SUSCEPTIBILITY TO OZONE-INDUCED PULMONARY INFLAMMATION: INVESTIGATING THE ROLE OF THE INNATE IMMUNE RESPONSE

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Toxicology

Chapel Hill
2006

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ABSTRACT

GILLIAN SWIFT BACKUS HAZZARD: Susceptibility to ozone-induced pulmonary inflammation: investigating the role of the innate immune response
(Under the direction of Dr. Steven R. Kleeberger)

Exposure to subacute levels of O_3 causes decreased lung function, and airway hyperresponsiveness in 10-20% of the human population. Interindividual responses to O_3 vary greatly, which suggests a genetic component. O_3-induced inflammation results from activation of the innate immune response via specific receptors such as Toll-like receptor 4 (TLR4). TLR4 activation leads to production of soluble mediators such as interleukins (IL)-12, IL-18, and IL-10 that can affect immune responses. However, the mechanism underlying TLR4-mediated responses as well as the contribution of variants in these specific innate immune response mediators have not been investigated in the inflammatory response to O_3. We hypothesized that specific innate immune response mediators confer differential susceptibility to O_3-induced inflammation in inbred mice.

Studies with *Tlr4*-mutated (C3H/HeJ) and *Tlr4*-sufficient (C3H/HeOuJ) mice verified a role for TLR4 in O_3-induced hyperpermeability and neutrophilic responses (Kleeberger et al., 2000) but the underlying mechanism remains unclear. The accessory molecule CD14 is critical for TLR4-mediated responses to LPS. However, results from studies using *Cd14* gene-deleted mice were negative and indicated that CD14 is not required for the response to O_3. The downstream mediator IL-12 was found to contribute to O_3-induced inflammation

while IL-18 receptor was protective. Moreover, O_3-induced neutrophilic inflammation was dramatically increased in mice lacking IL-10, an important anti-inflammatory cytokine, suggesting that IL-10 can potently attenuate neutrophilic influx in response to O_3. Together these results suggest that the mechanism underlying O_3-induced inflammation occurs independently of CD14 and that the cytokine milieu of the lung can alter the severity of inflammation in response to O_3. The complex interplay of multiple genetic contributors must be considered to gain further insight into how inflammation is regulated in response to O_3.

ACKNOWLEDGEMENTS

A dissertation is considered a solitary work but along the way, numerous people contribute to its successful completion. I sincerely thank my advisor, Dr. Steven R. Kleeberger for his excellent professional mentorship and my committee members Dr. David Peden, Dr. Ilona Jaspers, Dr. Scott Randell, and Dr. Donald Cook for their guidance on my dissertation project. NIEHS is a wonderful place to learn how to do research and I am indebted to many individuals for their technical expertise including Dr. Carl Bortner, Maria Sifre, Dr. Darlene Dixon, Brian Mills, Eric Steele, Louise Fitzgerald, Maureen Puccini, Julie Foley and Dr. Bob Maranpot. Dr. Daniel Morgan, Mr. Herman Price and all the animal technicians and engineers at Alion Environmental Science were invaluable in coordinating, monitoring, and helping with controlled animal exposures.

I would like to especially thank the members of the Kleeberger lab. Drs. Hye-Youn Cho, Alison Bauer, and Dianne Walters taught me every scientific tool I needed and provided constant guidance and editing assistance. I greatly enjoyed sharing laughter, research milestones, frustrations, ideas, and life stories with Laura Miller DeGraff, Jacqui Marzec, Wes Gladwell, Christopher Walker, Monica High, Katharine Holder, and Dr. Reuben Howden. They are wonderful friends and talented colleagues.

I am sincerely grateful to my sensational family and friends for their unending support. My parents have always encouraged me to follow my dreams; I could not ask for better cheerleaders. I especially thank my grandmother Elizabeth C. Newell, for her wisdom and love. My brother Brad consistently provided gentle and invaluable transatlantic encouragement and mentorship. In their lifetimes, my great aunt Dorothy Barwis, and my grandfathers C. Bradford Newell and Richard A. Backus taught me kindness, generosity, the value of hard work, and a love of the outdoors. I also thank my Circle: Shuly, Laurie, Lisa, and Christine who provided unconditional friendship and even a quiet place to work during the final stages. And finally, my heartfelt gratitude to my best friend and husband, Thomas A. Hazzard, who moved to North Carolina so that I could attend UNC-Chapel Hill. He has faithfully believed in our future; his constant love and support are a blessing. This work represents our collective hopes and dreams.

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
AP-1	Activated protein 1
APC	Antigen presenting cell
ARE	Antioxidant response element
BALF	Bronchoalveolar lavage fluid
CD14	Cellular differentiation factor 14
CD86	Cellular differentiation factor 86
CO	Carbon monoxide
FEV_1	Forced expiratory volume in 1 sec
FVC	Forced vital capacity
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPx	Glutathione peroxidase
GSH	Glutathione
GSK3-β	Glycogen synthase kinase 3-beta
GST	Glutathione S transferase
H_2O_2	Hydrogen peroxide
HBSS	Hanks Balanced Salt Solution
HSPs	Heat shock proteins
ICAM	Intercellular adhesion molecule
ICE	IL-1β converting enzyme
IFN-γ	Interferon-gamma
IL	Interleukin

Iκk	Inhibitory kappa kinase
iNOS	Inducible nitric oxide synthase
IRAK-1	IL-1 receptor associated kinase 1
IRF-3	Interferon regulatory factor 3
LOP	Lipid ozonation products
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MD-2	Myeloid differential protein 2
MHC II	Major Histocompatibility Complex II
MIP-2	Macrophage inflammatory protein 2
MyD88	Myeloid differentiation factor 88
NAAQS	National ambient air quality standards
NF-κB	Nuclear factor kappa B
NO_x	Nitric oxides
NQO1	NADP(H): quinone oxidoreductase –1
Nrf2:	Nuclear factor erythroid 2 related factor 2
O_2•	Superoxide radical
O_3	Ozone
•OH	Hydroxyl radical
PAF	Platelet activating factor
PAMPs	Pathogen associated molecular patterns
PGE2	Prostaglandin E2
PI(3)K	Phosphoinositol-3-kinase

PM_{10}	Particulate matter: diameter less than 10 μm
$PM_{2.5}$	Particulate matter: diameter less than 2.5 μm
PMN	Polymorphonuclear leukocyte (neutrophil)
ppm	Parts per million
QTL	Quantitative trait locus
ROO•	Peroxyl radical
ROS	Reactive oxygen species
SOCS-3	Suppressor of cytokine signaling – 3
SO_2	Sulfur dioxide
STAT-3	Signal transducer and activator of transcription 3
Th1	T-helper cell, type 1
Th2	T-helper cell, type 2
TIR	Toll-IL-1 related protein
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
TRAF-6	Tumor necrosis factor receptor-associated factor 6
TRAM	TRIF-related adapter molecule
TRIF	Toll/IL-1R domain-containing adaptor-inducing IFN-beta
UA	Uric acid
VOCs	Volatile organic compounds

CHAPTER 1: GENERAL INTRODUCTION

Background and Significance

Air pollution is a global concern. Beginning in the mid-20th century, industrialized nations began to notice decreases in air quality that correlated with adverse health effects. In two independent episodes, stagnant pollution-filled airmasses in two industrial cities Liege, Belgium (1930) and Donora, Pennsylvania, USA (1948) contributed to elevated morbidity and mortality rates (Bell and Davis, 2001; Nemery et al., 2001). From December 5-9, 1952 a thick, toxic smog reduced visibility to several feet and caused an estimated excess 12,000 deaths in London, England (Bell and Davis, 2001). This third event catapulted air pollution into the realm of public health and heightened the search for mechanisms by which polluted air might be linked with specific adverse health endpoints, including death. In the past 50 years, researchers have provided extensive evidence that acute exposures not only cause visible and immediate health effects such as reduced lung function and increased airway hyperresponsiveness but also are correlated with chronic health problems such as chronic cardiopulmonary disease, emphysema, and asthma (Pope, 2000). Subpopulations including children and the elderly are particularly susceptible to increased morbidity and mortality following acute exposures of air pollution (Pope, 2000).

Air pollution is a heterogeneous mixture of particles, and photochemical by-products such as ground level ozone (O_3). Epidemiological, clinical and basic research evidence has determined toxic effects of air pollution components in respiratory airways and prompted regulatory action. In 1970, the Clean Air Act gave the Environmental Protection Agency power to regulate six criteria air pollutants including nitric oxides (NO_x), sulfur dioxide (SO_2), particulate matter (PM_{10} and $PM_{2.5}$), lead, carbon monoxide (CO) and O_3. The current (1997) National Ambient Air Quality Standard (NAAQS) for O_3 is an 8 hr standard. The 8-hr

standards are met when the three-year average of the annual fourth highest daily maximum 8-hr average concentration is less than 0.08 parts per million (ppm).

Despite governmental regulation in this and other countries, photochemical pollution continues to be a major public health concern. As of 2004, 124 U.S. cities routinely had O_3 levels that exceeded the NAAQS. Air Pollution and Health: a European Approach (APHEA) found that increases of 50 $\mu g/m^3$ of O_3 (1 hr max) was correlated with a 2.9% increase in total mortality across 6 European cities in 1997 (Touloumi et al., 1997). Air quality issues are evident on nearly every continent. Los Angeles, Mexico City, Buenos Aires, and Beijing are among the most polluted cities in the world for O_3 (http://www.sbg.ac.at/ipk/avstudio/pierofun/mexico/air.htm, 06-08-2006). Moreover, there is increasing evidence of intercontinental pollution transport from Central America and Asia to the U.S., as well as transboundary air pollution from Mexico to the U.S. and between the U.S and Canada. Intercontinental air quality issues are just beginning to be addressed (http://www.epa.gov/airtrends/international.html, 06-14-06). Effectively solving air pollution problems, including those caused by O_3, requires continued scientific research as well as political initiatives, economic incentives, public health awareness, and global cooperation.

The biochemistry of tropospheric O_3

Since O_3 was first identified as a prevalent component of photochemical smog in the Los Angeles, CA basin in the 1940's, it has been known as the most active and toxic gaseous pollutant in the breathable atmosphere of many large cities in industrialized countries, especially during hot summer months. Ground level O_3 forms largely as a secondary

byproduct of photochemical reactions with nitrous oxides (NO_x) and volatile organic compounds (VOCs) (Figure 1.1A). NO_x and VOCs are generated from commercial/industrial and natural sources such as motor vehicles, aircraft, power stations, forest fires, trees and vegetation. NO_x and VOCs react with singlet oxygen ($^1\Delta gO_2$) and molecular oxygen (O_2) in the presence of ultraviolet (UV) light, particularly on hot, stagnant summer days. As a powerful oxidant, environmental O_3 leads to cyclical and self-generating oxidative reactions. When VOCs and NO_x react with normally harmless air components such as H_2O and O_2 in the presence of UV light, highly unstable radical and non-radical reactive oxygen species (ROS) including superoxide (O_2•), hydrogen peroxide (H_2O_2), the hydroxyl radical (•OH), and O_3 are formed (Figure 1.1B).

Because O_3 is highly reactive, it likely does not penetrate cells. Instead, the unstable O_3 molecule reacts further with various electrophiles to form more ROS such as the hydroxyl radical (•OH) and peroxyl radical (ROO•), which attack electron-containing biomolecules including proteins (e.g. prostaglandin synthase and elastase), lipids (e.g. lipid peroxidation chain reactions) and nucleic acids (e.g. DNA damage). O_3 can react directly with membrane lipids and lung lining fluid lipids to form lipid ozonation products (LOPs) including aldehydes and hydroxyhydroperoxides. LOPs induce release of proinflammatory mediators and injury-associated markers from damaged tissue that may trigger further cellular damage (Bulut et al., 2002; Gupta et al., 1998; Okamura et al., 2001; Wong et al., 1996).

Antioxidants are distributed throughout the airways in both extracellular and cellular compartments and have scavenger and enzymatic activities (Table 1). Inhaled oxidants interact initially with antioxidants such as glutathione, ascorbic acid and uric acid located in the epithelial lining fluid. When the oxidant burden exceeds the capacity of these

antioxidants, inducible secondary antioxidant enzymes (e.g. glutathione peroxidase, superoxide dismutases (SOD)) participate in antioxidant defense (Mudway and Kelly, 2000) (Figure 1.2). Phase 2 detoxifying enzymes (e.g. glutathione-S-transferase isozymes (GST), NADP (H): quinone oxidoreductase (NQO1)) assist these proteins and small molecules by facilitating elimination or detoxification of oxidized biomolecules. The expression of these antioxidant proteins is controlled by redox-sensitive transcription factors (e.g. activator protein (AP)-1, nuclear factor (NF)-κB, and nuclear factor erythroid 2 (NF-E2) related factor 2 (Nrf2)).

Antioxidant defenses can be overwhelmed by high doses of oxidants, increased ventilation during exercise, or decreased antioxidant production and lead to oxidative stress. Individuals most at risk for oxidant injury include those with lower amounts or differential distribution of antioxidants, either by genetics or through preexisting disease (Mudway and Kelly, 2000). In mouse studies, antioxidant levels have been associated with differential susceptibility to O_3 (Johnston et al., 2000) (Jang et al., 2005). In asthmatic children, polymorphisms in the gene encoding GST (GSTM1) were correlated with increased susceptibility to O_3-induced decrements in respiratory flow (Romieu et al., 2004).

Adverse health effects of O_3

That O_3 and other components of air pollution have been associated with increased morbidity and mortality emphasizes the critical negative effects of these pollutants on the lung (Kleeberger, 2005). Experimental and epidemiological studies have associated O_3 with decrements in pulmonary function (FEV_1, FVC and increased airway resistance) (Hazucha et al., 1989), increased airway hyperresponsiveness (Mudway and Kelly, 2000), pulmonary

inflammation (Driscoll et al., 1993), hyperpermeability (Longphre et al., 1999), epithelial cell damage and proliferation (Johnston et al., 2002), and immune system effects (Jakab et al., 1995). For a given exposure to O_3, interindividual variation in the magnitude of the pulmonary response suggests an intrinsic factor(s) that confer(s), susceptibility. This is cause for concern particularly in patients whose airways are already compromised by obstructive pulmonary disease or sensitized by allergens and may therefore be at risk for exacerbated injury in the presence of O_3 (Mudway and Kelly, 2000). Epidemiology studies support an association between O_3 exposure and aggravated asthmatic symptoms (Bayram et al., 2001; Gilliland et al., 2001) and increased incidence of asthma (McConnell et al., 2002).

Airway inflammation is the predominant toxicity caused by O_3 in humans as well as multiple animal models (Bascom, 1996). In response to O_3, activated airway cells release inflammatory mediators including tumor necrosis factor alpha (TNF-α) (Cho et al., 2001), macrophage inflammatory protein 2 (MIP-2) (Driscoll et al., 1993; Johnston et al., 1999), platelet activating factor (PAF) (Longphre et al., 1999), Interleukin (IL-) 8, and IL-6 (Devlin et al., 1994). These mediators contribute to the hallmark phenotypes of O_3-induced inflammation – neutrophilic infiltration and hyperpermeability (Figure 1.3). In addition, the hyperpermeability response correlates with inducible nitric oxide synthase (iNOS) mRNA levels (Kleeberger et al., 2001). O_3-damaged cells also release mediators such as heat shock proteins (HSPs) and fibronectin. Fibronectin has been detected in response to O_3 in cultured airway cells (Devlin et al., 1994) and in rodent airways (Gupta et al., 1998). HSPs have also been associated with O_3 exposure in rat (Wong et al., 1996) and guinea pig models (Su and Gordon, 1997). Fibronectin (Okamura et al., 2001) and HSPs (Bulut et al., 2002; Vabulas et al., 2001) are putative ligands of Toll-like receptor 4, an innate immune receptor responsible

for inflammatory mediator production. The interaction between HSPs and fibronectin and specific innate immune receptors provide a possible indirect mechanism of O_3-induced inflammation.

Exposure to subacute doses of O_3 causes decreased lung function and airway hyperresponsiveness in 10-20% of the human population (Mudway and Kelly, 2000). These effects are determined in part by environmental factors such as duration, frequency and concentration of exposure, and other extrinsic factors such as socioeconomic factors and pre-exposures to other airway irritants and toxics, in combination with various intrinsic factors such as genetics, gender, health status, age, and diet (Balmes et al., 1996; Kleeberger, 2005). The genetic mechanism underlying differential susceptibility to O_3 has been studied for the past decade using murine models. This dissertation project investigated underlying genetic susceptibility to O_3-induced pulmonary inflammation by focusing on the role of specific innate immune genes that control the inflammatory response in mice.

O_3 and the Pulmonary Immune Response

The lung is primarily considered a gas exchange organ. Because the lung is continuously exposed to mixtures of antigens, toxicants and oxidants present in ambient air, it also must be immunologically active (Strieter et al., 2002). Moreover, the pulmonary immune response must be carefully regulated in order to preserve the integrity of the fragile, highly vascularized epithelial surfaces in the organ, particularly those at which gas exchange occurs (Holt, 2000).

Oxidative damage due to O_3 exposure is limited by numerous anti-oxidant molecules and enzymes present in the upper and lower respiratory tract. However, when anti-oxidant

defenses are overwhelmed, oxidative damage and subsequent injury and inflammation occur. O_3 has been known to alter macrophage function and immunologic competence in the lung (Gilmour et al., 1993). In the lung parenchyma, alveolar macrophages and other antigen presenting cells (APCs) are the first line of immune defense and are accompanied by neutrophilic (PMN) infiltration (Mudway and Kelly, 2000) as well as injury markers (e.g. HSPs, fibronectin, nitric oxide) that can heighten an immune response and cause further damage. Activated APCs produce cytokines that propagate the inflammatory response and simultaneously activate the adaptive immune response by driving differentiation of T-cells into one of two mutually exclusive subsets: Th1 or Th2. Other factors including arachidonic acid metabolites, coagulation factors, complement, acute-phase proteins, and antimicrobial peptides are also involved in the innate immune response, but cytokines are by far the largest and most pleiotropic of the innate immune mediators. Moreover, cytokines contribute to all phases of the innate immune response – initiation, maintenance, and resolution (Strieter et al., 2002).

Successful innate defense responses in the lung lead to the eventual transition and development of adaptive immunity (Strieter et al., 2002). In addition to antigen presentation, macrophages and other APCs upregulate co-stimulatory molecules such as CD86. CD86 interacts with CD28 on T-cells. In the absence of co-stimulation, T-cells are primed but not activated (anergic). Moreover, APCs produce cellular mediators that act in paracrine or autocrine fashion to alter the cytokine milieu. IL-12 and IL-18 are two such mediators produced by innate immune cells and have been shown to control Th1 production. IL-10 is a potent anti-inflammatory cytokine produced by macrophages, T-cells and B-cells. IL-10 can inhibit inflammation by acting either on macrophages or on T-cells, and thus has dual

functions in modulating both innate and adaptive immune responses. The interplay between the adaptive and innate arms of the immune system is critical for proper immune function but is not well understood, particularly in response to O_3.

There are several lines of evidence to suggest that O_3 can modulate the adaptive immune response. Epidemiology studies have shown that O_3 exposure can exacerbate allergic responses in humans (Kunzli et al., 2003; McConnell et al., 2002). Studies in animal models have confirmed that when antigenic stimulation occurs during O_3 exposure, allergic responses are enhanced (Holz et al., 2002; Jakab et al., 1995). Taken together, these studies provide indirect evidence that O_3 may influence immune responsiveness by "priming" the adaptive response.

Studies to assess the effect of O_3 on T-cells suggest immunosuppressive and immunoactivating functions. Holz *et. al.* (2001) reported that the numbers of CD4+ lymphocytes increase as do proportions of T-helper cells after single exposures to 0.2 ppm O_3 in normal human subjects (Holz et al., 2001). In cell culture, however, nonspecific clonal expansion of T cells was inhibited by direct exposure to 0.1 to 1.0 ppm O_3 (Becker et al., 1989). Koike *et. al.* (1998) found that, in response to a 3-day exposure of 1.0 ppm O_3, suppression of lymphocyte proliferation by alveolar macrophages could be inhibited by the O_3-induced release of soluble factors that inhibit nitric oxide (Koike et al., 1998). When T-cell function was suppressed by cyclosporine A in mice, O_3-induced lesions were greater than O_3 alone and implicate a role for T-cells in suppressing the response to 0.7 ppm O_3 (Bleavins et al., 1995). In another T-cell depletion study using an anti-Thy1.2 antibody, the numbers of inflammatory cells in the bronchoalveolar lavage fluid (BALF) significantly decreased after 0.3 ppm O_3 compared to untreated mice (Chen et al., 1995) and suggest that

T-cells actively participate in the inflammatory response. While an immunomodulatory effect has been implicated in response to O_3, different dose regimens and different measured endpoints make it difficult to compare studies and assess human risk. In addition, different T-cell subsets may have distinct functions – some attenuate the immune response (T regulatory cells) while others promote enhanced responses (T helper cells). Further research is required to uncover the underlying mechanism for the observed immunomodulatory effects.

Influence of innate immune response genes on pulmonary susceptibility to O_3

Significant interindividual differences to O_3 sensitivity suggest a genetic component that has been investigated in mouse models and humans (Goldstein et al., 1973; Kleeberger et al., 1993b; Kleeberger et al., 1997). Kleeberger *et. al.* (1997, 2000) used segregant populations of differentially susceptible mice to study the genetics of O_3-induced pulmonary neutrophilic inflammation and hyperpermeability (Kleeberger et al., 1997; Kleeberger et al., 2000). Using linkage analysis, Kleeberger *et. al.* (2000) identified a significant quantitative trait locus (QTL) for susceptibility to O_3-induced hyperpermeability on murine chromosome 4 (Figure 1.4) (Kleeberger et al., 2000). This locus contains the candidate gene, toll-like receptor 4 (*Tlr4*). Originally discovered in *Drosophila melanogaster*, the highly conserved TLRs belong to the IL-1 superfamily of innate immune receptors. Studies using *Tlr4*-mutant (C3H/HeJ) and *Tlr4*-normal (C3H/HeOuJ) strains of mice verified a role for TLR4 in O_3-induced hyperpermeability (Figure 1.5) (Kleeberger et al., 2000). Initial research showed that TLR4 altered O_3-induced hyperpermeability via inducible nitric oxide synthase (iNOS)

(Kleeberger et al., 2001). However, further mechanistic studies are needed to determine detailed mechanisms underlying *Tlr4*-mediated pulmonary O_3 toxicity.

TLR4 structure and function

TLRs are essential mediators of host defense by recognition of pathogen associated molecular patterns (PAMPs) (Qureshi et al., 2006). Of the 11 mammalian TLRs, only TLR4 recognizes the non-variant Lipid A component of lipopolysaccharide (LPS), a surface glycolipid of most gram-negative bacteria. To transduce an LPS signal, the lipid A moiety binds to lipopolysaccharide binding protein (LBP) that interacts with CD14 and MD2, two TLR-associated receptors (Kurt-Jones et al., 2000). These accessory cell-surface molecules cannot initiate signaling due to the lack of transmembrane domains. In LPS responsiveness, the bound ligand complex is required for dimerization with TLR4 and subsequent initiation of downstream signaling. In the absence of *Cd14*, response to LPS is severely attenuated in mice (Haziot et al., 1996). However, the role of CD14 in O_3-induced pulmonary injury and inflammation has not been investigated.

TLR4-mediated signaling occurs via myeloid differentiation factor 88 (MyD88)-dependent and -independent pathways (Figure 1.6). The MyD88 pathway requires the adaptor molecules TNF receptor associated factor (TRAF)-6 and IL-1 receptor associated kinase (IRAK)-1 to lead to nuclear translocation of nuclear factor kappa B (NF-κB), a transcription factor commonly implicated in upregulation of pro-inflammatory gene expression. The MyD88-independent pathway utilizes the Toll/IL-1R domain-containing adaptor-inducing Interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) adaptor molecules and leads to interferon regulatory factor (IRF)-3 activation. TLR4 activation

results in upregulation of important pro-inflammatory cytokines such as TNF-α and macrophage inflammatory protein (MIP)-2. Independently, studies have shown that TNF-α and TNF receptor contribute to O_3-induced inflammation in rodents (Bhalla et al., 2002; Cho et al., 2001). MIP-2 also increases in response to O_3 in rats (Bhalla and Gupta, 2000). LPS can induce IL-12 and IL-18 expression *in vitro* via the c-Rel subunit of NF-κB (Mason et al., 2002; Sanjabi et al., 2000), which suggests a role for TLR4 in the expression of these regulatory cytokines. TLR4 also controls inflammation by induction of anti-inflammatory cytokines such as IL-10 (Higgins et al., 2003).

IL-12 Structure and Function

IL-12 is a heterodimeric protein (70 kD) consisting of p35 (IL-12α) and p40 (IL-12β) subunits (Gately et al., 1998; Trinchieri, 2003). IL-12, produced by B-cells and macrophages, promotes Th1 responses, and suppresses Th2 responses by stimulating naïve T cells to differentiate into interferon gamma (IFN-γ) secreting T-cells (Gately et al., 1998). Murine *Il12α* shares 75% sequence homology with human *IL12A*. It is located on chromosome 3 in both species while the murine *Il12β* gene is located on chromosome 11 and shares 82% sequence homology with human *IL12B* on chromosome 5. Polymorphisms in *IL12B* and concomitant altered IL-12β structure and function have been associated with autoimmune diabetes in non-obese diabetic (NOD) mice (Adorini, 2001), hepatitis C virus infectivity (Houldsworth et al., 2005), and atopic asthma (Hirota et al., 2005; Morahan et al., 2002; Randolph et al., 2004). Single nucleotide polymorphisms in human *IL12A* and five other genes have been associated with a significant decrease in total white blood cell counts in response to occupational benzene exposure but has not been confirmed using gene-

targeted animal models to mimic this phenotype (Lan et al., 2005). The polymorphism data suggests that of the two IL-12 subunits, the IL-12β (p40) subunit may be more important in terms of modulating inflammatory disease than IL-12α (p35).

IL-12 is produced by LPS-stimulated macrophages, suggesting a role for TLR4 (Trinchieri, 2003) as well as activated B-cells. A *TLR4* polymorphism (Asp299Gly) associated with asthma was also implicated in reduced LPS-induced production of IL-12p70 in Swedish children (Fageras Bottcher et al., 2004). TLR4-dependent IL-12α production appears to occur via MyD88-independent pathways (Goriely et al., 2006). However, the contribution of IL-12 in the pulmonary pathogenesis of O_3-induced injury and inflammation has not been determined.

IL-18 Structure and Function

IL-18, initially called IFN-γ inducing factor, is an 18 kD single polypeptide that shares structural homology with IL-1 (Okamura et al., 1995). It is derived from the enzymatic cleavage of a 23kD precursor by IL-1β converting enzyme (ICE) that is activated by LPS (Schumann et al., 1998). IL-18 is expressed in macrophages, T-cells, B-cells and airway epithelium (Cameron et al., 1999). The gene encoding IL-18 is located on chromosome 1 (humans) and chromosome 9 (mice), and is 75% homologous. IL-18 is involved in production of pro-inflammatory mediators including IL-8, TNF-α and intercellular adhesion molecule (ICAM)-1.

IL-18 has been associated with a number of inflammatory diseases including rheumatoid arthritis (Liew, 2001), LPS-induced liver injury (Sakao et al., 1999), and asthma (Wild et al., 2000). IL-18 overexpression in the skin aggravated allergic cutaneous inflammation and was

accompanied by high expression of Th1 and Th2 cytokines (Kawase et al., 2003). Low levels of IL-18 have been associated with asthma and sarcoidosis (Ho et al., 2002). Elevated circulating levels of IL-18 have been correlated with HIV-1 seroconversion. Significant associations have been found between polymorphisms in *IL18* and asthma (105:G/C) (Higa et al., 2003), allergic rhinitis (-607, promoter) (Lee et al., 2006), atopic eczema (+113:G/C, +127:G/C, -137:G/C, -133:G/C) (Novak et al., 2005), mite allergens (-148:G/C)(Shin et al., 2005), and latex allergy (-656, promoter) (Brown et al., 2005). Taken together, these studies suggest that IL-18 has pro-inflammatory functions and can critically modulate inflammation in response to infection, autoimmune disease, and allergic responses.

IL-18 production increases in response to LPS (Joshi et al., 2002; Rouabhia et al., 2002) and IL-18 neutralizing antibodies decrease LPS-induced myocardial dysfunction (Raeburn et al., 2002), suggesting a role for TLR4 in IL-18 production. This effect may depend on prior immune status, however. Sakao *et. al.* (1999) found that *Il18* deficient mice primed with a low dose (200 μg) of heat-killed *Propionbacterium acnes* were resistant to LPS-induced liver injury but were highly susceptible when injected with a high dose (1 mg) of heat-killed *P. acnes* prior to LPS challenge (LPS-induced endotoxin shock) (Sakao et al., 1999). Moreover, few studies have used a *Tlr4*-deficient mouse model to directly correlate IL-18 production with TLR4. IL-18 may work in an autofeedback loop to control TLR4 levels or activation. Radstake *et. al.* (2004) determined by flow cytometry that IL-18 together with IL-12 can activate IFN-γ-mediated *Tlr2* and *Tlr4* expression in rheumatoid arthritis patients though the mechanism of this effect remains unknown(Radstake et al., 2004).

The heterodimeric IL-18 receptor (IL-18R1) is a 62 kD protein that specifically binds IL-18, and is expressed on macrophages as well as naïve and activated T- and B-cells. It is

composed of a ligand binding subunit (IL1Rrp or IL-18Rβ) and a signal transducing unit (IL-18acPL or IL-18Rα) (Sergi and Penttila, 2004). IL-18R1 shares structural homology with receptors of the IL-1 superfamily including IL-1R and TLR4. The human IL18 Receptor 1 gene (*IL18R1*) shares 75% homology with the murine form, and is located on chromosome 2 along with a cluster of IL-1 related genes. *IL18R1* promoter polymorphisms have recently been identified at –69 and –638 (Cardoso et al., 2004) but functional relevance to human disease has not yet been established. The IL-18R-α subunit leads to transcriptional activation of NF-κB through the MyD88, IRAK-1, and TRAF-6 pathway and suggests that IL-18R1 shares a common pathway with TLR4. Its structural homology to other innate immune receptors and its ability to activate a common pro-inflammatory pathway suggest that IL-18R1 may contribute to O_3-induced inflammation.

Taken together, the pro-inflammatory activities of IL-12, IL-18, and IL-18R1 have been well documented. IL-12 may be a downstream effector of TLR4, while IL-18R1 shares structural homology to TLR4 and a common signaling pathway leading to inflammatory cytokine expression. Moreover, functional polymorphisms in *IL12B*, and *IL18* have been correlated with differential responses in a variety of inflammatory disorders and may serve as important genetic markers for susceptibility to pulmonary inflammatory diseases.

IL-10 Structure and Function

IL-10 is a homodimeric protein product of *Il10* that is located on human and murine chromosome 1 (Kim et al., 1992). Human and murine genes share 81% sequence homology. IL-10 is a pleiotropic immunoregulatory and anti-inflammatory cytokine produced by activated monocytes, helper T cells, and B cells, amongst others. IL-10 attenuates

inflammation by inhibiting pro-inflammatory cytokines and chemokines (Lang et al., 2002; Standiford et al., 1995), cell injury markers (Cunha et al., 1992; Tulic et al., 2001), and activation of pro-inflammatory transcription factors (Saadane et al., 2005). IL-10 also affects the adaptive immune response by downregulating T-cell activation via decreased macrophage CD86 expression (Ding et al., 1993) and inhibition of CD28 tyrosine phosphorylation (Akdis et al., 2000) leading to anergic adaptive immune cells (Flores Villanueva et al., 1994).

A number of models suggest, indirectly and directly, an association between TLR4 and IL-10. LPS-induced lethality in mice increased significantly when endogenous IL-10 bioactivity was neutralized (Standiford et al., 1995). Similarly, intranasally administered recombinant murine IL-10 reduced lung inflammation and bronchopulmonary hyperreactivity caused by LPS exposure (Deleuze et al., 2004). In an LPS-challenged murine model of cystic fibrosis, recombinant IL-10 attenuated excessive inflammation (Saadane et al., 2005). In addition, TLR4-mediated IL-10 production has been shown to confer resistance to *Bordetella pertussis*, a gram-negative bacteria, by inhibiting inflammatory pathology (Higgins et al., 2003).

IL-10 secretion from LPS-stimulated blood cultures varied greatly and led to structural analysis of the human *IL10* gene to positively identify functional promoter polymorphisms (Eskdale et al., 1998a; Eskdale et al., 1998b). *IL10* polymorphisms have been associated with inflammatory disorders including sudden infant death due to infection (-592:A/C) (Summers et al., 2000), and rheumatoid arthritis (-2849:A/G) (Lard et al., 2003).

Hypothesis and Specific Aims

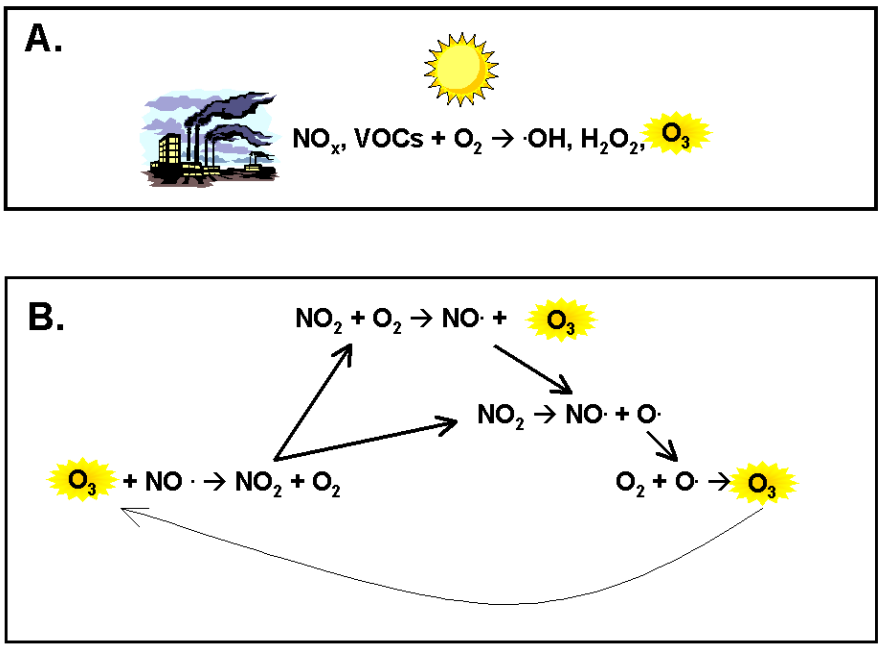
Exposure to environmental O_3 has been shown to induce pulmonary inflammation, and cause altered immune responses particularly in susceptible individuals (Jakab et al., 1996; Kleeberger, 1995; Mudway and Kelly, 2000). The underlying genetic contributions to these responses are not well understood. Although previous studies demonstrated the role of TLR4 in the pathogenesis of O_3-induced pulmonary injury, details of molecular mechanisms underlying TLR4-mediated responsiveness and the role of TLR4-inducible cytokines are unclear. We hypothesized that differential response to O_3 may be regulated by innate immune genes at the receptor level (*Cd14*) and downstream of TLR4 (*Il12α, Il12β, Il18, Il18r1, Il10*). Our specific aims were: 1) to characterize the role of the Toll-like receptor 4 accessory molecule CD14 in modulating receptor level events to the response to O_3; 2) to assess the downstream effector-level contribution of IL-12 and IL-18 to O_3-induced inflammation and; 3) to elucidate the putative anti-inflammatory role of IL-10 in a subacute O_3 model. We utilized mice genetically deficient in *Cd14, Il12α, Il12β, Il18, Il18r1*, and *Il10* to assess whether these genes specifically contribute to O_3-induced pulmonary pathogenesis.

Table 1.1: Distribution of anti-oxidants in the respiratory tract.

Name	Location	Reactant	Mechanism
Uric Acid	Lung lining fluid, upper airways	Hydroxyl radical	Scavenger
α-tocopherol	Lung lining fluid, distal airways	Peroxyl radical	Scavenger, chain breaking, terminates lipid peroxidation,
Glutathione (GSH)	Ubiquitous: Lung lining fluid, distal airways	H_2O_2	Thiol for oxidant-antioxidant equilibrium
Ascorbic Acid	Lung lining fluid, distal airways	Superoxide, peroxyl, H_2O_2, singlet O	Scavenger
Glutathione peroxidase (GSH-Px)	Lung lining fluid and cellular, lavage cells, Epithelial cells, macrophages	Organic hydroperoxides, including H_2O_2	Enzyme
Superoxide dismutase (SOD)	Lung lining fluid, produced by epithelial cells (EC-SOD), type II alveolar cells, macrophages, neutrophils	$O_2\cdot$	Enzyme
Catalase	Ubiquitous: Lung lining fluid, macrophages, fibroblasts, type II pneumocytes	H_2O_2	Enzyme
NADP(H):quinone oxidoreductase (NQO1)	Intracellular	Quinones	Phase II detoxifying enzyme
Glutathione-S-transferase (GST)	Intracellular	Quinones, aldehydes, epoxides, H_2O_2	Phase II detoxifying enzyme

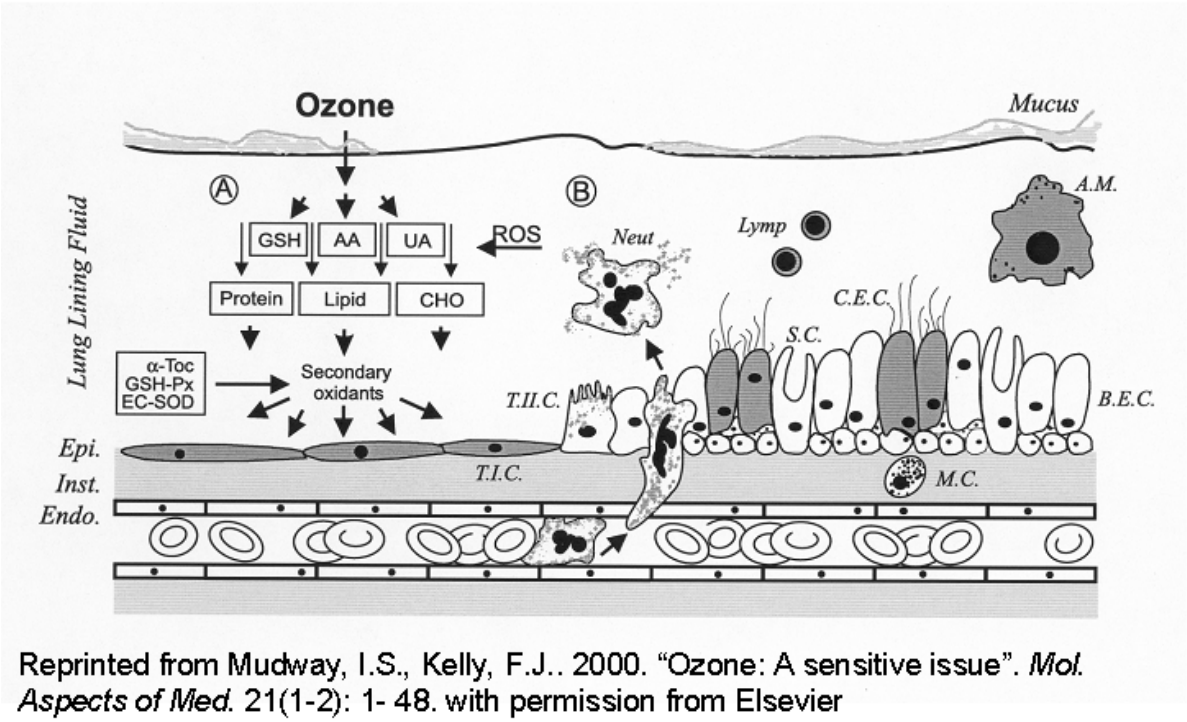
Antioxidants vary in their distribution, substrate (reactant) and mode of action (mechanism).

Figure 1.1: Tropospheric ozone (O_3) chemistry.



(A) O_3 forms in the troposphere when nitric oxides (NO_x) and volatile organic compounds (VOCs), formed from combustion reactions react with molecular O_2 in the presence of ultraviolet light (UV) to form byproducts such as the hydroxyl radical (·OH), hydrogen peroxide (H_2O_2) and O_3. **(B)** NO_2 and O_2 can produce nitrogen radicals (NO·) as well as O_3. Singlet oxygen (O·), formed from light-catalyzed degradation of NO_2, can react with molecular O_2 to form O_3. O_3 can react with NO· to regenerate NO_2 and O_2. Thus, O_3 formation reactions are cyclical.

Figure 1.2: Ozone (O_3) interacts with antioxidants in lung lining tissue and may interact with numerous tissue targets.



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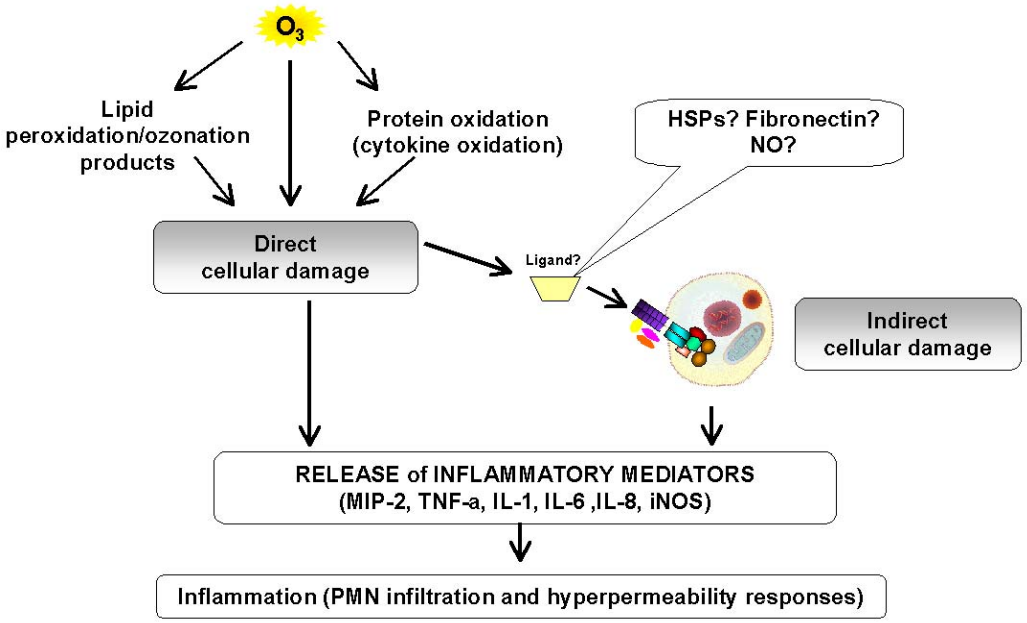
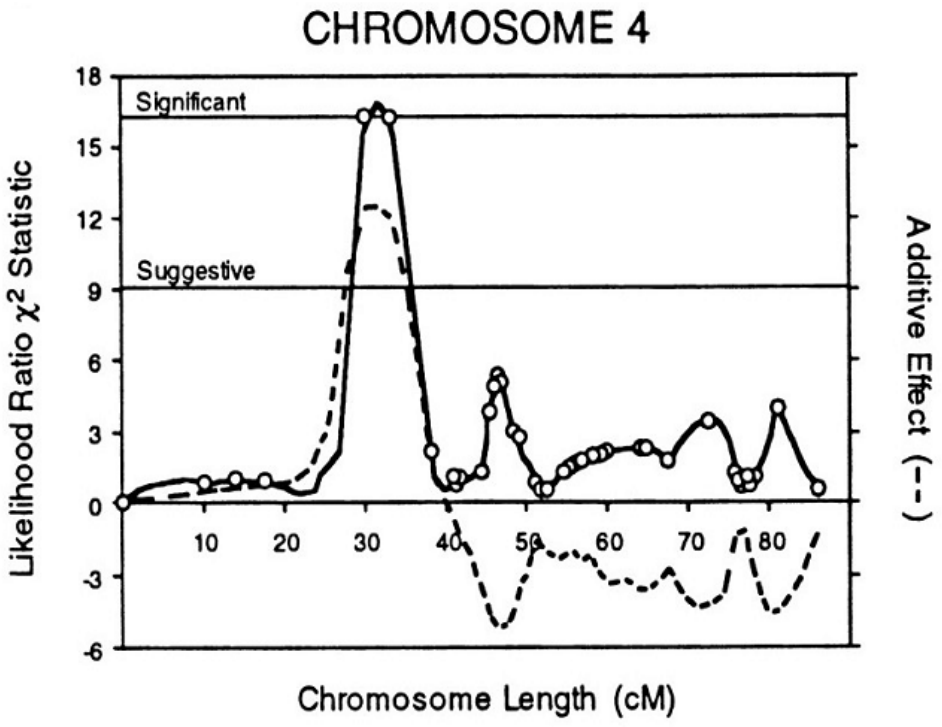
Water-soluble glutathione (GSH), ascorbic acid (AA), and uric acid (UA) are present in the lung lining fluid and are thought to interact directly with O_3. When antioxidant defenses are insufficient, O_3 reacts with proteins and lipids to potentially inactivate enzymes and form lipid ozonation products (LOPs), respectively. Secondary antioxidants such as α-tocopherol (α-toc) and glutathione peroxidase (GPx) counteract lipid autooxidation products and extracellular superoxide dismutase (EC SOD) removes superoxide formed as a by-product. O_3 promotes neutrophilic infiltration. Neutrophils (Neut) produce their own toxic reactive oxygen species (ROS) and contribute to the total lung oxidant burden. Lymphocytes (Lymp), mast cells, alveolar macrophages (A.M.), Ciliated Epithelial Cells (C.E.C.), and Type I Cells (T.I.C.) are all affected by exposure to ROS. Other abbreviations: Epi, epithelium; Inst, interstitium; Endo, endothelium; TIIC, Type II Cell; BEC, bronchial epithelial cell (Mudway and Kelly, 2000). Reprinted from Mudway, IS and Kelly, FJ. "Ozone: A Sensitive Issue", Mol. Aspects of Med., Vol 21, issue 1-2, pp. 1-48, 2000 with permission from Elsevier.

Figure 1.3: Direct and indirect cellular effects due to O_3.



Once inhaled, O_3 as well as O_3-induced lipid peroxidation, lipid ozonation products (LOPs), and oxidation of proteins can cause direct cellular damage. Oxidized soluble proteins may contribute to altered intercellular communication as well. In addition, injured cells may release endogenous cell injury markers such as heat shock proteins (HSPs), fibronectin, and nitric oxide (NO) that may perpetuate cell damage or initiate cellular responsiveness. Damaged and activated lung tissue produce pro-inflammatory mediators such as macrophage inflammatory protein (MIP)-2, tumor necrosis factor (TNF)-α, Interleukin (IL-) 6, and inducible nitric oxide synthase (iNOS). These mediators signal neutrophilic infiltration and hyperpermeability responses, the hallmarks of O_3-induced inflammation.

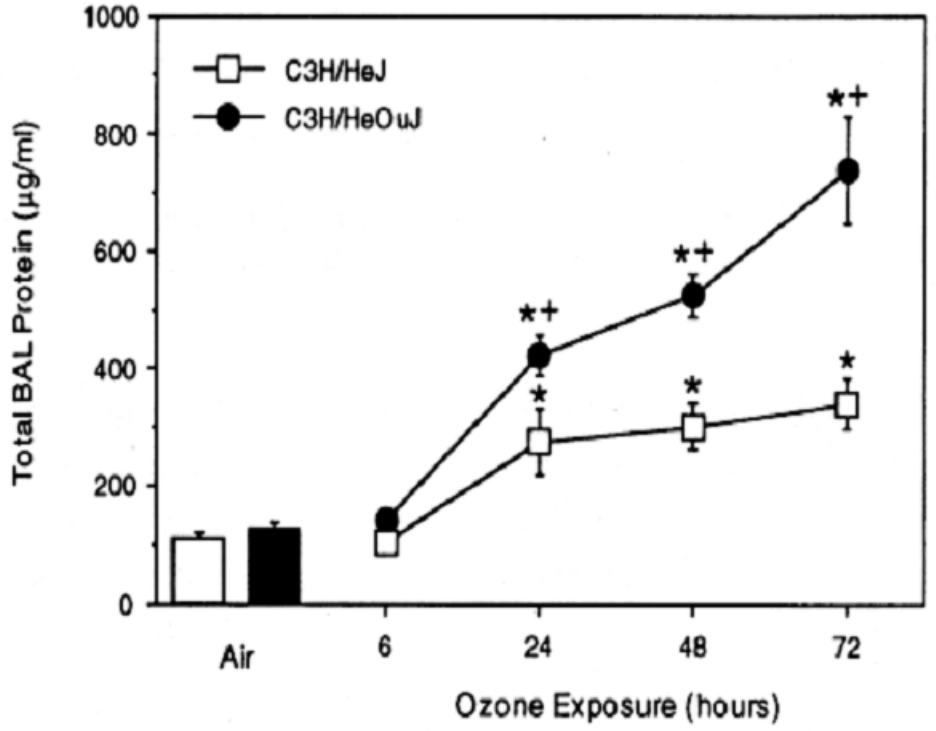
Figure 1.4: A quantitative trait locus (QTL) for O_3-induced hyperpermeability.



Kleeberger, SR *et. al.*, 2000. *Am. J. Respir. Cell Mol Biol.* 22(5): 620- 627.

A genome wide scan using recombinant inbred mouse strains derived from O_3-resistant (C3H/HeJ) and O_3-susceptible (C57BL/6) mice phenotyped for hyperpermeability responsiveness identified a significant quantitative trait locus (QTL) located on murine chromosome 4. Plot of the likelihood ratio χ^2 statistic (solid line) and additive regressive coefficient (dashed line). Interval mapping was done by simple linear regression with Map Manager QTb27 to generate χ^2 and regression coefficient values. The upper and lower horizontal lines in each plot represent significant and suggestive linkage thresholds, respectively, as determined by permutation test (Kleeberger et al., 2000).

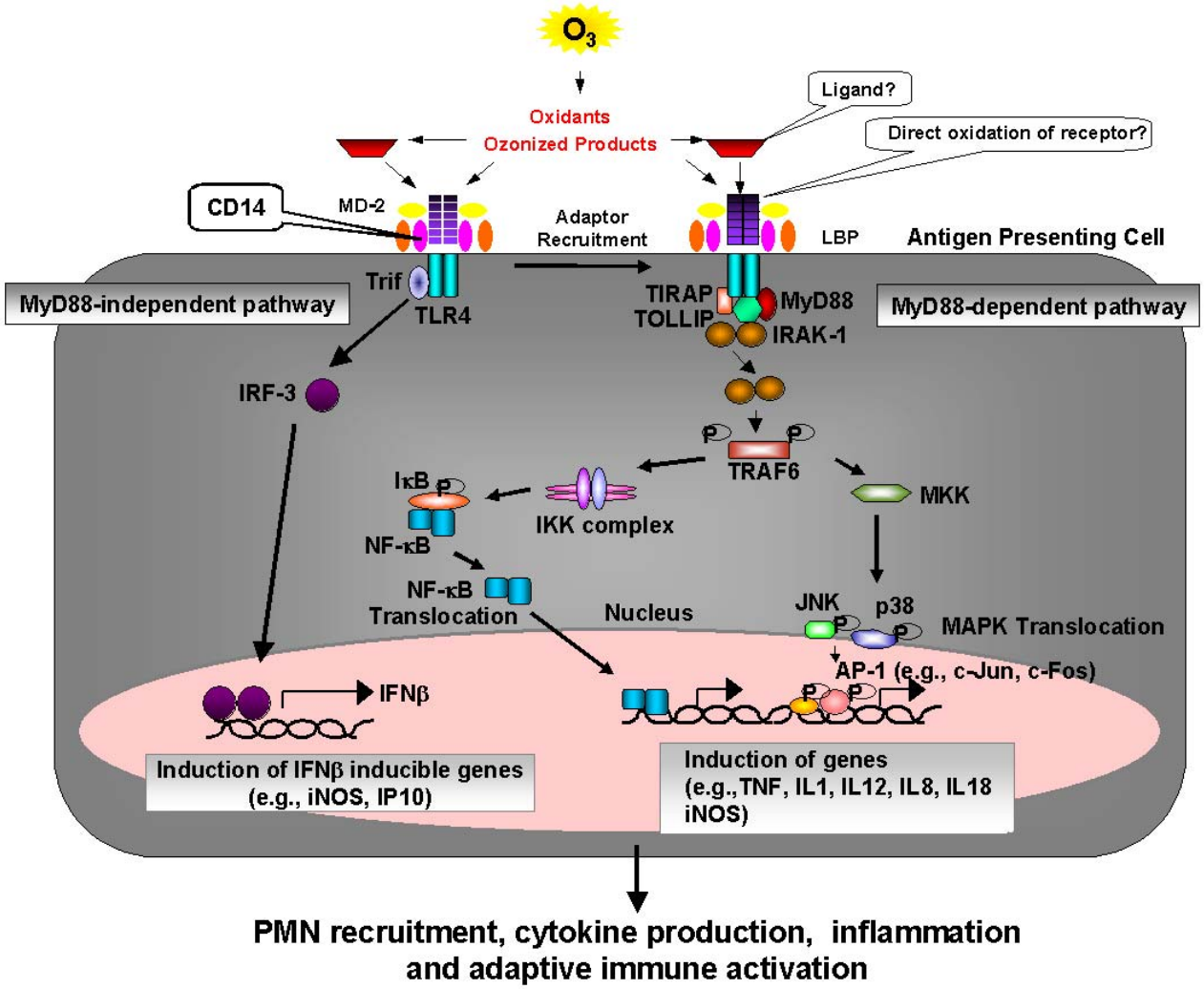
Figure 1.5: TLR4 mediates pulmonary protein permeability caused by O_3.



Kleeberger, S.R. *et. al.*, 2000. *Am. J. Respir. Cell Mol Biol.* 22(5): 620- 627.

Total BAL protein responses were compared in *Tlr4*-mutated (C3H/HeJ) and *Tlr4*-normal (C3H/HeOuJ) mice after 6, 24, 48 and 72 h exposure to 0.3 ppm O_3. Air controls were not statistically different from each other. Data are presented as means ± SE (n = 5 - 16) * $p < 0.05$ air vs. O_3. + , $p < 0.05$ C3H/HeJ vs. C3H/HeOuJ (Kleeberger et al., 2000).

Figure 1.6: Proposed TLR4 signaling pathways in response to O_3.



Oxidation of TLR4 or other oxidants or ozonized by-products initiate TLR4 activation directly or indirectly. The TLR4 complex includes MD-2 and lipopolysaccharide binding protein (LBP) in addition to CD14. TLR4 activation is followed by adaptor recruitment and results in activation of ether the myeloid differentiation factor (MyD)-88-independent or MyD88-dependent signaling pathways. The MyD88-independent pathway leads to DNA binding by interferon regulatory factor (IRF)-3 that transcriptionally activates interferon-beta (IFN-β) inducible genes. MyD88-dependent signaling requires IL-1 receptor associated kinase (IRAK)-1 and Toll-related activation factor (TRAF)-6 and leads to activated protein (AP)-1 and nuclear factor-kappa B (NF-κB) nuclear transcriptional activation and subsequent induction of pro-inflammatory gene products such as tumor necrosis factor (TNF), interleukin (IL-)1, IL-12, IL-18, IL-8, and inducible nitric oxide synthase (iNOS). These mediators may direct PMN recruitment and cytokine production leading to increased inflammatory and adaptive immune responses.

CHAPTER 2: OZONE-INDUCED LUNG HYPERPERMEABILITY AND INFLAMMATION ARE INDEPENDENT OF CD14

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Supported by the National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services

Running Head: CD14 and O_3-induced inflammation

Abstract

Previous work has shown that a dominant negative mutation of the innate immune receptor toll-like receptor 4 (TLR4) decreases pulmonary hyperpermeability and inflammatory responses to ozone (O_3) in the mouse. However, the mechanism through which TLR4 mediates the effect of O_3 remains unknown. TLR4 works in conjunction with several extracellular accessory molecules, including CD14. CD14 is critical for TLR4 signaling in response to lipopolysaccharide (LPS) and respiratory syncytial virus. However, the role of CD14 in O_3-induced inflammation has not been investigated. We hypothesized that O_3-induced pulmonary inflammation and hyperpermeability are dependent on CD14. To test this hypothesis, *Cd14*-deficient ($Cd14^{-/-}$) and *Cd14*-sufficient ($Cd14^{+/+}$) mice were exposed to 0.3 ppm O_3 or filtered air. Mean total protein concentration and numbers of polymorphonuclear leukocytes (PMN) in bronchoalveolar lavage fluid were significantly increased in $Cd14^{+/+}$ and $Cd14^{-/-}$ mice after 48 and 72 hr exposure to O_3 compared to air controls. Lung mRNA expression of IL-6 and TNF-α increased in $Cd14^{+/+}$ and $Cd14^{-/-}$ mice after O_3. However, no significant differences between strains were observed in lung injury parameters and TLR4-inducible inflammatory gene expression. Total numbers of PMNs increased in response to LPS in $Cd14^{+/+}$ mice but not $Cd14^{-/-}$ mice. Results suggest that TLR4-mediated pulmonary injury responses to O_3 are independent of CD14 and are therefore different from LPS responsiveness. The mechanism underlying O_3-induced pulmonary inflammation is unique from that of LPS

Introduction

Ozone (O_3) is a prevalent constituent of air pollution. Experimental and epidemiological studies have associated O_3 exposures with exacerbation of asthma (Bayram et al., 2001; Hiltermann et al., 1997; McConnell et al., 2002; Neuhaus-Steinmetz et al., 2000) and increased mortality and morbidity (Touloumi et al., 1997). Animal studies have shown that O_3 induces airway inflammation, hyperpermeability, and airway hyperresponsiveness (Cho et al., 2000; Kleeberger, 1995; Plopper et al., 1998; Smith, 1986; Toward and Broadley, 2002). In turn, these responses are correlated with O_3-induced production of inflammatory mediators tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and macrophage inflammatory protein 2 (MIP-2) (Driscoll et al., 1993; Longphre et al., 1999) and their functional roles have been established (Cho et al., 2001; Johnston et al., 2005).

We performed a genetic linkage analysis for susceptibility to O_3-induced lung hyperpermeability that identified toll-like receptor 4 (*Tlr4*) as a candidate gene (Kleeberger et al., 1997). C3H/HeJ (HeJ) mice possess a missense mutation in the third exon of the *Tlr4* gene that renders them resistant to lipopolysaccharide (LPS) (Poltorak et al., 1998) and we found that the mutation also confers resistance to O_3-induced hyperpermeability compared to *Tlr4*-normal C3H/HeOuJ (OuJ) mice (Kleeberger et al., 2000). However, the mechanism of TLR4-mediated responses to O_3 remains unknown.

The 11 known mammalian TLRs belong to the IL-1 receptor superfamily and function as innate immune receptors. TLR4 responds to the non-variant lipid A component of LPS, a surface glycolipid of most gram-negative bacteria. The lipid A moiety binds to LPS binding protein (LBP) that interacts with the membrane-anchored protein CD14 and MD2 (Akira and Takeda, 2004). The LPS-bound LBP-CD14-MD2 complex interacts with TLR4 to initiate

downstream signaling via its intracellular toll-IL-1 receptor (TIR) domain. Adaptor molecules, including myeloid differentiation factor-88 (MyD88) and interleukin-1 receptor associated kinase-1 (IRAK-1), bind to the TIR domain and phosphorylate tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF-6) leading to nuclear factor kappa B (NF-κB) nuclear translocation. In the nucleus, NF-κB binds promoter sequences of proinflammatory cytokines (e.g., IL-6).

CD14 and TLR4 are required for LPS-mediated pulmonary inflammation (da Silva Correia et al., 2001; Haziot et al., 1996; Wright et al., 1990). CD14 and TLR4 are also required to respond to respiratory syncytial virus (RSV) (Kurt-Jones et al., 2000) though this mechanism is independent of LBP. However, the role of CD14 in TLR4-mediated inflammation induced by O_3 has not been investigated. The present study was designed to test the hypothesis that CD14 contributes to O_3-induced lung injury and hyperpermeability. CD14-sufficient ($Cd14^{+/+}$) and CD14-deficient ($Cd14^{-/-}$) mice were exposed to 0.3 ppm O_3 or air (48 or 72 hr). O_3-induced alterations in pulmonary injury phenotypes and TLR4-downstream signal transducers were compared between the two mouse genotypes.

Materials and Methods

Animals. Female and male (6-8 weeks) *Cd14*$^{+/+}$ (C57BL/6) and *Cd14*$^{-/-}$ (B6.129S-*Cd14*^{tm1Jm}) were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were provided water and mouse chow (NIH-31) *ad libitum*. All experimental procedures were conducted in accordance with approved NIEHS Animal Care and Use protocols.

O_3 Exposure. Mice were exposed to 0.3 ppm O_3 or filtered air for 48 or 72 hr, 23.5 hr/day. O_3 was generated from ultra-high purity air (<1 ppm total hydrocarbons; National Welders, Inc., Raleigh, NC) using a silent arc discharge O_3 generator (Model L-11, Pacific Ozone Technology, Benicia, CA). Constant chamber air temperature (72 ± 3° F) and relative humidity (50 ± 15%) were maintained. Water and mouse chow were provided during the exposure.

LPS Aspiration. Mice were lightly anesthetized with 3 % Isofluorane/Oxygen for 2 min. We administered 1.0 μg/kg of LPS (0111:B4, Sigma, St. Louis, MO) in a 50 μL volume or phosphobuffered saline (PBS, 50 μL/mouse) by oropharyngeal aspiration. Mice were allowed to recover on warm wax pads for 24 hrs.

Necropsy and Bronchoalveolar Lavage (BAL) Analysis. Mice were euthanized (sodium pentobarbital, ip., 104 mg/kg) immediately following O_3 exposure and up to 24 hrs after LPS exposure. The right lung was lavaged four times with ice-cold Hanks' balanced salt solution (HBSS) (26.0 ml/kg, pH 7.2-7.4). The left lung was snap frozen in liquid nitrogen. Recovered bronchoalveolar lavage fluid (BAL) was centrifuged (500 × g, 4°C, 10 min.).

BAL analysis followed standard laboratory protocols (Cho et al., 2001). Briefly, mean total protein was determined from the supernatant via the Bradford Assay (BioRad, Hercules, CA). Cell pellets were resuspended in 1 mL ice-cold HBSS and total cell numbers were counted with a hemacytometer. A 200 μL aliquot was used to make cytospin slides (300 rpm for 10 min) (Shandon Southern Products, Pittsburgh, PA). Slides were stained with Wright-Giemsa stain (Diff-Quik; Baxter Scientific Products, McGaw Park, IL) for differential cell analysis.

Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR). Total mRNA was isolated from lung homogenates using TRIzol reagent (Life Technologies, Gaithersburg, MD). Five hundred ng of RNA from individual mice were reverse transcribed according to previously published protocols (Cho et al., 2001). PCR amplification was performed with an aliquot of cDNA (10 μl). Forward and reverse primers specific for mouse *Il6*, *TNFα*, *TLR4*, *Mip-2*, and *Cd14* were used. β-actin served as an internal control. All primers were initially incubated at 94^{o} for 5 minutes prior to a three-step cycle consisting of denaturation, annealing, and extension segments (Table 2.1). Reactions were electrophoresed in 1.2 % agarose gels containing ethidium bromide. cDNA Bands were visualized and quantitated with a Bio-Rad Gel Doc 2000 system (Bio-Rad Laboratories, Hercules, CA). The ratios of *Il6*, *Mip-2*, or *Tnfα* to β-actin cDNA were determined.

Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (Q-RT-PCR). Total RNA was isolated from left lung homogenates using the RNAeasy Midi Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. One μg of total RNA was

reverse transcribed into cDNA (50 μl reaction). The ABI 7000 Sequence Detector (Applied Biosystems, Foster City, CA) was used to detect TNF-α and CD14 gene expression using qPCR TaqMan ® Gene Expression Power Syber Assays (Applied Biosystems, Foster City, CA). Briefly, two μL of cDNA was amplified in a 20-μl reaction containing 10 μL of 2x PowerSyber Master Mix (Applied Biosystems, Foster City, CA) and 1 μL of custom specific primers for *Tnf-α* and *Cd14* (Invitrogen, Carlsbad, CA). 18s ribosomal RNA was served as an internal control. PCR was performed by 10 min incubation at 95 ^{o}C followed by 40 cycles of 95 ^{o}C-15 sec and 60^{o}C-1 min (n = 9, data not shown).

Western Blot. Total lung protein was extracted from lung tissue homogenates in ice-cold RIPA buffer with phosphatase inhibitors of 100 nM sodium orthovanadate (10 μL/L), aprotinin (0.2 μL/mL), PMSF (10 μL/mL), leupeptin (50 μg/mL, 5 μL) and pepstatin (25 μg/mL, 9 μL). Aliquots of total proteins (30-80 μg) were subjected to Western blot analysis with specific primary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) to TLR4 (SC-16240), CD14 (SC-9150), MyD88 (SC-8197), IRAK-1 (SC-7883), and TRAF-6 (SC-7221) with gentle agitation overnight at 4 ^{o}C. Detection agents (SuperSignal West Pico Chemiluminescent Substrate, Pierce, IL) were added to the blot and exposed to BioMaxMR x-ray film (Kodak, Rochester, NY). Intensity of Western blot bands was quantitated using the BioRad Gel Doc 2000 System.

Enzyme-Linked Immunosorbant Assay. Protocols followed manufacturer's instructions (R&D Systems, Minneapolis, MN). Briefly, 50 μL assay diluent and 50 μL of either cell-free BAL fluid or MIP-2 cytokine standards (500 – 7.8 pg/mL) were added to murine MIP-2 pre-

coated 96-well ELISA plates. All samples were performed in duplicate and allowed to incubate for 2 h at room temperature. Plates were washed and incubated with MIP-2 conjugate for 2 h at room temperature. Following further washes, 100 μL of substrate solution was added. The reaction was stopped following a ½ hr incubation at room temperature in the dark and plates were measured at 450 nm on a Bio-Rad microplate reader (Hercules, CA). The minimum detectable dose of murine MIP-2 was less than 1.5 pg/mL.

Nuclear Protein Isolation and Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared from right lung lobes following 48 hr of either air or 0.3 ppm O_3 using a Nuclear Extraction Kit (Active Motif, Carlsbad, CA). An aliquot (6 μg) of nuclear protein was incubated in a binding buffer (10mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5 % glycerol, 1 mM DTT, 0.2 μg PolydI-dC, 1 mM PMSF) in a total volume of 19 μl. After 15 min incubation, 1 μl (3 x 10^4 cpm) of [γ^{32}P] dATP end-labeled oligonucleotide containing a NF-κB consensus sequence was added to the reaction and followed by 30 min incubation at room temperature. The mixture was subjected to electrophoresis on a 4 % SDS-PAGE with 0.25X TBE buffer. The gels were autoradiographed at –70 °C. To analyze specific binding of NF-κB subunits, nuclear proteins (6 μg) were pre-incubated with either 4 μg of anti-p65 NF-κB antibody (sc-372X; Santa Cruz) or 4 μg of anti-p50 NF-κB antibody (sc-1190X; Santa Cruz), and processed for gel shift assay as described above.

Statistics. All data were expressed as group means ± SE. The data were transformed, if necessary, to ensure normal data distribution and equal variance. Two-way ANOVA was used to evaluate the effects of O_3 exposure on pulmonary toxicity. The factors were exposure (air or O_3) and genotype (*Cd14*$^{+/+}$ or *Cd14*$^{-/-}$). The dependent variables were lung

protein concentration, PMN cell counts, and cytokine mRNA and protein expression. The Student Newman Keuls *a posteriori* test was performed to compare group means. All statistical analysis was performed using a commercial statistics package (SigmaStat, Systat Software, Point Richmond, CA). Statistical significance was accepted at $p < 0.05$.

Results

CD14 is required for the PMN response to LPS. To confirm the importance of CD14 to the pulmonary response to LPS and to serve as the positive control for the O_3 experiments, we compared the inflammatory response to LPS in $Cd14^{+/+}$ and $Cd14^{-/-}$ mice. No significant differences in mean numbers of PMNs and mean total protein recovered in BAL were found between $Cd14^{+/+}$ and $Cd14^{-/-}$ mice in response to saline (Figure 2.1). Mean numbers of PMNs were significantly increased in $Cd14^{+/+}$ mice 24 hr after aspiration of LPS compared to saline (Figure 2.1A). However, mean numbers of PMNs were not significantly different between LPS- and saline-treated $Cd14^{-/-}$ mice. Numbers of PMNs from LPS-treated groups were significantly higher in $Cd14^{+/+}$ compared to $Cd14^{-/-}$ mice (Figure 2.1A). Compared to corresponding saline controls, LPS did not cause a significant increase in total lung protein concentration in either genotype (Figure 2.1B).

CD14 is not required for the PMN response to O_3. We tested the hypothesis that CD14 contributes to the inflammatory response to O_3 by exposing $Cd14^{+/+}$ and $Cd14^{-/-}$ mice to filtered air or 0.3 ppm O_3 for up to 72 hrs continuously followed by assessment of inflammatory endpoints. No significant differences in mean numbers of PMNs and total protein concentration recovered in BAL were found between $Cd14^{+/+}$ and $Cd14^{-/-}$ mice in

response to air (Figure 2.2). Mean numbers of PMNs and mean protein concentration were significantly elevated in *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice after 48 and 72 hrs of 0.3 ppm O_3 compared to genotype-matched air controls (Figure 2). However, we did not find a statistically significant interaction of treatment and strain on mean numbers of PMNs at 72 hr although an increase in the number of animals may help to further clarify this trend (Figure 2.2A). Protein hyperpermeability did not differ significantly between *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice after O_3 at either time point (Figure 2.2B).

CD14 is not required for activation of TLR4 signaling intermediates in response to O_3. To confirm that *Cd14* was not present in *Cd14*$^{-/-}$ mice, we determined *Cd14* mRNA and CD14 protein levels in response to air and O_3 in both strains of mice. As expected, neither *Cd14* mRNA nor CD14 protein were expressed in *Cd14*$^{-/-}$ mice after air or O_3 (data not shown). We next determined that TLR4 and TLR4-associated signaling molecules (MyD88, IRAK-1, TRAF-6) were still intact in *Cd14*$^{-/-}$ mice. *Tlr4* mRNA expression was not different between the two genotypes of mice after air or O_3 exposure (Figure 2.3A). In addition, basal protein levels of MyD88, IRAK-1 and TRAF-6 were not different between *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice. In response to O_3, MyD88 expression increased significantly compared to air in *Cd14*$^{+/+}$ mice and was also significantly greater in *Cd14*$^{+/+}$ compared to *Cd14*$^{-/-}$ mice (Figure 2.3B). This trend is interesting but should be interpreted with caution since the MyD88 antibody used did not yield consistent results, despite an n of 6 and numerous replicates. No effect of O_3 on IRAK-1 or TRAF-6 protein levels was detected in *Cd14*$^{+/+}$ or *Cd14*$^{-/-}$ mice (Figure 2.3C and 2.3D).

CD14 does not influence DNA binding activity of nuclear factor kappa B (NF-kB) in response to O_3. The transcription factor NF-κB is an effector of TLR4 and is activated in response to O_3. NF-κB controls induction of proinflammatory genes IL-6 and TNF-α. We expected NF-κB levels to increase in response to O_3. In addition, we hypothesized if CD14 contributed to O_3-induced inflammation, then NF-κB activation would be attenuated in *Cd14*$^{-/-}$ compared to *Cd14*$^{+/+}$ mice. Relative to air exposure, total NF-κB as well as specific p50 and p65 binding were enhanced in *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice after O_3. However, no differences in either total NF-κB, or supershifted p50 or p65 binding were found between genotypes (Figure 2.4).

LPS but not O_3 induces differential MIP-2 production in CD14$^{+/+}$ *and Cd14*$^{-/-}$ *mice.* The neutrophil chemoattractant MIP-2 is known to increase in response to LPS and to O_3. To confirm that LPS-induced MIP-2 production is dependent on CD14, we challenged *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice with either LPS or saline. MIP-2 expression was not different between *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice after saline. MIP-2 levels were 4.5 fold higher in *Cd14*$^{+/+}$ mice compared to *Cd14*$^{-/-}$ mice after LPS (Figure 2.5A). We then hypothesized that if CD14 contributed to the inflammatory response to O_3, MIP-2 would be attenuated in *Cd14*$^{-/-}$ compared to *Cd14*$^{+/+}$ mice after O_3. MIP-2 production increased significantly in response to O_3 compared to air in both genotypes. MIP-2 levels were, however, not significantly different between *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice after O_3 (Figure 2.5B).

CD14 does not influence production of cytokines MIP-2, TNF-α, and IL-6 associated with TLR4 in response to O_3. MIP-2, TNF-α and IL-6 are pro-inflammatory cytokines that

increase in response to O_3. Moreover, TNF-α and IL-6 are induced via TLR4-mediated NF-κB activation. We hypothesized that mRNA expression of these cytokines would be significantly higher in *Cd14*$^{+/+}$ compared to *Cd14*$^{-/-}$ mice in response to O_3. No differences in mRNA expression of MIP-2, TNF-α, and IL-6 were found between *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice after air exposure (Figure 2.6). O_3 significantly increased expression of these inflammatory mediators in *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice over the corresponding air control levels. However, no significant CD14 effect was detected in the O_3-induced expression of these genes.

Discussion

O_3 inhalation can increase the susceptibility of mice to bacterial infection and thus can alter the immune response in the lung, possibly by modulating alveolar macrophage function (Gilmour et al., 1993). We previously demonstrated that the innate immune receptor TLR4 contributes to O_3-induced hyperpermeability in a mouse model (Kleeberger et al., 2000). Inasmuch as CD14 is required for TLR4-mediated signaling by LPS and RSV, the current study was designed to test the hypothesis that CD14 is also essential in O_3-induced pulmonary hyperpermeability and inflammatory responses. In contrast to our hypothesis, we found that O_3-induced increases in mean numbers of PMNs and total protein were not significantly different between *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice. Furthermore, we observed that the expression of downstream proinflammatory cytokines MIP-2, IL-6 and TNF-α was not different in *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice after exposure to O_3.

Clinical studies have demonstrated that constitutive CD14 levels correlate with the magnitude of PMN influx in response to small doses of inhaled LPS (Alexis et al., 2001).

Tasaka *et. al.* (2003) and Jiang *et. al.* (2005) confirmed that CD14 is critical for the TLR4-mediated response to LPS via MyD88-dependent and MyD88-independent pathways (Jiang et al., 2005; Tasaka et al., 2003). On the cell surface, CD14 acts in concert with lipopolysaccharide binding protein (LBP) to bring the lipid A portion of LPS into close proximity to MD2 that, in turn, complexes to the extracellular domain of TLR4 (Schumann, 1992; Wright et al., 1990). CD14 increases the sensitivity of the TLR4-MD2 complex to LPS (Akashi et al., 2000; da Silva Correia et al., 2001). In addition, Kurt-Jones *et. al.* established that CD14 and TLR4 are required for response to RSV (Kurt-Jones et al., 2000) and suggest that CD14 and TLR4 do not require LBP in some protocols. Signaling causes nuclear translocation of NF-κB and activated protein-1 (AP-1) to induce proinflammatory gene expression.

In the present study, IRAK-1 and TRAF-6 expression did not differ significantly between *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice after O_3. Similarly, O_3-induced NF-κB binding did not differ between *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice. These results suggest that TLR4 downstream signaling and activation in response to O_3 occurs independently of CD14. We also determined that *Cd14* gene disruption did not affect transcription or translation of TLR4. TLR4 can activate MyD88-dependent and MyD88-independent signaling cascades (Akira and Hoshino, 2003; Kawai et al., 2001; Kovarik et al., 1998). Although we did not assess whether O_3-induced MyD88-independent signaling requires CD14, it is unlikely that CD14 functionally affects the MyD88-independent pathway given that differential expression of downstream effectors and inflammation were not observed between *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice in response to O_3.

Our findings are consistent with those of Hollingsworth *et. al.* (2004) where no differences in O_3-induced inflammation were found between *Tlr4*-sufficient and *Tlr4*-deleted

mice (Hollingsworth et al., 2004). The *Tlr4*-deleted and *Cd14*$^{-/-}$ mice were on the same genetic background (C57BL/6) and both genes have previously been shown to be critical to the response to LPS (Haziot et al., 1996; Hollingsworth et al., 2004). Genetic background may modulate the response to O_3. The role of CD14 should be tested in a C3H model, the same background as the C3H/HeJ (*Tlr4*-mutated) strain that has shown differential susceptibility to O_3 (Kleeberger et al., 2001). To test this hypothesis, we would generate CD14-deficient mice on the C3H background by repeated backcross matings and retest our current hypothesis. A second possibility would be to administer an *in vivo* CD14-depleting antibody to C3H mice and reassess inflammatory responses to O_3 or air in comparison to C3H animals that received sham antibody.

We found that LPS-induced PMN inflammation was significantly greater in *Cd14*$^{+/+}$ mice compared to *Cd14*$^{-/-}$ mice, and confirmed that LPS-induced neutrophilia is dependent on CD14. Haziot *et. al.* demonstrated that LPS-resistant *Cd14*$^{-/-}$ mice produce less TNF-α and IL-6 compared to control mice in response to LPS (Haziot et al., 1996). Consistent with these observations, we found that LPS-induced levels of the neutrophil chemoattractant MIP-2, a mouse analog of IL-8, differed significantly between *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice. However, MIP-2 protein production as well as IL-6 and TNF-α mRNA were not different between the two mouse genotypes after O_3. These results suggest that CD14 does not functionally contribute to O_3-induced inflammatory mediator production.

Polymorphisms in *CD14* are associated with differential responsiveness to LPS (Baldini et al., 2002) and increased risk for atopy in childhood (Baldini et al., 2002; Kleeberger and Peden, 2005; Vercelli, 2002). Interestingly, Raby *et. al.* (2002) found that polymorphisms in *TLR4* (D299G) are not associated with asthma or atopy-related phenotypes while other

polymorphisms seem to be associated with increased severity to atopy (Raby et al., 2002). It is possible that the role of TLR4 in LPS-mediated atopy may be dependent on the dose or timing of exposure. Moreover, these studies suggest that CD14 may have a distinct role in the atopic response compared to TLR4 and suggest that these two cell-surface proteins may be stimulus-specific.

CD14-independent LPS recognition has been suggested in recent studies. One model describes a CD14-independent, MyD88 dependent pathway that leads to the induction of TNF-α and IL1-β (Perera et al., 1997). Taxol, an antitumor agent mechanistically linked to LPS, has also been shown to induce CD14-dependent and CD14-independent pathways in *Cd14*$^{-/-}$ mice (Tasaka et al., 2003). In addition, Triantafilou *et. al.* determined that TLR4 interacts with a heterogeneous activation cluster consisting of heat shock proteins 70 and 90 (HSP70, HSP90), chemokine receptor 4 (CXCR4), and growth differentiation factor 5 (Triantafilou et al., 2001). In particular, HSP70 has been associated with the response to O_3 (Su and Gordon, 1997; Wong et al., 1996) and is thought to be a potential ligand of TLR4 (Vabulas et al., 2001). It is possible that TLR4-mediated O_3-induced inflammation may occur when O_3 interacts directly or indirectly with HSP70 that then combines with other extracellular proteins to form a CD14-independent heterogeneous activation cluster. However, further studies are necessary to confirm this hypothesis.

Numbers of PMNs were elevated in *Cd14*$^{-/-}$ mice compared to *Cd14*$^{+/+}$ mice after 72 h O_3 but this increase was not statistically significant. We attribute this difference to a number of different factors. The soluble form of CD14 (sCD14) attenuates LPS-induced macrophage activation by competitively binding LPS (Le-Barillec et al., 1999; Wurfel et al., 1995) and is therefore thought to protect against inflammation. The elevated PMNs in *Cd14*$^{-/-}$ mice could

be due at least partially to the absence of sCD14 in the knockout animals. In addition, CD14 is part of a macrophage recognition system that leads to phagocytosis of apoptotic cells (Devitt et al., 2004; Schlegel et al., 1999). Apoptotic cells may occur as a result of oxidative damage sustained by exposure to O_3 (Kirichenko et al., 1996). The elevated number of PMNs in *Cd14*$^{-/-}$ mice could also be due to the reduced ability to clear apoptotic cells in these animals.

In conclusion, we have shown that TLR4-mediated lung injury occurs independently of CD14 in response to O_3. Our results suggest that molecular mechanisms of O_3 and LPS-stimulated TLR4 signaling are stimulus specific. Direct or indirect oxidative damage to the TLR4 receptor itself may contribute to differential response to O_3. Investigating mechanisms through which TLR4 signaling modifies susceptibility responses to O_3, will improve understanding of the innate immune response to airway injury.

Figure 2.1: Effect of targeted gene disruption of Cd14 on LPS-induced inflammation.

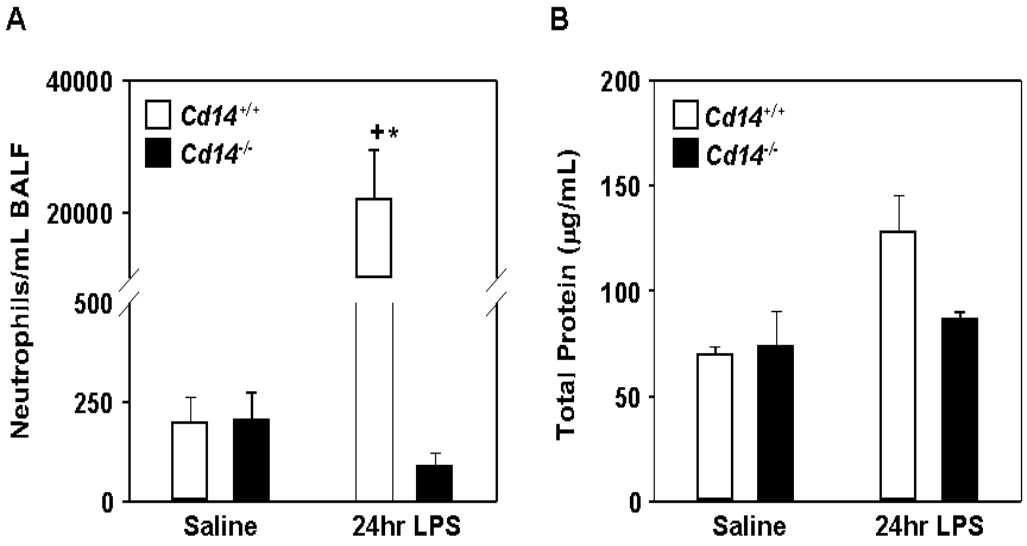


Figure represents changes in number of polymorphonuclear leukocytes (PMNs) (A) and total protein concentration (B) recovered in the bronchoalveolar lavage fluid (BALF) between air and 0.3 ppm O_3 in *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice. Values are means ± SE (n = 5/group). * $P < 0.05$, saline vs. 1.0 µg/kg LPS. + $P < 0.05$, *Cd14*$^{+/+}$ vs. *Cd14*$^{-/-}$.

Figure 2.2: Effect of targeted gene disruption of Cd14 on O_3-induced inflammation.

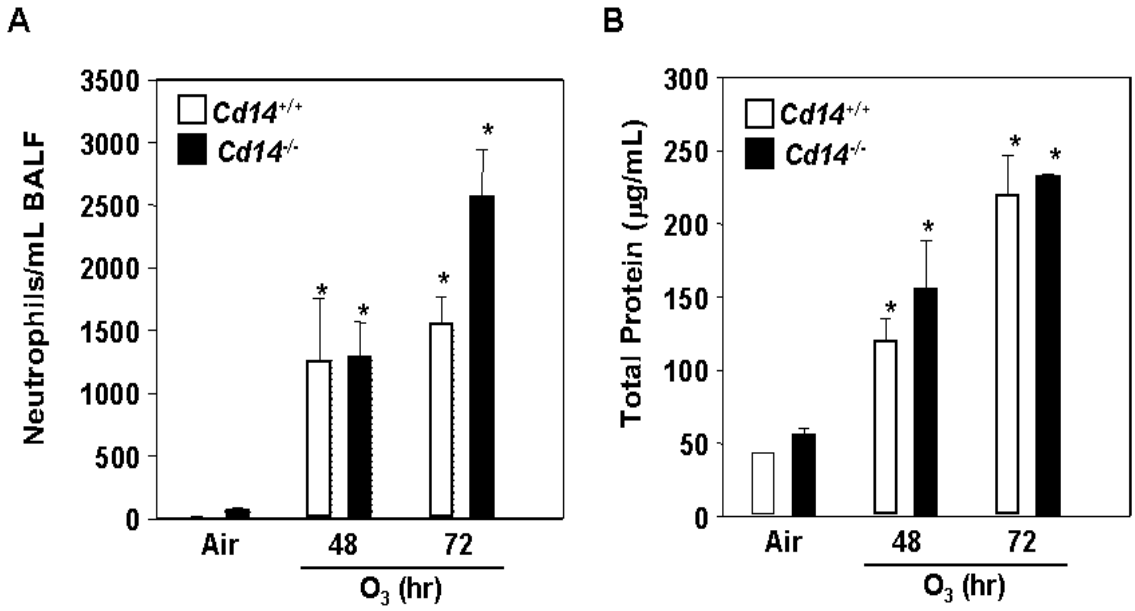


Figure represents changes in numbers of polymorphonuclear leukocytes (PMNs) (A) and total protein concentration (B) from recovered bronchoalveolar lavage fluid (BALF) of *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice exposed to air or 0.3 ppm O_3. Data are presented as means ± SE (n = 5 /group). * $p < 0.05$, air vs. 0.3 ppm O_3.

Figure 2.3: Effect of targeted gene disruption of Cd14 on TLR4-signaling molecules in response to O_3.

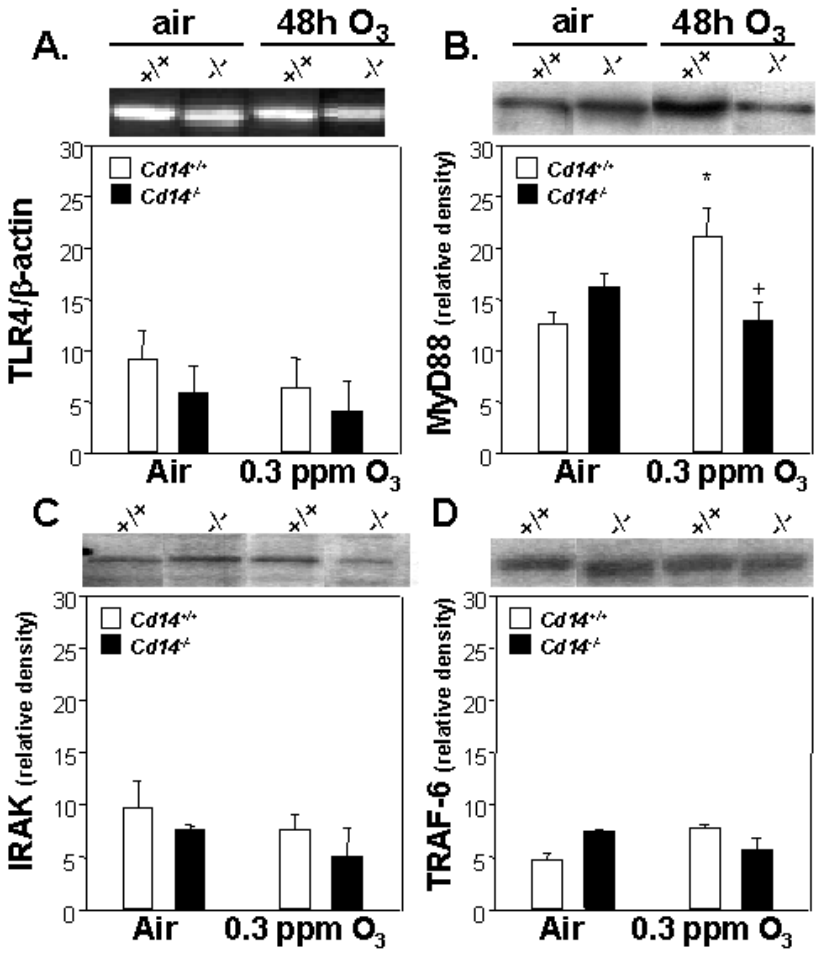


Figure represents protein levels of TLR4 (A), MyD88 (B), IRAK-1 (C), and TRAF-6 (D) in response to air or 0.3 ppm O_3 in *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice. Values are means ± SE (n = 6 per group). All values were normalized to actin. Air vs. O_3 * $P < 0.05$; *Cd14*$^{+/+}$ vs. *Cd14*$^{-/-}$ + $P < 0.05$.

Figure 2.4: NF-κB specific DNA binding in response to 0.3 ppm O_3 in $Cd14^{+/+}$ and $Cd14^{-/-}$ mice

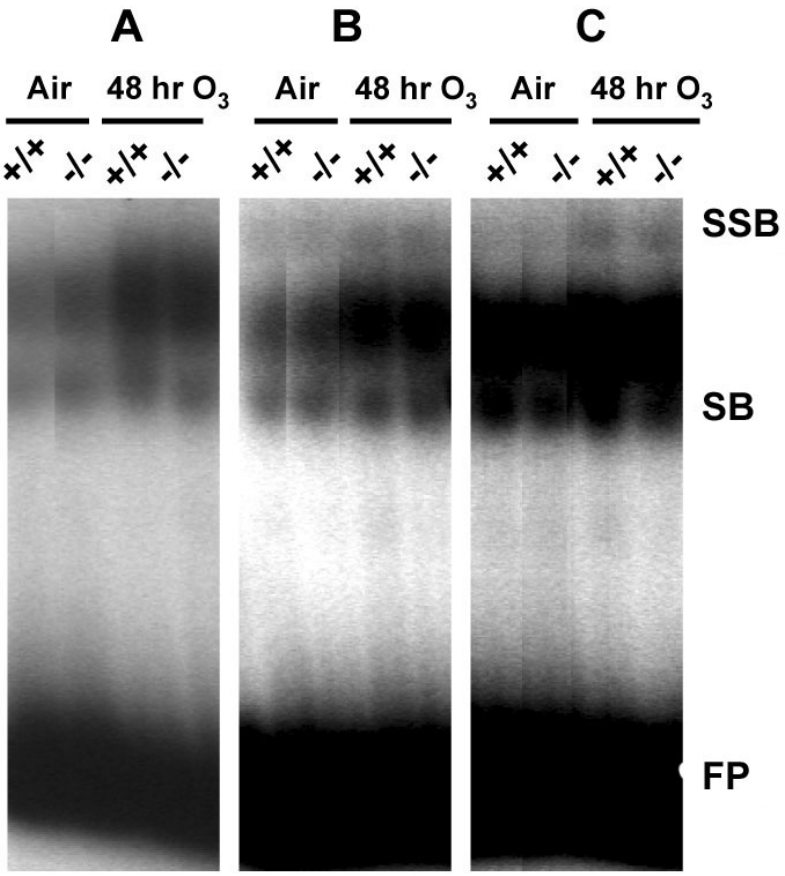


Figure represents NF-κB (A) and NF-κB-specific subunits p50 (B) and p65 (C) that bind to DNA in response to 0.3 ppm O_3 in *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice. Each lane represents nuclear protein pooled from three representative animals of each treatment group and is representative of three trials. SB, shifted band; SSB, supershifted band; FP, free probe.

Figure 2.5: MIP-2 levels in $Cd14^{+/+}$ and $Cd14^{-/-}$ mice in response to LPS or O_3.

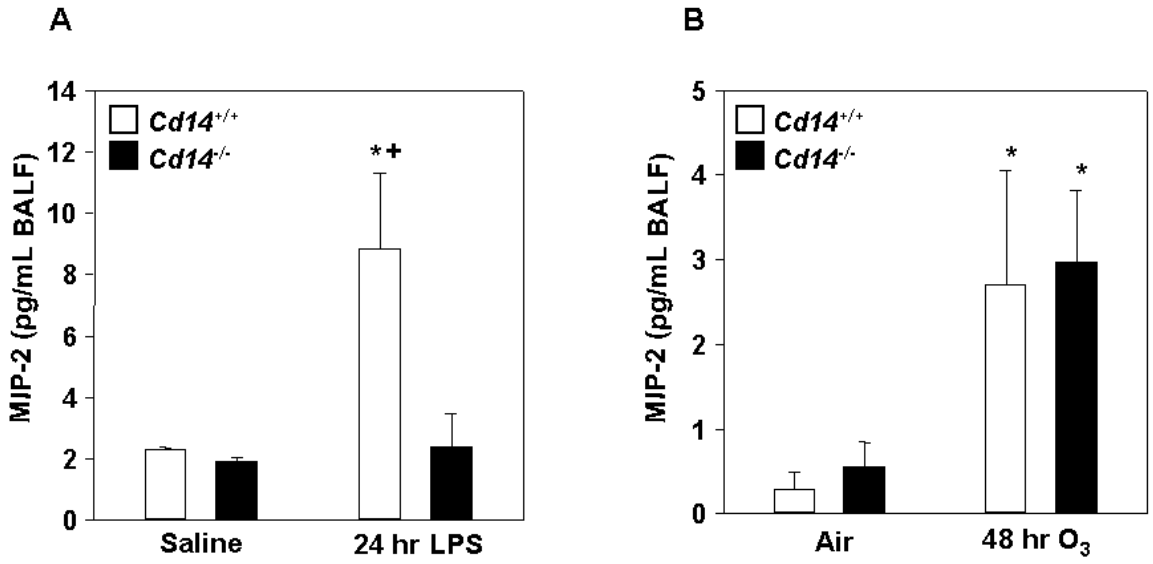
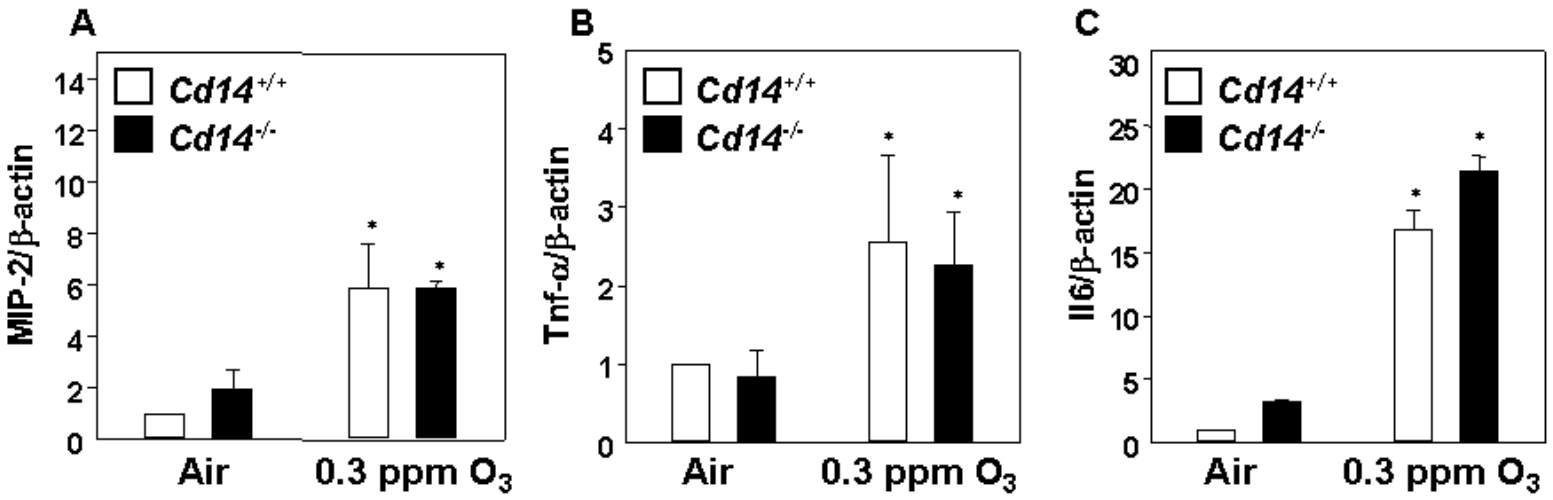


Figure represents MIP-2 levels in $Cd14^{+/+}$ and $Cd14^{-/-}$ mice in response to saline vs. 1.0 μg/kg LPS (A) or air vs. 0.3 ppm O_3 (B). Values are means ± SE (n = 5 per group). * $P < 0.05$, Air or saline vs. 1.0 μg/kg LPS or 0.3 ppm O_3, + $P < 0.05$, $Cd14^{+/+}$ vs. $Cd14^{-/-}$.

Figure 2.6: O_3-induced proinflammatory gene expression in $Cd14^{+/+}$ and $Cd14^{-/-}$ mice.



MIP-2 (A), *Tnf-α* (B), and *Il6* (C) mRNA expression in $Cd14^{+/+}$ and $Cd14^{-/-}$ mice after air or 0.3 ppm O_3 exposure. Data are presented as means ± SE (n = 3-5 per group). Air vs. 0.3 ppm O_3: *, $P < 0.05$.

Table 2.1: cDNA primer sequences and PCR conditions

Gene	Primer Sequences	GeneBank ID	Amplified Size (bp)	Annealing Temp (°C)	PCR Cycle No.
β-actin	Forward GTG GGC CGC TCT AGG CAC CA Reverse CGG TTG GCC TTA GGG TTC AGG	328504	221	55	27
Tlr4	Forward CCA ATT GAC TTC ATT CAG A Reverse CAT TTT TAA GTC TTC TCC A	21898	782	57	30
Tnf-α	Forward ATG AGC ACA GAA AGC ATG ATC Reverse TAC AGG CTT GTC ACT CGA ATT	21926	276	55	30
Cd14	Forward ATC CGA AGC CAG ATT GGT CCA Reverse AAC TGA GAT CCA CGA GCG TGA	12475	782	65	30
Il-6	Forward AAG AAC GAT AGT CAA TTC CA Reverse GAT CTC AAA GTG ACT TTT AG	16193	649	57	40
Mip-2	Forward AGC TGC GCT GTC AAT GCC TGA Reverse GGC ACA TCA GGT ACG ATC CAG	20310	288	57	30

TLR4, Toll-like receptor 4; IL, Interleukin; TNF, tumor necrosis factor; MIP, macrophage inhibitory protein; Temp, temperature

CHAPTER 3: IL-12 AND IL-18 RECEPTOR REGULATE OZONE-INDUCED LUNG INFLAMMATION

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Supported by the National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services

Running Head: IL-12 and IL-18 receptor-mediated O_3-induced inflammation

Abstract

Ambient ozone (O_3) induces airways inflammation and alters immune responses. Interleukins (IL-) 12 and 18 regulate production of inflammatory cytokines. Previous work has shown that the innate immune receptor, toll-like receptor 4 (TLR4) positively modulates O_3-mediated inflammation. The link between TLR4 and IL-12 and IL-18 production in response to O_3 has not been shown. We hypothesized that TLR4 can mediate O_3-induced *Il12* and *Il18* expression and that deficiencies in IL-12 and IL-18 would attenuate O_3-induced inflammation. *Tlr4*-normal (OuJ), *Tlr4*-mutated (HeJ), IL-12-alpha-deficient ($Il12\alpha^{-/-}$), IL-12-beta-deficient ($Il12\beta^{-/-}$), and IL-18 receptor-deficient ($Il18r1^{-/-}$) mice and corresponding wildtype (+/+) controls were exposed continuously to 0.3 ppm O_3 or filtered air for up to 72 hrs. Immediately following exposure, differential cell counts were assessed in bronchoalveolar lavage fluid (BALF). Tumor necrosis factor (TNF-α) gene expression was determined in whole lung homogenates. Compared to respective +/+ controls, no significant differences in BALF cells or total protein (a marker of lung permeability) were found between HeJ, $Il12\alpha^{-/-}$, $Il12\beta^{-/-}$, or $Il18r1^{-/-}$ mice after air exposures. *Il12* expression was not different at baseline between OuJ and HeJ mice. O_3-induced *Il12* expression was higher in OuJ than HeJ mice. *Il18* expression was elevated in OuJ compared to HeJ mice at baseline and after O_3. O_3 caused significant increases in BAL polymorphonuclear leukocytes (PMNs) and total protein at 48 and 72 hr in $Il12\alpha^{-/-}$, $Il12\beta^{-/-}$, and $Il18r1^{-/-}$ mice and +/+ controls, relative to respective air exposed control mice. However, compared to respective O_3-exposed +/+ mice, the number of PMNs was significantly lower in $Il12\beta^{-/-}$ mice, and significantly greater in $Il18r1^{-/-}$ mice after exposure to O_3. *Tnf*-α mRNA expression was also significantly increased in lung homogenates from O_3-exposed $Il18r1^{-/-}$ mice compared to

Il18r1$^{+/+}$ mice after exposure to O_3. Results demonstrated that TLR4 may control expression of the regulatory cytokines *Il12* and *Il18*. IL-12β contributes significantly to the pulmonary inflammatory response induced by exposure to 0.3 ppm O_3. In contrast, deletion of IL-18R1 enhanced O_3-induced inflammation, perhaps through upregulation of *Tnf-α* expression. Results suggest that IL-12 and IL-18R1 modulate pulmonary inflammation after oxidant injury.

Keywords: TLR4, IL-12, IL-18, IL-18R1, ozone, lung, inflammation

Introduction

Ozone (O_3) is a prevalent constituent of air pollution. Experimental and epidemiological studies have associated O_3 exposures with exacerbation and increased incidence of asthma (Bayram et al., 2001; Hiltermann et al., 1997; McConnell et al., 2002; Neuhaus-Steinmetz et al., 2000) and increased mortality and morbidity (Touloumi et al., 1997). Animal studies have shown that O_3 induces airway inflammation, hyperpermeability, and airway hyperresponsiveness (Kleeberger, 1995; Plopper et al., 1998; Smith, 1986; Toward and Broadley, 2002). O_3 inhalation can also increase the susceptibility of mice to bacterial infection and thus can alter the immune response in the lung (Gilmour et al., 1993). Pre-exposure to O_3 may sensitize individuals to a secondary stimulus, resulting in prolonged inflammation, and expression of cytokine and chemokine messages (Johnston et al., 2002). The regulatory cytokines interleukin (IL-) 12 and IL-18 have been proposed to correlate with O_3-induced inflammation and production of inflammatory mediators such as tumor necrosis factor alpha (TNF-α) (Cho et al., 2001). However, the functional role of these cytokines in the pathogenesis of O_3-induced lung injury has not been investigated.

Positional cloning identified toll-like receptor 4 (*Tlr4*) as a candidate gene for susceptibility to O_3-induced lung hyperpermeability (Kleeberger et al., 1997). C3H/HeJ (HeJ) mice possess a missense mutation in the third exon of *Tlr4* (Poltorak et al., 1998) and are resistant to O_3-induced hyperpermeability compared to *Tlr4*-normal C3H/HeOuJ (OuJ) mice (Kleeberger et al., 2000). LPS, a TLR4 ligand, can induce IL-12 and IL-18 expression *in vitro* via the c-Rel subunit of NF-κB (Mason et al., 2002; Sanjabi et al., 2000).IL-12 is a heterodimeric protein consisting of p35 (IL-12α) and p40 (IL-12β) subunits (Gately et al., 1998; Trinchieri, 2003) and enables communication between the innate and adaptive

responses. IL-12 is produced by B-cells and macrophages, promotes Th1 responses, and suppresses Th2 responses by stimulating naïve T cells to differentiate into interferon gamma (IFN-γ) secreting T-cells (Gately et al., 1998).

IL-18, initially called IFN-γ inducing factor, is an 18 kD single polypeptide that shares structural homology with IL-1 (Okamura et al., 1995). It is derived from the enzymatic cleavage of a 23kD precursor by the caspase IL-1beta converting enzyme (ICE) that is activated by LPS (Schumann et al., 1998) and is expressed in macrophages, T-cells, B-cells and airway epithelium (Cameron et al., 1999). IL-18 has been associated with rheumatoid arthritis (Liew, 2001), LPS-induced liver injury (Sakao et al., 1999), and asthma (Wild et al., 2000). In humans, low levels of IL-18 have recently been associated with asthma and sarcoidosis (Ho et al., 2002). The heterodimeric IL-18 receptor (IL-18R1) is expressed on macrophages and naïve T-cells and shares structural homology with other IL-1 superfamily members IL-1R and TLR4. IL-18R1 has a ligand binding subunit (IL1Rrp or IL-18Rβ) and a signal transducing unit (IL-18acPL, IL-18Rα). The IL-18 Receptor-α subunit uses the MyD88, IRAK, TRAF6, NF-κB pathway and cellular levels of this subunit are highest in activated Th1 cells (Akira, 2000).

IL-12 and IL-18 are regulatory cytokines thought to bridge innate and adaptive immune responses due to their roles in inflammatory and T-cell mediated responses (Hofstra et al., 1998; Lewkowich et al., 2005; Wang et al., 2004). The objective of this study is to determine whether these cytokines are under the regulatory control of TLR4 and, in turn, whether these cytokines contribute to innate responses (neutrophilic inflammation) in response to O_3. Because IL-12 and IL-18 have potential roles in TLR4-mediated responses to LPS (Dobashi et al., 2001; Kollet and Petro, 2006) and TLR4 also mediates inflammatory responses to O_3,

we hypothesized that these cytokines would be differentially expressed in *Tlr4*-normal and *Tlr4*-mutated mice and further, that these cytokines are important in O_3-induced pulmonary inflammatory responses.

Materials and Methods

Animals. Male (6-8 weeks) C3H/HeOuJ (OuJ), C3H/HeJ (HeJ), *Il12a*$^{-/-}$ (B6.129S1-*Il12*α^{tm1Jm}), *Il12*$\beta^{-/-}$ (B6.129S1-IL12β^{tm1Jm}/J), *IL18*$^{-/-}$ (B6.129P2-*Il18*^{tm1Aki}/J), *Il18r1*$^{-/-}$ (B6.129P2-Il-18R1^{tm1Aki}/J), and C57BL/6 (*Il12a*$^{+/+}$, *Il12*$\beta^{+/+}$, *IL18*$^{-/-}$, and *Il18r1*$^{+/+}$) were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were provided water and mouse chow (NIH-31) *ad libitum*. All experimental procedures were conducted in accordance with approved NIEHS Animal Care and Use protocols.

O_3 Exposure. Mice were exposed to 0.3 ppm O_3 or filtered air for 6, 24, 48 or 72 hr, 23.5 hr/day. O_3 was generated from ultra-high purity air (< 1 ppm total hydrocarbons; National Welders, Inc., Raleigh, NC) using a silent arc discharge O_3 generator (Model L-11, Pacific Ozone Technology, Benicia, CA). Constant chamber air temperature (72 ±3° F) and relative humidity (50 ± 15%) were maintained. Water and mouse chow were provided during the exposure.

Necropsy and Bronchoalveolar Lavage (BAL) Analysis. Mice were euthanized (sodium pentobarbital, ip., 104 mg/kg) immediately following O_3 exposure. The right lung was lavaged four times with ice-cold Hanks' balanced salt solution (HBSS) (26.0 ml/kg, pH 7.2-7.4). The left lung was snap frozen in liquid nitrogen. Recovered bronchoalveolar lavage

fluid (BALF) was centrifuged (500 × g, 4°C, 10 min.). BALF protein and cell analysis followed standard laboratory protocols (Cho et al., 2001). Briefly, mean total protein was determined from the supernatant via the Bradford Assay (BioRad, Hercules, CA). Cell pellets were resuspended in 1 mL ice-cold HBSS and total cell numbers were counted with a hemacytometer. A 200 μL aliquot was used to make cytospin slides (300 rpm for 10 min) (Shandon Southern Products, Pittsburgh, PA) and slides were stained with Wright-Giemsa stain (Diff-Quik; Baxter Scientific Products, McGaw Park, IL) for differential cell analysis.

Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR). Total mRNA was isolated from lung homogenates using a Qiagen Midi-RNA extraction kit (Valencia, CA). One microgram of total lung RNA from individual mice were reverse transcribed according to previously published protocols (Cho et al., 2001). PCR amplification was performed with an aliquot of cDNA (10 μl) using forward and reverse primers specific for mouse *Il12p40, Il18,* and *TNF*-α (Table 1). β-actin was amplified as an internal control. Reactions were electrophoresed in 1.2 % agarose gels. cDNA Bands were visualized and quantitated with a Bio-Rad Gel Doc 2000 system (Bio-Rad Laboratories, Hercules, CA). The cDNA intensity ratios of each gene to β-actin were determined.

Histological analysis: Left lungs were inflated with 10% formalin, removed *en bloc*, and immersed in 10% formalin. After 24 hrs, lungs were cut into three cross sections, and processed for microscopic slides. Five micron (μM) thick lung sections were stained with hematoxylin and eosin (H&E). The extent of peribronchiolar and perivascular inflammation

in *Il12β*$^{-/-}$ and *Il18r1*$^{-/-}$ compared to *Il12β*$^{+/+}$ and *Il18r1*$^{+/+}$ mice respectively was qualitatively assessed with the assistance of a pathologist.

Nuclear Protein Isolation and Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared from left lung lobes following 6, 24 or 72 hr exposure to 0.3 ppm O_3 or filtered air using a Nuclear Extraction Kit (Active Motif, Carlsbad, CA). An aliquot (6 μg) of nuclear protein was incubated in a binding buffer (10mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5 % glycerol, 1 mM DTT, 0.2 μg PolydI-dC, 1 mM PMSF) in a total volume of 19 μl. After 15 min incubation, 1 μl (3 x 10^4 cpm) of [γ^{32}P] dATP end-labeled oligonucleotide containing a NF-κB or AP-1 consensus sequence was added to the reaction and followed by 30 min incubation at room temperature. The mixture was subjected to electrophoresis on a 4 % SDS-PAGE with 0.25X TBE buffer. The gels were autoradiographed at –70° C. To analyze specific binding of NF-κB subunits, nuclear proteins (6 μg) were pre-incubated with either 4 μg of anti-p65 NF-κB antibody (sc-372X; Santa Cruz), and processed for gel shift assay as described above.

Statistics. All data were expressed as group means ± SE. The data were transformed, if necessary, to ensure normal data distribution and equal variance. Two-way ANOVA was used to evaluate the effects of exposure (air or O_3) or genotype (*Il12a*, *Il12β*, *Il18r1*$^{+/+}$ or *OuJ* vs. *Il12α*$^{-/-}$ *Il12β*$^{-/-}$, *Il18r1*$^{-/-}$ or *HeJ*) on pulmonary toxicity. The dependent variables were lung protein concentration, PMN cell counts, and cytokine mRNA and protein expression. The Student Newman Keuls *a posteriori* test was performed to compare group means. All statistical analysis was performed using a commercial statistics package

(SigmaStat, Systat Software, Point Richmond, CA). Statistical significance was accepted at $p < 0.05$.

Results

Il12β and Il18 expression in OuJ and HeJ mice. *Il12β* mRNA expression was not different between C3H/HeOuJ and C3H/HeJ mice after exposure to air (Figure 3.1A). However, *Il12β* mRNA expression was significantly higher in C3H/HeOuJ mice compared to C3H/HeJ mice after 24, 48 and 72 hr exposure to O_3. *Il18* mRNA expression was significantly higher in C3H/HeOuJ mice compared to C3H/HeJ mice after air exposure. *Il18* mRNA remained significantly higher in C3H/HeOuJ mice compared to C3H/HeJ mice during O_3 exposure, and O_3 significantly increased *Il18* mRNA at 72 hr (Figure 3.1B).

Contribution of IL-12 to the PMN response to O_3. To determine the role of IL-12 p35 (IL12α) and p40 (IL12β) subunits in O_3-induced inflammation, we compared BALF cell profiles and protein in $Il12\alpha^{+/+}$, $Il12\beta^{+/+}$, $Il12\alpha^{-/-}$, and $Il12\beta^{-/-}$ mice after exposure to air and O_3. No differences in mean numbers of PMNs and mean total protein recovered in BALF were found between $Il12a^{+/+}$ and $Il12a^{-/-}$ mice after exposure to filtered air. Relative to air controls, total numbers of PMNs and protein increased significantly in $Il12a^{+/+}$ and $Il12a^{-/-}$ mice after 48 and 72 hr exposure to O_3, however no significant differences were found between genotypes (Figures 3.2A and B). No differences in mean numbers of PMNs and mean total protein recovered in BALF were found between $Il12\beta^{+/+}$ and $Il12\beta^{-/-}$ mice after exposure to air. However, compared to respective air controls, total numbers of PMNs and protein increased significantly in $Il12\beta^{+/+}$ and $Il12\beta^{-/-}$ mice after 48 and 72 hr exposure to

O_3 (Figures 3.2C and D). Further, total numbers of PMNs were significantly decreased in *Il12β*$^{-/-}$ compared to *Il12β*$^{+/+}$ mice after 48 and 72 hr exposure to O_3 (Figure 3.2C). No differences in mean total protein were found between the two genotypes after O_3 (Figure 2D).

Peribronchiolar and perivascular inflammation in Il12β$^{+/+}$ *and Il12β*$^{-/-}$ *mice.* After exposure to air, no differences in lung histology were found between *Il12β*$^{+/+}$ and *Il12β*$^{-/-}$ mice. Compared to air-exposed *Il12β*$^{+/+}$ and *Il12β*$^{-/-}$ mice, perivascular and peribronchiolar inflammation was found in both genotypes after 48 and 72 hr exposure to O_3 (Figure 3.3). However, pulmonary inflammation was attenuated in *Il12β*$^{-/-}$ mice compared to similarly-exposed *Il12β*$^{+/+}$ mice (Figure 3.3).

Role of Il18 and Il18r1 in the PMN response to O_3*.* IL-18 is a ligand for IL18 receptor, a member of the IL-1 superfamily. To further characterize the functional role of IL-18 and its receptor, we compared pulmonary responses between *Il18*$^{+/+}$ and *Il18*$^{-/-}$ (Table 3.2) as well as between *Il18r1*$^{+/+}$ and *Il18r1*$^{-/-}$ mice exposed to filtered air or O_3 (Figures 3.4A and B). Mean numbers of PMNs were significantly increased in response to O_3 compared to air in *Il18*$^{+/+}$ and *Il18*$^{-/-}$ mice. However, there was no significant difference in O_3-induced neutrophilic inflammation between *Il18*$^{+/+}$ and *Il18*$^{-/-}$ mice (Table 3.2). Mean numbers of PMNs and mean protein concentration were significantly elevated in *Il18r1*$^{+/+}$ and *Il18r1*$^{-/-}$ mice exposed to O_3 for 48 and 72 hrs compared to genotype-matched air controls (Figures 3.4A and B). In addition, significantly greater numbers of PMNs were found in *Il18r1*$^{-/-}$ compared to *Il18r1*$^{+/+}$ mice after 72 hr exposure to 0.3 ppm O_3 (Figure 3.4A). Mean BALF

protein concentration did not differ significantly between *Il18r1*$^{+/+}$ and *Il18r1*$^{-/-}$ mice after 48 or 72 hr exposure to O_3 (Figure 3.4B).

Peribronchiolar and perivascular inflammation in Il18r1$^{+/+}$ *and Il18r1*$^{-/-}$ *mice.* In response to air, no inflammation occurred in *Il18r1*$^{+/+}$ and *Il18r1*$^{-/-}$ mice. Compared to respective air-exposed control mice, O_3 caused perivascular and peribronchiolar inflammation to increase in *Il18r1*$^{+/+}$ and *Il18r1*$^{-/-}$ mice. However, consistent with the BAL results above, inflammation was markedly enhanced in *Il18r1*$^{-/-}$ compared to *Il18r1*$^{+/+}$ mice (Figure 3.5).

TNFα levels in Il18r1$^{-/-}$ *compared to Il18r1*$^{+/+}$ *mice after 72 hr* O_3*.* We previously found that *Tnfα* accounts for a significant portion of the PMN response to O_3 in mice (Kleeberger et al., 1997). To determine whether differences in *Tnfα* expression may account for the differential inflammatory response in *Il18r1*$^{+/+}$ and *Il18r1*$^{-/-}$ mice, we measured *Tnfα* mRNA in lung homogenates in both genotypes after air and O_3. *Tnfα* expression did not differ significantly between the two genotypes after exposure to air (Figure 3.6). Relative to respective air-exposed controls, *Tnfα* expression was significantly increased after 72 hr of continuous O_3 in *Il18r1*$^{-/-}$ mice. After 72 hr O_3, *Tnfα* expression was significantly higher in *Il18r1*$^{-/-}$ compared to *Il18r1*$^{+/+}$ mice.

NF-κB activation in Il18r1$^{-/-}$ *compared to Il18r1*$^{+/+}$ *mice after exposure to* O_3*.* We previously found that O_3-induced inflammation mediated through TNF-α signaling involves NF-κB and AP-1 DNA binding (Cho et al., 2006). As a member of the IL-1 superfamily, IL-

18R1 shares many signaling components, including NF-κB̃. IL-18 signaling may also be mediated by MAPK signaling (Nakanishi et al., 2001), detectable by AP-1 activation. We hypothesized that NF-κB and AP-1 activation would be elevated in *Il18r1*$^{-/-}$ mice compared to *Il18r1*$^{+/+}$ mice after O_3 exposure, concurrent with increased neutrophilic inflammation in *Il18r1*$^{-/-}$ mice. NF-κB total binding was elevated in *Il18r1*$^{+/+}$ compared to *Il18r1*$^{-/-}$ at baseline and in response to O_3 (Figure 3.7A and B). However, specific p65 binding (indicated by supershifted bands) was increased in response to O_3 compared to air controls and O_3-induced specific p65 binding was higher in *Il18r1*$^{+/+}$ compared to *Il18r1*$^{-/-}$ mice (Figure 3.7B). AP-1 total binding was not different between the two genotypes at baseline. While AP-1 DNA binding was elevated in response to O_3 compared to air, no differences due to genotype were determined (Figure 3.7C).

Discussion

We previously demonstrated that TLR4 contributes to O_3-induced inflammation and hyperpermeability (Kleeberger et al., 2000). The molecular mechanisms underlying O_3-mediated hyperpermeability have been linked to iNOS (NOS2) and TLR4-associated *Nos2* mRNA expression (Kleeberger et al., 2001). However the TLR4 contribution to the innate immune response remains unclarified. IL-12 and IL-18 are regulatory cytokines thought to prime adaptive immune cells by modulating the Th1-Th2 cytokine profile (Kitasato et al., 2004). Both cytokines are produced by macrophages and have been associated with lung injury induced by LPS, a TLR4 ligand (Dobashi et al., 2001; Kollet and Petro, 2006; Sakao et al., 1999). The present study demonstrated that the production of *Il12* and *Il18* is dependent on TLR4. Moreover, inflammatory responses to O_3 in mice differ with targeted disruption of

Il12β and *Il18r1*, and suggest a pro-inflammatory role for IL-12 and an anti-inflammatory role for IL-18R1 in this model.

As a generalized receptor, TLR4 can initiate innate immune responses to LPS (Poltorak et al., 1998), respiratory syncytial virus (RSV) (Kurt-Jones et al., 2000), particles (Cho et al., 2005) and heat shock protein-mediated cell death (Triantafilou and Triantafilou, 2004). In addition, TLR4 is known to control activation of Th1 adaptive immune responses (Schnare et al., 2001) and probably Th2 driven responses to inhaled allergen (Eisenbarth et al., 2002). The TLR4 ligand LPS activates macrophages to release IL-12 (Brunda, 1994; Trinchieri, 2003) and IL-18 (Nakanishi et al., 2001). Together, these innate immune response effectors mediate protective immunity. In the present study, we showed that IL-12 and IL-18 mRNA expression was significantly higher in C3H/HeOuJ mice compared to C3H/HeJ mice in response to O_3. O_3-induced IL-18 protein levels were also dependent on TLR4 activation. Moreover, IL-18 mRNA expression was significantly elevated in C3H/HeOuJ mice compared to C3H/HeJ mice at baseline. These data support a role for TLR4 in production of IL-12 and IL-18, and suggest that these two cytokines may contribute to O_3-induced immune responses.

IL-12 stimulates interferon-gamma (IFN-γ) production, favors differentiation of T helper 1 (Th1) cells and has been linked to T-cell mediated diseases such as collagen-induced arthritis (Bradley et al., 2002; Malfait et al., 1998). IL-12 is known to bridge innate and adaptive immune responses as it is produced by macrophages, and binds to specific receptors expressed on activated T-cells (Brunda, 1994; Gately et al., 1998). Neutralization of IL-12 via anti-IL-12 antibody prevented lethality in an acute LPS model (Ozmen et al., 1994). We tested the functional relevance of IL-12 in O_3-induced inflammation by comparing

inflammatory responses in *Il12 p35*$^{-/-}$ (*Il12*$\alpha^{-/-}$) and *Il12 p40*$^{-/-}$ (*Il12*$\beta^{-/-}$) mice and their wildtype counterparts after exposure to air and 0.3 ppm O_3. O_3-induced neutrophilic inflammation was significantly attenuated in *Il12*$\beta^{-/-}$ but not in *Il12*$\alpha^{-/-}$ mice relative to wildtypes. The p35 subunit of IL-12 (IL-12α) does not transmit signals and is much less prevalent than the p40 (IL-12β) subunit (Wang et al., 2004), which may explain why a significant effect was observed in the *Il12*$\beta^{-/-}$ mice and not the *Il12*$\alpha^{-/-}$ mice. In addition, IL-12p40 forms heterodimers with either p19 or p28 to form the IL-12 family members IL-23 or IL-27, respectively (Kobayashi et al., 2003; Owaki et al., 2005). We therefore cannot exclude a possible pathological role for IL-23 and IL-27 for the observed O_3-induced neutrophilia.

Our results are consistent with those of Kawakami *et. al.* (Kawakami et al., 1999) who found a marked increase in inflammation when IL-12 was administered to *Cryptococcus neoformans*-infected mice compared to sham-administered controls and also those of Tanaka *et. al.* (Tanaka et al., 1996) who determined that *Il12*$\beta^{-/-}$ mice were resistant to liver injury in response to LPS compared to wildtype controls. Ours is the first report that IL-12 contributes to O_3-induced inflammation. In contrast, Wang *et. al.* (Wang et al., 2004) found that the absence of IL-12p40 led to marked increases in neutrophilic inflammation compared to wildtype mice exposed to respiratory syncytial virus (RSV). Thus, IL-12 may have different functional roles depending on the type or strength of the insult.

While best known for its synergistic effects with IL-12, IL-18 can act alone on immune cells. Elevated levels of IL-18 have been associated with rheumatoid arthritis, diabetes and Crohn's disease as well as lung diseases including asthma (Nakanishi et al., 2001; Tanaka et al., 2001; Wild et al., 2000). Moreover, IL-18 production has been correlated with TNF-α

levels in an endotoxin model (Sakao et al., 1999), and in rheumatoid arthritis patients (Liew, 2001). However, no studies have characterized the functional role of IL-18R1 in response to environmental exposures. We determined that IL-18 expression was suppressed in O_3-resistant, *Tlr4*-deficient C3H/HeJ mice, relative to the C3H/HeOuJ strain. We found that the total number of PMNs was significantly higher in *Il18r1*$^{-/-}$ compared to *Il18r1*$^{+/+}$ mice after 72 hr O_3 exposure. The protective effect of IL-18 has also been documented in response to mycobacterial infection (Mastroeni et al., 1999; Sugawara et al., 1999) and viral infection (Fujioka et al., 1999; Sareneva et al., 1998).

We have previously shown that TNF-α causes O_3-induced increases in PMNs (Cho et al., 2001). Moreover, IL-18 can induce TNF-α (Jordan et al., 2001). In the present study, O_3-induced *Tnf*-α expression was significantly greater in *Il18r1*$^{-/-}$ compared to *Il18r1*$^{+/+}$ mice. Our findings are consistent with those of Sakao *et. al.* (Sakao et al., 1999) who demonstrated that *Il18*$^{-/-}$ mice were highly susceptible to LPS-induced endotoxin shock, and that this phenotype was associated with markedly elevated levels of serum TNF-α.

The mechanism underlying the IL18/TNF-α relationship is not clear. NF-κB is commonly associated with induction of pro-inflammatory genes such as *Tnfα*. Moreover, IL-18R1 can activate NF-κB via the MyD88 pathway (Nakanishi et al., 2001). In this study, DNA binding of total NF-κB was reduced in the absence of IL-18R at baseline and DNA binding of the p65 subunit of NF-κB was reduced in the absence of IL-18R in response to O_3. These data suggest that O_3-induced IL-18R1 signaling is mediated at least partially through NF-κB pathways. However, this finding does not correspond with the observed *Tnfα* expression that was elevated in *Il18r1*$^{-/-}$ compared to *Il18r1*$^{+/+}$ mice. We propose that NF-κB activation and associated TNF-α production in *Il18r1*$^{-/-}$ mice appear to be independently

regulated. O_3-induced TNF-α production may be induced via compensatory upregulation of alternative signaling pathways associated with IL-1Receptor or related family members (Kleeberger et al., 2000; Park et al., 2004). Moreover, the discrepancy between O_3-induced effects of the p65 subunit and total NF-κB binding indicate the p65 may act independently of NF-κB in another function.

The p38 mitogen activated protein (MAP) kinase pathway can result in activated protein (AP-1) activation. In response to LPS, IL-18 activated the MAP kinase pathway to prime neutrophils in a concentration-, time-, and calcium-dependent manner (Wyman et al., 2002). The present study demonstrated that O_3-induced AP-1 activation occurs independently of IL18R1, at the concentration and time points tested however. IL-18R1 mediated MAP kinase signaling pathway activation may be dose-dependent and stimulus-specific. The role of IL-18-mediated p38 MAP kinase activation in O_3-induced pulmonary inflammation upstream of AP-1 remains to be elucidated.

IL-12 and IL-18 may work synergistically to prevent Th2-driven responses to allergen (Hofstra et al., 1998; Lewkowich et al., 2005) and to RSV infection (Wang et al., 2004). In addition, IL-18 selectively induces ICAM-1, and IFN-γ expression and production is enhanced by IL-12 (Okamura et al., 1995; Stuyt et al., 2003; Yoshimoto et al., 1998). It is also possible that IL-12 and IL-18R1 interact to control the pulmonary inflammatory response to O_3, but the exact mechanisms of this interaction remain to be clarified.

In conclusion, we have shown that the innate immune receptor TLR4 may at least partially control expression of IL-12 and IL-18 during the pathogenesis of O_3-induced lung injury and inflammation. The p40 subunit of IL-12 (IL-12β) contributes to O_3-induced inflammation while the IL-18 receptor may play a role in reducing the inflammatory response to O_3,

possibly through regulation of TNF-α. These novel observations provide insight into the regulatory processes that govern oxidant-induced inflammation and suggest possible links between innate and regulatory responses that may prime adaptive immune responses. Human polymorphisms in *IL12P40* and *IL18R1* may help to identify individuals who are differentially susceptible to O_3 and thus lead to individual-specific treatments.

Table 3.1: cDNA primer sequences and PCR conditions

Gene	Primer Sequences	Gene Bank ID	Amplified Size (bp)	Annealing Temp (°C)	PCR Cycle No.
β-actin	Forward GTG GGC CGC TCT AGG CAC CA Reverse CGG TTG GCC TTA GGG TTC AGG	11461	221	55	27
Il18	Forward ACT GTA CAA CCG CAG TAA TAC GG Reverse AGT GAA CAT TAC AGA TTT ATC CC	16173	434	55	30
Tnfα	Forward ATG AGC ACA GAA AGC ATG ATC Reverse TAC AGG CTT GTC ACT CGA ATT	21926	276	55	30
Il12	Forward ATC CGA AGC CAG ATT GGT CCA Reverse AAC TGA GAT CCA CGA GCG TGA	16159	782	65	30

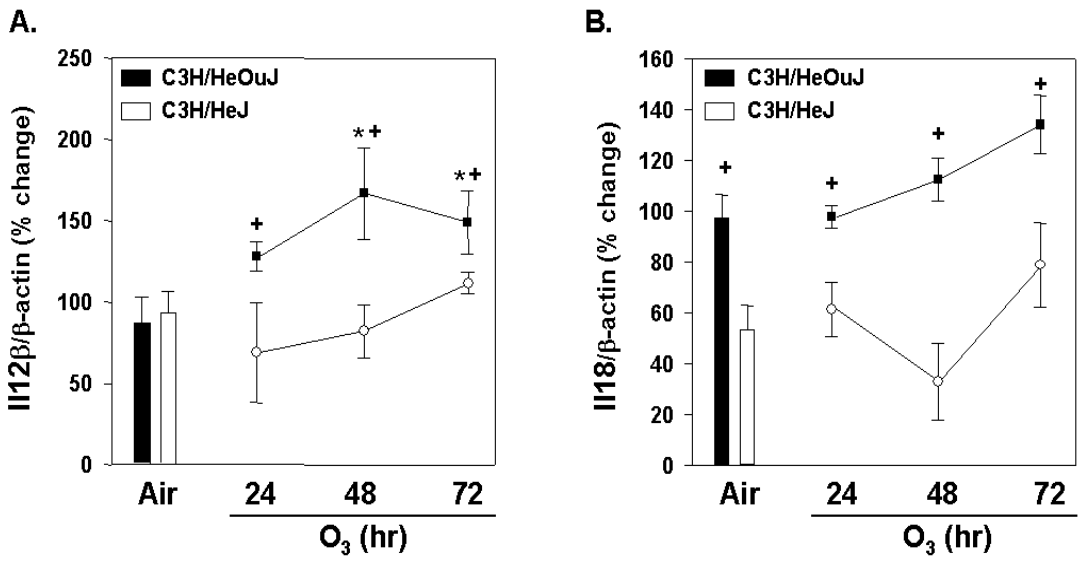
Il, Interleukin; TNF, tumor necrosis factor; Temp, temperature

Table 3.2: Effect of targeted disruption of Il18 on BALF inflammatory cells and total protein after exposure to filtered air or O_3.

Exposure	Genotype	Total Protein	Total Cells	Macrophages	Lymphocytes	Epith. Cells	PMNs
Air	*Il18*$^{+/+}$	65.2 ± 7.9	16,000 ± 1,730	15,500 ± 3,870	54 ± 17	342 ± 39	33 ± 13
	Il18$^{-/-}$	65.4 ± 6.1	16,800 ± 1,463	16,200 ± 3,191	132 ± 63	364 ± 70	23 ± 4
0.3 ppm O_3, 48 hr	*Il18*$^{+/+}$	275.2 ± 18.3	23,667 ± 12,004	21,500 ± 3,130	161 ± 15	429 ± 65	1,550 ± 139
	Il18$^{-/-}$	237.0 ± 30.3	22,273 ± 6,039	20,900 ± 2,211	113 ± 28	310 ± 37	1,190 ± 236
0.3 ppm O_3, 72 hr	*Il18*$^{+/+}$	321.8 ± 30.9	28,300 ± 3,537	26,000 ± 9,146	235 ± 141	332 ± 101	1,670 ± 208
	Il18$^{-/-}$	315.9 ± 20.4	24,300 ± 4,425	22,400 ± 6,421	79 ± 76	420 ± 136	1,390 ± 144

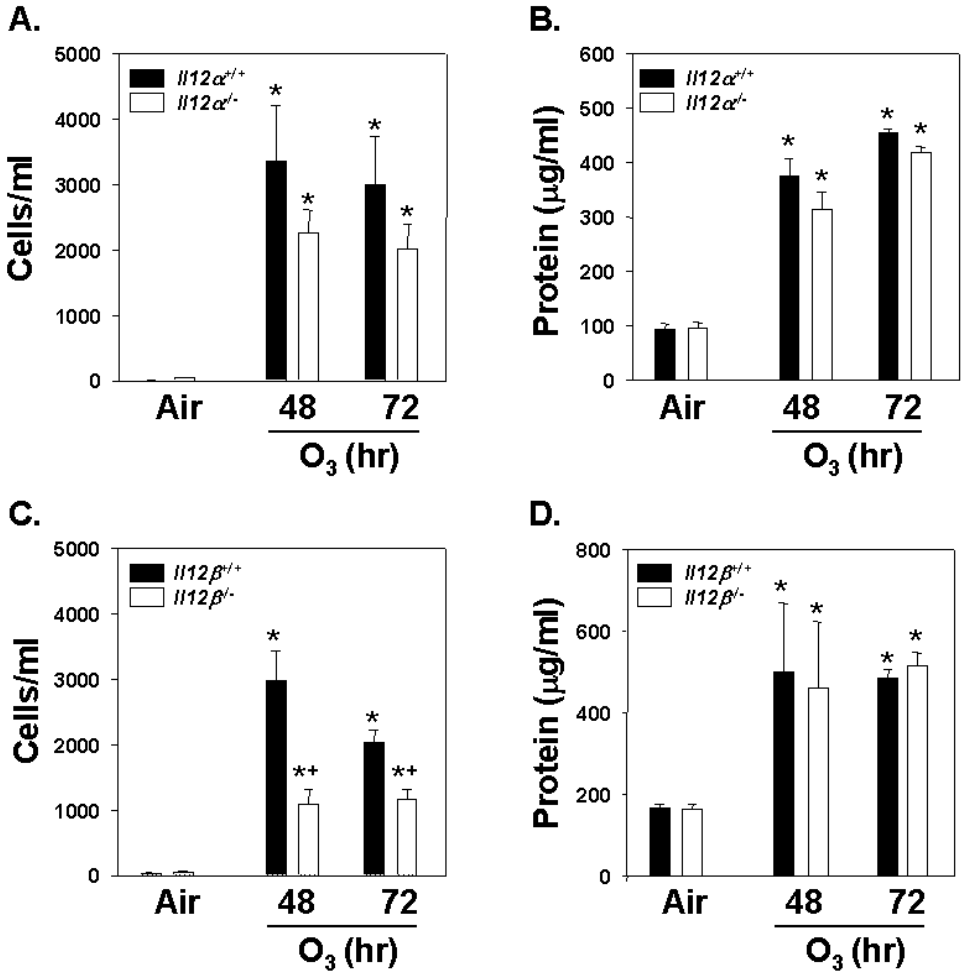
Table represents total protein, total cells, and specific inflammatory cells (macrophages, lymphocytes, epithelial cells, and neutrophils (PMNs)) from *Il18*$^{+/+}$ and *Il18*$^{-/-}$ mice in response to air or 0.3 ppm O_3.

Figure 3.1. Differential mRNA expression of Il12β and Il18 in C3H/HeJ and C3H/HeOuJ mice in response to O_3.



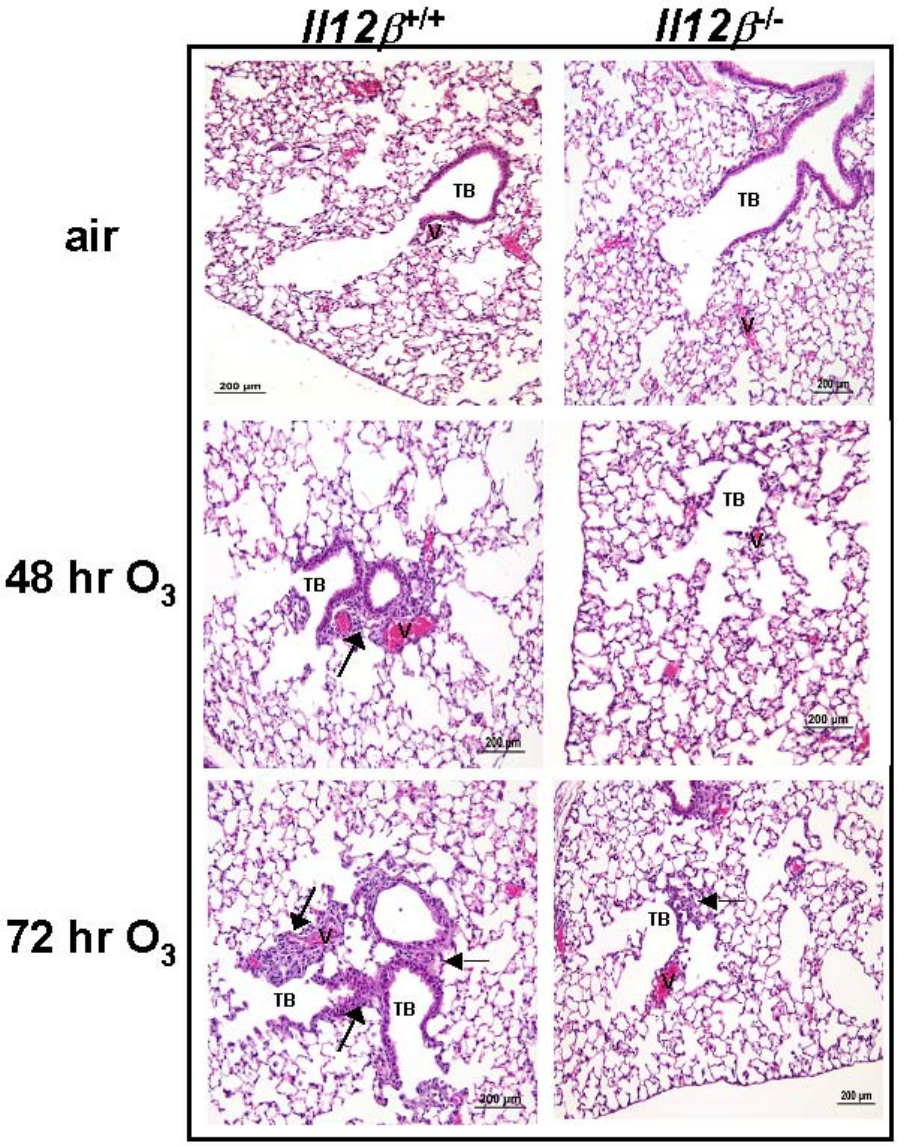
Differential expression of *Il12β* (A) and *Il18* (B) mRNA after exposure to air and 0.3 ppm O_3 in C3H/HeOuJ and C3H/HeJ mice. Values are means ± SE (n = 3 per group). * $P < 0.05$, air vs. 0.3ppm O_3. + $P < 0.05$, C3H/HeOuJ vs. C3H/HeJ.

Figure 3.2: Effect of targeted disruption of Il12 on O_3-induced inflammation.



O_3-induced inflammation and hyperpermeability responses in $Il12\alpha^{-/-}$ vs. $Il12\alpha^{+/+}$ mice (A and B) and in $Il12\beta^{+/+}$ and $Il12\beta^{-/-}$ mice (C and D) compared to air. PMN infiltration (A and C) and total protein (hyperpermeability) (B and D). Values are means ± SE (n = 3-6 per group). * $P < 0.05$ air vs. O_3; + $P < 0.05$, $^{+/+}$ vs. $^{-/-}$

Figure 3.3: Lung histology in Il12β$^{+/+}$ and Il12β$^{-/-}$ mice in response to air and O_3.



Perivascular and peribronchiolar neutrophilic inflammation (arrows) are prominent in O_3-exposed *Il12β*$^{+/+}$ compared to *Il12β*$^{-/-}$ mice. Terminal bronchiole (TB), Blood vessel (V). All images are magnified 32x. Histology is representative of n = 3 mice per group. Scale represents 200 µm.

Figure 3.4: Effect of targeted gene disruption of Il18r1 on O_3-induced inflammation.

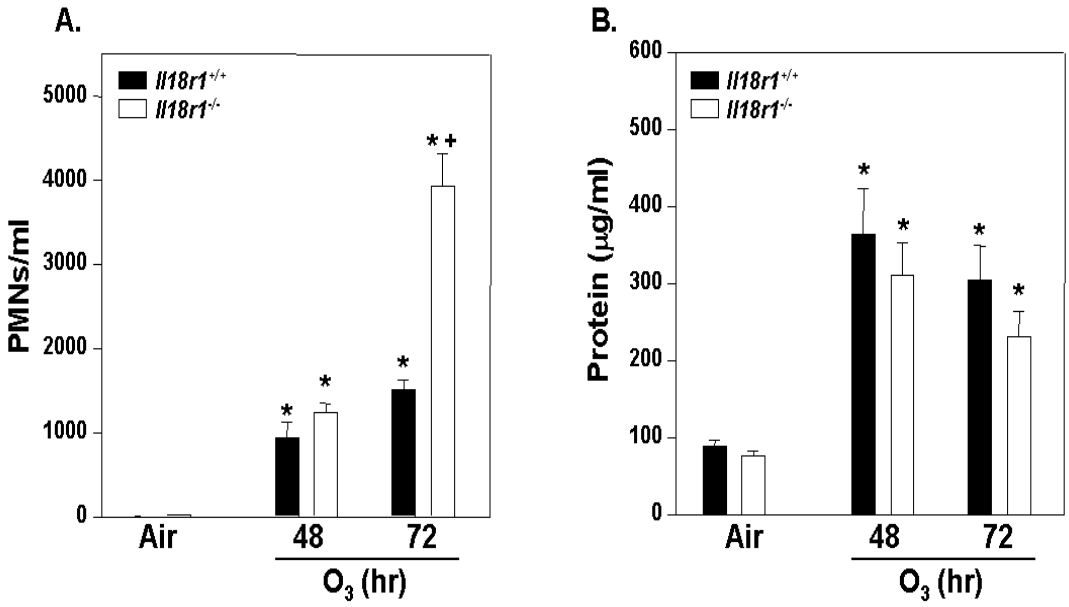
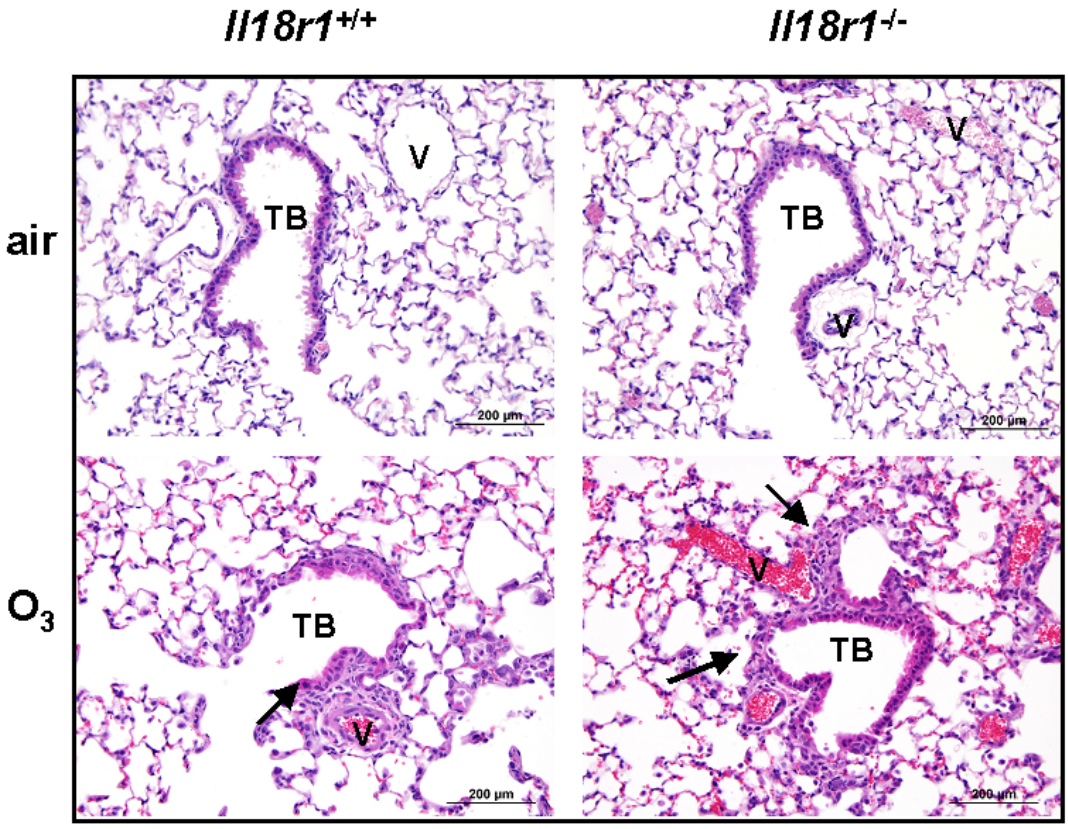


Figure represents PMN infiltration (A) and total protein (hyperpermeability) (B) in *Il18r1*$^{+/+}$ vs. *Il18r1*$^{-/-}$ mice exposed to air or 0.3 ppm O_3. Values are means ± SE (n = 7 per group for PMNs, n = 6-12 for total protein). * $P < 0.05$ air vs. O_3; + $P < 0.05$, *Il18r1*$^{+/+}$ vs. *Il18r1*$^{-/-}$

Figure 3.5: Lung histology in Il18r1$^{+/+}$ and Il18r1$^{-/-}$ mice in response to air and O_3.



Perivascular and peribronchiolar neutrophilic inflammation (arrows) in *Il18r1*$^{+/+}$ and *Il18r1*$^{-/-}$ mice in response to 0.3 ppm O_3 or air for 72 h. Inflammatory cells are prominent in O_3-exposed mice and are enhanced in O_3-exposed *Il8r1*$^{-/-}$ compared to *Il18r1*$^{+/+}$ mice. Terminal bronchiole (TB), Blood vessel (V). All images are magnified 32x and are representative of histology from individual mice (n = 6 – 12). Scale represents 200 µm

Figure 3.6: Differential Tnf-α expression in Il18r1$^{+/+}$ and Il18r1$^{-/-}$ mice in response to 0.3 ppm O_3.

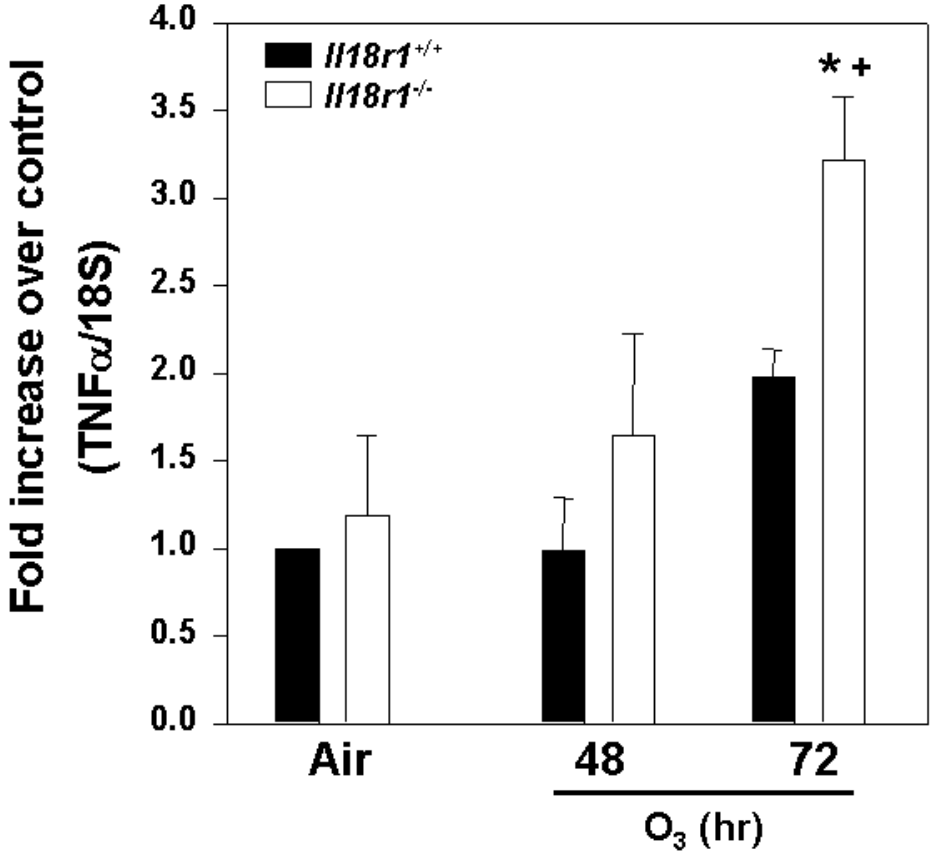


Figure represents *Tnfα* mRNA expression in *Il18r1*$^{+/+}$ vs. *Il18r1*$^{-/-}$ mice in response to air or 0.3 ppm O_3. Values are means ± SE and have been normalized to 18s (n = 3 per group). * P < 0.05 air vs. O_3; + P < 0.05, *Il18r1*$^{+/+}$ vs. *Il18r1*$^{-/-}$

Figure 3.7: Differential NF-κB and AP-1 binding in Il18r1$^{+/+}$ and Il18r1$^{-/-}$ mice in response to air and 0.3 ppm O_3.

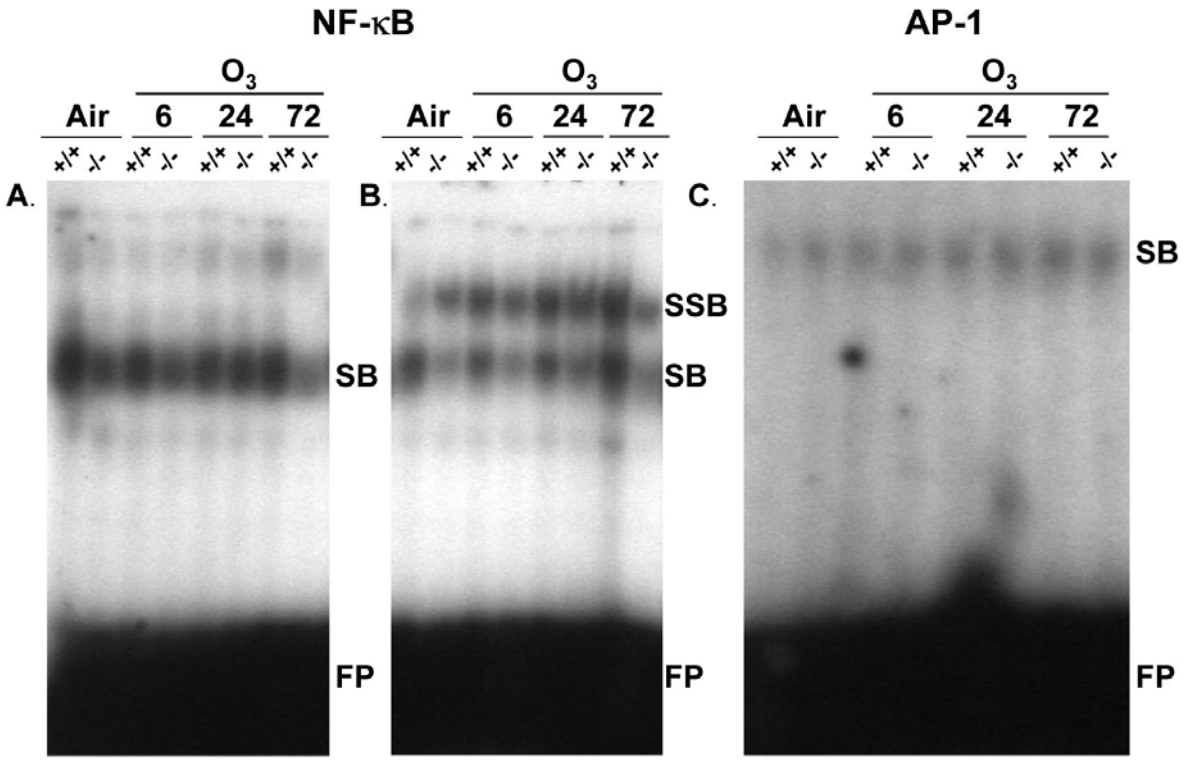


Figure represents total NF-κβ binding (A); specific subunit p65 supershift (B), total AP-1 (C) of *Il18r1*$^{+/+}$ vs. *Il18r1*$^{-/-}$ mice in response to air or 0.3 ppm O_3. Each lane represents nuclear protein pooled from three representative animals of each treatment group and is representative of three separate experiments. SB, shifted band; SSB, supershifted band; FP, free probe.

CHAPTER 4: PROTECTIVE ROLE OF IL-10 IN OZONE-INDUCED PULMONARY INFLAMMATION

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Supported by the National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services

Running Head: IL-10 and lung O_3 toxicity

Abstract

Pulmonary inflammation caused by ozone (O_3) is accompanied by increased inflammatory mediators including macrophage inflammatory protein (MIP)-2 and co-stimulatory molecules such as CD86. IL-10 is an anti-inflammatory cytokine that is known to inhibit MIP-2 and CD86 production. The current study was designed to test the hypothesis that murine IL-10 protects lungs from O_3-induced inflammation. *Il10*-deficient ($Il10^{-/-}$) and wild type ($Il10^{+/+}$) mice were exposed to 0.3-ppm O_3 or filtered air for 24, 48 or 72 hrs. Immediately following exposure, differential cell counts, and total protein (a marker of lung permeability) were assessed from bronchoalveolar lavage fluid (BALF). MIP-2 and CD86 levels were determined in lung homogenates. Transcriptional activation of nuclear factor kappa B (NF-κB) was determined in lung nuclear protein extracts. Mean numbers of BALF polymorphonuclear leukocytes (PMNs) were significantly greater in $Il10^{-/-}$ mice than in $Il10^{+/+}$ mice after exposure to O_3. O_3-enhanced production of MIP-2 and CD86 and nuclear NF-κB translocation was elevated in the lungs of $Il10^{-/-}$ compared with $Il10^{+/+}$ mice. Results indicated that IL-10 contributes to protection of O_3-induced pulmonary neutrophilic inflammation. These protective effects may be under the control of IL-10-mediated MIP-2 and CD86 production, and NF-κB activation.

Keywords: IL-10, ozone, lung, inflammation, NF-κB, MIP-2, CD86

Introduction

Ozone (O_3) is the most toxic oxidant gas in air pollution mixtures, and has been associated with numerous health effects including airway inflammation and hyperreactivity, (Plopper et al., 1998; Toward and Broadley, 2002), exacerbation of asthma (McConnell et al., 2002; Neuhaus-Steinmetz et al., 2000) and increased mortality and morbidity (Touloumi et al., 1997). O_3-induced lung inflammation is characterized by polymorphonuclear leukocyte (PMN) infiltration (Mudway and Kelly, 2000) and the release of a number of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α (Bhalla et al., 2002; Cho et al., 2001) and interleukin (IL)-6 (Johnston et al., 2005) and the PMN chemoattractant macrophage inflammatory protein (MIP)-2 (Driscoll et al., 1993). O_3 exposure induces inducible nitric oxide synthetase (iNOS) (Fakhrzadeh et al., 2002; Inoue et al., 2000; Kleeberger et al., 2001) that is thought to contribute to further injury. Lung injury due to either acute or subacute O_3 exposure may directly or indirectly affect adaptive immune responses such as T-cell proliferation (Jakab et al., 1995), response to allergen (Depuydt et al., 2002), and upregulation of co-stimulatory molecules such as CD86 (B7.2) that contribute to T-cell activation (Koike et al., 2001).

IL-10 is a pleiotropic anti-inflammatory cytokine produced by activated monocytes and macrophages, helper T cells, and B cells. Increased IL-10 production decreases TNF-α and IL-6 (Lang et al., 2002), and inhibits MIP-2 (Standiford et al., 1995). Moreover, the bioactive IL-10 homodimer exerts anti-inflammatory effects by suppressing macrophage CD86 expression on innate immune cells leading to anergic T-cells (Ding et al., 1993; Flores Villanueva et al., 1994). IL-10 also inhibits iNOS production in macrophages (Cunha et al., 1992) (Tulic et al., 2001), and blocks nuclear factor-kappa B (NF-κB) activation (Saadane et

al., 2005). Tyrosine phosphorylation of the intracellular domains of the IL-10 receptor is known to activate the signaling transducer and activator of transcription (STAT)-3 pathway (Donnelly et al., 1999).

Polymorphisms in *IL10* have been identified as a risk factor associated with the development of asthma (Chatterjee et al., 2005) and LPS sensitivity (Schippers et al., 2005). The functional role of pulmonary IL-10 has been studied in experimental animals in response to LPS (Deleuze et al., 2004; Standiford et al., 1995; Tulic et al., 2001), silica (Huaux et al., 1998), and various parasitic (Netea et al., 2004b) and infectious organisms (Higgins et al., 2003), and as a key mediator in autoimmune diseases (Gangi et al., 2005), allergy (Akdis et al., 2001) and cystic fibrosis (Saadane et al., 2005). Reinhart ***et. al.*** (1999) showed that intratracheal instillation of IL-10 significantly attenuated O_3-induced BALF albumin, fibronectin, and protein (Reinhart et al., 1999). However, the role of IL-10 in O_3-induced pulmonary cellular infiltration and its underlying mechanism has not been determined.

In the current study, we tested the hypothesis that targeted deletion of *Il10* in mice enhances O_3-induced pulmonary inflammation. O_3-induced alterations in pulmonary injury phenotypes and putative downstream molecular events were compared between *Il10*-sufficient ($Il10^{+/+}$) and *Il10*-deficient ($Il10^{-/-}$) mice. Differential neutrophilic (PMN) infiltration, chemoattractant expression, and nuclear NF-κB activation in the lung indicated that IL-10 has a protective role in O_3-induced airway inflammation in mice.

Materials and Methods

Animals. Male *Il10*$^{+/+}$ (C57BL/6) and *Il10*$^{-/-}$ (B6.129P2-*Il10*^{tm1Cgn}/J) mice (6-8 weeks) were purchased from Jackson Laboratories (Bar Harbor, ME). Gene-targeted knockout mice were backcrossed to C57BL/6 mice over ten generations. All mice were provided water and mouse chow (NIH-31) *ad libitum*. All experimental procedures were conducted in accordance with approved NIEHS Animal Care and Use protocols.

O_3 Exposure. Mice were placed in individual stainless-steel wire cages within a Hazelton 1000 chamber (Lab Products, Maywood, NJ) equipped with a charcoal and high-efficiency particulate air–filtered air supply. Mice had free access to water and pelleted open-formula rodent diet NIH-31 (Zeigler Brothers, Gardners, PA). Mice were exposed to 0.3-ppm O_3 or filtered air for 24, 48 or 72 hr (23.5 hr/day). O_3 was generated from ultra-high purity air (< 1 ppm total hydrocarbons; National Welders, Inc., Raleigh, NC) using a silent arc discharge O_3 generator (Model L-11, Pacific Ozone Technology, Benicia, CA). The O_3 concentration was continually monitored (Dasibi model 1008-PC, Dasibi Environmental Corp.). Parallel exposure to filtered air was done in a separate chamber for the same duration. Constant chamber air temperature (72 ± 3° F) and relative humidity (50 ± 15%) were maintained.

Necropsy and Bronchoalveolar Lavage Fluid (BALF) Analysis: Mice were euthanized (sodium pentobarbital, ip., 104 mg/kg) immediately following O_3 exposure. The right lung was lavaged with four separate aliquots (26.0 ml/kg, pH 7.2-7.4) of ice-cold Hanks' balanced salt solution (HBSS). The left lung was snap frozen in liquid nitrogen for later molecular analyses. Recovered BALF was centrifuged (500 × g, 4°C, 10 min.). BALF analysis

followed standard laboratory protocols (Cho et al., 2001). Briefly, total protein (a marker of lung permeability) was determined from BALF supernatants using a Bradford Assay. Cell pellets were resuspended in 1-mL ice-cold HBSS, and total cell numbers were counted with a hemacytometer. A 200 μL aliquot was used to make cytospin slides (300 rpm for 10 min) (Shandon Southern Products, Pittsburgh, PA). Slides were stained with Wright-Giemsa stain (Diff-Quik; Baxter Scientific Products, McGaw Park, IL) and differential cell counts (300 cells/slide) were determined using morphological criteria.

Histological analysis: Left lungs were inflated with 10% formalin, removed *en bloc*, and immersed in 10% formalin. After 24 hrs, lungs were cut into three cross sections, and processed for microscopic slides. Hematoxylin and eosin (H&E)-stained tissue sections were used to assess the extent of peribronchiolar and perivascular inflammation in *Il10*$^{-/-}$ and *Il10*$^{+/+}$ mice qualitatively with the assistance of a pathologist. Immunostaining for cellular proliferation was performed using an anti-Ki67 antibody and slides were qualitatively assessed by comparing the extent of Ki67 staining present in peribronchiolar, perivascular, and centriacinar areas of inflammation.

Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (Q-RT-PCR). Total RNA was isolated from left lung homogenates using the RNAeasy Midi Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. One μg of total RNA was reverse transcribed into cDNA (50 μl reaction). The ABI 7000 Sequence Detector (Applied Biosystems, Foster City, CA) was used to detect TNF-α and iNOS gene expression using qPCR TaqMan ® Gene Expression Power Syber Assays (Applied Biosystems, Foster City,

CA). Briefly, two μL of cDNA was amplified in a 20-μl reaction containing 10 μL of 2x PowerSyber Master Mix (Applied Biosystems, Foster City, CA) and 1 μL of custom specific primers for *Tnf-α* and *Nos-2* (Invitrogen, Carlsbad, CA). 18s ribosomal RNA was served as an internal control. PCR was performed by 10 min incubation at 95 ^{o}C followed by 40 cycles of 95 ^{o}C-15 sec and 60^{o}C-1 min.

Western Blot. Total lung protein was prepared in RIPA buffer containing protease and phosphatase inhibitors. Aliquots of total proteins (30-80 μg) were subjected to Western blot analyses with specific primary antibodies for CD86 (SC-19617; Santa Cruz Biotechnologies, Santa Cruz, CA), actin (SC-1615, Santa Cruz), phosphorylated STAT-3-tyr 701 (Cell Signaling, Danvers, MA), and STAT-3 (Cell Signaling). Blots were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and BioMaxMR x-ray film (Kodak, Rochester, NY). Intensity of Western blot bands was quantitated using the Bio-Rad Gel Doc 2000 System (Hercules, CA).

MIP-2 Protein Enzyme-Linked Immunosorbant Assay (ELISA). Fifty μL of BALF from each mouse was used for ELISA. The procedures were performed following manufacturer's instructions (R&D Systems, Minneapolis, MN). All samples were performed in duplicate, and absorbances were measured at 450 nm on a Benchmark Microplate Reader (Bio-Rad). The minimum detectable dose of murine MIP-2 was less than 1.5 pg/mL.

Nuclear Protein Isolation and Electrophoretic Mobility Shift Assay (EMSA) for p50 NF-κB. Nuclear extracts were prepared from right lung lobes using a Nuclear Extraction Kit (Active Motif, Carlsbad, CA). An aliquot (5 μg) of nuclear protein was incubated in a

binding buffer (10mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5 % glycerol, 1 mM DTT, 0.2 μg PolydI-dC, 1 mM PMSF) in a total volume of 19 μl for 15 min on ice. The reaction was then incubated with 1 μl of [γ^{32}P] dATP end-labeled oligonucleotide containing a NF-κB consensus sequence (3 x 10^4 cpm) at room temperature. After 30 min, the mixture was subjected to electrophoresis on a 4 % SDS-PAGE with 0.25X TBE buffer. The gels were autoradiographed at –70 °C. To analyze specific binding activity of NF-κB subunits, nuclear proteins (5 μg) were pre-incubated with 4 μg of anti-p50 NF-κB antibody (sc-1190X; Santa Cruz), and processed for gel shift assay as described above.

Statistics. All data were expressed as group means ± SE. The data were transformed, if necessary, to ensure normal data distribution and equal variance. Two-way ANOVA was used to evaluate the effects of O_3 and IL-10 on pulmonary toxicity. The factors were exposure (air or O_3) and genotype (*Il10*$^{+/+}$ or *Il10*$^{-/-}$). The dependent variables were BALF protein concentration, BALF PMN numbers, mRNA expressions, and protein levels. The Student Newman Keuls *a posteriori* test was performed to compare group means. All statistical analysis was performed using a commercial statistics package (SigmaStat, Systat Software, Point Richmond, CA). Statistical significance was accepted at $p < 0.05$.

Results

Lung inflammatory responses to O_3. To test the hypothesis that IL-10 contributes to the pulmonary inflammatory response to O_3, *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice were exposed continuously to filtered air or 0.3 ppm O_3 for 24, 48, and 72 hrs. No significant differences in mean numbers of PMNs were detected between *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice exposed to air. O_3-induced

mean numbers of BALF PMNs were significantly increased in *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice compared to corresponding air controls (Figure 4.1A). Compared to *Il10*$^{+/+}$ mice, mean numbers of PMNs were significantly greater (~3-fold) in *Il10*$^{-/-}$ mice throughout the O_3 exposure. Total numbers of cells recovered in the BALF were increased in response to O_3, and were aggravated in *Il10*$^{-/-}$ compared to *Il10*$^{+/+}$ mice. This difference was attributable to differences in total numbers of PMNs and not to other cell types in the BALF (Table 4.1). Mean total BALF protein concentrations increased significantly in both genotypes of mice, compared to air controls. However, no significant differences in O_3-induced increases in BALF protein concentration were found between the two genotypes ($p = 0.055$) (Figure 4.1B).

Centriacinar cellular proliferation. To confirm differential O_3-induced inflammation in *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice, H&E-stained lung tissue sections were observed by light microscopy. No inflammation was found in air-exposed lungs of *Il10*$^{-/-}$ and *Il10*$^{+/+}$ mice. O_3 caused perivascular, peribronchiolar, and terminal bronchial inflammation characterized by increased cellularity and neutrophilic infiltration in *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice (Figure 4.2A). O_3-induced pulmonary inflammatory infiltrates were more prominent in *Il10*$^{-/-}$ compared to *Il10*$^{+/+}$ mice. The number of Ki67-positive cells at distal perivascular and peribronchiolar areas and centriacinar regions increased after O_3 in both genotypes. Ki67-immunostaining was visible in Clara cells and alveolar macrophages (Figure 4.2B). O_3-induced Ki67-positive cells were more prominent in *Il10*$^{-/-}$ than in *Il10*$^{+/+}$ mice in these focal areas (Figure 4.2B).

Il10-deficiency and O_3-induced inflammatory mediator production. MIP-2 levels were determined in BALF as a downstream effector molecule of IL-10 in PMN recruitment. Lung MIP-2 levels increased after O_3 compared to air-exposed controls in both genotypes (Figure 4.3). O_3-induced increases in MIP-2 amounts were significantly higher in *Il10*$^{-/-}$ compared to *Il10*$^{+/+}$ mice at 24 hrs (Figure 4.3). However, these differential levels were not sustained at 48 and 72 hrs of O_3 exposure. TNF-α and iNOS mRNA expression was significantly enhanced by O_3 in both genotypes of mice, but no significant genotypic differences were determined in the O_3-induced message levels of these two inflammatory mediators (data not shown).

CD86 production and exposure to O_3. The co-stimulatory molecule, CD86, has been shown to increase in alveolar macrophages in response to O_3 (Koike et al., 2001) and can be inhibited by IL-10 (Ding et al., 1993; Flores Villanueva et al., 1994). We therefore tested whether O_3-induced CD86 protein production would be enhanced in *Il10*$^{-/-}$ compared to wildtype mice. Compared with *Il10*$^{+/+}$ mice, significantly higher levels of lung CD86 proteins were found in *Il10*$^{-/-}$ mice basally and after O_3 (Figure 4.4). However, O_3 exposure did not increase CD86 production above basal levels in either *Il10*$^{+/+}$ or *Il10*$^{-/-}$ mice.

Il10-deficiency potentiated O_3-induced nuclear NF-κB activity. NF-κB induces transcription of many inflammatory mediators including MIP-2 in response to O_3 (Driscoll et al., 1993). Total nuclear DNA binding activity of NF-κB (shifted bands, SB) was greater in *Il10*$^{-/-}$ mice than in *Il10*$^{+/+}$ mice basally and after O_3 (Figure 4.5A). Specific p50 κB activity was also basally higher in *Il10*$^{-/-}$ mice than in *Il10*$^{+/+}$ mice. p50 κB activity was enhanced by

O_3 in both genotypes, and the induced activity of the specific p50 subunit was higher in *Il10*$^{-/-}$ compared to *Il10*$^{+/+}$ mice (Figure 4.5B).

O_3-induced effects of Il10 occur independently of STAT-3 activation. STAT-3 signaling pathways have been associated with IL-10-mediated activation (Larner et al., 1993; Tan et al., 1993) (Donnelly et al., 1999). The *p*-STAT-3/STAT-3 protein ratio increased significantly after O_3 exposure compared to air controls in both genotypes (Figure 4.6). However, no genotype effects were observed in O_3-induced increases of *p*-STAT3/STAT-3 levels.

Discussion

The lung is a primary target for exposure to environmental pathogens and is immunologically active. IL-10 is a pleiotropic cytokine that potently suppresses inflammation and may direct adaptive immune responses. The current study investigated the role of IL-10 in O_3-induced airway neutrophilia in mice and mechanisms leading to its anti-inflammatory effects. Targeted deletion of *Il10* significantly enhanced O_3-induced pulmonary cellular inflammation in mice. O_3-induced nuclear activity of NF-κB and expression of CD86 proteins were also greater in the lungs of *Il10*$^{-/-}$ compared to *Il10*$^{+/+}$ mice. Our results indicated that IL-10 is essential in modulation of pulmonary inflammation caused by O_3 in the mouse.

The anti-inflammatory activity of IL-10 has been noted in a variety of pulmonary models. Intratracheal instillation of crystalline silica caused significantly greater increases in levels of lactate dehydrogenase, total protein, and numbers of inflammatory cells in *Il10*$^{-/-}$ than in

Il10$^{+/+}$ mice, suggesting that IL-10 can limit the amplitude of the inflammatory response (Huaux et al., 1998). In addition, LPS-induced lethality increased significantly when endogenous IL-10 bioactivity was neutralized (Standiford et al., 1995). Similarly, intranasally administered recombinant murine IL-10 reduced lung inflammation and bronchopulmonary hyperreactivity after airway-instilled LPS administration (Deleuze et al., 2004). Patients with cystic fibrosis (CF) and mice lacking the CF transmembrane conductance regulator protein (CF-KO) are deficient in IL-10. In response to LPS, *Il10*$^{-/-}$ and CF-KO mice had more profound neutrophilic inflammation than wildtype counterparts. Treatment with exogenous IL-10 in CF-KO mice attenuated excessive LPS-induced inflammation and suggests a therapeutic target for increased inflammation associated with chronic disease (Saadane et al., 2005). The immunosuppressive activity of IL-10 has also been associated with pulmonary *Candida albicans* infection (Netea et al., 2004b). The current study demonstrated that IL-10 has anti-inflammatory activity in response to subacute exposure to O_3. R.G. Reinhart ***et. al.*** (Reinhart et al., 1999) demonstrated that addition of recombinant IL-10 significantly attenuated by 25% protein permeability responses induced by acute O_3 exposure (0.8 ppm, 3 hrs) compared to controls. This attenuation was attributed to decreases in albumin and fibronectin levels. In the present study, we did not find any significant effect of *Il10* deletion on protein responses to 0.3 ppm O_3. The role of IL-10 in the hyperpermeability response to O_3 may be dose-dependent.

Neutralization of IL-10 prior to LPS administration can attenuate production of the PMN chemoattractant MIP-2 and associated neutrophilia (Standiford et al., 1995). We determined that O_3-induced increases in MIP-2 levels were potentiated in the absence of *Il10* at 24 hrs prior to the peak PMN influx. Upregulation of MIP-2 may increase initial neutrophilic

infiltration while other mediators such as intercellular adhesion molecule (ICAM)-1 may control sustained neutrophilia in response to O_3 (Takahashi et al., 1995). In addition to MIP-2, studies have shown that IL-10 inhibits TNF-α production (Conti et al., 2003) and inducible nitric oxide synthase (iNOS) to exert its anti-inflammatory effects (Cunha et al., 1992) (Tulic et al., 2001). Previous work in our lab has demonstrated that TNF-α (Cho et al., 2001) and iNOS (Kleeberger et al., 2001) contribute to O_3-induced inflammation. However, we found that deficiency in IL-10 did not contribute to O_3-induced increases in TNF-α and iNOS expression (data not shown). Others have found similar dissociations between IL-10 and TNF-α. Salez *et. al.* reported that LPS-induced IL-10 protein and associated TNF-α were not detectable in alveolar macrophages while LPS-induced production of these same mediators in peritoneal macrophages was robust (Salez et al., 2000). Deleuze *et. al.* found IL-10-mediated reductions in LPS-induced TNF-α production only when recombinant IL-10 was administered intranasally and not when generated *in vivo* after muscle electrotransfer of murine IL-10-encoding plasmid (Deleuze et al., 2004). The relationship between IL-10 and TNF-α may be dependent on tissue type and route of administration.

In addition to controlling inflammatory cytokine expression, IL-10 can inhibit LPS-induced co-stimulatory molecule CD86 expression on antigen presenting cells (APC) (Ding et al., 1993). Similarly, we found that lung CD86 protein levels were enhanced in $Il10^{-/-}$ compared to $Il10^{+/+}$ mice. This effect occurred independently of exposure to O_3. Koike *et. al.* found that approximately 8% of alveolar macrophages obtained from rats exposed to 1 ppm O_3 for 3 days were CD86-positive (Koike et al., 2001), and suggested that small increases in co-stimulatory activity may be sufficient for regulating O_3-induced immune responses. Our results corroborate that of Soltys *et. al.* who showed that CD86 in alveolar

macrophages was significantly enhanced in *Il10*$^{-/-}$ mice at baseline (Soltys et al., 2002). Soltys *et. al*. suggested that normally low levels of alveolar macrophage costimulatory molecule expression are due to constitutive IL-10 secretion. Our data similarly indicate that *Il10*-deficiency permits endogenous CD86 expression that would normally be suppressed by constitutive levels of IL-10 and could explain why subtle increases in CD86 due to O_3 were not detectable in *Il10*$^{-/-}$ mice.

IL-10 inhibits NF-κB (Saadane et al., 2005; Spight et al., 2005) and mitogen activated protein kinase (MAPK) pathways (Haddad et al., 2003; Ward et al., 2005). We previously showed that NF-κB actively binds DNA in response to O_3 (Cho, HY, et. al., 2006 Am. J. Crit Care Medicine in press). In accordance with our hypothesis, specific binding of p50 κB increased in response to O_3 in both genotypes but was enhanced in *Il10*$^{-/-}$ compared to *Il10*$^{+/+}$ mice. Specific binding of p65 was similar (data not shown). These data are consistent with IL-10 mediated protection against O_3-induced inflammation through inhibition of the NF-κB signaling pathway. The mechanism through which IL-10 exerts this effect on NF-κB is not clear, but may be through inhibition of inhibitory kappa kinase (IκK) as demonstrated in response to LPS (Saadane et al., 2005; Spight et al., 2005)or through the action of inhibitory p50 homodimers.

STAT-3 signaling is widely associated with IL-10-mediated activation (Lang et al., 2002; Takeda et al., 1999; Williams et al., 2004). In the lung, STAT-3 has been associated with inhibition of IL-10-mediated alveolar macrophage activation by LPS (Berlato et al., 2002). This signaling pathway depends on IL-10 receptor ligation to activate Janus-associated kinase (JAK) tyrosine kinases that result in phosphorylation and activation of STAT-3 (Riley et al., 1999). There were, however, no significant genotype-specific differences in *p*-STAT-

3/STAT-3 levels, which suggest that STAT-3 activation occurs in an IL-10-independent manner in response to O_3. To further isolate the cell types involved with this response, immunohistochemical analysis of lung tissue with an IL-10 specific antibody is warranted. IL-10-independent STAT-3 activation has been documented, previously. In response to *Toxiplasma gondii*, IL-10-deficient macrophages did not rescue parasite-induced STAT-3 phosphorylation, suggesting that STAT-3 phosphorylation occurred independently of IL-10 (Butcher et al., 2005). Murray *et. al.* used a TNF-α-specific targeting vector in *Il10*$^{-/-}$ mice to eliminate the potential confounding effect of TNF-α in IL-10 mediated signaling activation and determined that IL-10 primarily acts on STAT-3 indirectly (Murray, 2005). Other STAT-family members such as STAT-1 and STAT-5 could be involved in IL-10 signaling activated by O_3. Finbloom suggested that IL-10 may selectively regulate monocytes and T-cells by differential activation of different STAT proteins (Finbloom and Winestock, 1995). IL-10 mediated modulation of NF-κB and Iκk activity may also contribute to these effects but remains controversial (Spight et al., 2005).

In conclusion, this study highlights the protective effects of IL-10 on O_3-induced pulmonary inflammation. IL-10 deficiency results in enhanced neutrophilic inflammation in response to O_3 and increased lung injury at the centriacinar region of the lung. IL-10 has multiple immunosuppressive functions that operate to control inflammation. In response to O_3, our initial mechanistic work suggests that IL-10's effect may be mediated at least in part by MIP-2, CD86, and NF-κB. Differential response to O_3 was independent of activated STAT-3 signaling. Further investigation of the mechanisms through which IL-10 modifies responses to O_3 will elucidate the pathophysiological regulation of the response to pulmonary

O_3 toxicity and may provide a possible therapeutic target for attenuating the effects of O_3 in susceptible individuals.

Table 4.1: Effect of targeted deletion of Il10 on the inflammatory response to filtered air and 0.3 ppm O_3.

Exposure	Genotype	Total Cells	Macrophages	Lymphocytes	Epith. Cells
Air	*Il10*$^{+/+}$	43,833 ± 4,390	42,166 ± 4,191	39 ± 19	1,549 ± 238
	Il10$^{-/-}$	39,889 ± 2,815	40,881 ± 4,490	23 ± 11	1,737 ± 171
24 h O_3	*Il10*$^{+/+}$	42,333 ± 5,812	75,956 ± 19,413	24 ±11	4,106 ± 1168
	Il10$^{-/-}$	75,000 ± 4,340	38,829 ± 2,730	0 ± 0	1,718 ± 356
48 h O_3	*Il10*$^{+/+}$	75,000 ± 11,258	75,629 ± 13,039	123 ± 58	3,356 ± 1014
	Il10$^{-/-}$	124,200 ± 33,392	64,108 ± 8,273	1,229 ± 459	2,505 ± 1156
72 h O_3	*Il10*$^{+/+}$	80,333 ± 4,807	116,484 ± 23,607	9 ± 4	3,048 ± 923
	Il10$^{-/-}$	173,400 ± 34,156	128,429 ± 29,561	59 ± 49	1,724 ± 395

Table represents total cells and specific inflammatory cell types (macrophages, lymphocytes, epithelial cells) recovered in the BALF from *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice in response to air or 0.3 ppm O_3.

Figure 4.1: Effect of targeted disruption of Il10 on O_3-induced inflammation.

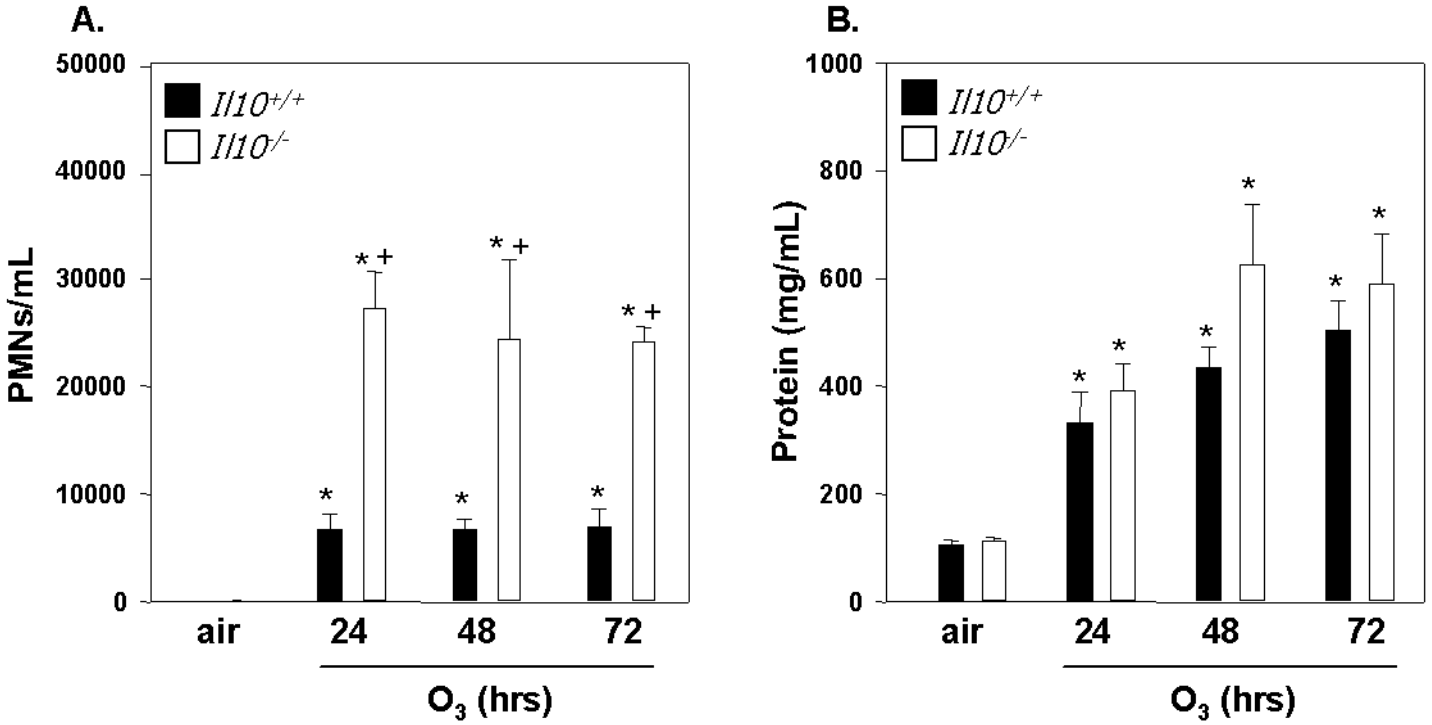
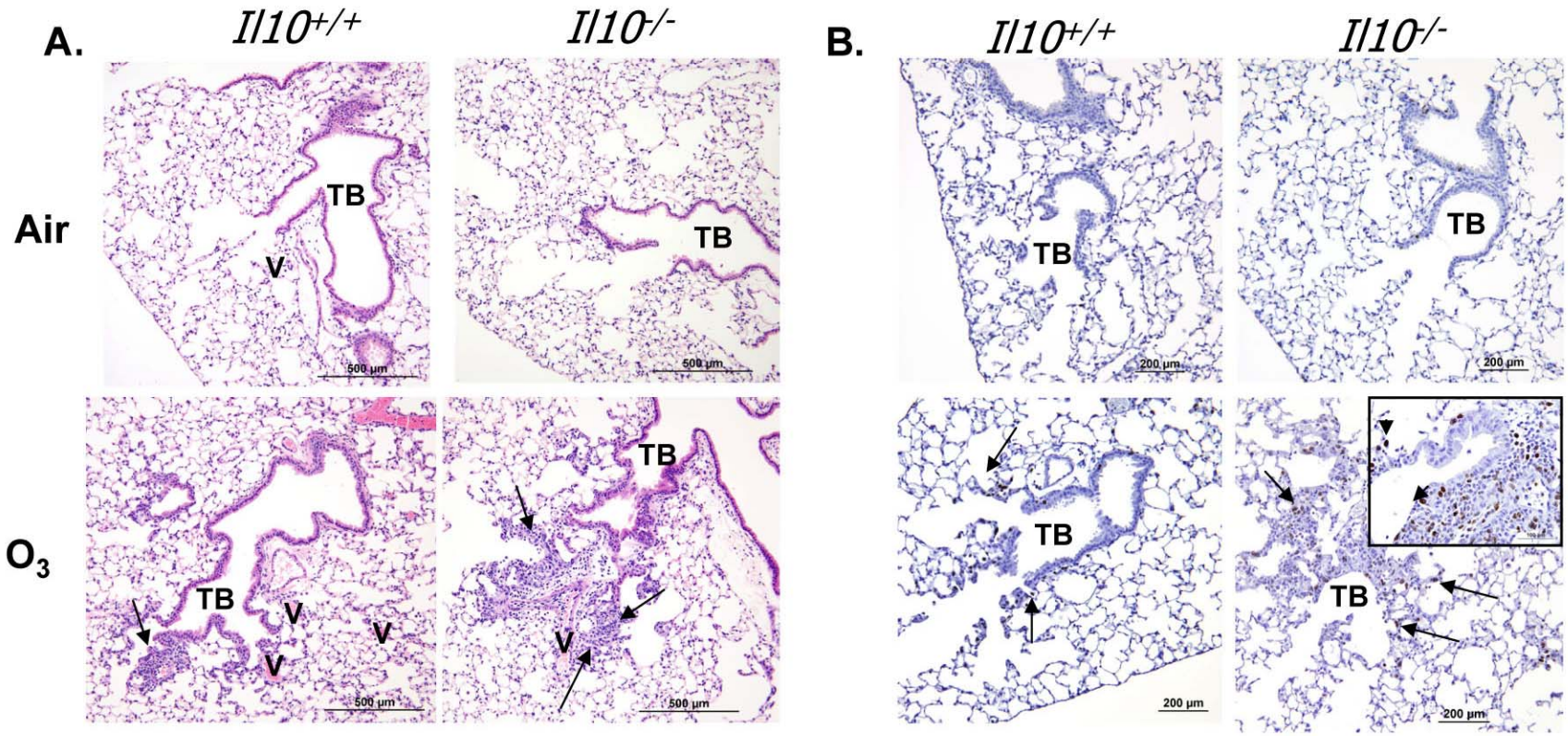


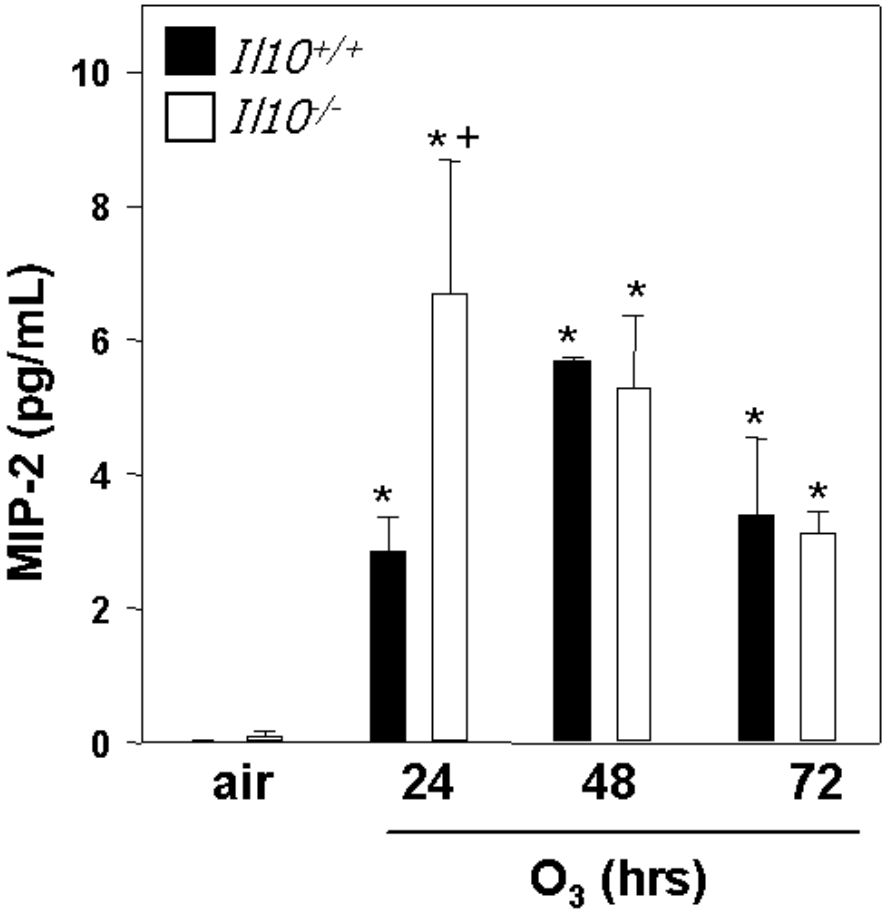
Figure represents changes in number of polymorphonuclear leukocytes (PMNs) (A) and total protein concentration (B) recovered in the bronchoalveolar lavage fluid (BALF) from *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice in response to air or 0.3 ppm O_3. Values are means ± SE (n = 8/group). *, $p < 0.05$, air vs. 0.3 ppm O_3. +, $p < 0.05$, *Il10*$^{+/+}$ vs. *Il10*$^{-/-}$.

Figure 4.2: Lung sections from Il10$^{+/+}$ and Il10$^{-/-}$ mice exposed to air and O_3.



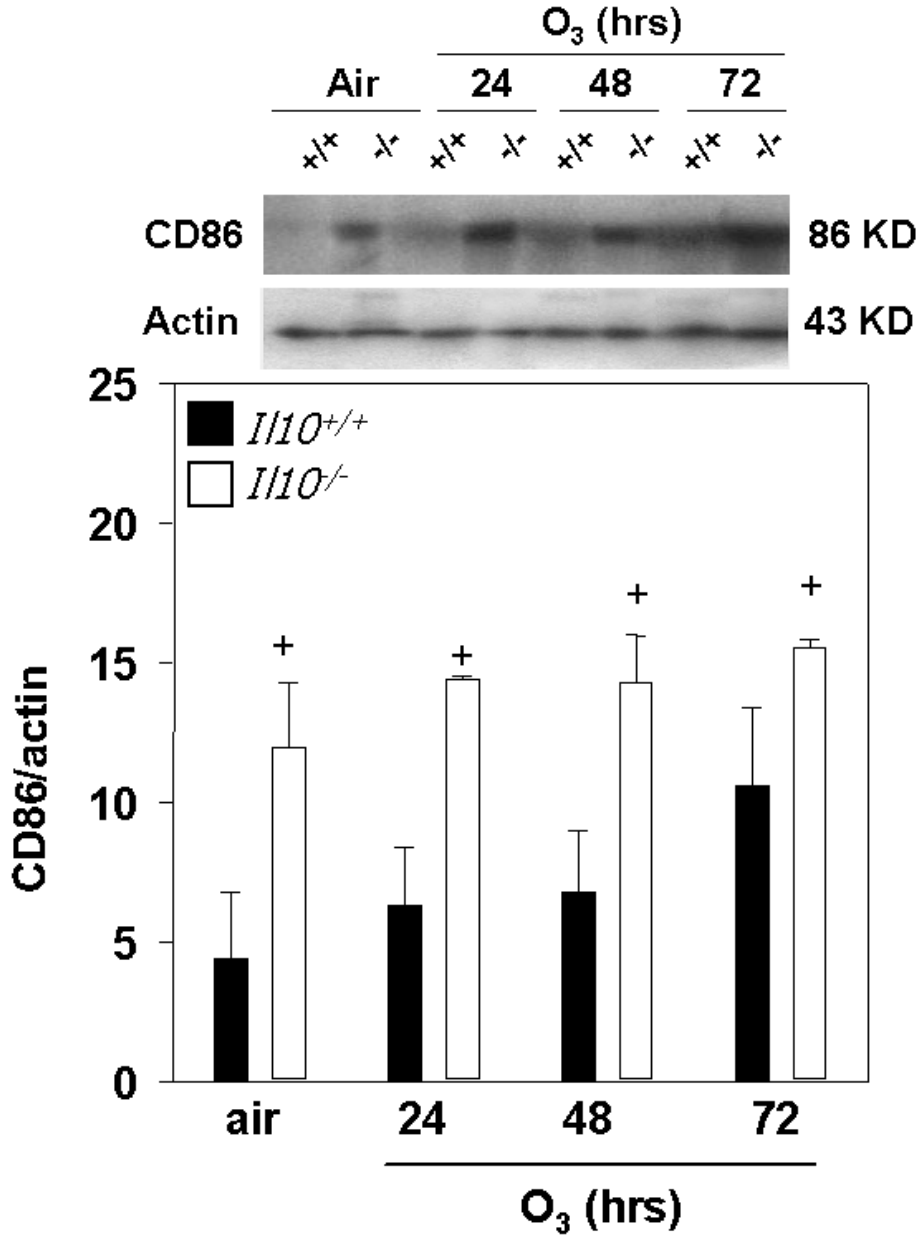
Lung sections from *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice in response to air or 0.3 ppm O_3 (72 h). Five micron lung sections were stained with H&E (A) and Ki67 immunostaining (B). (A) Arrows illustrates areas of greatest neutrophilic inflammation, particularly around terminal bronchioles (TB) and blood vessels (V). (B) Ki67 immunostaining (Brown dots, arrows) indicate foci of cellular proliferation. All sections are photographed at 20X and are representative of mice from each treatment group. Bar represents 500 µm for H&E sections and 200 µm (B)

Figure 4.3: IL-10-mediated MIP-2 production in response to O_3.



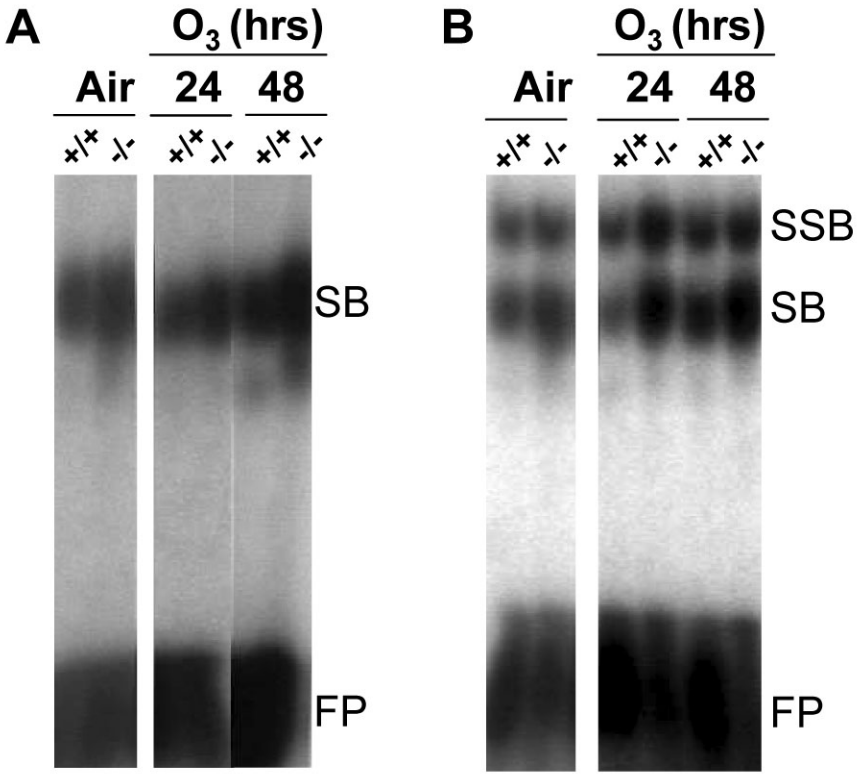
MIP-2 protein levels in BALF recovered from *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice exposed to air and 0.3 ppm O_3. Values are means ± SE (n = 4 per group). MIP-2 protein is expressed in pg/mL. * $p < 0.05$, air vs. O_3; + $p < 0.05$, *Il10*$^{+/+}$ vs. *Il10*$^{-/-}$.

Figure 4.4: IL-10 mediated production of CD86 in response to O_3.



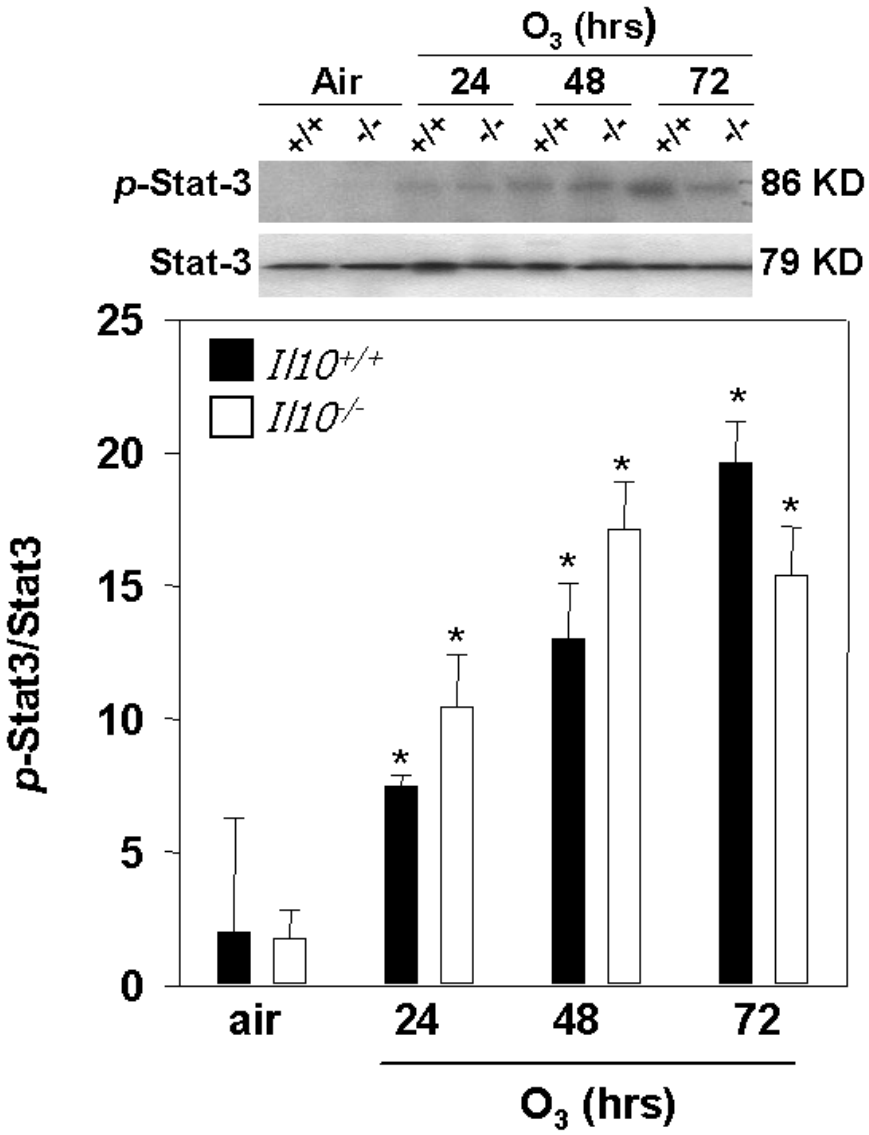
Lung CD86 protein expression in *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice exposed to air and 0.3 ppm O_3. Data are presented as means ± SE (n = 3-5 per group). CD86 expression was detected from whole lung homogenates by Western blot analysis. Blots were reprobed with actin for normalization. +, $p < 0.05$.

Figure 4.5: IL-10 mediated DNA binding by NF-κB in response to O_3.



Electromobility shift assay to determine DNA binding by total NF-κB (A) and specific p50κB (B) in response to 0.3 ppm O_3 in *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice. Each lane represents nuclear protein pooled from three representative animals of each treatment group and was repeated three times. S, shift; SSB, Supershifted Band; FP, free probe.

Figure 4.6: STAT-3 activation mediated by IL-10 in response to O_3.



Phosphorylated-STAT-3 (*p*-STAT-3) in *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice in response to air or 0.3 ppm O_3. The ratio of *p*-STAT-3 to total STAT-3 was determined from whole lung homogenates by Western blot analysis. Data are presented as means ± SE (n = 3 per group). *, $p < 0.05$, air vs. 0.3 ppm O_3.

CHAPTER 5: GENERAL DISCUSSION

Summary

The descriptive toxicology of ground level O_3 has been well characterized, and governmental legislation has reduced emissions leading to decreased O_3 production nationwide. However, O_3 concentrations have not yet reached legislated levels in many areas of the U.S. and cause persistent health effects, particularly in susceptible individuals during the hot summer months. In addition, the mechanism(s) underlying O_3-induced inflammation are still not well understood. The studies presented here focused specifically on understanding the role of innate immune mediators as putative susceptibility genes that may alter O_3-induced pulmonary inflammation.

Using linkage analysis, Kleeberger *et. al.* (2000) identified a significant quantitative trait locus (QTL) for susceptibility to O_3-induced hyperpermeability in mice (Kleeberger et al., 2000). This QTL contains the candidate gene, toll-like receptor 4 (*Tlr4*). Proof of concept studies in *Tlr*-mutant and *Tlr*-sufficient mouse models verified a role for TLR4 in O_3-induced hyperpermeability responses (Kleeberger et al., 2000).

We hypothesized that CD14, an accessory molecule to TLR4, as well as proposed downstream mediators (IL-12, IL-18, IL-10) would contribute to differential pulmonary inflammatory responses to O_3. Commercially available gene targeted knock out mice were used to carry out *in vivo* exposures to O_3 under controlled conditions. Our principal phenotypes were neutrophilia and hyperpermeability, derived from cellular analysis of the BALF and BALF total protein. Histological and molecular analysis confirmed our inflammatory phenotype and provided insight into the molecular mechanism of action.

Receptor-level events associated with TLR4

CD14 brings LPS into proximity with TLR4 that then activates intracellular signaling. Both genes have been shown to be critical to the response to LPS (Haziot et al., 1994; Hollingsworth et al., 2004; Poltorak et al., 1998). We tested the hypothesis that absence of CD14 would diminish O_3-induced inflammation. However, unlike the response to LPS, we found that O_3-induced inflammation was not different between *Cd14*$^{+/+}$ and *Cd14*$^{-/-}$ mice and suggested that neutrophilic and protein permeability responses were independent of CD14 in response to O_3. These negative findings are consistent with those of Hollingsworth ***et. al.*** (2004) who demonstrated that O_3-induced hyperreactivity but not inflammation was different between *Tlr4*-deleted and *Tlr*-normal C57BL/6 mice (Hollingsworth et al., 2004).

The contribution of regulatory cytokines

O_3-induced *Il12* and *Il18* expression levels were significantly lower in *Tlr4*-mutant compared to *Tlr4*-normal mice. Interestingly, *Il18* expression was also higher in *Tlr4*-normal compared to *Tlr4*-mutant mice in response to air. These results suggested that TLR4 could mediate production of regulatory cytokines and provided an initial indicator that TLR4 could serve as a link between innate and adaptive immune responses after oxidant injury.

We then tested the hypothesis that IL-12 and IL-18 would increase the inflammatory response to O_3. Neutrophilic inflammation was attenuated in *Il12*$\alpha^{-/-}$ and *Il12*$\beta^{-/-}$mice compared to wildtype controls in response to O_3 but only reached statistical significance when *Il12*$\beta^{-/-}$ were compared to *Il12*$\beta^{+/+}$ mice. While IL-12 is an important contributor to O_3-induced inflammation, the IL-12β (p40) subunit is more critical than the IL-12α (p35) subunit in modulating this response. Because p40 is also a component of IL-23 and IL-27,

we cannot eliminate the possibility that these two IL-12 family members may also contribute to the underlying pathophysiology of O_3-induced inflammation.

Surprisingly, we found no O_3-induced differences in inflammation between *Il18*$^{+/+}$ and *Il18*$^{-/-}$ mice suggesting that IL-18 does not contribute to pulmonary inflammation following O_3 exposure. IL-18 binds exclusively to the IL-18 receptor, leading us to hypothesize that perhaps O_3-induced inflammation was IL-18 receptor-dependent. Interestingly, we found that O_3-induced neutrophilic inflammation was enhanced in *Il18r1*$^{-/-}$ mice compared to wildtype counterparts suggesting that IL-18 receptor may be protective in the response to O_3. Further molecular analysis suggested that TNF-α may contribute to this phenotype though the mechanism remains unclear. The discrepancy in O_3-induced pulmonary responses between *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice suggests that IL-18 may not be the sole ligand for IL-18R1. Further research is necessary to determine if O_3-induced ligands interact with IL-18R1 or if the receptor is directly oxidized and structurally altered.

The contribution of anti-inflammatory mediators in controlling inflammation

Results from our IL-10 studies strongly implicate IL-10 as an important anti-inflammatory mediator in O_3-induced inflammation. O_3-induced neutrophilic inflammation was significantly enhanced in *Il10*$^{-/-}$ compared to *Il10*$^{+/+}$ mice, and these results were confirmed by qualitative histological analysis. MIP-2 levels were significantly enhanced after 24 hr O_3 in *Il10*$^{-/-}$ compared to *Il10*$^{+/+}$ mice. Molecular analysis showed that IL-10 potently decreased costimulatory molecule expression (CD86) and DNA binding of the pro-inflammatory transcription factor NF-κB independent of exposure. Differential response to O_3 was not

dependent on STAT-3 signaling. These results suggested that IL-10 downregulates inflammation in response to O_3.

The results of our studies confirm that specific innate immune mediators contribute to O_3-induced inflammation. *Il12* and *Il18* expression are at least partially dependent on TLR4. IL-12α and, to a greater extent, IL-12β contribute to O_3-induced neutrophilia while IL-18R and IL-10 are protective. O_3-induced inflammation occurs independently of CD14. Subsequent mechanistic analysis is required to more fully understand how these effects occur. Moreover these mediators may have overlapping and/or synergistic contributions that require sophisticated experimental designs in order to understand their interactions.

Research Implications and Unanswered Questions

Implication of CD14 and O_3-induced inflammation

Numerous studies have confirmed that CD14 is required for TLR4-mediated activation in response to LPS (Alexis et al., 2001; Haziot et al., 1994; Moreno et al., 2004). Moreover, polymorphisms in CD14 have been associated with differential responsiveness to LPS (Baldini et al., 2002) and increased risk for atopy in childhood (Kleeberger and Peden, 2005). Clearly, CD14 is important in inflammatory diseases, particularly those that are mediated by LPS. Given our previous finding that TLR4 could mediate the inflammatory response to O_3, we had strong rationale to hypothesize a putative role for CD14 as well. However, in response to subacute exposures to O_3, CD14 did not modulate O_3-induced pulmonary injury or inflammatory responses in C57BL/6J mice. Genetic background (C57BL/6) may influence the role of TLR4 as well as CD14 in response to oxidant injury. Further study is now required to assess the role of CD14 on a C3H genetic background, the same background

as the C3H/HeJ (*Tlr4*-mutant) strain that is resistant to the inflammatory response to O_3 compared to C3H/HeOuJ mice (Kleeberger et al., 2000). It is also possible that CD14 may become important only at high doses of O_3, albeit at levels that are not environmentally relevant.

Interactions between TLR4/IL-10

Experimental evidence suggests that potential interactions between TLR4 and IL-10 exist but were not directly tested in these studies. For example, Higgins *et. al.* (2003) found reduced IL-10 production in dendritic cells and T-regulatory (T-reg) cells in lungs of C3H/HeJ compared to C3H/HeN mice in response to *Bordetella pertussis* (Higgins et al., 2003) which may suggest TLR4-initiated control of T-reg cells via IL-10. DN Cook *et. al.* (2004) (Cook et al., 2004) suggested that people possessing the *TLR4* polymorphism D299G may have decreased IL-10 production. TLR4-mediated reductions in IL-10 production may explain the link between *TLR4* polymorphisms and its effect on asthma and atopic responses. In addition, polymorphisms in the proximal IL-10 promoter region were associated with *in vivo* and *ex vivo* LPS sensitivity, and *TLR4* polymorphisms were associated with slightly elevated IL-10 production in human cardiology patients (Schippers et al., 2005). In addition, TLR2 signals have been shown to mediate increased IL-10 production in a *Candida albicans* model (Netea et al., 2004b) and in response to *Mycobacterium tuberculosis* (Jang et al., 2004).

In these studies, IL-10 had an anti-inflammatory effect in response to O_3, while previous studies showed that functional TLR4 contributed to lung injury (Kleeberger et al., 2001). As a central innate immune receptor, TLR4 may limit IL-10 production. When TLR4 is mutated

or when inflammation reaches a critical threshold, this inhibition is released and IL-10 exerts anti-inflammatory effects. In support of this, Fernandez *et. al.* (2004) determined that the TLR2 ligand, lipoteichoic acid, and the TLR4 ligand, LPS, inhibit the ability of IL-10 to induce phosphorylation of STAT-3 in macrophages (Fernandez et al., 2004).

Interactions between TLR4/IL-12p40/IL-10

A potential interaction may exist among TLR4, IL-10, and IL-12p40. Though IL-10 and IL-12p40 have antagonistic effects, both cytokines are produced in response to LPS and to CpG oligonucleotides in the lung (Schwartz et al., 1999) and suggests that TLR4 and TLR2 are involved in these responses. Recent studies have provided compelling insights to confirm the interdependence among these three mediators. For example, Zhou *et. al.* (2004) reported that LPS-induced IL-10 inhibited IL-12p40 expression by blocking polymerase II (Zhou et al., 2004). In another study, IL-10-mediated suppression of LPS-induced IL-12p40 expression was ameliorated in *Stat3*-mutant macrophages, suggesting that aberrant STAT-3 signaling prevents the anti-inflammatory effects of IL-10 (Kobayashi et al., 2003). Preliminary data showing O_3-induced *Il12*β expression in *Il10*$^{-/-}$ mice is inconclusive and *Il10* expression in *Il12*β$^{-/-}$ mice has not been tested. Interestingly, a recent paper by Martin *et. al.* (2005) demonstrated that glycogen synthase kinase 3 (GSK3)-β regulated TLR-induced inflammatory responses as well as IL-10 and IL-12 production (Martin et al., 2005). The data suggest that GSK3-β may selectively augment anti-inflammatory cytokine production while also suppressing pro-inflammatory cytokine production in response to various TLR agonists. This paper provides a novel pathway that may link TLRs, IL-10 and IL-12 while providing a potential mechanism for how two antagonistic cytokines could be controlled by

the same innate immune receptor. The role of polymerase II, STAT-3 and GSK3-β in oxidant injury is not known. We hypothesize that polymerase II is differentially expressed in response to O_3 vs. air, and that O_3-induced inflammatory responses are different in *Stat3*$^{-/-}$ compared to *Stat3*$^{+/+}$ mice. Additionally, we hypothesize that GSK3-β could critically alter the pulmonary response to O_3 via differential expression of TLR4, IL-10 and IL-12p40. Early pro-inflammatory responses to O_3 that occur along the pro-inflammatory TLR4-GSK3-β axis and result in IL-12p40 production would be attenuated by anti-inflammatory *Il10* gene expression by the same axis.

However, the relationship between IL-12 and TLR4 became controversial since Merlin *et. al.* (2001) determined that C57BL/10ScCr mice, commonly thought to have an exclusive defect in the *Tlr4* gene, carry a second genetic defect that leads to IL-12 unresponsiveness (Merlin et al., 2001). IL-12p40 has been shown to be produced in response to numerous other infectious and inflammatory stimuli, including *Corynebacterium parvum*, CpG oligodeoxynucleotides, anti-CD40 and low molecular weight hyaluronic acid (Mason et al., 2002), and suggests that the relationship between IL-12p40 and TLR4 is not exclusive. These results imply, with caveats, a plausible relationship among TLRs, IL-10 and IL-12p40 via novel mechanistic targets including GSK3-β and a rationale for future studies in response to oxidant injury.

Interactions between IL-12 and IL-18

The ability of IL-12 and IL-18 to modify pulmonary immune responses after exposure to inflammatory and infectious stimuli is well documented. For example, IL-12 and IL-18 have been shown to prevent Th2-like cellular differentiation in allergic asthma (Akira, 2000;

Hofstra et al., 1998) and to sustain Th1 responses in rheumatoid arthritis (Gracie et al., 1999). In addition, IL-12p40 and IL-18 limit lung inflammation and Th2 effector molecules in response to respiratory syncytial virus in children and potentially lead to atopy and asthma later in life (Wang et al., 2004).

IL-12 and IL-18 stimulate IFN-γ in response to *Chlamydia pneumoniae* (Netea et al., 2004a) as well as LPS and concurrent viral infection (Raue et al., 2004). IFN-γ potentiates the respiratory burst and activity of phagocytes, enhances cellular generation of nitric oxide (NO), promotes expression of MHC class I and class II antigens, and induces intercellular adhesion molecule (ICAM)-1 expression (Strieter et al., 2002). In addition, new evidence has shown that induction of ICAM-1 was directly enhanced by recombinant IL-12 and IL-18 in peripheral blood mononuclear cells (Stuyt et al., 2003). Interestingly, NOS2 production and ICAM-1 expression occur in response to O_3 (Kleeberger et al., 2001; Mudway and Kelly, 2000), and provide rationale for IFN-γ and/or ICAM-1 mediated effects directed by IL-12 and IL-18 in response to O_3.

We showed that TLR4 controlled O_3-induced expression of *Il12* and *Il18*. Conversely, IL-12 and IL-18 could modify TLR activation since elevated IL-12 and IL-18 levels from the synovial tissue of rheumatoid arthritis patients were found to increase expression of TLR4 and TLR2 (Radstake et al., 2004). Thus, the immunomodulatory role of TLR4 and IL-12 and IL-18 may involve a possible autocrine feedback loop that remains to be fully tested. Clearly, IL-12 and IL-18 are important regulatory cytokines that have been implicated in inflammatory disease and, at least for IL-12p40, in response to O_3. TLR4-mediated IL-12 and IL-18 expression may lead to combinatorial effects that influence O_3-induced ICAM-1 and IFN-γ expression. ICAM-1 is an adhesion molecule that facilitates neutrophilic

migration into the airspaces while IFN-γ stimulates local inflammatory cells. IL-12 and IL-18 may then regulate TLR activation to control inflammation.

We found an inverse functional relationship between IL-12p40 and IL-18 receptor (IL-18R1) in response to O_3. The relationship between IL-12 and IL-18R1 is not well understood. Coexpression of both IL-18Rα and IL-18Rβ chains are required for IL-18 binding. Yoshimoto *et. al.* (1998) determined that expression of IL-18R1 and significant IFN-γ production occurred in splenic T-cells stimulated with IL-12 and IL-18 (Yoshimoto et al., 1998). In support of this, Nakahira *et. al*. (2001) reported that IL-12 activates STAT-4 and *Stat4*$^{-/-}$ mice lack IL-18 binding sites (Nakahira et al., 2001) and suggests that IL-12, acting through STAT-4, may control IL-18Rα and IL-18Rβ gene expression (Sergi and Penttila, 2004). However, Nakahira *et al.* (2001) also demonstrated that upregulation of the IL-18Rα chain could occur after IL-12 incubation in resting T-cells, but upregulation of the β chain in response to IL-12 stimulation occurred after a significant time delay (eight hrs) (Nakahira et al., 2001) that would make it improbable for the IL-18R1 heterodimer to be functionally active, at least in response to IL-12. A strong link between IL-12p40 and IL-18R1 therefore does not seem to exist.

Interactions between IL-18R1 and other IL-1R superfamily members

IL-18R1 consists of an α chain, critical for IL-18 binding, and a β chain, for signal transduction. This heterodimeric complex shares structural, intracellular signaling, and functional homology with the IL-1 Receptor (IL-1R). The α and β chains possess extracellular immunoglobulin folds and an intracellular signaling domain similar to TLR4, which are also members of the IL-1R superfamily (Sergi and Penttila, 2004). Once IL-18 is

bound to the α subunit and IL-18Rβ is recruited to the complex, IL-1 receptor associated kinase (IRAK) and MyD88 are recruited, in the same way as TLR4 and IL-1R signaling. Subsequent signaling steps culminate in nuclear NF-κB-dependent induction of pro-inflammatory gene expression including *Il18*, *Ifn-γ*, *Tnf-α*, and *Il1*.

Our studies showed a protective effect of IL-18R1 in response to O_3 at 72 hr that is in contrast to previous work with other IL-1R superfamily members that demonstrated that TLR4 promotes O_3-induced hyperpermeability responses (Kleeberger et al., 2001) and that an IL-1R antagonist attenuated O_3-induced airway hyperresponsiveness as well as TNF-α and MIP-2 production (Park et al., 2004). IL-18R1 may reduce inflammation in the later phases of exposure. There may also be interactive effects where the activity of TLR4 and IL-1R co-regulate the activation of IL-18R1. A kinetic analysis of these receptors in response to O_3 may improve understanding about their role in mediating inflammation during oxidant exposure. A third possibility is that in response to O_3, innate immune receptors IL-1R and TLR4 have pro-inflammatory effects to generate inflammation and initiate T-cell responses. To prevent excessive inflammation, activated T-cells may act via IL-18R1 to decrease inflammatory responses.

NF-κB as the inflammatory "master switch"

Numerous environmental and pathogenic triggers activate multiple receptors including TLR4, IL-1R, IL-18R1, and tumor necrosis factor receptors (TNFRs). Signaling through these receptors ultimately result in the release of p50 and p65 from NF-κB's inhibitory subunit, Iκk. In turn, NF-κB-dependent DNA binding induces production of numerous inflammatory cytokines. Thus, NF-κB is the "master switch" of inflammation. We have

recently shown that NF-κB actively binds DNA in response to 0.3 ppm O_3 (Cho, HY et. al., 2006, in press).

Within this group of NF-κB-activating receptors, TNFRs are directly associated with O_3-induced inflammation (Cho et al., 2001) and IL-1R is indirectly implicated via its known activation of O_3-associated target genes including NO Synthase (NOS2), and ICAM-1 (Fantuzzi, 2001). In addition, IL-18R1 and IL-10 modulated specific DNA binding by NF-κB in these studies and may have regulatory functions. IL-12p40-mediated differential NF-κB activation in response to O_3 is plausible since Mason *et. al.* (2002) found that the c-Rel subunit of NF-κB was required for IL-12p40 production by a range of infectious and inflammatory stimuli (Mason et al., 2002). Clearly, NF-κB plays a central role in mediating responses to oxidant injury and can be influenced by pro- and anti-inflammatory signals. The mechanisms through which this occurs are not well understood. Activation of specific inflammatory transcription factors may be one important regulatory factor and remains to be tested. The upstream adaptor molecule, MyD88, may be one link that leads to NF-κB activation since targeted disruption of MyD88 results in loss of IL-1 and IL-18-mediated functions (Adachi et al., 1998) as well as TLR4 responsiveness. Receptor clustering by lipid rafts, or perhaps a common ligand formed as a secondary oxidation product may determine novel and potential antagonistic effects amongst these receptors and mediators to regulate NF-κB function. IL-1R, TLR4, IL-18R1, TNFR, IL-10 and IL-12p40 have complex and antagonistic effects on NF-κB activation. Their multiple interactions, converging at NF-κB, modulate the inflammatory response to oxidant injury.

Ozone and the adaptive immune response

A successful innate defense response in the lung is critical to the transition and development of adaptive immune responses (Strieter et al., 2002). O_3 inhalation has been shown to affect respiratory tract defense mechanisms, and increased susceptibility to infection (Becker et al., 1991), implying that O_3 can induce nonspecific and specific immune responses.

Several studies have described O_3-induced effects on the innate and adaptive immune systems including altered phagocytic activity (Jakab et al., 1995), inability to clear infectious disease (Steerenberg et al., 1996), decreased lymphoid organ weights, enhanced T-cell proliferation (Jakab et al., 1995), and enhanced response to allergen (Depuydt et al., 2002). Several studies have shown a direct effect of $CD4^+$ T-cell depletion on O_3-induced inflammation (Chen et al., 1995) (Bleavins et al., 1995) while others have shown O_3-induced effects on B cells (Becker et al., 1991).

Recent work in our laboratory has identified a role for major histocompatibility complex (MHC) II genes in O_3-induced inflammatory responses (Bauer, A.K. ***et. al.***, in press). The MHC II complex is expressed on antigen presenting cells such as macrophages and dendritic cells and permits antigen presentation to T cells and subsequent adaptive immune activation. MHC II gene expression occurred only in O_3-susceptible B6 mice, compared to resistant C3 mice. Moreover, deficiency in MHC class II genes decreased O_3-induced neutrophilia, implicating these genes in oxidant-mediated pulmonary inflammation. These genes are located within a QTL on chromosome 17 that was previously established as a genetic susceptibility locus for O_3-induced lung inflammation (Kleeberger et al., 1997). MHC II

genes thus provide a link between innate and adaptive responses, and further work may implicate these genes in modulating T-cell effects.

We also demonstrated that innate immune genes *Il12p40*, *Il10* and *Il18r1* modulate O_3-induced inflammation. In addition to APCs, IL-18R1 and IL-12R expression levels are highest on activated T cells (Gately et al., 1998; Sergi and Penttila, 2004). The IL-10 receptor is located on T-cells and macrophages, and has paracrine and autocrine effects on both cell types (Akdis et al., 2001). These genes may directly or indirectly stimulate adaptive immune activation.

We found that O_3-induced inflammation was significantly reduced in the absence of IL-12p40, an IL-12 subunit that also forms part of IL-23. IL-23 (composed of IL-12p40 and p19) has been implicated in the production of a new T cell subset distinct from Th1, called Th-IL-17 (Langrish et al., 2004) produced by memory T-cells (Kolls and Linden, 2004). IL-23 can also lead to enhanced antigen presentation and IFN-γ production that can alter T-cell activities (Langrish et al., 2004). These findings suggest that IL-23 may prime the adaptive immune response for allergic responsiveness via IL-17-producing memory T-cells but remains to be tested experimentally in response to O_3.

In response to *Chlamydia pneumoniae,* IL-12 and IL-18 stimulate IFN-γ production (Netea et al., 2004a) and potentially activate immune responses without antigen receptor engagement (Nakanishi et al., 2001). Thus T-cell stimulation can occur without physical contact with activated innate immune cells. In addition, IL-12 and IL-18 have been shown to prevent Th2-like cellular differentiation in allergic asthma (Akira, 2000; Hofstra et al., 1998), to sustain Th1 responses in rheumatoid arthritis (Gracie et al., 1999), and activate $CD8^+$ T-cells in response to LPS (Raue et al., 2004). In children with respiratory syncytial virus, IL-

12p40 and IL-18 limit Th2 effector molecules and potentially lead to atopy and asthma later in life (Wang et al., 2004). This evidence provides rationale for further investigation into whether IL-12 and IL-18 contribute to adaptive immune activation in response to oxidant injury.

Of the cytokines we investigated, IL-10 is the most pleiotropic. *IL10* polymorphisms are associated with asthma phenotypes in children and indicate a role for adaptive immune responses (Lyon et al., 2004). IL-10 suppresses CD86 costimulatory molecule expression on APCs (Soltys et al., 2002), a critical signal for adaptive immune activation. Moreover, IL-10 directly blocks the CD28 co-stimulatory signal in T cells in allergic inflammation (Akdis et al., 2001). $CD4^{+}CD25^{+}$ regulatory T-cells secrete IL-10 to suppress immunity against *Candida albicans* (Netea et al., 2004b), *Bordetella pertussis* (Higgins et al., 2003), and autoimmune thyroiditis (Gangi et al., 2005). B-cells, dendritic cells, and other T-cells also produce IL-10 and multiple cell types from both innate and adaptive immune cells can induce a "stop" signal at high levels of inflammation via production of this cytokine (O'Garra et al., 2004). Koike *et. al.* (2001) reported that expression of CD86, (a costimulatory molecule on APCs) was enhanced in response to O_3 (Koike et al., 2001). We showed that CD86 was also enhanced in $Il10^{-/-}$ compared to $Il10^{+/+}$ mice. These findings indirectly suggest that T-cells, responding to CD86, may be primed in response to O_3 and may be under the control of IL-10. To further elucidate O_3-induced adaptive immune effects mediated by IL-10 and other mediators, we must investigate T-cell specific endpoints such as differential T-cell activation, CD28 expression, and T-cell activity.

Future Directions

IL-23 and IL-27 may contribute to O_3-induced inflammation

In assessing the role of the heterodimeric cytokine IL-12, we determined that one subunit, IL-12p40, contributes to O_3-induced inflammation. IL-12p40 also forms part of the newly characterized cytokines IL-23 and IL-27 (Hunter, 2005). The possible pathological roles of these two cytokines in oxidant-mediated lung injury have not been determined.

IL-12 and IL-23 are cytokines produced by activated macrophages within hours of stimulation. An IL-12p40-dependent but IL-12p70-independent mechanism controls mycobacterial and *Francisella tularensis* infections, suggesting novel and unique roles for the p40 subunit (Langrish et al., 2004). IL-12p40 has been implicated in pulmonary responses to *Mycobacterium tuberculosis* (Cooper et al., 2002) and was later specifically associated with IL-23 (Happel et al., 2005). Also TLR4 is required for induction of p19 subunit of IL-23 in response to *Klebsiella pneumoniae* (Happel et al., 2003). In a model of enterocolitis, TLR4-mediated recognition of microbial components triggered IL-12p40 production in *Stat3*-deficient mice (Kobayashi et al., 2003). The potential connection between TLR4 and IL-12p40/IL-23 cytokine production provides further rationale to determine whether IL-23 is differentially expressed in *Tlr4*-normal vs. *Tlr4*-mutant mice and, in turn, whether there is a possible pathological role for IL-23 in oxidant-mediated lung injury.

IL-27 limits the intensity and duration of adaptive immune responses (Hunter, 2005) and induces rapid ICAM-1 expression on naïve $CD4^+$ T-cells, leading to Th1 differentiation (Owaki et al., 2005). O_3-induced IL-27 production has not been tested in differentially

susceptible mouse strains. Based on its functionality, IL-27 may best be assessed at later timepoints using T-cell based assays.

Ozone-allergen studies:

Asthma is a chronic, inflammatory disorder with a complex etiology consisting of both polygenic and environmental factors (Backus-Hazzard et al., 2004). Asthma symptoms include wheeze, airway hyperresponsiveness, pulmonary inflammation, and mucus hypersecretion. Atopic asthmatics comprise approximately 75% of asthmatic patients and have a Th2 immunological profile that includes airway eosinophilia, IgE production, and increases in IL-4, IL-5, and IL-13 (Umetsu et al., 2002). Epidemiological and experimental evidence suggest that air pollutants such as O_3 exacerbate asthmatic symptoms and may contribute to increased asthma prevalence (Kunzli et al., 2003; McConnell et al., 2002; Vagaggini et al., 2002). O_3 may have a priming effect on allergen-induced responses and intrinsic inflammatory action in nasal airways of asthmatics (Peden et al., 1995). Further mechanistic work is needed to fully characterize the pathophysiology of O_3-induced inflammation in allergic and asthmatic animal models (Kleeberger and Peden, 2005).

The present studies have identified IL-10 and IL-12p40 as new cytokine targets that critically regulate O_3-induced inflammation. Polymorphisms in *IL10* have been associated with allergic asthma in a North Indian family-based study (Chatterjee et al., 2005). In a separate study, polymorphisms in *IL10* (3' Untranslated region (UTR)) were associated with asthma-specific phenotypes such as FEV_1, percent predicted (measured FEV_1 as a percent of predicted FEV_1) and IgE levels (Lyon et al., 2004). Polymorphisms in *IL12B* have been associated with childhood asthma in a Japanese population (Hirota et al., 2005), adult asthma

in a white population (Randolph et al., 2004), and may be linked to asthma severity rather than susceptibility (Morahan et al., 2002). Thus, IL-10, IL-12p40 can critically affect adaptive responses. Moreover, there is a potential interaction between these two cytokines and atopic asthma. In addition, our current findings suggest that these cytokines importantly contribute to O_3-induced inflammation. For these reasons, we hypothesize that IL-10 and/or IL-12p40 contribute to O_3-induced exacerbation of allergic responsiveness.

Gene Array studies

The complex pathophysiology of O_3-induced inflammation is modified by a number of interactive genes of which only a few were tested in these studies. Of particular interest was the finding that O_3-induced neutrophilic inflammation was significantly enhanced in *Il10*$^{-/-}$ mice over that produced by an already susceptible mouse strain, the C57BL/6. Further analysis revealed that IL-10 affected a number of important downstream factors including NF-κB, MIP-2, and CD86. These findings were among the first to implicate an anti-inflammatory cytokine as critical to the response to O_3. To further characterize the complex phenotype elicited by IL-10, RNA was isolated from lung homogenates of *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice after exposure to air or O_3 and was hybridized to Affymetrix Gene Array chips containing all known mouse genes. This gene array currently awaits analysis and will hopefully provide insightful information as to related genes that interact with IL-10 and provide new targets for functional confirmation.

Human Implications

These projects focused on specific innate immunity genes *Cd14*, *Il18r1*, *Il12p40* and *Il10* and their contribution to the response to O_3 in mice. Ultimately, these results must be extrapolated to humans to establish clinical relevance as susceptibility genes. Human variants in these genes exist and are functionally important in inflammation and atopic phenotypes. For example, the *CD14*-C159 T single nucleotide promoter polymorphism represents a well-studied example of a genetic influence on diseases associated with inflammation (atopy) (Baldini et al., 2002; Kleeberger and Peden, 2005; Vercelli, 2002). In humans, different polymorphisms in the promoter region of *IL10* have been identified as risk and protective factors associated with the development of asthma (Chatterjee et al., 2005). Other *IL10* promoter polymorphisms are associated with increased LPS sensitivity (Schippers et al., 2005). Similarly, polymorphisms in *IL12B* have been linked with hepatitis C virus infectivity (Houldsworth et al., 2005), and atopic asthma (Hirota et al., 2005; Morahan et al., 2002; Randolph et al., 2004). Thus, each of these cytokines has polymorphisms that have been implicated in inflammatory diseases, and *CD14*, *IL10* and *IL12B* have specifically been linked to asthma. In addition, *IL18R1* promoter polymorphisms have recently been identified (Cardoso et al., 2004) but functional relevance to human disease has not yet been established (Table 5.1). The relationship between human polymorphisms in *CD14*, *IL10*, *IL12B*, and *IL18R1* and O_3 susceptibility has not been established. If associations are found, they could provide new insights into potential genetic markers of susceptibility and novel therapeutic targets. Moreover, polymorphisms in these genes, if associated with O_3 susceptibility, could also provide insight into the genetic contribution to O_3-induced exacerbation of asthma. However, individual single nucleotide

polymorphisms may be insufficient by themselves to cause disease, but combinations of variations in the same gene or in gene-gene interactions may eventually generate enough "hits" to generate dysfunction (Vercelli, 2002).

Limitations

Inflammation is a highly complex process

The innate immune defense system consists of numerous layers that are regionally distinct. In the upper airways, the mucociliary escalator and epithelial barrier of the upper respiratory tract form primary defense barriers while innate immune cells such as macrophages, natural killer cells, mast cells, and dendritic cells as well as secreted mediators monitor the lower airways. All of these components are organized to sequester the invading pathogens, neutralize them and then repair the lesion or remodel the tissue. If these processes are not properly regulated, excessive tissue inflammation can lead to irreversible tissue damage (such as fibrosis) (Strieter et al., 2002). The present studies focused on airway inflammation mediated largely by macrophages. Specific cell types in the lower and upper airways were simultaneously affected by O_3 and contributed to an overall inflammatory phenotype.

Cytokines such as IL-12, IL-18, and IL-10 are instrumental in orchestrating inflammation and have been shown to be critical in modulating the pulmonary response to O_3 in particular. However, these cytokines do not act in isolation but rather in combination with a number of other factors including arachidonic acid metabolism, coagulation factors, complement, acute phase proteins, and antimicrobial peptides. The present studies did not incorporate consideration of the complex interaction between cell types, cytokines and other inflammatory mediators.

Genetic background

To study the genetic contribution underlying O_3-induced pulmonary inflammation, we utilized commercially available gene targeted knock out mice in a candidate gene approach. The advantages of using a mouse model include availability, quick generation time, genetic similarity between individuals, large numbers of progeny from controlled crosses, and the possibility of studying contributions from whole or segments of chromosomes using congenic and consomic strains.

However, genetic background contributes substantially to differential responses to exposure. In response to 0.3 ppm O_3, neutrophils were significantly elevated in C57BL/6 compared to C3H/HeJ mice (Kleeberger et al., 1993a). Other mouse strains respond to O_3 along a continuum from resistant to susceptible. To control for this potential confounding factor, all gene targeted knock-out strains used in our studies were generated on a similar, mixed C57BL/6 – 129 (B6.129) background (e.g. *Il12*$\alpha^{-/-}$. *Il12*$\beta^{-/-}$, *Il18*$^{-/-}$, *Il18r1*$^{-/-}$, *Il10*$^{-/-}$). Mice generated as B6.129 hybrid strains were repeatedly backcrossed to C57BL/6 mice for at least 10 generations (*Il18r1*$^{-/-}$: 10 generations, *Il18*$^{-/-}$: 10 generations, *Il12*$\beta^{-/-}$: 11 generations, *Il12a*$^{-/-}$: 11 generations, *Il10*$^{-/-}$: 10 generations) and are thus comparable to the C57BL/6 strain, used as a wildtype control. However, we cannot rule out the possibility that the 129 background may slightly influence the response in these strains. Moreover, the C57BL/6 strain is a high TNF-α responder and a high IL-10 responder (De Maio et al., 2005) and has a predisposition toward a Th1 cytokine profile. This basal profile influences the way these mice respond to O_3.

Gene-Environment interactions are complex

The etiology of complex lung diseases is difficult to determine because of the interaction between multiple genetic and environmental factors (Kleeberger and Peden, 2005). Moreover, an individual single nucleotide polymorphism (SNP) may be insufficient to cause disease but might become significant when combined with other SNPs or specific environmental exposures (Vercelli, 2002). Multiple genes (each with modest effects) operate to modify pulmonary inflammatory responses to O_3 and, in addition, these multiple genes interact with multiple environmental factors that contribute to disease pathogenesis (Kleeberger and Peden, 2005). The candidate gene approach used in these studies is an important tool for gaining insight into the genetic pathways that mediate pulmonary inflammation and can provide the information necessary to understand at a molecular level how these variants contribute to differential phenotypes (De Maio et al., 2005). However, the single gene approach does not permit investigation of the complex interplay of genetic and environmental factors that truly drive these responses. To address these concerns, the candidate gene approach can be used in a gene array to witness gene-gene interactions in response to single or relevant combinations of environmental exposure(s).

Conclusion

The negative effects of air pollution have received public attention in the U.S. since the early 20th century. O_3 damages lung tissue, aggravates lung disease, and increases susceptibility to respiratory infections particularly in children, the elderly and individuals with preexisting disease, such as atopic asthma (Mudway and Kelly, 2000). O_3-induced pulmonary inflammation is a tightly orchestrated process involving antioxidants as well as numerous cell types and gene products (De Maio et al., 2005). This project focused on the individual contribution of several important regulatory and anti-inflammatory cytokines, as well as a protein associated with the important innate immune receptor, TLR4, in response to the environmental pulmonary toxicant, O_3. These studies showed that O_3-induced inflammation is probably mechanistically distinct from other insults (such as LPS and RSV). In viewing the lung as immunologically active, this dissertation showed that innate immune response genes contribute to differential susceptibility to O_3-induced neutrophilic inflammation, and provided new targets for further research. In particular, these studies are the first to determine that an anti-inflammatory cytokine, IL-10, can attenuate O_3-induced inflammation. In addition, data presented here provided indirect evidence that IL-12p40 and IL-10, cytokines that have paracrine effects on adaptive immune cells, may modulate adaptive immune responses. These candidate genes should be tested in an O_3-allergen model to assess their putative role as susceptibility genes underlying O_3-induced exacerbation of allergic responsiveness. Overall, this dissertation contributed to an increased understanding of the complex gene(s)-environment interactions involved in pulmonary oxidant injury.

Table 5.1: Human polymorphisms in innate immune genes and disease associations

Gene	Polymorphism	Disease Association	Reference
CD14	-260: C/T	House dust endotoxin	Zambelli-Weiner, A et. al., 2005
		Myocardial infarction	Hubacek, AJ et. al., 1999
	-159: C/T	Cardiovascular mortality in dialysis pts	Losito, A et. al., 2005
		Risk of atopy	Baldini M et. al., 2002
			Kleeberger, SR and Peden, DB., 2005
IL12B	Polymorphism in the 3' UTR	Autoimmune diabetes	Adorini L., 2001
	3' UTR, +1188: A/C	Hepatitis C infectivity	Houldsworth, A et. al., 2005
	3" UTR, 10841: C/A	Atopic asthma	Hirota, T. et. al., 2005
	3' UTR +1188 A/C		Morahan, G. et. al., 2002
	+4237: G/A		Randolph, AG et. al., 2004
IL12A	+8685: G/A	Occupational benzene exposure	Lan, Q et. al., 2005
IL18	+105: G/C	Asthma	Higa, S et. al., 2003
	-607: A/C	Allergic rhinitis	Lee, HM et. al., 2006
	+113: G/C, +127: G/C, -137: G/C, -33: G/C	Atopic eczema	Novak, N et. al., 2005
	-148: G/C, +13925:A/C	Mite allergen	Shin, HD et. al., 2005
IL18R1	-69: C/T -638: C/T	N/A	Cardoso, SP et. al., 2004
IL10	Promoter, IL10 R/G haplotype	LPS sensitivity	Eskdale, J et. al., 1998
	IL10.R microsatellite	Rheumatoid arthritis	Eskdale, J et. al. 1998
	-592: *A or *C	SIDS	Summers, AM et. al., 2000
	-2849: A/G	Rheumatoid arthritis	Lard, LR et. al., 2003

Known polymorphisms in human innate immune genes (*CD14*, *IL12A*, *IL12B*, *IL18*, *IL18R1*, and *IL10*) are associated with numerous inflammatory diseases including atopy, allergy, and asthma.

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