# The Impact of Macrophage Migration Inhibitory Factor on Cigarette Induced Emphysematous Disease Severity

**By**

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# Thesis Abstract

Cigarette smoke (CS) is the most common cause of chronic obstructive pulmonary diseases (COPD) including emphysema. Emphysema is a debilitating disease characterized by oxidative stress, apoptosis, and alveolar tissue loss. CS, which contains and enhances reactive oxygen species (ROS), impacts all cell types within the lung parenchyma and airways, causing alveolar tissue destruction through four mechanisms: 1) oxidative stress, 2) inflammation, 3) protease-induced degradation of the extracellular matrix, and 4) enhanced alveolar epithelial and endothelial cell (EndoC) apoptosis. Importantly, these mechanisms all cause apoptosis through intrinsic mitochondrial pathways or through extrinsic death receptor-mediated pathways. With emphysema progression, oxidative stress is continuously exacerbated in response to CS, contributing to enhanced apoptosis that by definition out paces repair processes. Recognizing that about a fifth of all smokers will develop emphysema, suggesting differential genetic susceptibilities, we focused on intrinsic modifiers of disease that may be altered by chronic CS exposure.

Macrophage migration inhibitor factor (MIF), a pleiotropic cytokine with intrinsic oxidoreductase activity is one potential factor conferring cytoprotection from CS-induced damage. Studies in human pulmonary EndoC demonstrate that MIF antagonizes CS-induced apoptosis. Here we show for the first time that MIF is significantly reduced in patients with COPD. This is confirmed in our murine model of chronic CS exposure. The

absence of MIF leads to enhanced CS-induced DNA damage, lung EndoC apoptosis, and exacerbated alveolar airspace enlargement. These results highlight a crucial role for MIF in antagonizing CS-induced toxicity in the lung and resultant emphysematous tissue remodeling by maintaining EndoC homeostasis.

To further investigate the capacity and molecular mechanism(s) by which MIF modifies oxidant injury we aimed to understand the potential effect of MIF on xanthine oxidoreductase (XOR), a superoxide-generating enzyme that is increased in human COPD and mediates CS-induced DNA damage and EndoC apoptosis. Here we showed that superoxide concentrations are elevated following CS exposure in the absence of MIF. In addition this report demonstrates that XOR hyperactivation and apoptosis in the absence of MIF occurred via a p38 mitogen activated protein (MAP) kinase-dependent mechanism. This is the first report to show that MIF was sufficient to directly suppress ASK1 enzymatic activity, upstream of p38 activation, and providing one possible mechanism by which MIF suppresses CS-mediated cytotoxicity in the lung.

We uncovered another potential and novel mechanism whereby MIF confers protection from oxidant injury, by positively regulating nuclear factor erythroid 2-related factor 2 (NRF2), a major regulator of the antioxidant (AO) response element (ARE). We demonstrated that the downstream targets of NRF2 are significantly decreased following CS exposure in MIF deficient animals. This report further links MIF to the

stabilization of NRF2 through a mechanism involving c-Jun activation domain binding protein-1 (JAB1), a recognized intracellular receptor for MIF.

Taken together, the results of this study provide strong support for a novel role for MIF as a determinant of emphysema disease severity in mouse and man, impacting on pulmonary EndoC and alveolar homeostasis, and regulating both oxidant and antioxidant production, that in the absence of MIF contribute to oxidative stress, cell damage, and apoptosis.

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# Abbreviations

| | |
|---|---|
| 8OhdG | 8-hydroxy-2'-deoxyguanosine |
| AKT | Protein kinase B |
| AP1 | Activator protein 1 |
| APAF1 | Apoptotic protease activating factor 1 |
| ARE | Antioxidant response element |
| ATF1 | Cyclic AMP-dependent transcription factor |
| ATM | Ataxia telangiectasia mutated |
| BAL | Bronchoalveolar lavage |
| BNIPL | Bcl-2/adenovirus E1B 19 kDa-interacting protein 2-like |
| BPD | Bronchopulmonary dysplasia |
| CDK | Cyclin dependent kinase |
| COPD | Chronic obstructive pulmonary disease |
| CPFE | Combined pulmonary fibrosis and emphysema |
| CREB | Cyclic AMP response element-binding protein |
| CS | Cigarette smoke |
| CSE | Cigarette smoke extract |
| CT | Chest tomography |
| CXCR | Chemokine receptor |
| Cys | Cysteine |
| DFCO | Diffusing factor for the lung for carbon monoxide |
| DLCO | Diffusing capacity for the lung for carbon monoxide |
| DR | Death receptor |
| DSB | Double stranded DNA breaks |
| ECM | Extracellular matrix |
| EndoC | Lung endothelial cells |
| EpiC | Epithelial pneumocytes |
| ERK | Extracellular signal-regulated kinases |
| FEV1 | Forced expiratory volume in 1 second |
| FGF | Fibroblast growth factor |
| FLICE | Caspase 8 |
| FLIP | FLICE-like inhibitor protein |
| FVC | Forced vital capacity |
| GCR | Glucocorticoid receptor |
| GOLD | Global Initiative for Chronic Obstructive Lung Disease |
| $H_2O_2$ | Hydrogen peroxide |
| HAT | histone acetyltransferases |
| HDAC2 | Histone deactylase 2 |
| HGF | Hepatocyte growth factor |
| HIF1 | Hypoxia inducible factor 1 |
| HMVEC | Human microvascular endothelial cells |

| | |
|---|---|
| HPO | Hepatopoietin |
| HRE | Hypoxia response element |
| I/R | Ionizing radiation |
| JAB1 | c-Jun activation domain binding protein-1 |
| JNK | Jun-N-terminal kinase |
| KEAP1 | Kelch-like ECH-associated protein 1 |
| MAPK | mitogen-activated protein kinases |
| MHC | Major histocompatibility complex |
| MIF | Macrophage migration Inhibitory factor |
| Mif -/- | MIF deficient |
| MMP | Matrix metalloprotease |
| NEDD8 | Neddylin |
| Nf-kappaB | Nuclear factor-kappa B |
| NM23-H1 | Nucleoside diphosphate kinase 1 |
| NOS | Nitric oxide synthase |
| NRF2 | Nuclear factor erythroid 2-related factor 2 |
| p53 | Transformation-related protein 53 |
| PAG | Proliferation associated gene |
| PCNA | Proliferation cell nuclear antigen |
| PKA | Protein kinase A |
| PLA2 | Phospholipase A2 |
| PR | Progesterone receptor |
| Pro | Proline |
| PTM | Post-translational modification |
| RA | Rheumatoid arthritis |
| RAG | Recombination activating genes |
| RMVEC | Rat microvascular endothelial cells |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SCID | Severe combined immunodeficient |
| SNO | S-nitrosylated |
| TGF | Transforming growth factor |
| TNF | Tumor necrosis factor |
| TPM | Total particulate matter |
| TPOR | thiol-protein oxidoreductase |
| Treg | Regulatory T cells |
| TRX | Thioredoxin |
| TSP | Total suspended particles |
| UBC12 | Ubiquitin-conjugating enzyme E2M |
| VEGF | Vascular endothelial growth factor |
| XOR | Xanthine oxidoreductase |

# Chapter 1

## Introduction

# Chapter 1: Introduction

## 1.1 COPD

*"…The slow space…dominates the behavior of the lung in obstructive disease. [This space] applies to a large group of alveoli whose location is unknown but which are probably scattered throughout the whole of both lungs. They washout nitrogen very slowly when oxygen is breathed. The properties of the slow space, its ventilation, volume, and blood flow are most important in the understanding of the severity of the obstructed lung."*
*Briscoe and Nash 1965*

### *1.1.1 A Brief History of COPD*

The term COPD, which stands for chronic obstructive pulmonary disease, was not coined until 1965 by Briscoe and Nash at the 9th Aspen Emphysema Conference (Briscoe and Nash 1965, Petty 2006); however the chronic nature and devastating emphysematous tissue destruction inherent to the disease were long appreciated. The earliest written descriptions of capacious and over-inflated lungs in patients with difficulty breathing dates back to the late 1600's and 1700's (Bonet 1679, Ruysch 1691, Morgagni, 1769). In the early 1800's several disease manifestations were investigated; Badham described patients with chronic cough and persistent mucus hypersecretions (Badham 1814)

and Laennec, the originator of the stethoscope, described varying degrees and types of emphysema, pulmonary or interlobular, both resulting in lungs that did not empty well even after post-mortem dissection (Laennec and Forbes 1829). While improving procedures with the stethoscope Laennec noted that there is “no disease that illustrates the importance and necessity of percussion more than [emphysema],” because diagnosis was often inaccurate using the stethoscope alone. In 1846, Hutchinson, further advanced diagnostic proficiency to detect emphysematous lung damage with the advent of spirometry and although underutilized, it provided the ability to detect precise differences in total lung capacity in the context of health and disease (Hutchinson 1846).

Very few novel diagnostic procedures and devices were improved upon until almost 100 years after the invention of the spirometer. Before this time smoking was uncommon and susceptibility was often limited to genetic factors and increasing industrial pollution (Laennec and Forbes 1829). However, with the rise of the cigarette in the early twentieth century, it was not long before COPD burden increased and interest in diagnostics and treatment were reestablished. By 1944, leading physicians began to appreciate that the separate and individualistic etiologies of COPD, including emphysema and bronchitis, were part of the same disease process (Christie 1944). The medical recommendation henceforth was to complete a thorough review of patient history as well as physical examination for proper diagnoses. Several leading experts

published on the various clinical presentations and case reports of each disease manifestation to aid in better analysis and treatment (Christie 1944, Oswald, Harold et al. 1953, Noehren, Barach et al. 1964). In the 1950's, Donhurst, further described the classic phenotypic patients as "blue bloaters" and "pink puffers" (Dornhorst 1955). In these extreme depictions of COPD, blue bloaters were cyanotic from bronchial inflammation and related hypoxia, and were often obese or visibly swollen due to water retention and increased red blood cells or polycythemia; pink puffers were often older and thin with barrel-shaped chests, pursed lips, and clear dyspnea from emphysema. While these phenotypes were described as independent disease manifestations, signifying the prominence of one disease indicator over the other, upon autopsy many blue bloaters were described as having emphysematous damage, and many pink puffers as having airway hypersecretions. By the late 1970's genetic susceptibility was hypothesized as driving the differential and pronounced disease manifestations (Mountain, Zwillich et al. 1978).

Over this same time, Tiffeneau pioneered key spirometric advances, namely the inclusion of a timed vital capacity to analyze airflow (Tiffeneau and Pinelli 1947); and by 1951 Dayman had published on spirometric and air flow differences observed between healthy patients and those with emphysema, recognizing airway collapse as a possible contributing factor in altered lung function examinations in symptomatic

patients (Dayman 1951). These early studies into the presentation, pathophysiology, and treatment of emphysema or bronchitis during this era culminated in the commencement of two international symposia (Ciba Guest Symposium 1959, American Thoracic Society 1962), where the definitions of these chronic obstructive diseases were discussed and designated, providing the substance for which the current COPD definitions are based. The burgeoning issue of emphysema also led to the birth of the Aspen disease conferences, named for the meeting location in Aspen, Colorado in 1959. Over the next decade the discussions at these conferences formed the foundations for COPD therapy and related treatments (Aspen Emphysema Conference 1965). It was around this time that Briscoe elegantly described a new technique called the nitrogen washout to study patients with emphysema; nitrogen in healthy subjects was washed out in minutes, whereas it took substantially longer in those with emphysema (Briscoe, Cree et al. 1960). This technique was further improved upon the following year to yield quantitative data used to determine fractional alveolar ventilation and pulmonary capillary blood flow (Finley 1961).

In the context of a genetic impact on disease, the discovery of alpha-1 antitrypsin deficiency in 1962 proved to be groundbreaking in 2 aspects: 1) it signaled the beginning of new therapies for some patients with idiopathic lung disease, and 2) it provided a platform for understanding the cause of emphysematous tissue destruction (Laurell

and Eriksson 1963). From a public health perspective, over the first 60 years of the twentieth century smoking consumption increased precipitously, escalating from 54 cigarettes per person in 1900 to 4345 cigarettes in 1963 (Giovino 2002). An important event in the medical history of COPD occurred the following year; the Surgeon General officially reported that smoking was the main cause of COPD, correlating smoking history with chronic bronchitis and emphysema onset, marking the beginning of preventative measures and perhaps models of disease development and/or progression (Wellmann 1964). By 1967, Petty and Nett were publishing on clinical aspects of CS related lung alterations, and new effective treatments (Nett and Petty 1967, Petty, Ryan et al. 1967). In the 1970's Fletcher established methodologies to better utilize spirometric data and in fact was the first to associate smoking with enhanced deteriorations in the forced expiratory volume in 1 second (FEV1), further publishing data showing that smoking cessation would stem the rapid decline (Fletcher et al. 1976). In the late 1970's CS was officially linked to disruption of anti-proteases, including alpha-1 antitrypsin, greatly impacting the understanding of CS-induced lung disease (Gadek, Fells et al. 1979). It was not until the 1980's that the ratio of FEV1 to forced vital capacity (FVC) was used to detect the early functional changes and airway obstruction, a key element for improving treatment (Burrows, Knudson et al. 1987). The advent of CT imaging to detect pathological alterations in COPD would not become practical and

commonplace until much closer to modern times with the invention of high-resolution computed tomograpgy (CT).

### *1.1.2 A Current Understanding of COPD*

COPD is a term used for a group of pulmonary and respiratory ailments that by definition obstruct airflow, making it hard to breathe. COPD is treatable to a degree but is associated with an irreversible decline in airflow limitation, and tissue loss. CS causes approximately 90% of COPD cases domestically, but other occupational factors, such as dust and fumes, biomass fuel sources in less developed countries, and genetic variants also lead to disease (Blanc, Iribarren et al. 2009, Lamprecht, McBurnie et al. 2011, Po, FitzGerald et al. 2011). The onset of disease is variable, with a long asymptomatic period followed by characteristic clinical symptoms, including chronic cough, worsening dyspnea, mucus hypersecretion and sputum production, difficultly exercising, wheezing, chest tightness, and altered mental status, usually emerging once the disease has already progressed to mild and advanced stages. Exertional dyspnea is the most commonly reported early symptom (Rennard, Decramer et al. 2002). As in the early descriptions of COPD, the most common iterations of the disease are emphysema and chronic bronchitis, although the occurrence of either form is not mutually exclusive, but variably concomitant in most patients suffering from the disease. The

destruction of the pulmonary tissues with emphysema is debilitating, making it progressively harder to properly exhale or draw in fresh air, and this process can be further compounded by swelling of the bronchi.

While this definition for COPD has remained relatively simple since 1965, it is important to underscore that heterogeneity of COPD presentation and progression between individuals is still a key focus in continuing work. This could not be more evident than in patients who stop smoking but continue to suffer from enhanced deteriorations in lung function, while other patients may return to a more normal rate of lung function decline as observed with aging in non-smokers (Vestbo, Edwards et al. 2011). This also raises questions as to the underlying nature of disease progression in the absence of the main stimulus. Nonetheless, basic lung function examinations are used to analyze the FEV1/FVC and screen most patients with suspected COPD. This is in addition to a thorough evaluation of smoking and inhalational exposure histories as set forth after the first lung conferences in the 1960's.

### *1.1.3 The Natural History of COPD*

The natural history of COPD clearly differs on an individual basis, but in general terms onset of symptoms occurs decades after the disease initiation (Anto, Vermeire et al. 2001). Physical exams of patients in the early stages of disease typically reveal prolonged expiration during the

forced expiration maneuver. These patients often describe a sedentary lifestyle and fatigue, probably due to the avoidance of activity-induced dyspnea (Rennard, Decramer et al. 2002). With increasing disease severity, patients may present with hyperinflation as determined by increased resonance with percussion, decreased breath sounds, wheezing, chronic cough, and in the most sever cases, an increased chest diameter, a depressed and stiff diaphragm, Hoover's sign (inward movement of the lower rib cage during inspiration), cyanosis, and an enlarged liver due to right heart failure (Badgett, Tanaka et al. 1993, Garcia-Pachon and Padilla-Navas 2006). Increasing dyspnea appears to coincide with a more persistent and purulent cough, which begins and is worst upon waking but progresses to an intermittent occurrence throughout the day (Kessler, Partridge et al. 2011). Also early disease and exertional dyspnea can occur in tandem with weight gain due to activity avoidance, but as dyspnea worsens, late stages of COPD can often be associated with weight loss, which carries a worse prognosis

With disease progression it is currently understood that the decline in lung function is not a smooth curve from onset through severe COPD but more closely resembles a staircase. This step-wise decline in lung function is due to the progressively worse and more frequent exacerbations experienced by COPD patients, especially those still actively smoking (Vestbo, Prescott et al. 1996, Kanner, Anthonisen et al. 2001). These exacerbations can be caused by numerous stimuli

interacting with host factors, including bacteria, viruses, and changes in air quality that generate amplified inflammatory responses in the lower airway and compound the effects of CS (White, Gompertz et al. 2003, Au, Bryson et al. 2009). In addition hypoxemia, which is progressively worse with disease, can be acutely severe with exacerbation, and accompanied by hypercapnia (Kent, Mitchell et al. 2011)

### *1.1.4 Diagnosis of COPD*

Unfortunately there is currently no laboratory test whereby alterations in the serum or plasma can be used to diagnose COPD. Accurate diagnosis usually relies of the occurrence of indicative symptoms, exposure history, and family history, while confirmation of the disease is determined mainly using spirometry and chest CT scan. The incomplete reversibility of FEV1 with inhaled bronchodilators is also a confirmatory measure, especially in the absence of other, alternate explanations for airflow limitation (Qaseem, Wilt et al. 2011). To further aid in the clinical classification and treatment of COPD, the hallmark decline in lung function as determined by FEV1, the frequency of exacerbation, patient history, and the severity of symptoms are part of the new classification system for COPD, termed the Global Initiative for Chronic Obstructive Lung Disease, or GOLD stages (Global Initiative for Chronic Obstructive Lung Disease. 2011) (Table 1-1). As indicated in the table GOLD stages

1-4 are associated with an FEV1/FVC of less than 70%, indicating airflow obstruction, compared to above 80% for healthy individuals. The FEV1 shifts from around the normal 80% predicted in GOLD stage 1, to less than 30% in GOLD stage 4.

It is important to reiterate the importance of patient history and presentation for accurate diagnosis as several other disease processes, such as acute heart failure or carcinoma of the central bronchi, may share similar associated symptoms (Price, Yawn et al. 2010). Usually, in cases where the cause of disease is not understood, or a better understanding of the disease is needed, chest radiography and CT are employed. Radiography is often used to exclude certain alternate diagnoses or other diseases and is generally sensitive enough to detect COPD related characteristics including, vascular shadows, increased transparency of the lung, and a flat diaphragm, in 50% of patients with mild to moderate disease (Wallace, Winter et al. 2009). CT, especially high resolution CT, is considered more sensitive for detecting emphysema, particularly loss of capillary beds and distal vasculature in undiagnosed smokers, but it is not necessary for diagnosis, carries a high cost, and is associated with high radiation exposure (Miniati, Monti et al. 2008, Estepar, Kinney et al. 2013). CT is usually employed for better understanding of disease complications and exacerbations or for extreme treatment (Mair, Miller et al. 2009). However depending on the radiographic criteria others have shown that radiography is a simple test,

yielding 90% sensitivity for patients with emphysema (Miniati, Monti et al. 2008).

An additional and highly effective index for the diagnosis of moderate to severe emphysema is the diffusing capacity of the lung for carbon monoxide (DLCO) (diffusing factor or DFCO in animal models). The DLCO is taken into consideration with the measurement of blood gases, indicating hypoxia. While the blood gas measurements do not indicate alteration in alveolar ventilation, the DLCO can detect with reasonable certainty the loss of alveolar surface area indicative of structural losses due to emphysema (Macintyre, Crapo et al. 2005, Fallica, Das et al. 2011). However it is not considered to be overly specific for mild emphysema, especially in current smokers due to already persistent carboxyhemoglobin levels and possible thickening of the air-liquid interface and/or the endothelial cell (EndoC) and pneumocyte (EpiC) barrier (Graham, Mink et al. 2002). Comorbidities may also further confound the accuracy of DLCO in the absence of other diagnostic tools (Morrison, Abboud et al. 1989, Gould, Redpath et al. 1991, Saydain, Beck et al. 2004)

### *1.1.5 Treatments for COPD*

The overwhelming majority of COPD cases can be linked to smoking, and therefore the first line of defense is to recommend smoking

cessation for those still actively using cigarettes. Secondly, the GOLD classification system, with recent modifications, provides a platform for treatment, which generally attempts to reduce declines in lung-function, exacerbations, hospitalization, and increase quality of life, largely by improving upper airway symptoms versus treating the underlying cause (Qaseem, Wilt et al. 2011). Early treatments include short-acting bronchodilators on an as-needed basis for all stages and respiratory rehabilitation, including exercise training, education, nutritional intervention, and psychosocial support, starting in stage 2 (Balkissoon, Lommatzsch et al. 2011). Also beginning as early as Stage 2, long-acting inhaled beta-agonists, bronchodilators, or anticholinergics like formoterol, salmeterol, and tiotropium, are prescribed alone or together in varying combinations depending on each patients specific symptoms and GOLD stage, and are generally associated with a significant improvement in annual FEV1 declines, occurrence of dyspnea, and exacerbation frequency (Qaseem, Wilt et al. 2011). Antibiotics are usually prescribed for the treatment of acute exacerbations following sputum cultures, and as the disease progresses to include repeated yearly exacerbation, long-term corticosteroids are considered the next step in treatment. In advanced stages of COPD, where hypoxemia is a significant factor, and possibly with acute and or severe exacerbations, oxygen therapy is recommended and several studies show improved survival in patients receiving long-term supplemental oxygen therapy (Stoller, Panos

et al. 2010). In patients with confirmed and extensive upper lobe emphysema, removal of 25-35% of the poorly functioning lung tissue via lung volume reduction surgery has been shown to improve breathing function and quality of life (Miller, Berger et al. 2005)

### *1.1.6 Epidemiology of COPD*

COPD is currently the fourth leading cause of morbidity and mortality in the developed world and in 2012 was the third leading cause of death. On a global scale as much as 10% of the population was projected to be in GOLD stage 2 or higher, a prevalence that also increased significantly with age (Buist, McBurnie et al. 2007). This is a striking estimate given that GOLD stage 1 was excluded to avoid age–related biases. In the United States COPD prevalence has increased 183% since 1965, while other major causes of death steadily decline (Doherty 2003). From 1999-2009 COPD was the third leading cause of death in the United States (American Lung Association 2013). In 2009 alone over 130,000 people, or about 41 individuals per 100,000, died of COPD, of which just over half were women (Murphy, Xu et al. 2013). In 2008, over 12 million people were estimated to have diagnosed COPD in the United States, while an additional 12 million people are projected to have some degree of undiagnosed COPD based on lung function examinations (American Lung Association 2013). Of those diagnosed with COPD, emphysema was

the main diagnosis in approximately 5 million patients, but accounted for 15 times more deaths than chronic bronchitis (Centers for Disease Control 2011). From 1979-2001 it was estimated that over 8.5% of all hospital discharges were for COPD patients, where COPD was the primary or secondary diagnosis (Holguin, Folch et al. 2005). In addition cigarette smoking diseases, including emphysema, contribute to an estimated 2.5 million deaths worldwide and a healthcare cost approaching 38.8 billion dollars annually in the United States alone (Foster, Miller et al. 2006).

## 1.2 Emphysema

*"Emphysema of the lungs is a much more frequent disease than is commonly imagined. I meet with it constantly in practice, in some intermediate degree between what may be called its first stage, the dry catarrh, and its complete development..."* (Laennec 1819)

### *1.2.1 Defining Emphysema*

Emphysema, a word of Greek origin meaning inflation, is currently defined pathologically as the atypical and permanent enlargement of airspaces that are distal to the terminal bronchioles coincident with destruction of the alveolar tissue in the absence of apparent fibrosis. This pathologic definition has changed very little in the past 30 years (National Heart, Lung, and Blood Institute, Division of Lung Diseases 1985). This debilitating disease is further characterized by oxidative stress, apoptosis, and alveolar destruction (Demedts, Demoor et al. 2006, Boutten, Goven et al. 2010, Mohamed Hoesein, Zanen et al. 2013). This destructive process is described as the heterogenous pattern of airspace enlargement which thereby alters the orderly appearrance of the acinus, or cluster of air sacs and alveolar ducts including the associated capilaries. This specific acinar distinction is key in the comprehension of

emphysema, as airspace enlargement alone can be observed in other disease states with no apparent tissue destruction (Yamada, Nakanishi et al. 2013).

### *1.2.2 Histopathological Description of Emphysema*

There are three main histopathological classifications of emphysema that vary in their relation to the changing lobule structure containing the acini. The acinar structures are not readily distinguished in gross dissection, therefore the types of emphysema generally reference the damage of the acini within secondary lobules, circumscribed by septal tissue. Centrilobular emphysema is the most frequently described manifestation and it clearly correlates with smoking history, which has long been described (Thurlbeck 1963, Satoh, Kobayashi et al. 2001). In centrilobular emphysema, lesions occur in the proximal acini, and generally in the center of the acinus where the terminal bronchioles are situated. This type of emphysema predominates in the upper lobes of the lungs, may be an early manifestation of CS-induced disease, and is hypothesized to alter the microstructures of the aveoli and alveolar pores (Desplechain, Foliguet et al. 1983, Thurlbeck and Churg 1995, Fishman, Elias et al. 2008). In contrast panlobular emphysema is worse in the lower lobes and often discovered in histological examinations of patients with alpha-1 antitrypsin deficiency. However both manifestations are

observed in the later stages of emphysema in COPD (Fishman Elias et al. 2008, Sverzellati et al. 2014). The third type of emphysematous destruction is paraseptal in nature, referring to lesions of the peripheral lobules along the septal lines, usually adjacent to the pleura and occurring concurrently with fibrosis (Anderson and Foraker 1973, Fishman, Elias et al. 2008, Takahashi, Fukuoka et al. 2008). In all three instances there is clear pruning and distortion of the microvasculature and an absence of alveolar walls around large emphysematous airspaces; all three types of tissue destruction are apparent and the distinction is blurred in later stages of COPD (Fishman, Elias et al. 2008, Jindal and Vijayan 2013). The loss of the microvascular structures would undoubtedly have adverse affects on the surrounding tissues, as the cognate circulation is necessary for bathing the acini with nutrients and removal of the products of metabolism.

### *1.2.3 Emphysema, Cigarette Smoke, and Alveolar Maintenance*

As noted above, emphysema alone is a significant cause of COPD related mortality and, while the main cause of emphysema is understood to be CS, the underlying molecule pathogenesis of emphysema is not fully understood. The pathologic observation with CS-induced tissue destruction has lead to several emerging hyoptheses into disease development including increased oxidative stress, persistent

inflammation, and a protease/anti-protease imbalance, all of which promote and point towards an overarching phenotype of increased alveolar-capillary apoptosis and therefore alveolar maintenance as a therapeutic approach (Rahman and MacNee 1996, Kasahara, Tuder et al. 2000, Tuder, Zhen et al. 2003, Petrache, Natarajan et al. 2006, Tuder, Yoshida et al. 2006).

To understand the impact of CS, it is necessary to consider its impact on the breathing process. Airflow velocity and breath size are often increased, disturbing laminar flow but also recruiting alveoli to receive the increased volume. Air that reaches the alveolar space is polluted with a mix of extrinsic factors and oxidants capable of altering cell fate, including carbon monoxide, sulphur dioxide, nitric oxide, hydrocarbons, volatile organic compounds, and particulate matter (Fowles and Dybing 2003, Rodgman and Perfetti 2013). This polluted air is often held in the alveoli, increasing the incubation time of CS with alveolar EndoC and EpiC, the major structural components of the alveoli, both of which are susceptible targets of CS-induced oxidative stress and emphysematous remodeling (Thorley and Tetley 2007, Damico, Simms et al. 2011, Guarino, Cantarella et al. 2011). Hence, the concept of alveolar maintenance and homeostasis is becoming more appreciated as it pertains to the protection of these delicate microvascular structures from CS induced oxidative damage. More specifically, new hypotheses as to

the root of emphysematous destruction are linked with altered EndoC death and repair.

### *1.2.4 Emphysematous Progression*

After smoking cessation, emphysema continues to progress in a persistent cascade of tissue destruction (Mohamed Hoesein, Zanen et al. 2013). Some research indicates that the rates of progression do not differ between current and former smokers, while others show a slight improvement in the rate of decline in former smokers (Soejima, Yamaguchi et al. 2000, Bellomi, Rampinelli et al. 2010, Coxson, Dirksen et al. 2013). Recent work and computer modeling indicates that redistributed mechanical stress resulting from alveolar wall and extracellular matrix destruction may also overload the surrounding alveolar tissue increasing the likelihood of further destruction (Suki, Jesudason et al. 2011). The snowball effect of this mechanical breakdown may be a culmination of alveolar-capillary apoptosis due to oxidative stress, proteolysis, and/or failed repair, but its persistence is also suggestive of altered gene expression and behavior. Just as genetic factors play a role in disease susceptibility, it is also becoming more apparent that CS exposure is playing a role in dramatically altering intrinsic determinants of alveolar cell fate and disease progression. This may be due to underlying alterations in gene expression through

epigenetic modifications such as DNA methylation, histone modification, and microRNA expression but more work is needed to understand these context-dependent mechanisms. Still, there are several key proteins involved in cell fate, oxidative stress, and gene expression that are differentially expressed with disease, like vascular endothelial growth factor (VEGF), histone deactylase 2 (HDAC2), tumor suppressor protein p53, macrophage migration inhibitory factor (MIF), xanthine oxidoreductase (XOR), and nuclear factor erythroid 2-related factor 2 (NRF2), whose dysregulation has been implicated in abnormal alveolar apoptosis and repair in emphysematous tissue destruction (Voelkel, Vandivier et al. 2006, Sussan, Rangasamy et al. 2009, Kabesch and Adcock 2012, Kim, Serebreni et al. 2013).

### *1.2.5 Emphysema and Protease Imbalance*

The early discovery and subsequent research regarding spontaneous emphysema in patients with alpha-1 antitrypsin deficiency, a potent neutrophil elastase inhibitor, led to an initial interest in protease/antiprotease imbalances and effects on the extracellular matrixes, mainly of EndoC and EpiC, the key structural components of the acinus and new potential therapeutic targets. Intriguingly most therapies for COPD target airway constriction and inflammation; there is currently no specific treatment for emphysema or its progressive tissue

destructive, including proteolytic processes. The spontaneous airspace enlargement and loss of alveolar tissue in alpha-1 antitrypsin deficiency largely ignores the impact of CS on alveolar integrity and yields different pathologic features as previously noted (Abboud and Vimalanathan 2008, Fishman, Elias et al. 2008). Nonetheless, proteases may be increased in COPD patients, especially current smokers or patients experiencing exacerbations (Fera, Abboud et al. 1986, Fujita, Nelson et al. 1990, Ilumets, Rytila et al. 2008). Whether this imbalance is due to more protease production or activation and or less production of antiproteases remains uncertain and the specific effect on lung function has not been extensively studied in humans. Both increased proteases and their inhibitors are detected in the sputum of COPD patients, particularly those experiencing an exacerbation, but these increases do not correlate with FEV1 responses (Cawston, Carrere et al. 2001, Mercer, Shute et al. 2005, Calikoglu, Unlu et al. 2006). Some evidence links the overproduction of proteases in response to CS to the dramatic, transient and long-lasting increases in the source of many proteases, innate immune cells such as macrophages and neutrophils (Barnes 2004, Hoenderdos and Condliffe 2013). Other evidence suggests that CS, and the associated oxidative stress, can inactivate antiproteases, specifically the elastolytic effects of alpha-1 antitrypsin in the bronchoalveolar lavage (BAL) of smokers, through post-translation modifications (PTM) (Gadek,

Fells et al. 1979, Carp, Miller et al. 1982, Hubbard, Ogushi et al. 1987, Petrache, Fijalkowska et al. 2006).

### *1.2.6 Emphysema, Oxidative Stress, and DNA Damage*

The imbalance of antioxidants and oxidants, that by definition leads to oxidative stress, is linked with 1) numerous manifestations of COPD, 2) alterations in the underlying mechanisms of lung function decline, including alveolar-capillary damage, alveolar-capillary apoptosis, extracellular matrix (ECM) remodeling, inactivation of antiproteases, membrane lipid peroxidation, and mitochondrial respiration, and 3) the poor clinical and therapeutic efficacy of corticosteroids in patients with COPD (Abboud, Fera et al. 1985, Dalle-Donne, Giustarini et al. 2003, Abboud and Vimalanathan 2008, Ciencewicki, Trivedi et al. 2008, Louhelainen, Rytila et al. 2009, Merkwirth and Langer 2009). Further markers of oxidative stress, including hydrogen peroxide ($H_2O_2$), nitrotyrosine, 8-isoprostanes, malondialdehyde, 4-hydroxynonenal and 4-hydroxy-2-nonenal are increased in in the lung tissue, breath, sputum and blood of patients with COPD (Ichinose, Sugiura et al. 2000, Paredi, Kharitonov et al. 2000, Rahman, van Schadewijk et al. 2002, Drost, Skwarski et al. 2005, Montuschi 2005, Kluchova, Petrasova et al. 2007, Barreiro, Peinado et al. 2010). The main cause of increased amounts of these reactive oxygen species (ROS) in COPD is CS, essentially a harmful

mix of inhaled oxidants. However the dramatic imbalance between oxidants and antioxidants that results can persist long after smoking cessation (Zhou, Yan et al. 2000). As evidenced by the persistence of extracellular and intracellular ROS detected in former smokers, ROS is also produced by mitochondrial respiration, and various inflammatory, airway, and alveolar-capillary cells (Le, Damico et al. 2008, Aguilera-Aguirre, Bacsi et al. 2009, Louhelainen, Rytila et al. 2009, Damico, Zulueta et al. 2012, Kim, Serebreni et al. 2013). Important pulmonary and microvascular sources of ROS are xanthine oxidoreductase (XOR), uncoupled nitric oxide synthase (NOS), and NAD(P)H oxidase (Cai, Griendling et al. 2003, Birukov 2009, Damico, Zulueta et al. 2012). More specifically, XOR is a source of superoxide and hydrogen peroxide within pulmonary EndoC and it is elevated in human CS-induced COPD and emphysema (Pinamonti, Muzzoli et al. 1996, Pinamonti, Leis et al. 1998). Superoxide and hydrogen peroxide are abundantly produced, and while they are considered to be weak oxidizers, their increased production along with reactive nitrogen species (RNS) can parlay into the formation of more destructive radicals, which are increased with disease severity (Ichinose, Sugiura et al. 2000, Janssen-Heininger, Persinger et al. 2002, Van Beurden, Wielders et al. 2003, Marin-Corral, Minguella et al. 2009, Domej, Oettl et al. 2014, Antus and Kardos 2015).

Oxidative stress and increased ROS are implicated in DNA damage, histone modification, and therefore alterations in repair processes,

expression of cytoprotective proteins, and production of pro-inflammatory mediators in lung tissue and pulmonary vasculature. In particular the non-coding guanine rich segments of DNA, including promoter regions, are very susceptible to oxidative damage, and are repaired less efficiently (Makris, Tzanakis et al. 2008, Thomou, Paraskakis et al. 2009, Samara, Tzortzaki et al. 2010, Neofytou, Tzortzaki et al. 2012). In addition, emphysema patients and current smokers often have more oxidative DNA damage in the lung, as evidenced by the increased 8-hydroxy-2'-deoxyguanosine (8OHdG) double stranded DNA breaks (DSB), common by-products of oxidative DNA damage (Igishi, Hitsuda et al. 2003, Caramori, Adcock et al. 2011, Woodbine, Brunton et al. 2011, Dizdaroglu 2012, Tzortzaki, Dimakou et al. 2012, da Silva, da Rosa et al. 2013, Paschalaki, Starke et al. 2013). Single-stranded DNA lesions are more common than DSB, especially in non-replicating cells, but with extensive ROS-induced single-stranded lesions on opposite strands, the simultaneous removal of these bistranded lesions can give rise to DSB, before base-excision repair of only one lesion. This type of clustered DNA damage is very prominent in response to ionizing radiation (I/R), and occurs at a substantially lower frequency in response to endogenous ROS, making it more difficult to study under oxidative stress conditions (Dizdaroglu 2012). However recent work reveals that the repair mechanisms involving proteins like Ataxia telangiectasia mutated (ATM) kinase and Artemis, as well as the

kinetics of repair processes are fundamentally indistinguishable in ROS-induced and I/R induced DSB, suggesting that the underlying characteristics of DSB formation in each condition is also similar (Shackelford, Innes et al. 2001, Riballo, Kühne et al. 2004, Woodbine, Brunton et al. 2011).

In addition, DSB are very common in replicating cells where extensive or unrepaired single stranded DNA lesions, potentially from increased endogenous ROS, stall the DNA replication fork and trigger fork collapse (Pfeiffer, Goedecke et al. 2000, Chapman, Taylor et al. 2012). This may be an important aspect in emphysematous tissue loss where cell damage and death by definition outpace repair and proliferation. Further, DNA repair mechanisms such as DSB repair are increasingly inefficient with CS-induced disease severity possibly due to 1) increased ROS-induced DNA lesions that delay or inhibit cell proliferation, or 2) genetic polymorphisms, causing apoptosis, senescence, and inflammation (Pfeiffer, Goedecke et al. 2000, Sancar, Lindsey-Boltz et al. 2004, Aoshiba, Zhou et al. 2012, Chapman, Taylor et al. 2012, Neofytou, Tzortzaki et al. 2012, da Silva 2013, Kirkham and Barnes 2013, Paschalaki, Starke et al. 2013, Antus and Kardos 2015). Notably, the promoter of vascular endothelial growth factor (VEGF) and the hypoxia response element (HRE) are prone to increased 8OHdG in lungs of COPD patients, suggesting that oxidative stress may also modify

cell survival and transcription in EndoC and other lung cells through additional and alternative mechanisms (Pastukh, Zhang et al. 2011).

Recent research also indicates that continuous ROS production during and after CS is a key factor in driving inflammation and disrupting repair processes through the activation of redox-sensitive mitogen-activated protein kinases (MAPK) and transcription factors, such as Jun-N-terminal kinase (JNK), p38 MAPK, NRF2, and nuclear factor-kappa Beta (NF-kappaB) (Xia, Dickens et al. 1995, Huot, Houle et al. 1997, Ichijo, Nishida et al. 1997, Li and Karin 1999, Low, Liang et al. 2007, Boutten, Goven et al. 2011, Kirkham and Barnes 2013). NF-kappaB expression and activation correlates with airflow limitation, is increased in COPD, and is a known positive regulator of pro-inflammatory mediators (Di Stefano, Caramori et al. 2002, Edwards, Bartlett et al. 2009, Lawrence 2009). Activation of p38 is linked with inflammatory processes, apoptosis, activation of XOR, and as with NF-kappaB, is also increased with emphysema severity (Schieven 2005, Cai, Chang et al. 2006, Low, Liang et al. 2007, Le, Damico et al. 2008, Barnes 2013, Marumo, Hoshino et al. 2014). Emerging data also suggests that NRF2-regulated antioxidant activity, is reduced with CS-induced emphysema (Goven, Boutten et al. 2008, Malhotra, Thimmulappa et al. 2008, Suzuki, Betsuyaku et al. 2008, Malhotra, Thimmulappa et al. 2009). Signals to these proteins are propagated through key upstream

redox sensitive cysteine residues on MAPK or susceptible protein modifiers, and in turn alter cell fate and gene transcription.

Although it is incompletely understood, CS and ROS are also tied with abnormal histone regulation and therefore gene expression, possibly through PTM of proteins that regulate histone activity (Barnes, Ito et al. 2004, Rahman, Marwick et al. 2004). DNA wraps around a core of histone proteins, which can be modified by acetylation and or deacetylation to allow the transcriptional machinery to access specific gene sequences on the DNA. To counteract and perhaps balance histone acetylation by histone acetyltransferases (HAT) are several histone deacetylases (HDAC) and in COPD, HDAC activity is significantly reduced, most likely due to increased ubiquitination as a result of PTM (Barnes, Adcock et al. 2005, Ito, Ito et al. 2005). In contrast, histone acetylation is significantly increased, specifically around the promoter regions of pro-inflammatory genes (Ito, Ito et al. 2005).

### *1.2.7 Emphysema and Inflammation*

Chronic inflammation in the small airways and airspaces in the form of macrophages and neutrophils is a recognized COPD characteristic. Interestingly, the direct inhibition of alpha-1 antitrypsin is directly linked with a significant reduction in primary human EndoC death, suggesting an increased burden of neutrophils may be associated with capillary

pruning and EndoC caspase 3-dependent apoptosis (Petrache, Fijalkowska et al. 2006). Conversely elastolytic activity is increased in macrophages from patients with primary emphysema compared to patients with other primary diagnoses such as bronchitis, although this increased activity may purely reflect the increase in total phagocytes, as activity on a per cell basis does not differ (Muley, Wiebel et al. 1994, Abboud, Ofulue et al. 1998). Further some studies show that the degree of emphysema is associated with macrophage and not neutrophil burden (Finkelstein, Fraser et al. 1995). This is evidenced by the significant increase in macrophage-derived cytokines and chemokines, like tumor necrosis factor (TNF) alpha, and Il-8, which are increased with COPD severity (Keatings, Collins et al. 1996, Hirani, Antonicelli et al. 2001). However the burden and or persistence of macrophages in the airspace may also be skewed by defective clearance mechanisms. Healthy macrophages in the airspaces are thought to be cleared through a lymphatic or mucocilliary route, both of which are increasingly impaired due to mucus cell hyperplasia and production, and destruction of the pleura or lymphatic vessels respectively, although there is data to suggest the latter is increased in severe emphysema as part of an adaptive immune response (Hardavella, Tzortzaki et al. , Bhowmik, Chahal et al. 2009, Mori, Andersson et al. 2013).

Macrophages were once hypothesized to quench extracellular neutrophil elastase, further diminishing the impact of neutrophils on

disease progression, but others have since shown that macrophages are a source of elastase as well as other proteinases such as collagenase and gelatinase (Betsuyaku, Yoshioka et al. 1995, Finlay, O'Driscoll et al. 1997, Belvisi and Bottomley 2003, Dollery, Owen et al. 2003, Barnes 2004). These macrophage-derived sources of ECM degradation, including the collagenases matrix metalloprotease (MMP)1 and MMP8, the gelatinases MMP2 and MMP9, the elastase MMP-12 and the cysteine proteases or cathepsins, have been implicated in emphysema progression, as they are all increased in lung tissue, BAL, or ex vivo and BAL derived alveolar macrophages of human COPD patients, albeit at varying degrees depending on the studied cohort, possibly indicating variance due to genetic susceptibilities (Finlay, O'Driscoll et al. 1997, Betsuyaku, Nishimura et al. 1999, Segura-Valdez, Pardo et al. 2000, Imai, Dalal et al. 2001, Russell, Culpitt et al. 2002, Russell, Thorley et al. 2002, Culpitt, Rogers et al. 2005, Molet, Belleguic et al. 2005).

The strongest evidence for a causal role for MMPs in emphysema development comes from a study in 1997 by Hautamaki et al., where MMP12 deletion in mice provided protection from CS-induced emphysema development (Hautamaki, Kobayashi et al. 1997). This finding has also been validated in a similar guinea model, and there may be polymorphisms in the MMP12 promoter that participate in differential phenotypes in humans; however research in human emphysema patients indicates an absence of MMP12 in the later stages of COPD, and

suggests MMP2 and MMP9 may be key elastolytic participants (Finlay, O'Driscoll et al. 1997, Ohnishi, Takagi et al. 1998, Betsuyaku, Nishimura et al. 1999, Culpitt, Maziak et al. 1999, Imai, Dalal et al. 2001, Churg, Wang et al. 2007, Korytina, Akhmadishina et al. 2008). However MMP12 is known to cleave membrane bound TNF-alpha, a potent neutrophil chemoattractant, which is still considered a major player in ECM remodeling (Churg, Zay et al. 2002, Churg, Wang et al. 2003, Churg and Wright 2005, Lucattelli, Bartalesi et al. 2005, Elias, Kang et al. 2006). Intriguingly MMP12 is recently implicated in vascular injury and remodeling in the lung and in other disease states like scleroderma, suggesting it may be a contributor in dysregulated alveolar maintenance (Petrache, Natarajan et al. 2005, Stawski, Haines et al. 2014). Further the suspected longitudinal transition of MMP expression in COPD patients, from MMP12 to MMP9 for example, could be the result of evolving epigenetics and immune response to chronic CS and or persistent lung degradation. MMP9 is known to cleave latent transforming growth factor (TGF)-beta, a factor that is tied with the regulation of adaptive immune responses, endothelial apoptosis, and small airway fibrosis—a recognized characteristic of late stage emphysema often termed combined pulmonary fibrosis and emphysema (CPFE) (Wiggins, Strickland et al. 1990, Lanone, Zheng et al. 2002, Morris, Huang et al. 2003, Leksa, Godar et al. 2005, Wan and Flavell

2008, Ferrari, Cook et al. 2009, Katzenstein, Mukhopadhyay et al. 2010, Jankowich and Rounds 2012).

### *1.2.8 Emphysema and Abnormal Clearance of Apoptotic Cells*

In addition to macrophage and neutrophil recruitment in response to CS, there is also an effect of CS on the infiltrating and resident alveolar cells that is linked with abnormal EndoC homeostasis (Vandivier, Henson et al. 2006, Voelkel, Vandivier et al. 2006, Thorley and Tetley 2007). CS impacts on macrophage phagocytic function which may lead a continuous cycle of infiltrating naïve macrophages and neutrophils due to dysregulated clearance of bacteria and dead or dying EndoC, EpiC, and neutrophils (Vandivier, Henson et al. 2006, Harvey, Thimmulappa et al. 2011). Specifically regarding vascular homeostasis, macrophages involved in the clearance of apoptotic EndoC and EpiC cells are also an important source of the EndoC survival factor, VEGF. This process, termed efferocytosis, is necessary for the initiation or continuation of the tissue repair processes but is significantly reduced in response to CS, oxidative stress, and in macrophages from COPD patients with a history of heavy chronic but not current smoking history (Hodge, Hodge et al. 2003, Vandivier, Henson et al. 2006, Hodge, Hodge et al. 2007, Voelkel and MacNee 2008, Belchamber, Singh et al. 2014, Hamon, Homan et al. 2014). This further suggests that the predominant cell infiltrate,

macrophages, are necessary for alveolar maintenance, whereas the sufficiency of the related pro-inflammatory or proteolyitc response of macrophages to CS on emphysematous destruction remains controversial. Poor efferocytosis also leads to secondary necrosis of uncleared apoptotic cells partly explaining the persistent inflammation (Silva 2010, McCubbrey and Curtis 2013). Conversely, tissue density determined by CT imaging, which is a means to quantify alveolar structure and inflammation intensity, is reduced in former smokers suggesting that inflammation or related secretions may not drive emphysema progression, although it may participate (Ashraf, Lo et al. 2011, Mohamed Hoesein, Zanen et al. 2013, Karimi, Tornling et al. 2014).

### *1.2.9 Emphysema and Autoimmunity*

As emphysema progresses there is a shift from an inflammatory environment dominated by innate immune cells like macrophages and neutrophils, to an enhanced adaptive immune response characterized by increased T lymphocytes (Aoshiba, Koinuma et al. 2004, Curtis, Freeman et al. 2007). This discovery has recently led to a vast expansion in hypotheses involving a developed autoimmunity that is pathologic in nature. Grumelli et al. showed that emphysema patients not currently suffering an exacerbation or an active infection had increased CD4 T

cells (Grumelli, Corry et al. 2004). Others showed that certain autoimmune and CD4 chemokines were increased in lungs of COPD patients (Panina-Bordignon, Papi et al. 2001). Interestingly memory T cells from smoker with COPD have a recalled immune response to elastin fibers, suggesting that autoimmunity may develop from the breakdown of the alveolar ECM (Lee, Goswami et al. 2007). In supporting models, Taraseviciene-Stewart et al. showed CD4 t cells target EndoC causing centrilobular emphysema, suggesting adaptive immune reactions may participate in the breakdown of the acini and endothelial apoptosis (Taraseviciene-Stewart, Scerbavicius et al. 2005).

Whether this is the case in human emphysema is still unknown. This uncertainty remains in part because there is a disconnect between mouse models and human samples regarding the suspected subset of pathogenic CD4 T cells, termed Th17 cells, which produce interleukin (IL)-17 and proinflammatory mediators, that recruit innate immune cells, and also the subset of regulatory T cells (Treg), which may or may not have altered function or presence in response to CS or with disease progression (Grumelli, Corry et al. 2004, Lee, Goswami et al. 2007, Barcelo, Pons et al. 2008, Shan, Cheng et al. 2009, Dancer and Sansom 2013, Hou, Sun et al. 2013). This hypothesis is further complicated in viral infections when other adaptive immune cells, like CD8 T cells, predominate and actively destroy infected alveolar-capillary cells (Gadgil and Duncan 2008).

The complicated multifactorial etiology of autoimmunity has led to high-throughput screening to discover the underlying genetic changes that may regulate autoimmune responses and the related inflammatory mediators in chronic lung and other diseases (Curtis, Freeman et al. 2007, Gregersen and Olsson 2009, Pillai, Ge et al. 2009, Regan, Hokanson et al. 2010). The persistence of infections and the differential causes of exacerbations may also play a role in how genes are regulated and/or autoimmunity develops (White, Gompertz et al. 2003). However in stark contrast to these findings, CS-exposure in mice devoid of B and T cells still causes emphysema (D'Hulst A, Maes et al. 2005). Taken together, the experimental and clinical findings support a role for a dysregulated adaptive immunity and related inflammation in the progression of emphysema in genetically susceptible smokers, although its sufficiency and or necessity in human disease still remain controversial.

## 1.3 Apoptosis in Emphysema

Emphysema development is propelled by alveolar-capillary apoptosis (Yokohori, Aoshiba et al. 2004, Demedts, Demoor et al. 2006, Park, Ryter et al. 2007). Increased oxidative stress, inflammation and protease imbalances, and the associated alterations in growth factors, cell cycle, ECM, immune cell regulation, and extracellular death ligands, can all cause apoptosis through intrinsic mitochondrial pathways or through extrinsic death receptor-mediated pathways. Death receptor (DR) related apoptosis is initiated through various DR ligands including TNF alpha, which is increased with COPD severity (Strasser, O'Connor et al. 2000, Park, Ryter et al. 2007). The cascade of activation following phosphorylation of the intracellular receptor domain culminates in the cleavage and activation of FLICE (aka caspase 8) and finally caspase 3 and or 7. The intrinsic activation of apoptosis in EndoC and EpiC is categorically tied with oxidative stress and subsequent DNA damage (Roos and Kaina 2006, Morissette, Parent et al. 2009, Damico, Simms et al. 2011).

There is a considerable amount of apoptotic related research into emphysematous tissue destruction and remodeling focusing on the loss and repair of both EndoC and EpiC comprising the alveolar wall. There are several reports of increased alveolar wall apoptosis in patients with COPD compared to non-smokers and in animal models of CS-induced

emphysema (Yokohori, Aoshiba et al. 2004, Calabrese, Giacometti et al. 2005, Imai, Mercer et al. 2005, Kanazawa and Yoshikawa 2005, Demedts, Demoor et al. 2006, Henson, Cosgrove et al. 2006, Marwick, Stevenson et al. 2006, Petrache, Fijalkowska et al. 2006, Petrache, Fijalkowska et al. 2006). This alveolar wall apoptosis further correlates with increases in surface area and mean airspace size but interestingly it is also associated with enhanced proliferation of both EndoC and EpiC, suggesting that the repair process is overwhelmed by CS-induced tissue destruction and general alveolar cell apoptosis (Yokohori, Aoshiba et al. 2004).

### *1.3.1 Emphysema and p53*

DNA damage is linked to the activation of p53 and both are increased in the acini of smokers and emphysema patients (Roos and Kaina 2006, Siganaki, Koutsopoulos et al. 2010, Caramori 2011, Aoshiba, Zhou et al. 2012, Neofytou, Tzortzaki et al. 2012). Polymorphisms in p53 also correlate with disease onset and severity (Lee, Chen et al. 2006, Arif, Vibhuti et al. 2008). Depending on the degree of damage and its phosphorylation status, p53 can directly modulate members of the Bcl-2 family of mitochondrial membrane regulators, such as the pro-apoptotic BAX and BAK, which are both increased in emphysema, or p53 can act as a transcription factor, enhancing the gene expression of apoptotic

protease activating factor 1 (APAF1), mediators of cell cycle arrest, and the pro-apoptotic Bcl-2 proteins, tBID, BAX NOXA, and PUMA (Haupt, Berger et al. 2003, Morissette, Vachon-Beaudoin et al. 2008, Morissette, Parent et al. 2009). These pro-apoptotic proteins converge on the mitochondrial membrane with cleaved tBID, inducing pore formation, disrupting the membrane potential, and causing the release of cytochrome c (Degenhardt, Sundararajan et al. 2002, Haupt, Berger et al. 2003, McDonnell, Wang et al. 2003). In addition free cytosolic p53 can also mediate the oligomerization of the subunits comprising the pore channel or directly disrupt mitochondrial membrane integrity (Chipuk, Bouchier-Hayes et al. 2005, Wolff, Erster et al. 2008). Cytochrome C, which normally is a part of the mitochondrial respiratory chain, once in the cytoplasm can activate caspase 9 and disrupt endoplasmic reticulum potential (McDonnell, Wang et al. 2003, Jin, Zhao et al. 2004). Caspase 9 will then activate caspase 3 and or 7, ending in programmed cell death as in the extrinsic pathway. Activation of p53 is also associated with the enhanced expression and membrane trafficking of the extrinsic pathway associated DR, further linking p53 to many mechanisms of apoptosis in COPD (Bennett, Macdonald et al. 1998, Wu, Kim et al. 2000).

### *1.3.2 Apoptosis in Emphysema: An Endothelial Focus*

Both Endo and EpiC are key structural components of the alveoli and main targets of CS induced apoptosis (Morissette, Parent et al. 2009, Damico, Simms et al. 2011, Guarino, Cantarella et al. 2011). EpiC form the inner wall of the alveolar surface and have therefore been an appealing focus regarding CS related damage, considering the close proximity to noxious gas mixtures and proteases released by infiltrating immune cells (Mercer, Lemaître et al. 2006, Thorley and Tetley 2007). Moreover, type II EpiC are reportedly an important source of VEGF, which is necessary for EndoC maintenance and can also influence EpiC growth (Voelkel, Vandivier et al. 2006, Lee, Chen et al. 2007). EndoC line the vessels and provide structural support to the microcirculation in the lung, a system obligatory for bathing all alveolar components with nutrients. Gordon and colleagues have reported that circulating markers of pulmonary EndoC apoptosis are observed at higher levels in smokers with diminished DLCO, a physiologic manifestation of diminished alveolar surface area, thus providing evidence that pulmonary EndoC apoptosis is an early manifestation of CS-induced lung injury (Gordon, Gudi et al. 2011). Further the unbalanced loss of EndoC in these microcirculatory structures is both necessary and sufficient for disease progression and this would undeniably impact EpiC homeostasis (Kasahara, Tuder et al. 2000, Tuder, Zhen et al. 2003, Petrache, Natarajan et al. 2005).

### *1.3.3 Apoptosis and Growth Factors*

Human emphysema is associated with diminished growth factor secretion in the lung, including VEGF, hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and TGF-beta. HGF and FGF secretion and or receptors, which are necessary for alveolar repair processes, are increased with disease severity, suggesting that they may have an impact on vascular remodeling (Kranenburg, de Boer et al. 2002, Sauleda, Noguera et al. 2008). TGF-beta, which is also increased in COPD, is an active component in regulating inflammation, and aspects of lung remodeling such as angiogenesis, and apoptosis of EndoC (Leksa, Godar et al. 2005, Wan and Flavell 2008, Ferrari, Cook et al. 2009, Pastukh, Zhang et al. 2011). Further genetic polymorphisms in TGF-beta are associated with COPD susceptibility (Silverman, Speizer et al. 2000, Hersh, DeMeo et al. 2008, Konigshoff, Kneidinger et al. 2009). VEGF is decreased in the sputum of COPD patients and inversely correlates with disease severity, suggesting that impaired EndoC growth and survival may influence susceptibility in humans (Kanazawa and Yoshikawa 2005).

It has been postulated that decreased VEGF and/or signaling would be sufficient to induce disease (Kasahara, Tuder et al. 2001, Kanazawa, Asai et al. 2003). By using in situ end labeling, Segura-Valdez and colleagues determined that apoptotic cells in smokers with emphysema

were mainly EndoC, implicating both increased TGF-beta or decreased VEGF (Segura-Valdez, Pardo et al. 2000). In addition smoking may disrupt the expression or signaling of the VEGF-VEGF receptor complex (Marwick, Stevenson et al. 2006). In animal studies, there is more direct evidence of a causal relationship between EndoC apoptosis and development of emphysema. Kasahara and Tuder, reported that VEGF receptor blockade led to an apoptosis-dependent emphysematous remodeling; this was in the absence of inflammation but with a clear dependence on oxidative stress (Kasahara, Tuder et al. 2000). Taken together, EpiC death is hypothesized to decrease VEGF expression, ultimately disrupting endothelial homeostasis and resulting in emphysema.

However, not to understate the importance of EpiC derived VEGF, the proposed dependence of EndoC apoptosis on EpiC apoptosis has not been directly shown. To the contrary the key finding in blocking VEGF or its receptors highlights the importance of EndoC homeostasis on EpiC death. Further in limited models where apoptosis and proliferation were studied simultaneously in human emphysema lung specimens, general alveolar wall apoptosis was increased but the majority of cells undergoing proliferation, as determined by proliferation cell nuclear antigen (PCNA), were EpiC, suggesting that EpiC VEGF production and or autocrine and paracrine signaling may be less prone to CS-induced alterations (Yokohori, Aoshiba et al. 2004, Takahashi, Nakamura et al.

2008). In addition, macrophages, which are also an important source of VEGF and the predominant cellular infiltrate into the lungs of emphysema patients are known to be poorly efferocytic in COPD patients, a process normally sufficient to enhance macrophage-derived VEGF (Vandivier, Henson et al. 2006, Wan, Huang et al. 2015). It should also be noted that until recently EndoC were not thought to produce VEGF, but recent evidence suggest they do and it is sufficient for steady-state EndoC homeostasis, further highlighting the importance of EndoC integrity on alveolar and Endo maintenance (Maharaj, Saint-Geniez et al. , Lee, Chen et al. 2007). Taken together, these data highlight the potential contributions of non-EpiC derived VEGF, and therefore other potential sources of reduced VEGF leading to EndoC death with increasing disease severity.

## 1.4 Animals Models of Emphysema

There are several animal models of emphysema that have been developed and implemented to recapitulate various aspects of the complicated etiologies of disease onset, alveolar-capillary apoptosis, and progression. While some mouse strains and specific gene knockout mice, the VEGFR null or NRF2 null mice for example, develop spontaneous emphysema with age, exposures to many stimuli, including CS and proteases, are often used to induce disease, sometimes in tandem with postulant genetic deletions, with the prospect of simulating what we know about human emphysema, and understanding the impact of certain susceptibility genetic factors or candidate protein mediators.

### 1.4.1 Elastase Model

With the discovery of emphysema in patients with alpha-1 antitrypsin deficiency, enhanced protease expression has long been considered a driving factor in alveolar-capillary apoptosis, tissue degradation, and disease development (Laurell and Eriksson 1964, Carp, Miller et al. 1982, Fujita, Nelson et al. 1990). The main protease that is degraded by alpha-1 antitrypsin is neutrophil elastase, and it degrades elastin fibers in the ECM of acinar cells (Hubbard, Ogushi et al. 1987). In order to

mimic the increase in protease production, intratracheal instillation of a plant protease called papain, human neutrophil elastase, or porcine pancreatic elastase is mainly performed on mice, rats, hamsters, and guinea pigs (Qian and Mitzner 1989, Tseng, Qian et al. 1992, Corteling, Wyss et al. 2002, Borzone, Liberona et al. 2009, Vecchiola, de la Llera et al. 2011, Craig, Scott et al. 2013). In mice, porcine elastase yields consistent and striking emphysematous damage accompanied with airspace enlargement, diminished alveolar surface area for gas exchange, declines in lung function, and immune function, which in part validate the model regarding relevance to human disease processes (Kuhn, Yu et al. 1976, Gillot, Masy et al. 1997, Fallica, Das et al. 2011, Ganesan, Faris et al. 2012).

Intratracheal or nasopharyngeal installation of elastase to induce emphysematous lesions is much different than the slow onset of human disease and may involve other mediators. This model may provide insights into the nature of the step-wise decline in lung function observed with increasingly worse exacerbations in human cases, as the progression that occurs after elastase clearance may be mediated by host inflammatory proteases (Kaplan, Kuhn et al. 1973, Kuhn and Starcher 1980). However a large dose of elastase gives a clear picture of severe disease. In addition, it is a low-cost method, usually requiring one bolus dose of elastase to induce characteristics of human panacinar emphysema (Antunes and Rocco 2011). This method is often employed to

study the relationship of structure and function in the lung and to develop and or improve morphometric and stereological techniques (Lande and Mitzner 2006, Mitzner 2007, Fallica, Das et al. 2011, Munoz-Barrutia, Ceresa et al. 2012, Limjunyawong, Kearson et al. 2014, Limjunyawong, Fallica et al. 2015). Further, given the rapid onset of emphysema, this is an attractive model to test specific genes and or proteins as well as potential therapeutic interventions that target, reverse, or protect against alveolar-capillary destruction (Takahashi, Nakamura et al. 2008). This also an appealing model in that the instilled elastase is cleared from the lung in 2-3 days but the alveolar damage persists thereafter and increases for several weeks, raising the possibility of developed autoimmunity as a result of ECM breakdown (Gillot, Masy et al. 1997, Le Guennec, Pestre et al. 2012, Kurimoto, Miyahara et al. 2013).

### *1.4.2 Autoimmune Model*

It is recognized that innate immunity responds to early exposure to CS and is prevalent during exacerbations but new research suggests that as emphysema progresses, autoimmunity is increased and dysregulated (Aoshiba, Koinuma et al. 2004, Curtis, Freeman et al. 2007). Evidence for autoimmunity in COPD was recently established, in which CD4 T cells from the blood of COPD patients showed a dramatic inflammatory

response to elastin fibers, partly validating a focus on autoreactive T cells as well as the protease based model (Gillot, Masy et al. 1997, Lee, Goswami et al. 2007, Le Guennec, Pestre et al. 2012, Kurimoto, Miyahara et al. 2013). In support of this role for autoimmune CD4 T cells in human emphysema, Taraseviciene-Stewart et al. show that rats immunized with human endothelial cells develop antibodies towards EndoC and within in weeks have signs of centrilobular emphysema, suggesting adaptive immune reactions may participate in the breakdown of the acini and endothelial apoptosis (Taraseviciene-Stewart, Scerbavicius et al. 2005). Interestingly the adoptive transfer of CD4 T cells from these immunized rats, into naive immunocompetent animals also results in emphysema, implicate a role for CD4 T cells in the targeted destruction of the alveolar EndoC. Therefore this model may fill the gap in knowledge as to the continued alveolar wall and EndoC destruction after smoking cessation. However the root cause of CD4 T cells initiating immune responses to self EndoC remains speculative, although the PTM of certain proteins, possibly due to CS, is recently implicated (Anderton 2004, Ryan, Nissim et al. 2014). The drawback of this model is that it largely avoids the slow initiation of disease onset, innate inflammation, and oxidative stress observed in human emphysema. In addition, emphysema can occur in murine models with recombination activating gene (RAG1) deficient mice and severe combined immunodeficient (SCID) mice suggesting processes involving

autoimmune responses in emphysema may contribute to the disease but are not necessary for its development.

### *1.4.3 Cigarette Smoke Model*

Chronic CS exposure is the main cause of human emphysema and therefore a frequently used exposure method in animal models. In general the onset of detectable emphysema, defined by airspace enlargement accompanying decreased alveolar surface tissue, in mice takes months to a year and therefore has substantial associated operating costs (Fehrenbach 2006, Antunes and Rocco 2011, Leberl, Kratzer et al. 2013). Other species of animals including guinea pigs and rats are also used given there increased susceptibility to emphysematous remodeling, but due to the wide availability of genetic mutations, mice, particularly the C57BL/6, Balb/c, and A/J strains, are commonly used species (Leberl, Kratzer et al. 2013, Laucho-Contreras, Taylor et al. 2015). In these mouse strains there are several varying approaches for smoke exposure based on ease of delivery and cost.

A widely used method for exposing mice is whole body exposure; however there are other strategies utilizing individual mouse restrainers for direct CS exposure to the nose which also understandably incurs a higher labor cost (Reduction, Bondurant et al. 2001, Leberl, Kratzer et al. 2013). In either case the smoke delivered to the mice is a mixture of side

and mainstream smoke. With whole body exposure the mixture is approximately 80% side stream and 20% mainstream; with the direct nose exposure this estimated ratio is flipped (Moritsugu 2007, Leberl, Kratzer et al. 2013). In addition the use of modern equipment, such as the Teague exposure chambers, allows for total suspended particles (TSP) or total particulate matter (TPM) to be quantified, in order to insure regular dosing, and more accurate recapitulation of human main and side stream CS exposure (Rangasamy, Cho et al. 2004, Rangasamy, Misra et al. 2009, Sussan, Rangasamy et al. 2009, Simet, Sisson et al. 2010, Leberl, Kratzer et al. 2013). While this is theoretically the closest model for mimicking human disease, there is a lack of consensus in the literature regarding the length of exposure, the "dose" of CS, particularly the TSP, TPM, and the amount of cigarettes used per mouse or per chamber per day, the type of cigarettes utilized, and the type of smoke, main or side stream, being delivered to the mice (Leberl, Kratzer et al. 2013). Given that CS is a mixture of over 4000 potentially hazardous compounds in a gaseous form, the lack of consensus on these features could be a source of differential responses (Fowles and Dybing 2003, Rodgman and Perfetti 2013, John, Kohse et al. 2014). In addition due to the varying length of time it takes mice to develop CS-induced emphysema, the natural age-dependent alteration is lung structure may also make for difficult interpretation regarding the impact of CS

(Fehrenbach 2006, Takahashi, Fukuoka et al. 2008, Yao and Rahman 2012).

Standardized reference cigarettes are available to aide in consistency in the field with dosing and TPM per cigarette, but other cigarettes are still used and the TSP and or TPM vary greatly from one experiment to the next. In addition, because the levels of certain oxidants and compounds differ in main and side stream CS, the type of smoke could significantly impact the patterns of inflammation, proteotlytic damage, declines in lung function, and the associated detection of emphysema (Finkelstein, Fraser et al. 1995, Nikula, March et al. 2000, Reduction, Bondurant et al. 2001, DeMarini 2004, Moritsugu 2007, Guarino, Cantarella et al. 2011, Rinaldi, Maes et al. 2012, John, Kohse et al. 2014). Despite these pitfalls, acute exposure in mice generally recapitulate many aspects of human disease processes, including DNA damage, dramatic increases in innate immune cell infiltration, alterations in immune cell function, enhanced proteolytic activity, enhanced oxidative stress, and increased alveolar-capillary apoptosis. This continues with chronic CS resulting in the irreversible loss of alveolar tissue, ECM destruction, pulmonary hypertension, and associated late stage fibrosis that characterizes human COPD (Nikula, March et al. 2000, Reduction, Bondurant et al. 2001, Rangasamy, Cho et al. 2004, Abboud and Vimalanathan 2008, Churg and Wright 2009, Sussan, Rangasamy et al. 2009, Barreiro, Peinado et al. 2010, Simet,

Sisson et al. 2010, Aoshiba, Zhou et al. 2012, Pellegrino and Antonelli 2012, Rinaldi, Maes et al. 2012, Leberl, Kratzer et al. 2013).

### *1.4.4 Other Inhalation Models*

Oxidative stress in response to cigarette or its continuance after smoking cessation has lead to other inhalation models, including exposure to zone, sulfur dioxide, or nitrogen dioxide, exclusively exploring the effects of environmental oxidants, production of ROS or RNS, and therefore oxidative damage on emphysema development (Holroyd, Eleff et al. 1997, Ichinose, Sugiura et al. 2000, Janssen-Heininger, Persinger et al. 2002, Persinger, Poynter et al. 2002, Meng, Qin et al. 2003, Groneberg and Chung 2004, Triantaphyllopoulos, Hussain et al. 2011, Uh, Kim et al. 2011). Further, these noxious gases are hypothesized to be a significant source of exacerbated symptoms. These are interesting models, but require high concentrations that far exceed those detected in the environment for observable effects. Acute exposure to high concentration leads to increased alveolar wall permeability and a robust inflammatory response predominated by neutrophils, while longer exposures can cause the development of similar histopathological alterations and diffuse emphysematous tissue destruction observed in the later stages of COPD (Farone, Huang et al. 1995, Haddad, Salmon et al. 1996, Holroyd, Eleff et al. 1997, Kodavanti, Jackson et al. 2000, Mudway and Kelly 2000,

Persinger, Poynter et al. 2002, Triantaphyllopoulos, Hussain et al. 2011). The impact of ozone in particular seems to be on the small airways, causing enhanced airway hyperactivity and eventually signs of obstruction, as well as increases in protease production with subsequent loss of alveolar surface area (Tsukagoshi, Haddad et al. 1995, Mudway and Kelly 2000, Triantaphyllopoulos, Hussain et al. 2011). However ozone exposure is specifically linked with differential proteomic alterations compared with CS, suggesting its pathway to destroying tissue may not be consistent with human or murine emphysema (Uh, Kim et al. 2011). In addition there is a specific effect of all three gases on airway cells, causing hyperplasia, damage to cilia, and mucus hypersecretion, suggesting it may be an important model for bronchitis, although the use of mice may confound the translational value since they lack a dedicated bronchial circulation (Verloop 1949, Mitzner, Lee et al. 2000, Groneberg and Chung 2004). Nonetheless these noxious gases and their secondary oxidation products provide key perspective as to the involvement of the oxidative stress component in disease progression but do not provide a full picture of emphysema or COPD. Further the different interspecies and intraspecies alterations may implicate underlying genetic susceptibilities but the duration and extent of exposures varies widely.

### *1.4.5 Genetic Models*

There is overwhelming evidence that connects susceptibility of developing emphysema with underlying alterations to genetic factors, including genetic polymorphisms and epigenetic silencing (Martorana, Brand et al. 1993, Holroyd, Eleff et al. 1997, Wesselkamper, Prows et al. 2000, Hoffmann 2005, Reinhard, Meyer et al. 2005, Elias, Kang et al. 2006, Silverman 2006, Shapiro 2007, Hersh, DeMeo et al. 2008, Regan, Hokanson et al. 2010). Therefore the deletion of candidate genes that may regulate CS-induced disease and or result in spontaneous emphysema development is widely used. The targeted genetic deletions in mice are best selected to mirror genes that are silenced or proteins that are significantly decreased in human COPD (Holroyd, Eleff et al. 1997, Silverman, Chapman et al. 1998, Reinhard, Meyer et al. 2005, de Andrade 2012, Kabesch and Adcock 2012). This is perhaps recently exemplified in studies where the VEGF receptor and NRF2 have been individually knocked out in mice, to further understand their respective roles on aging and more specifically on EndoC, alveolar maintenance, and the role of antioxidants in regulating CS-induced oxidative stress (Kasahara, Tuder et al. 2000, Rangasamy, Cho et al. 2004). The spontaneous nature of emphysema in these and other genetic models further implicates differential gene expression in the aging process, suggesting that the increased severity of emphysema observed with the

tandem approach of genetic deletion with CS exposure is accelerating the aging process (MacNee 2009).

### *1.4.6 Summary of Mouse Models*

The animal models of emphysema development discussed above represent the most promising approaches to recapitulate what we know about human COPD. The elastase model is useful for understanding the potential contribution of protease/antiproteases imbalances but leads to slightly different histopathology and utilizes a time frame inconsistent with COPD development. The autoimmune model is an interesting approach to study the potential impact of altered immunity on disease progression observed after smoking cessation or with chronic CS, but similarly avoids the key aspects of disease onset and duration. The acute and chronic exposure of animals to CS is used to closely mimic many disease processes in human emphysema. Inhalation models other than tobacco smoke may provide information as to the specific effects of oxidative stress on disease development and or progression, as well as provide insight into the nature of non-infectious exacerbations.

Each of theses models carry value in understanding the multifactorial etiologies of emphysematous disease onset and or progression, but the tandem approach of CS exposure and genetic deletion was selected for this thesis project for several reasons. First this

exposure model represents the main cause of emphysema in humans and has been validated extensively in the literature. Secondly it allows accurate modeling of the acute and chronic effects of CS exposures on the alveolar compartment. Third it allows for the study of specific factors through genetic deletion, such as MIF, that may have an impact on emphysema, over the natural course of disease development. Thus understanding the underlying mechanisms of CS in tandem with potential novel intrinsic determinants of disease would propel the development and advancement of emphysema therapies, for which there are currently none. Fourth, MIF is an ideal candidate for targeted therapies, as the loss of MIF activity is implicated in normal airspace enlargement observed with aging, several disease processes of lung, and cytoprotection from CS induced apoptosis in vitro (Rosengren, Bucala et al. 1996, Kleemann, Kapurniotu et al. 1998, Damico, Simms et al. 2011, Mathew, Jacobson et al. 2013). The incorporation of MIF and MIF deficient (*Mif* $^{-/-}$) mice in this model is further described in the following section.

## 1.5 Macrophage Migration Inhibitory Factor (MIF)

Historically MIF was one of the first cytokines to be discovered, and much research described its in vitro effects on macrophage function and motility, hence the common name alluding to this arresting behavior (Calandra and Roger 2003). In addition, MIF was thought to be secreted mainly from activated T lymphocytes. It was not until some 30 years after its discovery that MIF was cloned and still another few years before the recombinant protein was readily obtainable to study its structure and function. We now appreciate that MIF is constitutively expressed, stored, and secreted by many cells types that interact with the host environment, including macrophages, a main source during inflammatory responses, EndoC, EpiC, parenchymal cells, tumor cells, eosinophils, and glandular pituitary cells (Baugh and Bucala 2002, Bucala 2012). In addition MIF is found in the serum under basal conditions, indicating it may exert regulatory function. MIF is also secreted by a nonclassical ER/Golgi-independent pathway, and its release from preformed pools is regulated by cellular stress and inflammation (Weiser, Temple et al. 1989, Bernhagen, Calandra et al. 1993, Merk, Baugh et al. 2009). Further, while MIF that is present on the extracellular surface of EndoC can promote monocyte arrest under flow conditions, and despite its name, MIF is generally recognized as promoting the chemotactic transmigration of leukocytes, namely

monocytes, into tissues (Ren, Tsui et al. 2003, Bernhagen, Krohn et al. 2007, Simons, Grieb et al. 2011, Bucala 2012).

MIF is now redefined as a 12.5kDa evolutionarily conserved pleiotropic cytokine, chemokine, hormone, and enzyme with intrinsic thiol-protein oxidoreductase (TPOR) activity, tautomerase activity and pro-inflammatory activity (Rosengren, Bucala et al. 1996, Kleemann, Kapurniotu et al. 1998, Chesney, Metz et al. 1999, Baugh and Bucala 2002, Calandra and Roger 2003). In humans the MIF gene is located in region 1 of the q arm of chromosome 22 (22q11.2), which shares genetic homology with the MIF containing chromosome 10 in mice (Weiser, Temple et al. 1989, Calandra and Roger 2003). The MIF gene encodes a short 800 base mRNA that is translated into a 114 amino acid protein in which the sequence is 90% conserved and function is indistinguishable from mice to humans, suggesting that MIF has significant biological functions (Weiser, Temple et al. 1989, Bernhagen, Mitchell et al. 1994). Among these functions MIF is implicated in mechanisms that regulate MAPK activation, prevent apoptosis, promote cell survival, stimulate the expression of pro-inflammatory mediators, and block glucocorticoid activity, all of which are dysregulated with emphysema disease progression (Finkelstein, Fraser et al. 1995, Fujimoto, Kubo et al. 1999, Vignola, La Grutta et al. 2002, Drost, Skwarski et al. 2005, Schieven 2005, Demedts, Demoor et al. 2006, Mercer and D'Armiento 2006, Low, Liang et al. 2007, Elsby, Donn et al. 2009, Barreiro, Peinado et al. 2010,

Shan, Yuan et al. 2012, Barnes 2013, Marumo, Hoshino et al. 2014, Antus and Kardos 2015). Because MIF has oxidoreductase and redox activity it is an attractive candidate in understanding its effect on oxidative stress a main component of emphysema progression. MIF is acutely increased in response to CS, suggesting it may have important protective effects (Damico, Simms et al. 2011). And lastly the anti-apoptotic effects of MIF may be important in understanding the dysregulated EndoC death in emphysema.

### *1.5.1 MIF in Endothelial Homeostasis*

EndoC are key components of the alveolar environment, providing the rest of the acini with nutrients, and they are destroyed during emphysema development (Kasahara, Tuder et al. 2001, Morissette, Parent et al. 2009, Gordon, Gudi et al. 2011, Guarino, Cantarella et al. 2011). MIF is unique in that it can both antagonize EndoC apoptosis and potentiate their growth and proliferation. Exogenous MIF is an intrinsic, negative regulator of lipopolysaccharide (LPS)- and CS-induced apoptosis in human pulmonary EndoC, suggesting that the protective effects of MIF may be altered with disease development (Damico, Chesley et al. 2008, Damico, Simms et al. 2011). In addition, MIF is increased in response to acute CS extract (CSE) and functions to antagonize EndoC apoptosis by blocking death signals, like TNF-alpha, from both the DR

and mitochondrial apoptotic pathways (Damico, Chesley et al. 2008, Damico, Simms et al. 2011). Specifically, MIF is a novel positive regulator of the FLICE-like inhibitor protein (FLIP), which functions to antagonize signals via the DR pathway in response to LPS (Damico, Chesley et al. 2008). Additionally, MIF functions to suppress CS-induced p53 expression and p53-dependent activation of the mitochondrial death pathway, including the uplregulation and cleavage of BID and BAX, in CSE-challenged EndoC (Baumann, Casaulta et al. 2003, Damico, Chesley et al. 2008, Damico, Simms et al. 2011). Thus, MIF antagonizes death signals from multiple apoptotic pathways in EndoC, by stabilizing intrinsic inhibitors and suppressing promoters of the apoptosis cascade.

In addition to the anti-apoptotic effects, MIF can enhance endothelial proliferation in an Extracellular signal-regulated kinase (ERK) and or Protein kinase B (AKT) dependent manner (Amin, Volpert et al. 2003, Lue, Thiele et al. 2007, Wadgaonkar, Somnay et al. 2008). Further, increased and sustained ERK1/2 activation leading to proliferation, in response to exogenous MIF, is dependent on a mechanism involving protein kinase A (PKA) and phospholipase A2 (PLA2), linking this effect of MIF to an extracellular receptor (Mitchell, Metz et al. 1999). This is an intriguing aside because PLA2 is a main target for glucocorticoids, endogenously produced or medically prescribed, and this type of treatment is often ineffective in current and former patients with varying degrees of emphysema or COPD (Mitchell, Metz et al. 1999, Barnes 2002,

Hersh, DeMeo et al. 2008, Elsby, Donn et al. 2009). This suggests that the acute increase in CS-induced MIF and its stabilizing effect on PLA2 may in part explain MIFs control over glucocorticoid activity and their ineffectiveness in disease treatment.

Exogenous MIF is also sufficient to upregulate the expression of VEGF, which is necessary for EndoC maintenance and repair, but reduced with disease severity (Kasahara, Tuder et al. 2000, Ren, Chan et al. 2004, Marwick, Stevenson et al. 2006). This may be through ERK dependent transactivation of hypoxia inducible factor 1 (HIF1) alpha, an important regulator of VEGF (Ren, Chan et al. 2004, Maity and Koumenis 2006, Oda, Oda et al. 2008). This relationship is most apparent in studies of the angiogenic potential of tumors with and without MIF and in neonatal respiratory distress syndrome (Kevill, Bhandari et al. 2008, Bucala 2012). Further, increased MIF enhances the chemotactic and pro-inflammatory potential of macrophages into areas of damage or stress and this may have 2 main effects, 1) The local increase of MIF counterbalances oxidative stress and prevents cell death, or 2) cells that die are engulfed by the infiltrating macrophages, which is sufficient to increase macrophage derived VEGF production (Kleemann, Kapurniotu et al. 1998, Lind, Trindade et al. 1999, Gregory, Morand et al. 2006, Vandivier, Henson et al. 2006, Damico, Simms et al. 2011, Fan, Hall et al. 2011).

### *1.5.2 MIF and Altered Immunity*

MIF has well-established pro-inflammatory functions including macrophage and T cell recruitment, enhancing expression of adhesion molecules on EndoC, and upregulating the expression of pro-inflammatory cytokines from multiple cell types (Calandra, Bernhagen et al. 1995, Donnelly, Haslett et al. 1997, Bozza, Satoskar et al. 1999, Mitchell, Liao et al. 2002, Calandra and Roger 2003, Gregory, Morand et al. 2006, Bernhagen, Krohn et al. 2007, Harrison 2007, Zernecke, Bernhagen et al. 2008). While these functions are beyond the scope of this thesis, the potential dysregulation of MIF in disease could partly explain the altered innate and adaptive immune responses observed in emphysema, or other disease where MIF is altered, and validate the study of MIF in the modeling of emphysematous disease progression (Finkelstein, Fraser et al. 1995, Cosio and Guerassimov 1999, Vignola, La Grutta et al. 2002, Drost, Skwarski et al. 2005, Pridgeon, Bugeon et al. 2011, Hou, Sun et al. 2013). The acute CS-induced increase in MIF could therefore explain influx of predominately macrophages, which correlates with disease progression (Damico, Simms et al. 2011). MIF dysregulation with disease progression also correlates with dramatically distorted adaptive immunity; in disease processes like respiratory distress syndrome, where MIF is decreased, T cell populations are significantly increased as observed in later stages of emphysema

(Aoshiba, Koinuma et al. 2004, Rosen, Lee et al. 2006, Hodge, Nairn et al. 2007, Kevill, Bhandari et al. 2008, Chang, Nadigel et al. 2011, Dancer and Sansom 2013, Eppert, Wortham et al. 2013, Hou, Sun et al. 2013). In cases where MIF is significantly increased like rheumatoid arthritis (RA), its overexpression is coincident with the onset of autoimmune responses (Leech, Metz et al. 1999, Baugh, Chitnis et al. 2002, Morand, Leech et al. 2002, Denkinger, Metz et al. 2004). In addition and in possible contrast to the idea that MIF expression is protective toward CS-induced cellular damage, MIF has been implicated as a positive regulator of metalloproteases (MMPs) including collagenases (MMP13 and MMP1) and gelatinases (MMP2, and MMP9) but not elastase (MMP12) (Meyer-Siegler 2000, Onodera, Nishihira et al. 2002, Kong, Huang et al. 2005). Of the known MMPs responsive to MIF, MMP2 and MMP9, are elevated in human COPD and animal models of emphysema (Betsuyaku, Nishimura et al. 1999, Seagrave, Barr et al. 2004, Boschetto, Quintavalle et al. 2006).

### *1.5.3 MIF in Aging and Other Diseases*

MIF protein decreases in the lungs of aging mice, indirectly linking it in age-related airspace enlargement, a well-documented process (Verbeken, Cauberghs et al. 1992, Mathew, Jacobson et al. 2013, Papaioannou, Rossios et al. 2013). It is also recognized that genetically engineered

strains of mice, hypersensitive to CS, can also demonstrate evidence of accelerated age-dependent lung remodeling (Morris, Huang et al. 2003, Ruwanpura, McLeod et al. 2011). This suggests MIF may influence lung homeostasis, development, and or oxidative damage resulting from continued or prolonged smoking. In neonatal mice exposed to hyperoxia, both surpluses and deficits of MIF can alters neonatal alveolar development, suggesting that MIF concentrations are tightly regulated for optimal lung maturation (Sun, Choo-Wing et al. 2013). In premature babies increased MIF levels correlate with a polymorphism in the MIF promoter and is thought to confer protection from neonatal respiratory distress syndrome which leads to bronchopulmonary dysplasia (BPD) (Prencipe, Auriti et al. 2011). In contrast and highlighting the importance of MIF levels, MIF overexpression, which is central in the pathogenesis of RA, is associated with hyperplasia and overgrowth of the synovium and increased burden of pro-inflammatory macrophages (Santos and Morand 2006, Kasama, Ohtsuka et al. 2010). Further in cases of sepsis, increased levels of MIF are associated with increased risk of lethal shock (Bozza, Satoskar et al. 1999). On the opposite end of the MIF spectrum, animal models of BPD and other disease states like ischemic cardiac injury and radiation-induced lung injury demonstrate that loss of MIF is associated with increased tissue damage (Qi, Hu et al. 2009, Prencipe, Auriti et al. 2011, Mathew, Jacobson et al. 2013). Further, MIF polymorphisms are present in other chronic lung diseases, including

asthma and cystic fibrosis, and correlate with disease pathogenesis, raising the likelihood that similar polymorphisms may confer protection or contribute to emphysema progression, and in fact may underlie some of the genetic susceptibilities suspected in CS-induce emphysema onset (Hoffmann 2005, Mizue, Ghani et al. 2005, Plant, Gallagher et al. 2005, Wu, Fu et al. 2009).

### *1.5.4 MIF Expression and Secretion*

MIF is constitutively expressed and stored in preformed pools by many cells in the lung including EndoC, EpiC, and alveolar macrophages (Calandra and Roger 2003, Roger, Ding et al. 2007, Cheng, McKeown et al. 2010). The MIF promoter has two key response elements that can mediate its expression or upregulation observed with inflammation or oxidative stress— a sequence that binds cyclic AMP response element-binding protein (CREB) and cyclic AMP-dependent transcription factor (ATF1), and a hypoxia response element (HRE) respectively (Baugh, Gantier et al. 2006, Elsby, Donn et al. 2009). In response to hypoxia, a recognized component of CS induced emphysema, HIF1-alpha will upregulate MIF expression through the HRE located in the MIF promoter. The activity of CREB or ATF1 on the MIF promoter may in part explain the constitutive nature of MIF expression. While polymorphisms may also be a source of altered MIF expression there are few other molecular

mechanisms that can suppress MIF transcription. MIF is susceptible to epigenetic silencing, by microRNA miR-451, which is upregulated in the circulation of human smokers suggesting one potential molecular mechanism of MIF suppression in COPD (Bandres, Bitarte et al. 2009, Liu, Jiang et al. 2013, Takahashi, Yokota et al. 2013). The MIF promoter is also repressed by histone deacetylase (HDAC) inhibitors, and HDAC expression and activity are reduced in human COPD (Yang, Chida et al. 2006, Lugrin, Ding et al. 2009).

### *1.5.5 MIF Structure and Function*

MIF has a complex structural dynamic that may help illuminate the many functions, binding partners, and enzymatic reactions attributed to it. The crystallographic structure of MIF reveals that it exists as a barrel-shaped 37.5kDa homotrimer with a solvent accessible core (Kato, Muto et al. 1996, Sun, Bernhagen et al. 1996, Dobson, Augustijn et al. 2009). However it is recently speculated that physiologically relevant quantities of MIF contain a mix of monomers, homodimers, or homotrimers, a balance that may be heavily influenced by its concentration (Sun, Swope et al. 1996, Bernhagen, Calandra et al. 1998, Mischke, Kleemann et al. 1998). During inflammatory response, for example, extracellular MIF is increased which may enhance the probability of trimer formation and therefore shift the balance of the

three configurations (Swope and Lolis 1999, Fan, Rajasekaran et al. 2013). The structure of the monomer consists of six beta strands, four of which form a sheet, and two alpha helices (Sun, Bernhagen et al. 1996, Dobson, Augustijn et al. 2009). In forming the trimeric structure two of the beta strands in the sheet of one monomer interact with beta strands on an adjacent monomer, stabilizing the complex with 3 intertwining beta sheets (Kato, Muto et al. 1996, Sun, Bernhagen et al. 1996). The homotrimer is further stabilized by hydrophobic interactions between a leucine residue in the beta-3 strand of one monomer with a pocket of other hydrophobic amino acids on the beta-2 strand of another monomer (El-Turk, Fauvet et al. 2012). Given these interactions, the homotrimer is thought to be the most stable form of MIF and functional research utilizing locked-trimer mutants of MIF also suggests that the active form of MIF that binds its putative receptors is a homotrimer (Fan, Rajasekaran et al. 2013). Further, the homotrimer contains catalytic tautomerase sites between the subunits; whether or not a biologically relevant substrate for this site exists remains unknown, but alterations to a key terminal proline (Pro2) residue within the site can reduce the binding capacity of MIF, suggesting this region in the trimer complex is necessary for at least some biological functions of MIF (Lubetsky, Swope et al. 1999, Lubetsky, Dios et al. 2002, Senter, Al-Abed et al. 2002, Fingerle-Rowson, Kaleswarapu et al. 2009). In Contrast, the monomer and dimer forms may be capable of binding other proteins or receptors

and exerting differential effects. This is perhaps exemplified in studies involving the reactive cysteine residues on MIF.

While MIF does not belong to a structural class of proteins, it is functionally homologous with the TPOR family of proteins, which most notably contains thioredoxin (TRX), which are important in redox signaling (Kondo, Ishii et al. 2004, Kudrin and Ray 2007). MIF contains a redox sensitive TPOR motif with two reactive cysteine residues, Cys57 and Cys60, separated by two amino acids (CALC) (Mischke, Gessner et al. 1997, Kleemann, Kapurniotu et al. 1998, Kleemann, Mischke et al. 1998, Kleemann, Kapurniotu et al. 1999). In the trimeric form of MIF, these residues are not readily accessible suggesting that the trimer may not have oxidoreductase activity (Sun, Bernhagen et al. 1996, Dobson, Augustijn et al. 2009, Luedike, Hendgen-Cotta et al. 2012). Further in studies utilizing single or double mutations at these sites, loss of Cys60 resulted in total loss of redox activity, while loss of Cys57 only resulted in a partial reduction (Nguyen, Beck et al. 2003). Of these two cysteine residues, Cys60 has more surface accessibility in the monomeric form (Sun, Bernhagen et al. 1996, Dobson, Augustijn et al. 2009). In addition, monomeric MIF is approximately the same size as TRX with a remote structural resemblance although the fold containing the TPOR motif is distinctly different (Kudrin and Ray 2007). This suggests that the MIF monomer may actively participate in redox signaling and may have similar disulfide formation, protein interactions, and redox signaling as

other TPOR family members (Kleemann, Kapurniotu et al. 1998, Kleemann, Mischke et al. 1998, Kondo, Ishii et al. 2004, Nadeau, Charette et al. 2007). There is also a third cysteine residue on MIF, Cys81, which is modifiable in the monomer or trimer form (Kleemann, Kapurniotu et al. 1999, Jung, Seong et al. 2008, Dobson, Augustijn et al. 2009, Luedike, Hendgen-Cotta et al. 2012). In either case modification of Cys81, can alter the conformation of MIF thereby altering its function. For example when Cys81 is S-nitrosylated (SNO), oxidioreductase capacity of MIF is enhanced (Luedike, Hendgen-Cotta et al. 2012). Taken together, these cysteine residues are key to some of MIF's biological functions, although more work is needed to understand the in vivo conformations of MIF and their impact on downstream signaling.

### *1.5.6 MIF Protein-Protein Interactions*

In addition to altering and conferring function, the reactive cysteine residues on MIF are also sufficient for protein-protein interactions. MIF can bind to intracellular proteins as an endogenous protein, or via internalization into the cell. The recognized intracellular receptor for MIF is the cysteine-rich c-Jun activation domain binding protein-1 (JAB1), also known as subunit 5 (CSN5) of the COP9 signalosome, which through its downstream target, activator protein 1 (AP1), can modulate cell proliferation, differentiation, and apoptosis (Hwang, Ryu et al. 2004,

Burger-Kentischer, Finkelmeier et al. 2005, Lue, Thiele et al. 2007). The peptide region on MIF spanning amino acids 50-65, which contains the TPOR region, is necessary for the JAB1 interaction (Burger-Kentischer, Finkelmeier et al. 2005). Some research suggests that the Cys60 residue may be key for a strong interaction but not essential for binding (Kleemann, Hausser et al. 2000, Nguyen, Beck et al. 2003). Recent research also suggests that Cys81 allosterically regulates the binding capacity of this TPOR spanning region on MIF. Mutating or deleting Cys81 results in significantly less JAB1 binding and SNO-Cys81 enhances it (Luedike, Hendgen-Cotta et al. 2012). Cys81 is further implicated in the ability of MIF to directly bind and inhibit p53 (Jung, Seong et al. 2008). Although there is evidence that JAB1 also binds p53 and may act as a molecular bridge, recent data suggests that Cys81 forms a disulfide linkage with Cys242 on p53 (Chamovitz and Segal 2001, Lee, Oh et al. 2006, Oh, Lee et al. 2006, Jung, Seong et al. 2008, Jung, Seong et al. 2008). However given the importance of Cys81 on MIF structure, conformational changes have not yet been ruled out in MIF-p53 binding. Interestingly, nucleoside diphosphate kinase 1 (NM23-H1), which binds to Cys60 on MIF, negatively regulates the ability of MIF to suppress p53, suggesting that NM23-H1 binding is competitive for the same region on MIF (Jung, Seong et al. 2008). Nonetheless these cysteine residues are critical for many intracellular protein interactions with MIF and while this is still an ongoing area of research, cysteine interactions

are further implicated in MIF binding with the proapoptotic Bcl-2/adenovirus E1B 19 kDa-interacting protein 2-like (BNIPL), the thiol specific antioxidant proliferation associated gene (PAG), and the hepatotrophic growth factor hepatopoietin (HPO) (Jung, Kim et al. 2001, Shen, Hu et al. 2003, Li, Lu et al. 2004).

Binding of extracellular MIF to its cognate receptors is thought to be independent of the cysteine residues, and dependent on the trimeric structure. This is most evident in studies utilizes a mutant form of MIF that is locked in a trimeric state through an intersubunit disulfide bond; the locked trimer of MIF binds CD74 and activates downstream signals (Fan, Rajasekaran et al. 2013). Interestingly CD74 is the extracellular invariant chain of major histocompatibility complex II (MHCII), which normally binds and facilitates shuttling of MHCII to the surface, and MIF shares structural similarities with the peptide-binding region of MHCs (Thiele and Bernhagen 2005, Bucala 2012). In this cognate role CD74 interacts with MHCII as a trimer. In this trimeric state CD74 shares rotational symmetry with the MIF trimer, and this may help shed light on the importance of the MIF intersubunit tautomerase sites on CD74 binding (Lubetsky, Swope et al. 1999, Dobson, Augustijn et al. 2009, Fingerle-Rowson, Kaleswarapu et al. 2009, Schwartz, Lue et al. 2009). Further MIF may contribute in class II antigen presentation by binding HLA-DP molecules and processing antigen peptides (Potolicchio, Santambrogio et al. 2003). The MIF monomer also shares remote

structural similarities with the chemokine dimer CXCL8, implicating extracellular binding of different forms of MIF on intracellular signaling pathways; MIF is known to bind the chemokine receptors CXCR2 (CXCL8 receptor) and CXCR4, which is sufficient to induce leukocyte recruitment, tying the secretion of MIF to inflammatory responses (Bernhagen, Krohn et al. 2007, Bucala 2012, Schwartz, Kruttgen et al. 2012).

Conversely more studies are needed to elucidate whether these effects may also be mediated through the efficient endocytosis of MIF into certain cells. Intriguingly, MIF has been recently shown to bind its TPOR family member TRX on the extracellular surface, which mediates its internalization and inhibits some of the MIF-associated pro-inflammatory activity (Son, Kato et al. 2009). However the formation of chemokine receptor heterodimers, or dimerization with CD74, specifically CD74/CXCR4, is also implicated in MIF endocytosis involving clathrin and dynamin-dependent processes (Schwartz, Kruttgen et al. 2012). More work is needed to elucidate this complicated picture of MIF structural dynamics and the resulting differential binding capacities or behaviors with the formation, binding, and internalization capacities of the extracellular receptors, which may also vary depending on the stimulus or type of response being elicited.

### *1.5.7 MIF and Transcription Factors*

Through its effects on cytosolic p53, intracellular MIF can ultimately alter cell fate decisions by modulating the stability of nuclear p53 and other transcription factors, including HIF1 alpha (Lee, Oh et al. 2006, Jung, Seong et al. 2008, Oda, Oda et al. 2008). Further, through JAB1 binding, MIF can alter HIF1-alpha, p53, AP1, Progesterone receptor- (PR), and glucocorticoid receptor (GCR)-related transcription and may also enhance the major transcriptional regulator of the antioxidant response element (ARE), NRF2, leading to secondary effects on antioxidant gene expression and regulatory activity towards oxidative stress (Bianchi, Denti et al. 2000, Chauchereau, Georgiakaki et al. 2000, Kleemann, Hausser et al. 2000, Chamovitz and Segal 2001, Bae, Ahn et al. 2002, Hwang, Ryu et al. 2004, Lee, Oh et al. 2006, Oh, Lee et al. 2006, Villeneuve, Lau et al. 2010). The antagonism of p53 by MIF, leads to a reduction in the pro-apoptotic genes regulated by p53 (discussed above in section 1.3.1) and therefore has a pro-survival effect. In addition to the hypoxic upregulation of MIF expression, MIF can stabilize HIF1-alpha by preventing the p53-dependent ubiquitination of HIF1-alpha (Oda, Oda et al. 2008, Simons, Grieb et al. 2011). This stabilizing effect on HIF1-alpha may be sufficient to maintain vascular homeostasis in the lung, as VEGF and glucose transport are increased as a result of is transcriptional activity (Xiao, Zeng et al. 2006, Oda, Oda et al. 2008, Song, Wang et al. 2009). The effect of MIF on JAB1 dependent AP1

transactivation is also straightforward; JAB1 stabilizes c-jun protein binding to AP1 sites, thereby acting as a coactivator for AP1 transcription, and MIF binding to JAB1 antagonizes this function, altering cell growth, proliferation, and or apoptosis-related protein expression depending on the cellular context (Bianchi, Denti et al. 2000, Kleemann, Hausser et al. 2000, Hwang, Ryu et al. 2004). JAB1 also directly interacts with PR, GCR, and to an extent NF-kappaB by stabilizing their association with nuclear receptor coactivator 1 (NCOA1), but the effects of MIF on JAB1 regarding these transcription factors, while plausible, remains ill defined (Claret, Hibi et al. 1996, Kleemann, Hausser et al. 2000, Chamovitz and Segal 2001).

The hypothesis of MIF binding JAB1 to affect NRF2 stability is still controversial. The COP9 signalosome, to which JAB1 associates, is involved in the deneddylation of proteins (Schmaler and Dubiel 2010). Neddylin (NEDD8) stabilizes cullins, allowing for activation of associated ubiquitin ligases (E3) (Lyapina 2001, Cope 2002, Zheng 2002, Yamoah, Wu et al. 2005). Kelch-like ECH-associated protein 1 (KEAP1) tethers NRF2 to these cullin scaffolds, and when neddylated, the E3 ligase targets NRF2 for ubiquitination (Villeneuve, Lau et al. 2010). In order for this complex to efficiently degrade more cytosolic NRF2 it must cycle apart and back together (Chamovitz and Segal 2001, Pintard 2003). JAB1, as part of the signalosome, is hypothesized to remove NEDD8, destabilizing the cullin complex in order for KEAP1 to bind another NRF2

(Cope 2002, Villeneuve, Lau et al. 2010). In the presence of ubiquitin-conjugating enzyme E2M (UBC12) the complex will be re-neddylated, stabilizing the complex once again, allowing the cycle to continue (Chamovitz and Segal 2001, Schmaler and Dubiel 2010, Villeneuve, Lau et al. 2010). However, it is not known whether MIF interacts with JAB1 as a single protein or as part of the multi-protein complex (COP9 signalosome) or how MIF may modulate these relative pools of JAB1. Under the premise that MIF antagonizes JAB1 activity, this scenario paints a picture whereby MIF would stabilize NRF2, by preventing its rapid targeting for proteosomal degradation. Taken together intracellular MIF can modulate gene transcription by regulating several key transcription factors involved in cell fate.

### *1.5.8 MIF and Intracellular Signaling Pathways*

As described above MIF can alter fundamental pathways involved in cell-fate through extracellular reception, as an endogenous protein, or through internalization via an ill-defined endocytic route. Depending on how MIF is received by a given cell may determine the response, which could also be functionally distinct and or conflicting within a single cell type or between cell types. For example, CD74, in conjunction with CD44, is capable of transducing the extracellular binding of MIF into activation of intracellular kinase cascades involved in cell survival,

including JNK, p38, ERK1/2, and AKT (Santos, Lacey et al. 2004, Shi, Leng et al. 2006, Starlets, Gore et al. 2006, Qi, Hu et al. 2009, Sanchez-Nino, Sanz et al. 2009). However CD74 has not been identified on EndoC and other cells, suggesting the impact of MIF, at least on EndoC survival is through a different pathway or absent in these cell types. In addition p38 is likely still modified in MIF dependent manner but by means other than CD74. XOR for example, which is increased with disease severity and an important source of ROS, is activated downstream of p38 in human microvascular EndoC (HMVEC) which lack CD74 (Pinamonti, Muzzoli et al. 1996, Pinamonti, Leis et al. 1998, Le, Damico et al. 2008, Kim, Serebreni et al. 2013). Although the internalization of MIF, either through receptor ligand interaction or pinocytosis when its concentrations are high, is not well studied, MIF is however hypothesized to act freely in the cytosol versus in an endolysosomal compartment because its oxidoreductase activity is optimal at neutral pH (Potolicchio, Santambrogio et al. 2003, Kudrin and Ray 2007). Theoretically free cytosolic MIF could then interact with redox sensitive MAP3K and the associated pathways. However, the resulting pathway activations may be entirely different given that extracellular stimulation of CD74 is likely with a MIF trimer and the intracellular alterations are likely mediated through the TPOR site and oxidoreductase activity of MIF.

The ability of MIF to antagonize JAB1 may also allude to one possible mechanism by which MIF exerts its protective effects, through

the NRF2 related mechanism discussed above for example. However there is conflicting evidence that may in part be due to the context of this cellular interaction between MIF and JAB1. They both stabilize HIF1-alpha, albeit through different mechanisms, but this raises the question of whether their relationship is purely antagonistic (Bae, Ahn et al. 2002, Oda, Oda et al. 2008, Simons, Grieb et al. 2011). JAB1, independent of the COP9 signalosome, inhibits the cyclin dependent kinase (CDK) inhibitor p27KIP1, which can induce G1 cell cycle arrest (Tomoda, Kubota et al. 1999). Exogenously applied MIF is internalized where it binds with cytosolic JAB1 near the plasma membrane and inhibits the control of JAB1 over p27KIP1, essentially stabilizing p27KIP1 and G1 cell cycle arrest (Tomoda, Kubota et al. 1999, Kleemann, Hausser et al. 2000). In addition, perhaps tying in with the JAB1-related regulation of AP1 transcription, JAB1 enhances JNK activity and downstream c-jun phosphorylation; MIF blocks this JAB1–dependent effect, possibly disrupting cell growth and or proliferation. However the exact mechanism remains ill-defined, as MIF and JAB1 do not possess kinase activity and do not directly associate with JNK (Tomoda, Kubota et al. 1999, Kleemann, Hausser et al. 2000). Further this regulation of the MAPK pathways contradicts signaling mediated through extracellular receptors since exogenous MIF is mostly reported as a positive regulator of cell survival and proliferation. Nonetheless, MIF, through its TPOR motif may alter the function of the MAPK kinase kinases (MAP3K), which are known

to be sensitive to oxidative stress. This hypothesis could also partially explain JAB1 control over JNK activity, as JAB1 can also directly associate with TRX a known regulator of MAP3K (Burger-Kentischer, Finkelmeier et al. 2005, Kudrin and Ray 2007).

JAB1 appears to have promiscuous cell behavior and binding, not only modulating transcription factors in the nucleus and protein activity in the cytosol, but redundancy in binding the known MIF-binding partners, like p53, and HPO (Wang, Lu et al. , Lu, Li et al. 2002, Lee, Oh et al. 2006). JAB1 can bind p53 and as part of the COP9 signalosome it can mediate the degradation of p53 (Bech-Otschir 2001, Lee, Oh et al. 2006). HPO appears to stabilize JAB1 effects on AP1, which is in contrast to MIF. Intriguingly the MIF-JAB1 complex is detectable in the cytosol, but it is not known whether this complex, while together, has signaling or binding functionality as a cytosolic and or transcription factor similar to the individual capacities of MIF and JAB (Kleemann 2000, Bech-Otschir 2001, Chamovitz and Segal 2001, Lee, Oh et al. 2006, Kudrin and Ray 2007). Given the differential regulation of JAB1 by MIF and HPO it is conceivable this may be due to differential binding. In addition the promiscuous activity of JAB1 may underlie its reportedly divided role in inducing or protecting cells from apoptosis (Tomoda, Yoneda-Kato et al. 2004, Hallstrom and Nevins 2006, Liu, Pan et al. 2008, Damico, Simms et al. 2011). Further, MIF may also have the capacity for multiple and simultaneous interactions as both the TPOR motif and Cys81 are

involved in binding. However further work is needed to uncover the complex networking of these cysteine-rich and redox-sensitive proteins when their cognate intracellular locations are in flux, especially in the context of specific stimuli like CS, which provokes the associated redox reactions.

### *1.5.9 MIF Deficient Mice*

Deletion of the MIF gene from the mouse genome was completed in 1999 by Bozza et al, in order to better understand the role of MIF in sepsis (Bozza, Satoskar et al. 1999, Calandra and Roger 2003). It was reported that *Mif* $^{-/-}$ mice are healthy, lacking any apparent abnormalities or growth and developmental deficiencies. Since then *Mif* $^{-/-}$ mice have been utilized extensively to better understand its role in many inflammatory, autoimmune, and exposure-related disease processes especially where human MIF polymorphisms correlate with disease severity. In recent studies, we now understand that MIF may be involved in normal lung development and antagonize the natural aging of the lung (Mathew, Jacobson et al. 2013). Given the role of MIF polymorphism in other chronic lung diseases, in vitro data implicating MIF in pulmonary vascular homeostasis, and the potential cytoprotection conferred through its oxidoreductase activity we have employed *Mif* $^{-/-}$ mice to elucidate its role in CS-induced lung injury, hypothesizing that MIF expression is

diminished in emphysema thereby altering the lung structure and EndoC homeostasis.

## 1.6 Thesis Summary

Emphysema is a common form of COPD that contributes to significant morbidity, mortality, and healthcare cost in the United States. CS is the most common cause of emphysema but there are clear genetic risk factors, such as alpha-1 antitrypsin deficiency, that inform our understanding of the cellular and molecular mechanisms of emphysematous tissue destruction. However the relationship between intrinsic determinants of disease and CS exposure history is not well understood. In addition there are currently no therapies directed at ameliorating or curtailing the persistent nature of alveolar tissue loss observed in emphysema.

Endo and EpiC are key structural components of the alveoli and principal targets of CS-induced apoptosis leading to emphysematous tissue destruction. This alveolar wall apoptosis correlates with increases in surface area and mean airspace size but is associated with enhanced proliferation of both EndoC and EpiC, suggesting that the repair process is overwhelmed by CS-induced tissue destruction and apoptosis. This disproportionate apoptosis is necessary for emphysema and EndoC apoptosis is sufficient to induce emphysematous lung destruction. In addition, the associated increases in oxidative stress, inflammation, and a protease/anti-protease imbalance all promote EndoC apoptosis and all

are implicated in loss of acinar structures, further emphasizing apoptosis as a nodal point CS-induced emphysema pathogenesis.

Recognizing CS is a mixture of many extrinisic factors and oxidants that may alter and or contribute to emphysema development and severity, the identification of the pathways and intrinsic determinants of CS-induced apoptosis may further the understanding of disease pathogenesis for therapeutic interventions. However, apoptosis persists after cessation of chronic CS, indicating that protective mechanisms may be significantly or irreversibly impaired. This is evident from the continued exacerbations of oxidative stress in former smokers and patients with emphysema. In addition oxidative stress may underlie many of the disease manifestations like protease imbalance and inflammation. Therefore, understanding functional relationships between apoptosis, oxidative stress, and intrinsic determinants of emphysema pathogenesis is critical for therapy.

MIF is one intrinsic factor that is acutely increased in response to CS in vitro and antagonizes CS-induced EndoC apoptosis. This suggests that the cytoprotective effects of MIF may be altered or diminished with disease severity. The multiple functions of MIF as a cytokine and enzyme reveal a critical role for MIF in enhancing acute CS-induced inflammatory responses and modulating oxidant and antioxidant reactions through its redox sensitive cysteine residues, both key characteristics in emphysema pathogenesis. MIF also interacts with

many proteins and directly or indirectly modulates transcription factors that are linked to and are dysregulated in emphysema. In addition there are other lung diseases where polymorphisms in the MIF promoter correlate with disease severity suggesting that the levels of MIF may be critical in maintaining lung and alveolar EndoC maintenance. Therefore the study of emphysema utilizing a combination of genetic and exposure models may uncover a novel role for MIF in disease pathogenesis and open the door for new therapeutic strategies targeting oxidative stress and tissue destruction.

### *1.6.1 Hypothesis and Aims*

The overarching hypothesis is that MIF deficiency will exacerbate CS-induced oxidative stress, EndoC apoptosis, and alveolar tissue destruction.

### *1.6.2 Specific Aims*

**<u>Aim 1:</u> To test the hypothesis that MIF impacts on the severity of CS-induced EndoC injury *in vivo* and influences emphysematous tissue remodeling.**

To disrupt MIF signaling, *Mif* $^{-/-}$ mice were examined following acute and chronic CS-exposure. To examine the effects of MIF deficiency immunohistochemistry, apoptosis-related protein expression, and DNA-damage were assessed in comparison to wild type animals.

**<u>Aim 2</u>: To test the hypothesis that MIF impacts on CS-mediated cytotoxicity in lung by regulating ROS production, in part, via XOR.**

*Mif* $^{-/-}$ mice were exposed to acute CS and examined for alterations in XOR activity and expression. A combination of in vitro, in vivo, and cell free functional assays and immunohistochemistry were employed to uncover mechanisms by which MIF alters XOR activity.

**<u>Aim3:</u> To test the hypothesis that MIF regulates antioxidant responses by impacting on NRF2 stability**

*Mif* $^{-/-}$ mice were exposed to acute CS and examined for alterations in NRF2 activity and related gene expression. In vitro protein expression and functional assays were also utilized to uncover alterations in NRF2 activity due to MIF or MIF protein-protein interactions.

## Tables 1-1

| GOLD stage | Description | FEV1 (% Predicted) | FEV1/FVC (%) |
|---|---|---|---|
| 1 | Mild | 80 | <70 |
| 2 | Moderate | 50-80 | <70 |
| 3 | Severe | 30-50 | <70 |
| 4 | Very Severe | <30, or <50 with severe chronic symptoms | <70 |

Adapted from (Global Initiative for Chronic Obstructive Lung Disease. 2011)

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# Chapter 2

## Materials and Methods

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## 2.1 Experimental Models

### *2.1.1 Human Subjects*

All study protocols related to human studies were approved by the Institutional Review Board of the Johns Hopkins University. Written, informed consent was obtained from all participants at the time of sample collection. Patients with COPD were recruited from clinical populations at the Johns Hopkins Hospital, Johns Hopkins Bayview Medical Center and from the NHLBI Lung Tissue Research Consortium. Healthy, non-smoking subjects supplied serum for control reference.

### *2.1.2 Animals and Treatments*

All animal protocols were conducted as approved by the Johns Hopkins University Institutional Animal Care and Use Committee. $MIF^{-/-}$ mice with a C57BL/6 background were generated as previously described (Bozza, Satoskar et al. 1999). $Mif^{-/-}$ and $Mif^{+/+}$ C57BL/6 mice were housed under controlled conditions for temperature and humidity using

a 12-hour light/dark cycle. At 8-10 weeks of age, mice were exposed to CS or filtered air. CS exposure was carried out as previously described (Sussan, Rangasamy et al. 2009) for 5 hours/day, 5 days/week, for 3 days, or 6 months. Briefly, 2R4F reference cigarettes (Tobacco Research Institute, University of Kentucky) were burned using a smoke machine (Model TE-10, Teague Enterprises). The smoke machine was adjusted to produce a mixture of side stream and mainstream smoke as previously described (Rangasamy, Cho et al. 2004) with minimal adjustments. Chamber atmosphere was monitored for total suspended particulates and carbon monoxide, with concentrations of 150 mg/m$^3$ and 750 ppm, respectively. In pharmacologic studies mice were injected intraperitoneally with 2 mg/kg of a selective pyridinyl imidazole inhibitor, SB203580 (LC8 Laboratories, Woburn MA), dissolved at 0.4 mg/ml in 1% DMSO in sterile saline or carrier alone (vehicle) as previously described (Le, Damico et al. 2008), with minor modifications.

### *2.1.3 Cells and Reagents*

Human and rat lung microvascular EndoC (HLMVEC, CC2527, Lonza, MD and RLMVEC, RA6011, Cell Biologics, IL, respectively) were cultured and maintained in EGMV-2 (Lonza, MD) or (Basal Media supplemented with M1266, Cell Biologics, IL) from passage 4-9. Recombinant MIF (rMIF, R&D Systems, Minneapolis, MN), On-target control siRNA and MIF

siRNA (Dharmacon, Lafayette, CO), ASK1 siRNA (Dharmacon, Lafayette, CO), and cigarette smoke extract (CSE) were used as previously described (Damico, Simms et al. 2011). Briefly, at 50% confluence, cells were washed with PBS, and bathed with optimem reduced serum media (Life Technologies, Grande Island, NY) for transfection of siRNAs. Geneporter 2 transfection reagent (GenLantis, San Diego, CA) was optimized for lipid based transfections and after 4 hours, complete media was added to the cells. Naïve cells, transfected cells or cells receiving rMIF were exposed to CSE for 5-45minutes, 4, 8, 24 and 48hrs hours, after which lysates and supernatants were collected for analysis.

## 2.2 Lung Mechanics and Morphometry

Fifteen hours after the last CS exposure, mice were anesthetized with ketamine/xylazine (75mg/kg, 15mg/kg). Under deep anesthesia, mice were tracheostomized, and the lungs mechanically ventilated with a Flexivent (SCIREQ, Montreal, Quebec, Canada) to measure lung resistance and elastance. Once under mechanical ventilation, mice received a bolus of the paralytic succinylcholine intramuscularly. Mice were ventilated at 150 breaths per minute, and lung function parameters were determined during a 2 second breath hold with a 2.5-Hz sinusoidal oscillation.

Subsequent to lung mechanics, bronchoalveolar lavage was performed on the right lung, which was removed immediately, rinsed in cold PBS, and flash frozen for RNA and protein analysis. The left lung was inflated with a warm 1% agarose under pressure (30 cm $H_2O$) and submerged in zinc buffered formalin (Z-fix, Anatech LTD, Battle Creek, MI) for 2 days. The fixed lung volumes were measured by water displacement after which the left lung was divided into 3 sections as previously described (Mitzner, Fallica et al. 2008). Briefly, the cranial 1-2mm was removed from the left lung cutting along the transverse plane. Moving down the cranial-caudal axis, 2-3mm thick transverse sections were cut, removing approximately 1mm in between each section. The caudal-most portion was discarded with the apex and 1mm thick spacers, resulting in three 2-3mm thick lung sections, cranial, middle, and caudal, which were embedded cut side down in paraffin for thin sectioning via microtome.

For morphometric analysis, lung slices were stained with hematoxylin and eosin (H&E), and systematic random sampling of each section was performed to obtain unbiased representative pictures of each lung. A line probe was employed to assess tissue ratio and mean chord length using the stereology tool, STEPanizer (Tschanz, Burri et al. 2011). The ends of the probes were used to estimate air to tissue ratios. The number of intercepts, or points where septal tissue bordering airspace intersects the line probe, was also recorded. After calibrating for the

length of the line probe, we used a ratio of points hitting airspace ($P_a$) to intercepts to estimate mean chord length. The number of intercepts counted is proportional to the alveolar surface density and, therefore, we were able to estimate whole lung alveolar surface area as previously described (Knudsen, Weibel et al. 2010).

## 2.3 Immunohistochemistry

Cleaved Caspase-3 ± Thrombomodulin or ± Tomato Lectin: Unstained thin lung sections from paraffin blocks were deparaffinized using xylenes and slowly hydrated with washes of decreasing ethanol percentage. The lung sections were blocked with a 10% donkey serum with BSA and Tween for 1hour. After blocking rabbit anti-mouse cleaved caspase 3 (#9661, Cell Signaling, Boston, MA) was added to the sections with or without goat anti-mouse thrombomodulin (#AF3894, R&D Systems, Minneapolis, MN) as previously described (Le, Damico et al. 2008) and incubated overnight in a BSA/triton solution. After thoroughly washing, the secondary antibody, donkey anti-rabbit Alexa Fluor 594, was added for 2 hours at room temperature and for co-staining investigations, donkey anti-goat Alexa Fluor 488 or the endothelial specific marker tomato lectin (DL-1177, Vector Labs, Burlingame, CA) was also added. Lung sections were washed and rinsed with SlowFade anti-fade kit

(Invitrogen/ Life Technologies, Carlsbad, CA) after which Vectashield with DAPI (Vector Labs, Burlingame, CA) was applied to each slide immediately before adding a coverslip. Lung slices were immediately imaged using a Nikon microscope with a mounted camera. All images were acquired and analyzed in a blinded fashion.

γH2AX ± Isolectin GS-$IB_4$: Thin lung sections from paraffin blocks were heated for 1hr at 60°C, deparaffinized using xylenes, and hydrated using washes of decreasing ethanol percentage and deionized water. For antigen retrieval (Vector reagent H3300, Burlingame, CA) the slides were placed in a steam cooker for 40 minutes. After heating, temperatures were normalized and slides were further cooled in distilled water. Each slice of tissue was outlined with a pap pen and incubations were completed in accordance with a mouse on mouse kit (MOM kit, Millipore Corp., Billerica, MA). Mouse Ig Blocking Reagent was applied for 1 hr. After washing, slides were incubated with MOM diluent first, and then incubated with anti-mouse γH2AX in MOM diluent overnight. Again washing was performed and the sections were incubated with goat anti-mouse Alexa Fluor 488 antibody for 1 hour or overnight with isolectin GS-$IB_4$ conjugated with Alexa Fluor 568. Hoechst stain (Invitrogen/ Life Technologies, Carlsbad, CA) was carried out during the wash phase for the detection of nuclei. SlowFade was applied to each slide immediately before adding a coverslip. Lung slices were immediately imaged using a

Nikon microscope with a mounted camera. All images were acquired and analyzed in a blinded fashion.

Phospho-p38: Unstained thin lung sections from paraffin blocks were deparaffinized using xylenes and slowly hydrated with washes of decreasing ethanol percentage. The lung sections were blocked with a 10% donkey serum with BSA and Triton for 1 hour. After blocking mouse lung sections, rabbit anti-mouse phospho-p38 (SC-17852-R, Santa Cruz Biotechnology, Santa Cruz, CA) was added to the sections and incubated overnight in a BSA/triton solution. After thoroughly washing, the secondary antibody, donkey anti-rabbit Alexa Fluor 488, was added for 1 hour at room temperature.

XOR ± PECAM: For human lung sections, rabbit anti-human XOR (SC-20991, Santa Cruz Biotechnology, Santa Cruz, CA), and goat anti-human PECAM (SC-1506, Santa Cruz Biotechnology, Santa Cruz, CA) were added as the primaries, whereas, donkey anti-goat Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 594 were used as secondaries. DAPI (Invitrogen, Eugene, Oregon) was added to the wash and lung sections were preserved with SlowFade (Invitrogen/ Life Technologies, Carlsbad, CA) immediately before adding a coverslip. Lung slices were immediately imaged using a Nikon microscope with a mounted camera. All images were acquired and analyzed in a blinded fashion.

## 2.4 ROS Sensitive Indicators

Dihydroethidium (D-1168, Life Technologies, Carlsbad, CA) staining was performed as previously described (Jankov *et al.* 2007). Mice lungs were flushed with saline and inflated with Tissue-Tek CRYO-OCT compound diluted 1:3 in PBS containing 20% w/v sucrose. Inflated lungs were cryo-frozen in molds containing OCT compound and stored at -80C.

Transverse lung sections were mounted on super frost plus slides (Fischer Scientific, Waltham, MA), incubated with PBS or appropriate drugs (50uM diphenylene iodonium, Cat. # D2926, or 2mM *N*-Acetyl-L-cysteine, Cat. # A7250, Sigma Chemical Co., St. Louis, MO) for 30 minutes. Drugs were washed off with PBS, and slides were incubated with 10uM dihydroethidium (DHE) for 30 minutes after which slides were mounted with Slow Fade Gold (# S36936, Life Technologies, Carlsbad, CA) and imaged immediately. Pictures of parenchymal tissue were taken using a Zeiss fluorescent microscope. 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA, # D-399, Life Technologies, Carlsbad, CA) staining was performed on HLMVEC per manufactures recommendations. Briefly, HLMVEC were loaded with $H_2$DCFDA (10mM in Hanks with 10mM HEPES, pH7.4) for 45 minutes and subsequently incubated with rMIF (100ng/ml), carrier, or NAC (2mM). post-CS extract (20%V/V) addition, fluorescent intensity (495 /520 nm) was assessed every 5 minutes. An increase in DCF fluoresces was quantified as the ratio of the post-stimulus fluoresce over the baseline (Delta F/F0).

## 2.5 ELISA

Supernatants from Human microvascular EndoC exposed to 4, 8, 24, and 48 hrs CSE or PBS were analyzed using a solid phase sandwich MIF ELISA (R&D Systems, Minneapolis, MN) as previously described (Fallica, Boyer et al. 2014) and per manufacturer's specifications.

## 2.6 Western Blotting

For tissue derived protein quantification, lungs were washed in cold PBS and placed in lysis buffer (Cell Signaling, Boston, MA) with protease inhibitors (Sigma Chemical Co., St. Louis, MO; P8340). Tissue lysis beads (Next Advance, NY, USA; PINKE5) were added to the lung samples and proteins were liberated via homogenization. SDS–PAGE separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). Specific proteins were detected with the following antibodies: caspase-3 (# 9662, Cell Signaling, Boston, MA), anti-cleaved caspase-3 (# 9661, Cell Signaling, Boston, MA), anti-p53 (# 9662, Cell Signaling, Boston, MA), γH2AX (# 05–636, Millipore Corp., Billerica, MA), HSP90 (4874, Cell Signaling, Boston, MA), p-p38 (9215s, Cell Signaling, Danvers, MA), p38 (9212s,

Cell Signaling, Danvers, MA), ASK1 (8662s, Cell Signaling, Danvers, MA), XOR (22006, Santa Cruz Biotechnology, Santa Cruz, CA) Cleaved caspase (9661s, Cell Signaling, Danvers, MA), MIF (20121, Santa Cruz Biotechnology, Santa Cruz, CA) and beta actin (13157, Santa Cruz Biotechnology, Santa Cruz, CA). HRP-conjugated secondary antibodies were used to amplify the primary signal and the protein/antibody complexes were detected by chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

## 2.7 RNA Extraction and qPCR

Total RNA was isolated and purified from lung tissue using TRIzol reagent (Invitrogen/ Life Technologies, Carlsbad, CA), and components of an RNeasy kit (Qiagen, Valencia, CA) respectively. Complementary DNA was generated by using an RT$^2$ FirstStrand cDNA kit (SAbioscience/ Qiagen, Valencia, CA). Gene expression was measured by utilizing mouse primers (IDT, San Jose, CA) for MIF (5'-TGGGTCACACCGCGCTTTGT-3',5'-TGCGATGTACTGTGCGGGCT-3'), p53 (5'-ACAGCAGGGCTCACTCCAGCTACC-3', 5'-AGCAGAAGGGACCGGGAGGATT-3'), heme oxygenase-1 (HO1) (5'-GGCCACCAAGGAGGTACACAT-3',5'-TGGGGCATAGACTGGGTTCT-3'), NADPH dehydrogenase quinone 1 (NQO1) (5'-AGCCAATCAGCGTTCGGTAT-3',5'-GTAGTTGAATGATGTCTTCTCTGAAT-3'), glutathione peroxidase 2 (GPX2) (5'-

CCCTTCCGTCGCTACAGCCG-3', 5'-TCCCAGGGTCTCCCGAGGGTA-3') beta actin (5'ACGGCTCCGGCATGTGCAAA-3', 5'-ACCATCACACCCTGGTGCCT-3') and human primers Glyceraldehyde 3-phosphate dehydrogenase (5'-AATGCCAGCCCCAGCGTCAAA-3', 5'-TGTCCCCACTGCCAACGTGTCA-3') with the SYBR green method.

Human oxidative stress and antioxidant defense PCR array (SAbioscience/ Qiagen, Valencia, CA) were used per manufacturer's specifications.

## 2.8 Pterin Assay

The measurement of XOR enzymatic activity was performed on mouse lung homogenates using a fluorometric assay based on the conversion of pterin to its fluorescent product isoxanthopterin as previously described (Kayyali, Budhiraja et al. 2003). Lung tissue was homogenized with pterin buffer containing 50mM $KH_2PO_4$, 10mM DTT, and 0.18mg/mL PMSF. Lysates were centrifuged at 13,000g for 15 minutes at 4°C and the supernatants were used in the assay immediately before measurement.

## 2.9 Kinase Assay

MIF and ASK1 interactions were tested using a cell free ASK1 kinase enzyme system (Promega, Madison, WI), whereby ASK1 in the presence of a substrate protein MBP, and ATP will phosphorylate MBP, converting ATP to ADP. After quenching any remaining ATP following a reaction period, the remaining ADP is converted back to ATP and coupled to a luciferase/luciferin reaction producing light (ADP-Glo assay, Promega, Madison, WI). The manufacturer's protocol was followed for setting up controls and determining the $IC_{50}$ values for inhibitors. With this assay system rMIF was titrated across several dilutions in triplicate with ASK1 and the formation of ADP was subsequently measured.

## 2.10 Caspase 3 Activity Assay

After CS exposure *Mif*$^{+/+}$ and *Mif*$^{-/-}$ lung homogenates were collected and directly plated in triplicate on a white-bottom 96-well plate. Homogenates were probed for caspase 3/7 activity using the Caspase-Glo 3/7 assay (G8091, Promega, Madison, WI) following manufacturers guidelines. Briefly the Caspase-Glo substrate and buffer were combined and equal volumes of this Glo reagent and lung sample were combined in each well. In the presence of the Glo reagent, active caspase 3/7 will cleave from the substrate and generate luminescent signal, produced by luciferase.

## 2.11 NRF2 Dual Luciferase Assay

An antioxidant response element (ARE) reporter, containing a mixture of a Nrf2-responsive luciferase construct and a constitutively expressing Renilla construct were transfected into rat microvascular EndoC. The Nrf2-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of the ARE transcriptional response element. After a 24 hour period cells were lysed with a passive lysis buffer, and subjected to a one freeze-thaw cycle before NRF2 reporter activity was analyzed using the manufacturers guidelines for the Dual-Luciferase Reporter Assay System (E1910 Promega, Madison, WI). In separate approaches NRF2 activity was measured in the presence of rMIF (100ng/ml) or in the presence of siRNA targeting JAB1.

## 2.12 Statistical Analysis

For comparisons among groups of normally distributed data sets, the student's *t* test or ANOVA with post hoc Bonferroni correction was used. For non-normally *distributed* data, the Mann Whitney or Kruskal-Wallis test with post-hoc Bonferonni correction was used. Values are presented as means ± standard error.

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Le, A., et al. (2008). "Alveolar cell apoptosis is dependent on p38 MAP kinase-mediated activation of xanthine oxidoreductase in ventilator-induced lung injury." J Appl Physiol **105**(4): 1282-1290.

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# Chapter 3

## Macrophage Migration Inhibitory Factor (MIF) is a Novel Determinant of Cigarette Smoke-induced Lung Damage

**This work has been modified from the previously accepted publication:**

Fallica, J., et al. (2014). "Macrophage migration inhibitory factor is a novel determinant of cigarette smoke-induced lung damage." Am J Respir Cell Mol Biol **51**(1): 94-103.

# Chapter 3

## 3.1 Abstract

Cigarette smoke (CS) is the most common cause of chronic obstructive pulmonary diseases (COPD) including emphysema. CS exposure impacts all cell types within the airways and lung parenchyma, causing alveolar tissue destruction through four mechanisms: 1) oxidative stress, 2) inflammation, 3) protease-induced degradation of the extracellular matrix, and 4) enhanced alveolar epithelial and endothelial cell (EndoC) apoptosis. Studies in human pulmonary EndoC demonstrate that macrophage migration inhibitor factor (MIF) antagonizes CS-induced apoptosis. Here we used human microvascular EndoC, an animal model of emphysema (mice challenged with chronic CS), and patient serum samples to address both the capacity of CS to alter MIF expression and the effects of MIF on disease severity. We demonstrate significantly reduced serum MIF levels in patients with COPD. In the murine model, chronic CS exposure resulted in decreased MIF mRNA and protein expression in the intact lung. MIF deficiency (*Mif*$^{-/-}$) potentiated the toxicity of CS exposure *in vivo* via increased apoptosis of EndoC resulting in enhanced CS-induced tissue remodeling. This was linked to MIF's capacity to shield against double stranded DNA damage and suppress p53 expression. Taken together, MIF appears to antagonize CS-induced toxicity in the lung and resultant emphysematous tissue remodeling by

suppressing EndoC DNA damage and controlling p53-mediated apoptosis, highlighting a critical role of MIF in EndoC homeostasis within the lung.

## 3.2 Introduction

In humans, pulmonary EndoC apoptosis represents an early event in cigarette smoke (CS)-induced lung pathology, preceding other physiologic manifestations of obstructive disease (Gordon, Gudi et al.). Numerous studies have identified increased EndoC apoptosis in human COPD/emphysema, and animal studies support a causal link between cell death and airspace remodeling (Calabrese, Giacometti et al. 2005, Kanazawa and Yoshikawa 2005, Henson, Cosgrove et al. 2006). Importantly, the targeted induction of EndoC death is sufficient to promote apoptosis of both EndoC and type II alveolar epithelial cells within the alveolar-capillary unit, with the net result being emphysematous tissue remodeling (Giordano, Lahdenranta et al. 2008). Thus, there is a critical interdependence between alveolar epithelial and microvascular EndoC in the maintenance of airspace structure, and loss of EndoC within the lung directly contributes to emphysematous remodeling. Despite these clinical and pre-clinical observations, understanding of the molecular basis of CS-induced EndoC apoptosis remains incomplete. We sought to identify and characterize intrinsic molecular regulators of alveolar-capillary homeostasis and define their contribution to disease severity and CS-induced tissue destruction.

Macrophage migration inhibitor factor (MIF) is a pleiotropic cytokine, hormone, and enzyme with oxidoreductase and tautomerase

activities (Rosengren, Bucala et al. 1996, Kleemann, Kapurniotu et al. 1998). It is constitutively expressed by multiple cell types including pulmonary microvascular EndoC (Damico, Chesley et al. 2008), vascular smooth muscle (Zhang, Shen et al. 2012), fibroblasts (Zhang, Talwar et al. 2012) and bronchial epithelium (Kobayashi, Nasuhara et al. 2006) within the lung. Further, it is produced by multiple leukocytes, including both B and T cells (Bloom and Shevach 1975) and macrophages (Calandra, Bernhagen et al. 1994), and is secreted by both the anterior pituitary gland (Bernhagen, Calandra et al. 1993) and pancreas (Waeber, Calandra et al. 1997). MIF is found in the serum under basal conditions and elevated circulating levels of MIF have been observed in humans in both acute and chronic inflammatory disease states (Morand 2005). Extracellular MIF is capable of engaging multiple recognized cell surface receptors, functioning in an autocrine or paracrine manner to activate signaling kinases, such as extracellular signal-related kinase (ERK), AKT, and AMP-Activated Protein Kinase (AMPK). Each of these kinases has been linked to pro-survival pathways. Additionally, intracellular pools of MIF modulate the stability of transcription factors, including hypoxia inducible factor 1 alpha (HIF1a) (Oda, Oda et al. 2008), p53 (Jung, Seong et al. 2008), and, as recent research shows, possibly nuclear factor (erythroid-derived 2)-like 2 (NRF2) (Mathew, Jacobson et al. 2013), leading to secondary effects on gene expression. These three transcription factors are critical regulators of cell fate in the context of

environmental stimuli (i.e. hypoxia, DNA damage, and increased reactive oxygen species, respectively) and thus are critical determinants of homeostatic responses to intracellular or extracellular stressors. Animal models of ischemic cardiac injury, bronchopulmonary dysplasia (BPD), and radiation-induced lung injury (Qi, Hu et al. 2009, Prencipe, Auriti et al. 2011, Mathew, Jacobson et al. 2013) demonstrate that loss of MIF is associated with increased tissue damage. We have previously identified MIF as a modulator of the sensitivity of CS-induced human lung EndoC apoptosis *in vitro* (Damico, Simms et al. 2011) antagonizing p53-dependent activation of the mitochondrial apoptotic pathway. Thus, we hypothesize MIF will impact on the severity of CS-induced EndoC injury *in vivo* and thus influence emphysematous tissue remodeling through its effects on alveolar-capillary homeostasis.

The present study identifies MIF as a novel modifier of disease severity in CS-induced injury and emphysematous remodeling and provides evidence that MIF is a critical determinant of lung EndoC homeostasis in the face of oxidative injury. Further, it shows that MIF acts predominantly in EndoC within the alveolar-capillary unit to antagonize DNA damage and to suppress subsequent p53 induction; thus preventing EndoC cell death and apoptosis-mediated tissue remodeling. Here, we demonstrate for the first time that serum MIF is significantly decreased in COPD patients, most markedly in patients with

the most severe disease, implicating relative MIF deficiency in the severity and/or susceptibility of humans with CS-induced lung disease.

## 3.3 Results

### *3.3.1 MIF levels are significantly reduced in humans with COPD*

We have previously demonstrated that MIF modifies EndoC injury *in vitro* in response to acute CS exposure by antagonizing p53-mediated mitochondrial apoptosis (Damico, Simms et al. 2011). To explore if alterations in MIF occur in the context of human COPD, we quantified circulating MIF levels in the serum of normal controls (n=18) and in subjects with COPD. This was defined by irreversible airflows obstruction (i.e., FEV1/FVC ratio of less than 0.7 and an FEV1 less than 90% predicted, n=32). Patient demographics are in Table 3-1. Serum MIF levels were significantly lower in patients with COPD compared to healthy, non-smoking controls (950 pg/ml vs. 1541 pg/ml, $P$=0.03) (Figure 3-1A).

To begin to address the relationship between MIF and disease severity, the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria was used to further classify patients in COPD cohort as previously described (Mannino and Braman 2007). In this cross-sectional analysis, patients with severe COPD (GOLD Stage IV) had significantly lower circulating MIF than subjects with less severe COPD (GOLD II and III) ($P$=0.03) (Figure 3-1B). Thus, we observed significantly lower circulating MIF in patients with COPD and this was most pronounced in those with severe disease, implicating MIF in human disease severity.

### *3.3.2 Chronic smoke alters MIF expression in the intact lung*

To address the effects of chronic CS exposure on MIF expression *in vivo*, wild type C57BL/6 mice were randomized to exposure with filtered air or CS for 6 months. Whole lung homogenates were analyzed for MIF mRNA and protein. Chronic CS exposure resulted in a significant decrease in total lung MIF mRNA (Figure 3-2A). Similarly, MIF protein expression was significantly decreased with chronic CS relative to air controls (Figure 3-2B and C). These changes in MIF expression were associated with enhanced activation of caspase-3 (Figure 3-2D), a marker of CS-induced apoptosis in the lung. Thus, MIF mRNA and protein expression were significantly decreased in the setting of chronic CS exposure in the intact murine lung coincident with activation of the apoptotic pathway.

### *3.3.3 MIF suppresses cigarette smoke-induced DNA damage in vivo*

Cell culture, animal data (Kim, Serebreni et al. 2013), and human pathology (Pastukh, Zhang et al. 2011) demonstrate double stranded DNA breaks (DSB) as one mechanism of CS-induced cellular injury in the lung. DNA damage results in cell cycle arrest, senescence, or cell death

in the form of apoptosis, orchestrated by the transcription factor, p53, a major effector of the DNA damage response (Niida and Nakanishi 2006). In response to DSB, histone H2AX is phosphorylated (termed γH2AX) by the ataxia telangiectasia mutated (ATM) kinase; making γH2AX a marker of both DSB and ATM kinase activity. To test if MIF functions to suppress DNA damage in the face of CS, *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were randomized to air or CS for 0.5 months, and lung γH2AX levels were analyzed by immunohistochemistry and Western blot. In air-exposed mice, basal *in situ* staining for γH2AX was minimal and similar between genotypes. In contrast, *Mif*$^{-/-}$ animals demonstrated significantly increased numbers of γH2AX positive cells in lung parenchyma compared to *Mif*$^{+/+}$ mice following exposure to CS (13.6±2.2 vs. 2.5±1.7 cells/high power field, $P<0.05$) (Figure 3-3A and B). This was confirmed by Western blot analysis (Figure 3-4A and B).

Microvascular EndoC are susceptible to CS-induced DSB *in vitro* (Kim, Serebreni et al. 2013), thus we evaluated the extent of DNA damage in EndoC *in vivo*. Using lectins which bind alveolar (microvascular) EndoC and not extraalveolar (macrovascular), we co-stained microvascular EndoC and γH2AX by immunofluorescent microscopy (King, Hamil et al. 2004). *In vivo*, the majority of cells with CS-induced DSB in *Mif*$^{-/-}$ mice were microvascular EndoC (Figure 3-3C and D). This differed significantly with *Mif*$^{+/+}$ both in absolute numbers and as a proportion of damaged cells identified ($P<0.05$). DSB were not observed in

large blood vessels (identified on phase contrast). Therefore, MIF deficiency potentiated CS-induced DSB and ATM kinase activation within microvascular EndoC in the intact lung.

### *3.3.4 MIF suppresses cigarette smoke-mediated p53 expression in vivo*

ATM kinase is a well-established positive regulator of p53 expression. We next asked if the enhanced ATM kinase activity, as reflected by increased γH2AX (Figure 3-3), was associated with increased p53 expression *in vivo*. Protein and mRNA were extracted from the lung of CS-exposed *Mif*$^{-/-}$ and *Mif*$^{+/+}$ mice and p53 expression was quantified via Western blotting and qPCR (Figure 3-5) both were significantly enhanced in CS-exposed *Mif*$^{-/-}$ mice relative to CS-exposed *Mif*$^{+/+}$ or air-exposed animals. CS-induced p53 expression is ATM-kinase-dependent (Kim, Serebreni et al. 2013) and p53 triggers activation of pro-apoptotic BAX and EndoC death via the mitochondrial apoptotic pathway (Damico, Simms et al. 2011). MIF functions upstream of ATM kinase. Thus, ATM kinase provides a molecular link between MIF effects in response to CS and p53 expression.

### *3.3.5 MIF suppresses cigarette-smoke induced apoptosis in vivo*

Based on the observed effects of MIF on p53-dependent human EndoC apoptosis in response to CS *in vitro* (Damico, Simms et al. 2011), we rationalized that increased p53 would be linked to increased cell apoptosis *in vivo*. To test this, lung sections from air and CS-exposed (0.5 and 6 months) mice were stained for cleaved caspase-3, the enzymatic effector of apoptosis (Figure 3-6A and B). *In situ* quantification demonstrated a significantly increased number of cleaved caspase-3 positive cells in the parenchyma of $Mif^{-/-}$ mice challenged with CS compared to $Mif^{+/+}$ animals exposed in parallel (upper panels of Figure 3-6C and D). There was no difference in the frequency of cleaved caspase-3 positive cells between genotypes under basal conditions (air-exposed) when comparing age-matched mice. In the presence of MIF, short-term exposure to CS was sufficient to increase apoptosis above air-exposed, age-matched mice (3.4/HPF vs. 7.1/HPF, $P$=0.02) (Figure 3-6C upper panel). This increase in cell death was lost by 6 months of CS exposure (5.4 /HPF vs. 6.2/HPF, $P$=0.2) (Figure 3-6D upper panel) with basal frequencies of apoptosis being higher in the lungs of these older mice. In contrast, there was a significant increase in CS-induced apoptosis within the parenchyma of $Mif^{-/-}$ mice at 0.5 months (2.5/HPF vs. 15/HPF, $P$<0.0001) (Figure 3-6C upper panel), which trended down by 6 months (Figure 3-6D upper panel), but remained elevated above air-exposed littermates (5.2/HPF vs. 7.3/HPF, $P$=0.002). Thus, the absence of MIF was associated with increased and persistent cell death following CS

exposure without demonstrable differences in air-exposed animals, further implicating MIF in cytoprotective responses in the lung following oxidative challenge.

### *3.3.6 MIF antagonizes cigarette-smoke induced endothelial apoptosis in the intact lung*

To address the contribution of MIF to EndoC apoptosis, we performed co-immunohistochemistry for both cleaved caspase-3 and the EndoC marker, thrombomodulin (Kasahara, Tuder et al. 2000) (Middle panels of Figure 3-6C and D). In the presence of MIF (*Mif*$^{+/+}$ animals) there was a relative resistance of microvascular EndoC following subacute exposures to CS with no significant difference in EndoC apoptosis observed relative to age-matched air-exposed animals. After prolonged exposure to CS (6 months), there was a trend towards an increase in microvascular EndoC apoptosis, which did not meet statistical significance (1.8/HPF vs 2.7/HPF, *P*=0.07) (Figure 3-6D middle panel). In the absence of MIF, there were no differences at baseline (air-exposed) after 0.5 months or 6 months exposure relative to *Mif*$^{+/+}$ mice (Figure 3-6D middle panel). In contrast, exposure to both short-term and prolonged CS resulted in significant increases in microvascular EndoC apoptosis in *Mif*$^{-/-}$ mice when compared to *Mif*$^{+/+}$ mice (1.8/HPF vs. 9.8/HPF at 0.5 months, *P*=0.001, and 2.6/HPF vs. 4.1/HPF at 6 months, *P*=0.02) (Middle panels

of Figure 3-6C and D).

Non-EndoC apoptosis was significantly increased after 0.5 months of CS exposure (1.6/HPF vs. 5.5/HPF, *P*=0.01); however there was no difference between genotypes (5.5/HPF vs. 5.1/HPF, *P*=0.8). Despite the apparent lower mean in the knockout mice, there was not a statistically significant difference between genotypes at baseline (1.6/HPF vs. 0.2/HPF, *P*=0.07) (Figure 3-5C lower panel). After 6 months, the frequency of apoptosis in non-EndoC did not differ between exposures or genotypes (Figure 3-6D lower panel). Thus, MIF deficiency preferentially enhanced microvascular EndoC sensitivity to CS-induced apoptosis in the intact lung.

### *3.3.7 MIF-deficiency enhances cigarette smoke-induced tissue remodeling*

In order to test the hypothesis that MIF modifies the severity of CS-induced emphysematous tissue remodeling, *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were randomized to prolonged CS exposure (6 months, 5 days/week) or filtered air for the same duration of time. Lung tissue was subjected to morphometric analysis under basal conditions (air-exposed, age-matched animals) and in the setting of chronic CS. Using systematic random sampling caudal, middle, and cranial left lung regions were analyzed for

alterations in tissue architecture, with quantification of airspace enlargement defined by increased mean chord length ($L_m$).

Baseline analysis revealed a modest, but statistically significant, increase in the cranial and middle $L_m$ in *Mif*$^{-/-}$ mice relative to age-matched *Mif*$^{+/+}$ mice (29.7 vs. 32.0, *P*=0.01, and 30.2 vs. 32.4, *P*=0.02, respectively) (Figure 3-7). Thus, in the absence of MIF there were statistically significant differences in airspace morphology under basal conditions, which may reflect a contribution of MIF in normal lung development and/or in the maintenance of lung structure with aging.

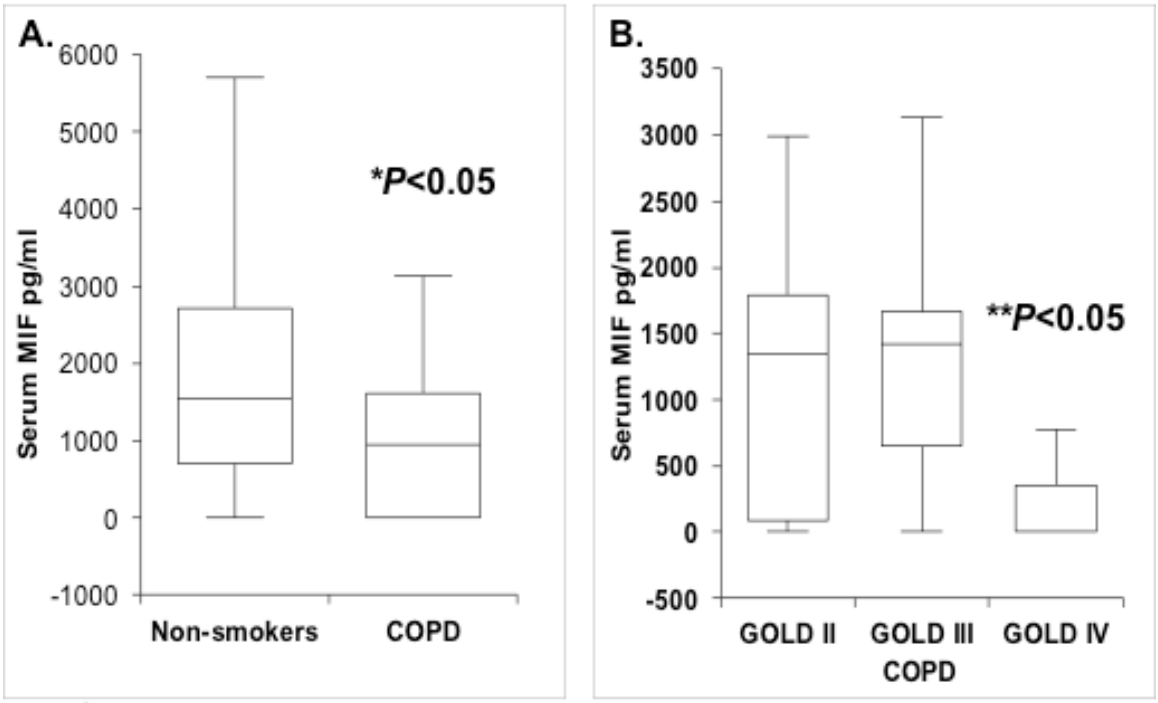
In response to CS, airspace enlargement was visually detected in caudal regions regardless of genotype (Figure 3-8A-D and Figure 3-9) and morphometric analysis confirmed that tissue remodeling in response to CS predominated in the caudal regions, highlighting a heterogeneous distribution of airspace enlargement and enhanced lower lung damage due to CS in this model (Figure 3-8A-D). In response to CS, airspace enlargement in *Mif*$^{+/+}$ mice was significant in the cranial and middle lung regions (29.7 vs. 31, *P*=0.03, and 30.3 vs. 32.5, *P*=0.04, respectively) (Figure 3-7 and Figure 3-9), and tended to be enlarged in the caudal regions (31.0 vs. 33.2, *P*=0.07) relative to air controls.

Regional remodeling was significantly enhanced in the absence of MIF. In *Mif*$^{-/-}$ mice, the caudal lung demonstrated significant increases in $L_m$ when compared with CS-exposed *Mif*$^{+/+}$ mice (33.2 vs. 36.5, *P*=0.007) or air-exposed littermates (32.9 vs. 36.5, *P*=0.002) (Figure 3-8 and Figure

3-9). Even after adjusting for multiple comparisons, *Mif*$^{-/-}$ mice exposed to CS demonstrated a significant increase in the absolute $L_m$ relative to smoke challenged *Mif*$^{+/+}$ mice ($P$<0.008) (Figure 3-7 and Figure 3-8). In addition to the effects observed in absolute $L_m$ in response to CS, quantification of the relative change in $L_m$ highlighted the effects of both MIF deficiency and CS exposure on airspace enlargement (Figure 3-8F). The calculated mean whole lung alveolar surface area ($S_{av}$) (Demedts, Demoor et al. 2006) did not differ between genotypes at baseline and was lower in *Mif*$^{-/-}$ mice following smoke. This did not reach statistical significance secondary to large variability in the values ($P$=0.09) (Table 3-2). Importantly, the validity of the calculated $S_{av}$ requires homogeneity of remodeling throughout the lung, a condition present at baseline but not observed following CS-induced remodeling.

| Table 1. Patient Demographics | | | | |
|---|---|---|---|---|
| | **Healthy controls** | **Group I-II** | **Group III** | **Group IV** |
| **Patients (n)** | 18 | 18 | 10 | 7 |
| **Mean Age** | 50 | 55 | 61.5 | 60.7 |
| **95% CI** | 46.5-53.6 | 50.6-59.4 | 53.7-69.3 | 55.9-65.5 |
| **Males, n** | 10 | 9 | 6 | 5 |
| **% Male** | 55 | 50 | 60 | 71 |

| Table 2. CS-related airspace Lung Characteristics | | | | |
|---|---|---|---|---|
| | ***Mif*** $^{+/+}$ **Air** | ***Mif*** $^{-/-}$ **Air** | ***Mif*** $^{+/+}$ **CS** | ***Mif*** $^{-/-}$ **CS** |
| **$S_{av}$±SEM ($cm^2$)** | 369.6 ± 8.2 | 374.6 ± 23.6 | 375.5 ± 30.8 | 318.8 ± 14.9* |
| **$V_L$ ± SEM (g)** | 0.305 ± 0.05 | 0.28 ± 0.01 | 0.272 ± 0.01 | 0.302 ± 0.03 |
| n | 8 | 8 | 6 | 6 |




C.

| Groups | number | median pg/ml | 95% CI | |
|---|---|---|---|---|
| Controls/non-smokers | 19 | 1541 | 676 | 2738 |
| COPD | 32 | 951 | 0 | 1530 |
| GOLD II | 15 | 1345 | 0 | 1820 |
| GOLD III | 10 | 1418 | 0 | 1921 |
| GOLD IV | 7 | 0 | 0 | 775 |

**Figure 3-1. Serum MIF concentrations are reduced in patients with severe COPD.** Serum MIF levels were significantly reduced in subjects with COPD when compared with non-smokers (left panel). In patients with COPD, serum MIF was significantly reduced in severe, GOLD IV disease (right panel). Values are expressed as median and 95% CI. $P<0.05$.

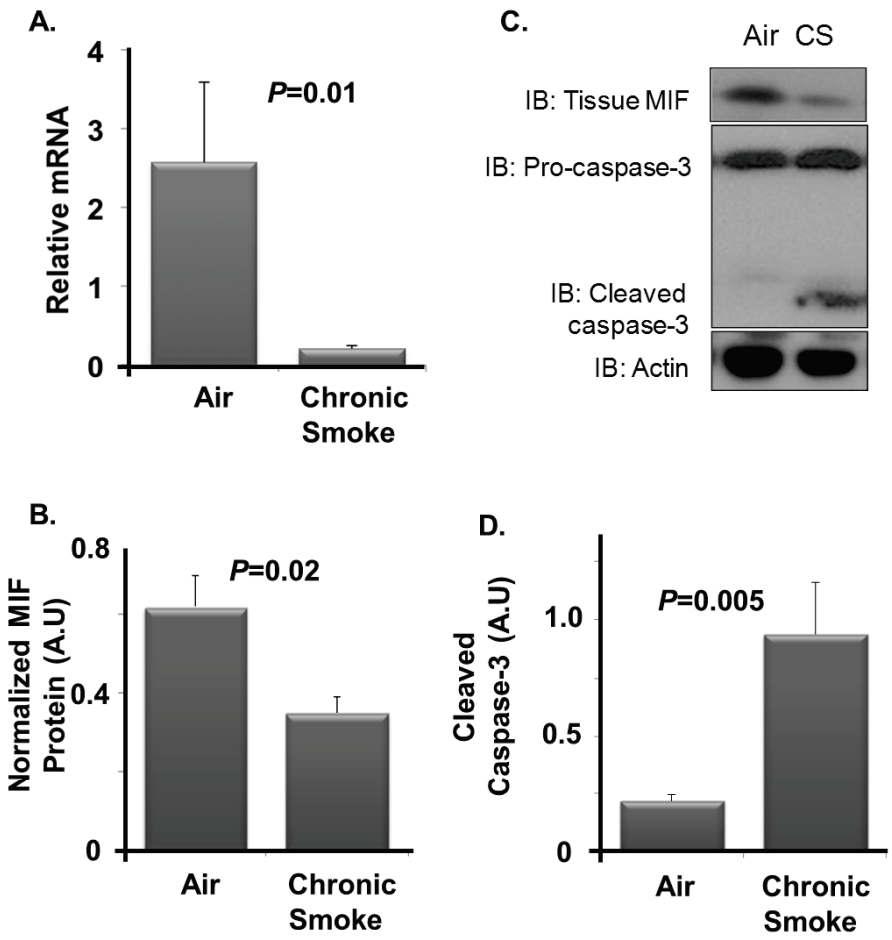



**Figure 3-2. Chronic smoke alters MIF expression in the intact lung.** Wild type C57BL/6 mice were exposed to CS or filtered air. Gene expression and Western blotting were utilized to study alterations in MIF and cleaved caspase-3. Lung MIF mRNA was significantly reduced following exposure to CS (A). Tissue MIF was significantly decreased with chronic CS relative to air controls (B). The resulting protein intensities were normalized to beta actin (C). Chronic CS was associated with enhanced cleavage of caspase-3 relative to air controls (D). n=5 per arm. Values are expressed as mean ± SEM.

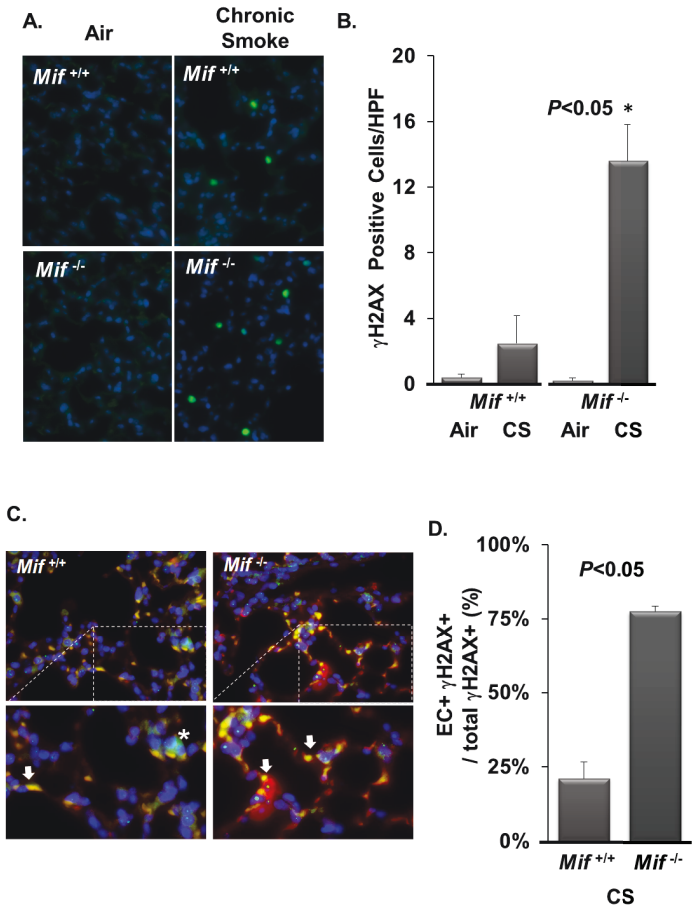



**Figure 3-3. MIF antagonizes CS-induced EndoC double stranded DNA breaks (DSB) *in vivo*.** Lungs from *Mif*$^{+/+}$ and *Mif*$^{-/-}$ animals exposed to filtered air or CS for 0.5 months were harvested and sectioned for immunohistochemistry (A and C). MIF deficient mice exposed to CS demonstrated a significant increase in γH2AX positive parenchymal cells relative to air-exposed *Mif*$^{-/-}$ and CS challenged *Mif*$^{+/+}$ mice (A and B). *Mif*$^{-/-}$ mice had significantly higher γH2AX positive EndoC as detected by co-localization of isolectin staining (red) (C and D). n=5 per arm. Values are expressed as mean ± SEM.

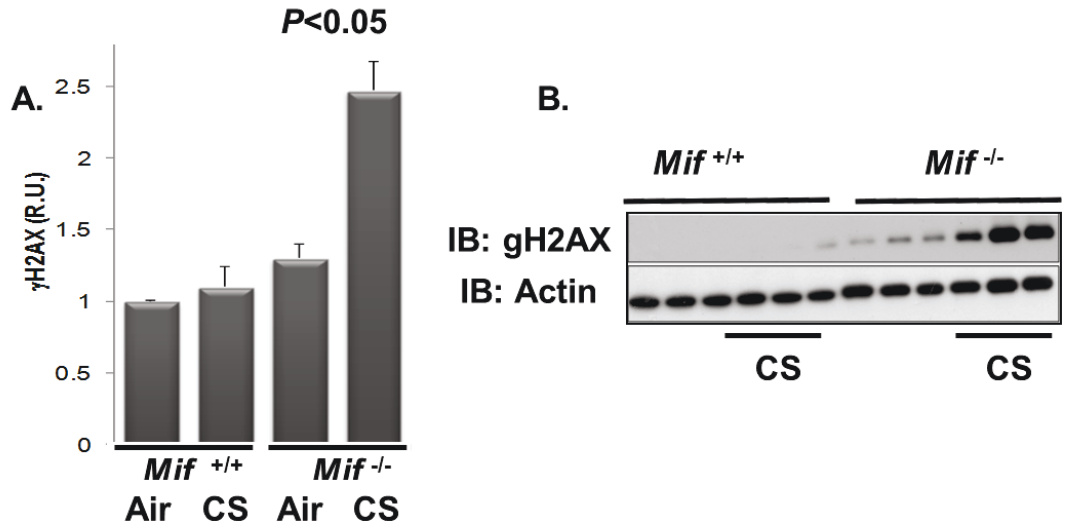



**Figure 3-4. MIF antagonizes CS-induced EndoC double stranded DNA breaks (DSB) *in vivo*.** Lungs from *Mif*$^{+/+}$ and *Mif*$^{-/-}$ animals exposed to filtered air or CS for 15 days were harvested and homogenized for Western blot analysis (B). Densitometric analysis showed samples probed with anti-γH2AX antibody had a significant increase in γH2AX levels after CS exposure in *Mif*$^{-/-}$ animals relative to *Mif*$^{+/+}$ (A). n=5 per arm. Values are expressed as mean ± SEM.

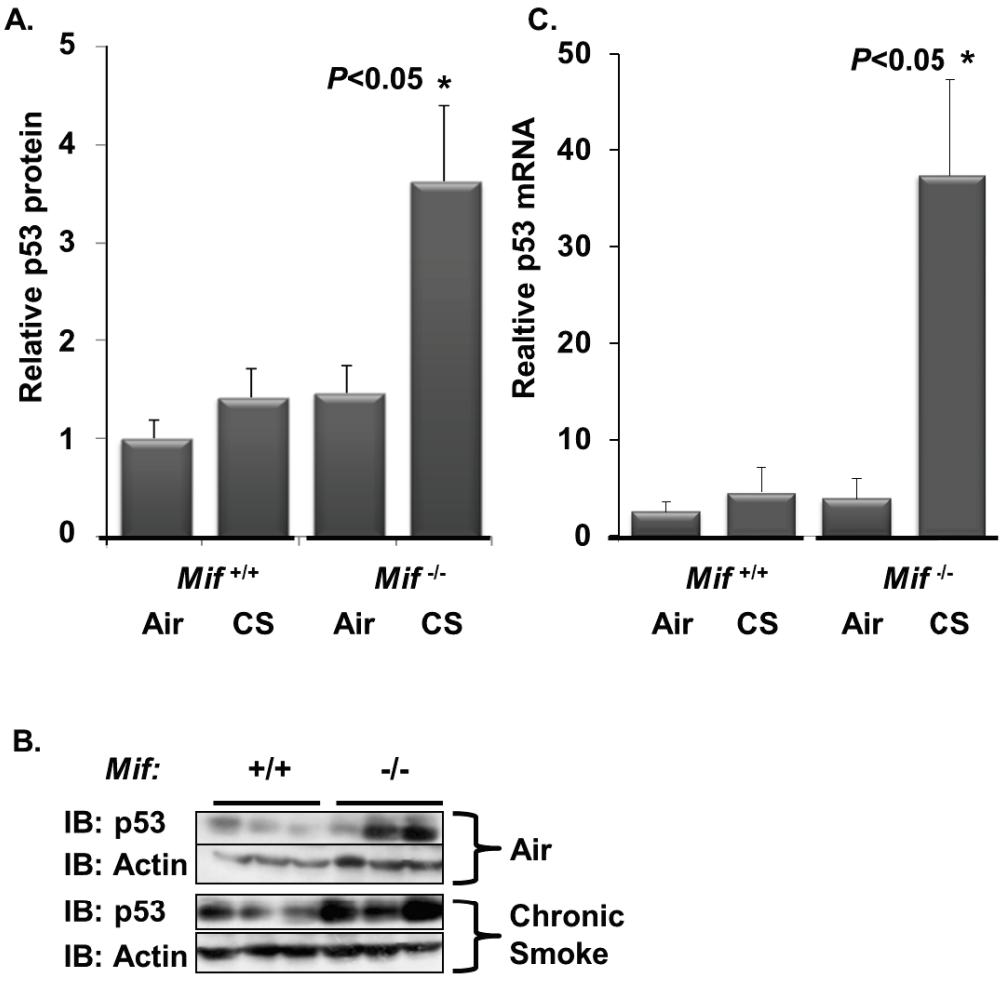



**Figure 3-5. CS-induced p53 expression is increased in the absence of MIF.** Lungs from *Mif*$^{+/+}$ and *Mif*$^{-/-}$ animals exposed to filtered air or CS for 0.5 months were harvested and homogenized for Western blotting (A and B) and gene expression (C). Relative p53 protein expression was increased in *Mif*$^{-/-}$ mice exposed to CS (A). Representative Western blot (B). n=5-6 per arm. There was a significant increase in p53 mRNA in *Mif*$^{-/-}$ animals in response to CS as assessed by comparative qPCR (C).

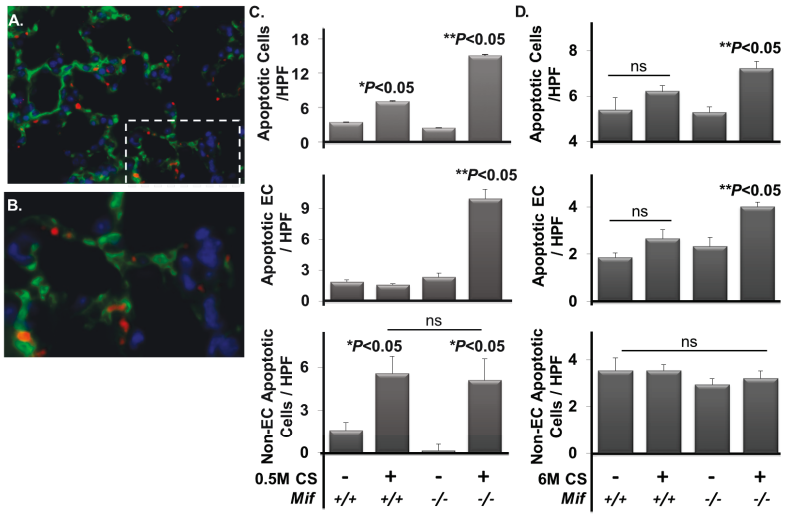



**Figure 3-6. Caspase-3 expression is increased in the absence of MIF with sub-acute and chronic CS exposure.** Lungs from *Mif*$^{+/+}$ and *Mif*$^{-/-}$ animals exposed to filtered air or CS for 0.5 or 6 months were harvested and sectioned for immunohistochemistry. Representative fluorescent microscopy images are shown of cleaved caspase-3 (red) and thrombomodulin (green) in the lung (A and B). The frequency of cleaved caspase-3 positive parenchymal cells was significantly increased in *Mif*$^{-/-}$ vs. *Mif*$^{+/+}$ mice exposed to 0.5 months CS ($P$<0.05) (C upper panel), and with 6 months of CS ($P$<0.05) (D upper panel). The majority of caspase-3 positive cells in *Mif*$^{-/-}$exposed to 0.5 or 6 months CS were EndoC (both with $P$<0.05) (middle panels of C and D). Non EndoC were enhanced with 0.5 months with exposure ($P$<0.05) and this did not differ with genoptype (lower panels of C and D). No differences were observed under basal conditions independent of genotype (n=5-8 per arm) Nuclei were stained with DAPI (blue). Values are expressed as mean ± SEM.

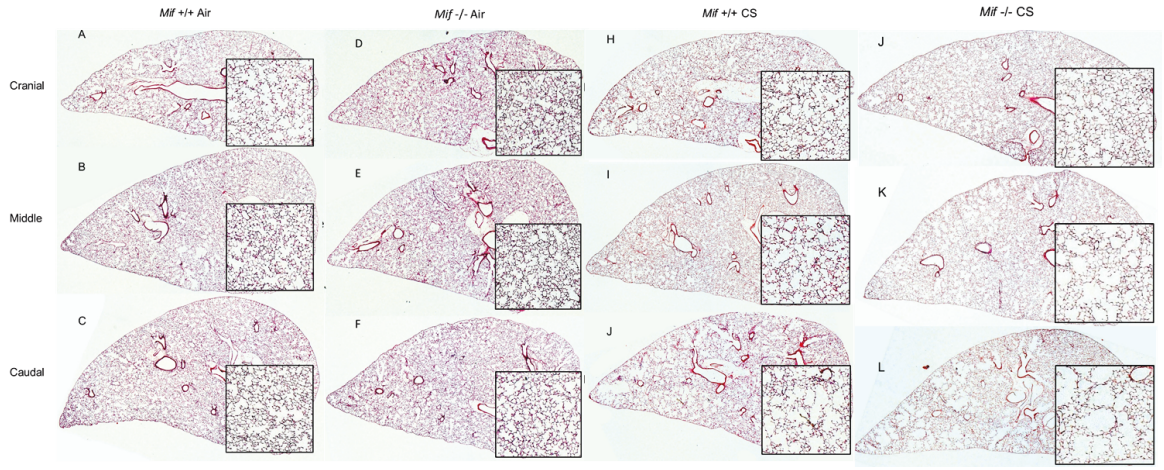



**Figure 3-7. Regional differences in cigarette smoke-induced emphysematous tissue remodeling *in vivo*.** Representative images of lung regions in CS-exposed mice highlight the significant increase in caudal airspace remodeling. *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were randomized to filtered air or chronic CS and harvested for morphometric analysis as detailed in Materials and Methods. In air exposed animals, there were no significant regional differences in $L_m$ between the cranial and caudal zones or between genotypes (A&C, D&F). Following CS exposure, caudal $L_m$ was significantly different from cranial $L_m$ and this difference from cranial $L_m$ was more pronounced in the middle and caudal *Mif*$^{-/-}$ lung (G&I, J&L).

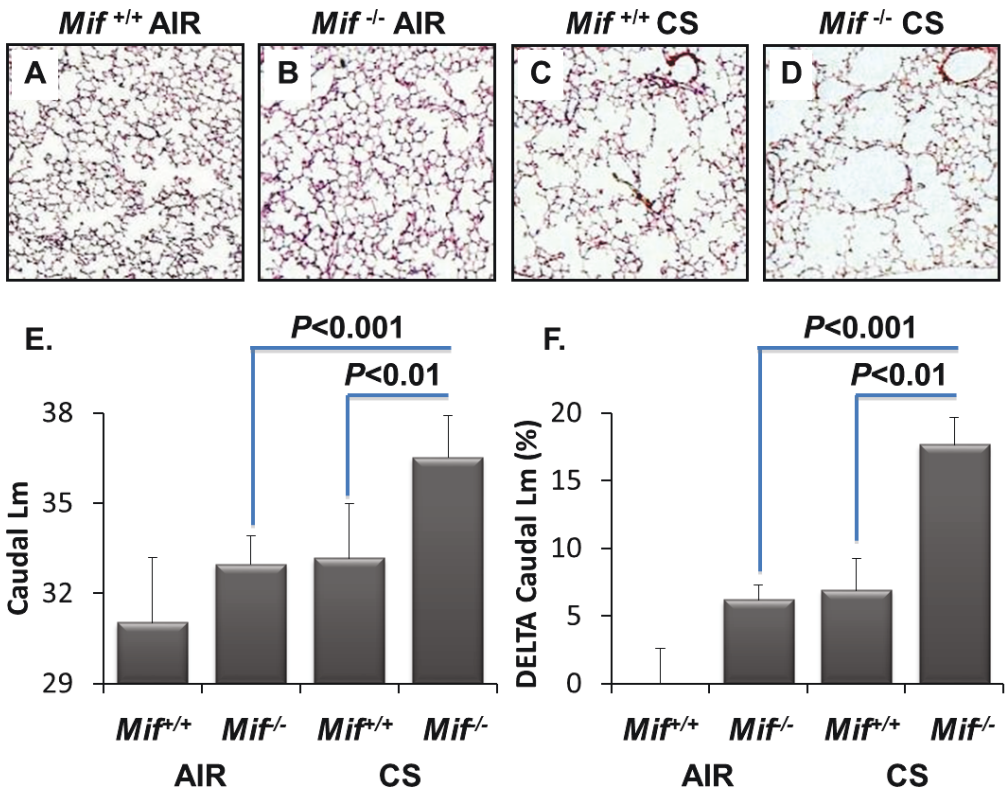



**Figure 3-8. MIF deficiency leads to increased sensitivity to emphysematous remodeling *in vivo*.** Lungs from *Mif*$^{+/+}$ and *Mif*$^{-/-}$ animals exposed to filtered air or CS for 6 months were sectioned for morphometry. Differences in alveolar remodeling in *Mif*$^{+/+}$ vs. *Mif*$^{-/-}$ mice are visually apparent in CS exposed caudal lung regions (C & D respectively) compared with air-exposed mice (A & B respectively). *Mif*$^{-/-}$ mice had significantly higher $L_m$ in the caudal lung regions than *Mif*$^{+/+}$ when exposed to CS (E) and the change in $L_m$ from baseline was significant in *Mif*$^{-/-}$ mice (F). Values are expressed as mean ± SEM.

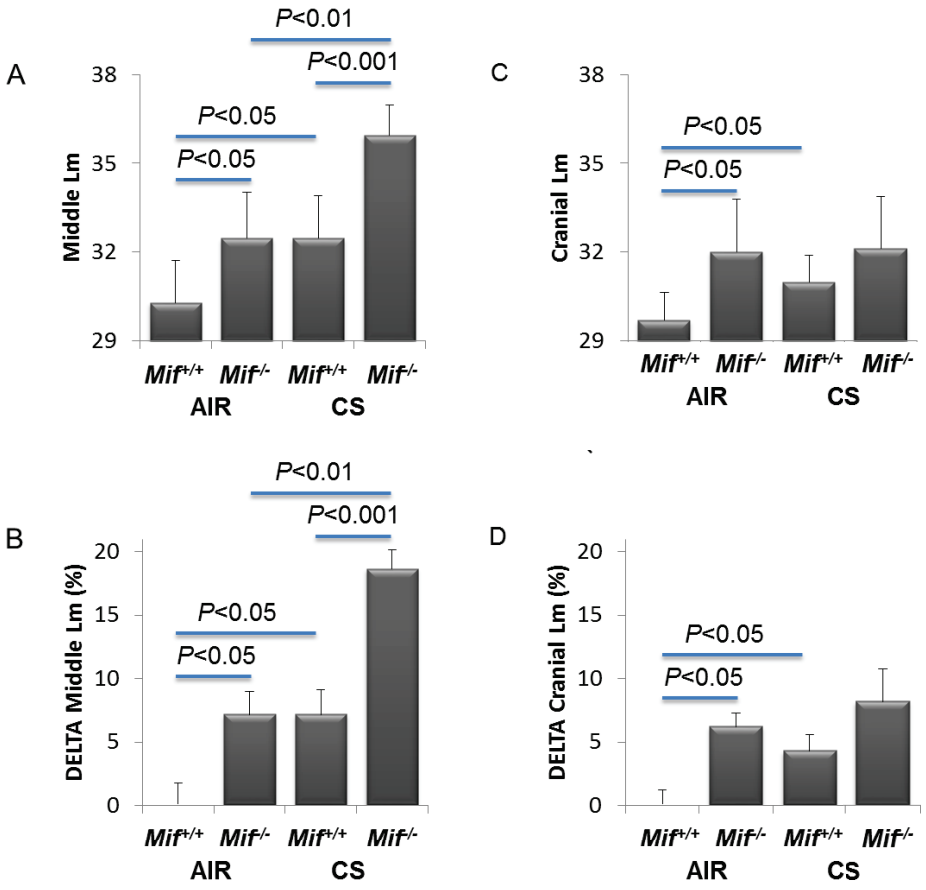



**Figure 3-9. Effects of MIF loss and CS on the $L_m$ of cranial and middle lung regions.** Lungs from *Mif*$^{+/+}$ and *Mif*$^{-/-}$ animals exposed to filtered air or CS for 6 months were sectioned for morphometry. By simple comparisons, cranial and middle lung regions were different at baseline between genotypes. In response to CS, middle lung regions had significantly increased $L_m$ in *Mif*$^{+/+}$ ($P<0.05$) and *Mif*$^{-/-}$*(*$P<0.001$*) mice.* CS-induced increases in *Mif*$^{-/-}$ middle lungs displayed more than an additive effect of MIF deficiency and CS. No effect was observed in the cranial regions in *Mif*$^{-/-}$ mice, while there was a small effect in *Mif*$^{+/+}$ mice ($P<0.05$).

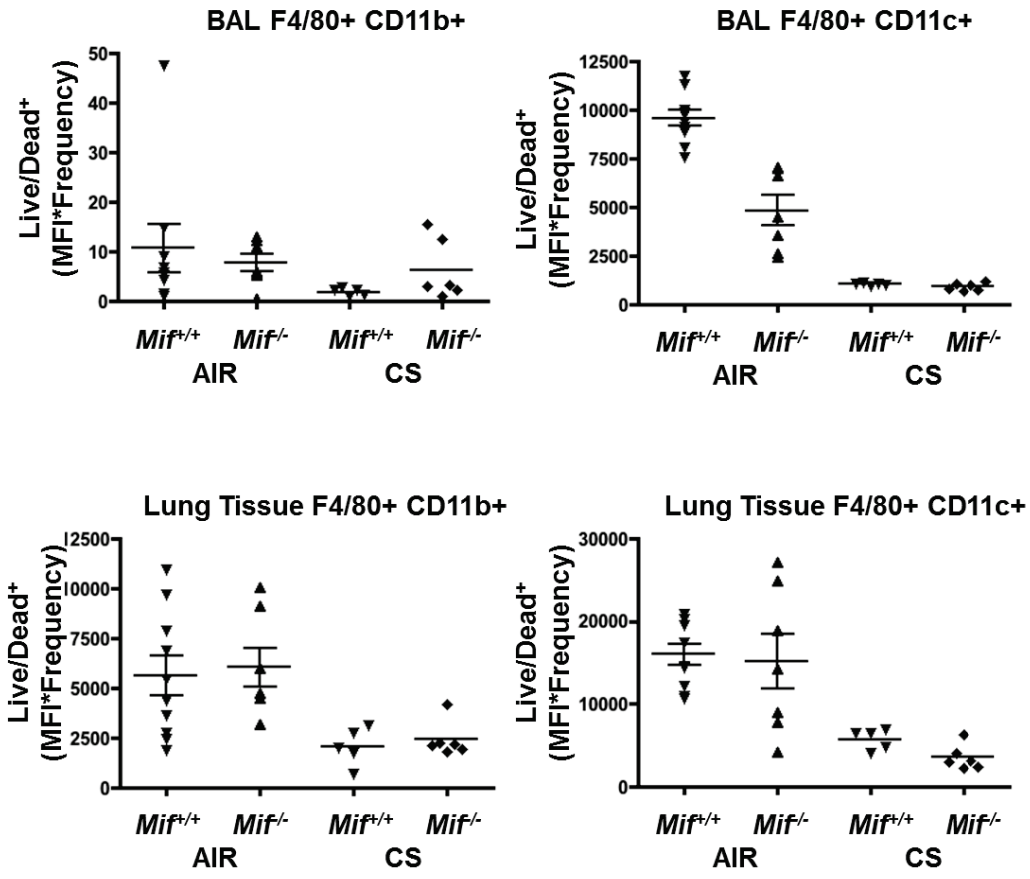



**Figure 3-10. Sub-acute CS does not differentially alter macrophage viability or frequency in the absence of MIF**. *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were exposed to 15 days of air or CS, after which BALF and lung tissue were processed for analysis via flow cytometry.

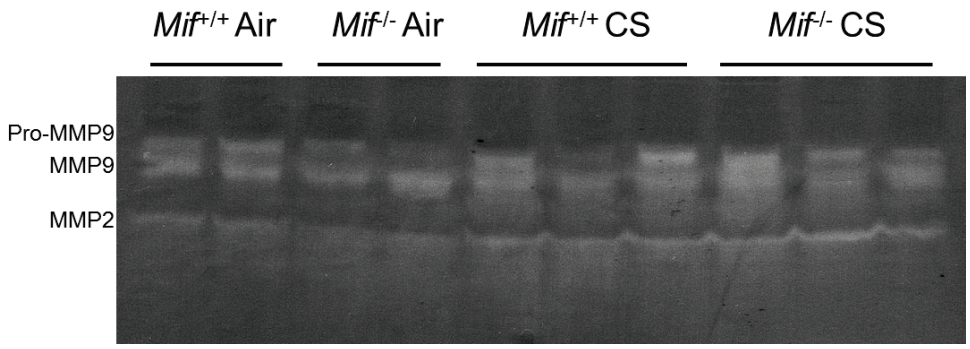



**Figure 3-11. Matrix Metalloproteinases are not regulated by MIF in response to CS.** Serum from *Mif*$^{+/+}$ and *Mif*$^{-/-}$ animals exposed to filtered air or chronic CS were harvested and homogenized for zymogram analysis.

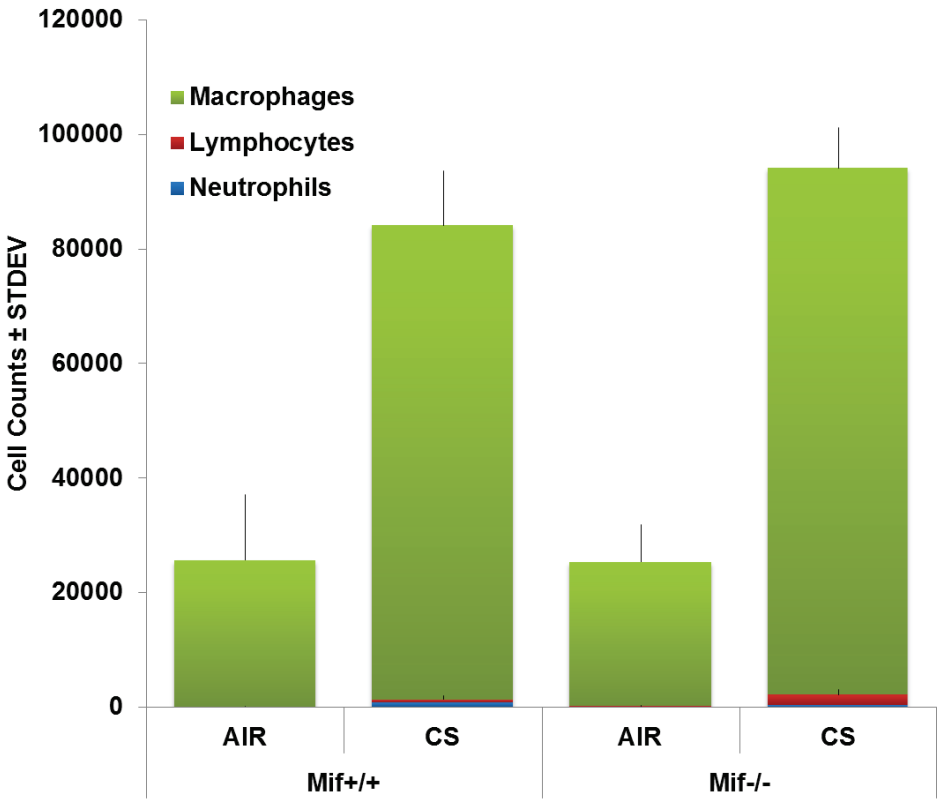



**Figure 3-12. BALF cell count and differentials in *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice exposed CS.** *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were exposed to 3 days of air or CS, after which BALF was extracted and analyzed for differential cell counts. There was a significant effect of CS exposure on total cell count with a vast predominance being macrophages. N=3 per arm.

## 3.4 Discussion

Emphysema, a common form of COPD, contributes to an estimated 2.5 million deaths and a health care cost approaching 38.8 billion dollars annually in the United States alone (Foster, Miller et al. 2006). It is a debilitating disease characterized by the irreversible destruction of the lung architecture with enlargement of the airspaces driven by enhanced apoptosis (Demedts, Demoor et al. 2006). Targeted EndoC apoptosis is sufficient to induce emphysema (Giordano, Lahdenranta et al. 2008) while pharmacologic blockade prevents disease, arguing that EndoC apoptosis is both sufficient and necessary for development of emphysema (Kasahara, Tuder et al. 2000, Tuder, Petrache et al. 2003). Further, loss of EndoC viability is sufficient to induce apoptosis of type II alveolar epithelial cells (Giordano, Lahdenranta et al. 2008), highlighting the contribution of EndoC homeostasis in the maintenance of normal alveolar structure. We set out to identify new molecular determinants of EndoC apoptosis and survival in response to CS (Damico, Simms et al. 2011, Kim, Serebreni et al. 2013) predicting that such factors could represent novel determinants of disease severity and pathologic tissue remodeling. The capacity of MIF to regulate the sensitivity of human pulmonary EndoC to CS *in vitro* made it an attractive candidate. Our work provides evidence of altered MIF expression in murine and human CS-induced lung disease and links enhanced disease severity to the loss of MIF's capacity to suppress DNA damage and apoptosis in

microvascular EndoC within the lung, potentiating CS-induced tissue remodeling.

Here, we provide the first evidence that patients with COPD have diminished serum MIF relative to normal controls (non-smokers). Further, we demonstrate that patient with severe disease (GOLD stage IV COPD) have markedly lower circulating MIF than those with milder disease. It remains to be determined if this is a cause or consequence in human disease, which cannot be specifically addressed in a cross-sectional study design such as this. However, in our animal and human EndoC studies, MIF levels inversely correlate with cellular injury and tissue remodeling, implicating a causal relationship in humans rather than an epiphenomenon. Our results suggest the possibility that individual variability in MIF expression, which is genetically determined (Baugh, Chitnis et al. 2002), could account for the clinical heterogeneity in susceptibility to CS-induced emphysematous pathology in humans.

In our animal model, we have demonstrated CS-induced DNA damage in the lung parenchyma that is antagonized by MIF. Further, our data indicated that MIF functions predominantly to maintain homeostasis of microvascular EndoC. In the absence of MIF, there was increased DSB, leading to increased ATM kinase-dependent p53 expression. As a consequence, MIF deficiency exacerbated CS-induced lung cell death. The cytoprotective effects of MIF in the context of CS impact on the microvascular EndoC within the lung, as these are the

primary targets of DSB and apoptosis in the absence of MIF. CS-induced EndoC apoptosis was observed rapidly (0.5 months), at a higher frequency (approaching 5-fold great), and persistently (6 months) in *Mif*$^{-/-}$ mice compared to *Mif*$^{+/+}$ animals. This occurred without evidence of basal differences in age-matched mice. Importantly, there was no difference between the frequencies of CS-induced non-EndoC death between genotypes, again pointing to the enhanced dependence of microvascular EndoC on MIF in the setting of CS. The enhanced EndoC apoptosis, was linked to increased emphysematous tissue destruction manifested by a significant increase in mean chord length in the lower lung zones (i.e. middle and caudal regions).

The alveolar-capillary structure is composed of multiple cell types. In addition to the contribution of microvascular EndoC, the alveolar wall is composed predominantly of type I and II epithelial cells (pneumocytes). Homeostasis of both EndoC and pneumocytes are necessary for maintenance of normal alveolar structure. Both cell types are also destroyed during human emphysematous remodeling. Our *in vivo* data indicates microvascular EndoC represent the major cellular target of DNA damage and apoptosis in the absence MIF. Others have shown that targeted killing of EndoC in the lung is sufficient to trigger apoptosis of type II pneumocytes (Giordano, Lahdenranta et al. 2008). Thus, it is possible that loss of these cells in the alveolus may be a consequence of EndoC death in our model or a direct effect of CS exposure. Importantly,

while we observed apoptosis in other cell types in the lung parenchyma, this did not differ as a function of MIF expression. MIF deficiency has also been linked to decreased macrophage viability (Mitchell, Liao et al. 2002) and macrophage numbers are significantly altered in both COPD patients and in animal models of CS-induced lung disease (Holloway and Donnelly 2013). Thus, we specifically assessed macrophage numbers, activation status, and/or viability. While we observed an increase in macrophage frequency in the bronchoalveolar fluid (BALF) following short-term CS exposure, this did not differ between genotypes (Figure 3-10). Viability of both naive ($CD11b^+$) and activated ($CD11c^+$) f4/80+ macrophages in the BALF and lung tissue increased following CS (Figure 3-11) and again was not altered by MIF expression. Thus, MIF's effects on macrophage number, viability, or activation status do not contribute to CS-induced tissue injury or remodeling in our model.

Under normal physiologic conditions, MIF is detectable in the circulation. It is produced and secreted by numerous cell types and tissues and functions via autocrine and paracrine effects. While we demonstrate that MIF expression decreases in the lung with chronic smoke and is lower in the circulation of patients with COPD, at this point it remains to be determined if MIF expression is reduced systemically or in a subset of cells/tissues in the context of disease. Such information will be critical to define the mechanism(s) by which MIF is decreased in this disease state. Irrespective of the basal cellular and tissue derived

sources of MIF, low circulating levels predictably impact EndoC which are in direct contact with the blood. Our murine data indicates that MIF mRNA is globally reduced in the lung following chronic smoke exposure. There are few defined molecular mechanisms to suppress MIF transcription. MIF is however sensitive to epigenetic silencing. MicroRNA miR-451 can suppress MIF expression (Bandres, Bitarte et al. 2009) and intriguingly this microRNA is upregulated in the circulation of human smokers (Takahashi, Yokota et al. 2013) suggesting one potential molecular mechanism of MIF suppression in COPD. Additionally, the MIF promoter is repressed by histone deacetylase (HDAC) inhibitors (Lugrin, Ding et al. 2009). Both HDAC expression and activity are reduced in human COPD (Yang, Chida et al. 2006) suggesting a second potential molecular mechanism to account for the decline in MIF observed.

MIF has well-established pro-inflammatory functions and has been implicated as a positive regulator of metalloproteases (MMPs) including collagenases (MMP13 (Onodera, Nishihira et al. 2002) and MMP1 (Kong, Huang et al. 2005)) and gelatinases (MMP2 (Meyer-Siegler 2000), and MMP9 (Onodera, Nishihira et al. 2002)) but not elastase (MMP12). Of the known MMPs responsive to MIF, MMP2 and MMP9, are elevated in human COPD (Betsuyaku, Nishimura et al. 1999, Boschetto, Quintavalle et al. 2006) and animal models of emphysema (Seagrave, Barr et al. 2004). Despite these potential molecular targets of MIF in CS-induced disease, we were unable to identify significant differences of the pro-

inflammatory cytokine tumor necrosis factor alpha (TNFa) expression or MMP2/MMP9 in our model by qPCR (data not shown) or zymography, respectively (Figure 3-12) based on genotype. Further, we did not detect altered total BAL cell counts between genotypes, though lymphocyte numbers were differentially affected by CS (Figure 3-10). Thus, our loss-of-function and clinical data argue against a model in which the pro-inflammatory functions of MIF promote COPD pathology.

Airspace enlargement is a well-documented age-related process (Verbeken, Cauberghs et al. 1992, Papaioannou, Rossios et al. 2013) and it is recognized that genetically engineered strains of mice, hypersensitive to CS, can also demonstrate evidence of accelerated age-dependent lung remodeling (Morris, Huang et al. 2003, Ruwanpura, McLeod et al. 2011). We observed differences in baseline $L_m$ in *Mif*$^{-/-}$ compared to *Mif*$^{+/+}$ mice housed in a filtered air environment. This suggests MIF may influence lung homeostasis and/or development. MIF protein decreases in the lungs of aging mice (Mathew, Jacobson et al. 2013) indirectly implicating it in age-related changes. In neonatal mice exposed to hyperoxia, both excess and insufficient MIF alters neonatal alveolar development, suggesting that there is an ideal "dose" of MIF for optimal lung maturation (Sun, Choo-Wing et al. 2013). Despite the basal difference in airspace morphology, the effects of CS-exposure on MIF deficient animals were more than an additive. Since all studies used age-matched controls, aging cannot account for the differences observed between treatment

arms. Moreover, we did not observe differences in basal DSB or apoptosis indicating that the effects of MIF on DNA damage and EndoC apoptosis in adult life are unlikely to account for the basal differences in alveolar morphology. Further studies will be necessary to determine if the observed basal differences in the adult lung are a result of premature aging and/or abnormal lung development.

From a clinical and therapeutic perspective, our data suggest that relative MIF deficiency may predispose patients to COPD/emphysema and that normalization of MIF could have a therapeutic advantage. Our preclinical and *in vitro* data (Damico, Simms et al. 2011) implicate the capacity of MIF to antagonize p53 expression in its cytoprotective effects against CS. While p53-induced apoptosis predisposes to pathologic remodeling, p53 is also a tumor suppressor and prolonged and/or global p53 suppression would predictably increase risk of malignancies, especially in the face of chronic carcinogen exposures such as CS. Importantly, the effects of MIF deficiency were observed in the microvascular EndoC. This cell type rarely gives rise to tumors (i.e. angiosarcomas) in the lung and these tumors are not epidemiologically linked to CS exposure. Moreover, our data indicate that MIF does not directly impact on p53, but functions upstream of CS-induced DSB. Thus, we predict that restoring physiologic MIF would reduce CS-induced DNA damage, promoting homeostasis and eliminating the driving force for CS-induced p53 expression in EndoC.

Limitations of our analysis are imparted by the animal model used which must condense a prolonged exposure in humans into a restricted timeline. Consequently airspace remodeling is relatively mild. Second these studies were performed in a relatively resistant background strain, C57BL/6, to take advantage of a genetic model. While MIF expression was suppressed by chronic CS in this strain, it remained detectable within the lung after 6 months. Thus, the reduced DSB, delayed EndoC apoptosis (observed at 6 months not 0.5 months), and a diminished airspace enlargement observed in *Mif*$^{+/+}$ mice, relative to Mif$^{-/-}$, demonstrate the persistent cytoprotective role of MIF in response to CS exposure.

From a methodologic standpoint, the most reliable and accurate stereologic methods to quantify emphysematous remodeling in small animal models are highly debated. We used an unbiased approach including systematic random sampling and the indirect/point counting method of unadulterated lung images to estimate $L_m$. This is in contrast to the mean linear intercept or direct method, which can substantially bias the $L_m$ estimations, favoring larger values. Thus, in the face of heterogeneous tissue destruction, $L_m$ derived from the point counting method represents the more accurate assessment of regional alveolar remodeling.

Finally, our serum analysis has the following limitations; 1) it is a cross-sectional analysis, 2) we have insufficient clinical data to address

the relationship between MIF and other clinically relevant variables such as emphysematous remodeling quantified by high-resolution chest tomography, and 3) it does not define MIF levels directly in the lungs of patients. Prior clinical studies suggest that the lung is a major source of circulating MIF (Sakuragi, Lin et al. 2007); making it a reasonable clinical surrogate. Despite these limitations, this represents the first clinical link between MIF and COPD and provides the framework for understanding the role of this potential modifier in disease severity.

Despite acknowledged environmental risks, it is well recognized that individual susceptibility and severity of COPD/emphysema is heterogeneous. While rare heritable risk factors have been identified as typified by alpha-one anti-trypsin deficiency, the contribution of other molecular antagonists of tissue destruction in emphysema is nascent. Our results provide strong support for a novel role for MIF as a determinant of disease severity in mouse and man, impacting on pulmonary EndoC apoptosis and alveolar remodeling. It establishes a role for MIF in controlling DNA damage and p53-derived apoptotic responses to smoke, by regulating DSB and caspase-3 activation in microvascular EndoC. The identification of this, and other such factors, may provide additional therapeutic targets directed at ameliorating tissue obliteration in COPD/emphysema.

## 3.5 Acknowledgments

We would like to thank Dr. Robert Wise for his support and generosity.

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# Chapter 4

## Macrophage Migration Inhibitory Factor (MIF) is a Novel Inhibitor of ASK1-p38-XOR-Dependent Cigarette Smoke-Induced Apoptosis

**This work is currently under peer review for publication in AJRCMB**

# Chapter 4

## 4.1 Abstract

Cigarette smoke (CS) exposure is the leading cause of emphysema. CS mediates pathologic emphysematous remodeling of the lung via apoptosis of lung parenchymal cells resulting in enlargement of the airspaces, loss of the capillary bed, and diminished surface area for gas exchange. Macrophage migration inhibitory factor (MIF), a pleiotropic cytokine, is reduced both in a preclinical model of CS-induced emphysema and in patients with chronic obstructive pulmonary disease, particularly those with the most severe disease and emphysematous phenotype. MIF functions to antagonize CS-induced DNA damage, p53-dependent apoptosis of pulmonary endothelial cell (EndoC) and resultant emphysematous tissue remodeling. Using primary alveolar EndoC and a mouse model of CS-induced lung damage, we investigated the capacity and molecular mechanism(s) by which MIF modifies oxidant injury. Here, we demonstrate that both the activity of xanthine oxidoreductase (XOR), a superoxide-generating enzyme obligatory for CS-induced DNA damage and EndoC apoptosis, and superoxide ($O_2^-$) concentrations are increased following CS exposure in the absence of MIF. Both XOR hyperactivation and apoptosis in the absence of MIF occurred via a p38 mitogen activated protein (MAP) kinase-dependent mechanism. Further, a MAP kinase kinase kinase family member, apoptosis signal-regulating kinase

1 (ASK1) was necessary for CS-induced p38 activation and EndoC apoptosis. MIF was sufficient to directly suppress ASK1 enzymatic activity. Taken together, MIF suppresses CS-mediated cytotoxicity in the lung, in part by antagonizing ASK1-p38-XOR dependent apoptosis.

## 4.2 Introduction

Five million Americans suffer from emphysema (Centers for Disease and Prevention 2012), a morbid disease characterized histologically by the irreversible destruction of gas-exchanging surfaces in the lung. Cigarette smoke (CS) exposure is the main cause of emphysema (Forey, Thornton et al. 2011) and triggers apoptosis within the lung parenchyma (Rennard, Togo et al. 2006, Tsuji, Aoshiba et al. 2006, Yoshida and Tuder 2007) resulting in enlargement of the airspaces, loss of the capillary bed, and diminished surface area for gas exchange. Endothelial cell (EndoC) apoptosis represents an early (Gordon, Gudi et al.), necessary (Kasahara, Tuder et al. 2000), and sufficient (Giordano, Lahdenranta et al. 2008) event in emphysematous tissue destruction. Thus, we set forth to identify novel regulators of EndoC apoptosis and survival in the context of CS exposure postulating that these would represent potential determinants of disease severity and needed therapeutic targets.

Emphysema is characterized by increased oxidative stress. CS amplifies the volume of inhaled oxidants (Pryor and Stone 1993) and also increases reactive oxygen species (ROS) produced by cells of the airway, alveoli, and immune system (Moodie, Marwick et al. 2004, Yang, Chida et al. 2006, Zhang, Venardos et al. 2006). The superoxide ($O_2^-$) and hydrogen peroxide generating enzyme xanthine oxidoreductase (XOR) is an important cellular source of oxidative stress. XOR activity is elevated

in patients with emphysema compared to controls (Pinamonti, Muzzoli et al. 1996, Heunks, Vina et al. 1999) and XOR activity is increased in the lungs of animals exposed to CS (Kim, Serebreni et al. 2013). Importantly, XOR is obligatory for CS-induced DNA damage, specifically double-stranded DNA breaks (DSB), p53 expression, and EndoC apoptosis (Kim, Serebreni et al. 2013).

Macrophage migration inhibitory factor (MIF) is significantly reduced in the serum of patients with severe emphysematous COPD (Fallica, Boyer et al. 2014) and in the lungs of mice exposed to chronic smoke (Fallica, Boyer et al. 2014, Sauler, Leng et al. 2014). MIF is an endogenous regulator of CS-induced EndoC apoptosis (Damico, Simms et al. 2011) with the capacity to antagonize CS-induced p53 expression and p53-dependent apoptosis. Acute exposure to CS is associated with a rise in total lung and lung microvascular EndoC associated MIF (Figure 4-1) (Damico, Simms et al. 2011, Sauler, Leng et al. 2014) suggesting a potential cytoprotective role that is lost over time. MIF deficiency (*Mif*$^{-/-}$) exacerbates the toxicity of CS *in vivo*, accelerating emphysematous tissue remodeling, and preferentially sensitizes EndoC to CS-induced DNA damage and apoptosis (Fallica, Boyer et al. 2014). Thus, the cytoprotective effects of MIF act upon EndoC to modify emphysema severity. The mechanism(s) by which MIF antagonizes CS-induced DNA damage are unknown. Since MIF deficiency exacerbates DNA damage, EndoC apoptosis, and potentiates airspace enlargement (Fallica, Boyer et

al. 2014) and XOR activity is obligatory for these events, we hypothesize that MIF impacts on CS-mediated cytotoxicity in lung by regulating ROS production, in part, via XOR.

In the present study, we demonstrate that MIF deficiency potentiates superoxide generation and XOR activation in response to CS. This occurs via a p38 MAPK-dependent mechanism. Further, we demonstrate that the apoptosis signal-regulating kinase 1 (ASK1), is upstream of p38 in response to CS and both kinases are required for EndoC apoptosis. Importantly, MIF suppresses CS-induced p38 activation and directly antagonizes ASK1 kinase activity. Thus, MIF antagonizes ROS formation and subsequent EndoC apoptosis by repressing ASK1-p38 kinase signaling upstream of XOR activation and increased ROS production.

## 4.3 Results

### *4.3.1 MIF is a determinant of CS-induced XOR activity and ROS production*

ROS are produced by multiple cellular sources in the lung, including the pulmonary vascular EndoC, the major target of CS-induced damage in the context of MIF deficiency (Fallica, Boyer et al. 2014). Important vascular sources of ROS are XOR, uncoupled nitric oxide synthase (NOS), and NAD(P)H oxidase (Cai, Griendling et al. 2003, Birukov 2009, Damico, Zulueta et al. 2012). More specifically, XOR is a source of superoxide and hydrogen peroxide within pulmonary EndoC. Importantly, XOR is elevated in human CS-induced lung disease (Pinamonti, Muzzoli et al. 1996, Pinamonti, Leis et al. 1998) and here we demonstrate that its increase is evident in EndoC and other lung cells from a patient with COPD (Figure 4-2). We have previously established that C57BL/6 mice exposed to CS have increased pulmonary XOR activity compared to filtered air-exposed controls (Kim, Serebreni et al. 2013). Further, in *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice exposed to chronic CS, increased XOR was detected in alveolar-capillary cells, comprising EndoC and non-EndoC (Figure 4-3). In order to test the hypothesis that MIF modifies CS-induced XOR activation, lung protein homogenates were collected from *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice exposed to 3 days of CS or filtered air and XOR

activity was measured as detailed in Materials and Methods. XOR activity was increased in response to CS in both genotypes relative to air controls; however activity was significantly greater in *Mif*$^{-/-}$ mice in comparison with their *Mif*$^{+/+}$ counterparts (Figure 4-4A). XOR activity did not differ at baseline between genotypes. In addition, XOR protein levels, determined by Western blotting, did not differ at baseline or in response to acute CS (Figure 4-5A & B), suggesting that the increases in XOR activity observed in response to CS were unlikely due to the effects of MIF on XOR protein expression. Rather, these data provide evidence that MIF antagonizes CS-induced increases in XOR activity by modifying the activation status of XOR.

In further support of this, intracellular $O_2^-$ concentrations were also assessed qualitatively using the lipophilic cell-permeable dye, dihydroethidium (DHE). In the presence of MIF (*Mif*$^{+/+}$ wild-type mouse lungs), 72 hours of CS exposure resulted in a mild increase in DHE fluorescence (Figure 4-4B). In contrast, CS exposure produced a robust increase in DHE staining in *Mif*$^{-/-}$ animals. Air-exposed *Mif*$^{-/-}$ mice had higher basal superoxide concentrations than wild-type controls despite similar basal XOR activity suggesting an XOR-independent mechanism to account for this difference. *Ex vivo* treatment with the thiol donor NAC antagonized DHE fluorescence demonstrating specificity (data not shown). Thus, the absence of MIF alters the redox status within the lung

and potentiates CS-induced ROS production concurrently with increased XOR activity.

### *4.3.2 The Role of p38 in CS-induced XOR activity*

An established mechanism for post-translation activation of XOR is via p38-dependent phosphorylation (Kayyali, Donaldson et al. 2001, Le, Damico et al. 2008). p38 MAPK activity has been linked to enhanced apoptosis (Cai, Chang et al. 2006), CS-induced endothelial permeability changes (Low, Liang et al. 2007), human COPD (Barnes 2013) and emphysema susceptibility in mice (Marumo, Hoshino et al. 2014). In addition, p38 activation is increased with chronic CS in our pre-clinical model. Using IHC directed at phosho-p38, we detect increased activation of p38 in the microvasculature of the lungs in both genotypes, with more intensive staining observed in the *Mif* $^{-/-}$ mice exposed to chronic CS (Figure 4-6). The contribution of the p38 signaling pathway in XOR activation in response to CS *in vivo* has not yet been established. We postulated that the increased XOR activation in *Mif* $^{-/-}$ mice exposed to CS may be mediated by p38. To test the contribution of the p38 signaling pathway in XOR activation, we exposed *Mif* $^{+/+}$ and *Mif* $^{-/-}$ mice to CS for 3 days, after treatment with the p38 inhibitor (SB203580) or its vehicle given intraperitoneally before CS exposure. Dose and route were chosen based on the literature (Le, Damico et al. 2008). Subsequently lung

homogenates were collected and XOR was measured as detailed in Materials and Methods. In response to CS, *Mif*$^{-/-}$ mice had significantly higher XOR activity compared to *Mif*$^{+/+}$ mice, which was completely abrogated with SB203580 treatment (Figure 4-7), returning to levels comparable to *Mif*$^{+/+}$ mice. Thus, the enhanced XOR activity observed in the absence of MIF is dependent on p38 MAPK activity.

### *4.3.3 MIF antagonizes CS-induced and p38 dependent EndoC apoptosis*

Activation of p38 and other MAP kinases, such as the c-Jun N-terminal protein kinase (JNK), are mediated through stress-inducing signals, including oxidative stress (Huot, Houle et al. 1997), which is a key component of CS-induced cellular injury. In addition, MIF deficiency potentiates p38 signaling responses in the context CS *in vivo,* as demonstrated by enhanced XOR activation and ROS production, suggesting a protective role for MIF as a modifier of ROS. To test the hypothesis that p38 is activated in response to CS and to evaluate the potential role of MIF in blocking CS-induced activation of p38, we used an *in vitro* approach. EndoC were pre-incubated with recombinant MIF protein or its carrier, treated with CS extract (CSE), and probed for p38 phosphorylation by Western blot. In response to CSE, p38 activation, determined by its phosphorylation status, in primary human lung

microvascular EndoC (Figure 4-8A) was rapid and transient. Similar findings were observed in primary rat pulmonary EndoC (Figure 4-9A). Total p38 protein did not differ across time points or with rMIF treatment. Pretreatment with exogenous MIF (rMIF) compared to carrier significantly abrogated the phosphorylation of p38 in EndoC, independent of species (Figure 4-8B and Figure 4-9B).

To understand the functional outcome of p38 activation *in vitro*, EndoC were subjected to CSE exposure in the presence or absence of the p38 inhibitor SB203580 and analyzed for alterations in nuclear morphology consistent with apoptosis via Hoescht staining as previously described (Damico, Simms et al. 2011). The observed increase in p38 activation in response to CSE was linked with increased apoptotic cell death (Figure 4-10) that was prevented by pharmacologic blockade of p38.

Using a complementary approach to evaluate the antagonism of MIF on p38, EndoC were transfected with siRNA directed against MIF or non-targeting siRNA (Control, Ctrl siRNA) as previously described (Damico, Chesley et al. 2008), and exposed to CSE in the presence of SB203580 or its vehicle. MIF deficient EndoC were sensitized to CS-induced apoptosis *in vitro* (Figure 4-8C). Pre-incubation with SB203580 was sufficient to significantly diminish CSE-induced apoptosis to the level observed with Ctrl siRNA. SP600125, a JNK inhibitor did not have an effect on CSE-induced apoptosis (Figure 4-11), showing specificity

and a dependence on p38 kinase activity. Together these data provide strong evidence that 1) exogenous MIF blocks p38 activation and 2) increased CSE-induced apoptosis in the absence of MIF is p38-dependent.

### *4.3.4 p38 inhibition antagonizes DNA damage in MIF deficient mice*

Our *in vitro* analysis revealed that cellular apoptosis in response to CSE is regulated by p38. Further, we have previously shown that DNA damage and EndoC apoptosis are exacerbated in the absence of MIF *in vivo* (Fallica, Boyer et al. 2014). To address the role of p38 *in vivo* and in the context of MIF deficiency and CS exposure, $Mif^{+/+}$ and $Mif^{-/-}$ mice were exposed to CS for 3 days with or without SB203580. Lung homogenates were collected and caspase 3/7 activity was measured as a marker of apoptosis. Baseline activity did not differ by genotype. In response to CS, both $Mif^{+/+}$ and $Mif^{-/-}$ mice had significantly increased caspase 3/7 activity; however, this activity was significantly higher in CS-exposed $Mif^{-/-}$ mice compared to their $Mif^{+/+}$ counterparts (Figure 4-12A). The increase in caspase 3/7 activity in $Mif^{-/-}$ mice was significantly reduced in animals treated with SB203580, suggesting that these observed cellular alterations in the absence of MIF are potentiated by a p38-dependent mechanism.

To further test the contribution of this pathway specifically on EndoC, lung sections were probed for caspase-3 (green) in tandem with the microvascular endothelial marker, tomato lectin (red). Apoptotic EndoC were increased in CS-exposed *Mif*$^{-/-}$ mice compared to their *Mif*$^{+/+}$ counterparts as determined by increased overlay of the above markers (orange) (Figure 4-12B).

### *4.3.5 CSE-induced p38 activation is ASK1 dependent*

Having established that MIF antagonizes p38 phosphorylation we questioned whether the ability of MIF to block p38 activation occurred by a direct or indirect interaction. There is no evidence that MIF possesses kinase/phosphatase activity. However, it is well established that p38 activation is mediated by the MAP kinase kinases (MKK), MKK3 and MKK6 (Zarubin and Han 2005), which are in turn regulated by MAP kinase kinase kinases (MAP3K). The MAP3K, ASK1, is activated and auto-phosphorylated in a redox-sensitive fashion (Adler, Yin et al. 1999). In quiescent cells, the redox sensitive thioredoxin (Trx) protein, which possesses a thiol-protein oxidoreductase (TPOR) motif similar to MIF is known to bind and inhibit ASK1 (Saitoh, Nishitoh et al. 1998, Nadeau, Charette et al. 2007). In the face of increased ROS, TPOR undergo oxidation, thereby releasing ASK1. Based on functional homology and oxidoreductase activity, we postulated that MIF may be a novel inhibitor

of ASK1 activity. To test this hypothesis, we transfected EndoC with ASK1 or Ctrl siRNA and treated cells with CSE or its vehicle, PBS. Sufficient knockdown of ASK1 was achieved by 24 hours (Figure 4-13A) as demonstrated by Western blotting. In response to CSE, p38 phosphorylation was not observed in ASK1 deficient EndoC (Figure 4-13B), whereas cells derived from the control transfections showed rapid p38 phosphorylation.

To test the functional consequence of diminished ASK1, apoptotic responses to CS were quantified by nuclear morphology in EndoC transfected with Ctrl or ASK1 siRNA. Knockdown of ASK1 led to decreased CS-induced apoptosis compared to Ctrl siRNA transfectants (Figure 4-13C). The reduction in p38 activation in the absence of ASK1 (Figure 4-13) mimics the effects of pre-treatment with exogenous rMIF (Figure 4-8) suggesting that MIF may be acting to antagonize CS-induced p38 activation by blocking its upstream activator, ASK1.

### *4.3.6 MIF has the capacity to directly block ASK1 kinase activity*

It is well recognized that MIF can bind to or affect intracellular proteins 1) as an endogenous protein, 2) via pinocytosis into the cell, or 3) through extracellular receptor ligand interactions (Kleemann, Hausser et al. 2000, Leng, Metz et al. 2003, Nguyen, Beck et al. 2003, Son, Kato et al. 2009, Schwartz, Kruttgen et al. 2012). In order to test the

hypothesis that MIF alters p38 through a direct interaction with ASK1, we utilized a cell free assay designed to measure the kinase activity of ASK1 in the presence or absence of rMIF. In this assay ASK1, ATP, and myelin basic protein (MBP, a non-specific MAPK substrate), concentrations were held constant while rMIF was added in a dilutions series. The addition of rMIF was sufficient to block ASK1 kinase activity, as determined by a reduction in ADP formation, in a dose dependent manner (Figure 4-14). Further, the half maximal inhibitory concentration ($IC_{50}$) of MIF was approximately 1.6 nM, well within the known biologically active range for MIF (Bruchfeld, Carrero et al. 2009). These data indicate a mechanism by MIF, internalized or endogenous, directly inhibits ASK1 auto-activation, thereby diminishing downstream signaling including p38 phosphorylation and XOR activity.

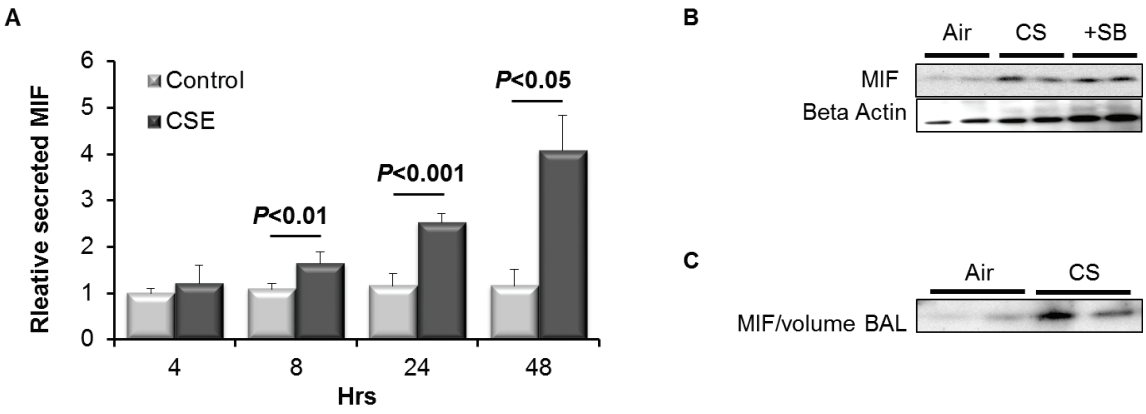
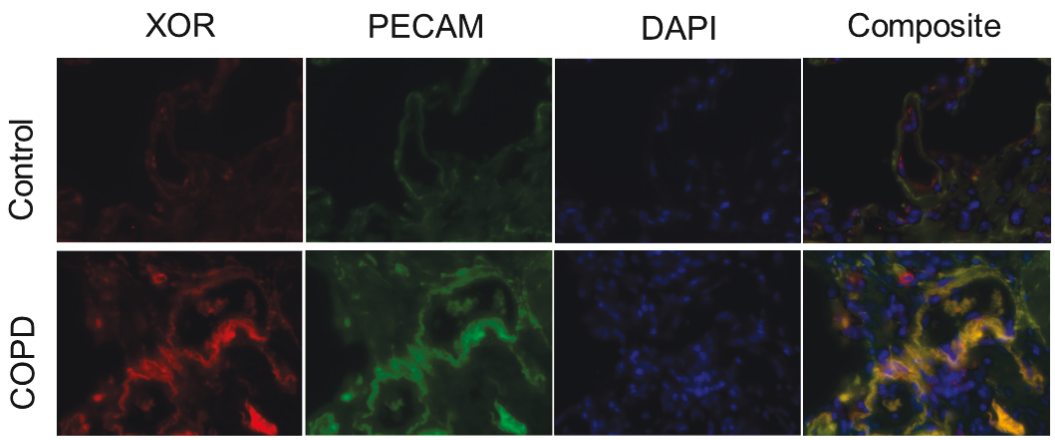



**Figure 4-1. Acute CS induces MIF in vitro and in vivo.** Primary human lung microvascular EndoC were exposed to PBS or CSE for 4, 8, 24, and 48 hrs and the supernatants were collected and analyzed via ELISA for MIF. Alternatively, *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were exposed to acute CS, after which lung homogenates were collected and subsequently probed for Western blotting. In vitro MIF secretion from EndoC was significantly increased in response to CSE by as early as 8hrs (A). In vivo, total MIF protein significantly increased in the tissue (B) and BAL (C). n=2-5 per condition




| Patient | Age | Gender | $FEV_1$ | $FEV_1$/FVC (% predicted) |
|---|---|---|---|---|
| **Control** | 56 | Female | 2.85 | 86 |
| **COPD** | 59 | Female | 1.69 | 61 |

**Figure 4-2. Increased XOR is detected in human COPD lung tissue.** Human Lung tissue sections were obtained from a smoker and non-smoker and analyzed via IHC. XOR positive EndoC were increased in the COPD patient compared to the control. Patient demographics highlight airflow limitations in the COPD patient, indicative of mild disease.

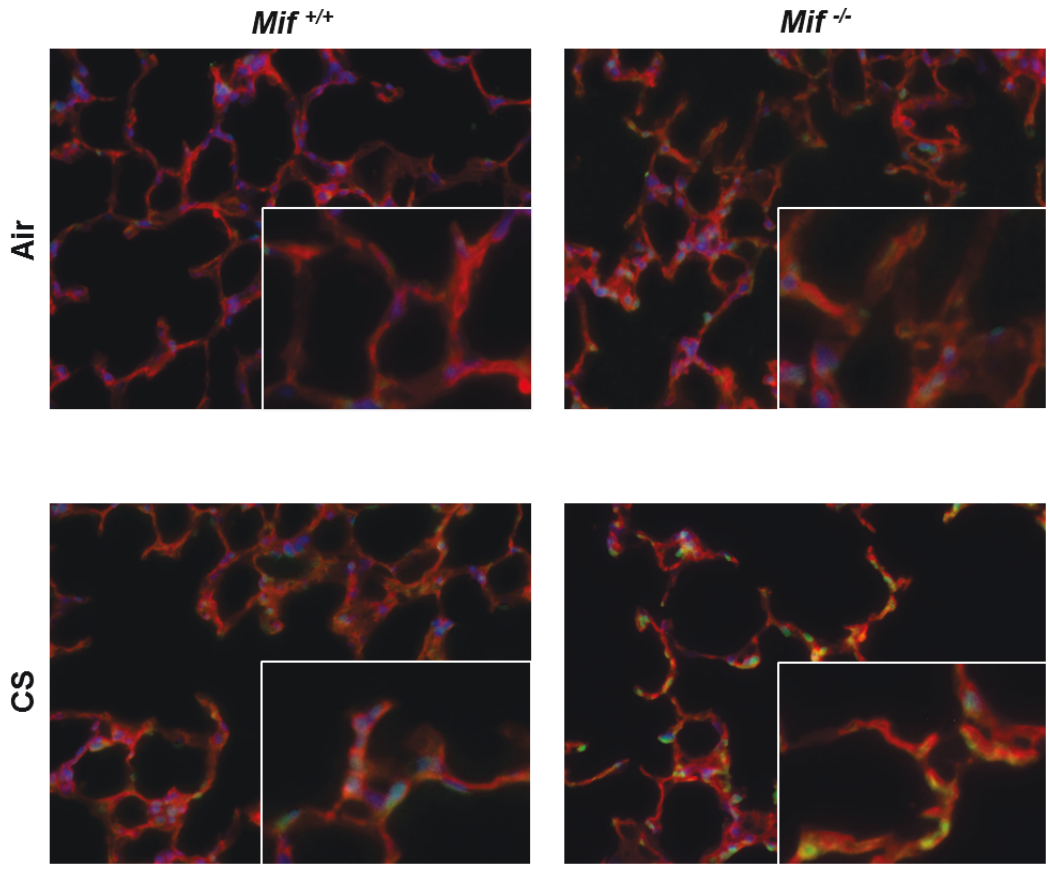



**Figure 4-3. Chronic CS exposure results in increased XOR.** Thin lung tissue sections from *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice chronically exposed to air or CS were analyzed by IHC. Lung tissue sections were probed for XOR (green) in the presence of the EndoC marker, tomato lectin (red). Both genotypes displayed noticeable EndoC (orange) and non-EndoC (green) XOR expression, with CS-exposed *Mif*$^{-/-}$ mice exhibiting a visible increased intensity in co-staining signifying increased XOR in EndoC. Nuclei are counterstained blue. n=3 per condition.

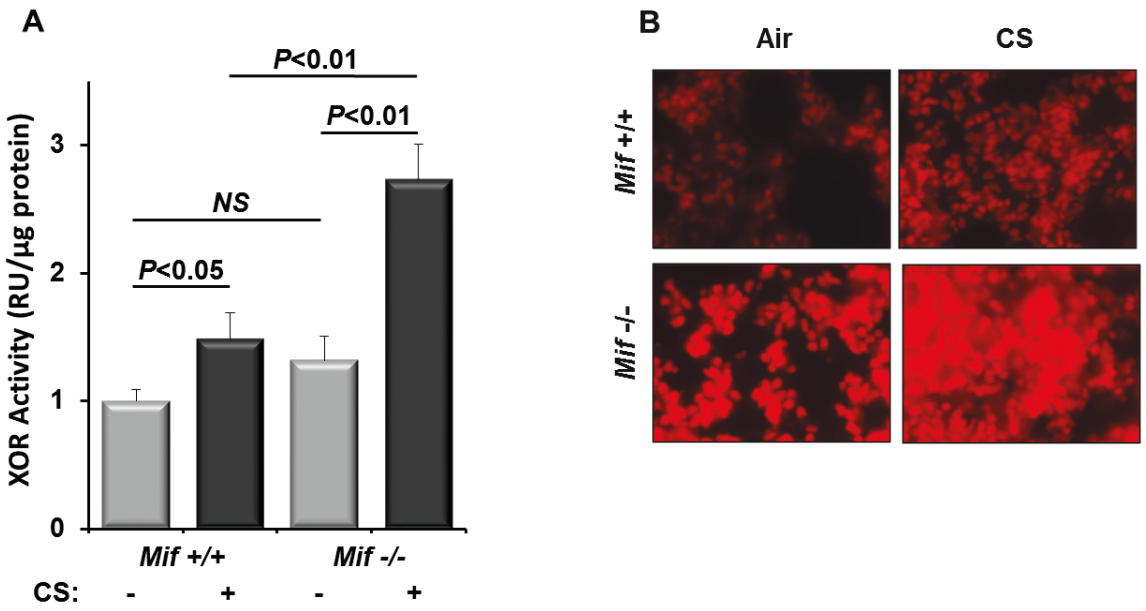



**Figure 4-4. MIF is a determinant of CS-induced XOR activity and ROS production.** *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were exposed to 3 days of CS, after which lung homogenates or fresh frozen lung cross sections were respectively collected in buffer and immediately probed for XOR activity using a fluorometric readout or histologically probed for DHE as a measure of intracellular ROS. There was significant interaction between genotype and exposure, 2 way ANOVA *P*=0.02. Both genotypes displayed significant increases in XOR activity in response to CS; however *Mif*$^{-/-}$ mice responded with a significantly higher XOR activity (A). NS indicates no significant difference, P=0.1 vs *Mif*$^{+/+}$ mice. While *Mif*$^{+/+}$ mice showed a modest increase in CS-induced DHE staining, *Mif*$^{-/-}$ mice had higher baseline and more robust CS-induced DHE staining when compared to wild-type mice (B). n=5 for each condition.

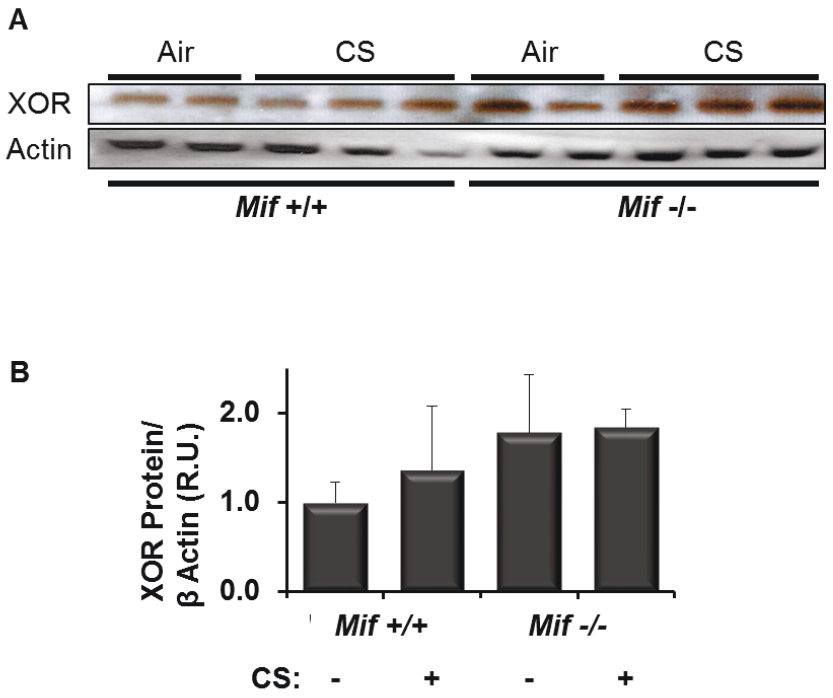



**Figure 4-5. MIF and CS do not alter total XOR protein.** *Mif*$^{+/+}$ and *Mif* $^{-/-}$ mice were exposed to 3 days of CS, after which lung homogenates were collected and subsequently probed by Western blotting (A). Normalized total XOR protein did not differ between genotypes or with CS-exposure (B). n=5 per condition.

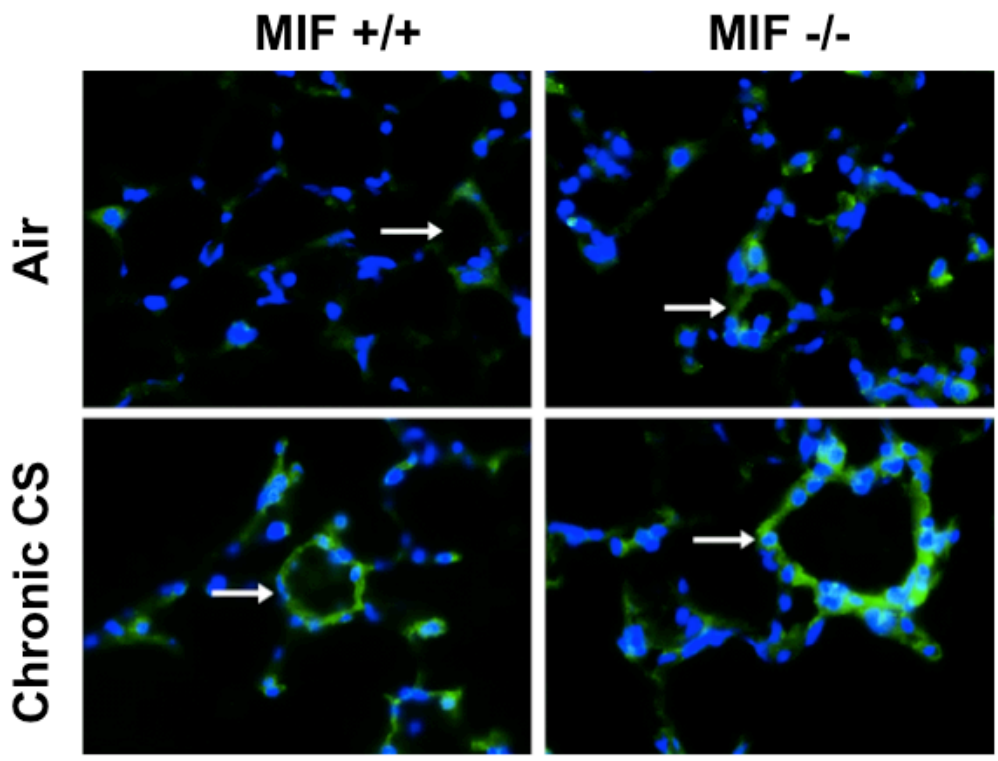



**Figure 4-6. Chronic CS exposure results in Increased p38 activation.** Thin lung tissue sections from *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice chronically exposed to air or CS were analyzed by IHC. Lung tissue sections were probed for phosphorylated p38 (green) and examined for perivascular (white arrows) differences. p38 activation is present at baseline and increased in both genotypes, with increased intensity observed in *Mif*$^{-/-}$ mice. Nuclei are counterstained blue. n=3 per condition.

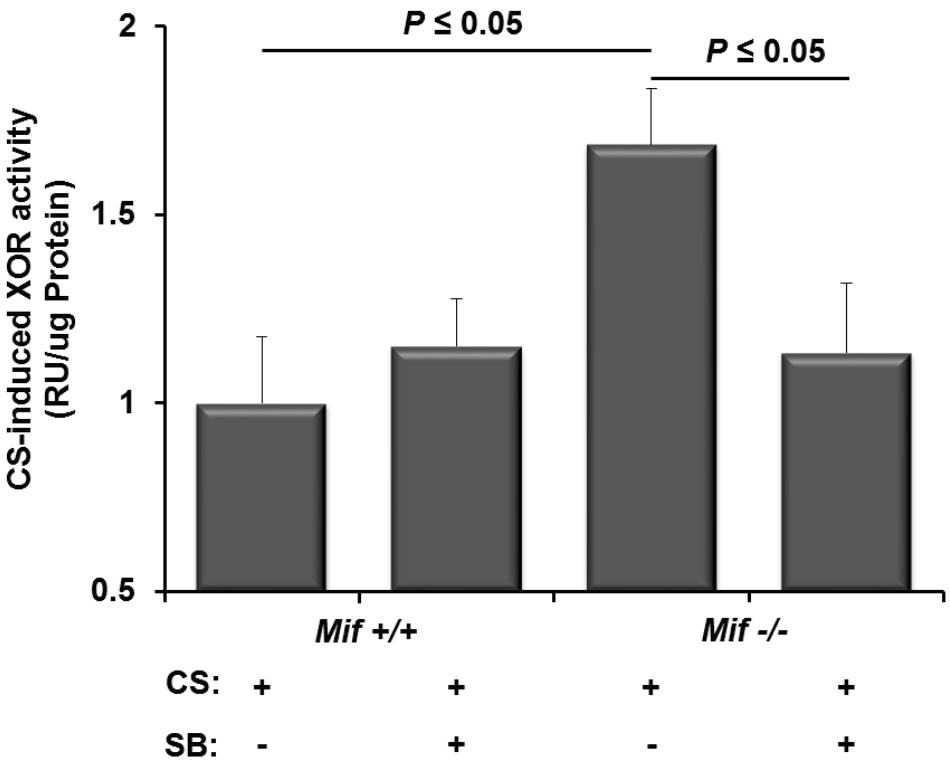



**Figure 4-7. The role of p38 in CS-induced XOR activity.** *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were treated with SB203580 or its vehicle and exposed to 3 days of CS, after which lung homogenates were collected and immediately probed for XOR activity. Data was normalized to the response of CS treated *Mif*$^{+/+}$ mice. *Mif*$^{-/-}$ mice had a significant increase in XOR activity and SB203580 treatment reduced this XOR activity to levels observed in *Mif*$^{+/+}$ mice. n=5 for each condition.

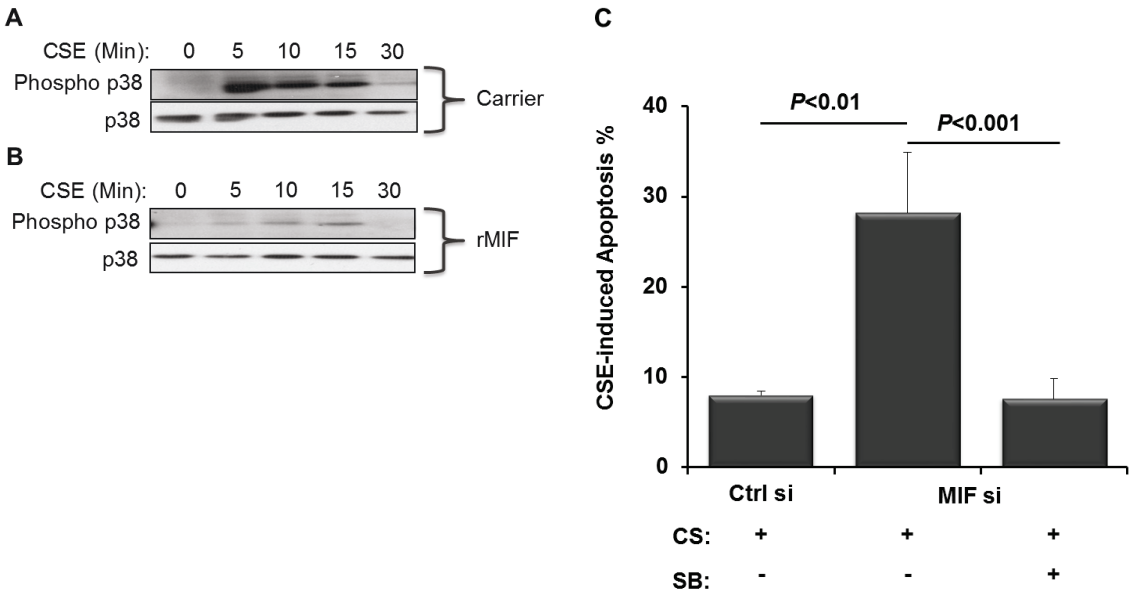



**Figure 4-8. MIF antagonized CSE-induced and p38-dependent apoptosis**. Primary human lung microvascular EndoC were exposed to CSE for 5, 10, 15, and 30 minutes in the presence of rMIF or its carrier and subsequently harvested for Western blot analysis or analyzed with Hoechst stain for apoptosis in the presence or absence of SB203580 (SB). In response to CSE p38 activation (Phospho p38) was rapid and transient (A). The addition of rMIF was sufficient to block the phosphorylation of p38 (B). Total p38 was unchanged. Pharmacological blockade of p38 with SB resulted in significantly reduced apoptosis (C). n=3-5 per condition.

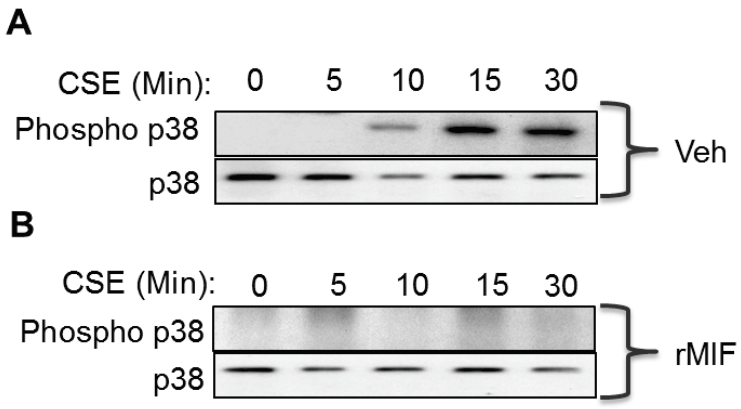



**Figure 4-9. MIF antagonized CS-induced p38 activation in rat EndoC**. Primary rat lung microvascular EndoC were exposed to CSE for 5, 10, 15, and 30 minutes in the presence of rMIF or its carrier and subsequently harvested for Western blot analysis. In response to CSE, p38 activation (phospho p38) was rapid and transient (A). The addition of rMIF was sufficient to block the phosphorylation of p38 (B). n=3-5 per condition.

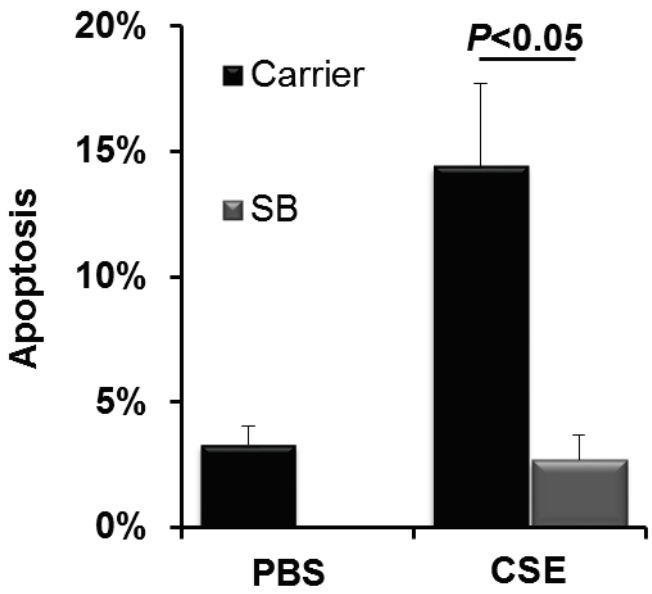



**Figure 4-10. CS-induced apoptosis is p38-dependent**. Primary human lung microvascular EndoC were subjected to CSE exposure in the presence or absence of the p38 inhibitor SB203580 (SB) and analyzed for alterations in nuclear morphology consistent with apoptosis via Hoescht staining. The observed Increase in CSE-induced apoptotic cell death was significantly reduced to baseline levels with SB treatment. n=3-5 per condition.

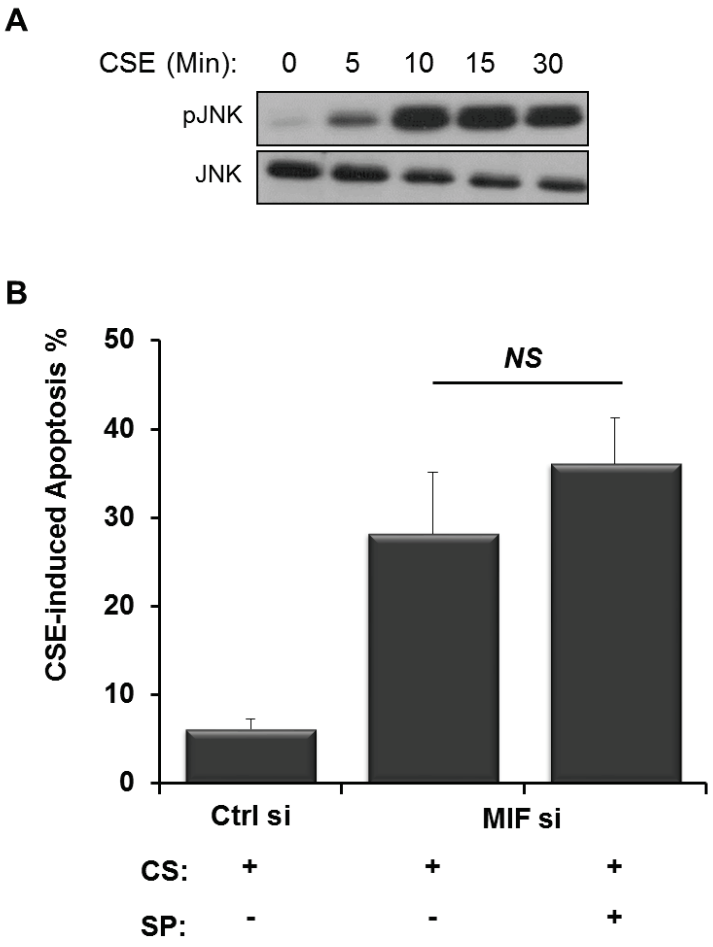



**Figure 4-11. CS-induced apoptosis in the absence of MIF is JNK-independent.** Primary human lung microvascular EndoC were exposed to CSE for 5, 10, 15, and 30 minutes and harvested for Western blot analysis. Alternatively, cells were transfected with MIF or Ctrl siRNA, treated with CSE or PBS in the presence or absence of the JNK inhibitor SP600125 (SP) and analyzed with Hoechst staining. In response to CSE, JNK activation (pJNK) was rapid and transient (A). Total JNK was unchanged. The pharmacological blockade of pJNK with SP had no effect of apoptosis (B). n=3-5 per condition.

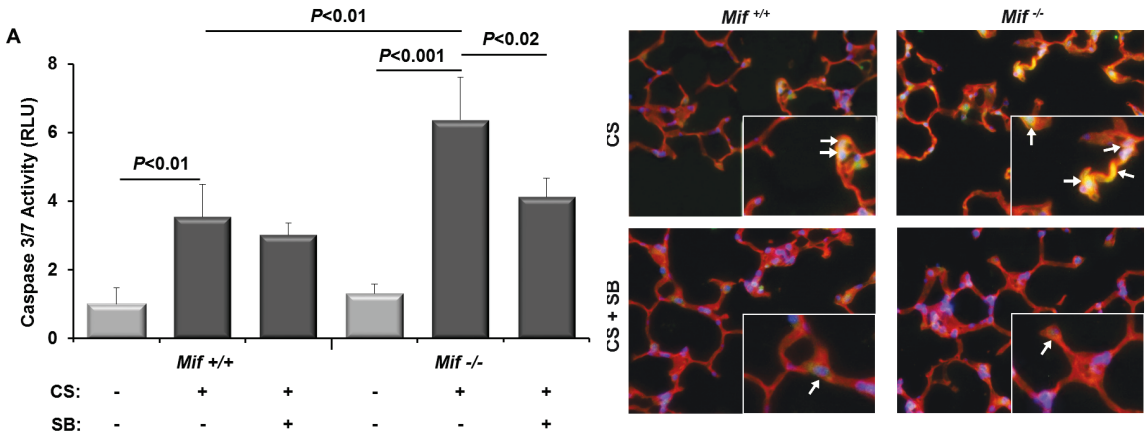



**Figure 4-12. p38 inhibition antagonizes apoptosis in *Mif*$^{-/-}$ mice.** *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were exposed to CS for 3 days after treatment with SB203580 (SB) or its vehicle. Lung homogenates and tissue sections were collected and subjected to a luminescent caspase 3/7 activity assay, or IHC, respectively. In response to CS, both *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice had significantly increased caspase 3/7 activity (A). Activity was significantly higher in CS-exposed *Mif*$^{-/-}$ mice compared to their wild-type counterparts. Caspase 3/7 activity was significantly reduced with SB in *Mif*$^{-/-}$ mice. n=5 per condition. Lung tissue sections were also probed for cleaved caspase 3 (green) in the presence of the EndoC marker, tomato lectin (red) (B). Both genotypes displayed noticeable EndoC (orange) and non-EndoC (green) apoptosis with *Mif*$^{-/-}$ mice exhibiting a visible increase in Endo death (white arrows), which was dramatically abrogated by SB treatment. Nuclei are counterstained blue. n=3 per condition.

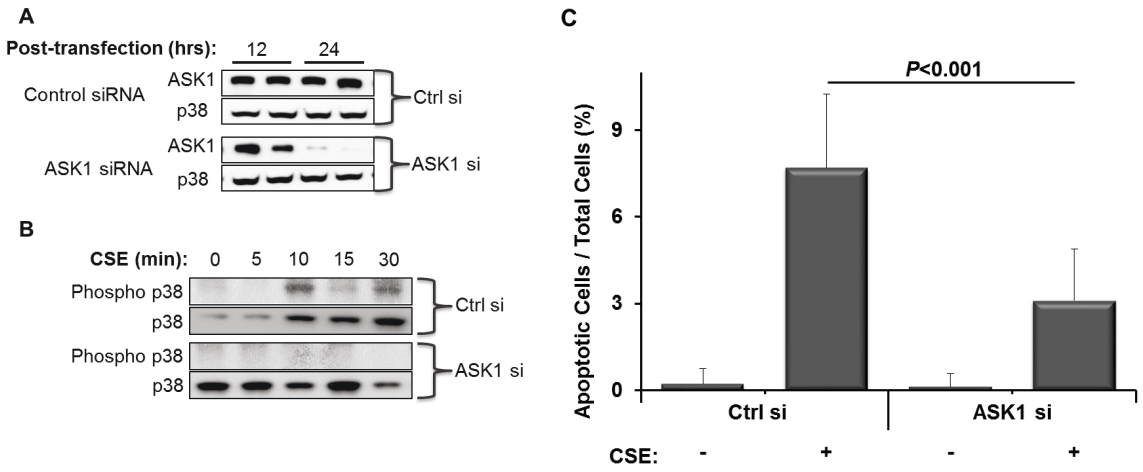



**Figure 4-13. CSE-induced p38 activation is ASK1 dependent.** Primary rat microvascular EndoC were transfected with ASK1 or Ctrl siRNA, treated with CSE or PBS, and probed via Western blotting or analyzed with Hoechst staining. Sufficient knockdown of ASK1 was achieved by 24 hours (A). In response to CSE, EndoC derived from the control transfections showed rapid and transient p38 phosphorylation which was not observed in ASK1 deficient cells (B). There was a significant reduction in apoptosis ASK1 deficient cells compared to Ctrl transfectants (C). n=3-5 per condition.

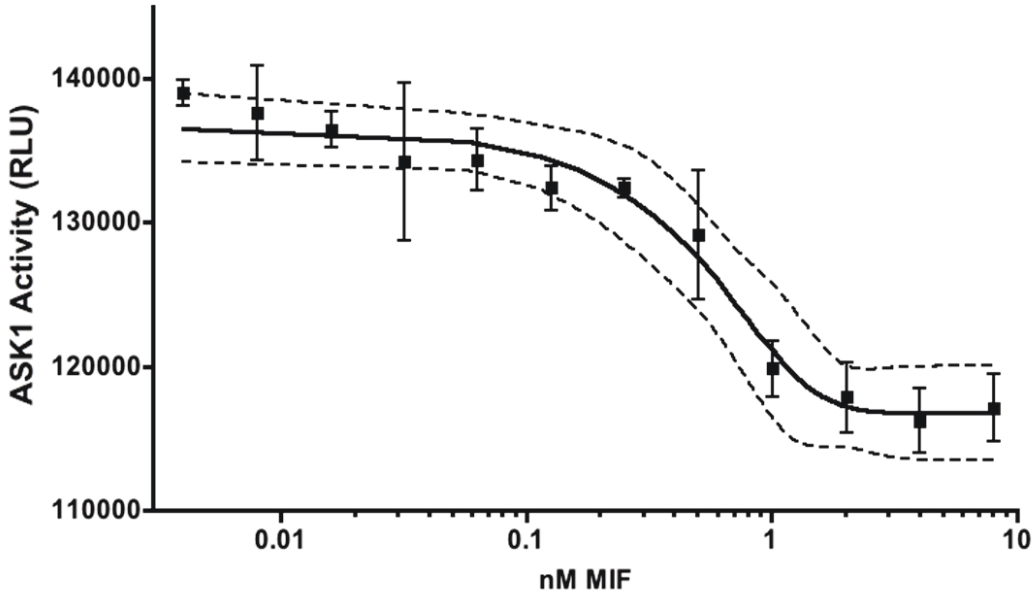



**Figure 4-14. MIF antagonizes ASK1 kinase activity.** A cell free assay was used to measure the kinase activity of ASK1 in the presence or absence of rMIF. ASK1, ATP, and myelin basic protein (MBP, a non-specific MAPK substrate) concentrations were held constant while rMIF was added in a dilution series. rMIF was sufficient to block ASK1 kinase activity (ADP formation) in a dose dependent manner. MIF $IC_{50}$ =1.6 nM. ASK1 activity in the presence of ATP and MBP was used as a buffer control.

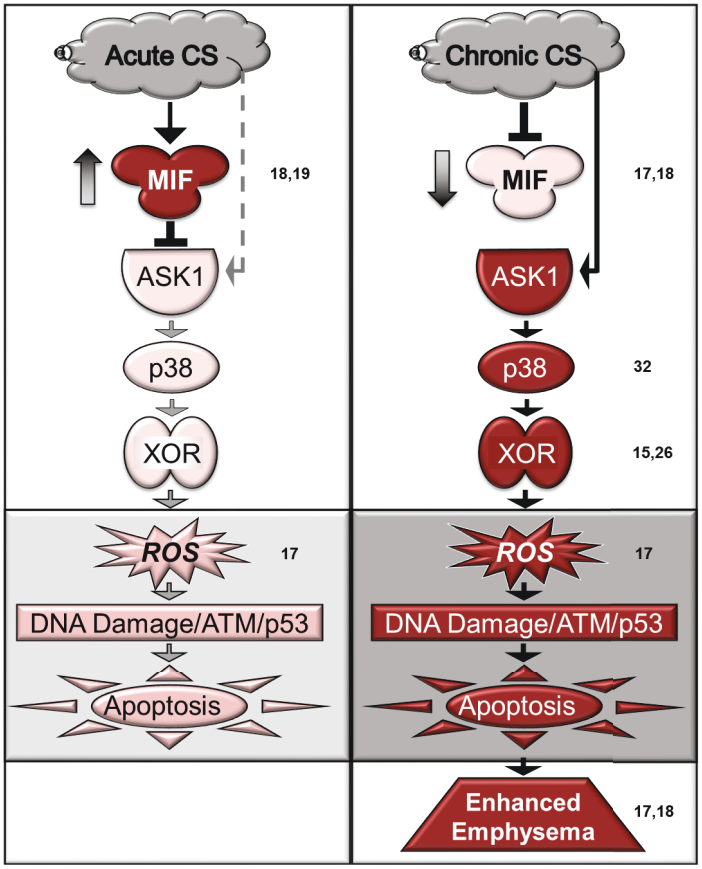



**Figure 4-15. The role of MIF in CS-induced activation of the ASK1-p38-XOR pathway.** Acute CS exposure resulted in increased MIF expression ((Damico, Simms et al. 2011, Sauler, Leng et al. 2014), Figure 4-1). This functions, in part, to antagonize CS-induced ASK1-p38-XOR activation and resulting ROS, p53 expression, and EndoC apoptosis. Chronic CS is associated with loss MIF (17, 18). XOR expression and activation of p38 are also increased in human and murine CS-induced lung disease, as we show here and others previously (15, 26, 32). When MIF is lost, we postulate that the ASK1-p38-XOR pathway is hyper-activated, resulting in increased EndoC apoptosis and

## 4.4 Discussion

Cigarette smoke (CS)-induced lung diseases, including emphysema, contribute to an estimated 2.5 million deaths worldwide and a healthcare cost approaching 38.8 billion dollars annually in the United States alone (Foster, Miller et al. 2006). Emphysema is a debilitating disease characterized by the irreversible destruction of the lung architecture with enlargement of the airspaces driven by enhanced EndoC apoptosis (Demedts, Demoor et al. 2006). The molecular regulators of EndoC fate in response to CS exposure are potential therapeutic targets and modifiers of disease phenotype. Despite this, no current therapies directly targets tissue destruction in emphysema. Further, our understanding of host factors predisposing to the emphysematous phenotype in humans is nascent (Ahmed, Jiang et al. 2014). Here we show that MIF acted to blunt the rise in intracellular ROS caused by CS exposure and antagonized CS-induced XOR activation and EndoC apoptosis in an ASK1-p38-dependent manner.

MIF is a 12.5 kDa, evolutionarily conserved protein constitutively expressed by multiple cell types in the lung including pulmonary microvascular EndoC (Damico, Chesley et al. 2008). MIF is found in the serum under basal conditions and is elevated in acute and chronic inflammatory disease states (Morand 2005). MIF expression is altered in human COPD/emphysema. Specifically, serum MIF levels are significantly lower in patients with COPD compared to healthy, non-

smoking controls and vary according to the severity of COPD as estimated by the GOLD criteria. Thus, patients with severe COPD (GOLD Stage IV) have significantly lower circulating MIF than subjects with less severe COPD (GOLD II or III), implicating MIF in human disease severity and/or susceptibility(Fallica, Boyer et al. 2014). Similar findings are observed in a separate COPD cohort (Sauler, Leng et al. 2014), independently validating this association. Thus, human COPD represents a relative MIF deficient state and there appears to be an inverse relationship between disease severity and MIF levels. Preclinical data demonstrates that MIF deficient mice have accelerated CS-induced emphysema (Fallica, Boyer et al. 2014, Sauler, Leng et al. 2014). In the absence of MIF, alveolar EndoC are predisposed to increased CS-induced DNA damage and apoptosis (Fallica, Boyer et al. 2014). We have identified MIF as an intrinsic, negative regulator of lipopolysaccharide (LPS)- and CS-induced apoptosis in human pulmonary EndoC (Damico, Chesley et al. 2008, Damico, Simms et al. 2011) MIF functions to antagonize EndoC apoptosis by blocking death signals from both the death-receptor and mitochondrial apoptotic pathways (Damico, Chesley et al. 2008, Damico, Simms et al. 2011). Specifically, MIF is a novel positive regulator of the short isoform of the FLICE-like inhibitor protein (i.e. FLIPshort), which functions to antagonize signals via the death-receptor pathway in response to LPS (Damico, Chesley et al. 2008). Additionally, MIF functions to suppress CS-induced p53 expression and

p53-dependent activation of the mitochondrial death pathway in CSE-challenged EndoC (Damico, Simms et al. 2011). Thus, MIF has the unique capability of antagonizing death signals from multiple apoptotic pathways in EndoC, acting to stabilize intrinsic inhibitors of the apoptosis cascade and suppressing promoters of apoptosis.

CS contains abundant oxidants and reactive oxygen species (ROS), including short-lived superoxide radicals, nitric oxide, and longer lived hydroquinones which contribute to the persistent oxidative stress observed following smoke exposure (Church and Pryor 1985). CS exposure modifies intracellular redox by altering both the expression and/or activity of pro- and anti-oxidant enzymes (Rangasamy, Cho et al. 2004). Thus, we hypothesized that MIF could exert its cytoprotective effects by modifying redox responses in the lung in the context of CS exposure. An important endogenous source of CS-induced oxidative stress is XOR. XOR is elevated in human and murine CS-induced lung disease (Pinamonti, Muzzoli et al. 1996, Pinamonti, Leis et al. 1998, Kayyali, Budhiraja et al. 2003, Kim, Serebreni et al. 2013). Moreover, XOR is necessary for CS-induced EndoC DNA damage, p53 induction, and apoptosis, implicating it as a critical mediator of EndoC cytotoxicity in response to CS. Here we demonstrated that XOR activity increased significantly in *Mif*$^{-/-}$ mice exposed to CS compared to their wild-type (*Mi*$^{+/+}$) counterparts without significant differences in XOR protein, implicating a post-translational mechanism for acute XOR activation.

With longer exposures this acute activation may provide a feed forward mechanism by which XOR expression increases with disease severity; increased XOR activity induces p53, and a p53-binding site is located in the promoter of XOR (predicted by SABioscience Text Mining Application, UCSC Genome Browser). This suggests that in the absence of MIF CS-induced acute hyperactivation of XOR enhances caspase-3 dependent apoptosis, as we show here, and that significant increases in CS-induced p53 expression detected after 15d CS in MIF deficient animals, as we previously showed, may lead to long-term increases in XOR expression (Figures 4-2 & 4-3) and therefore activity (Fallica, Boyer et al. 2014). Given the persistent nature of ROS and RNS activation is emphysema, XOR expression is tied with its increased activity, as both are increased in human COPD (Pinamonti, Muzzoli et al. 1996, Pinamonti, Leis et al. 1998, Ichinose, Sugiura et al. 2003)

XOR hyperactivation occurred via a p38-dependent mechanism. The link between XOR activity and p38 signaling is established in other models including hypoxia (Kayyali, Donaldson et al. 2001) and ventilator-induced lung injury (Le, Damico et al. 2008) where p38 signaling is linked to EndoC apoptosis and dysfunction (Cai, Chang et al. 2006, Le, Damico et al. 2008). Despite this established link between p38 signaling and XOR hyperactivation, potential upstream regulators of p38 in these models have not been established. Here we showed that the enhanced CS-induced apoptosis observed in the lungs of *Mif*$^{-/-}$ mice occurred via a

p38-dependent mechanism as demonstrated by its inhibition with the p38 inhibitor, SB inhibitor SB203580. *In vitro* studies demonstrated that CS exposure is sufficient to activate p38, as assessed by its phosphorylation status, in lung microvascular EndoC. Antagonism of p38 prevented CS-induced apoptosis of EndoC and was sufficient to prevent CS-mediated death of MIF-deficient EndoC *in vitro*. Thus, MIF deficient EndoC undergo apoptosis via a p38-dependent mechanism both *in vivo* and *in vitro*. In addition during the preparation of this manuscript, support for this potential p38 dependent mechanism was provided in epithelial cells (EpiC) (Lin, Yang et al. 2014). Although these EpiC were immortalized cells, this and our data in primary EndoC underlines the importance of this pathway on alveolar homeostasis.

Additionally, we demonstrated that exogenous MIF was sufficient to suppress p38 phosphorylation in response to CS. Since MIF does not possess intrinsic kinase/phosphatase activity we speculated that MIF acts upstream of p38 to modify its activation state. ASK1 is a well-recognized ROS-responsive kinase upstream of p38 MAPK and recently only speculated to be a therapeutic target for COPD due to its regulation of p38 (Millan, Bunnage et al. 2011, Barnes 2013). To assess the role of ASK1 in CS-induced p38 activation and apoptosis, we used a loss-of-function approach. Using siRNA directed against ASK1, we demonstrated that ASK1 was necessary for phosphorylation of p38 and EndoC apoptosis in response to CS *in vitro*. Thus, in EndoC ASK1 lies upstream

of p38 in response to CS and is obligatory for CS-mediated cytotoxicity. ASK1 activity in quiescent cells is regulated by protein-protein interactions which mask its capacity to auto-phosphorylate and activate the kinase. One recognized negative regulator of ASK1 is thioredoxin (Trx), which possesses a TPOR motif similar to MIF (Saitoh, Nishitoh et al. 1998, Nadeau, Charette et al. 2007). In the face of increased ROS, the TPOR residues undergo oxidation causing disassociation from ASK1, disinhibiting the kinase. Based on functional homology between MIF and Trx, we predicted that MIF may represent a novel direct inhibitor of ASK1 and, thus, modify downstream signaling intermediates including p38 MAPK. In support of this, we demonstrated that MIF was sufficient to directly inhibit the kinase activity of ASK1 at physiologically relevant concentrations. Collectively this work supports a model in which MIF protects EndoC in the lung to CS-toxicity by inhibiting signaling via a ASK1-p38-XOR pathway, thereby identifying additional potential therapeutic targets and disease modifiers.

In potential contrast to our findings in EndoC and the intact lung exposed to CS, others examining the relationship between MIF and the p38 axis implicate exogenous MIF as a positive regulator of p38 via CD74-dependent mechanism (Santos, Lacey et al. 2004, Sanchez-Nino, Sanz et al. 2009). CD74, in conjunction with CD44, is capable of transducing the extracellular binding of MIF into activation of intracellular kinase cascades, including JNK, ERK1/2, and AKT (Shi,

Leng et al. 2006, Starlets, Gore et al. 2006, Qi, Hu et al. 2009). We have not been able to detect CD74 expression on human lung EndoC (data not shown) and there is limited published data suggesting CD74 plays a role in EndoC signaling during inflammation(Morane Le Hiress 2014). Multiple factors may contribute to the differential role of MIF in the p38 axis under these experimental conditions including differences in the cellular targets, role of ASK1 in the model, differences in both MIF receptors and/or p38 isoform expression in the target cell(s), dose of recombinant MIF, and kinetics of the analysis. Importantly, here we demonstrate that CS-induced p38 activation was ASK1-dependent and that MIF directly blocked ASK1 kinase activity, suggesting the effects of MIF on ASK1 can occur independently of CD74.

While MIF has many potential intracellular binding partners, a direct interaction with ASK1 has yet to be demonstrated. The classic ASK1 inhibitor, Trx, is a redox-sensitive protein containing a TPOR motif (Saitoh, Nishitoh et al. 1998) similar in structure to MIF. The TPOR site consists of 2 reactive cysteine residues in a CXXC motif, which responds to cellular stress. In MIF, the intact CXXC motif is necessary for its biological function but does not appear to be required for binding to its intracellular receptor CSN5/Jab1 (Nguyen, Beck et al. 2003). Intriguingly, Trx has been demonstrated to bind to CSN5/Jab1 (Hwang, Ryu et al. 2004), and although both proteins contain reactive cysteine residues, it is currently not known if these residues contribute to this

interaction. These observations may suggest potential molecular redundancy with MIF and Trx, as has been implied in studies of lymphocytes (Kondo, Ishii et al. 2004). Further studies examining the mechanism(s) by which these TPOR containing proteins inhibit and interact with ASK1 are needed but are currently beyond the scope of this manuscript.

Another mitogen-activated protein kinase which lies down stream of ASK1, implicated both in ROS signaling and in CS-mediated EndoC dysfunction, is JNK (Schweitzer, Hatoum et al. 2011). We observed phosphorylation of JNK in response to CSE (Figure 4-11A) but inhibition of JNK was insufficient to abrogate CS-induced EndoC apoptosis (Figure 4-11B). Thus, while JNK may contribute to EndoC signaling, the activity of this kinase is not required for EndoC apoptosis.

Limitations of this study revolve around the kinetics of the molecular events as they relate to later markers of injury and remodeling. ASK1 auto-activation and p38 phosphorylation occur rapidly and transiently, making both justifiably difficult to assess *in vivo*. With such rapid activation of this pathway, the kinetics of activation in the presence of exogenous MIF could be altered or delayed rather than completely inhibited. The importance of this limitation is lessened by the use of complementary approaches, which demonstrated that ASK1 and p38 were obligatory to CS-induced cytotoxicity and that MIF directly antagonized ASK1 kinase activity. Inhibition of this pathway rescues MIF

deficiency *in vitro* and *in vivo* collectively arguing that these molecules interface on a common pathway. Other limitations may arise in that we utilize an acute CS-model with genetic knockouts to identify molecular mechanisms exacerbated by chronic CS exposure of wild type animals and in humans. Our preclinical and *in vitro* models of MIF deficiency represent a critical point in emphysema progression, where the cytoprotective effects of MIF are diminished, and cell damage/death by definition exceed repair. Understanding that MIF has pro-inflammatory activity and that acute versus chronic inflammation may differ, we have probed for several inflammatory endpoints and did not detect acute genotypic differences aside from increased lymphocytes in *Mif* $^{-/-}$ mice (Fallica, Boyer et al. 2014). While with chronic CS exposure macrophages are increased in both genotypes, and increased lymphocytes are detected in *Mif* $^{-/-}$ mice (18), the lack of other inflammatory differences 1) argue against a mechanism by which the proinflammatory effects of MIF play a role in COPD pathology and 2) fail to account for the EndoC specific alterations detected with chronic CS-exposure (Fallica, Boyer et al. 2014).

Redox signaling and more specifically an imbalance in oxidant and antioxidant production is a well-recognized contributing factor to disease progression. Our results provide strong support for a novel role for MIF as a determinant of ROS production in response to smoke exposure, impacting on the ASK1-p38 kinase cascade regulating the activity of the

ROS-generating enzyme XOR and antagonize ASK1-p38-dependent EndoC apoptosis (Figure 4-15). In our model, the acute production of MIF in response to CS acts to partially repress signaling via the ASK1-p38-XOR pathway accounting for its cytoprotective effects. Following chronic exposure to CS, MIF expression is diminished (Fallica, Boyer et al. 2014, Sauler, Leng et al. 2014), potentiating injury signaling via this pathway and exacerbating emphysematous remodeling. In summary, this study establishes a role for MIF in controlling ROS responses to smoke by regulating p38-depedent XOR activity, and it specifically links MIF as a direct inhibitor of ASK1, upstream of p38 in CS-induced EndoC injury. The identification of this pathway provides additional therapeutic targets directed at ameliorating ROS production and EndoC apoptosis, driving forces behind tissue injury and remodeling in emphysema.

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# Chapter 5

## Macrophage Migration Inhibitory Factor (MIF) regulates NRF2 antioxidant responses through its intracellular receptor JAB1

# Chapter 5

## 5.1 Abstract

Macrophage migration inhibitory factor (MIF), a pleiotropic cytokine, is reduced both in a preclinical model of CS-induced emphysema and in patients with chronic obstructive pulmonary disease, particularly those with the most severe disease and emphysematous phenotype. In addition oxidative stress is continuously exacerbated in response to CS or with diease progression. MIF functions to antagonize CS-induced DNA damage, p53-dependent apoptosis of pulmonary endothelial cell (EndoC), xanthine oxidoreductase dependent oxidant production, and subsequent emphysematous tissue destruction. Using primary alveolar EndoC and a mouse model of CS-induced lung damage, we investigated the capacity and molecular mechanism(s) by which MIF may further modify oxidant injury. Here, we demonstrate that the downstream targets of nuclear factor erythroid 2-related factor 2 (NRF2), a major transcription factor and regulator of the antioxidant (AO) response element (ARE) and therefore AO production, are decreased following CS exposure in the absence of MIF. Both the production of ROS and the activity of the AO are regulated by a MIF dependent mechanism. MIF was sufficient enhance NRF2 gene expression and stabilize NRF2 protein. Further the deletion of the intracellular MIF receptor, c-Jun activation domain binding protein-1 (JAB1), was sufficient to increase ARE activity. Taken

together, MIF suppresses CS-mediated cytotoxicity in the lung by enhancing NRF2 activity in a JAB1 dependent manner.

## 5.2 Introduction

Cigarette smoke (CS) exposure is the main cause of emphysema, a disease effecting five million people in the United States, initiates apoptosis within the lung parenchyma, and is linked to dramatic increases in oxidative stress (Rennard, Togo et al. 2006, Tsuji, Aoshiba et al. 2006, Yoshida and Tuder 2007, Forey, Thornton et al. 2011, Centers for Disease and Prevention 2012). CS-induced oxidative damage results in enlargement of the airspaces, loss of the capillary bed, and diminished surface area for gas exchange. CS is a mixture of thousands of extrinsic factors that dramatically augment the amount of oxidants in the lung in addition to increasing the production of endogenous reactive oxygen species (ROS) by cells of the airway, alveoli, and immune system (Pryor and Stone 1993, Moodie, Marwick et al. 2004, Yang, Chida et al. 2006, Zhang, Venardos et al. 2006). Exacerbation of oxidative stress is linked with Endothelial cell (EndoC) apoptosis, which represents an early, necessary, and sufficient event in emphysematous tissue destruction (Kasahara, Tuder et al. 2000, Giordano, Lahdenranta et al. 2008, Gordon, Gudi et al.). Thus, we set out to identify novel regulators of EndoC apoptosis and survival in the context of CS exposure postulating that these would represent potential determinants of disease severity and needed therapeutic targets.

The transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) is an important regulator of the antioxidant (AO) response element (ARE), AO production, and intracellular protection from increased ROS (Rangasamy, Cho et al. 2004, Boutten, Goven et al. 2010). Importantly NRF2-regulated antioxidant activity is reduced in human CS-induced emphysema (Goven, Boutten et al. 2008, Suzuki, Betsuyaku et al. 2008). In addition NRF2 deficient mice are susceptible to enhanced emphysematous tissue destruction (Rangasamy, Cho et al. 2004, Sussan, Rangasamy et al. 2009). The loss of NRF2-dependent transcripts is also coincident with the loss of macrophage migration inhibitory factor (Mathew, Jacobson et al. 2013).

MIF, a pleotropic cytokine with thiol-protein oxidoreductase (TPOR) activity, is significantly reduced in the serum of patients with severe emphysematous COPD and diminished in the lungs of mice exposed to chronic CS (Fallica, Boyer et al. 2014, Sauler, Leng et al. 2014). In addition, MIF is increased in response to acute CS, is an endogenous regulator of CS-induced EndoC apoptosis and DNA damage, and has the capacity to antagonize CS-induced p53 expression and p53-dependent apoptosis, suggesting a potential cytoprotective role that is lost over time (Damico, Simms et al. 2011, Fallica, Boyer et al. 2014, Sauler, Leng et al. 2014). The antagonism of p53 by MIF is multifactorial but it can specifically be through direct interaction mediated by reactive cysteine residues on each protein (Mitchell, Liao et al. 2002, Lee, Oh et

al. 2006, Jung, Seong et al. 2008, Jung, Seong et al. 2008, Damico, Simms et al. 2011). In addition, through this interaction with p53, MIF is recognized as a modulator of transcription factors such as hypoxia inducible factor 1 (HIF1)-alpha (Oda, Oda et al. 2008, Simons, Grieb et al. 2011). Our previous data linking MIF to altered XOR activity is also likely mediated through upstream MAP3K interactions involving the oxidoreductase activity of MIF (Chapter 4).

The TPOR spanning region on MIF is necessary for binding c-Jun activation domain binding protein-1 (JAB1), a main intracellular receptor for MIF (Burger-Kentischer, Finkelmeier et al. 2005). Interestingly, JAB1 is a key enzymatic component of the COP9 signalosome, which is implicated in the stabilitiy of the cullin/E3 ligase that targets NRF2 for degradation (Chamovitz and Segal 2001, Lyapina 2001, Cope 2002, Zheng 2002, Pintard 2003, Yamoah, Wu et al. 2005, Schmaler and Dubiel 2010). Since MIF deficiency exacerbates oxidative stress, EndoC apoptosis, potentiating airspace enlargement, and diminished NRF2 is coincident with these alterations, we hypothesize that MIF impacts on CS-mediated cytotoxicity in lung by regulating NRF2 activity in part through its interaction with JAB1 (Fallica, Boyer et al. 2014).

In the present study, we demonstrate that in response to CS, MIF deficiency results in diminished antioxidant responses downstream of NRF2. In vitro exogenous MIF blocked ROS production similar to NAC, showing specificity for a role in antioxidant production. We also

demonstrate that MIF was sufficient to increase ARE activity. Further MIF increased or stabilized the gene and protein expression, respectively, of NRF2. Importantly, JAB1 was shown to be a negative regulator of NRF2 dependent ARE activity placing JAB1 downstream of MIF. In addition alterations in MIF expression did not have an effect on JAB1 expression. Thus, MIF antagonizes ROS formation by stabilizing NRF2 dependent antioxidant responses in a JAB1 dependent manner.

## 5.3 Results

### *5.3.1 MIF is a determinant of CS-mediated expression of antioxidant transcripts in vivo*

Cellular defense against oxidant stress occurs via the activation of antioxidant genes through a cis-acting sequence known as the anti-oxidant response element (ARE) (Li and Jaiswal 1992). The transcription factor NRF2 binds and mediates expression of ARE containing gene products (Marini, Chan et al. 1997). We have previously established that MIF has the capacity to regulate the ROS generating enzyme XOR. Recognizing the capacity of MIF to bind JAB1, which may be involved in NRF2 ubiquitination, we rationalized that the observed changes in intracellular ROS could also result from a decrease in anti-oxidant and detoxifying gene products. We targeted our analysis on well-recognized transcriptional targets of the NRF2-ARE pathway. To test the effect of MIF on CS-induced redox responsive and regulator gene products in the intact lung, *MIF*$^{+/+}$ and *Mif*$^{-/-}$ mice were exposed to filtered air or CS for 3 consecutive days, and total lung RNA expression was characterized by quantitative PCR (qPCR). CS exposure resulted in an increase in mRNA for NADPH dehydrogenase, quinone 1 (NQO1), the prototypic NRF2-ARE target (Jaiswal 2000), and glutathione peroxidase 2 (GPX2), the major NRF2-dependent glutathione peroxidase in the lung (Singh, Rangasamy

et al. 2006). However, we were unable to detect changes in heme-oxygenase 1 (HO-1) mRNA in response to CS (data not shown). Therefore an additional mechanism of higher intracellular ROS observed in these lungs (Figure 4-1), CS-induced NQO1 or GPX2 mRNA expression was significantly depressed in the absence of MIF (Figure 5-1) implicating MIF in efficient NRF2-ARE signaling responses to CS and optimal anti-oxidant gene expression *in vivo*.

### *5.3.2 Exogenous MIF antagonizes CS-induced ROS accumulation and potentiates NRF2-ARE in EndoC in vitro*

Based on the direct and/or indirect effects of MIF deficiency on CS-induced ROS *in vivo*, we predicted that exogenous MIF would alter CS-induced ROS production *in vitro*. Increases in intracellular oxidative stress were measured using 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) which is cleaved by intracellular esterases to form the non-fluorescent and redox-sensitive dye $H_2$DCFDA. We have previously established that EndoC are major targets of CS-induced damage in the absence of MIF. Therefore exposure of primary human lung microvascular EndoC (HLMV EndoC) to CS extract (CSE) induces a rapid increase in intracellular ROS (Figure 5-2A) as assessed by an increase in $H_2$DCFDA fluorescence. Pretreatment of EndoC with the anti-oxidant and reduced thiol donor, NAC, efficiently abrogates this increase

demonstrating specificity of the reaction. Importantly, pretreatment of these cells with rMIF (100ng/ml) significantly antagonizes CSE-induced increases in intracellular ROS. Thus, exogenous MIF is sufficient to alter the redox responses of human alveolar EndoC to CS.

To determine if extracellular MIF is sufficient to influence transcriptional activity via effects on ARE containing promoters (present in both the promoters of NQO1 and GPX2), we transfected rat lung microvascular EndoC with an ARE-reporter plasmid. Cells were subsequently exposed to exogenous recombinant MIF protein (rMIF 100ng/ml) or carrier and lysates were examined for luciferase activity per manufacturer's recommendations (Materials and Methods). Treatment with exogenous MIF results in a 50 percent increase in ARE-driven transcriptional activity (Figure 5-2B). Thus, exogenous MIF can increase transcription of NRF2-mediated ARE-containing promoters.

### *5.3.3 MIF stabilizes CS-induced NRF2 expression*

The effect of MIF on NRF2-dependent ARE activity could arise through a direct effect of MIF on ARE promoters or through a mechanism whereby MIF modulates the stability and or the translocation of NRF2 to the nucleus. MIF itself is not known to directly impact on gene transcription or mRNA stability, although it is implicated in the stabilization of other transcription factors like HIF1-alpha. To

understand how MIF acts to increase NRF2-dependent antioxidant responses we exposed rat lung microvascular EndoC to exogenous rMIF (100ng/ml) or carrier and lysates were examined for alteration in normalized NRF2 protein. Treatment with exogenous MIF resulted in a 2-fold increase in NRF2 protein expression compared to carrier alone (Figure 5-3B). We also performed the complementary experiment, transfecting EndoC with siRNA targeting MIF or scrambled siRNA (CTRL) and analyzing NRF2 expression following a 24hr period. Near complete knockdown of MIF was achieved. In addition the siMIF-transfected EndoC had diminished NRF2 expression (Figure 5-4A). The addition of low dose rMIF (10ng/ml) did not have a substantial effect. Thus, exogenous MIF stabilizes NRF2 protein suggesting that the increased ARE activity is in part regulated by the capacity of MIF to enhance its promoter.

### *5.3.4 Jab1 negatively regulates NRF2 activity*

The recognized intracellular receptor for MIF is JAB1. JAB1 through its association with a larger complex, the COP9 signalosome, can deneddylate cullin proteins, a process that may be necessary for the continuation of cullin-associated E3 ligase activity that targets cytosolic proteins for ubiquitination. NRF2 is held in the cytosol by its repressor Kelch-like EndoCH-associated protein 1 (KEAP1), which associates with

the cullin scaffolding. Further, MIF antagonizes JAB1 through a direct interaction, inhibiting JAB1 related protein interactions and downstream signaling. To test whether the effects of MIF of NRF2 are dependent on JAB1, we first transfected EndoC with siRNA targeting JAB1. After 24 hours transfect EndoC were analyzed for JAB1 and NRF2 expression. Partial knockdown of JAB1 was observed, and this associated with a clear increase in NRF2 as compared to CTRL transfectants (Figure 5-4B). The addition of low dose rMIF (10ng/ml) appeared to have a small effect in increasing NRF2 protein similar to JAB1 knockdown.

Next we transfected EndoC with an ARE-reporter plasmid in tandem with siRNA targeted against JAB1. After 24 hours ARE activity was determined, showing a near 3 fold and significant increase in ARE activity in the absence of JAB1. Taken together JAB1 appears to be a negative regulator NRF2-dependent ARE activity, by reducing NRF2 protein expression.

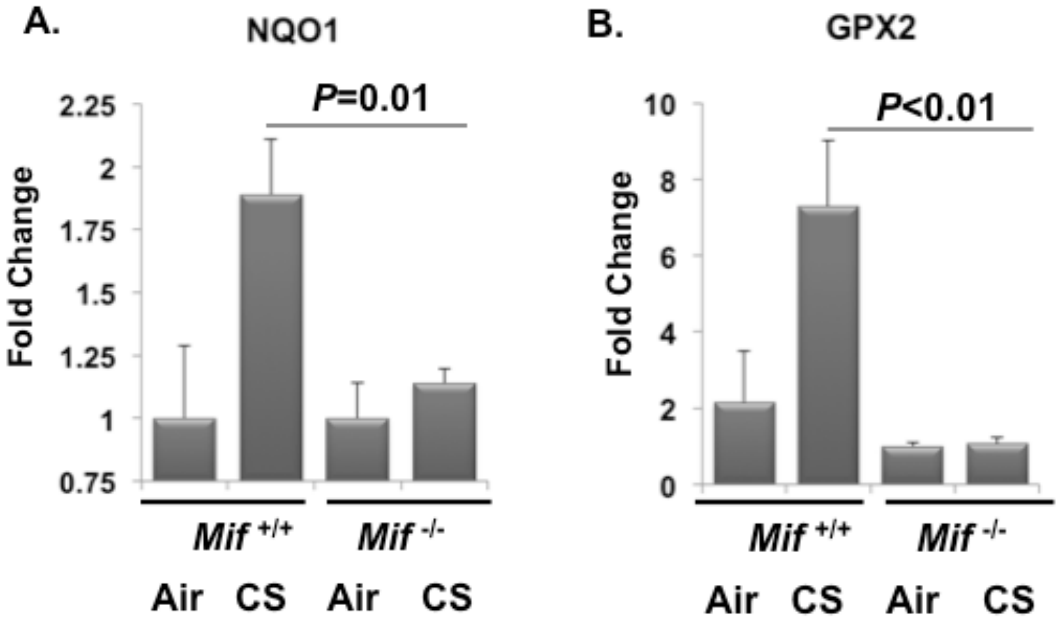


**Figure 5-1. MIF is a determinant of CS-mediated expression of antioxidant transcripts in vivo**. *Mif*$^{+/+}$ and *Mif*$^{-/-}$ mice were exposed to 3 days of CS and lungs were probed for NRF2-dependent gene expression by qPCR. In *Mif*$^{+/+}$ mice, CS induced a significant increase in NQO1 (A) and GPX2 (B), whereas this response was absent in *Mif*$^{-/-}$ mice. n=3 to 5 per arm. Values are expressed as mean ± SEM.

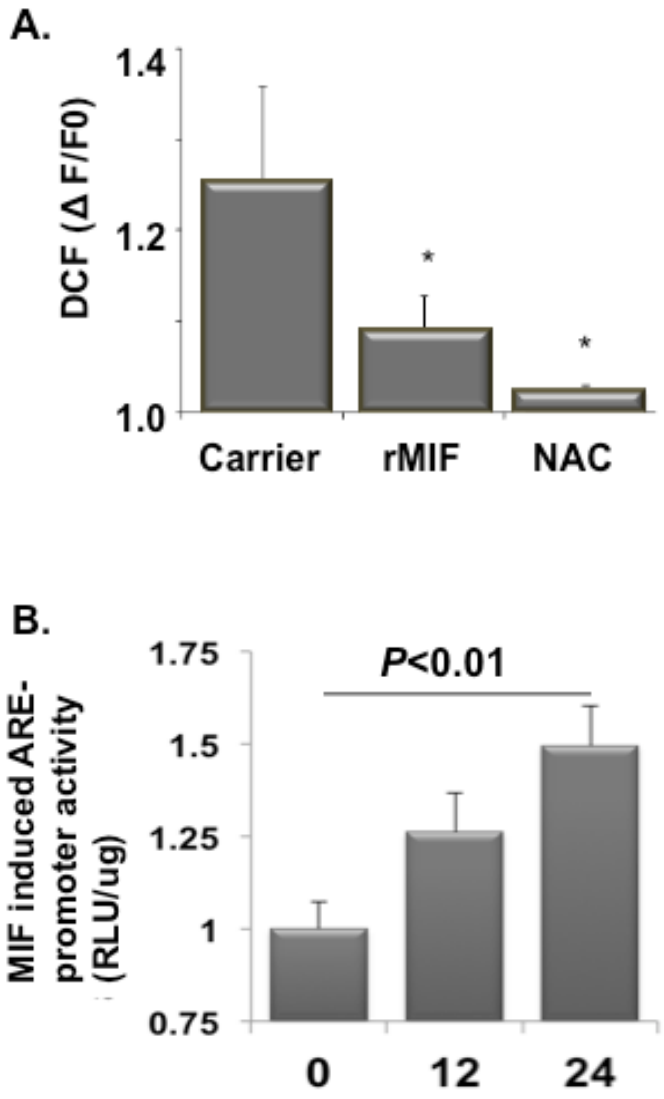


**Figure 5-2. Exogenous MIF antagonizes CS-induced ROS accumulation and potentiates NRF2-ARE in EndoC in vitro.** Rat microvascular Endo were pretreated with rMIF and examined for ROS production or ARE-activity. rMIF significantly antagonized CSE-induced changes in DCF fluorescence as does NAC (A). n=6 per treatment. **P*<0.05 vs. carrier. rMIF treatment was also sufficient to significantly increase ARE reporter activity over a 24hr period (B).

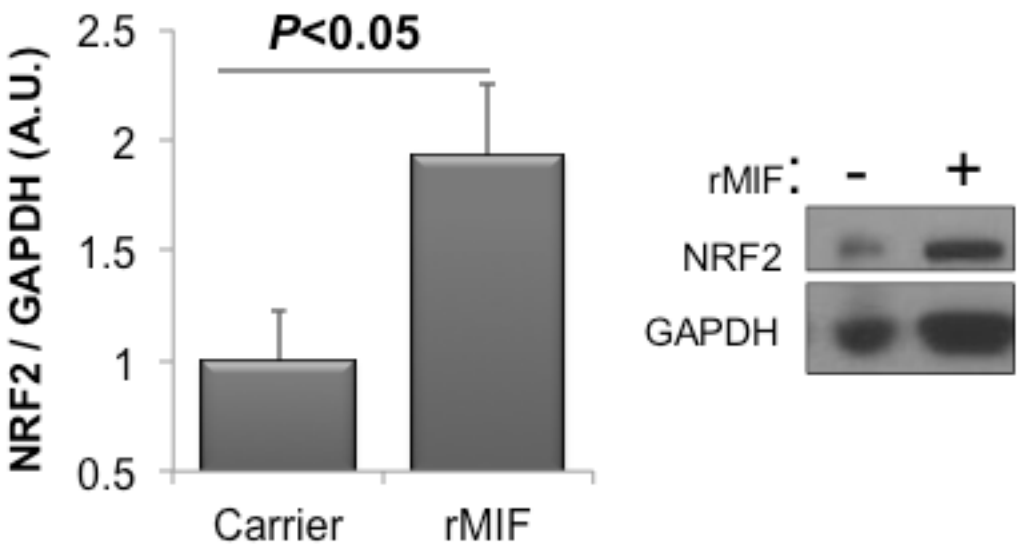


**Figure 5-3. MIF stabilizes CS-induced NRF2 expression**. Parallel rat microvascular Endo lysates were analyzed by Western blotting probing for NRF2 and normalized to GAPDH. Treatment with rMIF was sufficient to increase EC NRF2 protein expression. n=3 per arm. Values are expressed as mean ± SEM.

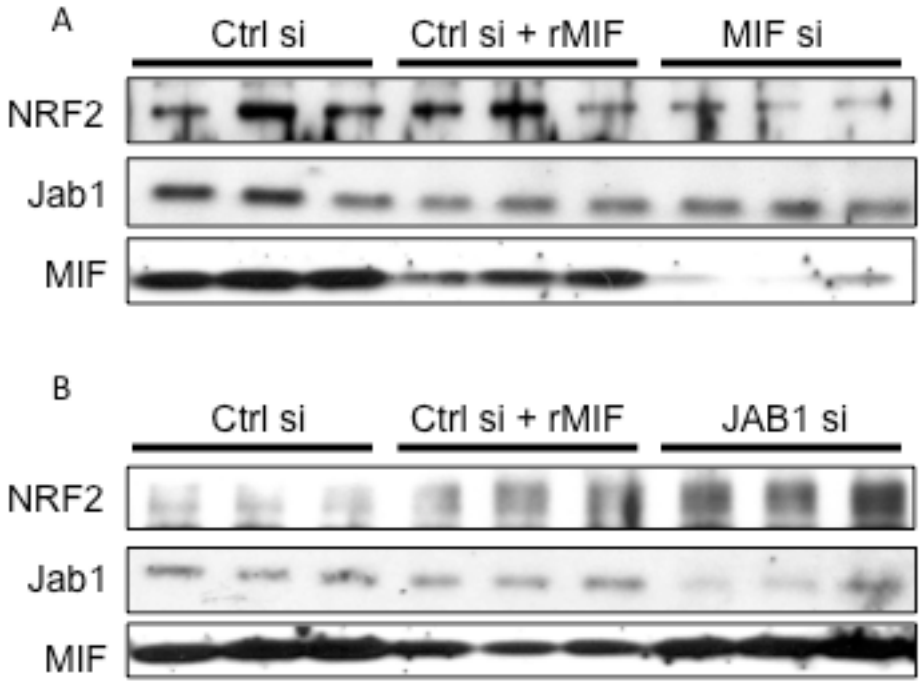


**Figure 5-4. The regulation of NRF2 protein expression by MIF and JAB1 is opposite and reciprocal**. Human microvascular EndoC were transfected with siRNA targeting MIF or JAB1 and anyalzed via Western blot, probing for NRF2, JAB1, and MIF. After 24 hours partial to complete knockdown of MIF or JAB1 was achieved. In the absence of MIF, NRF2 protein was diminished (A). In the absence of JAB1 NRF2 protein expression was potentiated. n=3 per arm.

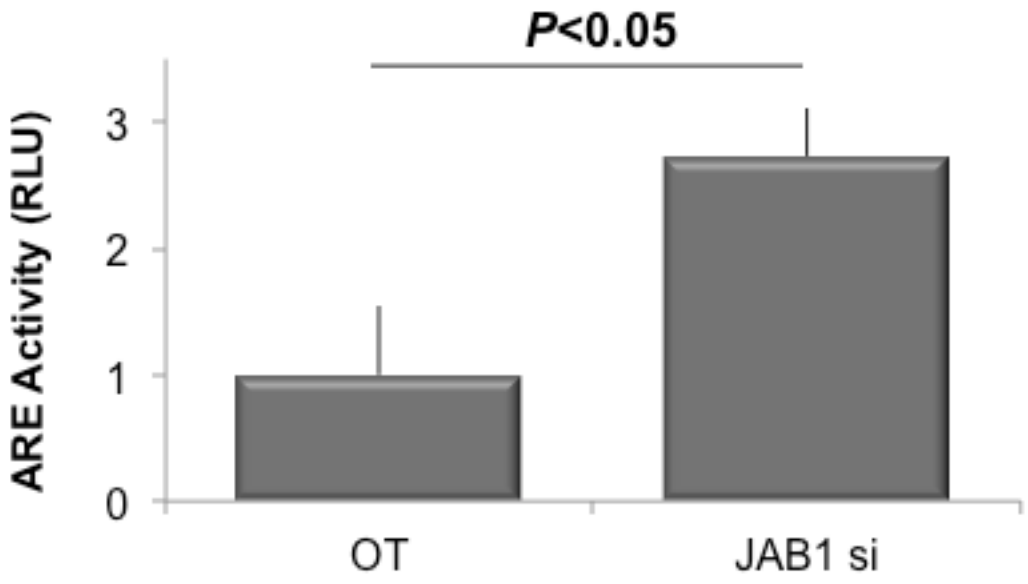


**Figure 5-5. Jab1 negatively regulates NRF2 activity.** Human microvascular EndoC were transfected with siRNA targeting JAB1 and an ARE-containing promoter. After 24 hours ARE activity was analyzed with via luminescence. In the absence of JAB1, NRF2-ARE activity was significantly increased. n=3 per arm

## 5.4 Discussion

Emphysema, a common form of COPD, contributes to an estimated 2.5 million deaths and a health care cost approaching 38.8 billion dollars annually in the United States alone (Foster, Miller et al. 2006). It is a debilitating disease characterized by irreversible destruction of the lung architecture with enlargement of the airspaces driven by enhanced oxidative stress and alveolar cell apoptosis (Demedts, Demoor et al. 2006). We have previously shown EndoC to be a major target of CS-induced cell damage in the absence of MIF (Fallica, Boyer et al. 2014). In addition we have strong evidence that MIF impacts of ROS generation (Chapter 4). Recognizing the multifactorial potential of MIF as a redox sensor and determinant of EndoC fate, we thus set out to identify new molecular determinants of cell fate in response to CS, predicting that such factors could represent novel determinants of oxidative stress with disease severity (Damico, Simms et al. 2011, Kim, Serebreni et al. 2013).

Here, we provide evidence that MIF influences the redox response to CS in primary human and rat EndoC, and in the intact murine lung. The increase in intracellular ROS observed (Chapter 4) in the absence of MIF could arise from loss of its intrinsic oxidoreductase activity, failed detoxification and/or increased production. In addition there are multiple enzymatic sources of ROS in the lung such NADPH oxidase and XOR (Damico, Zulueta et al. 2012). XOR is induced *in vitro* and *in vivo* by CS exposure (Kim, Serebreni et al. 2013). Importantly, in the absence of

MIF there is a decreased antioxidant (*NQO1* and *GPX2*) response that is coincident with increased XOR and ROS production (Chapter 4), suggesting that MIF is impacting both the generation and/or the clearance of ROS. MIF was sufficient to increase NRF2-ARE signaling in EndoC *in vitro* and was necessary for optimal expression of NRF2 targets in the response to CS *in vivo*. We have previously demonstrated that MIF, under the same conditions, antagonizes CS-induced DNA damage and p53-dependent apoptosis, linking these observations together (Fallica, Boyer et al. 2014). Thus in the absence of MIF both increased ROS, partly mediated through p38-dependent activation of XOR, and diminished detoxification appear to be driving acinar apoptosis in response to CS. Further our previous data linking MIF to altered XOR activity suggests that the effects of MIF are mediated through upstream MAP3K interactions involving the oxidoreductase activity of MIF (Chapter 4). Similarly, the reactive cysteine residues on MIF, that confer its oxidoreductase activity, are also sufficient for MIF protein-protein interactions with other intracellular proteins (Jung, Kim et al. 2001, Shen, Hu et al. 2003, Li, Lu et al. 2004, Burger-Kentischer, Finkelmeier et al. 2005, Jung, Seong et al. 2008, Jung, Seong et al. 2008).

Intracellular MIF is not known to directly impact on gene transcription or mRNA stability. However through its effects on cytosolic p53, MIF can alter cell fate decisions by modulating the stability of nuclear p53 and other transcription factors, including HIF1 alpha (Lee,

Oh et al. 2006, Jung, Seong et al. 2008, Oda, Oda et al. 2008). Further, through JAB1 binding, MIF can alter several other transcription factors and their downstream gene-products (Bianchi, Denti et al. 2000, Chauchereau, Georgiakaki et al. 2000, Kleemann, Hausser et al. 2000, Chamovitz and Segal 2001, Hwang, Ryu et al. 2004, Lee, Oh et al. 2006, Oh, Lee et al. 2006, Villeneuve, Lau et al. 2010). Here we show MIF enhances the activity of the major transcriptional regulator of the ARE, NRF2, in a JAB1 dependent manner, leading to secondary effects on antioxidant gene expression and regulatory activity towards oxidative stress. The loss of MIF correlated with diminished NRF2 protein, while the loss of JAB1 potentiated NRF2 protein and activity. In addition the effects of JAB1 and MIF on NRF2 were independent of each one altering the expression of the other. Further, Nrf2 mRNA is constitutively expressed independent of inducers, implicating a post-transcriptional mechanism for the observed alterations in Nrf2 activity. Mechanistically, activation of Nrf2 is generally controlled at the protein level, whereby Nrf2 activity is limited under basal conditions through repressors like Kelch-like ECH-associated protein 1 (KEAP1) and activated by inducers or oxidative stress that disrupt repressor binding.

JAB1 can interact with other transcription factors by stabilizing their association with coactivators, but the effects of MIF on JAB1 regarding these transcription factors, while plausible, remains ill defined (Claret, Hibi et al. 1996, Kleemann, Hausser et al. 2000, Chamovitz and

Segal 2001). In addition, there is no indication that JAB1 stabilizes transcriptional repressors through similar nuclear interactions suggesting its role in NRF2 activity is also at the protein level. Further, the TPOR spanning region on MIF is necessary for binding JAB1 and the capacity of MIF to block JAB1 activity has been previously established (Burger-Kentischer, Finkelmeier et al. 2005). JAB1 acts as a coactivator for AP1 transcription, and MIF binding to JAB1 antagonizes this function, altering cell growth, proliferation, and or apoptosis-related protein expression depending on the cellular context (Bianchi, Denti et al. 2000, Kleemann, Hausser et al. 2000, Hwang, Ryu et al. 2004). Specifically JAB1, through its association with the COP9 signalosome has been implicated in the cycle of protein degradation and here, to extend we validate the plausibility of JAB1 explicitly regulating NRF2. However given the complex nature of this cycle more work is needed to further elucidate the exact mechanism.

The COP9 signalosome, to which JAB1 associates, is involved in the deneddylation of proteins (Schmaler and Dubiel 2010). Neddylin (NEDD8) stabilizes cullins, allowing for activation of associated ubiquitin ligases (E3) (Lyapina 2001, Cope 2002, Zheng 2002, Yamoah, Wu et al. 2005). KEAP1 tethers NRF2 to these cullin scaffolds, and when neddylated, the E3 ligase targets NRF2 for ubiquitination (Villeneuve, Lau et al. 2010). In order for this complex to efficiently degrade more cytosolic NRF2 it must cycle apart and back together (Chamovitz and

Segal 2001, Pintard 2003). JAB1, as part of the signalosome, is hypothesized to remove NEDD8, destabilizing the cullin complex in order for KEAP1 to bind another NRF2 (Cope 2002, Villeneuve, Lau et al. 2010). In the presence of ubiquitin-conjugating enzyme E2M (UBC12) the complex will be re-neddylated, stabilizing the complex once again, allowing the cycle to continue (Chamovitz and Segal 2001, Schmaler and Dubiel 2010, Villeneuve, Lau et al. 2010). Therefore other potential sources by which MIF exerts an effect on JAB1 or NRF2 are KEAP1 and UBC12. Intriguingly, both of these proteins have redox sensitive cysteine residues, raising the likelihood that MIF may have a direct or indirect effect on their signaling and or function. There is limited evidence this may be the case, as exogenous MIF may enhance the KEAP1 repressor, and NRF2 activator, DJ1 (Pei, Wu et al. 2014). In addition the association of UBC12 with NEDD8 is redox sensitive; Under oxidizing conditions a modified cysteine residue on UBC12 prevents NEDD8 association, and therefore the cullin is not activated and the target protein is not degraded (Downs, Kumar et al. 2013). However our data here argue against this having a significant effect, as the in vitro experiments were carried out under basal conditions in the absence of an oxidizing stimulus such as CSE. This JAB1 Nonetheless, under the premise that MIF antagonizes JAB1 activity, this scenario paints a picture whereby MIF would stabilize NRF2, by preventing its rapid targeting for proteosomal degradation (Mathew, Jacobson et al. 2013).

Additionally, through its effects on JAB1 function, MIF can also alter the expression of HIF1-alpha and p53, which are both targeted by specific E3 ligases for proteasomal-degradation (Bae, Ahn et al. 2002, Nemajerova, Mena et al. 2007)

It is not known whether MIF interacts with JAB1 as a single protein or as part of the multi-protein complex (COP9 signalosome) or how MIF may modulate these relative pools of JAB1. Intriguingly the MIF-JAB1 complex is detectable in the cytosol, but it is not known whether this complex, while together, has signaling or binding functionality as a cytosolic and or transcription factor similar to the individual capacities of MIF and JAB (Kleemann 2000, Bech-Otschir 2001, Chamovitz and Segal 2001, Lee, Oh et al. 2006, Kudrin and Ray 2007). In addition both MIF and JAB1 stabilize HIF1-alpha, albeit through different mechanisms, but this raises the question as to whether their relationship is purely antagonistic (Bae, Ahn et al. 2002, Oda, Oda et al. 2008, Simons, Grieb et al. 2011). JAB1 inhibits the cyclin dependent kinase (CDK) inhibitor p27KIP1, which can induce G1 cell cycle arrest (Tomoda, Kubota et al. 1999). Exogenously MIF binds with cytosolic JAB1 and inhibits its control over p27KIP1, stabilizing cell cycle arrest (Tomoda, Kubota et al. 1999, Kleemann, Hausser et al. 2000). In contrast, JAB1 enhances JNK activity and downstream c-jun phosphorylation and MIF blocks this JAB1–dependent effect, possibly disrupting cell growth and or proliferation. However the exact mechanism

remains ill-defined, as MIF and JAB1 do not possess kinase activity and do not directly associate with JNK (Tomoda, Kubota et al. 1999, Kleemann, Hausser et al. 2000). This regulation of the MAPK pathways also contradicts MIF signaling mediated through extracellular receptors, which positively regulates cell survival and proliferation.

One limitation of this study is we did not include a second stimulus in order to test the function outcome of JAB1 deficiency, such as increased or decreased cell death. However given the extensive interconnections of redox sensitive and cysteine containing proteins, the addition of CSE into our analysis may confound the interpretational value of absolute JAB1 effects on NRF2. In addition there is currently a lack of consensus as to the role of JAB1 on cell fate depending on context (Tomoda, Yoneda-Kato et al. 2004, Hallstrom and Nevins 2006, Liu, Pan et al. 2008, Damico, Simms et al. 2011).

A second limitation arises in that we did not explicitly address the specific antagonism of MIF on JAB1. The combination of NRF2 protein loss observed in MIF deficient cells and NRF2 potentiation observed in JAB1 deficient cells, in addition to the previously observed antagonism of JAB1 by MIF, implies a direct mechanism by which MIF regulates JAB1 and NRF2 protein stability. Despite a lack of evidence to suggest a direct effect of MIF on the ARE, future experiments should be directed at understanding whether MIF may have a direct effect on ARE responses in the absence of JAB1. Alternatively, mutation of Cys60 on MIF reduces

the interaction of MIF and JAB1 and could also be employed to further examine the implicated direct interaction on NRF2 stability (Kleemann, Hausser et al. 2000, Nguyen, Beck et al. 2003).

A third limitation is that we have not defined the expression of all of the suspected factors that may be involved in cycling of NEDD8 and NRF2 degradation, like UBC12 and KEAP1 respectively. While MIF has not been directly implicated in binding or altering these proteins, MIF may have the capacity for multiple and simultaneous interactions as both the TPOR motif and Cys81 are involved in binding (Chamovitz and Segal 2001, Burger-Kentischer, Finkelmeier et al. 2005, Lee, Oh et al. 2006, Oh, Lee et al. 2006, Jung, Seong et al. 2008, Jung, Seong et al. 2008, Luedike, Hendgen-Cotta et al. 2012). However further work is needed to uncover the complex networking of these cysteine-rich and redox-sensitive proteins when their cognate intracellular locations are in flux, especially in the context of specific stimuli like CS, which provokes the associated redox reactions. In addition there is binding redundancy, overlap in function, and the possibility for alternative compensation amongst these cysteine rich redox sensitive proteins, exemplified by the inter- and intra- relationship of MIF and thioredoxin (TRX). For example JAB1 also has the capacity to bind TRX (Hwang, Ryu et al. 2004).

We also used a germ-line transgenic animal in our initial observations. Thus, we cannot rule out the possibility of secondary compensation. In addition limitations of our analysis are imparted by the

animal model itself. First, the model must condense pathologic events that take decades to develop in humans into a restricted timeline. Further, there are clear interspecies differences in inflammatory responses (Seok, Warren et al. 2013), susceptibility to oxidant injury (Frank, Bucher et al. 1978), and anatomy and respiratory physiology. This is not unique to our study but true of all pre-clinical studies using mice. These limitations are arguably balanced by the capacity to assess the causal relationships not easily addressed in human studies.

Oxidative stress is a well-recognized contributing factor to emphysema disease progression. Our results support a novel role for MIF as a determinant of ROS clearance in response to smoke exposure, impacting on JAB1-dependent NRF2 protein degradation. In our model, the acute production of MIF in response to CS acts to partially enhance the NRF2 pathway by blocking JAB1, accounting for its cytoprotective effects in wild type animals. Following chronic exposure to CS, MIF expression is diminished coincidently, and likely causally, with NRF2 expression, potentiating injury and exacerbating emphysematous remodeling (Mathew, Jacobson et al. 2013, Fallica, Boyer et al. 2014, Sauler, Leng et al. 2014). In summary, this study establishes a role for MIF in modulating ROS detoxification in response to smoke by regulating NRF2, and it specifically links MIF to enhanced antioxidant production in a JAB1 dependent manner. The identification of this pathway provides additional therapeutic targets directed at ameliorating ROS production

and EndoC apoptosis, driving forces behind tissue injury and remodeling in emphysema.

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# Chapter 6

## Thesis Discussion

# Chapter 6

## 6.1 Discussion

### *6.1.1 Summary*

At the genesis of this project we sought to identify and characterize intrinsic molecular regulators of alveolar-capillary homeostasis and define their contribution to disease severity and CS-induced tissue destruction. Recent work by our lab, sheds light on the significance of intrinsic modifiers of EndoC homeostasis as well as cytoprotection from CS-induced damage, typified by MIF. We were the first to show that MIF expression is significantly reduced in human emphysematous COPD, and this observation has since been validated in a separate cohort (Fallica, Boyer et al. 2014, Sauler, Leng et al. 2014). Next we showed that MIF deficient mice were susceptible to enhanced emphysematous tissue destruction in response to chronic CS. These chronic outcomes correlated with amplified sub-acute CS-induced DNA damage, ATM kinase activation, and pulmonary EndoC apoptosis. Thus we established a novel role for MIF in controlling DNA damage and p53-derived apoptotic responses to CS, by regulating DSB and caspase-3 activation in microvascular EndoC.

Recognizing that oxidative stress is a major characteristic of emphysema and an important inducer of apoptosis, our subsequent research sought to uncover a role for MIF in oxidant and antioxidant

imbalances that by definition contribute to oxidative stress. Our lab had previously shown that XOR, an important cellular source of oxidative stress, is obligatory for CS-induced DNA damage, p53 expression, and EndoC apoptosis (Kim, Serebreni et al. 2013). In addition XOR activity is elevated in patients with emphysema and CS exposed animals compared to controls, which is concurrent with declines in MIF expression, suggesting a potential interfacing of pathways or activity (Pinamonti, Muzzoli et al. 1996, Pinamonti, Leis et al. 1998). In our ensuing work we showed that the complete loss of MIF potentiates ROS production and XOR activation in response to CS. Both wild-type and MIF deficient mice have significant increases in XOR but this increase is substantially higher in the MIF null mice. This suggests that XOR has a basal response to CS and or may have other potential activators in the face of CS. However the substantial difference between CS-exposed genotypes is dependent on p38 activation. This p38-dependent activation is also sufficient to significantly increase apoptosis in the alveoli compared to CS-exposed wild-type mice. Because MIF does not possess kinase activity and given the potential impact of MIF in modulating redox reactions through its oxidoreductase activity, we suspected MIF was impacting on redox sensitive MAP3K upstream of p38. We demonstrated that ASK1 is one potential MAP3K upstream of p38 and also necessary for apoptosis. Importantly, MIF suppresses CS-induced p38 activation and directly antagonizes ASK1 kinase activity. Thus, MIF antagonizes ROS formation

and subsequent EndoC apoptosis by repressing ASK1-p38 kinase signaling upstream of XOR activation and increased ROS production.

In further understanding the role of MIF in oxidative stress we also uncovered a novel potentiation of the major antioxidant transcription factor, NRF2, by MIF in a JAB1 dependent manner. Through its oxidoreductase activity, MIF binds and in some cases inhibits JAB1, which is suspected in the degradation or NRF2 through its association with the COP9 signalosome (Chamovitz and Segal 2001, Burger-Kentischer, Finkelmeier et al. 2005, Lue, Thiele et al. 2007). In addition, coincident with deficits in MIF production and increased XOR, NRF2 is significantly diminished with human emphysema progression (Boutten, Goven et al. 2011). In this part of our investigation we demonstrated that in response to CS, MIF deficiency results in diminished antioxidant responses downstream of NRF2 suggesting that MIF is a positive regulator of NRF2 activity. MIF had the capacity to block ROS production similar to NAC, showing specificity for enhanced antioxidant production. Next we demonstrated that MIF was sufficient to increase NRF2-dependent ARE activity in EndoC. In addition MIF sufficiently stabilized the gene and protein expression of NRF2. Importantly, we showed JAB1 was a negative regulator of NRF2-dependent ARE activity, placing JAB1 downstream of MIF. In addition alterations in MIF expression did not have an effect on JAB1 expression. Thus, MIF antagonizes ROS formation by stabilizing NRF2 dependent antioxidant responses in a

JAB1 dependent manner. These results, in addition to previous findings, indicate that with loss of MIF, JAB1 has a negative effect on NRF2 antioxidant production, contributing to oxidative stress with emphysematous disease progression.

### *6.1.2 MIF deficiency as a model for human emphysema*

CS-induced lung disease manifestations and onset of symptoms are multiple and invariably inconsistent which likely reflects the variance in genetic susceptibility. However in investigating the role of MIF in emphysema development there are several intriguing observations to suggest the dysregulation of MIF may be a major nodal point in the advancement of emphysematous processes. MIF has been shown to be directly involved in the regulation—or its absence is directly associated with the dysregulation—of several processes contributing to tissue destruction, including the enhanced oxidative stress, apoptosis, and alveolar airspace enlargement we present here, but also the transition from a predominantly innate immune response to an adaptive one, bacterial clearance and susceptibility to infections (Das, LaRose et al. 2014).

The nodal point in disease progression may therefore be defined as a point whereby the cytoprotective and inflammatory effects of MIF are diminished, and cell damage and death by definition exceed repair, and

adaptive responses are enhanced, respectively. Regarding cytoprotection, MIF notably increases acutely in response to CS and its associated hypoxic conditions suggesting that the role of MIF may be protective in nature. In looking at the balance of antioxidants and oxidants, we show here that MIF regulates the hyperactivation of XOR, which is increased in human disease. In addition MIF is a positively regulator of NRF2, both of which are decreased with disease. This protective effect is also apparent in considering our in vitro and in vivo data, which provides strong support for MIF in blocking CS-induced p53 dependent apoptosis, specifically in EndoC; p53 is directly regulated by MIF and exacerbated with human disease (Morissette, Vachon-Beaudoin et al. 2008, Damico, Simms et al. 2011). However, acute increases in MIF, through paracrine or autocrine loops, also stabilizes HIF1A, which is necessary for VEGF production and EndoC homeostasis, both of which are diminished in human emphysema (Kanazawa and Yoshikawa 2005). Further MIF as well as VEGF, through extracellular receptor signaling can enhance survival through AKT signaling, which is also dysregulated in human COPD (Bozinovski, Vlahos et al. 2006, Lue, Thiele et al. 2007, Prakash Muyal, Kumar et al. 2014). This suggests MIF is important in enhancing cell survival on multiple levels and regulating oxidative stress, both of which are significantly dysregulated, skewing cell fate towards death in human disease progression.

While it is not addressed in this thesis, MIF has clearly been

implicated in controlling or enhancing innate immune responses, which are persistently more irregular with disease progression. MIF is sufficient to enhance macrophage phagocytotic activity and migration, which are both acutely increased in response to CS in mouse and man. This acute increase in MIF is further associated with proper clearance of bacteria, efferocytosis, and an increase in MMPs, and importantly all of these processes are dysregulated in emphysema (Betsuyaku, Nishimura et al. 1999, Meyer-Siegler 2000, Onodera, Nishihira et al. 2002, Patel, Seemungal et al. 2002, Hoi, Morand et al. 2003, Seagrave, Barr et al. 2004, Kong, Huang et al. 2005, Boschetto, Quintavalle et al. 2006, Roger, Delaloye et al. 2013, Das, LaRose et al. 2014). The relationship between MIF and MMPs may be counterintuitive because MIF can be a positive regulator; however MIF is not the only MMP regulator and the association of MMPs with lung disease is mainly described in current smokers or in COPD patients with mild disease, suggesting the positive association between MIF and MMPs is likely in the acute phase of responses to CS. And lastly there is limited evidence that MIF loss dramatically enhances T cell chemotaxis and infiltration in the lung, which is a defining characteristic in diseases with MIF deficiency and the later stages of COPD (Aoshiba, Koinuma et al. 2004, Rosen, Lee et al. 2006, Hodge, Nairn et al. 2007, Kevill, Bhandari et al. 2008, Chang, Nadigel et al. 2011, Dancer and Sansom 2013, Eppert, Wortham et al. 2013, Hou, Sun et al. 2013, Jonathan, Benjamin et al. 2013, Jonathan,

Benjamin et al. 2014).

These latter gaps in knowledge regarding MIF may highlight a couple of limitations with the model. First, there are clearly differences in inflammation regarding the acute versus chronic nature of exposure. However as highlighted in Chapters 3 and 4, these differences are minimal between genotypes and our loss-of-function and clinical data argue against a model in which the pro-inflammatory functions of MIF promote COPD pathology. Secondly, in human disease there is likely 3 phases of MIF expression—the acute increase, the chronic decline, and the transition phase between. Our model therefore represents a clear picture of processes in the later stages of COPD characterized by diminished MIF. However regarding therapeutic approaches, our model will be useful for addressing the nodal point, or transition phase, where the cytoprotective effects of MIF are lost and apoptosis outpaces repair. That is, the acute differences between genotypes would expose potential proteins and novel pathways altered during this phase, and therefore interesting candidates for pharmaceutical intervention. This proposal is further substantiated as subacute CS exposure in MIF expressing animals alters the post-translational modification of MIF (data not published), suggesting that the loss of MIF function may precede reductions in MIF expression, conceivably implicating this transition phase as a principal area of importance and future direction.

The other overarching limitation with our model encompasses the acute nature of our CS exposures for uncovering the molecular underpinnings of MIF regulatory activity. Repeating the chronic exposures needed to mimic human disease and produce detectable emphysema, are costly and inherently inefficient. We therefore utilize acute and sub-acute exposure periods to better understand the underlying molecular pathways altered by MIF in the face of CS. In many ways this limitation is also a strength. Notably, we can examine and correlate the potential significant acute CS-induced findings or differences with chronic alterations. This greatly enhances our ability to target points in the molecular pathway regulated by MIF for therapeutic intervention. For example XOR activity is acutely increased and its expression is enhanced chronically suggesting that the ROS derived from XOR persists and may in fact get worse. However we can disrupt or block XOR through pharmaceutical intervention as another plausible future direction.

### *6.1.3 Conclusion*

The study of MIF in emphysema and COPD is only in its infancy. MIF has multiple functions, numerous binding partners, regulates cell fate, and has potential variability in the structure-function relationship with varying concentrations and cellular contexts. Therefore the full impact of

MIF on lung homeostasis is likely through multiple cellular and molecular paths that are collectively not well understood in the context of CS-induced lung disease. This thesis provides strong evidence that the cytoprotective effects of MIF are crucial for balancing increases in CS-induced oxidative stress, cell damage, and apoptosis, the major underlying interconnected causes of persistent emphysematous tissue destruction. Thus uncovering this and other MIF pathways is promising for future therapeutic interventions that ameliorate or perhaps reverse these continuous and debilitating features of emphysema.

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# Curiculum Vitae

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## Education

**Environmental Health Science PhD** May 2015
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
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**Bachelor of Science - Cum Laude** May 2006
Washington College Chestertown, MD
Major: Biology; Minors: Chemistry and Pscychology
Honors: Order of Omega, Maryland Science and Technology Scholar (4 year merit)

## Experience

**Dissertation Research**
Johns Hopkins Bloomberg School of Public Health 2012-Current
Advisor: Rachel Damico

Develop and implement murine and *in vitro* models of exposure induced lung diseases to further understand molecular and immune alterations observed in patients with disease. Project highlights:

- **Led project development** and published research uncovering a novel determinant of human and murine emphysematous tissue destruction, utilizing clinical samples and a genetic mouse model
- **Directed and taught laboratory procedures** dedicated to understanding potential immunological regulators, molecular modifiers, and mechanisms of lung disease and endothelial dysfunction
- **Designed** flow cytometry panels and ex vivo assays to detect immune alterations to various exposures
- **Developed technical publications** to enhance the translational value of mouse lung function testing as it relates to human clinical exams
- **Acquired a broad range of skills** for understanding physiological effects, molecular/cellular alterations and immunological changes, including tissue/cell isolation and preparation, viability assays, IHC, 12-color flow cytometry, qPCR, western blotting, cell culture, cell

transfections, cell free protein assays, ELISAs, functional assays, and proper statistical analyses

**Animal Research and Pulmonary Function Specialist**
Johns Hopkins University 2006-2010
PI: Wayne Mitzner

- **Implemented** procedures measuring responses to vast array of direct exposures (cigarette smoke, aerosols, acetylcholine etc.), disease states (malaria, obesity, asthma etc.), and potential inhibitors
- **Designed and implemented computer programs and protocols** for accurately measuring and understanding lung architecture using stereological principles
- **Performed cardiac perfusions** directed at properly fixing the lung microcirculatory structures
- **Developed and published** murine lung function methodologies to reflect human tests
- **Performed analyses** dedicated to annealing measured lung function parameters with theoretical models of dynamic and static motions
- **Maintained** mice colonies and **performed** many murine surgeries including telemeter placement

## Teaching Experience

**Teacher's Assistant**
Johns Hopkins Bloomberg School of Public Health 2011-2014
Aided 3 classes of 40+ students in graduate level human physiology
Aided 2 classes of 250+ students in environmental health

## Awards

Travel award, Annual Retreat of the International Graduate Program "Molecular Biology and Medicine of the Lung" Universities of Giessen and Marburg Lung Center, Germany, 2013

Red Alert for an outstanding paper by a junior investigator, *American Journal of Respiratory Cell and Molecular Biology, July 2014*

Outstanding paper award nominee for junior investigator, *American Thoracic Society, to be decided May 2015*

## Publications

**Fallica J,** Varela L, Johnston L, Kim B, Serebreni L, Wang L, Damarla M, Kolb TM, Hassoun PM, Damico R. Macrophage Migration Inhibitory Factor (MIF) is a Novel Inhibitor of ASK1-p38-XOR-Dependent Cigarette

Smoke-Induced Apoptosis. *American journal of respiratory cell and molecular biology*. Accepted with Revisions 2014.

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## Presentations

Macrophage Migration Inhibitory Factor (MIF) is a Novel Regulator of Cigarette smoke Induced Oxidative Injury and Emphysema Severity. American Thoracic Society International Conference in San Deigo, USA, **Poster Discussion** May 2014

Macrophage Migration Inhibitory Factor (MIF) Alters Regulatory T Cells In Response To Cigarette Smoke. American Thoracic Society International Conference, San Diego, USA, **Poster Discussion** May 2014

Potential Role of MIF in CS-induced Emphysema. Annual Retreat of the International Graduate Program "Molecular Biology and Medicine of the Lung" Universities of Giessen and Marburg Lung Center, Giessen, Germany, **Oral Presentation** June 2013

The Loss of MIF Alters the Immune Response to Cigarette Smoke: A Role for Tregs? American Thoracic Society International Conference in Philadelphia USA, **Oral Presentation** May 2013