overall, ADA/A<sub>2B</sub>R double knockout mice have enhanced lung inflammation and tissue destruction when compared to ADA-deficient mice that express the A<sub>2B</sub>R. These observations contradict those seen in this same model following A<sub>2B</sub>R antagonism (57). In the A<sub>2B</sub>R antagonism study, ADA-deficient mice were kept on Peg-ADA enzyme therapy till Day 21. Three days after the removal of ADA enzyme therapy, the A<sub>2B</sub>R antagonist CVT-6883 was given intraperitoneally. CVT-6883-treated ADA-deficient mice exhibited diminished pulmonary inflammation, fibrosis, and alveolar airspace destruction, suggesting the pro-inflammatory functions of the A<sub>2B</sub>R. Clearly, discrepancies exist while characterizing the anti- or pro-inflammatory functions of the A<sub>2B</sub>R. In addition to the finding from A<sub>2B</sub>R antagonism in ADA-deficient mice show A<sub>2B</sub>R's tissue-destructive roles, engagement of the A<sub>2B</sub>R promotes the differentiation of

pulmonary fibroblasts into myofibroblasts and hence increases the deposition of collagen and fibrosis (30). A<sub>2B</sub>R activation also mediates the direct expression of pro-inflammatory cytokines such as IL-8, IL-4 and IL-13 from human HMC-1 cells (52, 53, 56). Stimulation of A<sub>2B</sub>Rs up-regulates IL-6 production from mouse macrophages (75), IL-13 and VEGF secretion from mouse bone marrow mast cells (56). Inconsistent with these findings, A<sub>2B</sub>R plays an anti-inflammatory role that tissue damage and injury could be amplified in the absence of A<sub>2B</sub>R signaling. A<sub>2B</sub>Rs are required for airway surface liquid (ASL) volume homeostasis in human airways and therapies directed at inhibiting the A<sub>2B</sub>R may lead to depleted ASL volume and mucus stasis (74). The ablation of the A<sub>2B</sub>R causes low-grade inflammation, augmentation of pro-inflammatory cytokines, such as TNF- $\alpha$ , and a consequent activation of NF- $\kappa$ B signaling pathway (58). Loss of this Gs-coupled receptor on mouse bone marrow-derived mast cells results in decreased levels of cAMP and enhances mast cell activation (59).

The functions of A<sub>2B</sub>R signaling are also controversial in other organs. The reno-vascular A<sub>2B</sub>R protects the kidney from ischemia that ischemic preconditioning (IP) protection from ischemia was abolished in A<sub>2B</sub>R<sup>-/-</sup> mice while treatment with the selective A<sub>2B</sub>R agonist dramatically improved renal function and histology following ischemia injury (76). Blockade or deletion of the A<sub>2B</sub>R in a mouse model of colitis showed reduced clinical symptoms, histological scores, IL-6 and KC levels in the supernatants of colonic organ cultures (77, 78). Interestingly, although colonic inflammation was attenuated in A<sub>2B</sub>R<sup>-/-</sup> mice compared with their wild type counterparts, A<sub>2B</sub>R<sup>-/-</sup> mice actually showed

increased susceptibility to systemic Salmonella infection (77). It seems that in most cases pharmacological blockage of the  $A_{2B}R$  by antagonists is beneficial while gene deletion of the  $A_{2B}R$  generally enhances the phenotypes seen in multiple organs and systemically after injury. The discrepancies between the pharmacological block and gene knockout studies may be related to the nature of injury, the specific organ environment, the levels and duration of adenosine elevation, and the timing of  $A_{2B}R$  antagonism. The  $A_{2B}R$  may have a bi-phasic effect on inflammation that the  $A_{2B}R$  antagonist could be beneficial only when it is given after the disease status is established, while the  $A_{2B}R$  plays an important protective role before the chronic stage of the disease. Moreover, the discrepancy between these two studies could be due to the fact that the antagonist may only have a partial affect in blocking the receptor activity while genetic knockout completely abolishes the receptor signaling. Additionally, it has been shown in the literature that the  $A_{2B}R$  could be recruited to the plasma membrane to form receptor dimers or a complex with other proteins, such as ezrin, and compartmentalize E3KARP (79). For this reason, Ryzhov et al. argued that mast cell activation and the increase in basal and LPS-stimulated TNF- $\alpha$  release observed in  $A_{2B}R^{-/-}$  mice is not directly related to adenosine signaling through the  $A_{2B}R$ , but it may represent a loss of other unidentified functions of this protein or reflect a developmental adaptation to the  $A_{2B}R$  gene ablation (75).

In conclusion, the results of the present study demonstrated that the genetic removal of the  $A_{2B}R$  from ADA-deficient mice enhanced pulmonary



inflammation and injury induced by increased adenosine levels. These findings are inconsistent with the previous pharmacological study and the hypothesis that A<sub>2B</sub>R signaling contributes to the pro-inflammatory and tissue-destructive activities present in chronic lung diseases. The A<sub>2B</sub>R may have a bi-phasic effect on inflammation that the A<sub>2B</sub>R antagonist may be beneficial only when it is given after the disease status is established. Thus, cautions must be taken when designing A<sub>2B</sub>R antagonist treatment strategies in human chronic lung disease. The results of the present study demonstrated that the genetic removal of the A<sub>2B</sub>R from ADA-deficient mice resulted in enhanced pulmonary inflammation and injury induced by increased adenosine levels. The underlying mechanism for enhanced tissue damage is thought to be excessive neutrophil infiltration caused by loss of pulmonary vascular barrier function. These findings suggest that during the acute stage of lung injury, A<sub>2B</sub>R plays a homeostatic and protective role such as barrier function, but it plays a detrimental role during the chronic stage of diseases such as pulmonary fibrosis. Moreover, inhibition of the A<sub>2B</sub>R may result in collapsed airway surface liquid and mucus stasis that could potentially accentuate disease status. Further studies are needed to understand the complicated role of the A<sub>2B</sub>R, which will be critical for developing A<sub>2B</sub>R-based therapies for the treatment of various diseases. In the next chapter, specific experiments were designed to determine the potential mechanisms of the destructive and protective components of A<sub>2B</sub>R signaling.

# **CHAPTER 4**

## **THE ROLE OF THE A<sub>2B</sub>R IN ACUTE AND CHRONIC STAGES OF BLEOMYCIN-INDUCED LUNG INJURY**

In the previous chapter, I demonstrated that the genetic removal of the A<sub>2B</sub>R from ADA-deficient mice results in enhanced pulmonary inflammation, fibrosis and alveolar airspace enlargement. Enhanced pulmonary vascular permeability due to the lack of the A<sub>2B</sub>R may be responsible for the increased damage in the lung. This finding, together with the previous findings that A<sub>2B</sub>R signaling promotes the expression of pro-inflammatory and pro-fibrotic mediators (32, 53, 57), suggests that A<sub>2B</sub>R plays a protective role during the acute phase of lung injury and promotes destructive features during chronic stages of the disease. Studies described in this chapter focus on understanding the distinctive roles of the A<sub>2B</sub>R at different stages of lung injury in another model of pulmonary injury: the model of bleomycin-induced pulmonary injury.

### **INTRODUCTION**

#### **Bleomycin induced pulmonary fibrosis**

Pulmonary fibrosis is characterized by extracellular matrix deposition, aberrant fibroblast proliferation, and inflammation, which are distinctive features

of interstitial lung diseases. These tissue remodeling processes result in distortion of lung architecture and compromised pulmonary function (10). Bleomycin is a glycopeptide antibiotic produced by the bacterium *Streptomyces verticillus* and is a widely used anti-cancer agent. It functions by catalytically generating double strand DNA breaks and results in apoptosis of affected cells (80). The expression of bleomycin hydrolase, the enzyme that degrades bleomycin, is particularly low in the lung and skin (81). As a result, the most serious side effect of bleomycin is pulmonary fibrosis and impaired lung function (82). About 1% of patients suffer from irreversible pulmonary fibrosis following bleomycin exposure (83).

This side effect has been used to investigate the mechanisms of fibrosis. Bleomycin results in similar lung parenchyma injury and interstitial fibrosis in mice as is seen in humans (84). Lungs that have been exposed to bleomycin show evidence of injury and inflammation, which involves an infiltration of granulocytes, lymphocytes and monocytes (85). Ultimately, increased deposition of extracellular matrix such as collagen and fibronectin in the alveolar wall compromise pulmonary function (86, 87). Pulmonary injury in mice induced by bleomycin delivered intratracheally or intraperitoneally are two well-established animal models of lung fibrosis (88, 89); however, the route of delivery results in different degrees of acute injury.

## **IT versus IP bleomycin treatment**

In the standard intratracheal (IT) bleomycin induced pulmonary injury model, 10-week old female mice are exposed to a single dose of 2.5 U/kg bleomycin IT. Mice develop significant pulmonary fibrosis at 21 days after challenge. However, since bleomycin is instilled directly into the respiratory tract, the acute damage caused by bleomycin results in extensive apoptosis of airway epithelial cells, followed by an acute phase of pulmonary injury resulting in a large amount of infiltrating granulocytes around Day 7 after the challenge. Most granulocytes are resolved by Day 21 and macrophages and lymphocytes remain in the airways. At this stage, mice develop severe fibrosis due to a failed wound healing response. In contrast, in the intraperitoneal (IP) bleomycin model, 6-week old male mice are treated with a higher dose of bleomycin IP twice a week for four weeks. Since drug is delivered systemically into the peritoneal cavity and the lung is not directly exposed to bleomycin directly, there is not excessive acute injury or inflammation in the lung. However, when mice are sacrificed a week after the last treatment on day 33, they do develop a significant amount of pulmonary fibrosis.

Thus, IT bleomycin is a model of acute injury, followed by fibrosis, and IP bleomycin is a model of mild injury and progressive pulmonary fibrosis.

## **Experimental Rational and Goals**

In the previous studies, when ADA-deficient mice were treated with the A<sub>2B</sub>R antagonist 3 days after the cessation of Peg-ADA treatment, they showed

less pulmonary inflammation, fibrosis, and alveolar airspace enlargement compared to ADA-deficient mice, suggesting pro-inflammatory and pro-fibrotic functions of the A<sub>2B</sub>R. However, the genetic removal of the A<sub>2B</sub>R from ADA-deficient mice resulted in significantly enhanced pulmonary inflammation, fibrosis and alveolar airspace enlargement, in which enhanced pulmonary vascular permeability due to the lack of A<sub>2B</sub>R likely played an important role. As a result, these studies provide the proof of concept that the A<sub>2B</sub>R is fundamental in maintaining vascular barrier function during the acute phase of pulmonary injury, while it is tissue-destructive during the chronic phase.

In order to further address this concept in another model of pulmonary injury, we took the genetic approach to investigate the role of A<sub>2B</sub>R in the model of bleomycin-induced pulmonary injury. We utilized the two different drug delivery methods described above to determine the functions of the A<sub>2B</sub>R at different stages of lung injury. We hypothesized that in the IP bleomycin model, since no acute damage and inflammation are involved, A<sub>2B</sub>R<sup>-/-</sup> mice will show significantly less pulmonary fibrosis compared to wild type mice; however, in the IT bleomycin model, enhanced acute injury and inflammation will be observed in A<sub>2B</sub>R<sup>-/-</sup> mice due to their compromised vascular barrier function. This may counteract the pro-fibrotic functions of the A<sub>2B</sub>R and there will not be a significant difference when assessing fibrosis at Day 21 endpoint in the IT model.

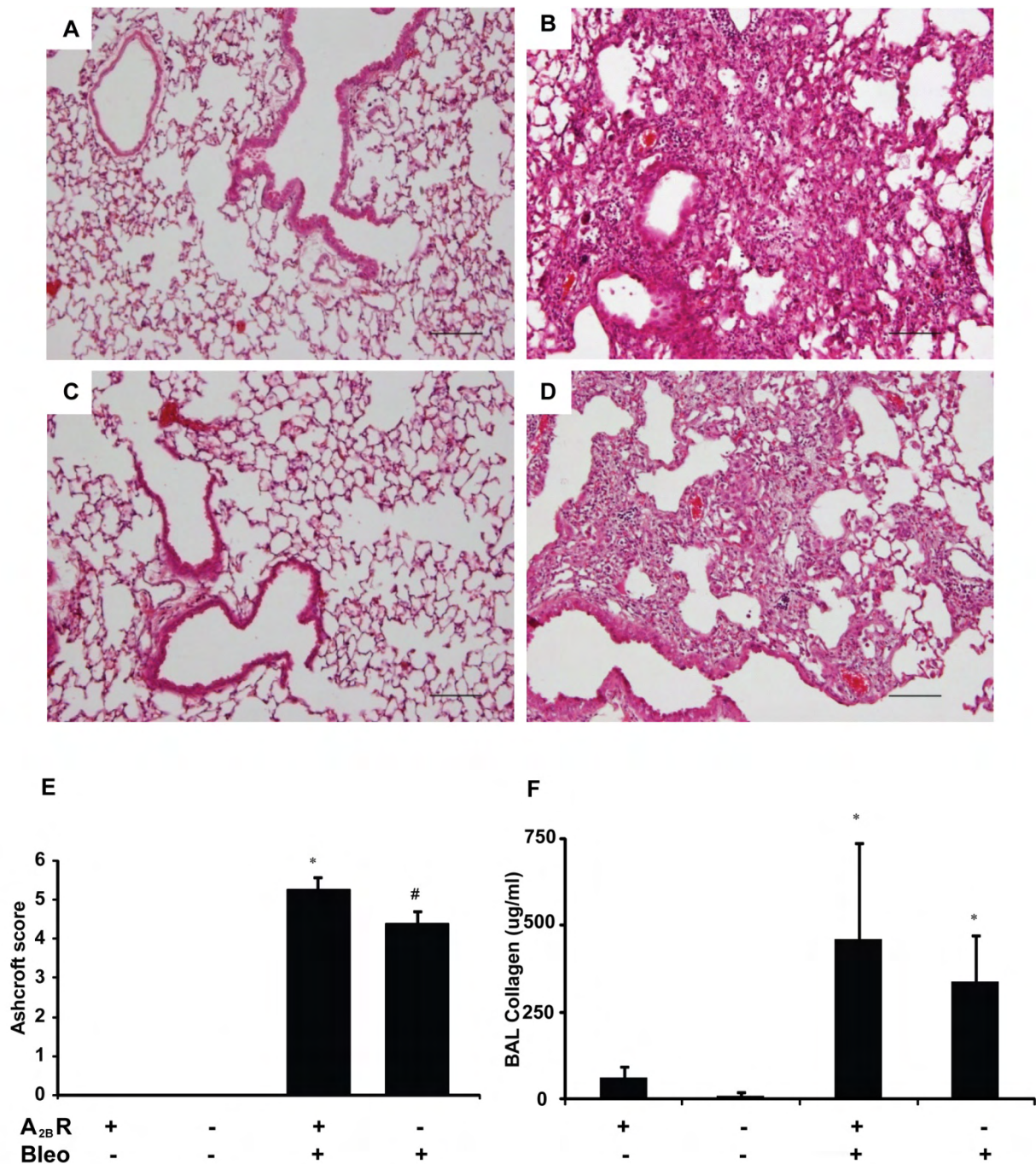
## RESULTS

### Fibrosis in the IT bleomycin model

To determine the role of the  $A_{2B}R$  in IT bleomycin-induced pulmonary injury, wild type and  $A_{2B}R^{-/-}$  mice were exposed to a single dose of 2.5 U/kg bleomycin IT and fibrosis was assessed 21 days after bleomycin exposure. Mice exposed to saline were used as controls and they showed no sign of fibrosis on Day 21 (Figure 4.1, A and C). Mice exposed to bleomycin exhibited significant pulmonary fibrosis and extracellular matrix deposition; however, there did not appear to be a difference in pulmonary histopathology when comparing wild type mice (Figure 4.1B) to  $A_{2B}R^{-/-}$  mice (Figure 4.1D) exposed to bleomycin. To assess the degree of fibrosis in these mice, the Sircol assay was used to measure soluble collagen in BAL fluid, and Ashcroft scores were determined on lung sections. Analysis showed that bleomycin exposure resulted in the same or slightly decreased degree of fibrosis in  $A_{2B}R^{-/-}$  compared to wild type mice (Figure 4.1, E and F). These results suggested that, surprisingly, the genetic removal of the  $A_{2B}R$  did not lead to diminished nor enhanced development of pulmonary fibrosis in the IT model.

### Fibrosis in the IP bleomycin model

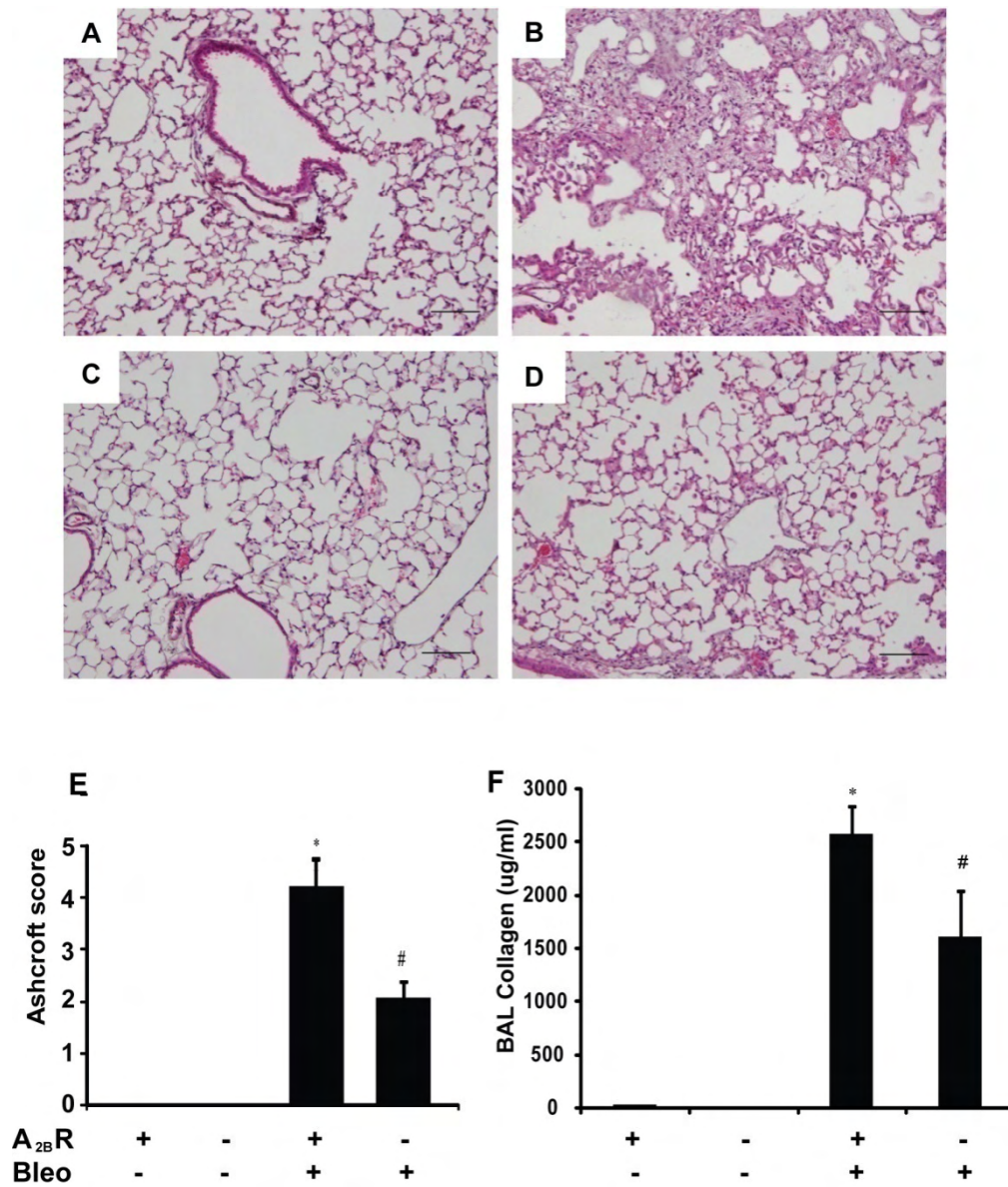
To investigate whether the route of bleomycin exposure affects the role of the  $A_{2B}R$  in bleomycin-induced injury, mice were treated with 8 doses of bleomycin IP twice a week for 4 weeks. Fibrosis was assessed a week after the last bleomycin exposure. Mice exposed to PBS were used as controls and



**Figure 4.1**

Histopathology of the lungs. Lungs were collected 21 days after bleomycin exposure and prepared routinely for sectioning and H&E staining. (A) Lung section from an A<sub>2B</sub>R<sup>+/+</sup> mouse treated with saline. (B) Lung section from an A<sub>2B</sub>R<sup>+/+</sup> mouse treated with bleomycin. (C) Lung section from an A<sub>2B</sub>R<sup>-/-</sup> mouse treated with saline. (D) Lung section from an A<sub>2B</sub>R<sup>-/-</sup> mouse treated with bleomycin. Sections are representative of 6-8 different mice from each genotype. Scale bars = 100  $\mu$ m. (E) Ashcroft scoring was conducted to determine the extent of fibrosis. (F) Soluble collagen protein levels in BAL fluid were quantified using the Sircol assay. Results are presented as mean collagen levels  $\pm$  SEM. \*,  $p \leq 0.05$  vs ADA<sup>+</sup> mice treated with saline; #,  $p \leq 0.05$  vs A<sub>2B</sub>R<sup>+/+</sup> mice treated with bleomycin.  $n = 6$  (saline treated),  $n = 8$  (bleomycin treated).





**Figure 4.2**

Histopathology of the lungs. Lungs were collected 33 days after the first bleomycin exposure and prepared routinely for sectioning and H&E staining. **(A)** Lung section from an A<sub>2B</sub>R<sup>+/+</sup> mouse treated with PBS. **(B)** Lung section from an A<sub>2B</sub>R<sup>+/+</sup> mouse treated with bleomycin. **(C)** Lung section from an A<sub>2B</sub>R<sup>-/-</sup> mouse treated with PBS. **(D)** Lung section from an A<sub>2B</sub>R<sup>-/-</sup> mouse treated with bleomycin. Sections are representative of 6-8 different mice from each genotype. Scale bars = 100  $\mu$ m. **(D)** Ashcroft scoring was conducted to determine the extent of fibrosis. **(E)** Soluble collagen protein levels in BAL fluid were quantified using the Sircol assay. Results are presented as mean collagen levels  $\pm$  SEM. \*,  $p \leq 0.05$  vs ADA<sup>+</sup> mice treated with PBS; #,  $p \leq 0.05$  vs A<sub>2B</sub>R<sup>+/+</sup> mice treated with bleomycin.  $n = 6$  (saline treated),  $n = 8$  (bleomycin treated).



showed no signs of fibrosis (Figure 4.2, A and C). In contrast, wild type mice exposed to bleomycin exhibited significant pulmonary fibrosis and extracellular matrix deposition (Figure 4.2B). Interestingly,  $A_{2B}R^{-/-}$  mice exposed to bleomycin had a diminished degree of fibrotic histopathology compared to wild type mice (Figure 4.2D). To quantitate the degree of fibrosis in these mice, Ashcroft Scoring was conducted and revealed that  $A_{2B}R^{-/-}$  had diminished fibrosis compared to wild type mice (Figure 4.2E). The Sircol assay confirmed that levels of soluble collagen in BAL fluid are significantly lower in  $A_{2B}R^{-/-}$  mice compared to that in wild type mice (Figure 4.2F). These results suggested that the genetic removal of the  $A_{2B}R$  resulted in diminished pulmonary fibrosis in the IP model of bleomycin-induced pulmonary injury.

### **Inflammation in the IT and IP bleomycin models**

To examine the effects of the  $A_{2B}R$  on pulmonary inflammation following bleomycin-induced injury, the numbers of inflammatory cells recovered from BAL fluid were quantitated at both the acute and chronic phase to determine the status of pulmonary inflammation at early and late stages of the disease.

In the IT bleomycin model, direct damage to the lung results in an acute inflammation. Indeed, our findings suggest that the total inflammatory cell numbers recovered from BAL fluid was significantly increased 7 days after bleomycin exposure. Interestingly, the number of total inflammatory cells was significantly higher in  $A_{2B}R^{-/-}$  compared to wild type mice (Figure 4.3A, left panel). Cell differentials suggested that increased numbers of macrophages and

neutrophils were responsible for the total cell number increase. In animals treated with bleomycin, the most significant difference between  $A_{2B}R^{-/-}$  mice and wild type mice was that there were more infiltrated neutrophils in  $A_{2B}R^{-/-}$  mice (Figure 4.3A, middle and right panels). At the chronic phase of the IT bleomycin model, we still observed an increase in total cell number in mice treated with bleomycin on Day 21. However, there was no difference in the number of total cells between wild type and  $A_{2B}R^{-/-}$  mice (Figure 4.3B, left panel). Moreover, the numbers of macrophages, lymphocytes and neutrophils were not altered in  $A_{2B}R^{-/-}$  mice compared to wild type animals (Figure 4.3B, middle and right panels). This is consistent with our observation that there was no difference in fibrosis between wild type and  $A_{2B}R^{-/-}$  mice. These findings suggest that IT bleomycin treatment in  $A_{2B}R^{-/-}$  mice is associated with enhanced acute pulmonary inflammation and neutrophil infiltration compared to wild type mice, whereas lacking the  $A_{2B}R$  at the chronic phase has no effect on pulmonary inflammation.

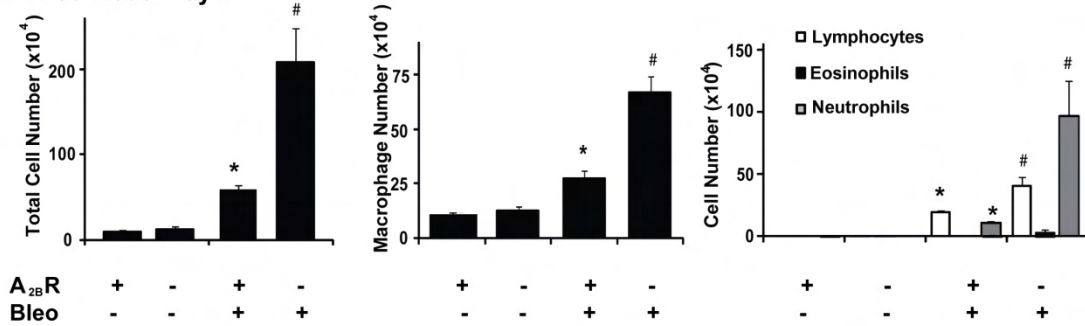
Examination of pulmonary inflammation in the IP bleomycin model was also determined by counting the number of inflammatory cells recovered from BAL fluid. At 10 days after the initiation of IP bleomycin treatments, the number of total inflammatory cells was significantly increased. However, there was no difference in the number of total cells between wild type and  $A_{2B}R^{-/-}$  mice (Figure 4.3C, left panel). Cell differentials suggested that most cells recovered were macrophages. Importantly, there was no increased neutrophil infiltration in this model. Moreover, there was no difference in the numbers of each cell types between wild type and  $A_{2B}R^{-/-}$  mice (Figure 4.3C, middle and right panels). At the

chronic phase of the IP bleomycin model, an increase in total cell number on Day 33 was also observed in mice treated with bleomycin. However, there was no difference in the numbers of total cells and macrophages between wild type and  $A_{2B}R^{-/-}$  mice (Figure 4.3D, left and middle panels). Interestingly, the numbers of lymphocytes and neutrophils were significantly decreased in  $A_{2B}R^{-/-}$  mice compared to wild type mice (Figure 4.3D, right panel). These findings suggest that the IP bleomycin treatment was not associated with an acute inflammation and neutrophil infiltration at the acute phase; even though decreased pulmonary fibrosis was observed in  $A_{2B}R^{-/-}$  mice compared to wild type mice following bleomycin exposure, lacking the  $A_{2B}R$  at the chronic phase had only mild effect on pulmonary inflammation.

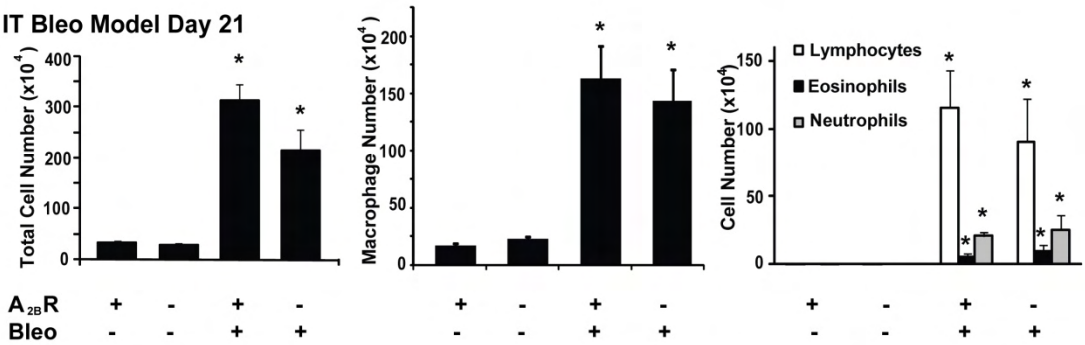
### **Vascular permeability in the IT and IP bleomycin models**

Acute lung injury is commonly associated with the loss of pulmonary vascular barrier function, which can contribute to enhanced neutrophil infiltration into the lung. Previous studies have shown that the  $A_{2B}R$  plays a protective role in the maintenance of pulmonary barrier function in various models of acute lung injury (14, 39). Findings in chapter 4 of this dissertation suggest that the enhanced loss of vascular barrier function in  $ADA/A_{2B}R$  double knockout mice resulted in enhanced pulmonary inflammation. Here I show that the IT bleomycin model where an acute damage is involved, there is an increase in the number of infiltrated inflammatory cells, specifically neutrophils, in  $A_{2B}R^{-/-}$  mice compared to wild type mice at the acute phase of the injury. To determine if the loss of

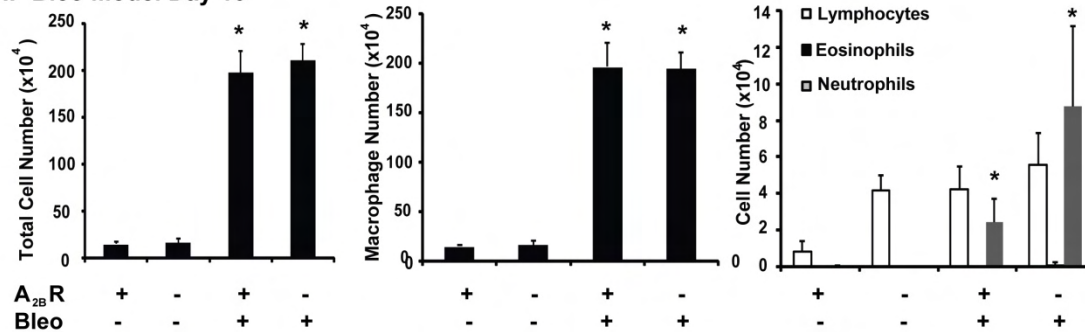
### A. IT Bleo Model Day 7



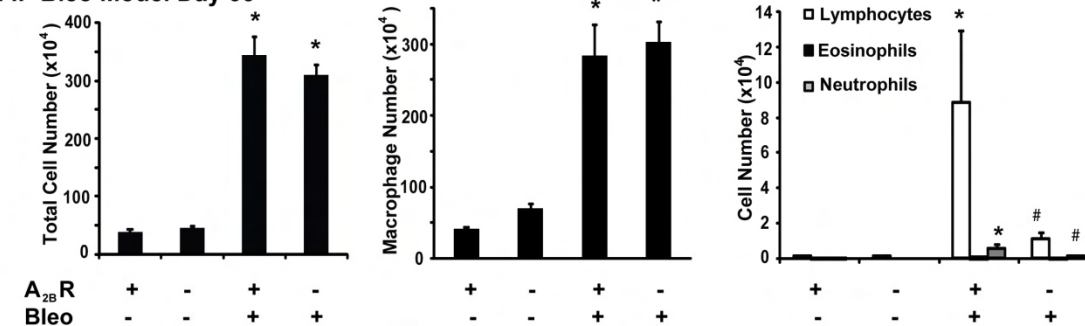
### B. IT Bleo Model Day 21



### C. IP Bleo Model Day 10

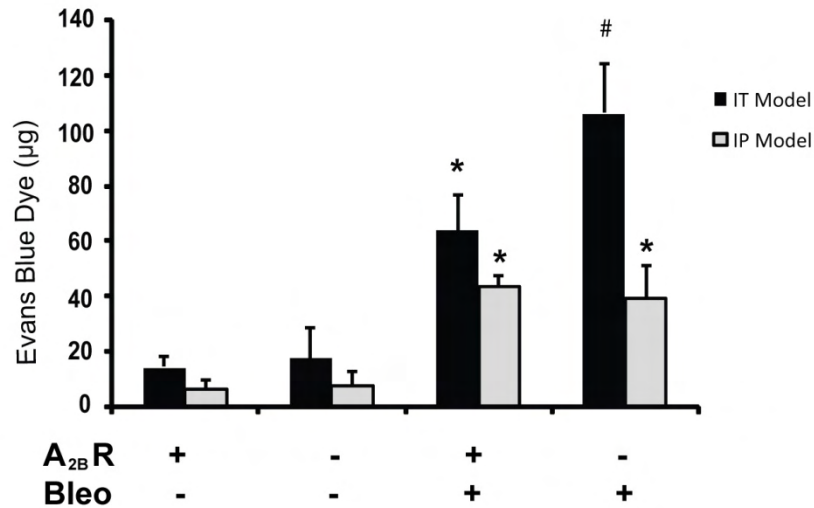
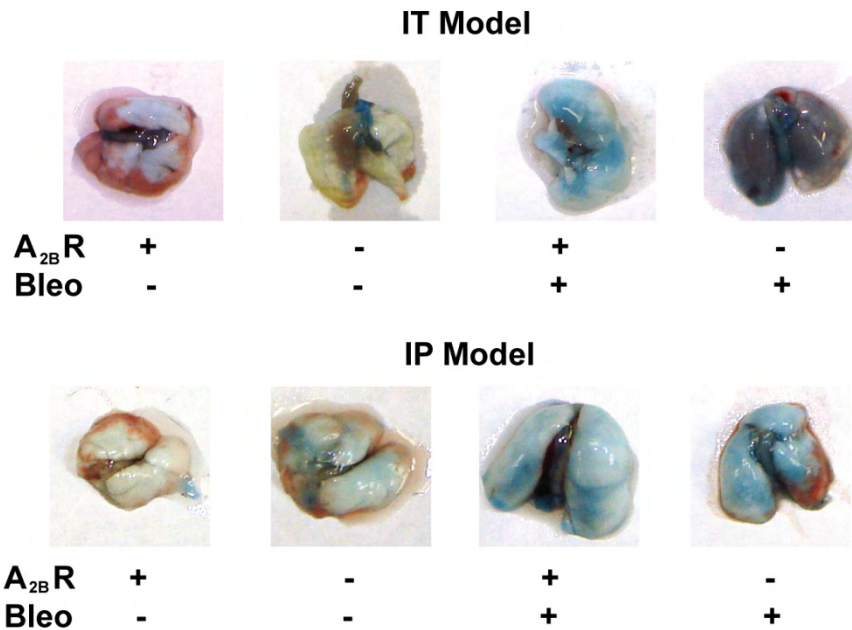


### D. IP Bleo Model Day 33



**Figure 4.3**

Pulmonary inflammation in the IT and IP bleomycin models. (A) BAL fluid was collected 7 days after IT bleomycin exposure. (B) BAL fluid was collected 21 days after IT bleomycin exposure. (C) BAL fluid was collected 10 days after IP bleomycin exposure. (D) BAL fluid was collected 33 days after IP bleomycin exposure. Total cell numbers were determined. Then BAL cells were cytopspun and stained with Diff-Quick, allowing for determination of cellular differentials. Data are mean cell counts  $\pm$  SEM. \* $p \leq 0.05$  versus mice treated with saline or PBS; # $p \leq 0.05$  versus A<sub>2B</sub>R<sup>+/+</sup> mice treated with bleomycin;  $n = 8$ .

**A****B****Figure 4.4**

Vascular permeability in the IT and IP bleomycin models. **(A)** 7 days after IT bleomycin exposure or 10 days after the first IP bleomycin exposure, mice were administered intraperitoneal Evan blue dye (0.2 mL of 0.5% in PBS) and sacrificed 4 hours later. Lungs were harvested and Evan blue dye concentrations were quantified as described in "Methods". **(B)** Representative images of lungs. Data are mean dye amount  $\pm$  SEM. \*p  $\leq$  0.05 versus mice treated with saline or PBS; #p  $\leq$  0.05 versus  $A_{2B}^{+/+}$  mice treated with bleomycin; n = 6.

vascular barrier occurs in  $A_{2B}R^{-/-}$  mice, Evans blue dye extravasations were quantitated four hours after injecting the dye intraperitoneally. Results revealed that there was enhanced vascular leakage in  $A_{2B}R^{-/-}$  mice compared to that in wild type mice after bleomycin induced injury (Figure 4.4, A and B). In contrast, there was only mild damage at the acute phase of the IP bleomycin model. Evans blue dye extravasations revealed that although vascular permeability was enhanced following bleomycin exposure, there was no further loss of vascular barrier function in mice lacking the  $A_{2B}R$  (Figure 4.4, A and B).

These findings suggest that IT bleomycin exposure results in an acute damage, making vasculature the fundamental barrier in preventing neutrophil infiltration and injury to the lung. Thus, there is enhanced loss of vascular barrier function and pulmonary neutrophilia in  $A_{2B}R^{-/-}$  mice at the acute stage of the disease. In contrast, IP bleomycin model does not involve an acute damage and there is only mild vascular damage in mice exposed to bleomycin and no further loss of barrier function in  $A_{2B}R^{-/-}$  mice at the acute stage of the disease.

## DISCUSSION

In the previous chapter, we observed that the genetic removal of the  $A_{2B}R$  results in loss of vascular barrier function in ADA-deficient mice. This may contribute to the enhanced pulmonary injury at the acute stage of disease in ADA/ $A_{2B}R$  double knockout mice, leading to the precocious death of these animals compared to ADA-deficient mice. However, in the same model,  $A_{2B}R$  antagonism resulted in diminished pulmonary inflammation and fibrosis,

suggesting a tissue-destructive role of this receptor at chronic stage of the disease. The discrepancy between these two studies could be due to the fact that the A<sub>2B</sub>R antagonist was given after the disease status has been established, while the genetic knockout totally abolished the receptor activity from birth of the animals. Therefore, in ADA-deficient model, the A<sub>2B</sub>R may have a bi-phasic effect on disease progression that it plays an important role in maintaining vascular barrier function during acute injury and serves a pro-inflammatory and pro-fibrotic role during the chronic phase of the disease. In order to further address this concept, I chose bleomycin-induced pulmonary injury as the model to investigate the distinctive roles of the A<sub>2B</sub>R at different stages of the disease.

The IT bleomycin model involves a single dose of bleomycin exposure directly into the lung. The local exposure of the toxic drug results in a severe apoptosis of epithelial cells and damage to the lung after it is delivered. In addition to the result that mice develop extensive pulmonary fibrosis in the interstitial areas of the lung, an acute inflammation characterized by neutrophil infiltration is involved before the fibrotic changes. The instant damage caused by bleomycin makes vasculature maintenance fundamentally important in preventing the infiltration of inflammatory cells. When A<sub>2B</sub>R<sup>-/-</sup> mice were exposed to bleomycin intratracheally, loss of vascular barrier function at the early stage of the disease (Day 7) contributed significantly to the enhancement of pulmonary inflammation. Furthermore, this enhanced inflammation counteracted the pro-

fibrotic role of the A<sub>2B</sub>R and there was no difference in appearance of the lungs of wild type and A<sub>2B</sub>R<sup>-/-</sup> mice after the development of fibrosis.

The generation of adenosine and subsequent A<sub>2B</sub>R signaling play tissue-protective roles during acute lung injuries (90). Previous studies have supported the protective roles of adenosine and A<sub>2B</sub>R signaling. For example, the levels of CD39 and CD73, the enzymes to generate adenosine, are elevated in the lungs following LPS exposure (37). Moreover, the accumulation of neutrophils following LPS stimulation is enhanced in mice deficient in CD39 or CD73. Similar findings have been demonstrated in mouse models of hypoxia-induced and ventilator-induced lung injuries. The levels of CD39 and CD73, and the expression of the A<sub>2B</sub>R are increased during ventilator-induced and hypoxia-induced lung injuries (3, 91). In addition, the A<sub>2B</sub>R's protective roles were revealed by pharmacological and genetic approaches. Enhanced pulmonary inflammation, edema, and attenuated gas exchange were seen in mice treatment with an A<sub>2B</sub>R antagonist and mice with the A<sub>2B</sub>R gene deletion (14, 92). In contrast, treatment with an A<sub>2B</sub>R agonist attenuated the injuries and protected the lungs from drying out (39). The A<sub>2B</sub>R's protective role was also demonstrated in other organs. A<sub>2B</sub>R blockage or gene deletion resulted in increased weight loss, colonic shortening, and disease activity indices in a mouse colitis model, implicating a protective role of the A<sub>2B</sub>R on intestinal epithelial cells (93). Furthermore, the A<sub>2B</sub>R provides potent protection in an intestinal ischemia/reperfusion injury model (94). Interestingly, the A<sub>2B</sub>R plays a protective role at the acute phase of the lung injury even in a mouse model of chronic lung disease. Studies described in the



previous chapter showed that ADA/A<sub>2B</sub>R double knockout mice exhibited enhanced pulmonary damage and inflammation compared to ADA-deficient mice (60). The predominant interface between acute damage and the organs is the vascular endothelium. Neutrophils penetrate the endothelium following acute damage and vascular endothelial cells create a potential endothelial barrier to prevent neutrophil infiltration, loss of fluid and edema formation. All studies described above revealed a critical role of the A<sub>2B</sub>R in attenuating vascular leakage through endothelial paracellular barriers. Hence, loss of vascular barrier function due to A<sub>2B</sub>R deficiency is responsible for the enhanced pulmonary inflammation and damage in A<sub>2B</sub>R<sup>-/-</sup> mice following acute lung injuries.

The mechanism by which the A<sub>2B</sub>R prevents vascular leakage is unknown. However, it has been proposed that signaling pathways through cAMP, subsequent phosphorylation of VE-cadherin and vasodilator-stimulated phosphoprotein, and alterations in cellular cytoskeleton are responsible for the enhanced vascular barrier function (3, 68, 95, 96). In addition, a recent study revealed that the neuronal guidance molecule netrin-1 can engage the A<sub>2B</sub>R on neutrophils, providing an unexpected mechanism by which the A<sub>2B</sub>R may play a protective role (97). More studies are needed to further investigate how the A<sub>2B</sub>R plays a protective role in maintaining vascular integrity.

In contrast to the IT bleomycin model where bleomycin was exposed directly to the lung, the IP bleomycin model involved 8 doses of bleomycin treatments into the peritoneal cavity. Only mild acute injury occurred in this model and we did not observe extensive pulmonary neutrophilia throughout the

protocol. An acute phase inflammatory response was not as robust in this model as that in the IT bleomycin model. In addition, although there was still vascular damage and increased vascular permeability after bleomycin treatments at the early stage, vascular barrier function was not a determinant factor in preventing inflammation and damage in the lung. Mice did develop fibrosis in the lung and pulmonary fibrosis was assessed on Day 33.  $A_{2B}R^{-/-}$  mice showed diminished pulmonary fibrosis compared to wild type mice, suggesting the  $A_{2B}R$  is playing a pro-fibrotic role. This finding suggests that the  $A_{2B}R$  only helps in maintaining vascular integrity when an acute damage is involved; it plays a pro-fibrotic role at the chronic stage of the diseases.

The observation in the IP bleomycin model is consistent with the findings in  $A_{2B}R$  antagonism study in ADA-deficient mice, where a pro-fibrotic and tissue-destructive role of the  $A_{2B}R$  was revealed (57). Numerous studies support the  $A_{2B}R$ 's detrimental roles. The activation of the  $A_{2B}R$  promotes fibroblasts differentiation into myofibroblasts and hence promotes collagen production and deposition (30). The engagement of the  $A_{2B}R$  on human mast cells mediates the expression of various pro-inflammatory cytokines such as IL-4, IL-8 and IL-13 (52, 55, 56, 98, 99).  $A_{2B}R$  activation up-regulates the production of IL-6 from mouse macrophages (75). These findings implicate a detrimental effect of the  $A_{2B}R$  in promoting the damage and injury at the chronic stage of the disease.

In conclusion, the results of the present study demonstrate that  $A_{2B}R$  plays a protective role in maintaining the integrity of vascular barrier when an acute injury is involved. On the other hand, the activation of  $A_{2B}R$  promotes the

production of pro-fibrotic cytokines and chemokines and it plays a pro-fibrotic and tissue-destructive role at the chronic stage of the disease. As a result, adenosine and the  $A_{2B}R$  regulate both the protective and detrimental aspects in diseases.  $A_{2B}R$  antagonist could be beneficial in treating chronic lung disease, especially at the later stages;  $A_{2B}R$  agonist could be useful in treating patients with acute respiratory distress and protecting the vascular barrier. Further studies are needed to understand the status of adenosine metabolism and  $A_{2B}R$  signaling in specific patients of these diseases in order to design adenosine-based therapies to treat these patients. This will be the focus of the next chapter.

# **CHAPTER 5**

## **ALTERATIONS OF ADENOSINE METABOLISM AND SIGNALING IN PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE AND IDOPATHIC PULMONARY FIBROSIS**

Findings in the previous chapters suggest that the  $A_{2B}R$  plays a protective role in acute lung injury but a destructive role in chronic lung diseases. It is critical to better understand adenosine metabolism and signaling in each specific disease in order to design potential adenosine-based therapeutics. Studies described in this chapter focus on investigating adenosine metabolism and signaling, specifically  $A_{2B}R$  signaling, in two types of human chronic lung diseases.

### **INTRODUCTION**

#### **Adenosine and Human Chronic Lung Disease**

The production of adenosine in the lungs of asthmatics and COPD patients suggests that it may regulate aspects of lung disease (1, 100). There is substantial clinical and scientific evidence to support this hypothesis. Adenosine can directly influence cellular and physiological processes in the lungs of asthmatics and patients with COPD (24, 100). For example, exogenous

adenosine can elicit acute bronchoconstriction in patients with asthma or COPD (101, 102), while no effect was found on normal individuals, suggesting a fundamental difference regarding adenosine signaling in these patients. Receptor binding studies to analyze adenosine receptor expression revealed the significantly decreased affinity of the A<sub>1</sub>R, A<sub>2A</sub>R, and A<sub>3</sub>R in patients with COPD compared with controls, whereas increases in density was observed. The density of A<sub>2B</sub>R was decreased in patients with COPD compared with the control group, but the affinity was not altered (103). The signaling pathways of adenosine can influence the activity of multiple cell types that play a critical role in chronic lung disease including, mast cells (104), eosinophils (35), macrophages (105), epithelial cells (36), fibroblasts (30), and smooth muscle cells (106). Recent studies directly implicate adenosine in the regulation of pulmonary fibrosis. Exposure of human pulmonary fibroblasts to adenosine promotes differentiation into myofibroblasts through mechanism that involved the A<sub>2B</sub>R (30). Moreover, A<sub>2B</sub>R engagement promotes the production of fibronectin from type II epithelial cells (107), a process that can impact pulmonary fibrosis. Lastly, it has been shown that the expression levels of A<sub>2B</sub>R are elevated in remodeled airway epithelial cells when comparing rapidly progressing idiopathic pulmonary fibrosis patients to slowly progressing patients (108). Collectively, these studies demonstrate that adenosine can regulate processes that influence pulmonary fibrosis.

## **Purinergic Remodeling in Animals Models of Chronic Lung Disease**

Examination of adenosine levels in animal models of chronic lung disease support these findings in humans. Transgenic mice that over express the TH2 cytokines IL-4 or IL-13 in the lungs develop progressive pulmonary inflammation and injury characterized by eosinophilic and monocytic infiltrates, airway fibrosis and alveolar airspace destruction in association with increases of adenosine levels in the lungs (66, 109). In addition, adenosine levels are elevated in the lungs of mice exposed to the fibrosis inducing agent bleomycin (110) or following chronic ovalbumin exposure (111). Lastly, there are correlations between the degree of inflammation and damage and adenosine accumulations in ADA-deficient mice (47). Therapeutic strategies to lower adenosine levels or disrupt adenosine receptors lead to improvement in pulmonary pathologies in these models. We have made the observation that the levels of key components of adenosine metabolism and signaling are altered in these mouse models where adenosine-based therapeutics are most effective. These changes include a selective down-regulation of ADA, the major enzyme for adenosine metabolism, the up-regulation of ecto-5'-nucleotidase (CD73), the major enzyme of adenosine production, the down-regulation of adenosine kinase (AK) and equilibrative nucleoside transporters (ENT) (Figure 1). In addition, we have observed up-regulation of pro-inflammatory and pro-fibrotic adenosine receptors in these models (47, 66, 109, 110). This is a process we call purinergic remodeling.

## **Experimental Rational and Goals**

These findings raise the possibility that adenosine-based therapeutics may be beneficial in the treatment of chronic lung disease such as COPD and IPF. If changes in purinergic metabolism and signaling also exist in humans with COPD and IPF, it would provide proof of concept information that these disorders may benefit from adenosine-based therapeutics. The focus of the study described in this chapter was to utilize tissues and from the Lung Tissue Research Consortium (LTRC) to determine if “purinergic remodeling” exists in patients with COPD and/or IPF. Patients were classified as Stage 0 COPD, Stage 4 COPD, Mild IPF and severe IPF according to spirometry, pathological specimen and high resolution CT scan (Figure 5.1). Our hypothesis was that purinergic metabolism and signaling components are altered in a manner that promotes adenosine production in tissue samples from patients with COPD and IPF. Our results demonstrate that CD73 and the  $A_{2B}R$  are elevated in lung biopsy samples from patients with Stage 4 COPD and Severe IPF compared to patients with preserved lung function. This was associated with significant alterations in expressions of pro-inflammatory mediators known to be driven by  $A_{2B}R$  signaling in animal models, and ex vivo studies demonstrated that activation of the  $A_{2B}R$  can influence the production of key inflammatory and fibrotic mediators from macrophages isolated from these patients. This study provides novel and important information into the potential involvement of adenosine signaling in human chronic lung diseases, and provides insight into potential mechanisms for screening patients for trials in adenosine-based therapies.

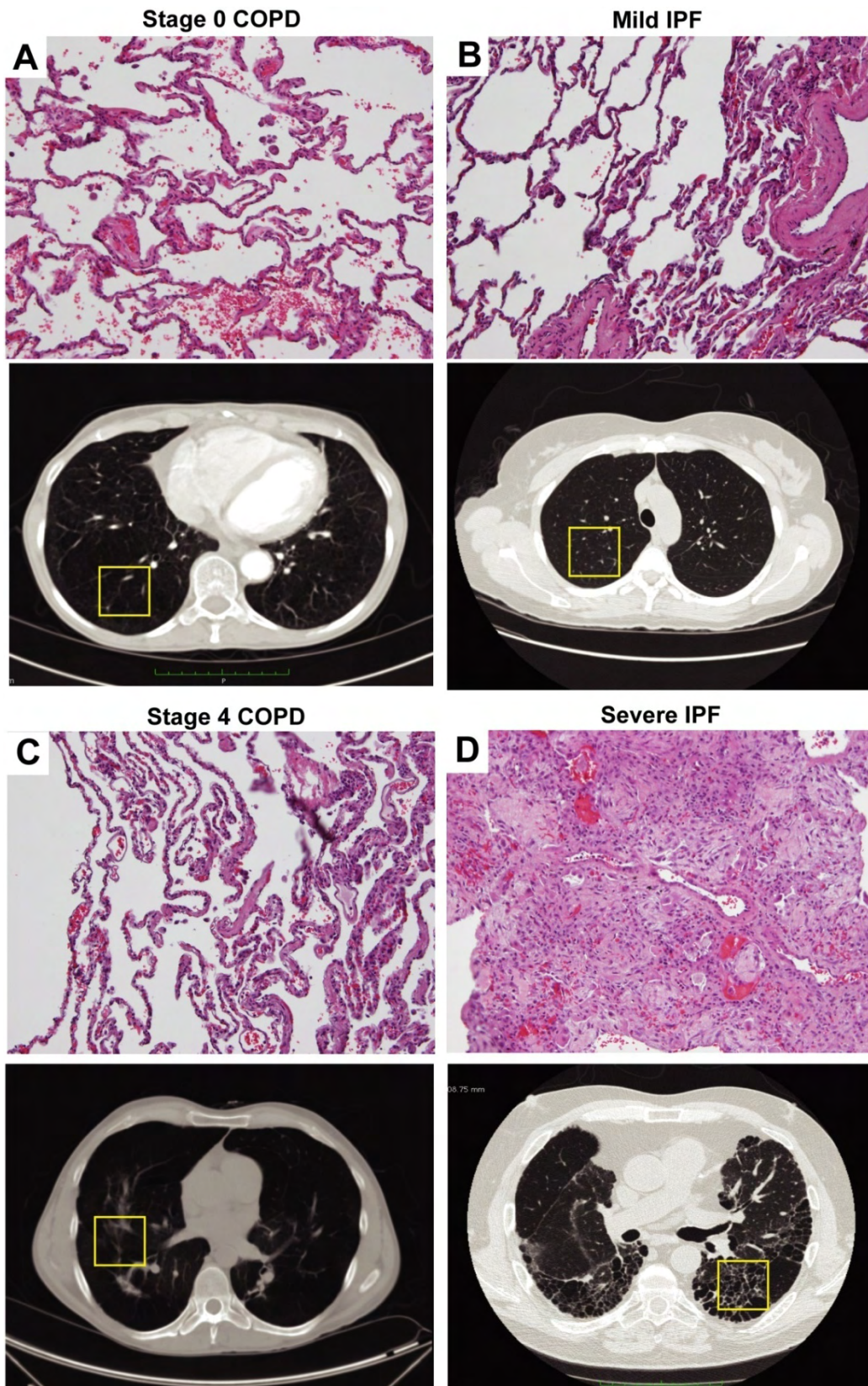
## **Transcript Levels of Components of Adenosine Metabolism and Signaling Are Altered in the Lungs of COPD and IPF Patients**

Components of adenosine metabolism and signaling are altered in mouse models of chronic lung disease in association with elevated levels of adenosine (47, 66, 109, 110). Total RNA was isolated from lung biopsy specimens and real-time RT-PCR was performed to quantify key components of adenosine metabolism and signaling. Results demonstrated a 3 fold increase of CD73 transcripts in whole lung RNA extracts from Severe IPF and Stage 4 COPD patients compared to Mild IPF and Stage 0 COPD patients with preserved lung function (Figure 5.2A). However, the transcript levels of ADA, AK and ENT1 were not altered in Severe IPF and Stage 4 COPD patients (Figure 5.2, B-D). All four adenosine receptors were detectable in subjects with preserved lung function (Figure 5.2E). Transcript levels for the A<sub>2B</sub>R were significantly increased in Severe IPF and Stage 4 COPD patients (Figure 5.2E). These findings demonstrate elevations in components of adenosine production and A<sub>2B</sub>R signaling in the lungs of patients with progressive chronic lung disease.

## **CD73 and ADA enzymatic activities are altered in the lungs of COPD and IPF patients**

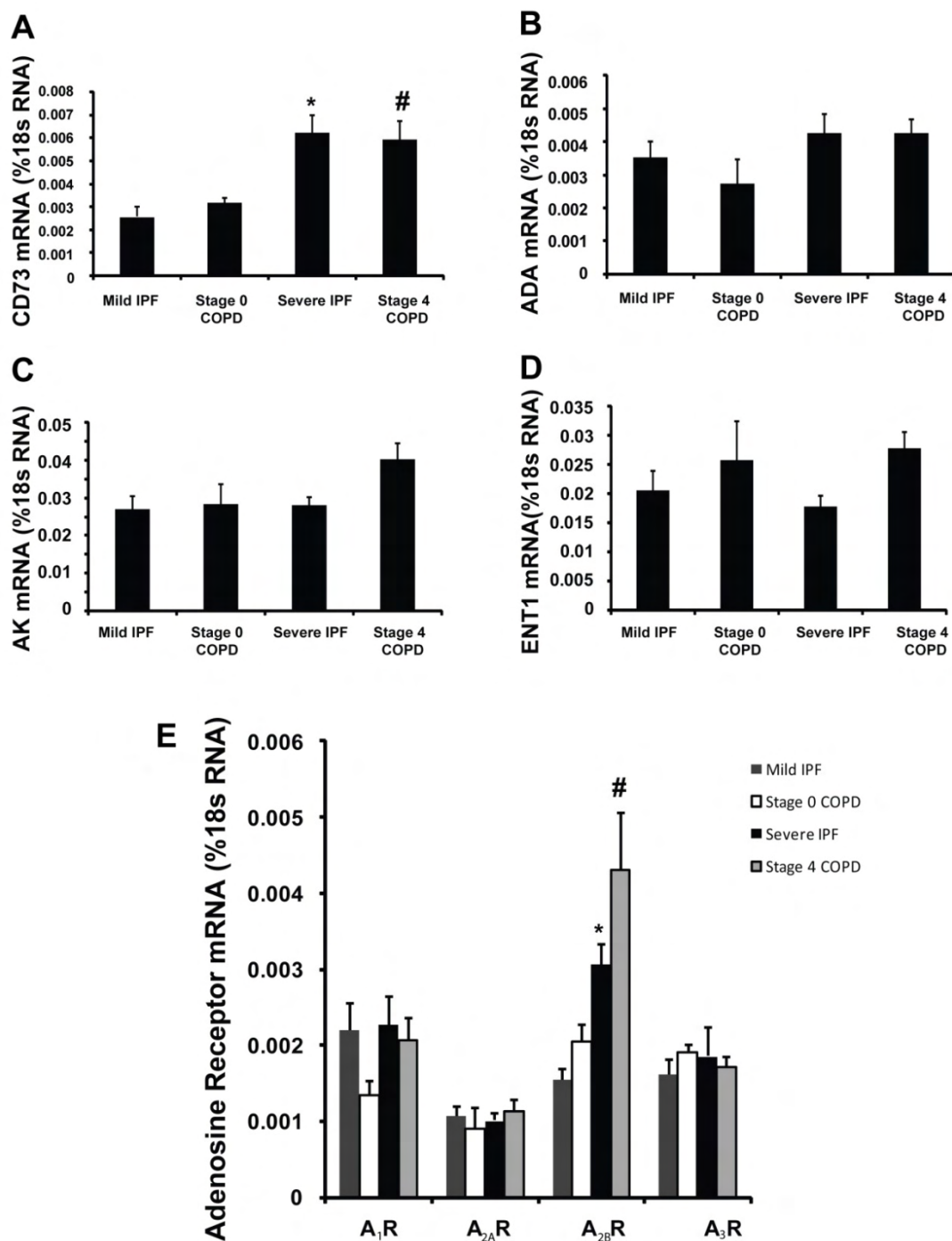
To examine alterations in enzymatic activities of the key enzymes of adenosine metabolism, protein extracts were made from lung biopsy specimens and enzymatic activities of CD73 and ADA were quantified using HPLC (Figure 5.3). The enzymatic activity of CD73 was increased by 2 fold and 2.5 fold,





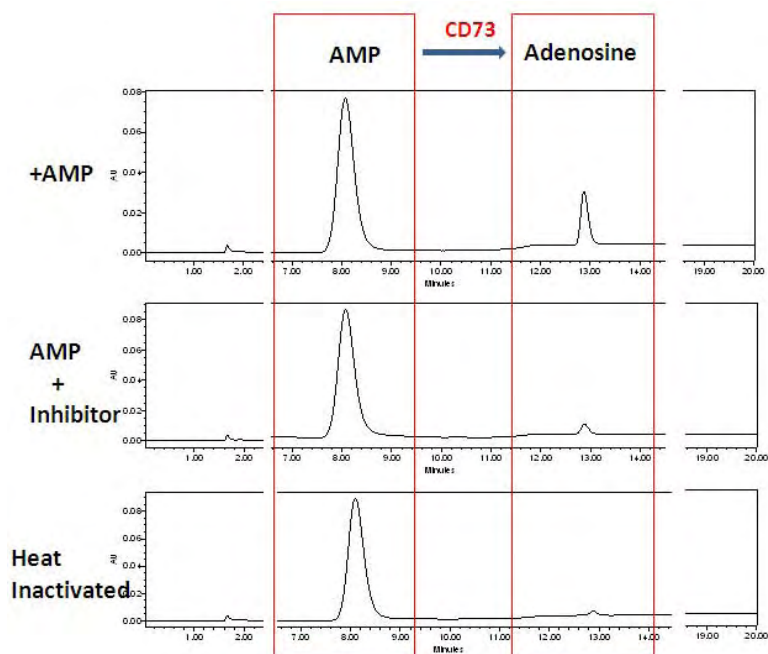
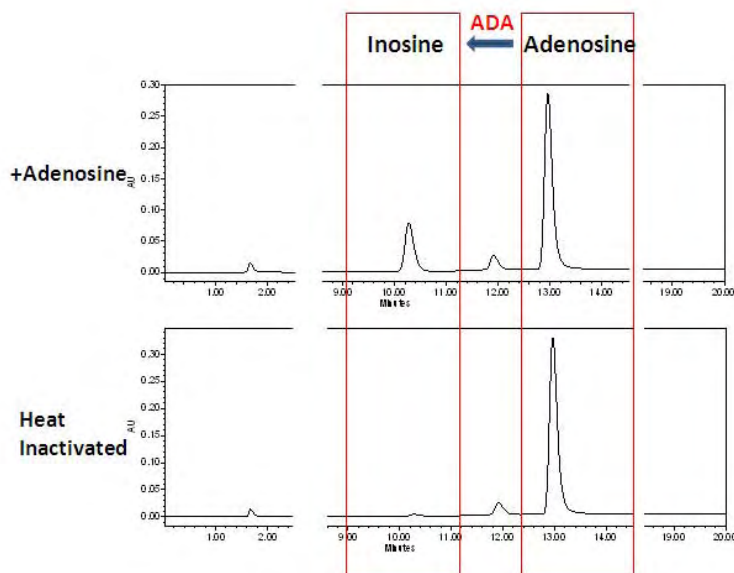
**Figure 5.1**

Lung histopathology and high-resolution CT scans. Representative H&E staining and high-resolution CT scan images showing: (A) A Stage 0 COPD patient with preserved lung function. (B) A Mild IPF patient with preserved lung function. (C) A Stage 4 COPD patient. (D) A Severe IPF patient. Sections are representative of 10-14 different patients from each group.



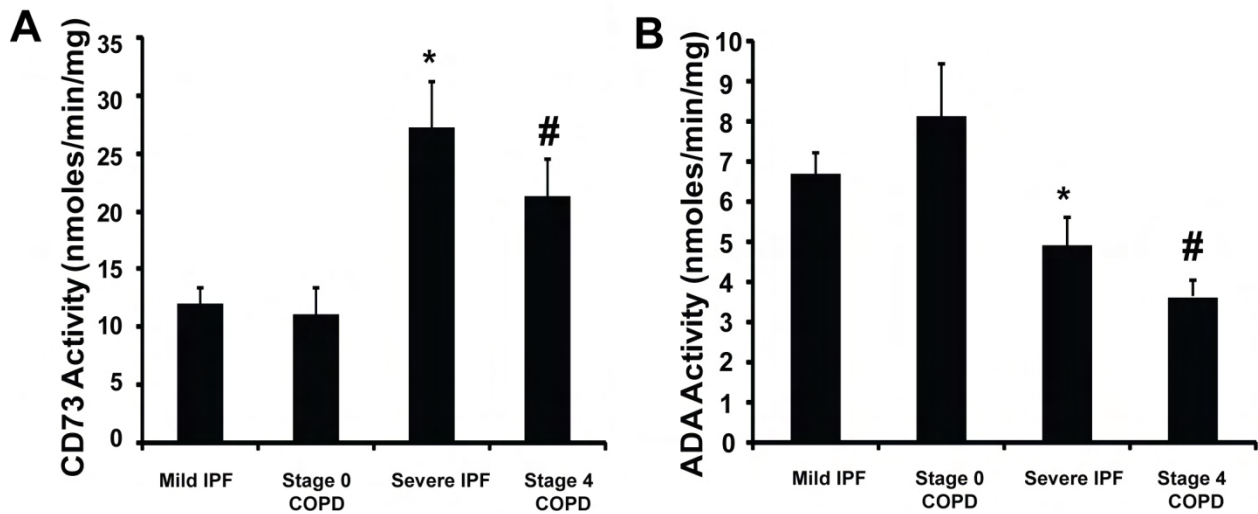
**Figure 5.2**

Expression of components of adenosine metabolism and signaling. Transcript levels of various enzymes in adenosine metabolism, and adenosine receptors were quantified in lung RNA extracts from patients using quantitative RT-PCR. Shown are levels of (A) CD73, (B) ADA, (C) AK, (D) ENT1, (E) Adenosine receptors. Results are presented as mean percentage of 18sRNA transcripts  $\pm$  SEM. \* $p \leq 0.05$  versus Mild IPF. #  $p \leq 0.05$  versus Stage 0 COPD.  $n = 4$  (Stage 0 COPD),  $n = 10$  (Mild IPF),  $n = 8$  (Severe IPF and Stage 4 COPD).

**A****B****Figure 5.3**

Determination of enzyme activity and representative chromatograms. **(A)** CD73 enzyme activity. Adenosine was quantitated by reverse phase HPLC, measuring absorbance at 260nm. Chromatogram in the upper panel represents a typical curve from a nucleotidase assay. The AMP peak is seen at 8 min, with adenosine detection at around 13 min. Samples that have been preincubated with the enzyme inhibitor or heat inactivated were used as controls (middle and lower panel). **(B)** ADA enzyme activity. Inosine was quantitated by reverse phase HPLC, measuring absorbance at 260nm. Chromatogram in the up panel represents a typical curve from an ADA assay. The Adenosine peak is seen at 13 min, with inosine detection at around 10 min. Samples that have been heat inactivated were used as controls (lower panel).





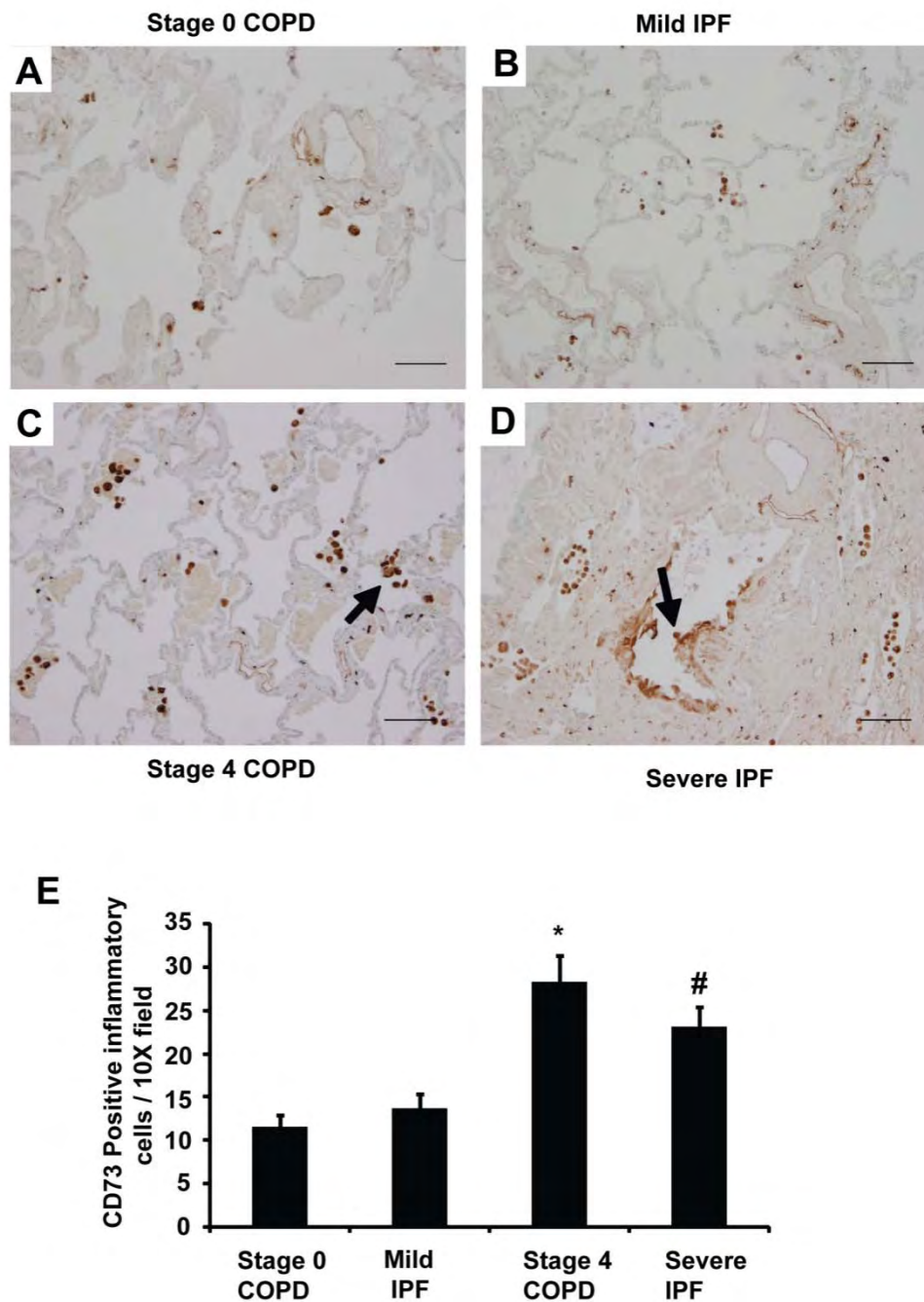
**Figure 5.4**

CD73 and ADA enzymatic activity. CD73 (**A**) and ADA (**B**) enzyme activity were quantified in lung protein extracts from patients. Reaction mixtures were separated, identified, and quantified by HPLC. Data are presented as mean nanomoles of substrate converted to product per min per milligram of protein  $\pm$  SEM. \* $p \leq 0.05$  versus Mild IPF. #  $p \leq 0.05$  versus Stage 0 COPD.  $n = 4$  (Stage 0 COPD),  $n = 10$  (Mild IPF),  $n = 8$  (Severe IPF and Stage 4 COPD).

respectively, in Stage 4 COPD and Severe IPF patients compared to subjects with preserved lung function (Figure 5.4A). This finding is consistent with increased CD73 RNA levels and suggests that its expression is altered to promote adenosine generation in patients with severe chronic lung disease. There was a significant decrease in ADA enzymatic activity in Severe IPF patients and Stage 4 COPD patients compared to subjects with preserved lung function (Figure 5.4B). These observations also suggest that purinergic remodeling exist in these patients and it promotes adenosine elevations.

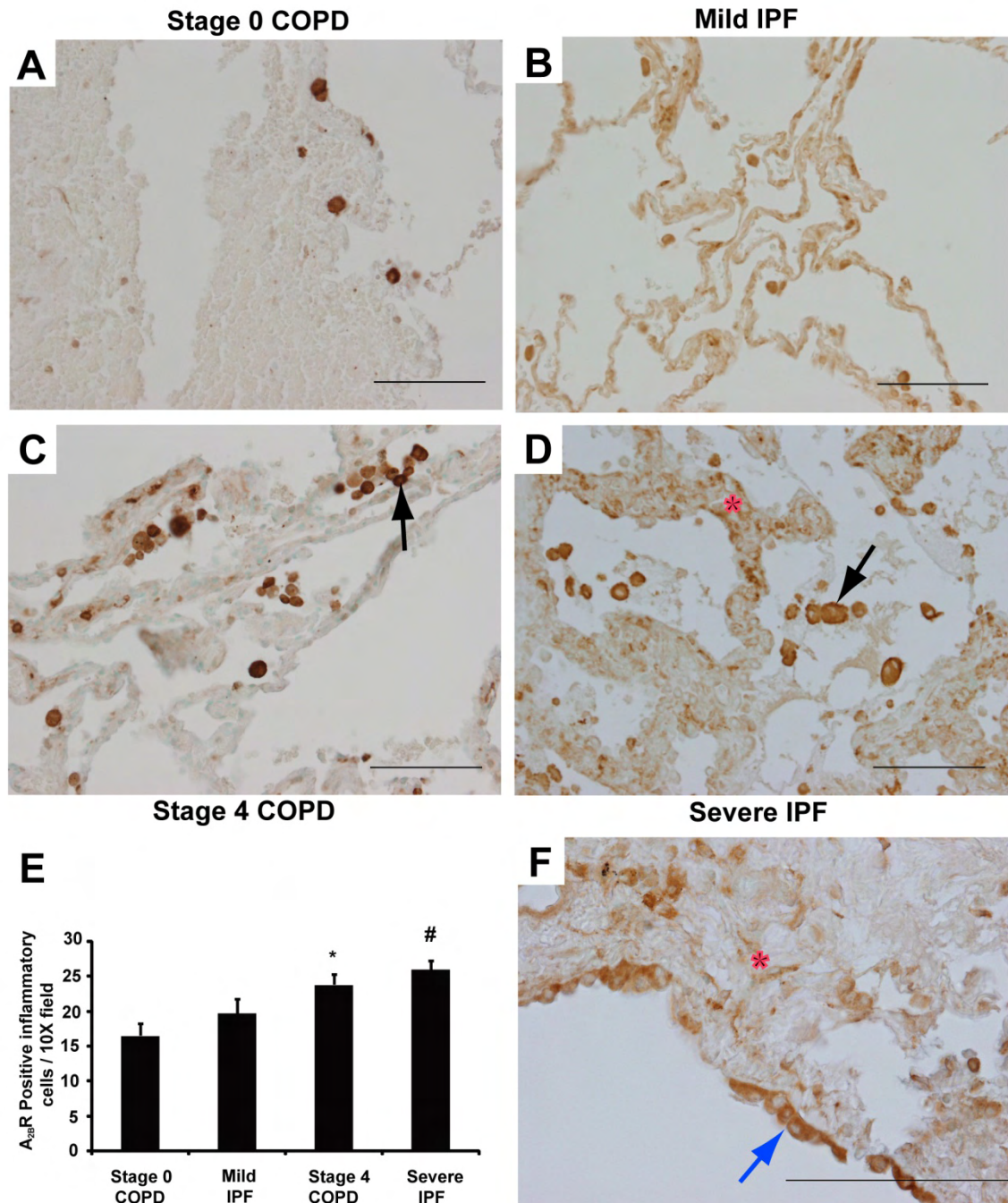
### **Cellular localization of CD73 and A<sub>2B</sub>R expression in lung tissue from COPD and IPF patients**

To identify the cellular localization of key components of adenosine metabolism and signaling in normal and diseased subjects, tissue sections were stained with antibodies against CD73 and A<sub>2B</sub>R. In Mild IPF and Stage 0 COPD subjects with normal lung function, CD73 was expressed on inflammatory cells as well as endothelial cells (Figure 5.5, A and B). In Stage 4 COPD patients, CD73 was expressed on inflammatory cells and endothelial cells (Figure 5.5C, arrow). In Severe IPF patients, CD73 was localized to inflammatory cells, endothelial cells and remodeled airway epithelial cells (Figure 5.5D, arrow). A<sub>2B</sub>Rs were localized on inflammatory cells, mostly macrophages in both Stage 0 and Stage 4 COPD patients (Figure 5.6, A and C). In IPF patients, A<sub>2B</sub>Rs were expressed on inflammatory cells and stromal cells (Figure 5.6, B and D) as well as hyperplastic cells in remodeled airways (Figure 5.6F).



**Figure 5.5**

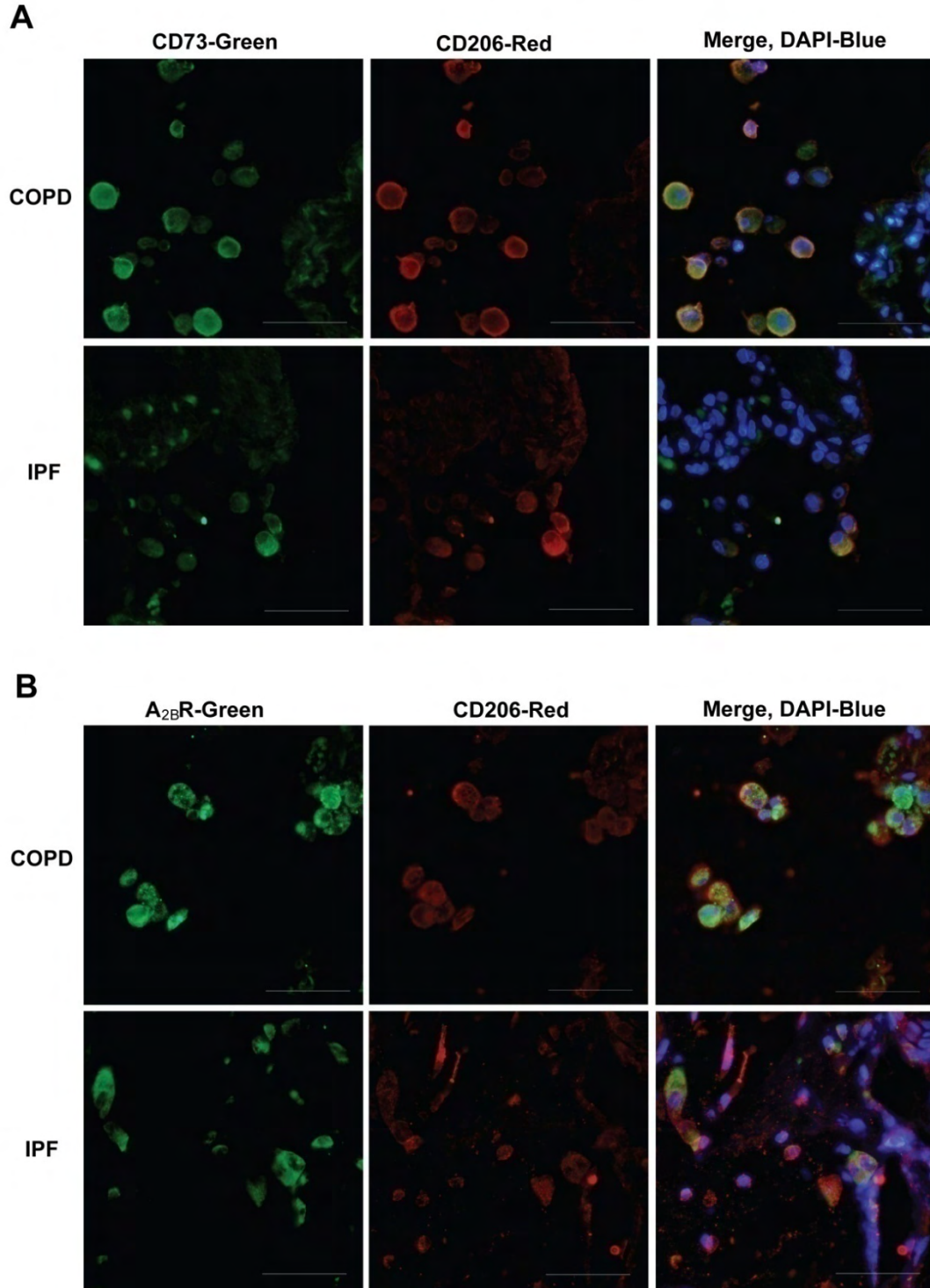
Localization of CD73. Lung sections were stained with antibodies against CD73. (A) Lung section from a Stage 0 COPD patient. (B) Lung section from a Mild IPF patient. (C) Lung section from a Stage 4 COPD patient. (D) Lung section from a Severe IPF patient. Sections are representative of 10-14 different patients from each group. Scale bars=100  $\mu$ m. (E) CD73 positive inflammatory cells were quantified in 20 images. Data are presented as mean number of positive cells per 10X field  $\pm$  SEM. \* $p \leq 0.05$  versus Stage 0 COPD. # $p \leq 0.05$  versus Mild IPF.  $n = 4$  (Stage 0 COPD and Mild IPF),  $n = 8$  (Stage 4 COPD and Severe IPF).



**Figure 5.6**

Localization of the A<sub>2B</sub>R. Lung sections were stained with antibodies against the A<sub>2B</sub>R. (A) Lung section from a Stage 0 COPD patient. (B) Lung section from a Mild IPF patient. (C) Lung section from a Stage 4 COPD patient. (D) Lung section from a Severe IPF patient. Sections are representative of 10-14 different patients from each group. Scale bars=100 µm. (E) A<sub>2B</sub>R positive inflammatory cells were quantified in 20 images. (F) Lung section from an IPF patient. Data are presented as mean number of positive cells per 10X field ± SEM. \*p ≤ 0.05 versus Stage 0 COPD. #p ≤ 0.05 versus Mild IPF. n = 4 (Stage 0 COPD and Mild IPF), n = 8 (Stage 4 COPD and Severe IPF).





**Figure 5.7**

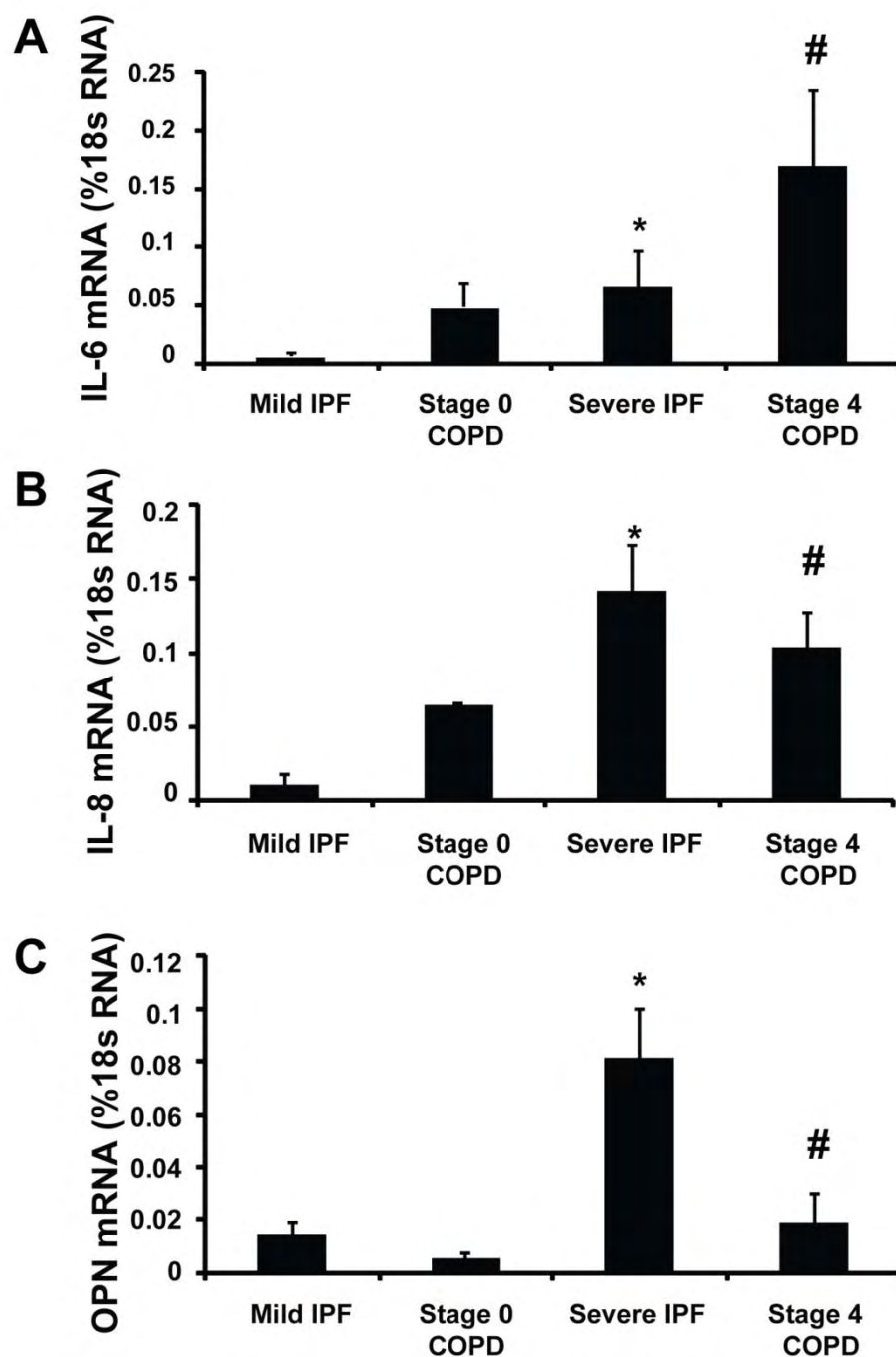
Expression of CD73 and the A<sub>2B</sub>R in M2 macrophages. Lung sections from COPD or IPF patients were reacted with antibodies against CD73 (**A**, green) or the A<sub>2B</sub>R (**B**, green) together with the M2 macrophage marker CD206 (red). In the merged images, yellow represents co-localization of CD73 or the A<sub>2B</sub>R and the M2 marker, blue is dapi stained nuclei. Sections are representative of 10-14 different patients from each group. Scale bars=100  $\mu$ m.



Alternatively activated macrophages, known as M2 macrophages, are involved in microenvironments with prolonged inflammation and fibrosis (112, 113). To determine if M2 macrophages express CD73 and the A<sub>2B</sub>R, co-localization studies using an M2 macrophage marker, CD206 and CD73 or A<sub>2B</sub>R antibodies were conducted on sections from these patients. Results demonstrated that M2 macrophages express high levels of CD73 (Figure 5.7A) and A<sub>2B</sub>R (Figure 5.7B) in Stage 4 COPD and Severe IPF patients.

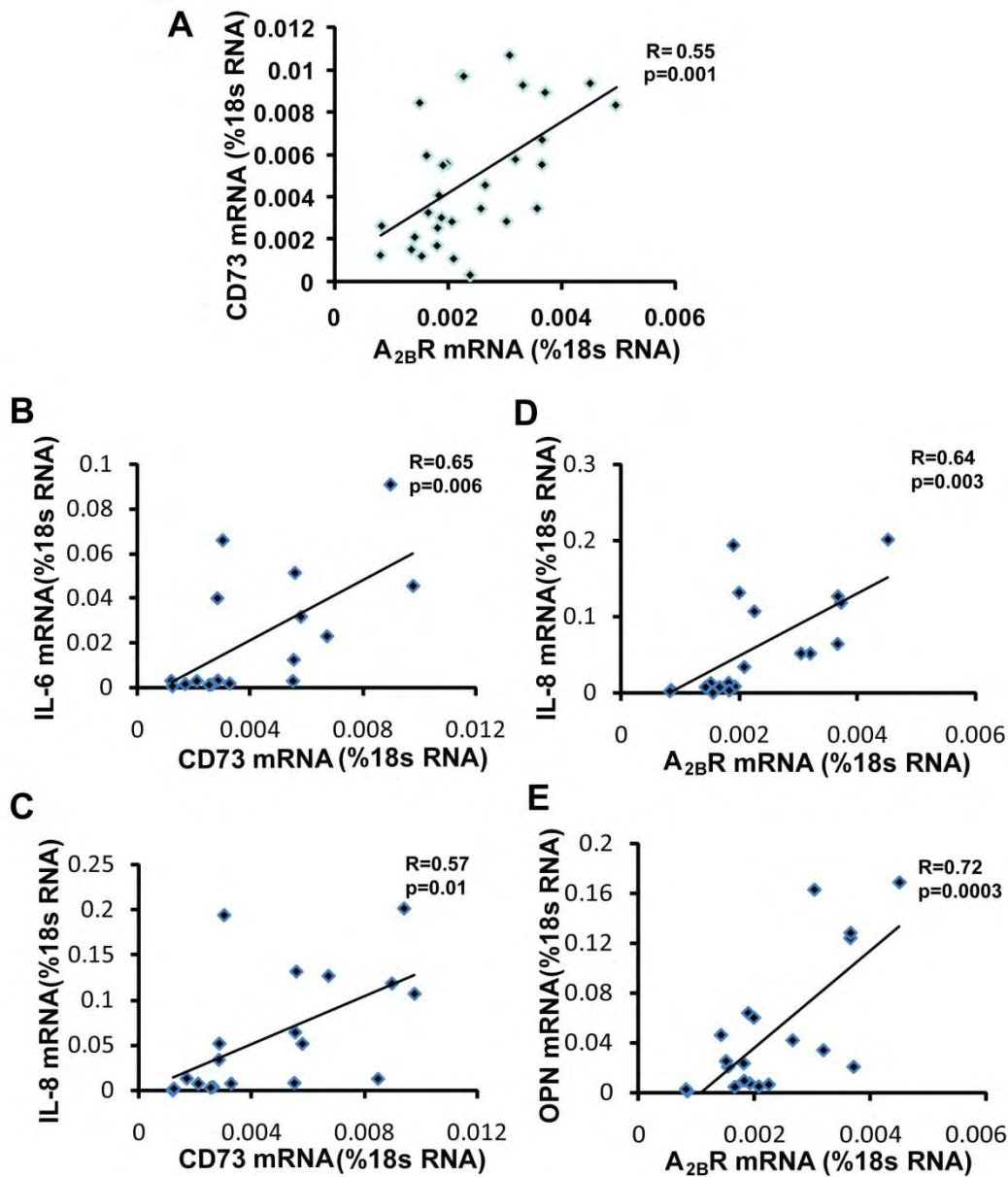
### **Transcript levels of pro-inflammatory mediators downstream of adenosine signaling are altered in the lungs of COPD and IPF patients**

Adenosine A<sub>2B</sub>R signaling has been shown to regulate the expression of certain inflammatory and fibrotic mediators in cell types and lung tissue associated with chronic lung disease (53, 56-58, 70, 75). Total RNA was isolated from lung biopsy specimens and real time RT-PCR was performed to quantify key mediators that have been shown to be regulated by A<sub>2B</sub>R signaling. Results demonstrate that transcript levels of IL-6, IL-8 and osteopontin are all increased in both Stage 4 COPD and Severe IPF patients compared to subjects with normal lung function (Figure 5.8, A-C). These findings suggest that the expression of these pro-inflammatory and pro-fibrotic mediators that might be regulated by A<sub>2B</sub>R signaling is elevated in these patients.



**Figure 5.8**

Expression of pro-inflammatory mediators. Transcript levels of various cytokines and chemokines were quantified in lung RNA extracts from patients using quantitative RT-PCR. Shown are levels of (A) IL-6, (B) IL-8, (C) OPN. Results are presented as mean percentage of 18sRNA transcripts  $\pm$  SEM. \* $p \leq 0.05$  versus Mild IPF. #  $p \leq 0.05$  versus Stage 0 COPD.  $n = 4$  (Stage 0 COPD),  $n = 10$  (Mild IPF),  $n = 8$  (Severe IPF and Stage 4 COPD).



**Figure 5.9**

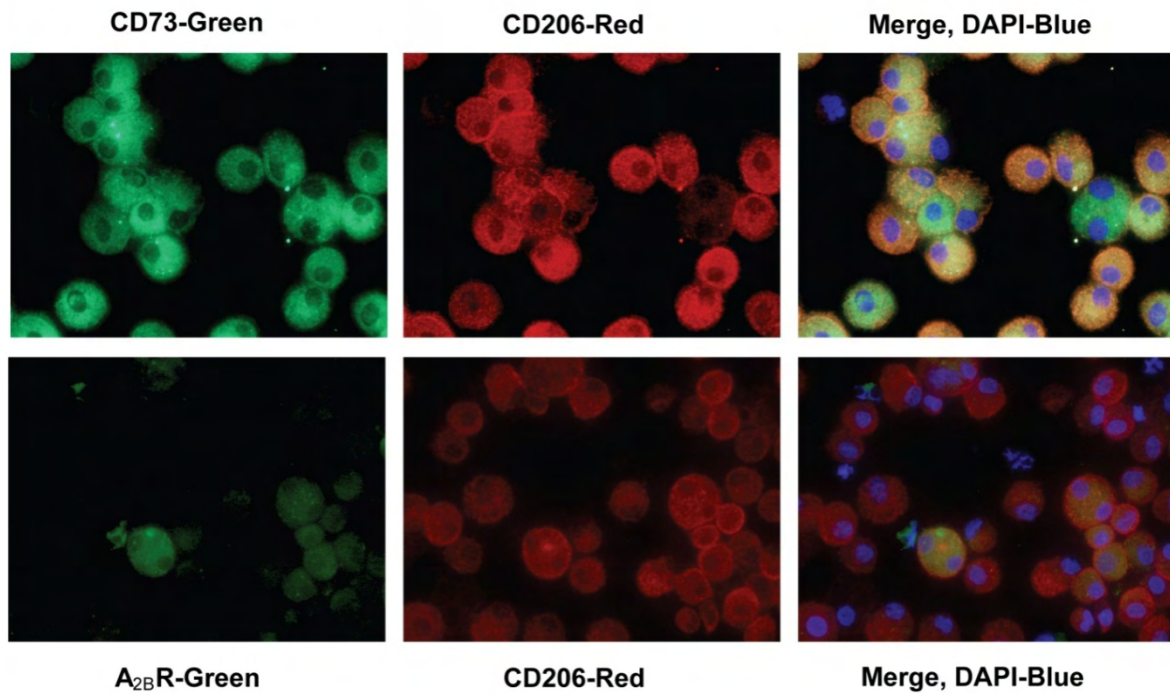
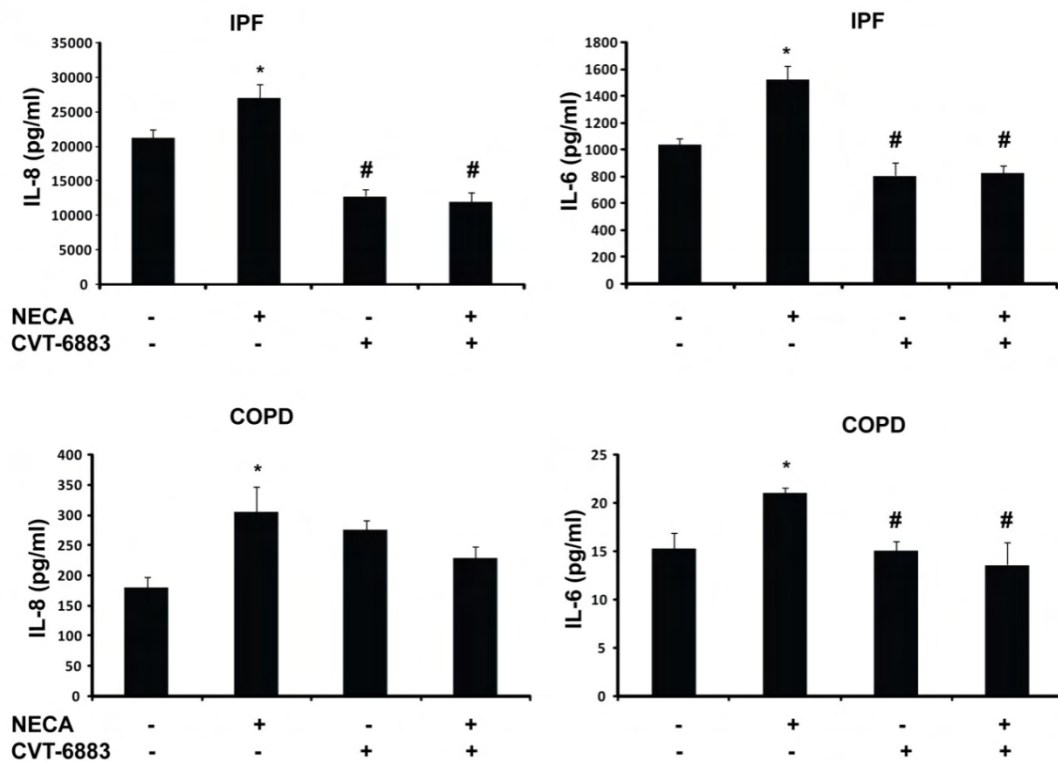
Associations between the expression of CD73, A<sub>2B</sub>R and inflammatory mediators. The transcript levels of CD73 in individual COPD and IPF patients were significantly associated with transcript levels of the A<sub>2B</sub>R (**A**). In IPF patients, the transcript levels of IL-6 (**B**) and IL-8 (**C**) were significantly correlated with the transcript levels of CD73; the transcript levels of IL-8 (**D**) and OPN (**E**) were significantly correlated with the transcript levels of A<sub>2B</sub>R.

### **Correlations between the transcript levels of A<sub>2B</sub>R, CD73, and inflammatory/fibrotic mediators**

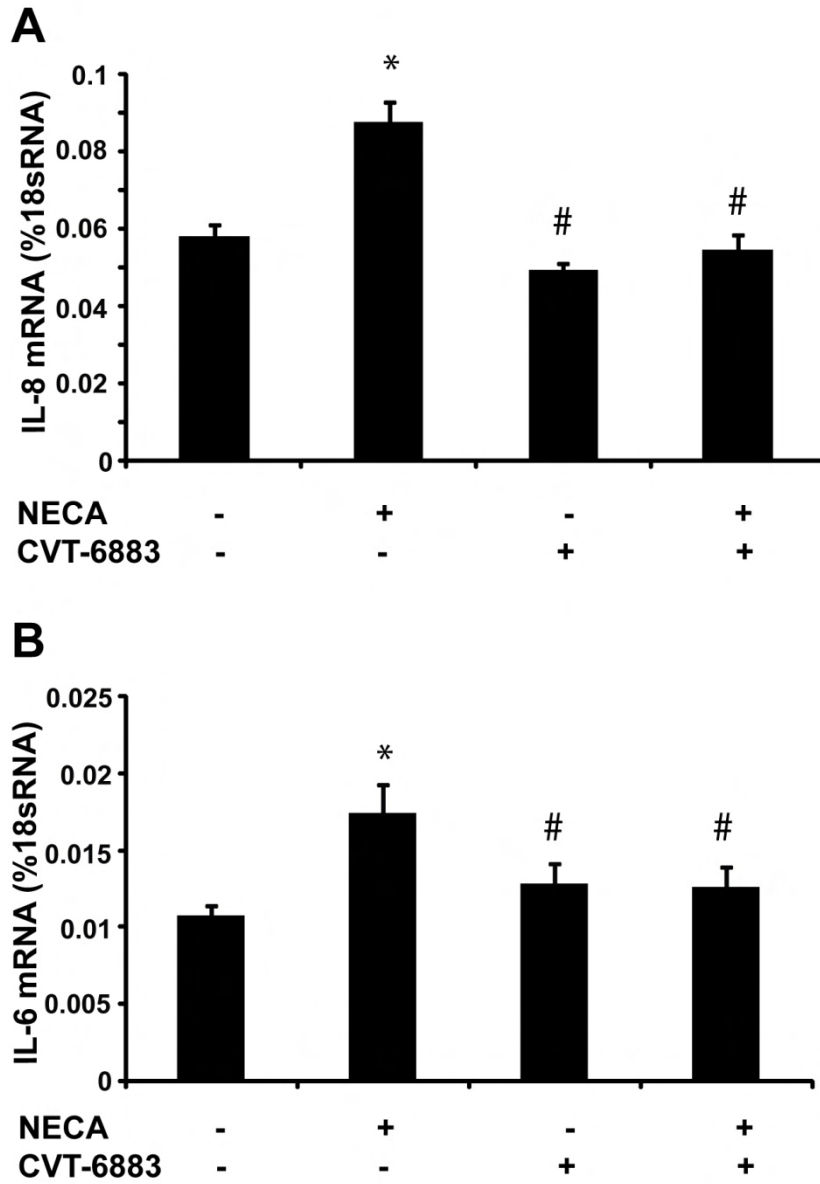
To investigate the associations between CD73, the A<sub>2B</sub>R and mediators, correlations were determined using linear regression. Among all the subjects analyzed, the transcript levels of CD73 significantly correlated with the transcript levels of A<sub>2B</sub>R (Figure 5.9A). Although significant correlations were not found in COPD patients, in IPF patients, the transcript levels of IL-6 and IL-8 significantly correlated with the transcript levels of CD73 (Figure 5.9, B and C); and the transcript levels of IL-8 and OPN significantly correlate with the transcript level of A<sub>2B</sub>R (Figure 5.9, D and E).

### **A<sub>2B</sub>R Signaling in M2 macrophages contributes to the production of IL-8 and IL-6**

To determine if the A<sub>2B</sub>R on M2 macrophages is directly involved in the induction of pro-inflammatory/fibrotic mediators, human primary alveolar macrophages were isolated from the BAL fluid of Severe IPF and Stage 4 COPD patients. Co-localization studies using an M2 macrophage marker, CD206 and either CD73 or A<sub>2B</sub>R antibodies were conducted on cells of an IPF patient. Results demonstrated that the macrophages were M2 macrophages, which expressed both CD73 and the A<sub>2B</sub>R (Figure 5.10A). Primary alveolar macrophages were then pretreated with an A<sub>2B</sub>R antagonist (CVT-6883) before NECA exposure. This resulted in a loss of NECA-stimulated increases in IL-8 and IL-6 protein levels in the media (Figure 5.10B). Interestingly, A<sub>2B</sub>R inhibition

**A****B****Figure 5.10**

A<sub>2B</sub>R-dependent IL-8 and IL-6 expression in human primary alveolar macrophages. **(A)** Cells from an IPF patient were reacted with antibodies against CD73 (A, upper panel, green) or the A<sub>2B</sub>R (A, lower panel, green) together with the M2 macrophage marker CD206 (red). In the merged images, yellow represents co-localization of CD73 or the A<sub>2B</sub>R and the M2 marker, blue is dapi stained nuclei. **(B)** ELISA measurements of IL-8 and IL-6 production from macrophage cultures of IPF and COPD patients. Results are presented as mean concentrations of cytokines ± SEM. \*p ≤ 0.05 versus cells without any treatment. #p ≤ 0.05 versus cells treated with NECA alone. n = 6.



**Figure 5.11**

$A_{2B}R$ -dependent IL-8 and IL-6 mRNA expression in human primary alveolar macrophages from IPF patients. Real-time PCR measurements of IL-8 (**A**) and IL-6 (**B**) mRNA expression from macrophage cultures of IPF patients. Results are presented as mean percentage of 18sRNA transcripts  $\pm$  SEM.. \* $p \leq 0.05$  versus cells without any treatment. # $p \leq 0.05$  versus cells treated with NECA alone.  $n = 6$ .

can decrease the base line production of IL-8 and IL-6 in cell cultures from the IPF patient, suggesting these cells are already activated and adenosine produced from these cells can activate the A<sub>2B</sub>R and contribute to IL-8 and IL-6 production (Figure 5.10B). Consistent to the levels of protein release into the macrophage culture media from IPF patients, IL-8 and IL-6 mRNA levels are altered in a similar pattern in response to NECA and/or CVT-6883 (Figure 5.11), suggesting that A<sub>2B</sub>R signaling directly regulates gene transcription rather than protein release. These findings demonstrate that IL-8 and IL-6 expression is regulated by engagement of the A<sub>2B</sub>R on alveolar macrophages isolated from the lungs of IPF and COPD patients.

## **DISCUSSION**

Adenosine is a nucleoside and signaling molecule that is generated in response to cell stress or damage. Substantial evidence indicates that there are elevated adenosine levels in patients with chronic lung disease. Elevation of adenosine levels were seen in BAL fluid collected from asthmatics (25), in the exhaled breath condensate of patients with allergic asthma (114), in plasma in asthmatic subjects following bronchial provocation with allergen (115), and patients with exercise-induced asthma (116). In addition, elevated adenosine levels have been found in sputum samples from patients with cystic fibrosis (117). Consistent with that, elevated adenosine levels are found in various mouse models exhibiting features of chronic lung disease (66, 109, 110), suggesting adenosine is sufficient to access pathways that lead to the

development of the features of chronic lung disease. In addition, the levels of key components of adenosine metabolism and signaling are altered in these mouse models to promote adenosine accumulation and tissue-destructive adenosine receptor signaling, a process known as purinergic remodeling. Our hypothesis was that purinergic remodeling also exists in patients with COPD and/or IPF. This would provide direct evidence that chronic lung diseases such as COPD and IPF may benefit from treatment with adenosine-based therapeutics.

CD73 is the major extracellular enzyme for adenosine production. CD73 levels were found to be up-regulated in the lungs of ADA-deficient mice and mice exposed to bleomycin (47, 110). In addition, bronchial cultures from patients with cystic fibrosis exhibited 3-fold higher CD73 activity than normal cultures (118). These findings suggest that the up-regulation of CD73 is an important purinergic remodeling response in lung where adenosine-dependent injury is present. In the current study, we observed that the transcript levels of CD73 are elevated in both Severe IPF and Stage 0 COPD patients compared with subjects with normal lung functions. Consistent with this, the enzymatic activity of CD73 is significantly increased in Severe IPF and Stage 4 COPD patients, suggesting there is an increased capacity for adenosine production in these patients compared to normal subjects. To localize CD73 in the lung, immunostaining showed CD73 is expressed on endothelial cells, inflammatory cells in normal lungs and endothelial cells, inflammatory cells and remodeled airway epithelial cells in diseased lungs. This demonstrates that at the sites of inflammation and tissue remodeling, there is excessive amounts of adenosine generated.



ADA is the major adenosine metabolic enzyme that deaminates adenosine to inosine both intracellularly and extracellularly. Mice over expressing the TH2 cytokine IL-4 or IL-13 in their lungs showed down-regulated ADA transcripts and enzymatic activity along with elevated lung adenosine levels and severe pulmonary inflammation, fibrosis and alveolar destruction (66, 109). Similar findings were observed in the lungs of mice treated with bleomycin (110). Our findings suggest that although the transcript levels of ADA are not changed in IPF and COPD patients, the enzymatic activity of ADA is significantly reduced in Stage 4 COPD and Severe IPF patients compared to subjects with normal lung function. Post-transcriptional regulations of ADA mRNA could contribute the discrepancy between the transcript and protein levels of ADA. Nonetheless, there seems to be less adenosine consumption, which could also contribute to higher levels of adenosine compared to normal subjects.

Adenosine regulates numerous cellular activities by engaging cell surface adenosine receptors. Work from our lab and others have provided evidence that adenosine receptor expression is altered in models of lung disease. The A<sub>1</sub>R, A<sub>2B</sub>R, A<sub>3</sub>R have been shown to be elevated in the lungs of models exhibiting pulmonary fibrosis and alveolar destruction (48, 66, 109, 119). The A<sub>2A</sub>R, which is down-regulated in these models, is thought to be largely an anti-inflammatory receptor. The differential expression of these receptors in these models of lung disease provides insight into the potential role of adenosine signaling in COPD and pulmonary fibrosis. In the current study, although all four adenosine receptors are detectable, the transcript level of the A<sub>2B</sub>R is the only adenosine

receptor that is elevated in both Stage 4 COPD and Severe IPF patients compared to subjects with normal function. This receptor is mostly localized on inflammatory cells as well as lung stromal cells such as airway epithelial cells and fibroblasts.

The A<sub>2B</sub>R has the lowest affinity for adenosine and is therefore likely activated under pathological conditions where adenosine levels are increased. A<sub>2B</sub>R activation has both anti-inflammatory and pro-inflammatory actions and both pro- and anti-fibrotic roles (54). Despite the receptor's homeostatic and protective functions during the acute phase of lung injury (14, 58), several studies in mouse models have shown that signaling through the A<sub>2B</sub>R can contribute to the pathology and progressive nature of chronic lung diseases such as IPF and COPD (30, 57, 70). Substantial evidence has indicated A<sub>2B</sub>R's pro-fibrotic functions. Studies have shown that engagement of the A<sub>2B</sub>R promotes their differentiation of pulmonary fibroblasts into myofibroblasts and hence increases the deposition of collagen and fibrosis (30). Engagement of the A<sub>2B</sub>R can mediate the direct expression of pro-inflammatory cytokines such as IL-4, IL-8 and IL-13 from human HMC-1 cells (53, 55), IL-13 from mouse mast cells (56), IL-6 and osteopontin from mouse macrophages (48, 57, 75). Consistent with these findings, the transcript of levels of IL-6, IL-8 and osteopontin downstream of A<sub>2B</sub>R signaling are elevated in both Severe IPF and Stage 4 COPD patients compared to subjects with normal lung function. In IPF patients, the elevation of these mediators is in close association with increases in A<sub>2B</sub>R and CD73. In addition, we showed that treatment with a selective A<sub>2B</sub>R antagonist can prevent

the release of IL-6 and IL-8 from macrophages isolated from Severe IPF or Stage 4 COPD patients. These findings suggest that indeed there is enhanced A<sub>2B</sub>R signaling in these patients and the A<sub>2B</sub>R antagonist can be useful in treating patients with IPF and COPD.

Substantial evidence in mouse models of chronic lung disease suggests that adenosine-based therapeutics may be beneficial for the treatment of chronic lung disease where alveolar destruction and fibrosis are prominent. ADA replacement therapy consists of intra-peritoneal or intranasal treatment with ADA conjugated to polyethylene glycol. This therapy is able to prevent elevations in adenosine, or lower the levels of elevated adenosine in various models of lung disease (45, 48, 66, 109). Preventing elevations in adenosine using this therapy in ADA-deficient mice, IL-13 transgenic mice or IL-4 transgenic mice can prevent and reverse TGF- $\beta$ 1 production and fibrosis in the lungs. In addition, lowering adenosine levels in the lungs of mice treated with bleomycin leads to reduced fibrosis in this model (unpublished data). Thus, adenosine elevations play a direct role in accessing pathways that lead to alveolar airway destruction and pulmonary fibrosis. The ADA-deficient model of adenosine-dependent pulmonary disease has provided a useful mechanism for examining the contribution of individual adenosine receptors to the inflammation and damage seen in the lung in response to adenosine elevations. A recent study used a selective A<sub>2B</sub>R antagonist (CVT-6883) to block the engagement of this receptor in ADA-deficient mice. Treating ADA-deficient mice with this antagonist prevented the production of numerous proteases and TGF- $\beta$ 1 from alveolar macrophages, thus preventing

the development of alveolar airspace enlargement and pulmonary fibrosis (57). Treatment with CVT-6883 also prevented pulmonary fibrosis in the bleomycin induced fibrosis model (57). These findings demonstrate that elevations in adenosine in the lung can promote airway enlargement and fibrosis in part by engaging the  $A_{2B}R$ . These findings in mouse models raised the possibility that ADA replacement therapy and  $A_{2B}R$  antagonist treatment may benefit patients with COPD and pulmonary fibrosis.

$A_{2B}R$  signaling can have distinctive roles in different diseases. It serves a tissue-protective role in acute lung injuries, whereas it plays a detrimental role in chronic lung diseases. It would be helpful to understand adenosine metabolism and signaling in each specific type of disease. Our findings in this chapter suggest that adenosine metabolism and signaling components are altered in a manner that promotes adenosine production in patients with COPD and IPF. These changes include the up-regulation of CD73, a down-regulation of ADA activity, and elevated in the  $A_{2B}R$ . It provides proof of concept information that human COPD and IPF patients may benefit from adenosine-based therapeutics. In addition, these changes could help to predict patient populations, where adenosine levels are elevated along with up-regulations of CD73, down-regulations of ADA and enhanced  $A_{2B}R$  signaling, may be suitable for therapies such as ADA-replacement therapy and  $A_{2B}R$  antagonist treatment.

# **CHAPTER 6**

## **OVERVIEW, FUTURE DIRECTIONS AND CONCLUSION**

### **Overview**

Adenosine is a nucleoside signaling molecule produced in response to cell stress or damage (120). By signaling through one of the four G-protein coupled adenosine receptors, adenosine can influence cellular physiology and intracellular signaling pathways through a variety of mechanisms (52, 121, 122). One of the most prominent areas of study is the effects of adenosine on immune regulation. During acute tissue injury, elevated adenosine levels generally play anti-inflammatory and tissue-protective functions. In contrast, sustained adenosine elevations in persistent inflammatory conditions, including chronic lung diseases such as IPF and COPD, appear to access tissue-destructive and pro-inflammatory pathways. The differential activities of adenosine receptors have been shown to contribute to the dichotomous role of adenosine. Among the four adenosine receptors, the A<sub>2B</sub>R draws a lot of attention because it has the lowest affinity for adenosine. Thus, it can be activated under pathological conditions when adenosine levels are elevated and may play important roles in disease. Work conducted in this dissertation was designed to better understand the role of the A<sub>2B</sub>R in various acute and chronic aspects of pulmonary disorders.

The ADA-deficient mouse model demonstrates that elevated levels of adenosine in the mouse is sufficient to induce lung disease with many of the pathological features seen in human chronic lung diseases, including pulmonary inflammation, mucus hypersecretion, airspace enlargement, fibrosis and airway remodeling. A previous study in our lab utilized this animal model and showed that treatment with a selective A<sub>2B</sub>R antagonist results in decreased pulmonary inflammation, alveolar destruction and pulmonary fibrosis, suggesting the A<sub>2B</sub>R is playing a tissue-destructive and pro-fibrotic role. In Chapter 3 of this dissertation, I took the genetic approach and deleted the A<sub>2B</sub>R gene from ADA-deficient mice to further investigate the role of the A<sub>2B</sub>R. Surprisingly, ADA/A<sub>2B</sub>R double knockout mice died precociously and exhibited enhanced pulmonary inflammation, airspace enlargement, mucus metaplasia and pulmonary fibrosis. A major finding in this study was the enhanced vascular permeability in ADA/A<sub>2B</sub>R double knockout mice compared to ADA-deficient mice. We concluded that enhanced loss of vascular barrier function in mice lacking the A<sub>2B</sub>R is associated with enhanced pulmonary neutrophilia and enhanced pulmonary damage. Although the A<sub>2B</sub>R has been proven to have tissue-destructive functions, it thus appeared to also play an important protective role at early stage of the disease in the ADA-deficient model.

In Chapter 4 of this dissertation, I sought to further address the concept of A<sub>2B</sub>R's bi-phasic functions. Two bleomycin-induced pulmonary fibrosis models were utilized to investigate the A<sub>2B</sub>R's distinctive functions at different stages of the disease. The IT bleomycin model involves a single bleomycin exposure

directly into the trachea and this local delivery is associated with acute damage to the lung. When  $A_{2B}R^{-/-}$  mice were exposed to bleomycin IT, enhanced loss of endothelial barrier function during the early stages of the pulmonary injury resulted in enhanced neutrophil infiltration into the lung and enhanced pulmonary damage. This acute inflammation counteracted the pro-fibrotic effect of the  $A_{2B}R$  so  $A_{2B}R^{-/-}$  mice were not protected from bleomycin-induced pulmonary fibrosis. In contrast, the IP bleomycin model does not involve extensive acute lung damage or inflammation, and in this case,  $A_{2B}R^{-/-}$  mice were protected from bleomycin-induced pulmonary fibrosis. These results demonstrate that the  $A_{2B}R$  plays a protective role in attenuating vascular leakage at the acute phase of the disease, whereas it exhibits pro-inflammatory and pro-fibrotic roles in chronic lung diseases. These findings highlight the complexity of  $A_{2B}R$  signaling and suggest that it would be critical to investigate the status of  $A_{2B}R$  in specific diseases.

In different mouse models of pulmonary fibrosis, molecules involved in adenosine metabolism and signaling are altered to promote adenosine production (22, 66, 109-111, 123, 124). In Chapter 5 of this dissertation, human samples from IPF and COPD patients were utilized to examine the status of adenosine metabolism and signaling to determine if these changes exist in human chronic lung diseases. Findings suggested that components of adenosine metabolism and signaling are altered in a manner that promotes adenosine production in patients with COPD and IPF. These changes included the up-regulation of CD73, a down-regulation of ADA activity, and elevated  $A_{2B}R$  expression. In addition, mediators that are regulated by the  $A_{2B}R$ , such as IL-6,

IL-8 and osteopontin were elevated in these samples and activation of the A<sub>2B</sub>R on macrophages isolated from the airways of COPD and IPF patients was shown to directly induce the production of these mediators. This study provides proof of concept information that A<sub>2B</sub>R signaling can promote the production of inflammatory and fibrotic mediators in patients with these disorders and these disorders may benefit from adenosine-based therapeutics such as exogenous ADA enzyme therapy or A<sub>2B</sub>R antagonist treatment.

Various studies in cellular and animal models provide substantial evidence that adenosine signaling through adenosine receptors can serve both protective and detrimental roles (90) (Figure 6.1). Adenosine and A<sub>2B</sub>R's tissue-protective role partially comes from its role in the maintenance of pulmonary barrier function (3, 14, 15). In lung injuries where the damage to the lung is accompanied by disruption of endothelial barriers, the A<sub>2B</sub>R plays an important role in maintaining vascular endothelial cell junctions and creating a potential endothelial barrier to prevent neutrophil infiltration. Studies described in this dissertation revealed a critical protective role of the A<sub>2B</sub>R in attenuating vascular leakage through endothelial paracellular barriers. In addition, A<sub>2B</sub>R signaling may also promote the normal function of CFTR, contribute to the maintenance of airway surface liquid height in airway epithelial cells, and prevent mucus stasis in the airways.

The local cytokine milieu in different diseases may play an important role in regulating the anti- or pro-inflammatory actions of adenosine. Acute lung injuries are associated with elevations in IL-12, IFN $\gamma$  and TNF $\alpha$  and a TH1 dominated environment. Adenosine signaling usually serves to down-regulate the

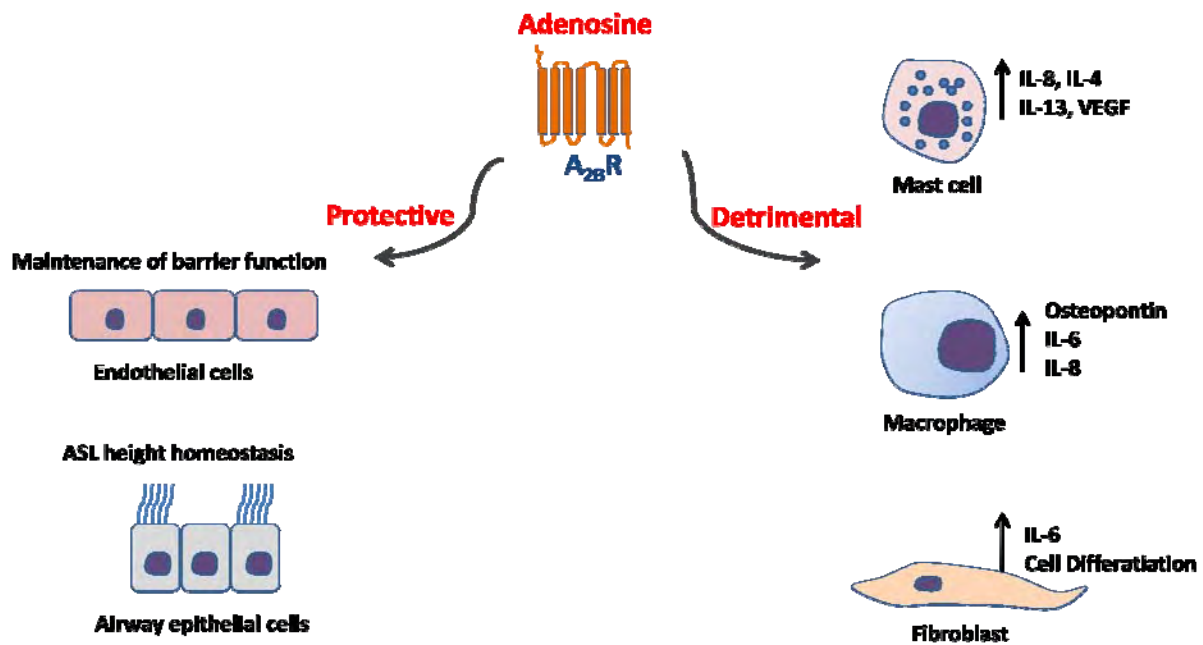


production of TH1 cytokines (125). In contrast, a lot of chronic lung diseases such as asthma, COPD, and interstitial lung diseases are associated with elevations in TH2 cytokines such as IL-4, IL-5, IL-8 and IL-13 (2). Adenosine acting through the low affinity  $A_{2B}R$  can increase the production of TH2 cytokines from various cell types (53, 55, 56, 99) (Figure 6.1). In mast cells,  $A_{2B}R$  activation can stimulate the production of the TH2 cytokines that regulate both airway constriction and inflammation. In addition, the  $A_{2B}R$  can promote the production of mediators from macrophages. Previous studies have shown that  $A_{2B}R$  activation stimulates the production of IL-6 and osteopontin from mouse macrophages (126). My result was the first evidence to show it can also promote the production of IL-6 and IL-8 from human alveolar macrophages. Furthermore, activation of the  $A_{2B}R$  on fibroblasts promotes the release of IL-6 and stimulates the differentiation into myofibroblasts (30), which play a key role in the production of extracellular matrix and fibrotic remodeling. These findings suggest that adenosine signaling is detrimental and can contribute to the amplification or progression of chronic lung disease

Taken together, patients with either acute lung injury or chronic lung disease may both benefit from adenosine-based therapeutics. Compounds selectively targeting adenosine receptors are under development by pharmaceutical companies. For example, The selective  $A_{2B}R$  antagonist, CVT-6883 has completed phase I clinical trials with no adverse events reported (127); however, efficacy in phase II trials has not yet been shown. Due to the complication of adenosine receptor signaling in different aspects and at the

different stages of the disease, the development of adenosine-based therapeutics can be challenging. It is critical to determine the right adenosine receptor as the drug target, whether to activate or inhibit it in any particular type of disease. Moreover, the dosage and timing of the drug exposure can also be a determinant factor in order to prevent any unwanted side effects. In acute lung injuries where a TH1 environment is commonly involved, the A<sub>2B</sub>R plays a critical role in maintaining vascular barrier function; when these patients fail the wound healing response and develop chronic lung diseases, A<sub>2B</sub>R signaling promotes the release of pro-inflammatory cytokines and contributes to disease progression (Figure 6.2).

My findings in this dissertation suggest that A<sub>2B</sub>R agonist could be useful in treating patients with acute lung injuries; whereas A<sub>2B</sub>R antagonist can be used to treat chronic lung diseases. Timing of these treatments can be critical. A<sub>2B</sub>R agonist to treat acute lung injuries may only be beneficial at the acute phase of the disease; A<sub>2B</sub>R antagonist to treat chronic lung disease may only be effective at the late stage of the disease, whereas treatment early in the disease may cause vascular leakage and enhanced injury. In addition, my findings provide a novel method to identify the subset of patients with chronic lung disease that may benefit from adenosine-based therapeutics. It would be helpful to conduct bronchoscopy on patients with IPF or COPD, isolate alveolar macrophages and determine if the production of pro-inflammatory cytokines from these macrophages respond to A<sub>2B</sub>R agonist or antagonist. If macrophages



**Figure 6.1**

A<sub>2B</sub>R activities in pulmonary cells. Adenosine signaling through the A<sub>2B</sub>R can promote endothelial barrier function and serve important anti-inflammatory and tissue-protective roles. A<sub>2B</sub>R signaling also contributes to the maintenance of ASL height in airway epithelial cells. In contrast, the A<sub>2B</sub>R contributes to the production of IL-8, IL-4, IL-13, and VEGF from mast cells. A<sub>2B</sub>R engagement can also promote the production of osteopontin, IL-6, and IL-8 from macrophages; IL-6 release from fibroblasts and myofibroblasts differentiation.

Acute lung injury <b>A<sub>2B</sub>R: protective</b>	Chronic lung disease <b>A<sub>2B</sub>R: detrimental</b>
Th1 cytokine ↓ IL-12 IFN $\gamma$ TNF $\alpha$	Th2 cytokine ↑ IL-8 IL-6 IL-4 IL-13
↑ Barrier function	↑ Effector cell activity

**Figure 6.2**

Model of A<sub>2B</sub>R signaling in acute and chronic lung disease. In acute injury, adenosine and the A<sub>2B</sub>R can serve important anti-inflammatory and protective roles, such as inhibition of Th1 cytokine production and maintenance of vascular barrier function. In chronic lung disease, A<sub>2B</sub>R signaling contributes to the production of Th2 cytokines and promotes the activity of effector cells such as mast cells, macrophages and fibroblasts that can drive disease progression.

isolated from the patients express high levels of A<sub>2B</sub>R and the production of IL-8 and IL-6 from these cells can be promoted by A<sub>2B</sub>R agonist treatment and inhibited by A<sub>2B</sub>R antagonist treatment, it is very likely that these patients would benefit from exogenous ADA enzyme therapy or A<sub>2B</sub>R antagonists. Additional studies are needed to further investigate the specific mechanisms involved in adenosine signaling in various pulmonary disorders.

## **Future Directions**

### **Treatment of ADA-Deficient Mice with A<sub>2B</sub>R Agonists**

We have provided substantial evidence that the A<sub>2B</sub>R plays a protective role during the early stage of pulmonary injury when acute inflammation is involved, and a tissue-destructive/pro-fibrotic role during the chronic stage of the disease. Another direct way to verify this concept is to treat ADA-deficient mice with an A<sub>2B</sub>R agonist at different stages of the disease. A selective A<sub>2B</sub>R agonist BAY60-6583 is available from Bayer HealthCare. Treating ADA-deficient mice from birth with this compound may postpone the development of pulmonary phenotypes. However, if ADA-deficient mice are kept on PEG-ADA from birth till Day 21, and then treated with the A<sub>2B</sub>R agonist a week after the last PEG-ADA treatment, we may observe precocious injury and death of these animals compared to animals without the agonist treatment. Similarly, this method could be utilized in other models of chronic lung disease such as the bleomycin-induced pulmonary fibrosis model. These studies would raise the possibility that A<sub>2B</sub>R agonist could be used to treat diseases during acute phases.

### **Timing and Dosage of A<sub>2B</sub>R Antagonist Treatment**

Previous studies have shown that the treatment with the selective A<sub>2B</sub>R antagonist, CVT-6883, resulted in diminished pulmonary inflammation and damage in ADA-deficient mice and decreased pulmonary fibrosis in the model of IT bleomycin-induced pulmonary fibrosis. These results suggest that the A<sub>2B</sub>R plays a pro-inflammatory and pro-fibrotic role in these models. Notably in previous studies, the treatment of the A<sub>2B</sub>R antagonist did not start and the receptors were not blocked until the disease status had been established. In addition, other studies, and the findings in this dissertation, support a tissue-protective role of the A<sub>2B</sub>R during the acute stage of the disease. It would be helpful to treat these animal models with the A<sub>2B</sub>R antagonist from birth and determine if it could mimic the conditions of A<sub>2B</sub>R gene deletion. It is possible that although treatment of the antagonist later would improve pulmonary phenotypes, treatment from birth may accelerate the development of the pulmonary damage in these animals.

A discrepancy between pharmacological inhibition and genetic knockout is that the receptor's activity may not be totally inhibited with antagonist treatment, while genetic knockout absolutely abolishes the receptor's activity. Thus, genetic deletion may result in loss of homeostatic roles of the receptor that would not be revealed by pharmacological inhibition. It will be important to determine the dosage of the antagonist that is sufficient to block the tissue-destructive and pro-fibrotic role of this receptor, but conserves the homeostatic and tissue-protective

role. This could be done by treating animal models with different doses of the antagonist and monitoring the down-stream signaling pathways of the A<sub>2B</sub>R, such as cAMP levels, change of vascular permeability and gene transcription profiles.

### **A<sub>2B</sub>R Signaling and Mucociliary Clearance**

Previous studies have shown that the A<sub>2B</sub>R is an important regulator of cystic fibrosis transmembrane conductance regulator (CFTR) functions, and lacking this receptor may lead to dehydration of airway surface liquid volume and mucus stasis (74). Thus, A<sub>2B</sub>Rs are required for airway surface liquid (ASL) volume homeostasis and mucus clearance in the airways. This is consistent with our findings that there is enhanced mucus metaplasia in ADA/A<sub>2B</sub>R double knockout mice compared to ADA-deficient mice even though mucin gene transcription is decreased. Studies conducted with epithelial cells ex vivo would provide us a simplified system to investigate the A<sub>2B</sub>R signaling pathway in airway epithelial cells. A standard protocol could be used to isolate mouse primary tracheal epithelial cells from wild type and A<sub>2B</sub>R<sup>-/-</sup> mice and it would be helpful to compare airway surface liquid height, mucin gene expression, mucus production and secretion.

Mouse tracheal epithelial cells from wild type and A<sub>2B</sub>R<sup>-/-</sup> mice could be isolated and grown on transwells coated with rat tail collagen. Cells could then be maintained for at least 14 days to allow the differentiation and the formation of air-liquid interface.  $\beta$ -tubulin staining will be performed on cells from wild type and A<sub>2B</sub>R<sup>-/-</sup> mice to compare the number of ciliated cells that contribute to mucus

clearance. Airway surface liquid height will be determined by confocal microscopy. 10 ng/ml IL-13 could be used to treat tracheal epithelial cells to induce mucus metaplasia. Comparisons of mucus production and secretion between cells from wild type and  $A_{2B}R^{-/-}$  mice could be done by histological analysis (mucus staining), real-time RT-PCR analysis of mucin gene transcription, and mucus contents in apical and basolateral media after the treatment. NECA, an  $A_{2B}R$  agonist could be used to treat epithelial cells after IL-13 stimulation to determine the functions of  $A_{2B}R$  signaling and mucus secretion.

I expect that tracheal epithelial cells isolated from  $A_{2B}R^{-/-}$  mice will have fewer ciliated cells and collapsed airway surface liquid height. Both of these will contribute to diminished mucus clearance and enhanced mucus deposition on airway epithelium. IL-13 treatment will induce mucus metaplasia in wild type and  $A_{2B}R^{-/-}$  tracheal epithelial cells. Although the same levels of mucus production are found in both genotypes, there may be a significant decrease in mucus secretion in the apical media of cells isolated  $A_{2B}R^{-/-}$  mice, suggesting a mucus secretion defect of airway epithelial cells lacking the  $A_{2B}R$ .  $A_{2B}R$  activation by NECA will enhance mucus secretion only in wild type epithelial cells but not in  $A_{2B}R^{-/-}$  cells. These expected results will demonstrate that the  $A_{2B}R$  is important in maintaining airway surface liquid height, differentiation of ciliated epithelial cells, and mucus secretion.



## **Purinergic Remodeling in Normal Subjects in Comparison to Diseased Patients**

A major limitation to the study described in Chapter 5 of this dissertation is the lack of normal subjects in comparison to subjects with IPF and COPD. Although we concluded that adenosine metabolism and signaling in patients of IPF and COPD are altered to promote adenosine production, comparisons were done between Severe IPF versus Mild IPF, and Stage 4 COPD versus Stage 0 COPD. Moreover, our ex vivo experiments showing  $A_{2B}R$  activation can directly regulate IL-6 and IL-8 production were performed with cells isolated from Severe IPF or Stage 4 COPD patients. A more definitive approach would be to characterize purinergic remodeling using frozen tissue and tissue sections, and to isolate alveolar macrophages from normal subjects. I expect that the levels of CD73, ADA and  $A_{2B}R$  would be altered more dramatically in diseased subjects when compared to normal subjects. Levels of other enzymes and molecules in adenosine metabolism and signaling that did not change in the previous study may also be altered. In the ex vivo experiment, macrophages isolated from normal subjects are not activated, thus they do not express high levels of  $A_{2B}R$ . Baseline levels of IL-6 and IL-8 in media of these cells are lower than that of activated macrophages from diseased patients. NECA stimulation may change the characteristics of these cells, including the induction of M2 macrophage activation,  $A_{2B}R$  expression and production of pro-inflammatory/fibrotic mediators.

## **Adenosine Production in M2 Macrophages**

An interesting finding in the ex vivo experiment using primary macrophages is that the macrophages isolated from Severe IPF and Stage 4 COPD patients are already activated and they release high levels of IL-6 and IL-8 at base line. The A<sub>2B</sub>R antagonist treatment can significantly decrease the release of these mediators. Moreover, these macrophages express the M2 macrophage marker CD206 and they also express CD73. These findings suggest that CD73 in these cells is enzymatically active and adenosine is generated at base line. It would be helpful to verify the activity of CD73 by adding the inhibitor of CD73, AoPCP, into the cell culture. I expect that the inhibition of CD73 would decrease the release of IL-6 and IL-8 at base line, suggesting that adenosine is produced from M2 macrophages and it can engage A<sub>2B</sub>R to play a pro-inflammatory role.

## **Conclusion**

Extensive analysis of inflammation, alveolar destruction, and fibrosis in mouse models suggest that adenosine based therapeutics such as ADA enzyme therapy and A<sub>2B</sub>R antagonism would be beneficial in the treatment of human conditions such as COPD and IPF. Understanding the involvement of A<sub>2B</sub>R signaling pathways will provide novel and important information into the potential significance of adenosine signaling in human lung disease, as well as provide insight into potential mechanisms for screening patients for potential adenosine-based therapeutics that are currently being planned. The findings in this

dissertation suggest that the  $A_{2B}R$  is playing a homeostatic and protective role during the acute stage of lung injury; while it plays a pro-fibrotic and tissue-destructive function during the chronic stage of the disease. As a result, patients with acute lung injuries or chronic lung diseases can both benefit from adenosine and  $A_{2B}R$ -based therapeutics. However, the  $A_{2B}R$  may have a bi-phasic effect on inflammation and fibrosis and cautions must be taken when designing  $A_{2B}R$  agonist to treat patients with acute lung injuries, or  $A_{2B}R$  antagonist to treat human chronic lung diseases. For example, the  $A_{2B}R$  antagonist could be beneficial in treating patients with chronic lung diseases only when it is given after the disease status is established. Additional studies in models as well as clinical trials are needed to investigate the roles of adenosine and adenosine receptors in each particular disease, and the efficacy of new generations of adenosine receptor agonists and antagonists in the treatment of acute and chronic lung diseases.

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## **VITA**

Yang Zhou was born in Huaian, China on March 11, 1983, the son of Ping Zhou and Fangli Yang. After graduating from Huaiyin High School in 2001, he entered Nanjing University in China where he graduated with a B.S. in Biochemistry in June, 2005. In August of 2005, he entered the Ph.D. program in the Graduate School of Biomedical Sciences at the University of Texas Health Science Center at Houston.

### **Permanent Address:**

84 North Huaihai Road, Apt.1-1-302

Huaian, Jiangsu 223001

China