A PATHOPHYSIOLOGIC EVALUATION OF THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) IN THE LUNG

by

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Bachelors of Science in Chemistry, University of North Carolina at Chapel Hill, 2005

Submitted to the Graduate Faculty of
The School of Medicine in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

University of Pittsburgh

2009
UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin super-family of cell surface receptors whose activation has been suggested to contribute to various pathologies. RAGE has been primarily studied in diabetes where its upregulation has been linked to disease in the kidney, vasculature, and nervous system. This protein is highly expressed in the lung under normal conditions, but its function is unknown. We therefore investigated the normal function of RAGE in the lung and its pulmonary expression in two disease states.

Idiopathic pulmonary fibrosis (IPF) is a debilitating disease with both high morbidity and mortality. Unfortunately, there are currently no effective therapies for IPF necessitating mechanistic insight into the disease pathogenesis. We found that pulmonary fibrosis led to a depletion of RAGE in both animal models and tissue from patients with idiopathic pulmonary fibrosis. In contrast to other diseases in which RAGE signaling promotes pathology, we found that aged RAGE null mice spontaneously develop pulmonary fibrosis-like alterations and more severe fibrosis in response to asbestos injury. In addition, we found that RAGE null mice were fully protected from the fibrotic effects of bleomycin.

In addition, we investigated the expression of RAGE in the lungs of diabetic rodents. Diabetes has been shown to alter RAGE expression in a number of tissues that do not normally express RAGE. We hypothesized that diabetes would alter pulmonary RAGE expression and
contribute to the susceptibility to pulmonary injury. We found that pulmonary RAGE expression was unaltered in five rodent models suggesting that diabetes does not effect RAGE expression in the lung.

Lastly, we identified that RAGE has a very high affinity for components in the basement membrane of the lung. A few RAGE studies suggested that it might serve a role as an adhesion molecule. We found that RAGE extensively colocalized with the alveolar basement membrane and had very high affinity for collagen I, collagen IV, and laminin, but not fibronectin. These findings along with the fact that RAGE null mice spontaneously develop fibrosis suggest a potential homeostatic function of RAGE in the lung. This is in stark contrast to the vast majority of studies, which suggest that its expression is solely pathologic.
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PREFACE

ACKNOWLEDGMENTS

As my thesis project comes to a close, I have the opportunity to reflect on the countless number of people that have made it all possible. While I would love to claim much of the credit for my own success, the fact is that the deck was stacked in my favor. No matter where I turned I was always surrounded by a group of supportive advisors, colleagues, family and friends.

First and foremost I would like to thank my mentor and thesis advisor, Dr. Tim Oury. Tim provided the most outstanding training environment that I could ever ask for. He provided me a degree of freedom that allowed me to develop as a researcher and learn from my mistakes. Equally important, he was supportive when projects did not work out and my results contradicted the norm. He has spent countless hours not only teaching me how to conduct good science, but also discussing future goals and how best to attain them. It is very comforting to know that I will always have his support and mentorship. He has also provided me a model of mentorship that I can only hope to emulate in the future.

My thesis committee has also played a critical role in my scientific development. The knowledge and suggestions they provided to me at each meeting were invaluable for my project. They kept me pointed in the right direction and ensured that my project stayed on track without too much diversion. I can only hope to find as strong of a group of advisors as I move forward in my career.
Each and every day in the Oury Laboratory has been an adventure. This is in large part due to the fantastic group of co-workers who I have had the opportunity to work with. Thank you so much: Beth Ganis, Fei Gao, Corrine Kliment, Michelle Manni, Lasse Ramsgaard, Jacob Tobolewski, and Lauren Tomai. There is certainly no way that I could have accomplished nearly as much without all of your help and support.

This project has been the culmination of the work of a lot of people throughout the entire scientific community. The collaborative nature of research is one of the many aspects that have drawn me towards this career path. Having the opportunity to work closely with experts from around the globe has been one of the many benefits of this project. I certainly could not have done all of this without the help of everyone. I have annotated their contributions throughout the thesis.

Lastly, I would like to thank all of my friends and family for their support. A research career has afforded me the opportunity to create my own life-work balance. However, there were times when I found myself unable to go on a group trip or family event. Your understanding and support has made these difficult decisions significantly easier. For me, I found that the best way to balance life and work was to combine the two. I would like to extend a special thanks to Dr. Corrine Kliment for her intellectual, emotional and medical support throughout the trials and tribulations of my thesis research.
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<td>AGE</td>
<td>advanced glycation end product</td>
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<tr>
<td>ALCAM</td>
<td>activated leukocyte cell adhesion molecule</td>
</tr>
<tr>
<td>AT-I</td>
<td>alveolar epithelial cell type I</td>
</tr>
<tr>
<td>AT-II</td>
<td>alveolar epithelial cell type II</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>Bleo</td>
<td>bleomycin</td>
</tr>
<tr>
<td>CLP</td>
<td>cecal-ligation and puncture</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EC-SOD</td>
<td>extracellular superoxide dismutase</td>
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<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
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<tr>
<td>FPLC</td>
<td>fast performance liquid chromatography</td>
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<td>ILD</td>
<td>interstitial lung disease</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>IPF</td>
<td>idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>i.t.</td>
<td>intratracheal</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>mRAGE</td>
<td>membrane isoform of the receptor for advanced glycation end product</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>RAGE</td>
<td>receptor for advanced glycation end product</td>
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<td>sRAGE</td>
<td>soluble isoform of the receptor for advanced glycation end product</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor -beta</td>
</tr>
<tr>
<td>TiO₂</td>
<td>titanium dioxide</td>
</tr>
<tr>
<td>UIP</td>
<td>usual interstitial pneumonia</td>
</tr>
<tr>
<td>UUO</td>
<td>unilateral ureter obstruction</td>
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<td>WT</td>
<td>wild type</td>
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1.0 INTRODUCTION

The receptor for advanced glycation end-products (RAGE) has been studied for over 15 years. Despite more than 2000 papers on the subject, very few investigations have offered any explanation for the high endogenous expression of RAGE in the lung. This study focused on trying to better understand the physiologic and pathologic roles of RAGE in pulmonary tissue. Various in vitro and in vivo models were utilized to elucidate its biological function. Understanding RAGE’s physiologic function is essential for the progression of its modulation as a therapeutic option in disease.

1.1 RAGE BIOLOGY

1.1.1 The History of RAGE

The receptor for advanced glycation end-products (RAGE) was first isolated and characterized from bovine lung tissue in 1992.¹ In brief, Schmidt and colleagues hypothesized that a putative cell surface receptor for glucose modified proteins existed on endothelial cells which mediated their negative interaction with the endothelium. Previously, most of the studies investigating AGEs had focused on their potential interaction with monocytes.²⁻⁴ However, Schmidt et al. proposed that AGEs interacted with endothelial cells and that this interaction was a likely
mediator of diabetic vascular disease. Their rationale stemmed from the fact that AGEs were known to accumulate in the basement membrane of blood vessels at the sites of lesions. However, in order to substantiate this they needed to identify an AGE receptor in the endothelium. They successfully isolated two proteins from the lung that had AGE affinity, a novel 35-kDa protein and lactoferrin. They demonstrated that immunoglobulins from animals immunized with the 35-kDa protein were able to block AGEs interaction with the surface of endothelial cells. Prior to this discovery, numerous other AGE receptors had been discovered in the liver\textsuperscript{5}, kidney\textsuperscript{6}, and macrophages\textsuperscript{7-9}. However, a rapid follow-up publication, employing the catchy acronym RAGE for the newly discovered protein\textsuperscript{10}, all but guaranteed its scientific future and predominance as the most researched AGE receptor. The irony is that the authors were themselves skeptical of AGE being the true ligand of RAGE stating in their conclusions that “an important challenge for future studies will be to identify the natural ligand of RAGE,”\textsuperscript{10} all but admitting that they potentially misnamed their discovery. To date it is still unclear what that natural ligand might be, though many have been proposed and tested. Since 1992, more than 2000 scientific articles have been published on RAGE encompassing almost every disease process and tissue. Ironically, only a handful of those 2000+ manuscripts have any mention of the tissue from which it was originally isolated and in which it is most highly expressed: the lung.

1.1.2 RAGE Structure and Signalling

RAGE is a member of the immunoglobulin superfamily of cell surface receptors.\textsuperscript{10} Full-length or membrane RAGE is composed of 394 amino acids: 334 residues in the extracellular portion, a 19 amino acid hydrophobic transmembrane domain, and a cytosolic tail of 43 amino acids.\textsuperscript{10} The
extracellular domain is composed of three immunoglobulin-like domains: two constant or C-domains and one variable or V-domain at the amino-terminus that is responsible for ligand binding.\(^\text{10}\) RAGE is known to undergo post-translational modifications including two sites of N-glycosylation at Asn-2 and Asn-57 in the V-domain, which have been shown to be essential for its ability to bind ligands.\(^\text{11, 12}\) The cytoplasmic portion of the protein has been shown to be essential for intracellular signaling and RAGE lacking this portion of the protein acts in a dominant-negative fashion.\(^\text{13}\) This biochemically engineered version of the protein is often referred to as DN-RAGE throughout the literature and is used as a control for RAGE signaling, but does not naturally occur.

Upon activation of the receptor by ligand binding, a cell-signaling cascade is initiated via the cytoplasmic domain at the carboxy-terminus. RAGE is thought to potentially signal through multiple pathways via a variety of mediators: p21\(^{\text{Ras}}\) activation\(^\text{14}\), the direct binding of extracellular signal-regulated protein kinase-1 and -2 (ERK-1/2)\(^\text{15}\), binding of diaphanous-1\(^\text{16}\), and Rac activation\(^\text{13}\). While there is no real consensus in the initial step of the RAGE signaling pathway, most agree that the end result is translocation (activation) of the pro-inflammatory transcription factor NF-\(\kappa\)B to the nucleus.\(^\text{13-15, 17}\) This has been shown to lead to the upregulation of numerous pro-inflammatory mediators, as well as, RAGE itself. The promoter region of human RAGE was found to contain no TATA or CAAT boxes in its promoter, but instead expression was found to be regulated by two NF-\(\kappa\)B-like binding sites at positions -1518 and -671.\(^\text{18}\) Therefore, the presence of RAGE ligands create a positive feedback loop in which RAGE expression is perpetually elevated until the ligands are removed or the signaling cascade is interrupted (see Figure 1). This complex control of expression potentially explains how nearly undetectable levels of RAGE in normal tissue\(^\text{19}\) can lead to disease when ligands are present.
1.1.3 Soluble RAGE

A second isoform of RAGE exists, which lacks the transmembrane and cytoplasmic regions of the full-length protein, but contains an identical extracellular portion including the ligand binding V-domain. This protein is often referred to as soluble RAGE or sRAGE. This soluble isoform is thought to act as a non-signaling decoy receptor, as it has been shown to have the same ligand affinity as membrane RAGE. It is therefore thought to be protective because it is able to sequester RAGE ligands and prevent their negative interaction with membrane RAGE.

The mechanism by which this soluble isoform is synthesized remains controversial. Two groups investigating human sRAGE determined an alternative splice site existed in the gene leading to the formation of a secreted isoform.\textsuperscript{20,21} Analysis of murine sRAGE by Hanford et al. revealed that no splice site existed in the murine gene and that the soluble isoform was generated by proteolytic truncation.\textsuperscript{11} Recent studies have further substantiated the idea that sRAGE is formed by proteolytic cleavage of membrane RAGE. Two independent groups demonstrated that matrix metalloproteinases (MMPs) are capable of cleaving human membrane RAGE to form soluble RAGE providing a potential mechanism by which the ratio of membrane to soluble RAGE is controlled.\textsuperscript{22,23} Their analyses found that the majority of sRAGE in the plasma is formed by proteolytic cleavage and that the alternative splice product only comprised a small percentage of the total soluble content. In contrast to these findings, Kalea et al. found that numerous soluble splice variants of murine RAGE existed, though admittedly none were detectable in the plasma\textsuperscript{24}, bringing into question their biological relevance. Further confounding these studies, another group found a murine splice variant that resulted in the secretion of a RAGE protein containing the extracellular and signaling portions, but lacking the transmembrane domain. They chose to name their discovery endogenous secretory RAGE
Its contribution to the overall soluble RAGE content remains unknown. The one thing that these studies all agree on is that soluble RAGE is capable of binding ligands and preventing their interaction with membrane RAGE (Figure 1).

![Figure 1. Schematic Depiction of RAGE Isoforms and Signaling.](image)

Ligands bind with equal affinity to either soluble or membrane RAGE. Upon binding to membrane RAGE a signaling cascade is initiated that results in the translocation of NF-κB to the nucleus where it promotes the transcription of pro-inflammatory mediators and RAGE. Thus, when ligands bind to membrane RAGE the expression of RAGE is increased and cellular signaling is increased until the cycle is interrupted.

### 1.1.4 Where is RAGE?

The original survey of RAGE expression in 1993 found that protein expression was highest in skeletal muscle followed by lung, heart, liver, kidney, uterus, and brain. It should be noted that their western blots and immunochemistry were performed with an antibody made against a 30-kDa protein that they believed to be pure bovine sRAGE. Notably, work by our group and
others have shown that pure bovine sRAGE has a M, of ~ 45-kDa creating a certain degree of skepticism as to what was being detected in these western blots. Further skepticism of this study arises secondary to the studies observation that the size of RAGE was tissue specific and ranged from 23 – 48 kDa,\textsuperscript{19} a phenomenon which has not been demonstrated since this publication. Lastly, the authors noted that the protein levels did not even remotely correlate with Northern blots for RAGE mRNA, which shows strikingly that RAGE is expressed almost solely in the lung. The authors attribute this to the fact that mRNA does not always correlate with levels of protein, but this seems to be an understatement in this case.

Since this original description, numerous investigators have further explored the expression of RAGE at both the protein and mRNA levels. There is now consensus that RAGE protein and mRNA expression are highest in the lung.\textsuperscript{26-30} The lung is also the only tissue in which RAGE protein is readily detectable by standard immunoblotting techniques in a non-pathologic state.\textsuperscript{26, 31} A recent study found that RAGE mRNA expression in the normal lung was 3000-fold higher than an atherosclerotic aorta, a disease in which RAGE is thought to be upregulated, and more than 1000 times higher than any other normal tissue (magnitude of RAGE mRNA expression: lung > eye > spleen > ovary > kidney > brain > ileum > heart > liver > colon > aorta > skeletal muscle).\textsuperscript{32}

The challenge in determining where RAGE is expressed did not end with a survey of tissues. The lung is a heterogeneous organ with numerous cell types and structures and identifying which of them expressed RAGE proved to be as challenging as the tissue surveys. The initial study conducted by Katsuoka et al. utilized in-situ hybridization on lung sections and determined that RAGE mRNA was expressed in type II alveolar epithelial (AT-II) cells but not in alveolar macrophages.\textsuperscript{33} They substantiated their \textit{in-vivo} findings by isolating and culturing
AT-II cells and alveolar macrophages. They found RAGE mRNA was expressed in the AT-II cells in culture, but not the alveolar macrophages, peritoneal macrophages or human umbilical vein endothelial cells (other cell types thought to express RAGE). However, it should be noted that RAGE mRNA expression in the cultured AT-II cells was investigated at day 7, which is a time point now thought to contain cells that are primarily differentiated into type I alveolar epithelial- (AT-I) like cells. A study published the following year performed immunofluorescence and immunoelectron microscopy on human and rat lung and found that the RAGE protein was selectively expressed in AT-I cells. The high resolution of the EM studies allowed them to localize RAGE’s expression to the basolateral surface of AT-I cells. Most studies have now provided conclusive evidence and agree that RAGE expression is confined to AT-I cells. However, an occasional publication will still claim to find this elusive protein in the endothelial and bronchial cells of lung. Notably, the data in these studies are obtained from immunochemical staining and are not substantiated by any other biochemical analysis making the findings a likely result of non-specific reactivity. All of these results taken together indicate that AT-I cells are likely the only cell type which express RAGE protein under normal conditions. However, it is possible that RAGE mRNA expression can be found in AT-II cells as they begin to differentiate into AT-I cells and may actually be a factor required for differentiation. This theory has yet to be explored in detail.

1.1.5 RAGE Ligands and Disease

Despite naming the protein the receptor for AGEs, the authors of the original paper issued a challenge to future investigators to determine its “natural ligand.” Since then many ligands have been proposed and implicated in a wide variety of diseases. These proteins include
advanced glycation end-products (AGEs)\textsuperscript{1}, S100 proteins/calgranulins\textsuperscript{44}, HMGB1/amphoterin\textsuperscript{45}, and amyloid-β\textsuperscript{46}. Each of these ligands has been shown to bind RAGE and activate the receptor resulting in the overproduction of inflammatory mediators, as well as, upregulation of RAGE itself. As a result, these ligands have been therapeutically targeted in the prevention and amelioration of numerous disease states.

AGEs have been shown to accumulate in conditions of oxidative stress and hyperglycemia (Described in Section 1.5.2). Due to their overproduction in the diabetic-disease state they have been primarily implicated in RAGE-mediated diabetic complications including: atherosclerosis\textsuperscript{47}, neuropathy\textsuperscript{48}, impaired wound healing\textsuperscript{49}, and nephropathy\textsuperscript{50, 51}. Inhibiting the interaction of AGEs with RAGE has been a highly touted therapeutic modality for the prevention of diabetic complications. However, a study conducted by Novartis suggested that AGEs devoid of endotoxin were incapable of eliciting an inflammatory response via RAGE and brought into question their disease-causing potential\textsuperscript{52}. Currently, there are two clinical trials investigating the therapeutic potential of blocking AGEs in diabetes being conducted by Pfizer and Ruhr University of Bochum (ClinicalTrials.gov NCT00287183 & NCT00437008).

S100 proteins are small (9-13 kDa) calcium binding proteins that are expressed in a tissue and cell specific manner. Certain members of this family have been shown to interact with RAGE and contribute to numerous disease processes. These include S100B in inflammation\textsuperscript{17, 53}, S100A8/9 in cardiomyocyte dysfunction\textsuperscript{54} and tumor invasion\textsuperscript{55}, S100A11 in osteoarthritis\textsuperscript{56}, and S100A12 in acute-respiratory distress syndrome\textsuperscript{42, 57}. Most of these interactions have only been discovered in the past few years and follow-up studies will be crucial to understanding the importance of their interaction in causing disease.
HMGB1 is a highly conserved, 30-kDa DNA-binding protein that is constitutively expressed in cells under normal conditions. While in the confines of the cell it has been shown to promote nucleosome formation thereby facilitating transcription. However, in instances of tissue damage, the protein is released from dying cells where it is free to act upon TLR2, TLR4 and RAGE.\textsuperscript{58, 59} HMGB1 is probably the second most well-characterized RAGE ligand after AGE. It was initially described to mediate cell migration via the receptor\textsuperscript{45, 60, 61}, but more recent studies have focused on its role in causing disease. RAGE-HMGB1 interaction has now been implicated in the pathogenesis of ischemia/reperfusion injury of the liver\textsuperscript{62}, heart\textsuperscript{63}, and lung\textsuperscript{64}. Others have suggested a negative role for their interaction in colitis\textsuperscript{65}, peritonitis/systemic inflammation\textsuperscript{59}, brain ischemia\textsuperscript{66}, and arthritis\textsuperscript{67} just to name a few. Additionally, their interaction is thought to contribute to cancer metastasis and tumor invasion.\textsuperscript{68, 69} If these connections prove to be valid, a molecule that can prevent HMGB1 from interacting with RAGE will have vast therapeutic potential though none are currently in clinical trials for any of the diseases named above.

Amyloid-\(\beta\) is a peptide that has been shown to deposit in the brain tissue of patients with Alzheimer’s disease. These deposits can result in the destruction of neurons both directly and indirectly, through the activation of microglia. Yan et al. first described the interaction between RAGE and amyloid-\(\beta\), noting their co-localization in diseased brain tissue.\textsuperscript{46} The following year it was discovered that amyloid-\(\beta\) led to upregulation of macrophage-colony stimulating factor via a RAGE/NF-\(\kappa B\) dependent pathway.\textsuperscript{70} Numerous studies since have begun to try and elucidate the mechanisms by which RAGE’s interaction with amyloid-\(\beta\) might contribute to the disease.\textsuperscript{71, 72, 73} Notably, a Pfizer-sponsored phase II clinical trial is currently investigating the effect of a RAGE inhibitor on cognitive decline in Alzheimer’s patients (ClinicalTrial.gov NCT00566397).
1.1.6 Soluble RAGE and Disease

Soluble RAGE has been utilized as a potential therapeutic in many of the diseases for which RAGE ligands have been implicated. It is important to note that sRAGE may function by not only preventing ligands from binding to RAGE, but also by preventing those same ligands from binding to other receptors. Thus, sRAGE has been proposed as a promising therapeutic for almost every disease process.\textsuperscript{74, 75} The first \textit{in vivo} study utilizing sRAGE as a therapy found that intraperitoneal injection was able to prevent the development of atherosclerosis in mice.\textsuperscript{47} In addition, that study also found that intraperitoneal injection of the protein had adequate bioavailability and a half-life of 22 hrs, which was similar to an earlier pharmacokinetic study in rats.\textsuperscript{76} This initial publication began a phenomenon in which sRAGE was tested in almost every plausible disease, limited only by the availability of the protein. Follow-up publications have now found that sRAGE can protect against periodontitis-associated bone loss in diabetic mice\textsuperscript{77}, impaired wound healing\textsuperscript{49, 78}, ischemia/reperfusion injury of the liver\textsuperscript{79} and heart\textsuperscript{80}, acetaminophen-induced hepatotoxicity\textsuperscript{81}, diabetes-induced glomerulosclerosis\textsuperscript{51}, intestinal barrier dysfunction after hemorrhagic shock\textsuperscript{82}, and endotoxin-induced pulmonary injury\textsuperscript{83}. In addition, one study found that sRAGE protected against arthritis in mice.\textsuperscript{84} However, they found sRAGE was also chemotactic for neutrophils and triggered an inflammatory response in the peritoneal cavity. These results are highly suggestive of endotoxin contamination. Studies have shown that the simultaneous administration of two inflammatory injuries has the potential to dampen the response to each.\textsuperscript{85} Thus, it is plausible that many of the effects that have been seen from sRAGE administration are due to endotoxin contamination despite the assertion of investigators to the contrary.
In addition to its therapeutic role, sRAGE levels in the plasma have been proposed as a “novel” biomarker for even more diseases than it has been thought to ameliorate. The first study found that low sRAGE levels could be correlated with the degree of coronary artery disease in non-diabetic men.\textsuperscript{86} In the following four years, lower than normal levels of sRAGE have been suggested to correlate with rheumatoid arthritis\textsuperscript{87}, essential hypertension\textsuperscript{88}, Alzheimer’s disease\textsuperscript{89}, chronic hyperglycemia\textsuperscript{90}, RAGE gene polymorphisms\textsuperscript{91}, mortality risk after renal transplant\textsuperscript{92}, primary Sjogren’s syndrome\textsuperscript{93}, multiple sclerosis\textsuperscript{94}, high body-mass index and waist-to-hip ratio\textsuperscript{95}, amyotrophic lateral sclerosis\textsuperscript{95}, non-alcoholic steatohepatitis\textsuperscript{96} and idiopathic pulmonary fibrosis.\textsuperscript{97}

During this same period, higher than normal levels of sRAGE in the blood were correlated with acute lung injury\textsuperscript{30, 98}, impaired renal function\textsuperscript{99}, elevated levels of plasma AGEs\textsuperscript{100, 101}, coronary artery disease in diabetics\textsuperscript{102}, mortality in septic patients\textsuperscript{103}, severity of disease in systemic sclerosis\textsuperscript{104}, and graft dysfunction after lung transplant\textsuperscript{105}. What, if anything, all of these correlations mean has yet to be determined, but it suggests that sRAGE levels in the blood are subject to fluctuations in states of disease.

1.1.7 RAGE and Tissue Homeostasis

The first homeostatic role that was proposed for RAGE involved amphoterin-mediated neurite outgrowth.\textsuperscript{45} In that study, the authors hypothesized that RAGE might “mediate adhesive functions for normal development.”\textsuperscript{45} The authors set out to isolate a “natural ligand” of RAGE from the lung. They performed heparin-sepharose chromatography followed by RAGE-affinity chromatography on lung homogenate and isolated a protein that they identified to be amphoterin. Previous studies with amphoterin had demonstrated that it was expressed in the developing rat...
brain and capable of inducing differentiation of cultured embryonic neurons. The authors then tested the hypothesis that this function of amphoterin might be mediated by its interaction with RAGE. They conducted numerous in vitro studies utilizing sRAGE and RAGE blocking antibodies to show that RAGE is capable of mediating neurite outgrowth when stimulated with amphoterin. This outgrowth was inhibited by the presence of sRAGE and RAGE blocking antibodies.

The second proposed homeostatic role for RAGE investigated its function in the lung. Demling and colleagues found that RAGE expression is most pronounced in type-I alveolar epithelial cells. These cells require the ability to adhere tightly to the basement membrane and spread extremely thin in order to facilitate gas exchange. They proposed that RAGE might function as an adhesive molecule in the lung by binding to components of the basement membrane. They performed in vitro experiments to determine that RAGE had affinity for collagen I and IV and that transfection of RAGE into cells facilitated cellular spreading and adherence on these matrix components. Notably, both of these physiologic roles involved the mediation of cell adherence and not ligand sequestration.

1.1.8 RAGE Knockout and Transgenic Mice

A mouse lacking RAGE expression in all tissues was developed in 2001 by deleting exons 2-7 of the Ager gene. This has been the primary tool for implicating RAGE in various disease processes. These mice have been subjected to almost every known disease model in an attempt to show a causal role of RAGE. In 2001, Yamamoto et al. developed a mouse that overexpressed RAGE in the vascular cells. A similar mouse was generated in 2004 by Liliensiek et al. who created a mouse that overexpressed RAGE in all cells of hematopoietic and
endothelial origin. These overexpressing mice have been used significantly less often than the global knockouts. Two other groups have also generated RAGE knockout mice targeting various portions of the gene. The first group began their deletion of the Ager gene upstream of the translation initiation site through intron 1 while the second group removed exons 2-4 by Cre recombination. Both groups demonstrated that expression was globally eliminated by these gene manipulations. Notably, all of these mice are completely viable and have no obvious phenotype through 6 months of age.

1.2 RAGE: A MEDIATOR OF RENAL DISEASE

Diabetic renal dysfunction is one of the most well-studied RAGE mediated disease processes. It has been shown that diabetes can lead to an accumulation of both soluble and matrix AGEs in the kidney. RAGE serves as a natural connection between AGEs and renal dysfunction. The first study investigating RAGE in renal disease performed an immunohistochemical evaluation of normal and diseased kidneys. They found that upregulation of RAGE was present in diabetic kidneys, but that it was non-specific, as it was upregulated in all diseased kidneys. Notably, a study in 4-month old diabetic (db/db) mice published only two years later found that RAGE mRNA expression in the kidney was unaltered despite elevations of AGEs in the serum and kidney. Unwilling to give up on the RAGE connection, another group performed the same experiment in streptozotocin treated rats finding that RAGE was upregulated in the kidneys by AGEs. The next paper to broach the subject also found that AGEs induced upregulation of RAGE, as well as, increased collagen I production in renal fibroblasts. This catapulted RAGE back to the forefront as a mediator of renal fibrosis. Next, mice overexpressing RAGE in the
vasculature were crossed with diabetic mice and found to have a greater degree of renal
dysfunction, supporting the assumption that RAGE contributes to renal disease. The
mechanism by which RAGE was contributing to the disease remained largely unknown until a
paper by Oldfield and colleagues. They proposed that AGEs were able to induce epithelial-
myofibroblast transition (EMT) via interaction with RAGE. This mechanism of diabetes-
induced tubulointerstitial disease was further supported by a follow-up paper a few years later.115
These findings carried significance beyond RAGE in renal disease as EMT is a proposed
mechanism in the fibrosis of numerous other organs. To date, more than 100 publications have
focused on RAGE’s ability to cause renal disease and spurred countless investigations into the
disease processes of other tissues.

1.3 RAGE AND PULMONARY DISEASE

Despite the pulmonary origin of RAGE, very few studies have investigated its role in pulmonary
disease as compared to the number of studies in other tissues. The initial pulmonary study was
an investigation into changes in RAGE gene expression in lung cancer. Since then, RAGE has
also been studied in acute lung injury/sepsis and hyperoxia.

1.3.1 RAGE, Systemic Sepsis and Acute Lung Injury

Cecal ligation and puncture (CLP) is an experimental model utilized to recapitulate the features
of polymicrobial sepsis in animals. Notably, this lethal model is known to have pulmonary
dysfunction similar to that observed in septic patients. When RAGE knockout mice were
subjected to this model they had reduced levels of NF-κB in their lung tissue as compared to wild-type controls. In a follow up study, it was found that even a 50% reduction in RAGE was able to protect against CLP, promoting survival and decreasing markers of injury. In addition, RAGE null mice have also been shown to be protected against both viral and bacterial pneumonia caused by influenza A and Streptococcus pneumoniae, respectively. Lastly, animal models have demonstrated that intraperitoneal treatment with sRAGE can mitigate the effects of endotoxin-induced lung injury. All of these studies suggest a critical role for RAGE in the pathogenesis of acute lung injury.

However, in human disease the role of RAGE is less clearly understood. The first study found that sRAGE levels were elevated in the BAL fluid and plasma of patients with acute lung injury and served as a marker of AT-I cell injury. Follow-up studies have suggested that high levels of sRAGE in the plasma are predictive of poorer outcomes in lung injury patients receiving high-tidal volumes. This is likely not surprising if it is a marker of AT-I cell injury as it would suggest they have a greater extent of disease. However, it does suggest that administration of more sRAGE might not have any therapeutic potential in the disease as those with the highest levels (2-fold greater than normal) did worse. It remains to be determined whether alterations in RAGE are a cause or effect of the human disease, but modulation of the receptor does present an interesting therapeutic option.

1.3.2 RAGE in Hyperoxic-Lung Injury

The role of RAGE in hyperoxic lung injury has been investigated in three separate studies, but remains somewhat elusive. The first study by our lab found that hyperoxic injury of neonatal rats resulted in a lower protein expression of RAGE in the lung tissue. However, the study
also found that RAGE expression was increased during lung development. It was therefore unclear as to whether hyperoxia resulted in a loss of RAGE or solely prevented lung development. The next study found that sRAGE levels in the BAL fluid were increased in response to hyperoxic lung injury, but not in models of indirect lung injury. This study did not investigate the levels of RAGE in the lung tissue and there was no implication as to what role, if any, RAGE might be playing in the injury. The final study subjected RAGE null mice to hyperoxia and found that they were protected against injury as measured by survival, histologic evidence of injury and pro-inflammatory cytokine expression. They found that hyperoxia resulted in overexpression of RAGE in cultured AT-I cells and lung tissue from mice.

1.3.3 RAGE and Lung Cancer

Lung cancer was the first pulmonary disease in which alterations in RAGE expression were investigated. The group found that RAGE protein expression was almost entirely abolished in lung tumors (non-small cell lung cancer) as compared to normal matched tissue, but it was unclear what role this might play in the cancer. They further elaborated on their initial study by demonstrating that RAGE overexpression could inhibit tumor growth and suggested that RAGE might be a novel tumor suppressor. A few years later the group went on to discover a polymorphism in the RAGE promoter that occurred more frequently in patients with NSCLC than normal controls. The downregulation of RAGE in lung cancer has now been reproduced and validated by numerous other groups. However, any role beyond that of a biomarker remains to be determined. Interestingly, RAGE has been studied in prostate, colorectal, and gastric cancers where its overexpression has been attributed to more rapid growth and
invasive disease. This makes investigations into the proliferative and invasive effects of RAGE expression extremely challenging as they support one set of observations while negating another.

1.4 IDIOPATHIC PULMONARY FIBROSIS

IPF is a debilitating disease characterized histopathologically by a pattern of usual interstitial pneumonia (UIP).\textsuperscript{131} A typical presentation occurs in the fifth to sixth decade of life with a non-productive cough and shortness-of-breath. The incidence in the United States is 6.8-16.3/100,000, while the prevalence is around 14.0-42.7/100,000.\textsuperscript{132} The low prevalence rate is consistent with a disease associated with high mortality. Mean survival time after biopsy-confirmed diagnosis is just 3-5 years.\textsuperscript{133, 134} The disease course is progressive in nature. Continual loss of normal lung parenchyma to scarring is associated with a steady decline in pulmonary function. Patients typically die as a result of an unexplained rapid deterioration in pulmonary function called an acute exacerbation.\textsuperscript{135, 136} However, acute coronary syndromes, congestive heart failure, lung cancer, and infection also contribute to the mortality associated with IPF.\textsuperscript{137} Unfortunately, there are currently no effective therapies.\textsuperscript{138}

1.4.1 Diagnosis of Pulmonary Fibrosis

Making a correct diagnosis of IPF is challenging, but essential because of the implications for treatment and prognosis. Many of the diseases that resemble IPF have viable therapies and more favorable outcomes when treated properly.\textsuperscript{131} Therefore a degree of certainty should exist before diagnosing IPF. The criteria were defined by the American Thoracic Society and European
Respiratory Society in 1999 in hopes of better identifying, understanding and treating the disease.\textsuperscript{139} In order to have a definitive diagnosis of IPF the patient must have a surgical biopsy showing a histologic pattern of usual interstitial pneumonia (UIP) as well as meet all four major criteria. 1) All other causes of the fibrosis such as environmental causes (e.g. asbestosis), autoimmune and connective tissue diseases, and drug-induced lung disease must be excluded. 2) Pulmonary function testing must be consistent with a restrictive disease and the patient must have a decreased diffusion capacity. 3) Bibasilar reticular abnormalities with ground glass opacities must be present on high-resolution CT. 4) Transbronchial biopsy and/or BAL fluid analysis must not support any other diagnosis. In addition, three of the four minor criteria must be met. These include: 1) being older than 50 yrs of age, 2) having symptoms for more than 3 months, 3) inspiratory crackles on auscultative exam and 4) rapid onset of unexplained dyspnea.

Histologically there are three criteria for classifying a biopsy as UIP.\textsuperscript{131} The first is that there must be a patchwork pattern of parenchymal involvement. This is characterized by spatial heterogeneity in the sample showing areas of normal parenchyma next to fibrotic lung. The second is architectural distortion characterized by honeycomb change or scarring. Distortion of the alveolar spaces is a common result of the fibrotic change. The third and final is temporal heterogeneity. This means that there are areas of both immature and mature fibrosis. These are characterized histologically by the presence of fibroblastic foci and collagen deposition, respectively.

Based on the above criteria, the diagnosis is best made by a team of clinicians, pathologists, and radiologists who are familiar with interstitial lung diseases. Numerous studies have now found that academic centers that diagnose and treat larger numbers of ILD patients have more consistency in their ability to correctly diagnose IPF.\textsuperscript{140, 141}
1.4.2 Pathophysiology of Idiopathic Pulmonary Fibrosis

As the name eludes, the precise factors leading to the development of idiopathic pulmonary fibrosis are not fully understood. In particular, the initiating event remains largely unknown making prevention difficult. Smoking, certain medications, infection, and environmental exposures have all been associated with higher risks of developing IPF. Currently, the most well-studied and likely causes are genetic predisposition, epithelial cell dysfunction/impaired re-epithelialization, pro-fibrotic cytokines, and fibroblastic foci.

1.4.2.1 Genetics

A genetic link for the disease has been established by the observation of familial clustering of the disease.\(^\text{142}\) In these families, investigators have been able to identify multiple genetic mutations that they believe are responsible. The first mutation to be identified was in the gene encoding surfactant protein C (SP-C).\(^\text{143, 144}\) However, a follow-up study investigating this cause in a cohort of sporadic causes of IPF found that only 9.6% of cases had a polymorphism in the gene and only 1 of these cases resulted in an amino acid change.\(^\text{145}\) Thus, it is unlikely that this genetic mutation is responsible for sporadic cases of IPF.

Mutations in telomerase have been identified in approximately 15% of familial IPF patients.\(^\text{146-149}\) This enzyme is responsible for adding telomeres to the ends of DNA during replication. Dysfunction in this enzyme has been shown to promote cellular aging and senescence.

A genetic mutation in surfactant protein A2 has now been identified in some familial cases of IPF.\(^\text{150}\) Fortunately, these genetic mutations are known to have low penetrance.
suggesting that factors other than genetics contribute to the disease pathogenesis even in familial cases.

1.4.2.2 Epithelial cell dysfunction and impaired re-epithelialization

Normal alveoli are lined with two types of alveolar cells, AT-I and AT-II. AT-I cells cover ~ 95% of the alveolar surface and facilitate gas exchange. In contrast, AT-II cells cover only 5% of the alveolar surface and are essential for secreting surfactant into the alveolar space and differentiating and replacing AT-I cells when they are injured. In, IPF there is a loss of AT-I cells, but not AT-II cells. Investigators have therefore hypothesized that IPF may represent aberrant wound healing.151, 152 Under normal conditions, AT-II cells would proliferate and differentiate into AT-I cells in order to repair the damaged epithelium. However, in IPF this process is dysregulated and the lung heals by scarring.

1.4.2.3 Expression of pro-fibrotic cytokines

After the injury to the alveolar epithelium, expression of growth factors and cytokines are thought to stimulate fibrosis. This is supported by higher-than-normal levels of TGF-β in the lungs and BAL fluid of IPF patients.153 The expression of this cytokine has been shown to stimulate the proliferation of fibroblasts and increase the production of matrix proteins both of which are fundamental components of IPF.154 Notably, mice treated with antibodies against TGF-β are protected against bleomycin-induced fibrosis.155

TNF-α is a second cytokine thought to play a detrimental role in pulmonary fibrosis as it is increased in the BAL of IPF patients. TNF-α is a pro-inflammatory cytokine that can increase the production of other cytokines including TGF-β. Animal studies in mice deficient for the
TNF-α receptor have shown that they are protected against fibrotic disease.\textsuperscript{156, 157} However, a clinical trial with etanercept, a TNF-α receptor blocker, was found to have no benefit in IPF patients.\textsuperscript{158}

Numerous other cytokines have been implicated in the pathogenesis of IPF including IFN-γ, platelet-derived growth factor (PDGF), IL-1, IL-8, MCP-1, and MIP1-α. (Reviewed in \textsuperscript{159 and 160}). The exact mechanisms by which they contribute to the disease are still being elicited.

1.4.2.4 Fibroblasts and fibroblastic foci

Fibroblasts are a type of cell that synthesize proteins of the extracellular matrix (ECM). They are typically found in the stroma of tissues and are responsible for creating the supporting structure of organs. In addition, they are thought to play a beneficial role in the wound healing process by secreting new matrix. Numerous cytokines are capable of stimulating and inhibiting these cells. Dysregulation of these cells has been shown to lead to excessive collagen deposition, a known pathology of IPF.

Fibroblastic foci are a hallmark feature of IPF pathology. They are comprised of fibroblasts and actively proliferating myofibroblasts that produce large amounts of collagen. These cells are important in the normal wound healing process but typically disappear after a tissue is repaired in order to prevent excessive collagen deposition.\textsuperscript{161} However, for unknown reasons it appears that these cells do not disappear in IPF, but instead lead to chronic matrix overproduction.\textsuperscript{162} Some studies have suggested that the number of fibroblastic foci can be inversely correlated with survival\textsuperscript{163}, further suggesting their pathologic role. However,
controversy still surrounds their function as a recent paper suggested that they might be more beneficial than detrimental.\textsuperscript{164}

1.4.3 Treatment of Pulmonary Fibrosis

There are currently no medical treatments with a survival benefit for patients with IPF. Numerous studies have been conducted utilizing various therapeutic modalities, but unfortunately none of them have been successful. Most treatments are therefore considered supportive and focus on improving patients’ quality of life rather than extending their life. The one exception is lung transplantation. Studies suggest that there may be a survival-benefit for IPF patients who receive a lung transplant, but there is still controversy surrounding the best time to perform the surgery.\textsuperscript{165}

Anti-inflammatory therapy involving the use of corticosteroids was one of the first treatments for IPF. Historically, this treatment produced an objective response in only 10-20% of patients and has a minimal effect on overall survival.\textsuperscript{134, 166, 167} However, glucocorticoids are no longer recommended as a monotherapy, but instead as part of a combination therapy in conjunction with azathioprine and N-acetylcysteine.\textsuperscript{168} Recent studies suggest that this treatment can preserve pulmonary function, but has no effect on overall survival.

Two novel antifibrotic agents, pirfenidone and bosentan, have been recently investigated for their efficacy in treating IPF. Pirfenidone inhibits TGF-β-induced collagen deposition and fibroblast proliferation. In a phase II randomized, double-blinded, placebo-controlled trial pirfenidone was found to preserve lung function and prevent acute exacerbations, but it did not meet its primary endpoint of preserving oxygen saturation during an exercise test.\textsuperscript{169} It is currently being investigated in an open-label phase III trial. Bosentan is an endothelin receptor
 antagonist that was shown to have anti-fibrotic effects in a mouse model of bleomycin-induced pulmonary fibrosis.\textsuperscript{170} It also did not meet its primary endpoint in maintaining exercise capacity but did show trends in increasing survival and improving quality of life.\textsuperscript{171, 172} Notably, the study authors found that there was a more pronounced treatment effect of bosentan in patients with biopsy-proven IPF suggesting that the results might be confounded by misdiagnoses of the enrolled patients. Bosentan is currently undergoing further investigation in larger studies.

The only treatment with a proven survival benefit for IPF patients is lung transplantation. In 2000, only 15\% of lung transplants performed were for IPF patients.\textsuperscript{173} This increased to 26\% in 2006. Unfortunately, lung transplantation is still associated with poor outcomes for IPF patients. The mean time of survival for IPF patients receiving a transplant was only 3.9 years, which was significantly lower than transplants for every other pulmonary disease.\textsuperscript{173}

Many clinicians focus on providing supportive care to improve patients’ quality of life. A recent review made a list of recommendations for managing IPF patients.\textsuperscript{138} They suggested oxygen therapy, vaccination against pneumococcus and influenza, and maintaining a healthy body weight to name a few. In addition, clinical trials should be discussed with the patients as a potential therapeutic option. Clear explanations regarding the risks and benefits of entering a clinical trial should be provided. Ultimately, the treatment plan is up to the patient. This is especially important in diseases where there is a lack of sound evidence to support any treatment at all.
1.5 ANIMAL MODELS OF PULMONARY FIBROSIS

One of the inherent challenges in understanding human disease is the development of *in vitro* and *in vivo* models to investigate hypotheses and test therapeutic interventions. There is a strong reliance on animal models to recapitulate the features of a human disease in order to perform the studies in non-human subjects. Often times, these models are discovered by mistake or as a side effect of a current therapeutic. There are currently a number of models for inducing pulmonary fibrosis in animals including: bleomycin, asbestos, silica, and FITC. Each of these models has distinct advantages and disadvantages, but none fully replicate the pathogenesis of the human disease.

1.5.1 Asbestos-induced Pulmonary Fibrosis

The term asbestos is used to describe mineral fibers that are resistant to both thermal and chemical degradation. The virtual indestructibility and cheap costs of the material led to its extensive use as both an insulator and fire-retardant. There are two classifications of fibers based on their crystalline structure: amphiboles or straight fibers (crocidolite, amosite, tremolite, anthophyllite, and actinolite) and serpentine or curvy fibers (chrysotile). Despite the negative connotation associated with the word asbestos today, detailed analyses of its use have suggested that it likely saved more lives from fire-protection than it hurt through lung disease.

Asbestos is capable of causing a diffuse interstitial fibrosis in humans, called asbestosis. The disease typically presents 20-40 years after the initial exposure with cough and dyspnea. Pathologically the disease resembles idiopathic pulmonary fibrosis with the addition of asbestos bodies in the lung tissue on microscopic evaluation. However, clinically asbestosis is milder and
slower progressing than IPF.\textsuperscript{174} Unfortunately, asbestosis has been shown to increase the risk of developing lung cancer and/or mesothelioma, both of which are associated with high rates of morbidity and mortality.\textsuperscript{176} There are currently no effective therapies to inhibit the progression of asbestosis or prevent the development of lung cancer or mesothelioma after diagnosis. It is estimated that 27 million workers were exposed to asbestos between 1940 and 1970, during its routine use.\textsuperscript{177} While asbestos is rarely used in the United States today, it continues to cause disease due to its long incubation period and unrestricted use in poorer nations.

Researchers took advantage of the disease-causing potential of asbestos in order to create a novel model of human pulmonary fibrosis.\textsuperscript{178} Mouse models of asbestosis have been shown to have pathologic features and a progressive fibrosis similar to pulmonary fibrosis in humans.\textsuperscript{179, 180} It therefore provides an ideal \textit{in vivo} model for investigating the mechanisms of both asbestosis and IPF. Asbestos can be introduced into the lungs of animals by either inhalation or intratracheal instillation. Inhalation requires significantly more equipment, time and asbestos material as compared to a single intratracheal instillation.\textsuperscript{181} On the other hand, intratracheal instillation is subject to some variability due to the required skill of the technician performing the procedure. The type of asbestos fiber used needs to be considered as well. Studies have found that fibers less than 1 micron in diameter and up to 200 microns in length are capable of depositing into the lung parenchyma.\textsuperscript{174, 182} Crocidolite fibers possess these characteristics and have been shown to have disease causing potential in both animals and humans.

Intratracheal instillation of crocidolite asbestos results in an inflammatory response within 24 hrs that is characterized by the extravasation of leukocytes into the alveolar space.\textsuperscript{183} Collagen deposition in the lung parenchyma occurs in a temporally heterogenous pattern beginning as early as 7 days\textsuperscript{184} and progressing beyond 1 year (unpublished data from our lab).
Unlike the bleomycin model, asbestos-induced fibrosis does not undergo a spontaneous resolution over time. This is likely beneficial in studies evaluating novel therapeutics. In addition, asbestos fibers are readily detectable in the BAL fluid and lung interstitium of treated animals and serve as a confirmation of proper intratracheal instillation.

1.5.2 Bleomycin-induced Pulmonary Fibrosis

Bleomycin is a chemotherapeutic agent used in the treatment of squamous cell carcinomas, testicular germ line cancers, and lymphomas. The drug itself is a mixture of glycopeptides originally isolated from the bacteria *Streptomyces verticillatus*. It was found to have activity both as an antibiotic and antineoplastic agent against, *Escherichia coli* and squamous cell carcinomas, respectively.\(^{185, 186}\) Its cytotoxic effects were found to be a result of single-strand DNA breaks.\(^{187}\) Thus, dividing cells would likely be more sensitive to the effect. Interestingly, there is a naturally occurring cysteine protease called bleomycin hydrolase that is responsible for metabolizing and inactivating the drug *in vivo*. Increased amounts of this enzyme in certain cancers have been shown to confer resistance.\(^{188}\) Bleomycin was found to cause no myelosuppression in clinical studies and therefore became a promising candidate for the treatment of lymphomas.\(^{189}\) However, it was not long until the pulmonary toxicities of the medication became evident.\(^{190}\) Even more troublesome, was that patients were beginning to die of the pulmonary complications of the treatment instead of their disease.\(^{191-193}\) Subsequently, it was observed that bleomycin-treated animals developed an interstitial pneumonia that histologically and pathologically resembled human pulmonary fibrosis and resulted in the development of a novel method for studying the disease.\(^{194}\) Studies have now shown that the
lungs have low endogenous levels of bleomycin hydrolase rendering it highly sensitive to bleomycin’s toxic effects.\textsuperscript{195, 196}

Bleomycin injury can be caused by intratracheal (i.t.), intraperitoneal (i.p.), or intravenous (i.v.) injection of the medication. While the pathogenesis of the disease remains similar among instillation methods, the time course varies widely depending upon the method of injury. The first study histologically evaluated the effect of twice weekly i.p. injections at various doses.\textsuperscript{194} The first change they saw was at two weeks when endothelial dysfunction and edema was seen by electron microscopy. This dysfunction worsened until at 4 weeks necrosis of AT-I cells was seen. Interestingly, AT-II cells went undamaged throughout the time course. At 4 weeks, intraalveolar and septal fibrosis was noted and thought to be a result of fibrinous exudate that escaped through the damaged epithelium and endothelium. From eight weeks on, proliferation and metaplasia of AT-II cells was seen along with fibrosis, which progressed until 12 weeks when the experiment ended. I.V. injection was found to have a similar, but accelerated, pathologic course compared to multiple i.p. injections.\textsuperscript{197} Mice treated with a single i.v. injection of bleomycin were found to develop endothelial dysfunction by 2 days, AT-I cell necrosis by 7-10 days, and progressive fibrosis from 14-28 days when the experiment was halted.\textsuperscript{197} Intratracheal instillation of bleomycin was first found to cause fibrosis in hamsters.\textsuperscript{198} It is now the most-utilized method of inducing fibrotic lung disease in animals due to its accelerated time course.\textsuperscript{199} The mice develop an acute inflammatory response characterized by neutrophil infiltration during days 1-3 and fibrosis starting as early as day 7 and progressing through day 21.\textsuperscript{199} Notably, bleomycin-induced fibrosis has been shown to resolve spontaneously beyond 21 days unlike the human disease. Therefore, a certain degree of caution
must be utilized as many of the mechanisms responsible for bleomycin-induced fibrosis likely differ from that of the human disease.

### 1.6 DIABETES AND RAGE

Diabetes results in prolonged periods of hyperglycemia due to either a lack of insulin (Type I) or insulin-resistance (Type II). Excess glucose in the serum accelerates the non-enzymatic glycation of proteins and results in the increased formation of AGEs. While AGEs have been shown to form under normal conditions, the body is able to clear the products through renal elimination and macrophage-mediated degradation. However, in diabetes the body’s clearance mechanisms are overwhelmed, allowing AGEs to accumulate. This accumulation is thought to lead to the pathologic interactions between AGEs and RAGE.

#### 1.6.1 Formation of Advanced Glycation End-Products

The Maillard reaction was first described in 1912 by Louis-Camille Maillard. In his paper he described a reaction between amino-acids and non-reducing sugars which led to browning of his reaction solution. Unbeknownst to him at the time, this reaction would cause a significant amount of aggravation for those working in the food, textile, and pharmaceutical industries. The in vivo significance of this discovery became evident in 1968 with the discovery of glycated hemoglobin in diabetic patients. Since then the reaction has been extensively studied with the underlying goal of its prevention.
The first step occurs when an aldehyde of a reducing sugar (i.e. glucose, fructose, or lactose) reacts with a free amino group, most often on lysine, to form a Schiff base. This product then undergoes a spontaneous rearrangement to form an Amadori product. At this point, the reaction is still reversible. However, once the Amadori product is oxidized an advanced glycation end-product is irreversibly formed. In diabetes, the elevated levels of glucose and oxidative environment are both thought to contribute to the extensive formation of AGEs.

The terms glycation and glycosylation are often used interchangeably throughout the literature. However, glycation traditionally refers to the non-enzymatic attachment of sugars whereas glycosylation is the enzymatic attachment. Inhibitors of glycation exist and have been shown to prevent AGE formation. The most studied of these is aminoguanidines, which has been shown to prevent some diabetes-associated complications. However, aminoguanidine is also an inhibitor of reactive oxygen species (ROS). Thus, it is somewhat challenging to determine whether the benefit in the studies was from a reduction in AGEs or ROS. Novel inhibitors of AGE formation and compounds that promote their breakdown are currently being developed.

1.6.2 Animal Models of Diabetes

1.6.2.1 Ove26 Mice

Ove26 mice are a model of type 1 diabetes. These mice possess a mutation in the insulin promoter resulting in diabetes. They exhibit hyperglycemia within 24 hrs of birth and by 15 days of age they express only 28% of normal pancreatic insulin levels. In addition, they are known to develop complications from their diabetes by as early 8 weeks of age, do not require
insulin, and survive for more than a year.\textsuperscript{213} The mice have been backcrossed into the FVB genetic background, which is utilized as a non-diabetic control in experiments.

1.6.2.2 Diabetes Prone BB (DP-BB) Rat

In 1978, a colony of Bio Breeding (BB) rats was noted to spontaneously develop hyperglycemia.\textsuperscript{214, 215} These rats were further characterized and found to develop hypoinsulinemia, hyperglucagonemia and ketoacidosis.\textsuperscript{216} In addition, they were found to have an autoimmune reaction that targeted the islet cells of the pancreas. This reaction and subsequent disease were ameliorated by the administration of anti-lymphocyte antibodies.\textsuperscript{216} This phenotype is consistent with many of the characteristics of type I diabetes mellitus. Inbreeding of the diabetic and normal rats has led to the generation of two strains, the diabetes prone (DP-BB) and diabetes-resistant (DR-BB) BB rats. This model has served as an invaluable tool in the study of the complications and pathophysiology associated with type I diabetes.\textsuperscript{217} The DP-BB rats have been shown to have elevated levels of AGEs and develop cardiac dysfunction in a RAGE-dependent fashion.\textsuperscript{218}

1.6.2.3 \textit{db/db} Mice

In 1968, a metabolic syndrome was observed to occur in the C57BL/Ks mouse strain at Jackson Laboratory.\textsuperscript{219} At the time, the investigators were unaware of what caused the syndrome, but observed that the mice developed hyperglycemia and polyuria in an autosomal recessive fashion. The diabetic state was preceded by excessive fat deposition beginning at 3-4 weeks of age. Today, \textit{db/db} mice are utilized as a type II diabetic model. The mice are known to possess a point mutation in the leptin receptor rendering it defective. This results in obesity by 3-4 weeks, hyperinsulinemia by 2 weeks, and hyperglycemia by 4-8 weeks of age. These mice are known to
develop numerous diabetic complications due to elevated levels of AGEs in the plasma and tissues. The AGE-RAGE axis has been implicated in this mouse in glomerusclerosis\textsuperscript{51}, cardiomyopathy\textsuperscript{220}, and atherosclerosis\textsuperscript{221}.

1.6.2.4 Zucker Diabetic Fatty (ZDF) Rat

Zucker diabetic fatty (ZDF) rats are an insulin-resistant animal model of type II diabetes.\textsuperscript{222} They are known to have elevated plasma insulin and triglycerides by 7 weeks of age and develop hyperglycemia by 8 weeks.\textsuperscript{223} Their metabolic dysfunction is a result of a point mutation in the leptin-receptor gene\textsuperscript{224}, and they have been compared to the \textit{db/db} and \textit{ob/ob} mouse models. ZDF rats develop numerous diabetic pathologies including neuropathy\textsuperscript{225}, nephropathy\textsuperscript{226}, and arterial injury\textsuperscript{227} to name a few. Notably, in the arterial injury and nephropathy studies the investigators found both AGEs and RAGE were upregulated in the respective tissues. The Zucker lean rat is utilized as the euglycemic control.
2.0 RATIONALE AND HYPOTHESIS

The receptor for advanced glycation end products (RAGE) is expressed at low levels in most tissues and is up-regulated by its ligands in disease states. In contrast, the lung expresses high levels of RAGE under normal conditions, but little is known about RAGE expression in pulmonary disease. Some studies have suggested that RAGE is expressed as type II alveolar epithelial cells transdifferentiate into type I cells\textsuperscript{36}, a component of pulmonary re-epithelialization and repair after injury.\textsuperscript{37,38} This led us to investigate the function of pulmonary RAGE expression utilizing two different disease models.

First, we chose to study RAGE in a model of pulmonary fibrosis because of several prior observations. 1) Treatment of renal podocytes with AGEs has been shown to lead to epithelial to myofibroblast transition via RAGE.\textsuperscript{114} 2) RAGE activation in renal epithelial cells has been shown to upregulate TGF-β expression and collagen synthesis.\textsuperscript{115} 3) RAGE expression is essential for cellular adherence and spreading in type I alveolar epithelial cells.\textsuperscript{35} And 4) AGE levels are increased in the lungs of IPF patients.\textsuperscript{228}

In addition, we chose to study the effect of diabetes on pulmonary RAGE expression and susceptibility to fibrotic injury. These experiments were based on the above criteria, as well as, some additional observations: 1) Hyperglycemia present in diabetes results in the non-enzymatic glycation of proteins resulting in the increased formation of AGEs.\textsuperscript{200} 2) Diabetes has been
shown to alter RAGE expression in the kidney\textsuperscript{110, 113} and vasculature\textsuperscript{229}. And 3) Epidemiologic studies suggest that diabetics have an increased risk of developing IPF\textsuperscript{230, 231}

Based on these observations, we hypothesized that increased levels of AGEs present in IPF and diabetes would inhibit RAGE-mediated adhesion of type I alveolar epithelial cells to the basement membrane, resulting in increased susceptibility for de-epithelialization and/or impaired re-epithelialization culminating in an increased pulmonary fibrotic response after injury (Figure 2).

![Diagram](image)

**Figure 2. Hypothesis for how AGEs and RAGE interact to promote pulmonary fibrosis.** 1) Fibrotic insults like asbestos or bleomycin injure type I alveolar epithelial cells. 2) AGEs inhibit RAGE from binding to the basement membrane. This promotes de-epithelialization and impairs wound healing. 3) These synergistic interactions result in a more severe fibrotic response.
3.0 METHODS

3.1 PURIFICATION OF SOLUBLE RAGE

All chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Fast performance liquid chromatography was performed using an FPLC System, FPLC Director Software, and columns from Pharmacia (now GE Healthcare Life Sciences, Pittsburgh, PA).

3.1.1 Lung Tissue Homogenization

Sixty-five grams of fresh frozen mouse lungs (300 pairs, Pel-Freez Biologicals, Rogers, AR) were blended in 600 mL of ice-cold homogenization buffer (50 mM K$_2$HPO$_4$, 300 mM KBr, 3 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), 0.01 mM E-64 (trans-Epoxysuccinyl-leucylamido-[4-guanidino] butane), pH 7.4). Alternatively, 500 grams of trimmed bovine lung (Pel-Freez Biologicals) were blended in 2.5 L of ice-cold homogenization buffer. The homogenate was centrifuged in 250-mL polycarbonate centrifuge bottles at 20,000 x g for 20 min. at 4 °C to pellet insoluble material. The supernatants were then pooled. Polyethyleneimine was added to a final concentration of 0.01% and the homogenate was stirred for 10 min. at 4 °C to precipitate nucleic acids. The homogenate was centrifuged again as above. The supernatant
was vacuum-filtered through a Büchner funnel with a coarse (40-60 μm) fritted disc to remove non-pelleted debris.

### 3.1.2 Concanavalin A Sepharose Chromatography

This step was carried out in a cold room (4 °C) to limit proteolytic degradation. One hundred milliliters of Concanavalin A Sepharose (Sigma) was packed into a 5.0 x 30 cm empty column (Bio-Rad Laboratories, Hercules, CA) and rinsed with 400 mL of wash buffer (50 mM HEPES, 250 mM NaCl, pH 7.0). The lung homogenate was then loaded by gravity onto the column at a rate of 2-3 mL/min. The Con-A was washed with 1 L of ice-cold wash buffer until the absorbance of the flow through at 280 nm was < 0.05. The bound protein was eluted at 2-3 mL/min with 1 L of elution buffer (50 mM HEPES, 250 mM NaCl, 200 mM methyl α-D-mannopyranoside, pH 7.0) or until the Abs<sub>280</sub> < 0.05. The eluate was then filtered through a 0.22 μm vacuum filter.

### 3.1.3 Heparin Sepharose Chromatography

An XK-16 column (Pharmacia) was packed with 80 mL of Affi-Gel Heparin Sepharose (Bio-Rad Laboratories). Using a Pharmacia FPLC system, the column was washed with 250 mL of 80% Buffer A (20 mM Tris-HCl, 50 mM NaCl, pH 7.5) and 20% Buffer B (20 mM Tris-HCl, 1 M NaCl, pH 7.5). The Concanavalin A eluate (not dialyzed) was loaded onto the column by gravity at a flow rate of ~ 0.5 mL/min at 4 °C. After loading, the column was attached to an FPLC system and washed at 5 mL/min with 80% Buffer A / 20% Buffer B until the A<sub>280</sub> was 0 (~ 300 mL). The following elution profile was used: Flow Rate = 4.5 mL/min, Gradient = 1%
B/min starting at 20% Buffer B, Fraction Collector = 1 fraction/min. Fractions containing the 45 kDa sRAGE protein (19-41), as determined by coomassie blue staining of SDS-PAGE, were pooled (volume = 148.5 mL) and dialyzed into 10 L of Buffer A at 4 °C for 4 hrs and then switched to fresh Buffer A overnight. The eluate was removed from the dialysis tubing and filtered through a 0.22 μm vacuum filter to remove any debris and to degas the solution.

### 3.1.4 Anion Exchange Chromatography

A 5-mL HiTrap Q column (GE Healthcare) was attached to the FPLC and washed with 25 mL of Buffer A at 1 mL/min. Using a peristaltic pump (Pharmacia), the Heparin Sepharose eluate was applied to the column at 1 mL/min. The column was washed with 25 mL of 100% buffer A and then the protein was eluted with an isocratic profile (35% Buffer B) at 1 mL/min and the eluate was pooled from 3-20 min. (Figure 4). The protein was dialyzed into PBS.

### 3.1.5 Endotoxin Removal

Endotoxin was removed using a pre-packed 1-mL Detoxi-Gel Column (Pierce) according to the manufacturer’s protocol. Endotoxin was assayed using the Pyrotell® LAL Single Test Vial (Associates of Cape Cod, Inc., East Falmouth, MA) and the protein was considered to be void of endotoxin when it tested negative at a cutoff of 0.12 EU per 50 μg of pure protein. Batches that tested positive were re-applied to the Detoxi-Gel column as many times as needed until they tested negative.
3.1.6 Identification by sRAGE by MALDI-MS/MS analysis

An aliquot of the final pool of sRAGE was subjected to reducing SDS-PAGE followed by coomassie blue stain. The gel band of interest (~45 kDa) was excised and digested at 37 °C for 18 hours using sequencing-grade modified trypsin (Promega). The resulting tryptic peptides were isolated (ZipTip tip μ–C18, Millipore Corporation) and spotted onto a MALDI sample target using 1 μl matrix solution containing 70% (v/v) acetonitrile, 0.03% (v/v) trifluoroacetic acid (Protein Sequencer Grade), and 0.4% (w/v) recrystallized α–cyano-4-hydroxy-cinnamic acid (Sigma). The sample was analyzed by MALDI-MS peptide mass fingerprinting and MALDI-MS/MS of selected ions using a Q-Tof UltimaTM Global mass spectrometer (Waters, Micromass, Manchester, UK) operated under MassLynx 4.0. The spectra were combined, background subtracted, deisotoped (MaxEnt 3) and exported as a Mascot-searchable SEQUEST file. The files were merged and used to query all mouse Swiss-Prot and MSDB entries using the Mascot software package (Matrix Sciences, London, U.K).

3.1.7 Assessment of RAGE Bioactivity

The ability of RAGE to bind its ligands was assessed as previously described.35, 57 In brief, high-binding polystyrene 96-well plates (R&D Systems, Minneapolis, MN) were coated with either recombinant HMGB-1 (Sigma Alrich) or BSA at 5 μg/mL in PBS (50 μL/well) overnight at 4 °C. All subsequent incubations were carried out while rocking the plate at 37 °C. The wells were washed with PBS (3x, 300 μL/well). The wells were then blocked in PBS/10% BSA for 1 h. Decreasing concentrations of purified sRAGE (starting with 25 μg/mL) in PBS/10% BSA
were incubated for 90 min. Each reaction was performed in triplicate. The plate was washed again and then incubated with goat anti-mouse sRAGE antibody (5 μg/mL) in PBS/0.2% BSA for 1 h. The plate was subsequently washed and then incubated with donkey anti-goat HRP (1:20,000, Jackson ImmunoResearch, West Grove, PA) in PBS/0.2% BSA for 1 h. The plate was washed and then incubated with SigmaFast™ OPD solution (100 μL/well) for 30 min at room temperature. sRAGE binding (function) was assessed by reading the absorbance at 450 nm on a plate reader (SpectraMax, Molecular Devices) and comparing the differences in binding between the HMGB-1 and BSA coated wells.

3.2 GENERATION OF ANTI-RAGE ANTIBODIES

3.2.1 Rabbit anti-mouse RAGE antibody

A mouse RAGE specific rabbit polyclonal antibody was produced against residues 38-51 (sequence: CKGAPKKPPQQLEW, Genbank accession #Q62151) by GenScript Corporation (Scotch Plains, NJ). Rabbits were immunized 4 times with a KLH conjugated mouse RAGE peptide. Antisera containing the RAGE antibodies were collected at sacrifice. Titers against the peptide were performed to confirm immunogenicity. Antibody specificity for membrane and soluble RAGE was confirmed by the detection of only those two proteins on Western Blot analysis of lung homogenate.
3.2.2 Goat anti-mouse RAGE antibody

A RAGE specific goat polyclonal antibody was produced against full-length mouse sRAGE by GenScript Corporation. In brief, goats were immunized with 800 μg of mouse sRAGE in complete Freund’s adjuvant on day 0. Booster immunizations of 800 μg of mouse sRAGE were given in incomplete Freund’s adjuvant on days 14, 35, and 56. The goats were sacrificed on day 63 and antisera containing RAGE antibodies were collected. Antibody specificity and species cross-reactivity were determined by western blot analysis of lung homogenate.

3.2.2.1 IgG Purification

Goat antisera containing anti-RAGE antibodies was purified using Protein G sepharose (Sigma) according to the manufacturer’s instructions. In brief, 1 mL of Protein G sepharose (Sigma) was packed into an empty column. It was then washed with 10 mL of wash buffer (20 mM Sodium Phosphate, pH 7.0) to equilibrate. Three milliliters of antiserum was diluted into 12 mL of wash buffer and then centrifuged at 10,000x g for 10 minutes in order to pellet any particulate matter. The supernatant was collected and passed through a 0.22 μm syringe filter to remove any remaining debris. The filtered serum was then added to the column by gravity. The column was washed again with 10 mL of wash buffer in order to remove non-binding proteins. Elution buffer (100 mM glycine, pH 2.7) was added to the column and the eluate was collected into tubes containing neutralizing buffer (1 M Tris, pH 9.0) in a final ratio of 9:1 (eluate:neutralizing buffer). The concentration of the purified IgG was determined from the molar extinction coefficient, which states that an A280 of 1.4 is equal to 1 mg/mL of IgG. Purity of the IgG was assessed by the presence of 50 and 25 KDa bands (Ig heavy and light chains, respectively) on reducing SDS-PAGE and a lack of other molecular weight bands.
3.3 HUMAN LUNG TISSUE

Samples were obtained from the tissue bank of the Department of Pathology at the University of Pittsburgh. The use of archived tissue has been approved by the University of Pittsburgh Institutional Review Board. Diagnosis of IPF was supported by history, physical examination, pulmonary function studies, chest high-resolution computed tomography, and corroborated by histologic evaluation of open lung biopsy material. The morphologic diagnosis of IPF was based on typical microscopic findings consistent with this disease.232 The patients fulfilled the criteria of the American Thoracic Society and European Respiratory Society.139 Control samples included histologically normal lung samples resected from patients with lung cancer obtained from the University of Pittsburgh Department of Pathology Tissue Bank.

3.4 ANIMAL STUDIES

3.4.1 Animal Experiments

All animal experiments were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee or the committee at the institution where the experiments were carried out. Animals were given free access to food and water and were cared for according to guidelines set by the American Association for Laboratory Animal Care. Eight to ten week old mice were used for all studies involving fibrotic stimuli. Diabetic and RAGE null rodents were aged up to 16 months and are noted in each study.
3.4.2 Diabetic Animals

Ove26 and FVB mice were obtained from The Jackson Lab (Bar Harbor, ME) and maintained at the University of Pittsburgh until 8 weeks of age. Lungs from aged (7 month old) Ove26 mice were obtained from the laboratory of Dr. Anne Marie Schmidt (Columbia University). Lungs from DP-BB (diabetic) and DR-BB (wild-type) were obtained from Dr. Irina Smirnova (Kansas University Medical Center) from 4 month old animals. Db/db (diabetic) and db/m (wild-type) mice were aged to 9 months of age in the laboratory of Dr. Anne Marie Schmidt. Their lungs were removed, flash frozen, and then processed and analyzed in our lab. ZDF and Zucker lean (ZL) control rats were aged to 4 months in the laboratory of Dr. Irina Smirnova. Their lungs were removed, flash frozen in liquid nitrogen, and then processed and analyzed by our lab.

3.4.3 RAGE null mice

Founder RAGE-null mice were generously provided by Dr. Angelika Bierhaus (University of Heidelberg) in order to start a breeding colony. In brief, a global knockout was created by flanking exons 2-7 of the Ager gene with two loxP sites. Upon recombination by Cre, the exons were deleted positioning a thymidine kinase (TK) promoter in front of a now completed genomic sequence for enhanced green fluorescent protein (EGFP). Therefore, recombination led to the “knock-out” of RAGE and the “knock-in” of EGFP. The EGFP sequence was inserted in between exon 1 and exon 8 and RAGE expression was found to be absent in all tissues while EGFP expression was present. These mice were backcrossed for 10 generations into the C57BL/6 background. Therefore, C57BL/6 mice were subsequently purchased from Taconic (Germantown, NY) to use as wild-type controls.
3.4.3.1 Genotyping of RAGE null mice

Genomic DNA was isolated from a mouse tail tip using a Qiagen DNAeasy kit (Qiagen, Inc, Valencia, CA) according to the manufacturer’s instructions. The PCR reaction was performed in a total reaction volume of 25 μL. The reaction contained 1 μL of genomic DNA, 0.1 μM of forward primer (5’-CCTGGGTGCTGGTTCTTG-3’), 0.1 μM of reverse primer (5’–CTGAGGTCCGTGGCTAGG-3’), 1.5 mM dNTP’s (Invitrogen, Carlsbad, CA), 2 mM magnesium sulfate, 2.5 μL of 10x High-fidelity PCR buffer (Invitrogen), and 1 unit of Platinum® Taq DNA polymerase high fidelity (Invitrogen). PCR reactions were performed in a 96-well thermal cycler (Techne). A denaturation step at 94 °C for 5 minutes proceeded 30 PCR cycles, with each cycle undergoing denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and elongation at 72 °C for 3 minutes. After the last cycle an additional elongation step was carried out at 72 °C for 5 minutes before the samples were held at 4 °C. The PCR products were analyzed by agarose gel electrophoresis on 0.7% agarose gels and compared to a 1 kb DNA ladder (Promega). RAGE null PCR products ran at ~1750 kb while wild-type mice ran at ~2100 kb, this difference in size corresponds to the deleted exons in the knockout.

3.4.4 Asbestos-Induced Pulmonary Fibrosis

Mice were subjected to intratracheal instillation of asbestos as previously described. Crocidolite asbestos was baked at 180 °C overnight to remove endotoxin. It was then diluted in sterile saline to a final concentration of 1.43 mg/mL so that a 70 μl dose contained 100 μg of asbestos. Control mice were treated with 100 μg of titanium dioxide (inert particulate control) prepared in an identical fashion. For treatment, the mice were anesthetized with isofluorane.
Baxter Healthcare Corporation), intubated with a 22-gauge feeding needle attached to a 1 cc syringe, and the respective material was instilled. Mice were sacrificed by pentobarbital overdose at the indicated time points.

### 3.4.5 Bleomycin-Induced Pulmonary Fibrosis

Mice were subjected to intratracheal instillation of bleomycin as previously described. In brief, bleomycin (Hospira, Inc, Lake Forest, IL) was diluted in sterile saline to a final concentration of 0.571 U/mL such that a 70 µl dose contained 0.04 units of bleomycin (~1.6 units/kg). Control mice received an instillation of sterile saline. The mice were weighed daily to assess injury and health of the animals throughout the experiment. Mice were sacrificed at the indicated time points by pentobarbital overdose.

### 3.4.6 Unilateral Ureteral Obstruction (UOO) Induced Renal Fibrosis

Mice were subjected to unilateral ureteral obstruction (UOO) for 7 days as previously described. In brief, mice were anesthetized fully with pentobarbital injection 75 mg/kg and rendered unresponsive to pain. A midline incision was performed, the left ureter was exposed and completely occluded by double-ligation with 4-0 silk suture. Sham operations were performed in an identical fashion except the ureters were only manipulated and not occluded. The abdomen was closed with two layers of sutures. The mice were monitored daily for the duration of the experiment.

After 7 days the mice were sacrificed by pentobarbital injection and their left kidneys were removed. The kidneys were weighed and then cut into four parts for various analyses. The
first part was removed for hydroxyproline analysis. The second part was fixed in 10% formalin for histologic analysis. The third portion was embedded in OCT and frozen for cryosection. The fourth part was placed in a microtube and flash frozen in liquid nitrogen for protein and mRNA analysis.

3.4.7 Treatment with soluble RAGE

Mice received daily intraperitoneal injections of either 50 µg of bovine sRAGE or bovine serum albumin (BSA) control. Treatment with sRAGE was started 3 days prior to the pulmonary injury and dosed every 24 hours until the experiment’s completion. An alternative dosing method was performed by intratracheally instilling 50 µg of bovine sRAGE on days 4 and 8 post-fibrotic injury.

3.4.8 Processing of Animal Tissue and Samples

Mice were euthanized by overdose of pentobarbital (150 mg/kg). Blood was collected by cardiac puncture of the right ventricle with a 27-gauge needle. The blood was placed into serum separator tubes (BD Biosciences) and processed according to the manufacturer’s protocol. Samples were then stored at -80 °C until their use.

Bronchoalveolar lavage fluid (BALF) was obtained by inserting a 20-gauge luer stub adapter through a tracheotomy. The adapter was secured in place with 3-0 silk suture and 800 µL of sterile saline was instilled and recovered.
Lungs designated for homogenization or hydroxyproline analysis were removed immediately after lavage. Tissue for homogenization was flash frozen in liquid nitrogen while tissue designated for hydroxyproline analysis was placed into a labeled glass vial for drying.

Lungs for histology were fixed by instilling 1.0 mL of 10% buffered formalin through the luer-stub adapter and allowed to fix in the chest cavity for 10 minutes. The lungs were then excised placed into histology cassettes and incubated in formalin for an additional 4 hours before being processed and paraffin embedded by the university’s Research Histology Services.

3.5 BIOCHEMICAL ANALYSES

3.5.1 Tissue Homogenization

Soluble lung homogenates were prepared as described previously. Lungs were homogenized in an isotonic buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing protease inhibitors (2 mM ortho-phenanthroline, 10 μM trans-Epoxy-succinyl-l-leucylamido(4-guanidino)butane (E-64), and 100 μM dichloroisocoumarin (DCI)) to obtain all soluble proteins. The supernatant containing the soluble portion was removed and the insoluble portion was then pelleted by centrifugation and re-suspended in a buffer containing CHAPS detergent (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CHAPS) containing the same protease inhibitors, sonicated briefly, rocked for 2 hours at 4 °C, then centrifuged at 20,000 x g for 20 minutes at 4 °C. The membrane fraction supernatant was removed and stored at -80 °C until use. Alternatively, in some experiments the tissues were homogenized directly into the CHAPS containing buffer in order to obtain all proteins in a single fraction.
3.5.2 Protein Quantification

Protein concentrations were determined using Coomassie Blue Protein Reagent (Thermo Fisher) according to the manufacturer’s protocol. In brief, 10 μL of diluted samples and protein standards were mixed with 300 μL of coomassie reagent in a clear, flat-bottom 96-well plate. The plate was then incubated for 5 minutes at room temperature. The absorbance at 595 nm was then determined on a SpectraMax (Molecular Devices, Sunnyvale, CA) plate reader. Protein concentrations in the unknown samples were then determined from the linear regression of the standard curve.

3.5.3 Immunoblotting

Equal amounts of protein (5-30 μg) of each sample was separated by SDS-PAGE and transferred to PVDF membranes as described previously. The membranes were blocked overnight in 5% nonfat dry milk/PBST at 4 °C. The membranes were then incubated with primary and secondary antibodies for 1 h at room temperature according to the conditions in the following tables. The membranes were washed with PBST (3x for 10 min) after each antibody incubation.

Table 1. Primary Antibodies and Conditions for Immunoblotting

<table>
<thead>
<tr>
<th>Protein</th>
<th>Company</th>
<th>Product #</th>
<th>Dilution (in PBST)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human RAGE</td>
<td>Santa Cruz</td>
<td>sc-5563</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Mouse RAGE</td>
<td>Oury Lab</td>
<td>Rabbit anti-mouse RAGE</td>
<td>1:5,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Oury lab label - 9A)</td>
<td></td>
</tr>
<tr>
<td>Aquaporin-5</td>
<td>Sigma</td>
<td>A4979</td>
<td>1:500</td>
</tr>
<tr>
<td>ALCAM</td>
<td>R&amp;D Systems</td>
<td>AF1172</td>
<td>1:500</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sigma</td>
<td>A5316</td>
<td>1:5,000</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Sigma</td>
<td>T-4026</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Bleomycin Hydrolase</td>
<td>Abnova</td>
<td>H00000642-A01</td>
<td>1:1,000</td>
</tr>
</tbody>
</table>
Table 2. HRP conjugated Secondary Antibodies and Conditions for Immunoblotting

<table>
<thead>
<tr>
<th>Species</th>
<th>Company</th>
<th>Product #</th>
<th>Dilution (in PBST)</th>
<th>Milk Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Mouse</td>
<td>Jackson ImmunoResearch</td>
<td>315-035-003</td>
<td>1:10,000</td>
<td>0%</td>
</tr>
<tr>
<td>Anti-Goat</td>
<td>Jackson Immuno Research</td>
<td>705-035-003</td>
<td>1:10,000</td>
<td>3%</td>
</tr>
<tr>
<td>Anti-Rabbit</td>
<td>GE Life Sciences</td>
<td>NA934</td>
<td>1:5,000</td>
<td>1%</td>
</tr>
</tbody>
</table>

To visualize antibody binding, enhanced chemiluminescence was used (ECL, Amersham Biosciences and ECL Plus, GE Healthcare). Image capture and densitometric analysis were performed on a Kodak Gel Logic 2200 Imaging System and Kodak Molecular Imaging software, respectively (Kodak, Rochester, NY).

3.5.4 Hydroxyproline Assay

Both the left and right lungs were dried at 110°C for 48-72 hrs and then acid hydrolyzed in 2 mL of 6 M hydrochloric acid in nitrogen-flushed and vacuum-sealed vials. The sealed vials were incubated at 110°C for 24 hours and hydroxyproline content was quantified as described previously.\(^{180, 235, 236}\)

In brief, lung samples were diluted 40-fold in PBS and a set of 4-hydroxy-L-proline standards (0-5 μg/mL) were also made in PBS. A chloramine-T solution was added to each sample and standard and allowed to incubate at room temperature for 20 min. A 3.15 M perchloric acid solution was then added to each sample and incubated for an additional 5 minutes at room temperature. Finally, a p-diaminobenzaldehyde (P-DMABA) solution was added to each sample and standard, vortexed to mix, and incubated in a 60 °C water bath for 20 minutes for the color to develop. The developed solutions were added to a 96-well plate in triplicate and
read at 557 nm on a spectrophotometer (Molecular Devices). The sample concentrations were calculated from the linear regression of the standard curve.

### 3.5.5 RNA Isolation and Real Time RT-PCR

RNA was isolated from mouse lungs using a Qiagen RNAeasy kit according to the manufacturer’s instruction. Reverse transcriptase reactions were performed on 1 µg of RNA by adding 5 mM MgCl₂, PCR Buffer II, 1 mM nucleotide mix (Promega, Madison, WI), 1.0 U RNAsin, 2.5 U MuLV reverse transcriptase enzyme and 3 µg random primers to each sample. Reverse transcriptase reactions were carried out in a thermocycler (Techne, Staffordshire, UK) programmed for 42 °C for 40 min, 99 °C for 5 min, and 5 °C for 5 min.

Quantitative PCR was performed by adding Universal PCR Buffer and Taqman® primer/probe assay reagent for RAGE (Mm00545815_m1) to each reverse transcriptase reaction according to manufacturer’s protocol. Primers/probe for GAPDH (Mm99999915_g1) were used as a loading control for normalization. The default program was performed on an ABI Prism 7300 (Applied Biosystems) and consisted of 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15s and 60 °C for 1 min. Sequence Detection Software Version 1.4 (Applied Biosystems) was used to analyze the data and obtain relative quantities of mRNA expression for each sample based on the crossing threshold. The ΔΔCt method of relative quantitation was used for determining the expression values.²³⁷ The expression of the TiO₂ treated controls was set as 100% for comparison.
3.5.6 Bronchoalveolar Lavage Fluid Analysis

Cell counts were obtained by diluting 50 μL of recovered sample into 10 mL of Isoton® II (Beckman Coulter, Fullerton, CA) and then counting on a Z1 series Coulter Counter in triplicate. A cytospin slide was made using 100 μL of each sample. The slides were dried for >24 h, stained with Diff-Quik stain (Dade Behring), dried overnight and then cover slipped using Permount mounting media. Manual cell differentials were performed by counting 200 cells on each slide at 40X magnification in a blinded fashion.

After making cytospin preparations, the BAL samples were centrifuged for 20 min. at 10,000x g in order to pellet the remaining cells. The supernatant was removed, the protein concentration was determined by Bradford assay and the samples were stored at -80 °C for future studies.

3.5.7 sRAGE ELISA

Samples and sRAGE standards were diluted in TBS and incubated in high-binding polystyrene 96-well plates (R&D Systems, Minneapolis, MN) overnight at 4 °C. The wells were blocked with 3% BSA in TBS-T (Tris Buffered Saline/0.05% Tween-20) at 37 °C for 1.5 hrs. The wells were washed 3 times with TBS-T. A mouse RAGE specific antibody was diluted 1:1000 in 1% BSA/TBS-T and 100 μL was added to each well and incubated at room temperature for 2 hrs. The plate was washed again and 100 μl of biotin conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:10,000 in 1% BSA/TBS-T was added to each well and incubated for 1 hr at room temperature. The plate was once again
washed and streptavidin-alkaline phosphatase (Jackson ImmunoResearch Laboratories) was diluted 1:1000 in 1% BSA/TBS-T and 100 uL was added to each well for 1 hr at room temperature. The plate was washed and then developed with an alkaline phosphatase substrate kit (Bio-Rad, Hercules, CA) for 5 min. The plate was read at 405 nm on a SpectraMax 96-well plate reader (Molecular Devices, Sunnyvale, CA). The concentrations were calculated from the linear regression of the standard curve using SoftMax Pro software (Molecular Devices).

3.5.8 Biolayer Interferometry

Biolayer interferometry (BLI) was performed on a ForteBIO Octet (Menlo Park, CA) to determine the binding kinetics of RAGE for collagen I, collagen IV, laminin, and fibronectin according the manufacturer’s protocol and published methods. Purified mouse and bovine soluble RAGE were conjugated to amine-reactive sensor tips. The tips were then transferred into wells containing the matrix protein of interest in varying concentrations to promote association between the proteins. Binding was measured as a deflection in the wavelength. The tips were then transferred to PBS in order to allow dissociation of the interaction. To confirm specificity of the binding, binding studies were also performed with matrix proteins coupled to the sensor and sRAGE in solution. Binding curves were analyzed using the ForteBIO software, which performs a global fit according to the 1:1 Langmuir model, in order to obtain the kinetic rate constants for each condition.
3.6 HISTOLOGY

3.6.1 Hematoxylin and Eosin Staining

Four-micron thick lung sections were placed onto Superfrost Plus glass slides (Fisher Scientific). They were incubated overnight at 60 °C to enhance tissue adherence to the slides, deparaffinized in xylene (3x 10 min), and rehydrated in an ethanol series. They were then stained with Gill’s Hematoxylin (Vector Labs) for 1 min, counterstained with Eosin for 5 seconds, and dehydrated in an ethanol series. The slides were dipped into xylene and coverslips were fixed with Permount mounting media.

3.6.2 Sirius Red Staining

Sirius red was used to stain collagen and reticulin fibers as previously described. Sections of lung were deparaffinized with xylene and hydrated with an ethanol series, washed in tap water, and stained with a Sirius red/fast green solution for 30 min. at room temperature. They were washed again and dehydrated in an ethanol series and xylene.

3.6.3 Immunofluorescence

Four-micron thick lung sections were heated overnight at 50 °C. Paraffin was removed from the tissues by washing slides in xylene (3x, 10 min/wash). The slides were rehydrated in a series of ethanol washes (100, 95, 90, 80, and 70 %) for 2 min each. The slides were then incubated in water for 5 min. Antigen retrieval was performed by incubating the slides in a 1 mg/mL pepsin,
0.2 N HCl solution at 37 °C for 5 minutes. The slides were washed in PBS at room temperature (3x, 5 min/wash). The slides were then blocked for 1 hr at room temperature in a 2% BSA/PBS-T solution. The tissue was incubated with primary antibodies, goat anti-mouse full-length sRAGE (1:1000) and/or rabbit anti-collagen IV (1:100, Pierce, Cat # PA1-26148) diluted in 0.5% BSA/PBS-T for 1 h. Non-immune IgG and RAGE knockout lungs served as the control for RAGE staining while non-immune rabbit IgG was used as the control for collagen IV staining. The slides were washed three times in PBS-T and then incubated with secondary antibodies, donkey anti-rabbit AlexaFluor 488 conjugated (1:1,000, Invitrogen) and donkey anti-goat Cy3 conjugated (1:1,000, Jackson ImmunoResearch), diluted in 0.5% BSA/PBS-T for 1 h. The slides were washed and the nuclei stained with Hoescht (10 μg/mL, Invitrogen). The slides were washed in PBS and then cover-slipped with gelvatol mounting media and stored at 4 °C. All imaging was performed on an Olympus Fluoview 1000 confocal microscope (Center for Biologic Imaging, University of Pittsburgh). Capture conditions were kept constant for each slide so as to allow comparisons.

3.6.4 Histologic Scoring

Hematoxylin and eosin stained sections were scored by a pathologist (T.D.O) who was blinded to both treatment and strain as previously described. Individual fields were examined with a light microscope at 200X magnification. Every field in the entire lung was scored, starting peripherally. Each field had to contain >50% alveolar tissue/terminal bronchioles in order to be counted. Scoring was based upon the extent of interstitial fibrosis occupying the field according to the following scale: 0 = no fibrosis, 1 = 0-25%, 2 = 25-50%, 3 = 50-75%, and 4 = 75-100%. The pathologic index score was then reported as ratio of the sum of all the scores divided by the
total number of fields counted for each sample. Group scores were averaged for statistical analyses.

Table 3. Histologic Scoring Rubric.

<table>
<thead>
<tr>
<th>Histologic Score</th>
<th>Extent of Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No fibrosis</td>
</tr>
<tr>
<td>1</td>
<td>&lt; 25 %</td>
</tr>
<tr>
<td>2</td>
<td>25 - 50 %</td>
</tr>
<tr>
<td>3</td>
<td>50 – 75 %</td>
</tr>
<tr>
<td>4</td>
<td>&gt; 75 %</td>
</tr>
</tbody>
</table>

3.7 CELL CULTURE CONDITIONS AND ANALYSIS

3.7.1 Primary Alveolar Epithelial Cell Isolation

Primary murine type II alveolar epithelial cells were obtained from 6-7 week old female C57BL/6 and RAGE null mice as previously described.\textsuperscript{34, 241, 242} In brief, mice were sacrificed with a pentobarbital injection and exsanguinated by clipping the abdominal aorta. The lungs were perfused free of blood via the right ventricle with normal saline. A 20-gauge luer-stub adapter was inserted intratracheally and secured with a 3-0 silk suture. Two mL of a 50 U/mL Dispase (Gibco) solution was instilled into the lungs in order to enzymatically digest the cells away from the extracellular matrix. A single cell suspension was generated by filtering the cells through a 100-μm and 40-μm cell strainer (BD Biosciences) and then a 20 μm nylon mesh (Sefar, Depew, NY). Hematopoietic cells were removed by incubating the suspension in 100 mm\textsuperscript{2} cell culture plates coated with anti-CD32 (BD Biosciences) and anti-CD45 (BD Biosciences) antibodies at 37 °C / 5% CO\textsubscript{2} for 60 min. The non-adherent ATII cells were
removed by gentle washing and collected while the hematopoetic cells remained tightly adhered to the plates. The cells were pelleted by centrifugation at 130x g and then resuspended at the desired concentration. Typical experiments yielded 5-6 million cells per mouse with a purity of >95% as determined by modified Papanicolau staining. Cells were grown in DMEM (Hyclone, Logan, UT) containing 10% FCS (Gemini Bio Products, West Sacramento, CA) and 100 U/mL penicillin and 100 μg/mL streptomycin on collagen I coated flasks (BD Biosciences) to promote differentiation into ATI-like cells. Cells were maintained in cell incubators at 37 °C / 5% CO2 for all parts of the experiment. All experiments were conducted on cells that were 6-9 days old as RAGE expression was previously found to be highest at this point along with other markers of ATI cells and a lack of ATII cell markers.

3.7.2  In Vitro Scratch Assay

Wound healing assays were performed as described in detail previously. Briefly, wild-type and RAGE null primary cells were passaged on day 4 of culture and plated at 40,000 cells/well in a 24-well collagen IV coated plates (BD Biosciences) in growth media. The cells were grown for 2-3 additional days until a confluent monolayer was reached. At this point, the plates were scratched with a p-200 pipet tip to make a uniform wound in all the wells. The wells were washed two times with PBS to remove the scraped cells and a mark was placed on the underside of each well to ensure that images of each wound were captured at the same place. The initial (t = 0 hr) images of the wound were captured with phase-contrast microscopy and the PBS in the wells was replaced with 500 μl of treatment media. The following treatments were used: Media alone, Media containing 0.075 units bleomycin/mL, and Media containing 20 μg/mL crocidolite.
asbestos. When sRAGE was included in the experiments it was used at a final concentration of 25 µg/mL. Six wells were used for each treatment condition. The experiment was stopped when one of the treatments was fully healed. Images of the wells were captured at the same place as the initial image. The area of the wound was measured by outlining the areas without cells using Metamorph Software (Molecular Devices, Sunnyvale, CA). Percent healing was calculated as:

\[
\% \text{ Wound Healing} = \frac{\text{Area } t_{\text{initial}} - \text{Area } t_{\text{final}}}{\text{Area } t_{\text{initial}}} \times 100
\]

### 3.7.3 MTS Cell Viability Assay

Primary alveolar epithelial cells (6-9 days old) from C57BL/6 and RAGE null mice were plated at a cell density of 5,000 cells/well in a 96-well cell culture plate (Corning, Lowell, MA). They were allowed to adhere for 24 hours. The growth media was then replaced with 100 µL of treatment media containing various dilutions of asbestos (0 - 100 µg/mL) or bleomycin (0 – 0.3 units/mL). In addition, sRAGE was included in half of the treated wells at a concentration of 25 µg/mL. Each treatment and genotype was performed in triplicate. After 24 hrs in culture with the treatment, an MTS reagent (Cat# G5421, Promega, Madison, WI) was prepared according to the manufacturer’s protocol and 20 µL was added to each well. The absorbance of each well at 490 nm was read on a Molecular Devices Spectramax 96-well plate reader immediately after the addition of the MTS reagent. The plate was then read again at 4 hrs. Each well served as its own blank by subtracting the absorbance at 0 hrs from the absorbance at 4 hrs. This corrected for any absorbance that was due to the asbestos particles themselves. The percentage of viable cells was calculated by dividing the absorbance of the treatment by the absorbance of the untreated (media only) wild-type or RAGE KO cells.
3.8 STATISTICAL ANALYSIS

Paired samples were analyzed using Student’s $t$-test. ANOVA followed by a Tukey post-test was used for multiple comparisons to one control. Experiments with two variables (i.e., i.p. treatment and i.t. treatment or i.t. treatment and strain) were analyzed by two-way ANOVA. Values are reported +/-SEM, and p-values $<0.05$ were considered significant.
4.0 RESULTS – RAGE AND IPF

4.1 SOLUBLE RAGE PURIFICATION

Soluble RAGE was purified from lung tissue homogenates utilizing four columns. sRAGE was isocratically eluted from the Concanavalin A column in 1000 mL of buffer. This was directly applied to the heparin sepharose column which was then eluted using a sodium chloride gradient. Fractions 19-41 of the heparin sepharose elution were found to contain sRAGE and were pooled (Figure 3) before being loaded onto the HiTrapQ column. Protein that bound to this column was eluted with an isocratic profile of 35% buffer B. All of the protein was eluted within 20 minutes and pooled (Figure 4A). A single band was identified in the final product with a molecular weight of ~ 45 kDa (Figure 4 B,C). Both the mouse and bovine products were confirmed to be sRAGE by mass spectral analyses (performed by Dr. Jan Enghild). No other proteins were detectable by coomassie blue staining in the final product indicating very high purity, an improvement over previous studies (Figure 4). The protocol resulted in a 46% yield with the greatest loss of protein occurring during the Heparin Sepharose step (Table 4). The final product contained less than 0.12 endotoxin units/50 µg of protein of after passage over the Detoxi-Gel column as measured by the LAL Pyrotell test. The amounts of tissue and column volumes prevented oversaturation and loss of sRAGE in the column washes. Minimal amounts of
sRAGE were detected by western blot in the Heparin Sepharose and HiTrap Q washes (not shown). The final product was found to bind to recombinant HMGB1 (Figure 5).

Figure 3. sRAGE elution from Heparin Sepharose. The eluate from the Concanavalin A column was filtered and applied to a column containing Heparin Sepharose. (A) The bound protein was eluted with a NaCl gradient while measuring the absorbance at 280 nm. Fractions were collected every minute and samples were subjected to reducing SDS-PAGE. (B) The gel was stained with coomassie blue and fractions 19-41 contained sRAGE as well as a lower molecular weight contaminate. Fractions 19-41 were pooled.
Figure 4. sRAGE elution from HiTrapQ column. The pooled and dialyzed eluate from the Heparin Sepharose column was applied to a HiTrap Q anion exchange column. (A) The protein was completely eluted with an isocratic elution of 35% Buffer B between 3 and 20 minutes. No additional protein eluted with a subsequent gradient of 35-100% Buffer B. (B) SDS-PAGE of the final mouse sRAGE product contained as a single band of ~45 kDa. Fractions 3-20 were pooled. (C) Bovine sRAGE was purified in an identical fashion. Reducing SDS-PAGE revealed a single band of ~45 kDa.

Table 4. Summary of the results of each step in the sRAGE purification

<table>
<thead>
<tr>
<th>Step</th>
<th>sRAGE Concentration (μg/mL)</th>
<th>Pool Volume (mL)</th>
<th>Total sRAGE (mg)</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lung Homogenate</td>
<td>36.2</td>
<td>625</td>
<td>22.6</td>
<td>100</td>
</tr>
<tr>
<td>2. Concanavalin A</td>
<td>21.1</td>
<td>1000</td>
<td>21.1</td>
<td>93</td>
</tr>
<tr>
<td>3. Heparin Sepharose</td>
<td>97.5</td>
<td>148.5</td>
<td>14.5</td>
<td>64</td>
</tr>
<tr>
<td>4. HiTrap Q</td>
<td>762.7</td>
<td>18</td>
<td>13.7</td>
<td>61</td>
</tr>
<tr>
<td>5. Detoxi-Gel</td>
<td>604.8</td>
<td>17</td>
<td>10.3</td>
<td>46</td>
</tr>
</tbody>
</table>
Figure 5. Evaluation of sRAGE Bioactivity. The function of purified sRAGE was measured by its ability to bind HMGB1. ELISA plates were coated with HMGB1 or BSA and then sRAGE was added to each well. sRAGE that remained bound to HMGB1 was detected with anti-RAGE antibodies. sRAGE bound to HMGB1 suggesting that its function was unaltered by the purification.

4.2 PULMONARY RAGE EXPRESSION IS DOWNREGULATED AFTER ASBESTOS INJURY

To investigate the regulation of mRAGE and sRAGE expression in pulmonary fibrosis, an asbestos mouse model of pulmonary fibrosis was utilized. In this model, it was revealed that pulmonary mRAGE and sRAGE levels decrease significantly at 14 days (Figure 6 a,b). This loss corresponds with a 1.5-fold decrease in mRNA expression of the RAGE gene after 14 days of asbestos treatment as compared to a titanium dioxide treatment (Figure 6c). The loss of RAGE in the asbestos model corresponded with a similar loss of aquaporin-5, a marker of type I alveolar epithelial cells (Figure 6b).
Figure 6. mRAGE and sRAGE expression are decreased after asbestos injury. C57Bl/6 mice were treated with 0.1mg crocidolite asbestos or titanium dioxide (TiO₂) as an inert particulate control. Fourteen days later, lungs were extracted to obtain both membrane and soluble protein fractions. mRAGE, sRAGE, and aquaporin-5 expression were analyzed by western blot analysis (a). PVDF membranes were stripped and re-probed for β-tubulin as a loading control. Shown to the right is the densitometric analysis (b), where the protein of interest band intensities are normalized to β-tubulin band intensities for each lane. RNA was isolated from the lungs of mice 14 days after they were treated with asbestos and compared with normal lungs from mice treated with titanium dioxide for 24 hrs. RAGE mRNA expression was measured by real-time PCR and normalized to GAPDH. Results are reported as a percent relative quantity compared to the TiO₂-treated group (c). A 1.5-fold decrease in RAGE expression was seen after asbestos treatment. (**p<0.01 and *p<0.05 compared to TiO₂-treated controls)
4.3 LOSS OF MEMBRANE AND SOLUBLE RAGE EXPRESSION IN HUMAN IPF LUNGS

To determine if observations from the animal model corresponded with human disease, IPF tissue samples were obtained from diagnostic biopsies or patients undergoing lung transplantation and control lung specimens from histologically normal lung samples resected from patients with lung cancer. Microarray analysis performed on these human lung samples (performed by the lab of Dr. Naftali Kaminski) indicated that RAGE is the most down regulated gene transcript in IPF lungs compared to control lungs, demonstrating a 4.65-fold reduction in IPF lungs (Figure 7a).

To confirm the findings from the microarray experiments, protein expression in another set of human IPF and control lung specimens was examined. These studies demonstrate that pulmonary mRAGE and sRAGE protein levels are reduced in IPF lungs compared to control lungs (Figure 7b). Taken together, these data indicate a depletion of RAGE mRNA and protein expression in pulmonary fibrosis pathogenesis.
Figure 7. RAGE transcripts and mRAGE/sRAGE protein are down regulated in IPF lungs. Microarray analysis was performed on human IPF lung samples or control lung samples to compare expression of RAGE transcripts between IPF and control lungs. a) Plot demonstrating the RAGE transcript level in each patient sample. RAGE transcript levels are significantly decreased in IPF lungs compared to control lungs (p=0.0000054). b) Western blot analysis on a different set of patient samples demonstrates a reduction in mRAGE (* p < 0.05) and sRAGE (** p < 0.01) protein levels in fibrotic areas of IPF lungs. Densitometry is shown with band intensities of mRAGE and sRAGE normalized to β-actin as a loading control.

4.4 ABSENCE OF MEMBRANE AND SOLUBLE RAGE EXPRESSION LEADS TO PULMONARY FIBROSIS-LIKE ALTERATIONS.

To determine if mRAGE and sRAGE loss contributes to pulmonary fibrosis pathogenesis, RAGE knockout mice were examined. RAGE knockout mice were allowed to age without any injury (Histologic aging studies were performed by Dr. Michael Kasper and Dr. Angelika Bierhaus, University of Heidelberg). These aged knockout mice were found to spontaneously develop fibrosis-like alterations in their lungs. Sirius Red staining showed visually apparent increases in collagen staining in the knockout mice as compared to wild-type (Figure 8).
Figure 8 Sirius Red staining of Aged Mouse Lungs. Lung sections from 19-24 month old wild-type and RAGE knockout mice were subjected to Sirius Red staining for total collagen. RAGE knockout lungs show increased collagen (red) staining.

As another means of assessing fibrosis in the lungs of RAGE null mice, hydroxyproline quantification was performed on the entire right lung from 48-week old RAGE knockout mice or age-matched wild-type controls. Notably, hydroxyproline levels in the right lungs from RAGE knockout mice are significantly higher than those from control mice (Figure 9 B). No difference in hydroxyproline levels were seen between 8 and 12-week old RAGE knockout and wild-type mice.

Hydroxyproline quantification was performed on total lungs (right and left) from 6-month old RAGE knockout and wild-type mice (n=5 for both groups) (Figure 9A). A similar increase in collagen content of the lung was noted with an average hydroxyproline content of 260 µg/total lung in the wild-type versus 367 µg/total lung in the RAGE knockouts.
Figure 9. Aged RAGE KO mice have more hydroxyproline in their lungs. A) Total lungs (left and right) from 24-week old wild-type (n=5) and RAGE KO (n=5) mice were analyzed for hydroxyproline content. B) Right lungs from 48-week old wild-type (n=3) or RAGE knockout mice (n=4) were subjected to hydroxyproline analysis. Both 24-week and 48-week old RAGE KO mice have significantly increased levels of hydroxyproline compared to age-matched wild-type controls. (**p<0.01 and *p<0.05)

4.5 INTRAPERITONEAL ADMINISTRATION OF SOLUBLE RAGE

To determine if intraperitoneal treatment of soluble RAGE was capable of reaching the lung, RAGE knockout mice were treated with 50 μg of sRAGE once a day for three days. On the third day, the mice were sacrificed 1-hr post-treatment, saline was instilled and recovered to generate BAL fluid, and the lungs were removed for homogenization. The BAL fluid and lung homogenate were analyzed by western blot analysis for RAGE. No sRAGE was detected in the BAL fluid of the mice, however, sRAGE was detectable in the lungs of RAGE KO mice treated with sRAGE (Figure 10). Since RAGE KO mice lack both the membrane and soluble isoforms
this experiment confirmed that i.p. administration of soluble RAGE was capable of reaching the lung. However, it was noted that the levels obtained in the lung were lower than expected.

**Figure 10. I.P. sRAGE is Detectable in the Lung.** RAGE KO mice were treated for 3 days with 50 μg of sRAGE by i.p. injection. Exogenous sRAGE was detectable in their lungs by western blot.

### 4.6 INTRATRACHEAL ADMINISTRATION OF SOLUBLE RAGE IS RAPIDLY CLEARED FROM THE LUNG

Intraperitoneal administration of soluble RAGE had low bioavailability in the lung. To determine if intratracheal administration of sRAGE had a better pharmacokinetic profile, RAGE KO mice were treated with 50 μg of sRAGE via intratracheal instillation. Mice were sacrificed at 0, 1, 2, 4, 6, and 8 hours as well as at 1, 3, 5 and 7 days post-i.t. treatment. Upon sacrifice, the BAL fluid was obtained and the lungs were flash frozen. RAGE expression in the lung was analyzed by western blotting. No RAGE was detected beyond the 8 h time point in any of the samples. RAGE expression was high in the BAL fluid starting at the 0 h time point and then rapidly decreased until it was last detected at 8 h (Figure 11A). During this time period there was a simultaneous increase in the amount of sRAGE detectable in the lung homogenates (Figure 11B), suggesting that the sRAGE was capable of either entering or binding to the lung.
tissue. Unfortunately, by 24 h there was no sRAGE detectable in the lung tissue making this administration route a challenge for chronic treatment.

Figure 11. Pharmacokinetic profile of i.t. sRAGE. RAGE KO mice were treated with 50 µg of sRAGE. Equal volumes of BAL fluid (A) and equal amount of lung protein were separated by reducing SDS-PAGE. The proteins were transferred to PVDF membranes and analyzed for RAGE expression by western blot. sRAGE protein was detectable in the BAL fluid (A) and lung (B) up to 8 h post-treatment. The amount of sRAGE in the BAL fluid decreased post-treatment while simulatenously increasing in the lung tissue. All RAGE was gone from the BALF and lung by 24 hrs.

4.7 ABSENCE OF MEMBRANE RAGE EXPRESSION INCREASES THE SEVERITY OF PULMONARY FIBROSIS

To further examine the role of mRAGE and sRAGE in pulmonary fibrosis, 8-week old RAGE null mice and age-matched wild-type controls were treated with asbestos and the lungs were examined 14 days after this injury. These studies demonstrate that RAGE null mice develop more severe pulmonary fibrosis as measured by both histologic scoring (Figure 12a,b,c,d,e) and total lung hydroxyproline quantification (Figure 12f).
To determine if responses seen in the RAGE null mice were a result of the absence of sRAGE or mRAGE, RAGE null mice were treated with asbestos and given sRAGE by daily i.p. injections. There was no difference in the degree of fibrosis as measured by relative hydroxyproline quantification between those treated with sRAGE and those treated with bovine serum albumin as a control (Figure 12g).
Figure 12 RAGE knockout (KO) mice develop more severe fibrosis after asbestos treatment. RAGE KO mice and C57Bl/6 wild-type mice were treated with 0.1mg crocidolite asbestos or titanium dioxide (TiO\textsubscript{2}) as an inert particulate control. Fourteen days later, lungs were either inflation fixed with 10\% formalin for histology (n=3 per group) or removed and dried for hydroxyproline quantification (n=5 per group). Light microscopic analysis (scale bar = 30 \mu m (a.,b.) & 6 \mu m (c., d.)) demonstrates increased alveolar thickening in the RAGE-null mice (b., d.)
compared to wild-type (a., c.). (e.) Histologic scoring by a pathologist blinded to genotype and treatment demonstrates more severe fibrosis in the asbestos treated RAGE KO group compared to wild-type controls. No fibrosis was appreciated in either of the TiO2 treated groups. (f.) Asbestos treatment resulted in increased fibrosis in the RAGE KO mice as determined by hydroxyproline levels compared to wild-type mice. (g.) RAGE KO mice were subjected to asbestos treatment along with intraperitoneal injections of either bovine sRAGE or bovine serum albumin (50 µg/day). There was no difference in the degree of fibrosis between the groups (n=5 per group) as measured by total lung hydroxyproline. **p<0.01 and *p<0.05 compared to TiO2-treated controls.

4.8 EXOGENOUSLY ADMINISTERED SOLUBLE RAGE DOES NOT PROTECT AGAINST ASBESTOS-INDUCED FIBROSIS

To determine if RAGE ligands were responsible for the fibrotic injury in response to asbestos, wild-type mice were treated daily with either bovine sRAGE or bovine serum albumin (BSA) by i.p. injection. C57BL/6 mice received a single instillation of 100 µg of either asbestos or titanium dioxide. Starting 72 hours prior to injury, daily i.p. injections of bovine sRAGE or BSA were initiated. After 14 days, the animals were sacrificed and their lungs evaluated for hydroxyproline content. Asbestos treatment resulted in a significant increase in fibrosis, however, no differences were noted between the sRAGE and BSA treated groups (Figure 13A). The BALF was used as a second indicator of injury. Asbestos treatment resulted in increases in protein and cellular content of the BALF, however, sRAGE treatment had no effect on these parameters (Figure 13B,C). Asbestos injury also resulted in a significant weight loss, though no differences were seen with sRAGE treatment (Figure 13D).
Figure 13. Exogenous sRAGE administration does not protect against asbestos-induced fibrosis.

C57Bl/6 mice were treated with 100 μg of crocidolite asbestos (TiO₂ control) (n=6 for all groups). They received daily intraperitoneal injections of 50 μg of sRAGE or BSA vehicle control starting 3 days prior to pulmonary injury. sRAGE had no effect on any disease parameters in either model. RAGE KO mice were treated for 3 days with 50 ug of sRAGE by i.p. injection. (**p<0.01 and *p<0.05)

4.9 ABSENCE OF MEMBRANE AND SOLUBLE RAGE EXPRESSION PROTECTS AGAINST BLEOMYCIN-INDUCED FIBROSIS

To investigate the function of RAGE in bleomycin-induced fibrosis, C57BL/6 and RAGE null mice were treated with a single intratracheal instillation of 0.04 units of bleomycin and then monitored before sacrificing at either 14 or 21 days. The extent of fibrotic injury was assessed both histologically and by hydroxyproline analysis. It was revealed that the absence of mRAGE
and sRAGE offered significant protection against bleomycin-induced fibrosis. Histologic evaluation revealed very few fields containing any appreciable fibrosis in the RAGE null mice (Figures 14D & 15D). In contrast, the wild-type mice had extensive fibrosis extending from the bronchi out to the pleura (Figures 14C & 15C). The wild-type mice had significantly elevated protein and white blood cells in their BAL fluid at both time points compared to the RAGE knockout mice (Figure 14E,F & 15B,E).

Figure 14. Lack of RAGE protects against bleomycin injury at 14 days. C57Bl/6 and RAGE KO mice were treated with 0.04 units of bleomycin and sacrificed 14-days post exposure. Their lungs were removed and analyzed by hydroxyproline quantification (n = 6-7/group) (A) and histologic analyses (n = 3/group) (B). The extent
of fibrotic injury was muted in the mice lacking RAGE (D) compared to wild-type controls (C). Two markers of inflammation, total protein content (E) and cell counts (F), were also dampened in the RAGE KO mice. (**p<0.01 and *p<0.05)

Figure 15. Lack of RAGE protects against bleomycin injury at 21 days. C57Bl/6 and RAGE KO mice were treated with 0.04 units of bleomycin and sacrificed 21-days post exposure. The lungs were removed, inflation fixed, and analyzed by histologic evaluation (A) (n = 6-7/bleomycin group and n = 4/saline group). Blinded
evaluation of the lungs revealed that lack of RAGE protects against bleomycin-induced fibrosis (D) compared to wild-type controls (C). Inflammatory markers, protein concentration (B) and cells (E), were significantly reduced in the BALF of RAGE KO mice. No differences in the cellular population of the BALF were noted between strains (F). (**p<0.01 and *p<0.05)

4.10 INTRAPERITONEAL ADMINISTRATION OF SOLUBLE RAGE DOES NOT PROTECT AGAINST BLEOMYCIN-INDUCED FIBROSIS

To determine if ligand signaling through mRAGE was the mechanism of fibrosis in response to bleomycin, wild-type mice were treated daily with either sRAGE or bovine serum albumin (BSA) by i.p. injection. C57BL/6 mice were treated with an intratracheal instillation of 0.04 units of bleomycin or saline control. Starting 72 hours prior to the lung injury, daily i.p. administration of 50 μg of bovine sRAGE or BSA was initiated. The mice were monitored daily and sacrificed 14 days after bleomycin treatment. The extent of fibrotic injury was measured by hydroxyproline quantification. Significant increases in hydroxyproline content were noted in the lungs of the mice that received bleomycin treatment (Figure 16A). However, no difference in hydroxyproline content was seen between the sRAGE and BSA treated mice. As a second marker of injury we looked at protein and cellularity in the BAL fluid. sRAGE treatment did not have any effect on the amount of protein or number of cells in the BALF (Figures 16B,C). There were also no significant differences in the amount of weight loss between the groups. (Figure 16 D).
C57Bl/6 mice were treated with 0.04 units of bleomycin (n=6/group) or saline control (n= 3-5/group). They received daily intraperitoneal injections of 50 μg of sRAGE or BSA vehicle control starting 3 days prior to bleomycin injury. sRAGE had no effect on any disease parameter.

4.11 INTRATRACHEAL ADMINISTRATION OF SOLUBLE RAGE DOES NOT PROTECT AGAINST BLEOMYCIN-INDUCED FIBROSIS

C57BL/6 mice were treated with an intratracheal instillation of 0.04 units of bleomycin or saline control. On days 4 and 8 of the experiment, the mice received an intratracheal instillation of 50 μg of bovine sRAGE or BSA. The mice were monitored daily and sacrificed 14 days after bleomycin treatment. The extent of fibrotic injury was measured by hydroxyproline
quantification. Mice treated with bleomycin had significant increases in total collagen content of the lung, however, sRAGE administration had no effect on this (Figure 17A). Notably, sRAGE had no effect on the protein concentration (Figure 17C) of the BAL fluid, but increased the total number of cells in the saline-treated group (Figure 17B). Histologic evaluation of the lungs revealed a vasculitis of the saline treated mice that received sRAGE suggesting an immunological reaction (Figure 17E). Analysis of the white blood cell population in the BAL fluid further suggested this with the presence of a large population of eosinophils (Figure 17F). No differences in weight loss were noted in the BSA or sRAGE treated groups, but all bleomycin treated mice lost significantly more weight (Figure 17D).
Figure 17. Intratracheal sRAGE does not protect against bleomycin-induced fibrosis. C57BL/6 mice were treated with 0.04 units of bleomycin. On days 4 and 8 post-injury sRAGE or BSA was administered by i.t. instillation. The mice were sacrificed on day 14 of the experiment. Bleomycin treatment resulted in significant increases in hydroxyproline content of the lung, however sRAGE had no effect compared to BSA (A). I.t. administered sRAGE caused a significant increase in the total number of cells present in the BAL fluid compared to BSA (B). No differences in the protein concentration of the BAL fluid were seen between the sRAGE and BSA treated groups, though bleomycin resulted in a large increase (C). The bleomycin treated mice lost significantly more weight, however there was no difference between sRAGE and BSA treatment (D). Histologic evaluation of saline/sRAGE treated mice revealed an inflammatory reaction in the vessels of the lung (E, black arrows indicate inflammatory cells). Differential analysis of the WBC’s in the BAL fluid revealed a large population of eosinophils.
4.12 ABSENCE OF MEMBRANE AND SOLUBLE RAGE DOES NOT ALTER BLEOMYCIN HYDROLASE EXPRESSION

To determine if RAGE null mice were ultra-metabolizers of bleomycin the expression of bleomycin hydrolase in the lungs of wild-type and RAGE null mice was investigated. Eight-week old male mice were sacrificed and their lungs processed for protein analysis. Western blot analysis of bleomycin hydrolase revealed that there was no difference in expression between wild-type and RAGE null mice (Figure 18).

Figure 18. Bleomycin hydrolase is unaltered in RAGE KO mice. Eight-week old wild-type and RAGE KO lungs were homogenized. Total protein was separated by reducing SDS-PAGE and transferred to PVDF membrane. Proteins were detected with specific antibodies and visualized with enhanced chemiluminescence. RAGE null mice have no alterations in bleomycin hydrolase expression to account for their protection against bleomycin injury.
Primary alveolar epithelial cells were isolated to perform *in vitro* studies. The purity of the isolation was found to be > 95%, as characterized by the presence of mucin-staining (surfactant) vesicles in the cytoplasm on modified Papanicolau stain (Figure 19A). In addition, it was confirmed that these cells expressed RAGE in culture (Figure 19B). To determine if the paradoxical response to fibrotic injuries was due to differences in cell viability after injury, killing assays were performed on primary alveolar epithelial cells from wild-type and RAGE null mice. Additionally, soluble RAGE was administered to half of the wells to determine if RAGE ligands were being released and propagating the injury. It was found that both wild-type and RAGE null cells were equally susceptible to cellular death from the administration of bleomycin or asbestos (Figure 20 A, D). Furthermore, exogenous sRAGE administration neither protected nor augmented injury to either cell type (Figure 20 B,C,E,F).

**Figure 19.** Alveolar epithelial cells express RAGE in culture. Primary alveolar epithelial cells were isolated from wild-type (WT) and RAGE knockout (KO) mice. A modified Papanicolau stain demonstrates the...
presence of surfactant granules in the cytoplasm of the isolated product confirming their identity as AT-II cells (A).

The cells were grown in culture on collagen coated plates for 7 days. The cells were then lysed in 1% Triton X-100 buffer in order to obtain both soluble and membrane proteins. Equal amounts of protein were separated by SDS-PAGE, transferred to a PVDF membrane and probed with anti-RAGE antibodies. Expression of the RAGE protein was noted in the wild-type cell lysate (B).

Figure 20. RAGE expression of alveolar epithelial cells has no effect on cell death in response to bleomycin or asbestos. Primary alveolar epithelial cells were isolated from C57Bl/6 and RAGE KO mice. After 6 days in culture, they were treated with increasing concentrations of asbestos or bleomycin. sRAGE (25 µg/mL) was added to the media of half the treatments. Each treatment was run in triplicate. After 24 hours, an MTS reagent was added to each well to assess cell viability. Wild-type and RAGE KO cells were equally susceptible to bleomycin (A) and asbestos (D) injury. sRAGE had no effect on viability of either cell type or either injury (B,C,E,F).
4.14 LACK OF MEMBRANE RAGE IMPROVES RE-EPITHELIALIZATION IN RESPONSE TO BLEOMYCIN BUT NOT ASBESTOS

To investigate differences in re-epithelialization after injury, *in vitro* scratch assays were performed on wild-type and RAGE null primary alveolar epithelial cells in the presence of bleomycin and asbestos. In addition, sRAGE was administered exogenously to better distinguish the impact of each RAGE isoform. No inherent differences in the untreated cells were seen after injury (Figure 21A). However, it was consistently found that RAGE null cells healed significantly better than wild-type cells when injured with bleomycin (Figure 21B). In contrast, when the two different cell types were treated with asbestos both cell types had their ability to heal inhibited equally (Figure 21C). The only effect noted from the treatment of sRAGE was that it consistently impaired the ability of wounded RAGE KO cells to heal in the presence of asbestos or bleomycin (Figure 21B,C). However, sRAGE treatment of RAGE KO cells that were wounded without additional injury had no effect (Figure 21A).
Figure 21. Lack of RAGE expression improves re-epithelialization in response to bleomycin. Primary alveolar epithelial cells from C57Bl/6 and RAGE KO mice were subjected to a wound scratch assay in the presence of bleomycin or asbestos. In addition, sRAGE (25 μg/mL) was added to half of the wells. An n of 6 was used for each treatment condition. Images were captured at 0 and 14 hrs and the wound areas were measured. sRAGE treatment had no effect on the healing of wild-type and RAGE KO cells without asbestos or bleomycin (A). Bleomycin significantly impaired wild-type cells ability to heal (B), but not RAGE KO cells. Both wild-type and RAGE KO cells had impaired healing in the presence of asbestos (C). Representative wound healing images (D).
5.0 RESULTS - DIABETES, PULMONARY RAGE EXPRESSION AND PULMONARY FIBROSIS

5.1 TYPE I DIABETES IN RODENTS DOES NOT ALTER PULMONARY RAGE EXPRESSION

To investigate the effect of hyperglycemia and elevated AGEs on pulmonary RAGE expression, lungs were obtained from both young (8-week old) and old (7-month old, Dr. Anne Marie Schmidt, Columbia University) Ove26 mice that were untreated. These mice all had serum glucose levels above 600 mg/dL at sacrifice. In addition, lungs were obtained from DP-BB (Dr. Irina Smirnova, Kansas University Medical Center), a rat type-I diabetic model, that were 4 months of age at sacrifice. Soluble and membrane protein fractions were prepared from 50 mg of lung tissue. Equal amounts of protein were separated by reducing SDS-PAGE and transferred to PVDF membranes. The membranes were probed for RAGE, stripped, and then re-probed for beta-actin. No difference was seen in either soluble or membrane RAGE protein expression in any of the three diabetic models (Figure 22-24). In addition, there was no difference seen in RAGE mRNA expression in the 8-week old Ove26 mice (Figure 22C).
Figure 22. RAGE expression is unaltered in 8-week old Ove26 mice. The lungs of 8-week old untreated FVB and Ove26 mice were analyzed for RAGE protein and mRNA expression. Both isoforms of RAGE (A,B) and the mRNA (C) expression were unchanged by diabetes.
Figure 23. RAGE expression is unaltered in 7-month old Ove26 mice. The lungs of 7-month old untreated FVB and Ove26 mice were analyzed for RAGE protein. Both soluble (A) and membrane (B) RAGE expression were unchanged by chronic diabetes.
Figure 24. RAGE expression is unaltered in 4-month old DP-BB rats. The lungs of aged DR-BB and DP-BB rats were analyzed for RAGE protein. Both soluble (A) and membrane (B) RAGE expression were unchanged in rat model of chronic type-I diabetes.

5.2 Type II Diabetes in Rodents Does Not Alter Pulmonary RAGE Expression

To investigate the effect of hyperglycemia and elevated AGEs on pulmonary RAGE expression in a model of type II diabetes the lungs of 9-month old $db/db$ mice (Lab of Dr. Anne Marie Schmidt, Columbia University) and 4-month old Zucker diabetic fatty rats (Lab of Dr. Irina Smirnova, Kansas University Medical Center) were obtained. Soluble and membrane protein fractions were prepared from 50 mg of lung tissue. Equal amounts of protein were separated by reducing SDS-PAGE and transferred to PVDF membranes. The membranes were probed for RAGE, stripped, and then re-probed for beta-actin. Densitometry was performed on the bands of
interest and normalized net intensities were obtained by dividing the intensity of the beta-actin band from the RAGE band. There were no differences in either soluble or membrane RAGE protein expression in either of the type II diabetes models (Figure 25 & 26).

Figure 25. RAGE expression is unaltered in aged db/db mice. The lungs of 9-month old homozygous db/db (diabetic) and heterozygous db/m (non-diabetic control) mice were analyzed for RAGE protein. Both soluble (A) and membrane (B) RAGE expression were unchanged in this mouse model of chronic type-II diabetes.
Figure 26. RAGE expression is unaltered in aged Zucker diabetic fatty rats. The lungs of 4-month old Zucker diabetic fatty (diabetic) and Zucker lean (non-diabetic control) mice were analyzed for RAGE protein. Both soluble (A) and membrane (B) RAGE expression were unchanged in this rat model of chronic type-II diabetes.

5.3 TYPE I DIABETES IN MICE MITIGATES THE FIBROTIC RESPONSE TO ASBESTOS

AGEs have been previously shown to accumulate in the lungs of IPF patients. In order to investigate if AGEs contribute to the fibrotic process, euglycemic (FVB) and diabetic (Ove26) mice were treated with asbestos to induce fibrotic injury. It was revealed that diabetic mice had significantly less fibrosis than euglycemic controls at 14 days by both hydroxyproline and histologic analysis (Figure 27A,B). There was no difference in the protein concentration or cell population of the BAL fluid (Figure 27C,D). The wild-type animals treated with asbestos lost
significantly more weight than the diabetic animals (Figure 27E). This is consistent with the findings that the wild-type mice had more fibrosis.

**Figure 27. Diabetes dampens the fibrotic response to asbestos.** FVB (wild-type) and Ove26 (diabetic) mice were treated with 0.1 mg of crocidolite asbestos or TiO$_2$. After 14 days, the mice were sacrificed and samples were collected for analysis. The diabetic mice had reduced fibrosis as measured by hydroxyproline (A) and histologic (B) analysis from two separate experiments. White blood cell analysis of the BAL fluid of the asbestos-treated animals revealed no differences in inflammation (C). Asbestos-treated animals had more cells in their BAL fluid, but there was no difference between mouse strains (D). Asbestos-treated animals lost significantly more weight than TiO$_2$-treated and wild-type animals lost more weight than diabetics after asbestos injury (E).
6.0 RESULTS – RAGE AND THE EXTRACELLULAR MATRIX

6.1 RAGE CO-LOCALIZES WITH THE BASEMENT MEMBRANE IN THE LUNG

Previous studies have suggested that RAGE was expressed on the basolateral surface of type-I alveolar epithelial cells along the basement membrane. Unfortunately, many immunochemical studies have been confounded by the fact that the antibodies also react with tissue from RAGE knockout mice (Figure 28). A novel antibody against full-length mouse soluble RAGE was developed to investigate RAGE expression and localization. The new goat anti-RAGE antibody did not stain RAGE KO lung tissue when subjected to the same conditions as wild-type RAGE expressing tissue (Figure 29). RAGE staining was only found in the alveolar epithelium and no staining was seen in the vasculature or bronchial cells of the lung (Figure 29). Double-labeling studies with an antibody specific for collagen IV revealed extensive co-localization of RAGE expression with the alveolar basement membrane (Figure 30).
Figure 28. Many RAGE antibodies are non-specific. RAGE KO and wild-type lung homogenate were separated by reducing SDS-PAGE and transferred to a PVDF membrane. The membrane was then probed with a widely-used RAGE “specific” antibody. In addition to detecting mRAGE and sRAGE at the appropriate molecular weights (50 and 45 kDa), it also detects a doublet in the RAGE KO’s just above 50 kDa that looks very similar to the real RAGE doublet. This demonstrates that when investigating RAGE expression, KO tissue should be used as a negative control for most analyses.

Figure 29. RAGE immunofluorescent staining of normal lung. Wild-type and RAGE knockout lung sections were stained with anti-RAGE antibodies. Staining was visualized with a Cy3-conjugated secondary
antibody. Nuclei were visualized with Hoescht stain. There is extensive labeling of the alveolar epithelium in the wild-type lung that is not present in the RAGE KO section. In addition, there is a lack of staining in the bronchial epithelium of the wild-type lung (white arrows) (Magnification = 40X).

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<tr>
<th>Collagen IV</th>
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**Figure 30. RAGE co-localizes with collagen IV in the lung.** Lungs from wild-type and RAGE knockout (KO) mice were inflation fixed and embedded in paraffin. 5-µm thick sections were immunolabeled with anti-RAGE and anti-collagen IV antibodies along with their respective fluorophore conjugated secondary antibodies. Hoescht stain was used to visualize the nuclei. Pre-immune and non-immune sera were used as controls for the RAGE and collagen IV antibodies, respectively. Co-localization of RAGE and Collagen IV (yellow) is noted throughout the alveolar space. (Magnification = 100x)
6.2 RAGE BINDS TO PROTEINS OF THE EXTRACELLULAR MATRIX WITH HIGH AFFINITY

RAGE’s ability to bind to proteins of the extracellular matrix was analyzed using a FortéBIO® Octet (Menlo Park, CA) system according to the manufacturer’s protocol. This system has been found to be equivalent to surface plasmon resonance in obtaining binding kinetics for a ligand and its receptor. Soluble RAGE was found to have very high affinity for collagen I, collagen IV, and laminin, but not fibronectin. Kd values of 3.29 nM for collagen IV, 2.04 nM for collagen I, and 1.18 nM for laminin were obtained for studies with sRAGE(Figure 32 & 33). Notably, no binding was seen with fibronectin (Figure 33) indicating that RAGE does not have affinity for all fibrillar proteins and that this affinity for the other three proteins is likely specific. For studies involving collagen I and collagen IV, the affinities were obtained for both sRAGE as the ligand (sRAGE in solution; collagen on the surface) and as the receptor (sRAGE on the surface; collagen in solution). Notably, the position of sRAGE in the experiment did not have any effect on the binding kinetics further demonstrating the specificity of the measured interaction. Unfortunately, laminin and fibronectin were not able to be conjugated to the surface of the sensor and the data for those proteins was only obtained with them in solution and sRAGE on the sensor surface.
Figure 31. Conjugation of sRAGE, Collagen I, and Collagen IV to Amine-Reactive Sensors. Reaction conditions were optimized to obtain maximal binding of sRAGE, collagen I, and collagen IV to the surface of amine-reactive sensors in order to study their binding kinetics. The amount of protein bound to the surface is indicated by the deflection in the reflected light (y-axis).

Figure 32. RAGE binds to collagen I and IV with high affinity. Soluble RAGE affinity for collagen I and IV was assessed by biolayer interferometry. Collagen IV (A) and I (B) were conjugated to an amine-reactive
sensor. sRAGE was then allowed to bind to the collagen coated sensors and then dissociation was initiated by washing in PBS. Binding was measured as a deflection in the reflected light. The binding affinities were calculated by fitting the curves with kinetics analysis software. The process was repeated with sRAGE being conjugated to the sensor and collagen I (D) and IV (C) in solution.

Figure 33. RAGE has high affinity for laminin. Biolayer interferometry was used to study the affinity of soluble RAGE for laminin (A) and fibronectin (B). Soluble RAGE was conjugated to the surface of amine-reactive tips and then incubated with solutions containing laminin and fibronectin at various concentrations to allow association between the proteins. The tips were then washed in PBS to dissociate the interaction. The data demonstrates that sRAGE has high affinity for laminin, but no affinity for fibronectin. Kinetics software was used to model the binding curves and a $K_d$ of 1.18 nM was determined for the interaction between sRAGE and laminin.
7.0 RESULTS – RAGE AND THE OTHER STUFF

7.1 RAGE EXPRESSION DOES NOT EFFECT RENAL FIBROSIS IN RESPONSE TO UNILATERAL URETERAL OBSTRUCTION

Numerous studies have implicated RAGE in the pathogenesis of renal nephropathy. Most suggest that RAGE and RAGE signaling promote the deposition of collagen in the kidney by various mechanisms. However, all of these studies utilized a chronic diabetes model for their studies. Therefore, C57BL/6 and RAGE KO mice were subjected to the UUO model of renal fibrosis to see if RAGE contributed to the pathogenesis in a similar fashion. Mice had their left ureter occluded or a sham procedure. On day 7, the mice were sacrificed and their left kidney was removed for analysis. The kidney was weighed and then divided and processed as described in the methods. Hydroxyproline analysis revealed that the UUO procedure resulted in a significant increase in collagen content of the kidneys, however, there was no difference between the wild-type and RAGE KO mice (Figure 34A). Similarly, there was a significant increase in the wet weight of the obstructed kidneys as compared to the unoccluded ones with no strain difference (Figure 34B). This further suggests that the UUO resulted in injury, but that there was no difference between the RAGE-null and RAGE expressing mice.
Figure 34. RAGE expression does not alter renal fibrosis in a UUO model. Wild-type and RAGE KO mice were subjected to a UUO model of renal fibrosis for 7 days. Hydroxyproline analysis revealed that UUO resulted in a significant increase in the collagen content of the kidneys (A). However, the two strains responded in an identical fashion. The kidney to total weight ratio was also evaluated and found to corroborate the hydroxyproline analysis. The UUO procedure resulted in an increased kidney weight (marker of injury), but there was no difference between strains (B).

7.2 LACK OF RAGE EXPRESSION CAUSES OTHER ALTERATIONS IN GENE EXPRESSION

When conducting studies in genetically modified animals there is always the possibility that the process of knocking out gene expression may have independent effects. A BLAST search of the NCBI database was performed and revealed that RAGE has the greatest homology with a protein called, activated leukocyte cell adhesion molecule (ALCAM). The expression of ALCAM was therefore investigated in untreated C57BL/6 and RAGE KO mice. It was found that ALCAM expression was significantly increased in the lungs of RAGE KO mice by almost two-fold (Figure 35A). Lungs from ALCAM knockout mice were then obtained to see if there were any
differences in RAGE expression. Notably, no difference in RAGE expression was seen in the ALCAM KO mice (Figure 35B).

Figure 35. Lack of RAGE expression results in upregulation of ALCAM. Lungs were obtained from C57BL/6, RAGE KO and ALCAM KO mice. Lung homogenates were prepared and separated by reducing SDS-PAGE. Proteins were transferred to PVDF membranes and detected with specific antibodies for ALCAM, RAGE, and beta-actin. Results are reported as normalized net intensity (protein of interest/beta-actin). The genetic deletion of RAGE resulted in a significant upregulation of RAGE (A). However, the genetic deletion of ALCAM did not result in any alterations in RAGE expression (B).

In order to determine if ALCAM was behaving like RAGE in the lungs of the RAGE knockout mice, C57BL/6 and RAGE KO mice were treated with asbestos, bleomycin, and saline (control) for 14 days to look at expression of ALCAM in fibrosis. It was found that ALCAM protein expression was decreased by about 50% with both asbestos (Figure 36A) and bleomycin
(Figure 36B) injury. However, the expression of ALCAM in the wild-type mice was unaffected by the fibrotic injuries.

Figure 36. RAGE KO mice have decreased ALCAM expression after fibrotic injury. Wild-type and RAGE KO mice were treated with asbestos (A), bleomycin (B), or saline control. After 14 days, the mice were sacrificed and their lungs homogenized to obtain total protein. Lung protein was separated by reducing SDS-PAGE and transferred to PVDF membranes. ALCAM and beta-actin expression were detected with specific antibodies. ALCAM expression was normalized to beta-actin and the wild-type saline group was designated 100% expression. Notably, the RAGE KO mice have increased ALCAM expression that is lost to fibrotic injury. However, there is no decrease in ALCAM expression in the wild-type mice.
8.0 DISCUSSION AND FUTURE DIRECTIONS

8.1 DISCUSSION – SRAGE PURIFICATION

One of the challenges hindering the study of RAGE is the inability of investigators to obtain large quantities of pure, endotoxin-free, glycosylated sRAGE. Previous studies had focused on cell culture systems where the protein was expressed in insect cells\textsuperscript{76}, bacteria\textsuperscript{245}, and yeast.\textsuperscript{246} There are significant negative aspects to the utilization of \textit{in vitro} expression systems. The first is that it is somewhat cumbersome to clone the cDNA of sRAGE for each species, insert it into a plasmid, and then obtain a stably transfected cell line. The media must then still be purified by column chromatography to obtain the pure protein. In addition, \textit{in vitro} glycosylation patterns of non-mammalian cell lines do not necessarily mimic what occurs to sRAGE \textit{in vivo}. The two glycosylation sites that were identified on \textit{in vivo} sRAGE were found to be essential for its ligand-binding ability.\textsuperscript{12} It is therefore imperative that any expression system used to purify sRAGE preserve these modifications of the protein. We therefore sought to develop a more efficient and reliable method for purifying large quantities of soluble RAGE from lung tissue to promote its use in animal studies. We developed a novel method that has a yield of 10 mg of sRAGE from 65 g of murine lung tissue. In addition, the protocol is shorter than previously described methods allowing for completion in a 7-day period. Our final product ran as a pure, single band at 45 kDa on reducing SDS-PAGE consistent with previous reports for sRAGE.\textsuperscript{11}
The identity of the protein was also confirmed by peptide fingerprinting. Lastly, we confirmed the ability of the pure protein to bind to HMGB1, a known RAGE ligand. This confirmed that sRAGE maintained its functional integrity throughout the purification process.

In addition to providing a greater yield and purity, this protocol also has several other advantages over a previously published protocol.\(^{11}\) 1) We eliminated the concentration and dialysis step after elution from the Concanavalin A column. Direct application at the higher salt concentration actually reduces the number of contaminants in the heparin elution and increases the column’s binding capacity for sRAGE. 2) A faster gradient is used for the heparin elution shortening the duration of this step without reducing the separating capacity. 3) A much less expensive HiTrapQ column replaced the costly MonoQ column in the anion exchange chromatography step. 4) sRAGE was eluted from the HiTrapQ column with an isocratic elution, which increased the protein concentration of the final product and reduced the length of time for this step. 5) Lastly, an endotoxin removal step was added so that the purified protein could be administered to animals as a therapeutic and utilized in cell culture models.

This enhanced protocol is essential for improving investigators ability to more easily purify large quantities of sRAGE for \textit{in vitro} and \textit{in vivo} investigations. The significantly higher yield now makes it possible to purify enough of the protein for \textit{in vivo} studies requiring doses of 50 $\mu$g/day/animal for greater than two weeks. Additionally, this protocol yielded nearly identical results with bovine lung, a much less costly and readily accessible material, and likely provides a method to isolate sRAGE from the lung tissue of other animals. This would allow a researcher to obtain a species-specific sRAGE in 7 days. We are currently collaborating with another laboratory to investigate the differences between sRAGE purified from lung tissue and cell expression systems.
RAGE expression and signaling have been implicated in the pathogenesis of many non-pulmonary diseases. However, few studies have investigated RAGE expression and function in the lung. We found this fact surprising since RAGE expression is highest in the lung and therefore set out to understand its role in normal physiology and fibrotic disease. We found RAGE expression in the lung to be dysregulated by fibrosis. In contrast to non-pulmonary tissues, the current study found that RAGE expression was significantly decreased in fibrotic lungs from both humans and mice. This study specifically investigated RAGE expression in human IPF tissue and an asbestos model of pulmonary fibrosis. However, a previous study in our lab and a concurrent study by an independent group found that RAGE expression is decreased in a bleomycin-model of pulmonary fibrosis as well.27, 28 In addition, the other group also found that RAGE protein expression was decreased in a second sample set of IPF tissue samples compared to normal controls further confirming our original findings.28 Lastly, a third group found that sRAGE levels in the plasma were decreased in patients with IPF but not in other pulmonary diseases.97 These studies indicate that the loss of mRAGE and sRAGE is a conserved step in the pathogenesis of pulmonary fibrosis, as well as, animal models of the disease. Despite this consistency, we found that mRAGE expression protects against asbestos-induced fibrosis while promoting bleomycin-induced fibrosis. Unfortunately, we found that treatment with sRAGE had no effect on either model.

Our asbestos-model studies suggest a protective role of pulmonary mRAGE expression in fibrosis. This sharply contrasts with the view of RAGE as a propagator of disease. However, we found that mRAGE and sRAGE were significantly down regulated in pulmonary fibrosis as shown in both mouse models (Figure 6) and human IPF lungs (Figure 7). It is possible that this
loss of RAGE solely indicates a loss of type I alveolar epithelial cells. AT-I cell loss is a known mechanism of injury in pulmonary fibrosis and they are the primary RAGE expressing cell. Thus, a loss of this cell type would result in a corresponding reduction in RAGE expression. The expression of aquaporin-5, an additional marker of AT-I cells, was therefore investigated after asbestos-injury. This protein was found to be decreased to the same extent as RAGE suggesting that AT-I cell loss might be responsible for the decrease in RAGE expression (Figure 6).

RAGE knockout mice were obtained in order to determine if RAGE was a cause or effect of the fibrosis. Notably, these mice lack all isoforms of the protein further complicating studies with them. These studies showed that the absence of mRAGE/sRAGE expression in RAGE knockout mice led to an age-related pulmonary fibrosis that displays many of the same features as human IPF (Figure 8). These mice also began to have increases in collagen deposition in their lungs with age that were biochemically detected by hydroxyproline analysis at 6 months and 1 year of age compared to age-matched wild-type controls.

Since these mice spontaneously developed fibrosis with age, young mice were challenged with an asbestos injury to see if they would develop more severe fibrosis. These studies revealed that the absence of mRAGE and sRAGE in knockout mice led to enhanced fibrosis. This finding was confirmed by both hydroxyproline quantification and histologic analysis from two separate experiments. However, it was still unclear whether the increased fibrosis was a result of a lack of mRAGE, sRAGE or both. RAGE KO mice were therefore treated with asbestos and daily i.p. injections of sRAGE. Notably, sRAGE injections had no effect on the degree of fibrosis as compared to vehicle control. In addition, wild-type mice were treated with either asbestos or TiO₂ and i.p. sRAGE or BSA vehicle control. These studies revealed no effect on any disease
parameter. Both of these studies were in contrast to other RAGE related disease processes, including atherosclerosis$^{234}$ and acute lung injury$^{83}$, in which this same dosage and delivery of sRAGE prevented disease. This suggests that the absence of mRAGE in the knockout mice may be the primary mediator contributing to pulmonary fibrosis. This led to the conclusion that the lack of mRAGE was responsible for the increased susceptibility to fibrosis and that it was likely playing a homeostatic role in the lung.

Unfortunately, these findings were somewhat contradictory to the published literature of RAGE and took an extensive period of time to get published. During the many resubmissions, He et al. published a study in which they found that their strain of RAGE KO mice were protected against fibrotic injury from bleomycin.$^{247}$ The authors suggested that the protective effects were due to an inability of HMGB1, which was elevated after bleomycin treatment, to signal and cause epithelial to myofibroblast transition in the knockout mice.$^{247}$ These contradictory findings led to confusion as to what the role of RAGE is in the normal lung and in fibrotic disease (Reviewed in $^{248}$). It was important to repeat and expand upon the bleomycin studies for a few reasons. 1) The RAGE KO mice used in the asbestos and aging studies were from a different genetic background$^{107}$ than the ones used in the bleomycin study$^{108}$ and could potentially behave in a different fashion if the genetic manipulation resulted in effects independent of the deletion of RAGE. 2) The authors of the bleomycin study hypothesized that HMGB1 was mediating the fibrosis in the RAGE expressing mice. If this were true, treatment with sRAGE could offer a therapeutic benefit in the bleomycin model. 3) Repeat analysis of the bleomycin studies would be beneficial to determine if there really was a discrepancy between the role of RAGE on the different models of pulmonary fibrosis.
In contrast to the asbestos studies, but similar to the He et al. study, the repeat analysis of the bleomycin studies in the strain of RAGE KO mice used in the asbestos and aging studies demonstrate that the RAGE null mice were completely protected against the fibrotic effects of bleomycin at both 14 and 21 days post-exposure. Thus, additional experiments were performed to determine if scavenging RAGE ligands with sRAGE would protect mice against bleomycin-induced fibrosis. These studies demonstrated that i.p. sRAGE administration offered no protection against the fibrotic effects of bleomycin in wild-type mice nor any other disease parameter. This data suggests that scavenging RAGE ligands with sRAGE has no effect on the bleomycin disease course and does not appear to be a viable therapeutic intervention for the treatment of pulmonary fibrosis.

These findings led to a re-evaluation of how RAGE KO mice could have such drastically different responses to these two models of fibrosis. Notably, one of the main differences is that bleomycin is a single-hit which is enzymatically inactivated in the lung while asbestos is retained in the lung and causes a chronic and progressive fibrosis. This led to the hypothesis that RAGE KO mice are “ultra-metabolizers” of bleomycin as a result of the genetic manipulation rendering them less susceptible to the initiating injury. The expression of bleomycin hydrolase in the lungs of wild-type and RAGE knockout mice was therefore investigated. If the KO mice overexpressed bleomycin hydrolase it would serve as a logical reason for their protection. Unfortunately, no differences in the protein expression of bleomycin hydrolase in the wild-type or RAGE KO lungs were observed.

At this point we found ourselves in the conundrum that plagues the study of RAGE in cancer. RAGE was found to function as a tumor suppressor in the lung\textsuperscript{123}, but as an oncogenic protein in the prostate\textsuperscript{127}, colon\textsuperscript{128}, and stomach\textsuperscript{130}. These results make it extremely
challenging to study the general mechanisms of how RAGE expression promotes or inhibits cancer progression as they serve to support one disease model while negating the other. This is similar to the current studies on pulmonary fibrosis in which it is difficult to mechanistically explain how RAGE expression in the alveolar epithelium is contributing to fibrosis in one disease model and protecting in another. In an attempt to decipher differences on the effects of RAGE in the two models, the \textit{in vitro} effect of bleomycin and asbestos on primary alveolar epithelial cells was evaluated. Utilization of traditional assays with fibrotic stimuli (i.e. cytokines and growth factors) they would likely lead to the support of one model while contradicting the other. Secondly, it is difficult to find a cell line that expresses the RAGE protein. There has been a significant amount of controversy regarding what cells express RAGE.\textsuperscript{33, 249} Notably, the only cell that reproducibly expresses RAGE are primary alveolar type II cells that are differentiated into type I-like cells in culture. These cells are unfortunately somewhat difficult to isolate, require a significant amount of costly materials, and only express RAGE from days 5-9.\textsuperscript{35} This made large explorations into the activation of various cell signaling pathways prohibitive. There is no protein expression of RAGE in numerous cell types and lines including A549, LL-47, E10, HUVEC, alveolar macrophages, peritoneal macrophages, and bone-marrow derived macrophages. Therefore, all of the \textit{in vitro} studies were performed utilizing freshly-isolated AT-II cells from wild-type and RAGE KO mice that were allowed to differentiate into type I-like cells, with analysis performed between days 6-9 in culture.

The first experiment investigated if RAGE expression altered the susceptibility of alveolar cells to injury from asbestos or bleomycin. As described earlier, AT-I cell death is a known mechanism of injury in IPF and since AT-I cells express RAGE it served as a likely candidate for the potential differences in the two models. The cells were treated with asbestos or
bleomycin at increasing concentrations for 24 hrs. An MTS reagent was then added to each well and developed for 4 hrs to measure the remaining metabolic activity. Cell viability was measured as a correlate of the metabolic activity in each well. Treatment with both asbestos and bleomycin resulted in significant injury to the cells. Consistent with the in vivo studies, treatment with sRAGE had no effect on this injury. One could hypothesize that AT-I cell injury could have led to the release of RAGE ligands, such as HMGB1. In this case, sRAGE treatment or a lack of RAGE expression should have increased cell survival if RAGE ligands were promoting cell death. Unfortunately, RAGE expression and sRAGE treatment had no effect on the viability of alveolar epithelial cells exposed to bleomycin or asbestos suggesting that both caused equivalent injury to AT-I cells in wild-type and RAGE KO mice.

The second hypothesis investigated was to determine if there is a difference in the capacity of RAGE expressing and RAGE KO cells to re-epithelialize in a wound healing assay. Demling and colleagues\textsuperscript{250} have reported that RAGE expression enhances adherence of alveolar epithelial type I cells to the extracellular matrix and is essential for cellular spreading. RAGE also induces spreading of these cells on a collagen type IV surface.\textsuperscript{250} These observations are interesting given that thin spreading of AT-I cells on the basement membrane, which is composed of collagen type IV, is essential for normal lung physiology. Additionally, differentiation of alveolar type II cells to alveolar type I cells has been shown to be an important mechanism by which the lung repairs itself and RAGE expression has been shown to correlate with this differentiation.\textsuperscript{37} Thus, a lack of RAGE expression may result in an inability to re-epithelialize the alveoli after AT-I cell loss.

A wound scratch assay was performed to assess the healing capacity of the cells after injury. In addition, this was done in the presence of asbestos, bleomycin, and sRAGE. Wild-
type and RAGE KO cells were able to heal equally in control experiments suggesting no inherent differences. Consistent with the *in vivo* studies, these *in vitro* studies demonstrate that RAGE null alveolar epithelial cells were able to re-epithelialize significantly better than RAGE expressing cells when treated with bleomycin. These findings suggest that RAGE null mice are able to heal better in response to bleomycin injury which is consistent with the *in vivo* data indicating RAGE KO mice are resistant to bleomycin fibrosis. In contrast, these same differences were not observed in cells treated with asbestos. In the studies with asbestos, both wild-type and RAGE knockout cells were equally impaired in their ability to heal. This suggests that the RAGE null mice would have the same inability to re-epithelialize and repair the lung after asbestos injury as wild-type mice. This does not explain the finding that RAGE KO mice develop fibrosis with age or more severe fibrosis in response to asbestos, but does potentially explain one mechanism which may contribute to their resistance to bleomycin-induced fibrosis.

In summary, pulmonary RAGE expression is depleted in all animal models of pulmonary fibrosis and IPF. RAGE KO mice spontaneously develop pulmonary fibrosis with age, more severe fibrosis with asbestos injury, and no fibrosis with bleomycin injury. Treatment with sRAGE did not cause any measurable changes in any of the disease parameters in either fibrosis model. AT-I cells are equally susceptible to bleomycin and asbestos injury regardless of RAGE expression or treatment with exogenous sRAGE. RAGE expressing cells have an impaired wound healing ability in the presence of bleomycin compared to cells lacking RAGE. There is no difference in the wound healing capacity of the two cell types after asbestos injury. While all of these findings support a role for RAGE in the pathogenesis of pulmonary fibrosis it is still not fully clear what exactly that role is.
8.3 DISCUSSION – DIABETES AND PULMONARY RAGE EXPRESSION

Diabetes has been shown to lead to the excessive production of AGEs, which are then thought to pathologically interact with RAGE on blood vessels\textsuperscript{47}, nerves\textsuperscript{48}, skin wounds\textsuperscript{49}, and renal epithelial cells\textsuperscript{50, 51} to cause disease. In each of these studies, the investigators found expression of RAGE to be upregulated by excess AGEs in the diabetic disease state. Despite having the highest normal expression of RAGE, no studies have ever investigated what happens to pulmonary RAGE expression in diabetes. To investigate the effect of hyperglycemia on pulmonary RAGE expression lungs were obtained from untreated diabetic and euglycemic rodents to investigate the effect of AGEs on pulmonary RAGE expression.

The first animal model investigated was the Ove26 transgenic mouse, a model of type I diabetes. Normal lungs were obtained from both young (8-week old) and aged (7-month old) diabetic mice and age-matched controls. These mice are known to develop diabetic complications by as early as 8 weeks of age.\textsuperscript{213} Analysis of mRAGE and sRAGE protein expression revealed no difference between the diabetic and wild-type mice at either age. Despite no difference in the protein levels, it is possible that diabetes would alter RAGE mRNA expression. This was investigated in the 8-week old diabetic mice. This analysis found no difference in RAGE mRNA expression, which is consistent with the protein levels.

Unfortunately, no one had specifically utilized the Ove26 strain in studies involving altered RAGE expression before. Thus, additional studies were needed to investigate pulmonary RAGE expression in diabetic models that had previously been shown to have altered RAGE expression in other tissues. The DP-BB rat, a type I diabetic model, has been shown to have increased AGE and RAGE expression in cardiac tissue by 3 months of age.\textsuperscript{218} Analysis of these rats at 4 months of age revealed that diabetes did not lead to a difference in pulmonary mRAGE
or sRAGE expression in these rats. \textit{db/db} mice have been heavily utilized for the study of the interaction between AGEs and RAGE. RAGE has been shown to be upregulated in this diabetic mouse model in the kidney\textsuperscript{51}, heart\textsuperscript{220}, and vasculature\textsuperscript{221}. Unfortunately, analysis of pulmonary mRAGE and sRAGE expression in these \textit{db/db} mice showed no change in expression compared to control mice. The final model investigated was the Zucker Diabetic Fatty rat. This type II diabetic rat model has been shown to have upregulated RAGE in the kidney\textsuperscript{226} and vasculature\textsuperscript{227} in response to elevated AGES. However, the elevated AGES had no effect on pulmonary RAGE expression as it was completely unchanged in 4-month old rats.

In summary, analysis of five different diabetic rodent models revealed that pulmonary RAGE expression was unaltered in all of them. This is even more perplexing since three of these models have been shown to have altered RAGE expression in other tissues at similar ages. Notably, these other studies investigated RAGE expression in those tissues only by immunohistochemistry and offered no other biochemical analysis to support their conclusions. In the one study that looked at RAGE expression in diabetic kidneys by Northern blot, the investigators found that diabetes did not change RAGE expression in the kidney.\textsuperscript{111} In addition, the authors found that they had to expose their Northern blot for 10 days as compared to overnight for the normal lung. These findings bring into question the expression of RAGE in these other tissues and whether or not AGES have the ability to modulate RAGE expression at all. It is very possible that many of the conclusions drawn from previous studies were based on non-specific reactivity in their immunolabeling studies.

Unfortunately, the planned studies were based on the notion that diabetes would alter RAGE expression in the lung and that this altered expression would change how diabetics respond to fibrotic injury such as asbestos. This was clinically relevant as epidemiologic studies
of IPF were conflicting as to whether or not diabetes was a risk factor for the disease.\textsuperscript{230, 231, 251} Nevertheless, diabetic and control mice were treated with asbestos while performing the analyses of RAGE expression in diabetic lungs. Two separate experiments, one for hydroxyproline and one for histology, revealed that Ove26 mice developed significantly less fibrosis as compared to euglycemic FVB mice. As the initial hypothesis was incorrect regarding altered RAGE expression, it is unclear as to what factors are contributing to these results. However, a prospective study of septic patients found that diabetes resulted in a significant risk reduction in the development of ARDS.\textsuperscript{252} At the time, the authors could offer no explanation for their findings and created a bit of controversy with the notion that diabetes could protect against a disease. While counterintuitive to most studies, and likely even more controversial than the concept that AGEs do not alter RAGE expression, it is possible that hyperglycemia somehow protects the lung from injury. This study conclusively demonstrates that hyperglycemia in rodents does not cause alterations in the expression of RAGE in the lung, but suggests it may provide protection against fibrosis.

8.4 DISCUSSION – RAGE AND THE ECM

Despite thousands of studies investigating RAGE in disease, only two have shed any light on RAGE’s normal biological function. It would seem counterproductive for an organ to utilize so much energy and resources to make a protein that has no normal function. Our discovery that aged RAGE KO mice develop spontaneous pulmonary fibrosis along with the finding that RAGE bind’s to collagen I and IV\textsuperscript{35} led us to investigate RAGE’s interaction with the extracellular matrix. Notably, the only other study that attributed a homeostatic function to
RAGE found that it behaved like an adhesion molecule for neurite outgrowth.\textsuperscript{13} Thus, it seemed logical that RAGE could be acting as an adhesion molecule for AT-I cells in the lung. After all, it was localized to the basolateral surface of AT-I cells along the basement membrane.\textsuperscript{36}

A novel anti-RAGE antibody was generated against full-length mouse soluble RAGE in order to confirm the localization of RAGE expression in the lung. Analysis of previously utilized antibodies revealed that these other antibodies also stained the lung tissue from RAGE KO mice suggesting that their specificity for immunostaining is questionable at best. Characterization of the new anti-RAGE antibody demonstrated that it specifically stained only lung tissue from RAGE expressing mice (Figures 29 & 30). RAGE is expressed specifically in the alveolar epithelium, and not in the bronchial epithelium as some other studies have suggested. Double-labeling studies with collagen IV revealed extensive co-localization of RAGE with the basement membrane confirming the previous findings and further supporting the notion that it may interact with the basement membrane.

As RAGE immunochemistry indicated it co-localized with the basement membrane, it was important to next determine if RAGE had direct binding affinity to for various components of the basement membrane. Traditionally, most binding kinetics are obtained using surface plasmon resonance (SPR). Unfortunately, that system utilizes microfluidics for its measurements that can be clogged by the viscosity of solutions containing ECM proteins. Therefore, in order to conduct these binding studies, a new method called biolayer interferometry was utilized. This technique relies on the oscillation of a microplate instead of flow. This method has been shown to replicate the results of SPR.\textsuperscript{253} These studies demonstrate that sRAGE has a very high affinity for collagen I (\(K_D = 2.04\) nM), collagen IV (\(K_D = 3.29\) nM), and
laminin ($K_D = 1.18$ nM), but no affinity for fibronectin. Notably, the binding affinity of sRAGE for collagen I and IV was the same whether it was placed on the sensor or in solution.

These experiments demonstrate that RAGE extensively co-localizes with the basement membrane in vivo. In addition, these studies demonstrate that RAGE is capable of binding to components of the basement membrane with very high affinity suggesting it is acting as an adhesion molecule in the lung. This finding may shed some light onto why RAGE KO mice develop spontaneous pulmonary fibrosis with age as their AT-I cells are likely more susceptible to injury and de-epithelialization if they are missing an adhesion molecule that normally anchors them to the basement membrane. It will be essential for future studies to determine what portion of sRAGE is interacting with the basement membrane components. Also, it will be interesting to determine what portion of the ECM proteins are interacting with RAGE. These studies will likely serve as a starting point for the better understanding of RAGE’s high pulmonary expression.

8.5 DISCUSSION – RAGE AND THE OTHER STUFF

RAGE has been implicated in many diseases, but renal nephropathy has probably been the most extensively studied. RAGE activation by AGEs was shown to lead to epithelial to myofibroblast transition\textsuperscript{114}, as well as, overproduction of TGF-\(\beta\) and and collagen I.\textsuperscript{113} Both of these processes are central to the pathogenesis of renal fibrosis. This led to the hypothesis that RAGE null mice would develop less severe renal fibrosis in response to unilateral ureter obstruction (UUO). Wild-type and RAGE KO mice were treated with UUO or sham procedure for 7 days and then evaluated for fibrosis by hydroxyproline quantification. These studies found that RAGE
expression had no impact on the degree of fibrosis in the kidney. Subsequent analysis found that there was no RAGE expression in the kidney even in the diabetic disease state. At that point, it was determined that even if different responses were observed, it would have been questionable what it would mean without expression of the protein. Future investigations into this project were therefore halted.

Researchers often utilize transgenic and knockout animals to evaluate the function of a protein. In many of these cases investigators underestimate the biological redundancy and the ability for animals to compensate for these alterations. We were initially approached by Dr. Cory Hogaboam at the University of Michigan who wanted to look at the expression of proteins in the TLR family in RAGE KO mice as compared to controls. He found that expression of one of these members, TLR-7, was upregulated by approximately 50 fold in the KO animals. This convinced us to investigate other potential alterations in the RAGE KO mice. A BLAST search of the RAGE sequence revealed that RAGE has the most homology with a protein called ALCAM. We therefore investigated the protein expression of ALCAM in the RAGE KO mice. Suprisingly, expression of ALCAM was increased by about two-fold in the RAGE KO mice. Lungs from ALCAM KO mice (courtesy of Dr. Joshua Weiner, University of Iowa) were then obtained to see if there were alterations in RAGE expression as this would suggest some sort of co-regulation of their expression. No differences in RAGE expression in the ALCAM KO mice were observed. However, this was not all that surprising as there are numerous other proteins that are much more similar to ALCAM than RAGE, but not vice versa.

The next step was to investigate what happens to this protein in models of fibrosis. If the protein were compensating for RAGE it would likely be lost in a fibrotic model. Notably, ALCAM expression was decreased only in RAGE KO mice after fibrotic injury, but remained
the same in wild-type mice. This loss of expression due to fibrosis is the same behavior that we observed for RAGE in wild-type mice. These findings suggest that embryonic deletion of RAGE leads to alterations in other genes. It is likely that a more macroscopic approach, such as microarray analysis, would identify significantly more adaptations in the RAGE KO mice.

8.6 SUMMARY AND FUTURE DIRECTIONS

These studies have begun to shed light on a very dark and confusing subject matter. The RAGE literature is fraught with controversy and unfortunately some of our studies will likely contribute to further controversy and confusion. However, we have clearly demonstrated that RAGE is lost in the lung in response to fibrosis and that this loss is likely both a cause and effect of the disease. It does not appear that sRAGE plays much of a role in the disease other than having its expression depleted. The vast majority of the contribution appears to be stemming from the expression of membrane RAGE. Notably, many of these conclusions were based on the use of knockout animals that have now been shown to have some other genetic alterations as a result of RAGE loss (i.e. increased ALCAM expression). In our future studies, we must address this fact and control for these alterations to ensure that what we report is truly RAGE dependent. We recently found that RAGE KO mice overexpress the protein ALCAM. Unfortunately, even less is known about the function of ALCAM in the lung. Most ALCAM studies have been performed in the field of cancer. One study found that ALCAM was capable of activating matrix metalloproteinase-2 (MMP-2) and that this promoted cancer metastasis. Notably, MMP’s have long been studied in pulmonary fibrosis for their ability to remodel the extracellular matrix. There is very little known about the specific role of MMP-2 in bleomycin and
asbestos injury. However, one study found that expression of MMP-2 after bleomycin injury in vivo was capable of inducing alveolar epithelial repair.\footnote{255} MMP’s in an asbestos model were thought to promote injury as demonstrated by the fact that a global MMP inhibitor conferred protection to asbestos treated mice.\footnote{184} Therefore, because ALCAM is overexpressed in RAGE KO mice and catalyzes the activation of MMP-2 it could potentially explain why RAGE KO mice have worse fibrosis in response to asbestos injury. In addition, higher levels of MMP-2 in RAGE KO mice could explain why they are resistant to bleomycin-induced fibrosis as the excess MMP-2 promotes alveolar repair compared to the normal levels in wild-type control mice. It therefore seems prudent to perform zymography studies on lung homogenates and BAL fluid of wild-type and RAGE KO mice to determine if there are differences in the amount of MMP-2 activation.

In addition, Jackson Labs is currently starting to breed an ALCAM KO mouse colony that will be commercially available within the next 6 months. We could breed these animals with our RAGE KO’s to create a double KO to see if it is truly playing a compensatory role. This could potentially lead to a non-viable animal if ALCAM is essential in the RAGE KO mice. In addition, we could subject the ALCAM KO and double KO mice to our models of fibrosis. If ALCAM were playing a compensatory role in the RAGE KO mice we would expect the double KO’s to be more susceptible to our fibrotic injuries or develop pulmonary fibrosis spontaneously at an earlier age.

Another important study that could be performed regarding ALCAM expression in the RAGE KO’s is a localization study. In order for our hypothesis of compensation to make sense, ALCAM must be expressed in the AT-I cells where RAGE is normally expressed. Notably, ALCAM expression in wild-type mice is thought to be confined to endothelial cells\footnote{256, 257}, so any
expression on AT-I cells would be consistent with this hypothesis. This could be determined by immunohistochemical comparison of normal wild-type and RAGE KO lungs.

We can also address the concern of genetic compensation by creating an inducible RAGE KO. Recently, investigators found that an acute loss of extracellular superoxide dismutase (EC-SOD) led to an ARDS-like injury with very high mortality within days of knocking out the gene. Notably, mice with embryonic deletion of EC-SOD are perfectly viable, though more susceptible to many pulmonary injuries when stressed. The authors of the inducible study found that embryonic deletion of EC-SOD resulted in alterations of 46 different genes that were likely compensating for its loss. We have recently begun a collaboration with Dr. Bernd Arnold at the University of Heidelberg to obtain mice with a floxed RAGE gene. We will then be breeding these with inducible-Cre mice that are commercially available from Jackson Labs. These mice will be bred again in order to obtain a homozygous floxed RAGE gene and a heterozygous inducible-Cre. We will then treat these mice with Tamoxifen at 12 weeks of age to induce recombination and deletion of RAGE. These mice will then be examined for phenotypes and susceptibility to various injuries. We hypothesize that they will better represent RAGE function in the lung as they will not have the compensation that exists in the embryonic RAGE KO’s, but more resemble the pathologic loss of RAGE that occurs in fibrosis. In addition, we can perform studies where RAGE is “knocked-out” at the time of injury or at a period during the re-epithelialization process in the bleomycin treated mice. This will more conclusively confirm or negate if lack of mRAGE promotes alveolar epithelial repair in the bleomycin model.

One of the most surprising aspects of this study was that RAGE expression in the lung was not altered by diabetes. I personally believe this should raise the question of whether or not AGEs actually modulate RAGE expression in any tissue. This became apparent when we were
unable to detect RAGE protein in the aorta or kidneys of diabetic mice, despite numerous publications to the contrary. Numerous studies have been allowed to base their conclusions solely on immunolabeling of RAGE. Unfortunately, the specificity of many of the RAGE antibodies is uncertain at best, and brings their results into question. The studies in this project likely confirm the need to abandon any connection between diabetes and pulmonary RAGE expression.

The concept of RAGE acting as an adhesion molecule will likely instigate many future studies. It is the only proposed RAGE function that explains the high normal expression in the lung. Notably, all tissues have basement membranes and the kidney has a basement membrane that, like the lung, is composed of collagen IV. However, in these other tissues the cells are not required to spread out to just few microns across in order to function. This is a unique feature of the alveolar epithelium and as RAGE is solely expressed in the alveolar epithelium, it serves as a promising candidate for this function. In order to better confirm these findings it must be demonstrated that these ECM proteins can activate RAGE and initiate some sort of cell signaling, which would lead to this thin spreading and adhesion. Unfortunately, there are currently no cell lines that express the RAGE protein and conducting these expedition-type studies in primary cells is prohibitive. The generation of a RAGE expressing cell line is essential to better understanding RAGE’s function. We are currently attempting to create a stable transfection of mouse alveolar epithelial cells with plasmids containing membrane RAGE. However, the immortilization of AT-I cells that naturally express RAGE would be significantly better for most studies as the normal feedback mechanisms would remain intact.

It is unlikely that the controversy surrounding RAGE will end any time soon. In our investigations to better understand RAGE function, we were most successful at generating more
questions. However, a valuable life lesson has been learned in these studies of RAGE. As a researcher, it is imperative that we understand the limitations of our animal models, *in vitro* systems, and reagent quality before generating conclusions that can have broad effects for many years to come.
REFERENCES


15. Ishihara K, Tsutsumi K, Kawane S, Nakajima M, Kasaoka T: The receptor for advanced glycation end-products (RAGE) directly binds to ERK by a D-domain-like docking site, FEBS Lett 2003, 550:107-113


18. Li J, Schmidt AM: Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products, J Biol Chem 1997, 272:16498-16506


52. Valencia JV, Mone M, Koehne C, Rediske J, Hughes TE: Binding of receptor for advanced glycation end products (RAGE) ligands is not sufficient to induce inflammatory signals: lack of activity of endotoxin-free albumin-derived advanced glycation end products, Diabetologia 2004, 47:844-852


72. Li M, Shang DS, Zhao WD, Tian L, Li B, Fang WG, Zhu L, Man SM, Chen YH: Amyloid beta interaction with receptor for advanced glycation end products up-regulates brain endothelial CCR5 expression and promotes T cells crossing the blood-brain barrier, J Immunol 2009, 182:5778-5788


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92. Gross S, van Ree RM, Oterdoom LH, de Vries AP, van Son WJ, de Jong PE, Navis GJ, Zuurman MW, Bierhaus A, Gans RO, Bakker SJ: Low levels of sRAGE are associated with increased risk for mortality in renal transplant recipients, Transplantation 2007, 84:659-663


96. Yilmaz Y, Ulukaya E, Gul OO, Arabul M, Gul CB, Atug O, Oral AY, Aker S, Dolar E: Decreased plasma levels of soluble receptor for advanced glycation endproducts (sRAGE) in patients with nonalcoholic fatty liver disease, Clin Biochem 2009, 42:802-807


113. Huang JS, Guh JY, Chen HC, Hung WC, Lai YH, Chuang LY: Role of receptor for advanced glycation end-product (RAGE) and the JAK/STAT-signaling pathway in AGE-induced collagen production in NRK-49F cells, J Cell Biochem 2001, 81:102-113


121. Lizotte PP, Hanford LE, Enghild JJ, Nozik-Grayck E, Giles BL, Oury TD: Developmental expression of the receptor for advanced glycation end-products (RAGE) and its response to hyperoxia in the neonatal rat lung, BMC Dev Biol 2007, 7:15


127. Ishiguro H, Nakaigawa N, Miyoshi Y, Fujinami K, Kubota Y, Uemura H: Receptor for advanced glycation end products (RAGE) and its ligand, amphoterin are overexpressed and associated with prostate cancer development, Prostate 2005, 64:92-100


129. Kuniyasu H, Chihara Y, Takahashi T: Co-expression of receptor for advanced glycation end products and the ligand amphoterin associates closely with metastasis of colorectal cancer, Oncol Rep 2003, 10:445-448


147. Herrmann M: [Idiopathic pulmonary fibrosis--telomerase mutation in the familial form], Pneumologie 2008, 62:121


193. Shimosato Y, Baba K, Watanabe S: [Pulmonary lesions produced by antitumor drugs--studies on autopsy cases], Gan No Rinsho 1971, 17:21-34


196. Lazo JS, Merrill WW, Pham ET, Lynch TJ, McCallister JD, Ingbar DH: Bleomycin hydrolase activity in pulmonary cells, J Pharmacol Exp Ther 1984, 231:583-588


204. Quan C, Alcala E, Petkovska I, Matthews D, Canova-Davis E, Taticek R, Ma S: A study in glycation of a therapeutic recombinant humanized monoclonal antibody: where it is, how it got there, and how it affects charge-based behavior, Anal Biochem 2008, 373:179-191


208. Soulis T, Thallas V, Youssef S, Gilbert RE, McWilliam BG, Murray-McIntosh RP, Cooper ME: Advanced glycation end products and their receptors co-localise in rat organs susceptible to diabetic microvascular injury, Diabetologia 1997, 40:619-628


212. Epstein PN, Overbeek PA, Means AR: Calmodulin-induced early-onset diabetes in transgenic mice, Cell 1989, 58:1067-1073


223. Etgen GJ, Oldham BA: Profiling of Zucker diabetic fatty rats in their progression to the overt diabetic state, Metabolism 2000, 49:684-688


235. Woessner JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid., Archives of Biochemistry and Biophysics 1961, 93:440-447


APPENDIX A: Curriculum Vitae

JUDSON M. ENGLERT
englert.judson@medstudent.pitt.edu

Education:
University of Pittsburgh – School of Medicine
Medical Scientist Training Program, MD/PhD Candidate
PhD: Department of Cellular and Molecular Pathology
PhD Graduation: December 2009
MD Graduation: May 2011 (anticipated)

University of North Carolina at Chapel Hill
Bachelors of Science in Chemistry with Highest Honors
Minor in Spanish
Graduation: May 2005

Positions:

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<th>ACTIVITY/OCCUPATION</th>
<th>BEGINNING DATE (mm/yy)</th>
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<tr>
<td>Graduate Student</td>
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<td>12/09</td>
<td>Pathology</td>
<td>University of Pittsburgh</td>
<td>Dr. Tim Oury</td>
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<td>MSTP Student</td>
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<td>5/11</td>
<td>MD/PhD</td>
<td>University of Pittsburgh</td>
<td>Dr. Clayton Wiley</td>
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<td>Intern Research Chemist</td>
<td>06/04</td>
<td>08/04</td>
<td>Chemistry</td>
<td>Merck Pharmaceuticals</td>
<td>Dr. Leigh Schultz</td>
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<tr>
<td>Teaching Assistant</td>
<td>1/04</td>
<td>5/05</td>
<td>Chemistry</td>
<td>UNC-Dept. of Chemistry</td>
<td>Dr. Todd Austell</td>
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<tr>
<td>Instructor</td>
<td>8/03</td>
<td>12/03</td>
<td>Mathematics</td>
<td>UNC-Math Help Center</td>
<td>Dept. of Mathematics</td>
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Academic and Professional Honors
1. University of Pittsburgh - 2009 Department of Pathology 9th Annual Retreat - Outstanding Poster Presentation, 2nd Prize (5/09)
2. AAAS Honorary Membership, Summer 2008 – Present
3. University of Pittsburgh - Graduate and Professional Student Association Travel Award (September 2008)
4. University of Pittsburgh - McGowan Institute for Regenerative Medicine Travel Award (September 2008)
5. University of Pittsburgh - Biomedical Graduate Student Association Travel Award (May 2008 & 2009)
6. MSTP Grant Recipient (Institutional, T32) - National Institutes of Health (September 2005-2006)
7. Phi Beta Kappa honor society (Inducted April 2004)
8. Merck Index Award for Academic Achievement in Chemistry (2005)
9. UNC Department of Chemistry Student Commencement Speaker (2005)
10. Dean's List 6 of 7 eligible semesters, UNC-CH
11. University of North Carolina at Chapel Hill Honors Program

**Grant Support**

**Ruth L. Kirschstein NRSA Fellowship Title:** "A Role for Advanced Glycation End-Products in Diabetic Lung Injury"

**Source/Project # or type (P.I.):** NIH / 1F30 ES016973-01 (Englert)

**Project Period:** 07/15/2008-07/14/2013

**Costs (Direct/Indirect)** $ 40,788 - 45,972 / $0

**Reviewer Participation**
Invited reviewer: *American Journal of Physiology – Lung Cellular and Molecular Physiology*

**Organizational Activities**

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<td>Univ. of Pittsburgh – Medical Scientist Training Program (MSTP)</td>
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<td>Univ. of Pittsburgh – School of Medicine</td>
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<td>University of North Carolina at Chapel Hill</td>
<td>Carolina Alumni Admissions Program</td>
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<td>Campus Tour Guide</td>
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**Professional Society Memberships**

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<td>American Association for the Advancement of Science (AAAS) – Honorary Member</td>
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<td>American Medical Association (AMA)</td>
<td>2005 - Present</td>
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<td>American Medical Student Association (AMSA)</td>
<td>2005 – Present</td>
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<tr>
<td>American Society of Investigative Pathology (ASIP)</td>
<td>2007-2008</td>
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Invited Talks

Peer-Reviewed Publications

Abstracts
2. Englert, JM; Kaminski, N; Bierhaus, A; Oury, TD. Advanced Glycation End-Products Increase Diabetics" Susceptibility to Pulmonary Fibrosis. 15th International Colloquium on Lung Airway Fibrosis, Sunset Beach, NC, USA, September 2008.