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Sheena Denise Brown

The Effect of Ethanol Consumption on Pulmonary Macrophage Maturation and Function

By

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Doctor of Philosophy

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The Effect of Ethanol Consumption on Pulmonary Macrophage Maturation and Function

By

Sheena Denise Brown
B.S. Spelman College, 2005

Advisor: Lou Ann Brown, Ph.D.

An abstract of
a dissertation submitted to the Faculty of the
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Abstract

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Alcohol addiction is a complex multifactorial disease that affects more than 70 million people and contributes to 1 in 25 deaths worldwide. Alcoholics have twice the incidence of intensive care unit-related morbidity and mortality, commonly caused by acute respiratory distress syndrome. This increased risk of respiratory infections is due, in part, to the detrimental effects of alcohol on alveolar macrophage maturation and function. Previous studies have shown that chronic ethanol ingestion leads to increased oxidant stress, increased TGF-β_1 production, increased fibronectin production, and decreased phagocytosis in alveolar macrophages. Alternative activation of alveolar macrophages also leads to similar alterations, as well as increased arginase production and activity, events that promote fibroblast proliferation and collagen production in the lung, leading to fibrosis. Our aim was to determine if the altered phenotype of the alveolar macrophage after alcohol abuse is related to alternative activation. Therefore, we hypothesized that chronic oxidant stress induced by chronic ethanol consumption leads to impaired maturation and alternative activation of alveolar macrophages. We also sought to examine the role of TGF-β_1, a key player in ethanol-induced lung dysfunction and IL-13 mediated fibrosis. To address this hypothesis, cell culture and rodent models of chronic alcoholism along with alveolar macrophages from otherwise healthy alcoholic subjects were used to examine the potential link between chronic alcohol-induced alterations of alveolar macrophage function and alternative activation. Collectively, these studies demonstrated that chronic ethanol ingestion leads to pulmonary macrophage dysfunction by delaying terminal maturation, impairing phagocytic function, inducing an alternative activation phenotype, increasing endogenous production of reactive oxygen species, and increasing production of TGF-β_1. Other studies demonstrated that TGF-β_1 was required for IL-13 induced alternative activation and deficits in phagocytic function. Supplementation with the antioxidant glutathione or its precursors prevented or reversed ethanol induced macrophage dysfunction, thereby suggesting ethanol-induced alveolar macrophage dysfunction was modulated by oxidant stress and antioxidant depletion.

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This dissertation is dedicated to my mother, Lillian M. Brown,

for her unconditional love and endless encouragement.

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World Health Organization (WHO)

Diagnostic and Statistical Manual-IV (DSM-IV)

Michigan Alcoholism Screening Test (MAST)

Short MAST (SMAST)

Alcohol Use Disorders Identification Test (AUDIT)

Glutathione (GSH)

Acohol Dehydrogenase (ADH)

Aldehyde Dehydrogenase (ALD2)

Acute respiratory distress syndrome (ARDS)

Transforming Growth Factor Beta$_1$ (TGF-β_1)

Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF)

Granulocyte Macrophage Colony-Stimulating Factor Receptor (GM-CSFR)

Reactive Oxygen Species (ROS)

Superoxide ($O_2^{\bullet -}$)

Hydrogen Peroxide (H_2O_2)

Hydroxyl Radicals ($^{\bullet}OH$)

Nicotinamide Adenine Dinucleotide Phosphate (NADPH)

Endoplasmic Reticulum (ER)

Superoxide Dismutase (SOD)

Chronic Obstructive Pulmonary Disease (COPD)

Oxidized Disulfide Form of GSH (GSSG)

γ-Glutamyl-Amino Acid (γ-glu-aa)

Human Immunodeficiency Virus (HIV)

Epithelial Lining Fluid (ELF)

Cystic Fibrosis Transmembrane Regulator (CTFR)

Acute Respiratory Distress Syndrome (ARDS)

T helper 1 (T_H1)

Staphylococcus aureus (*S. aureus*)

GM-CSF receptor α (GM-CSFR-α)

S-Adenosyl-Methionine (SAM)

Dulbecco's Modified Eagle's Medium (DMEM)

Fluorescein Isothiocyanate (FITC)

Relative Fluorescent Units (RFUs)

Phosphate Buffered Saline (PBS)

Tris Buffered Saline (TBS)

TBS with 0.2% Tween 20 (TBS-T)

Tetramethyl Rhodamine Iso-Thiocyanate (TRITC)

Ethanol (EtOH)

Tumor Necrosis Factor (TNF)

Nitric Oxide Synthase-2 (NOS-2)

Fetal Bovine Serum (FBS)

Radioimmunoprecipitation Assay (RIPA)

Dichlorofluorescein Diacetate (DCFH-DA)

Dichlorofluorescin (DCF)

TGF-β_1 Neutralizing Antibody (TGF-β_i)

Bone Morphogenetic Proteins (BMP)

Latency Associated Peptide (LAP)

Latent TGF-β Binding Proteins (LTBP)

Activin Receptor-Like Kinase (ALK)

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1. CHRONIC ALCOHOLISM.

1.1.1. Alcoholism: A Major Public Health Concern.

Alcohol abuse, the third leading cause of death within the United States, is a complex multifactorial disease that affects more than 13 million Americans and accounts for more than 10% of health care costs (Room et al., 2005). According to the World Health Organization (WHO), over 70 million people worldwide have alcohol use disorders that contribute to more than 1.5 million deaths worldwide (Jernigan et al., 2000). Alcohol use disorders are defined as disorders that are associated with ingestion of alcohol over a period o f time in ways that result in problems with one's health, personal relationships, school, and/or work, and include alcohol dependence, alcohol abuse, alcohol intoxication, and alcohol withdrawal. Alcohol consumption has both health and social consequences through intoxication, dependence or addiction, and a myriad of other physiological effects. Both the volume and pattern of drinking, especially binge drinking, affect the burden of alcohol abuse (Cook and Moore, 2002; Joshi and Guidot, 2007a; Moss and Burnham, 2003). Alcohol use disorders have been associated with more than 60 types of disease and injuries and are estimated to cause more than 20% of esophageal cancer, liver cancer, liver cirrhosis, epilepsy, and motor vehicle accidents worldwide. Alcohol use disorders have a much larger impact on health within developed countries (Rehm et al., 2009; Rehm et al., 2003b). The majority of persons with alcohol use disorders do not abuse other drugs; however, there is a higher rate of alcohol consumption and abuse among tobacco users (Room et al., 2005). Persons with alcohol use disorders have an increased risk of hospital admissions. Of all patients admitted to hospitals with the United States, approximately 20-40% have alcohol use disorders, with the highest incidence in people between the ages of 35 and 55. An even higher incidence of alcohol use disorders-related hospital admissions is seen in government-owned hospitals and academic medical centers (Boé et al., 2009; George et al., 1998; Joshi and Guidot, 2007a; Rehm et al., 2003b). Alcohol abuse is also associated with an increased risk of both intentional and unintentional injuries, such as burn injury, vehicular accidents, self-inflicted injuries, violent

attacks, and falls. Numerous studies indicate approximately one-half of trauma patients have detectable blood alcohol levels (Jernigan et al., 2000; O'Connor and Schottenfeld, 1998; Room et al., 2005).

The economic burden of alcohol abuse exceeds $150 billion annually within the United States. Total costs attributed to alcohol-related motor vehicle accidents that lead to mortality, and automobile and property damage are estimated to be $24.7 billion. An estimated $184.6 billion are used in healthcare costs, including alcohol detoxification programs and rehabilitation services. Approximately $68 billion are lost in workplace productivity annually, with $1 billion in costs due to fetal alcohol spectrum disorder and related mental handicaps (Casswell and Thamarangsi, 2009; Jernigan et al., 2000; Rehm et al., 2003b). Alcohol-related crimes have an estimated cost of $20 billion annually, which can be attributed to lost earnings from incarceration, criminal justice costs, and criminal victimization. A large portion of the economic burden of alcohol abuse falls on the shoulders of non-alcohol abusers. Alcohol use disorders cost the government in excess of 50 billion dollars annually (Rehm et al., 2009; Rehm et al., 2003a). Examining the mechanisms by which alcohol abuse induces organ injury and developing therapies to reverse these detrimental effects would prove help reduce the increased mortality and morbidity seen in alcohol abusers and alleviate some of the financial burden of this disease.

1.1.2. Defining Alcohol Abuse and Dependence.

Alcohol use disorders are defined as the disorders associated with continuous repetitive ingestion of alcohol in ways that have deleterious effects on psychosocial functioning and health, resulting in problems with personal relationships, school, and/or work (Maisto and Saitz, 2003). A standard drink has 14g of ethanol, the equivalent in one 12-oz beer, a 5-oz glass of table wine, or a 1.5-oz glass of spirits. According to the NIH, men who consume more than 4 drinks per day or 14+ drinks per week are characterized as having excessive alcohol consumption. For women, the limits are set at no more than 3 drinks per day and no more than 7 drinks per week (Hanson

and Li, 2003; Room et al., 2005). The *Diagnostic and Statistical Manual-IV (*DSM-IV) is a survey developed by the American Psychiatric Association for the clinical diagnosis of abuse and dependence. This survey is used clinically to define alcohol use disorders and to serve as a common language clinicians use to communicate with patients. The DSM-IV differentiates between alcohol abuse and dependence. Alcohol abuse, a precursor to dependence, occurs after a pattern of abuse has been established. The symptoms listed below are required for diagnosis of alcohol abuse. Dependence occurs after several physiological effects develop following alcohol intoxication. Countless studies have demonstrated the high reliability of DSM-IV for the diagnosis of alcohol dependence with less reliability for alcohol abuse (Association, 1994; Grant et al., 2004).

DSM-IV Criteria for Alcohol Abuse. The Diagnostic and Statistical Manual-IV defines alcohol abuse as: A maladaptive pattern of alcohol abuse leading to clinically significant impairment or distress, as manifested by one or more of the following, occurring within a 12-month period:

a) Recurrent alcohol use resulting in failure to fulfill major role obligations at work, school, or home (e.g., repeated absences or poor work performance related to substance use; substance-related absences, suspensions or expulsions from school; or neglect of children or household).
b) Recurrent alcohol use in situations in which it is physically hazardous (e.g., driving an automobile or operating a machine).
c) Recurrent alcohol-related legal problems (e.g., arrests for alcohol-related disorderly conduct).
d) Continued alcohol use despite persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the alcohol (e.g., arguments with spouse about consequences of intoxication or physical fights).

DSM-IV Criteria for Alcohol Dependence.. The *Diagnostic and Statistical Manual-IV* defines dependence as: A maladaptive pattern of alcohol use, leading to clinically significant impairment or distress, as manifested by three or more of the following seven criteria, occurring at any time in the same 12-month period:

1) Tolerance, as defined by either of the following:
    a) A need for markedly increased amounts of alcohol to achieve intoxicationt.
    b) Markedly diminished effect with continued use of the same amount of alcohol.
2) Withdrawal, as defined by either of the following:
    a) The characteristic withdrawal syndrome for alcohol (refer to DSM-IV for further details).
    b) Alcohol is taken to relieve or avoid withdrawal symptoms.
3) Alcohol is often taken in larger amounts or over a longer period than was intended.
4) There is a persistent desire or there are unsuccessful efforts to control alcohol use.
5) A great deal of time is spent in activities necessary to obtain/use alcohol; or recover from its effects.
6) Important social, occupational, or recreational activities are given up or reduced because of alcohol use.
7) Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the alcohol.

DSM-IV. American Psychiatric Association. (1994). Diagnostic and Statistical Manual of Mental Disorders (4th Ed.). Washington, DC.

1.1.3. Screening Methods for Diagnosing Alcohol Abuse.

Alcohol use disorders include alcohol dependence, alcohol abuse, alcohol intoxication, and alcohol withdrawal. Numerous brief screening tests have been developed to determine if a person has an alcohol use disorder. The most commonly used screening tests are the CAGE, the

Michigan Alcoholism Screening Test (MAST), and the Alcohol Use Disorders Identification Test (AUDIT) (Bush et al., 1987; Gibbs, 1983; Maisto and Saitz, 2003; Storgaard et al., 1994).

The Michigan Alcohol Screening Test (MAST), developed in 1971, is a 23 yes-or-no questions self-test used to indicate alcohol dependence. MAST is one of the oldest and most reliable alcohol screening tests available with 98% accuracy. Questions on the MAST examine a patient's self evaluation of social, work, and family problems that are commonly associated with heavy drinking and focus on possible alcohol problems throughout a person's lifetime (**Table 1.1**). The MAST is commonly used by courts to determine the appropriate sentencing and treatment for people convicted of alcohol-related offenses (Gibbs, 1983; Maisto and Saitz, 2003; Storgaard et al., 1994).

The AUDIT is an alcohol screening test developed by the WHO in 1982 that is commonly used to identify persons who are at risk of developing alcohol problems. AUDIT has been validated in six countries and is used internationally; importantly, AUDIT has been shown to be effective across age groups, ethnicities and gender. AUDIT is used to detect alcohol-related problems that have occurred within the last year. It is one of the most accurate alcohol screening tests available, rated as 92 percent effective in detecting hazardous or harmful drinking (**Table 1.2**). Questions 1-3 relate to the amount of alcohol consumed, 4-6 attempt to identify alcohol dependence and 7-10 evaluate alcohol related problems (Maisto and Saitz, 2003; Saunders et al., 1993).

The CAGE questionnaire is a mnemonic for attempts to cut back on drinking, being annoyed at criticisms about drinking, feeling guilty about drinking, and using alcohol as an eye opener. It consists of a four question screening test for alcoholism developed by John Ewing in 1984 (**Table 1.3**) (Bush et al., 1987; O'Connor and Schottenfeld, 1998). CAGE has been found to have a sensitivity of 93% and a specificity of 76% for the identification of problem drinkers. People that answer "yes" to two or more questions indicate that the respondent should be investigated further for alcohol abuse (Bush et al., 1987).

The MAST Test

The MAST Test is a simple, self-scoring test that helps assess if you have a drinking problem. Answer yes or no to the following questions:

1. Do you feel you are a normal drinker? ("normal" - drink as much or less than most other people)
YES or NO

2. Have you ever awakened the morning after some drinking the night before and found that you could not remember a part of the evening?
YES or NO

3. Does any near relative or close friend ever worry or complain about your drinking?
YES or NO

4. Can you stop drinking without difficulty after one or two drinks?
YES or NO

5. Do you ever feel guilty about your drinking?
YES or NO

6. Have you ever attended a meeting of Alcoholics Anonymous (AA)?
YES or NO

7. Have you ever gotten into physical fights when drinking?
YES or NO

8. Has drinking ever created problems between you and a near relative or close friend?
YES or NO

9. Has any family member or close friend gone to anyone for help about your drinking?
YES or NO

10. Have you ever lost friends because of your drinking?
YES or NO

11. Have you ever gotten into trouble at work because of drinking?
YES or NO

12. Have you ever lost a job because of drinking?
YES or NO

13. Have you ever neglected your obligations, your family, or your work for two or more days in a row because you were drinking?
YES or NO

14. Do you drink before noon fairly often?
YES or NO

Table 1.1 The MAST Test

15. Have you ever been told you have liver trouble such as cirrhosis?
YES or NO

16. After heavy drinking have you ever had delirium tremens (D.T.'s), severe shaking, visual or auditory (hearing) hallucinations?
YES or NO

17. Have you ever gone to anyone for help about your drinking?
YES or NO

18. Have you ever been hospitalized because of drinking?
YES or NO

19. Has your drinking ever resulted in your being hospitalized in a psychiatric ward?
YES or NO

20. Have you ever gone to any doctor, social worker, clergyman or mental health clinic for help with any emotional problem in which drinking was part of the problem?
YES or NO

21. Have you been arrested more than once for driving under the influence of alcohol?
YES or NO

22. Have you ever been arrested, even for a few hours because of other behavior while drinking?
(If Yes, how many times _____)
YES or NO
SCORING
Please score one point if you answered the following:
1. No
2. Yes
3. Yes
4. No
5. Yes
6. Yes
7 through 22: Yes
Add up the scores and compare to the following score card:
0 - 2 No apparent problem
3 - 5 Early or middle problem drinker
6 or more Problem drinker

Sources: National Council on Alcoholism and Drug Dependence of the San Fernando Valley. Michigan Alcohol Screening Test (MAST)

Table 1.1 The MAST Test Cont.

AUDIT Questionnaire

1. How often do you have a drink containing alcohol?
   - (0) Never (Skip to Questions 9-10)
   - (1) Monthly or less
   - (2) 2 to 4 times a month
   - (3) 2 to 3 times a week
   - (4) 4 or more times a week

2. How many drinks containing alcohol do you have on a typical day when you are drinking?
   - (0) 1 or 2
   - (1) 3 or 4
   - (2) 5 or 6
   - (3) 7, 8, or 9
   - (4) 10 or more

3. How often do you have six or more drinks on one occasion?
   - (0) Never
   - (1) Less than monthly
   - (2) Monthly
   - (3) Weekly
   - (4) Daily or almost daily

4. How often during the last year have you found that you were not able to stop drinking once you had started?
   - (0) Never
   - (1) Less than monthly
   - (2) Monthly
   - (3) Weekly
   - (4) Daily or almost daily

5. How often during the last year have you failed to do what was normally expected from you because of drinking?
   - (0) Never
   - (1) Less than monthly
   - (2) Monthly
   - (3) Weekly
   - (4) Daily or almost daily

Table 1.2 The AUDIT Questionnaire

6. How often during the last year have you been unable to remember what happened the night before because you had been drinking?

(0) Never
(1) Less than monthly
(2) Monthly
(3) Weekly
(4) Daily or almost daily

7. How often during the last year have you needed an alcoholic drink first thing in the morning to get yourself going after a night of heavy drinking?

(0) Never
(1) Less than monthly
(2) Monthly
(3) Weekly
(4) Daily or almost daily

8. How often during the last year have you had a feeling of guilt or remorse after drinking?

(0) Never
(1) Less than monthly
(2) Monthly
(3) Weekly
(4) Daily or almost daily

9. Have you or someone else been injured as a result of your drinking?

(0) No
(2) Yes, but not in the last year
(4) Yes, during the last year

10. Has a relative, friend, doctor, or another health professional expressed concern about your drinking or suggested you cut down?

(0) No
(2) Yes, but not in the last year
(4) Yes, during the last year

Add up the points associated with your answers above. A total score of 8 or more indicates harmful drinking behavior. See your doctor.

Table 1.2 The AUDIT Questionnaire Cont.

CAGE questionnaire:
Have you ever felt you should **C**ut down on your drinking?
Have people **A**nnoyed you by criticizing your drinking?
Have you ever felt bad or **G**uilty about your drinking?
Have you ever had a drink first thing in the morning to steady your nerves or get rid of a hangover (**E**ye-opener)?

Table 1.3 The CAGE Questionnaire.

1.1.4. Chronic Alcoholism and Disease Progression.

Due to its small molecular weight and miscibility, alcohol can readily be distributed to all organ systems and tissues within the body within minutes after it enters the bloodstream (Lands, 1998). Abuse or excessive consumption of alcohol has numerous detrimental effects on physical and mental health, and causes extensive damage to the brain, liver, heart, and lung. While some of these detriments are reversible, the majority are irreversible during long-term chronic alcohol abuse and contribute to increased mortality and morbidity among alcohol abusers (Boé et al., 2009; Cubero et al., 2009; Harper, 2009; Lands, 1998; Oscar-Berman and Marinkovic, 2003; Rehm et al., 2003a; Room et al., 2005).

Neurological Disorders. Approximately half of all alcohol abusers within the United States have cognitive impairments due to alcohol–induced structural changes in the brain. Susceptibility to alcohol-induced brain injury depends upon several factors including gender, drinking history and nutrition (Butterworth, 1995; Chan, 1996; Oscar-Berman and Marinkovic, 2003; Oscar-Berman and Marinković, 2007). These psychological changes, which can range from mild to severe, are generally related to executive functions and balance as well as negative effects on several mental functions (Harper and Blumbergs, 1982; Oscar-Berman and Marinkovic, 2003). Among these alterations are brain atrophy, dementia, and Wernicke-Korsakoff syndrome (Martin et al., 2003; Oscar-Berman and Marinkovic, 2003).

Over 400 studies have shown that alcoholics have reduced brain weight that correlates with rate and amount of alcohol consumed during the patient's lifetime (Butterworth, 1995; Harper, 2009; Harper and Blumbergs, 1982; Oscar-Berman and Marinkovic, 2003; Oscar-Berman and Marinković, 2007). The reduced brain weight is largely due to atrophy and decreased white matter volume within the cerebellum. Approximately 20-40% of all alcoholics exhibit cerebellar atrophy that correlates with deficits in performing executive function (Harper, 2009; Oscar-Berman and Marinkovic, 2003; Oscar-Berman and Marinković, 2007). Studies done by Pfefferbaum et. al show the volume of the corpus callosum, a brain structure involved in sensory,

motor and cognitive function, was significantly reduced in alcoholic patients, when compared to non-alcoholics (Pfefferbaum et al., 2006).

The most common neurological disorder associated with chronic alcohol abuse is alcohol-induced persisting amnesia, Wernicke–Korsakoff syndrome, which is characterized by permanent cognitive and emotional deficits (Harper, 2009). Wernicke-Korsakoff syndrome develops in two phases with the first being Wernicke's encephalopathy, a life-threatening short lived neurologic disorder. Approximately 80 to 90 percent of alcoholics with Wernicke's encephalopathy develop Korsakoff's psychosis, a chronic neuropsychiatric amnesic syndrome (Harper, 2009; Harper and Blumbergs, 1982; Martin et al., 2003). First described by Carl Wernicke in 1881, Wernicke-Korsakoff syndrome is caused by malnutrition that leads to deficiencies in thiamine (vitamin B_1), an essential nutrient required by all tissues. Since the body cannot endogenously produce thiamine, dietary intake is the sole mechanism by which the body has access to it (Martin et al., 2003; Paparrigopoulos et al., 2010). Thiamine deficiency may result from several factors including inadequate dietary intake, impaired absorption, reduced storage in the liver, and/or decreased conversion of thiamine to its active form. Within the brain and central nervous system, thiamine plays a vital role in glucose metabolism and synthesis of neurotransmitters (Butterworth, 1995; Mulholland, 2006; Nakagawasai, 2005). Thiamine supplementation in patients with Wernicke-Korsakoff syndrome has proven to reverse some of effects of this disease (Butterworth, 1995; Harper, 2009; Paparrigopoulos et al., 2010).

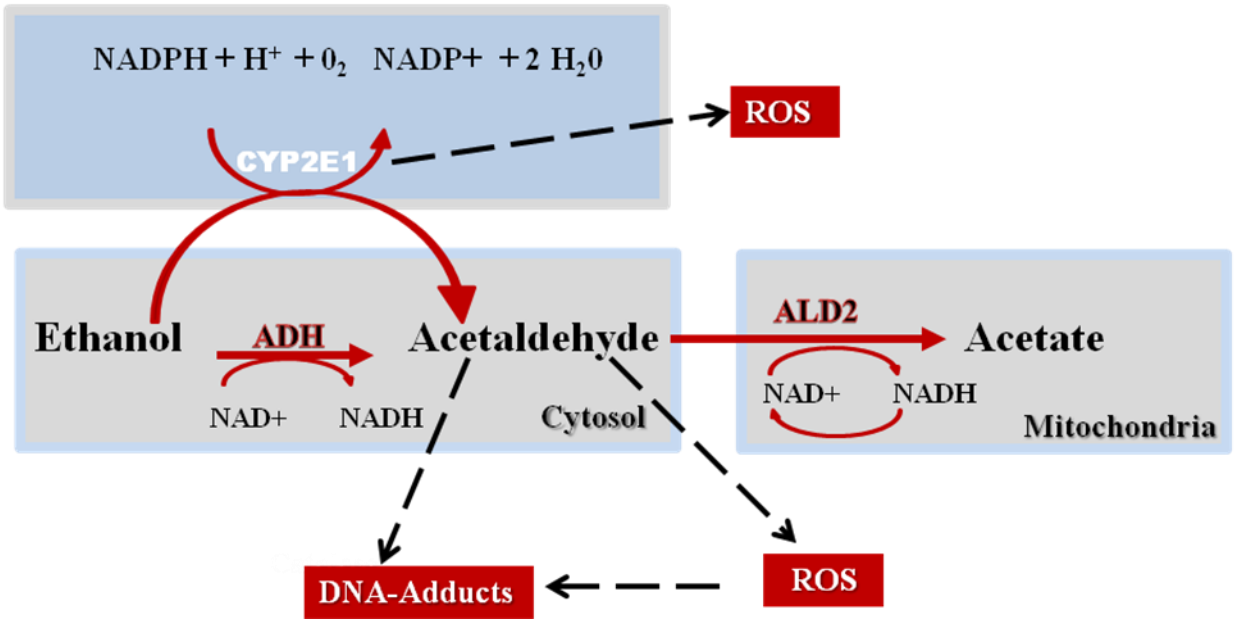
Liver diseases. Alcoholic liver disease, a hallmark of long-term alcohol abuse, represents a spectrum of progressive morphological changes within the liver that eventually render it non-functional (Diehl, 1998). Alcohol-induced liver injury primarily occurs due to the effects of alcohol metabolism and particularly the toxic effects of acetaldehyde, its primary metabolite. While alcohol can be metabolized in several organs, 90% of alcohol is metabolized in the liver via three major enzymatic pathways: (1) alcohol dehydrogenase in the cytoplasm, which is responsible for the bulk of ethanol oxidation; (2) the microsomal ethanol oxidizing system

catalyzed by CYP2E1 and (3) a non-oxidative pathway catalyzed by fatty acid ethyl ester synthase. Regardless of the pathway used to metabolize alcohol, acetaldehyde is produced as a toxic by-product. Acetylaldehyde has been shown to mediate lipid peroxidation and nucleic acid oxidation (Albano, 2008; Cubero et al., 2009; De Minicis and Brenner, 2008; Diehl, 1998; Gramenzi et al., 2006; Ishak et al., 1991; Lieber, 2004; Pessayre, 2007).

Once converted to acetaldehyde, aldehyde dehydrogenase quickly converts it into acetate through an irreversible reaction (Albano, 2008; De Minicis and Brenner, 2008; Diehl, 1998; Gramenzi et al., 2006). Acetate is then transported to extra-hepatic cells where it is converted to acetyl-CoA via acetyl-CoA synthetase and further oxidized to form water and carbon dioxide. In cases of intoxication, the excess amounts of acetaldehyde react with mitochondrial glutathione (GSH) and alter the redox status of the liver leaving it susceptible to further oxidant stress (**Figure 1.1)** (Albano, 2008; De Minicis and Brenner, 2008; Lieber, 2004; Wu et al., 2004).

Development of alcoholic liver disease begins with the development of fatty liver (steatosis), progresses to alcoholic hepatitis, and eventually leads to alcoholic cirrhosis. Approximately 15% of the patients who develop alcoholic cirrhosis also develop hepatocellular carcinoma (Fattovich et al., 2004). Steatosis, the most common consequence of alcohol abuse, is characterized by lipid accumulation within hepatocytes which is thought to be a direct result of the altered redox state of the liver induced by alcohol metabolism. While this step can be reversed by abstinence from alcohol abuse, continued use leads to the development of alcoholic hepatitis, which is characterized by marked hepatic inflammation, and necrosis (Albano, 2008; Sies, 1997). Approximately 10% of patients with hepatitis go on to develop cirrhosis, the final stage of alcoholic liver disease and one of the main causes of death among alcohol abusers (Diehl, 1998; Ishak et al., 1991; Lieber, 2004).

Figure 1.1 Ethanol Metabolism. Ethanol is metabolized to acetaldehyde by alcohol dehydrogenase (ADH) or by CYP2E1, leading to the production of ROS and DNA adducts. Once acetaldehyde is formed, aldehyde dehydrogenase (ALD2) quickly converts it into acetate through an irreversible reaction.


NADPH + H+ + 02 NADP+ + 2 H20
ROS
CYP2E1
Ethanol
ADH
Acetaldehyde
NAD+
NADH
Cytosol
ALD2
Acetate
NAD+
NADH
Mitochondria
DNA-Adducts
ROS

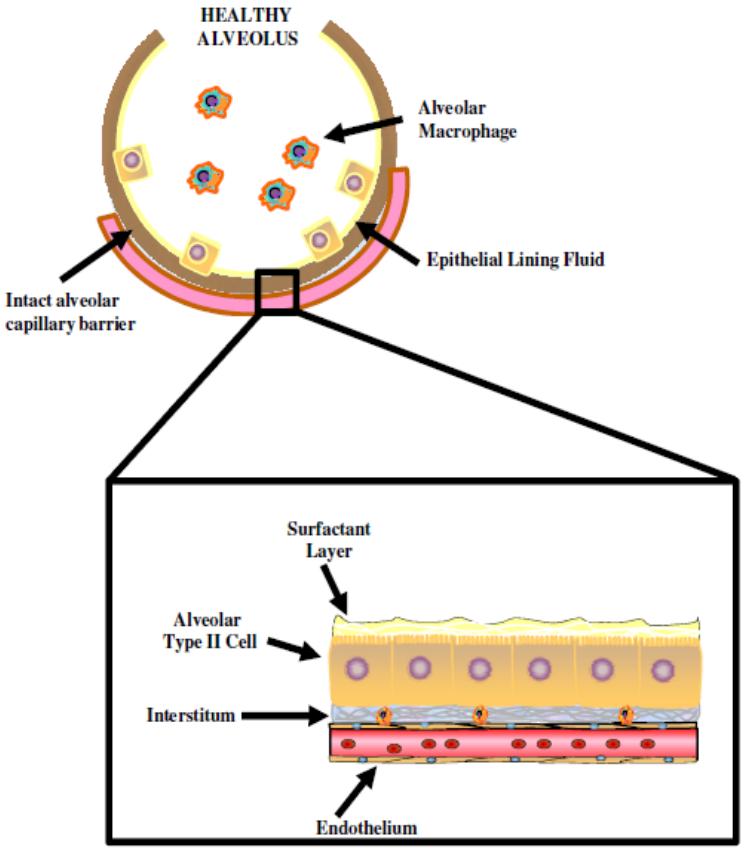
ALCOHOL ABUSE AND THE LUNG.

Acute respiratory distress syndrome (ARDS) is an acute, severe injury to the lungs that is characterized by disturbances in the pulmonary capillary-alveolar membrane that allows fluid to leak into the lung. This debilitating disease poses a large heath concern by affecting more than two thousand people annually within the United States (Frutos-Vivar et al., 2006). Patients with acute respiratory distress syndrome experience severe shortness of breath and often require mechanical ventilation (Boé et al., 2009). Acute respiratory distress syndrome is not a specific disease; instead, it is a severe condition that is associated with a myriad of diseases, such as pneumonia, shock, sepsis, and trauma (Esper A., 2006). During the acute phase of acute respiratory distress syndrome, there is sudden respiratory failure due to disturbances in the pulmonary capillary-alveolar membrane. This leads to rapid accumulation of fluid within the alveolar air sacs which impairs gas exchange, and results in respiratory distress. As a result of the air sacs filling with fluid, the lungs are unable to fill with air and the lungs become noncompliant, resulting in low blood oxygen levels, and the release of pro-inflammatory cytokines (**Figure 1.2**) (Frutos-Vivar et al., 2006; Kane and Galanes, 2004; Z. Sun, 2006). Onset of acute respiratory distress syndrome usually occurs within 24 hours to 3 days of the original illness or injury. The likelihood of developing acute respiratory distress syndrome depends on several predisposing disorders (Esper A., 2006; Ware and Matthay, 2000). Even though some patients can recover completely from acute respiratory distress syndrome, others develop lung fibrosis, have increased alveolar dead space, and a decline in pulmonary compliance. For those who survive acute respiratory distress syndrome, recovery can range from several weeks to years (Ware and Matthay, 2000).

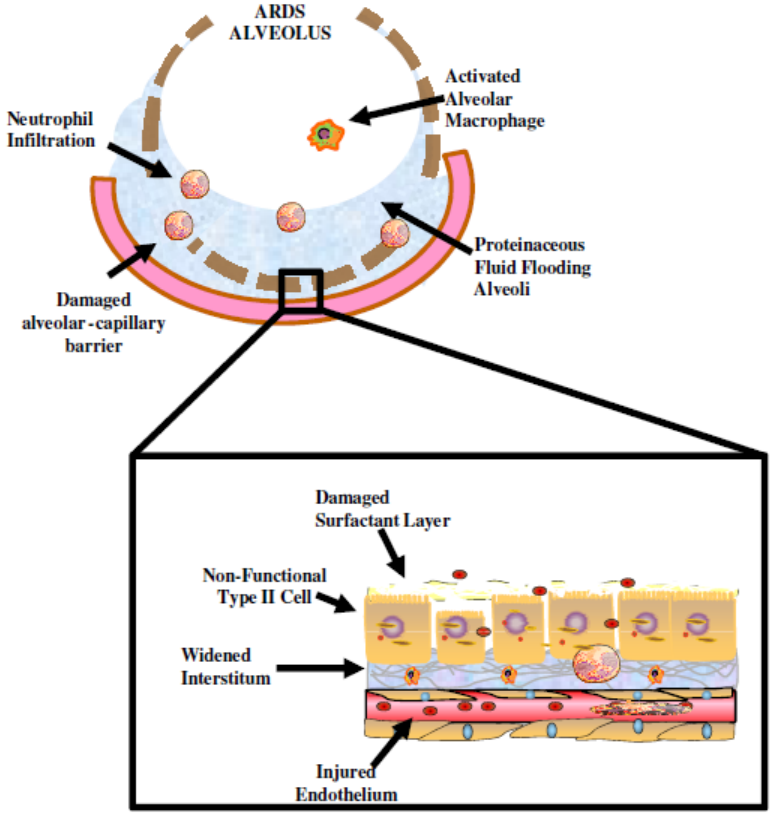
The incidence of acute respiratory distress syndrome is approximately 70% in patients with a history of alcohol abuse, compared to 30% in non-alcoholic patients (Moss et al., 1996). The outcome of alcoholic patients with acute respiratory distress syndrome is worse than in non-alcoholics as seen with a 30% increase in in-hospital mortality rates in patients with a history of

Figure 1.2 A Healthy and an ARDS Alveolus. ARDS is characterized by sudden respiratory failure due to disturbances in the pulmonary capillary-alveolar membrane that lead to rapid accumulation of fluid within the alveolar air sacs, impaired gas exchange, and respiratory distress. As a result of the air sacs filling with fluid, the lungs are unable to fill with air and the lungs become noncompliant, resulting in low blood oxygen levels, and the release pro-inflammatory cytokines

HEALTHY ALVEOLUS



ARDS ALVEOLUS



alcohol abuse. Alcoholism is specifically associated with sepsis-related acute respiratory distress syndrome induced by bacterial pneumonia (Moss, 2005). Alcoholic patients are more than three times as likely to develop pneumonia, most commonly due to community acquired pneumonia caused by Streptococcus pneumoniae. Once alcoholic patients develop community acquired pneumonia, they have a 2-fold increase in the risk of developing sepsis (Boé et al., 2009; Esper A., 2006; Moss et al., 2003). However, alcoholics also have increased susceptibility to pulmonary infections caused by other gram-negative bacteria, such as Klebsiella pneumoniae (Gamble et al., 2006). Within the Intensive Care Unit, alcoholic patients are also more susceptible to ventilator-associated pneumonia, the most common cause of nosicomial infections in critically ill patients (George et al., 1998). While there are numerous mechanisms by which alcohol abuse increases the susceptibility to ARDS in critically ill patients, previous studies suggest GSH depletion plays an integral role in derangements in alveolar type II cells and alveolar macrophages (Brown et al., 2004; Brown et al., 2007).

The epithelium of the lung consists of two distinct types of cells: alveolar type I cells, which covers the majority of the epithelium of the lung, and type II cells. Numerous studies have shown type II cells play a vital role in decreasing surface tension and maintaining lung compliance by producing surfactants, transporting fluids across the epithelial barrier, facilitating gas exchange, and regenerating damaged epithelium (Andreeva et al., 2007; Lucas et al., 2009). A hallmark of acute respiratory distress syndrome is damaged lung barrier function that leaves the lung unable to prevent fluid accumulation and facilitate gas exchange (Lucas et al., 2009; Moss and Burnham, 2003). In experimental models of alcoholism, 6 weeks of ethanol ingestion increased oxidant-mediated necrosis and decreased surfactant production, which was prevented and/or reversed by GSH supplementation(Brown et al., 2004; F. Holguin 1998). With ethanol, alveolar epithelial barrier function was attenuated, as seen by an increase in proteinaceous fluid in the alveolar space of the lung. Following endotoxin challenge, lungs isolated from alcohol-fed rats had increased activity of matrix metalloproteinases, enzymes that have been shown to

degrade the extracellular matrix of the lung. In addition, there was increased expression and activation of transforming growth factor beta$_1$ (TGF-β_1), a pluripotent cytokine shown to damage the alveolar epithelium and increase lung permeability (Crews et al., 2006; Frutos-Vivar et al., 2006; Joshi and Guidot, 2007a; Leask and Abraham, 2004; Moss and Burnham, 2003; Moss et al., 2003).

1.2.1. Pulmonary Macrophage Dysfunction.

Pulmonary macrophages are peripheral blood monocyte–derived mononuclear phagocytes that are found in the alveoli and in the interstitium of the lung. Peripheral blood monocytes pass through the pulmonary circulation and become sequestered in pulmonary capillaries. These sequestered monocytes migrate into alveolar and interstitial spaces, via interactions with the endothelial lining, diapedesis between the endothelial cells, and migration across the extracellular matrix (Forman and Torres, 2001b; Geiser, 2010; Gordon and Taylor, 2005; Gwinn and Vallyathan, 2006; Laskin et al., 2001). Monocytes are rich in adhesion molecules, such as members of the CD11 family (CD11a, CD11b, and CD11c), but they have diminished phagocytic ability. Mature pulmonary macrophages have minimal levels of such molecules but have higher phagocytic ability (Lundahl et al., 1996). Therefore, increased expression of these adhesion molecules is associated with an immature phenotype. Since monocytes must migrate through the pulmonary interstitium en route to the alveolus, the interstitial macrophage is thought to represent an intermediate stage of differentiation between monocytes and alveolar macrophages (Chandler and Brannen, 1990). Thus, differentiation and maturation of pulmonary macrophages encompass events that occur in both the interstitial and alveolar compartments of the lung (Chandler and Brannen, 1990; Laskin et al., 2001).

Granulocyte/macrophage colony-stimulating factor (GM-CSF) regulates alveolar macrophage differentiation through PU.1, a regulatory transcription factor required for normal hemopoietic cell development and the generation of both the innate and the adaptive immune

system (Berclaz et al., 2007; Lloberas et al., 1999; Scott et al., 1994). PU.1 promotes the proliferation and differentiation of myeloid progenitors by regulation of several genes (DeKoter et al., 1998; Lloberas et al., 1999; Rieske and Pongubala, 2001). Previous studies with GM-CSF knockout mouse have shown a lack of PU.1 prevents the differentiation of monocytes into mature macrophages and impairs alveolar macrophage function (DeKoter et al., 1998; Lloberas et al., 1999; Rieske and Pongubala, 2001). The reduced maturation and differentiation into alveolar macrophages is believed to be a result of a loss of expression of specific genes regulated by PU.1, including the following receptors: CD32, mannose receptor and granulocyte macrophage colony-stimulating factor receptor (GM-CSFR) (Egan et al., 1999; Neely et al., 2001; Trapnell and Whitsett, 2002). PU.1 also transcriptionally autoregulates its expression (Scott et al., 1994). These results suggest that PU.1 is required for the terminal maturation of alveolar macrophages in the lung. In previous experimental studies in a rat model, chronic ethanol ingestion has been shown to lead to decreased PU.1 expression within the alveolar epithelium and alveolar macrophages of the lung (Joshi et al., 2005a).

1.2.2. Alveolar Macrophage Phagocytosis.

The increased risk of respiratory infections in alcoholics is partially due to an impaired immune response of alveolar macrophages. Due to their ability to couple the internalization and degradation of inhaled pathogens to the release of inflammatory mediators, macrophages play a vital role in innate and acquired immunity (Berclaz et al., 2002; Gordon and Read, 2002; Shibata et al., 2001). Alveolar macrophages are readily recovered by washing the lung surface (termed bronchoalveolar lavage), while interstitial macrophages are embedded in the lung tissue. As the resident phagocyte in the airspace, alveolar macrophages provide a first line of host defense against inhaled particles. Upon exposure to pathogens, alveolar macrophages express cytokines that influence recruitment and activation of inflammatory cells and modify adaptive immune

responses. Interstitial macrophages function as the second line of defense and as antigen-presenting cells (Aderem and Underhill, 1999b; Chandler and Brannen, 1990).

Phagocytosis is initiated by the binding of specific receptors on the cell surface of the macrophage to ligands on the foreign pathogen. After attachment to the pathogen, macrophages begin to extend pseudopods to surround the pathogen and engulf the bacterium. The pathogen is then enclosed in a phagosome and is subsequently degraded once the phagosome fuses with a lysosome (Aderem and Underhill, 1999b). Pathogens can be recognized by a large variety of surface receptors on pulmonary macrophages, such as PU.1-dependent terminal differentiation/maturation markers for the phagocytosis of infectious particles (CD32, mannose receptor, and GM-CSFR)(Palecanda and Kobzik, 2001). Therefore, the degree of terminal differentiation is one mechanism that modulates the immune responses of alveolar macrophages.

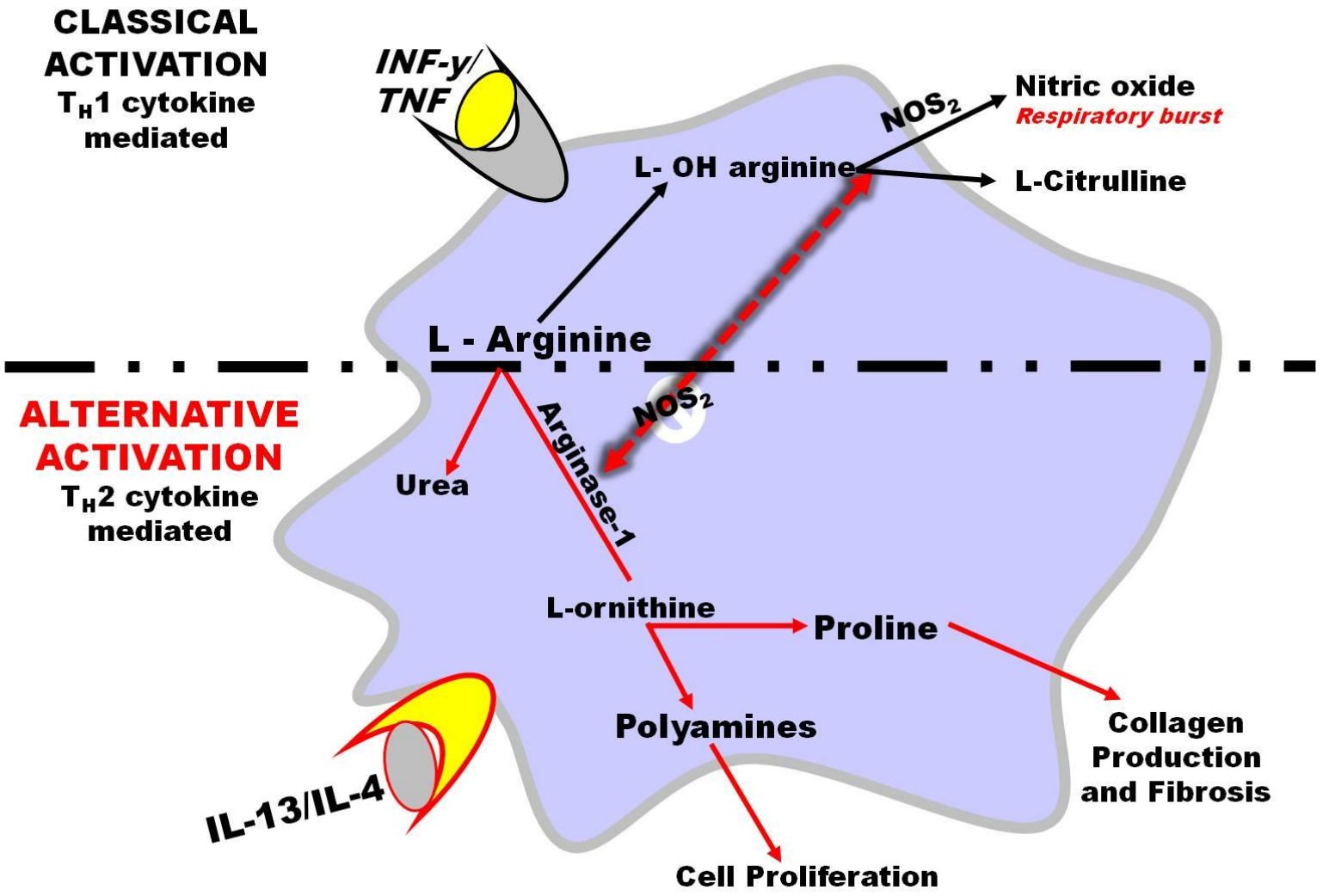
1.2.3. Alveolar Macrophage Activation.

In response to different stimuli within the microenvironment, such as microbial agents, cytokines, and antigen presenting cells, alveolar macrophages can become activated via distinct pathways that produce opposing effects on macrophage receptor expression, cytokine expression, and phagocytic function (Abramson and Gallin, 1990; Gordon, 2003; Gordon and Taylor, 2005; McKenzie et al., 1998; Muller et al., 2007; Taylor et al., 2005). Upon exposure to microbial stimuli such as lipopolysaccharide or T_H1 cytokines, such as IFN-γ, the alveolar macrophages are primed to become "classically activated" into effector cells (Downing et al., 1999; Nathan and Hibbs, 1991; Noda and Amano, 1997). IFN-γ stimulates the antimicrobial activity of infected alveolar macrophages which allows intracellular bacterial killing to occur via the production of ROS and reactive nitrogen intermediates (Downing et al., 1999; Nathan and Hibbs, 1991; Noda and Amano, 1997). Classical activation of alveolar macrophages also leads to upregulation of nitric oxide synthase-2, which generates nitric oxide and l-citrulline from L-arginine and molecular oxygen. Nitric oxide provides alveolar macrophages with antimicrobial

against foreign pathogens by inducing an inflammatory response that is required for phagocytosis as well as recruitment and activation of inflammatory cells (Aderem and Underhill, 1999a; Gordon, 2002; Gordon and Taylor, 2005; Song et al., 2000; Taylor et al., 2005). However, nitric oxide production is dependent on L-arginine availability (El-Gayar et al., 2003).

In response to T_H2 cytokines, such as IL-4 and IL-13, alveolar macrophages become alternatively activated and exhibit a phenotype that mediates allergic responses and promotes angiogenesis, tissue repair, and antiparasitic functions. Alternative activation is characterized by pronounced expression of arginase-1, which competes with nitric oxide synthase-2 for the substrate l-arginine (Munder et al., 1998). Arginase-1 hydrolyzes arginine to urea and L-ornithine, which produces polyamines and proline to promote cell growth and collagen production (**Figure 1.3**). As a result of decreased arginine availability and nitric oxide synthase-2 inhibition, the alveolar macrophage immune response is downregulated leading to increased susceptibility to lung infections (Hesse et al., 2001b; MacKinnon et al., 2008). This dampening of the alveolar macrophage immune response further promotes tissue repair (Gordon, 2002; Gordon, 2003; Gordon and Martinez; Gordon and Taylor, 2005; Martinez et al., 2009; Stein et al., 1992; Taylor et al., 2005). Alternative activation has also been shown to induce lung fibrosis via production of profibrotic factors such as fibronectin, matrix metalloproteinases, and TGF-β_1 expression (Isono et al., 2002; Leask and Abraham, 2004; Ruiz-Ortega et al., 2007; Wang et al., 2006). Several markers, such as arginase-1, galectin-3, mannose receptor, Ym1/2, TGF-β_1, and FIZZ-1, distinguish alternatively activated macrophages from classically activated macrophages (Fichtner-Feigl et al., 2006; MacKinnon et al., 2008; Nair et al., 2003; Stein et al., 1992). Since inhibition of galectin-3 blocks IL-13 and IL-4 induced alternative activation, this suggests that galectin-3 is necessary for alternative activation. Therefore, alternative activation is one mechanism by which the immune response or the pro-fibrotic response of alveolar macrophages is modulated.

Figure 1.3 Classical vs. Alternative Activation. The classically known macrophage activation pathway is induced by IFN-γ, which triggers the proinflammatory response necessary to kill intracellular pathogens. Classical activation of alveolar macrophages leads to upregulation of nitric oxide synthase- 2, which generates nitric oxide and l-citrulline from L-arginine and molecular oxygen. Classical activation is characterized by production of ROS and reactive nitrogen intermediates that participate in the respiratory burst. Macrophages also undergo alternative activation by T_H2 cytokines, IL-4 and IL-13, which trigger a unique phenotype seen in asthma, wound repair, and responses to parasitic infections. T_H2 cytokines antagonize classical activation, suppress the ROS produced during the respiratory burst and induce an alternative metabolic pathway for L-arginine. Rather than using L-arginine to generate nitric oxide, a key anti-microbial component, arginase-1 metabolizes L-arginine to L-ornithine, L-proline, and polyamine, which promote fibroblast proliferation, collagen production. This switch to an alternatively activated alveolar macrophage not only leads to suppression of the immune response but also contributes to lung fibrosis.


CLASSICAL ACTIVATION
T_H1 cytokine mediated
INF-y/ TNF
L- OH arginine
NOS_2
Nitric oxide
Respiratory burst
L-Citrulline
L - Arginine
NOS_2
ALTERNATIVE ACTIVATION
T_H2 cytokine mediated
Urea
Arginase-1
L-ornithine
Proline
Polyamines
Collagen Production and Fibrosis
IL-13/IL-4
Cell Proliferation

1.2.4. Alcohol Induced Alveolar Macrophage Dysfunction.

The increased risk of respiratory infections in alcoholics is partially due to an impaired immune response of alveolar macrophages (Rosseau et al., 2000). Previous studies have shown that chronic ethanol ingestion impairs their binding and internalization of inactivated *S. aureus.* Over 50% of the macrophages that were isolated from ethanol-fed animals were able to bind to the pathogen, but were unable to complete internalization (Brown et al., 2004). In other studies, chronic ethanol ingestion increased TGF-β_1 production within alveolar macropahges (Bechara et al., 2004). Increased TGF-β_1 expression is particularly important because it is associated with cellular dysfunction, increased pulmonary fibrogenesis, cell death, and antioxidant depletion (Khalil et al., 2001; Pulichino et al., 2008{Crews, 2006 #128).

1.3. OXIDATIVE STRESS AND ANTIOXIDANT DEFENSES.

1.3.1. Defining Reactive Oxygen Species and Oxidant Stress.

Free radicals are highly unstable molecules containing one or more unpaired electrons in atomic or molecular orbitals. Due to unpaired electrons, free radicals are highly reactive molecules that possess strong oxidizing capabilities (Gutteridge and Halliwell, 2000). Under normal conditions, free radicals and reactive oxygen species (ROS) play a vital role in cellular homeostasis by contributing to cellular proliferation, cellular respiration, protein folding, and modulating immune responses. However, dysregulation leads to increased production of free radicals and causes intracellular stresses to organelles and structures (Albano, 2006; Cadenas and Davies, 2000; Deneke and Fanburg, 1989; Elahi and Matata, 2006; Franco et al., 2007; Gutteridge and Halliwell, 2000; Pace and Leaf, 1995; Sies, 1997; Spector, 2000; Townsend et al., 2003; Valko et al., 2006; Wu et al., 2004). Major contributors of oxidative stress, such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$), are constantly generated and act as signaling intermediates during numerous cellular functions in aerobic

organisms (Forman and Torres, 2001a; Forman et al., 2002; Fuchs et al., 2010; Malhotra and Kaufman, 2007; Rojas et al., 2006).

Overproduction or inadequate removal of ROS contributes to oxidant stress by leading to an imbalance of pro-oxidants and antioxidants. Oxidant stress leads to modifications of cellular function, disruption of redox signaling, cellular damage, necrosis, and apoptosis due to oxidation of macromolecules, such as proteins, lipids, carbohydrates, and DNA. A common hallmark of ROS is increased peroxidation of polyunsaturated fatty acids within cell membranes. Peroxidation of membrane associated lipids can lead to altered membrane permeability and alter protein function (Albano, 2008; Pessayre, 2007; Sies, 1997). ROS mediated DNA damage can occur directly by inducing strand breaks or base and nucleotide modifications or indirectly by increasing intracellular calcium concentrations. Despite extensive DNA repair mechanisms, excessive ROS-mediated alterations can lead to irreversible permanent damage to the DNA that can prove to be detrimental to cellular function (Cadenas and Davies, 2000; Sedelnikova et al.; Valko et al., 2006). These alterations in cellular processes contribute to the pathogenesis of several diseases and countless disease processes; such as diabetes, liver disease, Alzheimer's disease, emphysema, Parkinson disease, HIV/AIDS, cystic fibrosis, aging and atherosclerosis (Guidot and Roman, 2002; Gutteridge and Halliwell, 2000; Halliwell, 1996; Pace and Leaf, 1995; Rahman et al., 2006; Spector, 2000; Valko et al., 2006; Ward, 2010).

There are numerous endogenous sources of ROS present in cell types. These sources include the mitochondrial electron transport chain, xanthine oxidase, cyclooxygenases, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, uncoupled nitric oxide synthases, and heme oxygenases (Inoue M., 2003). The mitochondrial electron transport chain has been shown to be a major source of intracellular ROS. During electron transport, approximately 5% of electrons escape to react with molecular oxygen to produce the highly reactive superoxide during oxidative phosphroylation (Cadenas and Davies, 2000; Inoue M., 2003; Pessayre, 2007; Sies, 1997). Once formed, superoxide is transferred to the outer membrane

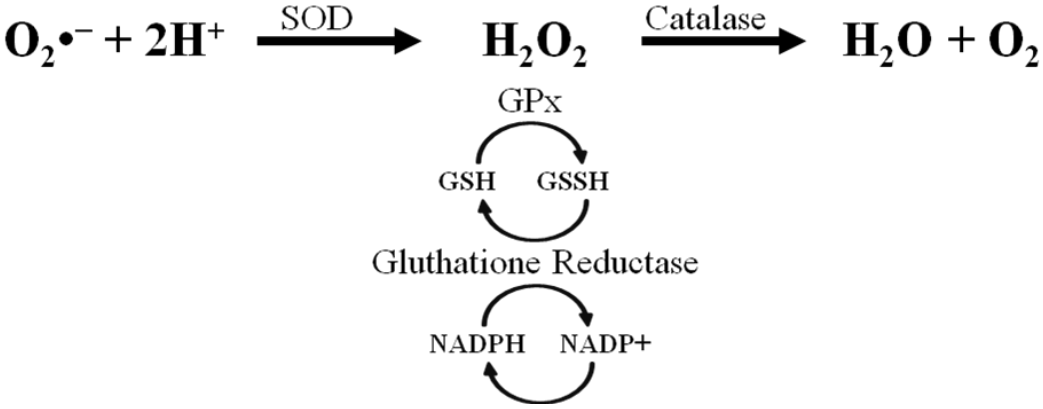
of the mitochondria where it is converted into hydrogen peroxide and other ROS. Since the mitochondria lack the antioxidant catalase, it is solely dependent on the GSH redox cycle to prevent hydrogen peroxide accumulation (Cadenas and Davies, 2000; Inoue M., 2003). ROS are also generated within the endoplasmic reticulum (ER) during protein folding, where an oxidized environment is required to support protein folding; during β-oxidation of fatty acids within the mitochondria; and by phagocytes during the process of phagocytosis (Forman and Torres, 2001a; Halliwell, 1994; Malhotra and Kaufman, 2007; Rojas et al., 2006; Ward, 2010).

1.3.2. Antioxidant Defenses.

Cellular defenses against the deleterious effects of ROS generation include both enzymatic (superoxide dismutases, catalases, glutathione peroxidases, peroxiredoxins, glutaredoxins, and thioredoxins) and non-enzymatic (vitamin A, vitamin C, vitamin E and GSH) antioxidants. The coordinated action of antioxidant enzymes is essential for efficient ROS detoxification and removal (Fridovich and Freeman, 1986; Gutteridge and Halliwell, 2000; Halliwell, 1994; Halliwell, 1996; Rojas et al., 2006). Antioxidants are compartmentalized based on the cellular localization of ROS generation.

Superoxide Dismutases. The enzymatic reduction of highly reactive superoxide to less reactive hydrogen peroxide is catalyzed by superoxide dismutase (SOD), a ubiquitous part of cellular antioxidant system present in all cell types (**Figure 1.4**). Three distinct isoforms of SOD exist and function to handle separate pools of superoxide: cytosolic copper/zinc superoxide dismutase (SOD_1), mitochondrial manganese superoxide dismutase (SOD_2), and extracellular superoxide dismutase (SOD_3). The activity of each of the superoxide dismutases is dependent upon specific metal ions and they vary in localization and ability to be induced (Beckman et al., 2002; Bruijn et al., 2004; Fridovich, 1975; Johnson and Giulivi, 2005). SOD_1, a 32.5 kDa homodimeric protein that requires both zinc and copper for stability and catalytic activity, is

Figure 1.4 Reactive Oxygen Species (ROS) and Antioxidant Defenses. Cellular defenses against the deleterious effects of ROS generation include both enzymatic and non-enzymatic antioxidant enzymes that are essential for efficient ROS detoxification and removal. The enzymatic reduction of reduced highly reactive superoxide to the less reactive hydrogen peroxide is catalyzed by superoxide dismutase (SOD). Catalase or glutathione peroxidase (GPX) catalyze the conversion of hydrogen peroxide to water and molecular oxygen. GSH works in concert with glutathione S-transferases and GPXs. GSH provides reducing equivalents for the GSH peroxidase-catalyzed reduction of hydrogen peroxide to water and molecular oxygen. As a result, GSH is transformed to its oxidized form GSSG. Glutathione reductase recycles GSSG back to GSH while oxidizing NADPH to NADP+.


$O_2\bullet^- + 2H^+$
SOD
H_2O_2
Catalase
$H_2O + O_2$
GPx
GSH
GSSH
Gluthatione Reductase
NADPH
NADP+

localized to the cytosol and plays a vital role in antioxidant defenses. Mutations in SOD_1 are found in patients with familial Lou Gehrig's diseases (amyotrophic lateral sclerosis), which is a progressive and fatal paralytic disease that is characterized by degradation of motor neurons in the brain stem and spinal cord (Beckman et al., 2002; Bruijn et al., 2004).

SOD_2 is an 88 kDa homotrimeric enzymatic protein that requires manganese for the two step dismutation of superoxide. SOD_2 is expressed in numerous cell types and tissues where it accounts for 10-15% of the total SODs. SOD_2 plays a vital role in cellular differentiation, aging, and cellular respiration by regulating ROS levels in the powerhouses of the cell, the mitochondria (Fridovich, 1975; Johnson and Giulivi, 2005). Mutations in SOD_2 have been associated with diabetes, Alzheimer's disease, and cardiomyopathy (Bäumer et al., 2000; Modlinger et al., 2004; Wiener et al., 2007).

SOD_3, a secretory tetrameric 135 kDa glycoprotein that requires copper and zinc for its activity, exhibits a high affinity for glycoaminoglycans, such as heparin. SOD_3 is localized to the interstitial spaces of selected tissues, plasma, lymph fluid, airways, and blood vessels (Nozik-Grayck et al., 2005). Numerous studies show that SOD_3 has a role in the modulation of vascular tone by blocking nitric oxide from reacting with superoxide within endothelial cells (Nozik-Grayck et al., 2005). Defects in SOD_3 expression have been found within the lungs of patients with chronic obstructive pulmonary disease (COPD) (Juul et al., 2006; Oberley-Deegan et al., 2009; Ward, 2010).

Catalase and Glutathione Peroxidase. Once SOD catalyzes the dismutation of superoxide to hydrogen peroxide, the enzymes catalase and glutathione peroxidase catalyze the conversion of hydrogen peroxide to water and molecular oxygen (Chan, 1996; Kinnula, 1999; Kinnula, 2005; Michiels et al., 1994) (**Figure 1.4**). Catalase is a peroxisomal tetrameric enzyme consisting of four 60 kDa subunits that reacts with hydrogen peroxide and hydrogen donors that possess peroxidase activity (Góth et al., 2004). Catalase is expressed in most aerobic cells, with high levels of expression in the liver and red blood cells, and decreased expression in the brain,

heart, and skeletal muscles (Wu et al., 2004; Zámocký and Koller, 1999). Catalase mutations are associated with the development of diabetes, hypertension, schizophrenia and atherosclerosis (Kinnula, 2005; Michiels et al., 1994). GSH peroxidases are a family of tetrameric selenium containing peroxidases that consist of one selenium-cysteine pair per subunit, which is required for its catalytic activity (Michiels et al., 1994; Mirault et al., 1994). GSH peroxidases exist in several different isoforms with varying levels of expression depending on the tissue and cell type. GSH peroxidase 1, the most common isozyome, is localized to the cytosol and mitochondria where it neutralizes hydrogen peroxide at the expense of GSH, a key regulator of intracellular redox and thiol status (**Figure 1.3**) (Day, 2009; Kinnula, 2005; Michiels et al., 1994; Mirault et al., 1994; Sies, 1997).

1.4. ROLE OF GLUTATHIONE IN CELLULAR HOMEOSTASIS AND DISEASE PROGRESSION.

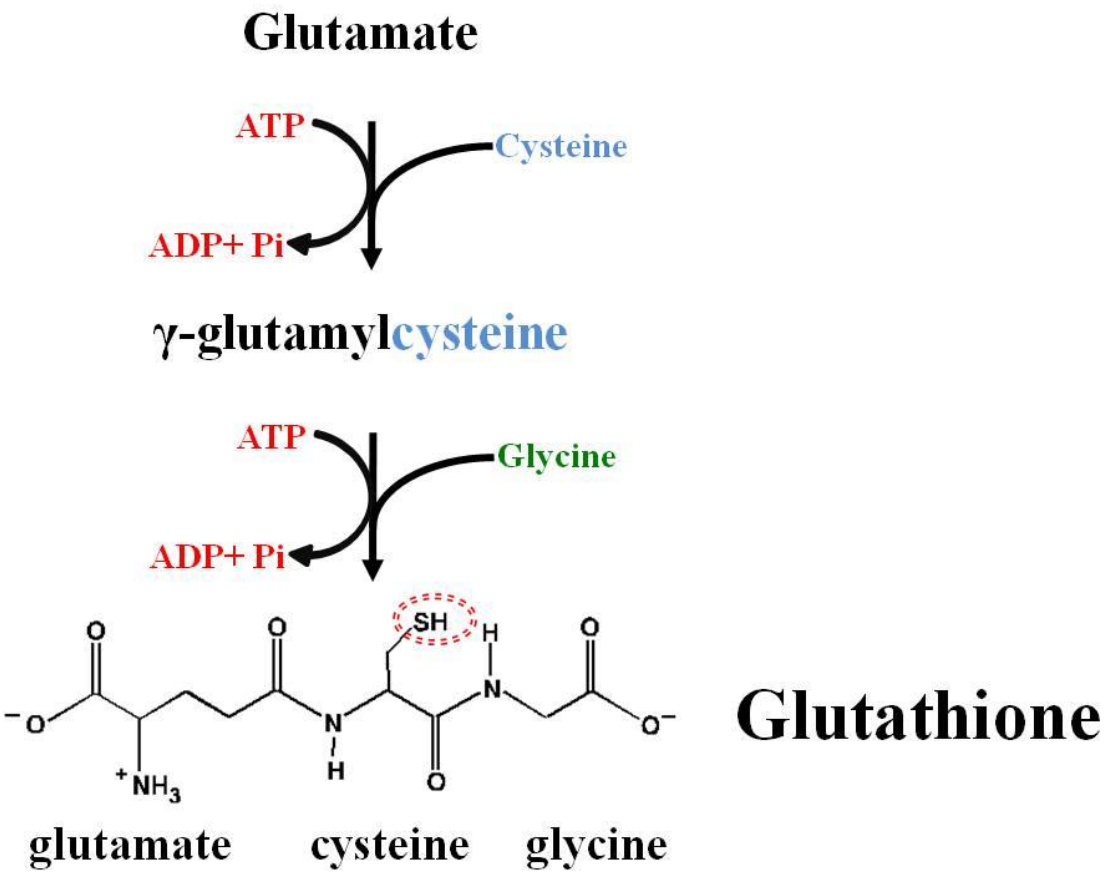
1.4.1. Glutathione Synthesis, Transport, and Compartmentalization.

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) is the most abundant thiol and antioxidant in mammalian cells. Its regulation plays a vital role in cellular homeostasis and pathogenesis of human disease (Lu, 2009; Rahman and MacNee, 2000; Townsend et al., 2003; Wu et al., 2004). GSH protects cells from ROS and reactive nitrogen species via detoxification by glutathione S-transferases and GSH peroxidases. Countless studies have shown that GSH acts as a regulator of cellular redox status and cellular signaling, as a reservoir of free cysteine, and in inflammatory responses. Dysregulation of GSH homeostasis has been implicated in the etiology of several disease states (Ballatori et al., 2009; Buhl et al., 1989; Deneke and Fanburg, 1989; Franco et al., 2007; Hudson, 2001; Huseby et al., 2003; Kinnula, 1999; Kinnula, 2005; Lash, 2005; Lu, 2009; Pace and Leaf, 1995; Rahman and MacNee, 2000; Sies, 1997; Sies, 1999; Sofic et al., 1992; Townsend et al., 2003; Wu et al., 2004).

GSH is a water soluble tripeptide (**Figure 1.4**) that is produced intracellularly in every cell type and organ system, but the majority of GSH synthesis occurs in the liver (Lu, 2009). GSH concentrations within the plasma are in the range of 0.01 mM. However, intracellular GSH concentrations are in the 1- 10 mM range. Intracellularly, GSH exists in a reduced form (GSH) or an oxidized disulfide form (GSSG). The reduced form, GSH, is the predominant form and accounts for 98% of the total intracellular pool (Townsend et al., 2003). Reduced GSH may also be protein-bound, but 90% of GSH is freely distributed within the cytosol, with a small percentage compartmentalized into organelles. Intracellular GSH concentrations are dependent upon a sensitive balance between synthesis, metabolism, and transport (Lu, 2009; Rahman and MacNee, 2000; Wu et al., 2004).

GSH can be synthesized *de novo* or via a salvage pathway. *De novo* synthesis of GSH begins with the formation of γ-glutamylcysteine, the rate-limiting step in GSH synthesis. The γ-glutamylcysteine synthase-catalyzed reaction combines glutamate and cysteine while consuming one molecule of ATP (Wild and Mulcahy, 2000). After this initial step, GSH synthase catalyzes the combination of γ-glutamylcysteine and glycine to form GSH (**Figure 1.5**) (Lu, 2009; Rahman and MacNee, 2000; Wu et al., 2004). Some cells can also synthesize GSH via salvage pathways that are involved in GSH catabolism and oxidation. Salvage of GSH through catabolism occurs across the outer membrane but is dependent on membrane-bound glutamyl transpeptidase, which transfers the γ-glutamyl moiety from extracellular GSH and GSH-conjugated compounds to different amino acid acceptors. Glutamyl transpeptidase forms γ-glutamyl-amino acid and cysteinylglycine or cysteinylglycine -conjugates which are further broken down by bipeptidase to form cysteine and glycine. The glycine and cysteine formed are taken up by specific transporters to preserve intracellular GSH and cysteine pools. The γ-glutamyl moiety is metabolized by γ -glutamyl cyclotransferase to form 5-oxoproline, the cyclical form of glutamate, and released as the conjugated amino acid (Ballatori et al., 2009; Franco et al., 2007; Lu, 2009; Rahman and MacNee, 2000; Townsend et al., 2003; Wu et al., 2004).

Figure 1.5 De Novo GSH Synthesis. De novo synthesis of GSH begins with the formation of γ-glutamylcysteine, the rate-limiting step in GSH synthesis. This reaction catalyzed by γ-glutamylcysteine synthase combines glutamate and cysteine while consuming one molecule of ATP. After this initial step, glutathione synthase catalyzes the combination of γ-glutamylcysteine and glycine to form GSH.


Glutamate
ATP
Cysteine
ADP+ Pi
γ-glutamylcysteine
ATP
Glycine
ADP+ Pi
SH
H
N
O
O
O
O
N
H
O
O
+NH3
glutamate
cysteine
glycine
Glutathione

GSH is synthesized exclusively in the cytosol in virtually all cells and then transported to intracellular organelles, where it acts as separate redox pools (Lu, 2009; Wu et al., 2004). Due to high levels of ROS produced during cellular respiration, the mitochondria depend heavily on GSH to prevent oxidative damage. Within the mitochondria, GSH is found predominately in its reduced form; mitochondrial GSH accounts for 10-20% of the total intracellular GSH pool. Nuclear GSH functions to protect protein sulfhydryl groups that are necessary to prevent ROS damage to DNA and acts as a hydrogen donor in DNA synthesis. Within the endoplasmic reticulum, GSH exists primarily in its oxidized form, GSSG. The higher level of GSSG is necessary for the formation of disulfide bonds to induce folding of nascent proteins formed by ribosomes (Rahman and MacNee, 2000; Sies, 1997).

1.4.2. Roles of GSH In Cellular Homeostasis.

GSH plays a vital role in the detoxification and reduction of numerous electrophilic compounds. GSH also plays an important role in numerous processes such as maintaining thiol status of proteins, metabolism of xenobiotics, thiol disulfide exchange reactions, regulation of cellular signaling, and acting as a reservoir for cysteine (Sies, 1999). As the most prevalent non-protein thiol in mammalian cells, GSH is required to regulate and maintain intracellular redox and thiol status. The ability of a cell to maintain optimal GSH:GSSG ratios is critical to regulate cell function and survival(Townsend et al., 2003; Wu et al., 2004). During detoxification, ROS are reduced via a reversible reaction that generates disulfide bonds between 2 molecules of GSH to form GSSG (e.g. $2GSH + H_2O_2 \leftrightarrow GSSH + 2\ H_2O$). Changes in the intracellular thiol-disulfide balance is used as an indicator of the redox potential of the cell (Jones, 2002).

All organisms produce physiological levels of ROS, and if not scavenged or detoxified, they can lead to cellular injury. GSH has been shown to scavenge a wide range of ROS, such as superoxide and hydrogen peroxide. In the presence of GSH peroxidase, ROS detoxification and reduction occurs with GSH acting as an electron donor, thereby becoming oxidized to GSSG. The

reduction of GSSG back to GSH is catalyzed by glutathione reductases at the expense of NADPH which is regenerated by the reduction of NADP+ in the pentose-phosphatase pathway (**Figure 1.4**) (Forman et al., 2002; Rahman and MacNee, 2000; Wu et al., 2004).

In numerous proteins, critical thiols are involved in cellular signaling but can have their function altered by redox modifications in response to ROS. These modifications can occur directly through interactions with ROS or indirectly via reactions with ROS byproducts (Ghezzi et al., 2002). Once modified by ROS, protein thiol groups form cysteine sulphenic derivatives through a reversible reaction but these sulphenic derivatives can be further oxidized through irreversible reactions to sulphinic acid derivatives. However, sulphenic derivatives can also react with GSH to form mixed disulfides, through a process known as glutathionylation (Gallogly and Mieyal, 2007). This is considered a reversible post-translational modification that prevents irreversible oxidation of protein thiols and regulates protein function (Ghezzi et al., 2002; Sies, 1999).

Due to its lack of stability, cysteine is rapidly oxidized to cystine and can lead to elevated production of ROS. Since GSH is more stable, it is a preferred cysteine storage form though the γ-glutamyl cycle involved in the salvage pathway of GSH synthesis. Transport of GSH to the extracellular compartment is essential for the transport and protection of cysteine (Wu et al., 2004). Due to its nucleophilic properties, GSH can also interact with electrophiles and xenobiotic compounds to regulate their activity and to eliminate these toxic compounds. GSH conjugation of xenobiotic compounds can occur non-enzymatically, or through GSH S-transferase catalyzed reactions. The GSH adducts are then secreted from cells via transporters, such as multidrug resistance proteins (Forman et al., 2009).

1.4.3. Diseases Associated With Altered GSH Levels.

Due to its vital role in regulation of ROS levels, alterations in GSH expression, transport, and/or catabolism have been implicated in numerous disease states such as cancer, HIV,

Parkinson's disease, liver disease, diabetes, and lung diseases (Buhl et al., 1989; Franco et al., 2007; Hudson, 2001; Lash, 2005; Sofic et al., 1992; Townsend et al., 2003; Wu et al., 2004). During cancer development and progression, large quantities of intracellular ROS are produced due to increased cellular metabolism and mitochondrial dysfunction. GSH works to defend the cells against the DNA damage induced by ROS (Brown et al., 1995; Sedelnikova et al.; Wu et al., 2004).

Cancer. Cancer, a disease that affects 1 in 3 people, is characterized by unrestricted growth of cells in general tissue or a specific organ. Numerous studies have linked polymorphisms in GSH S-transferase that alter its detoxifying activity and increase one's risk of developing skin, breast, kidney and liver cancers (Balendiran et al., 2004). Furthermore, transformed cancer cells have been shown to increase expression of GSH S--transferases and GSH peroxidases, which leads to an inhibition of apoptosis, cellular transformation, and increased resistance to chemotherapeutic agents and anti-neoplastic drugs (Sedelnikova et al.; Valko et al., 2006; Wu et al., 2004).

HIV. Human immunodeficiency virus (HIV), the virus that causes AIDS, is a retrovirus that attacks the human immune system. GSH levels are severely decreased in the plasma, epithelial lining fluid (ELF), and monocytes in asymptomatic HIV infected individuals (Buhl et al., 1989). These alterations in redox status are thought to potentiate HIV replication and progression. However, the precise mechanism by which GSH regulates HIV disease progression remains unclear (Pace and Leaf, 1995). In other studies, deficiencies in GSH have been associated with increased morbidity. In clinical studies, treatment with GSH precursors has proven to restore immunological functions and natural killer cell activity in HIV-infected individuals (Buhl et al., 1989; Franco et al., 2007; Ghezzi et al., 2002).

Liver Disease. Due to its detoxification functions, high levels of antioxidants are necessary for liver function (Cubero et al., 2009; Lieber, 2004; Mehta et al., 2002). GSH is the most abundant antioxidant in hepatocytes and plays a vital role in reducing ROS during phase I

liver detoxification. Thus, alterations in GSH levels within the liver have been associated with numerous pathologies (Han et al., 2006). For example, patients with chronic hepatitis C have depletions in GSH within the plasma, hepatocytes, and blood monocytes. Detoxification of drugs and xenobiotics, such as acetaminophen have been shown to deplete liver GSH levels as well (Mehta et al., 2002; Pessayre, 2007). Patients with alcoholic liver disease exhibit a depletion in both hepatic and plasma GSH levels. Furthermore, mitochondrial GSH becomes depleted leading to increased mitochondrial ROS and dysfunction. As the mitochondrial membrane integrity becomes compromised, apoptosis is initiated and eventually cirrhosis (Mehta et al., 2002; Wu et al., 2004).

1.5. GSH AND THE LUNG.

The lungs are exposed to numerous pollutants and pathogens during inhalation, and are therefore highly susceptible to oxidant injury. Furthermore, due to the oxygen in the ambient air, oxygen levels within the airways are more than three times higher than what is seen in other tissues (Deneke and Fanburg, 1989; Kinnula, 2005; Rahman et al., 2006). In response to inhalation of foreign pathogens and microorganisms, pulmonary macrophages are activated to produces large quantities of ROS during phagocytosis (Gordon and Read, 2002; Laskin et al., 2001). To combat these oxidants, the lung is a major source of GSH storage (Fridovich and Freeman, 1986; Rahman et al., 2006; Rahman and MacNee, 2000; Ward, 2010).

The lung imports and concentrates GSH via alveolar type II cells (Ballatori et al., 2009; Franco et al., 2007; Rahman and MacNee, 2000; Townsend et al., 2003). In the fluid lining the alveolar space, GSH levels (200–500 μM) are approximately 100 times higher than what is seen in the plasma (2–4 μM), with the majority of the GSH present in its reduced form (Brown et al., 2004; F. Holguin 1998). GSH within the epithelial lining fluid acts as a first line of defense to protect alveolar epithelial cells and alveolar macrophages from ROS and to maintain surfactant levels for alveolar stability. Alveolar macrophages maintain ten times higher GSH levels than

type II cells (Cantin and Bégin, 1991; Rahman and MacNee, 2000). Due to the impermeability of the alveolar-capillary barrier, there is limited release of GSH from the alveolar space into the vasculature. In addition to increased GSH, the lung also has 300-fold higher levels of γ-glutamylcysteine synthase when compared to other tissues. These factors contribute to an accumulation of GSH within the alveolar epithelial lining fluid (Ballatori et al., 2009; Franco et al., 2007; Townsend et al., 2003; Wu et al., 2004**).**

Deficits in GSH within the epithelial lining fluid have been shown to contribute to an altered redox status of the lung and to increase susceptibility to several pulmonary disorders such as idiopathic pulmonary fibrosis, acute respiratory distress syndrome, neonatal lung damage, and cystic fibrosis (**Table 1.4**) (Beeh et al., 2002; Morrison et al., 1999; Moss et al., 2000c). In these disease states, alterations in GSH levels are due to decreased GSH synthesis and increased lipid peroxidation within the lung (Cantin and Bégin, 1991; Kinnula, 1999; Kinnula, 2005; Rahman et al., 2006; Rahman and MacNee, 2000).

	GSH CONCENTRATIONS (μM)		
	Control Values	**Patients with Lung Disorders**	**Reference**
Alcohol Abuse	576	80	(Moss et al., 2000b), (Yeh et al., 2007)
ARDS	651	32	(Bunnel and Pacht, 1993)
Cystic Fibrosis	257	170	(Roum et al., 1993)
Idiopathic Pulmonary Fibrosis	429	97	(Beeh et al., 2002)

Table 1.4 GSH Concentrations in Pulmonary Diseases

In chronic smokers, the epithelial lining fluid GSH is increased when compared to non-smokers. However, this is not the case with mild smokers. This is believed to occur as a mechanism to protect alveolar cells from the high levels of ROS that occur within the lung of smokers (Kinnula, 2005). In idiopathic pulmonary fibrosis (IPF), a chronic inflammatory lung disease that is characterized by increased accumulation of inflammatory cells and increased macrophage production; there is an altered redox balance due to marked increases in release of ROS. Deficiency in epithelial lining fluid GSH levels amplifies this altered redox status, and further contributes to the pro-inflammatory environment (Hudson, 2001; Wu et al., 2004). Cystic fibrosis, a genetic disorder due to recessive mutations of the cystic fibrosis transmembrane regulator (CTFR) gene, is characterized by chronic lung inflammation. A key contributor to the airway derangements seen in cystic fibrosis patients is the increased production and release of ROS by inflammatory cells. In addition to serving as a chloride channel, the CFTR present on the apical side of lung epithelial cells also transports GSH into the epithelial lining fluid (Hudson, 2001). This mutation therefore leads to decreased GSH levels in lung epithelial lining fluid and blood plasma (Brown et al., 1995; Rahman and MacNee, 2000). During the development of acute lung injury, there is an increase in ROS production and imbalance in GSH homeostasis that leads to decreased antioxidant capacity of pulmonary tissues and a exacerbation of several disorders resulting in the development of acute respiratory distress syndrome (ARDS) (Kinnula, 1999; Rahman and MacNee, 2000; Ward, 2010; Wu et al., 2004). During hypoxia-mediated lung injury, there are decreased lung GSH levels along with a downregulation of glutamate cysteine ligase and GSH synthase, contributing to further injury (Fuchs et al., 2010; Juul et al., 2006; Touyz and Schiffrin, 2004).

1.6. SPECIFIC AIMS.

Patients with a known history of alcohol abuse have a 43% chance of developing ARDS, which frequently develops as a result of systemic infections caused by invading foreign pathogens, and is characterized by lung inflammation that leads to impaired gas exchange and release of pro-inflammatory cytokines. The most common cause of ARDS in alcoholic patients is community-acquired pneumonia (Jong et al., 1995a). The outcome of alcoholic patients with ARDS is worse than in non-alcoholics, as seen by a 30% increase in in-hospital mortality rates. The increased risk of respiratory infections in alcoholics is partially due to an impaired immune response of alveolar macrophages (Aytacoglu et al., 2006b; Brown et al., 2004; Joshi and Guidot, 2007b). Alveolar macrophages play a vital role in innate and acquired immunity due to their ability to couple the internalization and degradation of inhaled pathogens to the release of inflammatory mediators..

During classical "M1" IFN-γ dependent activation of alveolar macrophages by T helper 1 (T_H1) cytokines leads to a proinflammatory response that is required for the respiratory burst necessary for phagocytosis. However, alternative activation is induced by the T_H2 cytokines IL-4 and IL-12. This leads to unregulated ROS production, decreased phagocytosis, increased arginase production, and increased TGF-β_1 production. Increased arginase production/activity leads to proline and polyamine production which leads to fibroblast proliferation and collagen production in the lung.

Previous studies in experimental models have shown chronic alcohol treatment impairs alveolar phagocytosis of inactivated *Staphylococcus aureus* (*S. aureus)*. Moreover, *in vivo* treatment with precursors to GSH improves alveolar macrophage phagocytosis during chronic ethanol ingestion. These results suggest the decreased function of alveolar macrophages is due to decreased antioxidant availability. In previous studies, chronic ethanol ingestion has been shown to lead to increased oxidant stress, increased TGF-β_1 production, and increased fibronectin

production in the lung (Bechara et al., 2004; Brown et al., 2004; Guidot and Roman, 2002). Alternative activation of alveolar macrophages has also been shown to lead to similar alterations, as well as upregulation of: arginase-1, galectin-3, mannose receptor, and FIZZ1. Therefore, we hypothesized that chronic oxidant stress induced by chronic ethanol consumption leads to impaired maturation and alternative activation of alveolar macrophages. *In vivo* or *ex vivo* treatment with GSH or its precursor are expected to reduce the oxidant stress induced by chronic ethanol consumption and thereby reduce/reverse impairment/alterations of alveolar macrophage activation, maturation, and function. To address this hypothesis, cell culture and rodent models of chronic alcoholism along with alveolar macrophages from otherwise healthy alcoholic subjects were used to examine the potential link between chronic alcohol-induced alterations of alveolar macrophage function and alternative activation.

Aim 1: Determine the role of GSH in chronic ethanol ingestion-induced impairments of the maturation and function of alveolar and interstitial macrophages.

Aim 2: Determine if chronic alcohol ingestion alters expression markers of alternative activation and if in-vivo or ex-vivo GSH treatment can reduce/alter expression of markers of alternative activation.

Aim 3: Examine the role of TGF-β_1 in alternative activation of alveolar macrophages.

These studies will provide insight into the mechanism of chronic alcohol-induced macrophage malfunction and provide potential treatments to decrease the risk of infection, and thereby improve the compromised pulmonary function and mortality in alcoholics.

CHAPTER 2

CHRONIC ALCOHOL INGESTION IMPAIRS MATURATION AND FUNCTION OF ALVEOLAR MACROPHAGES

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2.1. ABSTRACT.

Alcoholic patients have an increased risk of respiratory infections, which is partially due to an impaired immune response of alveolar macrophages. The mechanisms by which alcohol impairs alveolar macrophage function are poorly understood. In this study, we demonstrated in a guinea pig model that chronic ethanol ingestion significantly impaired alveolar macrophage differentiation and function. Isolated alveolar macrophages were separated into 4 different subpopulations with varying densities and levels of maturation. Compared to control values, chronic ethanol ingestion decreased the percentage of alveolar macrophages in the mature fractions by 60%. Alveolar macrophage function in each subpopulation was determined by measuring phagocytosis of FITC-labeled *S. aureus*. Alveolar macrophages from ethanol-fed animals had 80% decrease in the phagocytic index. Western blot and immunohistochemical analysis of the differential markers GM-CSF receptor α (GM-CSFR-α), PU.1, CD11c, and CD11b verified that alcoholic macrophages displayed impaired terminal differentiation. While oral supplementation with the glutathione precursor S-adenosyl-methionine (SAM) did not alter the maturational status of control animals, SAM supplementation shifted the distribution of macrophages to more mature fractions, normalized the phagocytic index; as well as normalized expression of CD11c, CD11b, PU.1, and GM-CSFR-α. Chronic ethanol ingestion also impaired the differentiation status of interstitial macrophages which was normalized by SAM supplementation. This improvement in the maturational status suggested that ethanol-induced oxidant stress is a central feature in impaired terminal differentiation of macrophages in the interstitial and alveolar space. Therefore, strategies targeting pulmonary oxidant stress may restore macrophage differentiation and function even after chronic ethanol ingestion.

2.2. INTRODUCTION.

Patients with a known history of alcohol abuse have a 43% chance of developing acute respiratory distress syndrome (ARDS), an acute, severe injury to the lungs associated with a variety of risk factors (Ware and Matthay, 2000). ARDS is characterized by lung inflammation that leads to an impairment of gas exchange and the release of pro-inflammatory cytokines (Aytacoglu et al., 2006a). The incidence and severity of community-acquired pneumonia, the most common cause of ARDS in alcoholic patients, is associated with a 30% increase in the in-hospital mortality rate (Esper A., 2006; Jong et al., 1995b). This increased risk of bacterial pneumonia is partially due to impaired alveolar macrophage function (Rosseau et al., 2000).

Peripheral blood monocytes pass through the pulmonary circulation and become sequestered within pulmonary capillaries and then migrate into interstitial and alveolar spaces where they mature into pulmonary macrophages (Akagawa et al., 1988; Lloberas et al., 1999; Shibata et al., 2001). Monocytes are rich in adhesion molecules, such as members of the CD11 family (CD11a, CD11b, and CD11c), but possess diminished phagocytic ability. Since monocytes must migrate through the pulmonary interstitium en route to the alveolus, the interstitial macrophage is thought to represent an intermediate stage of differentiation between monocytes and alveolar macrophages (Laskin et al., 2001). As macrophages mature in the alveolar space, they increase in size and decrease in density. Compared to alveolar macrophages, interstitial macrophages are much smaller in size, have blunt pseudopodia, and closely resemble monocytes. Mature alveolar macrophages have minimal levels of such adhesion molecules but have significantly greater phagocytic capacity (Lundahl et al., 1996) and play a vital role in innate and acquired immunity (Trapnell and Whitsett, 2002).

Granulocyte/macrophage colony-stimulating factor (GM-CSF), a glycoprotein responsible for controlling the terminal differentiation of peripheral blood monocyte into terminally differentiated alveolar macrophages, promotes the proliferation and differentiation of myeloid progenitors (Baldwin, 1992; Burgess and Metcalf, 1980),(Berclaz et al., 2007; DeKoter

et al., 1998; Shibata et al., 2001). In the GM-CSF knockout mouse, alveolar macrophages displayed impaired surfactant expression, clearance, phagocytosis, as well as altered receptor expression (Berclaz et al., 2002; Trapnell and Whitsett, 2002). PU.1 is a regulatory transcription factor required for normal hemopoietic cell development, proliferation and differentiation of myeloid progenitors, and the generation of both the innate and the adaptive immune system (Lloberas et al., 1999; Scott et al., 1994). Previous studies have shown that GM-CSF regulates alveolar macrophage differentiation through PU.1. In a rat model, chronic ethanol ingestion decreased expression of PU.1 and the GM-CSF receptor (GM-CSFR) within the alveolar macropahge, suggesting decreased terminal differentiation (Joshi et al., 2005a).

Glutathione (GSH), the most abundant thiol in living organisms, is essential for protein synthesis, DNA synthesis and detoxification of oxygen radicals. In the lung, GSH is concentrated in the epithelial lining fluid through transport by alveolar type II cells (Brown et al., 2007). In clinical and animal studies, chronic ethanol ingestion depletes GSH within the alveolar space resulting in chronic oxidative stress and a loss of alveolar macrophage functions (Brown et al., 2004; Brown et al., 2007; F. Holguin 1998; Yeh et al., 2007). In animal studies, chronic ethanol-induced depletion of GSH in the lung impairs alveolar type II cell function (Guidot et al., 2000), impairs clearance of microbes and decreases alveolar macrophage cell viability (Aytacoglu et al., 2006a; Velasquez A, 2002).

Previous studies by this laboratory and others demonstrated that chronic ethanol ingestion impairs alveolar macrophage binding and internalization of inactivated *Staphylococcus aureus* (*S. aureus)* (Brown et al., 2004; Standiford TJ, 1997). Moreover, *in vivo* treatment with GSH precursors improved alveolar macrophage phagocytosis during chronic ethanol ingestion (Brown et al., 2007). These results suggested that the decreased functions of alveolar macrophages were due to decreased GSH availability and subsequent oxidant stress in the alveolar space. However, the precise mechanisms by which ethanol impairs alveolar macrophage function remain poorly understood.

Therefore, we hypothesized that chronic ethanol ingestion alters macrophage terminal differentiation and function and that these alterations were secondary to ethanol-induced decreases in antioxidant availability. To address this hypothesis, we examined the role of chronic ethanol consumption on the maturation and terminal differentiation of peripheral blood monocytes into pulmonary macrophages and their phagocytic capacity. Previous studies in a guinea pig model demonstrated that alveolar macrophages can be separated into subpopulations with distinct densities, as well as morphologic and functional properties (Chandler et al., 1986; Dauber JH, 1983; Laskin et al., 2001). In these experiments, we capitalized on this change in density to separate the alveolar macrophages by a percoll density gradient into four fractions with a range in terminal maturation. Assessment of these different fractions suggested that chronic ethanol ingestion impaired terminal differentiation of macrophages and that these effects were secondary to ethanol-induced oxidant stress.

2.3. MATERIALS AND METHODS.

Guinea Pig Model of Chronic Ethanol Ingestion. Since the adult guinea pig has well-defined subpopulations of resident alveolar macrophage that correspond directly to their level of phagocytic function (Dauber JH, 1983), we chose to examine the effects of chronic ethanol exposure on the maturation of the alveolar macrophage in this model. Pathogen-free 6-8 week old female Hartley guinea pigs weighing 400-500 grams were shipped from the vendor (Charles River). Three days after delivery to the Emory Animal Facility, animals were randomly assigned to drinking water with or without ethanol. The ethanol content of the drinking water was incrementally increased to 4% (Mato et al., 1999). For some of the control and ethanol-treated animals, the GSH precursor S-adenosyl-methionine (SAM) (0.04%) was also added to the drinking water. During the 25 days of treatment, the only access to drinking water was the experimental mixture. Solid diet was provided *ad libitum* to all four groups. All animals were used in accordance with NIH guidelines (*Guide for the Care and Use of Laboratory Animals*) as

described in protocols reviewed and approved by the Emory University Institutional Animal Care Committee.

Alveolar Macrophage Isolation. After pentobarbital anesthesia, the trachea was cannulated and the guinea pig lung underwent 4 bronchoalveolar lavages, consisting of 10 ml each, for a total of 40 ml of sterile phosphate-buffered saline (37°C, pH 7.4). The pooled lavage fluid was centrifuged at 500 g for 8 min and the cell pellet was resuspended in 2 ml of sterile Hank's Buffered Salt Solution (HBSS). After staining with Diff Quik (Dade Behring, Newark, DE) and counting on a hemacytometer, the cell population was determined to be 95% macrophages.

Alveolar Macrophage Separation. Alveolar macrophages were subfractionated by centrifugation on a continuous percoll density gradient as described by others (Chandler et al., 1986; O'Neill et al., 1984). After the iso-osmotic percoll was diluted to 49.2% with phosphate buffered saline, the pH was adjusted to 7.2 and 12 mL of the diluted Percoll was then added to a 15 ml glass centrifuge tube (Corning Glass, Corning, NY). Two identical density-based gradients of Percoll were generated for each animal. One tube was used for the separation of alveolar macrophages and the second tube was used for density calibration using marker beads of known density (Sigma-Aldrich, Saint Louis, MO). All of the tubes were centrifuged at 30,000 *g* for 90 min at 4°C to form a continuous gradient. After 2 ml of the resuspended alveolar macrophages was added to the Percoll tube and 2 ml of HBSS was added to the calibration tube, the tubes were centrifuged at 400 g for 20 min at 4 $^{\circ}$C. The gradient separated into 4 fractions with the following densities (*fraction 1*, <1.055 g/ml; *fraction 2*, 1.056-1.061 g/ml; *fraction 3*, 1.061-1.065 g/ml; and *fraction 4*, >1.065 g/ml). Starting with the top layer (layer 1), each gradient fraction was recovered by aspirating with an 18-guage spinal needle. Cells were then resuspended with 5 volumes of HBSS, centrifuged at 200 *g* for 20 min at 4°C and the resulting cell pellet from each layer was resuspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1%

penicillin-streptomycin solution and 10% fetal bovine serum. The cell number in each layer was determined via counting on a hemacytometer.

Phagocytosis Of Fluorescein Isothiocyanate - (FITC)-Labeled S. Aureus In Vitro. Alveolar macrophages from each fraction were plated at a density of 10^6 cells/mL in DMEM supplemented with 1% penicillin-streptomycin solution plus 10% fetal bovine serum and cultured at 37°C, 5% CO_2 for 1 hr. Fluorescein-labeled inactivated *S. aureus* (Molecular Probes, Eugene, OR) was then added in a 1:10 ratio (macrophage to bacteria). After a 4-hr culture period, alveolar macrophages were washed with DMEM and fixed with 3.7% paraformaldehyde. Phagocytosis of *S. aureus* was determined via quantitative digital analysis of fluorescence using confocal fluorescent microscopy (Olympus, Melville, NY) as previously reported (Brown et al., 2007; Ping et al., 2007). Using three-dimensional analysis, we evaluated the alveolar macrophages at 50% cell depth in the z plane to ensure that the *S. aureus* was internalized and not bound to the cell periphery (not within the outer 10 mm of the plasma membrane) (Brown et al., 2007). Fluorescence was determined via quantitative digital analysis using FluoView (Olympus, Melville, NY). Values are presented as mean relative fluorescent units (RFUs)/cell ± SEM, the mean percentage of cells fluorescently positive for internalization (per field) ± SEM and as the phagocytic index ± SEM (mean RFUs)/cell X percentage of cells fluorescently positive for internalization) as tallied from at least 15 experimental fields/set.

Interstitial Macrophage Isolation. To ensure removal of alveolar macrophages from the lung, two additional bronchoalveolar lavages with 10 mL of saline solution each were performed. After the lavages, the lungs were minced into 2-4 mm pieces with scissors and excess blood removed by rinsing the lung pieces with phosphate buffered saline and filtering through 70-µm nylon mesh cell strainer membranes (BD Falcon, Bedford, Mass.). The tissue pieces were then incubated with RPMI-1640 medium containing trypsin (166 mg/mL) and EDTA (66 mg/mL) for 40 min at 37°C.

To remove particulate matter, the cell suspension was filtered through gauze and then centrifuged for 10 min at 1,500 *g* at 4°C (Sansores et al., 1997). The cell pellet containing interstitial macrophages was resuspended in RPMI-1640 medium, washed twice, and then centrifuged. The cells were then resuspended in RPMI-1640 medium, counted, adjusted to a concentration of 5×10^5 cells/ml, and plated onto glass coverslips inserted in 15-mm 4-well culture plates before they were cultured at 37°C, 5% CO_2 for 1 hr. After this culture period, nonadherent cells were removed and the adherent cells were washed twice with phosphate buffered saline (PBS). The cells were then fixed with 2.7% paraformaldehyde before assessment by immunofluorescence techniques.

Western Blot Analysis. Alveolar macrophage and interstitial macrophage cell lysates were prepared by adding cell lysis buffer containing protease inhibitor cocktail (Roche) to isolated macrophages. Protein concentration was determined using the Micro BCA protein Assay (Pierce) Micro BCA Protein Assay Reagent Kit **(**Pierce**).** Fifty micrograms of total protein from each sample was separated by SDS-PAGE, and the gel was transferred on to polyvinylidene difluoride membranes, as previously described(Joshi et al., 2005b). Membranes were blocked at room temperature for 1 h in TBS with 0.2% Tween 20 (TBS-T) containing 5% nonfat dry milk in TBS-T. Primary antibodies at 1:50 dilution in 5% milk in TBS-T were added to the membranes and kept at 4°C overnight. After three washes with T-TBS for 10 min, membranes were incubated at room temperature with secondary antibodies coupled to a horseradish peroxidase (Santa Cruz Biotechnologies, Santa Cruz, CA) for 2 h. After adding ECL chemiluminescence reagent (Amersham Biosciences) to the membranes, bands were detected by exposure to radiographic film and quantified (via densitometry) using a densitometric scanning using a GS-800 calibrated laser densitometer (Bio-Rad).

Immunofluorescence Characterization. Alveolar macrophages and interstitial macrophages were isolated and cultured as described above. After the culture period, the macrophages were fixed with 3.7% paraformaldehyde and nonspecific binding blocked by incubating the cells with 1% bovine serum albumin for 1 hr at room temperature. Cells were then washed twice in PBS, and incubated for 2 hr at room temperature with the following primary antibodies at 1/100 dillutions: CD11b (Abcam, Cambridge, MA), CD11c (Antigenix America, Franklin Square, NY), the α subunit of the GM-CSF receptor (GM-CSFR α) (Santa Cruz Biotechnology, Santa Cruz, CA) or PU.1 (Santa Cruz Biotechnology, Santa Cruz, CA). The slides were washed three times with PBS and incubated with the appropriate secondary antibodies at 1:200 dilutions (all FITC- and Tetramethyl Rhodamine Iso-Thiocyanate (TRITC)-conjugated antibodies; Sigma-Aldrich) for 1 hr, followed by 2 washes with PBS. Immunofluorescence was determined via quantitative digital analysis of fluorescence (QImaging, Burnaby, BC, Canada) and data analysis performed using Image-Pro Plus for Windows (Jandel Scientific). Background fluorescence was determined and subtracted from the mean relative fluorescent units. Values are presented as the mean relative fluorescent units per cell (± SE) as tallied from at least 10 experimental fields per set.

Statistical Analysis. Statistical analysis was performed with Sigma Stat for Windows. The data are presented as means ± SE. Results were analyzed using one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test comparisons. A p-value of < 0.05 was considered significant.

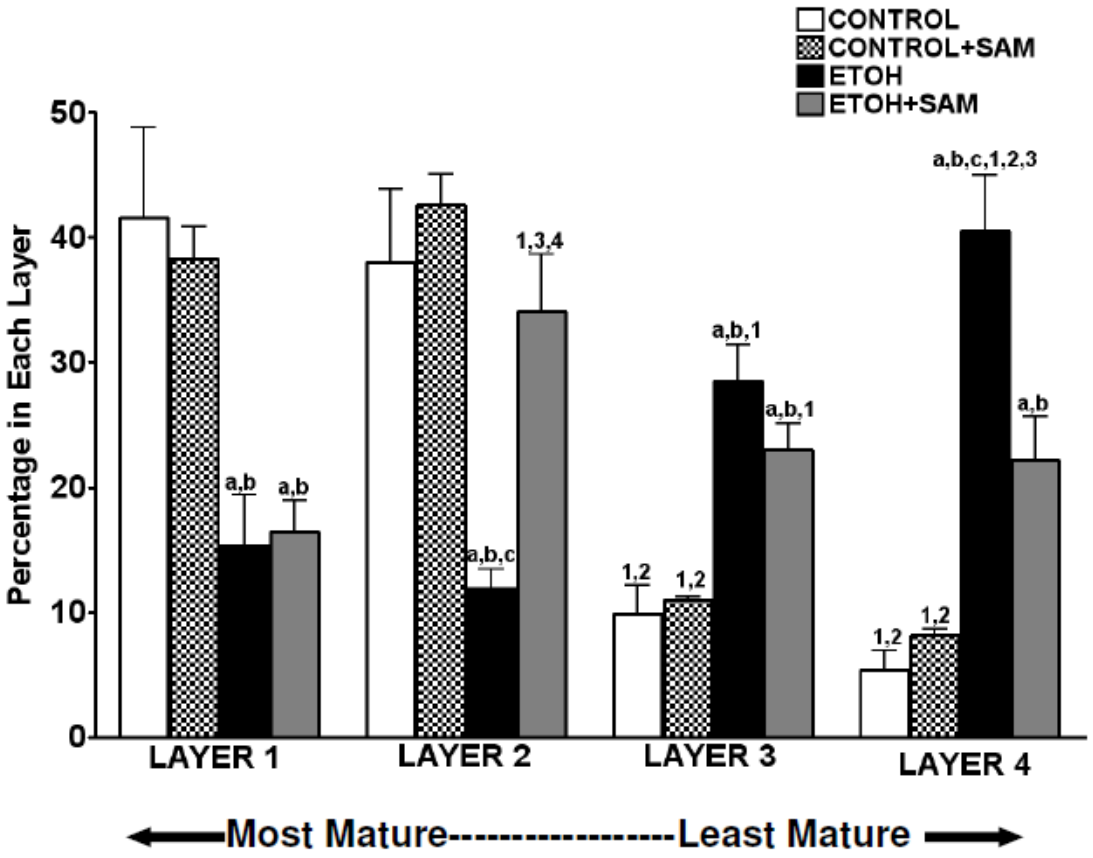
2.4. RESULTS.

Chronic Ethanol Ingestion Impaired Alveolar Macrophage Maturation. Pulmonary macrophages increase in size, gain cytoplasm and decrease in density as they mature. As a result, we were able to separate alveolar macrophages into various subpopulations based on densities that contained cells at different maturational stages. The most dense (high nuclear-to-cytoplasmic

ratio) alveolar macrophages represent the most immature cells and the least dense (low nuclear-to-cytoplasmic ratio) cells represent the most mature alveolar macrophages (Dauber JH, 1983). We determined 1) the total number of alveolar macrophages isolated by the bronchoalveolar lavage, 2) the number of alveolar macrophages in each of the four subpopulations, and 3) the distribution of alveolar macrophages across the four subpopulations. Across the four experimental groups, there were no significant differences in the total number of alveolar macrophages retrieved by the bronchoalveolar lavage (control 2.2 ± 0.5 $x10^6$/animal; control+ SAM 2.9 ± 0.3 $x10^6$; ethanol 1.6 ± 0.3 $x10^6$ /animal; ethanol + SAM 1.9 ± 0.4 $x10^6$ /animal). However, chronic ethanol ingestion altered the distribution of alveolar macrophages across the four layers of maturation. Over 75% of the control alveolar macrophages were located within the two most mature fractions (layer 1 and 2), while less than 30% of the ethanol-exposed alveolar macrophages were present in these fractions (**Figure 2.1, $^{a,b}p < 0.01$ vs. control and control + SAM).** When the GSH precursor SAM was added to the drinking water containing ethanol, the percentage of alveolar macrophages in the immature fraction decreased by 20% (Layer 4; **Figure 2.1, $^{c}p < 0.001$ vs. ethanol**), while the percentage of macrophages within layer 2 increased 2-fold (**Figure 2.1, $^{c}p < 0.01$ vs. ethanol**). Therefore, chronic ethanol ingestion resulted in a 60% decrease in the percentage of macrophages present in the two mature fractions but SAM supplementations normalized the alveolar macrophages to a more mature, control-like population.

Chronic Ethanol Ingestion Impaired Alveolar Macrophage Function In Defined Subpopulations. Since phagocytic function varies with the different subpopulations of guinea pig alveolar macrophages (Holian A, 1983), we evaluated the different subpopulations from the experimental groups to determine their phagocytic capacity. Alveolar macrophages from each subpopulation were incubated with inactivated FITC-labeled *S. aureus* in a 1:10 ratio (macrophage: bacteria) for 4 hr. Phagocytosis was evaluated using quantitative digital analysis of

Figure 2.1 Alveolar Macrophage Isolation and Distribution. Pathogen-free guinea pigs were randomly assigned to drinking water, ethanol (EtOH) in the drinking water (4%) or, EtOH + SAM in the drinking water. During the 25 days of treatment, the only access to drinking water was the experimental mixture. Solid diet was provided *ad libitum* to all three groups. After the appropriate period of experimental diet, the animals were lavaged and the cell pellet subfractionated on a Percoll gradient. A hemacytometer was used to count the percentage of cells obtained from each layer **(2.1.A)**. Bars represent means ± SE. [a] $p \leq 0.05$ compared to control, [b] $p \leq 0.05$ compared to control+SAM, [c] $p \leq 0.05$ compared to EtOH+SAM [1] $p \leq 0.05$ compared with layer 1 (within that group) and [2] $p \leq 0.05$ compared with layer 2 (within that group), [3] $p \leq 0.05$ compared with layer 3 (within that group). n=5 or more guinea pigs in each group.


CONTROL
CONTROL+SAM
ETOH
ETOH+SAM
Percentage in Each Layer
50
40
30
20
10
0
a,b
a,b
1,3,4
a,b,c
1,2
1,2
a,b,1
a,b,1
1,2
1,2
a,b,c,1,2,3
a,b
LAYER 1
LAYER 2
LAYER 3
LAYER 4
Most Mature----------Least Mature

fluorescence on the confocal microscopy. Representative photomicrographs at 60X magnification at 50% cell depth in the Z plane of the alveolar macrophages are shown in **Figure 2.2A**. As suggested in the confocal images (**Figure 2.2A**), the control alveolar macrophages in the two mature fractions (layers 1 and 2) had the greatest percentage of cells positive for internalization, 50% and 70%, respectively (**Figure 2.2B**). When the ingested RFU/cell was determined, macrophages isolated from layer 2 phagocytized the greatest amount of *S. aureus* (**Figure 2.2C**). When the percentage of cells positive for internalization was multiplied by the RFU/cell to obtain the phagocytic index, control and control + SAM cells localized to layer 2 were the most functional (**Figure 2.2D**).

As the confocal images suggested (**Figure 2.2A**), phagocytosis was compromised when the alveolar macrophages were derived from ethanol-fed guinea pigs. For alveolar macrophages in layers 1 and 2, there was a 60% decrease in the percentage of cells positive for internalization compared to control cells (**Figure 2.2B, $^{a}p < 0.05$**). Correspondingly, the amount of *S. aureus* phagocytized by cells in layer 2 was severely diminished as seen by more than a 40% decrease in RFU/cell (**Figure 2.2C, ^{a} p <0.01**) when compared to control alveolar macrophages located in layer 2. Therefore, the phagocytic index for control alveolar macrophages was 100,000 and 170,000 in layers 1 and layer 2 respectively, compared to only 25,000 in layers 1 and layer 2 respectively for ethanol-exposed alveolar macrophages (**Figure 2.2D, ^{a} p <0.05**). This translated to an 80% decrease in the phagocytic index for the entire pool of ethanol-exposed alveolar macrophages, when compared to controls.

As suggested in the confocal images (**Figure 2A**), SAM treatments during ethanol ingestion improved the percentage of cells present in layer 2 that were positive for internalization (**Figure 2.2B, ^{c} p <0.001**) to levels comparable to that observed in the control group. Importantly, the RFU/cell for the macrophages from the ethanol plus SAM group were comparable to the control cells present in layer 2 (**Figure 2.2C, ^{a,b} p >0.05**). SAM significantly improved overall phagocytosis of *S. aureus* (**Figure 2.2D, ^{c} p <0.01**) as seen by a partial, yet

Figure 2.2 Chronic Ethanol Ingestion Impaired Internalization of *S. aureus*. Alveolar macrophage subpopulations were incubated with inactivated FITC-labeled *S. aureus* in a 1:10 ratio (macrophage: bacteria) for 4 hr. Internalization by the alveolar macrophages in each subpopulation was evaluated using quantitative digital analysis of fluorescence on the confocal microscopy. Representative photomicrographs at 60X magnification at 50% cell depth in the Z plane of the macrophage are shown in **Figure 2.2.A**. Bar heights represent the % positive for fluorescence ± SE (**2.2.B**), the mean relative fluorescent units (RFU/cell) ± SE (**2.2.C**), and the Phagocytic Index ± SE (**2.2.D**). Bars in B-D represent means ± SE. Bars represent means ± SE. [a] $p \leq 0.05$ compared to control, [b] $p \leq 0.05$ compared to control+SAM, [c] $p \leq 0.05$ compared to EtOH+SAM [1] $p \leq 0.05$ compared with layer 1 (within that group) and [2] $p \leq 0.05$ compared with layer 2 (within that group), [3] $p \leq 0.05$ compared with layer 3 (within that group),and [4] $p \leq 0.05$ compared to layer 4 (within that group). n=8 or more fields per layer in each group.

Figure 2.2.A

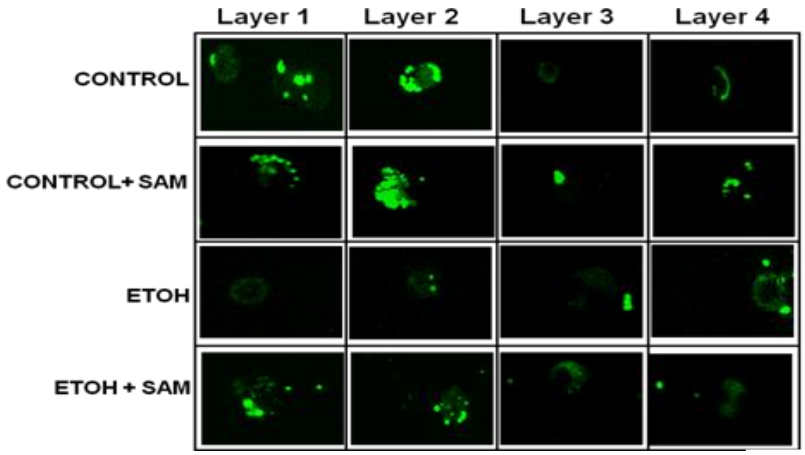


Figure 2.2.B

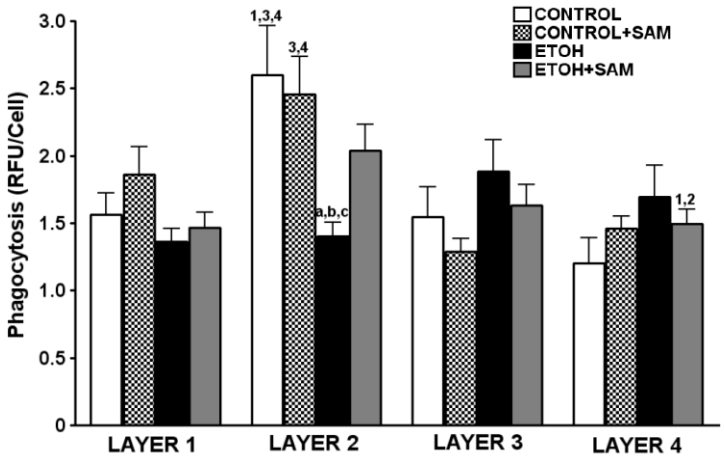


Figure 2.2.C

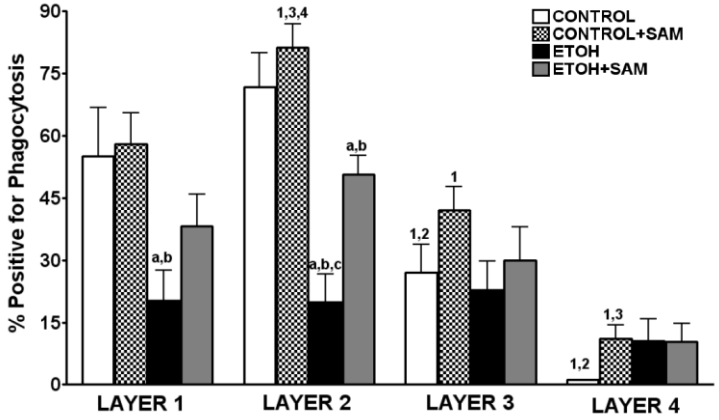
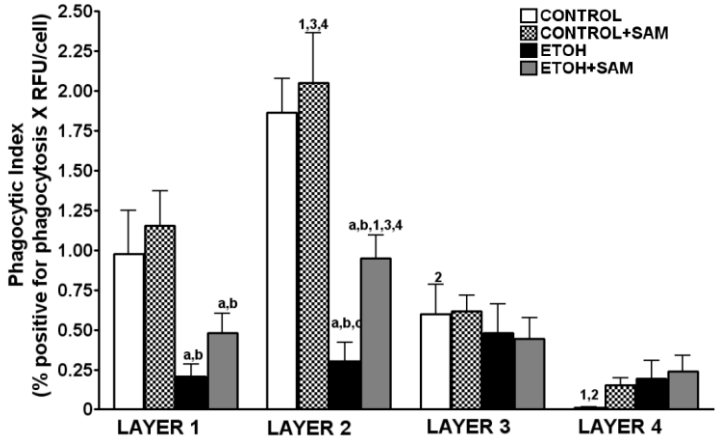


Figure 2.2.D



significant, recovery of the phagocytic index in layer 2. In all three treatment groups, the phagocytic index suggested that the cells present in layers 3 and 4 were relatively inactive.

Chronic Ingestion Altered Expression of PU.1 In Defined Subpopulation. As shown above, chronic ethanol ingestion resulted in a shift of the alveolar macrophages to a population with decreased terminal differentiation. To further examine our hypothesis, alveolar macrophage expression of the differentiation marker PU.1 was assessed. PU.1 was chosen because it is required for the terminal maturation of alveolar macrophages and increases as the cell becomes more terminally differentiated. As a result, an increased level of PU.1 is associated with a more terminally differentiated alveolar macrophage.

In control alveolar macrophages, PU.1 expression was highest in the mature layers (layer 1 and layer 2) (**Figure 2.3B**). Representative photomicrographs at 60X magnification are shown in **Figure 2.3A**. Chronic ethanol ingestion significantly decreased PU.1 expression in layer 1, layer 2, and layer 3, further supporting our hypothesis that ethanol exposure impaired alveolar macrophage maturation. In contrast, SAM supplementation during ethanol ingestion prevented the ethanol-induced alterations of PU.1 expression across the 4 layers (**Figure 2.3B, $^{a}p > 0.05$ vs. control**). PU.1 expression in alveolar macrophages from control animals supplemented with SAM did not differ from control values.

Chronic Ethanol Ingestion Severely Altered Expression of Maturational Factors. Based on the alveolar macrophage subpopulation data, chronic ethanol ingestion resulted in a shift of the alveolar macrophages to a population with decreased terminal differentiation. To further examine the effects of ethanol on terminal differentiation of alveolar macrophages, we determined expression of markers associated with alveolar macrophage maturation. GM-CSFR-α and PU.1 were chosen because they are both required for the terminal maturation of alveolar macrophages, with increased levels of GM-CSFR-α associated with a more terminally differentiated cell.

Figure 2.3 Quantification of PU.1 In Alveolar Macrophage Subpopulations. Representative photomicrographs at 60X magnification are shown in **Figure 2.3A**. PU.1 expressions were determined by staining alveolar macrophages with primary antibodies directed against PU.1 and then secondary antibodies directed against IgG labeled with FITC. The RFU/field was determined by computer analysis of the confocal microscopic images (**2.3B**). Bar height represents RFU/field ± SE and was tallied from at least 20 experimental fields. Bars represent means ± SE. ^{a} $p \leq 0.05$ compared to control, ^{b} $p \leq 0.05$ compared to control +SAM, and $^{c}p \leq 0.05$ compared to EtOH + SAM. n=5 or more fields per group.

Figure 2.3A

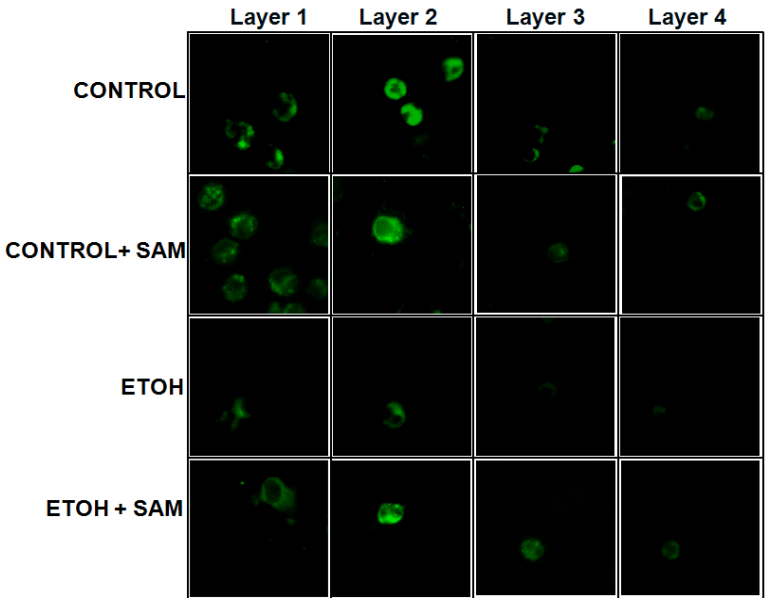
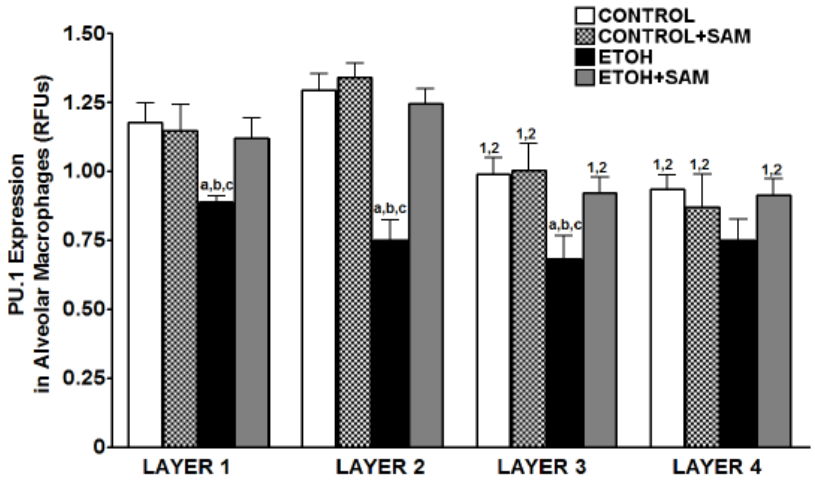


Figure 2.3B



Previous studies demonstrated in a rat model of chronic ethanol ingestion that expression of the α and β subunits of the GM-CSF receptor (GM-CSFR) were severely decreased in the plasma membrane of alveolar macrophages (Joshi et al., 2005a), suggesting a decrease in terminal differentiation. In the current study, total GM-CSFR-α expression was determined across the three treatment groups. When compared to control and control + SAM alveolar macrophages, alveolar macrophages from ethanol-fed animals displayed a 18% and 32% decrease in GM-CSFR-α expression, respectively **(Figures 2.4A and 2.5A, $^{a,b}p < 0.05$)**. PU.1 expression was also determined by western blot and immunohistochemical analysis. Compared to control alveolar macrophages, alveolar macrophages from ethanol-fed animals had a 31% decrease in PU.1 expression; a marker of maturity (**Figures 2.4B and 2.5B, $^{a,b}p > 0.05$**). SAM supplementation prevented the ethanol-induced depression of PU.1 expression (**Figures 2.4B and 2.5B, $^{a}p > 0.05$**).

We also assessed markers associated with immaturity including CD11b, and CD11c which are highly expressed in monocytes. For CD11b expression control and control + SAM alveolar macrophages displayed more than 35% less CD11b expression when compared to alveolar macrophages from ethanol-fed animals(**Figures 2.4C and 2.5C, $^{a,b}p < 0.05$**). SAM supplementation significantly improved Cd11b expression, as seen as a normalization to control values (**Figures 2.4C and 2.5C,$^{a}p > 0.05$)**. Similar to that observed for CD11b, chronic ethanol ingestion significantly increased expression of the monocytic marker CD11c expression when compared to control alveolar macrophages (**Figures 2.4D and 2.5D, $^{a}p < 0.05$**). When the ratio of GM-CSFR-α: CD11b expression was assessed, there were comparable ratios for the control, control + SAM, and ethanol +SAM alveolar macrophages (**Figure 2.4E**). In the ethanol-fed animals, the ratio of GM-CSFR-α: CD11b was also decreased, compared to the control groups. Furthermore, SAM supplementation prevented this decrease induced by ethanol (**Figure 2.4E, $^{a,b,c}p <0.05$**). Similar to what was observed with the ratio of GM-CSFR-α: CD11b, the ratio of PU.1:CD11c did not differ between the control, control + SAM, and ethanol +SAM alveolar

Figure 2.4 Quantification of GM-CSFR-α, PU.1, CD11b, and CD11c Expression In Alveolar Macrophages By Immunohistochemistry. GM-CSFR-α, PU.1, CD11c, and CD11b expressions were determined by staining alveolar macrophages with primary antibodies directed against GM-CSFR-α (**Figure 2.4A**), PU.1 (**Figure 2.4B**), CD11b (**Figure 2.4*C***), and CD11c (**Figure 2.4D**) and then secondary antibodies directed against IgG labeled with FITC or TRITC. The ratio of GM-CSFR-α**:** CD11b (**Figure 2.4E**) and PU.1:CD11c (**Figure 2.4F**) expression were also evaluated in each layer. The RFU/field was determined by computer analysis of the confocal microscopic images. Bar height represents RFU/field ± SE and was tallied from at least 20 experimental fields. Bars represent means ± SE. ^{a} $p \leq 0.05$ compared to control, ^{b} $p \leq 0.05$ compared to control + SAM, and $^{c}p \leq 0.05$ compared to EtOH + SAM. n=10 or more fields per group.

Figure 2.4A

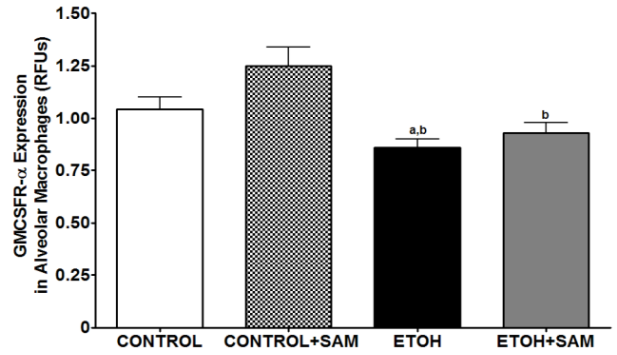


Figure 2.4B

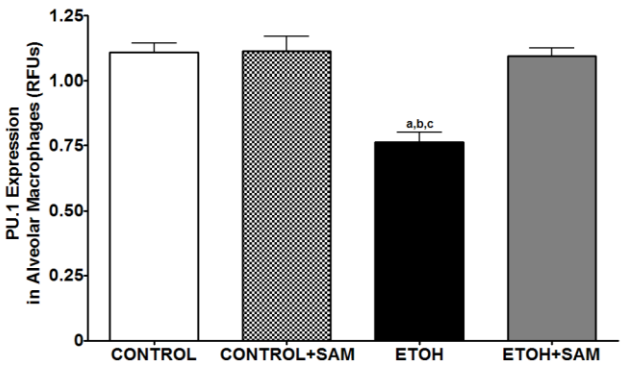


Figure 2.4C

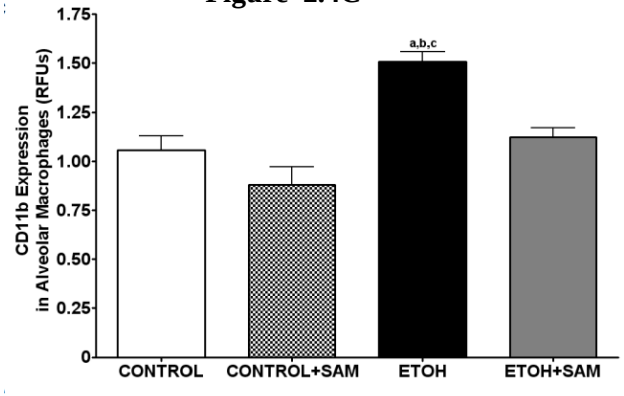


Figure 2.4D

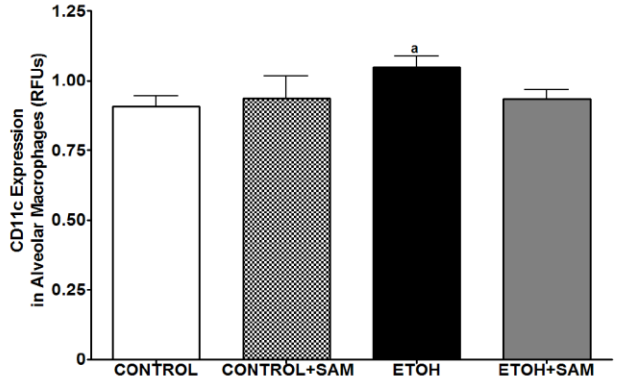


Figure 2.4E

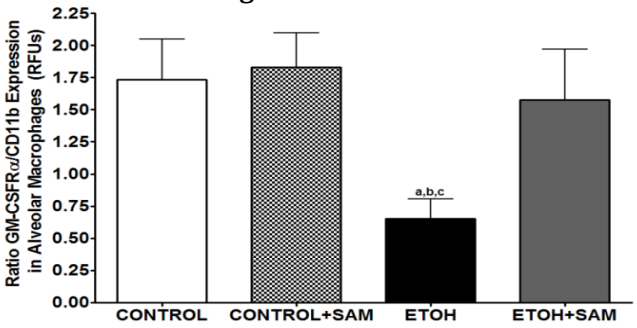


Figure 2.4F

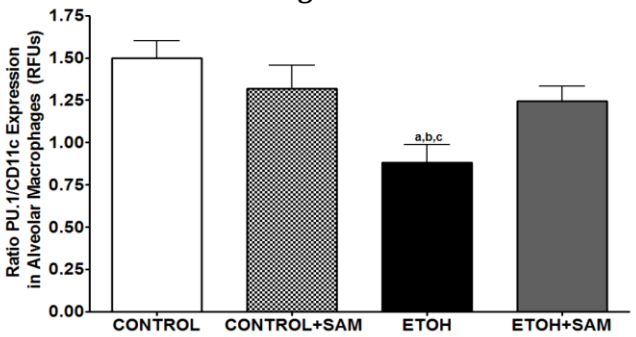


Figure 2.5 Quantification of GM-CSFR-α, PU.1, CD11b, and CD11c Expression In Alveolar Macrophages By Western Blot Analysis. GM-CSFR-α, PU.1, CD11c, and CD11b expressions were determined via western blot analysis. GAPDH, GM-CSFR-α (**Figure 2.5A**), PU.1 (**Figure 2.5B**), CD11b (**Figure 2.5*C***), and CD11c (**Figure 2.5D**) protein levels were detected by immunoblotting. Bars represent means ± SE. $^{a}p \leq 0.05$ compared to control, $^{b}p \leq 0.05$ compared to control + SAM, and $^{c}p \leq 0.05$ compared to EtOH + SAM. n=4 or more per group. Representative Western blots of GAPDH, GM-CSFR-α, PU.1, CD11c, and CD11b protein expressions in alveolar macrophages (**Figure 2.5E**).

Figure 2.5A

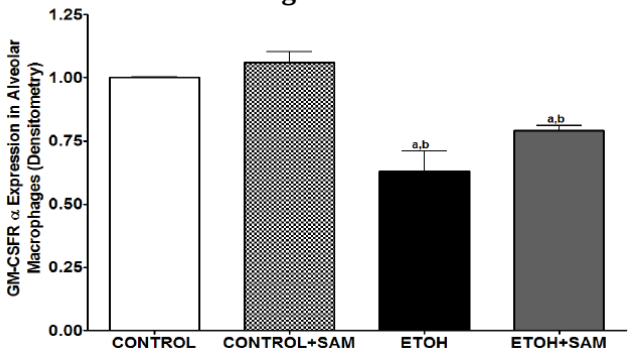


Figure 2.5B

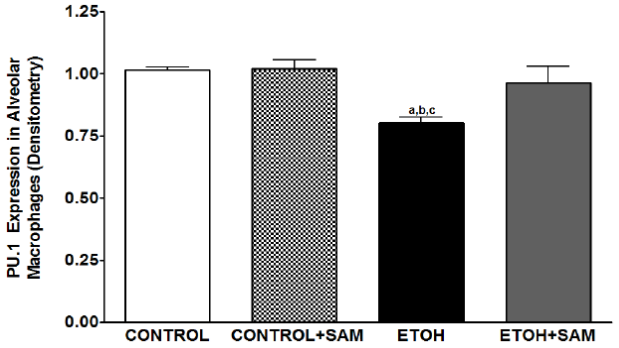


Figure 2.5C

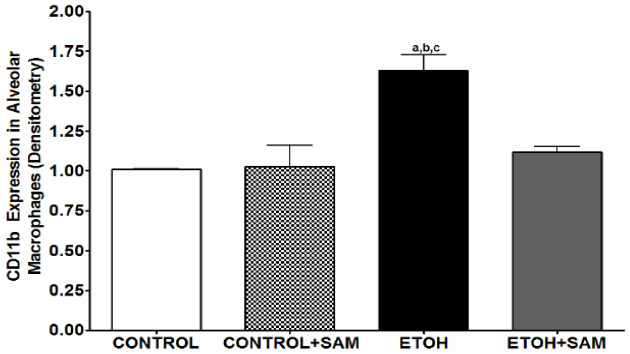


Figure 2.5D

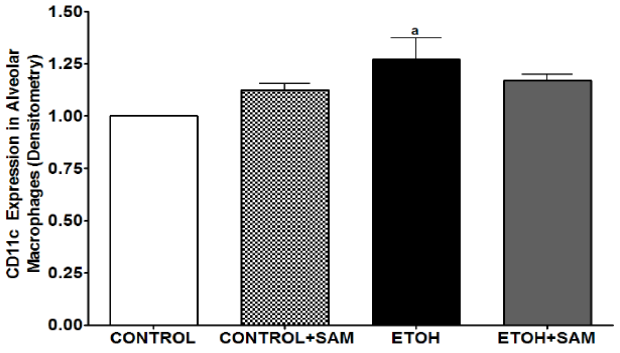
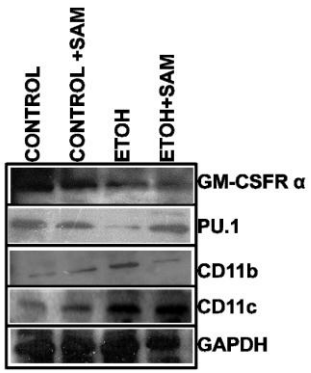


Figure 2.5E



macrophages. Similarly, ethanol-exposed alveolar macrophages had more than a 50% decrease in PU.1:CD11c expression (**Figure 2.4F, $^{a,b}p < 0.05$**).

Chronic Ethanol -Induced Defects Were Not Limited To The Alveolar Space. To determine if the ethanol-induced derangements in pulmonary macrophage maturation were limited to the alveolar space, the expressions of GM-CSFR-α, PU.1, CD11b, and CD11c, were determined in lung interstitial macrophages. For the interstitial macrophages, chronic ethanol exposure significantly decreased GM-CSFR-α expression when compared to control cells but SAM supplements failed to normalize GM-CSFR-α expression (**Figures 2.6A and 2.7A, $^{a,b}p < 0.05$).** PU.1 expression by the pool of interstitial macrophages was also significantly altered by chronic ingestion of ethanol (**Figures 2.6B and 2.7B $^{a}p < 0.05$**). When we examined the monocytic markers, chronic ethanol ingestion increased expression of CD11b by 65%, when compared to controls (**Figures 2.6C and 2.7C, $^{a,b}p < 0.05$).** As observed with alveolar macrophages, these effects of ethanol on monocytic markers were blunted by SAM supplements. When the ratio of GM-CSFR-α: CD11b expression was assessed, the control and control + SAM interstitial macrophages had comparable ratios. In the ethanol-fed animals, the ratio of GM-CSFR-α: CD11b also decreased when compared to control (**Figure 2.6E, $^{a,b}p <0.05$**). When the ratio of PU.1:CD11c expression was assessed, the interstitial macrophages from the control, control + SAM, and ethanol +SAM groups had comparable ratios (**Figure 2.6F**). In contrast, ethanol-exposed alveolar macrophages had an approximate 40% decrease in PU.1:CD11c expression (**Figure 2.6F, $^{a,b}p <0.05$**).

2.5. DISCUSSION.

The deleterious effects of chronic ethanol ingestion on lung physiology and function have been well characterized (Aytacoglu et al., 2006a; Joshi et al., 2005a; Moss et al., 2000c; Polikandriotis et al., 2006). Previous studies by this laboratory demonstrated chronic ethanol

Figure 2.6 Quantification of GM-CSFR-α, PU.1, CD11b, and CD11c Expression In Interstitial Macrophages By Immunohistochemistry. GM-CSFR-α, PU.1, CD11c, and CD11b expressions were determined by staining interstitial macrophages with primary antibodies directed against GM-CSFR-α (**2.6A**), PU.1 (**2.6B**), CD11b (**2.6C**), and CD11c (**2.6D**) and then secondary antibodies directed against IgG labeled with FITC or TRITC. The ratio of GM-CSFR-α**:** CD11b (**2.6E**) and PU.1:CD11c (**2.6F**) expression were also evaluated in each layer. The RFU/field was determined by computer analysis of the confocal microscopic images. Bar height represents RFU/field ± SE and was tallied from at least 20 experimental fields. Bars represent means ± SE. ^{a} $p \leq 0.05$ compared to control, ^{b} $p \leq 0.05$ compared to control + SAM, and ^{c} $p \leq 0.05$ compared to EtOH + SAM. n=10 or more fields per group.

Figure 2.6A

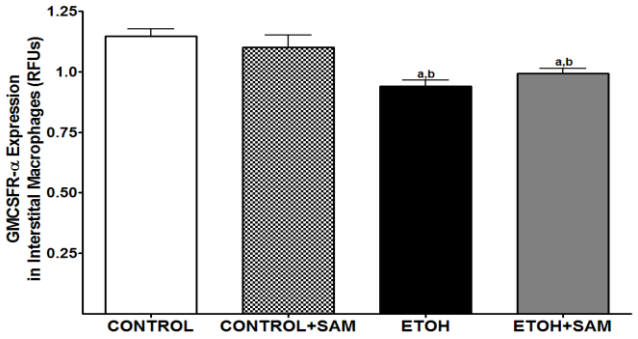


Figure 2.6B

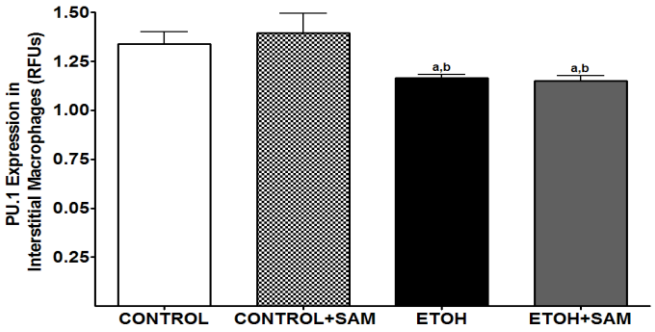


Figure 2.6C

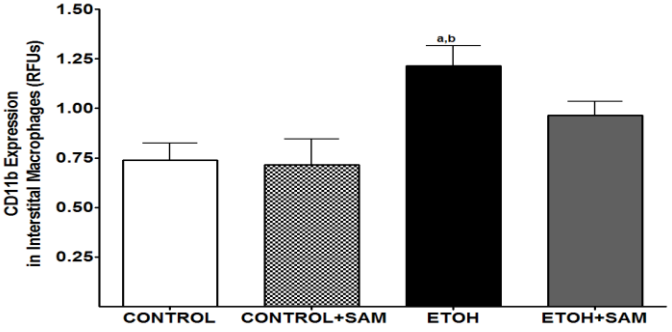
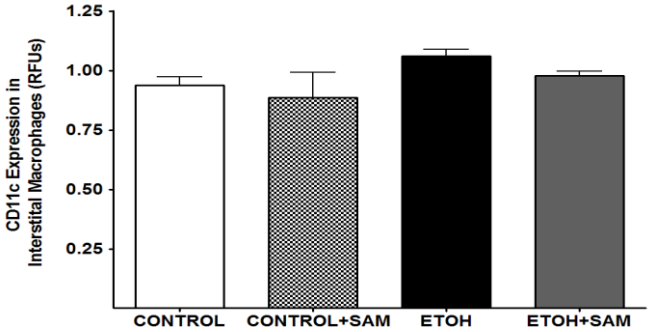


Figure 2.6D



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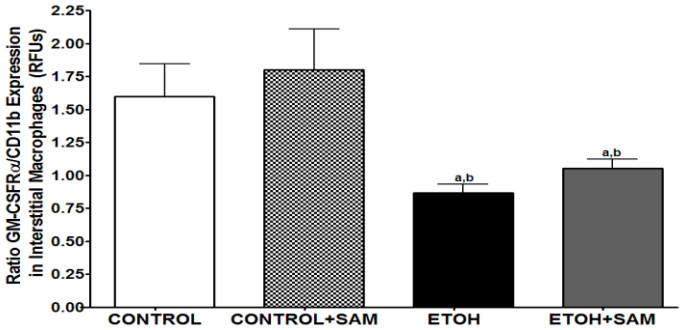


Figure 2.6F

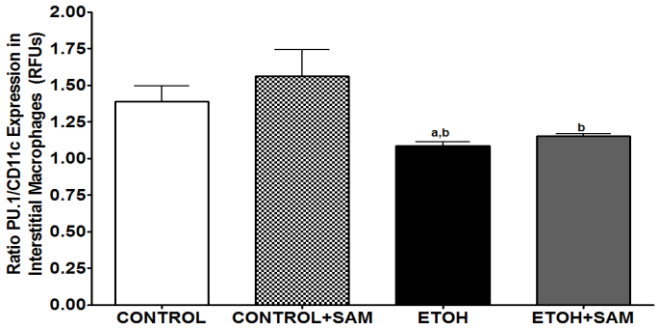


Figure 2.7 Quantification of GM-CSFR-α, PU.1, CD11b, and CD11c Expression In Interstitial Macrophages By Western Blot Analysis. GM-CSFR-α, PU.1, CD11c, and CD11b expressions were determined via western blot analysis. GAPDH, GM-CSFR-α (**2.7A**), PU.1 (**2.7B**), CD11b (**2.7*C***), and CD11c (**2.7D**) protein levels were detected by immunoblotting. Bars represent means ± SE. $^{a}p \leq 0.05$ compared to control, $^{b}p \leq 0.05$ compared to control + SAM, and $^{c}p \leq 0.05$ compared to EtOH + SAM. n=4 or more per group. Representative gels of GAPDH, GM-CSFR-α, PU.1, CD11c, and CD11b protein expressions in interstitial macrophages (**2.7E**).

Figure 2.7.A

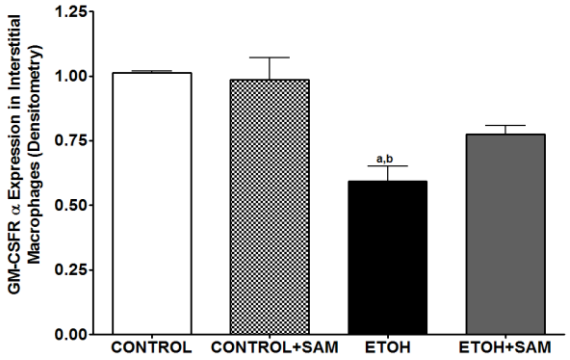


Figure 2.7.B

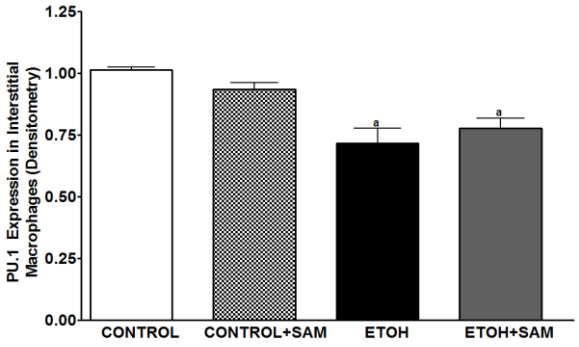


Figure 2.7.C

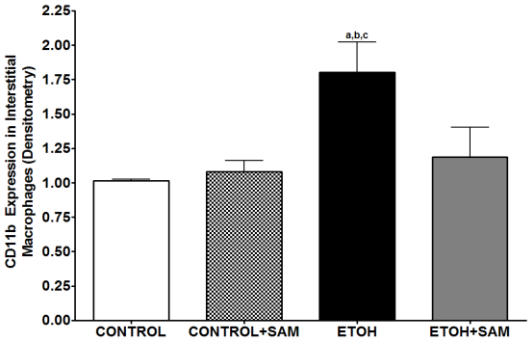


Figure 2.7.D

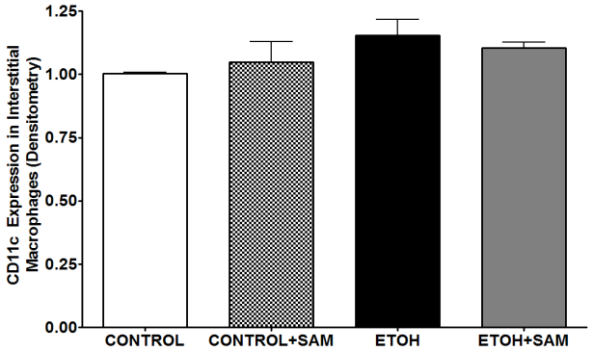
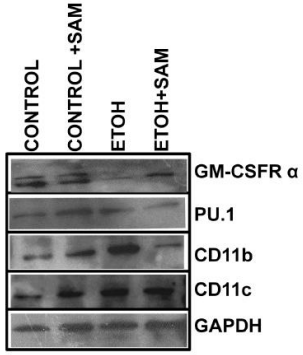


Figure 2.7E



ingestion and subsequent chronic oxidant stress impaired phagocytic functions of alveolar macrophages (Brown et al., 2007). In the current study, we examined the possibility that impaired phagocytosis after chronic ethanol ingestion was a result of impaired terminal differentiation. To address this question, we examined the effects of chronic ethanol ingestion on several different markers of alveolar macrophage differentiation including cell density, phagocytosis, and expression of maturational markers.

When cell density was used as a marker of differentiation, approximately 80% of the alveolar macrophages from control and control + SAM animals were equally distributed between the mature layers (layer 1 and layer 2). These two layers also displayed the highest phagocytic capacity based on the percentage of cells positive for phagocytosis and the quantification of bacteria ingested per cell. Thus, the majority of the control and control + SAM alveolar macrophages were terminally differentiated as assessed by cell density, phagocytic capacity, and maturational markers such as GM-CSFRα and PU.1.

As observed previously in an ethanol-fed rat model (Guidot et al., 2000), chronic ethanol consumption in a guinea pig model also severely decreased alveolar macrophage phagocytosis as evidenced by a drastic decrease in the percentage of alveolar macrophages positive for *S. aureus* internalization. Since the majority of the alcoholic macrophages were localized to the immature fractions, poor phagocytic capacity in these subpopulations was not surprising. However, a small percentage of ethanol-exposed alveolar macrophages had the cell size and density comparable to that observed for the majority of the control cells. Although these ethanol-exposed alveolar macrophages were located in the mature fractions, their phagocytic capacity was compromised. In addition, the cells located in the mature fractions had decreased expression of the maturational marker PU.1. These two diverse markers of maturation, cell density and PU.1 expression, suggest that chronic ethanol ingestion impaired terminal differentiation of the alveolar macrophages. However, we cannot rule out the possibility that chronic ethanol ingestion also alters the profile for receptor surface expression. Additional studies of receptor cell trafficking

are needed to determine why these ethanol-exposed cells present in the fractions and deemed to be mature based on cell density demonstrated altered expression of surface receptors and whether this altered receptor expression results in severely compromised phagocytic capacity.

Chronic ethanol ingestion has been shown to severely diminish GSH availability within the alveolar space (Foreman et al., 2002; Moss et al., 2000c; Velasquez A, 2002) as well as within the resident cells of the alveolar space, including alveolar type II cells (F. Holguin 1998) and alveolar macrophages (Brown et al., 2007). Previous studies in this laboratory have shown in the rat model of chronic ethanol ingestion that supplementation with GSH precursors procysteine or N-acetyl cysteine during ethanol ingestion maintained alveolar macrophage function, as well as normalized GSH levels (Brown et al., 2007). SAM supplementation has been shown to attenuate alcohol-induced liver injury in an animal model of chronic ethanol consumption and has clinically been shown to improve survival of liver transplantation patients with alcoholic liver cirrhosis (Lieber, 1990; Mato et al., 1999). This is the first study to examine the potential benefits of SAM supplementation to alveolar macrophages in an adult animal model of chronic ethanol ingestion. In the current study, SAM supplementation during chronic ethanol ingestion partially prevented the harmful effects of ethanol on alveolar macrophage maturation and function. More specifically, SAM supplementation led to a shift in the distribution of macrophages to a more mature fraction, as well as a significant increase in phagocytic capacity of the cells present in the mature fractions. However, SAM supplementation in control animals had minimal effects and this further supports the hypothesis that chronic ethanol ingestion leads to increased oxidant stress.

Studies done by Joshi et al. (Joshi et al., 2005a) reported in a rat model that chronic ethanol ingestion decreased membrane expression of GM-CSFR-α in alveolar macrophages. In the current study, SAM supplementation during ethanol ingestion increased expression of GM-CSFR-α and PU.1, markers of terminal differentiation. SAM supplementation during chronic ethanol ingestion also decreased expression of CD11b and CD11c, monocytic markers.

Therefore, SAM prevented the ethanol-induced alterations in alveolar macrophage density, terminal differentiation, and phagocytic capacity suggesting that ethanol-induced oxidant stress was a central feature in the impaired maturation of alveolar macrophages. Furthermore, SAM supplementation maintained phagocytic function in the small percentage of "mature" ethanol-exposed macrophages when cell density was used as the criteria. Additional studies are necessary to determine whether higher concentrations of SAM are needed to completely protect the alveolar macrophages from the negative effects of chronic ethanol ingestion.

In order to determine if altered maturation and function of pulmonary macrophages in response to chronic ethanol ingestion extended to interstitial macrophages, expressions of GM-CSFR-α, PU.1, CD11b, and CD11c were also determined in interstitial macrophages. Chronic ethanol ingestion led to a significant decrease in GM-CSFR-α expression. Similarly, expressions of CD11b and CD11b were also increased in interstitial macrophages from ethanol-fed animals. This suggested that chronic ethanol ingestion also impaired the differentiation of interstitial macrophages within the lung. SAM supplementation blunted these deleterious effects of ethanol, as seen by normalization of CD11b. It is also important to note this is the first study to examine the effects of ethanol on pulmonary interstitial macrophages.

In summary, this study demonstrated that chronic ethanol exposure decreased alveolar macrophage maturation, whether defined by decreased cell density, decreased expression of maturational markers, or persistent monocytic characteristics. These alterations were directly correlated to impaired phagocytic function. Since the majority of the ethanol-exposed alveolar macrophages were localized to the immature fractions, our study suggested the poor phagocytic capacity seen with these macrophages was primarily due to impaired terminal differentiation. For the limited percentage of cells that were present in the mature fractions, chronic ethanol ingestion also impaired phagocytic capacity. The capacity of SAM supplementation to normalize most of the detrimental effects of ethanol suggested ethanol-induced oxidant stress played a central role in the impaired terminal differentiation and dysfunction of lung interstitial and alveolar

macrophages. Additional studies are needed to determine whether GSH precursors will restore macrophage differentiation and phagocytosis as well as improve resistance to respiratory infections in this selected at-risk patient population.

CHAPTER 3

REDOX REGULATION OF ALCOHOL-INDUCED ALTERNATIVE ACTIVATION OF ALVEOLAR MACROPHAGES

3.1. ABSTRACT.

Previous studies have shown that chronic alcohol ingestion impairs alveolar macrophage function and increases oxidant stress within the lung. Alternative activation of alveolar macrophages has also been shown to lead to similar alterations. However, the potential link between alcohol induced dysfunction and alternative activation has yet to be examined. To determine if the chronic oxidant stress induced by chronic alcohol consumption impairs the phagocytic function of alveolar macrophages through alternative activation, NR8383 cells, a murine alveolar macrophage cell line, were treated with 0.08% ethanol ± the antioxidant glutathione for 5 days. Using a rodent model, primary alveolar macrophages were isolated from rats after 6 weeks of ethanol feeding. Following treatment, phagocytic capacity, ROS production, and expression of markers of alternative activation were assessed. In addition, alveolar macrophages isolated from otherwise healthy alcoholic and control subjects were examined for phagocytic function and expression of markers for alternative activation. Chronic ethanol ingestion or exposure greatly decreased alveolar macrophage phagocytic function, increased ROS production, and increased expression of markers of alternative activation. Glutathione supplementation during or following alcohol ingestion blocked or reversed these effects. Similar results were observed in macrophages from otherwise healthy alcoholics. Ethanol-induced alternative activation of alveolar macrophages but was ablated by glutathione supplementation. This suggested that the increased risk of respiratory infections associated with alcohol abuse occurs because the alveolar macrophages switch to an alternatively activated phenotype. Furthermore, these results suggested that antioxidant treatments could reverse alternative activation and improve the phagocytic function of the alveolar macrophages.

3.2. INTRODUCTION.

Excessive consumption of alcohol, the most commonly abused substance worldwide, contributes to 30% of hospitalizations within the United States and is the third leading cause of death (Joshi and Guidot, 2007b; Moss, 2005). The deleterious effects of alcohol abuse are broad ranging and affect numerous organ systems (Esper A., 2006; Moss et al., 2003). Alcohol-related incidences account for more than 40% of intensive care unit (ICU) admissions and alcoholic patients have a 2- fold increase in the incidence of ICU-related morbidity and mortality, commonly caused by acute respiratory distress syndrome (ARDS) (Jurkovich et al., 1993; Moss et al., 1996; O Brien et al., 2007).

ARDS is a severe form of lung injury characterized by lung inflammation, increased epithelial barrier permeability resulting in rapid fluid accumulation in the alveolar air sacs, impaired gas exchange, and the release of proinflammatory cytokines (Aytacoglu et al., 2006a; Bechara et al., 2005; Moss et al., 1996). There are several risk factors associated with the development of ARDS, such as pneumonia, sepsis, burn injury, aspiration, and trauma. However, community-acquired pneumonia is the most common cause of ARDS in alcoholic patients and numerous studies have demonstrated that chronic alcohol abuse is an independent risk factor (Fowler et al., 1983; Moss et al., 1996; Ware and Matthay, 2000). This subset of patients also have an increased severity of ventilator-associated pneumonia (Esper A., 2006; Moss et al., 1996) .

The increased incidence and severity of ARDS in alcohol abusers is partially due to a dampened immune response of the alveolar macrophages, the primary immune cell type within the alveolar air space (Rosseau et al., 2000; Standiford and Danforth, 1997). Alveolar macrophages provide the first line of cellular defense against inhaled microorganisms via the binding, internalization, and degradation of foreign pathogens (Aderem and Underhill, 1999b; Bonfield et al., 2003; Gordon and Taylor, 2005; Koay et al., 2002; Laskin et al., 2001; Taylor et al., 2005). In rodent models of alcohol abuse, alveolar macrophages display impaired

phagocytosis of *Staphylococcus aureus*, one of the primary causes of community-acquired pneumonia (Jurkovich et al., 1993). We have demonstrated in animal models that chronic ethanol ingestion also leads to decreased alveolar macrophage maturation, as determined by decreased cell density, decreased expression of maturational markers, and increased expression of monocytic markers (Brown et al., 2009). These alterations in alveolar macrophage maturational status directly correlated with impaired phagocytic function (Brown et al., 2009).

Previous studies have indicated that decreases in the antioxidant glutathione (GSH) within the alveolar lining fluid, alveolar type II cells, and alveolar macrophages is a key alteration induced by chronic alcohol ingestion (Brown et al., 2004; Brown et al., 2007; F. Holguin 1998; Guidot et al., 2000; Velasquez A, 2002). For alveolar macrophages, ethanol-induced impairment is due to severely diminished GSH availability, which leads to increased oxidant stress (ROS), an altered oxidant/antioxidant balance, impaired clearance of microbes and apoptosis (Brown et al., 2007; Brown et al., 2009; Jurkovich et al., 1993; Polikandriotis et al., 2006; Velasquez A, 2002). However, *in vivo* and *ex-vivo* treatments with GSH or its precursors improve alveolar macrophage function during and after chronic ethanol ingestion (Brown et al., 2007).

The classically known activation pathway for alveolar macrophages is induced by IFN-γ, which triggers the proinflammatory response required to kill intracellular pathogens. Classical activation mediated by T_H1 cytokines is characterized by high levels of tumor necrosis factor (TNF), ROS and nitric oxide synthase-2 (NOS-2)-dependent generation of reactive nitrogen intermediates (Aderem and Underhill, 1999b; Berclaz et al., 2002). However, T_H2 cytokines, such as IL-4 and IL-13, trigger alveolar macrophages to undergo alternative activation, a unique phenotype associated with the immune response to parasites, wound repair, asthma, and other diverse pathologies (Abramson and Gallin, 1990; El-Gayar et al., 2003; Muller et al., 2007). T_H2 cytokines antagonize classical activation, suppress NOS-2-mediated production of reactive nitrogen intermediates and induce an alternative metabolic pathway for L-arginine. In response to T_H2 cytokines, alternative activation promotes the upregulation of arginase activity in alveolar

macrophages so that L-arginine is metabolized to L-ornithine, L-proline, and polyamine (Gordon, 2003; Gordon and Martinez; Hesse et al., 2001b; MacKinnon et al., 2008; Martinez et al., 2009; Nair et al., 2003; Stein et al., 1992). With increased arginase activity, there is a shift from the production of the bactericidal nitric oxide to production of metabolites that promote fibroblast proliferation and collagen production which could lead to lung fibrosis. Alternative activation also downregulates the macrophage inflammatory response by severely diminishing phagocytic function and leads to an increased susceptibility to lung infections if the switch is sustained. In alveolar macrophages, T_H2 cytokine-dependent alternative activation is defined by upregulation of the markers arginase-1, galectin-3, scavenger receptor-A, and FIZZ1 (Gordon, 2003; Gordon and Martinez; Jurkovich et al., 1993; MacKinnon et al., 2008; Nair et al., 2003; Song et al., 2000; Stein et al., 1992).

Therefore, we hypothesized that alcohol-induced chronic ROS leads to alternative activation of alveolar macrophages and the subsequent impairment of phagocytosis. Correspondingly, antioxidant treatments were expected to restore the oxidant/antioxidant balance and thereby ablate alcohol-induced alveolar macrophage dysfunctions. To examine the potential link between chronic alcohol-induced alteration of alveolar macrophage function and alternative activation, we used a macrophage cell line with *in vitro* ethanol exposure, alveolar macrophages from an ethanol-fed rat model and human alveolar macrophages from otherwise healthy alcoholics. Furthermore, the capacity of GSH to reverse alternative activation and restore macrophage function were examined.

3.3. MATERIALS AND METHODS.

Cell Line Culture and Treatment: NR8383 cells, a rat alveolar macrophage–derived cell line (ATCC, Rockville, USA), were maintained in a humidified 5% CO_2 incubator at 37°C.with FK-12 media supplemented with 15% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (100 U/ml penicillin G sodium, 100 U/ml streptomycin, and

0.25 µg/ml amphotericin B). Cells were treated with 0.08% ethanol ± 500 µM GSH or 1.5 ng/ml IL-13 for 5 consecutive days. Cells treated with ethanol were maintained in a closed chamber in order to maintain the ethanol concentration and media were changed daily.

Rat Model of Chronic Ethanol Ingestion. Male Sprague-Dawley rats (Harlan, St. Louis, MO) were fed the Lieber-DeCarli liquid diet (Research Diets, New Brunswick, NJ) containing 36% ethanol *ad libitum* for 6 wk. Pair-fed controls were fed an isocaloric mixture of the liquid diet without ethanol. Where appropriate, 500 µM concentrations of GSH (Sigma; St. Louis, MO) diluted in 50 µl of PBS was instilled intranasally daily during the last week of ethanol feeding. All animals were used in accordance with NIH guidelines (*Guide for the Care and Use of Laboratory Animals*) as described in protocols reviewed and approved by the Emory University Institutional Animal Care Committee.

Alveolar Macrophage Isolation and Culture. After pentobarbital anesthesia, the trachea was cannulated and the rat lung underwent 5 bronchoalveolar lavages, consisting of 10 ml of sterile phosphate-buffered saline (37°C, pH 7.4). The lavage fluid was centrifuged at 500 *g* for 8 min, and the cell pellet was resuspended in Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum plus penicillin and streptomycin (100 U/l each) or preserved in radioimmunoprecipitation assay (RIPA) buffer. Cell count and viability was determined on the Countess™ Automated Cell Counter (Invitrogen; Carlsbad, CA). The cell-free bronchoalveolar lavage fluid was saved for further analysis. Rat primary alveolar macrophages were plated at 1 million cells/ml and cultured at 37°C and 5% CO_2 and allowed to adhere for 1 hr.

Subject Enrollment. Chronic alcoholics were recruited from the detoxification unit at the Veteran's Affairs Hospital in Atlanta, GA. Nonsmoking and smoking controls were enrolled from flyers postings at the different Emory University hospitals as well as in the community surrounding Emory Univeraity. Alcoholic status was confirmed by a score of > 3 on the SMAST

survey or a score of 0 for controls. Exclusion criteria included: a prior history of cardiac disease, liver dysfunction, kidney disease, diabetes mellitus, lung disease, human immunodeficiency virus infection, gastrointestinal bleeding, or concomitant illicit drug use. The details of the recruitment process and selection criteria were previously reported (Moss et al., 2000a). The demographics of the subjects are presented in **Table 3.1** (Control vs. alcoholic: 75% male vs. 100% male; median age of 50 vs. 45, and 0% vs. 66% smokers).

Bronchoscopy With Bronchopulmonary Alveolar Lavage (BAL). Patients were sedated with 5 mg/kg of 2% lidocaine and a bronchoscope was passed transnasally into a dependent segment of the lung and 3 aliquots of 50 ml sterile saline were sequentially instilled. The saline was then aspirated from the lung by hand into a sterile container. Cell count and viability was determined on the Countess™ Automated Cell Counter (Invitrogen; Carlsbad, CA). The BAL fluid was then centrifuged to separate the cells from the fluid. Once the cells were separated from the BAL, the fluid was retained and stored for future analysis. Cellular pellets were resuspended in DMEM medium supplemented with 2% fetal bovine serum plus penicillin and streptomycin (100 U/l each) and plated onto 8 well chamber slides at a density of 1 $X10^6$ cell/ml and allowed to adhere for 1 hr. Non-adherent cells were removed by washing each well twice with sterile phosphate buffered saline. Where appropriate, cells were then incubated with fresh media supplemented with 500 µM GSH for 24 hrs.

Phagocytosis of pHrodo Labeled *S. aureus.* NR8383 cells, rat primary alveolar macrophages, or human alveolar macrophages were plated onto 8 well chamber slides at a density of 1 $X10^6$ cell/ml and allowed to adhere for 1 hr. The cells were then washed twice with sterile phosphate buffered saline and incubated with pHrodo-labeled S. aureus (Invitrogen; Carlsbad, CA) for 2 hours at 1 mg/ml. After the media was removed, the cells were washed twice with phosphate buffered saline and fixed with 3.7% paraformaldehyde. Phagocytosis of pHrodo-

labeled *S. aureus* was determined via quantitative digital analysis of microscopic images using FluoView (Olympus; Center Valley, PA). Values are presented as mean relative fluorescent units (RFU)/cell ± SEM, the mean percentage of cells fluorescently positive for internalization (per

	Control Subjects	Alcoholic Subjects
Number	4	6
Male, n (%)	3 (75)	6 (100)
Age, yr (SD)	50 (7)	45 (10)
Smoker, n (%)	0 (0)	4 (66) *

* p = 0.05 (compared to controls).

TABLE 3.1. Subject Demographics

field) ± SEM, and phagocytic index ± SEM (RFU/cell X % positive cells) as tallied from at least 10 experimental fields/set.

RT-PCR: Total RNA was extracted by TRIzol Reagent and assayed by real time Q-PCR. Breifly, cells were collected and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNA-free reagent according to the manufacturers' protocols. Real-time quantitative PCR of mRNA templates was performed using the iScript SYBR Green One-Step RT-PCR Kit (Bio-Rad; Richmond, CA) and the ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). Briefly, PCR amplification of RNA (100 ng) was performed under the following conditions: cDNA synthesis at 50°C for 10 min, iScript reverse transcriptase inactivation at 95°C for 5 min, and PCR cycling and detection for 40 cycles at 95°C for 10 s followed by 60°C for 30 s. Values are expressed as relative mRNA expression that has been normalized to housekeeping GAPDH mRNA. The following primers were used: arginase-1 5-'AAAGCCCATAGAGATTATCGGAGCG-3' and 5'-AGACAAGGTCAACGGCACTGCC-3; CD32 5'- TGTCGCTGGAATTGCTGTAG-3' and 5'-AGGTCCTGGCCTTACTGGTT-3'; GAPDH 5'-TGAGAATGGGAAGCTG-3' and 5'-TTGGGGGTAGGAACAC-3' ; and Galectin-3 5'-AATGGCAGACGGCTTCTCACTT-3' and 5'-TAACACACAGGGCAGTTCTGGT-3'{Fukuda, 2001 #242(Mousavi et al., 2007; Schwartz et al., 2004; Seki et al., 2003).

Western Blot Analysis. Cell lysates were prepared by adding radioimmunoprecipitation assay (RIPA) buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Protein concentration was determined using the Micro BCA protein Assay (Pierce) Micro BCA Protein Assay Reagent Kit (Pierce). 30-50 µgs of total protein from each sample was electrophoresed on 4–15% gradient polyacrylamide gels at 200 V for 45 min and transferred to nitrocellulose membranes (Bio Rad Laboratories, Richmond, CA) at 100V for 1 hr. Membranes

were blocked at room temperature for 1 hr in Tris buffered saline (TBS) with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk. Membranes were then triple washed for 10 minutes each and probed with primary antibodies to arginase-1 (1:100) (Santa Cruz Biotechnologies, Santa Cruz, CA), CD32 (1:100) (Santa Cruz Biotechnologies), galectin-3 (1:500) (Santa Cruz Biotechnologies) or GAPDH (1:500) (Santa Cruz Biotechnologies) diluted in 5% milk in TBS-T and kept at 4^{o}C overnight. After 3 washes with T-TBS for 10 min, membranes were incubated at room temperature with secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnologies) for 1hr. After adding ECL chemiluminescence reagent (Denville Scientific, Metuchen, NJ) to the membranes, bands were detected and quantified via densitometry.

Detection of Extracellular Hydrogen Peroxide (H_2O_2). The release of H_2O_2 was determined using the oxidation of Amplex Red in the presence of horseradish peroxidase reagent according to the manufacturer's instructions (Invitrogen; Carlsbad, CA). Briefly, cells were treated with for 45 min at room temperature with 100 µl of Amplex Red solution (50 µM of Amplex Red reagent and 10 U/ml horseradish peroxidase in phosphate buffered saline). The H_2O_2 production was measured via a microplate reader by taking the absorbance at A_{560} and interpolating from a standard curve generated with known H_2O_2 concentrations.

Detection of Intracellular ROS Production. Intracellular ROS generation was assessed using 2,7-dichlorofluorescein diacetate (DCFH-DA, 10 µM; (Invitrogen; Carlsbad, CA). Once taken up by the cell, esterases cleave the acetate groups to DCFH-DA and trap the nonfluorescent DCFH probe. Oxidation by ROS then frees the fluorescent product dichlorofluorescin (DCF). Using an excitation wavelength of 480 nm and emission wavelength of 522 nm, DCF fluorescence was determined via quantitative digital analysis of microscopic images using FluoView (Olympus; Center Valley, PA). Background autofluorescence was determined and subtracted from the mean

relative fluorescent units. Values are presented as the mean relative fluorescent units per cell (± SE) as tallied from at least 10 experimental fields per set.

Detection of Intracellular H_2O_2 Production. Intracellular H_2O_2 production was determined using the Amplite Green Peroxidase Sensor (Invitrogen; Carlsbad, CA). Amplite green is a cell permeable sensor that fluoresces green when it reacts with H_2O_2. Using an excitation wavelength of 480 nm and emission wavelength of 522 nm, Amplite Green Fluorescence was determined via quantitative digital analysis of microscopic images using FluoView (Olympus; Center Valley, PA). Background autofluorescence was determined and subtracted from the mean relative fluorescent units. Values are presented as the mean relative fluorescent units per cell (± SE) as tallied from at least 10 experimental fields per set.

Immunofluorescence Characterization. Cells were isolated and cultured as described above. After the culture period, the cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.3% Triton X-100 and non-specific binding was blocked with 3% bovine serum albumin. Before incubating with the primary antibodies, cells were preincubated with Fc block® (Pharmingen, San Diego, CA) for 15 minutes at room temperature. Cells were then treated with the primary antibodies against arginase-1 or galectin-3 (1/100 dilution; 2 hrs; room temperature (Santa Cruz Biotechnology, Santa Cruz, CA). Cells that were to be examined for CD32 were not pre-incubated with Fc block®. The slides were washed three times with PBS and incubated with the Alexa-Fluor 488 conjugated donkey anti-rabbit IgG (Invitrogen) or Alexa-Fluor 568 conjugated chicken anti-goat (Invitrogen; Carlsbad, CA) for 1 hr at 1/200 dilution in 3% bovine serum albumin. Slides were then counter-stained with 4',6-diamino-2-phenylindole (DAPI) (Invitrogen; Carlsbad, CA) for 5 minutes to vizualize nuclear localization. Immunofluorescence was determined via quantitative digital analysis of microscopic images using FluoView (Olympus; Center Valley, PA). Background fluorescence was determined and

subtracted from the mean relative fluorescent units. Values are presented as the mean relative fluorescent units per cell (± SE) as tallied from at least 10 experimental fields per set.

Arginase Activity Measurements. Arginase activity was measured using the QuantiChrom Arginase Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions. All values were normalized to protein concentration.

Measurement of IL-13 by ELISA: IL-13 in cell lysates was detected by the commercially available ELISA kit Quantikine Rat IL-13 Immunoassay for IL-13 (Invitrogen; Carlsbad, CA). To measure production of IL-13, cell lysates and media were loaded in a 1:10 dilution onto 96 well plates. All values were normalized to the lysate protein concentration.

Statistical Analysis: Statistical analysis was performed with Sigma Stat for Windows. The data are presented as means ± SE. Results were analyzed using the student's t-test or one-way analysis of variance (ANOVA) where appropriate, followed by Student-Newman-Keuls test comparisons. A *p*-value of < 0.05 was considered significant.

3.4. RESULTS.

Ethanol Exposure Induced Alternative Activation In NR8383 Cells. We first examined the ability of the T_H2 cytokine IL-13 to induce alternative activation in NR8383 cells. As expected, IL-13 increased arginase-1 gene and protein expression by 4.5-fold **(Figure 3.1A; $^a p < 0.05$)** and 3-fold **(Figure 3.2A and 3.2B; $^a p < 0.05$),** respectively, as well as arginase activity by 3-fold **(Figure 3.3; $^a p < 0.05$)**. Similarly, 5 days of 1.5ng/ml IL-13 treatment increased the gene expression **(Figure 3.1B; $^a p < 0.05$)** and protein expression **(Figure 3.2A and 3.2C; $^a p < 0.05$),** respectively of galectin-3, another marker of alternative activation. Since IL-13 clearly induced alternative activation in these cells, we next examined whether ethanol induced expression of IL-13. In response to ethanol exposure, NR8383 cells exhibited a 3 fold increase IL-13 gene and

Figure 3.1 Chronic Ethanol Treatment of NR8383 Cells Promoted Gene Expression of Alternative Activation Markers. The effects of chronic ethanol treatment on gene expression of markers of alternative activation were examined in NR8383 treated with 0.08% ethanol ± 500 µM GSH for 5 days. Cells were also treated with IL-13 as a positive control. RNA was extracted from NR8383 cells and subjected to quantitative RT-PCR. Quantification of mRNA expression of arginase-1 (**A**) galectin-3**,** (**B**) and CD32 (**C**) in NR8383 cells was performed after 5 consecutive days of treatment. The results are given as the ratios of gene of interest expression to GAPDH mRNA. Bar heights represent mean ± SE. ^{a} $p \leq 0.05$ compared to controls, * $p \leq 0.05$ compared to ethanol n=4 or more per group.

Figure 3.1 A

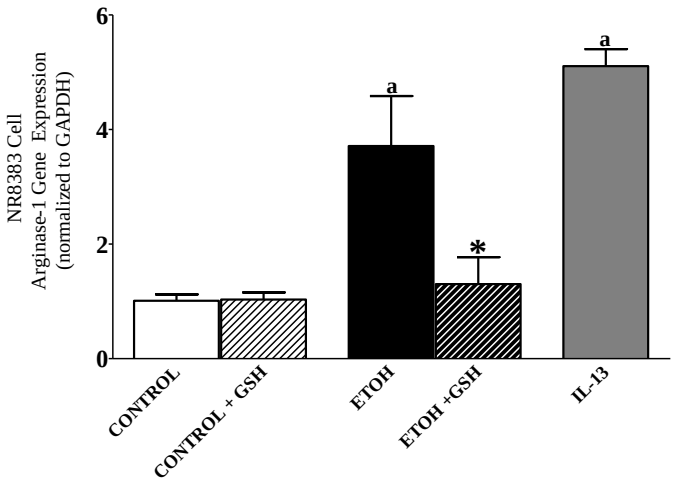


Figure 3.1B

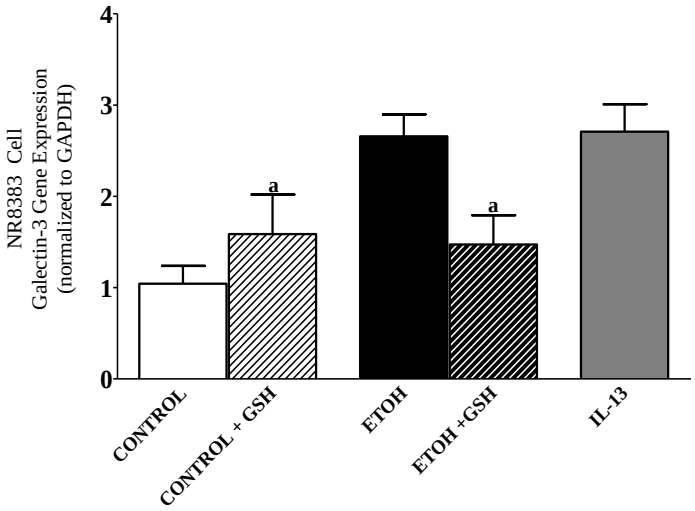


Figure 3.1C

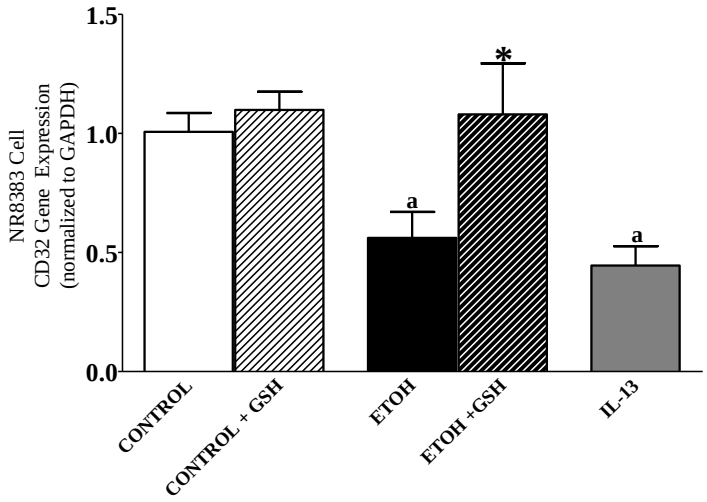


Figure 3.2. Chronic Ethanol Treatment of NR8383 Cells Increased Protein Expression of Markers of Alternative Activation. The effects of chronic ethanol treatment on protein expression of markers of alternative activation were examined in NR8383 treated with 0.08% ethanol ± 500 μM GSH for 5 days. Cells were also treated with IL-13 as a positive control. Representative immunoblots are shown in **A**. Expression of arginase-1 (**B**), galectin-3 (**C**), and CD32 (**D**) were determined via western blot analysis. Bar heights represent mean ± SE. ^{a} $p \leq 0.05$ compared to controls, * $p \leq 0.05$ compared to ethanol, n=3 or more fields per group.

Figure 3.2 A

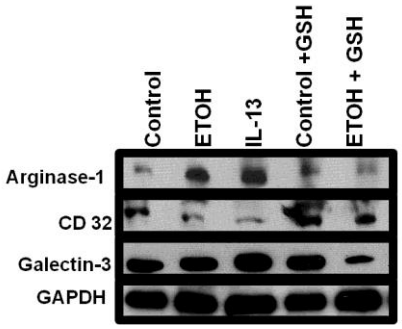


Figure 3.2 B

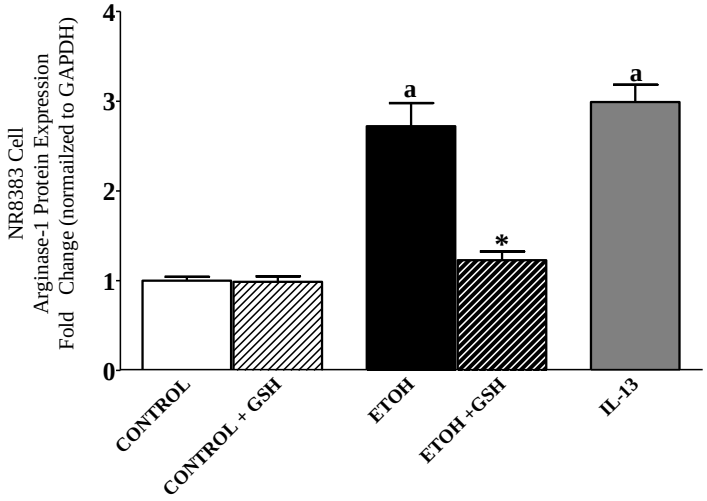


Figure 3.2 C

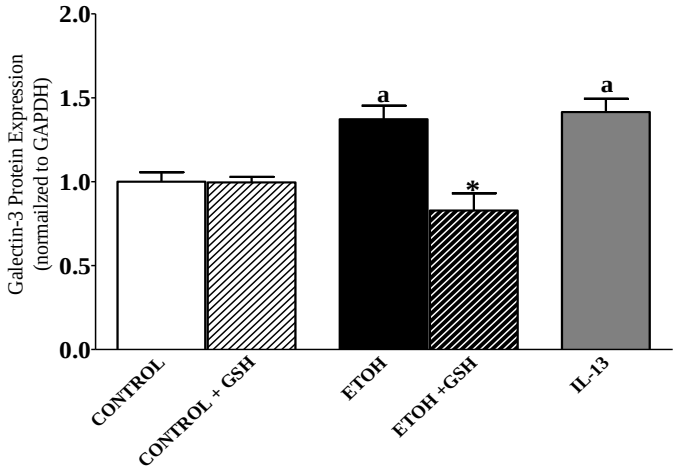


Figure 3.2 D

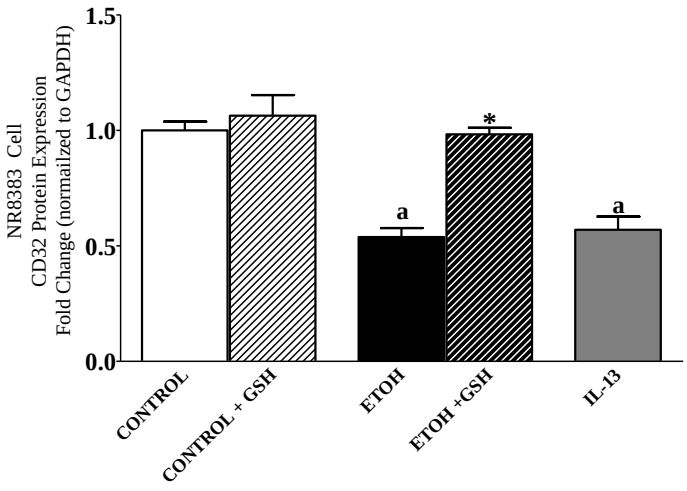
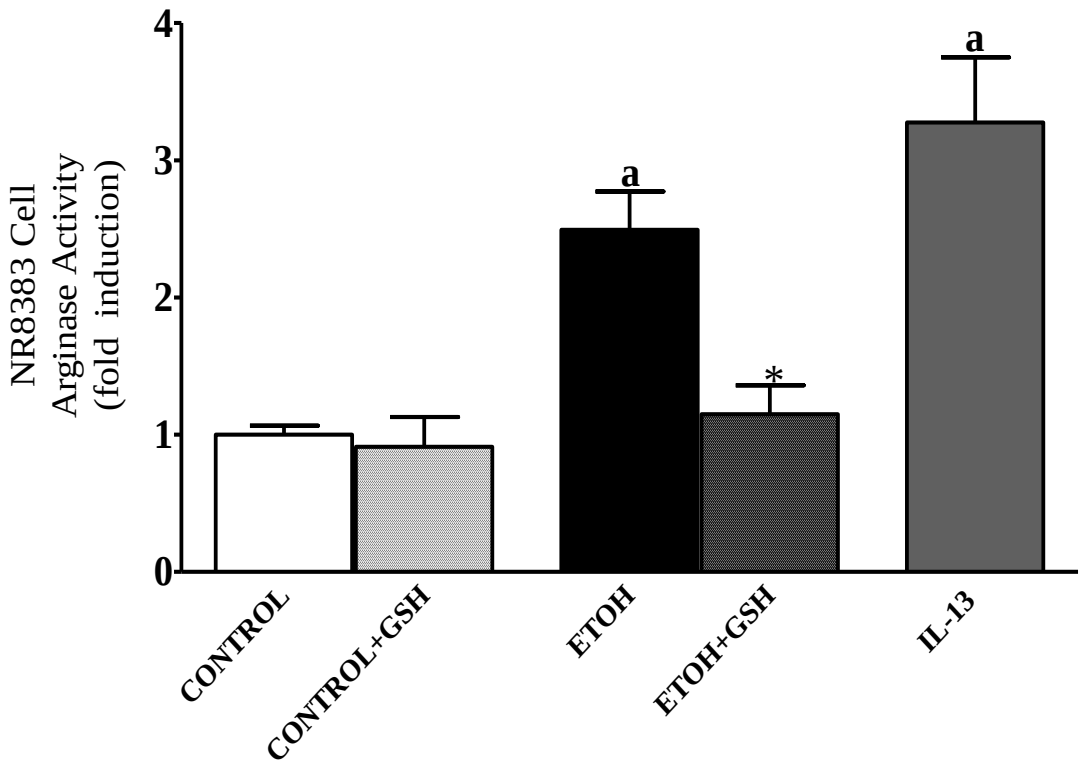


Figure 3.3. Chronic Ethanol Treatment of NR8383 Cells Increased Arginase-1 Activity. After appropriate treatments, NR8383 cells were lysed and prepared for arginase activity assays. Assessment of arginase-1 activity in control cells and cells treated with ethanol or IL-13 was determined and normalized to protein concentrations. Bar heights represent mean ± SE. ^{a} $p \leq 0.05$ compared to controls, * $p \leq 0.05$ compared to ethanol, n=4 or more fields.


NR8383 Cell
Arginase Activity
(fold induction)
4
3
2
1
0
a
a
*
CONTROL
CONTROL+GSH
ETOH
ETOH+GSH
IL-13

protein expression **(Figure 3.4A; [a] p <0.05 and Figure 3.4B and 3.4C; [a] p <0.05)**, respectively. In contrast, the gene and protein expression of the Fc gamma receptor IIa CD32, a marker of classical activation and maturation, was reduced by 45% in response to ethanol treatment **(Figure 3.1C, 3.2A, and 3.2D, [*]p <0.05).**

GSH Supplementation Prevented Ethanol-Induced Alternative Activation of NR8383 Cells. To investigate the role of GSH availability in ethanol-mediated alternative activation, NR8383 cells were treated daily with 500 µM GSH and ethanol, concomitantly. GSH supplementation normalized IL-13 gene expression to control values and significantly reduced IL-13 protein expression **(Figure 3.4A and 3.4B;[*]p <0.05).** GSH supplementation also prevented ethanol-induced increases in arginase-1 gene expression, protein expression and activity. **(Figure 3.1A, 3.2A, 3.2B and 3.3, respectively, [*]p <0.05).** GSH supplementation also normalized gene and protein expression of CD32 and galectin-3 **(Figure 3.1B-C and 3. 2A,C-D;[*]p <0.05)**.

Chronic Alcohol Treatment Altered NR8383 Cell ROS Production. Our previous studies demonstrated that chronic alcohol alters oxidant/antioxidant balance by decreasing GSH availability (Brown et al., 2007; Moss et al., 2000b). However, the effect of chronic ethanol treatment or ingestion on alveolar macrophage extracellular and intracellular ROS production has yet to be examined. To address this question, we determined the effects of chronic ethanol treatment on intracellular ROS as well as intracellular and extracellular H_2O_2 production of NR8383 cells. Treatment with 0.08% ethanol for 5 consecutive days led to a 6 fold increase in extracellular H_2O_2 levels as determined by the Amplex Red/horseradish peroxide assay **(Figure 3. 5A; [a] p <0.05),** a 16-fold increase in intracellular ROS production **(Figure 3.5B; [a] p <0.05)** and a 15-fold increase in intracellular H_2O_2 levels **(Figure 3.5C; [a] p <0.05).** Representative images for intracellular H_2O_2 production are shown in Figure 5D. Daily supplementations with 500 µM

Figure 3.4 Chronic Ethanol Treatment Increased IL-13 Expression in NR8383 Cells. The effects of chronic ethanol treatment on gene and protein expression of IL-13 was examined in NR8383 treated with 0.08% ethanol ± 500 µM GSH for 5 days. Cells were also treated with IL-13 as a positive control. RNA was extracted from NR8383 cells and subjected to quantitative RT-PCR. Quantification of IL-13 mRNA expression **(A) is** given as the ratio of the IL-13 gene to GAPDH mRNA**.** Representative micrographs of IL-13 expression are shown in **B**. IL-13 protein expression was measured via ELISA (**C**). Bar heights represent mean ± SE. ^{a} $p \leq 0.05$ compared to controls, * $p \leq 0.05$ compared to Ethanol, n=4 or more per group.

Figure 3.4A

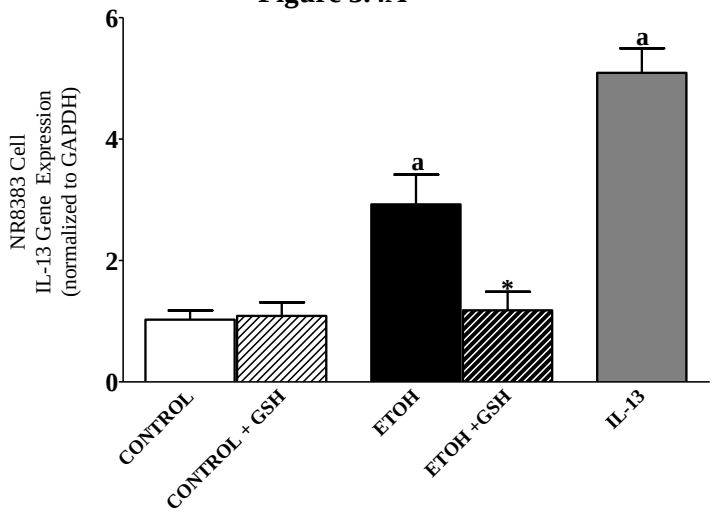


Figure 3.4B

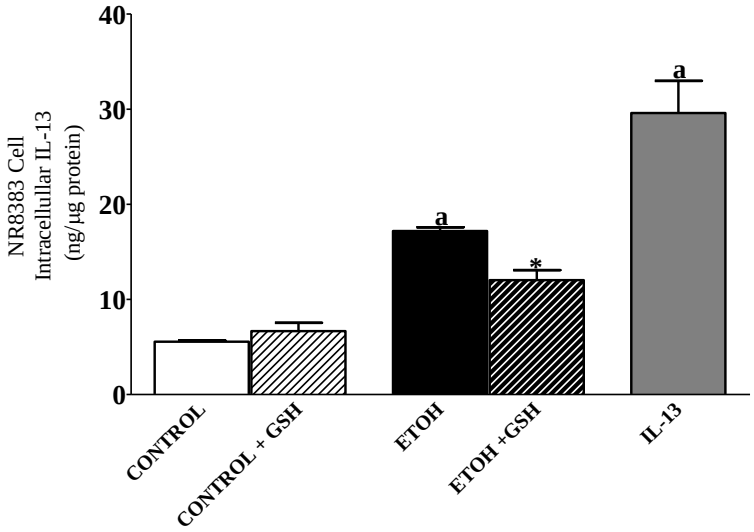


Figure 3.5 Chronic Ethanol Treatment Increased Intracellular and Extracellular ROS. NR8383 cells were treated with 0.08% ethanol ± 500 μM GSH for 5 consecutive days. The extracellular level of H_2O_2 produced by NR8383 cells was determined using amplex red (**A**). Intracellular ROS was determined using DCFH-DA (**B**). Intracellular H_2O_2 production was quantified via amplite green fluorescence (**C**). Representative confocal images are shown (**D**). Bars heights represent means ± SE. ^{a} $p \leq 0.05$ compared to controls, * $p \leq 0.05$ compared to the ethanol treatment. n=15 or more fields per group.

Figure 3.5A

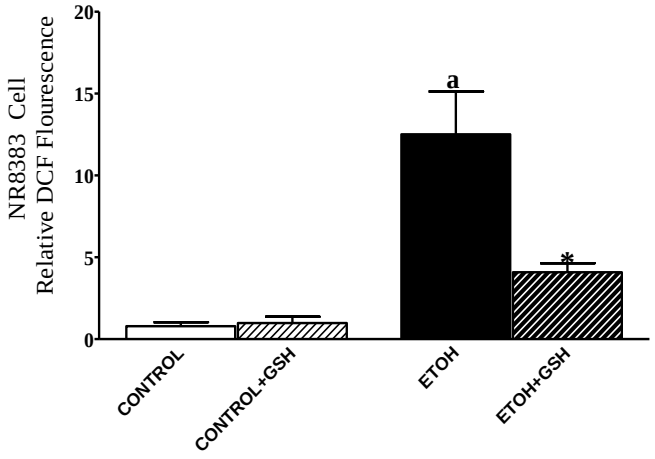


Figure 3.5B

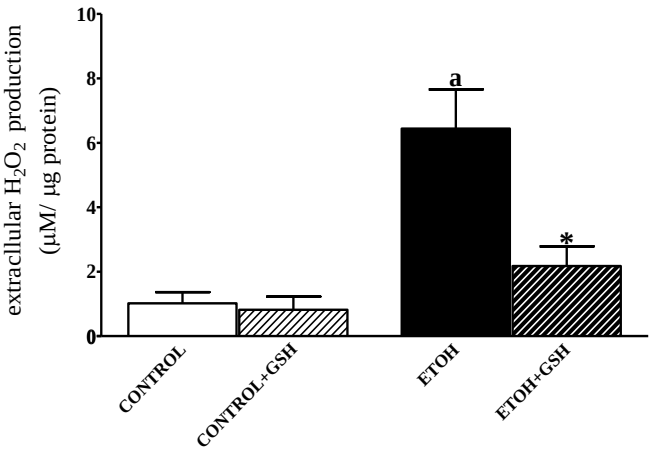


Figure 3.5C

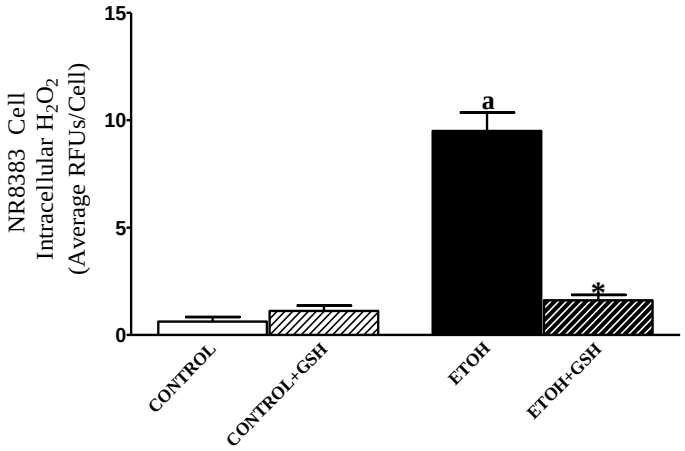
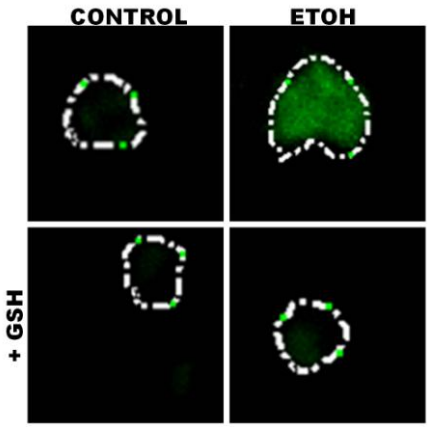


Figure 3.5D



GSH normalized extracellular H_2O_2 (**Figure 3. 5A**), intracellular ROS (**Figure 3.5B**) and intracellular H_2O_2 (**Figure 3. 5C and 3.5D**).

GSH Supplementation Prevented Ethanol–Induced Loss Of NR8383 Cell Phagocytic Function. To assess the effects of chronic ethanol exposure on phagocytosis, NR8383 cells (± ethanol treatment for 5 d) were cultured for 2 hrs with pHrodo red labeled *S. aureus*. The pH-sensitive rhodamine-based pHrodo dye is a specific sensor of phagocytic events; it is non-fluorescent at neutral pH and fluoresces red in acidic environments, such as phagolysosomes. Chronic ethanol exposure significantly decreased the internalization of pHrodo red labeled *S. aureus* as determined by a 60% decrease in the phagocytic index when compared to controls **(Representative images in Figure 3.6A; Figure 3.6B, ^{a} p <0.05).** For control cells, GSH supplementation did not significantly alter the phagocytic capacity. However, daily GSH supplementation prevented ethanol-induced decreases in NR8383 cell phagocytic function **(Figure 3.6 A-B, *p <0.05).**

Chronic Ethanol Ingestion Induced Alternative Activation in Primary Alveolar Macrophages But Was Reversed By Intranasal GSH. We next sought to determine if a similar switch to an alternatively activated phenotype occurred *in vivo* with chronic ethanol ingestion. Alveolar macrophages isolated from ethanol-fed animals displayed an alternative activation phenotype as evidenced by a significant increase in gene and protein expression of IL-13, a known inducer and marker of alternative activation **(Figure 3.7. A-C; ^{a}p <0.05)**. Corresponding to the ethanol-induced secretion of IL-13, there was increased gene expression of the alternative activation markers, arginase-1 and galectin-3 **(Figure 3.8A and B; ^{a}p <0.05).** There was also a corresponding increase in arginase-1 and galectin-3 protein expression **(Figure 3.9 A-C; ^{a}p <0.05)**. As expected, chronic ethanol ingestion significantly decreased CD32 gene and protein expression in the primary alveolar macrophages when compared to controls **(Figure 3.8C and**

Figure 3.6 Chronic Ethanol Treatment Impaired Phagocytosis of pHrodo-Labeled *S. aureus*. NR8383 cells were incubated with 1mg/ml pHrodo-labeled *S. aureus* for 2 hr. Internalization was evaluated using quantitative digital analysis of fluorescence from the confocal microscopic image. Representative photomicrographs at 20X magnification are shown in ***A***. The phagocytic index (***B***) was determined by multiplying the average RFUs/cell by the percentage of cells positive for internalization. Bars in B represent means ± SE. [a] $p \leq 0.05$ compared to controls, [*] $p \leq 0.05$ compared to ethanol, n=15 or more fields per group.

Figure 3.6A

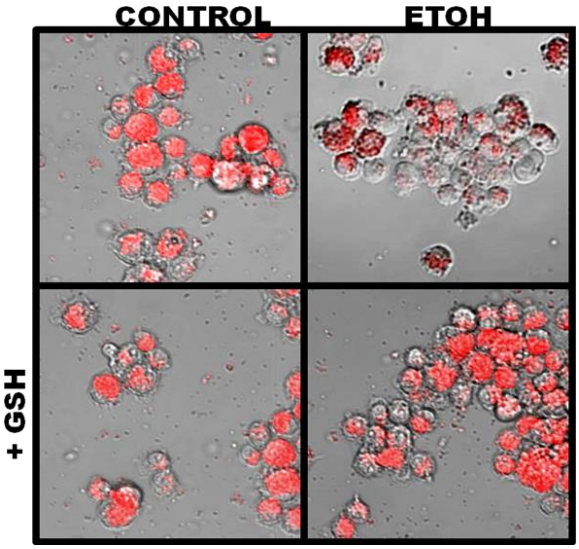


Figure 3.6B

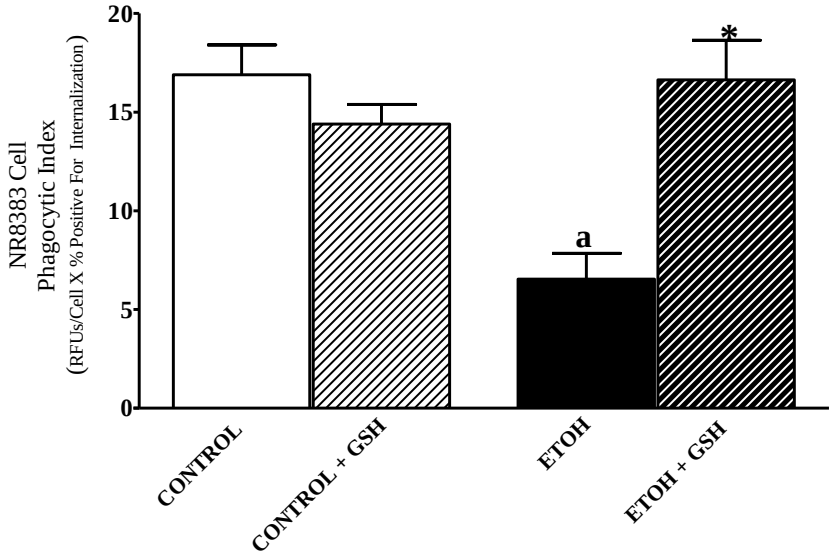


Figure 3.7. Rat Model of Chronic Ethanol Ingestion: Increased Gene and Protein Expression of IL-13. The effects of chronic ethanol ingestion on gene and protein expression of IL-13 were examined in rat alveolar macrophages after 6 weeks ethanol ingestion ± intranasal GSH treatment. Cells were also treated with IL-13 as a positive control. RNA was extracted from NR8383 cells and subjected to quantitative RT-PCR. Quantification of IL-13 mRNA expression (**A**) **is** given as the ratios of IL-13 expression to GAPDH mRNA**.** Representative micrographs of IL-13 protein expression are shown in **B**. IL-13 protein expression was measured via ELISA (**C**). Bar heights represent mean ± SE. ^{a} $p \leq 0.05$ compared to controls, * $p \leq 0.05$ compared to Ethanol, n=4 or more per group.

Figure 3.7A

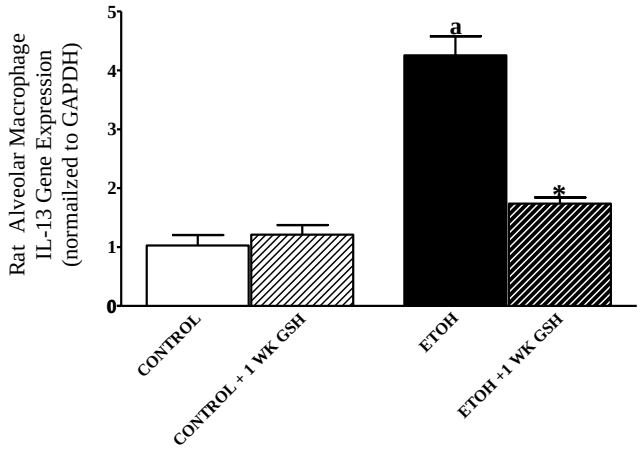


Figure 3.7 B

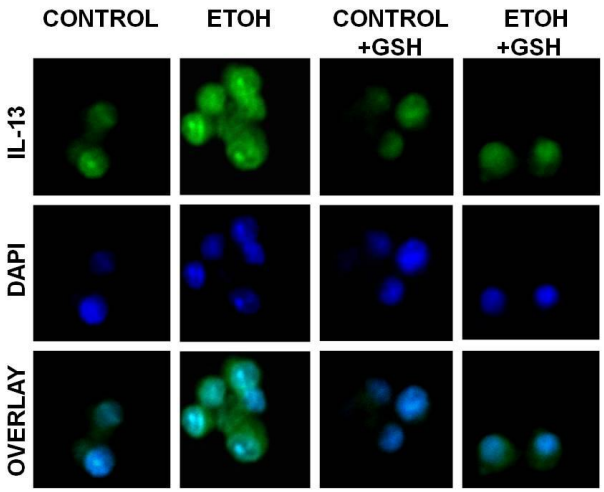


Figure 3.7C

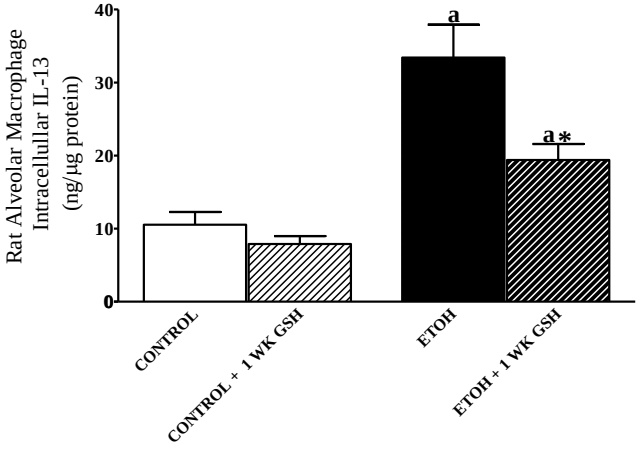


Figure 3.8. Rat Model of Chronic Ethanol Ingestion: Increased Gene Expression of Alternative Activation Markers. The effects of chronic ethanol ingestion gene expression on known markers of classical and alternative activation were examined in primary rat alveolar macrophages. RNA was extracted from primary rat alveolar macrophages and subjected to quantitative RT-PCR. Quantification of mRNA expression of arginase-1 (**A**) galectin-3 (**B**), and CD32 (**C**) was then performed. The results are given as the ratios of gene of interest expression to GAPDH mRNA. Bar heights represent mean ± SE. ^{a} $p \leq 0.05$ compared to control, * $p \leq 0.05$ compared to ethanol-fed animals, n=3 or more fields per group.

Figure 3.8A

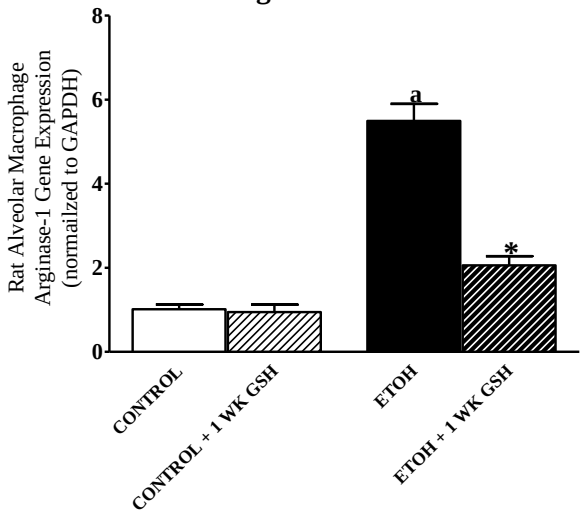


Figure 3.8B

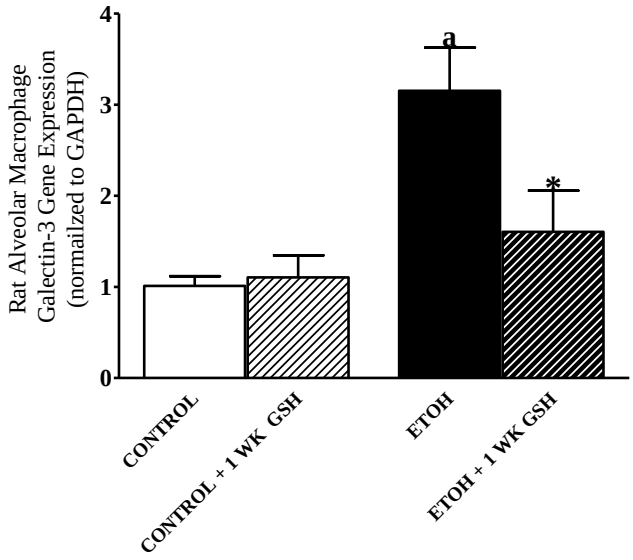
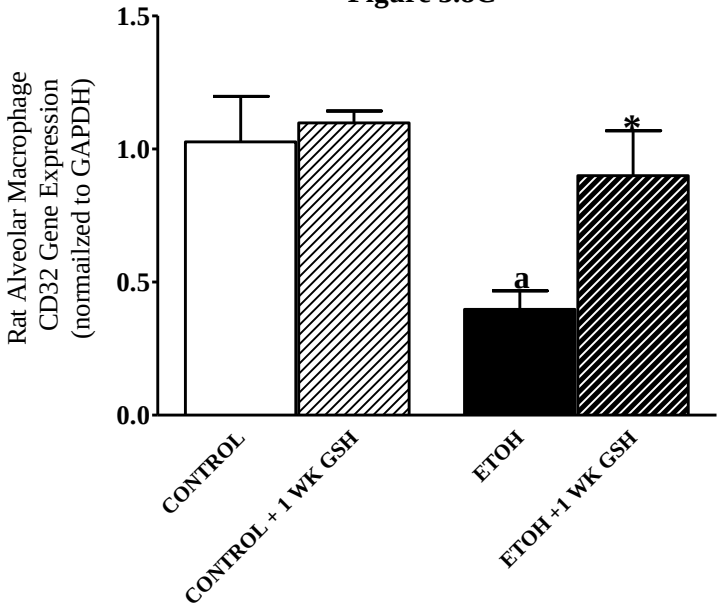


Figure 3.8C



3.9D; ^{a}p <0.05). Importantly, intranasal instillation of GSH during the last week of ethanol feeding reversed ethanol-induced alternative activation of alveolar macrophages as evidenced by normalization of IL-13, arginase-1, galectin-3, and CD32 but had no effect on alveolar macrophages in control animals. **(Figure 3.7-9, *p <0.05)**.

Chronic Ethanol Ingestion Increased ROS Production By Alveolar Macrophages But Was Reversed By Intranasal GSH. Previous studies by our laboratory demonstrated that chronic alcohol ingestion depletes GSH within the alveolar space and alters GSH homeostasis in alveolar macrophages (Brown et al., 2004; Brown et al., 2007; Yeh et al., 2007). Furthermore, GSH availability modulates alveolar macrophage function in a rat model of chronic ethanol ingestion (Brown et al., 2004; Gauthier TW, 2005). However, the effects of *in vivo* GSH supplementation on alveolar macrophage function and ROS production were not examined. In the current study, chronic ethanol ingestion induced a 17-fold increase in alveolar macrophage intracellular H_2O_2 production and 80% decrease in phagocytic function **(Figure 3.10B and 3.10D,^{a}p <0.05)**. Representative micrographs are shown in **Figure 3. 10 A and C**. However, intranasal delivery of GSH dramatically reduced intracellular H_2O_2 production by 70% and normalized phagocytic capacity **(Figure 3.10B and 3.10D,*p <0.05)**.

A History of Alcohol Abuse Induced Alternative Activation of Alveolar Macrophages in Humans. We next examined whether a history of alcohol abuse promoted alternative activation and whether GSH supplements could restore alveolar macrophage function. Based on computer analysis of the fluorescent images, alveolar macrophages from subjects with a history of alcohol abuse displayed a 2.5 fold increase in IL-13 expression, a 4 fold increase in arginase-1 expression and a 3 fold increase in galectin-3 expression **(Figure 3.11 B-E, respectively, ^{a}p <0.05),** when compared to control values. Conversely, CD32 expression was decreased by 70% in response to

Figure 3.9. Rat Model of Chronic Ethanol Ingestion: Increased Protein Expression of Alternative Activation Markers. The effects of chronic ethanol ingestion on protein expression of markers of alternative activation were examined in rat alveolar macrophages after 6 weeks ethanol ingestion ± intranasal GSH treatment. Quantification of arginase (**B**) galectin-3 (**C**) and CD32 (**D**) were determined via western blot analysis. Representative immunoblots are shown in **A**. Bar heights represent mean ± SE. ^{a} $p \leq 0.05$ compared to controls, * $p \leq 0.05$ compared to Alcohol, n=3 or more fields per group.

Figure 3.9 A

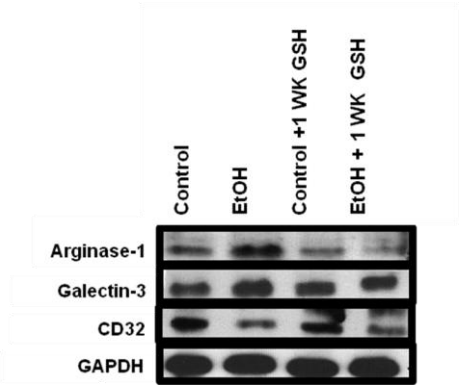


Figure 3.9 B

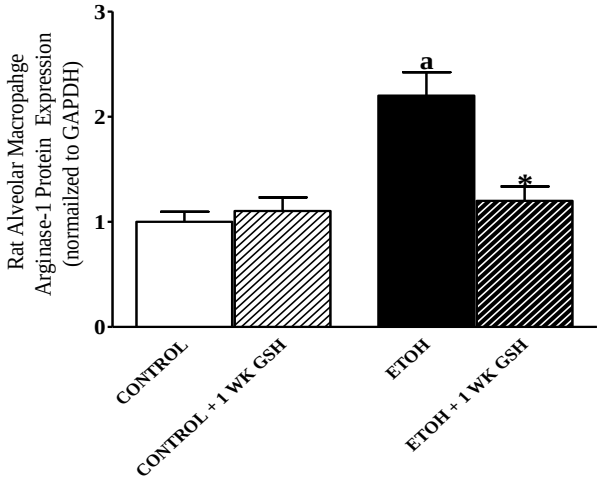


Figure 3.9 C

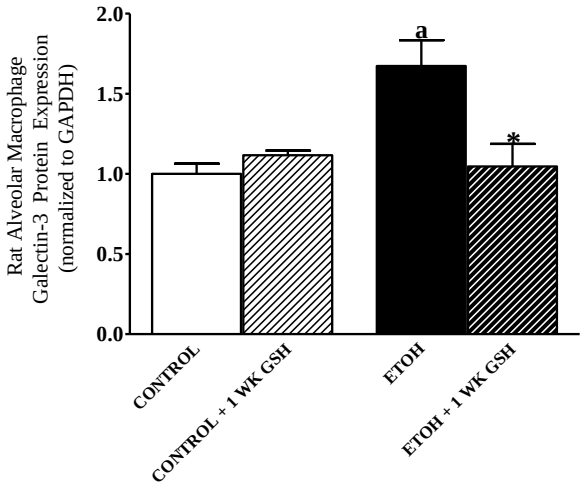


Figure 3.9 D

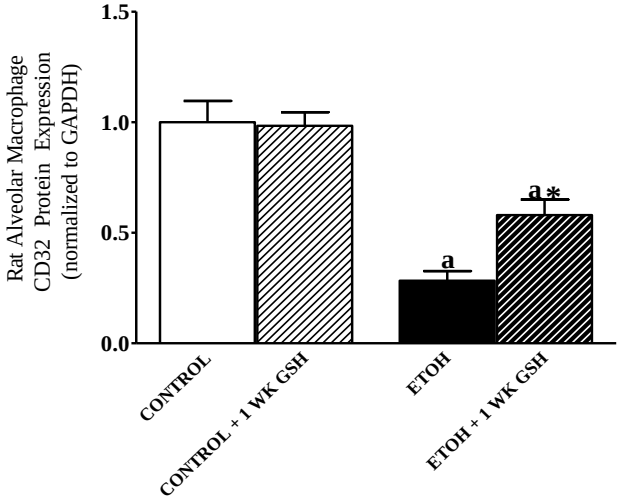


Figure 3.10. Rat Model of Chronic Ethanol Ingestion: Increased Intracellular H_2O_2 Production and Inhibition of Phagocytosis. After 6 weeks of ethanol feeding, rats were sacrificed and alveolar macrophages were isolated via bronchoalveolar lavage. Where appropriate, animals received intranasal delivery of 500 μM GSH during the last week of feeding. Freshly isolated alveolar macrophages were plated and allowed to adhere for 1hr. Intracellular H_2O_2 production using amplite green (**B**) and the phagocytic index for pHrodo labeled *S. aureus* (**D**) were then determined. Representative micrographs of intracellular H_2O_2 production and phagocytic function are shown in **A** and **C**, respectively. n=10 or more fields per group.

Figure 3.10 A

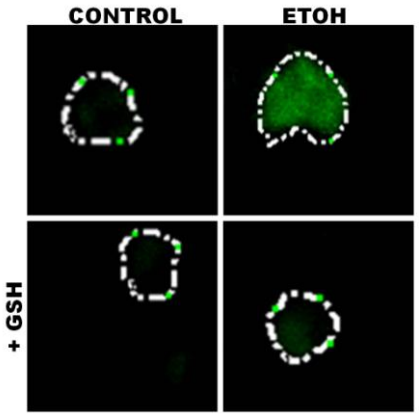


Figure 3.10 B

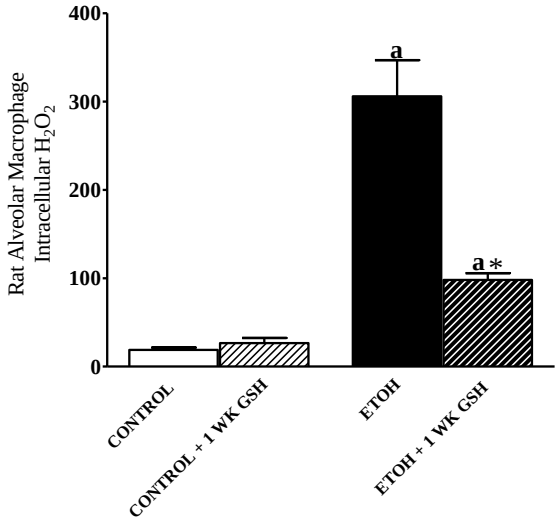


Figure 3.10 C

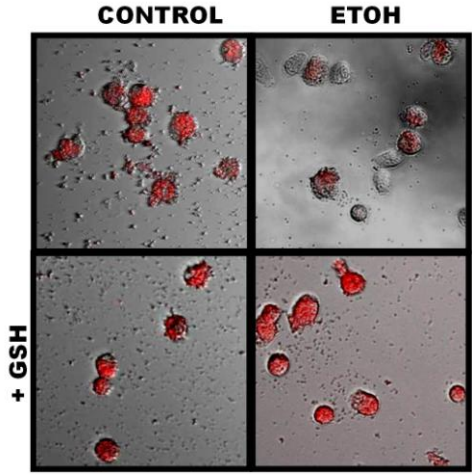


Figure 3.10 D

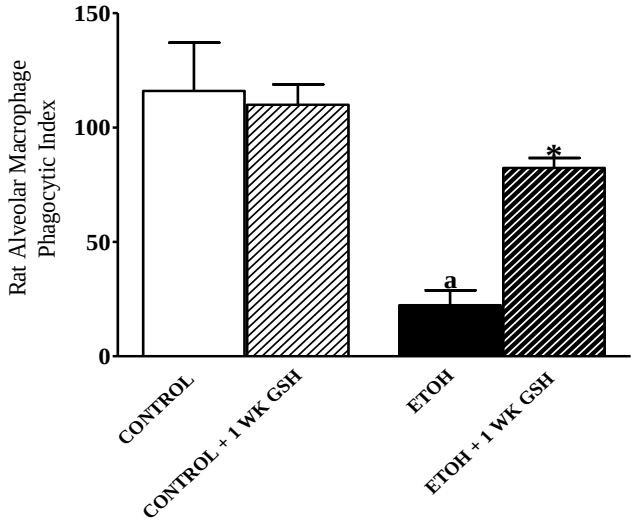


Figure 3.11.Otherwise Healthy Alcoholics: Induced Alternative Activation. The effects of chronic alcohol consumption on alternative activation were examined in freshly isolated primary human alveolar macrophages cultured for 24 hr ± *in vitro* 500 µM GSH . Protein expression was determined via quantification of immunofluorescence after incubation with primary antibodies directed against IL-13 (**C**), arginase-1 (**D**), galectin-3 (**E**), or CD-32 (**F**) and then secondary antibodies directed against IgG labeled with FITC or TRITC. Where appropriate, cells were also blocked with Fc block. Representative images of IL-13 and arginase-1 protein expression are shown in **A** and **B**, respectively. Bar height represents mean RFUs/cell ± SE. ^{a} $p \leq 0.05$ compared to control, * $p \leq 0.05$ compared to alcoholic, n=15 or more fields per group.

Figure 3.11A

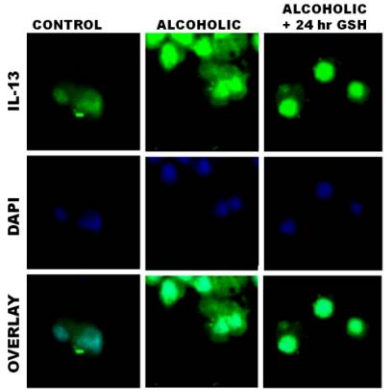


Figure 3.11B

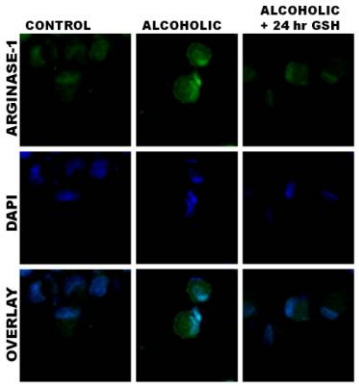


Figure 3.11C

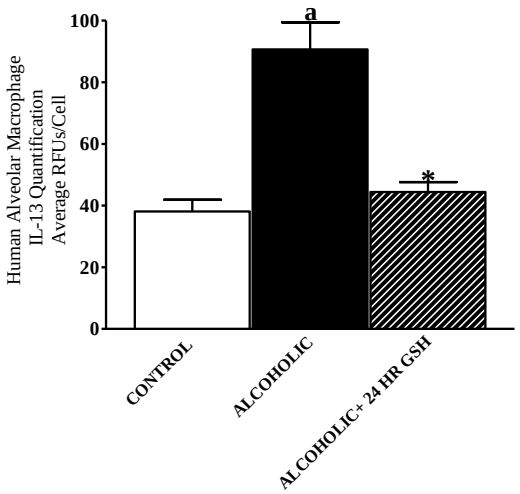


Figure 3.11D

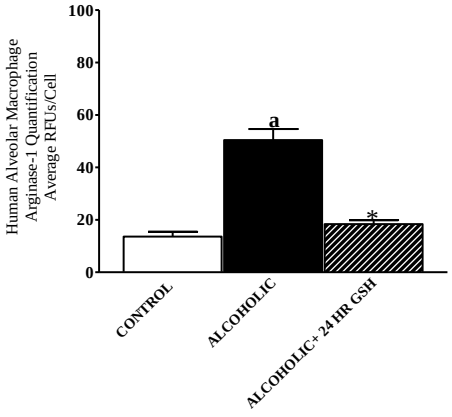


Figure 3.11E

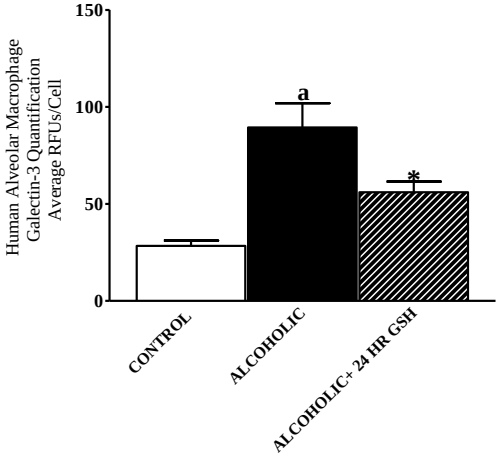
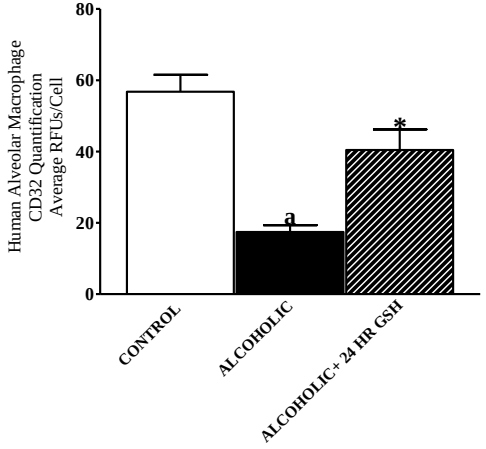


Figure 3.11F



in response to alcohol abuse **(Figure 3.11F, $^{a}p < 0.05$)**. Representative images of IL-13 and arginase-1 are shown in **Figure 3.11 A and B**, respectively. In the alveolar macrophages from otherwise healthy alcoholics, *in vitro* GSH treatments attenuated expression of IL-13, arginase-1 and galectin-3 **(Figure 3.11C-E, ^{a}p=NS, $^{*}p < 0.05$).** Correspondingly, CD32 expression was normalized when the macrophages from otherwise healthy alcoholics were treated with GSH **(Figure 3.11F, ^{a}p=NS, $^{*}p < 0.05$).** To evaluate alveolar macrophage phagocytosis, freshly isolated human alveolar macrophages cultured for 24 hr ± *in vitro* 500 µM GSH and then incubated with pHrodo red for 2 hrs. Phagocytic function was examined using quantitative digital analysis of the fluorescent microscopic images. Representative photomicrographs are shown in **Figure 3.12A**. A history of alcohol abuse severely blunted alveolar macrophage phagocytic capacity with a 70% decrease in alveolar macrophage phagocytic index when compared to alveolar macrophages from control subjects **(Figure 3.12B, $^{a}p < 0.05$)**. In contrast, the phagocytic index of the macrophages from alcoholic subjects was normalized in response to the *in vitro* GSH treatments **(Figure 3.12B, $^{a}p < 0.05$)**.

3.5. DISCUSSION.

Numerous studies have demonstrated that chronic alcohol abuse greatly diminishes alveolar macrophage phagocytosis of foreign pathogens and increases the risk of respiratory infections. We have previously demonstrated in clinical and animal studies that chronic alcohol ingestion decreases the critical antioxidant GSH in the alveolar lining fluid, shifts the redox potential of GSH/GSSG to a more oxidized state by 50 mV, and increases ROS generation within the alveolar space. In animal models, this decrease in lining fluid GSH was associated with decreased GSH in the alveolar macrophages (Brown et al., 2004; Brown et al., 2007; Guidot et al., 2000). Equally important, decreased GSH availability was demonstrated to be a central modulator of phagocytosis. However, the mechanisms underlying this impaired phagocytosis remain unclear.

Figure 3.12. Chronic Alcohol Consumption Severely Dampens Human Alveolar Macrophage Phagocytic Capacity. The effects of chronic alcohol consumption on alveolar macrophage function were examined in primary human alveolar macrophages ± 24 hr of *in vitro* 500 μM GSH treatment. Following 24hr incubation, human alveolar macrophages were incubated with 1mg/ml pHrodo labeled *S. aureus* for 2 hrs and then fixed with 4% paraformaldehyde. Representative photomicrographs at 20X magnification are shown in ***A***. Quantification of phagocytic capacity **(B)** was determined. Bars heights represent means ± SE. ^{a} $p \leq 0.05$ compared to controls, * $p \leq 0.05$ compared to alcoholic, n=15 or more fields per group.

Figure 3.12A

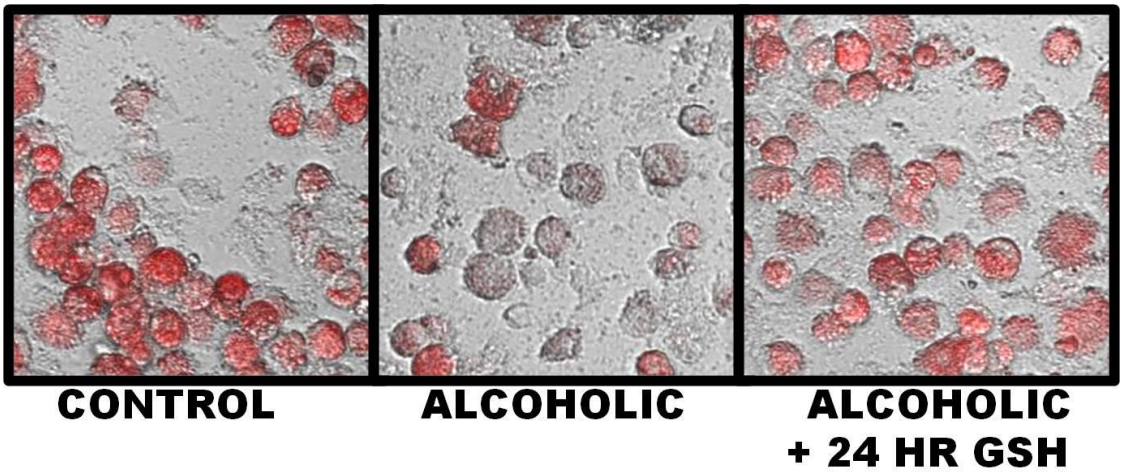
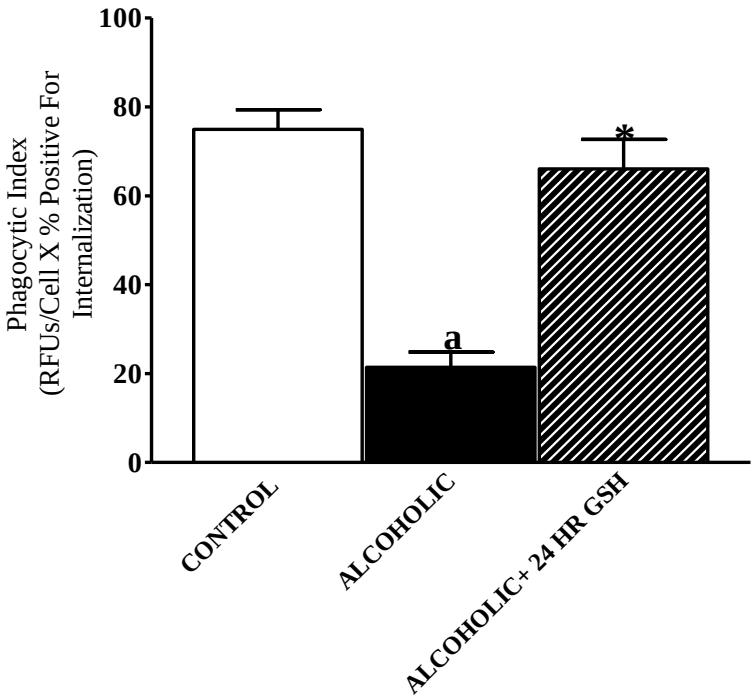


Figure 3.12B



Alveolar macrophages can be activated via two distinct pathways in response to T_H1 or T_H2 cytokines and these opposing pathways modulate the alveolar macrophage immune response. During T_H1 cytokine mediated classical activation; primed alveolar macrophages induce a cytotoxic, antimicrobial, and proinflammatory response necessary for phagocytosis. Exposure to T_H2 cytokines, such as IL-4 and IL-13, results in alternative activation of alveolar macrophages. This entails a downregulation of the alveolar macrophage inflammatory response by upregulation of the expression of arginase-1, an enzyme that acts as an endogenous inhibitor of NOS_2. This dampening of the alveolar macrophage immune response occurs to promote tissue repair via the promotion of cellular proliferation and collagen production (Gordon, 2002; Gordon, 2003; Gordon and Martinez; Gordon and Taylor, 2005; Martinez et al., 2009; Stein et al., 1992; Taylor et al., 2005). Alternative activation leads to unregulated ROS production and decreased phagocytosis; thereby, leading to an increased susceptibility to lung infections.

In the current study, we have provided evidence in a rat model of chronic ethanol ingestion, and in clinical samples, that chronic alcohol ingestion leads to alternative activation in alveolar macrophages. In NR8383 cells, *in vitro* ethanol exposure increased the synthesis of IL-13, a known inducer of alternative activation. This increase in IL-13 was associated with increased expression and activity of arginase-1, an endogenous inhibitor of classical activation. Expression of galectin-3, which is required for alternative activation, was also increased in response to chronic ethanol ingestion. Increases in these markers of alternative activation were associated with decreased expression of CD32, a marker of maturation and classical activation. In addition, ethanol increased the basal rate of ROS generation and release, a phenotype also associated with alternative activation. In the rat model, 6 weeks of chronic ethanol ingestion resulted in alveolar macrophages with increased gene and protein expression of IL-13, arginase-1, and galectin-3, suggesting alternative activation. For the maturation marker CD32, chronic ethanol ingestion downregulated gene and protein expression. Chronic ethanol ingestion also increased the basal rate of ROS generation and release. Likewise, a history of alcohol abuse

resulted in alveolar macrophages with a phenotype reflective of alternative activation as evidenced by decreased phagocytosis, increased protein expression of IL-13, arginase-1, and galectin-3 as well as decreased expression of CD32. Thus, alveolar macrophages from alcoholic subjects acquired an alternatively activated phenotype. Alternative activation may decrease the release of pro-inflammatory cytokines associated with a respiratory burst but the decreased phagocytic capacity may explain the increased risk of respiratory infections associated with alcohol abuse. Furthermore, the increased arginase production/activity associated with alternative activation leads to increased proline and polyamine production, precursors to fibroblast proliferation and collagen production in the lung.

Since our previous studies demonstrated that ethanol-induced suppression of phagocytosis could be reversed with GSH treatments, we also examined the effects of GSH on alternative activation. In the ethanol-treated NR8383 cells, the expression of the markers for alternative activation were normalized by co-treatment with 500 μM GSH, the concentration normally present in the alveolar lining fluid of healthy controls (Yeh et al., 2007). This was concomitant with restoration of phagocytosis. In the ethanol-fed rats, intranasal delivery of 500 μM GSH reversed the alternatively activated phenotype and restored phagocytosis. In the alveolar macrophages from alcoholic subjects, 24 hr treatments with GSH also reversed the alternatively activated phenotype and restored phagocytosis. It is important to note 66% of the alcoholic subjects were also smokers, while none of the control subjects were. Since smoking has also been shown to alter the alveolar macrophage phenotype, we cannot rule out the altered expression of alterative activation makers may be partially due to the smoking status of the subject. One potential mechanism for these beneficial effects of GSH treatments may be through attenuation of ROS generation as evidenced in the NR8383 cells and macrophages from ethanol-fed rats. Alternatively, changes in the GSH redox state can alter cell signaling and may also play a role (Go and Jones, 2005; Jones, 2006; Rahman and Adcock, 2006).

In conclusion, these studies demonstrated that chronic alcohol abuse induced alveolar macrophages to undergo a switch to an alternatively activated phenotype. As expected, this switch was associated with a severely blunted capacity for phagocytosis. GSH supplements reversed these effects, suggesting that GSH availability modulates alcohol-induced alterations in alveolar macrophages and, ultimately, phagocytic capacity. Preventing or reversing alternative activation may prove beneficial to alcoholic patients by leading to the development of novel therapies for patients with an increased risk of respiratory infections and ARDS due to chronic alcohol abuse.

CHAPTER 4

ROLE OF TGF-β_1 IN ALTERNATIVE ACTIVATION

4.1. ABSTRACT.

Depending on their microenvironment, alveolar macrophages can be activated by two distinct pathways that play divergent roles: classical, IFN-γ-dependent activation, or alternative activation mediated by T_H2 cytokines such as IL-4 and IL-13. Alternatively activated macrophages play a vital role in diverse pathologies such as wound repair, asthma, and parasitic infections. Numerous studies have demonstrated that T_H2 cytokine-mediated alternative activation leads to increased production of arginase-1, galectin-3 and other markers of alternative activation. In addition to alternative activation, IL-13 induces fibrosis during inflammation through increased TGF-β_1 production, but the role of TGF-β_1 signaling in alternative activation is unclear. In this study, we investigated the role of TGF-β_1 in IL-13-mediated alterations in alveolar macrophage immune responses. In response to IL-13, the alveolar macrophage cell line NR8383 cells upregulated expression of IL-13 itself as well as arginase-1 and galectin-3, markers of alternative activation. IL-13 also induced TGF-β_1 production, secretion, and signaling through smad 2/3 phosphroylation. This alternative activation was associated with impaired phagocytosis. Ablation of TGF-β_1 signaling significantly decreased expression of IL-13, alternative activation markers and prevented IL-13-induced alveolar macrophage dysfunction. Further studies showed that IL-13-enhanced fibrogenesis is dependent on TGF-β_1 signaling. Similarly, intratracheal delivery of IL-13 induced TGF-β_1 expression, alternative activation, and impaired phagocytosis. Inhibition of TGF-β_1 signaling *in vivo* ablated these effects of IL-13. These findings suggested TGF-β_1 signaling was an integral part of IL-13 mediated alternative activation of alveolar macrophages and promotion of fibrogenesis.

4.2. INTRODUCTION.

Due to their unique ability to couple internalization and degradation of inhaled pathogens with the release of inflammatory mediators, alveolar macrophages, the resident phagocytes within the lung, play a vital role in innate and acquired immunity (Aderem and Underhill, 1999a; Koay et al., 2002). They provide the first line of defense against inhaled foreign pathogens, bacteria and particles within the lung (Aderem and Underhill, 1999a; Ward, 1997). However, the functional and morphological phenotype of alveolar macrophages is modulated by exposure to different mediators within their microenvironment (Abramson and Gallin, 1990; Gordon, 2003; Gordon and Taylor, 2005; McKenzie et al., 1998; Muller et al., 2007; Taylor et al., 2005).

In response to exposure to T_H1 cytokines, such as IFN-γ or TNF-α, alveolar macrophages become primed to be "classically activated" into effector cells via the induction of antimicrobial activity (Downing et al., 1999; Nathan and Hibbs, 1991; Noda and Amano, 1997). Once primed, these alveolar macrophages become classically activated in response to exposure to foreign pathogens, such as LPS. Classical activation of alveolar macrophages leads to upregulation of arginine-derived nitric oxide synthase-2, which synthesizes nitric oxide in the presence of NADPH and O_2. Sustained nitric oxide production, which is dependent on L-arginine availability (El-Gayar et al., 2003), provides alveolar macrophages with a cytotoxic and antimicrobial inflammatory response against foreign pathogens. Nitric oxide also influences the recruitment and activation of inflammatory cells (Aderem and Underhill, 1999a; Gordon, 2002; Gordon and Taylor, 2005; Song et al., 2000; Taylor et al., 2005).

In the presence of T_H2 cytokines, such as IL-4 and IL-13, alveolar macrophages become alternatively activated and exhibit a phenotype that results in an anti-inflammatory immune response (Gordon, 2003; Gordon and Martinez; Gratchev et al., 2001; Martinez et al., 2009; Muller et al., 2007; Stein et al., 1992). Alternatively activated macrophages express proteins that are involved in cellular proliferation, angiogenesis, wound repair, and other diverse pathologies. Once activated, these macrophages produce and secrete anti-inflammatory mediators, such as IL-

10 and TGF-β_1, to downregulate the inflammatory response of the alveolar macrophages. This process is initiated by increased expression and activity of arginase-1, an inhibitor of nitric oxide synthase-2, which metabolizes L-arginine to L-ornithine, L-proline, and polyamine. Alternatively activated macrophages also produce fibronectin, and in concert with TGF-β_1 promote tissue repair by activating fibroblast proliferation and collagen production (Gordon, 2002; Gordon, 2003; Gordon and Martinez; Gordon and Taylor, 2005; Martinez et al., 2009; Stein et al., 1992; Taylor et al., 2005). Prolonged increases in fibronectin and TGF-β_1 expression, a known inducer of tissue remodeling (Isono et al., 2002; Leask and Abraham, 2004; Ruiz-Ortega et al., 2007; Wang et al., 2006), can eventually result in lung fibrosis.

Numerous studies have shown that IL-13 promotes fibroblast proliferation and collagen synthesis. In studies by Kolodsick et. al, IL-13$^{-/-}$ mice, but not IL-4$^{-/-}$ mice, were protected from pulmonary fibrosis through decreased collagen production by fibroblast. Lee et al demonstrated that IL-13 promotes TGF-β_1 activation in a rodent model of pulmonary fibrosis (Lee et al., 2001). In studies by Fichtner-Feigl et. al, IL-13 induced TGF-β_1 expression within macrophages via signaling through the IL-13α_2 receptor (Kolodsick et al., 2004). In a rodent oxazolone-induced colitis model, blocking IL-13 signaling by inhibiting IL-13α_2 receptor expression decreased TGF-β_1 expression, fibrosis, and collagen production (Fichtner-Feigl et al., 2006). However, the role of TGF-β_1 in alternative activation of alveolar macrophages has yet to be thoroughly investigated. In the current study, we used the alveolar macrophage cell line NR8383 cells, primary alveolar macrophages, and an *in vivo* model of IL-13-induced lung inflammation to examine the role of TGF-β_1-induced signaling in IL-13 induced alternative activation and alveolar macrophage function.

4.3. MATERIALS AND METHODS.

Reagents. Recombinant mouse IL-13 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). TGF-β_1 and the TGF-β receptor kinase inhibitor SB-431542 were purchased from Sigma

(Sigma Chemical; St. Louis, MO). pHrodo *Staphylococcus* aureus BioParticles conjugate was purchased from Invitrogen (Invitrogen; Carlsbad, CA) . Smad and p-smad antibodies, were purchased from Cell Signaling Technology (Cell Signaling Technology; Beverly, MA). Galectin-3, GAPDH, TGF-β_1, arginase-1, and galectin-3 primary antibodies were purchased from Santa Cruz (Santa Cruz Biotechnology; Santa Cruz. CA).

Cell Culture and Treatment.NR8383 cells, a rat alveolar macrophage cell line (ATCC, Rockville, USA), were maintained in FK-12 media supplemented with 15% heat-inactivated fetal bovine serum and1 % antibiotic-antimycotic solution (100 U/ml penicillin G sodium, 100 U/ml streptomycin, and 0.25 µg/ml amphotericin B) and incubated in a humidified 5% CO_2 incubator at 37°C. Cells were treated for 48 hrs with 20 ng/ml IL-13, with or without co-treatment with the TGF-β_1 signaling inhibitor SB-431542 (10 µM). Murine NIH/3T3 fibroblasts stably transfected with a fibronectin promoter upstream of a luciferase reporter gene (a generous gift from Dr. J. Roman, University of Kentucky) were maintained in a humidified 5% CO_2 incubator at 37°C and cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution.

Animal Model. Male Sprague-Dawley rats (225-275 g; Harlan, St. Louis, MO) were used in accordance with NIH guidelines (*Guide for the Care and Use of Laboratory Animals*) as described in protocols reviewed and approved by the Emory University Institutional Animal Care Committee. Animals were anesthetized by giving an intramuscular injection of a ketamine (30mg/kg) and xylazine (2mg/kg) mixture. The neck and the entire dorsal side of the body were cleaned with 70% ethanol and the trachea exposed via blunt dissection. In some animals, IL-13 (20 mg; 100 µl of saline) was injected into the trachea using a 26G 1/4 needle on two consecutive days. Controls received saline alone. Other animals also received an intra-peritoneal injection of SB-431542 (10 mg/kg; 100 µl of DMSO) or DMSO alone before IL-13 instillation.

Alveolar Macrophage Isolation and Culture. After pentobarbital anesthesia, the trachea was cannulated and the rat lung underwent 5 bronchoalveolar lavages consisting of 10 ml of sterile phosphate-buffered saline (PBS) (37°C, pH 7.4). The lavage fluid was centrifuged at 500 g for 8 min, and the cell pellet was resuspended in DMEM medium supplemented with 2% fetal bovine serum plus penicillin and streptomycin (100 U/l each) or preserved in RIPA buffer. Cell count and viability were determined using the Countess cell counter (Invitrogen; Carlsbad, CA). The cell-free bronchoalveolar lavage fluid was saved for further analysis. Rat primary alveolar macrophages were plated at 1 million cells/ml and allowed to adhere for 1 hr at 37°C and 5% CO_2.

RT-PCR. Total RNA was extracted by TRIzol Reagent and assayed by real time Q-PCR. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen; Carlsbad, CA) and treated with DNA-free reagent according to the manufacturers' protocols. Real-time quantitative PCR of mRNA templates was performed using the iScript SYBR Green One-Step RT-PCR Kit (Bio-Rad) and the ABI PRISM 7900 HT sequence detection system (Applied Biosystems; Foster City, CA). PCR amplification of RNA (100 ng) was performed under the following conditions: cDNA synthesis at 50°C for 10 min, iScript reverse transcriptase inactivation at 95°C for 5 min, and PCR cycling and detection for 40 cycles at 95°C for 10 s followed by 60°C for 30 s. Values are expressed as relative mRNA expression that has been normalized to housekeeping GAPDH mRNA. The following primers were used: TGF-β_1 5'-GGACTACTACGCCAAAGAAG-3 and 5'-CAAAAGACAGCCACTCAGG-3; GAPDH 5'-TGAGAATGGGAAGCTG-3' and 5'-TTGGGGGTAGGAACAC-3'; arginase 1 5-'AAAGCCCATAGAGATTATCGGAGCG-3' and 5'-AGACAAGGTCAACGGCACTGCC-3'; IL-13: 5'-AGTCTTCAGTTTAAGCCAGCTTAC-3' and 5'-TTTTCAATGGAAGGTACCACAGCGG-3; Galectin-3 5'-AATGGCAGACGGCTTCTCACTT-3' and 5'-TAACACACAGGGCAGTTCTGGT-3'.

Western Blot Analysis. NR8383 cell lysates were prepared by adding RIPA buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Lysates were separated by centrifugation (10000 g) and the protein concentration quantified. Fifty micrograms of total protein from each sample were electrophoresed on 4–15% gradient polyacrylamide gels at 200 V for 45 min and then transferred to nitrocellulose membranes (Bio Rad Laboratories; Richmond, CA) at 100 V for 1.5 hr. Membranes were blocked at room temperature for 2 hrs in tris buffered saline (TBS) with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk. Membranes were then probed with primary antibodies to smad (1:1000), p-smad (1:1000), arginase 1 (1:100), galectin-3 (1:500), or GAPDH (1:500) diluted in 5% milk in TBS-T and kept at 4^{o}C overnight. After 3 washes with TBS-T for 10 min, membranes were incubated at room temperature with secondary antibodies coupled to horseradish peroxidase (1:500) diluted in TBS-T containing 5% nonfat dry milk (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1hr. After adding ECL chemiluminescence reagent (Denville Scientific; Metuchen, NJ) to the membranes, bands were detected and quantified via densitometry.

Measurement of TGF-β_1 and IL-13 by ELISA. The TGF-β_1 and IL-13 present in the cell lysates or culture supernatants were quantitated by the commercially available ELISA kits TGF-β_1 Emax immunoassay system (Promega; Madison, WI) and the Quantikine Rat IL-13 Immunoassay for IL-13 (Invitrogen; Carlsbad, CA), respectively. To measure production and secretion of IL-13 and TGF-β_1, cell lyastes and culture media were loaded in a 1:10 dilution and a 1:2 dilution, respectively, into the 96 well plates. All values were normalized to the corresponding protein concentration.

Capacity to Activate Fibroblasts. To determine if alternatively activated alveolar macrophages promoted fibroblast activation, NIH/3T3 fibroblasts stably transfected with a fibronectin promoter

upstream of a luciferase reporter gene were used. These fibroblasts were seeded at a density of 10^6 cells/ml and allowed to adhere for 24 hrs. They were then incubated for 24 hr with 300 µl of cell-free supernatants from the treated NR8383 cells. At the end of the incubation, the cells were detached by scraping, washed with PBS lysed with 100 µl of reporter lysis buffer (Promega; Madison, WI), and then assayed for fibronectin transcription via luciferase activity using the Promega luciferase assay system (Promega; Madison, WI). Luciferase activity was normalized to total cellular protein.

Phagocytosis of pHrodo-labeled S. Aureus. NR8383 cells were plated onto 8 well chamber slides at a density of 10^6 cell/ml and allowed to adhere for 1 hr. After washing twice with sterile PBS, the cells were incubated with pHrodo-labeled *S. aureus* for 2 hours at 1 mg/mL . Briefly, the rhodamine-based pHrodo dye is pH-sensitive such that it is non-fluorescent at neutral pH and fluoresces red in acidic environments such as the phagolysosome. Thus, cells with red fluorescence indicated internalization, sequestration, and a respiratory burst within the phagolysosome. After incubation, the media was removed and the cells were washed twice with PBS before fixation with 4% paraformaldehyde. Phagocytosis of pHrodo labeled *S. aureus* was determined via quantitative digital analysis of microscopic images using FluoView (Olympus; Melville, NY). Values are presented as mean relative fluorescent units (RFU)/cell ± SEM, the mean percentage of cells fluorescently positive (per field) ± SEM, and phagocytic index ± SEM (RFU x % positive) as tallied from at least 10 experimental fields/set.

Immunofluorescence Characterization. NR8383 cells were isolated and cultured as described above. After the culture period, the cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.3% Triton X-100 and non-specific binding was blocked with 3% BSA. Before incubating with the primary antibodies, cells were preincubated with Fc block® (Pharmingen; San Diego, CA) for 15 minutes at room temperature. Cells were then stained with the appropriate primary

antibodies at 1/100 dilutions for 2 hrs at room temperature: The slides were washed three times with PBS and incubated with the Alexa-Flour 555 conjugated donkey anti-rabbit IgG (Invitrogen; Carlsbad, CA) for 1 hr at 1/200 dilution in 3% BSA. Slides were then counter stained with 4',6-diamino-2-phenylindole (DAPI) (Invitrogen) for 5 minutes to facilitate nuclear localization. Immunofluorescence was determined via quantitative digital analysis using FluoView (Olympus; Melville, NY). Background fluorescence was determined and subtracted from the mean relative fluorescent units. Values are presented as the mean relative fluorescent units per cell (± SE) as tallied from at least 10 experimental fields per set.

Statistical Analysis. Statistical analysis was performed with Sigma Stat for Windows. The data is presented as means ± SE. Results were analyzed using the students t-test or one-way analysis of variance where appropriate, followed by Student-Newman-Keuls test comparisons. A *p*-value of < 0.05 was considered significant.

4.4. RESULTS.

IL-13-Induced Alternative Activation Is Dependent On TGF-B_1 Signaling In NR8383 Cells. In order to determine the effects of IL-13 on alveolar macrophage TGF-β_1 expression, the rat NR8383 alveolar macrophage cell line was treated with 20 ng/ml of IL-13 for 48 hrs. Similar to previous reports (Fichtner-Feigl et al., 2006; Lee et al., 2001), IL-13 treatment led to a 5-fold and 2-fold increase in alveolar macrophage TGF-β_1 gene and protein expression, respectively (^{a} **p < 0.05; Figure 4.1A-B**). IL-13 also increased TGF-β_1 secretion from NR8383 cells by 2-fold (^{a} **p < 0.05; Figure 4.1C**). IL-13 treatments also increased gene expression and protein expression of IL-13 itself by 6-fold and 4-fold, respectively, when compared to control cells (^{a} **p < 0.05; Figure 4.2A-B**). In the media, after subtracting out the amount of IL-13 added to the media, there was an additional 2000 pg/ml IL-13 remaining in the IL-13 treated group, suggesting that IL-13 was also secreted by the cell (**Figure 4.2C**). IL-13 treatment also

Figure 4.1 IL-Treatment Increases TGF-B_1 Expression. NR8383 cells were treated with IL-13 (20 ng/ml) along with TGF-β_1 signaling inhibitor SB-431542 (10 μM) or DMSO for 48 hours and TGF-β_1 gene expression (**A**), TGF-β_1 production (**B**) and secretion (**C**) were measured. [a] Denotes $p \leq 0.05$ vs untreated control and [b] denotes $p \leq 0.05$ vs IL-13.n=4 or more per group.

Figure 4.1 A

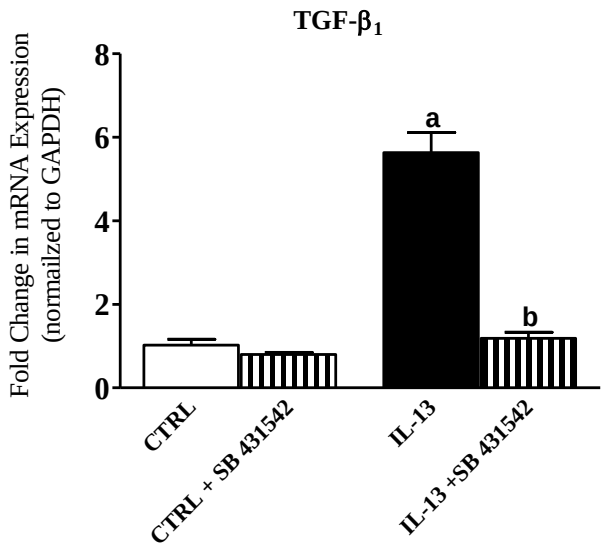


Figure 4.1 B

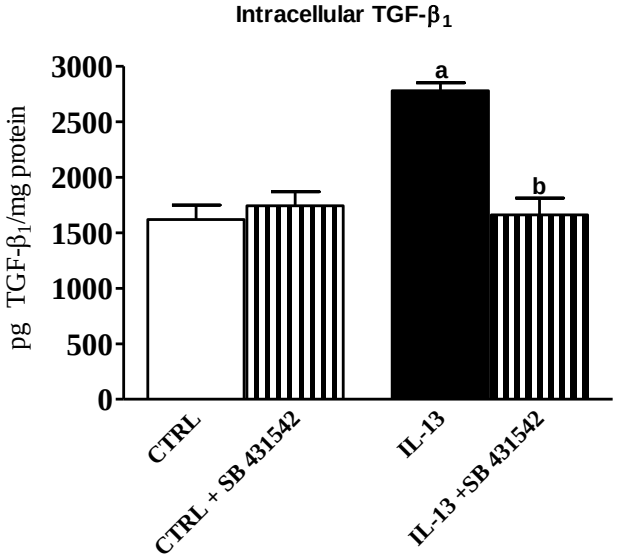


Figure 4.1 C

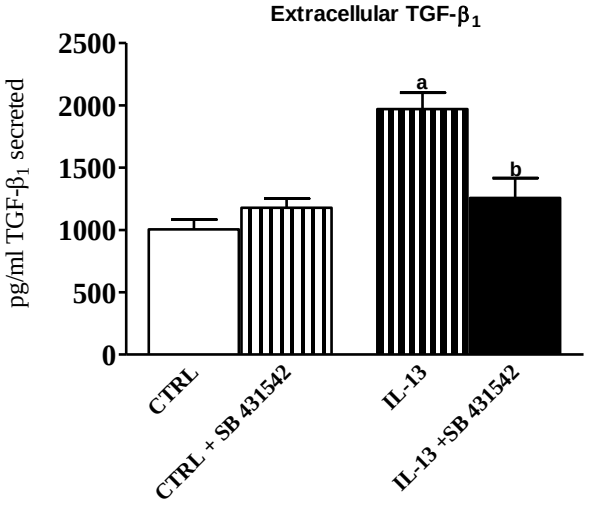


Figure 4.2. Blocking TGF-B_1 Signaling Inhibits The IL -13 Autocrine Loop . The effects of inhibiting TGF-β_1 signaling on the IL-13 autocrine loop were examined in NR8383 cells treated with IL-13 for 48 hrs. Quantification of mRNA expression of IL-13 gene expression (**A**), protein expression (**B**) and secretion (**C**) were determined. [a] Denotes $p \leq 0.05$ vs untreated control and [b] denotes $p \leq 0.05$ vs IL-13. 4 or more per group.

Figure 4.2 A

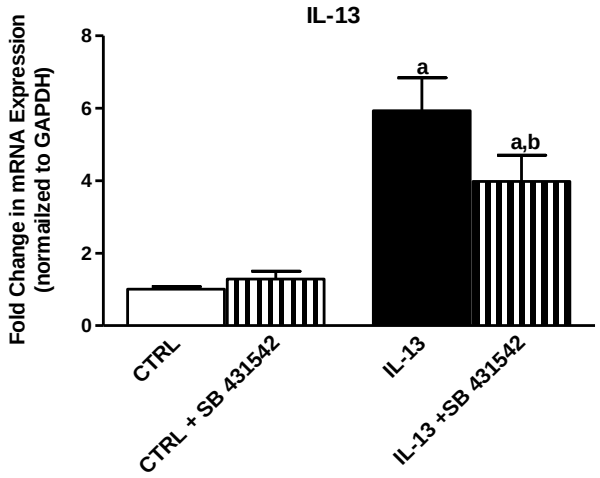


Figure 4.2 B

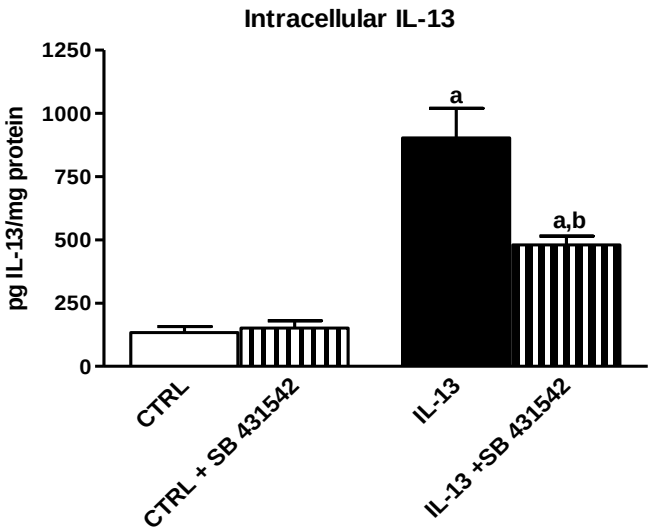
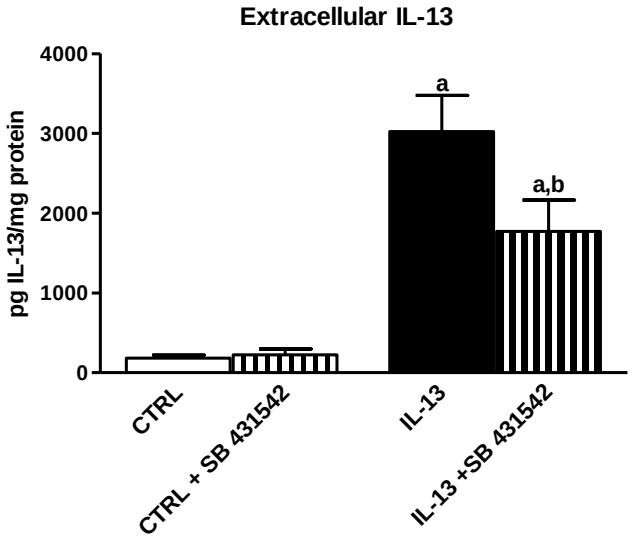


Figure 4.2 C



increased gene expression of markers associated with alternative activation including arginase-1 and galectin-3 by 6-fold and 4-fold, respectively, when compared to controls (^{a} **p < 0.05; Figure 4.3A-B**). Protein expression of arginase-1 and galectin-3 were increased 4-fold and 75%, respectively (^{a} **p < 0.05; Figure 4.3C-D**).

Given that IL-13 is associated with upregulation of TGF-β_1 expression and secretion, we examined the role of TGF-β_1 signaling in the IL-13-induced alternative activation. When the phosphorylation of smad 2/3 was used as a marker of TGF-β_1 signaling, IL-13 induced an 60% increase (**Figure 4.3E**). We next blocked TGF-β_1 signaling with SB-431542, a potent and selective inhibitor of TGF-β_1 activin receptor-like kinase (ALK) receptors. Inhibition of the TGF-β_1 receptor blocked TGF-β_1 signaling as evidenced by ablation of smad 2/3 phosphorylation (**Figure 4.3E**). Co-treatment with SB-431542 was also associated with suppression of IL-13 expression and secretion (**Figure 4.2**) as well as TGF-β_1 expression and secretion (**Figure 4.1**). SB-431542 also prevented IL-13 induced upregulation of gene and protein expression of markers of alternative activation arginase-1 and galection-3, but did not alter gene expression in control cells (**Figure 4.3A-D**).

IL-13 Inhibited NR8383 Phagocytosis. Using the pHrodo red labeled S. aureus as an indicator of phagocytosis and a respiratory burst, IL-13 treatments decreased the percentage of cells positive for pHrodo fluorescence by 14% but decreased the RFU/cell by 70%, when compared to controls (^{a}**p < 0.05; Figure 4.4 A-B**). Similarly, the phagocytic index, the percentage of cells positive for internalization multiplied by the RFUs/cell was significantly attenuated by the IL-13 (^{a}**p < 0.05; Figure 4.4 C,D**). However, SB-431542 treatment blocked the IL-13-induced attenuation of phagocytosis and returned the phagocytic index to control values (^{b}**p < 0.05; Figure 4.4 A-D**).

Figure 4.3 IL-13 Mediated Alternative Activation Is Dependent On TGF-B_1 Signaling. mRNA expression of (**A**) arginase-1 and (**B**) galectin-3 in NR8383 cells was quantitated after 48 hours of IL-13 treatment ± supplementation with SB-431542. The results are given as the ratios of expression of the gene of interest to GAPDH mRNA. Protein expression of arginase-1 (**C**), galectin-3 (**D**), and smad phosphorylation (**E**) were determined via western blot analysis. Representative immunoblots are shown in f. [a] Denotes $p \leq 0.05$ vs untreated control and [b] denotes $p \leq 0.05$ vs IL-13. n=4 or more per group.

Figure 4.3A

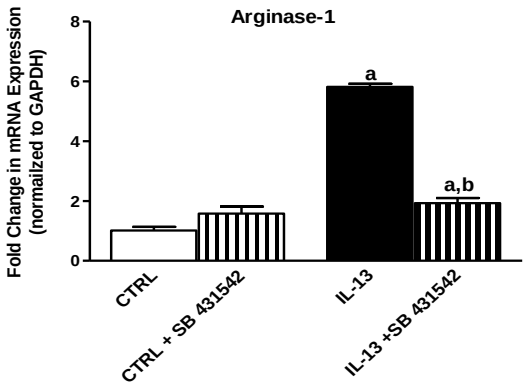


Figure 4.3B

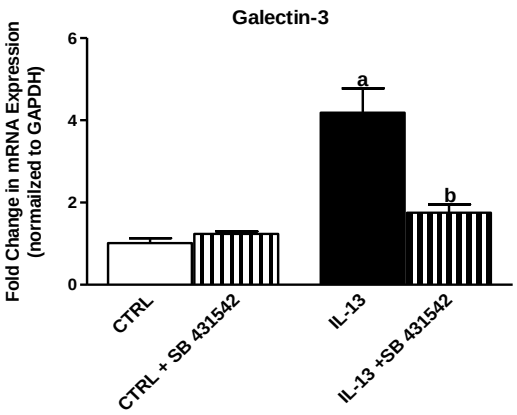


Figure 4.C

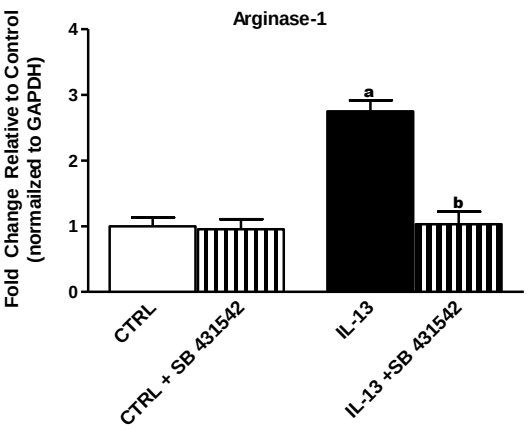


Figure 4.3D

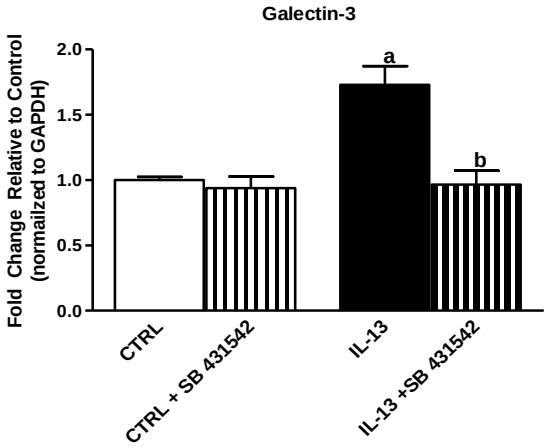


Figure 4.3E

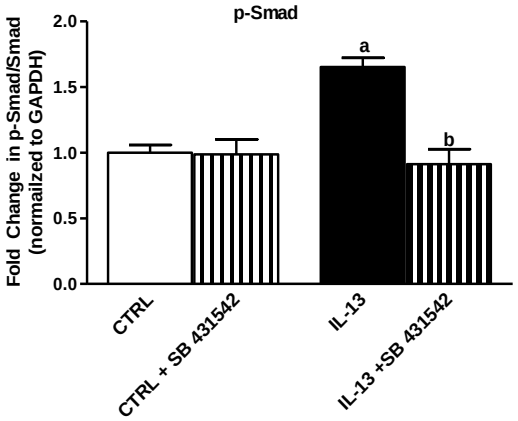


Figure 4.3F

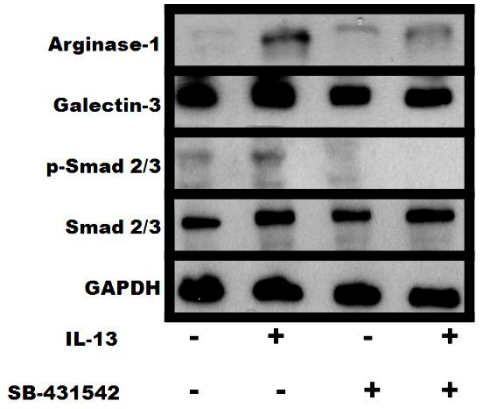


Figure 4.4 IL-13 Attenuation Of NR8383 Phagocytosis Is Through TGF-B_1 Signaling. NR8383 cells were incubated with pHrodo labeled *S. aureus* for 2 hr. Internalization was evaluated using quantitative digital analysis of fluorescence on confocal microscopic images. Representative photomicrographs at 20X magnification are shown in **Figure D**. Bar heights represent the % positive for fluorescence (**A**), the mean relative fluorescent units (RFU/cell) (**B**), and the phagocytic index (**C**). Bars in Figures A-C represent means ± SE. [a] Denotes $p \leq 0.05$ compared to controls, [b] $p \leq 0.05$ compared to IL-13. n=15 or more fields per group.

Figure 4.4A

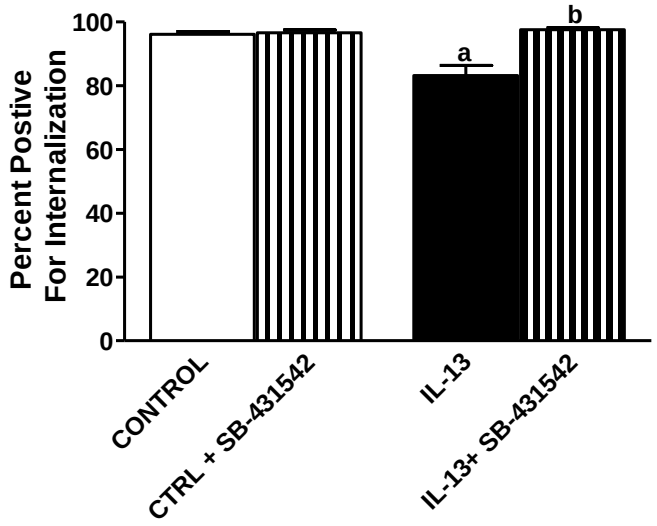


Figure 4.4B

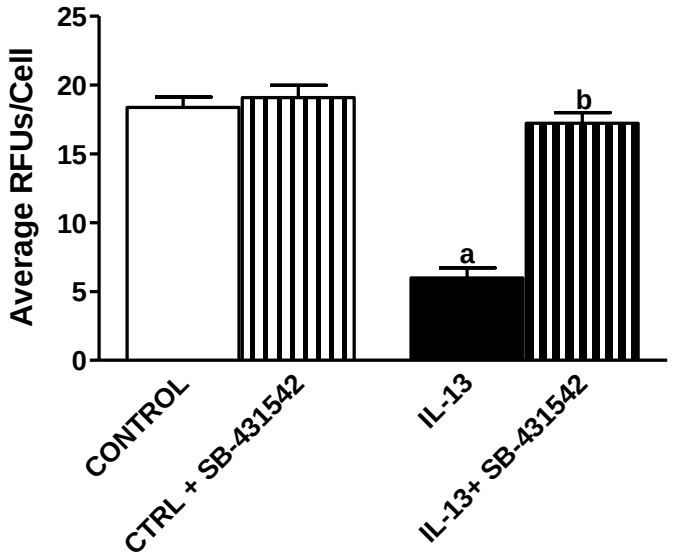


Figure 4.4C

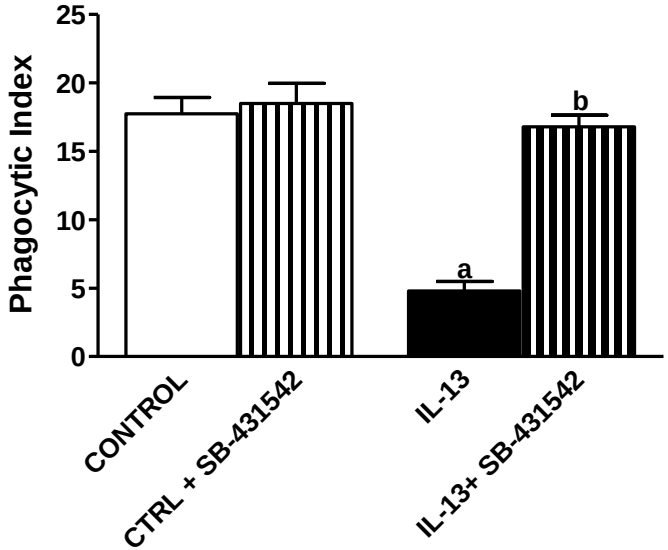
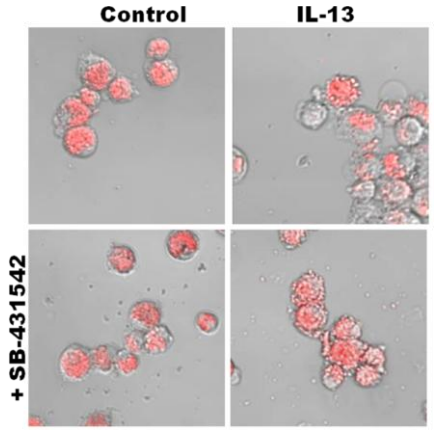


Figure 4.4D



IL-13 Treatment Of Macrophages Promoted A Pro-Fibrotic Phenotype. As discussed above, IL-13-induced alternative activation is associated with a macrophage phenotype that promotes fibroblast activation. To further examine the role of alveolar macrophagesin fibroblast activation, cell-free supernatants from IL-13-treated NR8383 cells were overlaid onto the 3T3 fibroblasts stably transfected with the human fibronectin promoter labeled with a luciferase reporter vector. Similar to that observed by Song et al (Song et al., 2000), the media from IL-13 treated NR8383 cells increased fibroblast proliferation, as seen by a 3-fold increase in fibronectin gene transcription, when compared to control **(^{a} $p < 0.05$; Figure 4.5)**. When TGF-β_1 signaling was blocked in NR8383 cells, IL-13 treatment of the NR8383 cells was unable to promote a macrophage phenotype that activated the fibroblasts **(^{b} $p < 0.05$; Figure 4.5)**.

IL-13 Induced Alternative Activation Via TGF-B$_1$ Signaling *In Vivo*. We next examined this relationship *in vivo* through intratracheal delivery of IL-13 for 48 hrs. Alveolar macrophages isolated from IL-13 treated animals displayed a robust increase in gene expression of arginase-1, galectin-3, TGF-β_1, and IL-13 when compared to controls **(^{a} $p < 0.05$; Figures 4.6A and 4.7)**. IL-13 also led to increased protein expression of TGF-β_1 and localization to the plasma membrane (**Figure 4.6B**). When TGF-β_1 signaling was inhibited with SB-431542 immediately before intratracheal IL-13 instillation, the increased expression of arginase-1 and galectin-3 gene were completely ablated (^{b}**$p < 0.05$; Figure 4.7 A and B**) (**Figure 4.7D**). Correspondingly, the IL-13 mediated increases in TGF-β_1 and IL-13 expression were also attenuated (^{b}**$p < 0.05$; Figure 4.6A and 4.7C**). As observed *in vitro*, IL-13 instillation resulted in a significant decrease in rat alveolar macrophage function as determined by the percentage of macrophages positive for internalization, average RFUs/cell, and phagocytic index (^{a}**$p < 0.05$; Figure 4.8**). Blockage of TGF-β_1 signaling normalized alveolar macrophage phagocytic function despite IL-13 treatment

Figure 4.5 TGF-β_1 Secretion By The Alternatively Activated Alveolar Macrophage Promoted Fibroblast Activation. Transfected 3T3 cells were treated with the cell-free supernatants from all NR8383 cell treatment groups for 24 hrs. The 3T3 cells were then washed and lysed before fibronectin gene transcription was measured by luminescence. Data are presented as average luciferase activity ± SD. [a]Denotes $p \leq 0.05$ vs untreated control and [b] denotes $p \leq 0.05$ vs IL-13. n=4 or more per group.

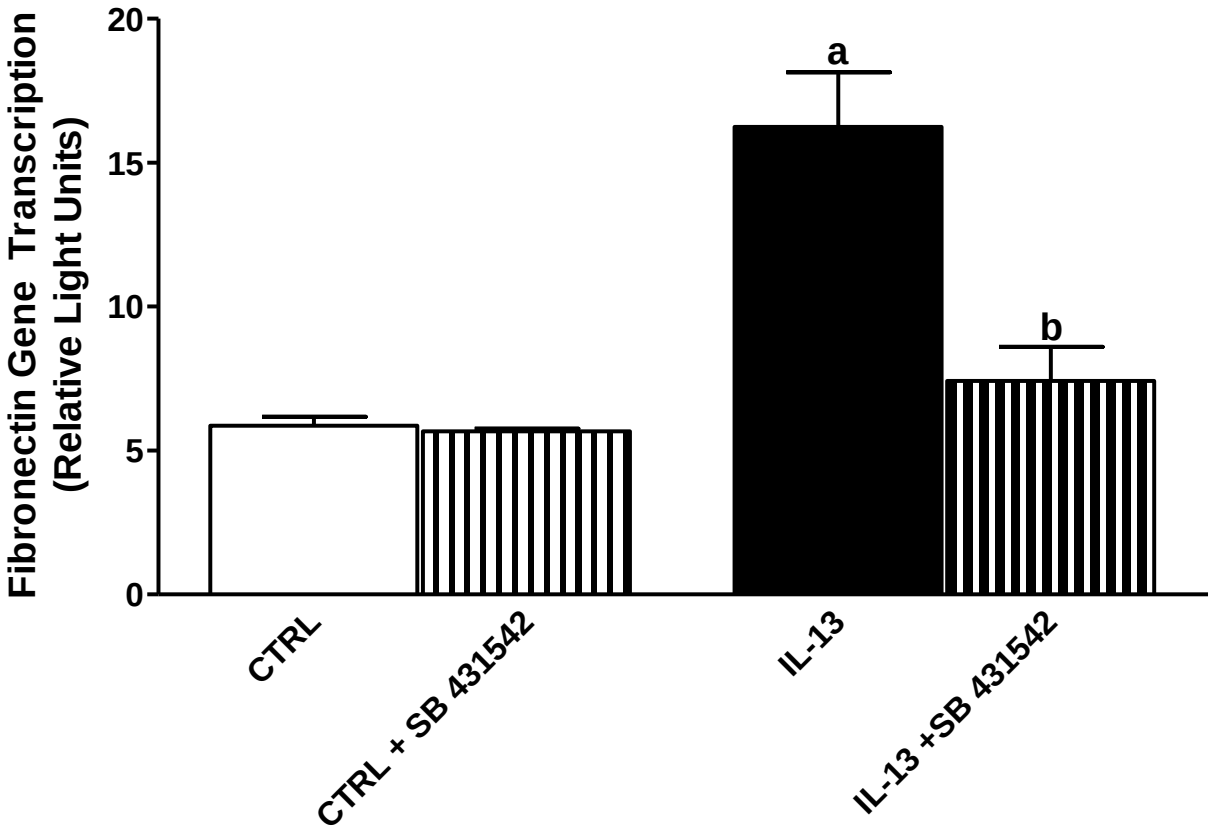

Fibronectin Gene Transcription
(Relative Light Units)
20
15
10
5
0
a
b
CTRL
CTRL + SB 431542
IL-13
IL-13 +SB 431542

Figure 4.6 Intratracheal IL-13 Induces TGF-B_1 Expression in Alveolar Macrophages. Quantification of mRNA expression of (**A**) TGF-β_1 in rat alveolar macrophage following intratracheal instillation of IL-13 or PBS along with an i.p. injection of the TGF-β_1 signaling inhibitor SB-431542 (10 μM) or DMSO. The results are given as the ratio of expression to GAPDH mRNA. Immunohistochemistry was used to determine localization of (**B**) TGF-β_1 (original magnification: 40×). a= $p \leq 0.05$ vs untreated control, b =$p \leq 0.05$ vs IL-13. n=3 or more per group.

Figure 4.6A

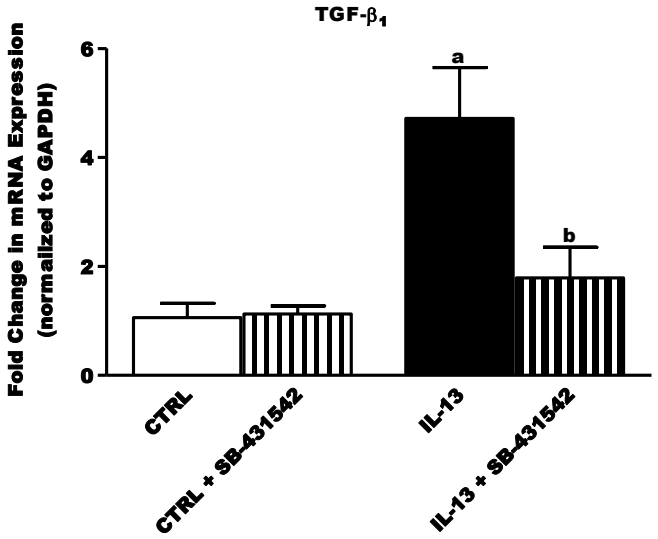


Figure 4.6B

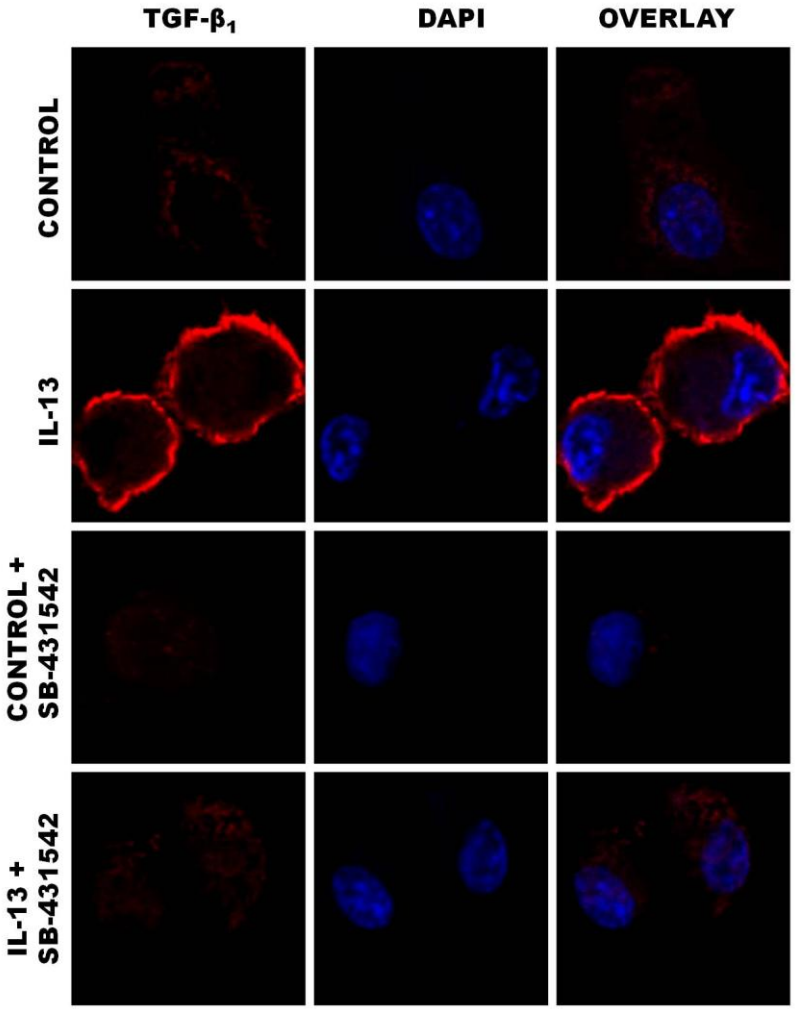


Figure 4.7 Intratracheal IL-13 Induced Alternative Activation Is Dependent On TGF-B_1. Quantification of mRNA expression of (**A**) arginase-1 (**B**) galectin-3, and (**C**) IL-13 in rat alveolar macrophage following intratracheal instillation of IL-13 or PBS along with an i.p. injection of the TGF-β_1 signaling inhibitor SB-431542 (10 μM) or DMSO. The results are given as the ratios of gene of interest expression to GAPDH mRNA. Immunohistochemistry was used to determine localization of (**D**) arginase-1 (original magnification: 40×). [a] Denotes $p \leq 0.05$ vs untreated control and [b] denotes $p \leq 0.05$ vs IL-13. n=4 or more per group.

Figure 4.7A

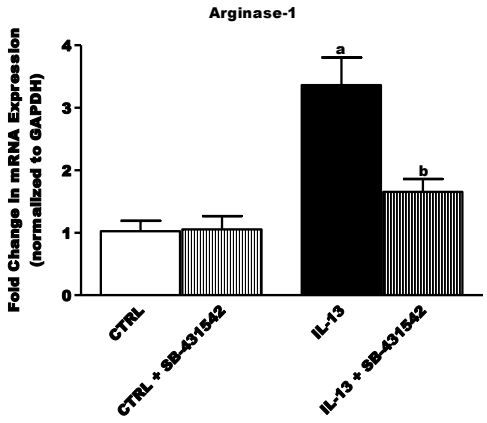


Figure 4.7B

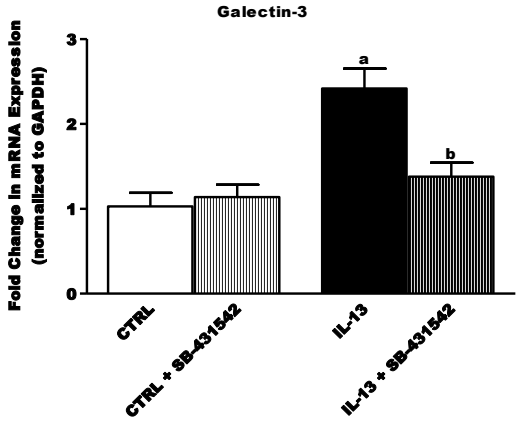


Figure 4.7C

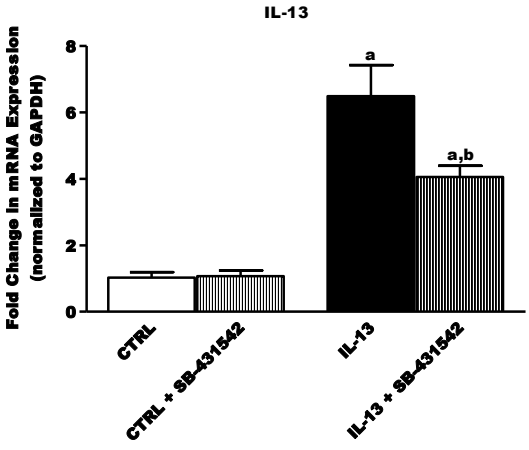


Figure 4.7D

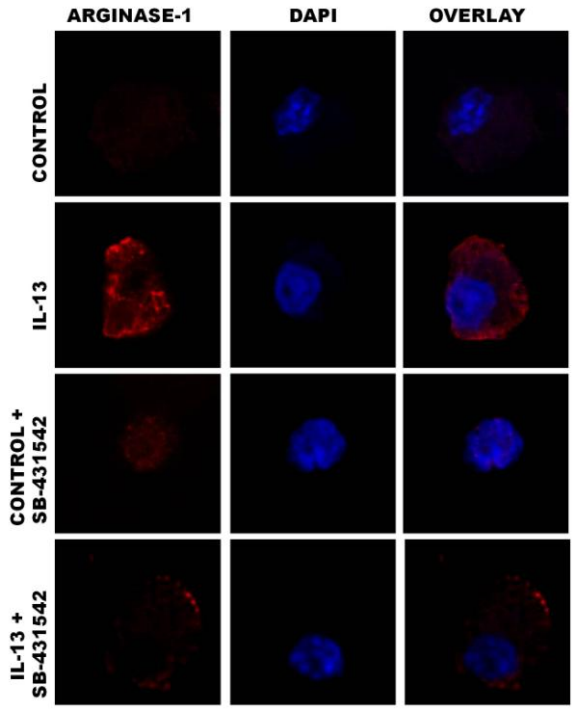


Figure 4.8 Intratracheal IL-13 Inhibited Alveolar Macrophage Phagocytic Function. Following isolation from the BAL fluid, rat primary alveolar macrophages were incubated with pHrodo labeled *S. aureus* for 2 hr. Internalization was evaluated using quantitative digital analysis of fluorescence in confocal microscopic images. Representative photomicrographs at 20X magnification are shown in **Figure 4.8D.** Bar heights represent the percent positive (**A**), the mean relative fluorescent units (RFU/cell) (**B**), and the Phagocytic Index (**C***)*. Bars in A-C represent means ± SE. [a] Denotes $p \leq 0.05$ compared to controls, [b] $p \leq 0.05$ compared to IL-13. n=15 or more fields per group.

Figure 4.8A

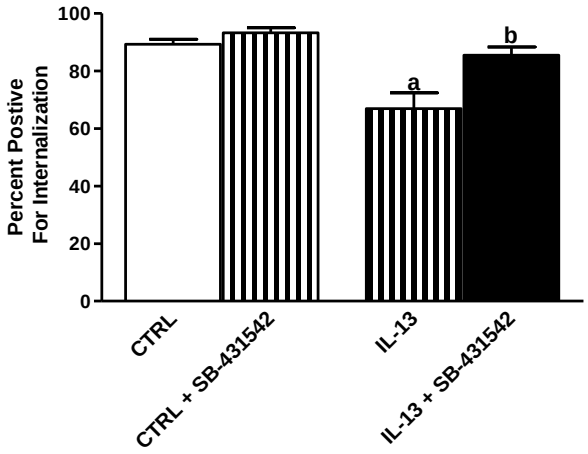


Figure 4.8B

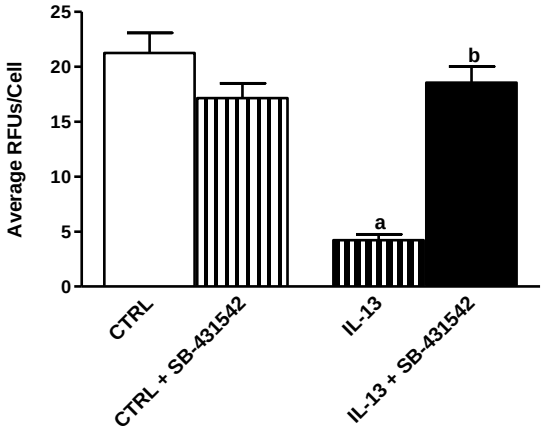


Figure 4.8C

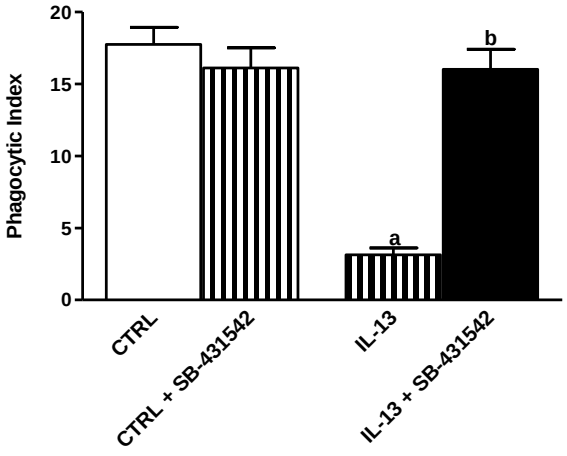
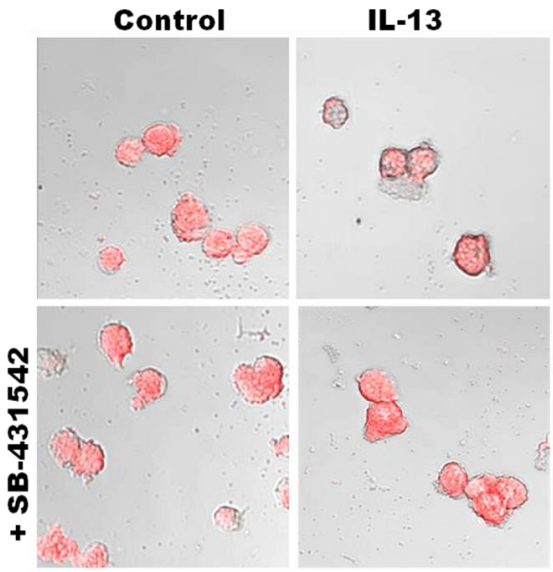


Figure 4.8D



and suggested TGF-β_1 played an integral role in IL-13 mediated alveolar macrophage dysfunction.

4.5. DISCUSSION.

Exposure to T_H2 cytokines such as IL-4 and IL-13 promotes alternative activation of macrophages as indicated by upregulation of proteins such as arginase-1, galectin-3, TGF-β_1 and mannose receptor (Gordon, 2003; Gordon and Martinez; Martinez et al., 2009; Stein et al., 1992). In previous studies with peritoneal macrophages, IL-13 stimulated galectin-3 expression and release whereas inhibition of galectin-3 blocked IL-13 and IL-4 induced alternative activation, suggesting a pivotal role for galectin-3 in alternative activation. The underlying mechanisms by which IL-13 induces alternative activation are unclear. In studies by Fichther-Feiglet et al. (Fichtner-Feigl et al., 2006), IL-13 induced TGF-β_1 promoter activity via increases in IL-13 Rα_2 expression in THP-1 cells, a monocytic cell line. However, IL-13 was insufficient to increase TGF-β_1 protein concentrations in the THP-1 cells. In contrast, Boutard et al. demonstrated that TGF-β_1 stimulates arginase activity in peritoneal macrophages (Boutard et al., 1995a) suggesting a role for TGF-β_1 in alternative activation.

In the current study, IL-13 induced alternative activation of the alveolar macrophage cell line NR8383 cells as evidenced by increased gene and protein expression of arginase-1 and galectin-3. IL-13 treatment also induced an autocrine loop to amplify IL-13 production and secretion by alveolar macrophages. IL-13-induced alternative activation of alveolar macrophages was further verified *in vivo* where intratracheal delivery of IL-13 induced arginase 1 and galectin-3 expression in alveolar macrophages. This was concomitant with increased expression of IL-13 in the alveolar macrophages, again, supporting an autocrine loop.

IL-13 treatments also upregulated gene and protein expression of TGF-β_1 in the NR8383 cells as well as increased TGF-β_1 secretion. This IL-13-induced TGF-β_1 secretion was also associated with increased smad 2/3 phosphorylation suggesting that IL-13 initiated TGF-β_1

signaling. A role for IL-13-induced TGF-β_1 signaling was further supported by the ability of the ALK 5 inhibitor SB-431542 to attenuate IL-13-induced smad2/3 phosphorylation. Furthermore, treatment with SB-431542 blocked IL-13 induced alternative activation and the IL-13 autocrine loop. When animals were treated with SB-341542 before the intratracheal IL-13 instillation, induction of IL-13, TGF-β_1, arginase 1 and galectin-3 were all attenuated. These results further suggested that that IL-13-induced alternative activation was secondary to upregulation of TGF-β_1 expression, secretion and signaling.

Induction of alternative activation has been implicated in diverse pathologies such as asthma, wound repair, and parasitic infections (Daley et al., 2009; MacKinnon et al., 2008; Mountford et al., 2001; Zhu et al., 2004). In addition, increased T_H2 cytokine expression has been associated with an increased susceptibility to infection via downregulation of the alveolar macrophage immune response. In studies done by Harris et. al, IL-13 inhibited autophagy-induced phagosome maturation (Harris et al., 2007). However, the direct effects of IL-13 on macrophage phagocytic function have yet to be determined. In our *in vitro* and *in vivo* models, IL-13 treatments significantly decreased the alveolar macrophage phagocytic capacity, as evidenced by a drastic decrease in the percentage of cells positive for *S. aureus* internalization, the amount internalized, and the phagocytic index. This suggested that the increased susceptibility to infection seen in response to T_H2 cytokines may be due to direct inhibition of alveolar macrophage phagocytosis through alternative activation. Our data also suggested that this IL-13-induced downregulation of phagocytosis was secondary to its upregulation of TGF-β_1.

When the fibroblasts were treated with media from the alternatively activated macrophages, the fibroblasts became activated as evidenced by activation of the fibronectin promoter. When the NR8383 cells were concomitantly treated with IL-13 and SB-431542, there was no fibroblast activation. This could have been due to the ablation of TGF-β_1 secretion by the NR8383 cells as suggested by other studies demonstrating that secretion of TGF-β_1 by alternatively activated macrophages induces fibroblasts to secrete factors that promote

extracellular matrix formation (Leask and Abraham, 2004; Mitaka et al.; Ruiz-Ortega et al., 2007; Zhao et al., 2002). However, we cannot rule out the possibility that the alternatively activated macrophage also secreted other mediators of fibroblast activation. For example, alternatively activated macrophages also contribute to fibroblast proliferation and collagen production via arginase-1 metabolism of L-arginine to l-ornithine, which is further metabolized to l-proline, and polyamines. Production of proline promotes extracellular matrix formation while polyamines induce cellular proliferation (Belperio et al., 2002; Endo et al., 2003; Gordon, 2003; Grasemann et al., 2005; Hesse et al., 2001a; Lee et al., 2001; Muller et al., 2007). While studies have demonstrated that IL-13 induced TGF-β_1 secretion led to increased fibroblast proliferation, this is the first study to suggest that TGF-β_1 is critical for alternative activation in the alveolar macrophage and secretion of fibrogenic factors that promote fibroblast activation and proliferation.

In conclusion, the present study demonstrated in both *in vitro* and *in vivo* models that IL-13 induced alternative activation of alveolar macrophages which resulted in downregulation of macrophage phagocytosis. Treatment with IL-13 was also associated with activation of an IL-13 autocrine loop resulting in increased IL-13 synthesis and secretion. Alternative activation was also associated with secretion of TGF-β_1 which promoted fibroblast activation. In both *in vitro* and *in vivo* studies, inhibition of TGF-β_1 signaling blocked IL-13 mediated alterations in the alveolar macrophage activation and immune responses by preventing alternative activation. This suggested that increased TGF-β_1 expression and signaling were central to IL-13 induced alternative activation, impaired phagocytosis, and the capacity of alveolar macrophages to contribute to fibroblast proliferation and pulmonary fibrosis.

CHAPTER 5

CONCLUSIONS, POSSIBLE MECHANISMS, AND STUDY LIMITATIONS

5.1. CONCLUSIONS.

Numerous studies demonstrate that patients with alcohol use disorders have an increased risk of respiratory infections, which is partially due to the effects of alcohol on alveolar macrophage immune cell function. The mechanisms by which alcohol impairs alveolar macrophage function are poorly understood (Boé et al., 2009; Esper A., 2006; Kane and Galanes, 2004; Moss et al., 1996; Moss and Burnham, 2003). However, previous studies suggest the majority of the negative effects of alcohol on alveolar macrophage dysfunctions are due to decreased GSH availability and subsequent oxidant stress within the alveolar space (Brown et al., 2004; Brown et al., 2007). In the current studies, we examined the effects of ethanol on pulmonary macrophage maturation, polarization, and function; and the role of oxidant stress in these ethanol-induced impairments. We also examined potential mechanisms by which alternative activation of alveolar macrophages occurs.

Our initial studies sought to determine the effects of chronic ethanol ingestion on terminal differentiation and function of pulmonary macrophages. To address this aim, we examined the following parameters: cell density, expression of maturational markers, and phagocytic function within defined subpopulations of alveolar macrophages derived from a rodent model of chronic ethanol ingestion. To examine the potential capacity of ethanol-induced macrophage dysfunction to extend to the interstitial space, expression of maturation markers in interstitial macrophages was also determined. As shown in Chapter 3, chronic ethanol ingestion leads to severely dampened alveolar macrophage maturation. This is seen by decreased cell density, decreased expression of maturational markers, and persistent monocytic characteristics. Interestingly, chronic ethanol ingestion also impaired the maturational status of interstitial macrophages. *In vivo* supplementation with the GSH precursor *S*-adenosyl-methionine during ethanol feeding prevented most of the detrimental effects of ethanol on both pulmonary macrophage populations, suggesting ethanol-induced oxidant stress played a central role in the impaired maturational status and dysfunction of pulmonary macrophages.

We next examined the effects of ethanol on alveolar macrophage activation and polarization. As discussed above, alveolar macrophages may be activated by T_H1 cytokines or T_H2 cytokines. Alternative-T_H2 cytokine driven- activation antagonizes classical-T_H1 mediated-activation, resulting in decreased alveolar macrophage phagocytosis, unregulated generation of reactive oxygen species, and increased expression of a multitude of prototypical markers (Gordon, 2003). Cell culture and rodent models of alcohol abuse along with human alveolar macrophages were used to examine the potential link between chronic alcohol-induced alteration of alveolar macrophage function and alternative activation. In both cell culture and animal models, chronic ethanol treatment increased expression of IL-13, a known inducer of alternative activation (Muller et al., 2007; Munder et al., 1998). Expression of established markers of alternative activation were also increased and correlated with decreased expression of markers of classical activation. Ethanol also greatly increased ROS generation and release while severely blunting the phagocytic capacity, two phenotypical markers of alternative activation. Similarly, a history of alcohol abuse resulted in clinical alveolar macrophages exhibiting decreased phagocytic function and manifestations of alternative activation.

Previous studies from the Brown laboratory demonstrated that in rodent models of alcohol abuse, ethanol-induced phagocytic dysfunction could be reversed with *ex-vivo* GSH supplementation (Brown et al., 2007). Our initial studied also showed that *in-vivo* GSH treatments prevented ethanol-induced pulmonary macrophage dysfunction. Therefore, we examined the effects of *in-vivo* and *ex-vivo* GSH supplementation on ethanol-induced alternative activation. GSH supplementation prevented and/or reversed the effects of ethanol on alveolar macrophage activation, ROS production, and function.

Prolonged overexpression of TGF-β_1 is a key feature is numerous fibroproliferative diseases, suggesting it plays a leading role in driving the fibrogenic process (Pulichino et al., 2008). Previous studies indicate that chronic ethanol ingestion leads to overproduction of TGF-β_1 within alveolar macrophages. In preliminary studies, blocking TGF-

β_1 signaling in alveolar macrophages during chronic ethanol treatments prevented detriments to cellular function, altered redox status, and ethanol-induced elevations in arginase-1 activity (**Figure 5.1**). Similarly, alternatively activated macrophages have been shown to have robust increases in TGF-β_1 production and secretion (Gordon, 2003; Gordon and Martinez, 2010; Martinez et al., 2009). Excess TGF-β_1 production has been shown to downregulate the classical activation pathway and is associated with alternative activation. While Lee et al demonstrated that IL-13 promotes TGF-β_1 activation in a rodent model of pulmonary fibrosis, other studies have shown TGF-β_1 directly increases arginase-1 activity and polyamine release (Boutard et al., 1995b; Lee et al., 2001). However, a key role for TGF-β_1 in IL-13-induced alternative activation has not been demonstrated.

Since TGF-β_1 is a key regulator of ethanol induced alveolar dysfunction and alternative activation, we next investigated the role of TGF-β_1 in alternative activation of alveolar macrophages. To address this aim, cell culture models and an *in vivo* model of IL-13-induced lung inflammation were used to examine the role of TGF-β_1 signaling in IL-13 induced alternative activation and alveolar macrophage function. In response to IL-13 treatment, alveolar macrophages exhibited increased expression of arginase-1and galectin-3, two prototypical markers of alternative activation, increased TGF-β_1 production, secretion, and signaling, as well and severely diminished phagocytic capacity. Ablation of TGF-β_1 signaling during IL-13 treatment resulted in decreased expression of alternative activation markers and prevented IL-13-induced alveolar macrophage dysfunction. Further studies showed that IL-13 induced fibroblast proliferation was dependent upon TGF-β_1 signaling. These findings suggested that TGF-β_1 signaling was an integral part of IL-13 mediated alternative activation of alveolar macrophages and promotion of fibrogenesis.

Figure 5.1 Inhibition of TGF-β_1 Signaling Prevents Ethanol Induced Alveolar Macrophage Dysfunction. Previous studies indicate that chronic ethanol ingestion leads to overproduction of TGF-β_1 within alveolar macrophages. We sought to determine the role of TGF-β_1 in ethanol-induced alveolar macrophage dysfunction. To test this hypothesis, NR8383 cells, a rat-derived alveolar macrophage cell line, were treated with 0.08% ETOH ± TGF-β_1 neutralizing antibody (TGF-β_i) for 5 days. Following treatment, the cells were analyzed for (**A**) phagocytic function, (**B**) ROS production, (**C**) arginase activity, and the ability to induce fibroblast proliferation (**D**), via fibronectin production by 3T3 cells, a fibroblast cell line. n=4 or more per group. Bars in A-D represent means ± SE. [a] Denotes $p \leq 0.05$ compared to controls, [*]$p \leq 0.05$ compared to ETOH. A-B n=15 or more fields per group. C-D n = 4 or more per group.

Figure 5.1 A

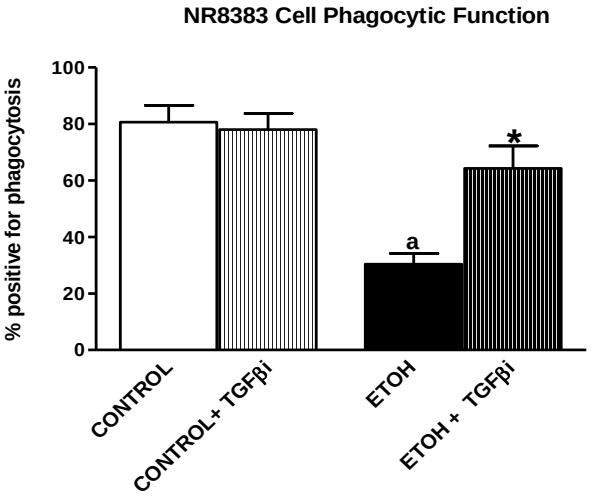


Figure 5.1 B

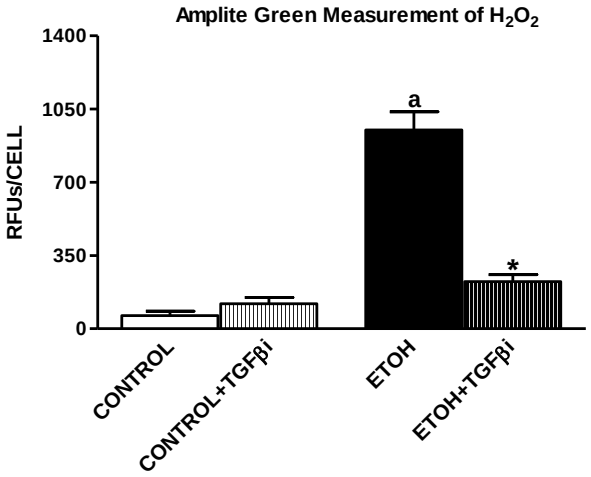


Figure 5.1 C

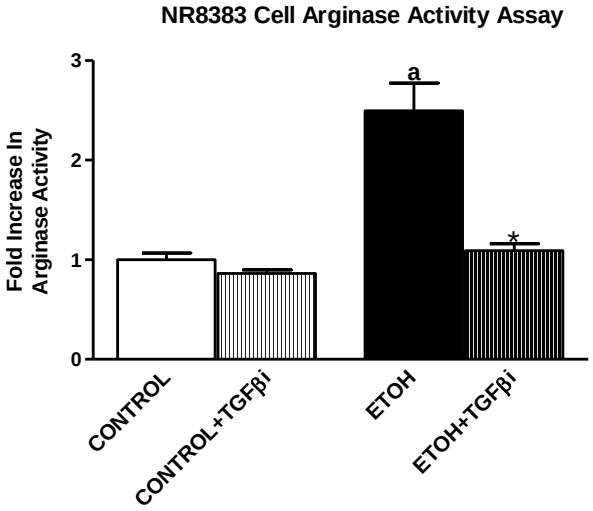
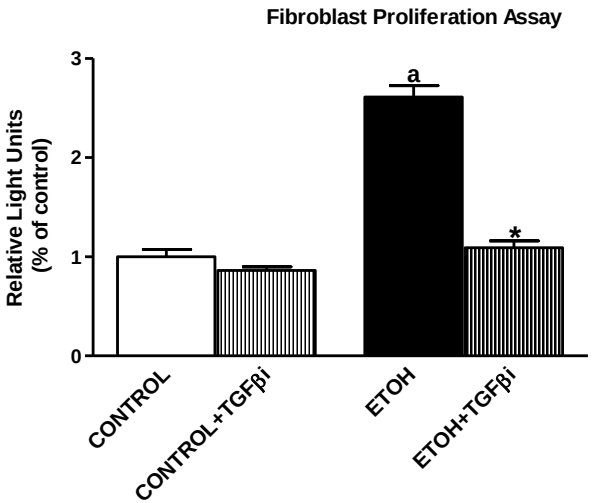


Figure 5.1 D



5.2 STUDY LIMITATIONS.

These studies demonstrated that chronic ethanol ingestion severely blunted alveolar macrophage maturation and function, and altered the alveolar macrophage activation pathway. Although numerous animal models of alcohol abuse exist to examine individual components of alcohol abuse, there are numerous limitations and they fail to mimic many aspects of the alcohol abuse phenotype in humans. This multifactoral disease has health and social consequences due to intoxication, dependence or addiction, and a myriad of other physiological effects. The volume and pattern of drinking especially binge drinking, and rate of alcohol metabolism affect the burden of alcohol abuse. Also, the susceptibility to alcohol abuse and/or tolerance depends on several factors including genetics, gender, ethnic background, drinking patterns, socioeconomic status, and nutrition (Hanson and Li, 2003; Jernigan et al., 2000; Room et al., 2005; Wallace Jr, 1999).

5.2.1. Genetics and Epidemiology of Alcohol Abuse.

Countless studies have shown that there is high individual and racial difference in ethanol metabolism. Genetic variations in ethanol metabolizing enzymes, especially alcohol dehydrogenases, can alter the rate of ethanol metabolism by 2 to 3 fold (Quertemont, 2004; Scarino et al., 2009). Polymorphisms in one or more of the multiple isoforms of alcohol dehydrogenase (ADH) can alter the rate at which acetaldehyde accumulates following alcohol consumption and this can alter the susceptibility to the acute and chronic effects of alcohol consumption as well as the development of alcohol use disorders (Quertemont, 2004). For example, genetic variants of ADH1B and ADH1C, commonly seen in Asian populations, leads to a faster rate of ethanol metabolism and buildup of acetaldehyde. The rapid accumulation of acetaldehyde leads to the “flush” feeling which becomes protective because it is associated with reduced alcohol use and decreased development of alcohol dependence (Higuchi et al., 2004).

The development of alcohol use disorders varies greatly across gender and racial/ethnic

groups but they are also dependent on environmental and/or socioeconomic factors. Controlling for age and race, men are more than twice as likely to develop alcohol abuse and dependence than women (Rehm et al., 2003b; van Oers et al., 1999). According to the 2007 National Survey on Drug Use and Health, alcohol use is most prevalent among Whites (60%), followed by Native American (48%), Hispanics (46%) , Blacks (44%), and finally Asian Americans (38%). Despite the fact that Whites are more likely to use and abuse alcohol, Hispanic and Blacks have a much higher risk of developing alcohol-related mortality and morbidity. This difference is more pronounced in persons with lower educational levels and socioeconomic status (Cook and Moore, 2002; van Oers et al., 1999). Other studies have shown children of alcoholic parents are more likely to develop alcohol dependence during adolescence and adulthood (Wallace Jr, 1999).

More than 80% of alcoholic subjects also smoke cigarettes, a rate that is more than three times higher than what is seen in non-alcoholics (Falk et al., 2006; Littleton et al., 2007). Countless studies have shown that alcohol abuse and smoking are independent risk factors for the development of numerous diseases and disorders, including acute respiratory distress syndrome. However, the concomitant use of alcohol and smoking results in an increased incidence and severity of multiple diseases (Rehm et al., 2009; Rehm et al., 2003b). This increased risk of disease is believed to be due to possible synergistic mechanisms of action between these two abusive substances (Falk et al., 2006; Littleton et al., 2007). While our current studies do not address the effects of smoking on alcohol induced macrophage dysfunction, several studies indicate ROS and GSH depletion play a role in both smoke and alcohol induced cellular dysfunction.

5.2.2 Alcohol Metabolism: Liver vs. the Lung. Alcohol abuse can directly lead to detriments in lung function that result in increased susceptibility to respiratory infections and pulmonary disorders (Aytacoglu et al., 2006b; F. Holguin 1998; Guidot et al., 2000). However, ethanol induced lung damage can also be due to the development of collateral circulation, which is due to

ethanol's effects on the liver (Albano, 2008; Cubero et al., 2009). Collateral circulation occurs in response to alcohol induced portal hypertension that induces the development of collateral vessels that connect directly to the portal blood vessel, thereby bypassing the liver. As a result of poor circulation, oxygen within the blood has a decreased affinity of hemoglobin and this leads to severely impaired lung function (Gramenzi et al., 2006). In addition, ethanol is primarily metabolized in the liver. The small percentage of ethanol that is not metabolized in the liver passes through the pulmonary circulation and is mainly metabolized within the lung. Metabolism of alcohol within the lung is increased in persons with alcohol use disorders (Brown et al., 2004; Karkoulias et al., 2008). Regardless of the organ or pathway used to metabolize alcohol; acetaldehyde is produced as a toxic by-product, resulting in antioxidant depletion, lipid peroxidation, and nucleic acid oxidation (Lieber, 2000). Therefore, we cannot rule out the possibility that the effects of ethanol on alveolar macrophage function may be secondary to the effects on the liver.

5.2.3. Liber-Decarli Model of Alcohol Abuse. Alcoholic patients get 36-50% of their calories from alcohol and these calories are considered empty calories since the metabolism of ethanol lead to no net energy production. As a result, alcoholic patients often develop deficiencies in proteins and vitamins that can contribute to liver disease, lung disease, and other alcohol-related disorder (Lieber, 2000; McDonough, 2003). Alcohol induced malnutrition can occur directly from decreased nutrient intake or indirectly by alcohol impairing the absorption of nutrients from the intestine (Martin et al., 2003; Oscar-Berman and Marinkovic, 2003).

The Liber-Decarli alcoholic diet is an ethanol-containing liquid diet that consists of 36% of its calories from ethanol. Importantly, this nutritionally complete liquid diet has been shown to be optimal for a high level of ethanol consumption in rodent models. Despite the fact that this model has been proven to mimic various complications observed in alcoholics, the direct effects of alcohol-induced malnutrition cannot be addressed with this model of alcohol ingestion. Since

malnutrition has been shown to impair alveolar fluid clearance and alveolar type II cell surfactant production, this model of ethanol ingestion may not encompass all of the detrimental effects of ethanol on alveolar macrophage function seen in human alcoholics (Guthmann et al., 2003; Sakuma et al., 2004). Despite these limitations, the current model is sufficient to induce impairments in alveolar macrophage function, alter ROS production, and blunt macrophage terminal differentiation.

5.2.4 SB-431542 Mediated Inhibition of TGF-β_1. The TGF-β superfamily consist of more than 40 multifuctional molecules, such as activins, inhibins, bone morphogenetic proteins (BMP), and three TGF- β isoforms (TGF-β_1, TGF-β_2, and TGF-β_3), that regulate diverse cellular processes such as cell growth, differentiation, immune responses, and tissue homeostasis (Bobik, 2006; Graham and Chun, 2006; Pulichino et al., 2008). While all three isoforms of TGF-β have been shown to be expressed in the lung, TGF-β_1 is the main isoforms secreted by monocytes and macrophages, and its expression is often upregulated during inflammation and injury. Macrophages have been shown to secrete and activate TGF-β_1 through the thrombospondin/CD36 receptor complex interacting with membrane-bound plasmin (Khalil et al., 2001; Koli et al., 2008; Pulichino et al., 2008). TGF-β_1, along with the other isoforms of TGF-β, is secreted in its inactive form due to it being in a complex with the latency associated peptide (LAP) and the latent TGF-β binding proteins (LTBP). The activation of TGF-β_1 is dependent on proteases, such as thrombospondin, and results in the release of active TGF-β and degradation of LAP. Active TGF-β1 then binds to the TGF-β type II receptor, a constitutively active cell surface serine/threonine kinase receptor, and this initiates the formation of a complex with the TGF-β type I receptor (activin-like kinase 5). Once the heteromeric complex is formed, the TGF-β type II receptor phosphorylates threonine residues in the GS domain of the TGF-β type I receptor to activate its serine/threonine kinase. TGF-β type I receptor then directly phosphorylates Smad2 and Smad3, which forms a complex with Smad4 and translocates to the nucleus to regulate gene

transcription (Bobik, 2006; Graham and Chun, 2006; Khalil et al., 2001; Koli et al., 2008; Pulichino et al., 2008; Waghabi et al., 2009).

Previous studies demonstrate TGF-β_1 expression and signaling is upregulated in alveolar macrophages during chronic ethanol ingestion and alternative activation (Bechara et al., 2004; Gordon, 2003). In other studies, inhibition of TGF-β_1 signaling protected against septic shock, liver fibrosis, and lung fibrosis (Chen et al., 2008; de Gouville et al., 2005; Fichtner-Feigl et al., 2006; Higashiyama et al., 2007) . To investigate the role of TGF-β_1 in alternative activation of alveolar macrophages, SB-431542, a potent small molecule inhibitor of TGF-β type I receptor was used. SB-431542 is an ATP-mimetic inhibitor of the kinase activity of the TGF-β_1 activin receptor-like kinase (ALK) family with selectivity for TGF-β type I receptor but also inhibits ALK4 (activin type I receptor), and ALK7 (nodal type I receptor), both of which have kinase domains similar to ALK5(Graham and Chun, 2006; Harrison et al., 2005). Previous studies demonstrate SB-431542 inhibits TGF-β_1 mediated activation of smad 2/3, mesenchymal cell proliferation, fibronectin production, and malignant glioma cell proliferation (Bonafoux and Lee, 2009; Graham and Chun, 2006; Harrison et al., 2005). In other studies, SB-431542 has been shown to have no effect on BMP signaling but to weakly inhibit p38 MAP kinase isoforms. The selectivity of SB-431542 for ALK5 can be seen in its ability to inhibit ALK5 at 100 times lower concentration than p38 MAP kinase isoforms. Treatment of cells with 2-10 μM SB-431542 had no effect on p38 MAP kinase activity, further demonstrating the specificity of SB-431542 for TGF-β1activin receptor-like kinases (Inman et al., 2002; Laping et al., 2002). Studies also indicate SB-431542 exhibits cellular toxicity at 100 μM in vivo (Inman et al., 2002; Laping et al., 2002).

In the current study, a concentration of 10mg/kg was administered via intra-peritoneal injection on two consecutive days before intratracheal instillation of IL-13. While no dose-response experiments were conducted to determine to optimal concentration of SB-431542, numerous studies have shown the concentration of 10mg/kg is sufficient to prevent LPS-

stimulated pro-inflammatory cytokine production, improve survival after septic shock, prevent heart damage, and to decrease the spread of bacterial infections (Chen et al., 2008; de Gouville et al., 2005; Higashiyama et al., 2007; Khalil et al., 2001; Laping et al., 2002; Liu et al., 2005; Pulichino et al., 2008; Waghabi et al., 2009; Wang et al., 2006). The effects of IL-13 -mediated lung dysfunction has been well documented in numerous disease states and been shown to affect numerous cell types within the lung. IL-13 has been shown to induce TGF-β_1 secretion from bronchial epithelial cells and to induce airway remodeling in a TGF-β_1 dependant manner (Hardy et al., 2010; Lee et al., 2001). Studies also show IL-13 drives TGF-β_1 expression in macrophages during alternative activation (Belperio et al., 2002; Zhu et al., 2004). The effect of IL-13 on TGF-β_1 expression in alveolar macrophages has not been documented previously, however we show in the current study that IL-13 treatment leads to a robust increase in alveolar macrophage TGF-β_1 gene and protein expression. Furthermore, inhibition of TGF-β_1 with SB-431542 blocked IL-13 mediated alterations in the alveolar macrophage activation and immune responses. We cannot rule out that these effects may be secondary to the effects of TGF-β_1 inhibition on other cell types within the lung however, alveolar macrophages have been shown to be the main cell type within the lung to produce and secrete active TGF-β_1 during lung inflammation and fibrosis (Ashcroft, 1999; Bobik, 2006; Chen et al., 2008; Vodovotz et al., 1993).

Despite these limitations, the current studies demonstrate chronic ethanol ingestion/exposure leads to pulmonary macrophage dysfunction by delaying terminal maturation, impairing phagocytic function, inducing an alternative activation phenotype, increasing endogenous ROS production, and increasing production of TGF-β_1. Other studies demonstrate TGF-β_1 is required for IL-13 induced alternative activation and deficits in phagocytic function. Supplementation with GSH or its precursors prevented or reversed ethanol induced macrophage dysfunction, thereby suggesting ethanol-induced alveolar macrophage dysfunction is modulated by oxidant stress and antioxidant depletion. Further elucidating the mechanism of alcohol induced alterations of pulmonary macrophage maturation and function will provide insight into

the mechanisms of alcohol-induced macrophage malfunction and provide potential treatments to decrease the risk of infection, thereby improving the compromised pulmonary function and mortality rates in alcoholics. In particular, these studies suggested that GSH supplements or attenuation of TGF-β_1 may improve the phagocytic function of alveolar macrophages and decrease the risk of community acquired or ventilator associated pneumonia in alcoholic subjects.

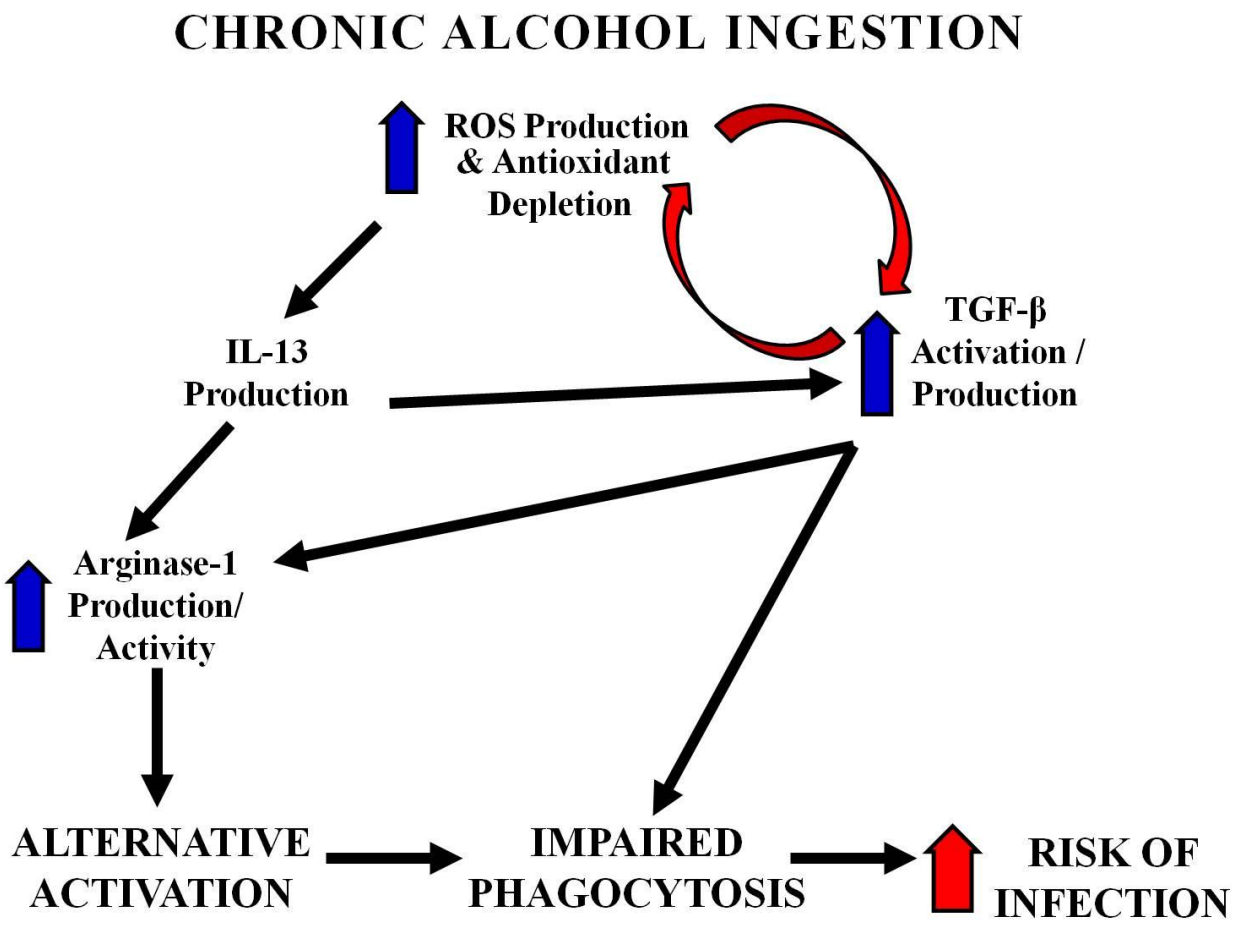
5.3. PROPOSED MECHANISMS.

Previous studies have shown that chronic ethanol ingestion increases TGF-β_1 production within alveolar macrophages. In other studies, chronic ethanol ingestion has been shown to decrease antioxidant availability (Bechara et al., 2004; Brown et al., 2004; Brown et al., 2007). In the current studies, we show chronic ethanol ingestion also leads to increased oxidant stress within the alveolar macrophage, as seen by a marked increase in intracellular and extracellular ROS production. In other studies, ROS have been shown to induce TGF-β_1 activation *in vivo* (Koli et al., 2008). Similarly, alternatively activated macrophages have robust increases in TGF-β_1 production and secretion (Martinez et al., 2009; Song et al., 2000; Stein et al., 1992). Once activated, TGF-β1 has been shown to deplete intracellular glutathione stores, and increase intracellular ROS production through NOX4, a novel NADPH oxidase, thereby, creating a vicious cycle of ROS generation. In vitro treatments with glutathione precursors have been shown to induce proteolysis of the TGF-β1 type II receptor and disintegration of TGF-β1(Koli et al., 2008). In other studies, TGF-β1 has been shown to directly activate arginase-1 activity and inhibit inducible NO synthase (Durante et al., 2001; Vodovotz et al., 1993). In preliminary studies, inhibition of TGF-β1 by neutralization of the TGF-β1 type II receptor during an *in vitro* model of alcohol abuse prevented ethanol-induced increases in arginase-1 activity and prevented ethanol-induced fibroblast proliferation (**Figure 6.1**). Also, studies by Mitchell et.al demonstrate chronic ethanol ingestion results in increased production of IL-13 and IL-13Rα_1 within the conducting airways(Mitchell et al., 2009). Similarly, our studies show ethanol ingestion increases

IL-13 expression within alveolar macrophages. In vitro studies suggest the increased expression of IL-13 can be ablated by treatment with TGF-β_1 inhibitors. **Therefore, we propose ethanol induced production of TGF-β_1 is central to ethanol induced impairments of alveolar macrophage function and induction of alternative activation** (**Figure 6.2**). Treatments with glutathione or its precursor are expected to reduce the oxidant stress induced by chronic ethanol consumption and thereby reduce ROS mediated activation of TGF-β1.

Figure 5.2 Proposed Schema of Ethanol Induced Alveolar Macrophage Dysfunction.

Previous studies have shown that chronic ethanol ingestion decreases antioxidant availability and increases TGF-β_1 production within alveolar macrophages. In the current studies, we demonstrated that chronic ethanol ingestion also leads to increased oxidant stress within the alveolar macrophage, as seen by a marked increase in intracellular and extracellular ROS production. Once activated, TGF-β_1 has been shown to deplete intracellular GSH stores and increase intracellular ROS production through NADPH oxidase 4, a catalytic subunit of NADPH oxidase. In addition, TGF-β_1 can directly activate arginase-1 activity and shift arginine from nitric oxide synthesis to proline synthesis. Based on these studies, we proposed that ethanol-induced production of TGF-β_1 was central to ethanol induced impairments of alveolar macrophage function and induction of alternative activation.


CHRONIC ALCOHOL INGESTION
ROS Production & Antioxidant Depletion
IL-13 Production
TGF-β Activation / Production
Arginase-1 Production/ Activity
ALTERNATIVE ACTIVATION
IMPAIRED PHAGOCYTOSIS
RISK OF INFECTION

CHAPTER 6

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