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Cysteine/Cystine Redox State in Lung Injury and Fibrosis

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CYSTEINE/CYSTINE REDOX STATE IN LUNG INJURY AND FIBROSIS

By

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MS., Purdue University, 2003

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ABSTRACT

Cysteine/cystine redox state in lung injury and fibrosis
By Smita S. Iyer

Acute lung injury (ALI) and idiopathic pulmonary fibrosis (IPF) are lung diseases with significant morbidity and mortality. Glutathione (GSH) is oxidized in both ALI and IPF, but little is known about the regulation of the precursor cysteine (Cys) pool. The studies described in this dissertation establish that the redox state (E_h) of the Cys/cystine (CySS) couple is a biomarker of oxidative stress in experimental ALI and IPF, and varies independently from the redox state of the GSH/glutathione disulfide (GSSG) couple during inflammation, injury, and fibrosis in the lung.

In the first study, we identified that plasma E_h Cys/CySS is highly oxidized relative to E_h GSH/GSSG during the acute phase of lung injury induced by endotoxin. *In vitro*, oxidized E_h Cys/CySS increased adhesion of leukocytes to the pulmonary endothelium, suggesting that early oxidation of Cys/CySS may potentiate neutrophil influx into the lungs.

To further understand the relationship between E_h Cys/CySS and inflammation, we modified extracellular E_h Cys/CySS and determined levels of the pro-inflammatory cytokine, interleukin (IL) -1β. Results showed that oxidized E_h Cys/CySS increased IL-1β levels in monocytes, and that dietary treatment to protect against plasma E_h Cys/CySS oxidation decreased plasma and lung levels of IL-1β in endotoxin-challenged mice. Analysis of E_h Cys/CySS and IL-1β in human plasma revealed a significant positive

association between oxidized E_h Cys/CySS and IL-1β, independent of age, gender, and BMI.

Additionally, we examined plasma Cys and GSH redox states during the resolution of inflammation in response to exogenously infused bone marrow derived mesenchymal stem cells (BMDMSC). Results showed a sequential preservation of systemic Cys and GSH pools in response to BMDMSC infusion.

Because lung injury is an upstream event in fibrosis, we assessed the dynamics of Cys redox state in a model of lung fibrosis. Results showed oxidation of plasma E_h Cys/CySS in the absence of changes to E_h GSH/GSSG. Furthermore, oxidation of E_h Cys/CySS in the lung lining fluid was associated with the induction of pro-fibrotic markers in the lung, suggesting that oxidation of Cys/CySS during lung injury may contribute to fibrosis.

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A mentor is an “artist of encouragement”, Marsha Sinetar, author

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involve lab members in all aspects of the life in the lab and outside the lab, such as seminars, journal club, and meetings with outside speakers. This has made me feel a valued member of his research team. Mauricio took the effort to make sure others were aware of my work, and actively encouraged me to present at national and international conferences. It has been an enormous privilege working with him and without any doubt; Mauricio will continue to be a mentor in all my future endeavors.

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LIST OF ABBREVIATIONS

AF	*ad libitum* fed
ALI	acute lung injury
ARDS	acute respiratory distress syndrome
AMD	age-related macular degeneration
BALF	bronchoalveolar lavage fluid
BMDMSC	bone marrow-derived mesenchymal stem cells
BSO	L-buthionine-S,R-sulfoximine
CDO	cysteine dioxygenase
Cys	cysteine
CySS	cystine
CySSG	mixed disulfide of cysteine and glutathione
DCF	dichlorofluorescein diacetate
ELF	epithelial lining fluid
EGF	epidermal growth factor
E_h	redox potential
E_o	standard redox potential
FACS	fluorescence assorted cell sorting
γ-Glu-Glu	γ-glutamyl glutamate
γ-GCS	γ-glutamyl cysteine synthetase
γ-GT	γ-glutamyl transpeptidase
GSH	glutathione
GS	glutathione synthetase
GSSG	glutathione disulfide

GST	glutathione-S-transferase
H&E	hematoxylin and eosin
H_20_2	hydrogen peroxide
IL-1β	interleukin-1β
IL-6	interleukin-6
iNOS2	inducible nitric oxide synthase2
IPF	idiopathic pulmonary fibrosis
LPS	lipopolysaccharide
MSC	mesenchymal stem cells
NAC	N-acetyl cysteine
PF	pair-fed
PBS	phosphate buffered saline
PCA	perchloric acid
ROS	reactive oxygen species
SAA	sulfur amino acids
SEM	standard error of the mean
SD	standard deviation
TNF-α	tumor necrosis factor-α
TGF-β1	transforming growth factor-β1
xCT	subunit of system x_c^-

CHAPTER I

INTRODUCTION

The inflammatory response to injury and the resolution of injury by repair is a highly regulated response in adult tissues. Unchecked, this process can cause tissue destruction which leads to deterioration in organ function. In the lung, an unregulated inflammatory response can lead to acute lung injury (ALI), while exaggerated repair results in idiopathic pulmonary fibrosis (IPF). Both diseases are associated with significant morbidity and mortality rates of greater than 30%. A better understanding of mechanisms that regulate inflammation and fibrosis will aid in better management strategies, and improve outcomes in patients with ALI and IPF.

As described in this dissertation, accumulating evidence indicates that the biological thiol/disulfide redox environment dictates responses to inflammatory and fibrotic stimuli. Cysteine (Cys) and its disulfide cystine (CySS) together with glutathione (GSH) and glutathione disulfide (GSSG) comprise the major low-molecular weight thiol/disulfide control systems. Much of our knowledge on thiol/disulfide redox imbalance in ALI and IPF is limited to GSH and very little is known about Cys. While Cys is a precursor for GSH, the redox state (E_h) of Cys/CySS is regulated independently of E_h GSH/GSSG, and has distinct regulatory functions. This dissertation is based on recent evidence that intracellular inflammatory and fibrotic pathways are influenced by extracellular E_h Cys/CySS in the absence of changes to cellular E_h GSH/GSSG. Therefore, investigating the role of E_h Cys/CySS in ALI and IPF will provide a better mechanistic understanding of thiol/disulfide redox imbalance in the disease process.

The first section of this introduction discusses aspects of thiol/disulfide redox control in health and disease, primarily with respect to Cys/CySS redox state. Section two describes the structure and function of the key cellular components in the alveolus that are altered in ALI and IPF. The epidemiology and pathogenesis of ALI and IPF are presented in section three and four, respectively. In section five, the role of oxidative stress and GSH in the pathogenesis of ALI and IPF is examined. Section six describes the potential role for Cys/CySS redox state in ALI and IPF which forms the basis for the research described. The statement of purpose is provided in section 7.

1. LOW-MOLECULAR WEIGHT THIOL/DISULFIDE REDOX SYSTEMS

1.1. Cysteine

The sulfur-containing amino acids cysteine (Cys) and methionine (Met) play critical roles in protein synthesis, structure, and function (Brosnan and Brosnan 2006). In human nutrition, Cys a conditionally-essential amino acid because it can be synthesized in the liver by transulfuration of Met (Stipanuk 1986). However, there is evidence that Cys becomes essential during infection and injury, where a greater need for protein and glutathione (GSH) synthesis places a higher demand on Cys supply (Grimble and Grimble 1998; Malmezat, Breuille et al. 2000).

Cys is synthesized in mammals by the transulfuration pathway. This involves transfer of sulfur from Met to serine in an irreversible process that is restricted mainly to the liver **(*Figure 1.2*)**. Because Cys is reactive and excess Cys is toxic, intracellular Cys concentrations are tightly regulated between 30 – 200 μM; among the lowest observed

for any of the amino acids (Griffith 1987). In rats, hepatic Cys levels are maintained within a 5-fold range between 20 to100 nmol/g (Stipanuk, Dominy et al. 2006).

Figure 1.1. Structure of the sulfur-containing amino acids

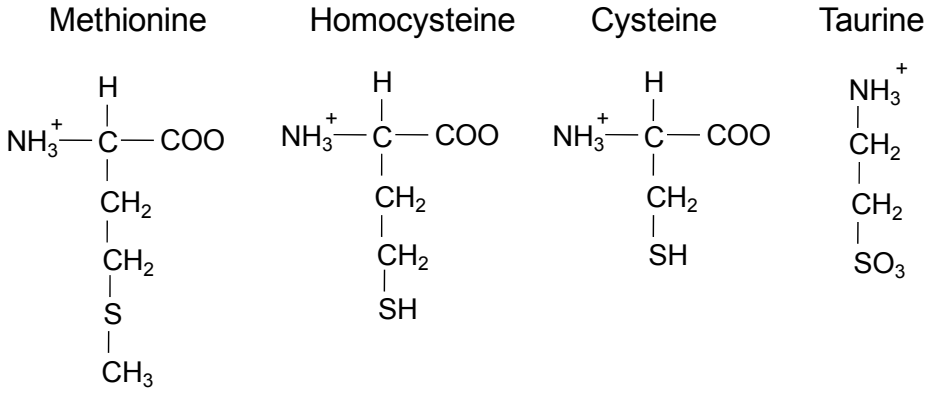


Figure 1.1. Structure of the sulfur-containing amino acids. Methionine (Met), Cysteine (Cys), Homocysteine, and Taurine are the most common sulfur-containing amino acids (SAA) in mammals. Met and Cys are the only SAA that are incorporated into proteins. Not shown is Cystine, the disulfide form of Cys.

The enzyme cysteine dioxygenase (CDO), an iron metalloenzyme, plays a central role in maintaining steady-state hepatic Cys levels. CDO catalyzes the oxidation of Cys to cysteine sulfinic acid, which is utilized in various metabolic pathways to generate pyruvate and synthesize taurine (Stipanuk, Dominy et al. 2006) ***(Figure 1.2, 1)***. Cys also serves as a precursor for GSH ***(Figure 1.2, 2)***, coenzyme-A, and inorganic sulfate (Brosnan and Brosnan 2006). Cys is present in almost all proteins. Some proteins have Cys as a major constituent; these include metallothionein, keap-1, and Cys-rich intestinal protein (Jones 2008). Cys also functions as an acceptor amino acid of selenium for the synthesis of selenoproteins and enzymes such as glutathione peroxidase (Fourquet, Huang et al. 2008).

Cys is transported in and out of cells by carrier-mediated mechanisms (Bannai 1984). Following export, Cys is rapidly oxidized to its disulfide form, cystine (CySS) ***(Figure 1.2, 3)***. Cys can also be oxidized to sulfenic acid (Cys-SOH) but this is unstable and only found in stabilized forms in some proteins. Higher oxidative forms, sulfinic ($Cys\text{-}SO_2H$) and sulfonic ($Cys\text{-}SO_3H$) acids are typically not reversible by reduction in mammalian systems (van Montfort, Congreve et al. 2003). These higher oxidation states do not constitute a major fraction of either protein or free Cys pools.

Analysis of the distribution of plasma Cys shows that approximately 23% of plasma cyst(e)ine is present in the protein-bound form (Lash and Jones 1985). Free-cyst(e)ine is largely present as CySS (83%). The mixed disulfide of CySS and glutathione, CySSG, comprises 10.8 % of the total cyst(e)ine pool and a minor fraction exists as the reduced form, Cys (5.9%). Plasma CySS is thought to be derived, almost exclusively, from plasma GSH and Cys, while plasma Cys is derived from sources other than GSH (Ookhtens and Mittur 1994).

Figure 1.2. Major pathways of Cys metabolism

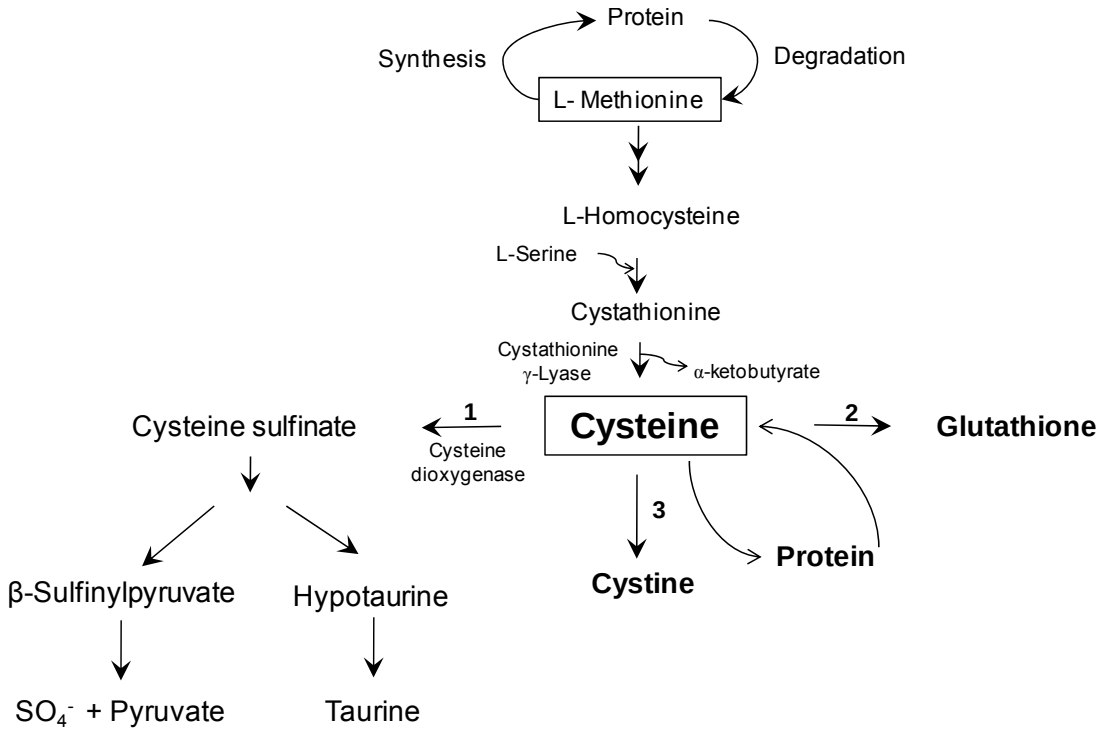


Figure 1.2. Major pathways of Cys metabolism. The first step in the synthesis of Cys is the activation of Met to S-adenosyl-L-methionine, which is subsequently demethylated to yield S-adenosyl-L-homocysteine (AdoHcy) (not shown). AdoHcy is hydrolyzed to homocysteine (Hcy) which can be re-methylated to Met or condensed with serine to form cystathionine. The latter is an irreversible reaction and commits Met to the transulfuration pathway. The major fates of intracellular Cys are oxidation to cysteine sulfinate, synthesis of proteins and GSH. Once exported, Cys is rapidly oxidized to CySS in the plasma.

1.2. Cysteine/Cystine redox state

Cys and CySS constitute the most abundant low molecular weight redox couple in the plasma and their concentrations are relatively stable in healthy individuals. The balance of Cys and CySS can be described in terms of the redox state or the steady-state redox potential, E_h. E_h provides a measure of the inherent tendency of the couple to accept or donate electrons (Schafer and Buettner 2001). E_h Cys/CySS can be estimated from the measured concentrations of Cys and CySS by the Nernst equation with the following terms in the equation (Jones, Carlson et al. 2000);

$$E_h = E_o + RT/nF \text{ X } \ln [\text{Cystine}]/[\text{Cysteine}]^2$$

where E_o (in mV) is the standard potential for the redox couple, R is the gas constant, T is the absolute temperature, n is the number of electrons transferred, and F is Faraday's constant. E_o value for the Cys/CySS couple is -250 mV at 25 ^{O}C, and a pH of 7.4. Due to the stochiometry for Cys oxidation to CySS, Cys enters the equation as a squared term. Therefore, E_h Cys/CySS is not a simply a function of the Cys/CySS ratio but is also dependent on the absolute concentrations of Cys and CySS. Thus, if the Cys/CySS ratio remains constant but the absolute concentrations of Cys and CySS change by a factor of 10, there will be a 30 mV change in E_h Cys/CySS.

Determination of E_h Cys/CySS in the plasma of healthy humans, aged 25-35 y, shows that Cys/CySS redox state is centered at -80 mV +/- 9 mV (Jones, Carlson et al. 2000). Remarkably, cultured cells actively regulate extracellular Cys and CySS levels to a value that approximates the E_h Cys/CySS in healthy humans, and variations in E_h Cys/CySS are associated with major cellular processes, such as proliferation, differentiation, and apoptosis (Jonas, Gu et al. 2003; Go and Jones 2005). This simple piece of evidence indicates that Cys/CySS redox state is a fundamental regulatory parameter in biological systems. Thus, mechanisms must exist to maintain extracellular Cys, CySS, and Cys/CySS redox state. Data from *in vitro* and *in vivo* studies show that plasma E_h Cys/CySS is maintained by multiple processes, including uptake and efflux of Cys and CySS, oxidation, degradation from GSH, substrate availability, and thiol-disulfide exchange reactions. ***Table 1.1*** illustrates how alterations in these regulatory mechanisms are associated with perturbations in the Cys/CySS pool.

Table 1.1. Mechanisms that regulate extracellular Cys, CySS and E_h Cys/CySS

Mechanism	Model	Compartment	Cys	CySS	CySSG	E_h Cys/CySS	Reference
Transport of CySS	Mice lacking xCT, component of CySS/glutamate transporter, system x_c^-	Plasma	No change	2-fold increase	1.6-fold increase	11 mV oxidation	(Sato, Shiiya et al. 2005)
Substrate availability	Rats fed SAA-deficient diet (0.25 X) for 7 d	Plasma	1.8-fold decrease	1.8-fold decrease	NA	10 mV oxidation	(Nkabyo, Gu et al. 2006)
	Rats fed SAA-excess diet (3X) for 7 d	Plasma	1.6-fold increase	No change	NA	15 mV reduction	
Thiol/disulfide exchange	HT-29 cells subjected to intracellular GSH depletion	Cell supernatant	No change	No change	2-fold decrease	No change	(Anderson, Iyer et al. 2007)
Degradation	γ-glutamyl transpeptidase deficiency	Plasma	540-fold increase		NA	NA	(Iida, Yasuhara et al. 2005)

Because extracellular E_h Cys/CySS is regulated within a narrow range in young, healthy adults, a relevant question is whether alterations in E_h Cys/CySS occur with age, nutritional and environmental factors, and disease. Multiple studies have shown that perturbations in Cys, CySS, and/or Cys/CySS redox state occur with age, and lifestyle factors such as smoking and alcohol abuse (Jones, Mody et al. 2002; Moriarty, Shah et al. 2003; Yeh 2007) ***(Table 1.2)***. This observation is of particular relevance to lung pathogenesis because smoking and chronic alcohol intake are risk factors for IPF and ALI, respectively. Interestingly, variations in the Cys/CySS pool correlate with biomarkers of pathology even in individuals without known disease. For instance, endothelial-cell function in healthy adults is negatively associated with increased plasma CySS levels (Ashfaq, Abramson et al. 2008).

Perturbations in Cys/CySS occur in numerous disease states. In patients with atrial fibrillation, plasma E_h Cys/CySS is oxidized by 10 mV compared to healthy controls. (Neuman, Bloom et al. 2007). CySS levels increase with high-dose chemotherapy (Jonas, Puckett et al. 2000), and oxidation of E_h Cys/CySS occurs with age-related macular degeneration (AMD) (Moriarty-Craige, Adkison et al. 2005). Mechanistic evidence in cell-culture systems supports a role for oxidized Cys/CySS in cardiovascular disease and AMD (Go and Jones 2005; Jiang, Moriarty-Craige et al. 2005). Thus, strategies to therapeutically prevent oxidation of E_h Cys/CySS in disease could be clinically important.

Table 1.2. Factors associated with variations in human plasma Cys, CySS and E_h Cys/CySS

Biological parameter	Cys	CySS	E_h Cys/CySS	Reference
Aging	Decreases by 0.06 µM/y after 19 y	Increases by 0.7 µM /y after 45 y	Oxidation by 0.2 mV/y after 19 y	(Jones, Mody et al. 2002)
Smoking	1.4-fold decrease	1.2-fold decrease	12 mV oxidation	(Moriarty, Shah et al. 2003)
Alcohol abuse	9.8-fold decrease	No change	60 mV oxidation	(Yeh, 2007)
Endothelial function in healthy adults	_	Inverse correlation with endothelial function , r = - 0.23	_	(Ashfaq, Abramson et al. 2008)
Atrial fibrillation	_	_	10 mV oxidation	(Neuman, Bloom et al. 2007)
High-dose chemotherapy in bone marrow transplant patients	No change	1.3-fold increase [a]	No change	(Jonas, Puckett et al. 2000)
Time of day	1.3-fold decrease between 0430-0630 compared to 2030	1.1-fold higher at 0130 compared to 1730	7 mV more oxidized at 0630 compared to 2130	(Blanco, Ziegler et al. 2007)
Zn supplementation in Age-related macular degeneration (AMD)	No change	1.2-fold decrease [b]	No change	(Moriarty-Craige, Ha et al. 2007)
Antioxidant supplementation in AMD	1.3 fold increase [b]	No change	7 mV reduced [b]	(Moriarty-Craige, Adkison et al. 2005)

[a], compared to pre-treatment levels
[b], compared to patients not taking antioxidants

The finding that diurnal variation in Cys,CySS, and E_h Cys/CySS occurs as a function of meal-timing suggests that plasma E_h Cys/CySS can be modulated, fairly rapidly, by the diet (Blanco, Ziegler et al. 2007). Experimentally, plasma E_h Cys/CySS can be made more reducing by adding Cys and Met to the diet (Nkabyo, Gu et al. 2006).

Clinically, disease-related oxidation of E_h Cys/CySS is attenuated in AMD patients taking antioxidant supplements (Moriarty-Craige, Adkison et al. 2005). Plasma CySS concenteration can also be decreased by dietary Zn supplements (Moriarty-Craige, Ha et al. 2007). Thus, dietary supplementation with Cys, Cys precursors, or antioxidants may be strategies to protect against oxidation of E_h Cys/CySS in disease.

In summary, *in vitro* and *in vivo* data show that E_h Cys/CySS serves as an important indicator of the extracellular redox environment. *In vitro,* changes in extracellular E_h Cys/CySS correlate with major cellular processes, and *in vivo,* E_h Cys/CySS is oxidized in association with age, disease, and disease-related risk factors. Of possible clinical significance is the observation that oxidation of E_h Cys/CySS in disease can be attenuated by dietary means. Together, these data indicate that E_h Cys/CySS is not only a sensitive biomarker of disease, but also represents an attractive therapeutic target.

1.3. Glutathione

Cys homeostasis is closely inter-linked to GSH metabolism. While Cys and GSH share a precursor-product relationship (Blanco, Ziegler et al. 2007), biochemical studies show that GSH can serve as a precursor for Cys (Griffith and Meister 1980). *In vivo*, degradation of plasma GSH by γ-glutamyl transpeptidase (γ GT) in the kidney yields Cys (Griffith and Meister 1980). However, it is important to note that despite the

dynamic precursor-product relationsip between Cys and GSH, the redox states of these systems are independently regulated (Anderson, Iyer et al. 2007).

GSH is the most abundant non-protein thiol within the cell, and represents one of the major intracellular redox control systems (Schafer and Buettner 2001). Almost 90% of cellular GSH is present in the cytosol at a concentration of 1 to 10 mM, an order of magnitude higher than intracellular Cys levels.

GSH is synthesized exclusively within the cell from its constituent amino acids; Cys, glutamate (Glu), and glycine (Gly). The first step in GSH synthesis is the formation of γ-glutamylcysteine from Glu and Cys in a reaction catalyzed by γ-glutamylcysteine synthetase (γ-GCS). GSH synthetase (GS) catalyzes the formation of a peptide bond between γ-glutamylcysteine and Gly to yield GSH (Snoke and Bloch 1952; Snoke, Yanari et al. 1953) ***(Figure 1.3).***

GSH can be synthesized in all mammalian cells; the liver and the skeletal muscle account for most of whole-body GSH homeostasis (Kretzschmar, Pfeifer et al. 1992). Approximately 50-80% of plasma GSH is derived from the liver (Ookhtens and Mittur 1994). Studies in rats have shown that lung GSH levels decrease after hepatectomy (Kretzschmar, Pfeifer et al. 1992). Thus, lung GSH levels depend on hepatic GSH homeostasis.

Steady-state hepatic GSH levels are achieved by a balance between the rates of GSH synthesis, the rate of utilization through oxidation of GSH to GSSG, conjugation of GSH to electrophiles, and GSH efflux (Kaplowitz, Aw et al. 1985). Fasting is associated with a decrease in hepatic GSH concentrations indicating that GSH homeostasis depends on dietary availability of Cys or Cys precursors (Leaf and Neuberger 1947).

Figure 1.3. Regulation of hepatic GSH homeostasis

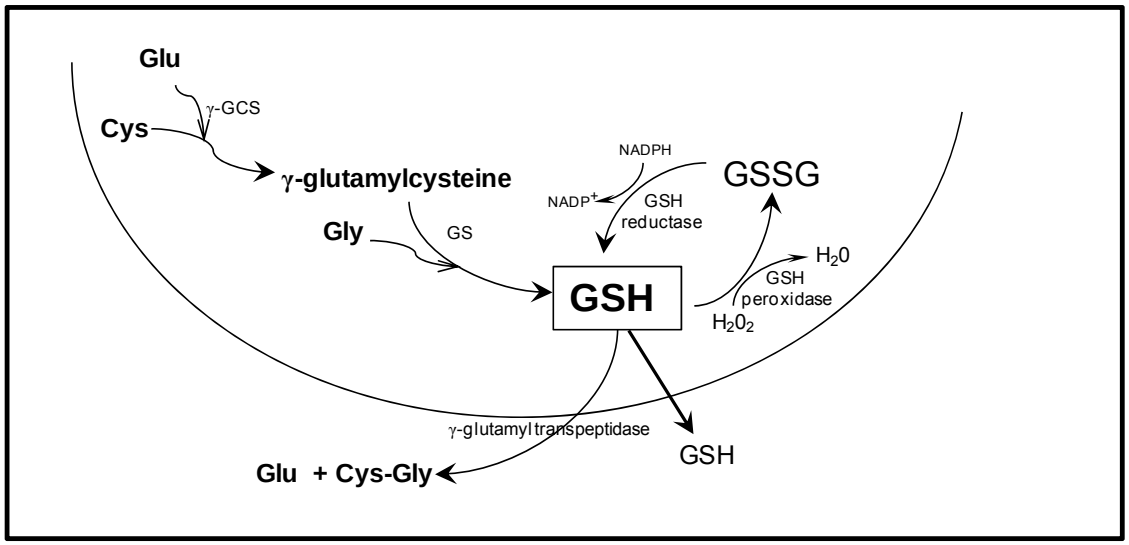


Figure 1.3. Regulation of hepatic GSH homeostasis. Hepatic GSH levels depend on GSH synthesis, utilization through redox and conjugating reactions, and GSH efflux. The ectoenzyme γ-glutamyl transpeptidase catalyzes the degradation of GSH to cysteinyl glycine (Cys-Gly) and glutamate. Cys-Gly is either cleaved to its constituent amino acids to supply plasma Cys or is shuttled into the cell via dipeptide transporters.

In the plasma, GSH largely exists in the protein-bound form (70%). A large fraction of the non-protein bound GSH is present as CySSG (62.6%), 28% is present as free GSH, and 9.5% as GSSG (Lash and Jones 1985). In human plasma, the total GSH equivalents are 10-fold lower than Cys equivalents (Jones, Carlson et al. 2000). GSH is also less-reactive than Cys; at a physiologic pH of 7.4, approximately 1% of GSH is ionized while Cys is 6% ionized (Torchinskii 1981). The greater relative reactivity of Cys together with its high concentrations in the plasma indicate that Cys may be proximal to extracellular oxidative events compared to GSH (Jones, Mody et al. 2002). In healthy adults, the steady-state redox potential of GSH/GSSG is 137 +/- 9 mV, approximately 60 mV more reduced than plasma E_h Cys/CySS (Jones, Carlson et al. 2000).

However, in the lung epithelial lining fluid (ELF) GSH is present at a concentration of 400-500 μM; among the highest known for any antioxidant in the extracellular compartment (Cantin, North et al. 1987). Because the lung is constantly exposed to environmental pollutants, the intracellular and extracellular GSH levels are critical in determining tolerance to oxidative insults (Rahman, Biswas et al. 2005). In the ELF, GSH is a crucial determinant of lung health and perturbations in ELF GSH levels are associated with numerous inflammatory and fibrotic disorders of the lung (Cantin, Hubbard et al. 1989; Cantin and Begin 1991).

Another important aspect of GSH metabolism relates to its role in immune cell function (Mihm, Galter et al. 1995; Brown, Ping et al. 2007). Cells from the innate and adaptive immune system are exquisitely sensitive to perturbations in GSH; alveolar macrophage phagocytosis and viability is impaired by GSH depletion (Brown, Ping et al. 2007), and lymphocyte proliferation and phenotype is sensitive to availability of GSH (Droge and Breitkreutz 2000). The role of Cys and GSH in immune cell function and lung disease in fully discussed in section five.

2. ALVEOLAR EPITHELIUM, ENDOTHELIUM, AND INTERSTITIUM

The lungs provide a large diffusible interface for oxygen supply to the blood. In order to generate this interface, the primordial lung must undergo extensive cell proliferation, branching morphogenesis, and alveolarization together with vasculogenesis and angiogenesis during development (Stenmark and Mecham 1997; Warburton, Schwarz et al. 2000). This process is regulated by interactions between epithelial cells, endothelial cells, and mesenchymal cells along with components of the extracellular matrix. In the adult lung, the interaction between these components maintains homeostasis under normal conditions (Warburton, Tefft et al. 2001; Demayo, Minoo et al. 2002; Torday and Rehan 2007). This section provides an overview of the biology of the alveolar epithelium, the pulmonary endothelium, and pulmonary interstitium in lung homeostasis. The role of the immune system in lung health is also considered.

2. 1. Alveolar Epithelium

The entire epithelial structure of the mammalian lung is derived from the primitive foregut endoderm (Warburton, Wuenschell et al. 1998). The alveolus is the primary site of gas exchange with the blood. The alveolar epithelium is composed of alveolar type I (ATI) and type II cells (ATII) that continuously line the terminal air spaces forming a barrier that is anatomically 1-cell thick (Johnson, Widdicombe et al. 2002).

ATI cells are large squamous epithelial cells with widely spread cytoplasmic extensions; a morphological adaptation which provides a short diffusion path for gas exchange. ATI cells constitute 90% of the alveolar surface area. Their cell volume is

typically 2.5 times and the surface area is approximately 60 times that of AT II cells (Crapo, Young et al. 1983). ATI cells are not metabolically active and are considered to be “inert” cells, providing mostly a barrier function. However, there is accumulating evidence that ATI cells participate in active ion transport and are critical in maintaining alveolar fluid balance and resolving air space edema (Johnson, Widdicombe et al. 2002; Johnson 2007)

Type II cells are metabolically more active than ATI cells, as evidenced by greater number of mitochondria (Mason 2006). ATII cells are cuboidal in shape, and have numerous microvilli on their apical surface. ATII cells serve many functions; they store and secrete surfactant, a phospholipid-rich component in the alveolar lining fluid that reduces surface tension and prevents collapse of the alveoli at low lung volumes (Mason and Williams 1977; Hamm, Fabel et al. 1992; Fehrenbach 2001). ATII cells also modulate alveolar fluid balance by transporting Na ions from the alveolar lumen into the interstitial space (Davis and Matalon 2007). Movement of Na^+ facilitates the passive transport of water through aquaporins, transcellular water channels located primarily on ATI cells (Matthay, Folkesson et al. 1996). ATII cells also maintain the pH of the epithelial lining fluid at 6.9 (Matalon 1991; Lubman and Crandall 1992), and serve as progenitors for ATI cells under normal conditions and during injury (Mason 2006). ATII cells also play a role in turnover of GSH in the lung. The high concentrations of GSH in the epithelial lining fluid are thought to result from the synthesis and efflux of GSH from ATII cells (van Klaveren, Demedts et al. 1997).

ATI and ATII cells form a tight barrier, which effectively separates the alveolar air-space from the vascular and interstitial spaces. The alveolar epithelium is an order of

magnitude less permeable to small solutes than the capillary endothelium (Taylor and Gaar 1970). Thus, injury to the pulmonary epithelium increases susceptibility to alveolar flooding. Multiple studies have shown that maintenance of alveolar integrity is critical in normal lung homeostasis, and alveolar barrier dysfunction is a key component in ALI and IPF (Matthay, Folkesson et al. 2002; Strieter 2008).

2.2. Pulmonary Endothelium

The lung microvascular bed is the single largest collection of endothelial cells in the human body. The anatomical location of the pulmonary microvascular bed allows it to receive the entire cardiac output, and its high metabolic activity allows it to modify the composition of the venous blood before it reaches the systemic circulation. A unique aspect of the lung capillary endothelium is its extreme thinness (0.2 to 0.4 micron), an adaptation to facilitate efficient gas exchange (Weibel 1973; West 2003). The presence of type IV collagen in the lamina densa of the endothelial basement membrane provides strength to withstand rise in capillary pressure with exercise (West 2003; Maina and West 2005). However, the capillary wall is extremely susceptible to injury (Ware 2006; Strieter 2008)

Apart from its structural functions, the pulmonary endothelium has numerous metabolic functions. The pulmonary endothelial cell helps in the regulation of pulmonary vascular tone by secretion of lipid mediators and by maintaining nitric oxide homeostasis (Mitchell, Ali et al. 2008). In addition, the endothelium participates in inflammatory reactions by upregulating adhesion molecules which facilitate the adhesion and intravasation of circulating leukocytes into the air-space (Rao, Yang et al. 2007).

Importantly, the endothelial cells maintain integrity of the alveolar-capillary membrane (Gladwin 2006). In all, the pulmonary endothelium possesses metabolic, structural, and immunological functions; each of which is disregulated during injury

2.3. Pulmonary Interstitium and Fibroblasts

Like the pulmonary endothelium, the pulmonary interstitium is derived from the embryonic mesoderm. The pulmonary interstitium comprises of extracellular matrix (ECM) components such as collagen, laminin, and fibronectin, and mesenchymal cells such as fibroblasts. Components of the ECM are involved in every stage of lung biology, from branching morphogenesis during lung development to maintenance of normal lung function, and in the resolution of lung injury (Strang 1977; Roman 1997). During development, the ECM serves not only as template to which cells anchor but also provides informational cues that cells respond to.

The thinness and hydration state of the interstitial layer between the alveolus and the endothelial cells is a critical determinant of gas exchange. The lymphatic network in the interstitium serves to drain excess fluid accumulating in the alveolar space. Accumulation of fluid in the air-space occurs when increased influx, arising either from increased capillary hydrostatic pressure or capillary leak, is not matched by lymphatic drainage (Lai-Fook 1986). Disturbances in matrix turnover and deposition lead to disregulation in steady-state fluid dynamics, which can progress towards severe lung disease as described in section four.

Interestingly, the composition of ECM can also influence cellular phenotype. Type II cells grown on laminin, an ECM component that constitutes the healthy lung,

undergo differentiation. However, an accelerated loss of form and function is observed when type II cells are cultured on fibronectin matrices (Rannels and Rannels 1989). Fibronectin is one of the major ECM components that increases markedly during tissue injury, and increased expression of fibronectin is observed in ALI and IPF (Limper and Roman 1992). As described in this dissertation, *in vitro* studies in fibroblasts show that fibronectin expression is increased in response to an oxidized Cys/CySS redox state (Ramirez, Ramadan et al. 2007).

2.4. Pulmonary Immune System

Because the lungs are constantly exposed to environmental pathogens, allergens, and toxicants; robust defenses are in place to protect the lung against the deleterious effects of such challenges (Green, Jakab et al. 1977). The mechanisms of pulmonary defense against infectious and non-infectious stimuli can be divided into non-specific defenses that act by physical removal of the stimulus, and specific humoral and cellular defenses that inactivate and prevent replication of the pathogen (Green 1968)

Mechanisms of physical removal include cough and expectoration, lymphatic flow from the alveolus to the lymph nodes, and mucociliary clearance (Green 1973; Lauweryns and Baert 1977). Cellular mechanisms include inflammatory cells such as alveolar macrophages, neutrophils, monocytes, lymphocytes, plasma cells, and eosinophils. In the lower respiratory tract, the alveolar macrophage represents the most important defense system both for non-specific phagocytic defense and for antigen triggered immunity via activation of T-lymphocytes (Newhouse, Sanchis et al. 1976; Bienenstock 1984). Sampling of the lower airway has revealed that about 90% of the

immune cells are macrophages and 10% are lymphocytes. In the healthy, non-inflammed lung; the alveolar macrophage is the primary sentinel cell which senses microbes and elaborates a cytokine response to recruit neutrophils, which are key effector cells of the innate immune system (Mizgerd 2008).

Neutrophil recruitment through the pulmonary capillaries and into the air spaces is initiated by the upregulation of adhesion molecules on pulmonary endothelial cells (Burns, Smith et al. 2003). Intravasated neutrophils phagocytose microbes and the ingested microbe is killed by reactive oxygen species, antimicrobial proteins, and degradative enzymes (Nathan 2006). Neutrophils also produce pro-inflammatory cytokines which recruit and activate other cells of the innate and adaptive immune system. Thus, neutrophils facilitate innate and adaptive immune responses against microbes, and defects in neutrophil function predispose to respiratory infections. However while inflammation is critical for host defense, it can injure the lungs (Mizgerd 2008). In the injured lung, the structure and function of the pulmonary endothelium, epithelium, and interstitum is altered and this underlies the pathogenesis of ALI and IPF.

3. ACUTE LUNG INJURY

Inflammation, defined by Celsus around AD40 as 'rubor, calor, dolor, tumor' (redness, heat, pain and swelling), is a highly evolved process that can arise in any tissue in response to pathogens, trauma, toxins, or autoimmune injury (Nathan 2002). Deficiencies in cellular and humoral components of the inflammatory response lead to an increased risk of infection and death (Biesma, Hannema et al. 2001). Thus, the ability to mount an inflammatory response is life-preserving. However, inflammation can injure the lungs. Acute lung injury (ALI) results from a dysregulated inflammatory response, and can progress to severe respiratory failure termed the acute respiratory distress syndrome (ARDS).

3.1. Historical Perspective

ARDS was first described by Ashbaugh and colleagues in 1967 in 12 patients wherein the onset of respiratory failure was preceded either by severe trauma, viral infection, or acute pancreatitis (Ashbaugh, Bigelow et al. 1967). Subsequently in 1971, Petty and Ashbaugh described 3 cases where gastric aspiration and bacterial pneumonia preceded ARDS (Petty and Ashbaugh 1971). Here, the authors proposed that ARDS was a result of a non-specific response to a variety of pulmonary/extra-pulmonary insults injures and its outcome was influenced by the degree of original injury, the extent of respiratory support, and by management strategies designed to prevent further injury.

Because the severity of lung injury determines survival, Murray and colleagues proposed a 4-point lung-injury scoring system based on measurements of lung function, lung compliance, and degree of pulmonary edema (Murray, Matthay et al. 1988).

However, the clinical usefulness of the 4-point scoring was limited because it could not be used to predict outcome in the first 24 to 72 hours after the onset of ARDS.

In 1994, the American-European consensus conference committee proposed defining clinical criteria that distinguished ALI and ARDS based on the extent of hypoxemia (Bernard, Artigas et al. 1994). In addition, the presence of bilateral infiltrates in the lung, on chest radiograph, in the absence of heart failure is considered another defining feature of ALI/ARDS (Ware and Matthay 2000).

3.2. Epidemiology, Risk Factors and Pre-Disposing Factors for ALI and ARDS

The first epidemiological study to use the 1994 consensus definition reported an annual incidence rate of 17.9 per 100,000 for ALI and 13.5 per 100,000 for ARDS in Denmark, Sweden and Iceland (Luhr, Antonsen et al. 1999). In the United States, the annual incidence of ALI is estimated to be 78 per 100,000 per year. The estimated cases of ALI are close to 200,000 per year, which are associated with 75,000 deaths (Rubenfeld, Caldwell et al. 2005).

Risk Factors for ALI and ARDS

In humans, ALI can develop from initiating events that are either pulmonary or extra-pulmonary in origin. Direct pulmonary injury can result from aspiration of gastric contents, respiratory infection, inhalation of toxic substances, and less commonly from near-drowning and fat embolism in the pulmonary capillary bed. Non-pulmonary risk factors for ALI include sepsis, severe trauma, and acute pancreatitis (Ware and Matthay 2000) ***(Table 1.3)***. The mortality rate of ARDS ranges between 40 to 50%, and sepsis is

associated with the highest risk of progression to ALI or ARDS (Doyle, Szaflarski et al. 1995). A prospective study identifying clinical predictors of ARDS in 136 ICU patients reported that 38% of patients with sepsis developed ARDS (Pepe, Potkin et al. 1982). Similar observations were made in a larger prospective study on 695 ICU patients, where the highest incidence of ARDS was in patients with sepsis (43%) (Hudson, Milberg et al. 1995).

Advanced age and chronic liver disease represent co-morbid conditions that can be used to predict the risk of death at the time of diagnosis (Suchyta, Clemmer et al. 1992; Zilberberg and Epstein 1998; Cooke, Kahn et al. 2008). A prospective cohort study on 256 ARDS patients identified from May 1987 to December 1990 found that mortality was significantly greater in patients older than 55 years (64%) compared to patients 55 years or younger (45%). However, the study also suggested that decisions to withdraw supportive care in elderly patients could influence outcomes (Suchyta, Clemmer et al. 1997). A more recent study in 343 patients who developed ARDS from 1993 to 2003 found that age had no significant effect on mortality, an outcome that may be reflective of improved supportive care in these patients (Rocco Jr, Reinert et al. 2001).

Pre-Disposing factors for ALI and ARDS

Chronic alcohol abuse substantially increases the risk for the development of ARDS (Moss, Bucher et al. 1996; Moss and Burnham 2003; Cooke, Kahn et al. 2008). A prospective cohort study in 351 ICU patients found that the incidence of ARDS was significantly higher in patients with a history of alcohol abuse, compared to patients who did not abuse alcohol (RR, 1.9, 95% CI, 1.32 to 2.85). Patients with sepsis who abused

alcohol had a greater risk of ARDS (RR, 2.59 95% CI, 1.29 to 5.12). Further, mortality rate from ARDS was higher in patients with a prior history of alcohol abuse (65%) compared to patients without a history of alcohol abuse (36%) (Moss, Bucher et al. 1996).

Table 1.3. Clinical disorders associated with the development of ALI and ARDS.

Direct lung injury	Indirect lung injury
Pneumonia	Sepsis
Aspiration of gastric contents	Severe trauma and multiple transfusions
Fat-embolism from multiple fractures	Multi-organ failure
Trauma	Acute pancreatitis

Table 1.3. The lung responds to acute injury from diverse stimuli via a series of common pathways that results in ALI, and can evolve to a more severe form, ARDS

3.3. Pathogenesis of ALI and ARDS

A critical underlying mechanism in the pathogenesis of ALI and ARDS is the disruption of the alveolar-capillary barrier (Ware and Matthay 2000). This can result either from direct epithelial injury e.g., gastric aspiration, or from injury to the pulmonary microvasculature due to a systemic inflammatory event eg., sepsis. The loss of alveolar-capillary barrier integrity contributes to alveolar flooding. Under normal conditions, excess fluid from the air-space is removed by the type II epithelial cells. However, injury to type II cells in ALI and ARDS impairs this process (Sznajder 1999). The resulting

pulmonary edema forms the basis for the physiological events of hypoxemia and reduced lung compliance in ARDS patients (Bigatello and Zapol 1996). The importance of intact alveolar fluid transport is demonstrated in studies showing that ARDS patients with maximal alveolar fluid clearance have a significantly decreased mortality (20%) compared to patients with sub-maximal or impaired clearance (60%) (Ware and Matthay 2001). Of relevance to the studies presented in this dissertation is the observation that malnourishment impairs alveolar fluid clearance. In rats that were nutritionally deprived for 5 days, alveolar clearance decreased by 38% leading to an 8% increase in lung wet/dry ratio, a marker of pulmonary edema (Sakuma, Zhao et al. 2004). Importantly, this impaired alveolar fluid clearance due to malnutrition was restored after 5 days of re-feeding indicating that optimal nutrition in patients with ALI and ARDS can improve pulmonary edema.

Apart from epithelial injury, damage to the pulmonary endothelium due to aberrant activation of the immune system, i.e., increase in neutrophils and cytokines, contributes to pulmonary edema in ALI and ARDS (Pittet, Mackersie et al. 1997). Increased interstitial and alveolar neutrophilic inflammation is evident during the acute phase of ARDS (Bachofen and Weibel 1982). Furthermore, ARDS patients with increased levels of granulocyte-colony stimulating factor (G-CSF) in the lung lining fluid, a neutrophil anti-apoptotic factor, have poorer outcomes (Wiedermann, Mayr et al. 2004).

Systemically, pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β are dramatically elevated in patients with ALI and ARDS. Experimental data support the role of increased TNF-α and IL-1β in mediating

endothelial damage and capillary leak. In response to the injury, the endothelium releases chemotactic factors such as interleukin-8 (IL-8), which cause recruitment of circulating neutrophils. In the air-space, TNF- α and IL-1β produced locally by the alveolar macrophages activate neutrophilic oxidative burst and this further contributes to alveolar damage (Pugin, Ricou et al. 1996).

Thus, systemic and local production of pro-inflammatory cytokines initiate and amplify the inflammatory response and the subsequent injury in ALI and ARDS. The resolution of injury occurs by mechanisms relating to decrease in pro-inflammatory cytokines and increase in anti-inflammatory mediators and growth factors. Because cytokine induction and signaling are sensitive to the thiol/disulfide redox environment (Peristeris, Clark et al. 1992), understanding the role of Cys/CySS redox state in the inflammatory process will aid in identifying potential therapies.

3.4. Animal models of acute lung injury

Modeling human disease in animals has contributed significantly to our understanding of lung physiology in health and in disease (Ware 2008). This section provides an overview of the commonly used animal models of ALI.

As previously discussed, ALI can develop from pulmonary or extra-pulmonary insults (***Table 1.3)***. In animals, each of these insults reproduces some, but generally not all of the three key pathological features of human ALI, also referred to as the ALI/ARDS pathological triad - 1) neutrophilic alveolitis 2) pulmonary edema 3) intravascular coagulation (Ware 2008).

Commonly used animal models of ALI are shown in ***Table 1.4***. Gastric aspiration is an important risk factor in the development of ALI/ARDS (Pepe, Potkin et al. 1982). The low pH of gastric contents is reproduced in animals by instilling HCL (0.1 N to 0.5 N) directly into the trachea or bronchi of an anesthetized, mechanically ventilated animal. This results in necrosis of the type I epithelial cells, followed by infiltration of inflammatory cells (Landay, Christensen et al. 1978). More recently, the role of oxidized phospholipids in mediating the inflammatory response in acid-induced ALI has been recognized (Imai, Kuba et al. 2008). Furthermore, increase in active transforming growth factor (TGF)-β1 may also be important because TGF-β1 is activated at low pH and is a potent neutrophil chemoattractant (Perng, Chang et al. 2007).

Another model reproduces damage caused by mechanical ventilation in ARDS patients by exposing animals to hyperoxia (95%-100% O_2) for more than 72 h. Hyperoxia-induced ALI is characterized by endothelial and epithelial cell death which results in increased alveolar capillary membrane permeability and neutrophilic alveolitis (Bhandari, Choo-Wing et al. 2007). A less-commonly used model of ALI involves depleting the lower-airway of surfactant by repeated saline (0.9%) lavages. While this is not sufficient to cause lung injury, it predisposes to mechanical ventilation-induced lung injury (Sommerer, Dembinski et al. 2001).

Models in which the lung is injured indirectly include cecal ligation and puncture (CLP), where the cecum is ligated and punctured 3-4 times with a needle (Walley, Lukacs et al. 1996). This results in the release of enteric bacteria into the blood stream which causes a systemic inflammatory response, leading to lung injury. Intravenous infusion of oleic acid is a model for fat embolism, a complication associated with

multiple fractures (Derks and Jacobovitz-Derks 1977). The formation of microemboli damages the pulmonary capillary endothelium and the alveolar cells, which results in alveolar capillary barrier dysfunction. Neutrophilic infiltration due to oleic acid-induced increase in adhesion proteins on the pulmonary endothelium is another salient feature of this model (Eiermann, Dickey et al. 1983; Syrbu, Thrall et al. 1995).

A commonly used model and the one used in the present dissertation research is endotoxin-induced model of ALI. Endotoxin/LPS is the structural component of the outer leaflet of the outer membrane of gram negative bacteria. LPS is a potent inflammatory stimulus, and E. coli LPS is the most potent endotoxin (Raetz and Whitfield 2002; Beutler and Rietschel 2003). Administration of endotoxin/ lipopolysachharide (LPS) to mice produces pathophysiologic changes similar to those seen in ALI in humans (Rojas, Woods et al. 2005). Intraperitoneal LPS activates the toll-like receptor (TLR)-4 on liver Kupffer cells. This results in the induction of pro-inflammatory cytokines, which when released systemically damage the pulmonary endothelium resulting in neutrophilic alveolitis.

Table 1.4. Animal models of ALI and ARDS (Ware and Matthay 2000)

Direct Lung injury	Indirect Lung injury
Acid aspiration	Cecal ligation and puncture
Surfactant depletion followed by high-volume mechanical ventilation	Oleic acid (I.V)
Hyperoxia	Mesenteric ischemia reperfusion
Lung ischemia reperfusion	Endotoxin (I.V, I.P)
Endotoxin (I.T)	

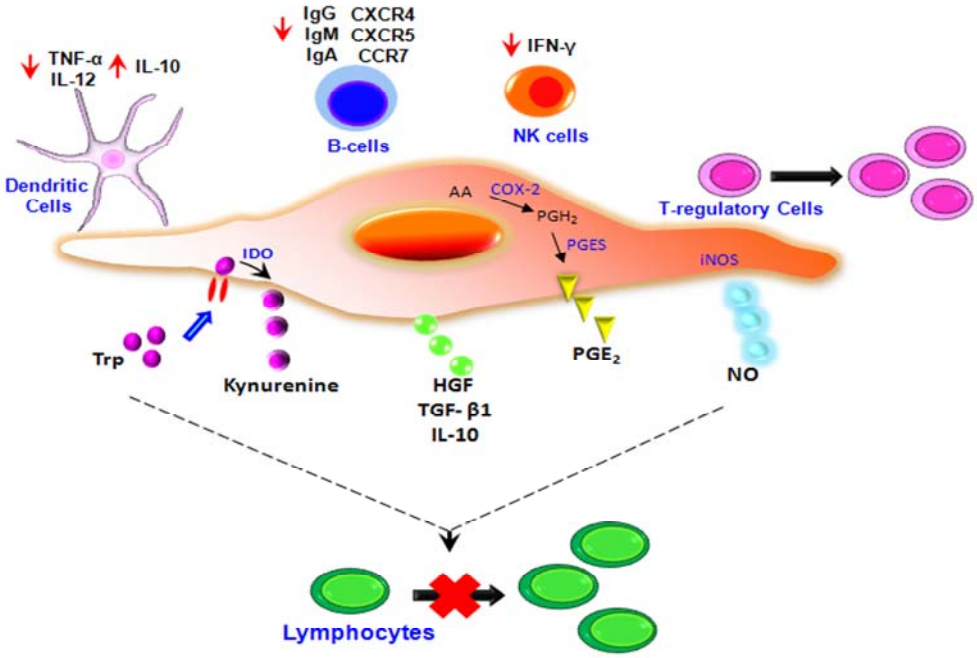
3.5. Bone marrow-derived Mesenchymal Stem Cells as a therapy in ALI

****NOTE: Parts of this chapter are presented as written for: Iyer S and Rojas M (2008) Expert Opin Biol Ther 8(5):569-81. Used with permission.**

Because disregulated inflammation causes lung injury, strategies to attenuate the inflammatory response in ALI and ARDS are of considerable therapeutic interest. Bone marrow-derived mesenchymal stem cells are emerging as a therapeutic modality in various inflammatory diseases (Iyer, Islam et al. 1961). Several studies have shown that exogenously infused mesenchymal stem cells (MSC) protect against endotoxin-induced inflammation and lung injury in mice (Rojas, Woods et al. 2005; Gupta, Su et al. 2007; Mei, McCarter et al. 2007).

MSC are multipotent cells that can be isolated from the bone marrow and expanded in culture relatively easily (Caplan 1991). Culture-expanded MSC have been used in the clinic to enhance hematopoietic stem-cell engraftment, and in tissue regeneration therapy (Horwitz, Gordon et al. 2002; Lazarus, Koc et al. 2005). MSC deploy a complex array of mechanisms to regulate proliferation, differentiation, and immune phenotype of cells from the innate and adaptive immune system ***(Figure 1.4)***. These mechanisms include secretion of regulatory molecules and growth factors, deprivation of tryptophan in the local milieu, and production of nitric oxide (Aggarwal and Pittenger 2005; Ryan, Barry et al. 2007; Sato, Ozaki et al. 2007).

Figure 1.4. Anti-inflammatory effects of MSC



MSC constitutively express tolerogenic factors such as hepatocyte growth factor (HGF), interleukin (IL)-10, transforming growth factor (TGF)-β1, and prostaglandin (PG) E_2, an enzymatic product of arachidonic acid metabolism. Production of these factors is dramatically increased after stimulation with pro-inflammatory cytokines such as TNF-α or IFN-γ (Ryan, Barry et al. 2007). MSC also adjust extracellular levels of tryptophan (Trp), an essential amino acid (Ryan, Barry et al. 2007). Local depletion of Trp and/or the accumulation of Trp metabolites inhibits T-cell proliferation (Moffett and Namboodiri 2003). Stimulation of MSC with IFN-γ induces the Trp-degrading enzyme, indoleamine 2, 3-dioxygenase (IDO), which metabolizes Trp to kynurenine. Accumulation of kynurenine occurs in a mixed lymphocyte reaction with MSC, and kynurenine alone can suppress T-cell proliferation (Ryan, Barry et al. 2007). Thus, in addition to the production

of cytokines, growth factors, and lipid mediators; MSC employ the restriction of local amino acid concentrations to regulate T-cell proliferation.

Aggarwal and Pittenger reported that MSC promote a shift from a pro-inflammatory to an anti-inflammatory phenotype in lymphocytes (Aggarwal and Pittenger 2005). By decreasing T-cell-mediated production of Interferon γ (IFN-γ), MSC induce a (T-helper 2) T_H2 shift in immature T-cells cultured under T-helper 1(T_H1) inducing conditions. MSC potentiate a T_H2 shift by increasing Interleukin 4 (IL-4) levels in T-cells cultured under T_H2 conditions (Aggarwal and Pittenger 2005). This interaction indicates that MSC can alter the outcome of an on-going inflammatory response by polarizing the cytokine profile of T-cell subsets to an anti-inflammatory phenotype. Of relevance to the ALI, MSC alter the phenotype of cells from the innate immune system. MSC decrease TNF-α, and increase IL-10 in different dendritic cells subsets (Jiang, Zhang et al. 2005).

While the precise molecular mechanisms that drive the immunomodulatory and immunosuppressive effects of MSC are yet to be unraveled, data generated in animal models of lung injury show that MSC elicit anti-inflammatory effects *in vivo* (Rojas, Woods et al. 2005; Gupta, Su et al. 2007). In a murine model of endotoxin-induced ALI, exogenous infusion of MSC prevented the systemic inflammatory response to endotoxin and thereby attenuated lung injury (Rojas, Woods et al. 2005). In this study, MSC were obtained from syngeneic donors expressing green fluorescent protein (GFP) to facilitate *in vivo* tracking of donor-derived MSC. The cells were expanded *in vitro* and were depleted of cells with hematopoietic and macrophage markers (CD45 and CD11b, respectively) prior to administration. MSC were infused intravenously (i.v), immediately

after intraperitoneal challenge with 1 mg LPS/kg body weight. Histological examination of lung sections demonstrated that infusion of MSC decreased numbers of neutrophils infiltrating into the lungs between 6 h and 48 h. Plasma levels of pro-inflammatory cytokines, IL-1β, IFN-γ, IL-6, and macrophage inflammatory protein-1α (MIP-1α) were significantly decreased with MSC infusion. The protection conferred by MSC was not related to clearance of endotoxin, and appeared to be at least partly independent of MSC engraftment into the lung. This indicates that MSC modulate the systemic environment. What remains unknown, however, is whether MSC infusion also protects against perturbations in the extracellular redox environment, associated with inflammation. Because the thiol/disulfide redox environment is intimately linked with inflammation and tissue injury, the role of MSC in modulating thiol disulfide redox status warrants consideration.

4. IDIOPATHIC PULMONARY FIBROSIS

The resolution of inflammation by repair is a highly regulated response in adult tissues. The repair program involves humoral factors such cytokines and growth factors, proteases, components of the extracellular matrix (ECM), and angiogenic factors which act in regulated and controlled ways to resolve the inflammatory response (Wynn 2004; Iredale 2007). However, persistent inflammation can lead to a disregulated repair response (Gross and Hunninghake 2001). This results in excessive deposition of ECM components such as collagen and fibronectin, which can contribute to fibrosis i.e., the formation of a permanent scar(Gross and Hunninghake 2001).

Fibrosis can affect all tissues and organs. Fibrotic tissue remodeling is a pathogenic component of numerous chronic diseases including, cardiovascular disease, age-related macular degeneration, renal dysfunction, and liver cirrhosis, an end-stage of liver fibrosis (Bataller and Brenner 2005; Wynn 2007). Of the fibrotic diseases in the lung, idiopathic pulmonary fibrosis (IPF) is the most common and the most deleterious (Coultas, Zumwalt et al. 1994).

4.1. Historical Perspective

IPF was originally described by Hamman and Rich in four patients who died of respiratory failure (Hamman and Rich 1944; Noble and Homer 2005). In these patients, the duration of the disease ranged from one to six months and the pathology was described by the authors as- “a diffuse thickening of the alveolar walls” and the “presence of small scars which had obliterated the architecture entirely” (Noble and Homer 2005).

Subsequently in 1957, Rubin and Lubliner described 54 cases meeting the criteria of the Hamman-Rich syndrome of diffuse interstitial fibrosis (Rubin and Lubliner 1957). The lungs were described as "cirrhotic, liver-like in consistency and presenting a cobblestone appearance" This cobblestone appearance of the lung is a key pathological feature in IPF and is presently used as a critical diagnostic tool (Hunninghake, Zimmerman et al. 2001; Flaherty, Thwaite et al. 2003). In contrast to the acute duration of the illness in the Hamman and Rich case report, Rubin and Lubliner reported up to a six year survival in their patients.

Subsequently, through the work of Sheridan and colleagues (Sheridan, Harrison et al. 1964), and Gross and co-workers (Gross 1962), an acute versus chronic group of the Hamman-Rich syndrome was recognized. However, pathological distinctions between the groups were not made then. We now know that patients with IPF exhibit distinct patterns of disease progression (Martinez, Safrin et al. 2005). While most patients undergo a slow, progressive decline in lung function (slow progressors- median survival time 3 to 5 years), a subset of patients experience a precipitous course of the disease (rapid progressors) and have a survival time of less than one year (Collard, Moore et al. 2007). Thus, the differences in the duration of disease described by Hamman and Rich versus Rubin and Lubliner may relate to clinical variants in IPF. Over the years, significant advances have been made in identifying important pathogenic modulators in IPF(Chapman 2004).

Of the known soluble mediators, transforming growth factor (TGF)-β1is the most potent (Border and Noble 1994), and *in vitro* studies show that TGF-β1 is induced in response to an oxidized extracellular Cys/CySS redox state (Ramirez, Ramadan et al.

2007). Thus, oxidation of Cys/CySS redox state could represent a pathogenic mechanism in lung fibrosis. The subsequent sections examine the pathogenesis of IPF in detail.

4.2. Epidemiology and Risk Factors

Age and gender

A recent retrospective cohort study, on data obtained from the United States Health Care Claims Database between January 2006 through December 2006, reported that the estimated prevalence rate of IPF was 14 per 100,000 persons, and the incidence rate was 7 per 100,000 (Raghu, Weycker et al. 2006). Substantially higher rates of prevalence and incidence were reported in individuals in the sixth and seventh decade of life. The study also reported that the prevalence of IPF was higher among older men than older women, an observation that is consistent with other reports which show a higher prevalence and higher mortality rate in males, with a relative risk of death in males ranging between 1.4 – 2.3 (Schwartz, Helmers et al. 1994; Mannino, Etzel et al. 1996).

Differences in lung function parameters are also observed between IPF males and females. An increase in the rate of oxygen desaturation during a six minute walk test (6MWT) correlates with increased mortality in IPF patients (Flaherty, Andrei et al. 2006). Measurements of serial changes in desaturation during a 6MWT over 1 y revealed a 2.8% increase per month for males and 1.4% increase for females (Han, Murray et al. 2008). *In vivo* experiments demonstrate that male mice have a greater decrease in static lung compliance with bleomycin, a pro-fibrotic drug, compared to female mice. Interestingly, treating female mice with dihydroxytestosterone (DHT)

decreases lung compliance to levels comparable to male mice (Voltz, Card et al. 2008), suggesting a mechanistic role for DHT in the fibrotic process.

Smoking and environmental factors

A history of cigarette smoking is associated with an increased risk for IPF (Baumgartner, Samet et al. 1997; Antoniou, Hansell et al. 2008). A multicenter case-control study on 248 clinically and histologically diagnosed IPF cases and 491 control subjects found that more cases (72%) than controls (63%) had a history of smoking. The odds ratio for ever-smokers was 1.6 (95% CI, 1.1 to 2.4) for IPF, and the risk was significantly higher in patients with a history of smoking (OR, 1.9; 95% CI, 1.3 to 2.9), and for smokers with 21 to 40 pack/y (OR, 2.3; 95% CI, 1.3 to 3.8) (Baumgartner, Samet et al. 1997). However, better survival is reported in current smokers compared to former smokers and this discrepancy is attributed to a "healthy smoker effect" i.e., patients with more advanced disease may stop smoking earlier. A recent cross-sectional study on 249 IPF patients (n=20 current smokers; n=166 former smokers; n=63 never-smokers) found a higher survival in never-smokers compared to former smokers (hazard ratio, 0.51; 95% CI, 0.41 to 0.83, after adjusting for disease severity (Antoniou, Hansell et al. 2008).

Exposure to environmental pollutants, especially particulates, is associated with increased risk for IPF. A case-control study found that occupations associated with dusty environments such as farming (OR, 1.6), tending to livestock (OR, 2.7) and stone cutting (OR, 3.9) were associated with increased risk for IPF after adjusting for age and smoking status (Baumgartner, Samet et al. 2000). Although a case-control study does not establish causality, data from animal models indicate that inhalation of air-borne pollutants triggers

lung inflammation and this could lead to fibrosis (Cassel, Eisenbarth et al. 2008; Dostert, Petrilli et al. 2008).

Infection

Multiple studies have found an association between Epstein-Barr virus (EBV), a herpes family virus, infection and IPF. A study based on EBV serology found elevated levels of antibodies against EBV in patients with IPF (n=13) compared to patients with other interstitial lung diseases (n=12) (Vergnon, Vincent et al. 1984). Another study analyzed lung tissue from IPF patients for evidence of EBV replication. 70% of IPF patients (14 of 20) were positive for EBV lytic cycle antigens compared with 21% in control patients. Immunohistochemical analyses of the lung revealed that viral replication was localized to type II epithelial cells in the lower respiratory tract (Egan, Stewart et al. 1995). Antibodies to the early nuclear and capsid antigens of EBV were also detected in the BALF of patients with IPF (Manika, Alexiou-Daniel et al. 2007).

In a mouse-model of fluorescein isothiocyanate-induced pulmonary fibrosis, gammaherpesvirus infection exacerbated pre-existing fibrosis (McMillan, Moore et al. 2008), suggesting that viral infection may play a role in the pathogenesis of IPF. Interestingly, infecting mice with γ-murine herpes virus (γ MHV) does not cause fibrosis unless the mice are biased towards a T helper 2 (T_H2) response by knocking out the IFN-γ receptor. Infection of IFN-γ -/- mice with γMHV leads to progressive fibrosis (Mora, Woods et al. 2005) and control of viral reactivation arrests fibrosis in this model (Mora, Torres-Gonzalez et al. 2007). Taken together, these data suggest viral infection can

contribute to fibrosis in the setting of modifier genes that bias towards a T_H2 response during infection.

Genetic predisposition

Familial IPF is defined as IPF occurring in two or more members of a family. Approximately 1 of every 50 patients with IPF has an affected first-degree family member. A study of familial IPF cases reported that familial patients are younger at diagnosis (mean age of diagnosis 55.5 compared to 67 y for sporadic IPF, but share the same clinical, radiological and pathological characteristics as sporadic IPF patients (Marshall, Puddicombe et al. 2000). More males are effected than females (male:female ratio – 1.75:1; $p<0.05$) and the inheritance pattern is autosomal dominant with incomplete penetrance (Allam and Limper 2006).

Mutations in the genes encoding the telomerase reverse transcriptase (TERT) and telomerase mRNA (TR), confer increased susceptibility to IPF suggesting that telomere shortening is involved in the pathogenesis of IPF (Tsakiri, Cronkhite et al. 2007). Polymorphisms in surfactant protein genes A and B have also been associated with IPF (Selman, Lin et al. 2003). Thus, numerous factors, both genetical and environmental, are associated with an increased risk for IPF and further studies are needed to establish a mechanistic link between these risk factors and fibrosis. Of relevance to research described in this dissertation is the observation that Cys/CySS redox state is oxidized with numerous risk factors associated with pulmonary fibrosis including age (Jones, Mody et al. 2002) and cigarette smoking (Moriarty, Shah et al. 2003). Thus, oxidation of Cys/CySS redox state could represent a pathogenic mechanism in fibrosis.

Table 1.5. Potential risk factors for Idiopathic Pulmonary Fibrosis

Age
Gender
Cigarette Smoking
Air-pollutants
Viral infection
Gastro-esophageal reflux disease
Telomerase deficiency
T_H2 biased immune response
Surfactant abnormalities

4.3. Pathogenesis of IPF

A critical aspect in understanding the pathogenesis of IPF lies in identifying both the similarities and the differences between fibrogenesis and normal tissue repair (Strieter 2008). Accumulating evidence indicates that three defining events distinguish the fibrotic process in IPF from normal lung repair: 1) epithelial and endothelial cell apoptosis/necrosis with loss of basement membrane integrity, 2) impaired re-epithelialization/re-endothelialization of the alveolar wall and 3) migration and proliferation of fibroblasts into the air-space with deposition of ECM components ***(Figure 1.5)*** (Strieter 2008). Together, these events result in air-space occlusion, loss of lung elasticity and surface area which leads to impaired lung function (Keane, Strieter et al. 2005; Strieter 2008).

The alveolar epithelium and endothelium are the primary site of damage in interstitial lung diseases. Ultrastructural analysis of lung tissue from IPF patients demonstrates injury to type I epithelial cells and endothelial cells. Bronchiolar and

alveolar epithelial cells in IPF lung stain positive for TUNEL, indicating presence of DNA damage (Kuwano, Kunitake et al. 1996). Additionally, the Fas ligand (FasL), which induces apoptosis of cells expressing Fas, is upregulated in inflammatory cells isolated from the lavage fluid of IPF patients (Kuwano, Miyazaki et al. 1999), and the Fas antigen is upregulated in the parenchyma of IPF lung. Further, elevated levels of serum soluble FasL in IPF patients with active disease compared to patients with inactive disease indicates that increased apoptosis may be associated with increased severity of fibrosis (Kuwano, Maeyama et al. 2002).

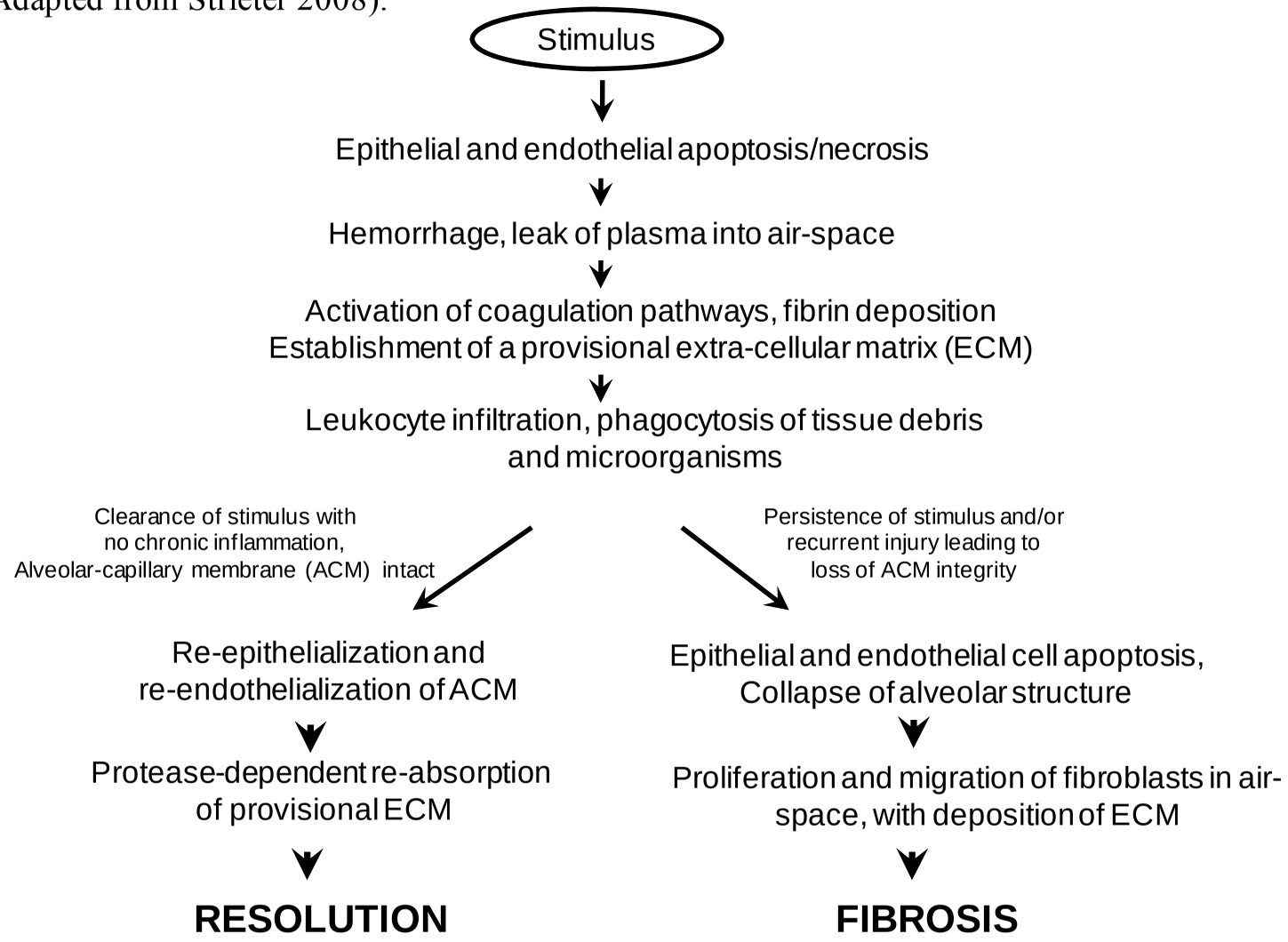
The importance of the endothelium in fibrosis is demonstrated in mice conditionally expressing the keratinocyte growth factor (KGF) transgene in type II pneumocytes. The selective expression of KGF protects the alveolar epithelium from hyperoxia-mediated injury (Ray, Devaux et al. 2003). However, due to injury to the pulmonary endothelium mortality in transgenic mice is comparable to wild-types. Thus, alveolar epithelial cells and pulmonary endothelial cells are both critical in maintaining the alveolar-capillary barrier.

Continual injury to the alveolar-capillary barrier impairs integrity of the basement membrane and current concepts hold that the loss of basement membrane integrity is critical in determining the "point of no return". Soluble mediators such as cytokines and growth factors, released and activated during injury, are critical in determining subsequent events leading to fibrosis (Chapman 2004).

A crucial growth factor in normal wound healing, and one whose sustained production has most consistently been linked to fibrosis, is TGF-β1 (Border and Noble 1994; Gauldie, Sime et al. 1999; Uhal, Kim et al. 2007). The actions of TGF-β1 are cell-

type dependent. TGF-β1 induces apoptosis of type I cells, induces type II cell hyperplasia and epithelial to mesenchymal transition, and induces fibroblast proliferation, myofibroblastic transdifferentiation, and ECM deposition (Blobe, Schiemann et al. 2000). Expression of TGF-β1 is detected in lung fibroblasts isolated from IPF patients (Uhal, Kim et al. 2007) and over-expression of active TGF-β1 in the air-space of rats, causes fibrosis (Sime, Xing et al. 1997). In rat lungs, transient over-expression of active TGF-β1 via an adenovirus vector led to monocytic infiltration into the airspace and rapid deposition of ECM proteins such as collagen, elastin and fibronectin with evidence for myofibroblastic differentiation (Sime, Xing et al. 1997). Thus, factors that induce TGF-β1 are likely to be involved in the pathogenesis of fibrosis. Studies in lung fibroblasts have shown that oxidized Cys/CySS redox state induces TGF-β1 expression (Ramirez, Ramadan et al. 2007). This raises the possibility that oxidation of Cys/CySS redox state may be a pathogenic component in fibrosis

Figure 1.5. Common and unique pathways in lung repair and lung fibrosis
(Adapted from Strieter 2008).



4.4. Animal models of lung fibrosis

While no single model recapitulates all aspects of human IPF (Gauldie and Kolb 2008), animal models of the disease have allowed dissection of mechanisms relevant to the fibrotic process (Moore and Hogaboam 2008). Among these, the bleomycin model is described in the detail. Other models are available but were not used in the present research and are briefly summarized.

Bleomycin-induced model of fibrosis

The bleomycin-induced model of lung fibrosis is the best characterized and most widely used model of lung fibrosis (Moore and Hogaboam 2008). Bleomycin is an antibiotic that was first isolated from the fungus, *Streptomyces verticillis* by Umezawa and colleagues (Umezawa, Maeda et al. 1966). Bleomycin has been used in adjunct chemotherapy in the management of squamous cell carcinomas and lymphomas (Zinzani, Pavone et al. 1997). Approximately 10% of patients treated with bleomycin develop interstitial pneumonitis and/or pulmonary fibrosis. The cytotoxicity of bleomycin is mediated by two main structural components; a bithiazole ring which partially intercalates into the DNA helix, and pyrimidine and imidazole moieties, which bind iron and oxygen to generate free radicals (Suzuki, Nagai et al. 1969). In patients with bleomycin-induced pneumonitis, increase numbers of lymphocytes and neutrophils are recovered from the lower airway.

The potential for bleomycin to induce experimental lung fibrosis was first demonstrated in dogs in 1971 (Hay, Shahzeidi et al. 1991). Subsequent studies show that intra-tracheal bleomycin induces fibrosis in a number of experimental animals including

hamsters, mice, and rats (Hay, Shahzeidi et al. 1991). Studies in mice have demonstrated that the C57/BL6 mice are prone to bleomycin-induced fibrosis while the Balb/c mice are resistant (Harrison and Lazo 1988). This strain-dependent sensitivity to bleomycin appears to depend on expression of the inactivating enzyme, bleomycin hydrolase. The susceptibility of the lungs compared to other tissues to bleomycin toxicity is also related to low levels of this enzyme in the lung (Schrier, Kunkel et al. 1983). The pharmacokinetic aspects of bleomycin are well-studied. Experiments investigating the pulmonary fate of tritiated bleomycin given intratracheally found that less than 1% of the radioactivity was recovered after 24 h suggesting that drug was rapidly metabolized (Lazo and Pham 1984). Thus, the fibrotic response to bleomycin is a result of its initial cytotoxic effect on alveolar epithelial cells which results in neutrophilic and lymphocytic alveolitis within 3-7 days (Janick-Buckner, Ranges et al. 1989). The subsequent development of fibrosis between 14-28 days is a result of the inflammatory response.

The strength of the bleomcyin model lies in the close resemblance of its histological characteristics, diffuse parenchymal inflammation, epithelial cell injury with basement membrane damage, and interstitial and intra-alveolar fibrosis, to the lesions in human IPF (Moore and Hogaboam 2008). However, unlike human IPF, bleomycin-induced fibrosis in mice is self-limiting. Furthermore, the short duration of the fibrotic response makes it a less amenable model to test therapies in the attenuation of established fibrosis. Nevertheless, the evolution of the fibrotic response is the best characterized in this model. This makes it useful to dissect the mechanisms involved in the fibrotic process.

Other models of IPF

Fluorescein isothiocyanate (FITC)-induced model of fibrosis involves instillation of FITC microbeads intratracheally (Roberts, Howie et al. 1995; Christensen, Goodman et al. 1999). The fibrotic response evolves in response to FITC-induced epithelial injury. Both BL/6J and Balb/c mice are susceptible to FITC-induced fibrosis. An advantage of the FITC model is the ability to visualize the deposition of FITC via immunofluorescence. Fibrotic changes are noted only in areas of FITC deposition. This makes it a robust model to objectively assess therapeutic regimens by comparing areas of comparable FITC deposition. Furthermore, the persistent nature of FITC-induced fibrosis (6 mo) makes it ideal for testing therapeutic strategies in established disease, a condition that is most relevant to the clinic (Dona, Dell'Aica et al. 2003).

An animal model that closely mimics occupational exposure to mineral dust and particulate dust is the silica-induced model of fibrosis. Because silica is not rapidly cleared from the lung, aerosolized installation results in a persistent inflammatory stimulus which causes the development of fibrotic foci at the areas of mineral deposition (Davis, Leslie et al. 1998).

Evidence for viral infection in IPF lungs has led to murine models investigating the role of virus infection in fibrosis. Mice infected with γ-murine herpesvirus 68 (γHV-68) 1 week before intra-tracheal bleomycin have an augmented fibrotic response (Lok, Haider et al. 2002), suggesting that viral infection can pre-dispose to fibrosis. Furthermore in mice biased to a T_H2 response, γHV-68 infection caused progressive fibrosis (Mora, Woods et al. 2005).

Thus, there are numerous models of fibrosis some of which eg., the FITC and virus model are useful to test therapeutic strategies to prevent progression of established fibrosis. The present work uses the bleomycin-induced model of fibrosis to characterize the response of Cys/CySS redox state. For studies addressing whether interventional strategies to ameliorate oxidation of Cys/CySS redox is useful in fibrosis, the utilization of the FITC or virus-induced model of fibrosis is recommended.

5. OXIDATIVE STRESS AND GSH DEFICIENCY IN ACUTE LUNG INJURY AND IDIOPATHIC LUNG FIBROSIS

This section summarizes our current understanding of oxidative stress and GSH deficiency in acute lung injury (ALI), and idiopathic pulmonary fibrosis (IPF). Because a excessive mediator production underlies the pathogenesis of ALI and IPF; I will first discuss aspects of thiol/disulfide redox control in cytokine and growth factor production. Next, I review clinical and experimental data demonstrating a role for oxidative stress and GSH deficiency in the etiology and progression of ALI and IPF. Within this scope, thiol antioxidants such as N-acetyl cysteine (NAC) represent a promising therapeutic strategy. However, demonstrating clinical benefits with NAC has proven difficult. The data on NAC are discussed and I and examine why the benefits seen in animal models have not translated into improvements in clinical outcomes. Finally, I discuss the role of Cys, a GSH precursor that has received remarkably little attention in ALI and IPF.

5.1. GSH and Cys as Determinants of Cytokine and Growth Factor Production.

Experimental manipulation of Cys and GSH levels alters cytokine production *in vitro* in numerous cell-types including endothelial cells (Ishii, Partridge et al. 1992), lymphocytes (Angelini, Gardella et al. 2002), and mononuclear cells (Gosset, Wallaert et al. 1999). In alveolar macrophages, lipopolysachharide (LPS)-induced production of tumor necrosis factor (TNF)-α and interleukin (IL)-6, major pro-inflammatory cytokines, is decreased by pre-treating cells with 1 mM N-acetyl cysteine (NAC) (Gosset, Wallaert et al. 1999). *In vivo,* oral pre-treatment of mice GSH and NAC decreases LPS-induced TNF-α in the circulation and in the spleen (Peristeris, Clark et al. 1992). In patients with

sepsis, oral supplementation with NAC significantly improved neutrophil phagocytosis and reduced oxidative burst activity (Heller, Groth et al. 2001). Thus, Cys and GSH are well-established determinants of cytokine production during activation of the immune system.

Cytokine production also impacts Cys and GSH homeostasis. In pulmonary endothelial cells, TNF-α decreases intracellular GSH and increases GSSG levels (Ishii, Partridge et al. 1992). Further, TNF-α mediated GSH depletion increases the sensitivity of pulmonary endothelial cells to peroxide-mediated apoptosis, a mechanism that could contribute to increased capillary leak in sepsis (Ishii, Partridge et al. 1992). During infection and injury, increased protein and GSH synthesis exert a greater demand on Cys supply (Sendelbach, White et al. 1990). In rats challenged with TNF-α, a greater fraction of the Cys pool is utilized for protein synthesis than GSH leading to a decrease in hepatic GSH content (Hunter and Grimble 1994). This decrease is exacerbated in rats fed a diet low in sulfur amino acids, indicating that adequacy of precursor availability is critical in maintaining GSH and Cys reserves during inflammation (Grimble and Grimble 1998).

Impaired Cys and GSH homeostasis can contribute to increased morbidity following infection and injury by two distinct mechanisms. The first relates to a suppression of immune function, which compromises the host's ability to mount an immune response. It is well-recognized that optimal thiol balance is critical for immune cells(Santangelo 2003). Lymphocyte proliferation in response to antigen challenge depends on Cys supply from antigen presenting cells such as dendritic cells and macrophages (Angelini, Gardella et al. 2002), and differentiation of monocytes to phagocytic macrophages is impaired when GSH levels are sub-optimal (Brown, Ping et

al. 2007). HIV patients have decreased levels of Cys and GSH, and significant improvements in immune function are seen after treatment with NAC (Breitkreutz, Pittack et al. 2000). Thus, Cys and GSH are critical for optimal immune function. The role of oxidative stress and Cys and GSH deficiency in the suppression of the immune response is not discussed further, but several references are available on this topic (Droge, Eck et al. 1991; Droge, Eck et al. 1992; Grimble 1998; Droge and Breitkreutz 2000).

The second mechanism relates to a disregulated inflammatory response i.e., when the inflammatory response has crossed the threshold from an adaptive to a maladaptive response. Evidence exists that a disregulated inflammatory response can be attenuated by antioxidants and thiol precursors. These data will be discussed in the subsequent sections.

5.2. Oxidative Stress and GSH Deficiency in Acute Lung Injury

5.2.1. Oxidative Stress in Acute Lung Injury

The idea that highly reactive oxygen metabolites, produced by activated leukocytes, caused tissue injury (Iyer, Islam et al. 1961) was advanced before the clinical description of ALI in 1967 (Ashbaugh, Bigelow et al. 1967). The acute pulmonary injury caused by these reactive oxygen species was believed to occur in a pathway that was parallel to the ongoing inflammatory response. We now know that ROS and redox signaling pathways, that are not strictly ROS-mediated, converge with cellular and humoral components of the immune system and this interaction is a key pathway in the pathogenesis of ALI.

Early evidence for such an interaction was presented by Cochrane and colleagues who found that α-1-antitrypsin (α-1-AT) recovered from the BALF of ARDS patients was oxidatively inactivated; rendering it ineffective as an inhibitor of neutrophil elastase (Cochrane, Spragg et al. 1983). The activity of the enzyme could be rescued by dithiothreitol and peptide methionyl sulfoxide reductase suggesting oxidation of a reactive site methionyl residue (Carp, Janoff et al. 1983). The source of oxidants was proposed to be the accumulation of activated leukocytes in the lower airway, a concept supported by prior *in vitro* and animal data (Sacks, Moldow et al. 1978; Johnson and Ward 1981; Shasby, Shasby et al. 1983). Subsequent studies reporting that peroxide levels in the exhaled breath condensate of ARDS patients were highly correlated with plasma lysozyme, a marker for neutrophil turnover, suggested that this indeed might be the case (Baldwin, Simon et al. 1986). More direct evidence was obtained from *in vitro* studies showing a direct inhibition of α-1-AT activity in alveolar macrophages obtained

from smokers in a reaction that could be inhibited by antioxidant enzymes, and by methionine (Hubbard, Ogushi et al. 1987).

Over the years, studies in humans have consistently shown two things. Firstly, patients with ALI and ARDS show increased levels of oxidative stress compared to healthy controls. For instance, levels of 8-iso-prostaglandin-F2 alpha, a non-enzymatic lipid peroxidation product, is increased by up to 10-fold in the exhaled breath condensate of patients who are with or at risk for ALI/ARDS (Carpenter, Price et al. 1998). Also, plasma antioxidants such as α-tocopherol, ascorbate, β-carotene, and selenium decrease with the progression of ARDS, and levels of malonyldialdehyde correspondingly increase (Metnitz, Bartens et al. 1999).

The second observation is that higher levels of oxidative stress in ARDS patients correlate with poorer outcomes. Elevation in plasma hypoxanthine, a substrate for superoxide and hydrogen peroxide, is associated with increased mortality in ARDS patients (Quinlan, Lamb et al. 1997). In this study, hypoxanthine was highly negatively correlated with loss of protein thiol groups in the plasma, indicating oxidative modification of extracellular protein thiols.

The redox state of thiol residues on extracellular proteins is regulated by two, low-molecular weight thiol/disulfide control systems, GSH and Cys. Therefore, understanding the regulation of GSH and Cys redox systems in ALI is important, both to dissect mechanisms of the disease process and to identify potential therapeutic targets.

5.2.2. GSH Deficiency in Acute Lung Injury

Measurements of GSH in the epithelial lining fliud (ELF), derived from the lower respiratory tract, of patients with sepsis and ARDS demonstrate a marked decrease in GSH levels (20 μmol compared to 90 μmol in controls) (Pacht, Timerman et al. 1991; Bowler, Velsor et al. 2003). Although the precise mechanisms contributing to perturbations in ELF GSH levels in ALI have not been clarified, evidence collected over the past 2 decades suggest that three general mechanisms may be important ***(Figure 1.6)***.

Alveolar type I cells constitute 90% of the alveolar surface area (Freeman, Mason et al. 1986), and are very susceptible to injury. In ALI/ARDS, denudation of the epithelial barrier together with damage to the pulmonary capillary endothelium leads to flooding of the alveolar space. In patients with ARDS, up to a 6-fold increase in ELF volume is documented (Cantin, Hubbard et al. 1989). This fluid influx can lead to a dilution of effective ELF GSH concentrations ***(Figure 1.6, 1)***.

Tissue injury also elicits influx of circulating neutrophils into the lower airways. The oxidative burst by activated neutrophils and resident macrophages helps in the clearance of infection and tissue debris, but also results in production of toxic oxygen metabolites such as peroxide, superoxide, and hypochlorous acid. GSH scavenges peroxide in a reaction catalyzed by glutathione peroxidase and is oxidized to glutathione disulfide (GSSG) (Meister 1995; Avissar, Finkelstein et al. 1996). Normally, GSSG is rapidly recycled back to GSH in an NADPH dependent process. However, excessive oxidant burden can overwhelm the recycling of GSH leading to a decrease in GSH levels and accumulation of GSSG ***(Figure 1.6, 2)***.

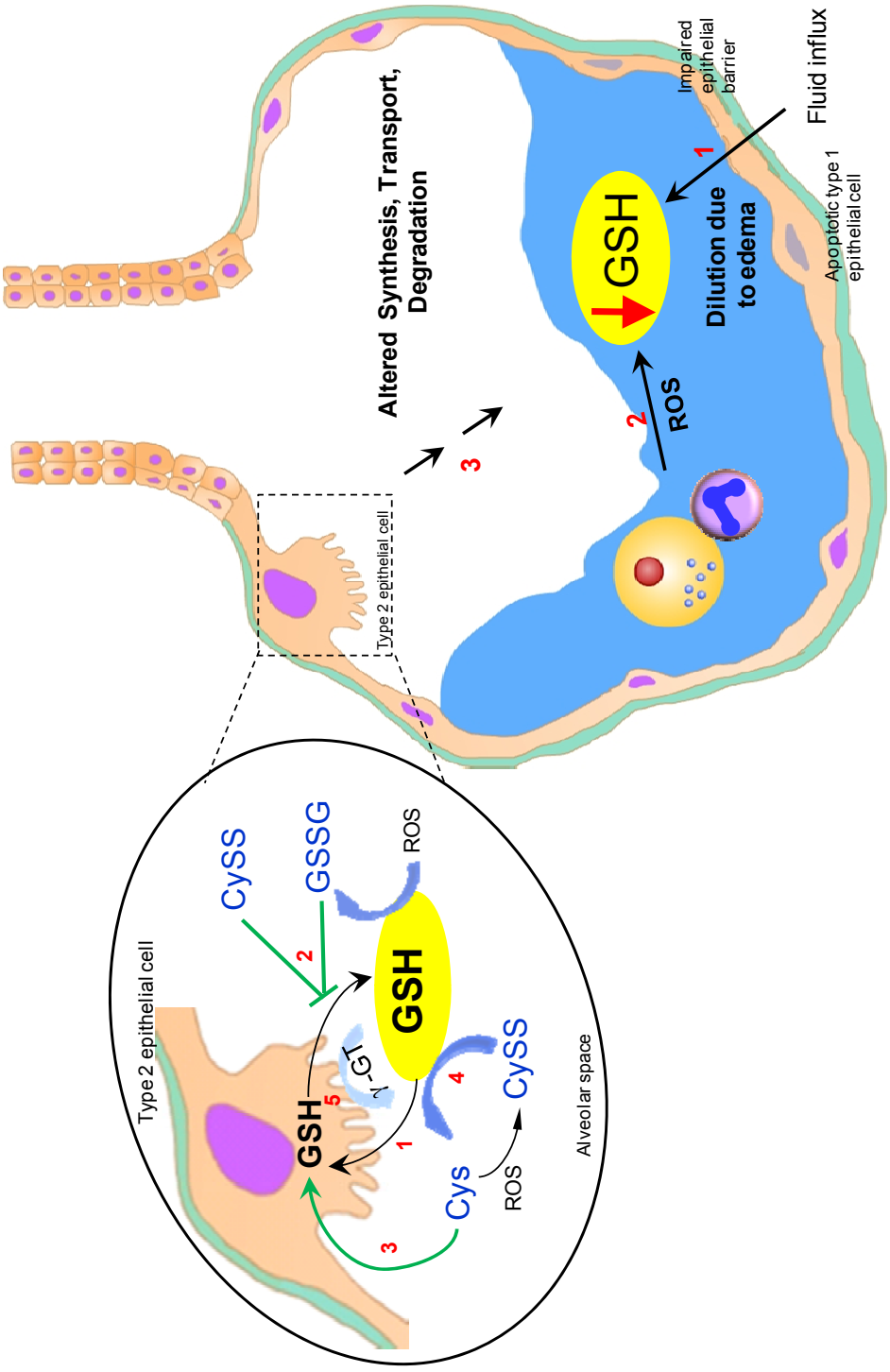


Figure 1. 6. Potential mechanisms contributing to decreased ELF GSH levels in ALI

A

nother important mechanism in the regulation of extracellular GSH levels in the lung is GSH turnover by the alveolar type II cells ***(Figure 1.6, 3)*** (van Klaveren, Demedts et al. 1997). Although alveolar type II cells make up only 10% of the alveolar surface area, they represent an important cell population in the lung. Type II cells synthesize and secrete pulmonary surfactant (Kikkawa, Yoneda et al. 1975), and regenerate type I cells during injury (Adamson and Bowden 1974; Freeman, Mason et al. 1986). Type II cells are relatively resistant to oxidant-mediated injury, a property conferred at least in part by increased activity of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase (Forman and Fisher 1981; Freeman, Mason et al. 1986). These cells also have the ability to import GSH by an active Na-dependent process during oxidant stress (Hagen, Brown et al. 1986), a mechanism that could contribute to decrease ELF GSH levels in ALI ***(Figure 1.6; inset, 1)***.

Although the origin of extracellular GSH in the ELF is not yet determined, GSH efflux by type II cells represents a potential mechanism (van Klaveren, Demedts et al. 1997; van Klaveren, Hoet et al. 1999). Characteriziation of the GSH efflux system in hepatocytes has demonstrated that GSH efflux is inhibited by elevated extracellular disulfide levels, while high extracellular thiol levels promote efflux of intracellular GSH. If the putative GSH efflux system in type II cells is similar to the sinusodal GSH transporter (Fernandez-Checa, Yi et al. 1996), then elevated levels of CySS and GSSG in the ELF can contribute to a decrease in GSH by inhibiting efflux ***(Figure 1.6; inset, 2)***.

GSH efflux would also depend on concentrations of intracellular GSH which, in turn, is determined by GSH synthesis. Under conditions of oxidative stress, Cys is rate

limiting in the synthesis of GSH. Therefore decreased Cys availability can impact GSH homeostasis ***(Figure 1.6; inset, 3)***.

Because type II cells transport Cys more efficiently than CySS, Cys availability for intracellular GSH synthesis depends on reduction of extracellular CySS to Cys by GSH (Deneke 2000); a process that may be limited at low ELF GSH concentrations ***(Figure 1.6; inset, 4)***. Furthermore, depletion of intracellular GSH in type II cells augments the cytotoxic effects of oxidants (Simon, DeHart et al. 1989; Jenkinson, Lawrence et al. 1994; Brown, Harris et al. 2001). Thus, increased apoptosis of type II cells can lead to impaired GSH homeostasis.

Finally, extracellular GSH levels also depend on the activity of γ-glutamyl transpeptidase (γ-GT); an ectoenzyme that degrades extracellular GSH (Meister 1988). In the adult lung, γ-GT is primarily expressed by the bronchiolar Clara cells and type II cells (Oakes, Takahashi et al. 1997), and its expression is increased with oxidative stress (Zhang, Forman et al. 2005). Increased activity of γ-GT in ALI can contribute to a decrease in extracellular GSH levels ***(Figure 1.6; inset, 5)***.

Thus, perturbations in ELF GSH levels in ALI result from mechanisms relating to oxidative stress, and altered transport, synthesis and degradation of GSH. It is also evident that availability and import of Cys, and CySS can have an important regulatory role in GSH homeostasis. Further research into elucidating these mechanisms will aid in designing effective strategies to counter the marked decrease in GSH levels in ALI. This is of critical importance because impaired GSH levels predispose to, and increase severity of ALI (Brown, Harris et al. 2004).

The role of GSH deficiency in increasing susceptibility to ALI is extensively studied in the setting of alcohol-induced lung injury. Chronic alcoholics are at an increased risk for ALI (Chawla, Lewis et al. 1984), and multiple clinical studies have shown a decrease in pulmonary and systemic GSH and Cys levels in this population (Chawla, Lewis et al. 1984; Moss, Guidot et al. 2000; Yeh 2007; Yeh, Burnham et al. 2007).

Mechanistic evidence for GSH depletion in ethanol-mediated susceptibility to ALI is demonstrated in animal models. In rats, chronic ethanol ingestion decreases lung and systemic GSH levels, and increases susceptibility to endotoxin-induced lung injury. Interventions to rescue ethanol-induced depletion of GSH protect against lung injury, indicating that increased susceptibility of alcoholics to ALI may be secondary to GSH depletion (Holguin, Moss et al. 1998).

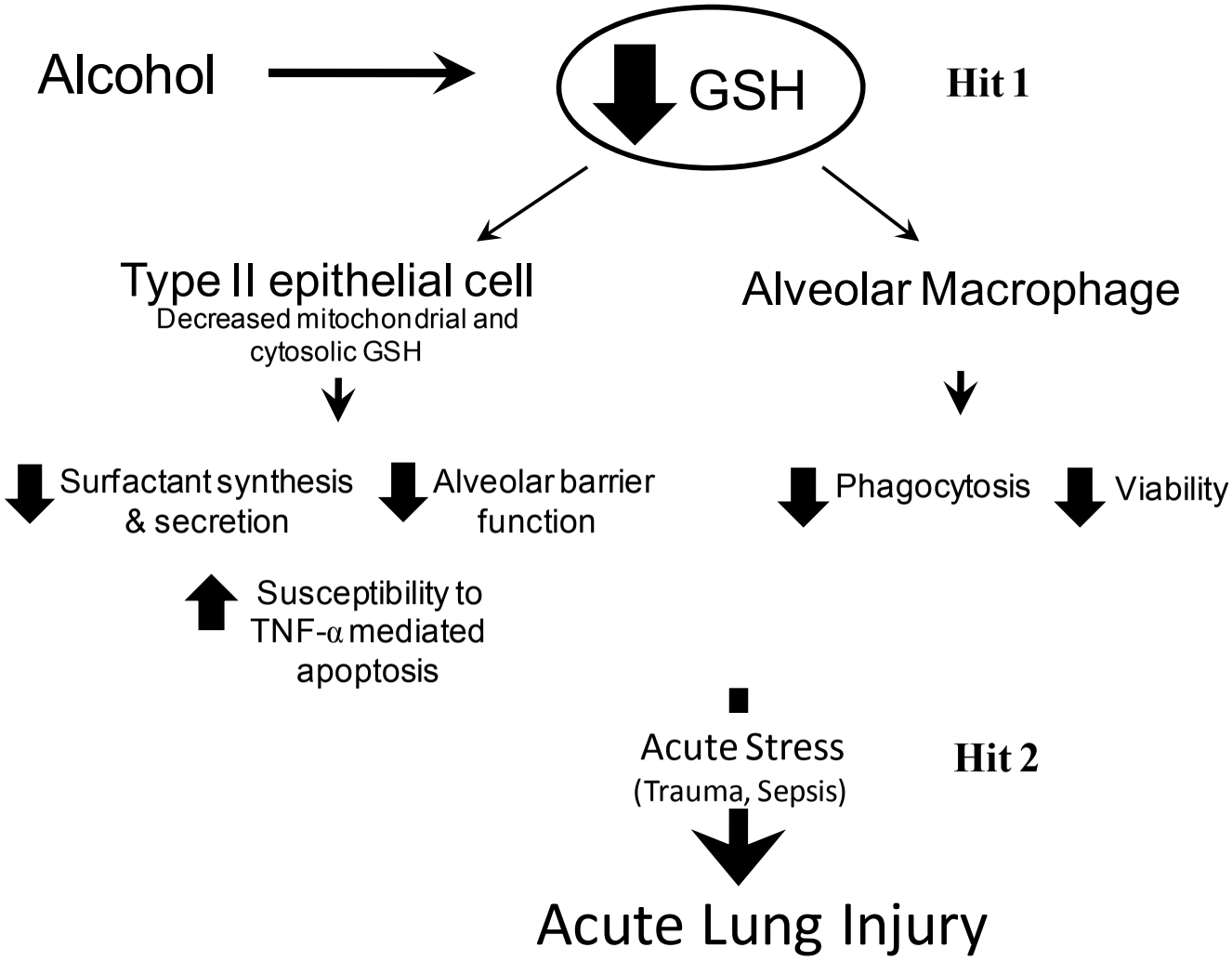
Data from cell-culture systems point to mechanisms by which altered GSH homeostasis in the alcoholic lung can predispose to lung injury ***(Figure 1.7)***. Chronic alcohol intake impairs surfactant secretion and synthesis in type II epithelial cells; an effect that is rescued by restoring mitochondrial and cytosolic GSH pools (Guidot and Brown 2000). Furthermore, GSH supplementation also protects type II epithelial cells against oxidant-induced attenuation of surfactant production (Brown 1994).

Epithelial barrier function is compromised with GSH deficiency; alveolar epithelial type II cells isolated from ethanol-fed rats form a more permeable monolayer *in-vitro* (Guidot, Modelska et al. 2000), and epithelial barrier function is preserved by supplementing the diet of alcohol-fed animals with L-2-oxothiazolidine-4-carboxylic

acid. GSH depletion also impairs type II cell viability by predisposing to TNF-α-mediated ROS production and apoptosis (Brown, Harris et al. 2001).

The alveolar macrophage represents another cell-type that is adversely affected by GSH depletion. In the alcoholic lung, GSH depleted alveolar macrophages fail to mature and demonstrate impaired phagocytosis. Supplementing alveolar macrophages isolated from the lungs of healthy alcoholics with GSH rescues the impaired phagocytosis and improves cell viability (Fischer, Pfalzgraf et al. 2006). Taken together, these data show that decreased GSH availability represents a predisposing state in the air-space that increases susceptibility to a "second-hit" such as sepsis or acute trauma. Thus, therapeutic targeting of GSH deficiency in ALI is of immense clinical interest. Because Cys is rate limiting in the synthesis of GSH, GSH deficiency could be secondary to deficiency of Cys.

Figure 1.7. "Two-hit" model for GSH depletion in contributing to ALI in chronic alcohol abuse



5.3. Thiol Antioxidants in Experimental and Clinical Acute Lung Injury

Administration of thiol precursors is an effective strategy to counter perturbations in GSH balance resulting either from diminished precursor availability and/or from increased generation of oxidants. Thiol compounds such as methionine, cysteamine, lipoic acid, N-acetylcysteine (NAC), L-2 oxothiazolidine-4-carboxylate (OTC), and glutathione esters have been given orally and intravenously under different toxicological and pathological conditions. Of these compounds, NAC is the most widely used. NAC supplies Cys necessary for GSH synthesis, and the sulfhydryl group of NAC allows for the direct scavenging of oxidants such as hydrogen peroxide. These properties have fuelled interest in the use of NAC in ALI where oxidant-mediated damage to the pulmonary endothelium and epithelium is a central pathological event.

The effects of NAC in experimental ALI and ARDS have been promising. In a sheep model of endotoxin-induced ARDS, intravenous NAC significantly attenuated endotoxin-induced increase in pulmonary vascular resistance resulting in improved lung compliance (Bernard, Lucht et al. 1984). NAC was administered at a loading dose of 150 mg/kg/h, 1 h prior to endotoxin challenge followed by a maintenance dose of 20 mg/kg/h over 5 h. Although measurements of oxidant stress were not made in this study, the effects were thought to be mediated by the antioxidant capacity of NAC. Direct evidence that NAC ameliorates endotoxin-induced oxidant stress came from Sprong et al who reported that continuous infusion of NAC at 275 mg/kg in rats 24 h before and after endotoxin, significantly decreased the early spike in circulating peroxide (Sprong, Winkelhuyzen-Janssen et al. 1998). More recently, Kao et al reported that intravenous drip infusion of NAC (150 mg/h for 1 h) given 10 minutes before challenge with

endotoxin decreased nitrates and nitrites in the BAL and plasma (Kao, Wang et al. 2006). There is also evidence that granulocytes from septic patients receiving high-dose NAC show reduced oxidative burst compared to patients on low-dose NAC (Heller, Groth et al. 2001). These data support the general conclusion that NAC ameliorates oxidative stress during endotoxemia.

Because oxidants enhance activation of nuclear factor (NF)- κB, a major pro-inflammatory transcription factor, a reduction in oxidant levels would be expected to decrease levels of pro-inflammatory cytokines. *In vitro* and *in vivo* studies have consistently shown that NAC attenuates LPS-mediated induction of pro-inflammatory cytokines, notably TNF-α and IL-1β (Peristeris, Clark et al. 1992; Gosset, Wallaert et al. 1999; Kao, Wang et al. 2006). Mice pre-treated with NAC orally prior to endotoxin challenge show decreased levels of TNF-α in circulation and in the spleen (Peristeris, Clark et al. 1992). Opposite effects are observed when DL-buthionine-(SR)-sulfoximine (BSO), a GSH synthesis inhibitor is used. LPS-mediated induction of TNF-α is potentiated by BSO and this effect is reversed by NAC, indicating that augmentation of thiol reserves is an important determinant in the anti-inflammatory effects of NAC. The effects of NAC may also extend to tempering pro-thrombotic lipid mediator production in ALI. The attenuation of pulmonary vasoconstriction by NAC (Bernard, Lucht et al. 1984) may relate to the inhibition of ROS-mediated induction of thromboxane-prostanoid receptors in the lung microvasculature (Wilcox 2005).

In all, experimental administration of NAC attenuates inflammation, and improves pulmonary hemodynamics leading to decreased lung injury and improved survival. Continuous infusion of NAC in rats (275 mg/kg 24 h before and after

endotoxin) resulted in survival of 83%, a value significantly higher than 53% survival rate in saline controls. Interestingly, in the same study the authors report that at a higher dose (950 mg/kg), NAC failed to protect against lung injury. At this high-dose, NAC did not protect against endotoxin-induced increase in plasma peroxides, and baseline levels of GSSG in the lung were elevated (Sprong, Winkelhuyzen-Janssen et al. 1998). There was also a trend for decreased survival (32%), compared to saline-controls indicating that high levels of NAC were deleterious. However, Blackwell et al reported that in rats, intraperitoneal NAC at 1000 mg/kg suppressed lung NF-κB activation and neutrophilic alveolitis (Blackwell, Blackwell et al. 1996). The explanation for the lack of adverse events at a similar dose may relate to the mode of administration.

Despite the short half-life of NAC in circulation (estimated to be 6.5 for oral NAC in human blood) (Holdiness 1991), a continuous intravenous infusion can lead to accumulation of NAC at high levels. On the other hand when given intraperitoneally, delivery of NAC into the peripheral circulation depends on its clearance by the portal system which may alter kinetics. Pharmacokinetic studies on the clearance of intraperitoneal NAC are required to better interpret the data. Nevertheless, the data suggest that in the presence of potent inflammatory/oxidative stimulus such as LPS, high systemic concentrations of NAC can be deleterious.

Indeed, there is evidence that high-dose NAC can potentiate the inflammatory response. In activated monocytes, high-dose NAC (5 mM) increased NF-κB mediated IL-1β release (Parmentier, Hirani et al. 2000). In pulmonary aortic endothelial cells, high-dose NAC (30 mM) activated NF-κB by inducing glutathionylation of the inhibitor protein IKK-α, (Mukherjee, Mishra et al. 2007). Furthermore, peroxide generation

increased with high-dose NAC.

It has been known for some time that at high concentrations, antioxidants can have prooxidant effects (Chan, Riches et al. 2001). For instance, autoxidation of thiols in the presence of transition metals generates the thiyl radical which rapidly reacts with O_2 to generate superoxide. Once initiated, these reactions can produce additional ROS such as peroxide and the hydroxyl radical, a potent and non-specific oxidant (Misra 1974). These reactions also appear to be influenced by the local redox mileu, with auto-oxidation being favored under oxidized thiol/disulfide redox states (Das, Lewis-Molock et al. 1995; Chan, Riches et al. 2001). Thus, the effects of NAC appear to depend on the route of administration, the dose, on the thiol/disulfide redox balance of the host, and the type of oxidant stimulus ***(Figure 1.8)***. The role of high-dose NAC and its metabolites, Cys and GSH, in chelating Zn and other heavy metals into urinary excretable complexes is another aspect that can contribute to toxicity of NAC (Brumas, Hacht et al. 1992).

Clinical evidence that high-dose NAC can be deleterious comes from a study in septic patients where NAC therapy, 24 h after admission to ICU, significantly increased mortality compared to placebo-treated group (60% versus 32%) (Molnar, Shearer et al. 1999). NAC was given as a bolus of 150 mg/kg followed by a continuous infusion of 12 mg/kg/hr for 24 hrs. In a different study, a lower maintenance dose of NAC (12.5 mg/kg/hr over 90 minutes) given 72 h after the onset of sepsis improved whole-body oxygen consumption (Spies, Reinhart et al. 1994). This dose-dependent, bimodal effect of NAC is an area that needs to be thoroughly investigated.

Figure 1.8. Potential mechanisms for toxicity of high-dose NAC

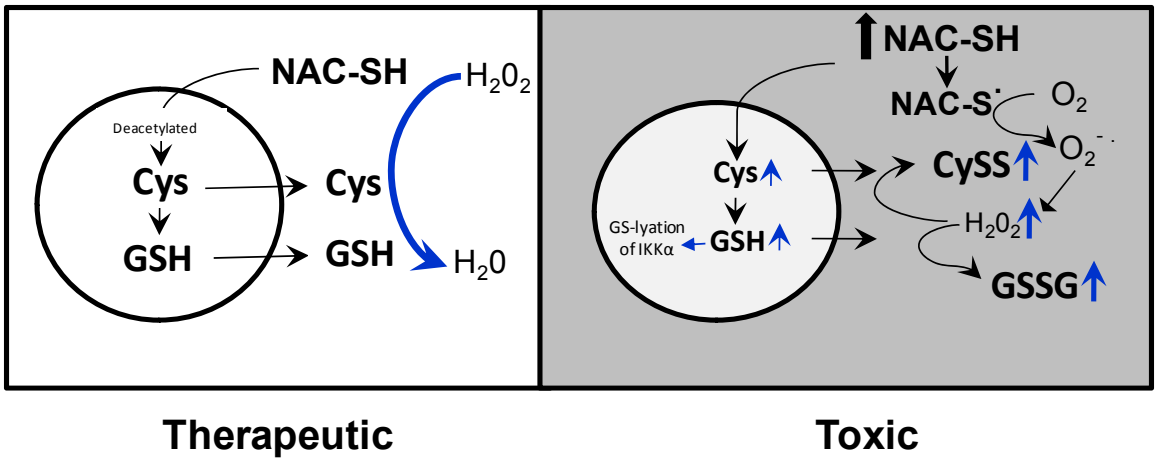


Figure 1.8. Potential mechanisms for toxicity of high-dose NAC. At high-doses, the autoxidation of NAC or Cys-derived from NAC results in the generation of ROS and free-radicals which can cause macromolecular damage. Oxidation of proteins may also occur as a consequence of elevated CySS and GSSG levels.

The effects of NAC in patients with established ALI and ARDS have been mixed. That NAC can improve Cys and GSH reserves in these patients is known, but how this correlates with clinical outcome is still an open question. The dose of NAC in sepsis is based upon the administration protocol in patients treated for acetominaphen overdose (Spapen 2004). Intravenous NAC given at 190 mg/kg/d for 3 days in patients in the early phase of ARDS significantly increased intracellular GSH stores in granulocytes but did not influence magnitude of oxidative burst or plasma elastase levels (Laurent, Markert et al. 1996). Jepsen et al investigated the effect of NAC in ICU patients with ARDS. Patients were randomized to receive either NAC (n=32) or placebo (n=34). NAC was given intravenously at a loading dose of 150 mg/kg for the first 30 minutes followed by continuous infusion of 20 mg/kg/h for 7 consecutive days. Although pulmonary compliance tended to be higher in the NAC group, no significant improvements in oxygenation index or progression of ARDS were observed (Jepsen, Herlevsen et al.

1992). Similarly, Domenighetti and colleagues found that in patients with established ARDS, intravenous NAC (90 mg/kg) did not improve systemic oxygenation or reduce the need for ventilator support (Domenighetti, Suter et al. 1997).

While NAC did not confer any benefits in patients with ARDS, Suter et al reported that in patients presenting with mild to moderate ALI, intravenous NAC significantly improved oxygenation index which led to an increase in ventilator-free days (Suter, Domenighetti et al. 1994). NAC was administered as a continuous infusion at 40 mg/kg/d for 3 days after admission to the ICU. Lung injury score significantly decreased in the NAC group. However, progression of ARDS was not affected and no significant differences in overall mortality between the NAC group and the placebo group were reported. Bernard et al investigated the effect of NAC or OTC in mechanically ventilated patients with ARDS. Patients received either NAC (70 mg/kg) or OTC (63 mg/kg) intravenously every 8 h over a 10-day period. OTC significantly decreased the number of days of acute lung injury and both NAC and OTC significantly improved cardiac index. However, no differences in mortality were observed between the treatment groups.

In all, the results from these four randomized, placebo-controlled clinical trials have been inconclusive. Two studies reported benefits relating to shortening the duration of lung injury and two failed to find any protection. The benefits observed in animal models have clearly not translated to improvement in clinical outcomes. The possible reasons are numerous and relate to the heterogeneity of ALI, a lack of understanding of the pharmacokinetic aspects of NAC, and to inherent limitations in the definition of oxidative stress (Jones 2006).

ALI is a heterogenous clinical entity that can result from pulmonary insults such

as aspiration of gastric contents and extra-pulmonary insults such as sepsis and pancreatitis (Ware and Matthay 2000). The heterogeneity of the patient population enrolled with regards to the underlying cause of ALI, as well as differences in the magnitude of initial lung injury can contribute to variations in outcome. It is possible that NAC may be beneficial in one subgroup of patients and may have no effect in others or may even be harmful. Clinical trials with a larger patient population that will enable stratification by cause and progression of ALI will provide more direct answers as to the efficacy of NAC. Furthermore, identifying and characterizing responders versus non-responders will aid in designing targeted therapies.

Finally, the simplistic view of oxidative stress as an imbalance of pro-oxidants over antioxidants has led to interventions attempting to rescue this imbalance by using high-dose antioxidants. However, the major thiol/disulfide control systems, GSH, thioredoxin, and Cys exist under non-equilibrium conditions and the redox states of these systems are regulated independently (Kemp, Go et al. 2008). These redox systems are distinctly modified by substrate availability (Go, Ziegler et al. 2007), chemical toxicants, and physiological (Blanco, Ziegler et al. 2007) and pathological stimuli (Moriarty, Shah et al. 2003; Hansen, Zhang et al. 2006; Go, Halvey et al. 2007). Thus, disruption in each of these systems could influence disease severity independently.

The way forward likely lies in identifying points of control in thiol/disulfide redox systems that regulate inflammation, and apply that knowledge to design targeted antioxidant interventions with tightly controlled dosing regimens.

5.4. Oxidative Stress and GSH deficiency in Idiopathic Pulmonary Fibrosis

5.4.1. Oxidative Stress in Idiopathic Pulmonary Fibrosis

A characteristic pathological feature in Idiopathic Pulmonary Fibrosis (IPF) is injury to the alveolar capillary barrier and fibrosis of the alveolar walls (Gross and Hunninghake 2001). Several lines of evidence implicate oxidative stress in the regulation of both these events**.**

In rats, intrapulmonary instillation of xanthine/xanthine oxidase or glucose/glucose oxidase, enzyme systems that generate hydrogen peroxide, causes lung injury which progresses to fibrosis (Johnson and Ward 1981). In human IPF, the cause of lung injury is unknown, but endogenous and exogenous stimuli eg., smoking and viral infection can cause epithelial injury via oxidant mediated pathways. Influx of inflammatory cells into the lower airway in response to epithelial damage can also contribute to an increase in oxidant burden. This may be especially true in a subset of IPF patients who present with acute exacerbations in respiratory status and experience a more precipitous course of the disease (Collard, Moore et al. 2007). These acute deteriorations are of unknown etiology, but rapid alterations in epithelial-cell integrity and cellular inflammation are thought to be important. Interestingly, recent microarray analysis in lung tissue from IPF patients displaying rapid disease progression demonstrate upregulation of genes involved in oxidative stress compared to slow progressors (Collard, Moore et al. 2007).

Whether persistent inflammation is a primary pathogenic mechanism in IPF is debatable. Nonetheless, the finding that alveolar inflammatory cells isolated from the lower respiratory tract of IPF patients spontaneously produce 2-fold greater superoxide

and 4-fold greater hydrogen peroxide than cells obtained from healthy controls (Cantin, North et al. 1987) indicates that when present, inflammatory cells can contribute significantly to the oxidant burden in the lower airway. Furthermore, ELF myeloperoxidase levels correlate significantly with the capacity of IPF ELF to enhance oxidant mediated cytotoxicity to epithelial cells. Levels of peroxide and 8-isoprostane, a biomarker of oxidant stress, are also significantly elevated in the exhaled breath condensate of IPF patients compared to healthy controls (Psathakis, Mermigkis et al. 2006). Thus, oxidant stress can contribute to IPF as a proximal mediator of epithelial damage and/or can potentiate epithelial damage in the setting of inflammation.

Activated fibroblasts and myofibroblasts represent yet another important source of oxidants, such as hydrogen peroxide, in the fibrotic lung. A characteristic feature in IPF is the accumulation of fibroblasts into "fibroblastic foci"; areas marked by excessive fibroblast proliferation, myofibroblastic differentiation, and deposition of matrix molecules such as collagen and fibronectin (Gross and Hunninghake 2001). Intriguingly, alveolar epithelial cells overlying fibroblastic foci undergo increased cell death, suggesting that proliferating fibroblasts produce pro-apoptotic mediator(s). Transforming growth factor (TGF)-β1, a predominant pro-fibrotic mediator, induces peroxide release in lung fibroblasts isolated from IPF patients. Interestingly, in a co-culture system, epithelial cells overlying TGF-β1 stimulated fibroblasts undergo apoptosis which is inhibited by catalase (Waghray, Cui et al. 2005). Together, these data indicate that both inflammatory and fibrotic mediators contribute to oxidant stress in lung fibrosis.

5.5.2. GSH Deficiency in Idiopathic Pulmonary Fibrosis

GSH is the major extracellular antioxidant in the lower respiratory tract. In healthy humans, GSH is present at a concentration of approximately 500 μM in the ELF, and decreases to 100 μM in IPF patients (Cantin, North et al. 1987). Interestingly, the marked perturbation in GSH in the lower respiratory tract is not reflected systemically. Teramoto et al reported that plasma GSH in IPF patients was not significantly different from healthy controls. However, GSSG levels were higher and the GSSG/GSH ratio was positively correlated to superoxide generated by whole blood (Teramoto, Fukuchi et al. 1995). Beeh et al reported a marked decrease in GSH concentrations in induced-sputum from IPF patients (1.4 μM compared to 5.8 μM in controls) (Beeh, Beier et al. 2002). A negative correlation between sputum GSH levels and disease severity was also observed, suggesting that GSH homeostasis worsened with disease progression. Because GSH levels in sputum reflect perturbations in GSH homeostasis in the lower airway, sputum may provide a safe, non-invasive way to monitor GSH levels in the lower respiratory tract.

While the pathophysiological mechanisms contributing to a decrease in ELF GSH levels in IPF are unclear, available evidence indicates that increased consumption and decreased synthesis may be important. Increased consumption of GSH due to increased oxidant burden, as outlined in Figure 1.6, can contribute to a decline in ELF GSH. There is also evidence suggesting that TGF-β1-mediated signaling events can deplete GSH.

Firstly, TGF-β1 induces peroxide formation in alveolar epithelial cells, lung fibroblasts, and endothelial cells (Thannickal, Hassoun et al. 1993). Utilization of GSH to scavenge peroxide can decrease GSH reserves. There is evidence that TGF-β1 inhibits

mRNA levels of γ-glutamylcysteine synthetase (γ-GCS) in A549 cells, a neoplastic alveolar type II cell (Arsalane, Dubois et al. 1997; Jardine, MacNee et al. 2002). This raises the possibility that excess TGF-β1 in the fibrotic lung can contribute to a depletion of GSH by inhibiting synthesis. TGF-β1-induced collagen accumulation in fibroblasts is inhibited by pre-treating cells with NAC or GSH, and collagen production is increased in cells treated with DL-buthionine-(SR)-sulfoximine (BSO), an inhibitor of GSH synthesis (Liu, Liu et al. 2004). Further, GSH supplementation restores collagen degradation in TGF-β1-stimulated fibroblasts by activating plasminogen (Vayalil, Olman et al. 2005). Additionally, extracellular GSH at a concentration found in the healthy ELF (500 μM) also inhibits lung fibroblast proliferation (Cantin, Larivee et al. 1990). Taken together, these data suggest that depletion of GSH can play a role in the progression of fibrosis by inducing fibroblast proliferation and by potentiating TGF-β1-mediated collagen deposition in the air-space ***(Figure 1.9)***.

Figure 1.9. Mechanisms for TGF-β1-induced GSH depletion in fibrosis

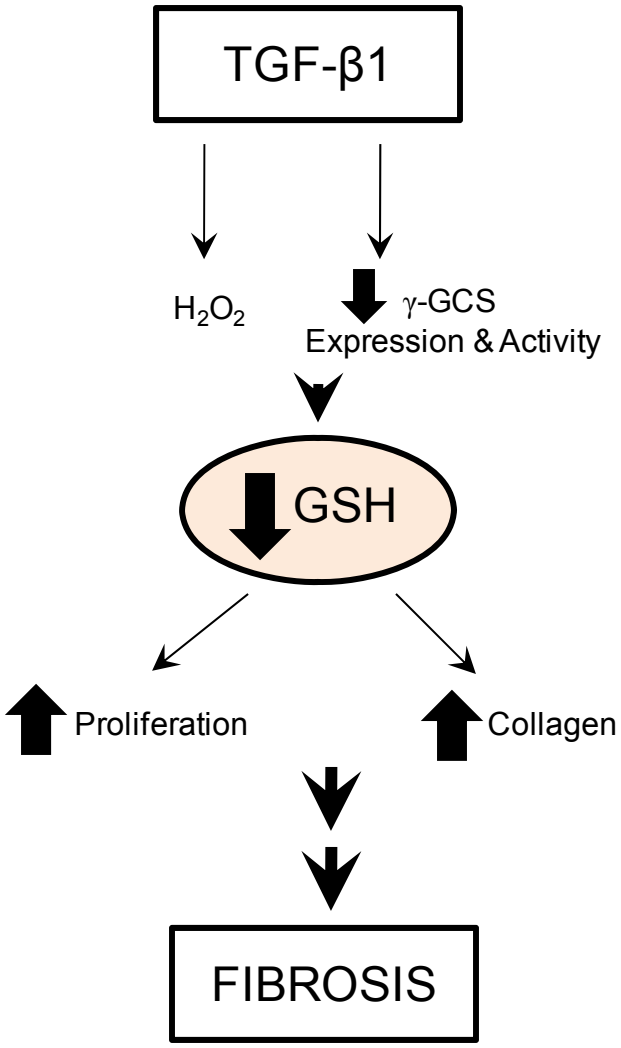


Figure 1.9. Mechanisms for TGF-β1-induced GSH depletion in fibrosis. Elevated TGF-β1 can decrease GSH levels by increased oxidative consumption of GSH and by inhibiting GSH synthesis. The resulting low GSH levels can contribute to the fibrosis by increasing fibroblast proliferation and potentiating TGF-β1-mediated collagen deposition.

Interestingly however, mice lacking the γ-glutamyl transpeptidase gene (γ-GT) demonstrate significantly reduced fibrosis in response to bleomycin, a pro-fibrotic agent (Selman, Lin et al. 2003). γ-GT -/- mice have lower levels of GSH and Cys in the lung at baseline and 72 h after bleomycin treatment, but are protected against fibrosis at 1 month.

Of note, the inflammatory response in γ-GT -/- mice comprises mostly of lymphocyte infiltration with little influx of neutrophils compared to the marked neutrophilic alveolitis in WT animals. Thus, the results can be interpreted in two ways: 1) reduced levels of GSH and Cys do not play a role in the progression of fibrosis or 2) when the inflammatory response preceding fibrosis is characterized by accumulation of neutrophils, as is seen in human IPF, depletion of thiol reserves may be important. Alternatively GSH and Cys in the ELF, which were not measured in the study, may be a more important determinant of fibrosis compared to intracellular levels.

Nevertheless, the decrease in GSH levels in the lower airway of IPF patients demonstrates an increased susceptibility to oxidative stress. The ability to augment GSH reserves in the ELF, relatively easily, has led to two major clinical trials which will be discussed in the next section.

5.5. Thiol Antioxidants in Experimental and Clinical Lung Fibrosis

The marked decrease in ELF GSH levels in IPF patients together with experimental evidence supporting a role for GSH depletion in the fibrotic process has led to considerable interest in targeting GSH deficiency in IPF. Numerous studies have demonstrated the feasibility of increasing ELF GSH levels via oral, aerosolized and intravenous administration of Cys and GSH precursors. Borok et al reported that aerosolized GSH in patients with IPF rescued GSH deficiency, albeit transiently, in the lower airway (Borok, Buhl et al. 1991). Aerosolized GSH (600 mg every 12 h for 3 days) increased GSH levels in the ELF by 4-fold at day 1, but not at day 3. The increase in GSSG levels at day 3 suggested that decrease in GSH was likely a consequence of oxidation to GSSG. Of note, the intervention decreased spontaneous production of superoxide in alveolar macrophages isolated from the lower airway. Thus, aerosolized GSH therapy transiently rescued GSH deficiency in the lower airway, and had favorable effects on macrophage activation.

Intravenous NAC is also shown to be effective in increasing GSH levels in the BAL of IPF patients. NAC (1800 mg) given intravenously for 3 days raised GSH levels in IPF ELF to near-normal values (Bernard, Lucht et al. 1984). Oral NAC represents a non-invasive and clinically attractive method of targeting ELF GSH. Meyer et al reported that high-dose oral NAC given at 1800 mg/day (600 mg, every 8 h) for 5 days significantly increased GSH levels in the BAL of IPF patients (Bernard, Lucht et al. 1984). The feasibility of augmenting ELF GSH reserves in IPF patients by oral NAC, led to the Idiopathic Pulmonary Fibrosis International Group Exploring N-Acetylcysteine I Annual (IFIGENIA) trial. The IFIGENIA trial was a double-blind, randomized, placebo

-controlled multicenter study that assessed the effectiveness of oral NAC (1800 mg/day for 1 year) as an adjunct therapy with prednisone (anti-inflammatory) and azathioprine (a purine analog that inhibits DNA synthesis) (Demedts, Behr et al. 2005). The results showed that addition of NAC to standard therapy in patients with IPF significantly slowed deterioration of vital capacity and single-breath carbon monoxide diffusing capacity, end points that reflect functional capacity of the lung. However, due to the small size of the study group, differences in survival could not be detected. Because azathioprine depletes liver GSH and NAC rescues this effect (Menor, Fernandez-Moreno et al. 2004), there is a possibility that the observed benefits of NAC relate to amelioration of azathioprine-induced toxicity rather than a direct effect on fibrosis. Nevertheless, this finding has prompted the initiation of the Prednisone, Azathioprine, and N-Acetylcysteine in People with Idiopathic Pulmonary Fibrosis (PANTHER-IPF) trial evaluating the efficacy of high-dose NAC for 60 weeks in combination and as a stand-alone therapy (NIH Clinical trials identifier, NCT00650091).

A pilot study evaluating the effects of aerosolized NAC (352 mg/day for 1 year) reported improvements in oxygenation saturation index during a 6-minute walk test and a decrease in lung occlusion as reflected by improvements in ground glass score on high resolution CT. However, no differences in overall survival were observed (Tomioka, Kuwata et al. 2005). In all, the clinical trials have demonstrated that NAC does augment GSH reserves, but definitive conclusions on the efficacy of NAC in progression of IPF will require larger, well-controlled studies.

While the efficacy of Cys and GSH precursors in human IPF remains to be established, evidence from animal models of fibrosis have consistently shown that thiol

antioxidants protect against fibrosis. Bleomycin is a commonly used drug to induce lung injury and fibrosis (Section 4, page 41). Mollar et al reported that intraperitoneal NAC (300 mg/kg/day) given 7 days before intratracheal bleomycin challenge (2.5U/kg) protected against fibrosis by ameliorating the inflammatory phase of the injury (Serrano-Mollar, Closa et al. 2003). Levels of TNF-α and IL-1β in the BAL were significantly attenuated and NF-κB activation in the lung was significantly reduced with NAC. While NAC did not protect against bleomycin-induced GSH depletion in the lung, the rise in GSSG was significantly attenuated. NAC also decreased upregulation of P-selectin in the lung which resulted in decreased neutrophil influx (Serrano-Mollar, Closa et al. 2002).

Oral NAC has also been shown to be effective; Cortijo et al reported that when given orally 1 week prior to intratracheal bleomycin (2.5 U/kg), NAC (3mmol/kg) decreased lung fibrosis by attenuating the inflammatory response to bleomycin (Cortijo, Cerda-Nicolas et al. 2001). Similar benefits have been reported with aerosolized NAC (Hagiwara, Ishii et al. 2000). Interestingly, NAC is protective only when given before or during the pro-inflammatory phase, i.e., up to 14 days after bleomycin treatment. When treatment with NAC is initiated during the pro-fibrotic phase of injury (14-28 days after bleomycin), no protection is observed (Hagiwara, Ishii et al. 2000).

This indicates that NAC ameliorates fibrosis by preventing inflammation. The therapeutic implications of this mechanism to human IPF are unclear, because inflammation does not always precede fibrosis in humans. Furthermore, the observation that NAC was without effect when administered during the pro-fibrotic phase indicates that NAC may not be protective in established disease. Another interpretation is that the bleomycin model provides too small of a therapeutic window for intervention; a scenario

that may not be relevant to the clinic. In all, the animal studies show that pre-treatment with NAC decreases the pulmonary inflammatory response to bleomycin, and consequently decreases fibrosis.

Testing the efficacy of NAC in animal models of progressive fibrosis, where the time window between inflammation and the development of fibrosis is protracted enough, will likely provide better insights into the role of NAC in fibrosis. However, because no animal model recapitulates all aspects of human IPF the results obtained from these interventions must be interpreted cautiously.

6. ROLE OF EXTRACELLULAR CYSTEINE/CYSTINE REDOX STATE IN ALI AND IPF

Much of our understanding on thiol/disulfide redox imbalance in ALI and IPF is limited to GSH. A consideration of Cys, CySS, and Cys/CySS redox state is warranted for the following reasons. Firstly, Cys is rate-limiting in the synthesis of GSH; therefore the decrease in GSH may be secondary to perturbations in Cys homeostasis. Secondly, oxidized extracellular E_h Cys/CySS induces intracellular inflammatory and fibrotic pathways independent of changes to cellular E_h GSH/GSSG. Therefore, investigating the role of E_h Cys/CySS will provide a better mechanistic understanding of thiol/disulfide redox imbalance in the disease process.

Observational data in humans indicate that E_h Cys/CySS may be important in the pathogenesis of ALI. In chronic alcoholics, a population with increased susceptibility to ALI, plasma Cys levels are decreased by 10-fold and E_h Cys/CySS is oxidized by 60 mV. This compares to a 20 mV oxidation in plasma E_h GSH/GSSG (Yeh 2007). This magnitude of oxidation in E_h Cys/CySS is sufficient to cause a greater than *10-fold* change in the ratios of reduced to oxidized forms of proteins with vicinal dithiols. Thus, substantial redox-dependent changes could occur for plasma or membrane proteins that interact with the Cys/CySS couple. Indeed, studies in cell-culture systems show that oxidized extracellular E_h Cys/CySS increases cellular production of ROS by oxidation of membrane-bound thiols (Go and Jones 2005).

Production of intracellular ROS is intimately associated with the induction of pro-inflammatory signaling pathways. In endothelial cells, oxidized E_h Cys/CySS increases ROS and leukocyte adhesion to the endothelium (Go and Jones 2005). Because influx of leukocytes into the air-space is a critical event in the pathogenesis of ALI, oxidized E_h

Cys/CySS could potentiate the inflammatory response to injury. Furthermore, evidence for activation of NF-κB by oxidized E_h Cys/CySS suggests that pro-inflammatory cytokine production may be increased by oxidized E_h Cys/CySS.

There is also evidence for a role of oxidized E_h Cys/CySS in the pathogenesis of fibrosis. Plasma E_h Cys/CySS is oxidized with age and smoking, risk factors for IPF. Additionally, lung fibroblasts show increased proliferation and upregulation of fibronectin expression in response to an oxidized extracellular E_h Cys/CySS. This occurs via the induction of TGF-β1 by oxidized E_h Cys/CySS (Ramirez, Ramadan et al. 2007). Thus, observational data in humans and *in vitro* data indicate that oxidized E_h Cys/CySS is likely to be important in inflammation and fibrosis association with ALI and IPF, respectively ***(Figure 1.10)***.

Figure 1.10. Oxidized extracellular E_h Cys/CySS induces intracellular pro-inflammatory and pro-fibrotic pathways

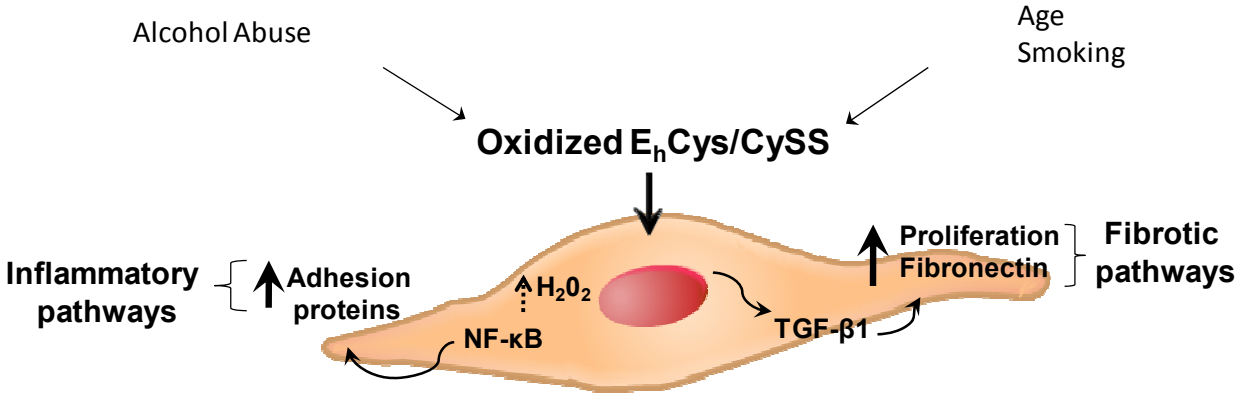


Figure 1.10. Oxidized extracellular E_h Cys/CySS induces intracellular pro-inflammatory and pro-fibrotic pathways. Oxidized E_h Cys/CySS can potentiate the inflammatory process in ALI by increasing adhesion of leukocytes to the pulmonary endothelium, and by increasing pro-inflammatory cytokine production. Oxidized E_h Cys/CySS can contribute to fibrosis by increasing TGF-β1 expression which leads to fibroblast proliferation and expression of fibronectin.

7. STATEMENT OF PURPOSE

Cys/CySS redox state is oxidized in association with risk factors for acute lung injury and idiopathic pulmonary fibrosis, including chronic alcohol abuse and cigarette smoking, respectively. *In vitro* studies show that oxidized Cys/CySS redox state modulates pro-inflammatory and pro-fibrotic signaling pathways. Together, these data indicate that oxidation of Cys/CySS redox state is likely to be an important pathogenic component in lung injury and fibrosis. The purpose of this dissertation was to investigate the dynamics of Cys, CySS and Cys/CySS redox state in experimental lung injury, repair, and fibrosis. Because GSH and Cys redox states vary independently in response to oxidative stress, physiological and pathological stimuli, a secondary purpose was to determine whether the two redox couples respond differently to inflammatory and fibrotic stimuli.

The first study was designed to evaluate variations in plasma Cys/CySS and GSH/GSSG redox states in a mouse model of LPS-induced acute lung injury. Because Cys and GSH homeostasis depends on food intake, control mice were pair-fed to intake of LPS-treated mice. This study allowed us to test two novel questions: first, whether E_h Cys/CySS is oxidized in association with E_h GSH/GSSG under pathophysiological conditions *in vivo*; and second, to determine the contribution of pro-oxidative changes induced by LPS, versus that of decreased food intake on oxidation of Cys and GSH redox states.

In the second study, we hypothesized that extracellular E_h Cys/CySS is a critical determinant of pro-inflammatory cytokine production. We tested this hypothesis by modifying extracellular E_h Cys/CySS and determining induction of interleukin (IL)-1β, a

prototypical pro-inflammatory cytokine, *in vitro* and *in vivo*. In the first set of experiments, monocytes were exposed to oxidized extracellular E_h Cys/CySS and induction of IL-1β was determined. The second set of experiments were designed to study whether preservation of plasma E_h Cys/CySS from oxidation, in response to LPS, was associated with a decrease in IL-1β levels. Thirdly, we analyzed plasma from healthy individuals to further test the association between oxidized E_h Cys/CySS and IL-1β.

In addition to understanding the role E_h Cys/CySS in inflammation, we wanted to determine the dynamics of E_h Cys/CySS in response to anti-inflammatory therapy using mesenchymal stem cells (MSC). MSC were isolated from the bone marrow, expanded *in vitro*, and infused intravenously into syngeneic LPS-treated recipient mice. Besides providing us with an opportunity to investigate the regulation of Cys/CySS and GSH/GSSG by MSC *in vivo*, this study enabled us to determine whether plasma GSH and Cys redox systems respond differently during the resolution of inflammation.

Finally, the fourth study was designed to study the temporal oxidation of E_h Cys/CySS with lung fibrosis. Mice were treated with bleomycin, a pro-fibrotic drug, and plasma E_h Cys/CySS was determined at the pro-inflammatory and pro-fibrotic phases of lung injury. Additionally, E_h Cys/CySS in the lung lining fluid was examined. This study enabled us to assess oxidation of Cys/CySS redox state in the lining fluid and plasma compartments during chronic lung inflammation and lung fibrosis.

Together, the four studies provide novel insights into the dynamics of extracellular E_h Cys/CySS and its association with E_h GSH/GSSG in lung injury, repair, and fibrosis. The findings described herein provide the foundation to investigate the

mechanistic role of Cys/CySS in ALI and IPF, and consider interventional strategies to prevent oxidation of Cys/CySS in these disease states.

CHAPTER II

SEQUENTIAL OXIDATION OF PLASMA CYSTEINE AND GLUTATHIONE REDOX STATES IN ENDOTOXIN-INDUCED LUNG INJURY

Sepsis and septic shock remain a major cause of morbidity and mortality in the intensive care unit. As described in Section 3 of Chapter I, patients with sepsis are an increased risk for ALI and ARDS. Because excessive and unregulated inflammation in sepsis injures the lungs, strategies to control the inflammatory response in septic patients are of therapeutic interest.

Cys and GSH are well established determinants of the inflammatory response, and experimental studies with thiol antioxidants such as N-acetyl cysteine and pro-cysteine have shown benefits. Nonetheless, very little known about the regulation of these thiol/disulfide redox states during sepsis induced ALI. Therefore, the present study was designed to investigate the dynamics of Cys/CySS and GSH/GSSG redox states during endotoxin-induced lung injury. A secondary objective was to determine whether nutrition is an important determinant of Cys and GSH homeostasis during inflammation and lung injury.

1. ABSTRACT

Several lines of evidence indicate that perturbations in the extracellular thiol/disulfide redox environment correlate with the progression, and severity of acute lung injury (ALI). Cysteine (Cys) and its disulfide cystine (CySS) constitute the most abundant, low-molecular weight thiol/disulfide redox couple in the plasma, and Cys homeostasis is adversely affected during the inflammatory response to infection and injury. While much emphasis has been placed on glutathione (GSH) and glutathione disulfide (GSSG), little is known about the regulation of the Cys/CySS couple in ALI. The purpose of the present study was to determine whether endotoxin administration causes a decrease in Cys and/or an oxidation of the plasma Cys/CySS redox state (E_h Cys/CySS) and to determine whether these changes were associated with changes in plasma E_h GSH/GSSG. Mice received endotoxin intraperitoneally, and GSH and Cys redox states were measured at time points known to correlate with the progression of endotoxin-induced lung injury. E_h in mV was calculated using Cys, CySS, GSH and GSSG values by HPLC and the Nernst equation. We observed distinct effects of endotoxin on the GSH and Cys redox systems during the acute phase; plasma E_h Cys/CySS was selectively oxidized early in response to endotoxin, while E_h GSH/GSSG remained unchanged. Unexpectedly, subsequent oxidation of E_h GSH/GSSG and E_h Cys/CySS occurred as a consequence of endotoxin-induced anorexia. Taken together, the results indicate that enhanced oxidation of Cys,

altered transport of Cys and CySS and decreased food intake each contribute to the oxidation of plasma Cys/CySS redox state in endotoxemia

2. INTRODUCTION

Acute lung injury (ALI) is initiated by an unregulated inflammatory response to a physical trauma or infection (Ware and Matthay 2000), most commonly sepsis (Doyle, Szaflarski et al. 1995), and often leads to severe respiratory failure termed the acute respiratory distress syndrome (ARDS) (Pittet, Mackersie et al. 1997; Ware 2006). Several lines of evidence indicate that perturbations in thiol status is related to the pathogenesis of ALI and ARDS (Quinlan, Evans et al. 1994; Quinlan, Lamb et al. 1997). In particular, levels of glutathione (GSH), a major cellular thiol antioxidant, are markedly decreased in the epithelial lining fluid (ELF) of patients with sepsis and ARDS (Pacht, Timerman et al. 1991), and impaired homeostasis of lung and plasma GSH predisposes to ARDS (Holguin, Moss et al. 1998). Numerous animal studies have addressed perturbations in the GSH system during endotoxemia (Jaeschke 1992; Payabvash, Ghahremani et al. 2006). However, GSH synthesis depends on the amino acid cysteine (Cys), and relatively little is known about the response of this precursor pool.

While GSH, and its oxidized form, glutathione disulfide (GSSG) constitute the most abundant cellular redox couple (Schafer and Buettner 2001), the cysteine/cystine (Cys/CySS) couple predominates in the plasma (Jones, Mody et al. 2002). Furthermore, the steady state redox potential (E_h) of Cys/CySS is regulated independently of E_h GSH/GSSG (Jones, Go et al. 2004; Anderson, Iyer et al. 2007; Banjac, Perisic et al. 2007), and plasma E_h Cys/CySS is oxidized in association with oxidative stress (Moriarty, Shah et al. 2003; Yeh, Burnham et al. 2007). Oxidized extracellular E_h

Cys/CySS induces upregulation of surface adhesion markers on endothelial cells (Go and Jones 2005), and conditions epithelial cells to apoptosis (Jiang, Moriarty-Craige et al. 2005). These cellular processes can contribute to the pathogenesis in ALI and activation of these processes by oxidized E_h Cys/CySS occurs in the absence of changes to the GSH pool. Additionally, the inconclusive outcomes from the therapeutic use of N-acetyl cysteine (NAC), a Cys precursor, in patients with ALI may be related to unknown variations in Cys/CySS redox state (Bernard 1991; Suter, Domenighetti et al. 1994; Domenighetti, Suter et al. 1997).

Experimental administration of bacterial lipopolysaccharide (endotoxin/LPS) to animals produces pathophysiologic changes similar to those seen in ALI in humans (Rojas, Woods et al. 2005) and allows for the dissection of regulatory events critical in the pathogenesis of ALI. The purpose of this study was to test whether endotoxin causes a decrease in Cys and/or an oxidation of the Cys/CySS redox state and to determine whether Cys/CySS redox state changes are associated with changes in GSH/GSSG redox state under these pathophysiological conditions. Mice were given endotoxin intraperitoneally and GSH/GSSG and Cys/CySS redox states were measured at time points known to correlate with the progression of lung injury (Rojas, Woods et al. 2005). Because food intake affects blood Cys concentrations (Nkabyo, Gu et al. 2006), PBS-treated mice were pair-fed to intake of mice receiving LPS. *Ad libitum* PBS-treated mice served as a separate set of controls. Here we showed that endotoxin had distinct effects on the GSH/GSSG and Cys/CySS redox systems during the acute phase of injury, and that sustained oxidation of Cys/CySS and GSH/GSSG redox states occurred as a result of LPS induced-anorexia.

3. MATERIALS AND METHODS

3.1. Materials. Except as indicated, all chemicals were purchased from Sigma Chemical Corporation (Sigma, St. Louis, MO). Distilled, deionized water was used for analytical purposes. HPLC quality solvents were used for HPLC.

3.2. Experimental Animals. Experiments were conducted using 10-14 week old, female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). While some strains such as the C3H/HeJ (Poltorak, He et al. 1998) are refractory to endotoxin, the C57BL/6 strain is a responsive strain (Johnston, Finkelstein et al. 1998; Kabir, Gelinas et al. 2002). The inflammatory response to endotoxin in the C57BL/6 strain is greater in older mice (Ito, Betsuyaku et al. 2007). We used 10-14 week old mice because the pathophysiology of lung injury in this model has been previously characterized by our group (Johnston, Finkelstein et al. 1998; Kabir, Gelinas et al. 2002; Rojas, Woods et al. 2005). For the current experiments, mice were housed individually for pair feeding and maintained on a 12-h light-12-h dark cycle at the Division of Animal Resources at Emory University. All animals were fed pelleted rodent food (Test Diet 5015, Lab Diet Inc., Richmond, IN) and had free access to water. Nestlets were presented daily to each mouse to compensate for the absence of other animals (Belz, Kennell et al. 2003). All experiments were initiated during the light cycle. Measurement of food intake in LPS-treated mice was performed by providing mice with a weighed food pellet at the initiation of the experiment and manually recording weight of the remaining food at timed intervals. The amount of food eaten by LPS-treated mice was averaged, and this amount was provided to pair-fed PBS-treated controls. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

3.3. LPS Administration. *Escherichia coli* O111:B6 LPS, dissolved in sterile PBS (100 μg/ml) was administered intraperitoneally at a dose of 1 mg LPS/kg body. Animals were sacrificed at 2, 6, 24 and 48 h post LPS. Pair-fed PBS animals received intraperitoneal injection of PBS and were sacrificed at corresponding time points. *Ad libitum* fed PBS controls were treated similarly.

3.4. Sample collection and analysis of Cys, CySS, GSH and GSSG. Mice were anesthetized by isofluorane inhalation (Baxter Pharmaceuticals, Deerfield, IL), and blood was collected by submandibular bleeding using a 4 mm mouse bleeding lancet (Medipoint, Inc. Mineola, NY). 0.18 ml of collected blood was immediately transferred to 0.02 ml of preservation solution containing gamma-glutamyl-glutamate (γ-Glu-Glu) as an internal standard (Jones 2002). Samples were centrifuged at 16000 g for 60 seconds to remove precipitated protein, and 0.1 ml of the supernatant was immediately transferred to an equal volume of ice-cold 10% (w/v) perchloric acid. Samples were immediately stored at -80 °C.

Bronchoalveolar lavage fluid (BALF) was obtained after mice were euthanized. Briefly, 0.6 ml of sterile PBS was instilled into the lung via a tracheal incision and withdrawn with gentle suction. This procedure took approximately 10 minutes to complete. The collected BALF was centrifuged at 200 g for 6-7 minutes. 0.15 ml of the cell-free supernatant was mixed with an equal volume of ice-cold 10% (w/v) perchloric acid containing 20 μM γ-Glu-Glu and stored at -80 °C until derivatization with iodoacetic acid and dansyl chloride.

For HPLC analysis (Gilson Medical Electronics, Middleton, WI), derivatized samples were centrifuged, and 50 μl (plasma) or 65 μl (BALF) of the aqueous layer was

applied to the Supercosil LC-NH_2 column (25 cm x 4.6 mm; Supelco, Bellefunk, PA). Derivatives were separated with a sodium acetate gradient in methanol/water and detected by fluorescence (Jones, Carlson et al. 1998). Concentrations of thiols and disulfides were determined by integration relative to the internal standard. Redox states (E_h) of the GSH/GSSG and Cys/CySS pools were calculated from concentrations of GSH, GSSG and Cys, CySS in molar units with the following forms of the Nernst equation for pH 7.4: GSH/GSSG, $E_h = -264 + 30 \log ([GSSG]/[GSH]^2)$, Cys/CySS, $E_h = -250 + 30 \log ([CySS]/[Cys]^2)$(Jones, Carlson et al. 2000).

3.5. Quantitative Real-Time PCR Analysis. Lung and liver samples were excised, snap frozen in liquid N_2 and stored at -80°C. Total RNA was extracted from tissue using an RNeasy Midi Kit (QIAGEN Inc., Valencia, CA) according to manufacturer's instructions. DNase treatment was performed to remove contaminating genomic DNA. RNA concentration was spectrophotometrically determined at 260 nm and 0.5 µg of total RNA was used to synthesize 20 µl of cDNA (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed on cDNA using gene-specific primers on an iCycler IQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primers were designed using Beacon Designer Software 4.00 (PREMIER Biosoft International, Palo Alto, CA) (Table 1). Samples containing serial dilutions of known concentrations of cDNA, encoding the gene of interest, were amplified in parallel. Data were analyzed using the iCycler Software, and starting quantities of message levels of each gene were determined from constructed standard curves. Melt curves were examined to ensure amplification of a single PCR product. Expression levels were

normalized to GAPDH, and data are presented as the fold change in LPS/PBS-treated animals compared with untreated controls.

Table 2.1. PCR primer sequences used for quantitative real time PCR

Target	Genbank accession number	Forward primer	Reverse primer	Product size, bp
IL-1β	NM_008361	ATCTCGCAGCAGCACATC	CAGCAGGTTATCATCATCATC	192
TNF-α	NM_013693	CGTGGAACTGGCAGAAGAG	ACAAGCAGGAATGAGAAGAGG	96
iNOS2	NM_010927	CAGAAGCAGAATGTGACC	GTAGTAGTAGAATGGAGATAGG	195
xCT	NM_011990	ACCATCAGTGCGGAGGAG	GAGCCGAAGCAGGAGAGG	119

3.6. Histopathology. Lungs were fixed by intra-tracheal instillation of neutral buffered formalin (10%). After further fixation overnight at room temperature tissue was embedded in paraffin, sectioned, and stained by hematoxylin and eosin (H & E). All sections were studied by light microscopy.

3.7. Cell culture. Human pulmonary arterial endothelial cells (EC) (HPAEC, Lonza Walkersville, Inc. Walkersville, MD, and HULEC-5A, SV-40 large T antigen-transformed line derived from human lung microvasculature) were maintained in EGM (Lonza), and were transferred to serum-free media 8-12 h prior to experimental manipulations. Human monocytic cells (U937, ATCC, Rockville, MD) and murine neutrophils were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Atlanta, Biologicals, Norcross, GA) and 10 U/ml penicillin and streptomycin

sulfate. Neutrophils were isolated from heparin-anticoagulated peripheral blood using the Ly-6G isolation kit (Miltenyi Biotec, Auburn, CA). Cells were maintained in a humidified 5% CO_2 incubator at 37°C.

To generate the desired range of extracellular redox states, the extracellular thiol/disulfide pool was altered by varying concentrations of Cys and CySS, added to cyst(e)ine-free DMEM (EC) or RPMI (monocytes and neutrophils), as previously described (Ramirez, Ramadan et al. 2007). Production of reactive oxygen species (ROS) was detected using 6-Carboxy-2′,7′-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) (Go and Jones 2005). EC, plated into 96-well plate, were washed with KRH buffer and incubated with 100 μM DCFH-DA for 30 minutes (37°C, 5% CO_2). Cells were washed and exposed to physiological (-80 mV) and oxidized (-46 mV) Cys/CySS redox media for 5 min at 37^{o}C. Oxidation of DCFH-DA to fluorescent DCF was measured on a microplate reader (excitation, 485 nm; emission, 530 nm) (Wang and Joseph 1999).

To examine the effect of oxidized Cys/CySS on adherence of neutrophils and monocytes to EC; EC were exposed to -80 and -46 mV redox media for 24-36 h. Media was changed every 12 h. Calcein-AM-labeled neutrophils or monocytes were incubated with endothelial cells at a cell density of 1 X 10^5 cells per well, of a 96-well plate. Cells were incubated in -80 and -46 mV redox media at 37°C. After 30 minutes, media was aspirated to remove unbound cells. Adherent cells were quantified by measuring fluorescent calcein on a microplate reader (excitation, 470 nm; emission, 530 nm).

3.8. Statistical Methods. Data are presented as means (n=3-5) + SEM. Statistical analysis was done using SAS v 9.1 (SAS Institute Inc., Cary, NC, US). Analyses were performed using a Generalized Linear Model, utilizing two-way ANOVA with time and treatment specified as the main effects and (time)·(treatment) as the interaction term. Data from in vitro experiments was analyzed using an unpaired t-test. Significance was set at a P value < 0.05 for all tests.

4. RESULTS

4.1. Cytokine expression and Immunohistochemistry. Because bacterial endotoxin is cleared by the liver and early responses to intraperitoneal endotoxin within the lung depends, in part, on hepatic release of inflammatory cytokines (Siore, Parker et al. 2005), we determined mRNA levels of TNF-α **(Figure 2.1A),** and IL-1β (**Figure 2.1B**) in the liver. TNF-α mRNA increased at 2 h ($P<0.001$) in the liver and subsequently declined while message levels of IL-1β gradually increased, reaching significance at 6 h ($P<0.05$) followed by a sharp decline at 24 h. In addition to the systemic inflammatory response, clearance of endotoxin by liver Kupffer cells triggers increased production of reactive oxygen and reactive nitrogen species. The inducible nitric oxide synthase (iNOS) system catalyzes the formation of nitric oxide (NO) from arginine, and in contrast to its constitutive isoforms, iNOS is synthesized de novo during inflammation (Bultinck, Sips et al. 2006). We observed up-regulation in i-NOS2 mRNA levels at 2 h and 6 h ($P<0.001$) (**Figure 2.1C**).

To evaluate the inflammatory response in the lung we conducted real-time PCR analysis of RNA to determine expression of TNF-α. TNF-α expression was up-regulated

13-fold at 2 h ($P<0.001$) and remained elevated up to 6 h after which message levels dropped (**Figure 2.1D**). Because message levels of TNF-α, IL-1β and iNOS2 were not up-regulated in pair-fed PBS controls, we conclude that these responses were induced specifically by LPS and not due to LPS-induced decrease in food intake.

Figure 2.1. Effect of LPS on liver TNF-α, IL-1β, iNOS2 mRNA and lung TNF-α mRNA

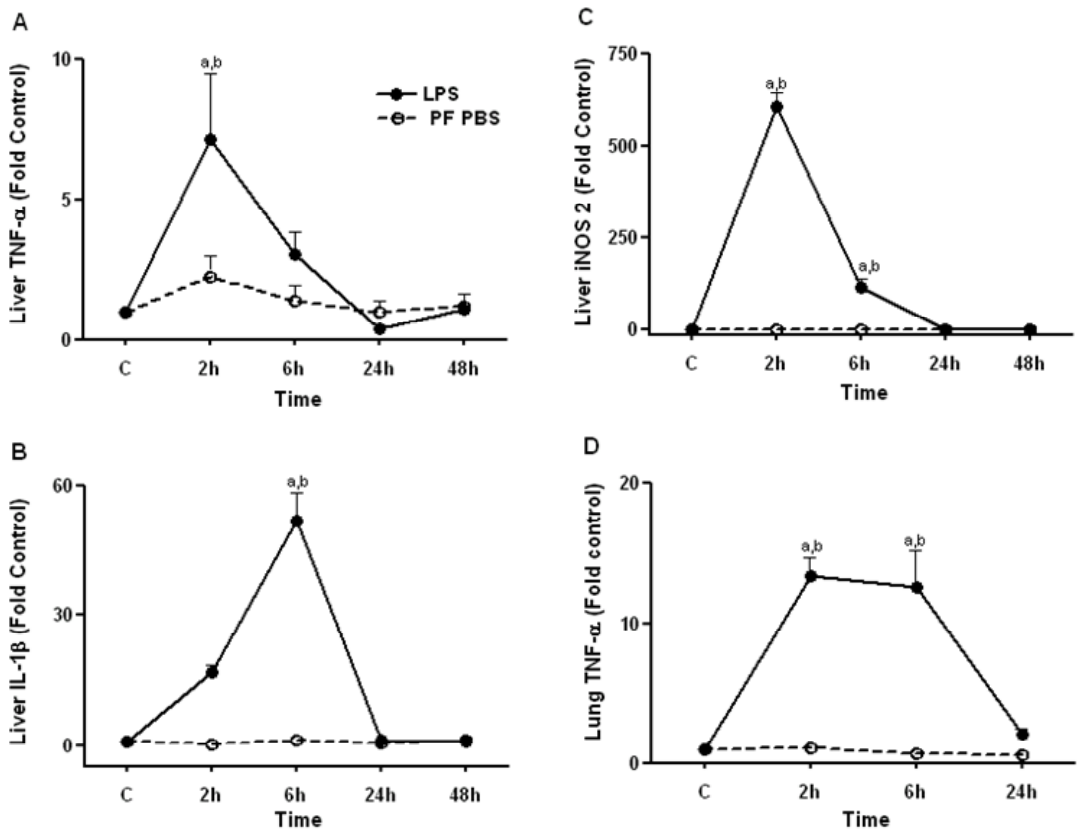


Figure 2.1. Effect of LPS on liver TNF-α, IL-1β, iNOS2 mRNA and lung TNF-α mRNA. C57BL/6 mice were treated with 1mg/kg i.p LPS or with PBS. At 2 h, 6 h and 24 h mice were sacrificed and lung and liver samples were obtained. Liver samples were also obtained at the 48 h time point. RNA was extracted from whole tissue and transcript levels of liver TNF-α (A), liver IL-1β (B), liver iNOS2 (C), and lung TNF-α (D) were quantified using quantitative RT-PCR. Data are expressed as means + SEM. [a]Values significantly different from un-treated controls, [b]Values significantly different from corresponding pair-fed PBS control.

Figure 2.2 shows photomicrographs of H & E-stained sections of the lung after LPS treatment. The pathophysiology of the lung in the endotoxin model has been previously characterized by our group; endotoxin causes lung edema and altered respiratory function at 2 h, and structural alterations in the lung between 6 h and 48 h (Rojas, Woods et al. 2005). To better define the time course of pathological changes in this model, we determined if there was evidence for lung injury at 2 h and whether injury persisted at 48 h. Results showed evidence for neutrophil influx in the lung at 2 h (Arrow, **Figure 2.2B**). As previously described, a diffuse inflammatory pattern was seen in the lung at 6 h and 24 h (Arrows, **Figure 2.2C**, **Figure 2.2D**). Injury largely resolved by 48 h but some cellularity and alveolar congestion was still evident (not shown).

Figure 2.2. Lung histopathology in response to endotoxin

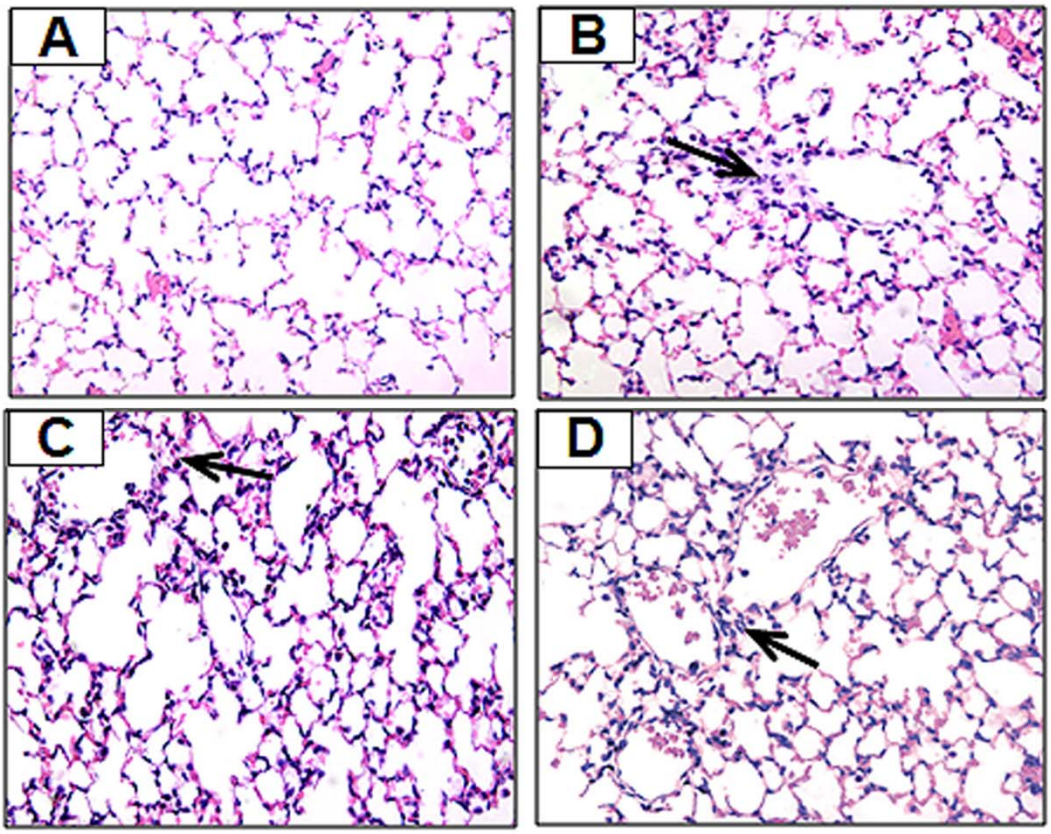


Figure 2.2. Hematoxylin and eosin stained sections of lung tissue from untreated controls (A), and endotoxin treated animals at 2 h (B), 6 h (C), and 24 h (D). Evidence for influx of neutrophils is observed near the airway (arrow) at 2 h. Diffuse inflammation is observed at 6 h and 24 h (arrows).

4.2. Effect of endotoxin on food intake and body weight. Because food intake affects blood Cys concentrations (Nkabyo, Gu et al. 2006), we designed the study to control for food intake by pair-feeding the control group to the LPS-treated group. LPS administration profoundly decreased food intake in the first 24 h of treatment. Food intake at 24 h was 3% of baseline body weight in LPS-treated animals compared to 30% of body weight in *ad libitum*-fed PBS controls (AF PBS) ($P<0.001$) (**Figure 2.3A**). Food intake increased 24 h after LPS treatment, and at 48 h, food intake in LPS-treated mice was 28% of body weight (measured at 24 h).

LPS-treated mice lost 13% of body weight within the first 24 h and regained 6% of the lost weight in the next 24 h (**Figure 2.3B**). Weight loss in pair-fed PBS controls (PF PBS) was comparable to LPS-treated animals at all time points. AF PBS controls did not lose weight at 24 and 48 h. The comparable weight loss observed in LPS and PF PBS animals support the conclusion that weight loss in LPS-treated animals was predominantly due to decreased food intake rather than changes in metabolic rate due to endotoxemia.

Figure 2.3. Effect of LPS on food intake and body weight

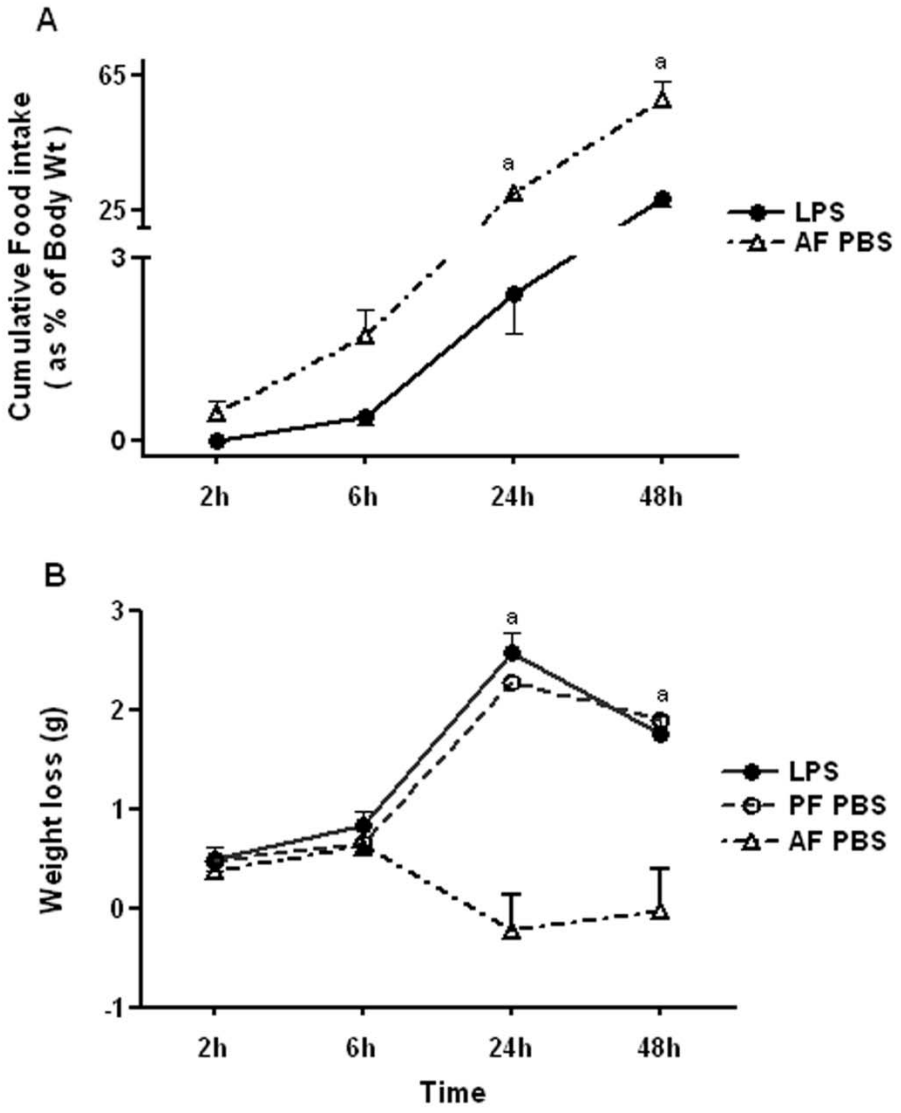


Figure 2.3. Effect of LPS on food intake and body weight. C57BL/6 mice were treated with 1mg/kg i.p LPS or with PBS. PBS-treated animals were either pair-fed (PF PBS) to intake of LPS-treated animals or were fed *ad libitum* (AF PBS). At 2 h, 6 h, 24 h and 48 h post-treatment, food intake was measured (A). Weight loss (B) in LPS-treated animals, time-matched pair-fed PBS, and *ad libitum* fed PBS controls is shown. Data are expressed as means + SEM. [a]Values significantly different from AF PBS controls**.**

4.3. Endotoxin-induced oxidation of plasma Cys/CySS redox state correlates with the initiation of lung injury. Because oxidative stress has previously been found to affect the Cys/CySS system and LPS induces oxidative stress, we hypothesized that LPS would cause an oxidation of the plasma Cys/CySS couple. We measured Cys and CySS concentrations in the plasma after administration of LPS. In these analyses, blood samples were collected into a preservation solution designed to prevent oxidation after collection (Jones, Carlson et al. 1998). LPS significantly decreased plasma Cys (**Figure 2.4A**) levels at 2 h ($P < 0.05$ compared to PF PBS and AF PBS controls). Cys levels also decreased in PBS-treated mice at 2 h ($P < 0.05$ compared to AF PBS controls). The latter effect may be due to cortisol-mediated clearance of plasma Cys in response to food removal or due to the small difference in food intake compared to AF PBS animals (0.4 g in AF PBS controls versus 0 g in PF PBS controls). In contrast to the decrease in Cys, plasma levels of the disulfide, CySS (**Figure 2.4B**) increased by an average of 20 μM in LPS-treated animals compared to PF PBS controls ($P< 0.05$) showing that a substantial shift in the balance of plasma Cys to CySS occurred at 2 h. The calculated value for plasma E_h Cys/CySS (**Figure 2.4C**) showed that LPS-treated animals were oxidized by an average of 10 mV compared to PF PBS controls ($P<0.01$), and by an average of 20 mV compared to AF PBS controls ($P<0.001$). Thus, the data show that LPS treatment caused a significant oxidation of the Cys/CySS couple in the plasma at 2 h.

Plasma Cys levels in LPS and PF PBS-treated animals remained below AF PBS controls at all subsequent time points studied ($P<0.01$). Interestingly, at 6 h and 24 h plasma CySS levels significantly decreased in LPS-treated animals compared to AF PBS

controls ($P < 0.001$). The decrease in CySS, in addition to the prevailing low concentrations of Cys contributed to a decline in the total plasma Cys pool at 6 h and 24 h ($P < 0.001$, compared to AF PBS controls). Despite the decrease in CySS, calculations of the redox state of Cys/CySS showed that it was considerably oxidized up to 48 h in LPS-treated animals. This change suggests that after the oxidation of plasma Cys/CySS at 2 h, induction of CySS uptake may protect against more extensive oxidation and also increase cellular supply of precursors for GSH synthesis.

Thus, the combination of data show that oxidation of plasma Cys/CySS at 2 h occurs without a change in the total pool size, and precedes maximal pathological changes in the lung. In contrast, oxidation of Cys/CySS redox state between 6 h and 48 h occurred with a decrease in total pool size, suggesting that the sustained oxidation occurred as a consequence of decreased precursor availability relative to tissue demand.

Figure 2.4. Effect of LPS on plasma Cys, CySS, and E_h Cys/CySS

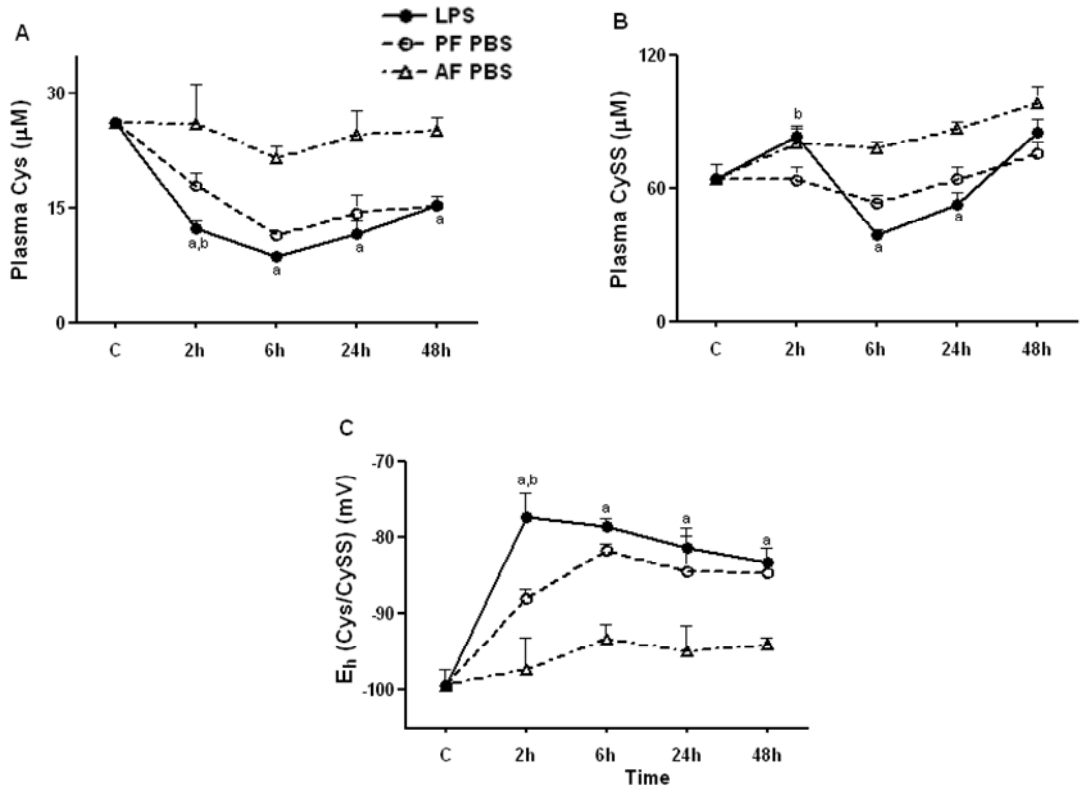


Figure 2.4. Effect of LPS on plasma Cys, CySS, and E_h Cys/CySS. C57BL/6 mice were treated with 1mg/kg i.p LPS or with PBS. At 2 h, 6 h, 24 h and 48 h mice were sacrificed and plasma was collected for HPLC analysis of Cys (A) and CySS (B). In (C), E_h Cys/CySS was calculated from the Cys and CySS concentrations using the Nernst equation. Total Cys equivalents (D) were obtained by adding together Cys + 2*CySS. Data are expressed as means + SEM. [a]Values significantly different from *ad libitum* fed PBS controls, [b]Values significantly different from corresponding pair-fed PBS control.

4.4. Oxidation of plasma GSH/GSSG redox state followed oxidation of plasma Cys/CySS redox state. Previous research shows that the effects of LPS on plasma GSH are time dependent. LPS induces an acute increase in plasma GSH, due to hepatic GSH efflux, followed by a decrease in GSH due to oxidation (Jaeschke 1992; Payabvash, Ghahremani et al. 2006). In the current study**,** analysis of GSH and GSSG showed that no significant changes were apparent at 2 h while a significant decrease in GSH without a corresponding increase in GSSG was present at 6 h (**Figure 2.5A, B**). The calculated E_h (GSH/GSSG) was significantly oxidized only at 6 and 24 h; no oxidation of GSH/GSSG redox state occurred at 2 h (**Figure 2.5C**). Compared to simultaneous measurements of Cys/CySS redox state in Fig 4, these results show that oxidation of GSH/GSSG redox state in plasma follows oxidation of plasma Cys/CySS.

Oxidation of GSH/GSSG occurred in pair-fed controls as well as in LPS-treated mice. At 6 h, the decrease in plasma GSH resulted in a 26 mV oxidation of E_h (GSH/GSSG) in LPS-treated animals compared to AF PBS controls ($P<0.001$) (**Figure 2.5C**). Unexpectedly, a similar decline in plasma GSH was observed in PF PBS controls, indicating that the decrease in plasma GSH at 6 h was related to the relative decrease in food intake rather than a direct consequence of LPS-induced oxidative stress. At 24 h, GSH levels remained significantly decreased in LPS-treated animals (10.1 ± 1.5 μM). Thus, the results show that oxidation of plasma E_h (GSH/GSSG) follows the early oxidation of Cys/CySS and is largely explained by a decline in food consumption rather than a direct oxidative process activated by LPS.

Figure 2.5. Effect of LPS on plasma GSH, GSSG, and E_h GSH/GSSG

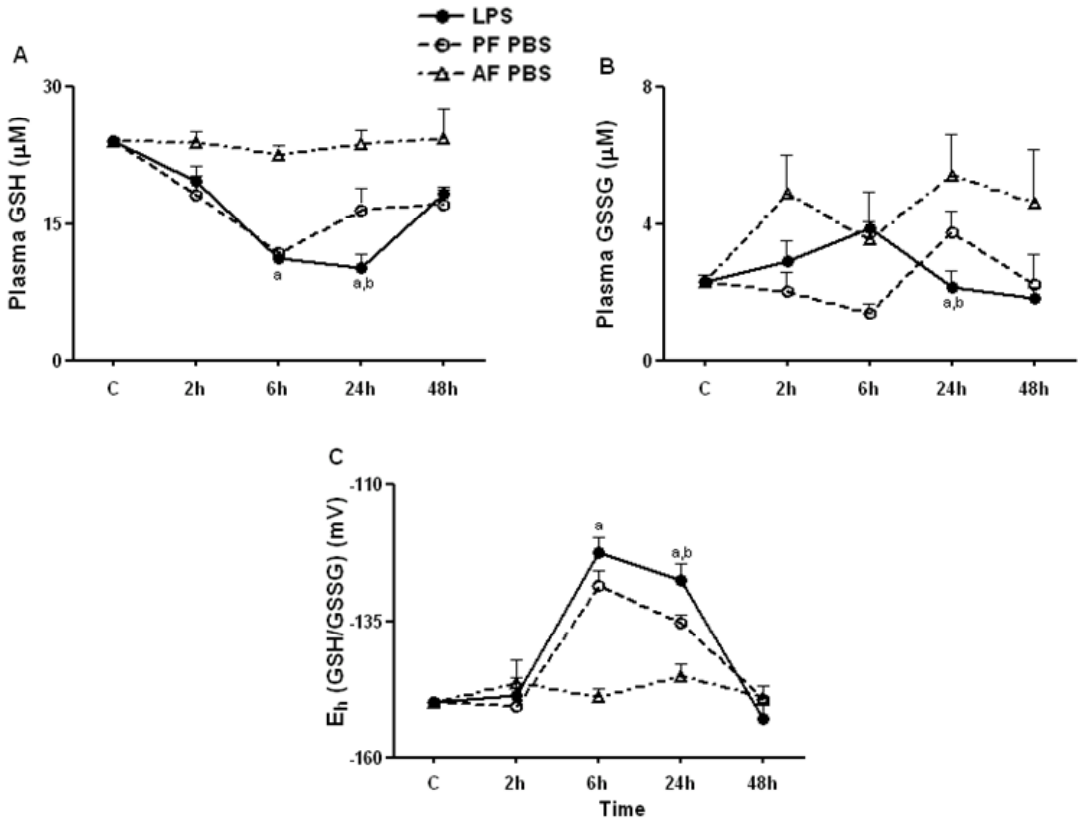


Figure 2.5. Effect of LPS on plasma GSH, GSSG, and E_h GSH/GSSG. C57BL/6 mice were treated with 1mg/kg i.p LPS or with PBS. At 2 h, 6 h, 24 h and 48 h mice were sacrificed and plasma was collected for HPLC analysis of GSH (A) and GSSG (B). In C, E_h GSH/GSSG was calculated from the GSH and GSSG concentrations using the Nernst equation. Total GSH equivalents (D) were obtained by adding together GSH + 2*GSSG. Data are expressed as means + SEM. [a]Values significantly different from *ad libitum* fed PBS controls, [b]Values significantly different from corresponding pair-fed PBS control.

4.5. Endotoxin induced oxidation of Cys/CySS redox state in lung epithelial lining fluid (ELF). Because GSH levels in the lung lining fluid are documented to be decreased post-endotoxin (Jacob, Porter et al. 2006), we hypothesized that levels of the precursor Cys pool would also be compromised. Concentrations of Cys and CySS in the ELF, obtained immediately after sacrifice, were estimated using the urea dilution factor. The dynamics of Cys in lung lining fluid closely mirrored that of plasma Cys (**Figure 2.6A**). At 2 h, Cys levels in LPS-treated animals was 7-fold lower than controls and remained significantly decreased up to 48 h ($P<0.001$). CySS levels markedly increased at 2 h, remained elevated up to 24 h ($P<0.001$) and dropped to control values at 48 h (**Figure 2.6B**). E_h Cys/CySS was oxidized by 50 mV at 2 h post-LPS ($P<0.001$; compared to untreated controls) and remained significantly oxidized up to 48 h (**Figure 2.6C**).

Figure 2.6. Effect of LPS on Cys and CySS levels in Epithelial Lining Fluid

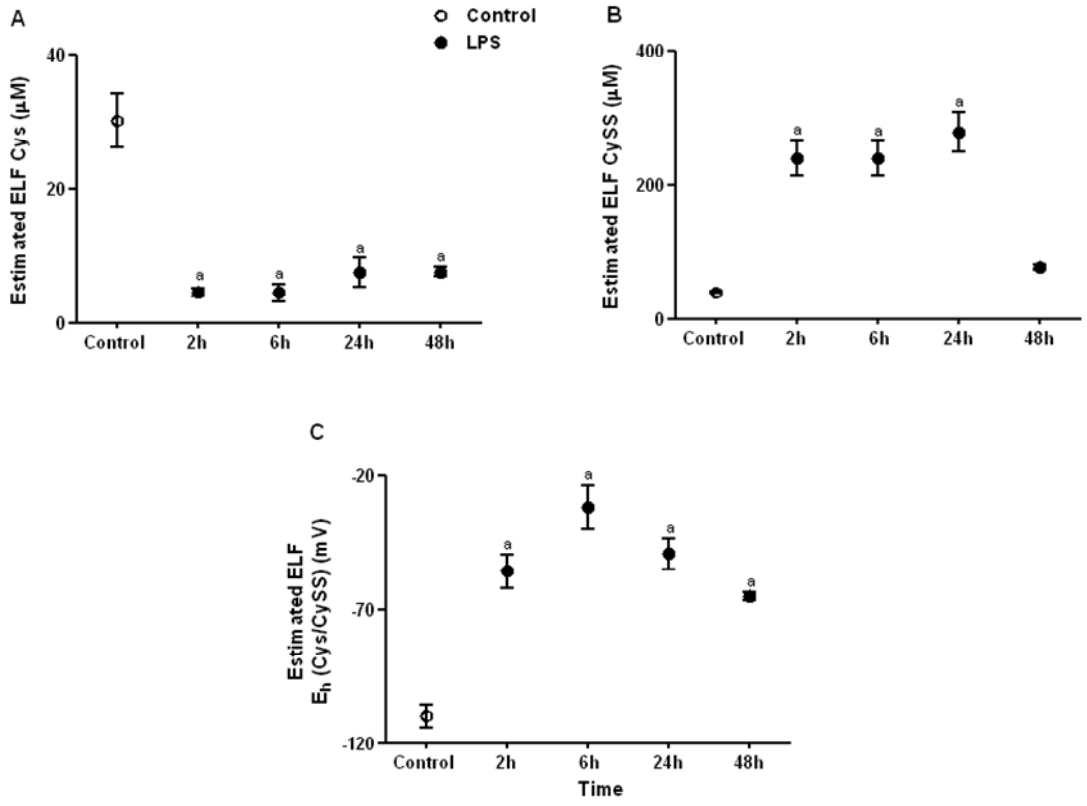


Figure 2.6. Effect of LPS on Cys and CySS levels in Epithelial Lining Fluid. Bronchoalveolar lavage was sampled from C57BL/6 mice treated with 1mg/kg i.p LPS and from untreated controls. Levels of Cys (A) and CySS (B) and E_h Cys/CySS (C) in epithelial lining fluid are shown after correction using a urea dilution factor. Data are expressed as means $\pm$ SEM [a]Values significantly different from untreated controls**.**

4.6. Endotoxin induced transcriptional upregulation of xCT in the lung.

LPS is known to increase the expression of the CySS transporter, system x_c^- in vitro (Sato, Fujiwara et al. 1995). Because the decline in plasma CySS at 6 h and 24 h could be a result of increased transport, we determined message levels of xCT, the subunit of system x_c^- responsible for transport activity, in the lung. RT-PCR analyses revealed that xCT was increased at 2 h and 6 h (**Figure 2.7**). Thus, the decline in plasma pools for Cys + CySS may result from increased CySS uptake due to induction of the xCT system.

Figure 2.7. Effect of LPS on xCT mRNA in the lung

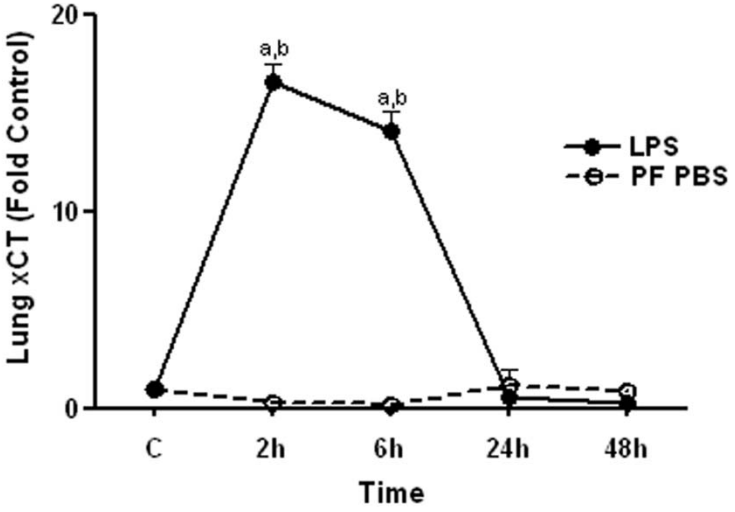


Figure 2.7. Effect of LPS on xCT mRNA in the lung. C57BL/6 mice were treated with 1mg/kg i.p LPS or with PBS. At 2 h, 6 h and 24 h, and 48 h mice were sacrificed and lung samples were obtained. RNA was extracted from whole lung and transcript levels of xCT were quantified using quantitative RT-PCR. Data are expressed as means + SEM. [a]Values significantly different from un-treated controls, [b]Values significantly different from corresponding pair-fed PBS control.

4.7. Oxidized extracellular Cys/CySS redox state enhances adhesion of leukocytes to the pulmonary endothelium. Previous studies have shown that oxidized extracellular Cys/CySS redox state stimulates the adhesion of monocytes to the vascular endothelium (Go and Jones 2005). This raises the possibility that early oxidation of plasma Cys/CySS, in response to endotoxin, increases adherence of leukocytes to the pulmonary vasculature. Because stimulation of intracellular peroxide is a proximal event in this process (Go and Jones 2005), we determined whether oxidized Cys/CySS redox state induced ROS production in pulmonary endothelial cells. Endothelial cells were pre-incubated with an ROS-sensitive dye, DCFH-DA (100 μM) for 30 min, before treating with Cys/CySS redox media for 5 minutes. As shown in **Figure 2.8A**, endothelial cells exposed to oxidized Cys/CySS redox (-46 mV) show a 3-fold increase in ROS production compared to cells exposed to a physiological redox state of -80 mV ($p< 0.001$). Pre-treating cells with 200 μM NAC for 30 min attenuated the increase in ROS production in both -80 and -46 mV treatments ($p < 0.05$).

To determine whether adherence of leukocytes to endothelial cells was augmented under oxidized Cys/CySS redox states, endothelial cells were exposed to -80 mV and -46 mV redox media for 24-36 h. Calcein-AM-labeled leukocytes were incubated with endothelial cells for 30 minutes and media was aspirated to remove unbound cells. Oxidized Cys/CySS redox state enhanced adherence of neutrophils (**Figure 2.8B**), and monocytes (**Figure 2.8C**) to endothelial cells ($p <0.01$). Thus, the data suggest that early oxidation of Cys/CySS during endotoxemia may contribute to subsequent events, including leukocyte influx into the lung

Figure 2.8. Oxidized extracellular Cys/CySS redox state enhances adhesion of leukocytes to the pulmonary endothelium

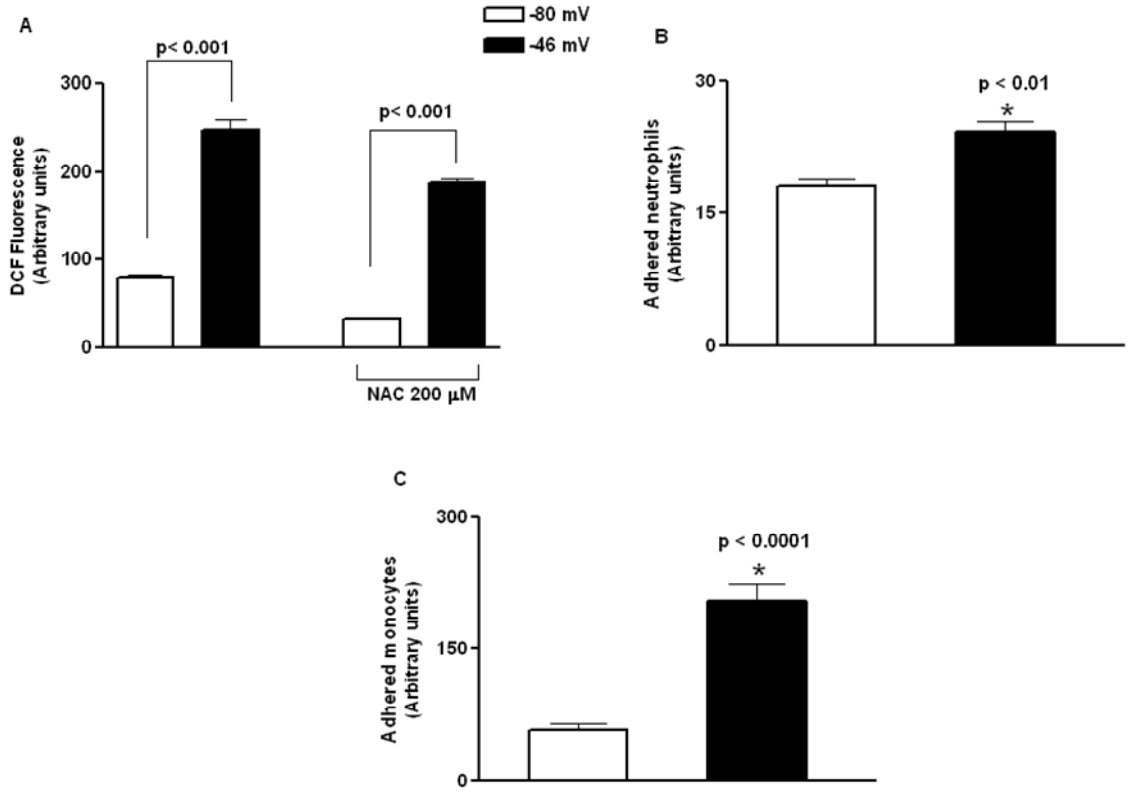


Figure 2.8. Effect of oxidized Cys/CySS redox state on leukocyte adherence to pulmonary endothelial cells. Pumonary endothelial cells exposed to oxidized Cys/CySS redox (-46 mV) show a 3-fold increase in ROS production, as measured by DCF fluorescence, compared to cells exposed to a physiological redox state of -80 mV (A). Pre-treating cells with NAC attenuates the increase in ROS at both -80 and -46 mV treatments ($p < 0.05$). Adherence of neutrophils (B) and monocytes (C) to endothelial cells is increased by 1.3-fold and 2-fold respectively at -46 mV compared to -80 mV ($p<0.01$). The values shown are mean + SEM from three replicates of a representative experiment.

5. DISCUSSION

In the present study, we describe the dynamics of plasma GSH/GSSG and Cys/CySS systems during endotoxemia. The findings on Cys/CySS redox state add to the understanding of thiol changes in sepsis. There is substantial evidence that LPS impairs GSH homeostasis. Jaeschke reported that hepatic efflux of GSH occurred during the first hour after endotoxin exposure, and the magnitude of efflux depended on endotoxin dose (Jaeschke 1992). More recently, Payabvash and colleagues reported that plasma (total) GSH significantly increased 1 h post-endotoxin (5 mg/kg) and returned to baseline values by 3 h (Payabvash, Ghahremani et al. 2006). In contrast, Minamiyama and co-workers found a 2-fold reduction in plasma GSH in rats as early as 2 h after endotoxin (20 mg/kg) (Minamiyama, Takemura et al. 1996). We found that plasma GSH and GSSG levels were comparable to baseline values 2 h after LPS. Therefore, the dynamics of plasma GSH during endotoxemia appear to depend on the dose of endotoxin and the time following administration when the measurement is made. Unexpectedly, we found that oxidation of GSH/GSSG redox state at 6 h and 24 h was largely a consequence of decreased food intake. Thus, the present data do not support a direct role for LPS in oxidation of GSH/GSSG redox state between 6 and 24 h but rather a role for LPS-induced anorexia in contributing substantially, to impaired GSH homeostasis. Since we did not make the measurements at earlier time points, a direct oxidative effect of LPS on GSH/GSSG redox state before 2 h cannot be excluded.

In contrast to GSH, we observed a significant decline in plasma Cys levels and a resulting oxidation of E_h Cys/CySS at 2 h post-endotoxin. This is consistent with the greater relative reactivity of Cys, compared to GSH, to hydrogen peroxide (Winterbourn

and Metodiewa 1999) which is produced early during endotoxin-induced lung injury (Minamiya, Abo et al. 1995). The pronounced decline in plasma Cys could be solely due to LPS-induced oxidative stress, an interpretation which is supported by an increase in plasma CySS at 2 h. However LPS increases activity of the hepatic Cys transporters (Inoue, Bode et al. 1995), and stimulated transport could contribute to a decrease in plasma Cys at 2 h. We found that the initial difference in plasma Cys between LPS-treated mice and pair-fed controls was not sustained over time, indicating a direct effect of reduced food intake on plasma Cys levels between 6 h and 48 h.

Interestingly at 6 h and 24 h, plasma CySS levels in LPS-treated mice decreased significantly. The increase in message levels of xCT suggests that this decline is due to increased CySS transport via the CySS/glutamate exchange transport system, system x_c^-. Increased CySS uptake is known to enhance extracellular Cys and GSH delivery which can support the transfer S-nitrosothiols across the cell membrane (Zhang and Hogg 2004). Thus, the balance of plasma Cys/CySS during endotoxemia appears to depend on mechanisms that include oxidation of Cys to CySS, transport of Cys and CySS, and dietary availability of Cys and Cys precursors **(Figure 2.9)**.

Figure 2.9. Potential mechanisms for endotoxin-induced oxidation of plasma Cys/CySS redox state

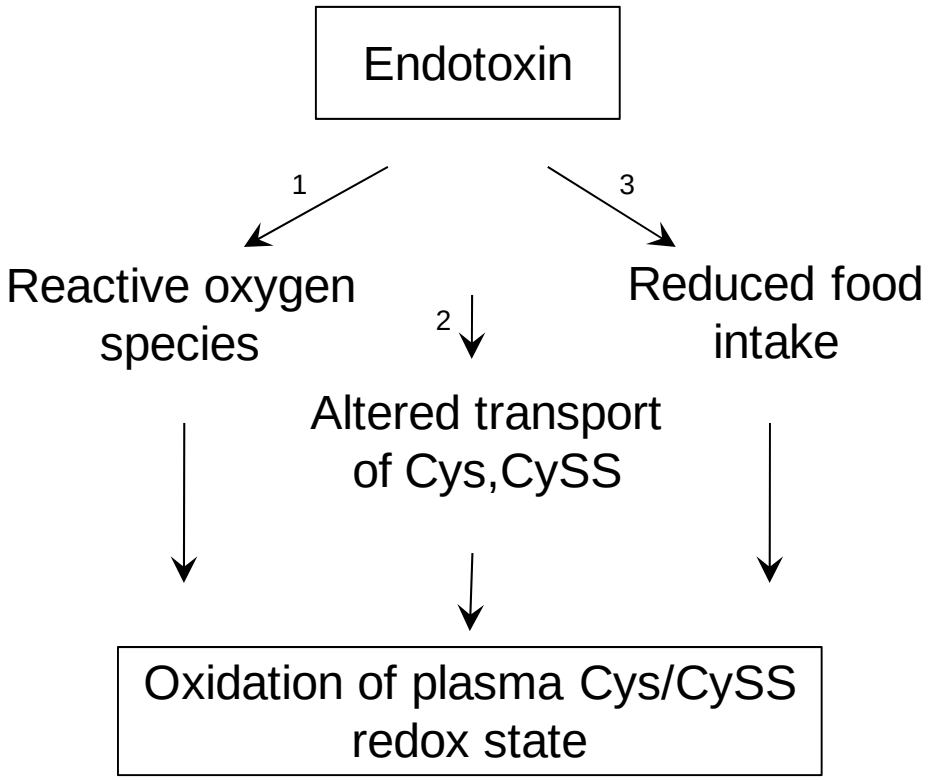


Figure 2.9. Endotoxin/LPS can induce oxidation of plasma Cys/CySS redox state by three mechanisms. Endotoxin-induced production of reactive oxygen species (1) is an early event that can contribute to the oxidation of Cys to CySS. (2) Increased transport of Cys can further potentiate the early decrease in Cys levels. CySS transport is also induced by endotoxin-mediated effects. In the present study we see that reduced food intake (3) contributes to LPS-induced decrease in Cys, and concominant oxidation of plasma Cys/CySS redox state at later time points.

The data show that E_h Cys/CySS in the plasma is more sensitive than E_h GSH/GSSG, to the acute effects of endotoxin, and that Cys/CySS redox state rebounds more slowly than GSH/GSSG redox state. While a precursor-product relationship exists between Cys

and GSH, the redox potentials of the couples are not in equilibrium (Jones, Mody et al. 2002). The redox state of plasma Cys/CySS is more oxidized compared to GSH/GSSG redox state in humans (-80 mV for Cys/CySS versus -137 mV for GSH/GSSG) (Jones, Mody et al. 2002) as well as in mice (-100 mV for Cys/CySS versus -150 mV for GSH/GSSG). The more oxidized redox state of Cys/CySS in the plasma is attributed to Cys being proximal to oxidative events in the extracellular fluid compared to GSH (Jones, Mody et al. 2002). For instance in humans, dietary restriction of sulfur amino acids leads to oxidation of plasma Cys/CySS redox state at 3 days, with no observable oxidation in the GSH/GSSG redox state (Mannery, Ziegler et al. 2007). Thus, the 10 mV oxidation of E_h Cys/CySS at 2 h after LPS, when E_h GSH/GSSG remains unchanged, is consistent with other studies of the dynamics of the redox state of the plasma Cys pool. Furthermore, the distinct effects of LPS on transport systems for GSH and Cys **(Table 2.2)** contributes to the disequilibrium in the redox potentials of the couples at 2 h. By 48 h, there is clearance of cytokines in the circulation and the concentrations of plasma GSH reflects re-feeding. Because a rapid increase in GSH synthesis occurs upon re-feeding (Leaf and Neuberger 1947), the disequilibrium in the redox potentials of Cys and GSH at 48 h is attributed to the utilization of Cys for GSH (and protein) synthesis. Experiments in endothelial cell demotrate that Here, we demonstrate that adherence of leukocytes to the pulmonary endothelial cells is increased under oxidized Cys/CySS redox states (**Figure 2.8**).

Table 2.2. Effect of LPS on GSH, CySS, Cys transport (systems)

Thiol/Transport System	Effects	Model	Mechanism	Putative Function	Ref
GSH/ Transporter unknown	300% increase in GSH efflux, 15 minutes post-LPS[1] (5 mg/kg body wt; i.v)	*In vivo,* hepatic sinusoids	Increase activity of GSH transporter by LPS-mediated induction of complement	Protect against extracellular oxidant stress	(Jaeschke 1992)
Cystine/ x_c^-	~3 fold increase in CySS influx, 6 h post-LPS (1 ng/ml)	*In vitro*, murine primary peritoneal macrophages	Transcriptional up-regulation of xCT	Increase cellular GSH	(Sato, Fujiwara et al. 1995)
Cysteine/ A, ASC	~ 2.7 fold increase in Cys influx 4 h post-LPS (7.5mg/kg body wt; i.p)	*Ex vivo*, hepatic membrane vesicles	Increase in V_{max} of System A and ASC by LPS-mediated induction of cortisol, TNF-α, and cyclooxygenase-derived products	Support gluconeogenesis and synthesis of acute phase proteins	(Inoue, Bode et al. 1995)

Cys/CySS redox state is now recognized as an independent extracellular redox control system (Jones, Go et al. 2004; Anderson, Iyer et al. 2007; Banjac, Perisic et al. 2007), and variations in extracellular E_h Cys/CySS can alter biological responses to growth factors such as Transforming growth factor-α and Transforming growth factor-β (Nkabyo, Go et al. 2005; Ramirez, Ramadan et al. 2007). Of significance to ALI, oxidized E_h Cys/CySS activates NF-κB and smad3, critical players in lung injury and in repair (Go and Jones 2005; Ramirez, Ramadan et al. 2007). Because plasma Cys/CySS is equilibrated with Cys/CySS in the pulmonary lymph (unpublished observations),

[1] *S. enteritidis LPS*

alterations in plasma Cys/CySS redox state can impact cellular processes in the lung. The early oxidation of ELF and plasma E_h Cys/CySS can potentiate the inflammatory response to injury, while the sustained oxidation of E_h Cys/CySS could play a role in modulating the repair process. Furthermore, because Cys is a rate limiting precursor in the synthesis of GSH, a decrease in Cys levels can compromise cellular GSH pools and thereby sensitize cells to acute stress (Brown, Harris et al. 2001). Mechanistic studies investigating the role of oxidized Cys/CySS redox state in lung injury will provide more direct answers on how perturbations in thiol status can contribute to the pathogenesis of ALI, and in doing so may help resolve the discrepancies associated with antioxidant therapies such NAC.

In summary, we find that early oxidation of plasma Cys/CySS redox state occurred due to LPS-treatment. Plasma GSH/GSSG showed no oxidation at 2 h. Subsequent oxidation of Cys/CySS and GSH/GSSG systems appeared to be due to anorexia because pair-fed controls showed equivalent oxidation. Consequently the combined observations indicate that enhanced oxidation of Cys, altered transport of Cys and CySS and decreased food intake each contribute to oxidized Cys/CySS redox state in endotoxemia.

CHAPTER III

CYSTEINE REDOX POTENTIAL DETERMINES PRO-INFLAMMATORY IL-1β LEVELS

In Chapter II, we demonstrated that Cys/CySS redox state is oxidized during the acute phase of endotoxin-induced lung injury. *In vitro* studies using a pulmonary vascular model system showed that oxidized Cys/CySS increased adhesion of leukocytes to the pulmonary endothelium, suggesting a causal role for oxidized Cys/CySS in the pathogenesis of ALI. The data also showed that nutrition is a critical determinant of plasma Cys/CySS redox state.

The present study was designed to investigate whether dietary supplementation with sulfur amino acids could attenuate the acute oxidation of plasma Cys redox state. Secondly, we determined whether preservation of Cys from oxidation decreased the acute inflammatory response to endotoxin. In addition, we performed *in vitro* experiments and analyzed human plasma to further delineate the relationship between Cys/CySS redox state and pro-inflammatory cytokines.

1. ABSTRACT

Cysteine (Cys) and its disulfide, cystine (CySS) represent the major extracellular thiol/disulfide redox control system. The redox potential (E_h) of Cys/CySS is centered at approximately -80 mV in the plasma of healthy adults, and oxidation of E_h Cys/CySS is implicated in inflammation associated with various diseases. The purpose of the present study was to determine whether oxidized E_h Cys/CySS is a determinant of interleukin (IL)-1β levels. Results showed a 1.7-fold increase in secreted IL-1β levels in U937 monocytes exposed to oxidized E_h Cys/CySS (-46 mV), compared to controls exposed to a physiological E_h of -80 mV ($P< 0.01$). In LPS-challenged mice, preservation of plasma E_h Cys/CySS from oxidation by dietary sulfur amino acid (SAA) supplementation, was associated with a 1.6-fold decrease in plasma IL-1β compared to control mice fed an isonitrogenous SAA-adequate diet ($P< 0.01$). Analysis of E_h Cys/CySS and IL-1β in human plasma revealed a significant positive association between oxidized E_h Cys/CySS and IL-1β after controlling for age, gender, and BMI ($P< 0.001$). These data show that oxidized extracellular E_h Cys/CySS is a determinant of IL-1β levels, and suggest that strategies to preserve E_h Cys/CySS may represent a means to control IL-1β in inflammatory disease states.

2. INTRODUCTION

Interleukin (IL)-1β is a pro-inflammatory cytokine that functions as a critical regulator of host defense in response to infection and injury. However when present in excess, IL-1β is extremely toxic (Dinarello and Wolff 1993). Elevated systemic levels of IL-1β cause hypotension during septic shock and induce capillary leak in acute lung

injury (Ganter, Roux et al. 2008). IL-1β is also involved in chronic inflammation associated with arthritis, lung fibrosis, and atherosclerosis (Kolb, Margetts et al. 2001; Waehre, Yndestad et al. 2004; Fan, Soder et al. 2007). Therefore, strategies to modulate IL-1β production in inflammatory diseases are of therapeutic interest.

IL-1β activation and induction are associated with inflammation, a process with enhanced generation of reactive oxygen and nitrogen species (Kolls 2006). These reactive species serve multiple biological functions, which include removal of cell debris and cell signaling necessary for host defense. Recent advances in redox signaling mechanisms have revealed that functional pathways utilize diffusible oxidants such as peroxide and redox-sensitive thiols in specific proteins as sensors (D'Autreaux and Toledano 2007). The redox states of these sensors are controlled by rates of oxidation of specific amino acid residues and their reduction by thiol/disulfide control systems.

The thiol/disulfide control systems are compartmentalized; glutathione/glutathione disulfide (GSH/GSSG) and thioredoxin provide control mechanisms within cells, while cysteine/cystine (Cys/CySS) and GSH/GSSG control the redox state of proteins in the extracellular space and on the cell surface (Hansen, Go et al. 2006). The Cys/CySS couple predominates in the extracellular fluid and the steady-state redox potential (E_h) of Cys/CySS is oxidized in acute and chronic inflammatory disease states (Moriarty-Craige, Adkison et al. 2005; Iyer, Jones et al. 2008). *In vitro,* oxidized E_h Cys/CySS induces upregulation of nuclear factor-kappa B (NF-κB) (Go and Jones 2005; Ramirez, Ramadan et al. 2007), increases adhesion of leukocytes to the endothelium (Go and Jones 2005; Iyer, Jones et al. 2008), and sensitizes epithelial cells to apoptosis (Jiang, Moriarty-Craige et al. 2005). Based on these observations, we

hypothesized that extracellular E_h Cys/CySS is a determinant of pro-inflammatory cytokine production.

We tested this hypothesis by modifying extracellular E_h Cys/CySS and determining IL-1β levels *in vitro* and *in vivo*. *In vitro* results showed that oxidized extracellular E_h Cys/CySS is sufficient to increase IL-1β levels in monocytes, and *in vivo* results showed that dietary treatment to protect against plasma E_h Cys/CySS oxidation is associated with decreased IL-1β levels in LPS-challenged mice. Analysis of E_h Cys/CySS and IL-1β in human plasma revealed a significant positive association between oxidized E_h Cys/CySS and IL-1β, independent of age, gender, and BMI. Together, the data show that oxidized extracellular E_h Cys/CySS is one of the determinants of IL-1β levels, and suggest that strategies to preserve E_h Cys/CySS may represent a means to control IL-1β in inflammatory disease states.

3. MATERIALS AND METHODS

3.1. Materials. Except as indicated, all chemicals were purchased from Sigma Chemical Corporation (Sigma, St. Louis, MO). Distilled, deionized water was used for analytical purposes. HPLC quality solvents were used for HPLC.

3.2. Cell culture. Human monocytic cells (U937, ATCC, Rockville, MD) were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) and 10 U/ml penicillin and streptomycin sulfate. Cells were transferred to 0.5% FBS media 8-12 h prior to experimental manipulations.

To generate the desired range of extracellular redox potentials, the extracellular thiol/disulfide pool was altered by varying concentrations of Cys and CySS, added to cyst(e)ine-free RPMI, as previously described (Ramirez, Ramadan et al. 2007). In these experiments, the total extracellular pool size of Cys + CySS was set at 200 μM, while concentrations of Cys and CySS were varied to obtain initial E_h values from -80 mV (physiological) to -46 mV (oxidized).

Production of reactive oxygen species (ROS) was detected using 6-Carboxy-2′,7′-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) (Go and Jones 2005). U937 cells, plated into 96-well plate, were washed with KRH buffer and incubated with 100 μM DCFH-DA for 30 minutes (37°C, 5% CO_2). Cells were washed and exposed to physiological (-80 mV) and oxidized (-46 mV) Cys/CySS redox media at 37^{o}C. Oxidation of DCFH-DA to fluorescent DCF was measured on a microplate reader (excitation, 485 nm; emission, 530 nm) (Kao, Wang et al. 2006).

3.3. Experimental animals and dietary intervention. Experiments were conducted using 10-14 week old, female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). Mice were housed in cages and maintained on a 12-h light-12-h dark cycle at the Division of Animal Resources at Emory University. All experiments were initiated during the light cycle. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Prior to the dietary intervention, all animals were fed pelleted rodent food (Test Diet 5015, Lab Diet Inc., Richmond, IN). Semi-purified diets were custom-prepared (Harlan-Teklad, Madison, WI, USA) in order to test the specific effects of sulfur amino

acid (SAA) supplementation (Nkabyo, Gu et al. 2006). The SAA-adequate and SAA-supplemented diets were isocaloric and isonitrogenous and contained adequate and identical quantities of energy, nitrogen, carbohydrate, fat, fiber, micronutrients and essential amino acids. The SAA and nitrogen content was controlled at the desired experimental levels by varying the amount of cystine and methionine and the non-essential amino acids L-alanine, L-aspartic acid, glycine and L-serine. The SAA-supplemented diet contained 3-fold cystine, and 1.8-fold methionine compared to the SAA-adequate diet. Animals had free access to water at all experimental time points.

3.4. LPS Administration. *Escherichia coli* O55:B5 LPS, dissolved in sterile PBS (100 μg/ml) was administered intraperitoneally at a dose of 1 mg LPS/kg body. Animals were sacrificed at 2 h post-LPS.

3.5. Sample collection and analysis of Cys, CySS, GSH and GSSG. Mice were anesthetized by isofluorane inhalation (Baxter Pharmaceuticals, Deerfield, IL), and blood was collected by submandibular bleeding using a 4 mm mouse bleeding lancet (Medipoint, Inc. Mineola, NY). 0.18 ml of collected blood was immediately transferred to 0.02 ml of preservation solution containing γ-glutamyl-glutamate (γ-Glu-Glu) as an internal standard (Jones 2002). Samples were centrifuged at 16000 g for 60 seconds to remove precipitated protein, and 0.1 ml of the supernatant was immediately transferred to an equal volume of ice-cold 10% (w/v) perchloric acid. Samples were immediately stored at -80 °C.

For HPLC analysis (Gilson Medical Electronics, Middleton, WI), derivatized samples were centrifuged, and 50 µl of the aqueous layer was applied to the Supercosil LC-NH_2 column (25 cm x 4.6 mm; Supelco, Bellefunk, PA). Derivatives were separated with a sodium acetate gradient in methanol/water and detected by fluorescence (Jones, Carlson et al. 1998). Concentrations of thiols and disulfides were determined by integration relative to the internal standard. Redox potential s (E_h) of the GSH/GSSG and Cys/CySS pools, given in millivolts (mV), were calculated from concentrations of GSH, GSSG and Cys, CySS in molar units with the following forms of the Nernst equation for pH 7.4: GSH/GSSG, $E_h = -264 + 30 \log ([GSSG]/[GSH]^2)$, Cys/CySS, $E_h = -250 + 30 \log ([CySS]/[Cys]^2)$(Jones, Carlson et al. 2000).

3.6. Quantitative Real-Time PCR Analysis. Lung samples were excised, snap frozen in liquid N_2 and stored at -80°C. Total RNA was extracted from tissue using an RNeasy Midi Kit (QIAGEN Inc., Valencia, CA) according to manufacturer's instructions. DNase treatment was performed to remove contaminating genomic DNA. RNA concentration was spectrophotometrically determined at 260 nm, and 0.5 µg of total RNA was used to synthesize 20 µl of cDNA (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed on cDNA using gene-specific primers on an iCycler IQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primers were designed using Beacon Designer Software 4.00 (PREMIER Biosoft International, Palo Alto, CA) (**Table 3.1**). Samples containing serial dilutions of known concentrations of cDNA, encoding the gene of interest, were amplified in parallel. Data were analyzed using the iCycler Software, and starting quantities of message levels of each gene were

determined from constructed standard curves. Melt curves were examined to ensure amplification of a single PCR product. Expression level of IL-1β was normalized to β-actin in monocytes and 18S ribosomal RNA in mouse lung tissue.

Table 3.1. Details of PCR primer sequences used for quantitative real-time PCR

Target	Genbank accession number	Forward primer	Reverse primer	Product size, bp
IL-1β human	NM_000576	TGATGGCTTATTACAGTGGCAATG	GTAGTGGTGGTCGGAGATTCG	140
β-actin human	NM_001101	GCGTGACATTAAGGAGAAG	GAAGGAAGGCTGGAAGAG	172
IL-1β mouse	NM_008361	ATCTCGCAGCAGCACATC	CAGCAGGTTATCATCATCATC	192
18S mouse	NR_003278	CGTCTGCCCTATCAACTTTCG	GCCTGCTGCCTTCCTTGG	130

3.7. Cytokine analysis. Levels of IL-1β in the cell-supernatant were detected by ELISA (R&D Systems, Minneapolis, MN), and are expressed relative to total protein content in the supernatant. To better assess the inflammatory status *in vivo*, levels of IL-1β, and tumor necrosis factor (TNF)-α were measured. IL-1β and TNF-α in human plasma, mouse plasma, and mouse lung homogenates were detected by immunofluorescence using a multiplex panel assay (R&D Systems, Minneapolis, MN). Briefly, the antibody coupled beads were incubated with the sample followed by incubation with a detection antibody. Fluorescence intensity was read on a Bioplex suspension array system (Bio-Rad, Hercules, CA).

3.8. IL-1β luciferase reporter assay. To evaluate IL-1β gene transcription, U937 monocytes were electroporated with a pIL-1 (4.0 kb) luciferase promoter construct, as previously described (Ritzenthaler and Roman 1998). Cells were plated in 6-well plate and were incubated for 8 h in specific redox media. Cells were lysed in reporter lysis buffer (Promega, Madison, WI) and assayed for luciferase activity. Measurements were done using a Labsystems Luminoskan Ascent Plate Luminometer. Results were recorded as luciferase units and were adjusted for total protein content.

3.9. Human subjects. This study was reviewed and approved by the Emory Institutional Review Board. A total of 16 healthy volunteers were recruited by posting fliers in public locations in the Atlanta/Emory University community. Following informed consent, participants were admitted to the outpatient unit of the Emory University Hospital General Clinical Research Center (GCRC), where potential subjects were screened using a medical history, physical examination, urinalysis, standard chemistry profile and a complete blood count. Eligibility was established based on the following criterion: absence of acute or chronic illness (other than a medical history of well-controlled hypertension), BMI < 30, non-smokers, and compliance in discontinuing nutritional supplements, if consumed, 2 weeks prior to study entry. Eligible participants were then scheduled for a 24 h inpatient visit within 2 weeks of screening in the GCRC. Characteristics of study subjects are presented in Table 2.

3.10. Human study protocol. Participants were instructed not to eat after 10 pm the night prior to the inpatient visit in order to standardize baseline levels of metabolites. Participants were admitted to the GCRC at 7.00 on Day 1 and a heparin-lock catheter was

placed in a forearm vein for blood sampling at 8.00. After a 30-minute supine resting period, 3 ml blood samples were drawn every hour for 24 consecutive hours. Participants were given breakfast at 9:30, lunch at 13:30, dinner at 17:30, an evening snack was provided at 21:30 immediately following the timed blood draw for that hour. The composition of the meals and snack was standardized for all subjects, as previously reported (Blanco, Ziegler et al. 2007). Water was provided *ad libitum* throughout the study period.

3.11. Statistical Methods. Data are presented as means + SEM. Statistical analysis was done using SAS v 9.1 (SAS Institute Inc., Cary, NC, US). Data from *in vitro* experiments were analyzed using an unpaired t-test. Data from *in vivo* murine experiments were analyzed using a one-way ANOVA with treatment specified as the main effect. To analyze data from human plasma, a linear mixed model was used. The association of Cys, CySS, and E_h Cys/CySS with IL-1β, and TNF-α was determined after controlling for time of day, BMI, age, and gender. Because none of the potential confounders had statistically significant regression coefficients, parameters for BMI, age, and gender were excluded to arrive at the most parsimonious model. The residuals for thiol/disulfide redox were normally distributed; therefore thiol/disulfide redox was specified as the response variable in all constructed models to improve the stability of the computed regression coefficients. The human study by its very design cannot establish a cause-effect relationship; causality is therefore not invoked by specification of thiol/disulfide redox as dependent and cytokine as the independent variable. The best model from all subsets was determined using the Akaike Information Criterion (AIC). Significance was set at a P value < 0.05 for all tests.

4. RESULTS

4.1. Oxidized extracellular E_h Cys/CySS increases IL-1β protein and IL-1β mRNA levels in human monocytes.

Measurements of Cys and CySS in human plasma show that E_h Cys/CySS is centered at approximately -80 mV in healthy young individuals (Jones, Carlson et al. 2000) and is oxidized in association with inflammatory disease states (Moriarty-Craige, Adkison et al. 2005). *In vitro*, oxidized extracellular E_h Cys/CySS activates nuclear factor (NF)-κB, a key pro-inflammatory transcription factor (Go and Jones 2005; Ramirez, Ramadan et al. 2007). Together, these data indicate that oxidized E_h Cys/CySS is likely to modulate pro-inflammatory cytokine production. Because peripheral blood monocytes are constantly exposed to the extracellular redox environment, we determined whether levels of IL-1β, a major monocyte-derived cytokine, are increased by oxidized extracellular E_h Cys/CySS.

U937 monocytes were exposed to E_h of -80 mV (physiological) or -46 mV (oxidized) for 8 h, and levels of secreted IL-1β in the cell-supernatant were quantified by ELISA. **Figure 3.1A** shows a 1.7-fold increase in IL-1β levels in cells treated with E_h of -46 mV compared to -80 mV ($P < 0.01$). To determine whether increased IL-1β protein in response to oxidized E_h occurred due to increase in mRNA abundance, IL-1β mRNA was quantified by real-time PCR. As shown in **Figure 3.1B**, IL-1β mRNA was more abundant in response to E_h of -46 mV, 4 h after treatment ($P < 0.05$). Experiments with U937 cells expressing the human IL-1β promoter fused to a luciferase reporter gene revealed significant increase in luciferase activity at -46 mV, suggesting transcriptional induction of IL-1β by oxidized E_h (**Figure 3.1C**; $P< 0.05$).

Figure 3.1. Oxidized extracellular E_h Cys/CySS increases IL-1β in monocytes

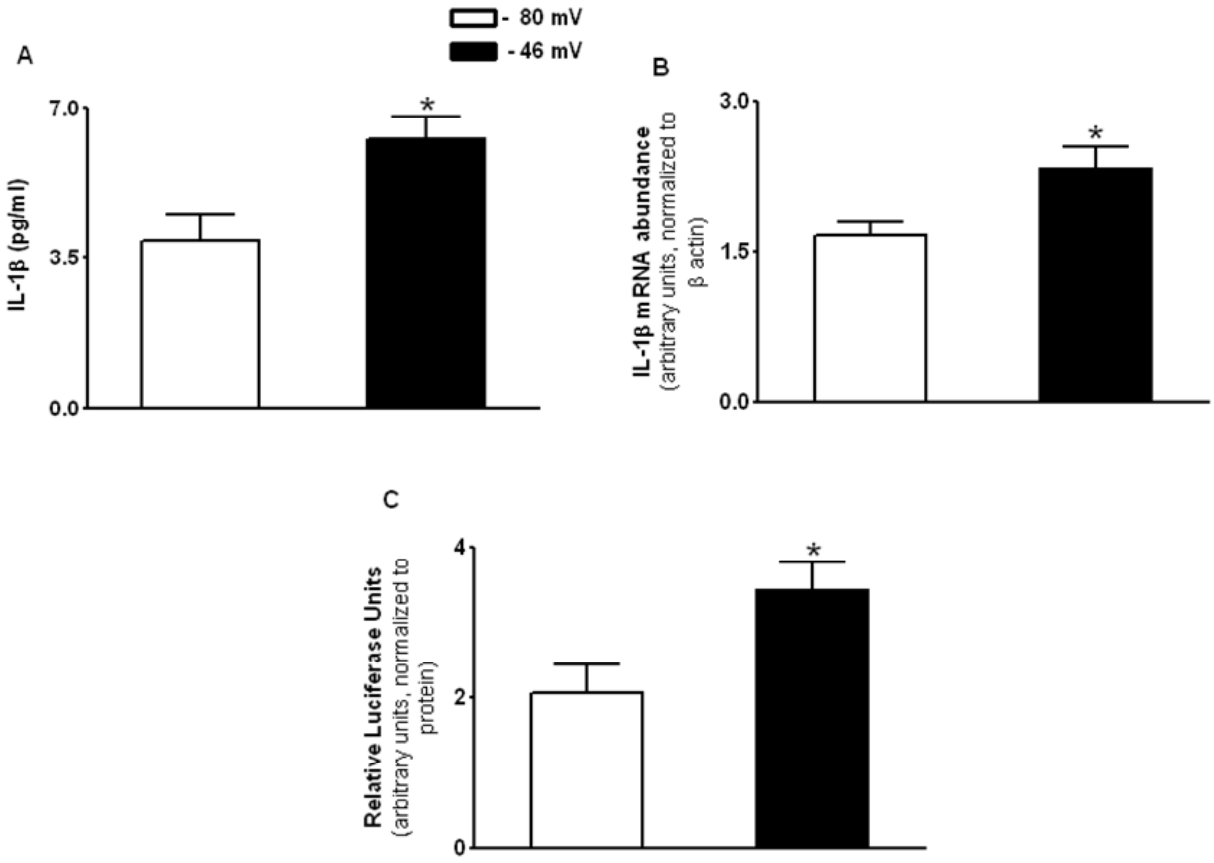


Figure 3.1. Oxidized extracellular E_h Cys/CySS increases IL-1β in monocytes. U937 cells were exposed to physiological (-80 mV) and oxidized (-46 mV) E_h Cys/CySS for 8h and levels of secreted IL-1β were quantified by ELISA. IL-1β levels are expressed relative to protein concentration in cell-supernatant (A). In (B), total RNA was extracted 4 h after treatment with redox media. Abundance of IL-1β mRNA was detected by real-time PCR and is normalized to β actin. In (C), U937 cells expressing the IL-1β-luciferase construct were exposed to given E_h for 12 h. Luciferase activity in cell-lysates is shown after normalization for total protein. Data are mean + SE of 3 replicates of a representative experiment repeated 3 times, * $P < 0.05$.

4.2. Oxidized extracellular E_h Cys/CySS induces intracellular ROS production in monocytes but has no effect on cellular E_h GSH/GSSG. Previous studies have shown that induction of IL-1β by extracellular stimuli, such as ATP, is associated with generation of ROS and up-regulation of genes involved in GSH synthesis (Cruz, Rinna et al. 2007). Therefore, we determined whether increase in IL-1β in response to oxidized extracellular E_h Cys/CySS is associated with intracellular redox changes. Measurement of cellular GSH (**Figure 3.2A**) and E_h GSH/GSSG (**Figure 3.2B**) revealed no significant differences between the -80 and -46 mV treatment groups at 2h and 8h.

Next, we examined whether oxidized extracellular E_h Cys/CySS stimulated cellular ROS production. Cells were pre-incubated with an ROS-sensitive dye, DCFH-DA, prior to treatment with -80 mV and -46 mV Cys/CySS redox media. Oxidation of DCFH-DA to fluorescent DCF was measured on a microplate reader. Cells treated with glucose oxidase served as positive controls. Within 5 minutes, cells exposed to oxidized E_h of -46 mV showed a 5-fold increase in DCF fluorescence compared to cells exposed to a physiological redox potential of -80 mV ($P< 0.001$) (**Figure 3.2C**). When measured 2-6 h after treatment, the magnitude of DCF fluorescence increased in both -80 and -46 mV conditions, with at least a 2-fold higher fluorescence at -46 mV ($P< 0.001$, data not shown). This indicates that oxidized E_h-induced ROS production was sustained over-time, and occurred in the absence of oxidation to cellular E_h GSH/GSSG.

We subsequently determined whether increase in ROS, in response to oxidized extracellular E_h Cys/CySS, was sensitive to the oxidation of redox-sensitive membrane-bound thiols. To this end, monocytes were pre-treated with 0.25 mM 4-acetamide-4'-

amleimidylstilbene-2,2'-disulfonic acid (AMS), a non-permeant alkylating agent, prior to exposing the cells to given E_h Cys/CySS. Pre-treatment with AMS decreased ($P< 0.001$), but did not completely inhibit, cellular ROS levels in response to oxidized E_h indicating that increase in cellular ROS occurs in part due to oxidation of membrane-bound thiols. Taken together these *in vitro* data show that oxidized extracellular E_h Cys/CySS induces a rapid and sustained increase in ROS in monocytes which could be involved in E_h mediated increase in IL-1β. Based on these *in vitro* findings, we wanted to determine whether increase in IL-1β during inflammation *in vivo* can be attenuated by preservation of E_h Cys/CySS from oxidation.

Figure 3.2. Oxidized extracellular E_h Cys/CySS induces ROS production in monocytes but has no effect on cellular E_h GSH/GSSG

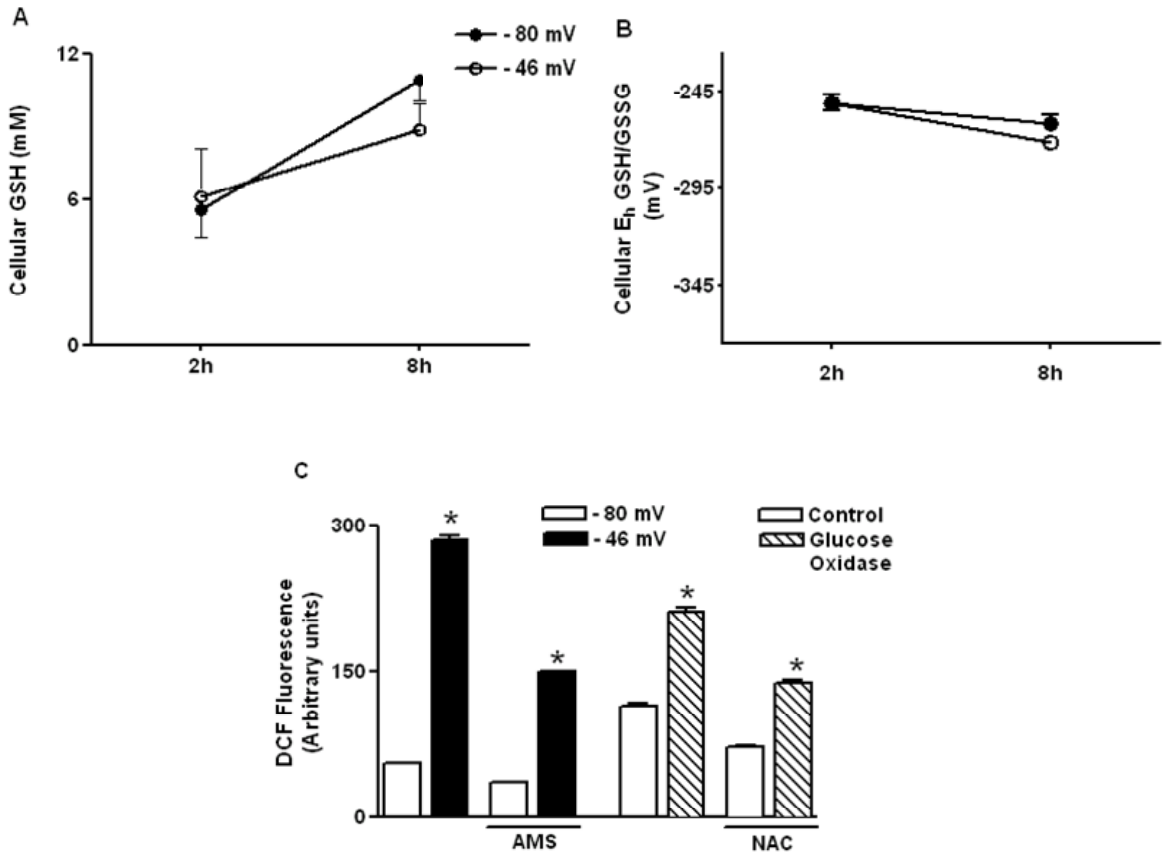


Figure 3.2. Oxidized extracellular E_h Cys/CySS induces ROS production in monocytes but has no effect on cellular E_h GSH/GSSG. U937 cells were lysed 2 h and 8 h after exposure to -80 mV and -46 mV Cys redox states. Cellular concentrations of GSH and GSSG were determined by HPLC**.** GSH levels (A) and E_h GSH/GSSG (B) were not significantly different between 80 mV and -46 mV treatments. In (C), U937 cells were pre-incubated with an ROS-sensitive dye, DCFH-DA (100 μM) for 30 min, before treating with -80 mV and -46 mV redox media for 5 min at 37 ^{o} C. Oxidation of DCFH-DA to fluorescent DCF was measured on a microplate reader. Cells exposed to oxidized Cys/CySS redox (-46 mV) show a 5-fold increase in ROS production compared to cells exposed to a physiological redox potential of -80 mV (*$P < 0.001$). Pre-treating cells with 0.25 mM 4-acetamide-4'-amleimidylstilbene-2,2'-disulfonic acid (AMS), a non-permeant alkylating agent, attenuated the increase in ROS production ($P < 0.001$). As a positive control, ROS production was measured in monocytes treated with glucose oxidase (2 units); an enzyme system that generates H_20_2 (*$P < 0.001$). NAC pre-treatment attenuated glucose oxidase-induced ROS production (*$P < 0.001$). Data are mean + SE of 4 replicates of a representative experiment repeated 3 times.

4.3. Dietary SAA supplementation protects against endotoxin-induced perturbations in plasma Cys, CySS and E_h Cys/CySS. Based on previous research showing that supplementation with sulfur amino acids (SAA) increases plasma Cys and shifts the E_h to a more reduced potential (Nkabyo, Gu et al. 2006), we applied this approach to a mouse model of inflammation in which LPS/endotoxin is administered to increase IL-1β (Rojas, Woods et al. 2005). In this design, mice were fed either a SAA-adequate diet or an isonitrogenous SAA-supplemented diet for 7 days prior to endotoxin challenge. Mice were sacrificed 2 h post-endotoxin, a time point that coincides with maximal IL-1β production (Rojas, Woods et al. 2005). Plasma was collected prior to sacrifice for analysis of Cys, CySS, GSH and GSSG by HPLC. Thiol/disulfide redox parameters in untreated controls, fed pelleted rodent chow, are shown for comparison.

SAA-supplementation protected against endotoxin-induced decrease in plasma Cys (**Figure 3.3A**; Cys (μM) - SAA supplemented, 19.6 $\pm$ 1.9; SAA-adequate, 13.5 $\pm$ 1.2; $P < 0.05$). The decrease in plasma Cys in response to endotoxin is likely due to clearance of Cys, and oxidation of Cys to CySS. *Ex vivo* studies in hepatic membrane vesicles show that LPS stimulates hepatic influx of Cys to support gluconeogenesis and synthesis of acute phase proteins (Inoue, Bode et al. 1995). LPS also increases peroxide levels (Minamiya, Abo et al. 1995), and this could decrease plasma Cys via oxidation to CySS, an interpretation supported by the increase in plasma CySS levels in mice fed SAA-adequate diet (**Figure 3.3B**).

The difference in plasma Cys between SAA-supplemented and SAA-adequate group is consistent with the predicted increase in Cys due to excess SAA intake, based on previous work done in our laboratory (Nkabyo, Gu et al. 2006). However, plasma CySS

levels were not affected by excess SAA intake (Nkabyo, Gu et al. 2006). Thus, the lack of increase in plasma CySS in the SAA-supplemented group, 2 h post endotoxin, suggests additional effects on ROS homeostasis due to SAA supplementation (CySS (μM) - SAA-adequate, 141 $\pm$ 9.7; SAA supplemented, 85.2 $\pm$ 1.2; $P< 0.01$). As a consequence of the higher Cys and decreased CySS, E_h Cys/CySS was on average 15 mV more reduced in response to endotoxin in the SAA-supplemented group compared to SAA-adequate group (**Figure 3.3C**; SAA-adequate, -76.4 $\pm$ 2.6; SAA supplemented, -91.5 $\pm$ 3.7; $P< 0.01$).

Measurements of plasma GSH, GSSG (data not shown) revealed that plasma E_h GSH/GSSG (**Figure 3.3D**) was not oxidized after endotoxin treatment, and was comparable across treatment groups. These results confirm that the *in vivo* model is adequate to test whether attenuating oxidation of plasma E_h Cys/CySS decreases plasma IL-1β levels in inflammation.

Figure 3.3. Effect of dietary SAA-supplementation on endotoxin-induced oxidation of plasma E_h Cys/CySS

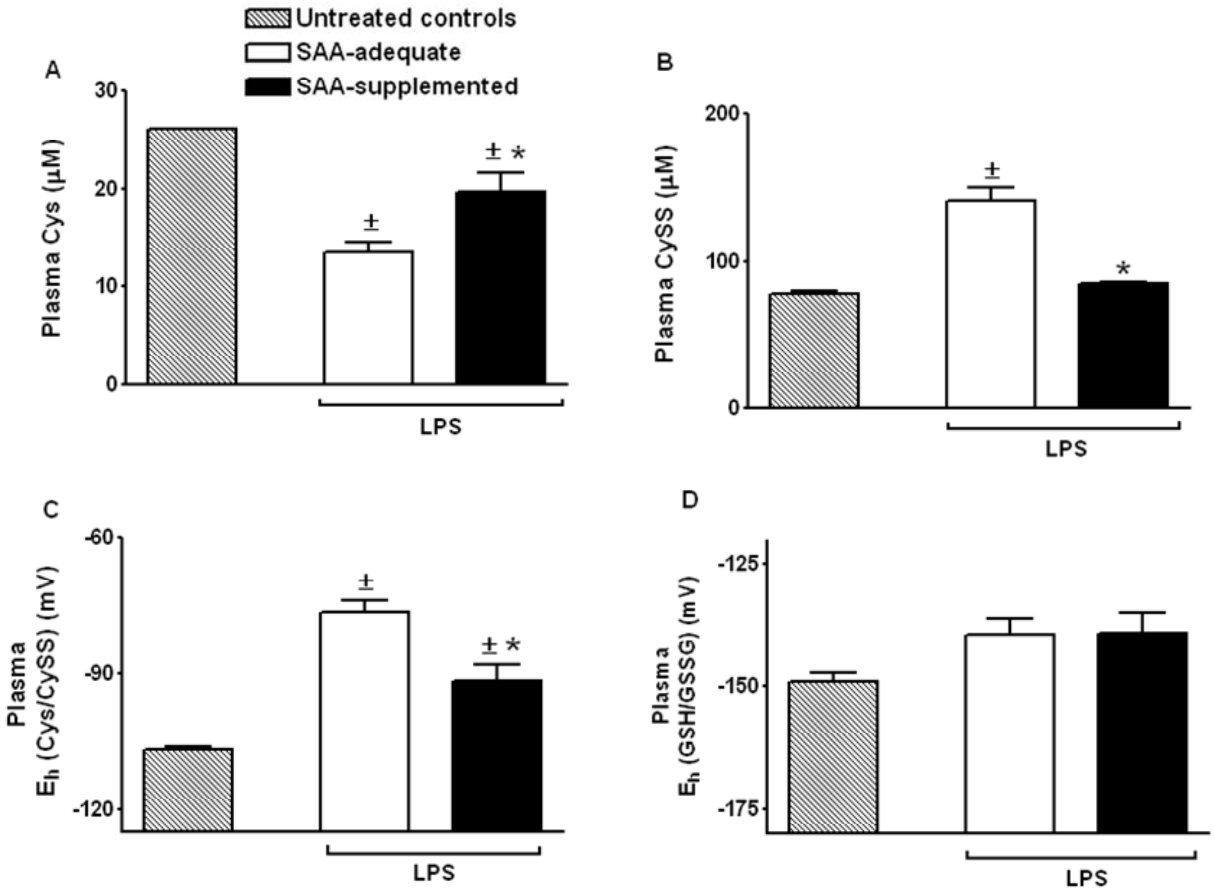


Figure 3.3. Effect of dietary SAA-supplementation on endotoxin-induced oxidation of plasma E_h Cys/CySS. C57BL/6J mice receiving either SAA-adequate diet or SAA-supplemented diet were treated with 1 mg/kg i.p endotoxin/LPS. At 2 h, mice were sacrificed and plasma was collected for HPLC analysis of Cys (A), CySS (B). In (C), E_h Cys/CySS was calculated from Cys and CySS concentrations using the Nernst equation. Plasma E_h GSH/GSSG is shown in (D). Data are mean + SE of 4 replicates of a representative experiment repeated 2 times. $^{\pm}$ Values significantly different from untreated controls, *Values significantly different from SAA-adequate group

4.4. Dietary SAA supplementation protects against endotoxin-induced IL-1β.

Next, we determined whether the more reducing plasma E_h Cys/CySS, in SAA-supplemented animals is associated with a decrease in plasma IL-1β, in response to endotoxin. Plasma IL-1β levels in untreated controls were un-detectable (not shown). As shown in **Figure 3.4A**, plasma IL-1β decreased by 1.6-fold in SAA-supplemented animals (IL-1β (pg/ml) - SAA adequate, 190 $\pm$ 22 ; SAA supplemented, 116 $\pm$ 12 ; $P < 0.01$). Induction of IL-1β in the lung is an early response to endotoxin (Iyer, Jones et al. 2008), so we determined whether a protective effect on IL-1β was also observed in the lung, in response to a reduced extracellular E_h Cys/CySS. Lung IL-1β levels were decreased by 2-fold in the lung homogenate from the SAA-supplemented group (**Figure 3.4B**; $P < 0.001$). Measurements of IL-1β message levels by quantitative real-time PCR revealed a 3-fold decrease in lung IL-1β mRNA levels (**Figure 3.4C**; $P< 0.05$). Thus, preserving extracellular E_h Cys/CySS during endotoxemia decreased tissue and circulating levels of IL-1β.

Figure 3.4. Effect of dietary SAA-supplementation on endotoxin-induced IL-1β

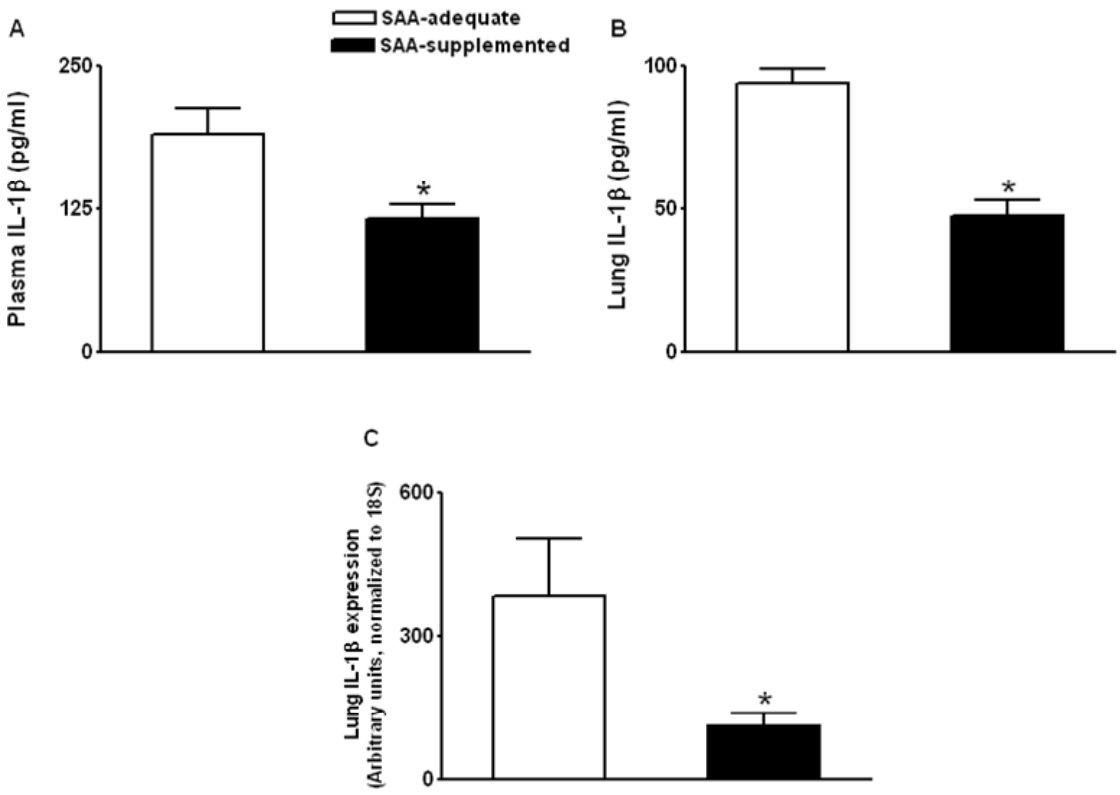


Figure 3.4. Effect of dietary SAA-supplementation on endotoxin-induced IL-1β. C57BL/6J mice receiving either SAA-adequate diet or SAA-supplemented diet were treated with 1 mg/kg i.p endotoxin/LPS. At 2 h, mice were sacrificed and plasma and lung samples were collected for analysis of IL-1β. Plasma IL-1β levels are shown in (A). IL-1β levels in lung homogenate are presented after normalization for total protein (B). In (C) RNA was extracted from whole lung and transcript levels of IL-1β were quantified by quantitative real-time PCR. Data are mean +SE of 4 replicates of a representative experiment repeated 2 times, * $P < 0.05$

4.5. Dietary SAA supplementation protects against endotoxin-induced TNF-α. Tumor necrosis factor (TNF)-α is another prototypical pro-inflammatory cytokine that is induced in concert with IL-1β in response to infection, injury, and immunological challenge (Dinarello 2000). To better assess the inflammatory status *in vivo*, we determined whether attenuation of TNF-α also occurred in SAA-supplemented mice. Results showed a greater than 2-fold decrease in plasma TNF-α levels with SAA-supplementation (**Figure 3.5A**; $P< 0.05$). TNF-α in the lung was also significantly decreased (**Figure 3.6B**; $P< 0.05$). Thus, the *in vivo* observations show that preservation of plasma E_h Cys/CySS during endotoxemia is associated with a decrease in IL-1β levels. These data extend the *in vitro* observations, and the combined findings support a mechanistic role for Cys redox potential in determining IL-1β levels.

Figure 3.5. Effect of dietary SAA-supplementation on endotoxin-induced TNF-α

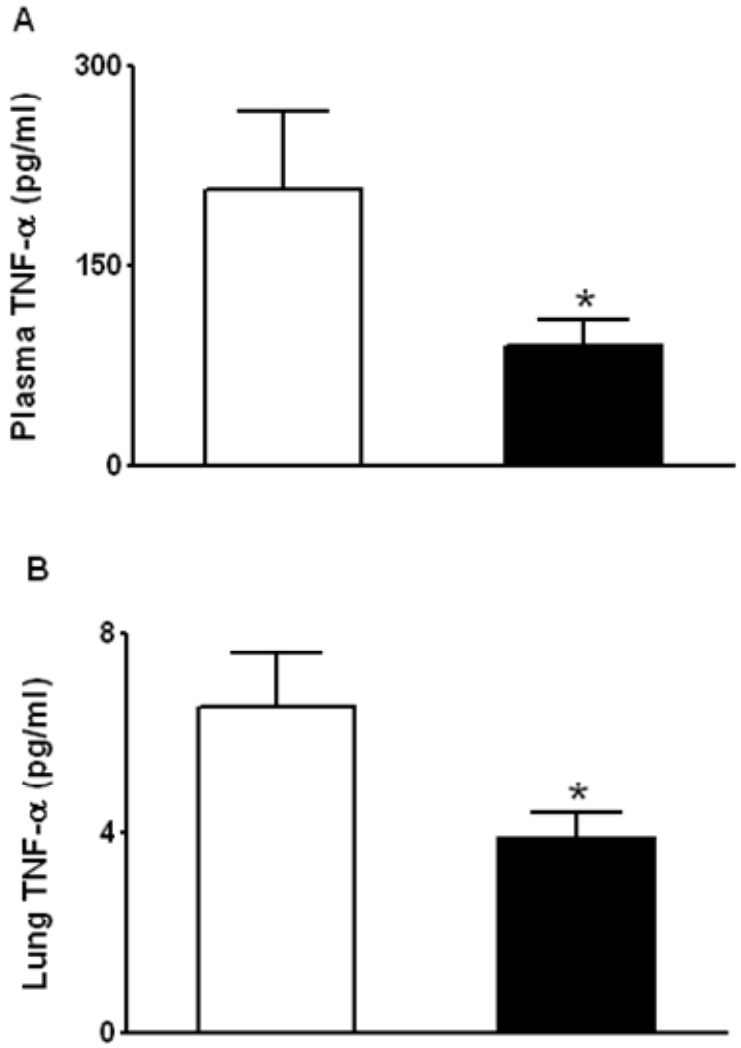


Figure 3.5. Effect of dietary SAA-supplementation on endotoxin-induced TNF-α. C57BL/6J mice receiving either SAA-adequate diet or SAA-supplemented diet were treated with 1 mg/kg i.p endotoxin/LPS. At 2 h, mice were sacrificed and plasma and lung samples were collected for analysis of TNF-α. Plasma TNF-α (A) and lung TNF-α (B) were determined. Data are mean +SE of 4 replicates of a representative experiment repeated 2 times, * $P < 0.05$

4.6. IL-1β and TNF-α in plasma of healthy adults are increased in association with oxidized E_h Cys/CySS. To investigate whether Cys redox potential could represent a determinant of pro-inflammatory cytokine levels in humans, we examined IL-1β, TNF-α, Cys, and CySS in plasma samples from 16 healthy adults. The characteristics of the study participants are shown in **Table 3.2**.

Table 3.2. Characteristics of study subjects

Demographics	
Age, mean ± SD	60.3 ± 17.9
BMI, mean ± SD, kg/m^2	24.2 ± 3.1
Females, %	56
Fasting markers, mean + SD	
Cys, μM	9.2 ± 2.8
CySS, μM	66.6 ±13.9
E_hCys/CySS, mV	(-) 71.7 ± 9.2
GSH, μM	1.2 ± 0.4
GSSG, μM	0.07 ± 0.02
E_hGSH/GSSG, mV	(-)122.8 ± 9
TNF-α, pg/mL	4.5 ± 3.7
IL-1β, pg/mL	3.6 ± 3.6

Because plasma Cys and E_h Cys/CySS, and cytokines exhibit well documented circadian rhythms (Petrovsky, McNair et al. 1998; Blanco, Ziegler et al. 2007), the study was designed to examine whether diurnal variation in E_h Cys/CySS was associated with variation in IL-1β and TNF-α. For this purpose, hourly samples were collected for an entire 24 h period from 16 individuals. Diurnal variation in plasma Cys and E_h Cys/CySS was closely related to meal intake, as reported previously (data not shown) (Blanco, Ziegler et al. 2007). However, due to considerable inter-individual variation in plasma IL-1β and TNF-α, we did not detect significant time-dependent variations in cytokine levels. Therefore, we used a secondary analysis to determine whether variation in E_h Cys/CySS correlated with variation in IL-1β, independent of time of day.

Because repeated measures were obtained from the same individual, we used a linear mixed procedure to model variation in redox parameters with cytokine levels controlling for time of day, BMI, age, and gender. As none of the potential confounders had statistically significant regression coefficients, parameters for BMI, age, and gender were excluded to arrive at the most parsimonious model. Cys, CySS and E_h Cys/CySS were specified as response variables in the analyses because the residuals for these biomarkers were normally distributed. Regression coefficients for the mixed model are presented in **Table 3.3.**

Table 3.3. Mixed model of redox parameters and IL-1β and TNF-α controlling for time of day

Biomarker	Regression coefficients		P
	IL-1β	**SE**	
Cys, μM	-0.19	0.09	< 0.05
CySS, μM	0.28	0.44	ns
E_hCys/CySS, mV	1.08	0.28	< 0.001
	TNF-α	**SE**	
Cys, μM	-0.06	0.04	ns
CySS, μM	0.41	0.17	< 0.05
E_hCys/CySS, mV	0.25	0.11	< 0.05

Examination of redox parameters for Cys redox potential revealed a strong positive association between E_h Cys/CySS and IL-1β (P< 0.001). As seen in **Figure 3.6A**, oxidized values of E_h Cys/CySS correlate with higher levels of plasma IL-1β. The regression parameter for IL-1β indicates that a 1 unit increase in plasma IL-1β is associated with a 1.1 mV oxidation of plasma E_h Cys/CySS. Analysis of the association between plasma Cys and IL-1β revealed a significant negative association indicating that low levels of plasma Cys correlate with high IL-1β levels (**Figure 3.6B**; P < 0.05). While an association does not establish causality, together with our *in vitro* and *in vivo* data the present findings strongly suggest that Cys redox potential is an important determinant of IL-1β. Plasma TNF-α was also significantly associated with E_h Cys/CySS (**Figure 3.6C**)

and CySS levels (**Figure 3.6D**) (P<0.05) suggesting that oxidized Cys redox potential is also a determinant of TNF-α.

Figure 3.6. IL-1β and TNF-α in plasma of healthy adults is increased in association with oxidized E_h Cys/CySS

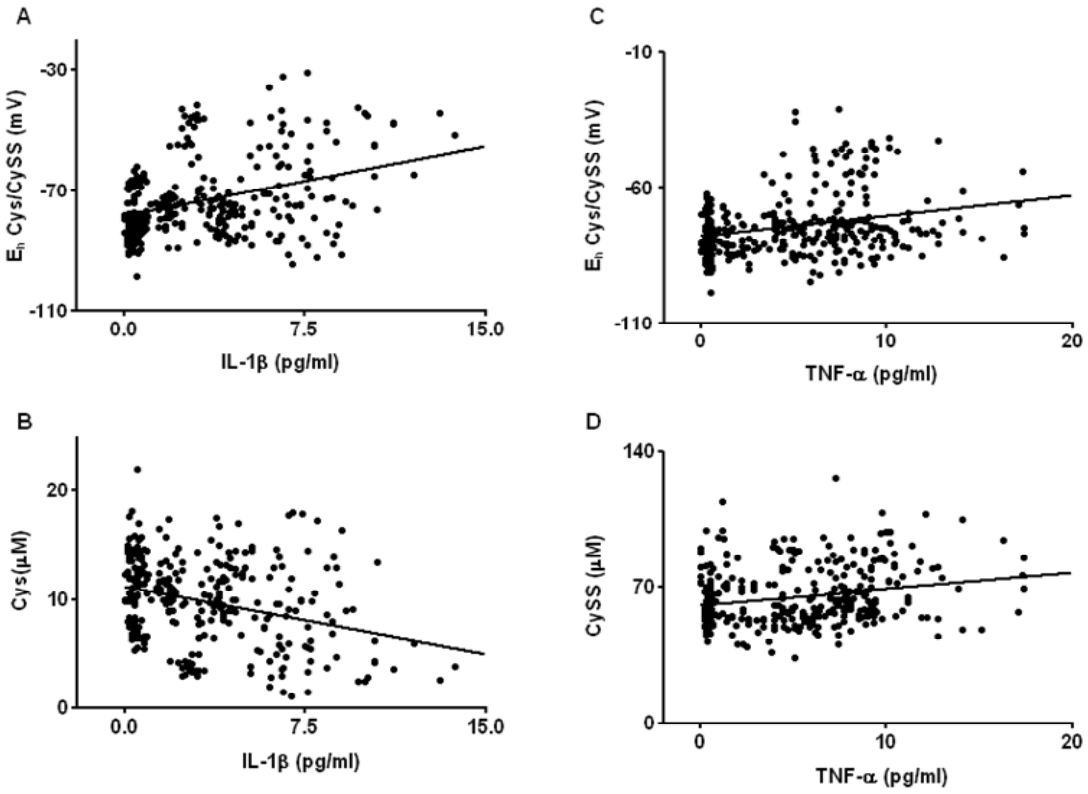


Figure 3.6. IL-1β and TNF-α in plasma of healthy adults is increased in association with oxidized E_h Cys/CySS. A linear mixed model was used to model variation in plasma E_h Cys/CySS and IL-1β and TNF-α. A strong positive association was observed between E_h Cys/CySS and IL-1β (A; P< 0.001). Plasma Cys was negatively associated with IL-1β (B; P<0.05). TNF-α was positively correlated with E_h Cys/CySS (C), and CySS (D) (P< 0.05).

5. DISCUSSION

The current study has three main findings: that oxidized extracellular E_h Cys/CySS is sufficient to induce IL-1β in a monocyte cell line; that preservation of plasma E_h Cys/CySS from oxidation during endotoxin-induced inflammation is associated with a decrease in circulating IL-1β levels in mice; and that oxidized E_h Cys/CySS is positively associated with circulating IL-1β levels in healthy humans. Together, these data suggest that plasma Cys redox potential is not only a biomarker of oxidative stress, but may also be a determinant of immune cell function. Because a number of dietary and behavioral risk factors for disease are known to oxidize Cys redox potential e.g., sulfur amino acid deficiency (Nkabyo, Gu et al. 2006), alcohol (Yeh 2007), and smoking (Moriarty, Shah et al. 2003); this provides a mechanistic basis to consider monitoring Cys redox potential as a risk factor for pro-inflammatory states, and consider interventional strategies to control oxidation of Cys redox potential in pro-inflammatory diseases.

Previous studies have shown that induction of IL-1β by non-infectious extracellular stimuli, such as ATP and cigarette smoke condensate, occurs by activation of membrane-bound receptors (Cruz, Rinna et al. 2007; Doz, Noulin et al. 2008). The mechanism by which oxidized extracellular E_h Cys/CySS induces IL-1β is unknown, but likely involves the generation of intracellular ROS signaled, in part, via oxidation of membrane-bound thiols. Studies in endothelial cells have revealed increased oxidation of cell-surface protein thiols, induction of intracellular ROS, and activation of NF-κB in response to an oxidizing extracellular E_h Cys/CySS (Go and Jones 2005). The rapid and sustained induction of cellular ROS by oxidized E_h in the present study is consistent with

the same type of mechanism in U937 cells, i.e., the increase in IL-1β by oxidized E_h could be signaled via the membrane and involve ROS-dependent activation of pro-inflammatory transcription factors such as NF-κB. Interestingly, the increase in ROS by oxidized E_h occurred in the absence of changes to intracellular E_h GSH/GSSG. This observation is, however, not unexpected. The major thiol/disulfide control systems GSH, thioredoxin, and Cys exist under non-equilibrium conditions and their redox states are distinctly modified by oxidative stress (Moriarty, Shah et al. 2003), substrate availability (Nkabyo, Gu et al. 2006; Anderson, Iyer et al. 2007; Go, Ziegler et al. 2007), physiological (Blanco, Ziegler et al. 2007) and pathological stimuli (Hansen, Go et al. 2006; Go, Ziegler et al. 2007). Indeed, the present research shows that the oxidation of plasma E_h Cys/CySS during acute endotoxemia occurs without the oxidation of plasma E_h GSH/GSSG.

The acute oxidation of E_h Cys/CySS by LPS is attenuated in mice supplemented with sulfur amino acids (SAA). This effect could be solely due to dietary augmentation of Cys reserves, an interpretation supported by the 1.4-fold higher plasma Cys concentrations in SAA-supplemented mice. However, plasma CySS does not increase in response to LPS suggesting the additional effects on ROS homeostasis due to SAA supplementation. The more reduced redox potential of the Cys/CySS couple in SAA-supplemented mice is associated with a significant decrease in circulating and tissue levels of IL-1β and TNF-α. These data suggest that Cys and associated Cys redox potential are critical determinants of cytokine production during activation of the immune system by LPS in mice.

In human nutrition, Cys is a conditionally essential amino acid because Cys requirements are normally met by the transulfuration of dietary methionine (Stipanuk, Dominy et al. 2006). However, Cys requirements increase during infection, injury, and in conditions associated with limited hepatic transulfuration (Hunter and Grimble 1994; Malmezat, Breuille et al. 2000). Because Cys is not routinely added to solutions used in parenteral therapy, patients with sepsis could be particularly susceptible to a deficiency of Cys. Studies in humans have shown that plasma Cys redox potential is modulated fairly rapidly by precursor availability (Blanco, Ziegler et al. 2007). Furthermore, in the present study we find that plasma Cys in humans in negatively associated with plasma IL-1β levels. Thus, nutritional supplementation with Cys or Cys precursors during early sepsis may be a strategy to alleviate acute inflammation and associated tissue injury.

In addition to the pathology associated with disregulated cytokine production in conditions such as sepsis; elevated cytokine levels in healthy individuals independently predict risk of chronic diseases such as type II diabetes and atherosclerosis (Cesari, Penninx et al. 2003; Spranger, Kroke et al. 2003). Therefore, the association between oxidized E_h Cys/CySS and IL-1β and TNF-α in otherwise healthy individuals suggests that oxidized E_h Cys/CySS may represent a risk factor for chronic inflammatory diseases. Accordingly, maintenance of Cys redox potential may be critical in protecting against subclinical inflammation in healthy individuals, and in ameliorating pathological processes associated with chronic inflammation. Large population studies with detailed measurements of known factors affecting redox potential along with pro-inflammatory cytokine markers and disease risk factors are needed to test this concept. Such data are critical because assays are available to assess plasma Cys redox potential in humans

(Jones 2002; Johnson, Strobel et al. 2008), and simple and inexpensive interventional strategies are available which could improve Cys redox potential (Moriarty-Craige, Adkison et al. 2005; Moriarty-Craige, Ha et al. 2007).

The association between oxidative stress and inflammation is well-recognized and multiple studies have shown that antioxidants such N-acetyl cysteine, have anti-inflammatory effects. The present observations identify a mechanistic link between oxidative stress and inflammation. The combined *in vitro* and *in vivo* observations show that oxidized extracellular E_h Cys/CySS is a previously unrecognized modulator of IL-1β. The findings suggest that strategies to preserve E_h Cys/CySS may represent a means to control IL-1β in inflammatory disease states

CHAPTER IV

OXIDATION OF EXTRACELLULAR CYSTEINE/CYSTINE REDOX STATE IN BLEOMYCIN-INDUCED LUNG FIBROSIS

Idiopathic Pulmonary Fibrosis (IPF) is a progressive fibrotic disorder characterized by structural alterations in the lung parenchyma which result in air space occlusion and reduced lung compliance. There are currently no effective therapies that can reverse the fibrotic process in IPF or prevent its progression. Thus, there is considerable interest in understanding the mechanisms responsible for triggering and sustaining lung fibrosis.

Oxidation of thiols is implicated in the pathogenesis of IPF, and strategies to attenuate lung fibrosis in IPF patients include administration of thiol antioxidants, such as N-acetylcysteine. However, the mechanisms linking oxidation of the thiol/disulfide redox environment to the fibrotic process remain incompletely understood. Recently, Ramirez and colleagues reported that lung fibroblasts showed increased proliferation and upregulation of fibronectin expression in response to an oxidized extracellular E_h Cys/CySS. Additionally in Chapter II, we find that Cys/CySS redox state is oxidized with lung injury, a purported upstream event in fibrosis. This raises the possibility that oxidation of E_h Cys/CySS may represent a pathogenic mechanism in fibrosis. In the present study, we investigated the dynamics of Cys/CySS redox state in a mouse model of bleomycin-induced lung fibrosis. This study is the first to our knowledge to investigate the regulation of the Cys pool in lung fibrosis.

****NOTE: This chapter is presented as written for: Iyer S, Ramirez A, Ritzenthaler J, Gonzalez-Torres E, Roser-Page S, Mora AL, Brigham K, Jones DP, Roman J, and Rojas M (2008) Am J Physiol Lung Cell Mol Physiol. Used with permission.**

1. ABSTRACT

Several lines of evidence indicate that depletion of glutathione (GSH), a critical thiol antioxidant, is associated with the pathogenesis of Idiopathic Pulmonary Fibrosis (IPF). However, GSH synthesis depends on the amino acid cysteine (Cys) and relatively little is known about the regulation of Cys in fibrosis. Cys and its disulfide, cystine (CySS) constitute the most abundant low-molecular weight thiol/disulfide redox couple in the plasma, and the Cys/CySS redox state (E_h Cys/CySS) is oxidized in association with age and smoking, known risk factors for IPF. Furthermore, oxidized E_h Cys/CySS in the culture media of lung fibroblasts stimulates proliferation and expression of transitional matrix components. The present study was undertaken to determine whether bleomycin-induced lung fibrosis is associated with a decrease in Cys and/or an oxidation of the Cys/CySS redox state, and to determine whether these changes were associated with changes in E_h GSH/glutathione disulfide (GSSG). We observed distinct effects on plasma GSH and Cys redox systems during the progression of bleomycin-induced lung injury. Plasma E_h GSH/GSSG was selectively oxidized during the pro-inflammatory phase, while oxidation of E_h Cys/CySS occurred at the fibrotic phase. In the epithelial lining fluid, oxidation of E_h Cys/CySS was due to decreased food intake. Thus, the data show that decreased precursor availability and enhanced oxidation of Cys each contribute to the oxidation of extracellular Cys/CySS redox state in bleomycin-induced lung fibrosis.

2. INTRODUCTION

Idiopathic Pulmonary Fibrosis (IPF) is a progressive fibrotic disorder characterized by structural alterations in the lung parenchyma due, in part, to excessive fibroblast proliferation and deposition of extracellular matrix components such as collagen and fibronectin (Gross and Hunninghake 2001). Several lines of evidence indicate that perturbations in glutathione (GSH) homeostasis are related to the pathogenesis of IPF. Observations in IPF patients demonstrate a marked decrease in epithelial lining fluid (ELF) GSH levels (Cantin, Hubbard et al. 1989; Rahman, Skwarska et al. 1999). Additionally, *in vitro* studies show that fibroblast proliferation is suppressed at optimal extracellular GSH levels, and inhibition of GSH synthesis stimulates transforming growth factor-β1 (TGF-β1)-mediated collagen synthesis (Cantin, Larivee et al. 1990; Liu, Liu et al. 2004). Together, these data support a mechanistic role for altered GSH homeostasis in the fibrotic process. However, GSH synthesis depends on the amino acid cysteine (Cys) and relatively little is known about the regulation of Cys in fibrosis.

Cys and its disulfide cystine (CySS), and GSH and glutathione disulfide (GSSG), comprise the major low-molecular weight thiol/disulfide redox control systems in mammals (Jones 2006). These control systems are compartmentalized; GSH/GSSG provides control mechanisms within cells, while Cys/CySS predominates in the extracellular fluid. Recent advances in redox signaling mechanisms have revealed that the redox states (E_h) of these couples are not in equilibrium with each other and have distinct regulatory functions (Jones, Go et al. 2004; Jones 2006; Jones 2008). We have shown that oxidized E_h Cys/CySS in the culture media of lung fibroblasts stimulates

proliferation and expression of transitional matrix components such as fibronectin, and this occurs in the absence of apparent changes to the GSH pool (Ramirez, Ramadan et al. 2007). Additionally, E_h Cys/CySS is oxidized in association with age and smoking, known risk factors for IPF (Jones, Mody et al. 2002; Moriarty, Shah et al. 2003). Thus, oxidation of Cys/CySS redox state may represent a hitherto unidentified pathogenic mechanism in lung fibrosis.

The purpose of the present study was to determine whether bleomycin-induced fibrosis is associated with a decrease in Cys and/or an oxidation of the Cys/CySS redox state, and to determine whether these changes were associated with changes in E_h GSH/GSSG. Mice received bleomycin intratracheally and GSH and Cys redox states were measured at time points known to correlate with the pro-inflammatory and pro-fibrotic phases of lung injury. To control for the effect of food intake on Cys concentrations, responses in bleomycin-treated mice were compared to pair-fed, saline-treated controls. We observed distinct effects on plasma GSH and Cys redox systems during the progression of bleomycin-induced lung injury. Plasma E_h GSH/GSSG was selectively oxidized during the pro-inflammatory phase, while oxidation of E_h Cys/CySS occurred at the fibrotic phase. Interestingly, E_h Cys/CySS in the epithelial lining fluid was substantially more oxidized than the plasma pool and was due, entirely, to bleomycin-related decrease in food intake. Thus, the data show that decreased precursor availability and enhanced oxidation of Cys each contribute to the oxidation of extracellular Cys/CySS redox state in bleomycin-induced lung fibrosis.

3. MATERIALS AND METHODS

3.1. Materials. All chemicals were purchased from Sigma Chemical Corporation (Sigma, St. Louis, MO), except where indicated. Distilled, deionized water was used for analytical purposes. HPLC quality solvents were used for HPLC.

3.2. Experimental animals and pair-feeding. Experiments were conducted using 10-14 week old, female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). Mice were housed individually for pair-feeding and maintained on a 12-h light-12-h dark cycle at the Division of Animal Resources at Emory University. All animals were fed pelleted rodent food (Test Diet 5015, Lab Diet Inc., Richmond, IN) and had free access to water. Nesting material was presented daily to each mouse to compensate for the absence of other animals (Belz, Kennell et al. 2003). All experiments were initiated during the light cycle. Measurement of food intake in bleomycin-treated mice was performed by providing mice with a weighed food pellet at day 0 and manually recording weight of the remaining food the next day. Fresh, weighed food was provided daily. The amount of food ingested by bleomycin-treated mice was averaged, and this amount was provided to pair-fed PBS controls. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Emory University.

3.3. Bleomycin administration. Bleomycin, dissolved in sterile PBS, was administered intratracheally at a dose of 3.2 U bleomycin/kg body weight under ketamine-xylazine anesthesia. Animals were sacrificed at 1, 3, 7, and 14 days post-

bleomycin. Pair-fed PBS animals received intratracheal injection of PBS and were sacrificed at corresponding time points.

3.4. Sample collection and analysis of Cys, CySS, GSH and GSSG. Samples were collected using a method optimized to minimize errors due to collection and processing (Jones, Carlson et al. 1998). Mice were anesthetized by isofluorane inhalation (Baxter Pharmaceuticals, Deerfield, IL) and blood was collected by submandibular bleeding using a 4 mm mouse bleeding lancet (Medipoint, Inc. Mineola, NY).

Because red blood cell GSH levels are approximately two orders of magnitude greater than plasma GSH levels, 1 % hemolysis can result in an increase of about 7 μM GSH in the plasma. To minimize artificial overestimation of GSH due to hemolysis, blood was collected into a heparin-coated eppendorf tube to inhibit coagulation. We have determined that for mouse plasma a > 0.2% hemolysis is visually detected. Consequently, samples were evaluated visually for hemolysis and hemolyzed samples were not included in the analysis.

The collected blood was (0.18 ml) was immediately transferred to 0.02 ml of preservation solution. The preservation solution included heparin, serine.borate to inhibit degradation of GSH by γ-glutamyltranspeptidase, bathophenanthroline disulfonate to inhibit oxidation of GSH and Cys, and iodoacetic acid to alkylate GSH and Cys. To facilitate quantification of the thiols and disulfides, γ-glutamyl glutamate (γ-Glu-Glu) was used an internal standard (Jones 2002). Samples were centrifuged at 16000 g for 60 seconds to remove precipitated protein, and 0.1 ml of the supernatant was immediately transferred to an equal volume of ice-cold 10% (w/v) perchloric acid. Samples were

immediately stored at -80 °C and were derivatized with dansyl chloride within 1 month. Stability tests have shown that non-derivatized samples are stable for at least 2 months at -80 °C (Jones, Carlson et al. 1998).

Bronchoalveolar lavage fluid (BALF) was obtained after mice were euthanized. Briefly, 0.6 ml of sterile PBS was instilled into the lung via a tracheal incision and withdrawn with gentle suction. This procedure took approximately 10 minutes to complete. The collected BALF was centrifuged at 200 g for 6-7 minutes. Cell-free supernatant (0.15 ml) was mixed with an equal volume of ice-cold 10% (w/v) perchloric acid containing 20 μM γ-Glu-Glu and stored at -80 °C until derivatization with iodoacetic acid and dansyl chloride.

For HPLC analysis (Gilson Medical Electronics, Middleton, WI), derivatized samples were centrifuged, and 50 μl (plasma) or 65 μl (BALF) of the aqueous layer was applied to the Supercosil LC-NH_2 column (25 cm x 4.6 mm; Supelco, Bellefunk, PA). Derivatives were separated with a sodium acetate gradient in methanol/water and detected by fluorescence (Jones, Carlson et al. 1998). Concentrations of thiols and disulfides were determined by integration relative to the internal standard. Redox states (E_h) of the GSH/GSSG and Cys/CySS pools were calculated from concentrations of GSH, GSSG and Cys, CySS in molar units with the following forms of the Nernst equation for pH 7.4: GSH/GSSG, $E_h = -264 + 30 \log ([GSSG]/[GSH]^2)$, Cys/CySS, $E_h = -250 + 30 \log ([CySS]/[Cys]^2)$(Jones, Carlson et al. 2000).

3.5. Urea measurements. Because urea diffuses readily through the body, plasma and ELF urea concentrations are identical (Rennard, Basset et al. 1986). Therefore,

estimates of Cys and CySS levels in the epithelial lining fluid were obtained after normalization for dilution using the urea dilution factor. The urea dilution factor is equal to $[urea]_{plasma}/[urea]_{BALF,}$ and estimation of BALF dilution using this method is considered to be reasonably accurate (Rennard, Basset et al. 1986; Yeh, Burnham et al. 2007). To minimize variability of recovery, dwell times for saline were maintained at less than 2 minutes under all experimental conditions. The concentration of urea in plasma and BALF was measured using a urea assay kit with a sensitivity range of 0.08 to 100 mg/dL (BioAssay Systems, Hayward, CA, USA).

3.6. Histopathology. Lungs were fixed by intratracheal instillation of neutral buffered formalin (10%). After further fixation overnight at room temperature, the tissue was embedded in paraffin, sectioned, and stained by hematoxylin and eosin (H&E). Masson's trichrome stain was used to detect collagen deposition. All sections were studied by light microscopy. For immunohistochemistry, fibronectin rabbit polyclonal antibody (Sigma), and anti-TGF-β1 antibody (Sigma) were used.

3.7. Cell culture. Primary fibroblasts were isolated from the lungs of 8-10 week old wild type C57BL/6 or from transgenic mice expressing the full length human fibronectin promoter-luciferase reporter gene construct as previously described (Tomic, Lassiter et al. 2005). Cells were maintained under 5% CO_2 at 37°C in DMEM supplemented with 10 U/ml penicillin, 10 μg/ml streptomycin, and 10% fetal bovine serum (MediaTech, Manassas, VA), but were transferred to serum-free media for 12-24 h prior to experimental manipulations.

3.8. Statistical Methods. Data are presented as means + SEM. Statistical analysis was done using SAS v 9.1 (SAS Institute Inc., Cary, NC, US). Analyses were performed using a Generalized Linear Model utilizing two-way ANOVA with time and treatment specified as the main effects and (time)·(treatment) as the interaction term. Tukey-Kramer post-hoc analysis was used for all comparisons with P value < 0.05.

4. RESULTS

4.1. Effect of bleomycin on lung histology, food intake, and weight loss. The pathophysiology of bleomycin-induced lung injury has been characterized in prior studies (Rojas, Xu et al. 2005; Moore and Hogaboam 2008). Endotracheal instillation of bleomycin leads to lung fibrosis and occurs in three stages. Bleomycin-induced cytotoxicity leads to apoptosis and necrosis of alveolar epithelial cells followed by an inflammatory phase that peaks at day 7 and is characterized by infiltration of neutrophils, and lymphocytes in the lung. An aberrant repair and remodeling process ensues, resulting in enhanced deposition of matrix molecules such as collagen which peaks at day 14.

Figure 4.1A shows photomicrographs of lung sections from untreated controls and at day 7 and day 14 after bleomycin treatment. In the left hand panel, lung sections from bleomycin-treated mice stained with hematoxylin and eosin (H&E) showed a progressive loss of normal pulmonary architecture with inflammatory cell infiltration and edema at day 7. By day 14, there is a partial clearance of inflammation. In the right hand panel, sections were stained with Masson's trichrome to highlight the presence of

collagen. At day 7 after bleomycin, modest amounts of collagen were present along with inflammatory cells around airways. Lungs from mice at day 14 after bleomycin stained extensively for aniline blue indicating increased collagen deposition.

Because food intake is an important determinant of Cys homeostasis (Nkabyo, Gu et al. 2006), we designed the study to control for food intake by pair-feeding the control group to the bleomycin-treated group. Bleomycin administration significantly decreased food intake within day 1 of treatment (**Figure 4.1B**; untreated controls, 5.11 $\pm$ 0.41 g versus bleomycin-treated mice at day 1, 1 $\pm$ 0.1 g; $P < 0.0001$), and intake remained substantially decreased at all subsequent time points measured (day 7, 3.5 $\pm$ 0.3 g; day 14, 3.2 $\pm$ 0.8g; $P < 0.01$, compared to untreated controls).

In **Figure 4.1C**, decline in body weight is shown for bleomycin-treated animals and pair-fed PBS controls. There was a linear decrease in body weight from day 1 to day 7 post-bleomycin (% weight loss at day 1, 6.38 $\pm$ 0.53 versus day 7, 19.01 $\pm$ 1.5; $P < 0.001$) and weight loss remained stable thereafter. No statistically significant differences in weight loss were observed between pair-fed control mice and bleomycin-treated animals (treatment effect, $P = 0.144$; time*treatment effect, $P = 0.45$), indicating that weight loss occurred as a consequence of decreased food intake.

Figure 4.1. Effect of bleomycin on lung histology, food intake, and weight loss

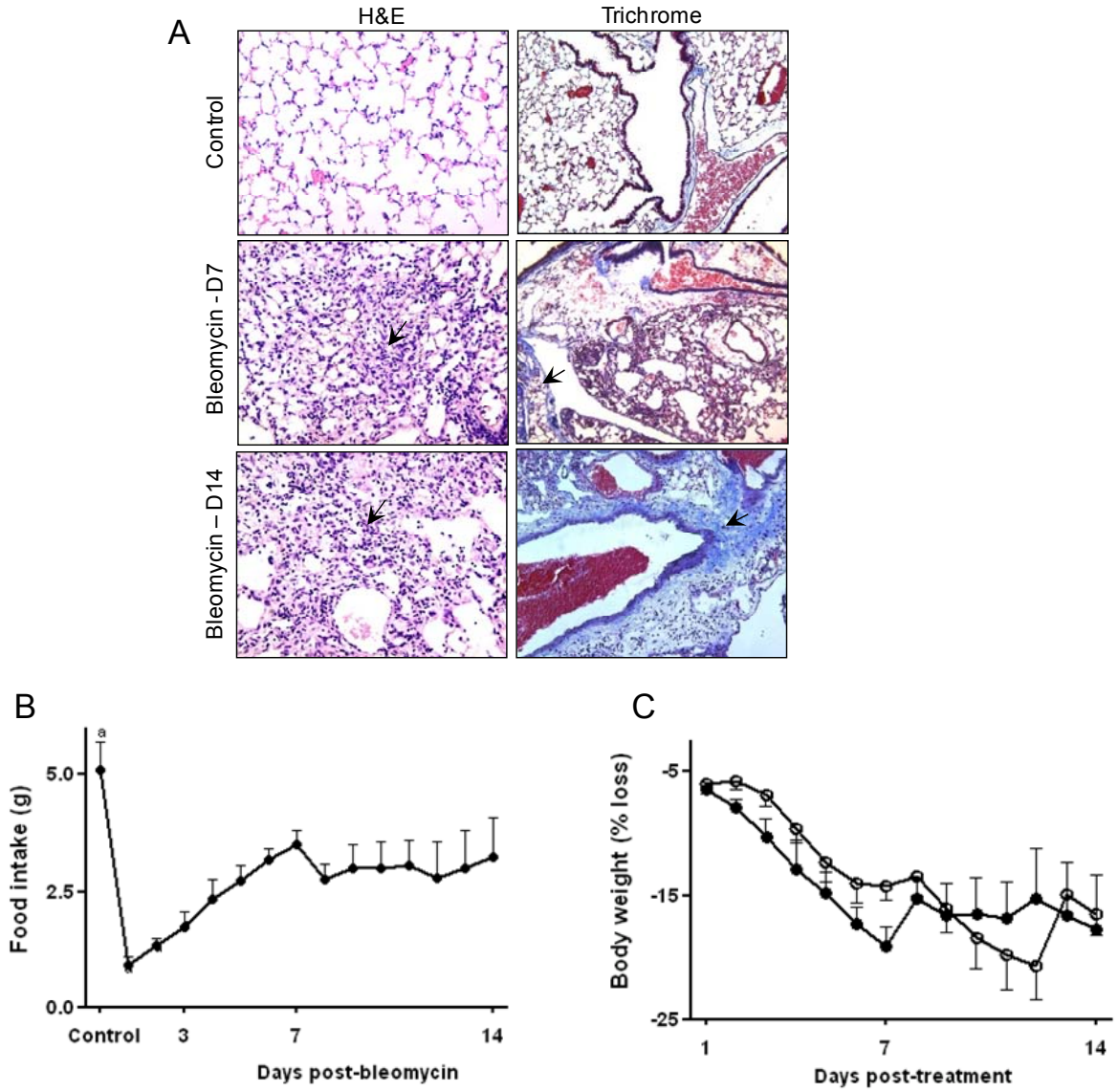


Figure 4.1. In A, lung sections show extensive inflammation and disruption of alveolar architecture in bleomycin-treated animals at day 7 (arrow, middle left hand panel). By day 14, there is a partial clearance of inflammation but cellular infiltrates are still visible (arrow, bottom left hand panel). Lung sections stain extensively for aniline blue day 14 after bleomycin, demonstrating extensive collagen deposition (arrow, bottom right hand panel) (magnification x40). Food intake was measured in bleomycin-treated mice daily for 14 days (B). Food intake in pair-fed controls was restricted to amount of food consumed by bleomycin-treated animals. In (C) weight loss in bleomycin-treated mice and time-matched pair-fed PBS controls is presented as % decline in body weight compared to weight at baseline. Data points and error bars correspond to the mean + SEM. [a] $P<0.001$ compared with bleomycin treated animals.

4.2. Oxidation of plasma GSH/GSSG redox state coincides with the inflammatory phase of bleomycin-induced lung injury. We next determined whether GSH levels are decreased after bleomycin administration, and whether similar responses are observed in pair-fed controls. A significant decline in plasma GSH levels was observed at day 7 coinciding with the pro-inflammatory phase of lung injury (**Figure 4.2A**; untreated controls, 24 $\pm$ 0.21 μM; pair-fed day 7, 22.5 $\pm$ 1.6 μM; bleomycin day 7, 13.5 $\pm$ 2.4 μM; $P < 0.05$ compared to untreated controls and pair-fed controls). The decrease in plasma GSH at day 7 caused a significant oxidation of E_h GSH/GSSG by an average of 15 mV (**Figure 4.2C**; untreated controls, -149 $\pm$ 0.3 mV; pair-fed day 7, -151.2 $\pm$ 1.1 mV; bleomycin day 7, -133.5 $\pm$ 4.9 mV, $P < 0.01$ compared to untreated controls and pair-fed controls). GSH levels completely recovered by day 14, leading to normalization of E_h GSH/GSSG.

Examination of the disulfide form, GSSG, revealed a trend for increase at day 1 and day 7 after bleomycin treatment. This increase was however not significant (**Figure 4.2B**; untreated controls, 2.3 $\pm$ 0.2 μM; bleomycin day 1, 3.5 $\pm$ 0.4 μM; bleomycin day 7, 3.6 $\pm$ 0.8 μM). At day 14, GSSG levels were 2.8 $\pm$ 0.5 μM. Thus, while GSH levels decreased significantly at day 7 with bleomycin treatment, no time or treatment-related effects were observed in plasma GSSG. The lack of significant increase in GSSG indicates that the activities of the γ-glutamyltranspeptidase and dipeptidases are sufficient to remove GSSG formed. Increased activity of these enzymes could also account for the decrease in plasma GSH at day 7. Examination of plasma GSH, GSSG, and E_h GSH/GSSG in pair-fed controls revealed no significant effect of decreased food intake on plasma E_h levels.

Figure 4.2. Oxidation of plasma GSH/GSSG redox state coincides with the inflammatory phase of bleomycin-induced lung injury

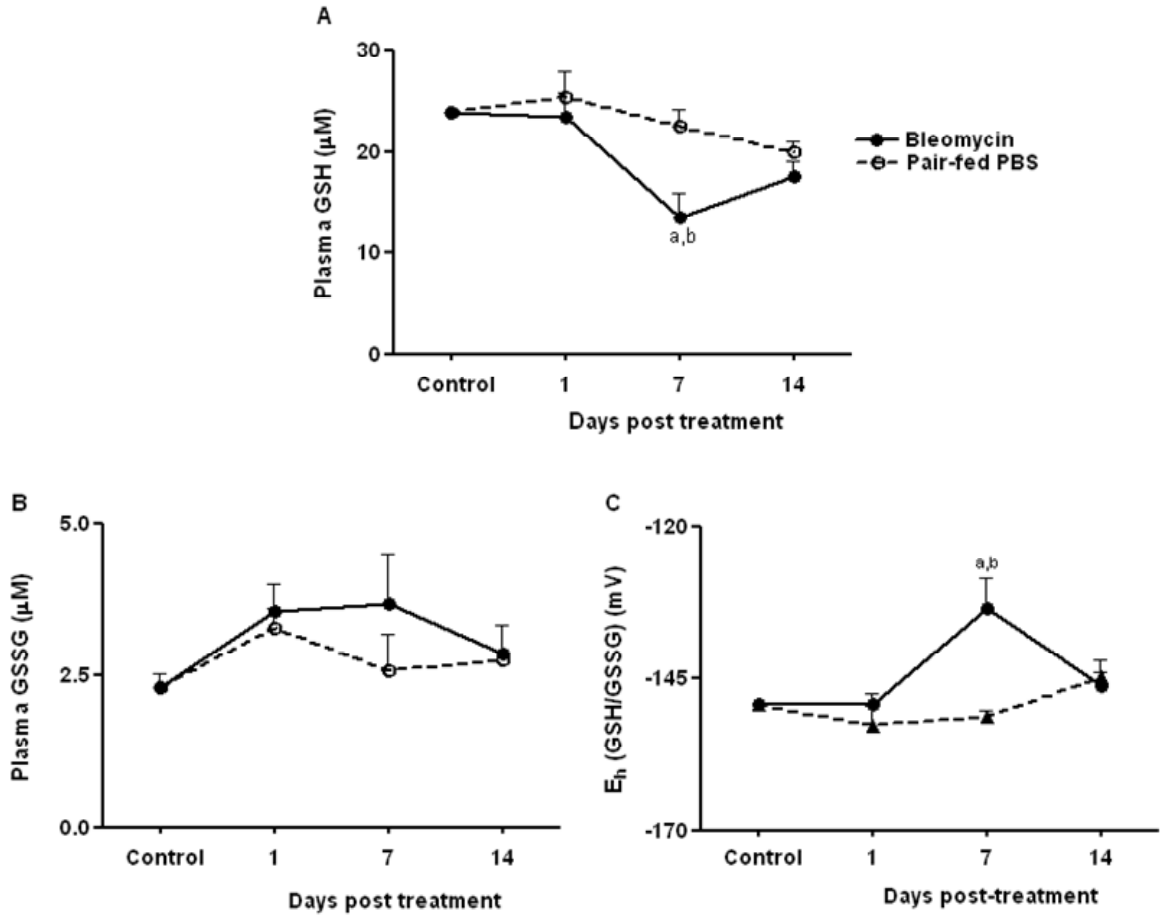


Figure 4.2. At day 1, 7 and 14 mice were sacrificed and plasma was collected for HPLC analysis of GSH (A) and GSSG (B). In (C), E_h GSH/GSSG was calculated from GSH and GSSG concentrations using the Nernst equation. Data are expressed as means + SEM. [a] Values significantly different from untreated controls**.** [b] Values significantly different from corresponding pair-fed controls

4.3. Oxidation of plasma Cys/CySS redox state coincides with the fibrotic phase of bleomycin-induced lung injury. Relatively little is known about the dynamics of the plasma Cys/CySS pool in fibrosis. Cys and CySS concentrations in the plasma were measured immediately after bleomycin administration at day 1, and at the peak pro-inflammatory (day 7) and pro-fibrotic phases (day 14) of lung injury. No significant differences in plasma Cys levels were observed at day 1 and day 7 post-bleomycin, but Cys levels dropped significantly at day 14 (untreated controls, 26.2 $\pm$ 0.08 μM; pair-fed day 14, 26.5 $\pm$ 1.2 μM; bleomycin day 14, 15.7 $\pm$ 1.3 μM, $P < 0.05$ compared to untreated controls and pair-fed controls) (**Figure 4.3A**). In contrast to the decrease in Cys, plasma levels of the disulfide, CySS, did not change with bleomycin treatment (**Figure 4.3B**; untreated controls, 73 $\pm$ 5.4 μM; bleomycin day 7, 66 $\pm$ 2.2 μM; bleomycin day 14, 64 $\pm$ 6.3 μM).

Despite the relative stability of plasma CySS, the decrease in plasma Cys resulted in oxidation of E_h Cys/CySS by an average of 20 mV at day 14 (untreated controls, -102.5 $\pm$ 1.9 mV; pair-fed day 14, 100 $\pm$ 1.7 mV; bleomycin day 14, -85 $\pm$ 3 mV, $P < 0.05$, compared to untreated controls and pair-fed controls) (**Figure 4.3C**). In pair-fed controls, E_h Cys/CySS did not change with time, except for an average 10 mV oxidation at day 1 (pair-fed controls day 1, 92 $\pm$ 1.2 mV; $P < 0.05$ compared to untreated controls) suggesting that oxidation of E_h Cys/CySS at day 14 was a consequence of the disease process and not related to food intake.

Figure 4.3. Oxidation of plasma Cys/CySS redox state coincides with the fibrotic phase of bleomycin-induced lung injury

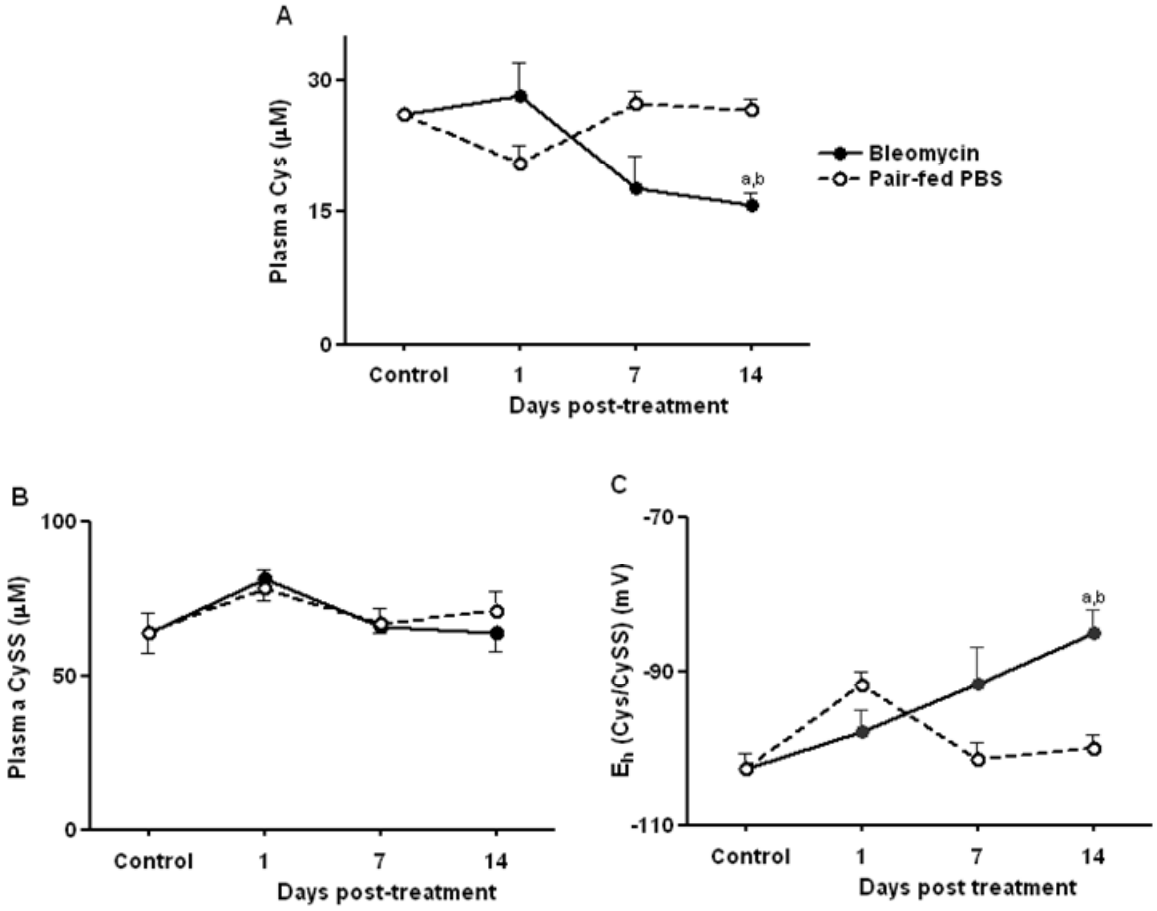


Figure 4.3. At day 1, 7 and 14 mice were sacrificed and plasma was collected for HPLC analysis of Cys (A) and CySS (B). In (C), E_h Cys/CySS was calculated from Cys and CySS concentrations using the Nernst equation. The total Cys pool is calculated as Cys+2*CySS (D). Data are expressed as means + SEM. [a] Values significantly different from untreated controls**.** [b] Values significantly different from corresponding pair-fed controls

4.4. Oxidation of E_h Cys/CySS in the lung epithelial lining fluid is due to bleomycin-related alterations in food intake. The decrease in GSH levels in epithelial lining fluid (ELF) in the bleomycin model is well-documented (Hagiwara, Ishii et al. 2000). Because Cys is a precursor for GSH and because the plasma pool and the ELF pool are independently regulated (Yeh, Burnham et al. 2007), we determined whether E_h Cys/CySS was oxidized in the ELF. Levels of Cys and CySS were determined in the ELF, immediately after sacrifice, at the peak pro-inflammatory and pro-fibrotic phases of bleomycin-induced lung injury. For these analyses, the ELF was collected into a preservation solution designed to prevent oxidation of Cys during storage. Levels of Cys and CySS are presented after correction for dilution using the urea dilution factor.

As shown in **Figure 4.4A**, Cys levels in the ELF rapidly declined within 24 hours, after bleomycin treatment (untreated controls, 33.6 $\pm$ 2.6 μM; bleomycin day 1, 2.5 $\pm$ 0.75 μM; $p < 0.0001$) and remained significantly below control values at all subsequent time points measured. No statistically significant differences in Cys levels were observed between bleomycin-treated animals and pair-fed controls at any time point (treatment effect, $P = 0.65$). The comparable decline in Cys in both treatment groups suggests that the decline in ELF Cys is a consequence of reduced food intake.

In contrast to Cys, levels of CySS increased after bleomycin-treatment (untreated controls, 40.4 $\pm$ 2.5 μM; bleomycin day 7, 267.8 $\pm$ 73.2 μM; bleomycin day 14, 362 $\pm$ 43 μM; $P < 0.0001$) (**Figure 4.4B**). CySS levels increased modestly in pair-fed controls (pair-fed day 7, 72 $\pm$ 9.4 μM; pair-fed day 14, 64.2 $\pm$ 7.2 μM) (Figure 4B). This increase was, however, not statistically significant suggesting that the increase in ELF CySS in

bleomycin-treated animals occurred as a consequence of the inflammatory and fibrotic process in response to lung injury.

Because the redox state of the Cys/CySS couple is driven by the absolute concentrations of Cys (Jones, Carlson et al. 2000), E_h Cys/CySS was oxidized equally in pair-fed controls and bleomycin-treated animals (**Figure 4.4C**; untreated controls, -108 $\pm$ 5.1 mV; bleomycin day 7, -37.2 $\pm$ 7.3 mV; bleomycin day 14, -60.9 $\pm$ 9.7 mV; pair-fed day 7, -55 $\pm$ 4.01 mV; pair-fed day 14, -52.5 $\pm$ 3.5 mV; $P < 0.0001$ compared to untreated controls).

Calculation of the total Cys pool (Cys + 2CySS) revealed a significant increase with bleomycin treatment at day 3 (**Figure 4.4D;** untreated controls, 113 + 6.1 μM; bleomycin day 3, 248 $\pm$ 32 μM; $P < 0.05$ compared to untreated controls), day 7 and 14 (bleomycin day 7, 541.5 $\pm$ 147 μM; bleomycin day 14, 742 $\pm$ 95.2 μM; $P < 0.0001$ compared to untreated controls and pair-fed controls).

Thus, oxidation of E_h Cys/CySS at day 7 and day 14 in bleomycin-treated animals was associated with an increase in total pool size, while in pair-fed controls; oxidation in E_h was associated without a major change in the total pool size. Taken together, the data show that plasma Cys/CySS is preserved in face of nutrient depletion, while in the lung lining fluid nutrient deprivation induces perturbations in Cys homeostasis.

Figure 4.4. Oxidation of E_h Cys/CySS in the lung epithelial lining fluid is due to bleomycin-related alterations in food intake

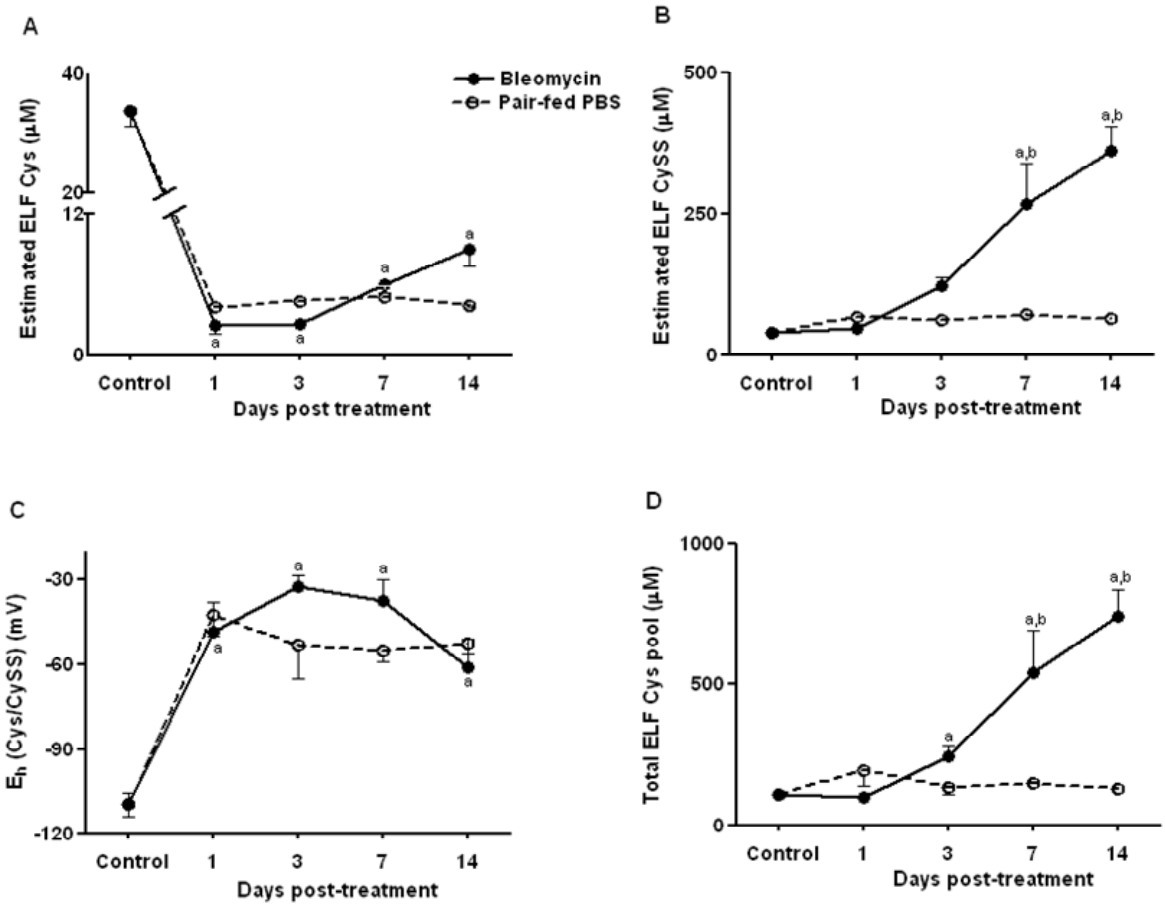


Figure 4.4. BALF was sampled from bleomycin-treated mice and pair-fed mice at time points shown. Levels of Cys (A) and CySS (B) in epithelial lining fluid are shown after correction using the urea dilution factor. In (C), E_h Cys/CySS was calculated from Cys and CySS concentrations using the Nernst equation. The total Cys pool is calculated as Cys+2*CySS (D). Data are expressed as means + SEM. [a] Values significantly different from untreated controls. [b] Values significantly different from corresponding pair-fed controls.

4.5. Temporal changes in lung fibrotic markers occur in bleomycin-treated mice, but not in pair-fed controls. Studies in pair-fed animals suggested that diet alone could result in an oxidized E_h Cys/CySS in the ELF. Therefore, we evaluated lung sections from pair-fed mice for fibrotic changes. As shown in **Figure 4.5A**, lung sections from pair-fed controls did not show increased staining for collagen compared to bleomycin treated animals at day 14. Furthermore, immunohistochemical analysis of lung sections showed no increase in fibronectin or TGF-β1 both at day 7 and day 14 (data not shown). As expected, lung sections from bleomycin-treated mice demonstrated a substantial increase in staining for fibronectin and TGF-β1 at day 14 (**Figure 4.5B**).

To confirm the lack of pro-fibrotic activity in the BAL of pair-fed controls, we examined fibronectin-inducing activity in the ELF of pair-fed versus bleomycin-treated animals. In these analyses ELF was not collected into a preservation solution, therefore the observed effects are not related to Cys/CySS redox state, but rather to the presence of pro-fibrotic mediators in the ELF. Fibroblasts transfected with a fibronectin luciferase reporter construct were stimulated with lavage fluid, and fibronectin expression was determined (**Figure 4.5C**). Fibronectin is one of the major extracellular matrix proteins involved in fibrosis, and increases substantially in the fibrotic lung (Limper and Roman 1992). Lavage fluid harvested from bleomycin-treated mice at day 7 and day 14 significantly increased activity of the fibronectin promoter ($P < 0.01$, compared to untreated controls and pair-fed controls). Furthermore, fibronectin bioactivity was greater at day 14 compared to day 7 after bleomycin ($P < 0.0001$). ELF obtained from pair-fed controls did not induce fibronectin expression.

Measurement of the pro-fibrotic cytokine interleukin (IL)-6 (Yoshida, Sakuma et al. 1995) in the BALF by ELISA revealed a significant increase in IL-6 at day 7 and day 14 in bleomycin-treated animals compared to untreated and pair-fed controls (**Figure 4.5D**; $P < 0.05$). In bleomycin-treated animals, IL-6 levels were greater at day 14 compared to day 7 ($P < 0.01$). We detected similar magnitude of differences in BALF fibronectin activity and IL-6 between bleomycin treated animals and pair-fed controls after normalization for dilution using the urea dilution factor. Thus, the data show that oxidation of E_h Cys/CySS in the ELF precedes maximal expression of collagen, fibronectin, TGF-β1 and IL-6 in bleomycin treated animals, but not pair-fed controls.

Figure 4.5. Temporal changes in lung fibrotic markers occur in bleomycin-treated mice, but not in pair-fed controls.

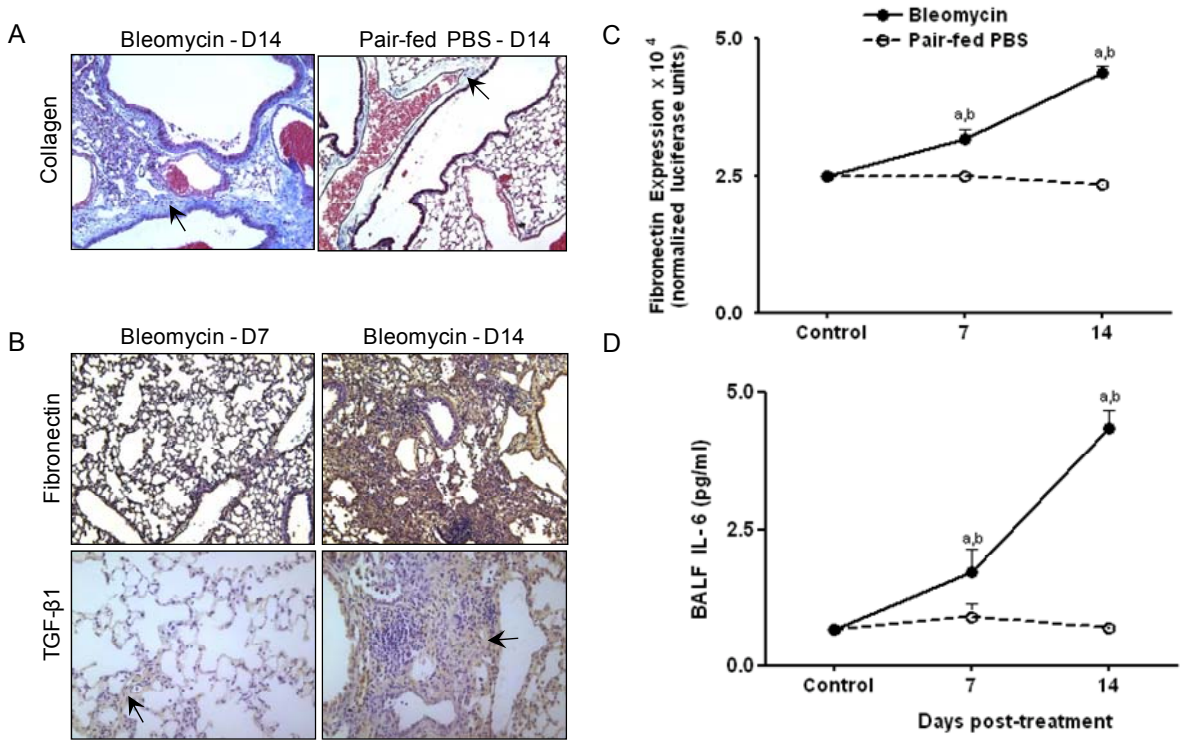


Figure 4.5. BALF was sampled from bleomycin-treated mice and pair-fed mice at time points shown. Levels of Cys (A) and CySS (B) in epithelial lining fluid are shown after correction using the urea dilution factor. In (C), E_h Cys/CySS was calculated from Cys and CySS concentrations using the Nernst equation. The total Cys pool is calculated as Cys+2*CySS (D). Data are expressed as means + SEM. [a] Values significantly different from untreated controls.
[b] Values significantly different from corresponding pair-fed controls.

5. DISCUSSION

Bleomycin is widely used to model lung fibrosis in mice (Moore and Hogaboam 2008). The cytotoxicity of bleomycin is mediated by two main structural components; a bithiazole ring which partially intercalates into the DNA helix and pyrimidine and imidazole moieties, which bind iron and oxygen to generate free radicals (Suzuki, Nagai et al. 1969; Yoshida, Sakuma et al. 1995). Studies investigating the pulmonary fate of intratracheal, tritiated bleomycin show that less than one percent of the radioactivity is retained at 24 h (Lazo and Pham 1984). This indicates that bleomycin is rapidly metabolized in the lung. Thus, the dynamics of GSH/GSSG and Cys/CySS redox states at days 7 and 14 (**Figures 4.2 – 4.4**) represent changes arising from bleomycin-induced lung injury rather than a direct oxidative effect of bleomycin.

The oxidation of plasma E_h GSH/GSSG at day 7, coinciding with the pro-inflammatory phase of lung injury, can be explained by perturbations in GSH homeostasis due to inflammation. A greater partitioning of sulfur amino acids for hepatic protein synthesis compared to GSH synthesis occurs during inflammation (Hunter and Grimble 1994), and could account for the decrease in plasma GSH at day 7. The role of GSH in the detoxification of reactive oxygen species (ROS) produced during the inflammatory response to injury could also lead to oxidation of E_h GSH/GSSG (Jaeschke 1992; Minamiyama, Takemura et al. 1996). Interestingly, the decrease in plasma GSH at day 7 is not associated with a corresponding decrease in the precursor Cys pool. This observation is, however, not unexpected. While Cys and GSH share a precursor-product relationship (Blanco, Ziegler et al. 2007), biochemical studies show that GSH can serve as a precursor for Cys (Meister 1988). *In vivo*, degradation of plasma GSH by γ-glutamyl

transpeptidase (γ GT) in the kidney yields cysteinyl glycine which is cleaved by dipeptidases to Cys (Meister 1988). γ GT is highly inducible by oxidative stress (Zhang, Forman et al. 2005), and increased degradation of GSH can contribute to a decrease in plasma GSH with an associated maintenance of plasma Cys.

By day 14, GSH levels recover leading to normalization of plasma E_h GSH/GSSG. The dynamics of the plasma GSH pool during the fibrotic phase of lung injury are in agreement with observations by Borok and Teramoto et al who reported that plasma GSH levels in IPF patients are not significantly different from healthy controls (Borok, Buhl et al. 1991; Teramoto, Fukuchi et al. 1995). While we observed no significant changes in plasma GSSG at day 14, GSSG levels are elevated in IPF patients (Teramoto, Fukuchi et al. 1995). This may be related to perturbations in GSH turnover, due to increased oxidant burden, associated with progressive fibrosis in human IPF.

In contrast to GSH, levels of Cys decrease at day 14. The decrease in plasma Cys and the associated oxidation of E_h Cys/CySS in bleomycin-treated animals, but not pair-fed controls indicates that perturbations in Cys homeostasis occur as a consequence of the fibrotic process. TGF-β1, a key fibrotic mediator, induces production of extracellular hydrogen peroxide in endothelial cells which could lead to oxidation of plasma Cys (Das and Fanburg 1991). However, plasma CySS levels do not change suggesting that mechanisms relating to increased transport of CySS into tissues may also be involved. Indeed, studies by Sato et al have shown the induction of specific CySS transporters in response to peroxide and other pro-oxidant stimuli (Bannai, Sato et al. 1991; Sato, Kuriyama-Matsumura et al. 2001). A role for TGF-β1-mediated transport of Cys into tissues to support increased protein synthesis cannot be excluded. In unpublished

observations, we have found that TGF-β1 induces depletion of extracellular Cys and CySS in a dose-dependent manner in lung fibroblasts. Indeed, increased protein synthesis during tissue repair places a higher demand on amino acid supply (Thomas 1997; Peng, Yan et al. 2005). Not-withstanding these possibilities, the decline in Cys levels resulted in a 20 mV oxidation of E_h Cys/CySS at day 14. This magnitude of oxidation is sufficient to cause a 5-fold change in the ratios of reduced to oxidized forms of proteins with vicinal dithiols (Jones 2002). Thus, substantial redox-dependent changes could occur for plasma or membrane proteins that interact with the Cys/CySS couple.

A significant observation is the disequilibrium in the redox states of plasma Cys/CySS and GSH/GSSG at day 7 and day 14. This suggests that the redox states of these major thiol/disulfide redox systems are regulated independently in the plasma, during inflammation and fibrosis. We recently reported that in cells subjected to Cys deficiency, oxidation of E_h Cys/CySS occurs in the absence of changes to E_h GSH/GSSG (Anderson, Iyer et al. 2007). Similarly, Banjac and colleagues found that over-expression of the cystine/glutamate antiporter, system x_c^-, protected lymphoma cells from apoptosis induced by GSH depletion, by a mechanism relating to increase in extracellular Cys, and not cellular GSH (Banjac, Perisic et al. 2007).

Interestingly, measurements of E_h Cys/CySS in the alveolar space reveal a more profound oxidation in the ELF compared to the plasma pool. Similar observations have been made in alcoholics where the E_h GSH/GSSG is oxidized by 40 mV in the lining fluid compared to a 20 mV oxidation in plasma (Yeh, Burnham et al. 2007). Additionally, E_h Cys/CySS in the ELF is oxidized prior to fibrosis. Together with previous observations in lung fibroblasts that oxidized E_h Cys/CySS stimulates fibronectin expression via

increase in TGF-β1, this indicates that oxidized ELF E_h Cys/CySS may contribute to lung fibrosis. However, ELF E_h Cys/CySS is also oxidized in pair-fed controls in the absence of fibronectin expression. These data would suggest that oxidized E_h Cys/CySS in the ELF is insufficient to promote fibrosis, but can contribute to fibrosis in the setting of injury, characterized by the destruction of basement membranes and the infiltration of fibroblasts into the alveolar spaces. Thus, oxidized E_h Cys/CySS may represent a predisposing state in the air-space that is active only after exposure to a "second hit" that compromises alveolar barrier integrity (**Figure 4.6**).

Figure 4.6. Potential mechanism by which oxidized Cys/CySS redox state can contribute to fibrosis in lung injury

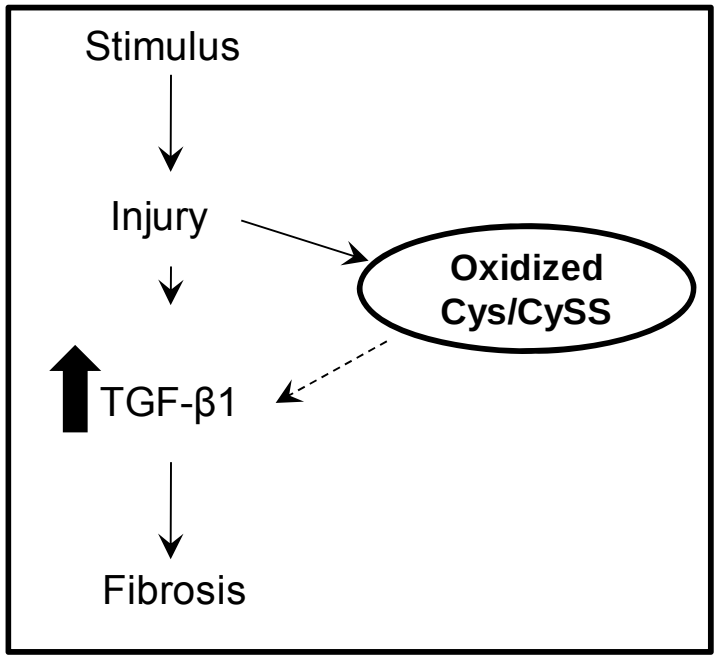


Figure 4.6. Oxidized E_h Cys/CySS may represent a target for "second hits" in the lung. Environmental factors such as poor nutrition trigger the oxidation of Cys/CySS redox state in the lung lining fluid. Age, smoking, alcohol, and disease represent potential contributing factors. While this is insufficient to induce the fibrosis *in vivo*, it sets the stage for a "second hit". This second hit, induced by lung injury, which also represents a stimulus for the oxidation of E_h Cys/CySS, is critical in dictating subsequent events including fibroblast proliferation and migration into the air-space. Here, fibroblasts come in contact with oxidized E_h Cys/CySS resulting in their activation, exaggerated production of TGF-β1 and transitional matrices such as fibronectin. Unopposed, this process can lead to lung fibrosis.

While E_h Cys/CySS is comparable between bleomycin-treated animals and pair-fed controls, an important distinction is the greater than 2-fold elevation in CySS levels with bleomycin treatment. The substantial increase in CySS is likely due to enhanced oxidation of extracellular Cys by ROS released from activated inflammatory cells sequestered in the air-space, and humoral factors such as tumor necrosis factor (TNF)-α

and TGF-β1 in the ELF (Rahman, Biswas et al. 2005). This elevation in CySS may be toxic to alveolar epithelial cells by mechanisms relating to oxidation of membrane and extracellular proteins and/or by increased efflux of intracellular glutamate via the x_c^- system (Bannai 1984; Jones 2006). However despite the elevation in CySS, the comparable decline in Cys in bleomycin-treated animals and pair-fed controls leads to a similar oxidation of E_h Cys/CySS. Because Cys enters the Nernst equation as a squared term, E_h Cys/CySS is largely driven by the absolute concentrations of Cys (Jones, Carlson et al. 2000). This indicates that dietary adequacy of Cys and Cys precursors is critical in maintaining the reducing capacity of the Cys/CySS redox couple in the ELF.

The decrease in Cys and GSH reserves is likely to play an important role in the inflammatory and fibrotic response to injury. Cys and GSH are well-established determinants of cytokine and growth factor production during activation of the immune system. For instance, oral pre-treatment of mice with GSH and N-acetyl cysteine (NAC), a Cys precursor, attenuates LPS-induced increase in TNF-α (Peristeris, Clark et al. 1992). Furthermore, alcohol-induced depletion of GSH is proposed to underlie the increased susceptibility of alcoholics to sepsis-induced acute lung injury (Holguin, Moss et al. 1998). The mechanistic role of E_h Cys/CySS in inflammatory and fibrotic cell responses is only beginning to be elucidated. *In vitro*, oxidized E_h Cys/CySS stimulates adhesion of leukocytes to the pulmonary endothelium (Iyer, Jones et al. 2008), sensitizes epithelial cells to apoptosis (Jiang, Moriarty-Craige et al. 2005), induces nuclear factor (NF)-κB in endothelial cells (Go and Jones 2005), and stimulates TGF-β1 expression in lung fibroblasts (Ramirez, Ramadan et al. 2007). Thus, oxidized E_h Cys/CySS can potentiate the inflammatory and fibrotic response to injury. The temporal association between

oxidation of ELF E_h Cys/CySS and induction of lung fibrotic markers in bleomycin-induced injury is consistent with this possibility. Consequently, maintenance of optimal Cys and GSH reserves during inflammation and fibrosis is likely to be beneficial. Indeed, multiple animal studies have consistently shown that NAC ameliorates the inflammatory and fibrotic response to bleomycin (Hagiwara, Ishii et al. 2000; Cortijo, Cerda-Nicolas et al. 2001; Serrano-Mollar, Closa et al. 2003). However, the early timing of the intervention and the lack of pharmacokinetic data on NAC complicates the interpretation of the results. Additional studies are required to confirm that the benefits of NAC in animal models relate to preservation of Cys and GSH redox systems.

In conclusion, the data show that decreased precursor availability and enhanced oxidation of Cys each contribute to the oxidation of extracellular Cys/CySS redox state in bleomycin-induced lung fibrosis. Consequently, oxidation of E_h Cys/CySS may be important in the pathogenesis of fibrosis. Further studies are needed to investigate whether preservation of E_h Cys/CySS represents a useful therapeutic target in IPF, and related fibrotic disorders.

CHAPTER V

EFFECT OF BONE MARROW-DERIVED MESENCHYMAL STEM CELLS ON ENDOTOXIN-INDUCED OXIDATION OF PLASMA CYSTEINE AND GLUTATHIONE

Bone marrow-derived mesenchymal stem cells (BMDMSC) are emerging as a therapeutic modality in various inflammatory disease states. A number of ongoing randomized Phase I/II clinical trials are evaluating the effects of BMDMSC infusion in patients with multiple sclerosis, graft versus host disease, Crohn's disease, and severe chronic myocardial ischemia. BMDMSC are also being considered as a potential therapy in patients with acute lung injury. Previous chapters in this dissertation highlight the intimate association between oxidation of plasma Cys and GSH redox systems and inflammation, during endotoxemia. In this final chapter, we examine whether BMDMSC infusion protects against endotoxin-induced oxidation of plasma Cys and GSH redox states.

1. ABSTRACT

An emerging therapy in various inflammatory diseases is the use of exogenously infused allogeneic or autologous bone marrow-derived mesenchymal stem cells (BMDMSC). Numerous studies have shown that BMDMSC attenuate systemic inflammation in response to endotoxin. As demonstrated in Chapter II in this dissertation, endotoxin induces a substantial oxidation of the extracellular thiol/disulfide redox environment.

In the present study we tested whether exogenously infused BMDMSC protect against endotoxin-induced oxidation of plasma Cys and GSH redox states. Mice received endotoxin intraperitoneally, followed by intravenous infusion of 5×10^5, CD 45-immunodepleted BMDMSC. Control mice received intraperioneal endotoxin followed by an equal volume of saline given intravenously. Results showed sequential preservation of plasma Cys and GSH redox states in response to BMDMSC infusion. The data provide the novel observation that BMDMSC infusion modulates thiol/disulfide redox status *in vivo.*

2. INTRODUCTION

The inflammatory response to pathogens, physical trauma, or toxic stimuli is critical in host defense, but excessive and unregulated inflammation can injure the lungs (Nathan 2002). In patients with gram negative sepsis; a disregulated inflammatory response to bacterial endotoxin increases the risk for acute lung injury (ALI), which can lead to severe respiratory failure termed the acute respiratory distress syndrome (ARDS) (Ware and Matthay 2000). ALI and ARDS are associated with significant morbidity and

mortality rates of greater than 30% (Ware and Matthay 2000). Consequently, strategies to attenuate the inflammatory response in ALI and ARDS are of considerable interest.

An emerging therapeutic modality in various inflammatory diseases is the use of bone marrow-derived mesenchymal stem cells (BMDMSC) (Iyer and Rojas 2008). BMDMSC are multipotent cells that can be isolated from the bone marrow and expanded in culture relatively easily. Several studies, including our own, have shown that exogenously infused BMDMSC protect against endotoxin-induced inflammation and lung injury in mice (Gupta, Su et al. 2007; Mei, McCarter et al. 2007; Xu, Woods et al. 2007). In these studies, the protective effects of BMDMSC are mediated by a decrease in circulating pro-inflammatory cytokine levels and appear to be independent of BMDMSC engraftment into the lung. Thus, BMDMSC infusion alters the systemic inflammatory milieu, increasing anti-inflammatory cytokines and mitigating pro-inflammatory cytokines. A hallmark of inflammation, and a consistent observation in patients with ALI, is a perturbation in the extracellular thiol/disulfide redox environment. However, little is known about the effects of BMDMSC on the systemic redox environment.

Cysteine (Cys) and its disulfide cystine (CySS) together with glutathione (GSH) and glutathione disulfide (GSSG) comprise the major extracellular thiol/disulfide redox control systems. Cys and GSH are important determinants of cytokine expression, and alteration in Cys and GSH metabolism is a central feature of inflammation. Because the thiol/disulfide redox environment is intimately linked to inflammation and tissue injury, the present study was undertaken to examine the effects of exogenous BMDMSC infusion on plasma Cys and GSH levels. Accumulating evidence shows that the redox states of Cys and GSH are independently regulated; therefore a secondary purpose of the

present study was to determine whether the two redox couples respond differently to BMDMSC infusion. Mice received endotoxin intraperitoneally, followed by intravenous infusion of 5 x 10^5, CD 45-immunodepleted BMDMSC. Results showed sequential preservation of plasma Cys and GSH redox states in response to BMDMSC infusion. The data provide the novel observation that BMDMSC infusion modulates thiol/disulfide redox status *in vivo.*

3. MATERIALS AND METHODS

3.1. Materials. Except as indicated, all chemicals were purchased from Sigma Chemical Corporation (Sigma, St. Louis, MO). Distilled, deionized water was used for analytical purposes. HPLC quality solvents were used for HPLC.

3.2. Experimental Animals. Experiments were conducted using 10-14 week old, female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). Mice were housed in cages and maintained on a 12-h light-12-h dark cycle at the Division of Animal Resources at Emory University. All animals were fed pelleted rodent food (Test Diet 5015, Lab Diet Inc., Richmond, IN) and had free access to water. All experiments were initiated during the light cycle. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

3.3. LPS Administration. Escherichia coli O55:B5 LPS, dissolved in sterile PBS was administered intraperitoneally at a dose of 1 mg LPS/kg body. Mice were subsequently anesthetized by isofluorane inhalation (Baxter Pharmaceuticals, Deerfield,

IL), and given either 5 x 10^5 BMDMSC in 0.1 ml PBS or 0.1 ml PBS intravenously using a 27-guage needle. Animals were sacrificed at 2, 6, and 24 h post LPS.

3.4. Sample collection and analysis of Cys, CySS, GSH and GSSG. Samples were collected using a method optimized to minimize errors due to collection and processing (Jones, Carlson et al. 1998). Mice were anesthetized by isofluorane inhalation (and blood was collected by submandibular bleeding using a 4 mm mouse bleeding lancet (Medipoint, Inc. Mineola, NY). To minimize artificial overestimation of GSH due to hemolysis, blood was collected into a heparin-coated eppendorf tube to inhibit coagulation. Samples that showed evidence of hemolysis were not included in the analysis. The collected blood was (0.18 ml) was immediately transferred to 0.02 ml of preservation solution. The preservation solution included heparin, serine.borate to inhibit degradation of GSH by γ-glutamyltranspeptidase, bathophenanthroline disulfonate to inhibit oxidation of GSH and Cys, and iodoacetic acid to alkylate GSH and Cys. To facilitate quantification of the thiols and disulfides, γ-glutamyl glutamate (γ-Glu-Glu) was used an internal standard (Jones 2002). Samples were centrifuged at 16000 g for 60 seconds to remove precipitated protein, and 0.1 ml of the supernatant was immediately transferred to an equal volume of ice-cold 10% (w/v) perchloric acid. Samples were immediately stored at -80 °C and were derivatized with dansyl chloride within 1 month. Stability tests have shown that non-derivatized samples are stable for at least 2 months at -80 °C (Jones, Carlson et al. 1998).

For HPLC analysis (Gilson Medical Electronics, Middleton, WI), derivatized samples were centrifuged, and 50 μl of the aqueous layer was applied to the Supercosil

LC-NH_2 column (25 cm x 4.6 mm; Supelco, Bellefunk, PA). Derivatives were separated with a sodium acetate gradient in methanol/water and detected by fluorescence (Jones, Carlson et al. 1998). Concentrations of thiols and disulfides were determined by integration relative to the internal standard. Redox states (E_h) of the GSH/GSSG and Cys/CySS pools, given in millivolts (mV), were calculated from concentrations of GSH, GSSG and Cys, CySS in molar units with the following forms of the Nernst equation for pH 7.4: GSH/GSSG, $E_h = -264 + 30 \log ([GSSG]/[GSH]^2)$, Cys/CySS, $E_h = -250 + 30 \log ([CySS]/[Cys]^2)$(Jonas, Farrell et al. 2000).

3.5. BMDMSC isolation and culture. Bone marrow-derived mesenchymal stem cells were obtained from the Tulane Center for Gene Therapy. BMDMSC were isolated from the tibias and femurs of C57BL/6J mice. The protocol for isolation and selective propagation of mesenchymal stem cells is described previously (Phinney, Kopen et al. 1999).

Cells were propagated in a 175 cm^2 flask in Iscove's modified Dulbeccos's medium (IMDM) containing 9% fetal bovine serum (FBS, Atlanta, Biologicals, Norcross, GA), 9% horse serum, and 1% penicillin-streptomycin in a humidified 5% CO_2 incubator at 37 ^oC. Cells were harvested at 70% confluency using 0.25% trypsin. Harvested cells were depleted of contaminating hematopoietic cells by magnetic immunodepletion with anti-CD45 conjugated to phycoerythrin (PE), followed by PE-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA). After negative depletion, cells were propagated for upto 10 passages. For *in vivo* infusion studies, cells were detached using 0.25% trypsin at 37 ^oC for 5 minutes. Trypsin was neutralized by adding IMDM with serum. The cell suspension was centrifuged and resuspended in sterile PBS (without Ca and Mg). After

two additional washes with PBS, cells were counted by trypan blue exclusion and were resuspended at 5 x 10^5 cells per 0.1 ml of PBS.

3.6. Flow cytometry. Flow cytometry for expression of a panel of surface markers was performed on a FACScan cytometer (Becton Dickinson) using standard techniques. BMDMSC were harvested by trypsinization, washed in PBS, and stained with the following antibodies: Fluorescein isothiocyanate (FITC) –anti-CD45, PE-anti-CD11b, PerCP-Cy5.5-anti-Sca-1 (Pharmigen). Acquired data was analyzed using FlowJo software (Tree Star, San Carlos, CA)

3.7. Differentiation assays. The ability of BMDMSC to differentiate into multiple mesenchymal lineages was determined using a mesenchymal stem cell functional identification kit (R&D Systems Minneapolis, MN USA). Adipogenic differentiation was induced by seeding cells for 14 days in α-MEM medium with 10% FBS, 1% penicillin-streptomycin, and adipogenic supplement containing hydrocortisone, isobutylmethylxanthine, and indomethacin. Oil droplets in the cultures were identified by staining cells with Oil Red O. Osteogenic differentiation was induced by culturing cells for 21 days in α-MEM medium with 10% FBS, 1% penicillin-streptomycin, and osteogenic supplement containing dexamethasone, ascorbate-phosphate, and β-glycerolphosphate. The calcium containing precipitates were visualized after staining with 2% Alizarin red adjusted to a pH of 4.4 with ammonium hydroxide. Chondrogenic differentiation was induced by pelleting cells in a 6-well plate and culturing in D-MEM/F-12 with 1% penicillin-streptomycin, and chondrogenic supplement containing

dexamethasone, ascorbate-phosphate, proline, pyruvate and TGF-β3. Pelleted cells were cultured for 14 days and stained with Alcian blue to visualize acid mucopolysachharides.

3.8. Statistical Methods. Data are presented as means + SEM. Statistical analysis was done using Graph Pad Prism v 4.01. An unpaired t-test was used to compare the LPS/Saline versus the LPS/BMDMSC groups at each of given time points. Significance was set at a P value < 0.05

4. RESULTS

4.1. Characterization of bone marrow-derived mesenchymal stem cells.

Enrichment of bone marrow-derived mesenchymal stem cells from crude bone marrow suspensions is achieved by selection for a plastic-adherent population that expresses neither hematopoietic nor endothelial cell surface markers but is positive for the expression of adhesion and stromal markers. However because a defined panel of unambiguous markers distinguishing MSC is lacking, a gold standard criterion for establishing MSC phenotype is a trilineage differentiation assay where the plasticity of MSC is confirmed by their ability to differentiate into adipocytes, osteocytes, and chondrocytes, on stimulation.

In the present study, we confirmed MSC phenotype by flow cytometry analysis and by differentiation assays. Analysis by flow cytometry demonstrated that the cells were uniformly negative for the hematopoietic markers, CD11b and CD45. Cells stained positive for Stem cell antigen (Sca)-1 (**Figure 5.1A-C**). Differentiation assays

demonstrated that BMDMSC retained their ability to differentiate into adipocytes, osteocytes, and chondrocytes (**Figure 5.1 D-E**).

Figure 5.1. Characterization of BMDMSC by flow cytometry and differentiation capacity

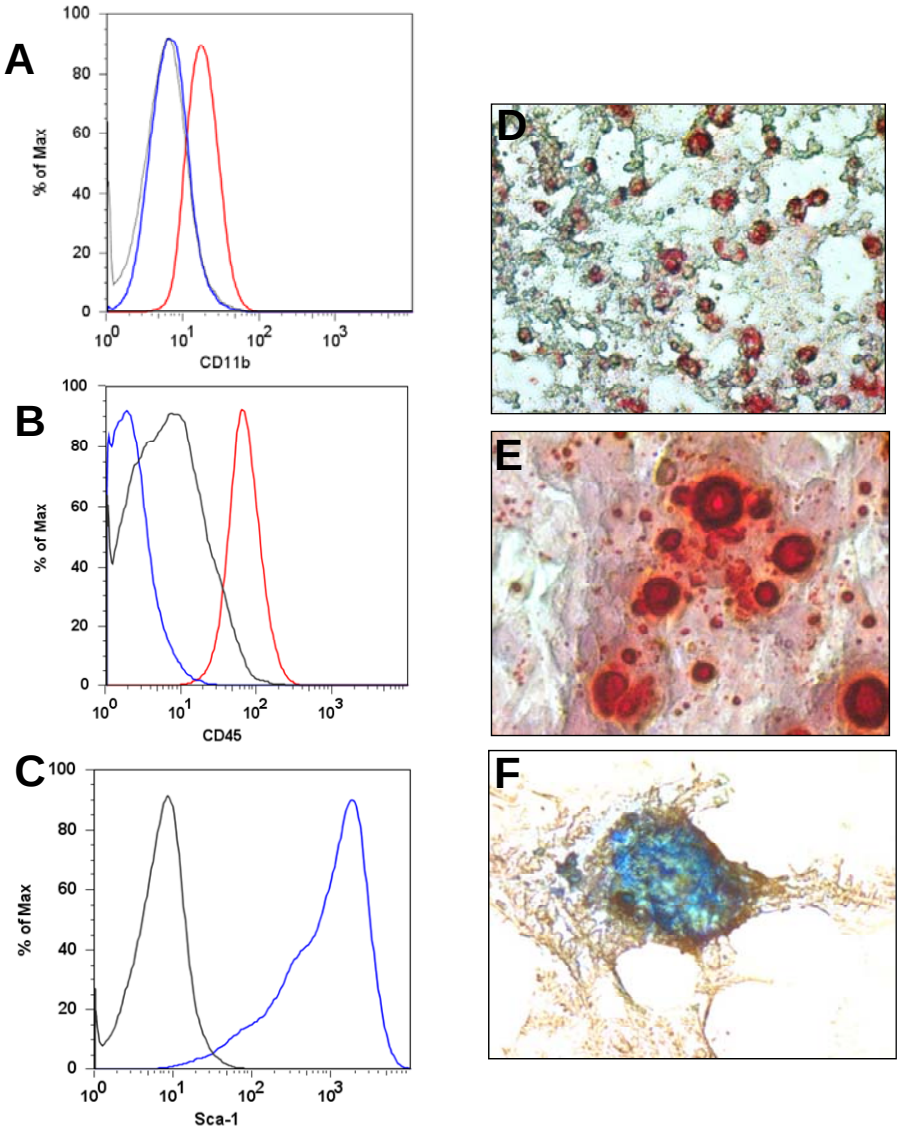


Figure 5.1. Characterization of BMDMSC by flow cytometry and differentiation capacity. Flow cytometry of BMDMSC demonstrated that cells did not express the hematopoietic markers, CD11b and CD45 (A-B; blue, BMDMSC; grey, isotype control, red, alveolar macrophages). BMDMSC stained positive for Sca-1 (C; blue, BMDMSC; grey, isotype control). In D-E, cells were examined for differentiation capacity. Fat droplets in cell preparations incubated in adipogenic medium for 14 days were stained with Oil Red O (D). Cells incubated with osteogenic medium for 21 days were stained Alizarin red to detect Calcium (E). Cartilage pellets were stained with Alcian blue to detect mucopolysachharides, after 14 days in chondrogenic medium (C)

4.2. Effect of BMDMSC infusion on endotoxin-induced weight loss. In Chapter II, we found that endotoxin - induced weight loss occurred due to a decrease in food intake. Furthermore, decreased food intake was an important determinant of plasma Cys and GSH levels. In the present study, we determined loss in body weight 24 h post-treatment in mice treated with LPS/ Saline versus mice given LPS/BMDMSC. Mice receiving intraperitoneal saline followed by intravenous BMDMSC served as controls.

As shown in **Figure 5.2**, mice treated with Saline/BMDMSC did not show a significant decline in body weight (body weight (g), 0 h, 19.5 $\pm$ 0.6; 24 h, 19.2 $\pm$ 0.6). As expected, LPS/Saline treated animals lost a significant amount of body weight (0 h, 20.3 $\pm$ 0.5; 24 h, 18 $\pm$ 0.6 ; $P < 0.05$). However, body weight did not significantly decrease in mice treated with LPS/BMDMSC (0 h, 21 + 0.9; 24 h, 18.9 $\pm$ 0.7; $P = 0.05$), suggesting protection due to BMDMSC infusion.

Figure 5.2. Effect of BMDMSC infusion on endotoxin-induced weight loss

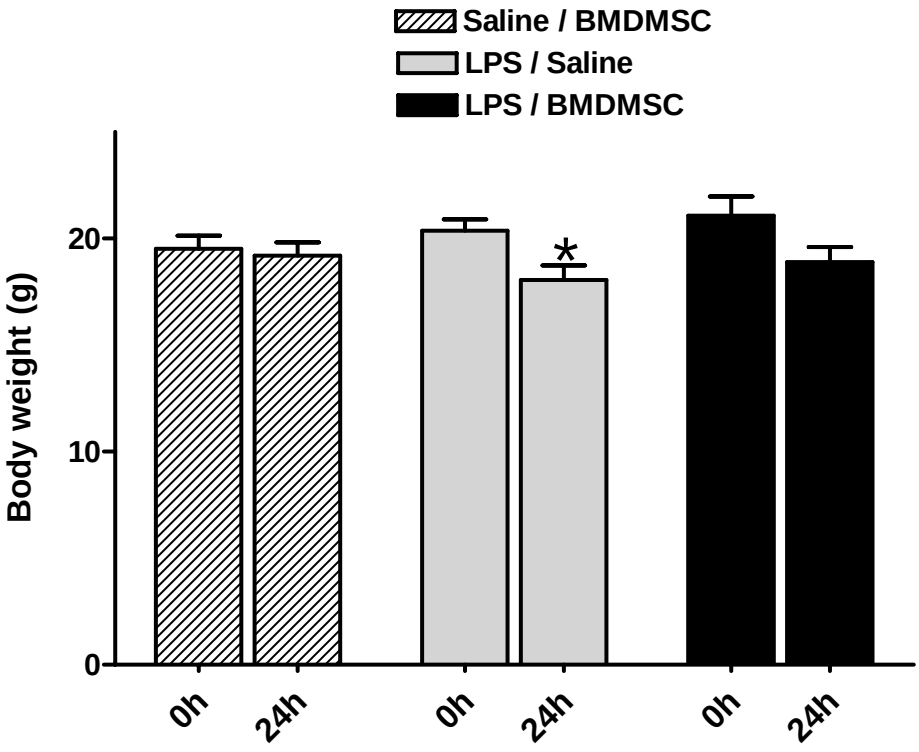


Figure 5.2. Effect of BMDMSC infusion on endotoxin-induced weight loss. C57BL/6 mice were treated with 1mg/kg i.p LPS or with saline. LPS-treated animals were either given intravenous Saline (LPS/Saline) or BMDMSC (LPS/BMDMSC). Saline treated mice were given intravenous BMDMSC. Body weight was measured at 0 h and 24 h post treatment. Data are expressed as means + SEM of n=4-5 mice per group. * Values significantly different from values at 0 h**.**

4.3. Effect of BMDMSC infusion on endotoxin-induced depletion of plasma Cys and oxidation of Cys/CySS redox state. The dynamics of plasma Cys and GSH redox state in response to endotoxin are previously described in Chapter II. In the present study we observed that plasma Cys and CySS levels recovered by 24 h, while in Chapter II plasma Cys and CySS remained significantly below control values until 48 h. These differences can be ascribed to serotype differences in LPS. In the present experiments, mice received serotype O55:B5, and serotype O111:B6 was used previously in Chapter II. However, we cannot exclude the possibility that these differences may have also resulted, in part, from variations in the dose of endotoxic LPS delivered.

The data showed that BMDMSC infusion had no effect on the early decline in plasma Cys levels at 2 h (**Figure 5.3A**; plasma Cys (μM), LPS/Saline, 12.4 $\pm$ 0.4; LPS/BMDMSC, 9.8 $\pm$ 0.7). At 6 h post-LPS, plasma Cys levels started to recover in LPS/BMDMSC treated group compared to LPS/Saline group (LPS/Saline, 9.1 $\pm$ 0.8; LPS/BMDMSC, 13.3 $\pm$ 1; $P < 0.05$) and by 24 h Cys levels normalized in both treatment groups.

Plasma CySS levels were not significantly different between the two treatment groups **(Figure 5.3B)**. However due to preservation of plasma Cys at 6 h in LPS/BMDMSC group, E_h Cys/CySS was, on average, 8 mV more reduced (**Figure 5.3C;** E_h Cys/CySS (mV), LPS/Saline, - 72.5 $\pm$ 3 ; LPS/BMDMSC, - 80.6 $\pm$ 2.4; $P< 0.05$) . Thus, the data show that BMDMSC infusion resulted in a modest but significant preservation of plasma Cys and E_h Cys/CySS at 6 h.

Figure 5.3. Effect of BMDMSC infusion on endotoxin-induced depletion of plasma Cys and oxidation of Cys/CySS redox state

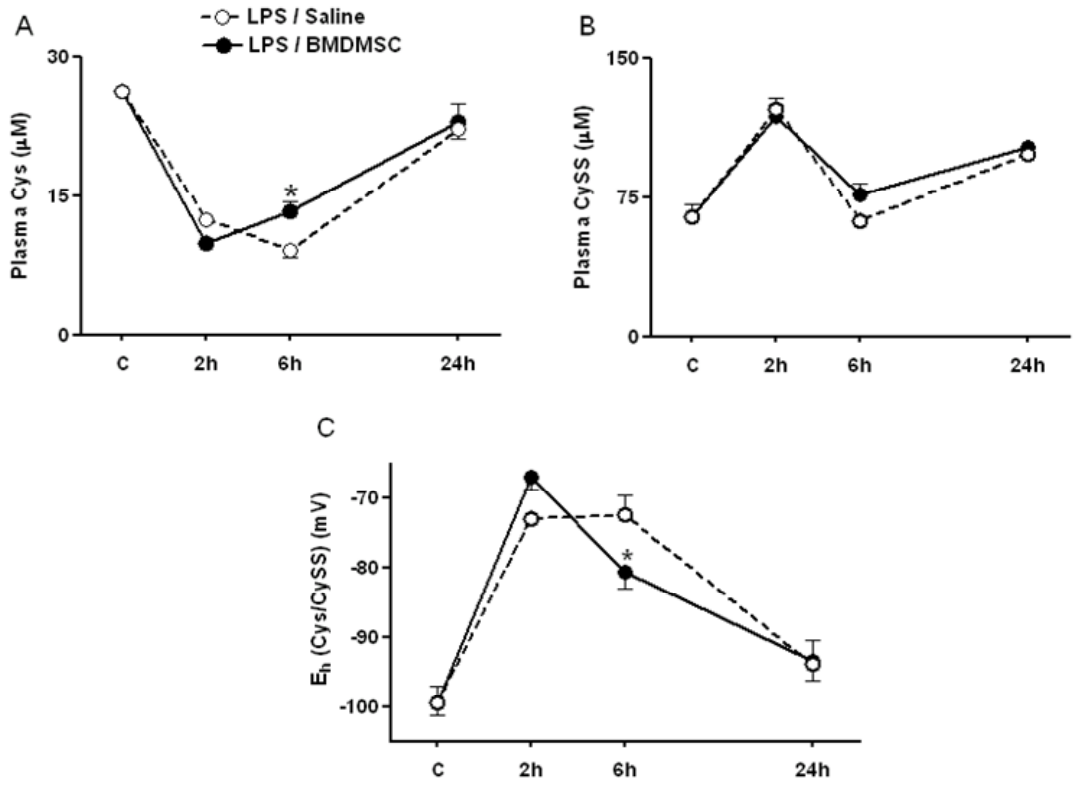


Figure 5.3. Effect of BMDMSC infusion on endotoxin-induced depletion of plasma Cys and oxidation of Cys/CySS redox state. C57BL/6 mice were treated with 1mg/kg i.p LPS followed by either i.v infusion of 500,000 BMDMSC in 0.1 ml saline or 0.1 ml saline alone. At 2 h, 6 h, and 24 h mice were sacrificed and plasma was collected for HPLC analysis of Cys (A) and CySS (B). In (C), E_h Cys/CySS was calculated from the Cys and CySS concentrations using the Nernst equation. Data are expressed as means + SEM. *Values significantly different from time-matched LPS/Saline group.

4.4. Effect of BMDMSC infusion on endotoxin-induced depletion of plasma GSH and oxidation of GSH/GSSG redox state. The data showed that BMDMSC infusion had no effect on the acute decline in plasma GSH at 2h **(Figure 5.4A;** plasma GSH (μM), LPS/Saline, 19.3 ± 0.9; LPS/BMDMSC, 20.8 ± 1.8) but protected against decline in GSH at 6 h (LPS/Saline, 22.7 ±1.1; LPS/BMDMSC, 26 ± 0.7; $P < 0.05$), and 24 h (LPS/Saline, 23.4 ± 0.4; LPS/BMDMSC, 26.8 ± 0.8; $P < 0.05$).

Calculation of GSH redox state using concentrations of GSH and GSSG (data not shown) revealed that E_h GSH/GSSG at 24 h was, on average, 8 mV more reduced in LPS/BMDMSC group compared to LPS/Saline group (**Figure 5.4B**; E_h GSH/GSSG (mV), LPS/Saline, -139.2 ± 0.7; LPS/BMDMSC, - 147.6 ± 0.3; $P < 0.01$). Together, the combined observations show that BMDMSC infusion protected against endotoxin-induced decline in plasma Cys and GSH at 6 h. The redox states of Cys and GSH couples were sequentially preserved in response to BMDMSC infusion at 6 h and 24 h, respectively.

Figure 5.4. Effect of BMDMSC infusion on endotoxin-induced depletion of plasma GSH and oxidation of GSH/GSSG redox state

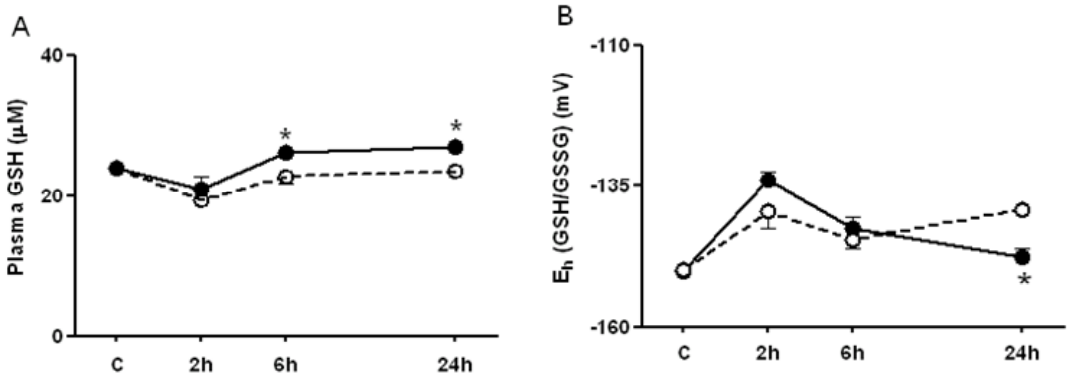


Figure 5.4. Effect of BMDMSC infusion on endotoxin-induced depletion of plasma GSH and oxidation of GSH/GSSG redox state. C57BL/6 mice were treated with 1mg/kg i.p LPS followed by either i.v infusion of 500,000 BMDMSC in 0.1 ml saline or 0.1 ml saline alone. At 2 h, 6 h, and 24 h mice were sacrificed and plasma was collected for HPLC analysis of GSH (A) and GSSG. In (B), E_h GSH/GSSG was calculated from the GSH and GSSG concentrations using the Nernst equation. Data are expressed as means + SEM. *Values significantly different from time-matched LPS/Saline group.

6. DISCUSSION

Several lines of evidence indicate that BMDMSC have immunomodulatory properties (Ortiz, Gambelli et al. 2003; Le Blanc, Rasmusson et al. 2004; Aggarwal and Pittenger 2005). *In vitro*, BMDMSC promote a shift from a pro-inflammatory to an anti-inflammatory phenotype in activated immune cells (Aggarwal and Pittenger 2005; Jiang, Zhang et al. 2005). *In vivo*, exogenously infused BMDMSC mitigate an ongoing inflammatory response and protect against tissue injury in numerous animal models of disease, including ALI (Parekkadan, van Poll et al. 2007). In the present model of acute inflammation induced by endotoxin, we demonstrate that infusion of BMDMSC protects against oxidation of Cys and GSH redox states at 6 h and 24 h, respectively. These findings provide the novel observation that BMDMSC infusion attenuates systemic oxidation of the thiol/disulfide redox environment, in response to endotoxin.

The potential mechanism by which BMDMSC infusion improves Cys and GSH homeostasis may involve increased efflux of Cys and GSH from tissues, increased recycling and/or increased GSH synthesis. This can be mediated by the secretion of soluble growth factors by BMDMSC, or by the interaction of BMDMSC with target host cells, or both. A candidate factor in this regard is keratinocyte growth factor (KGF). BMDMSC constitutively produce KGF (Chen, Tredget et al. 2008), and studies in a murine model of allogenic bone marrow transplant show that subcutaneous infusion of KGF improves hepatic GSH levels (Ziegler, Panoskaltsus-Mortari et al. 2001). Because 50-80% of plasma GSH is dependent on efflux from the liver (Ookhtens and Kaplowitz 1998), increase in hepatic GSH could increase plasma GSH levels. Furthermore, *in vitro* studies have shown that KGF modulates oxidized extracellular Cys/CySS to more

physiological values (Jonas, Gu et al. 2003). Further studies are needed to investigate the role of soluble factors such as KGF in the modulation of thiol/disulfide redox status *in vivo*.

Accumulating evidence indicates that the biological thiol/disulfide redox environment dictates responses to inflammatory stimuli. Experimental manipulation of Cys and GSH levels alters cytokine production *in vitro* in numerous cell-types including endothelial cells, lymphocytes, and mononuclear cells. *In vivo,* oral pre-treatment of mice with GSH and N-acetyl cysteine (NAC) attenuates LPS-induced TNF-α in the circulation and in the spleen (Peristeris, Clark et al. 1992). Thus, Cys and GSH are well-established determinants of cytokine production during activation of the immune system. Conversely, cytokine production also impacts Cys and GSH homeostasis. In pulmonary endothelial cells, TNF-α decreases intracellular GSH and increases GSSG levels (Ishii, Partridge et al. 1992). During infection and injury, increased protein and GSH synthesis exert a greater demand on Cys supply leading to a decrease in hepatic GSH content (Sendelbach, White et al. 1990) (Hunter and Grimble 1994). Thus, the thiol/disulfide redox environment is intimately related to inflammation.

However, in the present study the dynamics of E_h GSH/GSSG and E_h Cys/CySS indicates that modulation of thiol/disulfide redox status and pro-inflammatory cytokine levels by BMDMSC is likely occurring by independent mechanisms. This suggests that preservation of Cys and GSH by BMDMSC infusion may not be involved in the decrease in pro-inflammatory cytokine levels in the present model within the studied time frame. Nonetheless, because thiol/disulfide redox state is fundamental to basic cellular processes such as proliferation, differentiation, and apoptosis; the improvement in plasma Cys and

GSH indices may have therapeutic effects in attenuating lung injury and/or facilitating repair.

In conclusion, in a murine model of endotoxin-induced inflammation infusion of syngeneic BMDMSC improved plasma Cys and GSH redox indices at 6 h and 24 h, respectively. These studies are the first, to our knowledge, to demonstrate the antioxidant effects of BMDMSC *in vivo*. Further studies are needed to investigate the mechanistic basis of improvement in thiol/disulfide redox status by BMDMSC.

ACKNOWLEDGMENTS

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CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Studies investigating the role of the thiol/disulfide redox environment in lung injury and fibrosis have primarily focused on GSH. The data contained in this dissertation provide evidence for the role of Cys/CySS redox state in the pathogenesis of acute lung injury and lung fibrosis. The studies establish that Cys/CySS redox state is an important indicator of oxidative stress during inflammation, injury, and fibrosis. The data show that perturbations in the systemic thiol/disulfide redox environment under these pathological conditions occur not only due to an increase in oxidants, but also due to mechanisms relating to altered transport and decreased precursor availability.

The combined *in vitro* and *in vivo* data allow for six main conclusions related to Cys/CySS redox state in lung injury, repair, and fibrosis: 1) The redox state of plasma Cys and GSH vary independently during the acute phase of lung injury induced by endotoxin; 2) The acute oxidation of plasma Cys may contribute to subsequent events in acute lung injury including neutrophil influx into the lung; 3) Nutrition is a critical determinant of plasma Cys and GSH homeostasis during inflammation associated with lung injury; 4) Maintenance of a more reducing plasma Cys redox state by dietary sulfur amino acid supplementation decreases the acute inflammatory response to endotoxin; 5) In experimental lung fibrosis, Cys is oxidized relative to GSH in the plasma; and in the lung lining fluid, oxidation of E_h Cys/CySS is temporally associated with the induction of lung fibrotic markers; 6) The resolution of endotoxin-induced inflammation by the infusion of exogenous bone marrow-derived mesenchymal stem cells is associated with the sequential preservation of plasma Cys and GSH redox systems.

These data establish that the redox state of the Cys/CySS couple is a biomarker of oxidative stress, and varies independently from the redox state of the GSH/GSSG couple in acute lung injury and fibrosis. In the first study (Chapter 2), we identified that Cys/CySS is highly oxidized relative to GSH/GSSG during the acute phase of endotoxin-induced lung injury. Data obtained from an *in vitro* pulmonary vascular model system showed that oxidized Cys/CySS redox state increased adhesion of leukocytes to the pulmonary endothelium. Together, these data suggest a causal role for the acute oxidation of Cys/CySS in the pathogenesis of ALI i.e., oxidation of Cys/CySS can contribute to increased neutrophil influx into the lung. A second important finding was that adequate nutrition determines Cys and GSH homeostasis during endotoxin-induced lung injury. This suggests that nutritional interventions may represent a means to decrease oxidation of the systemic Cys and GSH pools in patients with sepsis and ALI.

Indeed in Chapter 3, we find that dietary sulfur amino acid supplementation augmented Cys reserves and prevented the acute oxidation of Cys/CySS redox state in response to endotoxin. This led to a decrease in plasma and lung levels of the major pro-inflammatory cytokines, IL-1β and TNF-α. *In vitro* studies in monocytes identified a mechanistic link between oxidized extracellular Cys/CySS redox state and the induction of IL-1β. Additionally, analysis of human plasma revealed a strong positive association between oxidized Cys/CySS redox state and IL-1β and TNF-α. Together, these data show that oxidized Cys/CySS redox state is a critical determinant of pro-inflammatory cytokine production.

In Chapter 4, we examined variations in Cys/CySS redox state in the extracellular compartments in a mouse model of bleomycin-induced lung injury and fibrosis. This

study identified that plasma Cys/CySS redox state is oxidized in the absence of changes to GSH/GSSG redox state in lung fibrosis. Furthermore, oxidation of E_h Cys/CySS in the lining fluid was associated with the induction of pro-fibrotic markers in the lung. Together with published data showing that oxidized Cys/CySS redox state increases TGF-β1-dependent fibronectin expression in lung fibroblasts, these findings indicate that oxidation of Cys/CySS during lung injury may contribute to fibrosis.

In the final chapter, we examined the effect of exogenously infused bone marrow-derived mesenchymal stem cells (BMDMSC) on the systemic Cys and GSH pools during endotoxemia. Results showed that preservation Cys/CySS redox state in endotoxemia was an acute response to BMDMSC infusion, while preservation of GSH/GSSG redox state occurred later at 24 h. These findings demonstrate that in addition to their well-documented anti-inflammatory effects, BMDMSC also have antioxidant effects *in vivo*. In all, the data validate a fundamental concept in the recent re-definition of oxidative stress i.e., the major thiol/disulfide redox systems, Cys and GSH are regulated independently of each other. The findings raise the possibility that targeting plasma Cys/CySS redox state may represent a therapeutic strategy in ALI and IPF.

Multiple studies have shown that Cys/CySS redox state represents a modifiable risk factor in human disease. Therefore, considerable opportunity exists to extend the present findings to the clinic to test whether interventions targeting Cys/CySS are beneficial in patients with ALI and IPF. At the same time, studies are needed to fill gaps in our understanding of the mechanistic role of Cys/CySS redox state in lung injury and fibrosis. *In vitro* studies are required to explore how changes in the extracellular Cys/CySS redox environment impacts membranal and intracellular signaling events

relevant to inflammation and fibrosis. Because numerous cytokine and chemokine membrane receptors contain conserved sulfhydryl residues on their extracellular domains, the extracellular Cys/CySS redox state can impact receptor activity. Methodologies such as redox proteomics will aid in the identification of such receptors and provide critical information on signaling pathways that are likely to respond to the extracellular Cys/CySS redox environment.

The approach to identifying such redox sensitive receptors can be furthered to examine lipid-raft associated proteins and their sensitivity to the extracellular Cys/CySS environment. From emerging evidence on the role of lipid rafts in redox signaling, one can speculate that the rapid and sustained induction of cellular peroxide by extracellular Cys/CySS in monocytes could be due to activation of raft-associated NADPH oxidase, downstream of membrane receptors such as Fas or TNFR1. Additionally, multiple redox-sensitive receptors such as EGF and integrins are associated with lipid rafts. Characterizing the redox state of raft and non-raft associated membrane proteins in response to oxidized and physiological Cys/CySS redox states may therefore be informative. Indeed, signaling through lipid rafts may represent another means of redox compartmentalization and specificity.

An important question raised by the findings included in this dissertation is whether oxidation of Cys/CySS redox state represents a predisposing factor in the pathogenesis of ALI and fibrosis. Transgenic mouse strains where plasma Cys/CySS redox state is constitutively oxidized can be utilized to study susceptibility to lung injury and fibrosis induced by endotoxin and bleomycin, respectively. Furthermore, characterizing the Cys/CySS redox state in the lung lining fluid in these strains will

provide information on the determinants of Cys/CySS redox state in the ELF. This is critical because very little is known about the regulation of Cys and CySS in the lung lining fluid.

Finally, the effects of BMDMSC in modulating Cys and GSH redox systems is an area that needs to be further investigated. BMDMSC represent not only an emerging therapeutic modality, but also a paradigm for the resolution of inflammation. Therefore, identifying the mechanisms by which BMDMSC modulate thiol/disulfide redox status will aid in understanding the regulation of these systems during the resolution of inflammation, and may unveil potential therapeutic targets. Furthermore, determining whether the anti-inflammatory effects of BMDMSC can be augmented by dietary or pharmacological interventions to preserve Cys/CySS redox state represents a therapeutic strategy that can be readily translated to the clinic.

In conclusion, the findings presented in this dissertation establish Cys/CySS state as a biomarker of oxidative stress during inflammation, lung injury, and fibrosis. The findings underscore the need for mechanistic studies to dissect the role of Cys/CySS redox state in lung injury and fibrosis. Together with information on the other important thiol/disulfide redox systems, GSH and thioredoxin; future studies should provide the necessary mechanistic framework to design targeted thiol based therapies in ALI and IPF.

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