LEUKOCYTE-DERIVED EXTRACELLULAR SUPEROXIDE DISMUTASE IN PULMONARY DISEASE

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The antioxidant enzyme extracellular superoxide dismutase (EC-SOD) is abundant in the lung and limits inflammation and tissue injury in response to many pulmonary insults. Previous studies reported a loss of full-length EC-SOD from the lung parenchyma with accumulation of proteolyzed EC-SOD in the airspace after interstitial lung injury. However, following airspace-only inflammation (pneumonia), EC-SOD accumulates in the airspace without a loss from the interstitium, suggesting this antioxidant may be released from an extrapulmonary source. Because leukocytes are known to express EC-SOD and are prevalent in the bronchoalveolar lavage fluid after injury, it was hypothesized that these cells may transport and release EC-SOD into airspaces. To investigate this, bone marrow chimeras were generated using wild-type and EC-SOD knockout (KO) mice. Following intratracheal treatment with asbestos, reconstituted mice without pulmonary EC-SOD expression, but with EC-SOD in infiltrating and resident leukocytes did not have detectable levels of EC-SOD in the airspaces. In addition, leukocyte-derived EC-SOD did not significantly lessen inflammation or early stage fibrosis.

Although these results indicate that leukocyte-derived EC-SOD is not influential in asbestos-induced interstitial lung injury, EC-SOD in these cells may play a role in attenuating pneumonias and other inflammatory diseases. To test this hypothesis, wild-type and EC-SOD KO mice were given *Escherichia coli* pneumonia. Notably, even though EC-SOD KO mice had greater pulmonary inflammation than wild-type mice, there was less bacterial clearance from their lungs following infection. While EC-SOD expression has been previously reported in

macrophages and neutrophils, its function and subcellular localization in these inflammatory cells is unclear. In this study, EC-SOD was found to be in membrane bound vesicles of phagocytes. This finding led to the hypothesis that inflammatory cell EC-SOD may play a role in antibacterial defense. To investigate this, phagocytes from wild-type and EC-SOD KO mice were evaluated. While macrophages lacking EC-SOD produced more oxidants than EC-SOD expressing cells after stimulation, they had significantly impaired phagocytosis and bacterial killing ability. Overall, these studies suggest that while EC-SOD inside leukocytes does not contribute to interstitial lung injuries, it plays a central role in mediating bacterial infections by facilitating bacterial clearance and limiting inflammation by promoting phagocytosis.

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PREFACE

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ABBREVIATIONS

ALI	acute lung injury
ARDS	adult respiratory distress syndrome
BALF	bronchoalveolar lavage fluid
BMC	bone marrow chimeric
CGD	chronic granulomatous disease
CMH	1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrrolidine
CuZn-SOD	copper/zinc superoxide dismutase
ECM	extracellular matrix
EC-SOD	extracellular superoxide dismutase
EGFP	enhanced green fluorescent protein
EPR	electron paramagnetic resonance
H_2O_2	hydrogen peroxide
IL	interleukin
ILD	interstitial lung disease
iNOS	inducible nitric oxide synthase
IPF	idiopathic pulmonary fibrosis
KO	knockout
LB	Luria-Bertani
LPS	lipopolysaccharide
MIP	macrophage inflammatory protein
Mn-SOD	manganese superoxide dismutase
MPO	myeloperoxidase

NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
$NO^{\bullet}$	nitric oxide
NOS	nitric oxide synthase
$O_2^{\bullet-}$	superoxide
$^{\bullet}OH$	hydroxyl radical
$ONOO^-$	peroxynitrite
PAMP	pathogen-associated molecular patterns
PMA	phorbol 12-myristate 13-acetate
PPH	1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine
PRR	pattern recognition receptor
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
SP	surfactant protein
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
UIP	usual interstitial pneumonia

1.0 INTRODUCTION

1.1 THE LUNG

The lung is a vital organ that is elegantly structured for its function in respiration and host defense. The major function of the lung is to exchange gases between the bloodstream and the air we breathe, oxygenating the blood while excreting carbon dioxide. In addition to respiratory gas exchange, the lung is constantly exposed to and challenged by foreign material as one breathes. Numerous immune and non-immune defense mechanisms exist in the respiratory system to efficiently remove these threats without damaging lung tissue.

1.1.1 Structure and Function of the Lung

When one breathes, inspired air enters the respiratory tract and travels through the airways, which consist of two zones: the conducting zone and respiratory zone[1]. With gradual reduction in diameter and multiple branching, the conducting airways begin at the trachea and divide into the bronchi, bronchioles, and finally down to the terminal bronchioles. At this point, the respiratory zone of the lower respiratory tract begins with the respiratory bronchioles and ends with alveoli, where gas exchange occurs[1]. The blood-gas barrier is extremely thin (approximately 0.2 μm thick) with a large surface area estimated at 80 to 140 m^2 (nearly the area of a single tennis court) making it well suited for exchanging gases by simple diffusion[2].

Specifically, this alveolated region of the lung facilitates the transport of oxygen to hemoglobin in the blood while carbon dioxide is released from the blood to the airspace to be exhaled (Figure 1).

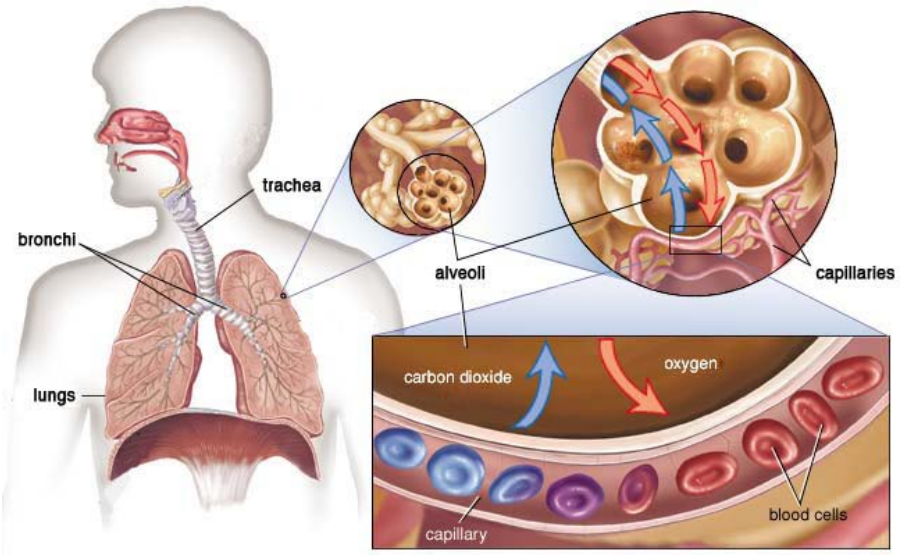

Figure 1: Respiratory Gas Exchange.

The major function of the lung is to exchange gases between the bloodstream and the air we breathe. Specifically, the alveoli of the lung facilitate the transport of oxygen to hemoglobin in the blood while carbon dioxide is released from the blood to the airspace to be exhaled. Figure modified from previous work[3].

Histologically, the lung parenchyma has a honeycomb-like morphology that is comprised of alveoli, pulmonary vessels, and bronchioles. The alveolar epithelium is comprised of three main cell types: type I epithelial cells, type II epithelial cells, and macrophages[2]. Type I cells have an extremely thin cytoplasm that stretches over the capillaries that are intertwined in the alveoli. The blood-air barrier is formed by the basal lamina of type I cells and the endothelium. Although type I cells comprise less than half of the lung's cell population, they occupy 90% of its surface area[2]. Type II cells are cuboidal, surfactant-producing cells that can serve as progenitor cells for injured type I cells. Lastly, alveolar macrophages are derived from monocytes circulating in the blood and can reside in alveoli for months or years. These resident alveolar macrophages function in immunological defense as they are responsible for sequestering

particles and foreign molecules, killing invading microorganisms, and recruiting other leukocytes in response to lung damage or infection, which will be further discussed in Section 1.1.2.2.

1.1.2 Host Defense in the Lung

Due to its extremely large surface area and the continual inhalation of air and its contents, the lung is constantly exposed to foreign particles and pathogens. Therefore, a plethora of immune and non-immune defense mechanisms exist to help remove foreign species from the respiratory system. In the lung, the barrier function and mucociliary clearance provided by the epithelium as well as the cellular innate immune response serve as the first line of defense against inhaled respiratory pathogens.

1.1.2.1 Barrier Function of the Epithelium

In the lung, the epithelium of the airways provides both a mechanical and chemical barrier against foreign material and pathogens[4]. Structurally, the surfaces of the upper and lower airways and of the alveoli are lined with an epithelial layer that rests on a basement membrane. The epithelial cells form intercellular tight junctions that provide a protective seal from the external environment[4, 5].

For additional protection, the airway epithelium is also involved in mucociliary clearance, which is designed to remove unwanted pathogens and foreign material. Ciliated cells and mucus-producing goblet cells of the airway epithelium are primarily involved in this defense mechanism. In the large airways, the submucosal glands, which lie beneath the epithelium and connect to the surface by ducts, and goblet cells of surface epithelium are both responsible for mucus production[5]. In the highly efficient mucociliary clearance system, mucus coats the

inhaled pathogen or particle preventing it from adhering to the epithelium and the movement of the cilia of the ciliated cells transports the contaminated mucus towards the oropharyngeal cavity, where it is removed by swallowing or expelled by coughing[5]. Mucus also contains antimicrobial peptides and proteins as well as high molecular weight mucin glycoproteins that assist in immunologic defense by directly targeting the microorganisms in the lung[6].

In addition to mucus, pulmonary surfactants also play a key role in the innate immune system by controlling inflammation and prevent microbial infection of the distal lung[7]. Type II alveolar epithelial and clara cells of the terminal bronchioles are the source of surfactant protein (SP)-A, SP-B, SP-C, and SP-D[2]. All pulmonary surfactants are vital for normal alveolar function. Among these surfactant components, SP-A and SP-D are well characterized and are known to be important in lung host defense. Specially, SP-A and SP-D bind and coat the surface of pathogens, inflict damage to microbial membranes, and mediate microbial phagocytosis (opsonization) as well as alter inflammatory responses by alveolar macrophages[8].

Epithelial cells function as more than just a mere physical barrier; they also have an important role in the innate immune response by producing a range of chemical substances to assist immune cells in removing the threat. The epithelium serves as a source of antimicrobial peptides and proteins and anti-inflammatory molecules that are important for host defense[5]. In addition to these effector molecules that directly target the foreign material, epithelial cells can also serve as orchestrators of innate immunity by secreting cytokines, chemokines, and growth factors, which further activate the innate immune response in the lung[9, 10].

1.1.2.2 Innate Immune Cells and Mediators

The innate immune system is an inherent, constantly active, and antigen-nonspecific defense mechanism that involves an initial cellular response to eliminate microbes and prevent infection.

The response of the innate immune system occurs immediately or within hours of exposure to the foreign substance[4]. Phagocytic cells, such as macrophages and neutrophils, play a key role in innate immunity because they recognize, ingest, and destroy invading foreign pathogens and particles. Such pathogens must overcome this defense to establish a pulmonary infection or induce pulmonary injury.

When inhaled pathogens and particles avoid the mucociliary clearance system and reach the epithelium of the lower respiratory tract, resident macrophages are present to provide additional defense and attempt to remove these substances. Macrophages are long-lived immune cells that are present in both the interstitium and the alveoli. These cells are continuously derived from monocytes produced from the bone marrow that migrate into the tissue and mature[5]. In most instances, macrophages eliminate threats from the lung without initiating an inflammatory response or activating host immune responses. In fact, up to 10^{10} particles per day reach the alveoli and are cleared unknowingly due to the efficient defense mechanisms of the epithelium and resident alveolar macrophages[11]. However, if the infection or particulate burden overwhelms this defense mechanism, alveolar macrophages and other pulmonary cells, such as epithelial cells, secrete cytokines and chemokines to initiate inflammation and to recruit neutrophils to the site of injury or infection to assist with the elimination of the microbe.

Neutrophils are short-lived phagocytes that serve as the second line of defense against the pulmonary threat. Although neutrophils are normally not present in the pulmonary airspaces, they are poised near the walls of the lung microvasculature to rapidly migrate into the lung when recruited during an infection or injury[4]. Therefore, the presence of neutrophils in the lung is indicative of inflammation and may promote severe lung injury. In fact, excessive neutrophil accumulation leads to acute lung injury and acute respiratory distress syndrome (ARDS)[12, 13].

Microbial recognition in innate immunity is mediated by pattern recognition receptors (PRRs) expressed by phagocytes and soluble proteins in the blood and extracellular fluids[14]. PRRs are present on the cell surface, in endosomal vesicles, and in the cytoplasm of phagocytes and are designed to recognize conserved structures that are characteristic of microbial pathogens, but not mammalian cells. The microbial substances that interact with PRRs are called pathogen-associated molecular patterns (PAMPs)[14]. Different classes of microbes (i.e. gram-positive bacteria, gram-negative bacteria, fungi, and viruses) express different PAMPs, such as unmethlyated CpG DNA, bacterial lipids and carbohydrates, and viral RNA[14]. Although the innate immune system has limited diversity when compared to the adaptive immune system, it is estimated that the innate immune system can recognize about 10^3 different PAMPs[14]. In addition to these cell-associated pattern recognition receptors, there are soluble recognition molecules in the blood and extracellular fluids (e.g. collectins in the alveoli) that facilitate clearance by enhancing uptake into cells via opsonization or by activated extracellular killing mechanisms.

There are several types of PRRs expressed on the cell surface that recognize PAMPs and opsonin-coated microbes. Some PRRs, such as toll-like receptors (TLRs), promote inflammatory responses to enhance killing of microbes; whereas other PRRs, including C-type lectins (e.g. mannose receptors), scavenger receptors, and Fc receptors, mainly participate in the uptake of microbes[14]. In addition to receptors on the plasma membrane, nucleotide-binding oligomerization domain-like receptors and caspase activation and recruitment domain-containing proteins are cytoplasmic receptors that recognize intracellular pathogens and activate inflammatory responses.

The direct binding of pathogens, particles, or opsonized microbes to cell surface receptors, such as C-type lectins, scavenger receptors, and Fc receptors, leads to phagocytosis (Figure 2). Phagocytosis is a dynamic and energy-dependent process by which neutrophils and macrophages engulf large particles (>0.5 μm in diameter)[14]. Once a microbe binds the receptors on the phagocyte, the plasma membrane in the region of the receptor begins to redistribute, and extends a cup-shaped projection around the microbe. By sequential receptor-ligand interactions, this protruding membrane extends beyond the diameter of the pathogen or particle and fuses at the apex of the foreign substance to form an intracellular vesicle. This vesicle, called a phagosome, contains the ingested foreign particle or pathogen and breaks away from the plasma membrane. Once inside the cell, phagosomes fuse with lysosomes to generate phagolysosomes in which the pathogen or particle is destroyed by proteolytic enzymes, reactive oxygen species (ROS), and reactive nitrogen species (RNS). Notably, ROS can be generated by a multi-component, membrane associated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in a process known as the respiratory burst[15]. Briefly, NADPH oxidase reduces molecular oxygen (O_2) into ROS, such as superoxide ($O_2^{\bullet -}$) radicals, with the reduced from of NADPH acting as a cofactor. In addition to ROS, macrophages produce RNS, mainly nitric oxide ($NO^{\bullet}$) by the action of the cytosolic enzyme, inducible nitric oxide synthase (iNOS). ROS and RNS generation for the purpose of host defense will be further discussed in Section 1.5.4.

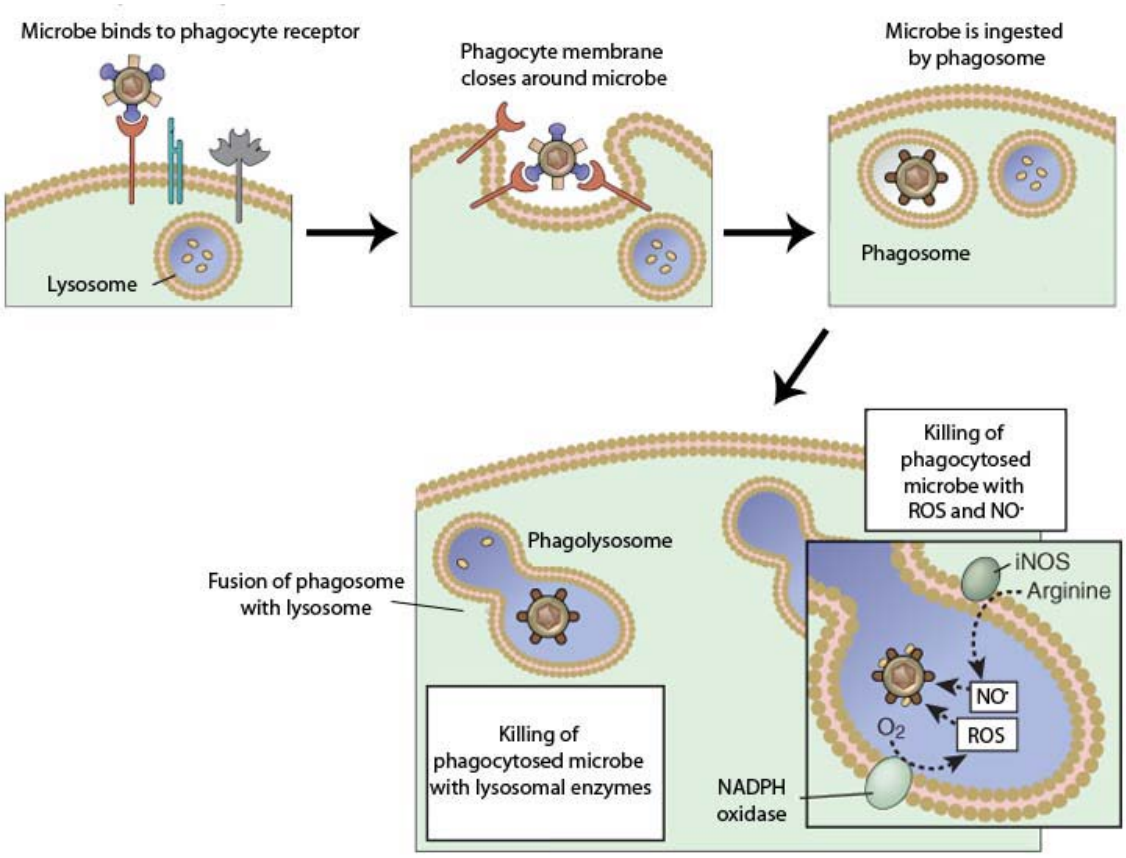

Figure 2: Phagocytosis and intracellular destruction of microbes.

The first step in phagocytosis is the recognition of the microbe or particle. Some cell surface receptors directly bind these substances and others recognize and bind opsonized microbes or particles. The foreign microbe or particle is internalized into a phagosome, which then fuses with the lysosome to form the phagolysosome. Once in the phagolysosome, the microbe is killed by reactive oxygen or nitrogen species (ROS and RNS, respectively) and proteolytic enzymes. (iNOS=inducible nitric oxide synthase, O_2=molecular oxygen, $NO^{\bullet}$=nitric oxide) Figure modified from previous work[14].

Aside from phagocytosis, other PRRs serve to signal microbial presence and mount the appropriate immune response. The most well studied and characterized PRRs are the TLRs that are expressed on various immune cells including epithelial cells, leukocytes, and lymphocytes[16]. Phagocytes have both plasma membrane-bound (TLR1, TLR2, TLR4, and TLR5) and endosome membrane-bound TLRs (TLR3, TLR7, TLR8, and TLR9) that are able to recognize bacterial pathogens in the lung[17, 18]. Ligand recognition by the TLRs stimulates a complex signaling cascade that leads to the activation of transcription factors (one such being nuclear factor-κB

(NF-κB)) and production of pro-inflammatory cytokines and chemokines. Specifically, activation and signaling through these receptors in response to microbial components leads to the release of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, IL-12, and tumor necrosis factor (TNF)-α, as well as the chemokines, such as IL-8, keratinocyte-derived chemokines, macrophage inflammatory protein (MIP)-2 from activated immune cells[4, 17, 18].

Successful engulfment and ingestion of a pathogen or particle is necessary to contain the antimicrobial arsenal in the phagolysosome. However, when neutrophils and macrophages are strongly activated or are unable to phagocytose the foreign pathogen or particle, these cells can release lysosomal enzymes, ROS, and RNS that can injure normal host tissue. Therefore, while the antimicrobial arsenal of phagocytic cells is beneficial in eradicating infections, it also can inadvertently damage the lung and lead to serious and lethal complications, such as sepsis and ARDS[19-21].

1.2 INTERSTITIAL LUNG DISEASE

Interstitial lung disease (ILD) is the general term for a group of potentially debilitating pulmonary disorders characterized by excessive deposition of collagen in the lungs. Thickening of the alveolar septa significantly decreases lung elasticity and severely impairs normal respiration. ILDs can occur spontaneously (idiopathic pulmonary fibrosis) or in response to known stimuli such as environmental, occupational, or chemical exposures (e.g. asbestosis, silicosis, bleomycin- or radiation-induced pulmonary fibrosis), secondary to other diseases (collagen vascular disease, scleroderma, and rheumatoid arthritis), or a familial syndrome[22, 23]. ILDs have high rates of morbidity and mortality due to both pulmonary and cardiovascular

complications. The pathogenesis of ILDs is not fully understood, but oxidant/antioxidant imbalances and inflammation are believed to contribute to these diseases.

1.2.1 Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is the most common ILD characterized by irreversible and progressive fibrosis. Currently, it is estimated that the prevalence of IPF is 14-42.7 per 100,000 people with an incidence of 6.8-14 cases per 100,000 in the general population[24]. The prognosis for IPF is poor and the median survival is 3 to 5 years after diagnosis[24, 25]. The prevalence of IPF is higher in men than women and is known to increase with age[26]. In fact, IPF most commonly presents in patients between the ages of 50 and 70. The median age at the time of diagnosis is 66 years old[24, 26].

1.2.1.1 Clinical and Pathology Features

IPF is a progressive, life-threatening ILD of unknown etiology. Patients usually present with a nonproductive cough and shortness of breath, or dyspnea, upon exertion[27]. As the disease progresses, these symptoms become more severe as the collagen deposition restricts the ability for the lung to fully expand. The majority of IPF patients eventually die from hypoxemia resulting from reduced oxygen exchange due to alveolar fibrosis and respiratory failure. However, pulmonary hypertension[28, 29], acute exacerbations[30, 31], and pneumonia[32] are also causes of ILD-associated death. Acute exacerbations occur in approximately 10-15% of all IPF patients and are characterized by rapid deterioration of lung function, increased hypoxemia, acute inflammation, and the absence of an infection, heart failure, and sepsis[30, 32].

IPF is diagnosed using information gathered from physical examination, chest radiography, pulmonary function tests, high-resolution computed tomography, and lung biopsy. The American Thoracic and European Respiratory Societies established specific diagnosis criteria for IPF, which includes: 1) surgical biopsy revealing a histologic pattern consistent with usual interstitial pneumonia (UIP, described below), 2) exclusion of known causes of ILDs (i.e. chemical and environmental exposures), 3) abnormal pulmonary physiology with evidence of restriction and impaired gas exchange, and 4) patchy areas of fibrosis in the peripheral, subpleural and basilar areas of the lung identified by high-resolution computed tomography and chest radiography[27].

IPF is characterized histopathologically by the presence of UIP. UIP is characterized by temporal and spatial heterogeneity meaning the histologic presence of areas of normal lung intermixed with immature/active and mature interstitial fibrosis[33]. This temporal heterogeneity with fibroblastic foci, or areas of active fibrosis characterized by aggregates of spindle-shaped myofibroblasts, is the hallmark of UIP. Moderate inflammation is also present mainly in fibrotic areas and is comprised of macrophages, neutrophils, lymphocytes, mast cells, and eosinophils[33, 34]. Upon gross pathological examination, the fibrosis in the lung is most pronounced in the lower lobes and in subpleural areas. As the disease progresses, the normal lung architecture becomes increasingly distorted and takes on a honeycomb-like appearance, which is indicative of end-stage disease[33, 34]. Bronchoalveolar lavage fluid (BALF) from the lungs also reveals the presence of inflammatory cells similar to what is seen histologically.

1.2.1.2 Treatments for IPF

Currently available therapies for IPF are inadequate as they provide little benefit to the patients. Established treatment modalities include the use of immunosuppressive/cytotoxic drugs

(cyclophosphamide and azathioprine), anti-inflammatory agents (corticosteroids, prednisone), anti-fibrotic agents (colchicine and interferon-γ), and antioxidants (N-acetylcysteine)[35]. Whether used alone or in combination, these drugs have limited to modest therapeutic success for IPF patients[36-38]. In addition, many treatments are limited by severe adverse side effects[39, 40].

Novel anti-inflammatory (etanercept) and anti-fibrotic (pirfenidone) agents for the treatment of IPF are currently under clinical investigation, but have not shown significant benefits to patients thus far[41]. To date, lung transplantation is the only effective treatment for IPF[42] and the 5-year survival rate after transplantation is approximately 50-60%[43]. With ineffective treatment options, the outcome for patients with IPF and other ILDs is dismal. Therefore, the need for new therapies to treat this debilitating disease is evident.

1.2.2 Asbestosis

Asbestosis is an ILD caused by the inhalation of asbestos fibers typically from environmental or occupational exposure. Asbestosis is further classified as a form of pneumoconiosis, or pulmonary disease caused by excessive amounts of dust in the lung[44]. In addition to asbestosis, asbestos exposure can lead to the development of other fatal non-malignant pulmonary diseases (pleural plaques and pleural effusions) as well as lung cancer (mesothelioma and bronchogenic carcinoma)[45, 46].

Asbestos-induced lung diseases are a significant health concern as approximately 27 million workers were exposed to aerosolized asbestos fibers between 1940 and 1979[47]. Specifically, miners, millers, and textile, insulation, and shipyard workers have the highest risk of asbestos-related diseases due to their heavy occupational asbestos exposure[48, 49]. Symptoms typically present 20 to 40 years after the initial exposure to asbestos[47].

1.2.2.1 Asbestos

Asbestos is a term for naturally occurring silica-containing mineral fibers that have high tensile strength and heat and chemical resistance making them ideal for commercial and industrial applications[49]. The types of asbestos are divided into two groups: serpentine and amphiboles. Chrysotile asbestos is the sole member of the serpentine group and the fibers are curly and flexible[50]. In the United States, approximately 95% of the asbestos used commercially was chrysotile asbestos[48]. Unlike the serpentine group, amphiboles are straight, rigid fibers and include crocidolite (blue asbestos), amosite (brown asbestos), tremolite, anthrophyllite, and actinolite[50]. Amosite and crocidolite asbestos were the primary amphiboles used commercially. Generally, amphiboles are considered to be more toxic than chrysotile asbestos as amphibole fibers are rigid, more durable, become easily lodged in the lung parenchyma, and are difficult to clear from the lung.

1.2.2.2 Clinical and Pathologic Features

The clinical, physiological, and pathological findings of asbestosis are similar to that of IPF. Clinically, patients present with dyspnea and dry cough and pulmonary function tests reveal defects in lung physiology[48]. Notably, asbestos exposure leads to irreversible and progressive fibrosis analogous to IPF. Histologic diagnosis of asbestosis requires the presence of diffuse and bilateral pulmonary interstitial fibrosis, with the classical UIP pattern (temporal heterogeneity) and the presence of asbestos bodies in the lung[44]. Under light microscopy, asbestos bodies appear as golden brown, segmented, dumbbell-shaped structures covering a fiber[51] (Figure 3). This iron-containing proteinous coating is thought to result from the inability of alveolar macrophages to effectively phagocytosis the fiber (frustrated phagocytosis) and increased exocytotic activity[52]. This coating makes the asbestos fiber nonfibrogenic and noncytotoxic

when compared to an uncoated fiber[51], suggesting that this is a compensatory host defense mechanism.

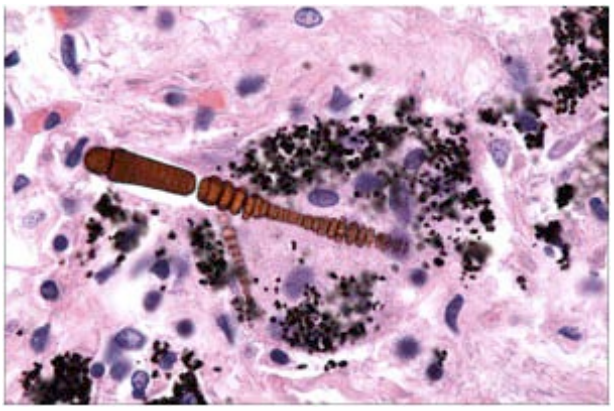

Figure 3: Asbestos body.

Asbestos bodies are asbestos fibers that have become covered with iron, protein, and polysaccharides and have a golden brown, dumbbell shaped appearance microscopically. The process of asbestos body formation is not well understood but is thought to involve the action of macrophages. Accumulations of black anthracitic pigments are also visible in this lung section. Image courtesy of Dr. Tim Oury.

Similar to IPF, gross pathological findings reveal that asbestosis presents as a diffuse interstitial fibrosis in the lower region of the lungs close to the pleura. Honeycombing is also often seen in advance disease[48]. However, asbestosis differs from IPF in that the clinical manifestations are less severe and the rate of disease progression is generally slower[44]. Studies have also shown that asbestosis increases the risk of lung cancer and mesothelioma. The usual therapeutic approaches for fibrotic lung diseases (discussed in Section 1.2.1.2) are also ineffective for the treatment of asbestosis[45].

1.2.3 Pathogenesis of IPF and Asbestosis

ILDs, like IPF and asbestosis, are largely considered diseases of abnormal wound repair that result in pulmonary fibrosis[53]. The underlying mechanisms behind the development of fibrosis are thought to involve alveolar re-epithelialization, myofibroblast differentiation/activation,

matrix remodeling, oxidative stress, and inflammation[54]. Particularly, the role of inflammation in disease pathogenesis is controversial as therapeutic strategies to suppress inflammation, such as corticosteroids, have not been successful for treating these conditions[36-38]. However, inflammation is still seen to be important in ILD pathogenesis as inflammatory cells and mediators are detected in BALF and lung biopsies of IPF patients and fatal acute exacerbations are characterized by an immense inflammatory cell influx[55]. Specifically, increased numbers of alveolar macrophages, neutrophils, and eosinophils are seen in the BALF of IPF patients[56-58] and have been associated with worse prognosis[59, 60]. In addition to inflammation in the airspaces, neutrophil infiltration is observed in the honeycomb-like fibrotic areas of lung biopsies from IPF patients and is correlated with decreased survival[61].

Both activated inflammatory cells and alveolar epithelial cells release oxidants, proteinases, cytokines, chemokines, and growth factors that can induce both inflammation and fibrogenesis in the lung. Numerous cytokines, chemokines, and growth factors are involved in chemotaxis and the activation of immune cells. More importantly, many pro-inflammatory cytokines and mediators are known to be elevated in lung tissue and fluids from IPF patients, including but not limited to IL-4[62], IL-5[62], IL-13[62], MIP-1α[63], monocyte chemoattractant protein-1[64], IL-8[64, 65], and macrophage colony-stimulating factor[66, 67]. In addition to inflammation, inflammatory cells are essential for the production and activation of mediators of fibrogenesis, such as transforming growth factor (TGF)-β [68, 69], matrix metalloproteinases, and platelet derived growth factor[70]. These pro-fibrotic mediators are also found to be increased in the lungs of IPF patients[68, 70]. Overall, these studies show that inflammation plays a role in the pathogenesis of ILDs by inducing tissue damage, contributing to a pro-fibrotic environment, and altering remodeling in the lung.

While studies have shown asbestosis has many of the same underlying molecular mechanism as IPF, asbestos fibers in the lung also directly contribute to the pathogenesis of this disease. As macrophages unsuccessfully attempt to phagocytose the asbestos fibers, this frustrated phagocytosis stimulates the release of copious amount of pro-inflammatory signals and ROS[71, 72] from these cells, which may contribute to the pathogenesis of this disease.

1.3 PULMONARY INFECTION

Globally, acute lung infections are the leading infectious cause of premature death with a greater disease burden than either cancer or HIV/AIDS, and its incidence is not affected by socioeconomic status[12]. In the United States, pulmonary infections in the form of pneumonia are responsible for one sixth of all deaths[73]. Despite advances in antimicrobial therapy, rates of pneumonia mortality have not decreased within the past 50 years[12, 13, 74]. In addition, the emergence of antibiotic-resistant strains and new organisms is constant and complicates the efficiency of new and current therapies. Severe forms of pneumonia can substantially compromise normal lung function and can lead to serious respiratory complications such as ARDS.

1.3.1 Causes and Diagnosis

Pneumonia is broadly defined as any infection located in the lung and affects people of any age. However, infection is more serious in patients who are very young, over 65 years of age, have underlying medical problems such as heart disease, diabetes, sickle cell disease, and chronic lung

disease, or are immunocompromised[75]. Pneumonia is commonly spread from person to person by contact with infected respiratory secretions.

Pneumonia is an infection of the gas-exchanging alveoli of the lung that is most commonly caused by bacteria, but can also be caused by viruses, fungi, parasites, and other infectious agents. It may present as an acute or chronic disease. In addition, this alveolar infection can manifest in an entire lobe of the lung (lobar pneumonia) or can have a patchy distribution that generally involves more than one lobe adjacent to the bronchi (bronchopneumonia)[76].

Routine testing for microbial etiology is not usually performed because empiric treatment is extremely effective[77]. However, microbial diagnosis, often through examination of Gram-stained sputum and blood cultures, is important for patients with pneumonia acquired in a clinical setting as these patients are usually already taking high doses of antibiotics. However, the presence of an infiltrate on plain chest radiograph is considered the standard for diagnosing pneumonia when clinical and microbiological features are also present[78].

Pneumonias are classified as typical or atypical depending on their clinical presentation[76]. Patients with typical pneumonia present with a sudden onset of high fever, chills, pleuritic chest pain, and a productive mucopurulent cough that are normally due to a bacterial pathogen[73]. On the other hand, atypical pneumonias often develop as the results of a viral infection and patients present with fever without chills and a non-productive cough with scant and watery sputum production, which preceded upper respiratory symptoms[76].

Pneumonias can also be classified either by the specific pathogen or by the clinical setting in which the infection was occurred. Even if a specific pathogen cannot be determined, pneumonia acquired from each distinct setting has common pathogens that are implicated in

disease pathogenesis. There are a number of pneumonia syndromes, but the most frequently seen are community-acquired acute and atypical pneumonias and nosocomial or hospital acquired pneumonia[73]. *Streptococcus pneumoniae* is the most common cause of community-acquired acute pneumonia leading to hospitalization in adults[79]. The histopathologic hallmark of acute pneumonia is the presence of neutrophils within the alveolar spaces. Unlike community acquired acute pneumonia, community acquired atypical pneumonias usually result from viral, *Chlamydia pneumoniae*, or *Mycoplasma pneumoniae* infections and histologically mononuclear leukocytes accumulate in the thickened alveolar wall instead of the airspaces[73]. Nosocomial or hospital acquired pneumonia is acquired by patients in a hospital setting and can result from mechanical ventilation referred to as ventilator-associated pneumonia[76]. Nosocomial infections are often caused by more resistant strains of microbes[73] (i.e. *Escherichia coli*, *Pseudomonas* species, and *Staphylococcus aureus*) as these patients are usually already taking high doses of antibiotics.

While pneumonias are usually treated successfully, severe lung infection can occur and lead to serious complications including tissue destruction and necrosis (lung abscess) and infection outside of the lung in the pleural space (empyema)[76]. Furthermore, primary pneumonia, which can be viral, bacterial, or fungal, is the major precursor of serious and lethal complications, such as ARDS and sepsis[80]. With no effective pharmacological therapy, ARDS is a debilitating and severe acute lung injury (ALI) with high morbidity and mortality due to sepsis and multiple organ failure[81]. In the United States, ALI/ARDS occur in approximately 190,000 people annually with an estimated mortality rate of around 40%[82]. Similar to ILDs, there are no effective pharmacological treatments for ALI/ARDS. However, limiting the severity of

pneumonias would likely be beneficial for preventing these complications, as pneumonia is the most common cause of ALI/ARDS.

1.3.2 Treatment

Patients with community-acquired pneumonias are primarily managed outside of the hospital with oral antibiotic treatment; however, elderly patients (over 65 years of age) represent approximately 60% of patients hospitalized for this condition[83]. Respiratory infections are typically treated with antibiotics given orally (for community-acquired pneumonias) or through inhalation (ventilator-acquired pneumonia) or intravenous administration (for nosocomial pneumonias). Systemic corticosteroid therapy is also being used, but has demonstrated limited success[84-86].

Although treatment for pneumococcal infections with penicillin and other antibiotics in most cases is effective, some stains of microbes have become resistant to these drugs. Therefore, prevention of pneumonia by vaccination has become increasingly important for high risk individuals, which include those who are less than two years old or more than 65 years old, who have long-term preexisting health problems, and who have weakened immune systems. Other risk factors include smoking and a previous diagnosis of asthma. Currently, a 23-valet polysaccharide pneumococcal vaccine (PPV-23) for use in adults and a 13-valet conjugate vaccine (PCV-13) for use in infants are available to protect against the most common strains of pneumococcal bacteria responsible for severe pneumococcal infections[87]. To date, the efficacy of the pneumococcal polysaccharide vaccine for preventing pneumococcal infection is controversial[88-92].

1.3.3 Pathogenesis of ALI/ARDS

Although the pathogenesis of ALI/ARDS is not fully understood, neutrophil-dependent endothelial and epithelial injury are thought to contribute to the accumulation of protein-rich edema seen in the lungs of ALI/ARDS patients[80]. Injury to the endothelium is thought to result from excessive neutrophil accumulation and activation in the microvasculature in the lung. Activated neutrophils extravasate across the endothelium into the injured tissue and release toxic agents, like proteases (matrix metalloproteinases and elastase), defensins, oxidants, and pro-inflammatory cytokines[80, 93]. In addition to activating neutrophils, these pro-inflammatory mediators stimulate the endothelium to increase expression of the potent neutrophil chemoattractants, IL-8, and leukocyte adhesion molecules on the cell surface[93]. These inflammatory mediators in turn increase vascular permeability and directly damage the endothelium. In addition to endothelial injury, transepithelial migration of neutrophils into the airspaces of the lung is also thought to be involved in the pathogenesis of ALI/ARDS. Under physiological conditions, neutrophils migrate across the epithelium into the airspaces without compromising the integrity of the epithelial surface[94, 95]. However, activated neutrophils are thought to damage the alveolar epithelium by releasing toxic proteases, ROS, and RNS that induce the dissolution of tight junctions and alveolar epithelium cell death[96].

1.4 MURINE MODELS OF PULMONARY DISEASE

Studying the pathogenesis of pulmonary diseases in humans is difficult and confounded by the fact that patients already have advanced disease when they present with symptoms and receive

medical care. Therefore, researchers use *in vivo* animal models to study ILDs and respiratory infections to elucidate and better understand the mechanisms underlying these conditions. There are advantages and disadvantages to each animal model and no one model accurately recapitulates human lung disease pathogenesis. However, animal models are essential to study the evolution of these conditions and have led to the discovery of many key components and mediators of disease pathogenesis in animals that are likely also involved in human disease. Specifically, the models of asbestos-induced interstitial lung injury and bacterial pneumonia in mice will be discussed further as these models were used to investigate the role of cellular extracellular superoxide dismutase (EC-SOD) in pulmonary inflammation.

1.4.1 Asbestos-induced Interstitial Lung Injury

Numerous animal models of ILD exist, such as bleomycin, asbestos, silica, irradiation, fluorescein isothiocyanate, transgenic models with pulmonary specific transgenes, and virus targeted transgene delivery[97]. Bleomycin and asbestos are the most commonly used agents and have been shown to cause acute inflammation followed by abnormal repair and fibrosis in the lung. Although there are numerous methods of administration, intratracheal instillation of these agents is an efficient method as it allows for exact dosing, uniform distribution through the lungs, and rapid inflammatory response and development of fibrosis as early as 7 days post-exposure.

Although bleomycin is the most prevalent model used, the fibrosis that develops following bleomycin treatment is self-limiting with a homogenous distribution in the lung. Notably, asbestos instillation in animals leads to irreversible and progressive fibrosis with temporal heterogeneity, which is histologically similar to IPF (described in Section 1.2.1.1).

Although controversial, the asbestos-induced interstitial lung injury method likely serves as the best model for studying the mechanisms underlying the pathogenesis of asbestosis and IPF. Another advantage to using the asbestos model is that the fibers can be seen in the BALF as well as in fibrotic areas in the lung providing objective evidence that the intratracheal instillation was successful.

The murine model of asbestosis produces a progressive peribronchial and alveolar septal fibrosis[98, 99]. In mice, crocidolite asbestos induces an acute inflammatory response that develops within 24 hours and remains during the first seven days following exposure[98]. Both interstitial and airspace inflammation are present. Typically, the inflammation is predominantly neutrophils, but macrophages are also present and are thought to play an important role in disease progression[98, 100, 101]. The development of fibrosis following crocidolite asbestos exposure is observed as early as seven days and continues to progress, never resolving.

1.4.2 Experimental Models of Bacterial Pneumonia

Human pneumonia is modeled with experimental infections in animals, most frequently mice. Bacterial inoculation in the lungs causes inflammation that leads to the accumulation of a large number of neutrophils in the airspaces, but does not lead to significant amounts of interstitial inflammation or fibrosis[102, 103].

Several methods exist to produce experimental pneumonia in mice, each with its own considerations. Common methods include, but are not limited to inhalation of aerosolized microorganisms, intranasal instillation, and intratracheal inoculation[104]. In particular, intratracheal instillation of a microbial suspension allows for direct inoculation of the lower

respiratory tract and is advantageous as it permits precise dosing to the lungs to each individual animal.

1.5 OXIDATIVE STRESS

Living organisms are constantly exposed to ROS and RNS as a consequence of normal metabolic processes or abnormal reactions and disease. Specifically, these reactive species can cause chemical modifications in lipids, proteins, DNA, and transcription factors that can seriously impair the biological functions of these molecules. To compensate for the presence of ROS and RNS, endogenous antioxidant defenses are necessary to remove these reactive species and prevent oxidative damage to the surrounding tissues. Oxidative stress results from a disruption of this balance either from increased production of ROS and/or RNS or decreased levels of antioxidants and other scavenging enzymes, and may lead to tissue damage and cellular dysfunction. The lung is especially vulnerable to oxidative damage due to its high oxygen tension and large epithelial surface area that is constantly bombarded by particulates (discussed in Section 1.1). Therefore, various antioxidants are present to scavenge these reactive species and maintain low levels of oxidants in the lung.

1.5.1 Reactive Oxygen Species

Oxygen is necessary for the biological processes within all aerobic organisms. Under normal conditions, molecular oxygen is a diradical as it has two unpaired electrons[105, 106]. The majority of cellular oxygen is consumed by the mitochondria, where it acts as a terminal electron acceptor

and undergoes a tetravalent reduction to water in a reaction catalyzed by cytochrome oxidase in the mitochondrial electron transport chain[107]. Oxygen can also be reduced via a non-enzymatic pathway through four sequential one-electron reductions[107].

When oxygen (O_2) undergoes four sequential one-electron reductions, a number of ROS are formed before conversion to water. These ROS include but are not limited to superoxide ($O_2^{\bullet-}$, Reaction 1), hydrogen peroxide (H_2O_2, Reaction 2), and hydroxyl radical ($^{\bullet}OH$, Reaction 3). Although molecular oxygen is not very reactive itself, these metabolites of oxygen rapidly

$$O_2 + e^- \rightarrow O_2^{\bullet-} \quad (1)$$

$$O_2^{\bullet-} + e^- + 2H^+ \rightarrow H_2O_2 \quad (2)$$

$$H_2O_2 + e^- \rightarrow OH^- + {}^{\bullet}OH \quad (3)$$

$$^{\bullet}OH + e^- + H^+ \rightarrow H_2O \quad (4)$$

participate in other chemical reactions that can initiate cell and tissue damage. Of these metabolites of oxygen, $O_2^{\bullet-}$ and $^{\bullet}OH$ are free radical species as they contain one unpaired electron, whereas H_2O_2 is not.

The $O_2^{\bullet-}$ radical anion is always produced as a result of a one-electron reduction of molecular oxygen (Reaction 1). However, $O_2^{\bullet-}$ can also be formed from the accidental leakage of electrons from the mitochondrial respiration chain and through enzymatic reactions (xanthine oxidase and NADPH oxidase). Specifically, activated macrophages and neutrophils, among many other cells, can produce $O_2^{\bullet-}$ using NADPH oxidase[107] that is necessary for host defense. $O_2^{\bullet-}$ is not able to diffuse freely across plasma membranes due to its charge, but can utilize anion channels to move in and out of cells[108]. In addition, $O_2^{\bullet-}$ can undergo further reduction to form additional ROS.

Although $O_2^{\bullet-}$ is a free radical, it is relatively stable and may act either as a reductant (electron donor) or as an oxidant (electron acceptor). Indeed, $O_2^{\bullet-}$ is seen to act as both an oxidizing and reducing agent in the spontaneous or enzymatically-calatyzed dismutation reaction by the antioxidant enzymes, superoxide dismutases (SODs)[108] (Reaction 5). In this dismutation reaction, half of the $O_2^{\bullet-}$ is oxidized to O_2, while the other half is reduced to H_2O_2.

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (5)$$

In addition to H_2O_2, $O_2^{\bullet-}$ is also a precursor for $^{\bullet}OH$. $O_2^{\bullet-}$ can produce $^{\bullet}OH$ through the direct reduction of H_2O_2 (Reaction 8). This reaction, known historically as the Haber-Weiss reaction, is thermodynamically unfavorable in biological systems without the presence of metal ions[109]. In the iron-catalyzed Haber-Weiss reaction, $O_2^{\bullet-}$ acts as a reductant by reducing ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (Reaction 6) followed by the Fenton reaction in which Fe^{2+} catalyzes the transformation of H_2O_2 into the most reactive radical species, $^{\bullet}OH$ (Reaction 7)[109].

Although other transition metal ions are capable of catalyzing this reaction, the iron-

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \qquad (6)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \qquad (7)$$

$$\text{Net Reaction: } H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + {}^{\bullet}OH + OH^- \qquad (8)$$

catalyzed Haber-Weiss reaction is thought to be one major mechanism by which the highly reactive $^{\bullet}OH$ is produced biologically[108, 110]. Free metals available to undergo oxidation/reduction and catalyze this reaction are rare as they normally found bound to carrier proteins and inaccessible for redox reactions. Therefore, this reaction is limited by the availability and location of metal ions in the body. However, this reaction can readily occur in response to the presence of exogenous metal ions. Notably, asbestos fibers are a source of free

iron that could catalyze ROS production[111] thereby contributing to the development of interstitial lung injury. One study noted that ROS generated by the iron in chrysotile and crocidolite asbestos, can activate the cytokine TGF-β_1, while addition of antioxidants (e.g. SODs) abrogated this effect [112].

$^{\bullet}$OH has a very short half-life and has the highest oxidation potential of all aerobic metabolites[105]. $^{\bullet}$OH is too reactive to traverse cell or organelle membranes[106] and therefore acts locally and will react with any biomolecules. In addition to the reaction of H_2O_2 with metal ions, the formation of $^{\bullet}$OH can also occur by the breakdown of peroxynitrite ($ONOO^-$, Section 1.5.2).

1.5.2 Reactive Nitrogen Species

Similar to oxidative stress, nitrosative stress occurs in disease states in which excess RNS leads to the modification of biological molecules. $NO^{\bullet}$ is a free radical capable of forming other powerful oxidants that modify and alter the function of proteins. $NO^{\bullet}$ is produced by nitric oxide synthases (NOSs) from L-arginine and oxygen[113]. Of the three isoforms, neuronal NOS and endothelial NOS produce low levels of $NO^{\bullet}$. The third isoform, iNOS can produce high levels of $NO^{\bullet}$, up to 20 times more than the other NOS enzymes[114]. iNOS was first identified and has been extensively studied in macrophages. iNOS is normally absent, but can be induced by bacterial lipopolysaccharide (LPS) and cytokines (TNF-α, IL-1β, and interferon-γ) to produce high levels of $NO^{\bullet}$[114, 115]. Indeed, production of $NO^{\bullet}$ is an essential part of the nonspecific immune defense system[116]. $NO^{\bullet}$ can easily diffuse in and out of cells. One electron reduction reactions of $NO^{\bullet}$ will form other nitrogen species: nitrite (NO_2^-), nitrogen dioxide ($NO_2^{\bullet}$), and finally nitrate (NO_3^-)[115].

The free radical $NO^{\bullet}$ can react with other species with unpaired electrons to form other powerful oxidants. $NO^{\bullet}$ can react very rapidly with $O_2^{\bullet-}$ (1.9×10^{10} $M^{-1}s^{-1}$) to produce peroxynitrite ($ONOO^-$, Reaction 9)[106] that can oxidize thiols and modify tyrosine residues on proteins. Notably, the reaction between $O_2^{\bullet-}$ and $NO^{\bullet}$ occurs more rapidly than the SOD-catalyzed dismutation of $O_2^{\bullet-}$. Although stable at an alkaline pH, $ONOO^-$ is easily protonated (pKa = 6.8[117]) to form peroxynitrous acid (ONOOH, Reaction 10) and then rapidly decomposes to form $^{\bullet}OH$ and $NO_2^{\bullet}$ in physiological or acidic conditions (Reaction 11).

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^- \quad (9)$$

$$ONOO^- + H^+ \rightarrow ONOOH \quad (10)$$

$$ONOOH \rightarrow {}^{\bullet}OH + NO_2^{\bullet} \quad (11)$$

1.5.3 Antioxidant Defenses in the Lung

The lungs are at risk for oxidant-mediated damage as they have an enormous epithelial surface area that is in direct contact with ambient air. Inhaled air may contain numerous oxidants, gases, particulates, toxicants and pollutants that can lead to tissue injury. Therefore, the lung has a multitude of antioxidant systems available to maintain normal pulmonary cellular function and control redox homeostasis. The high reactivity and toxicity of ROS and RNS are counterbalanced by both enzymatic and non-enzymatic endogenous antioxidant defenses.

Various antioxidant enzymes exist in the lung to scavenge different ROS. One family of antioxidant enzymes that rapidly scavenge $O_2^{\bullet-}$ are the SODs. There are three different SOD isozymes that exist in eukaryotes. First, copper/zinc-containing SOD (CuZn-SOD or SOD1) is present in almost all eukoaryotic cells in the cytosol and nucleus. Manganese-containing SOD

(Mn-SOD or SOD2) is present in the mitochondria. Lastly, extracellular SOD (EC-SOD or SOD3) is found in the extracellular matrix (ECM) and fluids. Specifically, EC-SOD is highly expressed in the lung[118] and will be discussed in more detail in Section 1.6.

Glutathione peroxidase and catalase are antioxidant enzymes that both scavenge H_2O_2 in the lung. Specifically, glutathione peroxidases are selenium-containing enzymes that utilize reduced glutathione (GSH) as an electron donor to reduce H_2O_2 to water[106]. Glutathione disulfide (GSSG) is formed in the course of this reaction and is reduced back to GSH by glutathione reductase. The ratio of GSH to GSSG is often used as an indicator of oxidative stress. Healthy, nonstressed cells are known to have a high intracellular GSH:GSSG ratio[107, 119]. In particular, alveolar macrophages are known to have high levels of GSH when compared to type II epithelial cells and Clara cells[120]. In addition to glutathione, most aerobic organisms have catalase located in peroxisomes to enzymatically catalyze the removal of H_2O_2 to water and oxygen[106, 107, 121]. Catalase is a tetrameric hemoprotein that is also found in the cytoplasm, mitochondria, and BALF[122, 123]. While glutathione peroxidase, coupled with reduced GSH, readily removes H_2O_2 at low concentrations, catalase is most effective in the presence of higher concentrations of H_2O_2[107].

In the lung, non-enzymatic antioxidants also exist to help prevent oxidative tissue damage. Vitamins, such as α-tocopherol (vitamin E), ascorbate (vitamin C), and β-carotene (vitamin A) and other small molecules and proteins (albumin, taurine, and uric acid) have antioxidant functions[119, 124]. Storage and transport proteins for transition metals, such as transferrin (iron), also have antioxidant capacity as they bind metal ions making them unavailable to participate in chemical reactions, thus preventing the formation of $^{\bullet}OH$ via Fenton type chemistry[121](as demonstrated in Reactions 6 and 7 above).

1.5.4 Oxidants and Host Defense

An integral part of host defense in the lung is the activation of phagocytes resulting from recognition and ingestion of the inhaled microbes and the production of the potent arsenal (large amounts of ROS) needed for efficient microbial killing. Activated phagocytes (macrophages, neutrophils, and eosinophils) are able to produce large amount of $O_2^{\bullet-}$ as they possess NADPH oxidase, an enzyme that catalyzes that production of $O_2^{\bullet-}$ from oxygen and NADPH (Reaction 12). Once the foreign microbe is internalized, phagocyte NADPH oxidase, assembled on the

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{\bullet-} + H^+ \qquad (12)$$

phagolysosomal membrane, reduces molecular oxygen into ROS, such as $O_2^{\bullet-}$. This process, known as the oxidative burst, plays an essential role in host defense, but these reactive species can also damage the surrounding tissue and alter cell functioning if released from the cell[125]. In fact, overproduction of lysosomal enzymes, ROS, and RNS during bacterial infections have led to serious lung-associated pathophysiological consequences, such as ARDS[19-21]. In addition, the importance of the production of $O_2^{\bullet-}$ via the NAPDH oxidase in host defense is clearly evident as patients with chronic granulomatous disease (CGD), an inherited disorder of NAPDH oxidase, suffer from chronic bacterial and fungal infections and excessive inflammatory responses.

In addition to oxidative species, neutrophils also release myeloperoxidase (MPO) that aids in microbial killing. Specifically, MPO uses H_2O_2 (produced from the dismutation of $O_2^{\bullet-}$) to produce the bactericidal oxidant hypochlorous acid or bleach (Reaction 13). Aside from host

$$H_2O_2 + Cl^- \rightarrow H_2O + OCl^- \qquad (13)$$

defense, the release of MPO can also be detrimental to the host tissue as MPO is known to use H_2O_2 and $NO_2^{\bullet}$ to generate reactive nitrogen species[126].

1.5.5 Oxidative Stress in ILD

Oxidant/antioxidant imbalances have been implicated in the pathogenesis of ILD[127-130]. Studies have shown that the BALF of IPF patients have decreased reduced glutathione levels[129] and increased markers of oxidative stress, such as lipid peroxidation[129] and 8-isoprostane[131]. In the lungs of IPF patients, oxidative stress is also observed as increased levels of nitrotyrosine, a $ONOO^-$-mediated protein modification, are detected in the lung tissue[132]. The antioxidant EC-SOD is also seen to be significantly decreased in the fibrotic areas of the lungs of patients diagnosed with UIP[133].

Similar to what has been observed in human pulmonary disease, oxidant/antioxidant imbalances are also important in the pathogenesis of animal models of ILD. It is well-known that injurious agents like bleomycin[134] and asbestos[99], utilized in animal models of ILD, induce the formation of ROS. Many studies have shown that asbestos exposure *in vitro* and *in vivo* leads to ROS formation that is detectable through markers of oxidative stress[71, 135, 136]. In the lung, exposure to asbestos can also lead to increased enzymatic production of $NO^{\bullet}$ by iNOS in macrophages[137-139] and lung epithelial cells[140]. The administration of an antioxidant SOD mimetic was shown to attenuate pulmonary fibrosis[141] while overexpression of EC-SOD was able to protect mice from radiation-induced[142] and bleomycin-induced interstitial lung injury[143]. Furthermore, studies have demonstrated that EC-SOD knockout (KO) mice have enhanced inflammation as well as increased lung damage and fibrosis when compared to wild-type mice

following bleomycin- and asbestos-induced pulmonary injury[99, 144]. Taken together, all of these studies suggest that oxidants play an important role in the development of interstitial pulmonary disease and that antioxidant enzymes, such as EC-SOD, may attenuate this damage.

1.5.6 Oxidative Stress in Pulmonary Infection

Oxidant/antioxidant imbalance has been reported in various respiratory diseases including pneumonia. During pneumonia, there is a massive influx of activated phagocytes into the lower airways to kill invading microorganisms using ROS, RNS, and lysosomal enzymes. This respiratory burst is an essential part of the defense system but may also result in oxidative stress. Indeed, increased markers of oxidative stress, such as lipid peroxidation and ROS production, and decreased levels of antioxidants have been found in the BALF and the blood of patients with pneumonia[145-147]. Furthermore, high concentrations of H_2O_2 were found in breath condensate of patients with ARDS or acute respiratory failure due to severe pneumonia[148].

Animal models of pulmonary infection have also noted oxidant/antioxidant imbalances in the lungs. For instance, Hennet and coworkers found that influenza virus-induced pneumonia in mice caused a decrease in total glutathione and in vitamin C and E[149]. Several other studies using animal models of infection noted that inhibition of oxygen radicals through administration of antioxidants or increased lung SOD levels significantly reduced lung injury and improved survival rate of infected animals[150-153]. Overall, these studies suggest oxidative stress may play a significant role in the pathogenesis of pulmonary infections.

1.6 EXTRACELLULAR SUPEROXIDE DISMUTASE

Endogenous antioxidant enzymes are normal cellular defenses that can protect against ROS-induced tissue injury. One family of antioxidant enzymes is the SODs that catalyze the conversion of $O_2^{\bullet-}$ radicals to H_2O_2 and molecular oxygen. There are three isozymes in this family that have been identified in mammals.

1.6.1 Historical Background

In 1969, Irwin Fridovich and Joe McCord discovered the first SOD enzyme in mammalian tissue, which was named copper/zinc SOD (CuZn-SOD) due to its metal content[154]. This enzyme with SOD activity was previously known as copper-containing human erythrocuprein. CuZn-SOD exists as a 32 kDa homodimer and is present in the cytoplasm and nucleus of every cell type examined, where it acts as a bulk scavenger of $O_2^{\bullet-}$[155]. A few years later in 1973, a second SOD enzyme, manganese SOD (Mn-SOD) was discovered in mitochondria of eukaryotes by Richard Weisinger and Irwin Fridovich[156]. Mn-SOD is a 96 kDa homotetramer and is located primarily in the mitochondrial matrix[156]. CuZn-SOD and Mn-SOD are not structurally similar and their biological activities can be distinguished based on cyanide sensitivity; the activity of Mn-SOD is unaffected, while CuZn-SOD is inhibited by cyanide[118].

A little over ten years after the original discovery of SOD, Stefan L. Marklund and his colleagues observed that CuZn-SOD only accounted for a small amount of the cyanide-sensitive SOD activity in human plasma[157]. Their further investigations revealed that a higher molecular weight protein that was distinctly different from CuZn-SOD with cyanide-sensitive activity was present in human extracellular fluids[158]. These observations led to the discovery of a third

mammalian SOD isoenzyme in 1982[118]. Marklund and coworkers termed this enzyme extracellular SOD (EC-SOD) as it was first detected in human extracellular fluids such as plasma, lymph, ascites, and cerebrospinal fluid[158]. In addition, EC-SOD was originally purified from human lung and was characterized as a homotetramer with three variants based on its affinity for heparin glycosaminoglycan[118]. These great discoveries have lead to over 40 years of research on SODs and their ability to prevent oxidant-induced tissue damage and disease.

1.6.2 Structure of EC-SOD

EC-SOD (EC 1.15.1.1) is a 135 kDa homotetrameric copper and zinc-containing glycoprotein that is comprised of two disulfide linked dimers[118, 159] (Figure 4). This intrasubunit disulfide bridge involved cysteines located in the carboxyterminal end of each subunit[160, 161]. The complete tetramer is formed by noncovalent interactions between N-terminal domains of all four subunits[162, 163]. Each subunit of EC-SOD contains one copper and one zinc atom[118, 164], which are required for its enzymatic activity, and a positively charged matrix-binding domain at the C-terminal region that localizes it to the ECM. The matrix-binding domains can be post-translationally cleaved without affecting enzymatic activity[165].

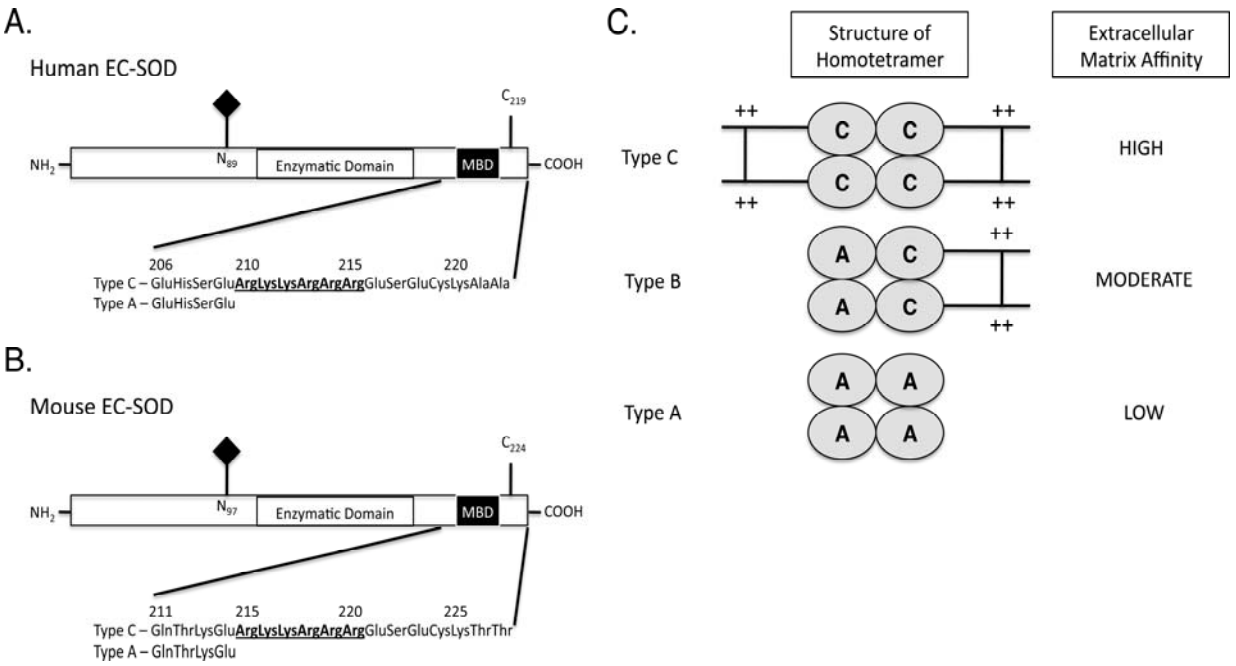

Figure 4: Schematic illustration of EC-SOD.

Each subunit of human EC-SOD (A) and mouse EC-SOD (B) is synthesized with a matrix-binding domain (bolded and underlined) at the C-terminus. Two identical subunits are linked via a disulfide bond formed between cysteines in the C-terminal region. EC-SOD functions as a homotetramer that is held together by noncovalent interactions at the N-terminal domains. (C) EC-SOD has different matrix affinity depending on the proteolysis of the positive charged matrix-binding domains. Type C EC-SOD, in which all found subunits contain a matrix-binding domain, has high matrix affinity and is predominantly found in the lung parenchyma. Proteolysis results in either partial (Type B) or complete (Type A) reduction of EC-SOD affinity and clearance of the protein from the tissue into the serum. Figure adapted from previous works[159, 160].

In humans, EC-SOD is a secretory protein as it is synthesized with an 18 amino acid signal peptide preceding the 222 amino acids of the mature enzyme (Figure 4A)[166]. Although the first 95 amino acids of the protein have no homology to either of the other SODs, amino acids 96-193, which contain the active site of the enzyme, show strong homology with eukaryotic CuZn-SOD[167]. Finally, amino acids 194-222 in the carboxyl-terminal domain of EC-SOD is strongly hydrophilic as it contains six positively charged amino acids with the sequence Arg-Lys-Lys-Arg-Arg-Arg[166]. This domain was originally termed the heparin-binding domain due to its strong affinity to heparin[118, 168, 169]. However, full-length EC-SOD is now known to bind to

other ECM components, like type I collagen[170] and hyaluronan[171], and therefore is more accurately referred to as the matrix-binding domain. EC-SOD is known to exist in three forms, which are characterized by matrix affinity[118, 168]: Type A (low affinity), Type B (moderate affinity) and Type C (strong affinity) (Figure 4C). Additionally, there is only one possible N-glycosylation site on EC-SOD (Asn-89 of human EC-SOD, Figure 4A and Asn-97 in mouse EC-SOD, Figure 4B) located in the N-terminus of the protein, which confers its affinity to concanavalin A[160]. Notably, both mouse and human EC-SOD share this overall structure (Figure 4).

The main function of EC-SOD is to scavenge $O_2^{\bullet-}$ and thereby lower levels of harmful oxidants in the body. EC-SOD catalyzes the dismutation of two $O_2^{\bullet-}$ anions to H_2O_2 and oxygen at a rate constant of 1×10^9 M^{-1} s^{-1} [118]. EC-SOD is a very stable glycoprotein and can withstand high temperature, pH extremes, and high urea and guanidinium chloride concentrations[172]. However, like CuZn-SOD, the activity of EC-SOD can be inhibited by cyanide, azide, H_2O_2, diethyldithiocarbamate, and sodium dodecyl sulfate[173].

Located on chromosome 4 (human) and chromosome 5 (murine), the EC-SOD gene is approximately 5900 base pairs long and comprised of three exons and two introns [174, 175]. The third exon contains the entire coding region for EC-SOD. The human EC-SOD gene is approximately 60% homologous to human CuZn-SOD with minimal homology to human MnSOD[167, 176]. The promoter region of the gene contains antioxidant response elements, activator protein-1 binding sites, xenobiotic response elements, and NF-κB motif[175].

1.6.3 Location and Function of EC-SOD

As its name implies, EC-SOD is located in extracellular fluid and in the ECM of tissues[169, 177-179]. Specifically, tissue EC-SOD is thought to account for 90-99% of the EC-SOD in the body[177, 178] and is mostly homoteterameric high matrix affinity type C protein variant[169, 180]. Although the tissue distribution of EC-SOD differs among species, generally the lung and kidney have the highest EC-SOD content[177, 181]. EC-SOD is abundant in lung tissue of humans and mice and is produced by type II epithelial cells, bronchial epithelial cells, macrophages, and neutrophils[179, 182, 183]. EC-SOD is also present in high concentrations in the arteries and plasma, where all three variants of EC-SOD are known to be present[181, 184].

The matrix-binding domain of EC-SOD plays an essential role in regulating tissue distribution and the proteolysis of this domain changes the location and function of EC-SOD in the body. Numerous proteinases have been shown to be able to cleave the matrix-binding domain of EC-SOD; however, the proteinases responsible for the cleavage of this domain *in vivo* are unknown. Indeed, full-length EC-SOD has a tissue half-life of approximately eighty five hours while partially (Type B) and completely proteolyzed EC-SOD (Type A) only have a half-life of twenty and seven hours, respectively[180].

Additionally, a single nucleotide polymorphism in *SOD3* gene (R213G) located in the coding region for the carboxyl-terminal domain is been shown to markedly decrease EC-SOD's binding affinity to the matrix[185]. Indeed, heterozygote carriers of this polymorphism have approximately 10-fold higher plasma concentrations of EC-SOD than non-carriers[186]. Patients with this R213G polymorphism are known to have increased risk of ischemic heart disease which is thought to results from the loss of EC-SOD from the arterial walls[187]. But, these patients also are protected against chronic obstructive pulmonary disease[188].

1.6.4 EC-SOD in Pulmonary Disease

Numerous studies have shown that the localization of EC-SOD to the lung parenchyma is essential for protecting it from ROS-mediated injury. Notably, EC-SOD is known to protect the lung against pulmonary injury from bleomycin[143, 144, 189], asbestos[98, 99], radiation[142, 190], hyperoxia[191-193], hemorrhage[194, 195], endotoxin[196, 197], and bacteria[103]. One mechanism by which EC-SOD is known to exert its protective effect in the lung is by directly binding to and preventing the oxidative fragmentation of type I collagen[144, 170], hyaluronan[171], and heparan sulfate proteoglycans[198, 199]. Therefore, loss of EC-SOD from the matrix to the airspace may result in increased oxidative fragmentation of matrix components and results in amplification of inflammation and fibrosis. The importance of endogenous pulmonary EC-SOD was also recently highlighted in a study by Gongora and colleagues, which showed that an acute loss of EC-SOD resulted in 85% mortality secondary to the spontaneous development of ARDS[200]. This indicates that EC-SOD is essential for protecting the lungs against inflammation and injury even in ambient air.

Prior studies have demonstrated that proteolysis of the heparin-binding domain of EC-SOD occurs in a number of ILD models including bleomycin[189], asbestos[99], and hyperoxia[193]. Following interstitial lung injury, the depletion of EC-SOD from the lung parenchyma and accumulation of its proteolyzed form (lacking its heparin-binding domains) in the BALF is observed [99, 189]. While this suggested that EC-SOD is being cleaved from the lung parenchyma and released into the alveolar lining fluid, further studies using an airspace-only model of inflammation (pneumonia) provided evidence that challenged this hypothesis[103].

In response to bacterial pneumonia, the accumulation of EC-SOD in the airspaces and the retention of EC-SOD in the lung parenchyma is observed. Numerous studies have also reported

the presence of EC-SOD in inflammatory cells of the lung[99, 143, 182]. Based on these findings, *it was hypothesized that inflammatory cells may transport and release EC-SOD into airspaces following inflammation resulting from interstitial lung injury or bacterial inoculation. In addition, this leukocyte-derived EC-SOD would help limit inflammation and subsequent lung injury or infection.* As these hypotheses were investigated, new insight into the functional role of EC-SOD inside leukocytes was uncovered.

2.0 LEUKOCYTE-DERIVED EXTRACELLULAR SUPEROXIDE DISMUTASE IN INTERSTITIAL PULMONARY INJURY

2.1 RATIONALE AND HYPOTHESIS

EC-SOD is the only extracellular SOD and is highly expressed in the lung compared to other tissues. Many studies have shown EC-SOD limits the development of fibrosis in experimental models and plays an important role in regulating inflammatory responses following pulmonary insult. Proteolysis of the matrix-binding domain of EC-SOD has also been noted in these ILD models and results in the depletion of EC-SOD from the lung parenchyma and accumulation of proteolyzed EC-SOD in the BALF[99, 189]. One mechanism by which EC-SOD is known to exert its protective effect in the lung is by directly binding to and preventing the oxidative fragmentation of type I collagen[144, 170], hyaluronan[171], and heparan sulfate proteoglycans[198, 199]. Therefore, loss of EC-SOD from the matrix to the airspace may result in increased oxidative fragmentation of matrix components and result in amplification of inflammation and fibrosis.

While the above studies suggest that EC-SOD is being cleaved from the lung parenchyma and released into the alveolar lining fluid, another study reported an accumulation of proteolyzed EC-SOD in the airspaces without a loss of EC-SOD from the lung parenchyma using an airspace-only model of inflammation (bacterial pneumonia)[103]. In addition, using EC-SOD transgenic mice that expressed both mouse and human EC-SOD in the lung, but only mouse EC-

SOD in inflammatory cells, only mouse EC-SOD was detected in the BALF[103]. These results suggested that inflammatory cells, which are known to contain EC-SOD[103, 143, 182], could transport and release this potent antioxidant into the airspaces after pulmonary injury. However, the contribution of EC-SOD from inflammatory cells in response to interstitial lung injury is still unknown.

Bone marrow chimeric (BMC) mice were generated to directly test the hypothesis that leukocytes contribute to EC-SOD accumulation in the alveolar lining fluid following asbestos-induced lung injury and to examine the impact of leukocyte-derived EC-SOD on inflammation and early stage fibrosis in response to asbestos injury.

2.2 MATERIALS AND METHODS

2.2.1 Generation of Bone Marrow Chimeric Mice

2.2.1.1 Animals

Seven-week-old male C57BL/6 (Taconic, Germantown, NY), C57BL/6 (B6.PL-Thy1a/CyJ, The Jackson Laboratory, Bar Harbor, Maine), and EC-SOD null mice (congenic with C57BL/6 background and bred in our animal facility) were used as donors with seven-week-old male C57BL/6 (B6.PL-Thy1a/CyJ, The Jackson Laboratory) wild-type mice and EC-SOD null mice as recipients. The EC-SOD null mice were originally derived by Dr. Stefan Marklund and were produced on the C57BL/6 background through the targeted disruption of the mouse *ec-sod* gene with a gene encoding neomycin resistance[191]. These mice are currently bred in our animal facility at the University of Pittsburgh. All animals were housed in pathogen free conditions in

microisolator cages and all animal experiments were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Donor mice were euthanized and bone marrow was extracted from the long bones of the hind legs. Recipient mice received 1000 R dose of whole-body irradiation and then were injected i.v. via tail vein with 100 µL of 1x Hank's Buffered Salt Solution (pH 7.1) containing 5 x 10^6 bone marrow cells. Recipient mice received 0.032 mg/ml of Baytril® (Enrofloxacin, Bayer Healthcare LLC, Shawnee Mission, Kansas) in their drinking water starting two days before and continuing until seven days after irradiation and transplantation.

2.2.1.2 Verification of Chimerism

Engraftment of donor cells was determined by flow cytometry using a BD FACS Vantage with DiVa and CellQuest analytical software (Becton Dickinson, Franklin Lakes, NJ). Peripheral blood was collected from the lateral saphenous vein in EDTA-coated anticoagulant tubes (Brinkmann Instruments, Westbury, New York) from of all BMC mice 33-35 days after transplantation[201]. All procedures were done in FACS Buffer (1x phosphate buffered saline with 1% bovine serum albumin). To verify chimerism, while blood cells were stained with the following antibodies for specific cell surface antigens: Fluorescein isothiocyanate-conjugated mouse anti-mouse CD 90.1 (Thy-1.1), Phycoerythrin-Cy5-conjugated rat anti-mouse CD8a, Phycoerythrin-conjugated rat anti-mouse CD 90.2 (Thy-1.2), and Allophycocyanin-conjugated rat anti-mouse CD4. Cells were then fixed in 2% paraformaldehyde for analyses the following day. BMC mice were allowed to recover for 15 days following this blood collection before intratracheal clodronate treatment.

2.2.2 Preparation and administration of liposomal clodronate.

Liposomal encapulation of dichloromethylene diphosphonate (clodronate) was performed as previously described[202]. In brief, 8 mg of cholesterol and 86 mg of dioleoyl-phosphatidylcholine (Avanti, Alabaster, AL) were mixed and dissolved in chloroform. The chloroform was then evaporated under nitrogen and further removed from the liposome preparation using a low vacuum speedvac. The clodronate solution was then prepared by dissolving 1.2 g dichloromethylene diphosphonic acid (Sigma-Aldrich Corp., St. Louis, MO) in 5 mL of sterile phosphate-buffered saline (PBS) and was added to the liposome preparation. The resulting solution was mixed, sonicated, and then ultracentrifuged at 10,000 g for 1 hour at 4°C. The resulting pellet of liposomes was then resuspended in PBS and ultracentrifuged again using the same conditions. The liposomes were removed, resuspended in PBS resulting in a final concentration of liposomal clodronate of 5 mg/mL. 75 µL of liposomal clodronate solution was administered by intratracheal instillation to the BMC mice.

2.2.3 Intratracheal Instillation of Asbestos

Four weeks following clodronate administration, BMC mice were anesthetized 20%v/v isoflurane in propylene glycol (Baxter Healthcare Corp., Deerfield, IL) using the open-drop exposure method[203] and then intratracheally instilled with 0.14 mg of crocidolite asbestos in 70 µl of 0.9% sterile saline. Crocidolite asbestos fibers were greater than 10 µm in length and a generous gift from Dr. Andrew Ghio at the Environmental Protection Agency, Research Triangle Park, NC. BMC mice were euthanized 150 mg/kg nembutol intraperitoneally at three and seven days post-treatment to assess inflammation and the beginning of the development of fibrosis.

BALF was also collected using the following procedure. Briefly, the trachea was surgically exposed and suture was placed underneath the trachea. Then, a small horizontal surface cut was made in the trachea and a 24-gauge lavage needle was placed into the trachea and secured with the suture. BALF was obtained via intratracheal instillation and recovery of 0.8 ml of 0.9% saline. The lungs were then inflation fixed with 10% buffered formalin (Fisher Scientific, Pittsburgh, PA) for at least 2 hours before incubation in 70% ethanol overnight. Lungs were paraffin embedded by Research Histology Services at the University of Pittsburgh.

2.2.4 Bronchoalveolar Lavage Fluid Analyses

To determine the number of white blood cells present in the BALF, triplicate counts of BALF samples were made using a Z1 Coulter Counter (Beckman Coulter, Fullerton, CA). To obtain a differential count, BALF samples were adhered to glass slides and stained using DiffQuik. The number of macrophages, neutrophils, lymphocytes and eosinophils were counted using a light microscope (200 cells/slide). The remaining BALF was centrifuged to remove the cellular content and stored at -80°C for later use.

2.2.5 Detection of Leukocyte-derived EC-SOD

Total protein in the BALF was determined using Commassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Equal concentrations of BALF protein (8 µg/sample) were boiled in a 50 mM dithiothreitol and 1% sodium dodecyl sulfate solution for 10 minute, subjected to SDS-polyacrylamide gel electrophoresis, and then blotted onto polyvinylidene fluoride membranes (PVDF, Millipore, Billerica, MA) at 500 mA for 50 minutes. As a loading control, the PVDF

membranes were then stained with Ponceau Red Stain (0.2% Ponceau S, 3% trichloroacetic acid) for 15 minutes and quickly destained (40% methanol, 7.5% glacial acetic acid in water) to visually verify equal loading of protein in all samples. The membranes were then blocked overnight in 5% milk in 1x phosphate buffered saline with 0.3% Tween-20 (PBST). Full-length and proteolyzed EC-SOD in the BALF was detected using a rabbit anti-mouse EC-SOD polyclonal antibody[198] (AnaSpec Corporation, San Jose, CA) at a 1:5,000 dilution in 1xPBST. After washing the membrane, the secondary antibody donkey anti-rabbit IgG conjugated to horseradish peroxidase was added to the blots at a concentration of 1:5,000 in PBST. ECL Plus Western Blotting Detection reagents (GE Healthcare, Piscataway, New Jersey) were utilized to visualize bands using the Kodak Gel Logic 2200 Imaging System.

2.2.6 Histological Analyses

Hematoxylin and eosin stained lung sections (5 μm thick) were obtained from Research Histology Services at the University of Pittsburgh and were scored by a pathologist (Tim D. Oury) who was blinded to the sample groups. Every field in the entire lung section was observed with a light microscope (x200 magnification) and scored if at least 50% of the field was alveolar tissue. Scoring was based on the percentage of alveolar tissue with interstitial fibrosis according to the following scale: 0= no fibrosis, 1= up to 25%, 2= 25-50%, 3= 50-75%, and 4= 75-100%. The pathological index score was then reported as a ratio of the sum of all the scores divided by the total number of fields counted for each sample.

2.2.7 Statistical Analyses

All comparisons between three or more groups were made using a one-way ANOVA with Tukey's post-test using GraphPad Prism 5 (GraphPad Software, San Diego, CA). All values shown are mean ± SEM. A p-value of less than 0.05 was considered statistically significant.

2.3 RESULTS

To determine if leukocyte-derived EC-SOD is the source of the EC-SOD that accumulates in the alveolar lining fluid in response to interstitial lung injury and to investigate its role in this injury, BMC mice were generated as shown in the schematic below (Figure 5). Wild-type C57BL/6 Thy-1.1 mice were irradiated and transplanted with bone marrow from EC-SOD KO mice (WT/KO) to create BMC mice that lack expression of EC-SOD in circulating leukocytes with normal expression of EC-SOD in the lung parenchyma. Conversely, EC-SOD KO mice were irradiated and received bone marrow from wild-type C57BL/6 Thy-1.1 mice (KO/WT) to generate mice with EC-SOD expression in leukocytes and no EC-SOD expression in the lung. Wild-type C57BL/6 Thy-1.1 mice were irradiated and received bone marrow from wild-type C57BL/6 Thy-1.2 mice (WT/WT) and EC-SOD KO mice were irradiated before receiving EC-SOD KO bone marrow (KO/KO) to serve as transplant controls.

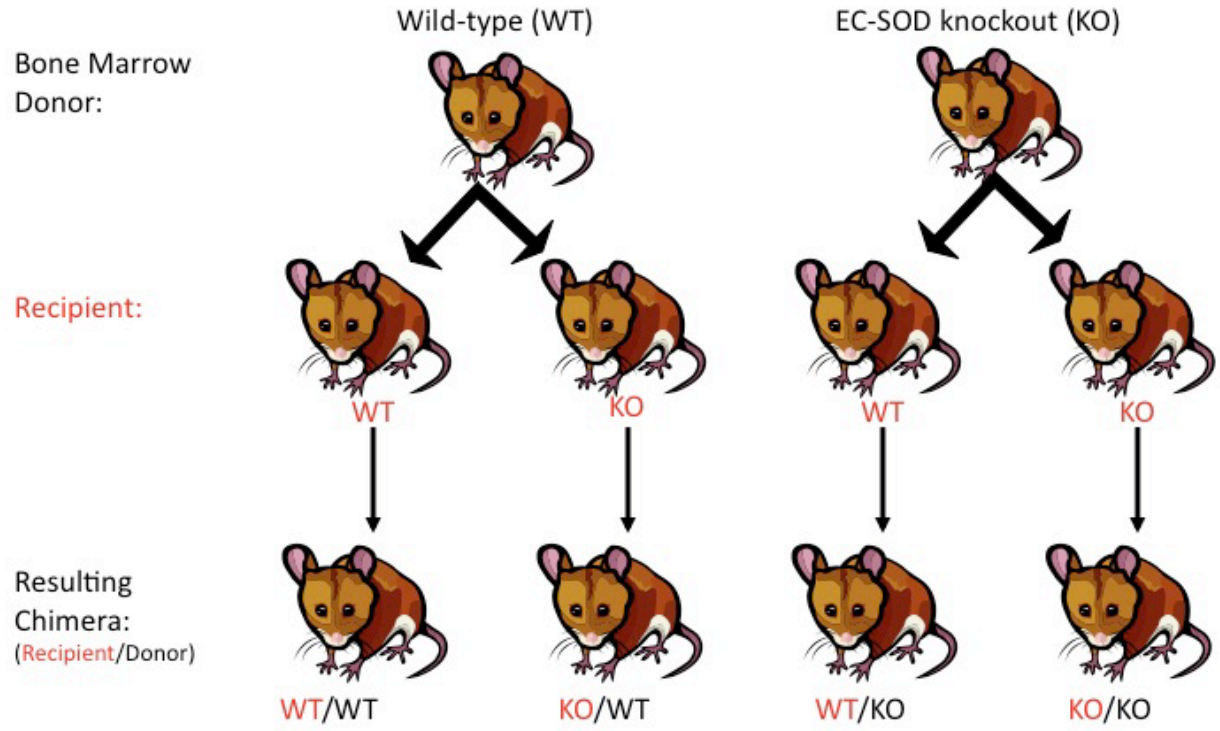

Figure 5: Generation of Bone Marrow Chimeric Mice.

Bone marrow chimeric mice were generated by the irradiation of wild-type and EC-SOD KO recipients and subsequent transplantation with wild-type and EC-SOD KO bone marrow from donor mice. From this process shown above, unique chimeras were created to definitively determine the role of leukocyte derived EC-SOD in asbestos-induced lung injury. WT/KO mice have EC-SOD expression in their lungs and not in their circulating leukocytes, while KO/WT mice that have no pulmonary EC-SOD expression, but EC-SOD inside their circulating cells. WT/WT and KO/KO were generated as controls.

Chimerism of the resulting BMC mice was confirmed by flow cytometry. Specifically, the presence of donor cells in recipient mice after transplantation was evaluated by assessing the percentage of total circulating T lymphocytes (Thy 1) from the blood that expressed either Thy 1.1 or Thy 1.2 alloantigen (Table 1). Values shown are the mean percentage of donor cells in total T lymphocytes ± standard deviation (n=8-12 per chimeric group).

Table 1. Verification of Chimerism.

	Control Mice		BMCs (Recipient/**Donor**)			
Type of Mice	C57BL/6 wild-type Thy 1.1	EC-SOD KO Thy 1.2	WT (Thy 1.1)/ **WT (Thy 1.2)**	WT (Thy 1.1)/ **KO (Thy 1.2)**	KO (Thy 1.2)/ **WT (Thy 1.1)**	KO (Thy 1.2)/ **KO (Thy 1.2)**
% Donor Cells (± standard deviation)	99.22 ± 0.18	99.04 ± 0.21	74.62 ± 6.91	80.33 ± 3.28	58.87 ± 6.93	99.08 ± 0.60

It is known that bone marrow transplantation alone is inadequate to repopulate the alveolar macrophages in the lung[202]. As alveolar macrophages are also known to express EC-SOD[103, 143, 182], these cells could also contribute to the EC-SOD that accumulates in the airspace. Therefore, in order to reconstitute the resident alveolar macrophages with donor cells, BMC mice were intratracheally treated with liposomal clodronate as previously described[202]. Using this approach, approximately 90% of the lung alveolar macrophages will be repopulated as previously reported[202]. All BMC mice were then intratracheally treated with asbestos and sacrificed either three or seven days post-treatment, as these time points were previously shown to coincide with maximal inflammation and the beginning of the development of fibrosis respectively [99]. A summary of the experimental design is illustrated below (Figure 6).

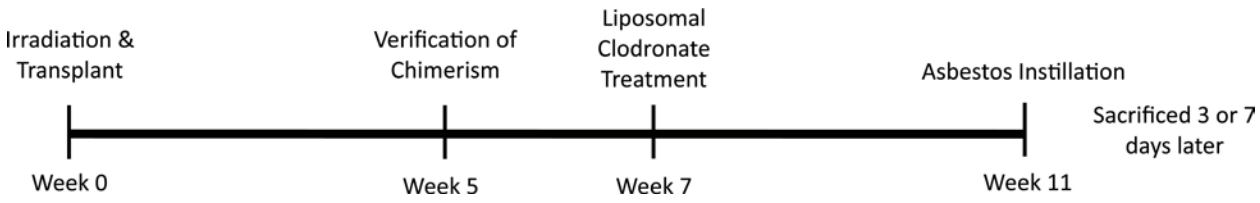

Figure 6: Summary of the Experimental Manipulations of Bone Marrow Chimeric Mice.

After irradiation and bone marrow transplantation, bone marrow chimeric mice undergo several procedures and treatments: verification of chimerism to ensure efficient reconstitution of donor bone marrow in the recipient mice, intratracheal instillation of liposomal clodronate to deplete resident alveolar macrophages and replace them with donor-derived cells, and finally intratracheal treatment with asbestos to induce interstitial lung injury.

2.3.1 Infiltrating inflammatory cells and resident macrophages are not the source of airspace EC-SOD

To determine whether leukocytes release EC-SOD into the airspaces following asbestos-induced interstitial lung injury, western blotting was performed on BALF from BMC mice at three and seven days after instillation of asbestos. From this analysis, an accumulation of EC-SOD was detected in the airspaces of WT/KO mice at both three and seven days post-asbestos exposure even though the leukocytes in these BMC mice did not express EC-SOD (Figure 7). Furthermore, there was a lack of EC-SOD observed in the BALF of KO/WT mice following asbestos injury (Figure 7). Together, these data demonstrate that leukocytes do not release detectable levels of EC-SOD into the airspace three or seven days following asbestos injury.

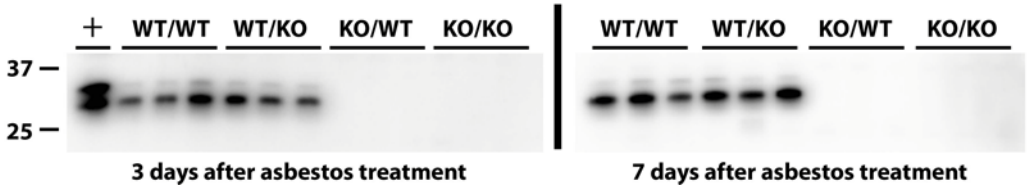

Figure 7: Absence of airspace EC-SOD in KO/WT mice at both three and seven days post-asbestos exposure.

Western blotting for EC-SOD was performed on equal protein amounts of BALF (8 µg) from bone marrow chimeric mice three and seven days following intratracheal treatment with asbestos. The absence of EC-SOD in the KO/WT BALF indicates that leukocyte-derived EC-SOD does not contribute to the EC-SOD that accumulates in the airspace after asbestos-induced lung injury. EC-SOD positive control (+) was 8 µg of wild-type lung homogenate.

2.3.2 Leukocyte-derived EC-SOD did not affect inflammation after asbestos exposure

Although significant amounts of EC-SOD from leukocytes could not be detected in the BALF after asbestos-induced pulmonary injury, it is still possible that these cells may be releasing EC-SOD that lessens the magnitude of pulmonary injury in the lung. To assess the amount of

inflammation, the total cells in BALF of the BMC mice were counted at both three and seven days following asbestos instillation. These measurements revealed that leukocyte-derived EC-SOD had no effect on inflammation resulting from asbestos treatment at either time point (Figure 8). Cell differential counting of BALF cells revealed that there were significantly more macrophages in the BALF of the KO/WT mice when compared to WT/KO mice seven days after asbestos exposure, but the biological significance of this observation is unclear. Other than this, there were no notable differences in the cellular composition of the BALF after injury (Figure 8).

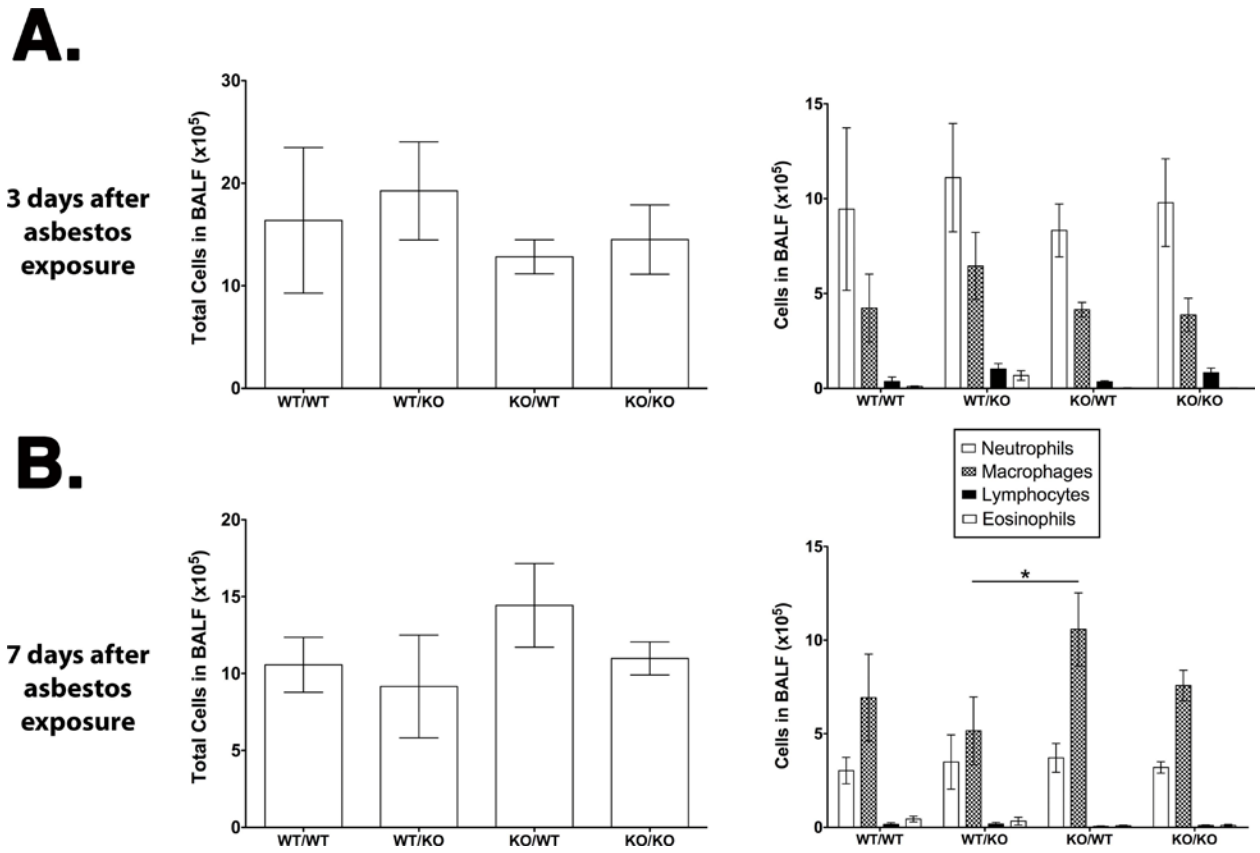

Figure 8: Leukocyte-derived EC-SOD does not alter leukocyte accumulation in response to asbestos.

Total white blood cells and cell differentials in the BALF of the bone marrow chimeras were measured three (A) and seven days (B) after asbestos treatment. Total cells were calculated by multiplying the cell concentration (cells/mL) by the total volume of BALF recovered. Data were analyzed using a one-way ANOVA with Tukey's post-test and are means (± SEM). *$p<0.05$ when WT/KO and KO/WT macrophages are compared, n=5-6 mice/group for three day time point and n=3-5 mice/group for seven day time point

2.3.3 Leukocyte-derived EC-SOD does not lessen the early fibrotic response to asbestos

Since EC-SOD is also known to be an anti-fibrotic agent, analyses were conducted to determine if leukocyte-derived EC-SOD could limit the early fibrosis seven days after asbestos treatment. To test this hypothesis, hematoxylin and eosin-stained lung sections were scored by a pathologist who was blinded to sample groups and experimental treatment. Individual fields were examined with a light microscope at ×200 magnification and scoring in each field was based on the percentage of alveolar tissue with interstitial fibrosis. Histological analyses of the lungs revealed that there were fibrotic areas present in the lungs of all mice (Figure 9A); however, there was not a significant difference between any of the BMC mice (Figure 9B).

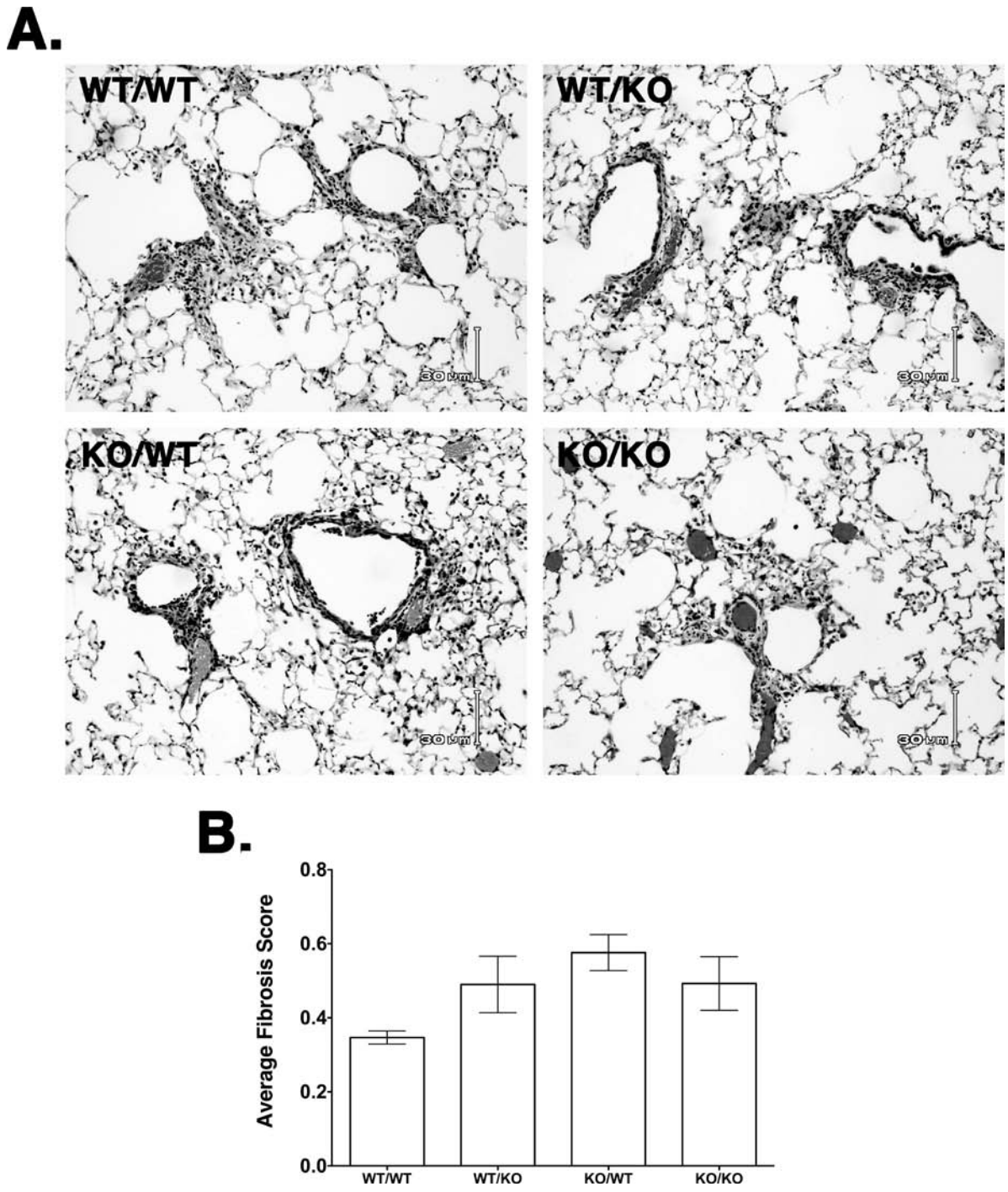

Figure 9: Leukocyte-derived EC-SOD does not alter early fibrosis in response to asbestos.

Bone marrow chimeras were euthanized seven days after intratracheal treatment with asbestos. Hematoxylin and eosin staining of lung sections revealed that leukocyte-derived EC-SOD did not attenuate asbestos-induced fibrosis as fibrosis in the lungs was similar for all mice (A). Representative images shown are at 10x magnification. Average histology score also showed no differences in fibrosis and was determined by a pathologist (T.D.O.) blinded to groups (B). Data shown are means (±SEM), n=3-5 mice/group

2.4 DISCUSSION

Prior to the current study, it was thought that the EC-SOD accumulating in the airspace following an interstitial lung injury was due to a combination of EC-SOD release from the interstitium as well as direct release of EC-SOD from the infiltrating inflammatory cells[103]. Bone marrow chimeras were generated to determine the source of airspace EC-SOD during inflammation following interstitial lung injury. The data in Figure 7 clearly shows that the EC-SOD, which accumulates in the airspace after pulmonary injury, originates solely from the lung parenchyma as infiltrating inflammatory cells and resident alveolar macrophages do not contribute detectable levels of EC-SOD.

Furthermore, leukocyte-derived EC-SOD did not appear to protect against asbestos-induced lung injury as it was incapable of lessening inflammation or early stage fibrosis *in vivo*. Previous work has shown that EC-SOD KO mice have more inflammation than wild-type mice twenty-four hours and twenty-eight days following asbestos treatment, while no significant difference was noted fourteen days after exposure[98]. Therefore, the current observations add to and are consistent with this previous data. Even though EC-SOD is known to be a potent antioxidant and anti-inflammatory agent, the EC-SOD produced by leukocytes is not abundant enough to lessen the inflammatory response induced by asbestos.

Prior studies noted that there was significantly more fibrosis in the lungs of EC-SOD KO mice when compared to wild-type mice as early as fourteen days following asbestos exposure[98]. Seven days after asbestos instillation is known to be the beginning of the fibrotic phase in this injury model. This early fibrotic time point may explain why there is not a significant difference in fibrosis between WT/WT and KO/KO mice, but only a trend toward the KO/KO mice having

more fibrosis than WT/WT mice. Overall, these results suggest that leukocyte-derived EC-SOD has no effect on the early fibrotic response to asbestos exposure.

Although the present findings strongly show that the lung parenchyma EC-SOD is being cleaved and release into the BALF following asbestos exposure, the possibility does exist that leukocytes do release EC-SOD at very low levels that are unable to be detected by western blotting. In addition, as the levels of EC-SOD in the BALF were only measured three and seven days following asbestos exposure, it is possible that the inflammatory cells do release EC-SOD that is quickly degraded or cleared into the circulation. Regardless, the overall significance of this leukocyte-derived release of EC-SOD in this model of ILD (if it does in fact occur) is trivial as leukocyte-derived EC-SOD does not alter inflammation or early stage fibrosis.

Although leukocytes are not the source of airspace EC-SOD in this lung injury model, previous work suggests that inflammatory cells may release EC-SOD into the airspaces after pulmonary bacterial infection[103]. In addition, EC-SOD KO mice have greater inflammation in their BALF when compared to wild-type mice following infection[103]. Therefore, it is still possible that the leukocyte-derived EC-SOD may be released into the BALF and may be effective at limiting inflammation following pulmonary infection.

3.0 EXTRACELLULAR SUPEROXIDE DISMUTASE IN BACTERIAL PNEUMONIA

3.1 RATIONALE AND HYPOTHESIS

Acute infection of the lower respiratory tract is the leading infectious cause of premature death with a greater disease burden than cancer[12, 13, 104]. In the lung, alveolar macrophages and neutrophils are phagocytic inflammatory cells that play a central role in the elimination of bacterial infections. As the first line of defense, macrophages and neutrophils produce potent molecules, such as ROS and proteases, in an attempt to eliminate microbes. While this antimicrobial arsenal is beneficial for eradicating the infectious organisms, these toxic agents can directly cause pulmonary damage and contribute to complications such as ARDS[19-21].

Endogenous antioxidant enzymes are normal cellular defenses that can protect against ROS-induced tissue injury. EC-SOD is a 135 kDa antioxidant enzyme that scavenges the free radical $O_2^{\bullet -}$[159]. This isozyme of the SOD family is highly expressed in the lung and arteries and is bound to the ECM via its positively charged heparin/matrix-binding domain[159-161, 181]. In addition to its localization on matrices and in extracellular fluids, EC-SOD is also present intracellularly in neutrophils and macrophages[103, 144, 182]. However, the localization and the role of EC-SOD in these inflammatory cells have not been investigated.

Few studies have investigated the role of this potent antioxidant in bacterial infections; therefore, the present study investigates the response of wild-type and EC-SOD KO mice to live *Escherichia coli* inoculation. To better understand the role of EC-SOD in infection, the subcellular localization and functional role of EC-SOD in inflammatory cells was also investigated. These findings are significant in that EC-SOD located in membrane bound vesicles in the cytoplasm of phagocytic cells is shown to be functionally important for innate host defense against gram-negative bacterial infections.

3.2 MATERIALS AND METHODS

3.2.1 Pneumonia Studies

Nine week old and sex-matched C57BL/6 mice (Taconic) and EC-SOD KO mice (congenic in the C57BL/6 background[191]) were used for all animal studies. Animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Mice were intratracheally instilled with ~$1x10^6$ CFUs live *E. coli* (ATCC 25922) in 50 μL of phosphate buffered saline[204] and euthanized immediately (0 hours), 6, or 24 hours after inoculation with 150 mg/mL pentobarbital. The thoracic cavity was opened by midline incision and the left lung was tied off using suture. The entire left lung was removed and placed in 1 ml sterile water for subsequent homogenization. BALF was collected via instillation and recovery of 0.6 ml of 0.9% saline from the right lung and the right lung was then inflation-fixed with 10% formalin for histological analyses.

3.2.2 Sample Processing

Cell counts and differentials in the BALF and cell differentials were performed as described previously (Section 2.2.4). BALF supernatants were stored at -70 °C until analyses. The left lung homogenates were separated into aliquots for quantitative bacterial cultures, western blot analysis, and MPO assays. In brief, for western blot analyses, buffer was added to an aliquot of lung homogenate so that the final concentration of buffer contained 0.5% Triton-X-100, 150 nM NaCl, 15 mM Tris, 1 mM Ca Cl_2, and 1 mM $MgCl_2$ (pH 7.4), incubated on ice for 30 minutes, and spun at 10,000 x g for 20 minutes. The supernatants were stored at -80ºC to use later. For MPO activity measurements, the lung homogenate was added to a buffer so that the final concentration of the solution was 50 mM potassium phosphate (pH 6.0), 5 mM EDTA, and 5% wt/vol hexadecyltrimethyl ammonium bromide. This mixture was then sonicated and spun down at 12,000 x g for 30 minutes. The supernatant was then stored at -80ºC until use.

3.2.3 Bacterial Recovery

Quantitative cultures of bacterial burden were performed by culturing serial 10-fold dilutions of lung homogenates in Luria-Bertani (LB) agar using the pour plate method to determine the number of colony-forming units.

3.2.4 Myeloperoxidase Activity Assay

Lung homogenates in 50 mM potassium phosphate (pH 6.0), 5 mM EDTA, and 5% wt/vol hexadecyltrimethyl ammonium bromide were assayed in triplicate at a 1:30 ratio (vol/vol) in

assay buffer (50 mM potassium phosphate buffer pH 6.0, 0.167 mg/ml o-dianisidine dihydrochloride, 0.0005% hydrogen peroxide, 0.01% hexadecyltrimethylammonium bromide) in a 96 well plate. Immediately after addition of assay buffer, MPO activity was monitored by measuring the absorbance at 460 nm over 2 minutes. Relative MPO activity was calculated as the change in absorbance over time per left lung (dA/min/left lung).

3.2.5 Isolation of Macrophages

Bone marrow-derived macrophages were isolated and cultured from wild-type mice and EC-SOD KO mice as previously described[205, 206]. Briefly, mice were euthanized and femurs were dissected from the animal and placed in ice cold 1x Hank's Buffered Saline pH 7.1 containing 1% penicillin/streptomycin and 1% fetal bovine serum. Bone marrow cells were then flushed from the bones with 1x Hank's Buffered Saline pH 7.1 with 1% fetal bovine serum using a 25-gauge needle until the bone had a translucent appearance. The cells were then filtered through a 40 μm cell strainer (BD, Franklin Lakes, NJ) and washed twice through centrifugation. Cells were plated on 24-well tissue culture grade plates at 1 x 10^6 cells/well (LPS stimulation) or on 100 cm VWR Petri dishes at around 4 x 10^6 cells/dish for all other assays and cultured for seven days in 10% L929 conditioned media. The next day, additional conditioned media was added to the culture and the media was then change every two days. Twenty-four hours prior to use, macrophages were washed and cultured with media lacking both antibiotics and L929 supplement. Peritoneal macrophages were isolated from the peritoneum via instillation and recovery of sterile phosphate buffered saline without calcium or magnesium. Alveolar macrophages were harvested via instillation and recovery of 0.8 ml of cold sterile PBS from the lungs. Both cell types were allowed to adhere at 37°C in a humidified 5% CO_2 atmosphere for 2

hours. Non-adherent cells were then removed by gentle washing and remaining cells were cultured overnight in media lacking both antibiotics and L929 supplement for experiments. The purity of the cell populations was verified by flow cytometry using macrophage marker PE-conjugated anti-mouse F4/80 (Molecular Probes, Eugene, OR). For detection of EC-SOD, cell lysates were collected in ice-cold lysis buffer with protease inhibitors (1% Triton, 25 mM HEPES, 150 mM NaCl, 5 mM EDTA, pH 7.5 with 100 μM 3,4-dichloroisocoumarin, 10 μM E-64 and 1 mM 1,10-phenanthroline).

3.2.6 Immuno-labeling

EC-SOD in human phagocytes in normal lung was localized using electron microscopy with immuno-gold labeling as previously described[179]. Dr. Tim Oury conducted this work in collaboration with Dr. James Crapo and Dr. Ling-Yi Chang while at Duke University.

3.2.7 Western blot Analyses

Western blot analysis for EC-SOD in BALF, cell lysate, or lung homogenates was done as previously described (Section 2.2.5). However, equal volume of sample was loaded for the western blots of BALF (26 μl/sample) and cell lysate (65 μl/sample). Equal protein loading was used for the lung homogenates (10 μg/sample). After probing for EC-SOD, the blots of cell lysate and lung homogenate were stripped and reprobed with antibody against β-actin at a 1:5000 dilution in 1xPBST as a loading control. Densitometry was performed by quantifying the band intensity and in some cases standardizing to β-actin using Kodak MI Software 4.5.1 SE.

3.2.8 EC-SOD Activity Assay

The activity of EC-SOD in the macrophages was determined using the Nitro Blue Tetrazolium Zymography assay as previously described[160]. Cell lysates of primary alveolar, peritoneal, and bone marrow-derived macrophages (90μl/sample) were collected and 21.2 μg CuZn-SOD (bovine liver, Alexis Biochemicals, San Diego, CA) and 2 μg of human EC-SOD were prepared as positive controls. Following non-denaturing PAGE, the gel was soaked, in the dark, in water containing 10.5 mg of Nitro Blue Tetrazolium, 1.2 mg of riboflavin and 220 μl of *N,N,N',N'*-tetramethylethylenediamine (100 ml final volume) for 40 min. The gel was then transferred to water and developed over a fluorescent light. Image of the gel was captured using the Kodak Gel Logic 2200.

3.2.9 Oxidant Production by Phagocytes

Electron paramagnetic resonance (EPR) spectroscopy was performed to determine the location and amount of oxidant production in wild-type and EC-SOD KO macrophages 10 minutes after stimulation with 15 μg/ml phorbol 12-myristate 13-acetate (PMA). Peritoneal macrophages isolated from wild-type and EC-SOD KO mice were resuspended (1 x 10^6 cells/ml) in Krebs HEPES buffer pH 7.4. Identification of ROS production ($O_2^{\bullet-}$, $ONOO^-$, and other downstream single electron reactive oxygen intermediates) was accomplished by exposing the stimulated cells to either 50 μM cell permeable (1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrrolidine (CMH)) or 50 μM cell impermeable (1 hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine (PPH)) spin probes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) and analyzed using a Bruker eScan Table-Top EPR spectrometer[207-209].

Various μM amounts of CM radical were measured to generate a standard curve to quantify the amount of oxidants produced by wild-type and EC-SOD KO cells. EPR settings were as follows: field sweep, 50 Gauss; microwave frequency, 9.78 GHz; microwave power 20 mW, modulation amplitude, 2 Gauss; conversion time, 327 ms; time constant, 655 ms; receiver gain, $1x10^5$. All buffers were treated with Chelex resin and contain 25 μM deferoxamine. Absence of transition metals was confirmed by the inability to detect the ascorbyl radical upon exposure of buffer to 100 μM ascorbic acid.

3.2.10 Pro-inflammatory Cytokine Measurements

Bone marrow-derived macrophages from wild-type and EC-SOD KO mice plated in 24-well plates were stimulated with 1 μg/ml LPS from *E. coli* (O26:B6, Sigma Aldrich). Cell culture supernatants were collected at various time points after stimulation from triplicate wells and stored at -80°C for further analysis. TNF-α was quantified in the supernatants using an Enzyme linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems, Abingdon, UK). The ELISA plates were read using a SpectraMax M2 plate reader, and the data was analyzed using SoftMax Pro software (Molecular Devices).

3.2.11 Culturing of Bacteria for *In vitro* Studies

Enhanced green fluorescent protein (EGFP)-expressing *E. coli* (BL21 strain)[210, 211] was grown overnight in LB broth with 50 μg/ml ampicillin at 37°C with continuous shaking. Cultures were refreshed and grown for 2 hours to reach log phase, washed with DPBS without calcium and magnesium, and used for phagocytosis and bacterial killing studies.

3.2.12 Bacteria Killing Assay

Bone marrow-derived macrophages from wild-type and EC-SOD KO mice (500,000 cells/tube) were incubated with EGFP-expressing *E. coli* at 37ºC (1:100 ratio of cells to bacteria). Wild-type and EC-SOD KO cells as well as bacteria alone were incubated as controls. After one hour, the bacteria remaining were cultured in LB agar using the pour plate method to determine the number of colony-forming units. The percentage of bacteria killed was calculated as the average CFUs of the bacteria alone minus the CFUs remaining after one hour incubation with cells divided by the average CFUs of the bacteria alone multiplied by 100.

3.2.13 Live Cell Microscopy of Phagocytosis

Bone marrow-derived macrophages (1×10^5 cells/dish) were plated onto 35mm collagen-coated glass bottom dishes (MatTek Corp, Ashland, MA). Multimode imaging (dimensions: X, Y, Z, time and color) was used to image cellular interactions between phagocytes and EGFP-expressing *E. coli* in a temperature-controlled chamber (Tokai-Hit, Tokyo, Japan) at 37°C. Movies were obtained using a Nikon Ti inverted microscope (40x magnification) running NIS-Elements AR 3.1 software (Melville, NY). Images were collected sequentially with a Photometrics CoolSNAP HQ2 camera (Tucson, AZ) using shuttered illumination in both fluorescence and differential interference contrast. Movies were analyzed using NIS-Elements software. Phagocytosis was quantified over 1 hour by an observer blinded to the experimental groups.

3.2.14 Endocytosis Analyses

Bone marrow-derived macrophages (250,000 cells/tube) were incubated with 5 µg/ml Alexa Fluor® 555 dextran (Molecular Probes, Eugene, OR) for 1 hour at either 4°C or 37°C. Cells were then fixed and flow cytometric analysis was done using a BD FACS Vantage with DiVa and CellQuest analytical software (Becton Dickinson, Franklin Lakes, NJ).

3.2.15 Cell Viability Assay

To determine if there were differences in cell viability between macrophages from wild-type or EC-SOD KO mice, bone marrow-derived macrophages were treated with various concentrations of PMA (Sigma), LPS (*E. coli* O26:B6, Sigma), and EGFP-expressing *E. coli*. Cell viability was measured using CellTiter 96 AQueous Non-radioactive Assay according to manufacturer's instructions (Promega, Madison, WI) or by visual assessment using trypan blue.

3.2.16 Statistical Analyses

Data were analyzed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). Animal experiments were analyzed by two-way analysis of variance with a Bonferroni post-test. Comparisons with one variable were analyzed using an unpaired t-test. The number of cells that phagocytosed bacteria were compared using a Fisher's exact test. All values are means (±SEM) unless otherwise noted. A P value less than 0.05 was considered to be statistically significant.

3.3 RESULTS

3.3.1 EC-SOD limits inflammation following *Escherichia coli* inoculation

EC-SOD has been shown to attenuate inflammation and oxidative injury in numerous pulmonary disease models; however, few studies have investigated its role in limiting injury in response to bacterial infections. To determine the role of EC-SOD in *E. coli* pneumonia, wild-type C57BL/6 and EC-SOD KO mice (congenic with the C57BL/6 strain) received an intratracheal instillation of *E. coli* and were killed immediately (0 hours) or at 6 or 24 hours after inoculation. The expression of EC-SOD in the airspace and lung parenchyma was evaluated in wild-type mice by western blot analyses of the BALF and lung homogenates. Consistent with previous work[103], bacterial pneumonia leads to a significant increase of EC-SOD in the alveolar lining fluid (Figure 10), but no loss from the lung parenchyma (Figure 11).

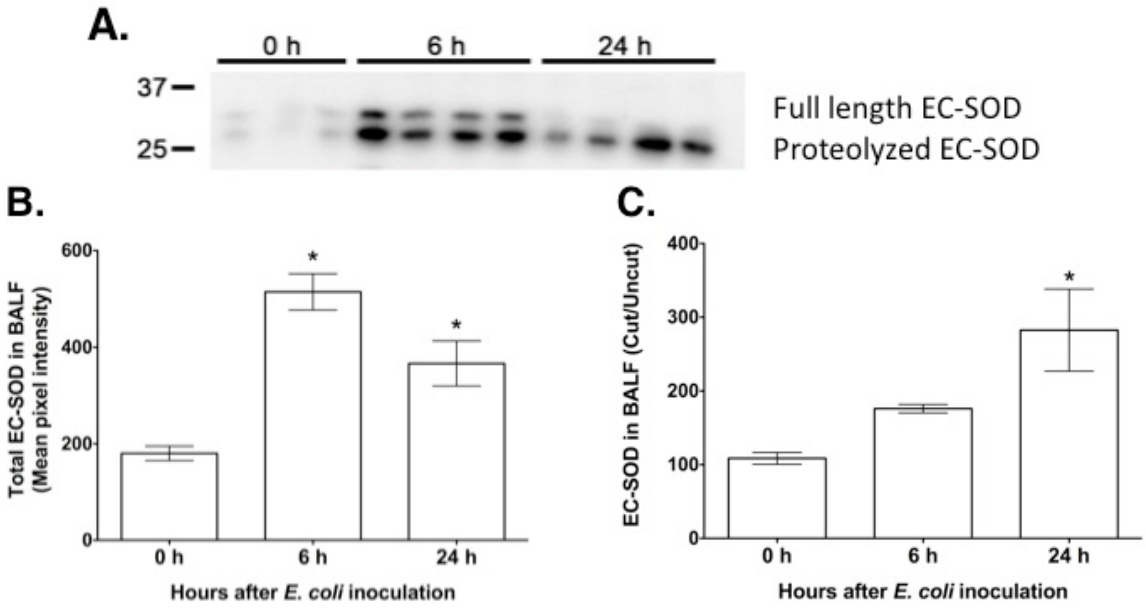

Figure 10: EC-SOD accumulates in the BALF after *E. coli* inoculation.

EC-SOD levels in the BALF (26 μl/sample) 6 and 24 hours after intratracheal instillation with *E. coli* were measured by western blotting (A). Densitometry showed increased EC-SOD at both 6 and 24 hours after exposure when compared to 0 hours. By 24 hours after inoculation, the majority of EC-SOD found in the BALF was proteolyzed EC-SOD. *$p<0.05$, one-way ANOVA

To evaluate the importance of EC-SOD in response to bacterial infection, pulmonary inflammation was assessed in the BALF and lung tissue of infected wild-type and EC-SOD KO mice. There was no significant difference in inflammation between wild-type and EC-SOD KO animals 6 hours after *E. coli* inoculation based on the cellular content of the BALF and the level of MPO and inflammation in the lung (Figure 12).

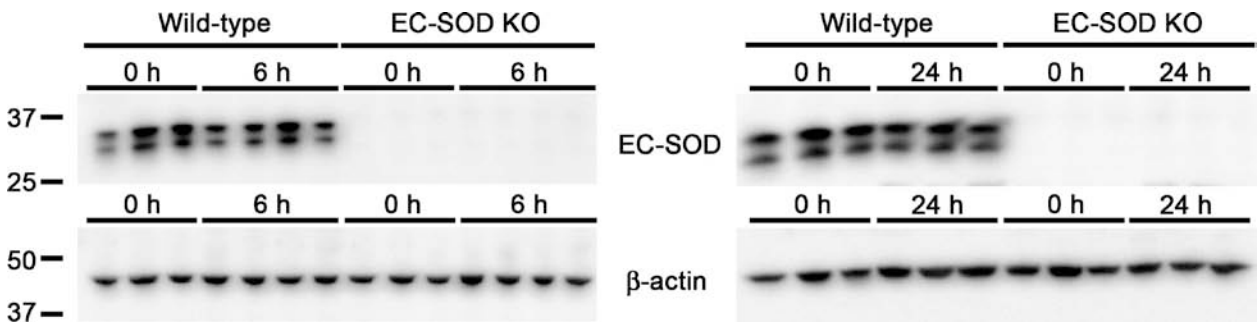

Figure 11: EC-SOD is not depleted from the lung after *E. coli* inoculation.

Western blotting was performed on equivalent protein amounts of left lung homogenates (10 μg) from mice exposed to *E. coli* via intratracheal instillation for 0, 6, or 24 hours. EC-SOD was found to be unchanged at both 6 and 24 hours after inoculation. Data shown is from the inoculated wild-type animals. As expected, no EC-SOD was detected in the BALF of the EC-SOD KO animals.

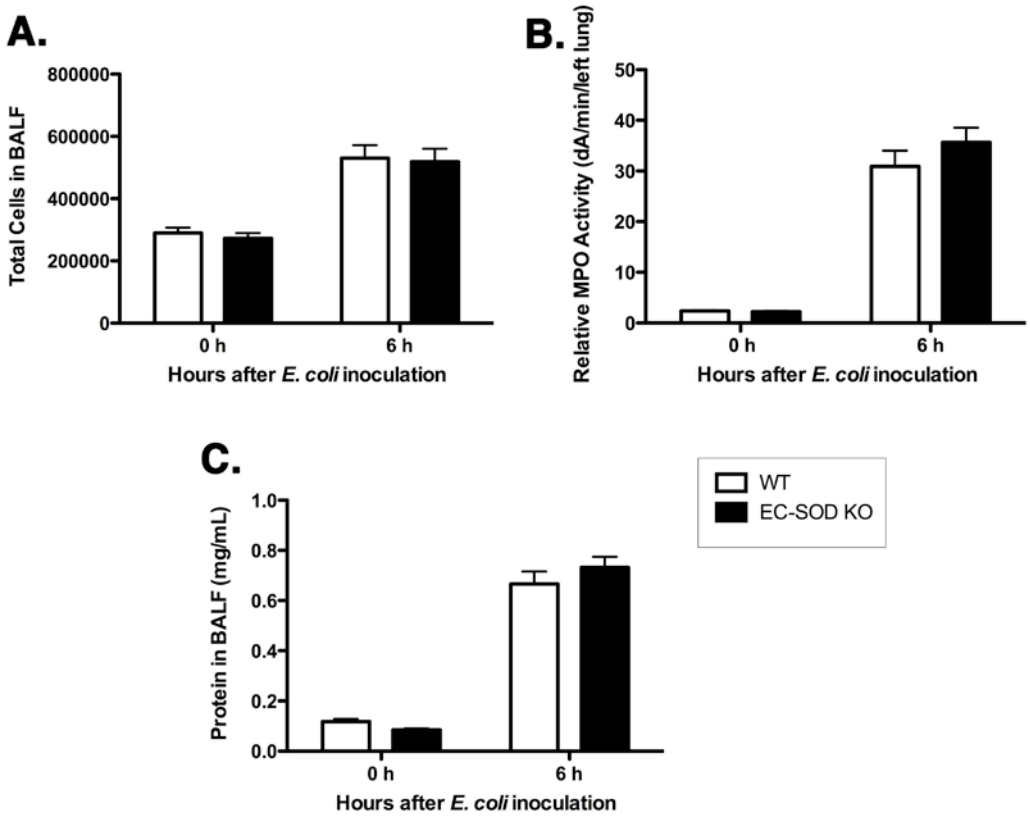

Figure 12: EC-SOD knockout and wild-type mice have similar inflammation 6 hours after infection.

There were a similar number of total cells in the bronchoalveolar lavage fluid (BALF) of EC-SOD knockout (KO) mice when compared to wild-type (WT) mice 6 hours after infection (A). Myeloperoxidase (MPO) activity in lung homogenates also showed that EC-SOD KO mice had similar neutrophilic inflammation when compared to WT mice after *E. coli* inoculation (B). Protein in the BALF was measured to assess lung injury and EC-SOD KO mice had comparable protein levels in the alveolar lining fluid than WT mice after infection (C). Data are means (±SEM) analyzed by two-way ANOVA with a Bonferroni post-test. (n=9-11/strain for each timepoint)

However, histological examination of the lungs 24 hours after inoculation with *E. coli* revealed an increased number of inflammatory cells in the EC-SOD KO mice when compared to the wild-type mice (Figure 13A). EC-SOD KO mice also had more inflammatory cells in the BALF than wild-type mice 24 hours after infection (Figure 13B). The majority of cells in the lung and BALF were neutrophils, although macrophages were also present. Indeed, there were significantly greater numbers of neutrophils in the BALF (Figure 13C, $9.604x10^5 \pm 1.625$ neutrophils in EC-SOD KO mice and $5.270x10^5 \pm 0.635$ neutrophils in wild-type mice) and higher MPO activity in the lungs (Figure 13D) of EC-SOD KO mice when compared to wild-type mice 24 hours following *E. coli* inoculation. EC-SOD KO mice also had significantly more protein in their BALF than wild-type mice 24 hours following infection (Figure 13E). These results are consistent with many prior studies, which have shown that EC-SOD expression in the lung inhibits inflammation in response to a wide variety of pulmonary injuries[98, 99, 103, 144].

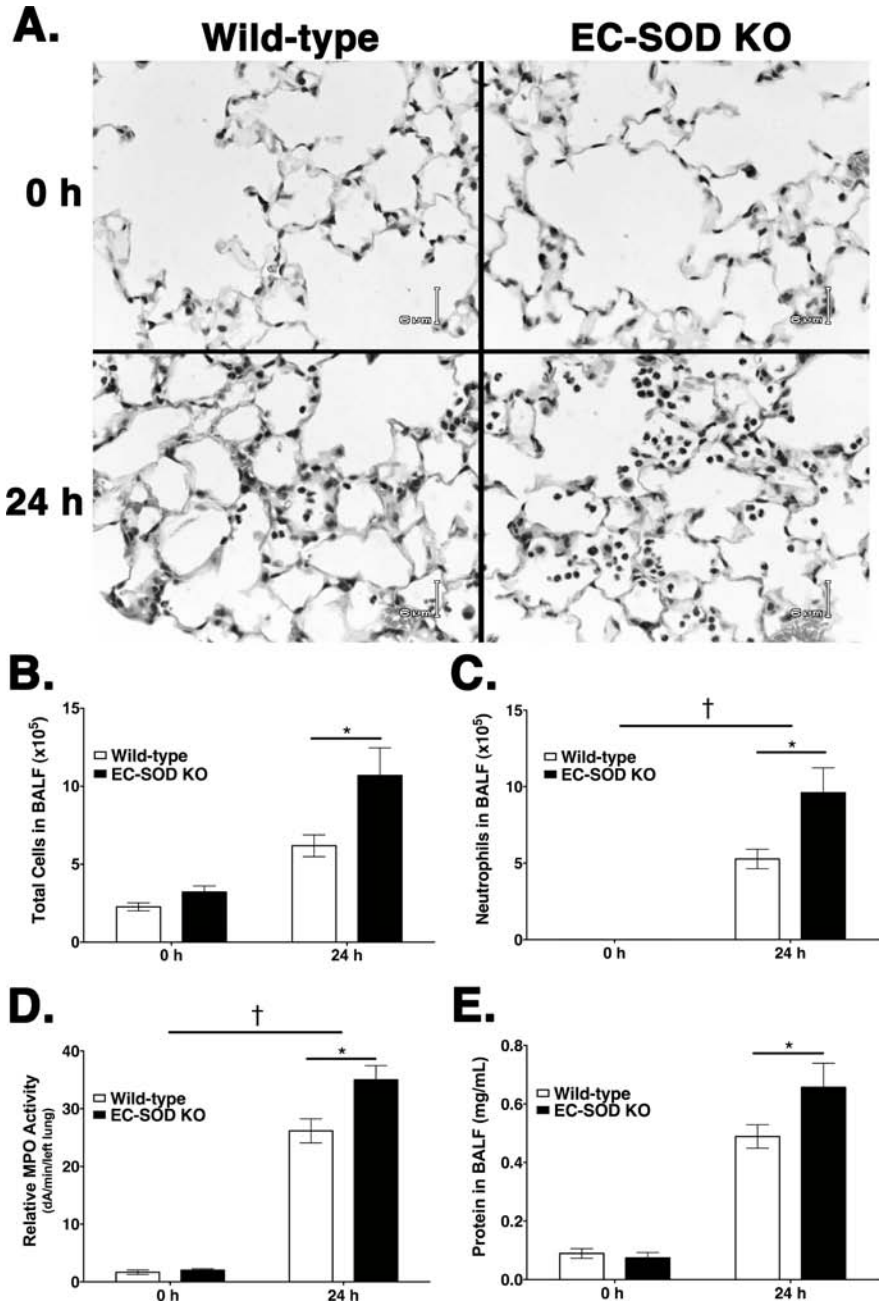

Figure 13: EC-SOD knockout mice have increased inflammation compared to wild-type mice.

Hematoxylin and eosin staining of lung sections from wild-type and EC-SOD knockout (KO) mice sacrificed at 0 and 24 hours after inoculation with *E. coli* shows increased acute inflammation in the EC-SOD KO compared to wild-type mice (A). There was more inflammation (total cells) in the bronchoalveolar lavage fluid (BALF) of EC-SOD KO mice when compared to wild-type mice 24 hours after infection (B). Differential counting demonstrates this inflammatory response was mainly due to an increase in neutrophils in the BALF (C). Myeloperoxidase (MPO) activity in lung homogenates also showed that EC-SOD KO mice had significantly greater neutrophilic inflammation when compared to wild-type mice after *E. coli* inoculation (D). Protein in the BALF was measured to assess injury and the lungs of EC-SOD KO mice had significantly more protein in the alveolar lining fluid than wild-type mice after infection (E). Data are means (±SEM) analyzed by two-way ANOVA with a Bonferroni post-test. *$p<0.05$ shows significant difference between groups, †$p<0.05$ shows interaction hence a significant difference between wild-type and EC-SOD KO (n=6/strain for each timepoint)

3.3.2 Mice lacking EC-SOD have a greater bacterial burden in the lung after pneumonia

Left lung homogenates from wild-type or EC-SOD KO mice at 0, 6, and 24 hours after *E. coli* inoculation were plated onto LB agar to determine the bacterial burden in the lungs. This burden is reported as the percentage of bacteria cleared from the lungs over time relative to the average initial burden at 0 h (± SEM). Wild-type mice cleared more bacteria than EC-SOD KO mice at both 6 hours (71.55 ± 4.694 versus 51.31 ± 6.637, n=11/strain) and 24 hours (99.90 ± 0.025 versus 99.80 ± 0.019, n=6/strain) following bacterial inoculation (Figure 14). Even though EC-SOD KO mice had greater inflammation compared to wild-type mice (Figure 13), the EC-SOD KO mice were found to have significantly more viable bacteria in the lungs when compared to wild-type mice (Figure 14). Although the differences between EC-SOD KO and wild-type mice were statistically significant at both 6 and 24 hours following bacterial inoculation (p=0.0217 and p=0.0095, respectively), the biological significance of this difference at 24 hours is unclear as the bacterial clearance of both strains at 24 hours is nearly 100%.

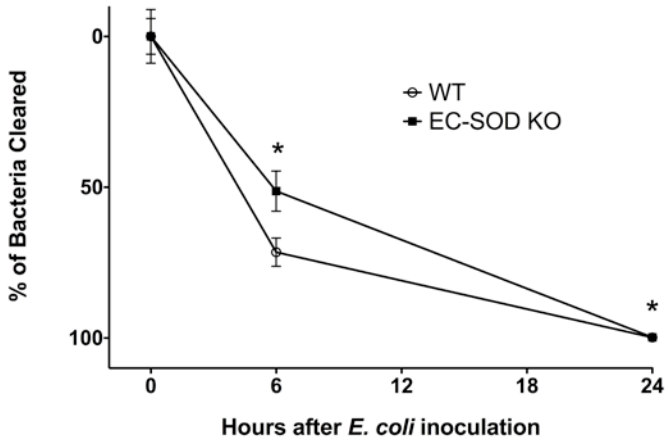

Figure 14: EC-SOD knockout mice have greater bacterial burdens than wild-type mice after *E. coli* inoculation.

Left lung homogenates from wild-type (WT) and EC-SOD knockout (KO) mice following exposure to *E. coli* were plated onto Luria-Bertani agar to determine the bacterial burden in the lungs. Percent of bacterial clearance for each strain was calculated as the average CFUs at 0 hours minus the CFUs remaining in the lungs at 6 and 24 hours divided by the average CFUs at 0 hours multiplied by 100. *$p<0.05$, unpaired t-test (n=11/strain at 6 hours; n=6/strain at 24 hours)

3.3.3 EC-SOD is present in human and murine phagocytic inflammatory cells

Prior studies have noted that EC-SOD is expressed in inflammatory cells[103, 144, 182]; however, the subcellular location and function are unknown. In the present study, electron microscopic immunolocalization of EC-SOD in human neutrophils and macrophages in human lung sections reveal that EC-SOD is located within membrane bound vesicles of these cells (Figure 15A-C). Similarly, western blotting of extracts from alveolar macrophages isolated from untreated wild-type and EC-SOD KO mice reveal the expression of both proteolyzed and full-length EC-SOD in wild-type cells (Figure 15D). In addition to protein expression in these cells, EC-SOD activity was detected in the cell lysate from alveolar, peritoneal, and bone marrow-derived macrophages using a Nitro Blue Tetrazolium SOD activity stain (Figure 16) as described in the methods (Section 3.2.8).

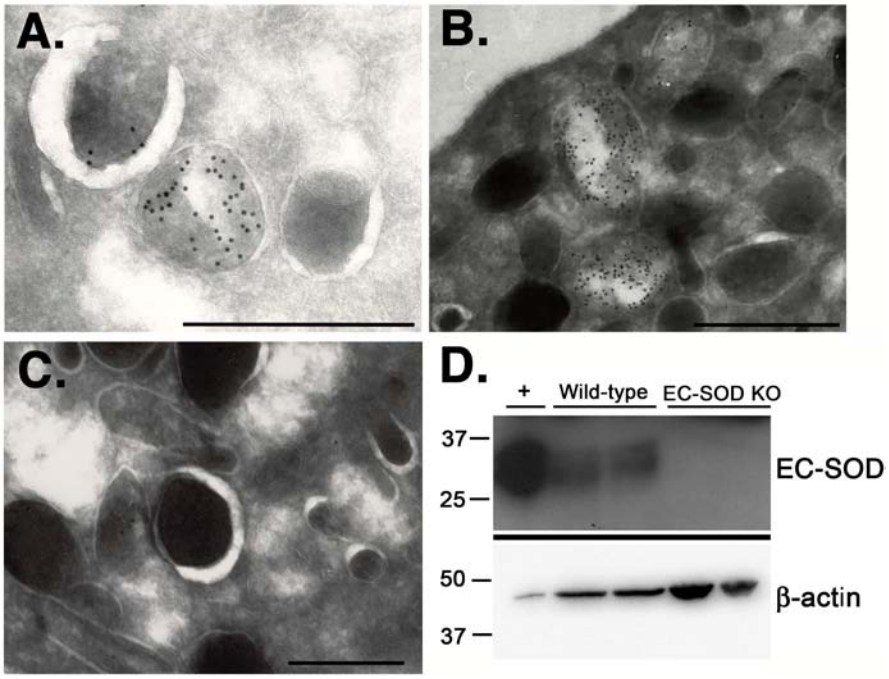

Figure 15: EC-SOD is present human and mouse phagocytic cells.

Immunogold labeling demonstrates the presence of EC-SOD in membrane bound vesicles of a polymorphonuclear leukocyte (A) and an alveolar macrophage (B). Black dots represent Protein-A gold (10 nm particles) bound to rabbit anti-human EC-SOD immunoglobulin. Non-immune rabbit IgG was used as a control and resulted in the absence of labeling (C). Western blot analysis of cell lysates from wild-type and EC-SOD knockout (KO) alveolar macrophages (D) revealed EC-SOD expression in wild-type, but not in EC-SOD KO alveolar macrophages. + = mouse EC-SOD positive control. Scale bar = 0.5 μm

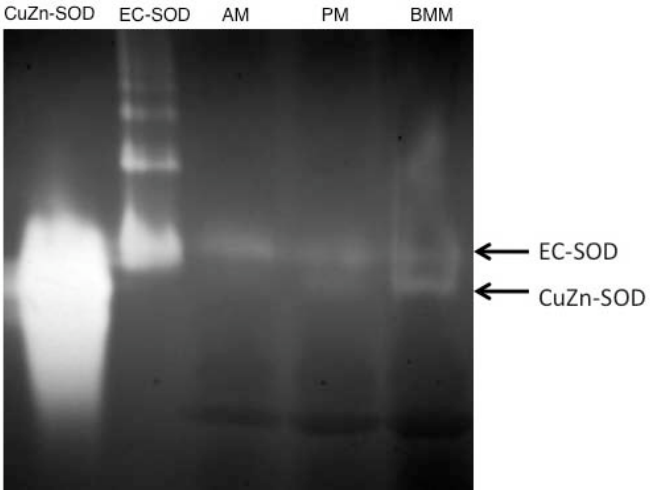

Figure 16: EC-SOD is active in mouse macrophages.

Cell lysate were collected from alveolar (AM), peritoneal (PM), and bone marrow-derived macrophages (BMM) and the EC-SOD activity was assayed using the Nitro Blue Tetrazolium Activity Gel. CuZn-SOD (21.2 μg) and EC-SOD (2 μg) were used as positive controls.

3.3.4 Cellular EC-SOD affects macrophage antimicrobial defenses

As phagocytic cell granules are known to contain a potent arsenal of compounds involved in antimicrobial defense, the novel finding that EC-SOD is stored inside and active in membrane bound vesicles in these cells suggests that EC-SOD may also play an important role in host defense against invading microbes. To study the role of EC-SOD in phagocytes, primary macrophages were isolated from the peritoneum or differentiated from bone marrow of wild-type and EC-SOD KO mice and the purity of the macrophage populations were determined by flow cytometry using Phycoerythrin-conjugated F4/80 (Table 2).

Table 2. Purity of Primary Phagocyte Populations.

	Percentage of F4/80 expressing cells average ± standard deviation from independent isolations (n)	
Type of Macrophage	Wild-type	EC-SOD Knockout
Peritoneal	87.9 ± 1.3 (n=3)	91.133 ± 2.318 (n=3)
Bone marrow-derived	95.688 ± 2.247 (n=4)	93.513 ±4.224 (n=4)

3.3.4.1 Macrophages lacking EC-SOD produce more intracellular oxidants

Electron paramagnetic resonance (EPR) spectroscopy was performed to determine the intracellular and extracellular oxidant production of wild-type and EC-SOD KO macrophages ten minutes after stimulation with PMA (Figure 17). In the presence of the cell permeable spin probe CMH, EC-SOD KO macrophages stimulated with PMA produced approximately 2.5-fold more oxidants than PMA-stimulated WT cells (2.691 μM ± 0.5989 versus 1.049 μM ± 0.2240, N=2/strain). Using the cell impermeable spin probe, PPH, there was no detectable extracellular oxidant production in PMA-stimulated wild-type or EC-SOD KO cells (Figure 17) suggesting

the oxidants produced by PMA stimulation were localized predominantly intracellularly. No oxidant production was detected with un-stimulated wild-type and EC-SOD KO macrophages exposed to either CMH or PPH spin probes (Figure 17).

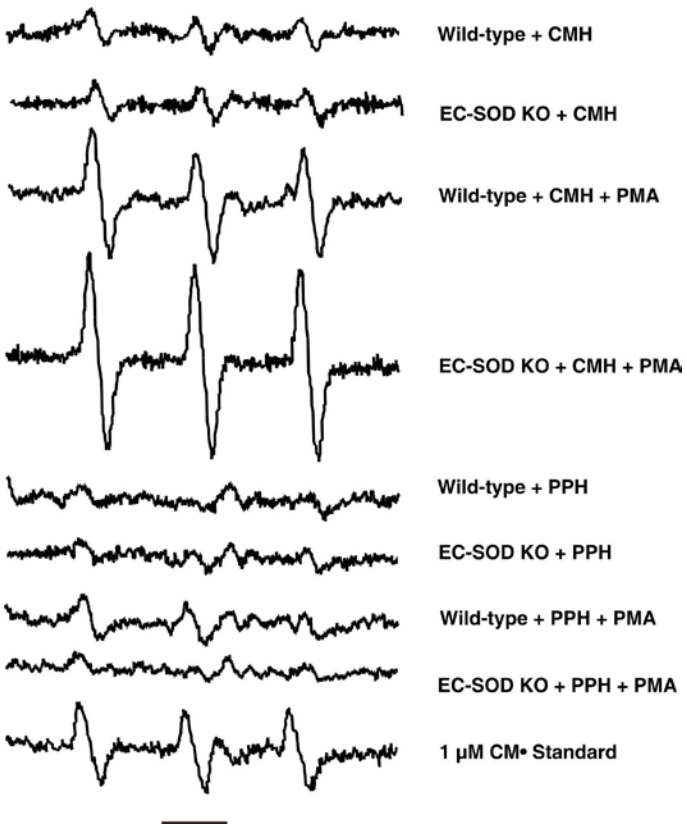

Figure 17: Macrophages lacking EC-SOD have more intracellular ROS production.

Peritoneal macrophages isolated from wild-type and EC-SOD knockout (KO) mice in Krebs HEPES buffer (pH 7.4) were exposed to 50 μM cell-permeable spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) or 50 μM non cell-permeable spin probe 1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine (PPH) and analyzed for the CM radical using a Bruker eScan Table-Top EPR spectrometer. Oxidant generation was measured in these cells after stimulation with 15 μg/ml PMA. 1 μM CM radical standard was also shown as a point of reference. Spectra represent 5 additive scans over 2 min at 37°C and are representative of three independent experiments. Bar=10 Gauss

The differences observed in oxidant production are not due to cell viability as cells that express and lack EC-SOD have similar viability when exposed to PMA (Figure 17). These data provide strong evidence that EC-SOD within macrophages reduces oxidant levels produced during the respiratory burst in response to stimuli.

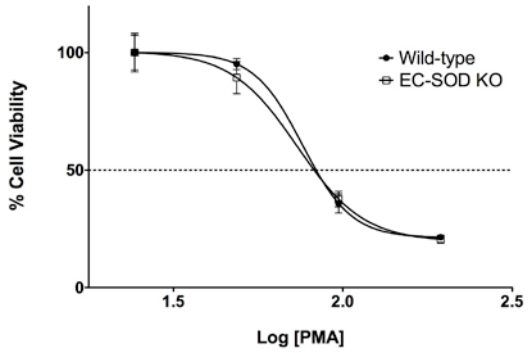

Figure 18: Cellular EC-SOD does not influence cell viability following PMA treatment.

Bone marrow-derived macrophages isolated from wild-type and EC-SOD knockout (KO) mice were treated with various concentrations of phorbol 12-myristate 13-acetate (PMA) and the cell viability was measured using either a Promega CellTiter 96 AQueous Non-radioactive Cell Proliferation Assay Kit.

3.3.4.2 Lack of EC-SOD in macrophages leads to altered pro-inflammatory cytokine expression

In addition, bone marrow-derived macrophages from wild-type and EC-SOD KO mice were isolated, cultured, and exposed to 1 μg/ml LPS. Media was removed from the cells at various times after LPS treatment and the pro-inflammatory cytokine TNF-α levels were measured by ELISA. EC-SOD KO macrophages released significantly more TNF-α 8 hours after treatment with endotoxin compared to wild-type macrophages with normal expression of EC-SOD (Figure 19). However, it was also observed that this trend was not the same at later time points and EC-SOD KO cells were even seen to produce less TNF-α than wild-type cells 48 hours after endotoxin treatment. Although perplexing, these results suggest that leukocyte-derived EC-SOD may alter pro-inflammatory cytokine production. However, additional cytokine profiling, especially in the BALF of EC-SOD KO and wild-type mice following bacterial inoculation, is necessary before any conclusions can be drawn from this finding.

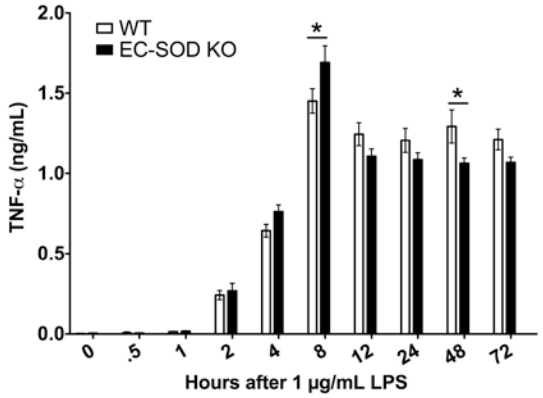

Figure 19: The presence of EC-SOD alters TNF-α secretion in response to LPS stimulation.

Bone marrow-derived macrophages were isolated and cultured from wild-type (WT) and EC-SOD knockout (KO) mice. Once differentiated, the cells were treated with lipopolysaccharide (LPS) and media was removed at various time points. TNF-α levels in the media were measured using an ELISA. *$p<0.05$

3.3.4.3 Cellular EC-SOD is necessary for efficient bacterial killing *in vitro*

While potential beneficial effects of releasing EC-SOD from activated neutrophils and macrophages for the surrounding host tissue are apparent, it is less clear what the role of EC-SOD will be on the actual anti-microbial function of these cells, however the *in vivo* data suggest that EC-SOD improved bacterial clearance (Figure 14). *In vitro* studies were performed to assess bacterial clearance in wild-type and EC-SOD KO cells. Bacterial killing assays revealed that macrophages lacking EC-SOD do not kill bacteria as efficiently as wild-type macrophages (Figure 20). Specifically, macrophages containing EC-SOD killed a significantly greater percentage of bacteria than EC-SOD-lacking cells (51.48 ± 8.756 versus 6.471 ± 6.587, $p= 0.0143$). These *in vitro* findings are consistent with the *in vivo* studies that demonstrate decreased bacterial clearance in EC-SOD KO mice in a model of bacterial pneumonia (Figure 14). These findings, in conjunction with the EPR studies above (Figure 17), suggest that EC-SOD might play an important role in macrophage-mediated bacterial clearance by regulating oxidants produced during the oxidative burst.

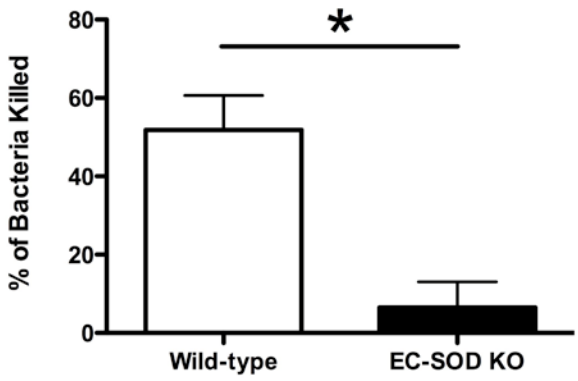

Figure 20: Macrophages lacking EC-SOD have decreased bacterial killing ability.

Bone marrow-derived macrophages from wild-type (WT) and EC-SOD knockout (KO) mice were isolated and incubated for one hour with EGFP-expressing *E. coli* to assess the bacterial killing ability of these cells. Following incubation, the bacteria remaining was determined by colony counting and the percentage of bacteria killed was calculated as the average CFUs of the bacteria alone minus the CFUs remaining after one hour incubation with cells divided by the average CFUs of the bacteria alone multiplied by 100. n=3/strain, unpaired t-test; *p=0.0143

3.3.4.4 Macrophages lacking EC-SOD have impaired phagocytic ability *in vitro*

To further delineate the role EC-SOD plays in macrophage-mediated bacterial clearance, live cell imaging was performed to visualize the dynamic interactions between EGFP-expressing *E. coli* and phagocytes expressing or lacking EC-SOD over a one hour period. Phagocytosis was assessed by enumerating the number of cells that phagocytosed at least one bacteria as well as the number of bacteria phagocytosed per cell in 10-15 random fields. These studies revealed that macrophages expressing EC-SOD (wild-type cells) are able to effectively phagocytose EGFP-expressing *E. coli*, while macrophages lacking this antioxidant enzyme (EC-SOD KO cells) have impaired phagocytosis (Figure 21).

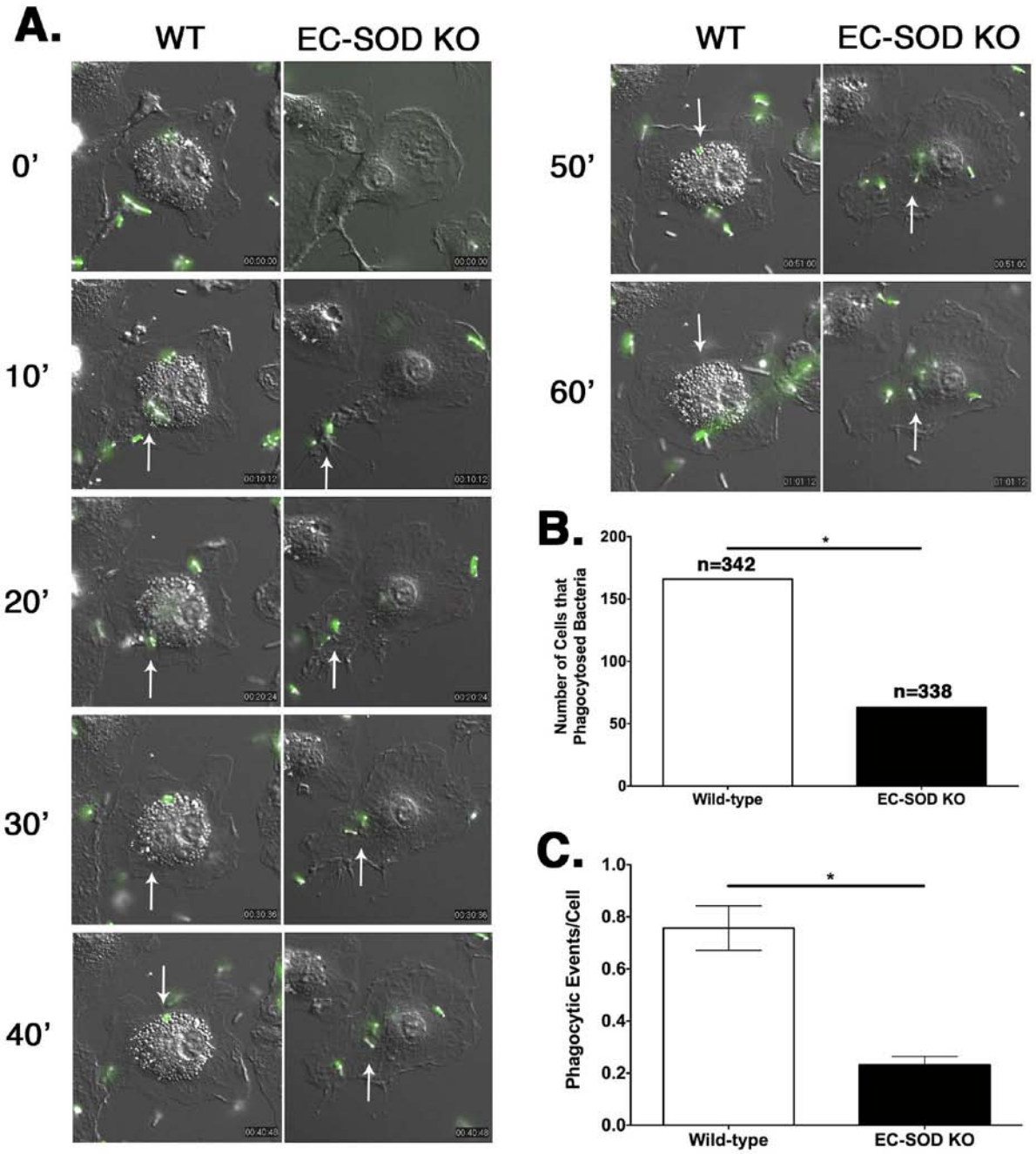

Figure 21: Lack of cellular EC-SOD impairs bacterial phagocytosis.

(A) Live cell imaging of bacterial phagocytosis was performed by incubating bone marrow-derived macrophages isolated from wild-type (WT) and EC-SOD knockout (KO) mice with EGFP-expressing *E. coli* (green). Images (10 min intervals, 40x magnification) show bacteria associating with and being phagocytosed by wild-type macrophages (arrows depict bacteria being internalized (phagocytosed) by the macrophages). However, although bacteria associate with EC-SOD KO macrophages, they are not phagocytosed as is evident by observing that the bacteria remain on the cell surface of these macrophages over 1 hour after addition of bacteria (arrows point to bacteria associating with the macrophage, but they are never phagocytosed). Phagocytosis was quantified as the number of macrophages that phagocytosed bacteria (B, Fisher's exact test) and the number of bacteria that were phagocytosed (phagocytic events) per cell (C, unpaired t-test). n=total cells analyzed; *$p<0.05$

This difference in macrophage function is not due to reduced cell viability as there is no difference in viability observed between cells that expressed or lacked EC-SOD following treatment with LPS (Figure 22) or EGFP-expressing *E. coli* (visual assessment with trypan blue). This effect was also found to be specific for phagocytosis as endocytosis of fluorescent-conjugated dextran over one hour was not affected by the absence of EC-SOD (Figure 23). These results suggest that macrophage expression of EC-SOD has an important function in promoting efficient bacterial phagocytosis.

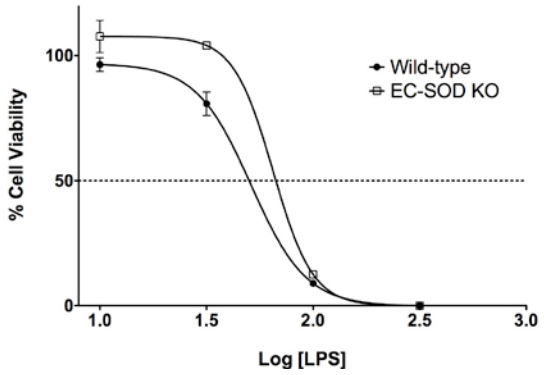

Figure 22: Cellular EC-SOD does not influence cell viability following exposure to LPS.

Bone marrow-derived macrophages isolated from wild-type and EC-SOD KO mice were treated with various concentrations of lipopolysaccharide (LPS) and the cell viability was measured using either a Promega CellTiter 96 AQueous Non-radioactive Cell Proliferation Assay Kit.

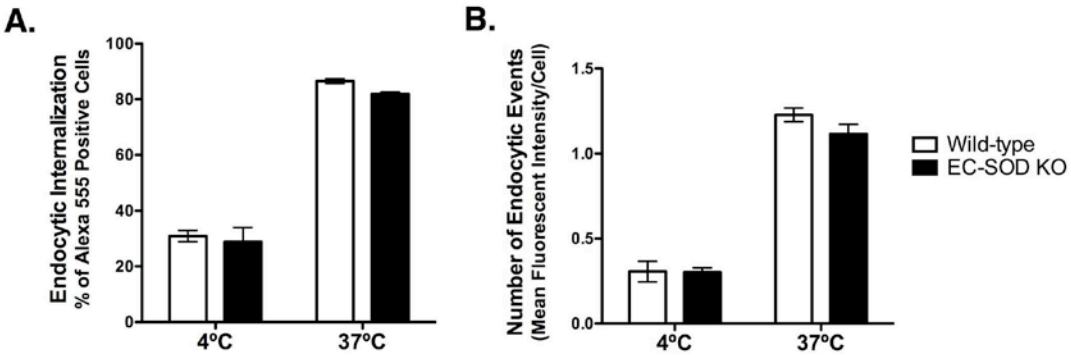

Figure 23: Endocytosis is not altered by intracellular EC-SOD in phagocytes.

Bone marrow-derived macrophages were cultured from wild-type and EC-SOD knockout (KO) mice and incubated with 5 µg/mL Alexa Fluor® 555 dextran for 1 hour. No differences in endocytosis (A and B) were observed between cells that express or lack EC-SOD. Results shown are from cells isolated and differentiated independently from four mice per strain.

3.4 DISCUSSION

Production of ROS is essential for antimicrobial defense, but this response needs to be carefully regulated to minimize the amount of damage to the surrounding tissue. The present study demonstrates that EC-SOD inhibits inflammation, while increasing bacterial clearance in a murine model of bacterial pneumonia. In addition, these studies indicate that one mechanism in which EC-SOD contributes to bacterial clearance is by promoting bacterial phagocytosis and killing by macrophages. These novel findings demonstrate that production of oxidants is not sufficient to eliminate an infection, but in fact the regulation of oxidant production by EC-SOD may be necessary for effective phagocytosis and killing of bacteria.

Indeed, recent studies have highlighted the importance of oxidant production in the induction and regulation of innate immunity, especially in the context of CGD. Patients with CGD are known to have defective phagocyte NADPH oxidase activity resulting in decreased $O_2^{\bullet-}$ generation, which strongly correlates with increased incidence of life-threatening infections and excessive inflammation. Macrophages from these patients are known to have abnormal function as these cells release higher levels of anti-inflammatory cytokines and lower levels of pro-inflammatory cytokines in response to bacterial stimuli[212]. Similarly, a study using a murine model of CGD further implicated oxidants as a critical regulator of the innate response as NADPH oxidase was shown to activate the anti-inflammatory transcription factor Nrf-2 and inhibit the pro-inflammatory transcription factor NF-κB thereby limiting inflammation[213]. Together, these findings show that oxidants produced via NADPH oxidase play a crucial role in regulating inflammation and host defense.

Numerous studies have also directly shown that antioxidant activity is necessary to regulate macrophage function. Specifically, EC-SOD has been shown to attenuate LPS-induced

inflammation in the lung by decreasing pro-inflammatory cytokine release from phagocytes[197]. Similarly, the antioxidant N-acetylcysteine has been shown to modulate the macrophage phagocytic response to endotoxin by decreasing the production of ROS and the release of the pro-inflammatory cytokine, TNF-α[214, 215]. This effect was found to be potentially due to its ability to undergo cellular uptake and therefore influence the oxidant-induced intracellular signal transduction activity within the phagocyte[215]. A more recent study found that hyperoxia reduces macrophage phagocytosis and that exogenous SOD treatment preserved actin cytoskeleton organization and phagocytosis of *Pseudomonas aeruginosa*[216]. These findings suggest that decreased $O_2^{\bullet-}$ scavenging may directly contribute to the defective phagocytosis observed in these cells. The present work further supports these previous studies in finding that regulation of oxidant production is important in promoting macrophage function. While prior studies found that administration of antioxidants or oxidative stress altered macrophage phagocytosis, the current study demonstrates that endogenous expression of EC-SOD inside membrane bound vesicles in these cells directly regulates oxidant production during the respiratory burst and is necessary to promote effective phagocytosis and bacterial killing. It is still unclear whether unregulated or elevated oxidant production directly affects the ability of phagocytes to recognize and/or ingest the microbes. However, the live cell imaging studies suggest bacteria are associating with macrophages lacking EC-SOD, but are not being phagocytosed. Further research will need to be conducted to fully understand how EC-SOD located inside phagocytic inflammatory cells modulates phagocytosis by controlling the oxidative environment in these cells.

A recent study has shown that an antioxidant mimetic, MnTE-2-PyP, was able to significantly decrease the number of *Mycobacterium abscessus* growing inside infected

macrophage-like cells by promoting increased fusion of *M. abscessus*-containing phagosomes with lysosomes[217]. Thus, promoting phagolysosome formation may be one mechanism in which EC-SOD stimulates bacterial clearance by macrophages.

The present data describe a novel and important function for EC-SOD inside membrane bound vesicles of macrophages and neutrophils in the innate inflammatory defense against bacterial infection. Western blot analysis of alveolar macrophage cell lysates from wild-type mice show the presence of both full-length and proteolyzed EC-SOD inside these phagocytes. While protease inhibitors were present during the isolation of proteins from these cells, macrophages contain high amounts of proteases. Therefore, it is unknown whether the EC-SOD in macrophages is all full-length EC-SOD that underwent adventitious proteolysis during cell lysis or if the western blot analysis is a true reflection that macrophages contain a mixture of both proteolyzed and full-length EC-SOD. Regardless, the current findings suggest that EC-SOD present in phagocytic inflammatory cells is enzymatically active and plays a central role in promoting bacterial killing by modulating phagocytosis. Although EC-SOD in phagocytic cells contributes to innate host defense against gram-negative bacterium, future investigations into the role of EC-SOD in other infection models will need to be conducted to determine whether its role is conserved for other bacterial infections as well.

In summary, these studies describe a novel and important function for EC-SOD inside of macrophages and neutrophils in the innate inflammatory defense against bacterial infection (Figure 24). These studies suggest that EC-SOD present in phagocytic inflammatory cells plays a central role in bacterial clearance. It is interesting that all models of lung injury examined to date result in a marked accumulation of EC-SOD in the alveolar lining fluid. This accumulation of EC-SOD in response to pulmonary injury may play an important role in defense against bacterial

infection (Figure 24). Thus, supplementation of EC-SOD may also be useful as an adjuvant to normal antibiotic therapy in the treatment of pneumonia and other bacterial infections.

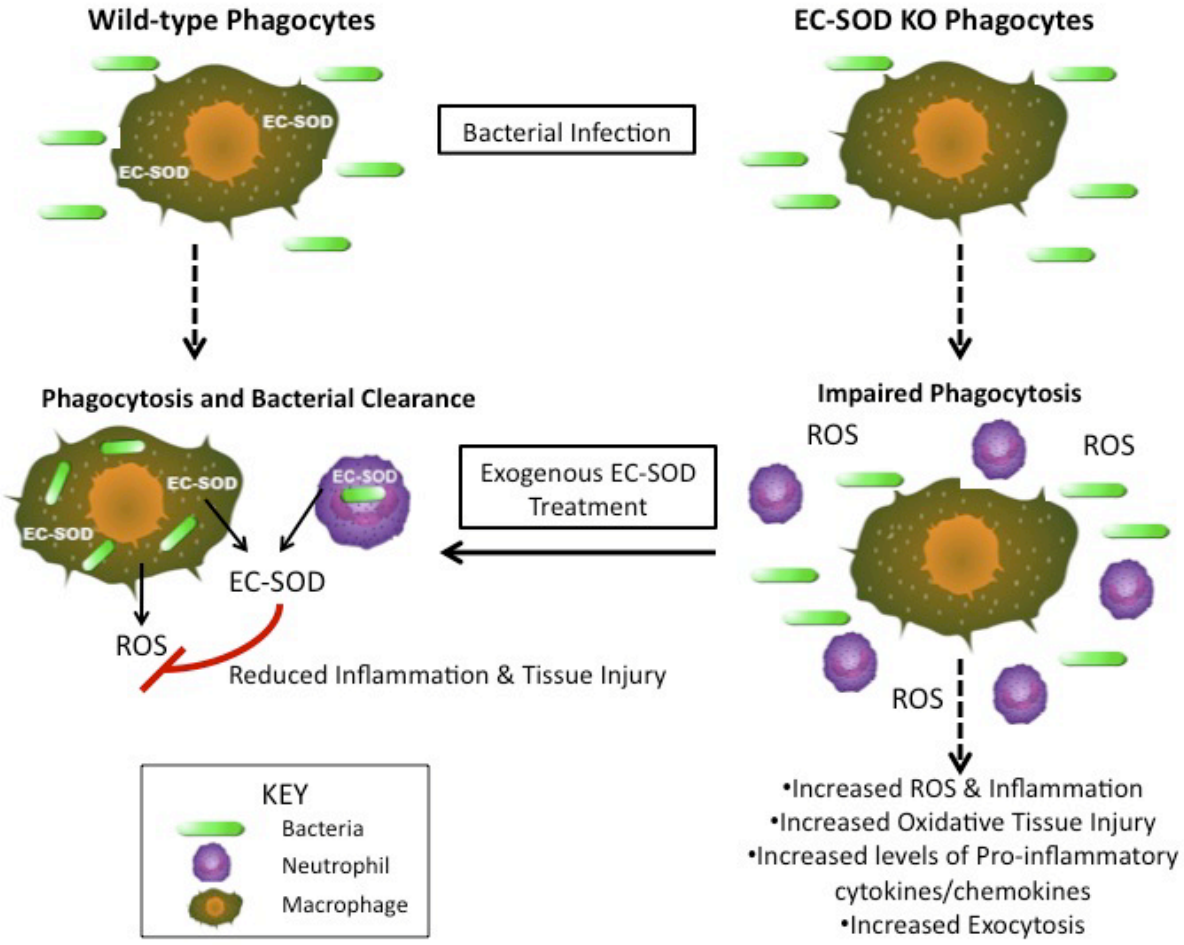

Figure 24: Proposed Role of EC-SOD within phagocytic cells.

EC-SOD in wild-type phagocytes promotes bacterial phagocytosis. This leads to effective clearance of bacterial pathogens and decreased oxidative injury, which in turn promotes efficient resolution of inflammation and minimal tissue injury. The absence of EC-SOD in inflammatory cells (EC-SOD KO) impairs bacterial phagocytosis and thus causes prolonged infection and the recruitment of additional inflammatory cells. This additional inflammation accentuates production of reactive oxygen species and release of proteases resulting in increased tissue injury. Based on its role in regulating inflammation and phagocytosis and killing, treatment with exogenous EC-SOD would likely stimulate more effective bacterial clearance while inhibiting further inflammation and oxidative tissue injury.

4.0 FINAL DISCUSSION

Endogenous antioxidant enzymes are normal cellular defenses that can protect against oxidant-induced tissue injury. EC-SOD is highly expressed in the lung and arteries and is bound to the ECM via its positively charged matrix-binding domain[159-161, 181]. Although it was well established that EC-SOD is lost from the lung interstitium and accumulates in the alveolar lining fluid in response to a variety of pulmonary insults and infections, the significance of this accumulation was unclear. In addition to its localization on matrices and in extracellular fluids, EC-SOD is also present intracellularly in neutrophils and macrophages[103, 144, 182]. However, prior to the studies described above, the localization and the functional role of EC-SOD in leukocytes were unknown.

Two different pulmonary disease models were employed to study the role of leukocyte-derived EC-SOD: the asbestos-induced ILD model and the bacterial pneumonia model. The ILD model shows effects primarily in the interstitium that are characterized by interstitial inflammation and fibrosis with some additional inflammation in the airspaces. In contrast, the bacterial pneumonia model demonstrates inflammation primarily in the airspaces in the absence of interstitial involvement. Although these model systems produce pathologically different injuries in the lung, there is commonality in that the biological function of EC-SOD in each injury largely depends upon its location in the lung.

4.1 DISCUSSION OF EC-SOD IN THE LUNG AND AIRSPACES

First, the localization of EC-SOD in the lung is crucial for protecting the interstitium from oxidative injury. In the normal lung, EC-SOD is bound to the ECM via its positively charged matrix-binding domain[160]. It is through this physical interaction that EC-SOD has been shown to prevent the oxidative fragmentation of numerous ECM components[170, 171, 198, 199]. The protection of the tissue matrix by EC-SOD has been shown to be one mechanism by which EC-SOD prevents inflammation and fibrosis following interstitial lung injury[144, 171, 198]. Overall, loss of EC-SOD from the lung is detrimental to the host tissue. A recent study using a Cre-Lox system to induce an acute loss of EC-SOD resulted in 85% mortality secondary to spontaneous development of acute pulmonary inflammation with a histologic appearance similar to ARDS[200]. This study shows that EC-SOD in the lung plays a vital role in continuously protecting it from injury even in ambient air.

It is interesting that all models of lung injury (interstitial lung injury and infection) examined to date result in a marked accumulation of EC-SOD in the alveolar lining fluid. It was originally thought that leukocytes could transport and release EC-SOD into the alveolar lining fluid in response to interstitial lung injury. However, the present findings confirm that airspace EC-SOD detected after interstitial lung injury is from the lung parenchyma and not an extrapulmonary source. As all the EC-SOD that accumulates in the BALF is from the interstitium, therapies to minimize the loss of EC-SOD from the lung may prove to be beneficial for the treatment of ILDs.

Although this depletion of full-length EC-SOD from the lung parenchyma is detrimental in this disease, proteolyzed EC-SOD is known to be enzymatically active[165]. Therefore, the retention of EC-SOD in the airspaces may be an adaptive and protective mechanism to limit

inflammation and injury to airway epithelial cells. This is evident by the fact that both inflammation and fibrosis are increased when EC-SOD is completely absent from the lung. Specifically, EC-SOD KO mice are reported to have increased inflammation and fibrosis when compared to wild-type mice in numerous models of interstitial lung injury[98, 144]. In addition, EC-SOD has been found to have a novel and important role in the regulation of innate immune defense by promoting the phagocytosis and killing of gram-negative bacterium by macrophages. In light of this current work, it is possible that airspace EC-SOD could increase macrophage phagocytosis and killing to help prevent the occurrence of bacterial infections (Further discussed in Section 4.3.3).

Although leukocytes are not the source of airspace EC-SOD following interstitial lung injury, the present findings in conjunction with previous work[103] strongly suggest that EC-SOD is released from inflammatory cells into the airspace following bacterial pneumonia. Indeed, many studies have noted that EC-SOD is present in leukocytes[103, 144, 182]. The present work furthers this current knowledge by showing that EC-SOD is contained in intracellular vesicles (Figure 15). This work also demonstrates that both full-length and proteolyzed EC-SOD are detected inside of murine alveolar macrophages. Therefore, this novel intracellular localization of EC-SOD may infer that EC-SOD could be released upon cell activation into the airspace. Interestingly, a mixture of full-length and proteolyzed EC-SOD was detected in the BALF of wild-type mice 6 hours after bacterial inoculation possibly suggesting that EC-SOD is released from these cells as the full-length protein. By 24 hours following bacterial inoculation, the majority of the EC-SOD found in the BALF was proteolyzed EC-SOD potentially due to proteolytic cleavage of this antioxidant enzyme that occurred in the extracellular fluids.

Once in the airspace, full-length EC-SOD has the capacity to interact directly with the bacterium via its positively charged matrix-binding domain as the surface coating (LPS and anionic lipids) and bacterial membrane (acidic phospholipids) of gram-negative bacterium make it negatively charged. Therefore, full-length EC-SOD could coat the bacterium and possibly serve as an "antioxidant-opsonizing agent" to enhance bacterial phagocytosis. In addition, full-length EC-SOD is known to be endocytosed into endothelial cells through clathrin-mediated pathway[218]. However, in a recent study, Pasupuleti and colleagues generated a peptide that corresponded to that C-terminal region of EC-SOD (RQAREHSERKKRRRESECKAA) and observed that this peptide was able to induce membrane leakage of negatively charged liposomes (such as gram-negative bacterium)[219]. Their results suggest the C-terminal domain of EC-SOD can directly kill the bacteria, however, these effects were abrogated at physiological salt conditions as well as in plasma. The current study challenges these findings as clear differences in phagocytic ability were observed between EC-SOD expressing cells and EC-SOD KO cells. Furthermore, incubation of *E. coli* with full-length EC-SOD did not result in decreased bacteria viability in our hands. Unlike in an *in vitro* system, it is also likely that the C-terminal domain is rapidly cleared or further degraded when it is proteolytically cleaved from EC-SOD *in vivo.* Overall, the presence of EC-SOD is clearly essential for its function; however whether or not EC-SOD confers matrix affinity might also influence its role in innate immunity.

4.2 DISCUSSION OF CELLULAR EC-SOD

As its location is essential for protecting the lung parenchyma, the intracellular location of EC-SOD in macrophages is now shown to be necessary for efficient phagocytosis. This work is the

first to report that EC-SOD plays a novel and important role in the regulation of innate immune defense by promoting the phagocytosis and killing of gram-negative bacterium by macrophages. In the lung, macrophages are the first inflammatory cells to encounter microbes and particulates that enter alveolar spaces and are continually eliminating these airborne substances without inducing inflammation. However, if they are unable to clear the microbes, macrophages recruit neutrophils into the lung to help eradicate the infection. Macrophages therefore are exposed to large quantities of ROS and RNS produced endogenously or by activated neutrophils, which may alter microbial recognition and phagocytosis via pattern recognition receptors. Indeed, a study by Bozeman and colleagues showed that exogenous and endogenously-produced H_2O_2 by neutrophils inhibited ligand binding to the mannose receptors on rat macrophages and decreased the number of macrophage mannose receptors on the cell surface[220]. Conversely, studies have also shown that oxidants can enhance phagocytosis. For instance, both exogenous and endogenously-produced H_2O_2 enhanced the phagocytic capacity of neutrophils[221]. In this same study, Pricop and others also show that human monocytes incubated in the presence of stimulated neutrophils showed increased Fcγ receptor signaling and amplified phagocytosis[221]. In this case, the reactive oxygen species generated in an inflammatory environment act in an autocrine and paracrine manner to rapidly increase Fcγ receptor-mediated phagocytosis. In the context of the current findings, leukocyte-derived EC-SOD may change the composition of oxidants during the respiratory burst creating a favorable environment for phagocytosis of gram-negative bacterium. EC-SOD in leukocytes may lower steady state levels of $O_2^{\bullet-}$ as EC-SOD KO cells produced more oxidants following PMA stimulation. However, EC-SOD in leukocytes may lower, raise, or not alter H_2O_2 levels depending on the redox state of the environment. Therefore, elucidating the oxidant composition of the respiratory burst and determining which

oxidative species inhibit phagocytosis in macrophages lacking EC-SOD may assist in determining the regulatory role of EC-SOD in phagocytosis.

In addition to affecting microbial recognition, ROS are capable of modulating mechanisms by activating transcription factors, increasing the levels of cytokines, chemokines, and other soluble pro-inflammatory factors, and altering the expression of cell surface adhesion molecules. Specifically, activation of the redox-sensitive transcription factor NF-κB has been shown to induce the expression of pro-inflammatory cytokines, chemokines, and adhesion genes. Under normal conditions, NF-κB is maintained in the cytosol in a complex with its inhibitor, IκB. Upon cell activation by cytokines or endotoxin, IκB is phosphorylated on specific serines, inducing a release from NF-κB, which can go to the nucleus and activate transcription of pro-inflammatory mediators. All protein-tyrosine phosphatases have a reactive cysteine residue in their active site, which must be reduced for catalytic activity. Therefore, ROS can inhibit activity of these phosphatases through reversible oxidation of their catalytic site, while antioxidants would protect these important regulators of intracellular signaling[222, 223]. Because ROS and RNS are shown to mediate cell-signaling events in inflammation, EC-SOD in leukocytes could play an important role in the inflammatory response by limiting the availability of these reactive species to participate in these reactions.

In agreement with the present work, other studies have noted the importance of intracellular antioxidants in modulating these inflammatory cell responses. Indeed, a study by Bulger and others examined the effect of intracellular and extracellular antioxidants on the alveolar macrophage response to endotoxin and found that only intracellular antioxidants were able to mediate the macrophage response to inflammatory stimuli[215]. Overall, intracellular signal transduction activity of ROS is crucial to macrophage pro-inflammatory activation and

intracellular EC-SOD would be vital for regulating these responses and this would potentially be one mechanism by which leukocyte-derived EC-SOD limits inflammation following bacterial infection.

Other studies have noted the importance of EC-SOD in modulating inflammation. For instance, LPS-induced lung inflammation was found to be exaggerated in EC-SOD KO mice while diminished in mice overexpressing the EC-SOD protein in their lungs[197]. In this study, lack of EC-SOD expression was correlated with increased pro-inflammatory cytokine release both *in vivo* and *in vitro* as well as increased expression of cell adhesion molecules necessary for neutrophil extravasation[197]. This LPS-mediated release of TNF-α and MIP-2 was also attenuated by EC-SOD treatment. As EC-SOD has been shown to regulate the release of cytokines, cytokines have also been shown to regulate EC-SOD. The pro-inflammatory cytokines, such as IL-1 and interferon-γ, have been shown to increase EC-SOD expression in both fibroblasts and type II epithelial cells[224, 225]. The presence of pro-inflammatory cytokines is associated with increased NF-κB activation and this increased in NF-κB activation has been shown to regulate EC-SOD transcription[175, 225]. Together, these studies suggest that the location of EC-SOD inside leukocytes would be a prime location for its role in the augmentation of oxidant-mediated induction of inflammation.

Although the underlying mechanism is still unknown, it is clear that leukocyte-derived EC-SOD promotes phagocytosis and limits inflammation. One potential mechanism in which EC-SOD functions in this capacity as suggested by the literature is by preventing oxidant-mediated damage/inactivation of cell surface receptors and excessive cell activation. Overall, the presence of EC-SOD inside phagocytic cells allows for efficient phagocytosis while the absence of this protein leads to impaired or frustrated phagocytosis and ultimately less bacterial

clearance. Impaired phagocytosis can augment inflammation and tissue injury due to constant stimulation of inflammatory and epithelial cells by the unresolved bacterial infection. This will lead to increased release of pro-inflammatory cytokines and chemokines that further induce inflammation. Indeed, studies have found that decreased phagocytic activity of neutrophils contributes to the development of sepsis in response to bacterial infections[226]. In this regard, the localization of EC-SOD in phagocytes is vital to promote innate immune responses and to limit inflammation and subsequent injury in the lung.

4.3 ADDITIONAL CONSIDERATIONS & FUTURE DIRECTIONS

This work is the first to describe that EC-SOD inside leukocytes promotes bacterial phagocytosis and killing of gram-negative bacterium. With this novel finding comes a plethora of new avenues of research to better understand how this antioxidant regulates the function of immune cells. The goal of this section is to present some additional considerations for the current work as well as highlight a few future experiments that will serve as tools to uncover the novel mechanism behind the regulation of leukocyte-derived EC-SOD in innate immunity.

4.3.1 Determine if leukocytes are source of airspace EC-SOD following bacterial inoculation

The present findings in conjunction with previous work[103] strongly suggest that EC-SOD is released from inflammatory cells into the airspace in response to bacterial inoculation and this leukocyte-derived EC-SOD limits inflammation by promoting bacterial phagocytosis and killing.

However, it is possible that the up-regulation of EC-SOD by type II alveolar epithelial cells could also account for the accumulation of EC-SOD in the alveolar lining fluid without a loss from the parenchyma as TNF-α and interferon-γ are potent inducers of EC-SOD expression in these cells[225].

To positively conclude leukocytes deliver and release EC-SOD to sites of active inflammation following bacterial inoculation, BMC mice will need to be generated as discussed in Chapter 2. If leukocytes are the sole source of EC-SOD in the airspace following bacterial pneumonia, EC-SOD will be detected in the BALF of KO/WT mice, which express EC-SOD only in their inflammatory cells. In addition, EC-SOD will not be present in the BALF of WT/KO mice that only have pulmonary EC-SOD expression. It is possible that both cell types could contribute to the EC-SOD present in the airspace. Therefore, the levels of EC-SOD in the BALF of WT/WT (total EC-SOD released), WT/KO (type II alveolar epithelial cells), and KO/WT (leukocytes) mice can be compared to deduce the relative contribution of each cell type. Regardless, the present *in vitro* experiments have shown that intracellular EC-SOD is important for macrophage function and thus leukocyte-derived EC-SOD is still essential for host defense against gram-negative bacterium.

The current findings also strongly suggest that leukocyte-derived EC-SOD reduces inflammation while promoting bacterial clearance by enhancing macrophage phagocytosis and killing. Therefore, it is hypothesized that these BMC studies will also show that EC-SOD expression in phagocytic cells plays an important role in augmenting bacterial clearance and limiting inflammation in bacterial pneumonia. This will be evident if there is decreased inflammation and increased bacterial clearance (decreased bacterial burden in the lungs) in BMC

mice that have EC-SOD in the inflammatory cells but no pulmonary expression of EC-SOD (KO/WT) when compared to BMC mice that completely lack EC-SOD expression (KO/KO).

4.3.2 Determine if EC-SOD promotes phagocytosis of all microbes

The current findings presented in this dissertation show that macrophages expressing EC-SOD (wild-type cells) display normal phagocytic and killing activity of the gram-negative bacterium *E. coli*. While on the other hand, macrophages lacking this antioxidant enzyme cannot efficiently phagocytose and kill this bacterium. These *in vitro* findings are also correlated *in vivo* as EC-SOD KO mice had greater inflammation and bacterial burden in their lungs than wild-type mice after bacterial inoculation. However, it is possible that EC-SOD is only necessary for the phagocytosis and killing of gram-negative bacterium. To test if leukocyte-derived EC-SOD promotes macrophage function in general, the ability for EC-SOD-expressing cells and cells that lack EC-SOD to phagocytosis different microorganisms, such as gram-positive bacterium, as well as opsonized particles will need to be assessed. Identifying whether or not this response is non-specific or exclusive for gram-negative bacterium will not only allow for a deeper understanding of the role of EC-SOD in host defense, but more importantly may reveal essential cell-surface receptors for regulating this response for future studies.

Despite whether or not EC-SOD promotes the phagocytosis and killing of all microorganisms equally, the current findings would suggest that all types of macrophages would respond similarly to gram-negative bacterium regardless of the host tissue as both bone marrow-derived and peritoneal macrophages were used in the *in vitro* studies presented in this dissertation. Therefore, these results may not only be relevant to pulmonary infections, but applicable for limiting inflammation and infection in other organs.

4.3.3 Dynamics of EC-SOD and macrophages

The present work is the first to report that EC-SOD is located in membrane bound vesicles inside macrophages and neutrophils. However, the exact subcellular location is still ambiguous. Without further classification, it is difficult to determine if EC-SOD is present in endocytic or exocytic vesicles. The *in vivo* bone marrow chimera studies described above (Section 2.2.1) could help make this distinction, but *in vitro* studies could also be conducted to determine if activated phagocytes release EC-SOD by measuring the levels of EC-SOD release into the media following cell stimulation.

As a study has observed that full-length EC-SOD is able to be endocytosed[218], the possibility still exists that phagocytes also have the capacity to uptake EC-SOD regardless of the presence of its matrix-binding domain. Fluorescently labeling EC-SOD and observing its behavior in the presence of stimulated and unstimulated cells using *in vitro* microscopy techniques would likely be valuable for testing this hypothesis. As macrophages and neutrophils are phagocytes, it is possible that they can uptake EC-SOD through normal processes and therefore store this protein in endocytic vesicles.

Preliminary experiments show that exogenous EC-SOD and CuZn-SOD can restore bacterial phagocytosis thereby promoting bacterial clearance (Figure 25). This suggests the enzymatic activity is what is important as CuZn-SOD and EC-SOD could restore phagocytosis. It is important to note, however, that EC-SOD appears to be a more potent inducer of phagocytosis as 10 fold less EC-SOD produces a more complete restoration when compared to CuZn-SOD. Overall, the movement and location of EC-SOD inside and outside the cell in response to bacterial stimuli will help better determine the dynamic role EC-SOD may play in regulating macrophage function and ultimately innate immunity. Although the underlying

mechanism is unclear, the location of EC-SOD in the alveolar lining fluid following bacterial inoculation is likely vital for its function in innate immune defense.

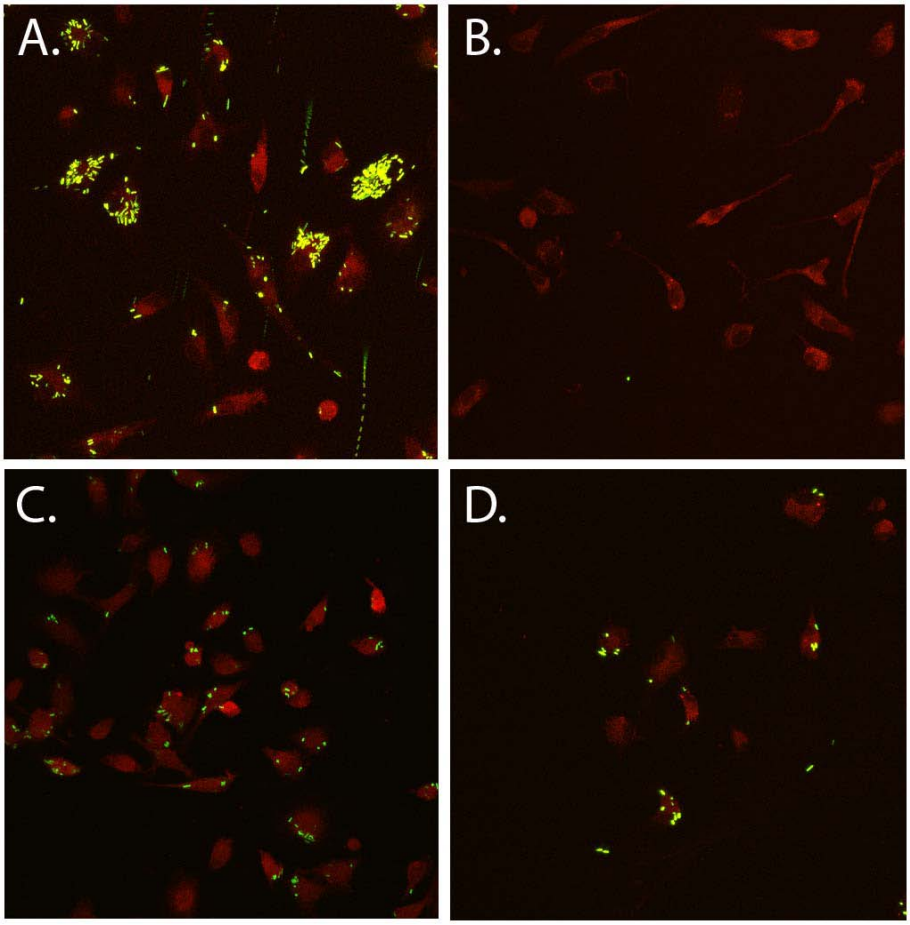

Figure 25: Exogenous SOD treatment promotes bacterial phagocytosis in EC-SOD KO macrophages.

Bone marrow-derived macrophages from wild-type and EC-SOD KO mice (stained with CellTracker Orange CMRA) were incubated with EGFP-expressing *E. coli* (green) for 2 hrs and in the presence of CuZn-SOD and EC-SOD. Phagocytosed bacteria were visualized by confocal microscopy. Wild-type macrophages phagocytosed the bacteria (A), while EC-SOD KO macrophages showed no evidence of bacterial phagocytosis (B). However, phagocytosis could be restored in EC-SOD KO macrophages by addition of 10 units of EC-SOD (C) or 100 units of CuZn-SOD (D). All images are maximum z projections (composite of 30 z sections, 2 μm apart).

4.3.4 Investigate oxidant-mediated inhibition of phagocytosis

The present data indicates that macrophages from EC-SOD KO mice do not phagocytose bacteria, which suggests that scavenging $O_2^{\bullet-}$ is necessary to promote bacterial phagocytosis and

killing. While lack of EC-SOD will increase the steady state concentration of $O_2^{\bullet-}$, it can also alter many other ROS downstream of $O_2^{\bullet-}$ in the reduction of oxygen to water. Thus, it is unclear which oxidative species are responsible for regulating EC-SOD dependent phagocytosis.

Since SODs are $O_2^{\bullet-}$ scavengers and preliminary results show administration of exogenous EC-SOD or CuZn-SOD restores bacterial phagocytosis in the EC-SOD KO macrophages (Figure 25), it is hypothesized that oxidants that are scavenged or prevented from forming by EC-SOD may interfere with normal phagocytosis and killing. If our hypothesis is correct and $O_2^{\bullet-}$ and $^{\bullet}OH$ are responsible for the deficient bacterial phagocytosis seen in EC-SOD KO cells, catalase (scavenging H_2O_2) should have no or some effect on restoration of phagocytosis in EC-SOD KO cells. However, we may find equivalent restoration of phagocytosis with catalase. Although we do not expect this result, this would suggest that either H_2O_2 itself is playing a major role in the altered phagocytosis seen in EC-SOD KO cells or that $^{\bullet}OH$ produced from iron catalyzed Haber-Weiss reactions[227] is the predominant mechanism by which the loss of EC-SOD inhibits phagocytosis. If there is only a partial recovery of phagocytosis with catalase treatment, it may suggest that $ONOO^-$ generation of $^{\bullet}OH$ is also contributing to this effect. As $^{\bullet}OH$ can be produced by the breakdown of $ONOO^-$, one would expect that inhibition of NOS with Nω-nitro-L-arginine might partially restore phagocytosis in EC-SOD KO cells. However, this would be in contrast to a prior study that showed inhibition of NOS inhibits bacterial phagocytosis[228]. This study suggests that $NO^{\bullet}$ itself is vital for phagocytosis and may indicate that all of EC-SOD's effects are due to its abilities to inhibit $O_2^{\bullet-}$-dependent inactivation of $NO^{\bullet}$[229]. If this is correct, we expect that EC-SOD treatment will restore phagocytosis while no other scavengers will.

4.3.5 Determine the role of EC-SOD in neutrophils

In addition to macrophages, EC-SOD is also known to be expressed in human and murine neutrophils. The current findings show that even though EC-SOD KO mice initially had more bacterial burden than wild-type mice at 6 hours, these mice were able to begin to clear bacteria by 24 hours (Figure 14). This is likely due to the fact that there is more neutrophilic inflammation and these cells produce large amounts of oxidants and other bactericidal substances that kill the bacteria, but in a way that may be harmful to the lung. Unfortunately, the oxidative stress in the lungs of these mice is still unknown, as this was not investigated in the current work. Furthermore, the effect of EC-SOD on neutrophil function (i.e. phagocytosis and killing ability) is still uncertain. Therefore, assessing the oxidative injury in the lung as well as examining the function of neutrophils *in vitro* could better indicate the role of neutrophil-derived EC-SOD in bacterial pneumonia.

It is important to note that studies were attempted to isolate neutrophils from wild-type and EC-SOD KO mice and to investigate the role of EC-SOD in neutrophils. However, preparation of mouse neutrophils from bone marrow using a three-layer Percoll gradient as described by Boxio and others was limited due to cell viability[230]. A second method using intraperitoneal injection of casein to elicit neutrophils to the peritoneum[231] was limited due to low purity as approximately 50% neutrophils and 50% macrophages were obtained from this isolation method. Recently, a long-term bone-marrow culture system for neutrophils was described and was reported to be able to overcome the purity and short life-span issues of other methods[232]. Therefore, this new method may be useful for future investigations involving neutrophils. As pneumonias are characterized by a large influx of neutrophils, it is important to pursue studies to determine the role of neutrophil-derived EC-SOD in host defense.

4.3.6 Summary

The current work is the framework for future investigations to better understand how EC-SOD regulates macrophage phagocytosis and killing. Based on the current findings and preliminary results shown in this dissertation, macrophage phagocytosis and activation is likely altered by the oxidative environment. Therefore, the exact localization of EC-SOD in a quiescent cell and its movement following cell activation will likely further reveal its role in macrophage immune function.

4.4 CLINICAL IMPLICATIONS

Acute lower respiratory infections are the leading cause of premature death with a greater disease burden than heart attacks, cancer, and HIV/AIDS[12, 13]. In addition, pneumonia is a prevalent disease that can lead to complications such as sepsis and acute respiratory distress syndrome and can also complicate many other diseases, such as chronic obstructive pulmonary disease, ILDs, cystic fibrosis, and HIV/AIDS. Despite advances in antimicrobial therapy, rates of pneumonia mortality have not decreased in the past 50 years[12, 74]. Therefore, improvements in therapy for pneumonia especially for those patients at higher risk of this disease have the potential to have a large clinical impact.

The novel finding that EC-SOD in leukocytes promotes bacterial phagocytosis and killing and limits inflammation supports the research and use of antioxidants for treatment of bacterial pneumonias. While antibiotic therapies have been a great resource, treatment with EC-SOD in conjunction with traditional antibiotics would likely result in more efficient bacterial

clearance and less oxidative damage to the host tissue. Furthermore, treatment with EC-SOD may be valuable for eliminating antibiotic-resistance strains. As bacterial infections are not limited to the lung, these findings are likely translatable to other bacterial infections as well.

IPF is a relentlessly progressive and fatal pulmonary disorder with unknown etiology. Like other ILDs, there are no effective treatments for IPF. The clinical course of IPF patients is generally marked by slow decline in pulmonary function over time, but a large number of patients have been recognized as having an acute, and often fatal, exacerbations characterized by a large influx of inflammatory cells into the BALF and lungs. Pirfenidone, a drug that is known to act as an anti-inflammatory, anti-fibrotic, and antioxidant, was shown to decrease the prevalence of acute exacerbations in IPF patients[233]. Although it was speculated that multiple biological functions of this drug may protect the fibrotic lung against the superimposed diffuse lung damage associated with acute exacerbations, significant adverse side effects were reported limiting its usefulness as a realistic prophylactic treatment option[233]. Based on the presented work, EC-SOD is known to function in all three of the same biological capacities. Therefore, although antioxidant therapies have been shown to be unsuccessful at preventing the progression of ILDs, it is likely antioxidant therapy could still represent a realistic therapeutic option in order to lower the prevalence of these exacerbations and increase survival of patients with IPF and other ILDs.

Aside from all this, it is essential to better understand the molecular mechanisms that explain how EC-SOD regulates bacterial phagocytosis and killing and to determine if this effect is general for all types of infections or specific to gram-negative bacterium. It is hoped that the research described in this dissertation will serve as a foundation for future research and will inspire diverse studies to better understanding the mechanism of how EC-SOD in leukocytes

attenuates infection. Overall, it is the hope that the findings presented in this dissertation will serve as the foundation for the development of new innovative translational applications for EC-SOD or other antioxidants in the treatment of bacterial pneumonia, non-pulmonary bacterial infections, and perhaps the prophylactic treatment for individuals at high risk of developing pneumonia and acute exacerbations.

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