ABSTRACT

GOWDY, KYMBERLY MAE. Increased Susceptibility and Severity of Influenza in Mice Exposed to Diesel Exhaust. (Under the direction of Dr. Ian Gilmour and Dr. Linda Martin.)

Epidemiological studies have noted an increase in adverse health effects with increasing levels of air pollution. One major area of concern is the incidence of respiratory infections, specifically influenza. An air pollutant that has raised concern in recent years is diesel exhaust (DE) due to an increase the amount of diesel engines in use. Previous laboratory studies have reported that DE exposure prior to an influenza infection increases viral titers but the mechanism of how this occurs is still unknown. Herein, studies were designed to investigate three main areas associated with DE enhanced influenza infection. 1) Assess whether DE affects host defense responses against pathogens, 2) Determine if pre-exposure to DE increases susceptibility to influenza *in vivo*, 3) Investigate whether exposure to DE increases the severity of an ongoing established influenza infection. DE exposure alone increased proinflammatory cytokines, adhesion molecules, decreased expression and production of surfactant proteins (SP)-A and D as well as clara cell secretory protein (CCSP). The molecules downregulated by DE are important for binding viral and bacterial pathogens therefore making the lung more susceptible to infection. This was confirmed when mice were exposed to DE and then subsequently infected with influenza A. One day post infection mice pre-exposed to DE had a significant increases in influenza induced inflammation and viral titers that were associated with a decrease in SP-A and SP-D. Mice exposed to DE during an

established influenza infection also had a significant increase in viral titers and pulmonary inflammation throughout the course of infection. This DE-enhanced influenza infection was associated with an upregulation of the Th2 cytokine IL-4 which has previously been shown to delay clearance. However with antioxidant treatment to combat the oxidative stress induced by DE, pulmonary inflammation and IL-4 expression returned to baseline levels. Taken together these data indicate that exposure to DE either before or during influenza infection has immunomodulatory effects that can be detrimental to the host with increased viral proliferation and morbidity associated with the disease.

Increased Susceptibility and Severity of Influenza in Mice Exposed to Diesel Exhaust

by
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DEDICATION

DEDICATED TO MY PARENTS AND ALEX

BIOGRAPHY

Written by Roxanne and Wayne Gowdy

Hard working, self-motivated and an intense love for animals are all words that describe Kym. She has a passion for wanting to know every detail of how things work and how they interact with one another.

1997- Graduates from East Catholic High with Honors. The focus of her studies was relative to the Life Sciences.

2001- Graduates from Virginia Tech with a Bachelor of Science Degree in Animal and Poultry Science. Her experiences included leadership positions in the Chemistry Fraternity and Poultry Science Club. Months before graduation Kym had already set her sights for the next challenge ….a Masters Program in Immunology and Poultry Science at North Carolina State University.

2004- Graduates from North Carolina State University with Masters Degree in Immunology and Poultry Science under the direction of Drs. Edens and Qureshi. Her research focused on the impact of selenium supplementation in avian immunology. Looking to expand the scope of her research, Kym contacted Dr David Thomas of the U.S. E.P.A. who had been doing similar research with mammals. Dr Thomas was so impressed by the techniques that she was using and the progress that she had made that he offered the use of his facility to expand her research.

2008- Graduates with a PhD in Immunology and Toxicology from North Carolina State University under the direction of Dr. Ian Gilmour. The basis of her PhD is relative to the impact of air pollution on the susceptibility to influenza infection.

Kym's unwavering desire to learn more and investigate further has carried her through these four years of PhD studies. She has had the support of her parents, Alex, Grandmother, immediate family and friends.

Knowing Kym's abilities, it is not hard to have high expectations for what she can and will accomplish with her life. We continue to be very proud of her, are amazed at her achievements and feel blessed to be part of her life.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Literature review

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As Host Defense and Immunotoxicology of the Lung

M.I. Gilmour and K.M. Gowdy

Chapter 1: Literature review

1. Introduction

Respiratory allergies and infections are the most common form of illness in the United States and Europe and account for more missed school and work days than any other disease (Akazawa *et al.*, 2003). A substantial body of experimental work has clearly shown that airborne toxicants such as tobacco smoke, ozone and other air pollutants can alter many aspects of the host defense network to either decrease resistance to infection, or exacerbate respiratory allergies and asthma (Cohen *et al.*, 2000). Exposure to air toxicants can suppress a number of key host defenses including mucociliary clearance in the airways, pulmonary macrophage function and development of specific immune responses such as IgG antibody production and cell mediated immunity. In contrast, immune stimulation in the form of increased T cell activity and IgE antibody formation has also has been shown to occur under some circumstances, resulting in increased incidence or severity of allergic lung disease.

These results continue to be confirmed in clinical, epidemiological and experimental studies while basic research activities seek mechanistic explanations for these effects. In particular, increased understanding of dendritic and T regulatory cell function, as well as the discovery of additional classes of antimicrobial molecules and pattern recognition receptors (PRRs) has inspired a new wave of interest in host-pathogen and host-allergen interactions, and provided an interesting link between innate and adaptive immunity. As toxicology studies begin to investigate the functionality of these cells and molecules, a fuller understanding of disease susceptibility following pollutant exposure will emerge. The

purpose of this chapter is to summarize new advances and understanding in pulmonary immunobiology, and to present recent information on how these processes are affected by inhaled pollutants.

2. Immune Function and Immunosuppression in the Lung

In a seminal review article, Green and colleagues (Green *et al.*, 1977) wrote that "despite the daily microbial assault that the respiratory tract experiences, the gas exchange area of the lung is maintained in a remarkably sterile condition by the combined antimicrobial activity of the mucociliary, phagocytic and immune systems". Much of this understanding was gained from experimental infections in rodents in which investigators reduced or eliminated specific defense components in order to demonstrate their anti-microbial value. Techniques involved monitoring the physical clearance of inhaled radio-labeled pathogens from the mouse lung and reduction in viable counts, as a means of comparing physical removal of the microbes to bactericidal activity.

Studies using isolated pulmonary macrophages from lung washes showed that exposure to various agents including ozone, nitrogen oxides, sulfur dioxide, metal compounds and tobacco smoke reduced the cells ability to ingest and/or kill bacteria through defects in bactericidal enzymes and impaired tumoricidal and anti-viral capability (Gardner, 1984). From this extensive body of work some air pollutants were described as immunotoxicants, because of their ability to suppress humoral and cellular host defenses and increase susceptibility to infection (Jakab *et al.*, 1995). During the 1980s and 90s, dose

response relationships for these and other compounds were developed, and the mechanistic basis for effects studied through assessment of cellular function, antibody production after immunization, T cell phenotype changes and host resistance assays.

At the same time that immunosuppression was being noted in a variety of different experimental systems, investigators were also reporting that air pollutant exposure, in tandem with antigen sensitization, could result in stimulation of IgE antibody and a subsequent increase in the number or severity of anaphylactic (allergic) type reactions (reviewed in (Granum and Lovik, 2002)). These observations were first noted with O_3 and NO_2 and more recently after exposure to combustion particles such as oil fly ash and diesel emissions.. While the mechanisms for these effects still need clarification, they likely lie at the level of antigen processing and presentation, at which stage the quality and quantity of specific immune responses is programmed. The following sections will describe the major components of the host defense network and how they may be compromised by air pollutant exposure, while the latter part of the chapter will review mechanisms for immuno-enhancement and allergic adjuvancy.

2.1 Mucociliary clearance mechanisms

The first line of airway defense against inhaled airborne particles and microbial pathogens is mucociliary clearance. This process involves coordinated beating of ciliated cells propelling a complex layer of mucus and surfactant up the airways. Mucus, which exists in a biphasic layer, is secreted by goblet cells on the surface epithelium and the

mucous and serous cells of the submucosal glands (Kim *et al.*, 1997). Four genes have been identified for airway epithelial mucins (MUC 1, MUC 2, MUC 4, and MUC5) (Kim *et al.*, 1997). Inhaled materials that land on the upper airways are propelled at a rate of 4-12 mm per minute up to the oropharynx where they are swallowed (Hofmann and Sturm, 2004). Particles that reach the lower airways and alveolar region of the lung are ingested by macrophages which then either migrate through interstitial pathways to the lymphatics, or are transported up to the mucociliary escalator.

Decreased mucociliary clearance has been observed in the lungs of smokers suffering from bronchitis (Nakagawa *et al.*, 2005). In addition, exposure to other agents such as sulfuric acid slows ciliary beat (Schlesinger *et al.*, 1983), while exposure to relatively low concentrations of ozone (in monkeys and rats) causes exfoliation of the ciliary surface (Hastie *et al.*, 1997). In general, respiratory infections as well as other forms of lung injury increase mucus production as a protective response. Despite this apparent benefit, over-production of mucin impairs ciliary beating, resulting in mucus and trapped particles staying in the lungs (Schiff and Graham, 1984). High levels of mucus are also present in airway diseases such as bronchitis, chronic obstructive pulmonary disease (COPD) and asthma (Prescott *et al.*, 1995; Vestbo *et al.*, 1996; Hasani *et al.*, 2005).

2.2 Antimicrobial Secretions

The respiratory tract is constantly bathed with a complex mix of surfactants and enzymes that maintains surface tension, facilitates gas exchange and provides protection

against reactive molecules and inhaled microbes. Some of the principal mediators, which are bactericidal or bacteriostatic, include lysozyme, complement components, collectins (surfactant proteins A and D), and the alpha and beta defensins (Table 1). Lysozyme is secreted by serous cells, macrophages and neutrophils, and has the ability to lyse and kill gram positive bacteria via enzymatic cleavage of peptidoglycan (Coonrod, 1986). Exposure to ozone has been shown to reduce lung lining fluid levels of lysozyme in rats (Shelley, 1994), while living in a highly polluted environment (New Delhi) has been associated with suppressed lysozyme production in the eyes (Gupta *et al.*, 2002).

The complement enzyme cascade is an important host defense mechanism in the lung, functioning to: kill microbes through the classical and alternate pathways; augment phagocytosis by opsonizing bacterial membranes; and recruit additional phagocytes via chemotactic fragments C3a and C5a. Complement molecules are produced locally by macrophages, type II pneumocytes and fibroblasts (Kaltreider, 1993), and inactivation with cobra venom factor, or through gene deletion, increases susceptibility to infection (Lukacs *et al.*, 2001; Tuite *et al.*, 2005). Expression of the receptors for C3a and C5a is increased by exposure to numerous biological contaminants such as lipopolysaccharide (LPS; bacterial endotoxin) and allergens (Drouin *et al.*, 2001), while several environmental agents including ozone, diesel exhaust, cigarette smoke and other particulates also activate complement or increase the deposition of C3 in the airway epithelium (Kanemitsu *et al.*, 1998; Park *et al.*, 2004) Specifically, cigarette smoke stimulates the complement pathway by cleaving an internal thiol ester bond in C3 (Kew *et al.*, 1987; Robbins *et al.*, 1991; Shima and Adachi,

1996). There are no reports showing a decrease in complement activity with air pollutant exposure, but rather an increase has been noted in a number of allergic systems, suggesting a relationship between air pollution-dependent increases in complement fragments and greater risk or severity of allergic airways disease (Walters *et al.*, 2002).

Alpha and beta defensins are small molecular weight peptides which have cidal activity against gram positive and negative bacteria, mycobacteria, fungi, and some viruses (Zhang *et al.*, 2000). In humans, six α defensins (HD 1-6) and two β defensins (HBD 1 and 2) have been characterized. Human α defensins 1-4 are synthesized by recruited neutrophils and released in response to stimuli such as proinflammatory cytokines or tissue injury (Ashitani *et al.*, 1998). HBD 1 and 2 genes are expressed in respiratory tract epithelial cells and HBD-2 is inducible by cytokines such as IL1-β (Zhao *et al.*, 1996; Singh *et al.*, 1998). Activated alveolar macrophages (AM's) produce defensins which can also act as chemoattractants for dendritic cells and T lymphocytes, thus encouraging generation of specific immune responses (Duits *et al.*, 2002). Defensins are inhibited by high salt concentrations as is seen in cystic fibrosis patients, (Kagan *et al.*, 1990; Goldman *et al.*, 1997) but presently there is no information on how exposure to lung toxicants affects defensin production and activity other than the fact they are upregulated in the lungs of smokers (Merkel *et al.*, 2005).

The pulmonary surfactant proteins A and D (SP-A and SP-D), termed collectins, are a family of carbohydrate binding proteins synthesized by alveolar type II cells and some non-ciliated bronchiolar epithelial cells (Crouch, 1998). Collectins are essentially opsonins that

enhance phagocytic activity of macrophages and neutrophils (Schagat *et al.*, 2001; Clark *et al.*, 2002), and activate cells via ligation with the heat shock protein/collectin receptor CD91 (Vandivier *et al.*, 2002) or through the binding of Toll-like receptors (Guillot *et al.*, 2002). Pulmonary collectins bind to various components of bacterial and fungal cell walls and viruses (LPS, lipoteichoic acids, lipoarabinomannans, polysaccharides, N-linked oligosaccharides in viral envelopes) through a carbohydrate recognition domain (CRD) (Crouch, 1998). SP-A -/- mice display delayed clearance of pathogens such as group B *streptococcus* (GBS) (LeVine *et al.*, 1997), *Haemophilus influenza* (LeVine *et al.*, 2000), RSV (LeVine *et al.*, 1999), *Pneumocystis carinii* (LeVine *et al.*, 2002) and *Pseudomonas aeruginosa* (LeVine *et al.*, 1998). The defect in clearance can be attributed to fewer alveolar macrophages ingesting and clearing the invading bacteria. SP-D -/- mice also show decreased alveolar macrophage phagocytosis of bacterial and viral pathogens (LeVine *et al.*, 2000; LeVine *et al.*, 2002). In addition however, these animals develop alveolar proteinosis and dilated distal airways as a result of increased levels of tissue and macrophage-associated metalloproteinases, macrophage derived oxidants, and phospholipids (Wert *et al.*, 2000). In terms of immunotoxicity, it is clear that cigarette smoke decreases the production of SP-A and SP-D which may explain in part increased infections in smokers (Honda *et al.*, 1996). Ozone exposure also affects the function of SP-A by decreasing its ability to modulate proinflammatory cytokine production by monocytes/macrophages (Janic *et al.*, 2005).

Other airway anti-microbial and anti-inflammatory soluble factors that protect the epithelium are: fibronectin, lactoferrin and cathelicidin. All are secreted to help the epithelial

surface eliminate inhaled materials. Fibronectin which is a cell adhesive glycoprotein important in tissue injury and repair, is secreted by lung fibroblasts after a broad variety of stimuli, including nicotine (Roman *et al.*, 2004). Lactoferrin in airway epithelial cells increases after exposure to catalytically active metals present in some air pollutants. These proteins transport and store metals and compete for free iron, thus diminishing oxidative stress and damage to the lung epithelium. Cathelicidins have multiple functions in normal lung homeostasis in addition to their known anti-microbial and LPS-neutralizing effects (Fahy and Wewers, 2005).

2.3 Resident Cells

Some forty cell types are found in the respiratory tract and virtually all have some form of host defense capability either through physical, regulatory, secretory, and phagocytic activities. The principal cells which keep the lung free of infection are the different types of epithelial and secretory cells, pulmonary macrophages, dendritic cells, neutrophils and lymphocytes, all of which interact in a complex and dynamic system. After exposure to an infectious or toxic agent, many of these cell types become activated (Figure 1) in order to respond and repair damaged tissue, and in some cases develop tolerance or immunity to a second insult.

Until recently, epithelial cells were considered to function solely as the ciliated barrier lining in the airways and as conduits for gas exchange at the air/blood interphase. As techniques have improved to isolate and culture these cells and measure their gene products,

it has become clear that they have a key role in lung defense and repair. Epithelial cells secrete a number of anti-microbial compounds and immunoregulatory cytokines (Knowles and Boucher, 2002), and are also capable of ingesting and killing bacteria (Beisswenger and Bals, 2005).

Epithelial cells differ in morphology and function depending on their location. The conducting airways have pseudostratified columnar (type II) epithelial cells which convert into cuboidal epithelial cells as the airways extend into branches. Embedded within the airway epithelium are secretory cells including goblet and clara cells. The epithelial lining in the conducting airways is an important barrier to external environmental stimuli, and when injured, has a variety of responses for rapid repair. Injury caused by inhaled pathogens or toxicants is characterized by a sloughing off of sheets of epithelial cells, leaving the underlying tissue more vulnerable to subsequent insult by those same or additional hazardous agents. Epithelial cells quickly proliferate and differentiate in order to maintain normal function. Chronic changes in the epithelial lining lead to limited airflow in conditions such as bronchitis, asthma and COPD (Mossman *et al.*, 2006).

The terminal and respiratory bronchioles (depending upon species) are enriched in secretory clara cells, whereas alveolar type I cells are the thin walled type I cells that facilitate gas exchange and are the primary producers of lung surfactant proteins and lipids. Clara cells are non-ciliated secretory epithelial cells in the airways that are distinct in morphology and release an anti-inflammatory molecule called the Clara cell 10 kD protein (termed CC10, CC16, or CCSP) (Singh and Katyal, 1997). Mice exposed to aged and diluted

cigarette smoke and/or ozone have much less CC10 in the airways, suggesting a loss of protection against inhaled toxicants (Yu *et al.*, 2002). In another example, CC10 was also decreased in the lungs of mice exposed to diesel engine emissions (DEE) and then infected with *Pseudomonas aeruginosa* compared to control animals (Harrod *et al.*, 2005). These reports indicate that air pollutants can clara cell function resulting in compromised host defenses in the conducting airways.

Pulmonary macrophages are the principal phagocytes in the lung and function to clear both cellular debris and inhaled particulates, and to engulf and kill microbes. They are a diverse, relatively long lived and dynamic cell population, and differences in activity may reflect stages of maturity, level of stimulation or perhaps even specific cell sub-populations arising from different precursors. Macrophages are highly mobile and able to mediate direct engulfment of particles through several pathways (Taylor *et al.*, 2005). They bear numerous receptors on their surface including the mannose, glucan and scavenger receptors that bind to conserved molecules on the outer surface of microbes. In addition, macrophages express various types of complement and Fc receptors that facilitate uptake of opsonized material through the binding of C3b and specific antibodies respectively. During phagocytosis the macrophages extend pseupodia around the receptor-ligand complex and engulf and internalize the bound microbe. The resulting internalized phagosome fuses with cytoplasmic lysozomal granules and undergoes a series of maturation steps; becoming increasingly acidic, generating toxic oxygen and nitrogen intermediates and hydrolases, leading to the enzymatic destruction of the foreign particle.

Macrophages are the best studied cell in the lung relating to immunotoxicity. For over two decades it has been appreciated that exposure to air pollutants including ozone, sulfuric acid, metals, and cigarette smoke components like acrolein suppress both uptake and killing of inhaled microbes (reviewed in (Li and Holian, 1998)). These effects have been shown after ozone exposure in both experimental animals and human subjects, as well as during side by side in vitro exposures of cells from different species including humans (Selgrade, 1999). Despite much research in this area, the actual mechanisms for these effects are not well understood. It is likely that some cells undergo direct membrane damage and loss of function, while others are influenced by the generation of immunosuppressive mediators such as $PGE_{2,}$ which reduces phagocytic activity (Canning *et al.*, 1991). Macrophages are also in themselves rich sources of prostaglandins, cytokines and other regulatory molecules, and as such, play a key role in initiating and maintaining inflammatory responses and in eventual tissue repair and resolution of injury.

Macrophages express an array of scavenger receptors which enhance uptake of low density lipoproteins (LDL), bacteria and other inhaled particles (Brown and Goldstein, 1983; Arredouani *et al.*, 2004). One molecule in particular, termed MARCO (MAcrophage Receptor with COllagenase structure), (Elomaa *et al.*, 1998),(Kodama *et al.*, 1990) binds to gram positive and gram negative bacteria like the other scavenger receptors, but does not perform the normal duties of mopping up acetylated and oxidized LDL (Hampton *et al.*, 1991; Elshourbagy *et al.*, 2000). In a recent study (Arredouani *et al.*, 2004), MARCO deficient mice were used to examine the role of scavenger receptors in alveolar macrophages

when faced with a pneumococcal infection and an acute exposure to TiO_2. The absence of MARCO impaired the ability of AM to clear the pneumococcal infection from the lungs, decreased opsonization of the environmental particles and enhanced pulmonary inflammation and cytokine release, indicating the importance of scavenger receptors in innate immune responses against inhaled air pathogens and pollutants.

Neutrophils are not present in large numbers in the normal healthy lung but infiltrate rapidly from the blood stream during inflammation and in response to infection and injury. Some of the principal chemo-attractive agents for neutrophils are complement components produced by macrophages, and pro-inflammatory cytokines such as the C-C and CxC chemokines, as well as the better known mediators like IL1β and TNF-α. Once recruited to the site of infection or injury, neutrophils readily engulf and deactivate microbes with a strong oxidative burst, involving the membrane NADPH oxidase complex (Becker *et al.*, 2002). A decreased neutrophil response, as is seen with alcohol exposure (Zhang *et al.*, 2002), leads to increased susceptibility to infection; however, defects in the function of these cells by air pollutants have not been reported (Dorio and Forman, 1988).

Dendritic cells (DCs) are the principal antigen presentation cells in the respiratory tract and are pivotal in the generation of specific immune responses against pathogens as well as allergens. DCs originate from the bone marrow and migrate around the body before becoming resident in specific tissues such as the lung, and additional cells may be recruited during antigen exposure, infection, or injury (McWilliam *et al.*, 1995). Immature DCs are generally regarded as being phagocytic, but after antigen sampling this activity is markedly

decreased, while specific surface markers including CD80, CD86, and MHC-II, are up-regulated (Banchereau and Steinman, 1998; Takahashi and Kobayashi, 2003). The DCs then migrate to secondary lymphoid organs where they present antigen and co-stimulatory signals to circulating lymphocytes (Steinman *et al.*, 1997).

Four distinct human DC types have been identified: classic tissue DCs, Langerhan cells, monocyte-derived DC, and plasmacytoid DC (Shortman and Liu, 2002). The first three DCs are termed myeloid because of their origin and similarities of function. Plasmacytoid DCs on the other hand are classified based on a very immature phenotype, Toll Like Receptor (TLR) 7 and 9 expression, and vigorous release of type I interferon in response to viruses (Gunn, 2003). Myeloid DCs express high levels of CD11c, CD11b, and MHC II, whereas plasmacytoid DCs have low levels of CD11c and no CD11b. It has been hypothesized that myeloid and. lymphoid DCs have different roles in the immune response: myeloid DCs sample Ag in the periphery, and travel to secondary lymph nodes, whereas plasmacytoid DCs regulate immune responses and are involved in immunological tolerance (Saunders *et al.*, 1996; Kadowaki and Liu, 2002)**.** Chronic cigarette smoke exposure has been shown to decrease the number of DC (MHC II/CD11c^{high}) in the lungs, but not the lymph nodes, of mice compared to control animals (Robbins *et al.*, 2004). Expression of the co-stimulatory molecule CD80 was also decreased, indicating a reduced capacity to present antigen. In vitro exposure of bone marrow derived DCs to nicotine has also been reported to inhibit cytokine production and T cell proliferation (Nouri-Shirazi and Guinet, 2003).

The respiratory tract is a rich source of intra- and sub-epithelial lymphocytes and has defined regions of bronchus associated lymphoid tissue (BALT) as well as numerous peripheral lymph nodes. T and B lymphocytes make up the effector arm of the specific immune response, generating cytotoxic and antibody mediated reactions which rid the lung of pathogens and prevent re-infection through the development of immunological memory. In addition to the classic helper (CD4) and cytotoxic (CD8) subsets, CD4 cells are also separated into T helper 1 and T helper 2 types depending on their cytokine profile and function (Kay, 2006). CD4/CD25 T regulatory cells are the most recent cell type to be defined as a distinct sub-population and are thought to control the development of immunological tolerance through the production of IL-10 (van Oosterhout and Bloksma, 2005).

Ozone is the most studied inhaled pollutant that suppresses lymphocyte function in the lung (reviewed in (Jakab *et al.*, 1995)), whereas diesel exhaust has been reported to have an apparent adjuvant effect that biases T cell function towards an allergic phenotype (Ohtani *et al.*, 2005). In general, ozone exposure causes a decrease in T cell function, as measured by proliferative responses to mitogens and delayed type hypersensitivity reactions after immunizations (Jakab, 1993). These decrements have also been associated with increased susceptibility to infections such as *Listeria monocytogenes*, which requires T cell mediated immune responses in order to clear the pathogen (Steerenberg *et al.*, 2004). The effect of ozone on antibody production is less clear. In some situations antibody levels are increased, while in others they are decreased depending upon; the route of antigen administration, the

exposure regimen and the class of antibody measured (Gilmour, 1995). The mechanisms for these effects are not understood although presumably altered T cell function changes communication between the afferent and efferent immune processes. It is known that skewed polarization between Th1 and Th2 immune responses drastically alters the quality and quantity of antibody responses as well as the potency and effectiveness of host defenses (Kimber, 1998). This is well demonstrated in the *Leishmania major* model in which polarization of Th1 or Th2 responses predicts survivability (Reiner and Locksley, 1995). For example, BALB/c mice are more susceptible to protozoal infection despite eliciting a strong Th2 response, while C57BL/6 mice control and overcome the disease through the generation of a prominent Th1 response (Sacks and Anderson, 2004). More recent studies using gene knock out mice have implicated the specific roles of IL-12 to control infection and IL-4 to exacerbate the disease, indicating that the cytokines ratios produced in the context of Th1/Th2 responses can profoundly influence the quality of immunity and disease protection (Stager *et al.*, 2003).

While reported changes in specific immunity, as measured by T and B cell effector function, are commonplace, the mechanisms underlying these effects are poorly understood. From the ozone literature it is known that adrenalectomy can substantially mitigate ozone dependent T cell suppression (Dziedzic and White, 1987), suggesting that endogenous corticosteroids and the stress response are involved. Several other possibilities exist, however. Exposure to immunotoxicants can change the mucosal milieu by causing inflammation and increased permeability of the epithelial barrier (Matsumura *et al.*, 1972). In

this way antigen can penetrate more deeply or in greater amounts towards sub-epithelial immune tissue. At the level of accessory macrophages and dendritic cells, immunotoxicants may also affect uptake and processing of antigen or alter production of influential immunoregulatory cytokines such as IL12 (Rusznak *et al.*, 1997).

NK cells are important effector non-T, non-B lymphocytes that recognize and bind to tumor and virus infected cells before mediating cytotoxicity through F porin production. In mice immature ($NK1.1^{+}Ly49^{-}$) and mature NK cells ($NK1.1^{+}Ly49^{+}$) leave the bone marrow and travel to various lymphoid compartments including those in the lung (Morris and Ley, 2004). Once resident in tissue, NK cells produce cytokines and chemokines (IFN-γ, TNF-α, CXCL8 (reviewed in (Raulet *et al.*, 2001)) and recognize target cells based on their absence of MHC class I or via receptors including NKG2D, Ly49s (mouse), and KARs (human) (Raulet *et al.*, 2001; Morris and Ley, 2004). This unique system of recognition allows NK cells to be one of the first lines of defense that is able to detect tumour cells and virus-infected cells. Exposure to ozone have been shown to decrease both pulmonary and systemic NK activity in animals (Burleson *et al.*, 1989) as well reduce circulating NK activity in humans (Harker *et al.*, 1990), although the mechanisms for these effects are not known.

2.4 Pattern Recognition Receptors (TLRs)

Until the late 1990s, the term innate immunity referred to non-specific host defenses such as bactericidal enzymes, macrophages and other recruited phagocytes. This arm of the immune system was classified as not having memory responses or specificity to particular

types and classes of pathogens. Toll-like receptors (TLRs) are conserved in both invertebrates and vertebrates. TLRs were first discovered in *Drosophila* as a genes important in embryonic development (Lemaitre *et al.*, 1996), and were later recognized as being important in resistance to bacteria and fungi (Michel *et al.*, 2001; Rutschmann *et al.*, 2002). This conserved pathway was later identified in human and mice through DNA sequence homolgies across various species. The TLRs recognize specific conserved microbial components termed pathogen associated molecular patterns (PAMPs) such as mannans from yeast cell walls, LPS from gram negative bacteria, lipoproteins and peptidoglycans from gram positive bacteria and bacterial DNA as characterized by presence of CpG motifs.

The PRRs (pattern recognition receptors) recognize PAMPS, with two principal reactions: mediation of acute phagocytosis (internalization) and rapid cellular and tissue activation with subsequent stimulation of specific immune responses. TLR often work in tandem with other co-receptors such as CD14, which is a required accessory signal for LPS. TLRs provide intracellular signaling through the TIR (Toll/Interleukin-1 receptor) domain via the MyD88–IRAK-TRAF 6 cascade which upregulates transcription factors including NFkB. In addition to this process, the receptors also utilize additional signaling pathways such as TRIF and TRAM which stimulate the expression of type I interferons (Chaudhuri *et al.*, 2005). Table 2 shows the principal recognition components of the TLR family and consequences for susceptibility to infection in animals with defects in TLR genes. The TLR receptors are found on a number of cells types, including macrophages, dendritic cells and epithelial cells. TLR 1, 2 and 6 recognize gram positive bacteria and yeast through

recognition of cell products including lipo-teichoic acids. TLR 3 is usually located intracellularly and recognizes double stranded (viral) RNA. TLR-4, in association with CD-14, recognizes LPS on gram negative bacteria. TLR5 recognizes bacterial flagellin. TLR 7 and 8 recognize viral nucleic acids. TLR9 recognizes CpG motifs present on bacterial DNA. TLR 10, 11 and 12 have been identified but their function is not yet known. While these receptors are clearly important in the generation of protective immune responses, their modulation during immunotoxicologic events needs to be investigated. Inappropriate stimulation of TLRs in organs such as the lung has been implicated in the pathogenesis of diseases. One of the first studies to examine TLRs in lung inflammation (Kleeberger *et al.*, 2000; Kleeberger *et al.*, 2001) reported that LPS resistant mice (C3H/HeJ) with a defect in TLR4 signaling had decreased pulmonary injury following ozone exposure, indicating a possible role of TLR4 in this effect.

3. Immunoenhancement and Adjuvancy in the Lung

As noted above, exposure to air pollutants in association with immunization with soluble protein antigens can result in stimulation of allergic immune responses through the promotion of T helper 2 cytokine responses and increased development of IgE antibody production (reviewed in (Peden, 1996)). This phenomenon was first described in mice (Gershwin *et al.*, 1981) and in monkeys (Biagini *et al.*, 1986) exposed to ozone, but has since been demonstrated following exposure to NO_2 (Gilmour *et al.*, 1996), and oil fly as (Lambert *et al.*, 2000), and most frequently in association with diesel exhaust particles (Diaz-Sanchez

et al., 2000). Animal experiments have since demonstrated that other types of particles including ambient particulate matter (PM), carbon black particles (CB), and polystyrene particles (PSP) can act as immunologic adjuvants when administered with an antigen via intraperitoneal, intranasal, intratracheal, and inhalation routes of exposure (Takafuji *et al.*, 1989; Fujimaki *et al.*, 1997; Maejima *et al.*, 1997; Lambert *et al.*, 2000; van Zijverden *et al.*, 2000; de Haar *et al.*, 2005; Nygaard *et al.*, 2005). In most cases the particles alone cause inflammation but when administered during sensitization they also stimulate the development of allergic immune responses (in the form of increased IgE antibody, TH2 cytokines). Upon repeated challenge with antigen, these animals exhibit increased severity of allergic type disease (pulmonary eosinophils, airway hyperresponsiveness, increased mucus production, etc.) compared to control animals which received antigen exposure and vehicle control in the place of the pollutant.

The general thought is that these exposures cause some form of oxidative stress resulting in redirecting or polarizing the immune response to a stronger Th2 phenotype (Nel, 2005). Although the mechanisms by which this occurs are still unclear, it is plausible that the tissue injury leads to cellular activation and immune stimulation. It is known that oxidative stress from inhaled air pollutants results in epithelial cell damage, increased lung permeability and the release of a broad spectrum of pro-inflammatory mediators. Epithelial damage and increased permeability enhances translocation of antigen to immunoreceptive sites in the submucosa, whereas increased cytokine output can profoundly affect

inflammatory and immune cells both locally and in areas remote for the site of damage. In addition, altered dendritic and macrophage cell number and function may affect immune signaling during both initial antigen recognition and secondary clonal expansion.

4. Air Pollution and Respiratory Viral Infections

A significant body of clinical observations and experimental research has unequivocally shown that exposure to ambient air pollutants and other toxicants can reduce host defenses and increase susceptibility to infection (Gilmour *et al.*, 1993; Yang *et al.*, 2001; Cohen *et al.*, 2002; Harrod *et al.*, 2005). There is also emerging evidence that certain exposures may result in greater incidence and severity of allergic lung disease and asthma (Sydbom *et al.*, 2001). These problems are still a reality in many highly populated urban areas of the U.S. and are of even greater issue in developing countries where uncontrolled combustion emissions are increasing at alarming rates. Respiratory infections remain a primary cause of morbidity and mortality worldwide, and any kind of exposure that might increase susceptibility to these agents is of public health significance. This is particularly important as additional infectious agents emerge, either through antibiotic resistance or from genetic shifts in, for example, the influenza virus.

Epidemiological data and laboratory research have suggested that DE a major contributor to air pollution increases susceptibility and response to respiratory viral infections. Hahon et al. published the first results of this interactions by examining the

effects of chronic exposure of mice to 2mg/m^3 of DE for 1, 3, or 6 months followed by infection with influenza (Hahon *et al.*, 1985). The results showed higher virus growth levels that was associated with a decreased type I interferon levels and influenza specific antibody production in the mice exposed to DE for 3 or 6 months although the mechanism of this effect was not discovered. A more recent study examined the effects of DE on the susceptibility to RSV infection in mice (Harrod *et al.*, 2003). This study reported that DE increased RSV gene expression, viral induced inflammation, and significantly decreased the expression of SP-A. They did not report a decrease in type I interferon production that Hahon et al. did, leaving the mechanism of DE immunomodulation still at large.

5. Influenza

Influenza infection causes significant morbidity and mortality in the United States each year (Klimov *et al.*, 1999). Influenza is characterized as a single stranded segmented RNA virus that is a member of the orthomixovirdae family. The influenza virus has 8 RNA segments that translate into different viral proteins including hemagglutinin (HA), neuraminidase (NA), polymerase proteins and nucleoproteins (Bender and Small, 1992). The virus particles are enveloped in a lipid bilayer that has HA and NA on the outer layer. HA and NA are responsible for influenza's entry and exit of the host cell which undergo major antigenic shift related to viral mutation and immune evasion. Human strains of influenza preferentially infect non-cilliated cells by recognition of 2,6- linked sialic aid residues whereas avian and

egg adapted strains of influenza bind 2,3 –linked sialic acid residues on ciliated cells (Matrosovich *et al.*, 2004)).

Although, influenza A viruses primarily target the respiratory epithelium it also has the ability to infect monocytes and macrophages. Once cells are infected, influenza virus elicits a strong cell mediated and humoral immune responses. Numerous immune cells play a role in viral clearance beginning with DCs in the lung that internalize the virus, migrate to local lymph nodes and present viral antigens to $CD4^+$ and $CD8^+$ T cells (Nonacs *et al.*, 1992). Previous studies have reported that migration of respiratory DCs (rDCs) from the lung to the draining lymph node occurs during the first 48 hours of an influenza infection (Legge and Braciale, 2005). These studies also noted that interaction of rDCs with naïve T cells may occur in the early stages of infection. Activated rDCs are also responsible for cytokines important in the generation of adaptive immunity such as IFN-γ, IL-10, and IL-12.

Influenza A viral infection results in the production of chemotactic (RANTES, MIP-1 alpha, MCP-1, MCP-3, and IP-10), pro-inflammatory (IL-1 beta, IL-6, IL-18, and TNF-alpha), and antiviral (IFN-alpha/beta) cytokines (reviewed in (Julkunen *et al.*, 2000)). Influenza A virus-induced IFN-alpha/beta is essential in host's defense by infected cells activating the expression of antiviral genes in uninfected cells in the first 48 hours of infection. IFN-alpha/beta also prolongs T cell survival, upregulates IL-12 and IL-18 receptor gene expression and together with IL-18 stimulates natural killer (NK) and T cell IFN-gamma production and the development of Th1-type immune responses (Sareneva *et al.*, 1998). However the production of Th2 cytokines such as IL-4 during an influenza infection

has been shown to inhibit antiviral immunity and delay the clearance of the virus (Moran *et al.*, 1996; Lopez *et al.*, 2002; Seneviratne *et al.*, 2005).

Along with adaptive immune responses being important for the clearance of an influenza infection, numerous innate pulmonary defenses are crucial for controlling infection. Multiple pulmonary cells employ various antiviral defense strategies to combat respiratory pathogens. Surfactant proteins (SP), which are members of the collectin family, belong to the IFN-independent defense responses. As stated earlier, SP-A and SP-D are secreted by alveolar type II cells and nonciliated bronchial epithelial cells, also known as Clara cells, in the lung (Madsen *et al.,* 2000; Madsen *et al.,* 2003). These proteins contribute to the innate defense responses against influenza through their ability to bind and neutralize the virus (Benne *et al.,* 1995; Hartshorn *et al.,* 1994). Previous studies have shown that uptake and clearance of Influenza A is reduced in SP-A or SP-D deficient mice, resulting in an increased inflammatory response and mortality (LeVine *et al.,* 2001; LeVine *et al.,* 2002). Other innate responses include stimulation of TLR3 and TLR7/8 in the epithelial cells and plasmacytoid DCs (reviewed in (Lopez *et al.*, 2002) which turns on downstream signaling of inflammation and type I interferon production.

Perhaps the most important immune mechanism to clear an influenza infection is the $CD8^+$ T cell response. The CTL response produce inflammatory molecules to lyse virally infected cells (Doherty *et al.*, 1996). Mice lacking functional $CD8^+$ T cells have significantly increased in mortality and inability to clear the influenza infection (Bender *et al.*, 1992). The generation of influenza specific $CD8^+$ T cells occurs around day 4 p.i. in the mediastinal

lymph node (MLN) (Lawrence and Braciale, 2004) and migration to the lung to further proliferate between day 5 and 7 p.i. (Lawrence *et al.*, 2005). While in the lung the influenza specific $CD8^+$ T cells produce IFN-γ, TNF-α, and IL-2 (Belz *et al.*, 2001). The production of these CTL mediators contributes to the clearance of the primary influenza infection (Baumgarth and Kelso, 1996).

6. Specific Aims and Significance of the Study

Epidemiological studies have noted a strong association between the level of air borne PM and incidence of respiratory infections more specifically influenza (Pope *et al.*, 2004). Since DE is a major contributor of air pollution with more than 40 of its components considered hazardous air pollutants (HAPs) by the US EPA (Environmental Protection Agency, 2002) it is of interest to develop an animal model to examine the health effects of this pollutant on the susceptibility and severity of an influenza infection. Although multiple epidemiological studies have noted this association and laboratory studies have mimicked it, the mechanisms behind the immunomodulation of the lung by DE have yet to be discovered. This study utilized a murine influenza model exposed to a variety of DE engines to test the hypothesis that DE alters expression and production of pulmonary host defense molecules and increase susceptibility and severity of an influenza infection. Data from these studies should provide insight into the mechanism of how pollutants modulate the pulmonary immune response and exacerbate infections.

Specific Aim #1. DE alone modulates pulmonary host defense mechanisms that may increase susceptibility to respiratory infections.

To test this hypothesis mice were exposed to filtered air or DE for 1 or 5 days and necropsied either immediately after or 18 hours post exposure to assess markers of inflammation and innate immune markers that are important in host defense. Both message and protein levels were assessed to evaluate which molecular component is affected by DE exposure.

Specific Aim #2. DE exposure increases the susceptibility to an influenza infection by modulation of pulmonary host defense markers.

This aim will build upon Aim #1 by exposing mice to filtered air or DE for 5 days and immediately after the last exposure mice were infected with influenza and necropsied 24 hours post infection to assess viral titers and pulmonary innate immune responses. This aim allows examination of known host defense molecules that were identified in aim 1 to be affected by DE and to assess if an influenza infection compensates for this defect or if DE increases susceptibility to the viral infection.

Specific Aim #3. Exposure to DE during an established influenza infection increases the severity of the disease.

This aim will evaluate the effects of an air pollutant on the ongoing course of a respiratory infection. To accomplish this aim, mice were infected with influenza and then exposed to filtered air or DE for 14 days to evaluate the effects of DE on the pulmonary innate and adaptive immune response to influenza and also viral clearance. Markers of inflammation

and pulmonary injury were measured to indicate the severity of the infection as well as viral titers and cytokine production important for clearing the virus.

Our studies are specifically aimed at determining the effects of DE on the essential pulmonary immune response that protects the sterile and delicate lower airways. The results of these experiments will not only aid in developing therapeutics strategies for influenza but also contribute to our understanding of how air pollutants can alter pulmonary immune responses thus increasing the incidence of respiratory infections.

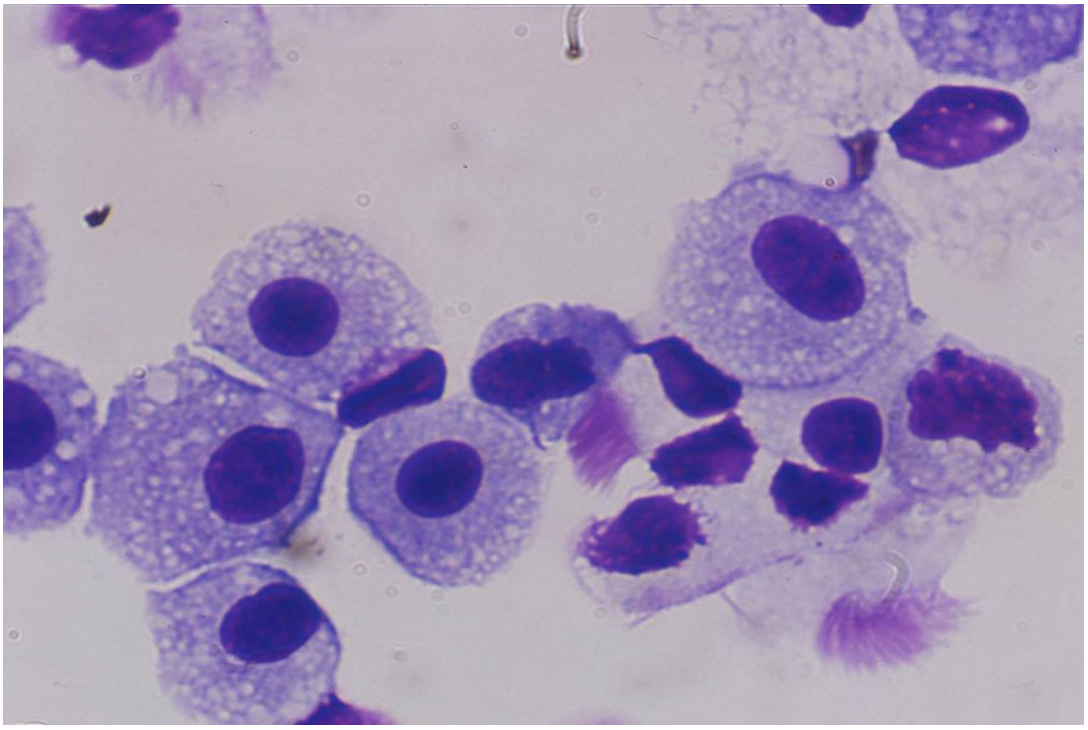

Figure 1: Differential cells in a rat adapted influenza infection

Pulmonary cells obtained from a rat lung wash 24 hour after infection with rat adapted influenza virus. The macrophages are heavily vacuolated indicating activation while ciliated epithelial cells are present indicating airway injury. Magnification x1000, Diff Quick stain.

Table1: Antimicrobial Defenses of the Lung

Antimicrobial defenses in lung	***Produced by***	***Target***	***Mechanism***	***Effects by Air Pollution***
Lysosyme	Serous cells, neutrophils, macrophages	gram positive bacteria	lyse and kill gram positive bacteria via enzymic cleavage of peptidoglycan	Decreased production after ozone exposure (Shelley *et al.*, 1991)
Complement (C3a, C5a)	macrophages, type II pneumocytes and fibroblasts	microbes	augment phagocytosis recruit phagocytes via chemotaxis	Increased C3 and C5 receptors after exposure to LPS and allergens; ozone, diesel, cigarette smoke activate complement or increase C3 in the airway epithelium (Kanemitsu *et al.*, 1998; Walters *et al.*, 2002)
Defensins HD 1-6; HBD 1 and 2	Neutrophils (HD1-4); epithelial cells (HBD 1 and 2); macrophages	bacteria, mycobacteria, fungi, and some viruses	cidal activity and chemotaxis	upregulated in the lungs of smokers (Merkel *et al.*, 2005)
Collectins	alveolar type II cells and non-ciliated bronchiolar epithelial cells	bacterial and fungal cell walls and viruses	Enhance phagocytosis of macrophages and neutrophils; activating cells	cigarette smoke decreases the production of collectin (Honda *et al.*, 1996); ozone decreases function (Janic *et al.*, 2005)

Table 2: Toll like receptors: Mechanism and Deficiencies

Toll Like Receptor	Ligand	Deficiency
TLR1	Heterodimerizes with TLR2 to recognize triacyl lipopeptides.	n/a
TLR2	Microbial components such as triacyl lipopeptides (heterodimerize with TLR1), diacyl lipopolypeptides (heterodimerize with TLR6), peptidoglycan from gram positive bacteria, glycophosphatidylinositol (GPI) anchors from malaria-causing parasites, zymosan from fungi, and forms of LPS structurally distinct from those recognized by TLR4 (Kopp and Medzhitov, 2003).	TLR2 -/- decreased clearance of *Borrelia burgdorferi* lipoproteins; greater susceptibility to *S. aureas* (Takeuchi *et al.*, 2000); *Streptococcus pneumoniae* (Echchannaoui *et al.*, 2002); and less resistant to *Mycobacterium tuberculosis* (Reiling *et al.*, 2002)
TLR3	Double stranded RNA; Poly (I:C); binds intracellularly (Alexopoulou *et al.*, 2001)	TLR3 -/- mice had a decreased response to pol(I:C); reduced production of TNF, IL-6, IL-12, and resistance to the lethal effect of poly(I:C) when sensitized to D-galactosamine (Alexopoulou *et al.*, 2001).
TLR4	LPS, F protein, heat shock protein (HSP) 60, HSP 70, RSV (Kopp and Medzhitov, 2003).	TLR4-/- and TLR4 lack of function mice (C3H/HeJ) have decreased clearance of *Haemophilus influenzae* (Wang *et al.*, 2002), *Salmonella* (Bernheiden *et al.*, 2001), *Mycobacterium tuberculosis* (Abel *et al.*, 2002), and RSV (Kurt-Jones *et al.*, 2000; Haeberle *et al.*, 2002); and are more susceptible to *Candida albicans* infection (Netea *et al.*, 2002).
TLR5	Flaggelin (constituent of bacterial flagella).	Polymorphism in TLR5 gene introduces a premature stop codon ($TLR5^{392STOP}$) and is correlated with increased susceptibility to *Legionella pneumophila* (Hawn *et al.*, 2003), but not typhoid fever (Dunstan *et al.*, 2005).
TLR6	Diacyl lipopolypeptides (heterodimerize with TLR2).	n/a
TLR7	Single stranded viral RNA and DNA.	IRAK-/- mice impair the TLR7 pathway and result in a deficient production of IFN α in serum when challenged with R-848 [(Uematsu *et al.*, 2005)
TLR8	Genomic material of viruses	n/a
TLR9	CpG DNA, Single stranded viral RNA and DNA.	IRAK-/- mice impairs TLR9 pathway and plasmacytoid DC can not produce IFN α in serum when challenged with CpG ODN (Uematsu *et al.*, 2005)
TLR10, 11, 12	TLR11- profiling from *T.gondi* (Yarovinsky *et al.*, 2005)	n/a

References:

Abel, B., N. Thieblemont, V. J. Quesniaux, N. Brown, J. Mpagi, K. Miyake, F. Bihl and B. Ryffel (2002). Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. *J Immunol,* **169**,(6), 3155-62.

Akazawa, M., J. L. Sindelar and A. D. Paltiel (2003). Economic costs of influenza-related work absenteeism. *Value Health,* **6**,(2), 107-15.

Alexopoulou, L., A. C. Holt, R. Medzhitov and R. A. Flavell (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature,* **413**,(6857), 732-8.

Arredouani, M., Z. Yang, Y. Ning, G. Qin, R. Soininen, K. Tryggvason and L. Kobzik (2004). The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. *J Exp Med,* **200**,(2), 267-72.

Ashitani, J., H. Mukae, M. Nakazato, T. Ihi, H. Mashimoto, J. Kadota, S. Kohno and S. Matsukura (1998). Elevated concentrations of defensins in bronchoalveolar lavage fluid in diffuse panbronchiolitis. *Eur Respir J,* **11**,(1), 104-11.

Banchereau, J. and R. M. Steinman (1998). Dendritic cells and the control of immunity. *Nature,* **392**,(6673), 245-52.

Baumgarth, N. and A. Kelso (1996). In vivo blockade of gamma interferon affects the influenza virus-induced humoral and the local cellular immune response in lung tissue. *J Virol,* **70**,(7), 4411-8.

Becker, S., J. M. Soukup and J. E. Gallagher (2002). Differential particulate air pollution induced oxidant stress in human granulocytes, monocytes and alveolar macrophages. *Toxicol In Vitro,* **16**,(3), 209-18.

Beisswenger, C. and R. Bals (2005). Antimicrobial peptides in lung inflammation. *Chem Immunol Allergy,* **86**,(55-71.

Belz, G. T., W. Xie and P. C. Doherty (2001). Diversity of epitope and cytokine profiles for primary and secondary influenza a virus-specific CD8+ T cell responses. *J Immunol,* **166**,(7), 4627-33.

Bender, B. S., T. Croghan, L. Zhang and P. A. Small, Jr. (1992). Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral

clearance and increased mortality after influenza virus challenge. *J Exp Med,* **175**,(4), 1143-5.

Bender, B. S. and P. A. Small, Jr. (1992). Influenza: pathogenesis and host defense. *Semin Respir Infect,* **7**,(1), 38-45.

Bernheiden, M., J. M. Heinrich, G. Minigo, C. Schutt, F. Stelter, M. Freeman, D. Golenbock and R. S. Jack (2001). LBP, CD14, TLR4 and the murine innate immune response to a peritoneal Salmonella infection. *J Endotoxin Res,* **7**,(6), 447-50.

Biagini, R. E., W. J. Moorman, T. R. Lewis and I. L. Bernstein (1986). Ozone enhancement of platinum asthma in a primate model. *Am Rev Respir Dis,* **134**,(4), 719-25.

Brown, M. S. and J. L. Goldstein (1983). Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu Rev Biochem,* **52**,(223-61.

Burleson, G. R., L. L. Keyes and J. D. Stutzman (1989). Immunosuppression of pulmonary natural killer activity by exposure to ozone. *Immunopharmacol Immunotoxicol,* **11**,(4), 715-35.

Canning, B. J., R. R. Hmieleski, E. W. Spannhake and G. J. Jakab (1991). Ozone reduces murine alveolar and peritoneal macrophage phagocytosis: the role of prostanoids. *Am J Physiol,* **261**,(4 Pt 1), L277-82.

Chaudhuri, N., S. K. Dower, M. K. Whyte and I. Sabroe (2005). Toll-like receptors and chronic lung disease. *Clin Sci (Lond),* **109**,(2), 125-33.

Clark, H., N. Palaniyar, P. Strong, J. Edmondson, S. Hawgood and K. B. Reid (2002). Surfactant protein D reduces alveolar macrophage apoptosis in vivo. *J Immunol,* **169**,(6), 2892-9.

Cohen, M. D., M. Sisco, K. Baker, Y. Li, D. Lawrence, H. van Loveren, J. T. Zelikoff and R. B. Schlesinger (2002). Effects of inhaled ozone on pulmonary immune cells critical to antibacterial responses in situ. *Inhal Toxicol,* **14**,(6), 599-619.

Cohen, M. D., J. T. Zelikoff and R. B. Schlesinger (2000). *Pulmonary Immunotoxicology*. Kluwer Academic Publishers,

Coonrod, J. D. (1986). The role of extracellular bactericidal factors in pulmonary host defense. *Semin Respir Infect,* **1**,(2), 118-29.

Crouch, E. C. (1998). Collectins and pulmonary host defense. *Am J Respir Cell Mol Biol,* **19**,(2), 177-201.

de Haar, C., I. Hassing, M. Bol, R. Bleumink and R. Pieters (2005). Ultrafine carbon black particles cause early airway inflammation and have adjuvant activity in a mouse allergic airway disease model. *Toxicol Sci,* **87**,(2), 409-18.

Diaz-Sanchez, D., M. Penichet-Garcia and A. Saxon (2000). Diesel exhaust particles directly induce activated mast cells to degranulate and increase histamine levels and symptom severity. *J Allergy Clin Immunol,* **106**,(6), 1140-6.

Doherty, P. C., D. J. Topham and R. A. Tripp (1996). Establishment and persistence of virus-specific CD4+ and CD8+ T cell memory. *Immunol Rev,* **150**,(23-44.

Dorio, R. J. and H. J. Forman (1988). Ethanol inhibition of signal transduction in superoxide production by rat alveolar macrophages. A proposed mechanism for ethanol related pneumonia. *Ann Clin Lab Sci,* **18**,(3), 190-4.

Drouin, S. M., D. B. Corry, J. Kildsgaard and R. A. Wetsel (2001). Cutting edge: the absence of C3 demonstrates a role for complement in Th2 effector functions in a murine model of pulmonary allergy. *J Immunol,* **167**,(8), 4141-5.

Duits, L. A., B. Ravensbergen, M. Rademaker, P. S. Hiemstra and P. H. Nibbering (2002). Expression of beta-defensin 1 and 2 mRNA by human monocytes, macrophages and dendritic cells. *Immunology,* **106**,(4), 517-25.

Dunstan, S. J., T. R. Hawn, N. T. Hue, C. P. Parry, V. A. Ho, H. Vinh, T. S. Diep, D. House, J. Wain, A. Aderem, T. T. Hien and J. J. Farrar (2005). Host susceptibility and clinical outcomes in toll-like receptor 5-deficient patients with typhoid fever in Vietnam. *J Infect Dis,* **191**,(7), 1068-71.

Dziedzic, D. and H. J. White (1987). Response of T-cell-deficient mice to ozone exposure. *J Toxicol Environ Health,* **21**,(1-2), 57-71.

Echchannaoui, H., K. Frei, C. Schnell, S. L. Leib, W. Zimmerli and R. Landmann (2002). Toll-like receptor 2-deficient mice are highly susceptible to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation. *J Infect Dis,* **186**,(6), 798-806.

Elomaa, O., M. Sankala, T. Pikkarainen, U. Bergmann, A. Tuuttila, A. Raatikainen-Ahokas, H. Sariola and K. Tryggvason (1998). Structure of the human macrophage MARCO receptor and characterization of its bacteria-binding region. *J Biol Chem,* **273**,(8), 4530-8.

Elshourbagy, N. A., X. Li, J. Terrett, S. Vanhorn, M. S. Gross, J. E. Adamou, K. M. Anderson, C. L. Webb and P. G. Lysko (2000). Molecular characterization of a human scavenger receptor, human MARCO. *Eur J Biochem,* **267**,(3), 919-26.

Environmental Protection Agency, U. S. A. (2002). Health risk assessment document for diesel exhaust.

Fahy, R. J. and M. D. Wewers (2005). Pulmonary Defense and the Human Cathelicidin

hCAP-18/LL-37. *Immunol Res,* **31**,(2), 75-90.

Fujimaki, H., K. Saneyoshi, F. Shiraishi, T. Imai and T. Endo (1997). Inhalation of diesel exhaust enhances antigen-specific IgE antibody production in mice. *Toxicology,* **116**,(1-3), 227-33.

Gardner, D. E. (1984). Alterations in macrophage functions by environmental chemicals. *Environ Health Perspect,* **55**,(343-58.

Gershwin, L. J., J. W. Osebold and Y. C. Zee (1981). Immunoglobulin E-containing cells in mouse lung following allergen inhalation and ozone exposure. *Int Arch Allergy Appl Immunol,* **65**,(3), 266-77.

Gilmour, M. I. (1995). Interaction of air pollutants and pulmonary allergic responses in experimental animals. *Toxicology,* **105**,(2-3), 335-42.

Gilmour, M. I., P. Park, D. Doerfler and M. K. Selgrade (1993). Factors that influence the suppression of pulmonary antibacterial defenses in mice exposed to ozone. *Exp Lung Res,* **19**,(3), 299-314.

Gilmour, M. I., P. Park and M. J. Selgrade (1996). Increased immune and inflammatory responses to dust mite antigen in rats exposed to 5 ppm NO2. *Fundam Appl Toxicol,* **31**,(1), 65-70.

Goldman, M. J., G. M. Anderson, E. D. Stolzenberg, U. P. Kari, M. Zasloff and J. M. Wilson (1997). Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell,* **88**,(4), 553-60.

Granum, B. and M. Lovik (2002). The effect of particles on allergic immune responses. *Toxicol Sci,* **65**,(1), 7-17.

Green, G. M., G. J. Jakab, R. B. Low and G. S. Davis (1977). Defense mechanisms of the respiratory membrane. *Am Rev Respir Dis,* **115**,(3), 479-514.

Guillot, L., V. Balloy, F. X. McCormack, D. T. Golenbock, M. Chignard and M. Si-Tahar (2002). Cutting edge: the immunostimulatory activity of the lung surfactant protein-A involves Toll-like receptor 4. *J Immunol,* **168**,(12), 5989-92.

Gunn, M. D. (2003). Chemokine mediated control of dendritic cell migration and function. *Semin Immunol,* **15**,(5), 271-6.

Gupta, S. K., V. Gupta, S. Joshi and R. Tandon (2002). Subclinically dry eyes in urban Delhi: an impact of air pollution? *Ophthalmologica,* **216**,(5), 368-71.

Haeberle, H. A., R. Takizawa, A. Casola, A. R. Brasier, H. J. Dieterich, N. Van Rooijen, Z. Gatalica and R. P. Garofalo (2002). Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways. *J Infect Dis,* **186**,(9), 1199-206.

Hahon, N., J. A. Booth, F. Green and T. R. Lewis (1985). Influenza virus infection in mice after exposure to coal dust and diesel engine emissions. *Environ Res,* **37**,(1), 44-60.

Hampton, R. Y., D. T. Golenbock, M. Penman, M. Krieger and C. R. Raetz (1991). Recognition and plasma clearance of endotoxin by scavenger receptors. *Nature,* **352**,(6333), 342-4.

Harker, W. G., C. Tom, J. R. McGregor, L. Slade and W. E. Samlowski (1990). Human tumor cell line resistance to chemotherapeutic agents does not predict resistance to natural killer or lymphokine-activated killer cell-mediated cytolysis. *Cancer Res,* **50**,(18), 5931-6.

Harrod, K. S., R. J. Jaramillo, J. A. Berger, A. P. Gigliotti, S. K. Seilkop and M. D. Reed (2005). Inhaled diesel engine emissions reduce bacterial clearance and exacerbate lung disease to Pseudomonas aeruginosa infection in vivo. *Toxicol Sci,* **83**,(1), 155-65.

Harrod, K. S., R. J. Jaramillo, C. L. Rosenberger, S. Z. Wang, J. A. Berger, J. D. McDonald and M. D. Reed (2003). Increased susceptibility to RSV infection by exposure to inhaled diesel engine emissions. *Am J Respir Cell Mol Biol,* **28**,(4), 451-63.

Hasani, A., N. Toms, J. E. Agnew, J. Lloyd and J. P. Dilworth (2005). Mucociliary clearance in COPD can be increased by both a D2/beta2 and a standard beta2 agonists. *Respir Med,* **99**,(2), 145-51.

Hastie, A. T., K. B. Everts, J. Zangrilli, J. R. Shaver, M. B. Pollice, J. E. Fish and S. P. Peters (1997). HSP27 elevated in mild allergic inflammation protects airway epithelium from H2SO4 effects. *Am J Physiol,* **273**,(2 Pt 1), L401-9.

Hawn, T. R., A. Verbon, K. D. Lettinga, L. P. Zhao, S. S. Li, R. J. Laws, S. J. Skerrett, B. Beutler, L. Schroeder, A. Nachman, A. Ozinsky, K. D. Smith and A. Aderem (2003). A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. *J Exp Med,* **198**,(10), 1563-72.

Hofmann, W. and R. Sturm (2004). Stochastic model of particle clearance in human bronchial airways. *J Aerosol Med,* **17**,(1), 73-89.

Honda, Y., H. Takahashi, Y. Kuroki, T. Akino and S. Abe (1996). Decreased contents of surfactant proteins A and D in BAL fluids of healthy smokers. *Chest,* **109**,(4), 1006-9.

Jakab, G. J. (1993). The toxicologic interactions resulting from inhalation of carbon black and acrolein on pulmonary antibacterial and antiviral defenses. *Toxicol Appl Pharmacol,* **121**,(2), 167-75.

Jakab, G. J., E. W. Spannhake, B. J. Canning, S. R. Kleeberger and M. I. Gilmour (1995). The effects of ozone on immune function. *Environ Health Perspect,* **103 Suppl 2**,(77-89.

Janic, B., T. M. Umstead, D. S. Phelps and J. Floros (2005). Modulatory effects of ozone on THP-1 cells in response to SP-A stimulation. *Am J Physiol Lung Cell Mol Physiol,* **288**,(2), L317-25.

Julkunen, I., K. Melen, M. Nyqvist, J. Pirhonen, T. Sareneva and S. Matikainen (2000). Inflammatory responses in influenza A virus infection. *Vaccine,* **19 Suppl 1**,(S32-7.

Kadowaki, N. and Y. J. Liu (2002). Natural type I interferon-producing cells as a link between innate and adaptive immunity. *Hum Immunol,* **63**,(12), 1126-32.

Kagan, B. L., M. E. Selsted, T. Ganz and R. I. Lehrer (1990). Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proc Natl Acad Sci U S A,* **87**,(1), 210-4.

Kaltreider, H. B. (1993). Hypersensitivity pneumonitis. *West J Med,* **159**,(5), 570-8.

Kanemitsu, H., S. Nagasawa, M. Sagai and Y. Mori (1998). Complement activation by diesel exhaust particles (DEP). *Biol Pharm Bull,* **21**,(2), 129-32.

Kay, A. B. (2006). The role of T lymphocytes in asthma. *Chem Immunol Allergy,* **91**,(59-75.

Kew, R. R., B. Ghebrehiwet and A. Janoff (1987). Characterization of the third component of complement (C3) after activation by cigarette smoke. *Clin Immunol Immunopathol,* **44**,(2), 248-58.

Kim, K. C., K. McCracken, B. C. Lee, C. Y. Shin, M. J. Jo, C. J. Lee and K. H. Ko (1997). Airway goblet cell mucin: its structure and regulation of secretion. *Eur Respir J,* **10**,(11), 2644-9.

Kimber, I. a. S., MJ (1998). *T Lymphocyte Subpopulations in Immunotoxicology*. John Wiley & Sons Ltd., West Sussex, England,

Kleeberger, S. R., S. Reddy, L. Y. Zhang and A. E. Jedlicka (2000). Genetic susceptibility to ozone-induced lung hyperpermeability: role of toll-like receptor 4. *Am J Respir Cell Mol Biol,* **22**,(5), 620-7.

Kleeberger, S. R., S. P. Reddy, L. Y. Zhang, H. Y. Cho and A. E. Jedlicka (2001). Toll-like receptor 4 mediates ozone-induced murine lung hyperpermeability via inducible nitric oxide synthase. *Am J Physiol Lung Cell Mol Physiol,* **280**,(2), L326-33.

Klimov, A., L. Simonsen, K. Fukuda and N. Cox (1999). Surveillance and impact of influenza in the United States. *Vaccine,* **17 Suppl 1**,(S42-6.

Knowles, M. R. and R. C. Boucher (2002). Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest,* **109**,(5), 571-7.

Kodama, T., M. Freeman, L. Rohrer, J. Zabrecky, P. Matsudaira and M. Krieger (1990). Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature,* **343**,(6258), 531-5.

Kopp, E. and R. Medzhitov (2003). Recognition of microbial infection by Toll-like receptors. *Curr Opin Immunol,* **15**,(4), 396-401.

Kurt-Jones, E. A., L. Popova, L. Kwinn, L. M. Haynes, L. P. Jones, R. A. Tripp, E. E. Walsh, M. W. Freeman, D. T. Golenbock, L. J. Anderson and R. W. Finberg (2000). Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol,* **1**,(5), 398-401.

Lambert, A. L., W. Dong, M. K. Selgrade and M. I. Gilmour (2000). Enhanced allergic sensitization by residual oil fly ash particles is mediated by soluble metal constituents. *Toxicol Appl Pharmacol,* **165**,(1), 84-93.

Lawrence, C. W. and T. J. Braciale (2004). Activation, differentiation, and migration of naive virus-specific CD8+ T cells during pulmonary influenza virus infection. *J Immunol,* **173**,(2), 1209-18.

Lawrence, C. W., R. M. Ream and T. J. Braciale (2005). Frequency, specificity, and sites of expansion of CD8+ T cells during primary pulmonary influenza virus infection. *J Immunol,* **174**,(9), 5332-40.

Legge, K. L. and T. J. Braciale (2005). Lymph node dendritic cells control CD8+ T cell responses through regulated FasL expression. *Immunity,* **23**,(6), 649-59.

Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart and J. A. Hoffmann (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell,* **86**,(6), 973-83.

LeVine, A. M., M. D. Bruno, K. M. Huelsman, G. F. Ross, J. A. Whitsett and T. R. Korfhagen (1997). Surfactant protein A-deficient mice are susceptible to group B streptococcal infection. *J Immunol,* **158**,(9), 4336-40.

LeVine, A. M., K. Hartshorn, J. Elliott, J. Whitsett and T. Korfhagen (2002). Absence of SP-A modulates innate and adaptive defense responses to pulmonary influenza infection. *Am J Physiol Lung Cell Mol Physiol,* **282**,(3), L563-72.

LeVine, A. M., K. E. Kurak, M. D. Bruno, J. M. Stark, J. A. Whitsett and T. R. Korfhagen (1998). Surfactant protein-A-deficient mice are susceptible to Pseudomonas aeruginosa infection. *Am J Respir Cell Mol Biol,* **19**,(4), 700-8.

LeVine, A. M., K. E. Kurak, J. R. Wright, W. T. Watford, M. D. Bruno, G. F. Ross, J. A. Whitsett and T. R. Korfhagen (1999). Surfactant protein-A binds group B

streptococcus enhancing phagocytosis and clearance from lungs of surfactant protein-A-deficient mice. *Am J Respir Cell Mol Biol,* **20**,(2), 279-86.

LeVine, A. M., J. A. Whitsett, J. A. Gwozdz, T. R. Richardson, J. H. Fisher, M. S. Burhans and T. R. Korfhagen (2000). Distinct effects of surfactant protein A or D deficiency during bacterial infection on the lung. *J Immunol,* **165**,(7), 3934-40.

Li, L. and A. Holian (1998). Acrolein: a respiratory toxin that suppresses pulmonary host defense. *Rev Environ Health,* **13**,(1-2), 99-108.

Lopez, C. B., T. M. Moran, J. L. Schulman and A. Fernandez-Sesma (2002). Antiviral immunity and the role of dendritic cells. *Int Rev Immunol,* **21**,(4-5), 339-53.

Lukacs, N. W., M. M. Glovsky and P. A. Ward (2001). Complement-dependent immune complex-induced bronchial inflammation and hyperreactivity. *Am J Physiol Lung Cell Mol Physiol,* **280**,(3), L512-8.

Maejima, K., K. Tamura, Y. Taniguchi, S. Nagase and H. Tanaka (1997). Comparison of the effects of various fine particles on IgE antibody production in mice inhaling Japanese cedar pollen allergens. *J Toxicol Environ Health,* **52**,(3), 231-48.

Matrosovich, M. N., T. Y. Matrosovich, T. Gray, N. A. Roberts and H. D. Klenk (2004). Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc Natl Acad Sci U S A,* **101**,(13), 4620-4.

Matsumura, Y., K. Mizuno, T. Miyamoto, T. Suzuki and Y. Oshima (1972). The effects of ozone, nitrogen dioxide, and sulfur dioxide on experimentally induced allergic respiratory disorder in guinea pigs. IV. Effects on respiratory sensitivity to inhaled acetylcholine. *Am Rev Respir Dis,* **105**,(2), 262-7.

McWilliam, A. S., D. J. Nelson and P. G. Holt (1995). The biology of airway dendritic cells. *Immunol Cell Biol,* **73**,(5), 405-13.

Merkel, D., W. Rist, P. Seither, A. Weith and M. C. Lenter (2005). Proteomic study of human bronchoalveolar lavage fluids from smokers with chronic obstructive pulmonary disease by combining surface-enhanced laser desorption/ionization-mass spectrometry profiling with mass spectrometric protein identification. *Proteomics,* **5**,(11), 2972-80.

Michel, T., J. M. Reichhart, J. A. Hoffmann and J. Royet (2001). Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature,* **414**,(6865), 756-9.

Moran, T. M., H. Isobe, A. Fernandez-Sesma and J. L. Schulman (1996). Interleukin-4 causes delayed virus clearance in influenza virus-infected mice. *J Virol,* **70**,(8), 5230-5.

Morris, M. A. and K. Ley (2004). Trafficking of natural killer cells. *Curr Mol Med,* **4**,(4), 431-8.

Mossman, B. T., K. M. Lounsbury and S. P. Reddy (2006). Oxidants and signaling by mitogen-activated protein kinases in lung epithelium. *Am J Respir Cell Mol Biol,* **34**,(6), 666-9.

Nakagawa, N. K., M. L. Franchini, P. Driusso, L. R. de Oliveira, P. H. Saldiva and G. Lorenzi-Filho (2005). Mucociliary clearance is impaired in acutely ill patients. *Chest,* **128**,(4), 2772-7.

Nel, A. (2005). Atmosphere. Air pollution-related illness: effects of particles. *Science,* **308**,(5723), 804-6.

Netea, M. G., C. A. Van Der Graaf, A. G. Vonk, I. Verschueren, J. W. Van Der Meer and B. J. Kullberg (2002). The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. *J Infect Dis,* **185**,(10), 1483-9.

Nonacs, R., C. Humborg, J. P. Tam and R. M. Steinman (1992). Mechanisms of mouse spleen dendritic cell function in the generation of influenza-specific, cytolytic T lymphocytes. *J Exp Med,* **176**,(2), 519-29.

Nouri-Shirazi, M. and E. Guinet (2003). Evidence for the immunosuppressive role of nicotine on human dendritic cell functions. *Immunology,* **109**,(3), 365-73.

Nygaard, U. C., A. Aase and M. Lovik (2005). The allergy adjuvant effect of particles - genetic factors influence antibody and cytokine responses. *BMC Immunol,* **6**,(11.

Ohtani, T., S. Nakagawa, M. Kurosawa, M. Mizuashi, M. Ozawa and S. Aiba (2005). Cellular Basis of the Role of Diesel Exhaust Particles in Inducing Th2-Dominant Response. *J Immunol,* **174**,(2412-2419.

Park, J. W., C. Taube, A. Joetham, K. Takeda, T. Kodama, A. Dakhama, G. McConville, C. B. Allen, G. Sfyroera, L. D. Shultz, J. D. Lambris, P. C. Giclas, V. M. Holers and E.

W. Gelfand (2004). Complement activation is critical to airway hyperresponsiveness after acute ozone exposure. *Am J Respir Crit Care Med,* **169**,(6), 726-32.

Peden, D. B. (1996). Effect of air pollution in asthma and respiratory allergy. *Otolaryngol Head Neck Surg,* **114**,(2), 242-7.

Pope, C. A., 3rd, R. T. Burnett, G. D. Thurston, M. J. Thun, E. E. Calle, D. Krewski and J. J. Godleski (2004). Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. *Circulation,* **109**,(1), 71-7.

Prescott, E., P. Lange and J. Vestbo (1995). Chronic mucus hypersecretion in COPD and death from pulmonary infection. *Eur Respir J,* **8**,(8), 1333-8.

Raulet, D. H., R. E. Vance and C. W. McMahon (2001). Regulation of the natural killer cell receptor repertoire. *Annu Rev Immunol,* **19**,(291-330.

Reiling, N., C. Holscher, A. Fehrenbach, S. Kroger, C. J. Kirschning, S. Goyert and S. Ehlers (2002). Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. *J Immunol,* **169**,(7), 3480-4.

Reiner, S. L. and R. M. Locksley (1995). The regulation of immunity to Leishmania major. *Annu Rev Immunol,* **13**,(151-77.

Robbins, C. S., D. E. Dawe, S. I. Goncharova, M. A. Pouladi, A. G. Drannik, F. K. Swirski, G. Cox and M. R. Stampfli (2004). Cigarette smoke decreases pulmonary dendritic cells and impacts antiviral immune responsiveness. *Am J Respir Cell Mol Biol,* **30**,(2), 202-11.

Robbins, R. A., K. J. Nelson, G. L. Gossman, S. Koyama and S. I. Rennard (1991). Complement activation by cigarette smoke. *Am J Physiol,* **260**,(4 Pt 1), L254-9.

Roman, J., J. D. Ritzenthaler, A. Gil-Acosta, H. N. Rivera and S. Roser-Page (2004). Nicotine and fibronectin expression in lung fibroblasts: implications for tobacco-related lung tissue remodeling. *Faseb J,* **18**,(12), 1436-8.

Rusznak, C., J. L. Devalia, J. Wang and R. J. Davies (1997). Pollution-induced airway disease and the putative underlying mechanisms. *Clin Rev Allergy Immunol,* **15**,(2), 205-17.

Rutschmann, S., A. Kilinc and D. Ferrandon (2002). Cutting edge: the toll pathway is required for resistance to gram-positive bacterial infections in Drosophila. *J Immunol,* **168**,(4), 1542-6.

Sacks, D. and C. Anderson (2004). Re-examination of the immunosuppressive mechanisms mediating non-cure of Leishmania infection in mice. *Immunol Rev,* **201**,(225-38.

Sareneva, T., S. Matikainen, M. Kurimoto and I. Julkunen (1998). Influenza A virus-induced IFN-alpha/beta and IL-18 synergistically enhance IFN-gamma gene expression in human T cells. *J Immunol,* **160**,(12), 6032-8.

Saunders, D., K. Lucas, J. Ismaili, L. Wu, E. Maraskovsky, A. Dunn and K. Shortman (1996). Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte/macrophage colony-stimulating factor. *J Exp Med,* **184**,(6), 2185-96.

Schagat, T. L., J. A. Wofford and J. R. Wright (2001). Surfactant protein A enhances alveolar macrophage phagocytosis of apoptotic neutrophils. *J Immunol,* **166**,(4), 2727-33.

Schiff, L. J. and J. A. Graham (1984). Pathologic changes induced by coal-fired fly ash in hamster tracheal grafts. *Toxicology,* **29**,(4), 307-13.

Schlesinger, R. B., B. D. Naumann and L. C. Chen (1983). Physiological and histological alterations in the bronchial mucociliary clearance system of rabbits following intermittent oral or nasal inhalation of sulfuric acid mist. *J Toxicol Environ Health,* **12**,(2-3), 441-65.

Selgrade, M. K. (1999). Use of immunotoxicity data in health risk assessments: uncertainties and research to improve the process. *Toxicology,* **133**,(1), 59-72.

Seneviratne, S. L., L. Jones, A. S. Bailey, R. V. Samuel, A. P. Black and G. S. Ogg (2005). Interleukin-4 induced down-regulation of skin homing receptor expression by human viral-specific CD8 T cells may contribute to atopic risk of cutaneous infection. *Clin Exp Immunol,* **141**,(1), 107-15.

Shelley, S. A. (1994). Oxidant-induced alterations of lung surfactant system. *J Fla Med Assoc,* **81**,(1), 49-51.

Shelley, S. A., J. E. Paciga and J. U. Balis (1991). Lysozyme is an ozone-sensitive component of alveolar type II cell lamellar bodies. *Biochim Biophys Acta,* **1096**,(4), 338-44.

Shima, M. and M. Adachi (1996). Effects of environmental tobacco smoke on serum levels of acute phase proteins in schoolchildren. *Prev Med,* **25**,(5), 617-24.

Shortman, K. and Y. J. Liu (2002). Mouse and human dendritic cell subtypes. *Nat Rev Immunol,* **2**,(3), 151-61.

Singh, G. and S. L. Katyal (1997). Clara cells and Clara cell 10 kD protein (CC10). *Am J Respir Cell Mol Biol,* **17**,(2), 141-3.

Singh, P. K., H. P. Jia, K. Wiles, J. Hesselberth, L. Liu, B. A. Conway, E. P. Greenberg, E. V. Valore, M. J. Welsh, T. Ganz, B. F. Tack and P. B. McCray, Jr. (1998). Production of beta-defensins by human airway epithelia. *Proc Natl Acad Sci U S A,* **95**,(25), 14961-6.

Stager, S., J. Alexander, K. C. Carter, F. Brombacher and P. M. Kaye (2003). Both interleukin-4 (IL-4) and IL-4 receptor alpha signaling contribute to the development of hepatic granulomas with optimal antileishmanial activity. *Infect Immun,* **71**,(8), 4804-7.

Steerenberg, P., A. Verlaan, A. De Klerk, A. Boere, H. Loveren and F. Cassee (2004). Sensitivity to ozone, diesel exhaust particles, and standardized ambient particulate matter in rats with a listeria monocytogenes-induced respiratory infection. *Inhal Toxicol,* **16**,(5), 311-7.

Steinman, R. M., M. Pack and K. Inaba (1997). Dendritic cells in the T-cell areas of lymphoid organs. *Immunol Rev,* **156**,(25-37.

Sydbom, A., A. Blomberg, S. Parnia, N. Stenfors, T. Sandstrom and S. E. Dahlen (2001). Health effects of diesel exhaust emissions. *Eur Respir J,* **17**,(4), 733-46.

Takafuji, S., S. Suzuki, K. Koizumi, K. Tadokoro, H. Ohashi, M. Muranaka and T. Miyamoto (1989). Enhancing effect of suspended particulate matter on the IgE antibody production in mice. *Int Arch Allergy Appl Immunol,* **90**,(1), 1-7.

Takahashi, M. and Y. Kobayashi (2003). Cytokine production in association with phagocytosis of apoptotic cells by immature dendritic cells. *Cell Immunol,* **226**,(2), 105-15.

Takeuchi, O., K. Hoshino and S. Akira (2000). Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. *J Immunol,* **165**,(10), 5392-6.

Taylor, P. R., L. Martinez-Pomares, M. Stacey, H. H. Lin, G. D. Brown and S. Gordon (2005). Macrophage receptors and immune recognition. *Annu Rev Immunol,* **23**,(901-44.

Tuite, A., M. Elias, S. Picard, A. Mullick and P. Gros (2005). Genetic control of

susceptibility to Candida albicans in susceptible A/J and resistant C57BL/6J mice. *Genes Immun,* **6**,(8), 672-82.

Uematsu, S., S. Sato, M. Yamamoto, T. Hirotani, H. Kato, F. Takeshita, M. Matsuda, C. Coban, K. J. Ishii, T. Kawai, O. Takeuchi and S. Akira (2005). Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-{alpha} induction. *J Exp Med,* **201**,(6), 915-23.

van Oosterhout, A. J. and N. Bloksma (2005). Regulatory T-lymphocytes in asthma. *Eur Respir J,* **26**,(5), 918-32.

van Zijverden, M., A. van der Pijl, M. Bol, F. A. van Pinxteren, C. de Haar, A. H. Penninks, H. van Loveren and R. Pieters (2000). Diesel exhaust, carbon black, and silica particles display distinct Th1/Th2 modulating activity. *Toxicol Appl Pharmacol,* **168**,(2), 131-9.

Vandivier, R. W., C. A. Ogden, V. A. Fadok, P. R. Hoffmann, K. K. Brown, M. Botto, M. J. Walport, J. H. Fisher, P. M. Henson and K. E. Greene (2002). Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. *J Immunol,* **169**,(7), 3978-86.

Vestbo, J., E. Prescott and P. Lange (1996). Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group. *Am J Respir Crit Care Med,* **153**,(5), 1530-5.

Walters, D. M., P. N. Breysse, B. Schofield and M. Wills-Karp (2002). Complement factor 3 mediates particulate matter-induced airway hyperresponsiveness. *Am J Respir Cell Mol Biol,* **27**,(4), 413-8.

Wang, X., C. Moser, J. P. Louboutin, E. S. Lysenko, D. J. Weiner, J. N. Weiser and J. M. Wilson (2002). Toll-like receptor 4 mediates innate immune responses to Haemophilus influenzae infection in mouse lung. *J Immunol,* **168**,(2), 810-5.

Wert, S., T. Jones, T. Korfhagen, J. Fisher and J. Whitsett (2000). Spontaneous emphysema in surfactant protein D gene-targeted mice. *Chest,* **117**,(5 Suppl 1), 248S.

Yang, H. M., J. M. Antonini, M. W. Barger, L. Butterworth, B. R. Roberts, J. K. Ma, V. Castranova and J. Y. Ma (2001). Diesel exhaust particles suppress macrophage function and slow the pulmonary clearance of Listeria monocytogenes in rats. *Environ Health Perspect,* **109**,(5), 515-21.

Yarovinsky, F., D. Zhang, J. F. Andersen, G. L. Bannenberg, C. N. Serhan, M. S. Hayden, S. Hieny, F. S. Sutterwala, R. A. Flavell, S. Ghosh and A. Sher (2005). TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science,* **308**,(5728), 1626-9.

Yu, M., X. Zheng, H. Witschi and K. E. Pinkerton (2002). The role of interleukin-6 in pulmonary inflammation and injury induced by exposure to environmental air pollutants. *Toxicol Sci,* **68**,(2), 488-97.

Zhang, P., G. J. Bagby, K. I. Happel, W. R. Summer and S. Nelson (2002). Pulmonary host defenses and alcohol. *Front Biosci,* **7**,(d1314-30.

Zhang, P., W. R. Summer, G. J. Bagby and S. Nelson (2000). Innate immunity and pulmonary host defense. *Immunol Rev,* **173**,(39-51.

Zhao, C., I. Wang and R. I. Lehrer (1996). Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. *FEBS Lett,* **396**,(2-3), 319-22.

Chapter 2:

Modulation of Pulmonary Inflammatory Responses and Antimicrobial Defenses in Mice Exposed to Diesel Exhaust.

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Abstract

Diesel exhaust (DE) is a major component of urban air pollution and has been shown to increase the severity of infectious and allergic lung disease. The purpose of this study was to evaluate the effects of DE exposure on pulmonary inflammation, mediator production and antimicrobial defenses in an exposure model that had previously been shown to increase susceptibility to influenza. BALB/c mice were exposed to filtered air, or to DE diluted to yield 0.5 or 2 mg/m^3 of diesel exhaust particles (DEP) for 4 hours per day for 1 or 5 days. Immediately and 18 hours after one or five diesel exposures mice were euthanized to assess both immediate and delayed effects. DE exposure for 5 days at either concentration caused higher neutrophil numbers and lesion scoring compared to air controls. Intracellular adhesion molecule-1 (ICAM-1), which recruits inflammatory cells and is an entry site for rhinoviruses was increased immediately after 1 or 5 days of DE exposure. Several inflammatory and immune cytokines (TNF-α, MIP-2, IL-6, IFN-γ, and IL-13) were also upregulated at various timepoints and concentrations. In contrast, clara cell secretory protein (CCSP), surfactant protein A (SP-A), and surfactant protein D (SP-D) which are important host defense molecules, were significantly decreased at both the message and protein level with DE exposure. We conclude that exposure to moderate and high occupational levels of DE caused an increase in lung injury and inflammation, and a decrease in host defense molecules, which could result in increased susceptibility to respiratory pathogens.

Introduction

Air pollution exposure has long been considered a risk factor for the development of respiratory infections and pneumonia. Diesel exhaust (DE) in particular has been reported to increase susceptibility to both bacterial and viral pathogens. (Hahon et al., 1985; Takizawa et al., 2000a; Takizawa et al., 2000b; Castranova et al., 2001; Harrod et al., 2003; Jaspers et al., 2005; Ciencewicki et al., 2007). Several studies have demonstrated that rodents exposed to high concentrations of re-entrained diesel exhaust particles (DEP) decreases phagocytosis and clearance of gram negative and gram positive bacteria (Yang et al., 2001; Steerenberg et al., 2004; Yin et al., 2004). Exposure to lower concentrations of fresh DE has also resulted in increased susceptibility to respiratory syncytial virus (RSV) and influenza infection (Hahon et al., 1985; Harrod et al., 2003), however the mechanisms for how this occurs are not fully understood.

Diesel exhaust consists of a mixture of hundreds of organic and inorganic compounds in both the gas and particle phases and is a major contributor to urban air pollution (Environmental Protection Agency, 2002). More than 40 of the compounds in DE are listed by the United States Environmental Protection Agency (U.S. E.P.A) as hazardous air pollutants (HAPs). Occupational exposures to particles where DE is the principal source have been measured at 1.28 mg/m^3 in mines and from 0.4 to 0.748 mg/m^3 in public transport depots (Environmental Protection Agency, 2002). The majority of the DEP in DE are found in the fine (0.1 – 2.5 μm) and ultrafine (<0.1 μm) size ranges, which when inhaled, readily

penetrate the lower respiratory tract (Li et al., 2002; Oberdorster and Utell, 2002; Riedl and Diaz-Sanchez, 2005).

Along with increasing susceptibility to pathogens, it has been widely demonstrated that exposure to DE causes significant pulmonary inflammation. Both animal and human studies have shown that exposure to DE increases neutrophil recruitment, nitric oxide production, and production of proinflammatory cytokines in the lung (reviewed in (Sydbom et al., 2001). These effects have been associated with increased expression of upstream leukocyte trafficking molecules including intracellular adhesion molecule-1 (ICAM-1), low-density lipoprotein (LDL) and platelet-activating factor (PAF) receptors (Ito et al., 2006). These adhesion molecules not only recruit cells to the site of injury, but they can also act as entry sites for respiratory pathogens.

The lung is kept in a remarkably sterile condition by the combined activities of the mucociliary apparatus, and a variety of specific and non-specific humoral and cellular defense mechanisms (Green et al., 1977). Of these components, collectins and other secretory proteins maintain surface tension, facilitate gas exchange and provide protection against inhaled pathogens (Hickling et al., 2004). Clara cells release a secretory molecule called the Clara cell 16 kD protein (termed CC10, CC16, or CCSP) (Singh and Katyal, 1997) which protects the airway epithelium from pathogens and inflammation. Previous studies have reported that CCSP is reduced after exposure to cigarette smoke, ozone (Yu et al., 2002) and DE with this latter effect being associated with delayed clearance of *Pseudomonas aeruginosa* (Harrod et al., 2005).

Surfactant protein A and D are members of the collectin family that bind to components of bacterial, viral and fungal cell walls (LPS, lipoteichoic acids, lipo-arabino-mannans, polysaccharides, N-linked oligosaccharides) by pattern recognition through a carbohydrate recognition domain (CRD), and facilitate opsonization (Crouch, 1998). Mice deficient in either of these collectins display delayed clearance of pathogens including group B *streptococcus* (GBS) (LeVine et al., 1997), *Haemophilus influenza* (LeVine et al., 2000), RSV (LeVine et al., 1999), *Pneumocystis carinii* (LeVine et al., 2002) and *Pseudomonas aeruginosa* (Korfhagen et al., 1998). Exposure to air pollutants such as cigarette smoke and ozone decreases the production or function of SP-A and SP-D (Honda et al., 1996; Janic et al., 2005) and recently it was reported that mice exposed to DE and subsequently infected with RSV infection had decrease levels of SP-A in the lungs (Harrod et al., 2003).

We have previously reported that exposure to exhaust from a stationary diesel engine used to power an air compressor increases the susceptibility to influenza infection in mice (Ciencewicki et al., 2007). In order to assess the early effects attributable to delayed viral clearance, we examined how DE altered the pulmonary inflammatory and host defense responses prior to infection. This study was designed to evaluate concentration and time-dependent pulmonary inflammatory responses following exposure to DE from an engine under steady load, and to determine whether these relatively low concentration, short term exposures affected production of anti-microbial host defenses molecules. We hypothesized that acute exposure to DE increases pulmonary inflammation and decreases the production of

host defense molecules, which creates a permissive environment that promotes the development of respiratory infections.

Materials and Methods

Animals

Pathogen-free BALB/c female mice, 10-12 wk old, weighing 17-20 g, were purchased from Charles River (Raleigh, NC). Once at the U.S. EPA animal care facilities (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care), animals were housed in groups of five in polycarbonate cages with hardwood chip bedding (Beta Chip, Northeastern Products, Warrensburg, NY), provided a 12-hour light (0600 hours) to dark (1800 hours) cycle, maintained at 22.3 ± 1.1°C and 50 ± 10% humidity, and given access to both food (5P00 Prolab RMH 3000, PMI Nutrition International, Richmond, IN) and water *ad libitum*. Animals were acclimated for at least ten days before the study began. Sentinel animals were housed in the same location. The studies were conducted after approval by the laboratory's Institutional Animal Care and Welfare Committee.

Diesel Exhaust Exposure and Monitoring

Diesel exhaust for exposure experiments was generated using a 30 kW (40 hp) 4-cylinder Deutz BF4M1008 diesel engine connected to a 22.3 kW Saylor Bell air compressor to provide a load. Diesel fuel was purchased from a local (Research Triangle Park, NC) service station and stored in drums. Replicate analysis (ultimate, elemental, heating value, and specific gravity) of multiple batches of fuel purchased over time indicated consistent fuel properties and composition (data not shown). Engine lubrication oil (Shell Rotella, 15W-40)

was changed before each set of exposure tests. The engine and compressor were operated at steady state to produce 0.8 m^3/min of compressed air at 400 kPa. This translates to approximately 20% of the engine's full-load rating. From the engine exhaust, a small portion of the flow (14 L/min) was educted by an aspirator (3:1 dilution) to a second cone diluter (10:1 dilution), and then through approximately 15 m of flexible food grade polyvinyl chloride (PVC) tubing (7.62 cm inside diameter) to two stainless steel 0.3 m^3 Hinners inhalation exposure chambers housed in an isolated animal exposure room. The dilution air used was drawn from the animal exposure room through a high efficiency particulate air (HEPA) filter. Target DEP concentrations in the two chambers were 2000 μg of PM/m^3 (high) and 500 μg of PM/m^3 (low). From here on the lower exposure will be referred to as 0.5 mg/m^3 of DE and the higher exposure as 2 mg/m^3 of DE. Control animals were housed in a third chamber supplied with the same HEPA filtered room air. DEP concentrations in the low (0.5 mg/m^3) chamber were achieved by additional dilution using HEPA filtered room air just prior to entering the chamber. All three chambers were operated at the same flow rate (142 L/min), which resulted in 28 full air exchanges per hour. Integrated 4 h filter samples (14.1 L/min) were collected daily from each chamber and analyzed gravimetrically to determine particle concentrations. In addition, triplicate 8 and 20 min quartz filter samples (14.1 L/min) were collected from the high and low chambers, respectively, and analyzed using a thermal/optical carbon analyzer (Sunset Laboratory Inc., model 107, Tigard, OR) to determine organic carbon/elemental carbon (OC/EC) partitioning of the collected DEP. Continuous emission monitors (CEMs) were used to measure chamber concentrations of PM

by tapered element oscillating microbalance (TEOM) (Rupprecht and Patashnick Co., series 1400, Albany, NY), oxygen (O_2) (Beckman Corp., model 755, La Habra, CA), carbon monoxide (CO) (Thermo Electron Corp, model 48, Franklin, MA), nitrogen oxides (NO_x) (Teledyne Technology Co., model 200A4, San Diego, CA), and sulfur dioxide ($SO_{2,}$) (Thermo Electron Corp, model 43c, Franklin, MA). Samples were extracted through fixed stainless steel probes in the exposure chambers. Gas samples were passed through a particulate filter prior to the individual gas analyzers. Dilution air was adjusted periodically to maintain target PM concentrations as measured by the TEOM. Particle size distributions were characterized during each exposure using a scanning mobility particle sizer (SMPS) (TSI Inc., model 3080/3022a, St. Paul, MN) and an aerodynamic particle sizer (APS) (TSI Inc., model 3321, St. Paul, MN). Chamber temperatures, relative humidity, and noise were also monitored, and maintained within acceptable ranges. Mice were exposed to HEPA filtered room air or diesel emissions diluted to yield 0.5, or 2.0 mg/m^3 of diesel emission particulate for 4 h/day for one or five consecutive days. To examine responses both immediately and 18 hrs post exposure mice were necropsied at either time point.

Bronchoalveolar Lavage

After 0 and 18 h post exposure, 5 mice from each treatment group were euthanized with sodium pentobarbital and the trachea was exposed, cannulated, and secured with suture thread. The left mainstem bronchus was isolated, clamped with microhaemostats after the trachea was cannulated. The right lungs lobes were lavaged 3 times with a single volume of warmed Hanks balanced salt solution (HBSS) (Invitrogen, Grand Island, NY) (35ml/kg).

The resulting lavage was centrifuged (717 x g, 15 min, 4° C) and 150 µl was stored at 4° C (for biochemical analysis) or -80° C (for cytokine measurement). The pelleted cells were resuspended in 1 ml of RPMI 1640 (Gibco, Carlsbad, CA) containing 2.5 % fetal bovine serum (FBS; Gibco, Carlsbad, CA). Total cell counts in the lavage fluid of each mouse were obtained with a Coulter Counter (Beckman Dickson, Fullerton, CA). Each sample (200 µl) was centrifuged in duplicate onto slides using a Cytospin (Shandon, Pittsburgh, PA) and subsequently stained with Diff Quik solution (American Scientific, McGraw Park, PA) for cell differentiation determination, with at least 200 cells counted from each slide. The left lobe was fixed n paraformaldehyde for immunohistochemistry or snap frozen in liquid nitrogen and subsequently stored at -80° C for isolation of RNA and protein.

Western Blots

Whole cell lysates were prepared by homogenizing the left lobe in 1x cell lysis buffer (Cell Signalling, Danvers, MA) containing protease inhibitors (Roche Diagnostics, Pemzberg, Germany). Whole cell lysates (10 µg) were separated by 10% bis-tris gels under reducing conditions (Invitrogen, Grand Island, NY). This was followed by immunoblotting using specific antibodies to surfactant protein A (1:2000; Chemicon, Temecula, CA, cat# AB3420), surfactant protein D (1:2000; Chemicon Temecula, CA, cat# AB3434), clara cell secretory protein (1:2000; Upstate, Temecula, Ca, cat# 07-623), or ICAM-1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, cat# sc-1511-R). β-actin was used as a loading control for all blots (1:2000; US Biological, Swampscott, MA, cat#A0760-40). Antigen-antibody complexes were stained with anti-goat or anti-mouse horseradish peroxidase-conjugated

antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). Chemiluminescent signals were acquired using an Alpha Innotech 8900 imaging station (San Leandro, CA) and visualized using the Fluorchem software (Alpha Innotech, San Leandro, CA). Densitometric analysis of optical densities was performed by the instrument software (Alpha Ease FC, San Leandro, CA).

Real Time PCR

Total RNA was extracted from lung tissue with TRIzol (Invitrogen, Grand Island, NY) as per the supplier's instructions. First strand cDNA synthesis and real-time RT-PCR were performed as previously described (Jaspers et al., 1999; Jaspers et al., 2001). Genbank mRNA primers were ICAM-1 NM_010493.2; IFN-γ NM_008337.1; MIP-2 NM_009140.1; IL-6 NM_031168.1; IL-13 NM_008355.1; CCSP NM_011681.1; SP-D NM_009160; 3', SP-A NM_023134.3 purchased from Applied Biosystems (Foster City, CA).

Immunohistochemistry

Lung tissue samples were fixed in 4% paraformaldhyde and embedded in paraffin. Five µm thick sections were placed on polylysine-coated slides and stained for SP-D (1:200; Chemicon, Temecula, CA), or CCSP (1:1000; Upstate Temecula, CA). Additional lung samples were also sent to Experimental Pathology Laboratories, Inc (RTP, NC) for processing and histopathological evaluation. Midsaggital lung sections were stained with hematoxylin and eosin for assessment of inflammatory changes. Lung sections were microscopically evaluated in lesion categories by open examination (House et al., 1992). These categories included particulate in macrophages, and perivascular mononuclear

infiltration. Lesions were scored for both severity of inflammatory cell infiltrate and distribution: 0 (normal), 1 (minimal), 2 (mild), 3 (moderate), 4 (moderately severe), 5 (severe). Total lesion score was derived by adding combined lesions scores from all categories from each sample and calculating the mean total lesion score for each treatment and time point.

Statistical Analysis

Data are expressed as mean fold changes ± SE over the air exposed controls. Data generated from experiments were analyzed using a nonparametric one-way ANOVA (Kruskal-Wallis test), followed by the Dunn's multiple comparison post hoc test. A value of $P<0.05$ was considered to be significant.

Results

DE Chamber Concentrations

Table 1 shows a summary of the 5-day average exposure data for the low (0.5 mg/m^3) and high (2.0 mg/m^3) DE concentrations. These target chamber concentrations, determined and adjusted based on continuous TEOM measurements were achieved with relatively low variability either within a particular 4 hour exposure or between different days. Chamber particle concentrations determined gravimetrically from integrated filter samples (one 4 h sample per exposure day), agreed with the TEOM measurements within 15%. CO and NO_x concentrations in the low and high chambers averaged 1.7 and 5.4 ppm and 2.0 and 7.4 ppm, respectively. SO_2 concentrations were very low and even below detection levels for the low

chamber. Particle number concentrations were relatively high, and corresponded to particle size distributions (PSDs) with a well established accumulation mode and little evidence of notable nuclei or coarse modes. Geometric median number and volume (assuming spherical particles) diameters of approximately 100 and 240 nm, respectively, were measured in both chambers. It should be noted, however, that the SMPS system (with long column) limited measurements to particles greater than approximately 15 nm, and a small increase in the number counts in channels less than 25 nm may indicate the presence of a small nuclei mode below the instrument's range. OC/EC wt ratios of 0.4 from both chambers indicate that approximately 28% of the DEP was comprised of organic carbon.

Neutrophil Recruitment and Pulmonary Inflammation

Differential cell counts from air or DE exposed mice were assessed in the BAL immediately and 18 hours after one or five daily exposures to DE (Fig 1A). A dose-dependent increase of neutrophils in the BALF was seen immediately after the single exposure to DE and this increase did not persist 18 hrs after exposure to the higher level of DE. The number of neutrophils immediately after 5 days of DE also showed a dose dependent increase, which persisted at the 18 hour time point in the high concentration group.

Histopathological examination showed that immediately after 1 day of DE, mice exposed to either level had an increase in lung injury and this effect persisted at the 18 hr time point (Figure 1B). After 5 days of DE, both exposure groups had an increase in lung

lesion scores when compared to the air control, however, the 2.0 mg/m^3 group had significantly more injury than the 0.5 mg/m^3 group.

Expression of ICAM-1

ICAM-1 is a cellular adhesion molecule that modulates the recruitment of leukocytes and can be upregulated during inflammation. To evaluate the effect of DE on ICAM-1 expression (Fig 2A) and production (Fig 2B), lung mRNA levels were assessed by real time PCR and protein levels by western blot. As shown in Figure 2, DE increased ICAM-1 expression at both the mRNA and protein level immediately after the 1 or 5 day exposure. However, 18 hrs after the 1 day exposure both DE groups returned to control levels. When ICAM-1 expression was assessed 18 hrs after 5 days of DE, the low DE exposed group had a sustained increase. This effect was also seen by western blot analysis of the protein.

Cytokine Expression

Pro-inflammatory cytokines are important for recruiting phagocytes and lymphocytes cells to the site of injury. Real Time PCR analysis of lung mRNA from air- or DE- exposed animals indicated that DE generally upregulated the expression of these mediators (Fig 3). Immediately after the first DE exposure IFN-γ was elevated ($p=0.09$) by exposure to both concentrations of DE while TNF-α was significantly increased with just the high DE (compared to air controls). After 18 hours, IL-6 and IFN- γ were elevated with exposure to either level of DE. Immediately after the 5 day exposure TNF-α, and IFN-γ were elevated with either level of DE compared to the air exposed animals. IL-6 message was only higher with mice exposed to the lower (0.5 mg/m^3) level of DE and this trend was also seen 18

hours post 5 day DE exposure as well as with TNF-α, and the Th2 cytokine IL-13. MIP-2 was significantly increased 18 hours post 5 day DE exposure in what appeared to be a dose-dependent pattern.

CCSP Expression and Production

Clara Cell Secretory Protein (CCSP also called CC10 or CC16) is an important anti-inflammatory host defense molecule produced by the airway epithelium and expression levels were assessed by real time PCR (Fig 4A), western blotting (Fig 4B) and immunohistochemistry (Fig 4C). Both levels of DE decreased the mRNA expression of CCSP in lung after one day of exposure to DE ($p=0.0665$) and this effect persisted through 18 hours. The same pattern was also seen after 5 days of DE with mRNA expression being significantly decreased at 18 hours compared to the air controls. Western blot analysis showed the same overall effect (Fig 4B) and immunohistochemical analysis of CCSP in the lung also confirmed reduced expression in the airways with the most prominent decrease 18 hours after 5 days of DE (Fig 4D).

Expression and Production of Surfactant Proteins

Surfactant proteins (SP) -A and –D are members of the collectin family and are produced by alveolar type II cells, Clara cells, and submucosal glands in the pulmonary airways (Balis et al., 1985; Crouch et al., 2000). These proteins contribute to alveolar surfactant homeostasis as well as innate defense responses by binding and neutralizing pathogens (Benne et al., 1995; Korfhagen et al., 1996; Korfhagen et al., 1998). SP-A mRNA showed a slight but non-significant decrease after 1 day of DE when compared to the air control (Fig 5A).

Immediately after 5 days of exposure to the lower concentration DE, SP-A mRNA (Fig 5A) and protein (Fig 5B and C) were decreased. This effect was significant at 18 hours post 5 days of DE compared to the air controls.

SP-D expression was similarly affected by exposure to DE (Fig 6). SP-D mRNA was decreased immediately after exposure to either level of DE although not significant, and 18 hours post exposure levels were still slightly decreased (Fig 6A). After 5 days post-DE, only the group exposed to the lower level of DE had a decrease in SP-D mRNA, and this decrease persisted at 18 hours post 5 days of DE although not significantly ($p=0.06$). Reduced lung production of SP-D was also evident by western blot analysis (Fig 6B and C) and staining in the airways and alveolar region (6D).

Discussion

Diesel inhalation is known to produce inflammation and lung injury, which under some circumstances is also associated with increased susceptibility to infection (Ciencewicki and Jaspers, 2007). Most of the inflammatory effects are attributed to enhanced chemo-attractant activity following oxidative stress-mediated injury, while the reduced host defenses are associated with damage to the airway epithelium, reduced phagocytosis, and generalized immunosuppression by a number of different mechanisms. We have previously shown that exposure to DE increased susceptibility to influenza infection in mice. To further investigate this phenomenon, this current study assessed pulmonary injury and inflammation, immediately and 18 hours after one or five daily exposures. In addition we examined a broad

range of cytokines to determine the kinetics and dose-dependent responses in relation to pulmonary injury and inflammation, and tested whether exposure affected the expression and production of adhesion and anti-microbial molecules. The results show that short-term exposure to DE caused an increase in expression of cytokines and adhesion molecules in association with lung inflammation and this was accompanied with decreased expression and production of host defense proteins.

To understand earlier chemotactic events responsible for DE-induced inflammation and injury, a number of different molecules were assessed. ICAM-1 is an adhesion molecule found on the vascular endothelium and airway epithelial cells, and participates in leukocyte recruitment and accumulation (Tosi et al., 1992). Both mRNA expression and protein levels of ICAM-1 were increased with DE, confirming previous *in vitro* and *in vivo* studies that showed upregulation of ICAM-1 in humans or rodents exposed to DEP (Salvi et al., 1999; Takizawa et al., 2000a; Ito et al., 2006). Pro-inflammatory cytokines can induce expression of ICAM-1 on the vascular and airway epithelium, and play a critical role in recruiting inflammatory cells to sites of tissue injury and infection (Martin et al., 1998; Takizawa et al., 2000a). ICAM-1 has also been shown to be a entry site for certain viruses, and previous studies have reported that increased ICAM-1 expression is associated with greater number of cells infected by rhinovirus (Ito et al., 2006). This may be a potential mechanism for how DE exposure predisposes the respiratory tract to viral infections.

The increases in cytokine expression, neutrophil recruitment and pulmonary injury are consistent with previous rodent studies at particle concentrations ranging from 0.35 to 7.0

mg /m^3 for 1 week up to 24 months (Henderson et al., 1988; Reed et al., 2004) suggesting that the effect is dependent on variables including type and strain of animal, engine, fuel and load conditions, and duration of exposure. As an example, we have previously shown that DEP samples obtained from different sources are not only chemically distinct but may also produce different toxicity and mutagenicity profiles (DeMarini et al., 2004; Singh et al., 2004) .

Despite the use of different engines, and variations in experimental protocols, the general observation of DE as a mild inflammatory agent confirms that this endpoint is a useful exposure/effect metric in both rodents and humans. Analysis of lung tissue and/or BAL fluid showed that DE exposure increased numerous cytokines including IL-6, MIP-2 and TNF–α, although the kinetics and dose-dependency varied across exposure concentration, duration, and time point. Dissecting out specific roles for each of these mediators is difficult since they operate in a complex response cascade with substantial redundancy. Nevertheless, it is known that TNF blockade can suppress pulmonary inflammatory responses to air pollution particles (Lambert et al., 2001) while IL-6 knock-out animals show reduced inflammation following diesel exposure (Lambert et al., 2001; Fujimaki et al., 2006). Many of the endpoints examined in this study showed effects at the lower (0.5 mg/m-3) concentration but no difference from controls at the higher level. Similar response patterns have also been observed in cells exposed to DEP and phorbol 12-myristate 13-acetate (PMA) whereby IL-8 expression was increased at lower doses of DEP, but decreased at higher doses (Ushio et al., 1999). In one of the first studies demonstrating

the adjuvant effects of DEPs, humans exposed via the nose with the intermediate 0.3 mg DEP concentration produced more IgE antibody while exposure to concentrations above and below that level (1.0 and 0.15 mg respectively) had no effect (Diaz-Sanchez et al., 1994). A possible explanation for these apparent bi-phasic response patterns is that DE induces a hierarchical oxidative stress response with antioxidant defense responses and inflammatory responses induced by lower DE concentrations and necrosis and apoptosis induced by higher levels of DE (Xiao et al., 2003).

As well as being markers of inflammation, certain cytokines play key roles in regulating specific immune responses, which can result in distinct disease phenotypes. It is generally accepted that animals instilled with experimental allergens and DEPs experience an immunological shift towards Th2 responses with a decrease in IFN-γ and an increase in IL-13 that culminates in enhanced IgE antibody production, pulmonary eosinophilia and airway hyper-reactivity (Diaz-Sanchez et al., 1994; Diaz-Sanchez, 1997; Diaz-Sanchez et al., 2000). To determine whether early Th2 skewing could be detected following inhalation exposure to DE in the absence of antigen, expression of IFN-γ and IL-13 was measured in control and DE exposed lungs. IFN- γ followed a dose dependent increase that correlated with the histological changes and neutrophil influx. IL-13 did not change until 18 hours after the 5 day exposure when it was increased with both DE concentrations. Since IL-13 has been associated with induction of eotaxin, bronchial hyperresponsiveness and mucin secretion, these data could explain why DE can create a priming environment to enhance Th-2 type immune responses. Increases in IL-13 have also been described in healthy human subjects

exposed to DE and provides another example of concordance between animal and human studies (Pourazar et al., 2004).

In addition to being characterized as an immunological adjuvant, diesel particles and accompanying gases such as NO_2 and SO_2 have been shown to decrease host defenses resulting in increased susceptibility to infection (Rudell et al., 1999; Groneberg-Kloft et al., 2006). While most of these studies have focused on bacterial clearance and macrophage function, the amount and activity of antimicrobial proteins such as CCSP and SP-A and D are also known to be crucial in host defense (Crouch et al., 2000). Previous studies have shown that in association with respiratory infections, mice exposed to DE have decreased amounts of CCSP and SP-A by immunohistochemical assessment (Harrod et al., 2003; Ciencewicki et al., 2007). The results presented here showed that DE alone decreased the expression of both CCSP and SP-A as well as SP-D, and could contribute to our observation of increased susceptibility to influenza infection (Ciencewicki et al., 2007).

When the diesel exposures were extended for 5 days, the decrease in CSSP remained low for both concentrations while the high dose animals showed recovery in expression and production of SP-A and D. It is known that prolonged exposure to air pollutants such as silica, NO_2, and O_3 induces hypertrophy of type II cells with subsequent increases in surfactant protein production (Evans et al., 1976; Miller and Hook, 1990; Barth et al., 1994) which could explain the recovery of SP-A and SP-D in the high dose group. The disparate recovery responses between CCSP and the surfactant proteins could be explained by the differential sensitivity of clara cells and type II alveolar cells to DEP exposure (Murphy et

al., 1999). For example CCAAT/enhancer binding proteins (CEBP) which are induced by DNA damage negatively regulate CCSP expression but positively regulate SP-A (He and Crouch, 2002, Ramsay et al., 2003).

In conclusion, this study has shown that mice exposed to DE experienced mild increases in inflammation, increased cytokine output and decreased anti-microbial protein expression and production. Importantly, similar inflammatory effects have been reported in human studies thus validating the use of rodents to predict potential health effects over a broad range of different exposure scenarios. The decrease in host defense molecules has not been reported in people as yet, but could explain reports of increased morbidity and mortality to respiratory infections associated with PM exposure (Pope et al., 2004). This experimental paradigm can be used to further explore how differences in engine, fuel, load characteristics and pollution control devices affect these health outcomes in association with respiratory infections.

References

Balis, J. U., Paterson, J. F., Paciga, J. E., Haller, E. M., and Shelley, S. A., 1985. Distribution and subcellular localization of surfactant-associated glycoproteins in human lung. Lab Invest 52, 657-669.

Barth, P. J., Uhlarik, S., Bittinger, A., Wagner, U., and Ruschoff, J., 1994. Diffuse alveolar damage in the rat lung after short and long term exposure to nitrogen dioxide. Pathol Res Pract 190, 33-41.

Behndig, A. F., Mudway, I. S., Brown, J. L., Stenfors, N., Helleday, R., Duggan, S. T., Wilson, S. J., Boman, C., Cassee, F. R., Frew, A. J., Kelly, F. J., Sandstrom, T., and Blomberg, A., 2006. Airway antioxidant and inflammatory responses to diesel exhaust exposure in healthy humans. Eur Respir J 27, 359-365.

Benne, C. A., Kraaijeveld, C. A., van Strijp, J. A., Brouwer, E., Harmsen, M., Verhoef, J., van Golde, L. M., and van Iwaarden, J. F., 1995. Interactions of surfactant protein A with influenza A viruses: binding and neutralization. J Infect Dis 171, 335-341.

Castranova, V., Ma, J. Y., Yang, H. M., Antonini, J. M., Butterworth, L., Barger, M. W., Roberts, J., and Ma, J. K., 2001. Effect of exposure to diesel exhaust particles on the susceptibility of the lung to infection. Environ Health Perspect 109 Suppl 4, 609-612.

Ciencewicki, J., Gowdy, K., Krantz, Q. T., Linak, W. P., Brighton, L., Gilmour, M. I., and Jaspers, I., 2007. Diesel exhaust enhanced susceptibility to influenza infection is associated with decreased surfactant protein expression. Inhal Toxicol 19, 1121-1133.

Ciencewicki, J., and Jaspers, I., 2007. Air pollution and respiratory viral infection. Inhal Toxicol 19, 1135-1146.

Crouch, E., Hartshorn, K., and Ofek, I., 2000. Collectins and pulmonary innate immunity. Immunol Rev 173, 52-65.

Crouch, E. C., 1998. Collectins and pulmonary host defense. Am J Respir Cell Mol Biol 19, 177-201.

DeMarini, D. M., Brooks, L. R., Warren, S. H., Kobayashi, T., Gilmour, M. I., and Singh, P., 2004. Bioassay-directed fractionation and salmonella mutagenicity of automobile and forklift diesel exhaust particles. Environ Health Perspect 112, 814-819.

Diaz-Sanchez, D., 1997. The role of diesel exhaust particles and their associated polyaromatic hydrocarbons in the induction of allergic airway disease. Allergy 52, 52-56; discussion 57-58.

Diaz-Sanchez, D., Dotson, A. R., Takenaka, H., and Saxon, A., 1994. Diesel exhaust particles induce local IgE production in vivo and alter the pattern of IgE messenger RNA isoforms. J Clin Invest 94, 1417-1425.

Diaz-Sanchez, D., Penichet-Garcia, M., and Saxon, A., 2000. Diesel exhaust particles directly induce activated mast cells to degranulate and increase histamine levels and symptom severity. J Allergy Clin Immunol 106, 1140-1146.

Environmental Protection Agency, U. S. A., 2002. Health risk assessment document for diesel exhaust.

Evans, M. J., Johnson, L. V., Stephens, R. J., and Freeman, G., 1976. Cell renewal in the lungs of rats exposed to low levels of ozone. Exp Mol Pathol 24, 70-83.

Fujimaki, H., Kurokawa, Y., Yamamoto, S., and Satoh, M., 2006. Distinct requirements for interleukin-6 in airway inflammation induced by diesel exhaust in mice. Immunopharmacol Immunotoxicol 28, 703-714.

Green, G. M., Jakab, G. J., Low, R. B., and Davis, G. S., 1977. Defense mechanisms of the respiratory membrane. Am Rev Respir Dis 115, 479-514.

Groneberg-Kloft, B., Kraus, T., Mark, A., Wagner, U., and Fischer, A., 2006. Analysing the causes of chronic cough: relation to diesel exhaust, ozone, nitrogen oxides, sulphur oxides and other environmental factors. J Occup Med Toxicol 1, 6.

Hahon, N., Booth, J. A., Green, F., and Lewis, T. R., 1985. Influenza virus infection in mice after exposure to coal dust and diesel engine emissions. Environ Res 37, 44-60.

Harrod, K. S., Jaramillo, R. J., Berger, J. A., Gigliotti, A. P., Seilkop, S. K., and Reed, M. D., 2005. Inhaled diesel engine emissions reduce bacterial clearance and exacerbate lung disease to Pseudomonas aeruginosa infection in vivo. Toxicol Sci 83, 155-165.

Harrod, K. S., Jaramillo, R. J., Rosenberger, C. L., Wang, S. Z., Berger, J. A., McDonald, J. D., and Reed, M. D., 2003. Increased susceptibility to RSV infection by exposure to inhaled diesel engine emissions. Am J Respir Cell Mol Biol 28, 451-463.

He, Y., and Crouch, E., 2002. Surfactant protein D gene regulation. Interactions among the conserved CCAAT/enhancer-binding protein elements. J Biol Chem 277, 19530-19537.

Henderson, R. F., Pickrell, J. A., Jones, R. K., Sun, J. D., Benson, J. M., Mauderly, J. L., and McClellan, R. O., 1988. Response of rodents to inhaled diluted diesel exhaust: biochemical and cytological changes in bronchoalveolar lavage fluid and in lung tissue. Fundam Appl Toxicol 11, 546-567.

Hickling, T. P., Clark, H., Malhotra, R., and Sim, R. B., 2004. Collectins and their role in lung immunity. J Leukoc Biol 75, 27-33.

Honda, Y., Takahashi, H., Kuroki, Y., Akino, T., and Abe, S., 1996. Decreased contents of surfactant proteins A and D in BAL fluids of healthy smokers. Chest 109, 1006-1009.

House, D. E., Berman, E., Seely, J. C., and Simmons, J. E., 1992. Comparison of open and blind histopathologic evaluation of hepatic lesions. Toxicol Lett 63, 127-133.

Ito, T., Okumura, H., Tsukue, N., Kobayashi, T., Honda, K., and Sekizawa, K., 2006. Effect of diesel exhaust particles on mRNA expression of viral and bacterial receptors in rat lung epithelial L2 cells. Toxicol Lett 165, 66-70.

Janic, B., Umstead, T. M., Phelps, D. S., and Floros, J., 2005. Modulatory effects of ozone on THP-1 cells in response to SP-A stimulation. Am J Physiol Lung Cell Mol Physiol 288, L317-325.

Jaspers, I., Ciencewicki, J. M., Zhang, W., Brighton, L. E., Carson, J. L., Beck, M. A., and Madden, M. C., 2005. Diesel exhaust enhances influenza virus infections in respiratory epithelial cells. Toxicol Sci 85, 990-1002.

Jaspers, I., Samet, J. M., and Reed, W., 1999. Arsenite exposure of cultured airway epithelial cells activates kappaB-dependent interleukin-8 gene expression in the absence of nuclear factor-kappaB nuclear translocation. J Biol Chem 274, 31025-31033.

Jaspers, I., Zhang, W., Fraser, A., Samet, J. M., and Reed, W., 2001. Hydrogen peroxide has opposing effects on IKK activity and IkappaBalpha breakdown in airway epithelial cells. Am J Respir Cell Mol Biol 24, 769-777.

Korfhagen, T. R., Bruno, M. D., Ross, G. F., Huelsman, K. M., Ikegami, M., Jobe, A. H., Wert, S. E., Stripp, B. R., Morris, R. E., Glasser, S. W., Bachurski, C. J., Iwamoto, H.

S., and Whitsett, J. A., 1996. Altered surfactant function and structure in SP-A gene targeted mice. Proc Natl Acad Sci U S A 93, 9594-9599.

Korfhagen, T. R., LeVine, A. M., and Whitsett, J. A., 1998. Surfactant protein A (SP-A) gene targeted mice. Biochim Biophys Acta 1408, 296-302.

Lambert, A. L., Selgrade, M. K., Winsett, D. W., and Gilmour, M. I., 2001. TNF-alpha enhanced allergic sensitization to house dust mite in brown Norway rats. Exp Lung Res 27, 617-635.

LeVine, A. M., Bruno, M. D., Huelsman, K. M., Ross, G. F., Whitsett, J. A., and Korfhagen, T. R., 1997. Surfactant protein A-deficient mice are susceptible to group B streptococcal infection. J Immunol 158, 4336-4340.

LeVine, A. M., Hartshorn, K., Elliott, J., Whitsett, J., and Korfhagen, T., 2002. Absence of SP-A modulates innate and adaptive defense responses to pulmonary influenza infection. Am J Physiol Lung Cell Mol Physiol 282, L563-572.

LeVine, A. M., Kurak, K. E., Wright, J. R., Watford, W. T., Bruno, M. D., Ross, G. F., Whitsett, J. A., and Korfhagen, T. R., 1999. Surfactant protein-A binds group B streptococcus enhancing phagocytosis and clearance from lungs of surfactant protein-A-deficient mice. Am J Respir Cell Mol Biol 20, 279-286.

LeVine, A. M., Whitsett, J. A., Gwozdz, J. A., Richardson, T. R., Fisher, J. H., Burhans, M. S., and Korfhagen, T. R., 2000. Distinct effects of surfactant protein A or D deficiency during bacterial infection on the lung. J Immunol 165, 3934-3940.

Li, N., Kim, S., Wang, M., Froines, J., Sioutas, C., and Nel, A., 2002. Use of a stratified oxidative stress model to study the biological effects of ambient concentrated and diesel exhaust particulate matter. Inhal Toxicol 14, 459-486.

Martin, L. D., Krunkosky, T. M., Voynow, J. A., and Adler, K. B., 1998. The role of reactive oxygen and nitrogen species in airway epithelial gene expression. Environ Health Perspect 106 Suppl 5, 1197-1203.

Miller, B. E., and Hook, G. E., 1990. Hypertrophy and hyperplasia of alveolar type II cells in response to silica and other pulmonary toxicants. Environ Health Perspect 85, 15-23.

Murphy, S. A., BeruBe, K. A., and Richards, R. J., 1999. Bioreactivity of carbon black and diesel exhaust particles to primary Clara and type II epithelial cell cultures. Occup Environ Med 56, 813-819.

Oberdorster, G., and Utell, M. J., 2002. Ultrafine particles in the urban air: to the respiratory tract--and beyond? Environ Health Perspect 110, A440-441.

Pope, C. A., 3rd, Burnett, R. T., Thurston, G. D., Thun, M. J., Calle, E. E., Krewski, D., and Godleski, J. J., 2004. Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. Circulation 109, 71-77.

Pourazar, J., Frew, A. J., Blomberg, A., Helleday, R., Kelly, F. J., Wilson, S., and Sandstrom, T., 2004. Diesel exhaust exposure enhances the expression of IL-13 in the bronchial epithelium of healthy subjects. Respir Med 98, 821-825.

Ramsay, P. L., Luo, Z., Major, A., Park, M. S., Finegold, M., Welty, S. E., Kwak, I., Darlington, G., and Demayo, F. J., 2003. Multiple mechanisms for oxygen-induced regulation of the Clara cell secretory protein gene. Faseb J 17, 2142-2144.

Reed, M. D., Gigliotti, A. P., McDonald, J. D., Seagrave, J. C., Seilkop, S. K., and Mauderly, J. L., 2004. Health effects of subchronic exposure to environmental levels of diesel exhaust. Inhal Toxicol 16, 177-193.

Riedl, M., and Diaz-Sanchez, D., 2005. Biology of diesel exhaust effects on respiratory function. J Allergy Clin Immunol 115, 221-228; quiz 229.

Rudell, B., Blomberg, A., Helleday, R., Ledin, M. C., Lundback, B., Stjernberg, N., Horstedt, P., and Sandstrom, T., 1999. Bronchoalveolar inflammation after exposure to diesel exhaust: comparison between unfiltered and particle trap filtered exhaust. Occup Environ Med 56, 527-534.

Salvi, S., Blomberg, A., Rudell, B., Kelly, F., Sandstrom, T., Holgate, S. T., and Frew, A., 1999. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. Am J Respir Crit Care Med 159, 702-709.

Salvi, S. S., Nordenhall, C., Blomberg, A., Rudell, B., Pourazar, J., Kelly, F. J., Wilson, S., Sandstrom, T., Holgate, S. T., and Frew, A. J., 2000. Acute exposure to diesel exhaust increases IL-8 and GRO-alpha production in healthy human airways. Am J Respir Crit Care Med 161, 550-557.

Singh, G., and Katyal, S. L., 1997. Clara cells and Clara cell 10 kD protein (CC10). Am J Respir Cell Mol Biol 17, 141-143.

Singh, P., DeMarini, D. M., Dick, C. A., Tabor, D. G., Ryan, J. V., Linak, W. P., Kobayashi, T., and Gilmour, M. I., 2004. Sample characterization of automobile and forklift diesel exhaust particles and comparative pulmonary toxicity in mice. Environ Health Perspect 112, 820-825.

Steerenberg, P., Verlaan, A., De Klerk, A., Boere, A., Loveren, H., and Cassee, F., 2004. Sensitivity to ozone, diesel exhaust particles, and standardized ambient particulate matter in rats with a listeria monocytogenes-induced respiratory infection. Inhal Toxicol 16, 311-317.

Sydbom, A., Blomberg, A., Parnia, S., Stenfors, N., Sandstrom, T., and Dahlen, S. E., 2001. Health effects of diesel exhaust emissions. Eur Respir J 17, 733-746.

Takizawa, H., Abe, S., Ohtoshi, T., Kawasaki, S., Takami, K., Desaki, M., Sugawara, I., Hashimoto, S., Azuma, A., Nakahara, K., and Kudoh, S., 2000a. Diesel exhaust particles up-regulate expression of intercellular adhesion molecule-1 (ICAM-1) in human bronchial epithelial cells. Clin Exp Immunol 120, 356-362.

Takizawa, H., Ohtoshi, T., Kawasaki, S., Abe, S., Sugawara, I., Nakahara, K., Matsushima, K., and Kudoh, S., 2000b. Diesel exhaust particles activate human bronchial epithelial cells to express inflammatory mediators in the airways: a review. Respirology 5, 197-203.

Tornqvist, H., Mills, N. L., Gonzalez, M., Miller, M. R., Robinson, S. D., Megson, I. L., Macnee, W., Donaldson, K., Soderberg, S., Newby, D. E., Sandstrom, T., and Blomberg, A., 2007. Persistent endothelial dysfunction in humans after diesel exhaust inhalation. Am J Respir Crit Care Med 176, 395-400.

Tosi, M. F., Stark, J. M., Smith, C. W., Hamedani, A., Gruenert, D. C., and Infeld, M. D., 1992. Induction of ICAM-1 expression on human airway epithelial cells by inflammatory cytokines: effects on neutrophil-epithelial cell adhesion. Am J Respir Cell Mol Biol 7, 214-221.

Ushio, H., Nohara, K., and Fujimaki, H., 1999. Effect of environmental pollutants on the production of pro-inflammatory cytokines by normal human dermal keratinocytes. Toxicol Lett 105, 17-24.

Xiao, G. G., Wang, M., Li, N., Loo, J. A., and Nel, A. E., 2003. Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. J Biol Chem 278, 50781-50790.

Yang, H. M., Antonini, J. M., Barger, M. W., Butterworth, L., Roberts, B. R., Ma, J. K., Castranova, V., and Ma, J. Y., 2001. Diesel exhaust particles suppress macrophage function and slow the pulmonary clearance of Listeria monocytogenes in rats. Environ Health Perspect 109, 515-521.

Yin, X. J., Dong, C. C., Ma, J. Y., Antonini, J. M., Roberts, J. R., Stanley, C. F., Schafer, R., and Ma, J. K., 2004. Suppression of cell-mediated immune responses to listeria infection by repeated exposure to diesel exhaust particles in brown Norway rats. Toxicol Sci 77, 263-271.

Yu, M., Zheng, X., Witschi, H., and Pinkerton, K. E., 2002. The role of interleukin-6 in pulmonary inflammation and injury induced by exposure to environmental air pollutants. Toxicol Sci 68, 488-497.

Table 1. Summary of concentrations and characteristics of the diesel exhaust particles and gases within the animal exposure chambers.

Constituent	*Units*	*Low exposure*	*High exposure*
Particle mass concentration (TEOM)	$\mu g/m^3$	514±3	2026±38
Particle mass concentration (filter)[b]	$\mu g/m^3$	540±29	1778±31
Particle number concentration[c]	$\#/cm^3$	5.2×10^5 $\pm2.4\times10^4$	1.4×10^6 $\pm5.2\times10^4$
Oxygen (O_2)	%	21.0±0.10	20.7±0.09
Carbon monoxide (CO)	ppm	1.7±0.15	5.4±0.07
Nitrogen oxides (NO_x)	ppm	2.0±0.36	7.4±0.61
Sulfur dioxide (SO_2)	ppm	0.0±0.0	0.4±0.3
Number median D_p[d]	nm	96±2	97±2
Volume median D_p	nm	238±2	249±2
OC/EC[e]	wt ratio	0.4±0.04	0.4±0.07

Tapered element oscillating microbalance (TEOM), O_2, CO, NO_x, and SO_2 data represent mean values from continuous measurements taken over the five day exposure ± SE. [b]Filter data represent mean values from one measurement per day taken over the five day exposure ± SE. [c]Particle number concentration data represent mean values from four measurements (low exposure) and six measurements (high exposure) taken over one representative exposure day ± SE. [d]D_p indicates particle geometric number and volume median diameters for a single representative particle size distribution ± geometric standard deviation. Note that volume information is calculated from number based mobility diameters and assume spherical particles. [e]OC/EC (organic carbon to elemental carbon ratio) data represent mean values from three measurement per day taken over the five day exposure ± SE. *Definition of other abbreviations:* O_2, oxygen; CO, carbon monoxide; NOx, nitrogen oxides; SO_2, sulfur dioxide.

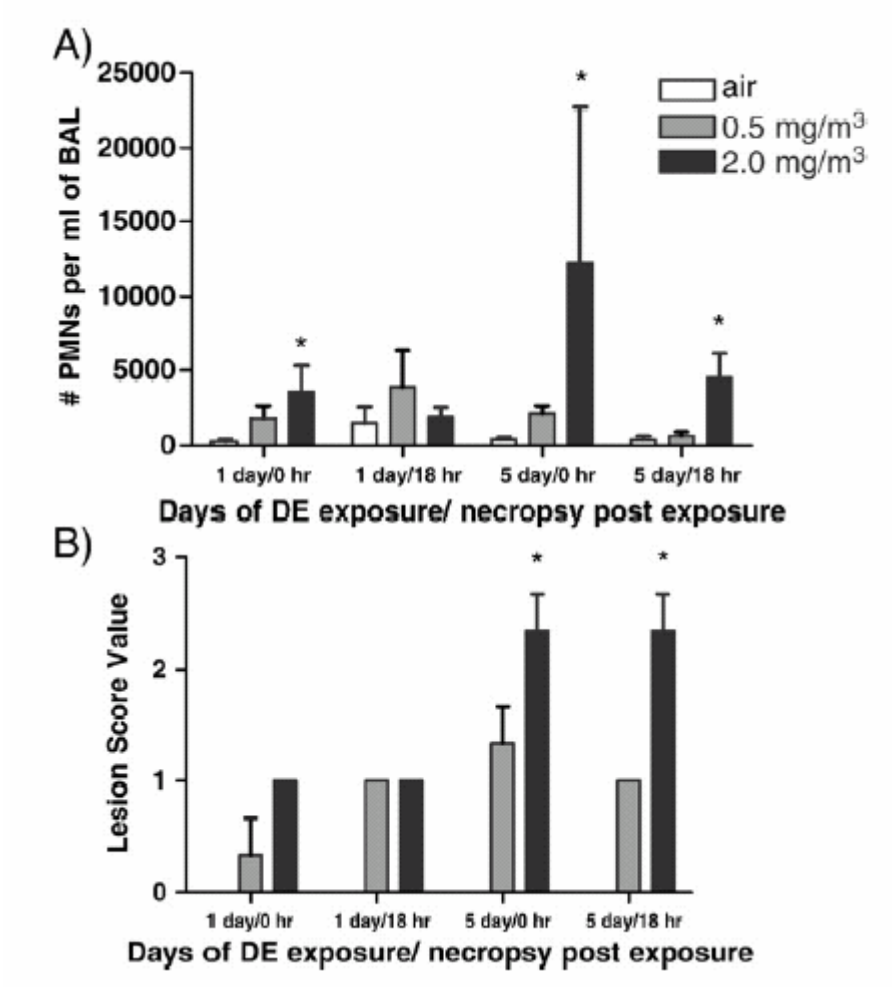

Figure 1. Exposure to DE enhances neutrophil recruitment and inflammation. BALF were obtained 0 hrs or 18 hrs. post 1 or 5 days of diesel exhaust exposure. **A)** Neutrophil counts per ml of BAL. **B)** Pathology scores of mouse lung sections were stained with H & E and visualized and scored using light microscopy. *significantly different from air exposed mice; $p<0.05$.

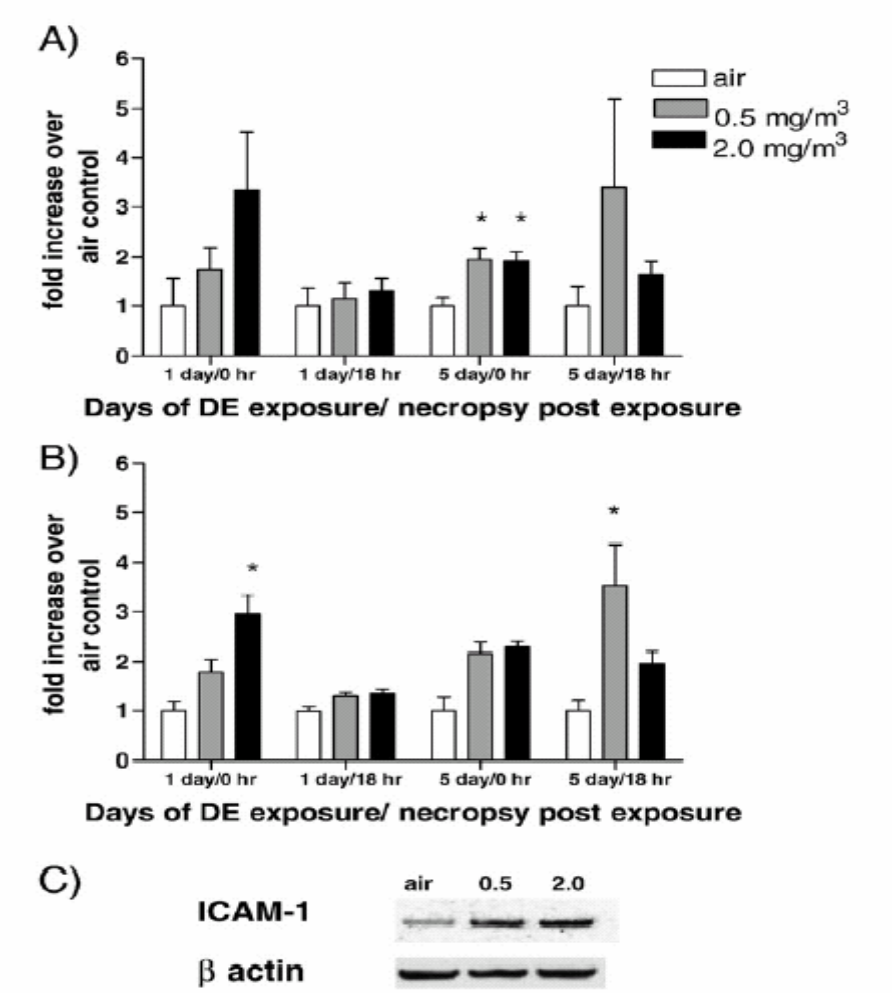

Figure 2. Exposure to DE increases the message and protein expression of adhesion molecule ICAM-1.

ICAM-1 expression was analyzed 0 hrs or 18 hrs. post 1 or 5 days of air or 0.5 and 2.0 mg/m^3 exposure and all data expressed as fold induction over air-exposed mice for each time point. **A)** Levels of ICAM-1 mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin. **B)** Lung homogenates collected 0 and18 hrs post 1 or 5 days of air or 0.5 and 2.0 mg/m^3 exposure were analyzed for ICAM-1 protein levels. Nitrocellulose membranes were stripped and re-probed with anti-β-actin antibody. Integrated Density Values (IDV) were derived from normalizing the level of ICAM-1 expression to the expression of the β-actin band. **C)** Representative western blot from 0 hr post 5 days of air or 0.5 and 2.0 mg/m^3 exposure. *significantly different from air exposed mice; $p<0.05$.

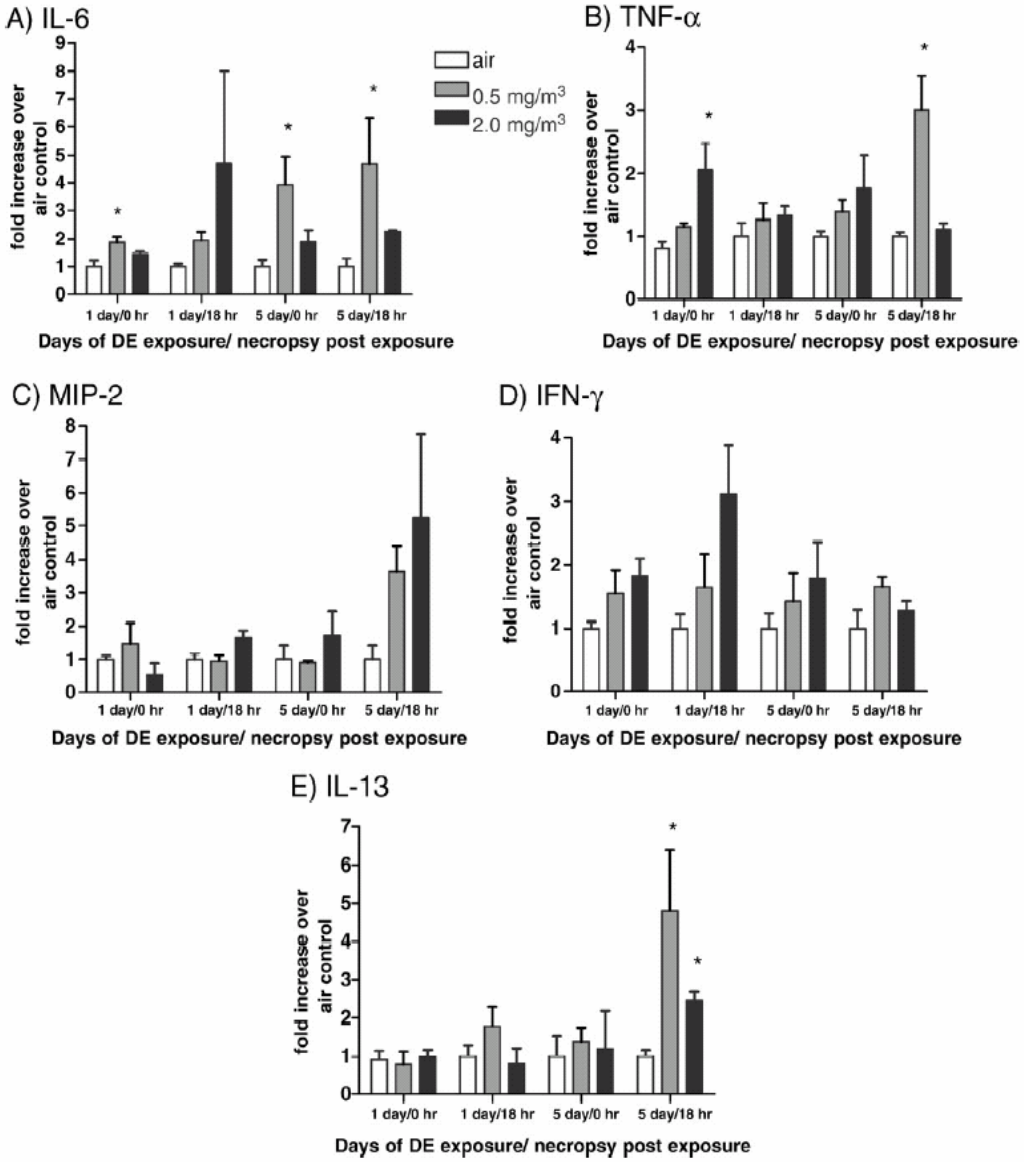

Figure 3. Exposure to DE increases the production of pro-inflammatory cytokines. Measurements were taken 0 hrs or 18 hrs. post 1 or 5 days of air or 0.5 and 2.0 mg/m^3 exposure and all data expressed as fold induction over air-exposed mice from each time point. Levels of all cytokine mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin. **A)** IL-6; **B)** TNF-α; **C)** MIP-2; **D)** IFN-γ; **E)** IL-13. *significantly different from air exposed mice; $p<0.05$.

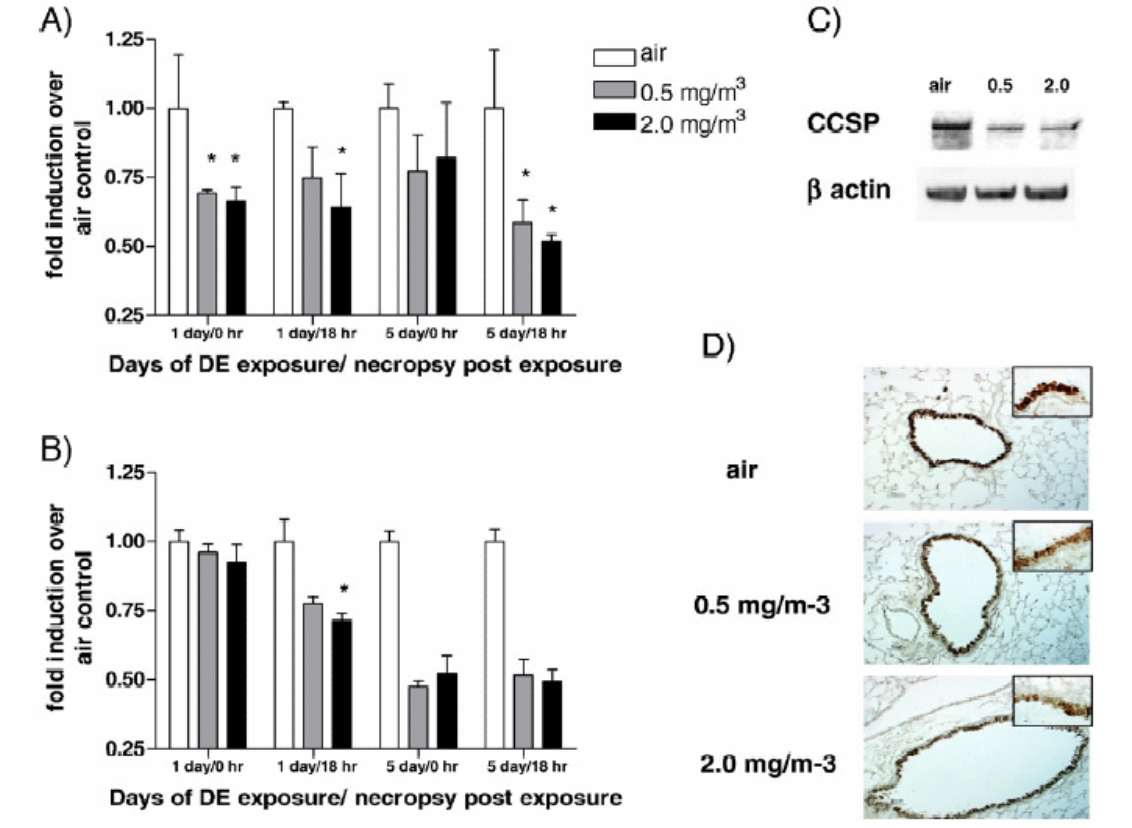

Figure 4. Exposure to DE decreases the message and protein expression of Clara cell secretory protein (CCSP).

Expression of CCSP was analyzed 0 hrs or 18 hrs. post 1 or 5 days of diesel exhaust exposure and all data expressed as fold induction over air-exposed mice from each time point. **A)** Levels of CCSP mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin mRNA. **B)** Lung homogenates collected 0 and18 hrs post 1 or 5 days of air or 0.5 and 2.0 mg/m^3 exposure were analyzed for CCSP protein levels. Nitrocellulose membranes were stripped and re-probed with anti-β-actin antibody. IDV values were derived from normalizing the level of CCSP expression to the level of β-actin expression. **C)** Representative western blot from 18 hr post 5 days of air or 0.5 and 2.0 mg/m^3 exposure. **D)** Mouse lung sections were immunohistochemically stained for CCSP and visualized using light microscopy; 40x magnification, a representative of expression at 18 hours post 5 day air or 0.5 and 2.0 mg/m^3 exposure shown. Insert is representative of 100x magnification. Brown staining on both images is indicative of positive cells. *significantly different from air exposed mice; $p<0.05$.

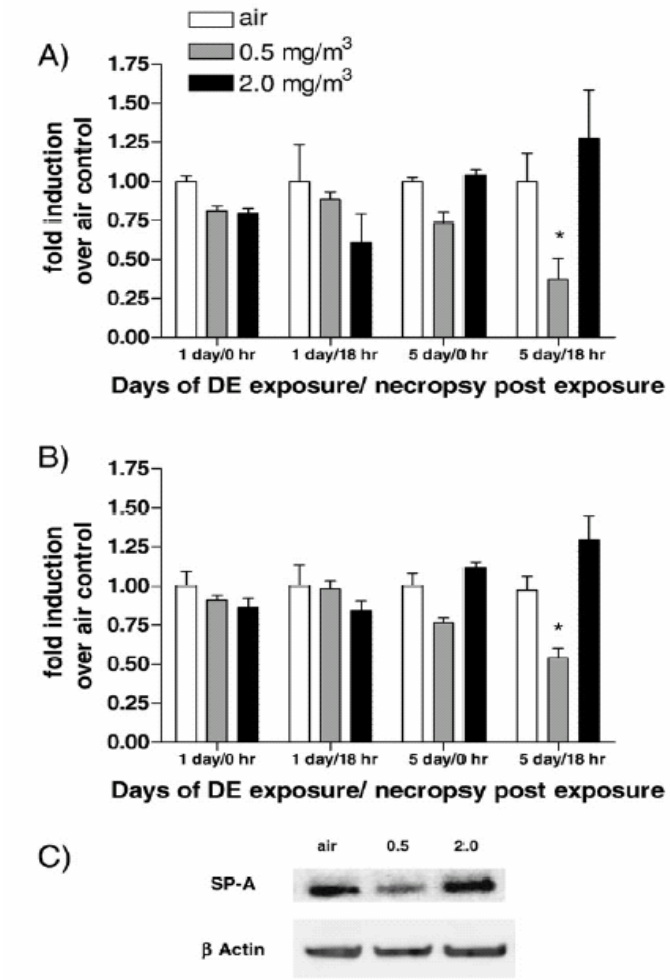

Figure 5. Exposure to DE decreases SP-A expression.
A) SP-A mRNA was quantified in lung homogenates 0 hrs or 18 hrs. post 1 or 5 days of air or 0.5 and 2.0 mg/m^3 exposure by real-time RT-PCR. Values are normalized to β-actin mRNA and expressed as fold induction over air-exposed mice for each time point. **B)** Lung homogenates collected 0 hrs or 18 hrs. post 1 or 5 days of air or 0.5 and 2.0 mg/m^3 exposure were analyzed for SP-A protein levels. Nitrocellulose membranes were stripped and re-probed with anti-β-actin antibody. IDV values were derived from normalizing the level of SP-A to the level of β-actin expression. **C)** Representative western blot from 18 hr post 5 days of air or 0.5 and 2.0 mg/m^3 exposure. *significantly different from air exposed mice; $p<0.05$.

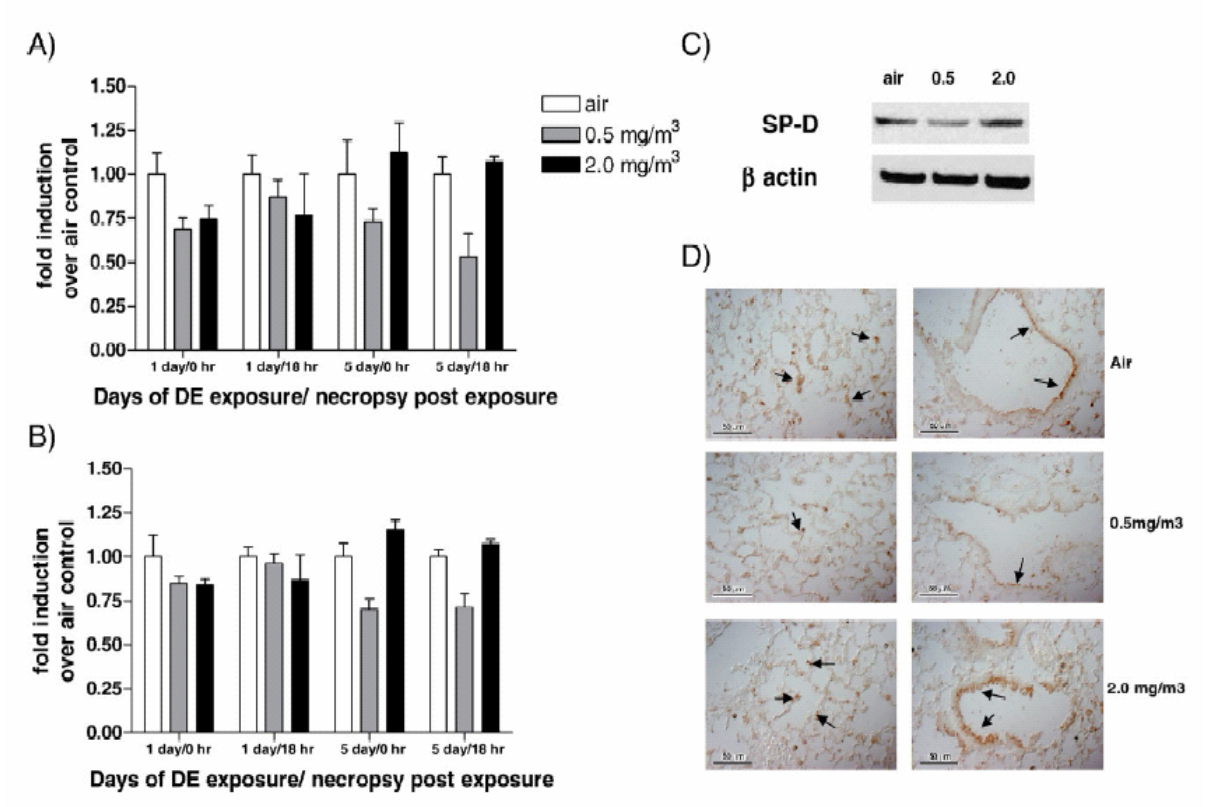

Figure 6. Exposure to DE decreases SP-D expression.
A) SP-D mRNA was quantified in lung homogenates 0 hrs or 18 hrs. post 1 or 5 days of air or 0.5 and 2.0 mg/m^3 exposure by real-time RT-PCR. Values are normalized to β-actin and expressed as fold induction over air-exposed mice for each time point. **B)** Lung homogenates collected 0 hrs or 18 hrs. post 1 or 5 days of air or 0.5 and 2.0 mg/m^3 exposure were analyzed for SP-D protein levels. Nitrocellulose membranes were stripped and re-probed with anti-β-actin antibody. IDV values were derived from normalizing the level of SP-D to the level of β-actin expression. **C)** Representative western blot from 18 hr post 5 days of air or 0.5 and 2.0 mg/m^3 exposure. **D)** Mouse lung sections were immunohistochemically stained for SP-D and visualized using light microscopy; 40x magnification, a representative of expression at 0 hours post 5 day air or 0.5 and 2.0 mg/m^3 exposure shown. Right panel shows the airways, left panel shows the alveolar region. Brown staining on both images is indicative of positive cells, and arrows point to positive cells.

Chapter 3

Diesel Exhaust Enhanced Susceptibility to Influenza Infection is Associated with Decreased Surfactant Protein Expression

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Abstract

We have previously shown that exposure of respiratory epithelial cells to diesel exhaust (DE) enhances susceptibility to influenza infection and increases the production of IL-6 and IFN-β. The purpose of this study was to confirm and expand upon these *in vitro* results by assessing the effects of DE exposure on the progression of influenza infection, and development of associated pulmonary immune and inflammatory responses *in vivo*. BALB/c mice were exposed to air, or DE containing particulate matter at concentrations of 0.5 or 2 mg/m^3 for 4 hours/day for 5 days and subsequently instilled with influenza A/Bangkok/1/79 virus. Exposure to 0.5 mg/m^3 (but not the higher 2 mg/m^3 dose) of DE increased susceptibility to influenza infection as demonstrated by a significant increase in hemagglutinin (HA) mRNA levels, a marker of influenza copies, and greater immunohistochemical staining for influenza virus protein in the lung. The enhanced susceptibility to infection observed in mice exposed to 0.5 mg/m^3 of DE was associated with a significant increase in the expression of IL-6, while anti-viral lung IFN levels were unaffected. Analysis of the expression and production of surfactant proteins A and D, which are components of the interferon-independent antiviral defenses showed that these factors were decreased following exposure to the to 0.5 mg/m^3 of DE but not the higher 2mg/m^3 concentration. Taken together, the results demonstrate that exposure to DE enhances the susceptibility to respiratory viral infections by reducing the expression and production of anti-microbial surfactant proteins.

Introduction

Diesel exhaust (DE) is an important contributor to particulate air pollution in urban air (Department of Health, 1995; United Nations Environment Program, 1994). Combustion of diesel fuel generates a mixture of hundreds of organic and inorganic compounds in both the gas and particle phase, and more than 40 of the compounds in DE are listed by the United States Environmental Protection Agency (U.S. E.P.A) as hazardous air pollutants (HAPs) (United States Environmental Protection Agency, 2002). Diesel exhaust particles (DEPs) refer to aerosols produced within the engine or formed by gas to particle transformation mechanisms occurring post-combustion. On an emitted mass basis, the majority of DEP contribute to an accumulation mode aerosol with aerodynamic diameters between 0.1 and 0.3 μm. These particles are typically comprised of chain agglomerates of primary particles containing an elemental carbon (EC) core that provide available surfaces for absorptive and adsorbtive condensation of a multitude of incompletely oxidized organic carbon (OC) species. On an emitted number basis, however, most of the DEP (>90%) contribute to a nuclei mode with diameters between 5 and 50 nm. These particles are believed to be formed via homogeneous nucleation of OC components or condensation of OC on inorganic nuclei such as sulfates. Once emitted, both the nuclei and accumulation mode DEPs contribute to an atmospheric particle loading that eventually produces an atmospheric accumulation mode aerosol between 0.1 and 1.0 μm diameter. DEPs are thus easily respirable and capable of being deposited in the lower airways and alveolar region of the lung (Li *et al.*, 2002b; Riedl *et al.*, 2005). Animal and human *in vitro* and *in vivo* studies have shown that exposure to

DEPs increases neutrophil recruitment, nitric oxide production, and production of pro-inflammatory cytokines (Hiura *et al.,* 1999; Kato *et al.,*1992; Nightingale *et al.,* 2000; Nordenhall *et al.,* 2000; Rudell *et al.*, 1999; Salvi *et al.,* 1999; Singh *et al.,* 2004).

Exposure to DE has been shown to have adverse effects on host immune responses. Both human and animal experiments have demonstrated that DEPs can act as immunologic adjuvants by increasing allergen specific IgE and the production of Th2 cytokines (Diaz-Sanchez *et al.,* 1997; Fujimaki *et al.,* 1997; van Zijverden *et al.,* 2000). Additionally, numerous studies have reported that DE increases susceptibility to respiratory infections. For example, several studies have demonstrated that DE decreases phagocytosis and clearance of both gram negative and gram positive bacteria (Castranova *et al.,* 2001; Saito *et al.,* 2002; Steerenberg *et al.*, 2004; Yang *et al.,* 2001;Yin *et al.,* 2004). In addition, reports by our laboratory as well as others' have shown that exposure to DE can increase susceptibility to respiratory virus infections, such as influenza and respiratory syncytial viruses (RSV) (Hahon *et al.,* 1985; Harrod *et al.*, 2003; Jaspers *et al.,* 2005).

Influenza infections in the USA account for approximately 36,000 deaths and over 100,000 hospitalizations each year, despite large-scale vaccination and antiviral treatments (Thompson *et al.,* 2003; Thompson *et al.,* 2004). The virus replicates primarily in the epithelial cells of the respiratory tract, but can also infect macrophages and monocytes. Epithelial cells recognize viral pathogens through receptors including Toll-like receptor (TLR) 3, TLR 7, and retinoic acid–inducible protein I (RIG-I), whose activation leads to the

expression of Type I interferons (IFNs) and inflammatory cytokines such as regulated upon activation, normal T-cell expressed, and secreted (RANTES), interleukin (IL)-6, IL-8, and tumor necrosis factor alpha (TNF-α). These cytokines recruit and activate immune cells, which ultimately clear the influenza infection. In addition, other innate immune defenses of the lung, such as calcium-dependent collagen-like lectins (collectins) bind and facilitate the phagocytosis of influenza, thereby inhibiting it from attaching and infecting pulmonary cells (Benne *et al.,* 1995; Hartshorn *et al.*, 1994). Surfactant proteins (SP), which are members of the collectin family, are part of the IFN-independent defense against influenza as well as other respiratory infections. SP-A and SP-D are secreted by alveolar type II cells and non-ciliated bronchial epithelial cells (clara cells) in the lung (Madsen *et al.,* 2000; Madsen *et al.,* 2003). Previous studies have shown that uptake and clearance of Influenza A is reduced in SP-A- and SP-D-deficient mice (LeVine *et al.,* 2001; LeVine *et al.,* 2002). Furthermore, mice exposed to DE had an increased susceptibility to RSV infection, and this effect was associated with decreased expression of SP-A in the lungs (Harrod *et al.*, 2003).

Given our previous findings, that demonstrated that exposure of respiratory epithelial cells to DE increases the susceptibility to influenza infections *in vitro*, we sought to confirm and expand upon these results using an *in vivo* murine model of DE exposure and subsequent influenza infection. Specifically, we analyzed whether repeated exposures to DE would increase the susceptibility to influenza infection and examined potential mechanisms mediating this effect. The results demonstrate that repeated exposure to DE increases the susceptibility to influenza infections in mice and this was associated with increased lung

injury and inflammatory cytokine levels. In addition, repeated exposures to DE decreased the expression of SP-A and SP-D in the lung, suggesting a potential mechanism for the observed effect.

Materials and Methods

Animals

Pathogen- free BALB/c female mice, 10-12 wk old, weighing 17-20 g, were purchased from Charles River (Raleigh, NC). Once at the U.S. EPA animal care facilities (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care), animals were housed in groups of five in polycarbonate cages with hardwood chip bedding (Beta Chip, Northeastern Products, Warrensburg, NY), provided a 12-hour light (0600 hours) to dark (1800 hours) cycle, maintained at 22.3 ± 1.1°C and 50 ± 10% humidity, and given access to both food (5P00 Prolab RMH 3000, PMI Nutrition International, Richmond, IN) and water ad libitum. Animals were acclimated for at least ten days before dosing began. The studies were conducted after approval by the laboratory's Institutional Animal Care and Welfare Committee.

Diesel Exhaust Exposure and Monitoring

Diesel exhaust for exposure experiments was generated using a 30 kW (40 hp) 4-cylinder Deutz BF4M1008 diesel engine connected to a 22.3 kW Saylor Bell air compressor to provide a load. Diesel fuel was purchased from a local (Research Triangle Park, NC) service

station and stored in drums. Replicate analysis (ultimate, elemental, heating value, and specific gravity) of multiple batches of fuel purchased over time indicate relatively consistent fuel properties and composition (data not shown). Engine lubrication oil (Shell Rotilla) was changed before each set of exposure tests. The engine and compressor were operated at steady-state to produce 0.8 m^3/min of compressed air at 400 kPa. This translates to approximately 20% of the engine's full-load rating. From the engine exhaust, a small portion of the flow (14 L/min) was educted by an aspirator (3:1 dilution) to a second cone diluter (10:1 dilution), and then through approximately 15 m of flexible food grade polyvinyl chloride (PVC) tubing (7.62 cm inside diameter) to two stainless steel 0.3 m^3 Hinners inhalation exposure chambers housed in an isolated animal exposure room. The dilution air used was drawn from the animal exposure room through a high efficiency particulate air (HEPA) filter. Target DEP concentrations in the two chambers were 2000 μg of PM/m^3 (high) and 500 μg of PM/m^3 (low). From here on the low exposure will be referred to as 0.5 mg/m^3 of DE and the high exposure as 2mg/m^3 of DE. Control animals were housed in a third chamber supplied with the same HEPA filtered room air. DEP concentrations in the low (0.5 mg/m^3) chamber were achieved by additional dilution using HEPA filtered room air just prior to entering the chamber. All three chambers were operated at the same flow rate (142 L/min) which resulted in 28 full air exchanges per hour. Integrated 4 h filter samples (14.1 L/min) were collected daily from each chamber and analyzed gravimetrically to determine particle concentrations. In addition, 8 and 20 min quartz filter samples (14.1 L/min) were collected from the high and low chambers respectively, to determine organic

carbon/elemental carbon (OC/EC) partitioning of the collected DEP. Continuous emission monitors (CEMs) were used to measure chamber concentrations of PM by tapered element oscillating microbalance, (TEOM, TSI Inc., St Paul, MN), oxygen (O_2, Beckman, La Habra, CA), carbon monoxide (CO, Thermo Electron Corp, Waltham, MA), nitric oxide (NO, Thermo Electron Corp, Waltham, MA), nitric dioxide (NO_2, Thermo Electron Corp, Waltham, MA), and sulfur dioxide (SO_2, Thermo Electron Corp, Waltham, MA). Samples were extracted through fixed stainless steel probes in the exposure chambers. Gas samples were passed through a particulate filter prior to the individual gas analyzers. Dilution air was adjusted periodically to maintain target PM concentrations as measured by the TEOM. Particle size distributions were characterized using a TSI Inc. (St. Paul, MN) scanning mobility particle sizer (SMPS) and aerodynamic particle sizer (APS).

Average concentrations (and standard deviations) for the CEM measurements from both PM chambers are presented in Table 1. Chamber temperatures, relative humidity, and noise were also monitored, and maintained within acceptable ranges. Mice were exposed to HEPA filtered room air or diesel emissions diluted to yield 0.5, or 2.0 mg/m^3 of diesel emission particulate for 4 h/day for 5 consecutive days.

Oropharyngeal Aspiration of Virus

Immediately after the last DE exposure mice were anesthetized in a small Plexiglass box using vaporized isofluorane (Webster Veterinary Supply Inc., Sterling, MA). The needle used for intratracheal injection was a 24 –gauge intragastric feeding needle, with a 1.25 mm-

diameter ball on tip, attached to a 1 ml syringe. The mice were then suspended vertically by their front incisors on a small wire attached to a support. The tongue was extended with forceps and 50 µl of either sterile saline (Hospira Inc., Lake Forest, IL) or 10 hemagglutination units (HAU) of influenza A/Bangkok/1/79 (H3N2 serotype) in 50 µl of saline was instilled into the oro-pharynx. The nose of the mouse was then covered, causing the liquid to be aspirated into the lungs.

Influenza Virus

The influenza A/Bangkok/1/79 (H3N2 serotype) used in this study was obtained from Dr. Melinda Beck (Dept. of Nutrition, University of North Carolina, Chapel Hill, NC 27514). The virus was propagated in 10-day-old embryonated hen's eggs. The virus was collected in the allantoic fluid and titered by 50% tissue culture infectious dose in Madin-Darby canine kidney cells and hemagglutination as previously described (Beck *et al.*, 2001). Stock virus was aliquoted and stored at -80° C until use. Each mouse was infected with 10 HAU diluted in sterile saline by intratracheal instillation as described above.

Bronchoalveolar Lavage

After 18 h post infection, mice from each treatment group (uninfected $n \geq 5$; infected $n \geq 11$) were euthanized with sodium pentobarbital and the trachea was exposed, cannulated, and secured with suture thread. The left mainstem bronchus was isolated, clamped with alligator clips after the trachea was cannulated. The right lungs lobes were lavaged 3 times with

three volumes of warmed Hanks balanced salt solution (HBSS) (Invitrogen, Grand Island, NY) (35ml/kg). The resulting lavage was centrifuged (717 x g, 15 min, 4° C) and 150 µl was stored at 4° C (for biochemical analysis) or -80° C (for cytokine measurement). The pelleted cells were resuspended in 1 ml of RPMI 1640 (Gibco, Carlsbad, CA) containing 2.5 % fetal bovine serum (FBS; Gibco, Carlsbad, CA). Total cell counts in the lavage fluid of each mouse were obtained with a Coulter Counter (Beckman Dickson). Each sample (200 µl) was centrifuged in duplicate onto slides using a Cytospin (Shandon, Pittsburgh, PA) and subsequently stained with Diff Quik solution (American Scientific, McGraw Park, PA) for cell differentiation determination, with at least 200 cells counted from each slide. The left lobe was then removed for RNA, protein isolation, or immunohistochemistry.

Cytokine Measurements

IL-6 concentrations in bronchoalveolar lavage (BAL) were measured by enzyme-linked immunosorbent assay (ELISA) with commercially available paired antibodies per manufacturer's instructions (Pharmingen, Franklin Lakes, NJ).

BAL fluid Biochemistry

Total protein, microalbumin (MIA), and *N*-Acetyl-B-D-glucosaminidase (NAG) were modified for use on the Konelab 30 clinical chemistry analyzer (Thermo Clinical Labsystems Espoo, Finland). Total protein concentrations were determined with the Coomassie plus protein Reagent (Pierce Chemical, Rockford, IL) with a standard curve prepared with bovine

serum albumin from Sigma-Aldrich (St. Louis, MO.). Microalbumin concentrations were determined with the MALB SPQ II kit (Diasorin, Stillwater, MN). NAG activity was determined from a commercially available kit from Roche Diagnostics (Pemzberg, Germany).

Western Blotting

Lung homogenates were prepared by homogenizing the tissue in 1x lysis buffer (Cell Signalling, Danvers, MA) containing protease inhibitors (Roche Diagnostics, Pemzberg, Germany). Lung homogenates (10 μg) were separated by 10% bis-tris gels under reducing conditions (Invitrogen, Grand Island, NY). This was followed by immunoblotting using specific antibodies to Surfactant Protein A (1:2000; Chemicon, Temecula, CA) or Surfactant Protein D (1:100; Chemicon, Temecula, CA). β-actin was used as a loading control for SP-A and SP-D (1:2000; US Biological, Swampscott, MA). Antigen-antibody complexes were stained with anti-goat or anti-mouse horseradish peroxidase-conjugated antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). Chemiluminescent signals were acquired using an Alpha Innotech 8900 imaging station (San Leandro, CA) and visualized using Fluorchem software (Alpha Innotech, San Leandro, CA). Densitometric analysis of optical densities was performed using software (Alpha Ease FC, San Leandro, CA).

Real Time PCR

Total RNA was extracted with TRIzol (Invitrogen, Grand Island, NY) as per the supplier's instructions. First strand cDNA synthesis and real-time RT-PCR were performed as previously described (Jaspers *et al.,* 1999; Jaspers *et al.,* 2001). The sequences for the primers and probes used in this study are as follow: HA: probe 5'-FAM-TGATGGGAAAAA CTGCACACTGATAGATGC-TAMARA-3';sense5' CGACAGTCCTCACCGAATCC- 3'; antisense 5'- TCACAATGAGGGTCTCCCAATAG -3'; IL-6: probe 5'-FAM-CCAGCAT CAGTCCCAAGAAGGCAACT-TAMRA-3'; sense 5'-TATGAAGTTCCTCTCTGCAAGA GA-3'; antisense 5'-TAGGGAAGGCCGTGGTT-3'; IFN-α: probe 5'-FAM-CTGCATCA GACAGCCTTGCAGGTCATT-TAMRA-3'; sense 5'-TGCAACCCTCCTAGACTCAT TCT-3'; antisense 5'-CCAGCAGGGCGTCTTCCT-3'; IFN-β: probe 5'-FAM-AGGGCGGA CTTCAAGATCCCTATGGA-TAMRA-3'; sense 5'-TGAATGGAAAGATCAACCTCACC TA-3'; antisense 5' CTCTTCTGCATCTTCTCCGTCA-3'; IFN-γ: probe 5'-FAM-CCTCA AACTTGGCAATACTCATGAATGCATCC-TAMRA-3'; sense 5'-AGCAACAGCAAG GCGAAAA-3'; antisense 5'-CTGGACCTGTGGGTTGTTGA-3'. The mRNA analyses for SP-A and SP-D were performed using commercially available primer/probe sets (inventoried Taqman® Gene Expression Assays) purchased from Applied Biosystems (Foster City, CA).

For all PCR analyses, the level of target mRNA was normalized to ß-actin mRNA after assuring that ß-actin mRNA levels did not significantly differ among the experimental groups. HA, IFN-α and IFN-β PCR was run in Dr. Ilona Jasper's lab by Jonathan Ciencewicki.

Immunohistochemistry

Lung tissue samples were fixed in 4% paraformaldhyde and embedded in paraffin. Five µm thick sections were placed on Superfrost/plus slides (Fisher Scientific) and stained for nucleoprotein of influenza A (clone IA52.9, 1:40; Argene, Varilhes, France), SP-A (1:4000; Chemicon, Temecula, CA), or SP-D (1:200; Chemicon, Temecula, CA). A no primary antibody control was also performed for each protein. The slides were evaluated under light microscopy. Five to ten fields of at least 2 sections per animal and 2 animals per experimental group were evaluated. Staining for influenza A was performed by Jonathan Ciencewicki.

Statistical Analysis

Data were pooled from three replicate exposure studies/experiments and are expressed as means ± SEM. For uninfected HBSS control animals a total of atleast 5 and for influenza-infected animals a total of atleast 11 were used. Data were analyzed using a nonparametric one-way ANOVA (Kruskal-Wallis test), followed by the Dunn's multiple comparison post hoc test. A value of $P<0.05$ was considered to be significant.

Results

Diesel exposure conditions

Data from the three five day exposure periods were pooled and presented in table 1. The target concentration of 2 mg/m^3 was achieved with very low variation either within a particular 4 hour exposure or between different days. Likewise the lower target concentration of 0.5 mg/m^3 was achieved through dilution air. While the operation and control of chamber concentrations was performed using time of flight TEOM data, particles were also collected onto filters for the duration of exposure and the mass values were in agreement with the TEOM results. CO levels were 5.4 ppm in the high chamber and 0.9 ppm in the low chamber which reflected a fivefold dilution. NO and NO_2 levels were 10.8 and 1.1 ppm respectively in the high chamber. Gas levels in the low concentration chamber were variable and fell below reliable detection limits of the analyzers which were set to span the high concentration chamber and the tailpipe stack emissions. SO_2 levels were also low.

Effect of DE exposure on the susceptibility to influenza infection

The first objective of this study was to determine if repeated exposure of mice to DE under the described conditions would also enhance the susceptibility to infection with Influenza A. To do this, RNA levels of hemagglutinin (HA), a marker of viral proliferation, in whole lung homogenates were measured 18 hours post-infection using real-time RT-PCR. Figure 1A shows that mice exposed to 0.5 mg/m^3 of DE had significantly greater levels of

HA mRNA compared to air exposed mice. Interestingly, HA levels were not significantly altered in mice exposed to 2.0 mg/m^3 of DE. To confirm this effect, lung sections were immunohistochemically stained for influenza using indirect immunofluorescence and visualized using epifluorescence microscopy. Figure 1B shows influenza staining in the airways of mice exposed to air or DE. Mice exposed to the low dose of DE show increased influenza staining, confirming the mRNA data. Thus, enhanced susceptibility to influenza infection following the low dose of DE exposure is illustrated by the increases in both HA mRNA and influenza staining observed in the lungs of mice.

Effect of DE exposure on the influenza-induced inflammatory response

Given the effect of DE on the susceptibility to influenza infection, the next objective was to determine what effect DE had on pro-inflammatory mediator production in influenza infected animals. Eighteen hours post-infection, the expression of IL-6 was analyzed. Levels of IL-6 expression were quantified in whole lung homogenates using real-time RT-PCR and protein levels were measured in the BAL fluid by ELISA (Figure 2). Figure 2A shows that levels of IL-6 mRNA were significantly greater in the lungs of mice exposed to 0.5 mg/m^3 of DE prior to infection compared to mice exposed to air. Similarly, there was a significantly greater amount of IL-6 protein in the BAL fluid of these animals compared to air exposed controls (Figure 2B). Exposure to DE in the absence of influenza infection had no significant effect on IL-6 mRNA or protein levels. Taken together, these data show that in addition to

enhanced susceptibility to influenza infection, DE also increased the virus-induced IL-6 response.

Effect of DE exposure on pulmonary injury

Since levels of the pro-inflammatory mediator IL-6, was increased in mice exposed to DE prior to infection, it was important to determine if there was also an increase in inflammatory cell infiltration into the lungs as well as increased lung injury resulting from the enhanced inflammatory response. The number of total cells in the BAL fluid of mice was quantified by coulter counter in addition to differential cell counts to quantify levels of polymorphonuclear cells (PMNs) BAL fluid 18 hours post-infection. As expected, Figure 3A shows that infection with influenza virus increases the levels of PMN in the BAL fluid. However, exposure to either dose of DE prior to infection had no statistically significant effects on the levels of BAL fluid PMNs. Surprisingly, exposure to DE alone had no effect on the levels of PMNs in the BAL fluid (fig 3A). Levels of protein in the BAL fluid, a marker of edema, were determined 18 hours post-infection. Neither exposure to DE nor infection with influenza significantly increased BAL fluid protein levels as compared to air exposed non-infected mice (fig 3B). Similarly, other markers of injury, such as NAG and MIA were not significantly affected by either DE exposure or influenza virus infections (data not shown).

Effect of DE exposure on the influenza-induced interferon response

Hahon et al. have shown that mice repeatedly exposed to DE particulate for 6 months had a decreased ability to produce interferon in response to influenza infection as well as increased viral multiplication (Hahon *et al.,* 1985). Therefore, it seemed likely that the increased viral load observed in the lungs of our DE-exposed mice was due to a decrease in interferon production and possibly other antiviral mediators. To investigate this, levels of interferon (IFN)-α, IFN-β, and IFN-γ mRNA were quantified 18 hours post-infection in whole lung homogenates using real-time RT-PCR. There was no significant change in IFN-α mRNA levels at either dose of DE (Figure 4A), however, mice exposed to 0.5 mg/m^3 of DE prior to infection had significantly greater levels of IFN-β mRNA compared to air controls (Figure 4B). Similarly, IFN-γ mRNA levels were elevated, albeit not significantly, in mice exposed to 0.5 mg/m^3 of DE prior to infection with influenza (Figure 4C) compared to controls. There was no effect on any of the IFNs observed in uninfected mice exposed to DE (data not shown).

Effect of DE exposure on surfactant protein expression

Since the enhanced susceptibility to infection observed in animals exposed to the 0.5 mg/m^3 dose of DE could not be explained by a decreased interferon response, the next objective was to examine the expression of interferon-independent antiviral defense molecules, such as surfactant proteins (SP) in the lung. Levels of SP-A mRNA in whole lung homogenates were quantified 18 hours post-infection by real-time RT-PCR. Influenza virus

infection alone significantly increased the expression of SP-A in the lung in mice exposed to air (Figure 5A). Mice, exposed to 0.5 mg/m^3 of DE prior to infection had a significant decrease in the levels of SP-A mRNA in the lungs, which was not observed in mice exposed to 2.0 mg/m^3 DE prior to infection. To determine if these effects also occurred at the protein level expression of SP-A protein was analyzed in whole lung homogenates by western blotting. Figure 5B shows that there was a decreased expression of SP-A protein in the lungs of mice exposed to 0.5 mg/m^3 of DE prior to infection. Localization of SP-A in lung sections following immunohistochemically staining showed that air exposed mice infected with virus strongly expressed SPA in the alveolar region. This expression was decreased in mice exposed to 0.5 mg/m^3 of DE prior to infection, but not in the mice pre-exposed to 2.0 mg/m^3.

In addition to SP-A, pulmonary expression of SP-D was also examined. Levels of SP-D mRNA in whole lung homogenates were quantified 18 hours post-infection using real-time RT-PCR. Similar to SP-A, levels of SP-D mRNA and protein were significantly decreased in the lungs of mice exposed to 0.5 mg/m^3 of DE prior to infection compared with mice exposed to air or 2.0 mg/m^3 DE prior to infection (figures 6A and 6B). Immunohistochemical analysis (Figure 6C) showed that in mice exposed to air prior to infection with influenza SP-D localized in the alveolar region (left panels) and airways (right panels). Similar to SP-A and confirming the observations made in figure 6A and B, Figure 6C demonstrates that exposure to 0.5 mg/m^3 of DE prior to infection with influenza decreased levels of SP-D, especially in the airways, while mice exposed to 2.0 mg/m^3 of DE prior to infection showed no significant difference as compared to the air-exposed mice.

Discussion

Various reports have linked DE to adverse effects on host immunity. Specifically, *in vitro* studies conducted in our laboratory as well as *in vivo* studies conducted by others have shown that exposure of human respiratory epithelial cells and repeated exposure of mice to DE increases the susceptibility to respiratory viral infections (Castranova *et al.,* 2001; Hahon *et al.,* 1985; Harrod *et al.,* 2003; Jaspers *et al.,* 2005). Given these findings, this study was designed to elucidate potential mechanisms by which exposure to DE enhances the susceptibility to infection with influenza virus *in vivo*. The overall hypothesis for the study was that prior exposure of mice to DE would enhance the susceptibility to influenza infection via suppression of innate immune defenses of the host, which is why we focused our analyses on changes occurring 18 hrs post-infection. The results obtained in this study demonstrate that repeated exposures of mice to 0.5 mg/m^3 of DE enhance the susceptibility to Influenza A infections and that these effects were linked with increased influenza-induced expression of IL-6 and IFN-ß. Interestingly, exposure of mice to 2.0 mg/m^3 of DE did not enhance the susceptibility to Influenza A infection. The DE-enhanced susceptibility to influenza virus infections was strongly associated with decreased expression of SP-A and SP-D in the lung, suggesting that modification of surfactant protein levels presents a potential mechanism for the effect of DE exposure on influenza virus infections.

Pulmonary cells employ various antiviral defense strategies to combat respiratory pathogens. Surfactant proteins (SP), which are members of the collectin family, belong to the

IFN-independent defense responses. As stated earlier, SP-A and SP-D are secreted by alveolar type II cells and nonciliated bronchial epithelial cells, also known as Clara cells, in the lung (Madsen *et al.,* 2000; Madsen *et al.,* 2003). These proteins contribute to the innate defense responses against influenza through their ability to bind and neutralize the virus (Benne *et al.,* 1995; Hartshorn *et al.,* 1994). Previous studies have shown that uptake and clearance of Influenza A is reduced in SP-A or SP-D deficient mice, resulting in an increased inflammatory response (LeVine *et al.,* 2001; LeVine *et al.,* 2002). It has also been demonstrated that exposure to air pollutants such as ozone and cigarette smoke decreases the expression or modulates the activity of pulmonary surfactant proteins (Honda *et al.*, 1996; Wang *et al.,* 2002). In this study a significant decrease in mRNA and protein expression of both SP-A and SP-D was observed in the lungs of mice exposed to 0.5 mg/m^3 of DE alone or prior to infection compared to mice exposed to air. No change in expression was observed in either SP-A or SP-D levels in mice exposed to 2.0 mg/m^3 of DE, which was an exposure level that also did not increase susceptibility to influenza virus infections. Harrod et al. observed an enhanced susceptibility to RSV infections and a decrease in SP-A expression in mice sub-chronically exposed (6 hrs/day for 7 days) to 0.03 or 1.0 mg/m^3 of DE (Harrod *et al.,* 2003). Despite the similarity in findings, this present study differs in certain aspects. For instance an off road diesel engine was used to generate the DE for this study, whereas Harrod et al. employed an on-road diesel engine, which likely resulted in different chemical composition of the diesel engine emissions. In addition, our studies examined the effects on influenza infections, while the study by Harrod et al. used RSV infection. Furthermore,

endpoints for this study were analyzed 18 hours post infection, which focuses entirely on the innate immune responses, whereas the analyses performed by Harrod et al. were 4 days post infection that would be during the development of specific immune responses. Despite their differences, these studies show that DE can affect the production of essential clearance mechanisms therefore increasing the susceptibility to respiratory viral infections. However, the mechanism whereby DE exerts its effect on surfactant protein expression is still unknown. One possibility is that SP expression is affected indirectly by DE, involving upstream signaling proteins that play a role in the transcriptional regulation of surfactant proteins. Previous studies have shown that the transcription factor NFAT (nuclear factor of activated T cells) regulates both SP-A and SP-D gene transcription (Dave *et al.,* 2004; Dave *et al.,* 2006), and NFAT in turn is negatively regulated by Akt (Yoeli-Lerner *et al.,* 2005). Previous studies in our laboratory have shown that exposure of respiratory epithelial cells to DE results in an increased expression of activated Akt (Ciencewicki *et al.,* 2006). Thus, DE-induced activation of Akt may result in increased inhibition of NFAT, thus decreasing the transcriptional activation of SP-A and SP-D.

In addition to influenza and RSV, SP-A and SP-D are also essential for the clearance of other respiratory pathogens such as group B *Streptococcus* (GBS), *Haemophilus influenza*, and *Pseudomonas aeruginosa* (LeVine *et al.,* 1997; LeVine *et al.,* 1998; LeVine *et al.,* 2000). Animal models deficient in either of these collectins have a significant increase in pro-inflammatory cytokines such as IL-6 and TNF-α, an increase in NFκB activation, as well

as an increase in superoxide production after microbial challenge (LeVine *et al.,* 1997; LeVine *et al.,* 1998; LeVine *et al.,* 2000; Yoshida *et al.,* 2001). Furthermore, studies have shown that decreased expression of SP-A and SP-D are associated with pulmonary disease states such as cystic fibrosis, acute interstitial pneumonias (ARDS) (Greene *et al.,* 1999; Postle *et al.,* 1999; Wang *et al.,* 1998). Taken together, these data demonstrate how a DE-induced decrease in SP expression could impact host immunity.

In addition to the enhanced susceptibility to infection and decrease in SP-A and SP-D expression observed, there was also an increase in the influenza-induced production of the pro-inflammatory mediator IL-6 observed in mice exposed to DE prior to infection. Levels of IL-6 mRNA and protein were significantly greater in mice exposed to the low dose of DE prior to infection with influenza. Similar results were obtained in the study by Harrod et al., which examined the effects of repeated DE exposures on the susceptibility and response to respiratory syncytial virus (RSV) infection. Mice exposed to 1.0 mg/m^3 of DE for 7 days were more susceptible to RSV infection and mounted a greater inflammatory response to infection (Harrod *et al.,* 2003). Inflammatory mediators are produced by infected cells to orchestrate an antiviral defense response aimed at clearing the invading pathogen. However, excessive inflammation is detrimental to the host, causing tissue injury and increasing the morbidity of a respiratory infection. Given the increase in IL-6 it was necessary to determine if this effect was associated with inflammatory cell recruitment and tissue injury. As expected, influenza infection increased the level of PMNs in the BAL fluid. However, exposure to either low or high level of DE did not further enhance influenza-induced PMN

influx. In addition, no changes in BAL fluid protein levels or other markers of injury were observed in mice exposed to either dose of DE prior to infection. Previous studies have demonstrated that levels of BAL fluid protein and other markers of injury in influenza-infected mice do not increase until about 3 days post-infection and peak even later than that (Bohn *et al.,* 2005;Le Goffic *et al.*, 2006). Thus, it is reasonable to speculate that although no significant effects of DE on influenza-induced lung injury were observed at the timepoint included in this study (18 hrs p.i.), it is still possible that increases in PMN influx and pulmonary injury may result at later timepoints post-infection. Further studies are currently underway to examine the effects of DE at markers of injury and adaptive immune responses occurring at later time points during the course of an influenza infection.

Previous studies have demonstrated that chronic exposure of mice to DE resulted in an increased susceptibility to influenza infection, which was correlated with decreased lung IFN levels (Hahon *et al.,* 1985). These data suggest that suppression of the host's IFN-dependent, innate antiviral response resulted in a reduced ability to limit and clear the invading pathogen. However, in the present study the enhanced influenza infection in mice exposed to 0.5 mg/m^3 of DE was not associated with any decrease in IFN expression and in fact, levels of IFN-β mRNA were significantly greater in the lungs of mice exposed to 0.5 mg/m^3 of DE and corresponded to the increased level of infection. These observations confirm our previous studies that demonstrated that respiratory epithelial cells exposed to DE had increased susceptibility to influenza virus without affecting the expression of IFN-ß (Jaspers *et al.,* 2005). The results are in accordance with previous studies, which reported that

sub-chronic exposure of mice to DE resulted in an enhanced susceptibility to RSV infection, but did not decrease lung IFN levels (Harrod *et al.,* 2003).

Many of the endpoints examined in this study showed an unusual response pattern to the 2 different exposure concentrations of DE. While there is no direct evidence to explain why this type of response was observed, there are several hypotheses that warrant further investigation. One possible explanation for this observed effect is that exposure to the high dose of DE causes an increase in iNOS expression, resulting in increased NO production. The virucidal activity of increased NO levels (Croen, 1993) could in turn create an antiviral environment within the lungs, resulting in less infectivity. This hypothesis is supported by a study conducted by Rao et al., which showed that instillation of rats with a high dose of DEPs, but not the low and mid doses, resulted in significantly greater levels of iNOS mRNA in lung cells obtained from the BAL fluid (Rao *et al.,* 2005). Thus, high level of DE but not the low level of DE exposure applied here might result in increased NO, thus generating an unfavorable environment for a viral infection. Another possible explanation is increased oxidative stress caused by exposure to the high levels of DE. Previous reports have suggested that exposure to DE induces a hierarchical oxidative stress response, with antioxidant defense responses and inflammatory responses induced by lower DE exposure levels and necrosis and apoptosis induced by higher levels of DE (Xiao *et al.,* 2003). While exposure to DE at a lower level leads to an inflammatory response, exposure to levels such as 2 mg/m^3 DE may be a dose which ultimately leads to cytotoxicity. Previous studies, including our own, have demonstrated that exposure to DEP results in a dose-dependent increase in oxidative stress as

well as cell death and these studies propose that as oxidative stress increases there is a progression from inflammation to toxicity ultimately resulting in apoptosis or necrosis (Jaspers *et al.,* 2005; Li *et al.,* 2002a; Li *et al.,* 2002b; Xiao *et al.,* 2003). Interestingly, apoptosis in epithelial cells creates an environment which is not conducive for infection and replication of influenza (reviewed in Barber, 2001). Thus, apoptosis in epithelial cells induced by the high DE exposure level may prevent efficient infection and replication of influenza virus and therefore neutralize the enhancing effects of DE on influenza virus infection. Lastly, biphasic dose response patterns are not all unusual in immune responses (for review see Calabrese, 2005). For example, *in vitro* exposure to DEP and phorbol 12-myristate 13-acetate (PMA) resulted in increased expression of IL-8 at lower doses of DEP, but a decrease in IL-8 expression was observed when cells were exposed to higher doses of DEP (Ushio *et al.,* 1999). Stanulis et al. showed that low doses of corticosterone resulted in an enhancement of the Th2 cytokines, IL-4 and IL-10, whereas higher doses had a suppressive effect in mice (Stanulis *et al.,* 1997). Most importantly, one of the first studies demonstrating that DEPs increase allergen-specific IgE production in the nasal mucosa in humans demonstrated that while challenging volunteers with either 0.15 or 1.0 mg DEP resulted in no increased IgE levels, while 0.3 mg DEPs significantly increased IgE production (Diaz-Sanchez *et al*., 1994). Taken together these as well as many others studies illustrate that a number of different immunological endpoints can display biphasic dose-responses caused by exposure to a variety of agents, including DEPs.

This study is representative of one type of DE engine. Characteristics of DE can vary based on engine type, load, fuel used, and the age of engine which can generate varying biological effects (DeMarini *et al.*, 2004; Singh *et al.*, 2004). Therefore even though this one DE engine increased influenza titers that is associated with a decrease in SP-A and SP-D this may not be the mechanism for this phenomenom. This same study was repeated using an on road DE powered truck (Appendix I; Fig 1). After a 5 day exposure to DE from the truck (TDEP) inflammatory markers were increased compared to the air/flu controls (Appendix I Fig 3), however no change in the HA mRNA was seen (Appendix I Fig 2). The expression and production of SP-A and SP-D was also unaltered with TDEP exposure (Appendix I Fig 4 and 5). This could be explained by the differences in the composition of the exhaust and the particles. Noxious gas levels such as NOx and CO were significantly higher in TDEP than CDEP (Appendix I Fig 1). The particles of TDEP also contained more organic carbon (OC) than the CDEP particles (Appendix I Fig 1) which has previously been shown to alter adjuvancy (Stevens *et al.*, 2008) and mutageniticity (DeMarini *et al.*, 2004). Therefore different DE engine may have varying effects on the susceptibility to influenza.

The data presented here provide further evidence that exposure to an air pollutant such as DE at levels which are seen in occupational settings, can enhance the susceptibility and response to respiratory viral infection through an alteration of the host's innate immune defenses. By decreasing the expression of surfactant proteins, exposure to DE increases the likelihood of an individual becoming infected with influenza. The enhanced viral load associated with the increased inflammatory response may result in increased inflammatory

cell recruitment, pulmonary injury, and changes in adaptive immune responses at later times during the course of an infection, which is currently the focus of another study in our laboratory. It will be of great interest and public concern to elucidate further conditions and mechanisms whereby DE can alter host susceptibility and response to respiratory viral infections as well as to identify effects on other aspects of host immunity.

References

1. Barber, G. N. (2001). Host defense, viruses and apoptosis. Cell Death Differ. **8**, 113-126.

2. Beck, M. A., Nelson, H. K., Shi, Q., Van Dael, P., Schiffrin, E. J., Blum, S., Barclay, D., and Levander, O. A. (2001). Selenium deficiency increases the pathology of an influenza virus infection. FASEB J **15**, 1481-1483.

3. Benne, C. A., Kraaijeveld, C. A., van Strijp, J. A. G., Brouwer, E., Harmsen, M., Verhoef, J., van Golde, L. M. G., and van Iwaarden, J. F. (1995). Interactions of surfactant protein A with influenza A viruses: binding and neutralization. J. Infect. Dis. **171**, 335-341.

4. Bohn, A. A., Harrod, K. S., Teske, S., and Lawrence, B. P. (2005). Increased mortality associated with TCDD exposure in mice infected with influenza A virus is not due to severity of lung injury or alterations in clara cell protein content. Chemico-biological Interactions **155**, 181-190.

5. Calabrese, E. J. (2005). Hormetic dose-response relationships in immunology: occurrence, quantitative features of the dose response, mechanistic foundations and clinical implications. Crit Rev Toxicol. **35**, 89-295.

6. Castranova, V., Ma, J. Y. C., Yang, H.-M., Antonini, J. M., Butteworth, L., Barger, M. W., Roberts, J., and Ma, J. K. H. (2001). Effect of exposure to diesel exhaust particles on the susceptibility of the lung to infection. Environ Health Perspect. **109**, 609-612.

7. Ciencewicki, J., Brighton, L., Wu, W.-D., Madden, M., and Jaspers, I. (2006). Diesel exhaust enhances virus- and poly(I:C)-induced Toll-like receptor 3 expression and signaling in respiratory epithelial cells. Am J Physiol Lung Cell Mol Physiol. **290**, 1154-1163.

8. Croen, K. D. (1993). Evidence for antiviral effect of nitric oxide. Inhibition of herpes simplex virus type 1 replication. J Clin Invest. **91**, 2446-2452.

9. Dave, V., Childs, T., and Whitsett, J. A. (2004). Nuclear factor of activated T cells regulates transcription of the surfactant protein D gene (Sftpd) via direct interaction with thyroid transcription factor-1 in lung epithelial cells. J Biol Chem. **279**, 34578-34588.

10. Dave, V., Childs, T., Xu, Y., Ikegami, M., Besnard, V., Maeda, Y., Wert, S. E., Neilson, J. R., Crabtree, G. R., and Whitsett, J. A. (2006). Calcineurin/Nfat signaling is required for perinatal lung maturation and function. J Clin Invest. **116**, 2597-2609.

11. Department of Health (1995). Particle dosimetry. Nonbiological particles and health. Committee on the Medical Effects of Air Pollutants 29-42.

12. Diaz-Sanchez, D., Dotson, A.R., Takenaka, H., and Saxon, A. (1994). Diesel exhaust particles induce local IgE production in vivo and alter the pattern of IgE messenger RNA isoforms. J Clin Invest. **94**, 1417-1425.

13. Diaz-Sanchez, D., Tsien, A., Fleming, J., and Saxon, A. (1997). Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human *in vivo* nasal ragweed-specific IgE and skews cytokine production to a T helper cell 2-type pattern. J Immunol. **158**, 2406-2413.

14. Fujimaki, H., Saneyoshi, K., Shiraishi, F., Imai, T., and Endo, T. (1997). Inhalation of diesel exhaust enhances antigen-specific IgE antibody production in mice. Toxicology **116**, 227-233.

15. Greene, K. E., Wright, J. R., Steinberg, K. P., Ruzinski, J. T., Caldwell, E., Wong, W. B., Hull, W., Whitsett, J. A., Akino, T., Kuroki, Y., Nagae, H., Hudson, L. D., and Martin, T. R. (1999). Serial changes in surfactant-associated proteins in lung and serum before and after the onset of ARDS. Am J Respir Crit Care Med. **160**, 1843-1850.

16. Hahon, N., Booth, J. A., Green, F., and Lewis, T. R. (1985). Influenza virus infection in mice after exposure to coal dust and diesel engine emissions. Environ Res. **37**, 44-60.

17. Harrod, K. S., Jaramillo, R. J., Rosenberger, C. L., Wang, S.-Z., Berger, J. A., McDonald, J. D., and Reed, M. D. (2003). Increased susceptibility to RSV infection by exposure to inhaled diesel engine emissions. Am J Respir Cell Mol Biol. **28**, 451-463.

18. Hartshorn, K. L., Crouch, E. C., White, M. R., Eggleton, P., Tauber, A. I., Chang, D., and Sastry, K. (1994). Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses. J. Clin. Invest. **94**, 311-319.

19. Hiura, T. S. , Kaszubowksi, M. P., Li, N., and Nel A.E. (1999). Chemicals in diesel exhaust particles generate reactive oxygen radicals and induce apoptosis in macrophages. J Immunol. **163**, 5582-5591.

20. Honda, Y., Takahashi, H., Kuroki, Y., Akino, T., and Abe, S. (1996). Decreased contents of surfactant proteins A and D in BAL fluids of healthy smokers. Chest **109**, 1006-1009.

21. Jaspers, I., Ciencewicki, J. M., Zhang, W., Brighton, L. E., Carson, J. L., Beck, M. A., and Madden, M. C. (2005). Diesel exhaust enhance Influenza virus infections in respiratory epithelial cells. Toxicol Sci.

22. Jaspers, I., Samet, J. M., and Reed, W. (1999). Arsenite exposure of cultured airway epithelial cells activates kappaB-dependent interleukin-8 gene expression in the absence of nuclear factor-kappaB nuclear translocation. J Biol Chem. **274**, 31025-31033.

23. Jaspers, I., Zhang, W., Fraser, A., Samet, J. M., and Reed, W. (2001). Hydrogen peroxide has opposing effects on IkK activity and IkB alpha breakdown in airway epithelial cells. Am J Respir Cell Mol Biol. **24**, 769-777.

24. Kato, A., Kyono, H., and Kuwabara, N. (1992). Electron-microscopic observations on rat lungs after long term inhalation of diesel emissions--non-neoplastic lesions. Japanese J Thoracic Dis. **30**.

25. Le Goffic, R., Balloy, V., Lagranderie, M., Alexopoulou, L., Escriou, N., Flavell, R., Chignard, M., and Si-Tahar, M. (2006). Detrimental contribution of the Toll-like receptor (TLR)3 to Influenza A virus-induced acute pneumonia. PLoS Pathog. **2**, 1-10.

26. LeVine, A. M., Bruno, M. D., Huelsman, K. M., Ross, G. F., Whitsett, J. A., and Korfhagen, T. R. (1997). Surfactant protein A-deficient mice are susceptible to group B streptococcal infection. J Immunol. **158**, 4336-4340.

27. LeVine, A. M., Hartshorn, K., Elliott, J., Whitsett, J., and Korfhagen, T. (2002). Absence of SP-A modulates innate and adaptive defense responses to pulmonary influenza infection. Am. J. Physiol. Lung Cell Mol. Physiol. **282**, L563-L572.

28. LeVine, A. M., Kurak, K. E., Bruno, M. D., Stark, J. M., Whitsett, J. A., and Korfhagen, T. R. (1998). Surfactant protein A-deficient mice are susceptible to Pseudomonas aeruginosa infection. Am J Respir Cell Mol Biol. **19**, 700-708.

29. LeVine, A. M., Whitsett, J. A., Gwozdz, J. A., Rixhardson, T. R., Fisher, J. H., Burhans, M. S., and Korfhagen, T. R. (2000). Distinct effects of surfactant protein A or D during bacterial infection on the lung. J Immunol. **165**, 3934-3940.

30. LeVine, A. M., Whitsett, J. A., Hartshorn, K. L., Crouch, E. C., and Korfhagen, T. R. (2001). Surfactant protein-D enhances clearance of influenza A virus from the lung in vivo. J. Immunol. **167**, 5868-5873.

31. Li, N., Kim, S., Wang, M., Froines, J., Sioutas, C., and Nel, A. (2002a). Use of a stratified oxidative stress model to study the biological effects of ambient concentrated and diesel exhaust particulate matter. Inhal Toxicol. **14**, 459-486.

32. Li, N., Wang, M., Oberley, T. D., Sempf, J. M., and Nel, A. E. (2002b). Comparison of the pro-oxidative and proinflammatory effects of organic diesel exhaust particle chemicals in bronchial epithelial cells and macrophages. J Immunol. **169**, 4531-4541.

33. Madsen, J., Kliem, A., Tornoe, I., Skjodt, K., Koch, C., and Holmskov, U. (2000). Localization of lung surfactant protein D on mucosal surfaces in human tissues. J Immunol. **164**, 5866-5870.

34. Madsen, J., Tornoe, I., Nielsen, O., Koch, C., Steinhilber, W., and Holmskov, U. (2003). Expression and localization of lung surfactant protein A in human tissues. Am. J. Respir. Cell Mol. Biol. **29**, 591-597.

35. Nightingale, J. A., Maggs, R., Cullinan, P., Donnelly, L. E., Rogers, D. F., Kinersley, R., Chung, K. F., Barnes, P. J., Ashmore, M., and Newman-Taylor, A. (2000). Airway inflammation after controlled exposure to diesel exhaust particulates. Am J Respir Crit Care Med. **162**, 161-166.

36. Nordenhall, C., Pourazar, J., Blomberg, A., Levin, J.-O., Sandstrom, T., and Adelroth, E. (2000). Airway inflammation following exposure to diesel exhaust: A study of time kinetics using induced sputum. Eur Respir J. **15**, 1046-1051.

37. Postle, A. D., Mander, A., Reid, K. B., Wang, J. Y., Wright, S. M., Moustaki, M., and Warner, J. O. (1999). Deficient hydrophilic lung surfactant proteins A and D with normal surfactant phospholipid molecular species in cystic fibrosis. Am J Respir Cell Mol Biol. **20**, 90-98.

38. Rao, K. M. K., Ma, J. Y. C., Meighan, T., Barger, M. W., Pack, D., and Vallyathan, V. (2005). Time course of gene expression of inflammatory mediators in rat lung after diesel exhaust particle exposure. Environ Health Perspect. **113**, 612-617.

39. Riedl, M. and Diaz-Sanchez, D. (2005). Biology of diesel exhaust effects on respiratory function. J Allergy Clin Immunol. **115**, 221-228.

40. Rudell, B., Blomberg, A., Helleday, R., Ledin, M. C., Lunback, B., Stjernberg, N., Horstedt, P., and Sandstrom, T. (1999). Bronchoalveolar inflammation after exposure to diesel exhaust: Comparison between unfiltered and particle trap filtered exhaust. Occup Environ Med. **56**, 527-534.

41. Saito, Y., Azuma, A., Kudo, S., Takizawa, H., and Sugawara, I. (2002). Effects of diesel exhaust on murine alveolar macrophages and a macrophage cell line. Exp Lung Res. **28**, 201-217.

42. Salvi, S., Blomberg, A., Rudell, B., Kelly, F., Sandstrom, T., Holgate, S. T., and Frew, A. (1999). Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. Am J Resp Crit Care Med **159**, 702-709.

43. Singh, P., DeMarini, D. M. D. C. A., Tabor, D. G., Ryan, J. V., Linak, W. P., Kobayashi, T., and Gilmour M.I. (2004). Sample characterization of automobile and forklift diesel exhaust particles and comparative pulmonary toxicity in mice. Environ Health Perspect. **112**, 820-825.

44. Stanulis, E. D., Jordan, S. D., Rosecrans, J. A., and Holsapple, M. P. (1997). Disruption of Th1/Th2 cytokine balance by cocaine is mediated by corticosterone. Immunopharmacology **37**, 25-33.

45. Steerenberg, P., Verlaan, A., De Klerk, A., Boere, A., Loveren, H., and Cassee, F. (2004). Sensitivity to ozone, diesel exhaust particles, and standardized ambient particulate matter in rats with a listeria monocytogenes-induced respiratory infection. Inhal Toxicol. **16**, 311-317.

46. Thompson, W. W., Shay, D. K., Weintraub, E., Brammer, L. , Bridges, C. B., Cox, N. J., and Fukuda, K. (2004). Influenza-associated hospitalizations in the United States. JAMA. **292**, 1333-1340.

47. Thompson, W. W., Shay, D. K., Weintraub, E., Brammer, L. , Cox, N., Anderson, L. J., and Fukuda, K. (2003). Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA. **289**, 179-186.

48. United Nations Environment Program (1994). Air pollution in the world's megacitites. Environment **36**, 5-37.

49. United States Environmental Protection Agency (2002). Health risk assessment document for diesel exhaust.

50. Ushio, H., Nohara, K., and Fujimaki, H. (1999). Effect of environmental pollutants on the production of pro-inflammatory cytokines by normal human dermal keratinocytes. Toxicol Lett. **105**, 17-24.

51. van Zijverden, M., van der Pijl, A., van Pinxteren, F. A., de Haar, C., Penninks, A. H., van Loveren, H., and Pieters, R. (2000). Diesel exhaust, carbon black, and silica particles display distinct TH1/TH2 modulating activity. Toxicol Appl Pharmacol. **168**, 131-139.

52. Wang, G., Umstead, T. M., Phelps, D. S., Al-Mondhiry, H., and Floros, J. (2002). The effect of ozone exposure on the ability of human surfactant protein a variants to stimulate cytokine production. Environ Health Perspect. **110**, 79-84.

53. Wang, J. Y., Shieh, C. C., You, P. F., Lei, H. Y., and Reid, K. B. (1998). Inhibitory effect of pulmonary surfactant proteins A and D on allergen-induced lymphocyte proliferation and histamine release in children with asthma. Am J Respir Crit Care Med. **158**, 510-518.

54. Xiao, G. G., Wang, M., Li, N., Loo, J. A., and Nel, A. E. (2003). Use of proteomics to demonstrate a hierarchal oxidative response to diesel exhaust particle chemicals in a macrophage cell line. J Biol Chem. **278**, 50781-50790.

55. Yang, H. M., Antonini, J. M., Barger, M. W., Butterworth, L., Roberts, B. R., Ma, J. K., Castranova, V., and Ma, J. Y. (2001). Diesel exhaust particles suppress macrophage function and slow the pulmonary clearance of Listeria monocytogenes in rats. Environ Health Perspect. **190**, 515-521.

56. Yin, X. J., Ma, J. Y., Antonini, J. M., Castranova, V., and Ma, J. K. (2004). Roles of reactive oxygen species and heme oxygenase-1 in modulation of alveolar macrophage-mediated pulmonary immune responses to Listeria monocytogenes by diesel exhaust particles. Toxicol Sci. **82**, 143-153.

57. Yoeli-Lerner, M., Yiu, G. K., Rabinovitz, I., Erhardt, P., Jauliac, S., and Toker, A. (2005). Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Mol Cell **20**, 539-550.

58. Yoshida, M., Korfhagen, T. R., and Whitsett, J. A. (2001). Surfactant protein D regulates NF-kappa B and matrix metalloproteinase production in alveolar macrophages via oxidant-sensitive pathways. J Immunol. **166**, 7514-7519.

Table 1

Constituent	Units	Low	High
Particle mass	mg/m^3	0.529±0.008	2.07±0.03
O_2	%	20.87±0.06	20.54±0.07
CO	ppm	0.9±0.19	5.4±0.24
NOx	ppm	1.09±0.31	10.3±0.7
NO_2	ppm	0.15±0.05	1.13±0.04
NO	ppm	0.25±0.13	10.8±0.51
SO_2	ppm	0.06±0.02	0.32±0.1
THC	ppm	11.1±1.6	9.8±0.7

Table 1. Summary of exposure concentrations of particle mass and gases.

Measurements shown are mean of three experiments ± SE. Definition of abbreviations: O_2, oxygen; CO, carbon monoxide; NOx, nitric oxide; NO_2, nitrogen dioxide; NO nitrogen oxide; SO_2, sulfur dioxide; THC, total hydrocarbon.

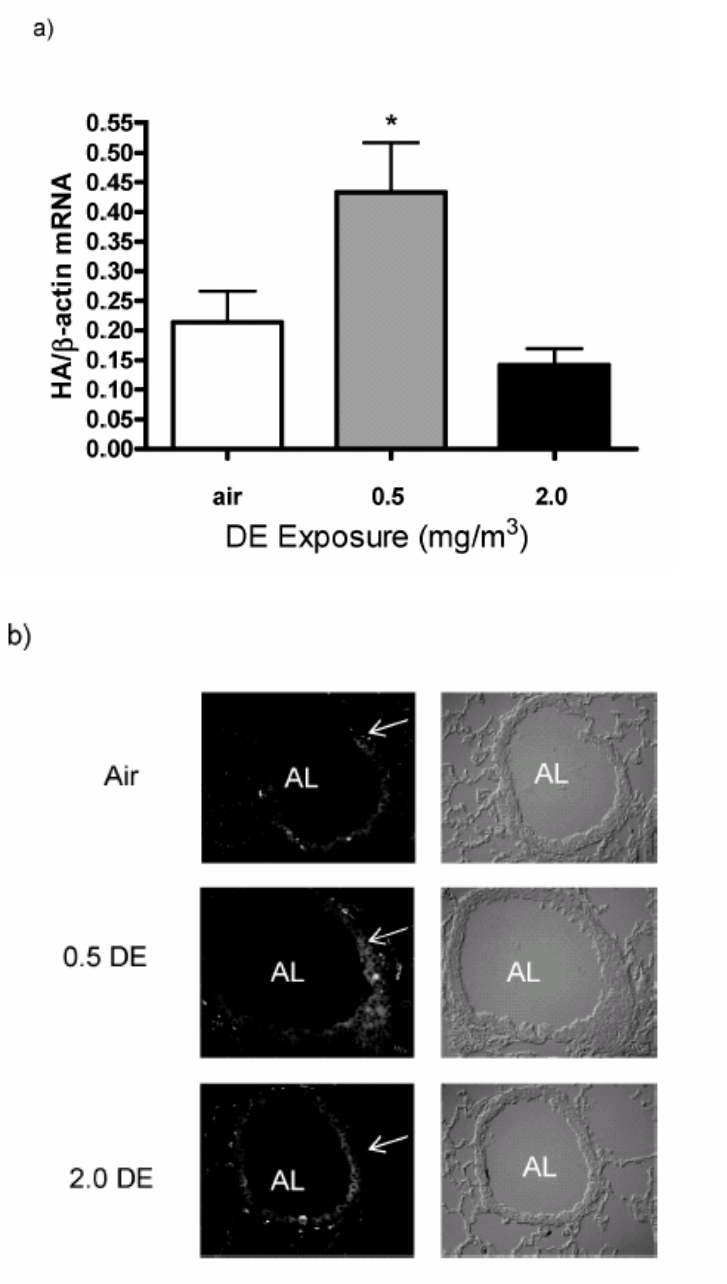


FIG. 1. Effect of DE on the susceptibility to influenza infection. (**a**) Levels of HA mRNA were quantified in lung homogenates 18 h after infection with influenza by real-time RT-PCR. Values are normalized to β-actin and expressed as mean ± SEM. Asterisk indicates significantly different from air/influenza mice, $p < .05$. (b) Representative image of mouse lung sections immunohistochemically stained for influenza and visualized using confocal microscopy. AL, airway lumen; arrows indicate bronchial epithelium.

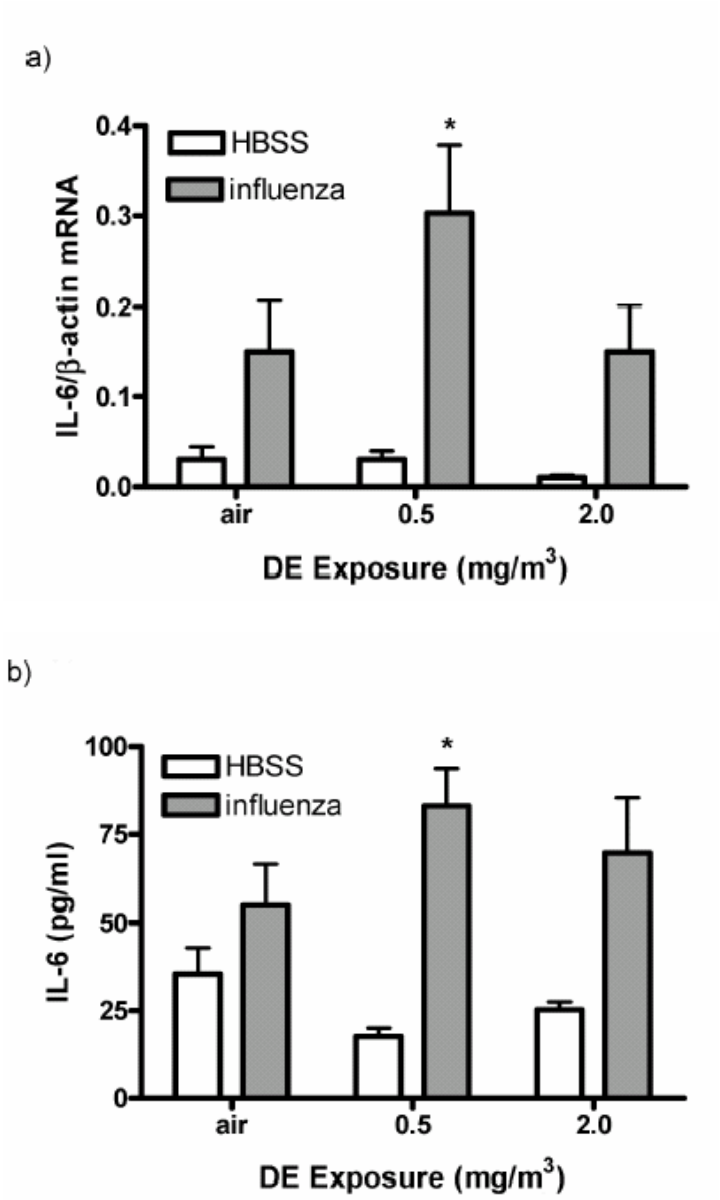


FIG. 2. Effect of DE on the influenza-induced inflammatory response. Measurements were taken 18 h postinfection and all data is expressed as mean ± SEM. (a) Levels of IL-6 mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin. (b) Levels of IL-6 protein were quantified in the BAL fluid by ELISA. Asterisk indicates significantly different from air/influenza mice, $p < .05$.

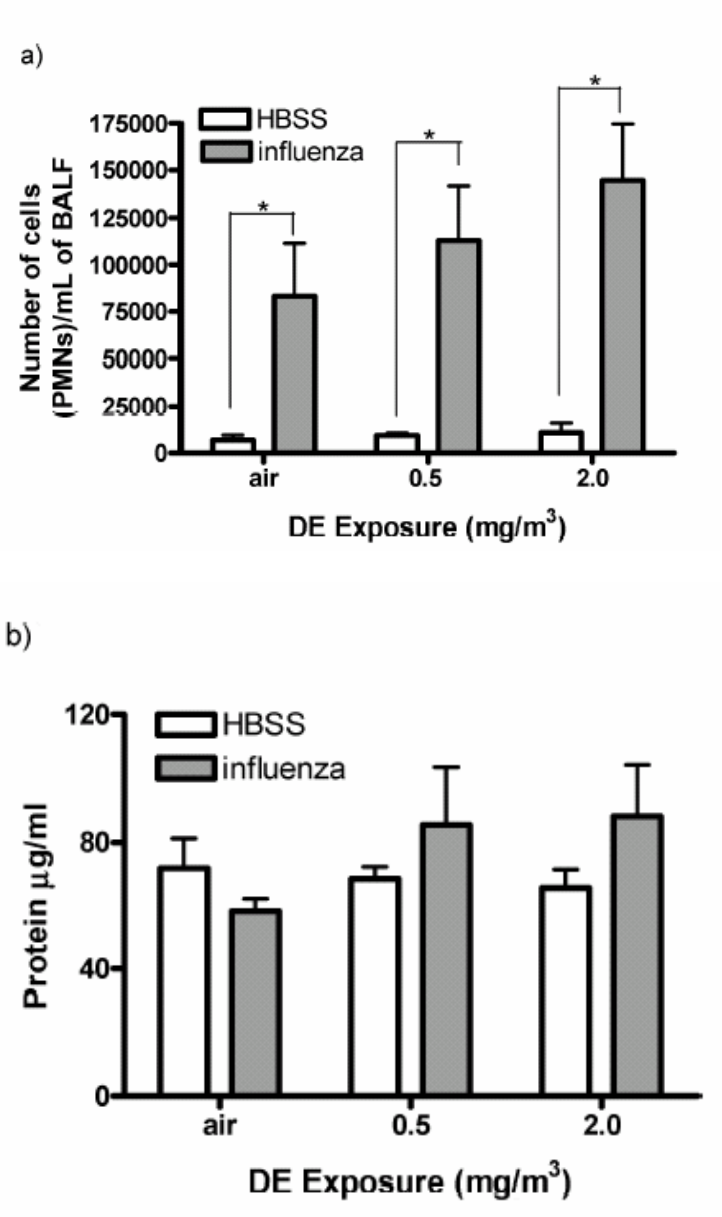


FIG. 3. Neutrophil recruitment and levels of protein in the BAL fluid. Measurements were taken 18 h postinfection and all data expressed as mean ± SEM. (a) The number of neutrophils was determined by differential cell counts. (b) Levels of protein were quantified in the BAL fluid. Asterisk indicates significantly different from indicated groups and # significantly different from all groups, $p < .05$.

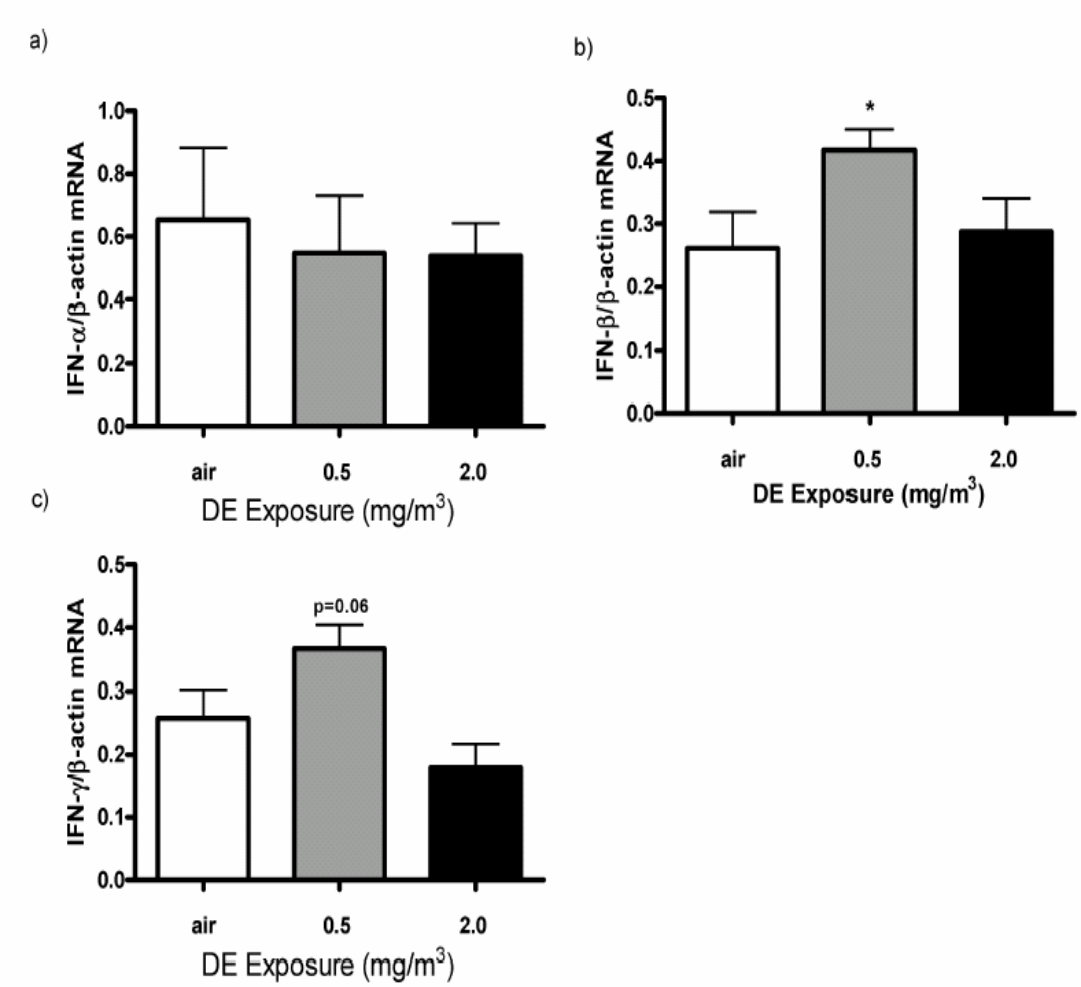


FIG. 4. Effect of DE on the influenza-induced interferon response. Levels of (a) IFN-α, (b) IFN-β, and (c) IFN-γ mRNA were quantified in lung homogenates 18 h after infection with influenza by real-time RT-PCR. Values are normalized to β-actin and expressed as mean ± SEM. Asterisk indicates significantly different from air/influenza mice, $p < .05$.

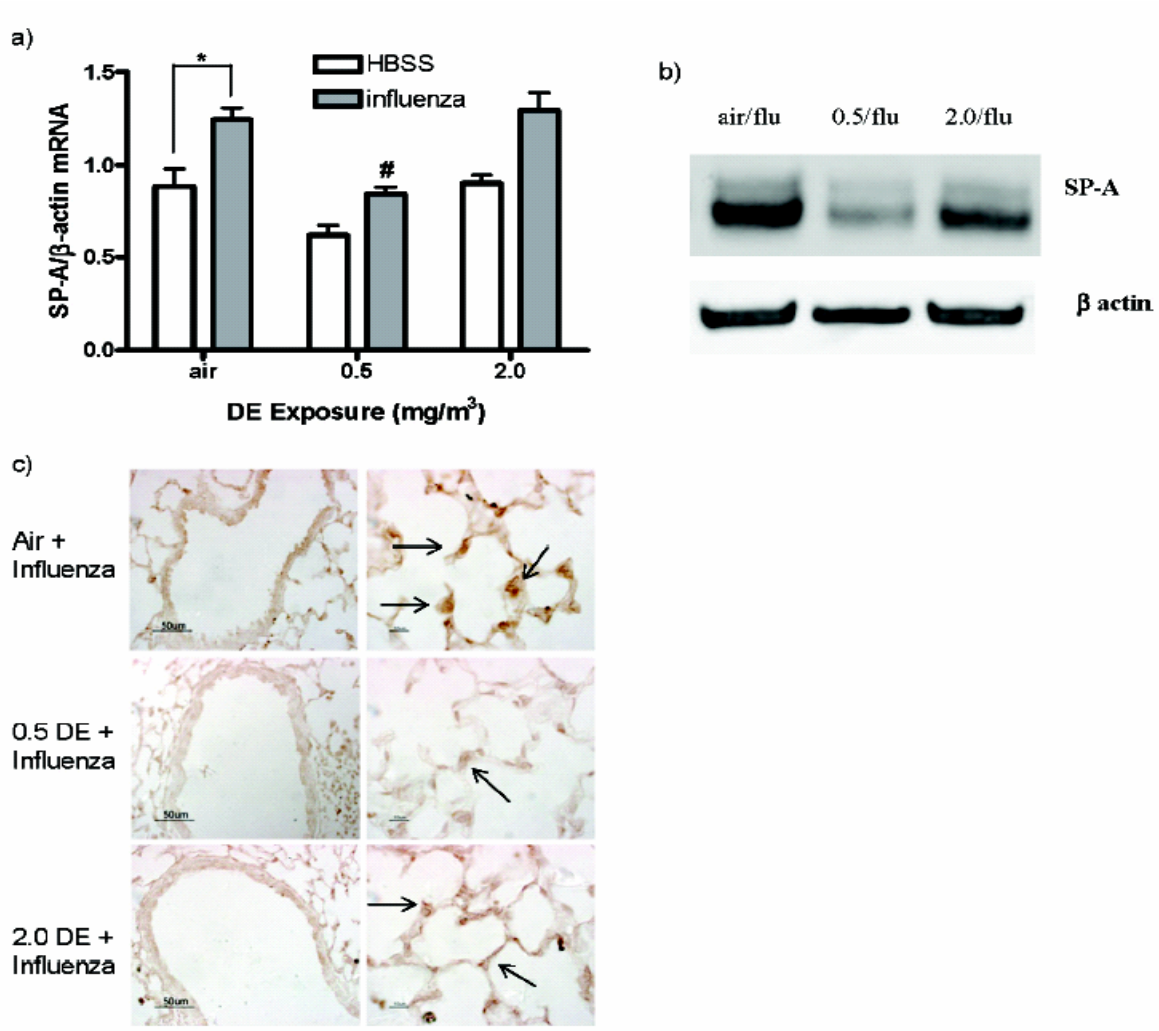


FIG. 5. Effect of DE on SP-A expression. (a) SP-A mRNA was quantified in lung homogenates 18 h after infection by real-time RT-PCR. Values are normalized to β-actin and expressed as mean ± SEM. Asterisk indicates significantly different from indicated group and # significantly different from air/influenza and 2.0-mg/m^3 DE/influenza mice, $p < .05$. (b) Lung homogenates collected 18 h postinfection were analyzed for SP-A protein levels. Nitrocellulose membranes were stripped and reprobed with anti-β-actin antibody. (c) Representative image of mouse lung sections immunohistochemically stained for SP-A and visualized using light microscopy: 40× magnification of airways (left panel) and 100× magnification of alveolar region (right panel). Arrows indicate type II epithelial cells positively stained for SP-A.

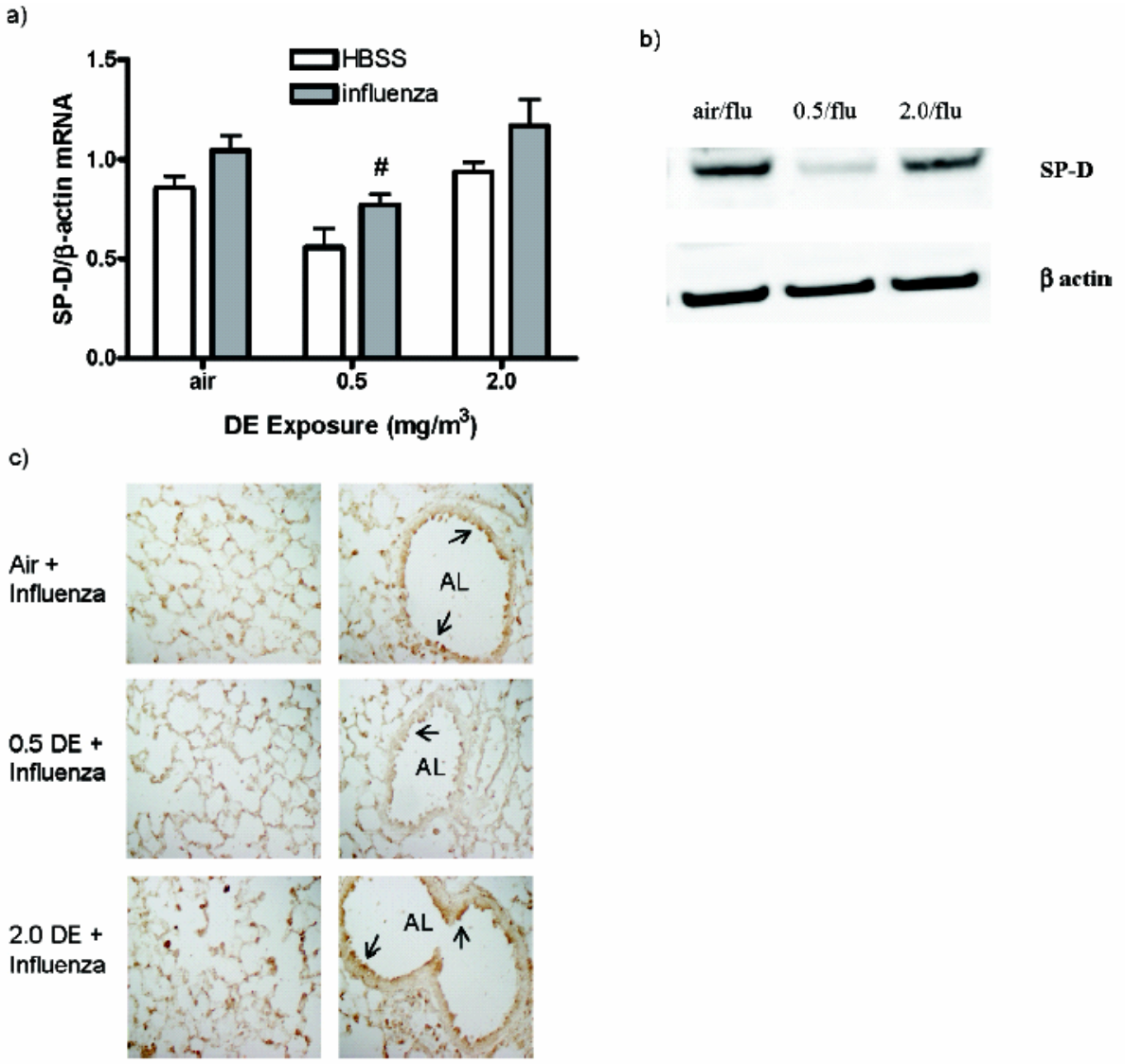


FIG. 6. Effect of DE on SP-D expression. (a) SP-D mRNA was quantified in lung homogenates 18 h after infection by real-time RT-PCR. Values are normalized to β-actin and expressed as fold mean ± SEM. Symbol # indicates significantly different from air/influenza and 2.0 mg/m^3 DE/influenza mice, $p < .05$. (b) Lung homogenates collected 18 h postinfection were analyzed for SP-D protein levels. Nitrocellulose membranes were stripped and reprobed with anti-β-actin antibody. (c) Representative image of mouse alveolar (left panel) and airway (right panel) lung sections immunohistochemically stained for SP-D and visualized using light microscopy. AL, airway lumen; arrows indicate positive staining of the bronchial epithelium for SP-D.

Chapter 4

Exposure to Diesel Exhaust Enhances the Severity of an Ongoing Influenza Infection.

Abstract

Numerous studies have shown that air pollutants including diesel exhaust (DE), alter host defense responses to decrease resistance to respiratory infection. The purpose of this study was to evaluate the effects of DE exposure on the severity of an ongoing influenza infection in mice. BALB/c mice were intra-tracheally instilled with 50 plaque forming units (PFUs) of A/HongKong/8/68 and immediately exposed to air or 0.5 mg/m^3 DE from a diesel truck (4 hrs/day, 14 days). Mice were necropsied at day 1, 4, 8 and 14 post-infection and lung tissue was assessed for virus titers by $TCID_{50}$ and mRNA expression of IFN-β, IL-4, IFN-γ and IL-12p40. Lung disease was measured by differential cell counts, protein levels in bronchoalveolar lavage fluid (BAL) and pulmonary responsiveness (PR) to inhaled methacholine. Exposure to 0.5 mg/m^3 DE during the course of infection caused an increase in viral titers at days 4 and 8 post-infection which was associated with a significant increase in neutrophils and protein in the BAL. PR was also significantly increased in the mice exposed to DE during influenza infection. Increased virus load was not caused by decreased interferon levels, since IFN-β levels were enhanced in these mice. However, the expression of IL-4 was significantly increased on day 1 and 4 p.i. and was associated with a subsequent decrease in Th1 cytokines IFN-γ and IL-12p40. Treatment with the antioxidant n-acetylcysteine (NAC) diminished the DE-enhanced inflammation and IL-4 expression levels but did not

affect viral titers. We conclude that exposure to moderate levels of DE (0.5 mg/m^3) during an influenza infection results in increased oxidative stress which promotes polarization of the local immune response to an IL-4 dominated profile in association with increased viral disease.

Introduction

Viral infections are a major cause of pulmonary-related illnesses in children, the elderly, and other susceptible populations such as asthmatics (Klimov *et al.*, 1999; Falsey and Walsh, 2000; Monto *et al.*, 2004). Epidemiological studies have noted an association between air pollution exposure and an increased rate of pulmonary infections (Pope and Dockery, 1992; Pope *et al.*, 2004). Laboratory research has also shown that exposure to airborne particulate matter (PM) increases susceptibility to both bacterial and viral pathogens (reviewed in Ciencewicki and Jaspers, 2007). In particular, diesel exhaust (DE) is a significant contributor to urban air pollution and has been shown to alter pulmonary immune responses (Hahon *et al.*, 1985; Takizawa *et al.*, 2000; Takizawa *et al.*, 2000; Castranova *et al.*, 2001; Harrod *et al.*, 2003; Jaspers *et al.*, 2005; Ciencewicki *et al.*, 2007), however the mechanisms that underlie this process are not fully understood. Several laboratories have demonstrated that rodents exposed to high concentrations of re-entrained diesel exhaust particles (DEP) have decreased phagocytosis and reduced clearance of gram negative and gram positive bacteria (Yang *et al.*, 2001; Steerenberg *et al.*, 2004; Yin *et al.*, 2004). Exposure to lower concentrations of fresh DE has also resulted in increased susceptibility to respiratory syncytial virus (RSV) and influenza infection (Hahon *et al.*, 1985; Harrod *et al.*, 2003; Ciencewicki *et al.*, 2007), however, these reports only examined how DE altered the pulmonary environment before infection and did not consider the immunomodulatory effects of DE exposure during viral illness.

Influenza is a respiratory virus that accounts for approximately 36,000 deaths and over 100,000 hospitalizations each year, despite large-scale vaccination and antiviral treatment (Thompson *et al.*, 2003; Thompson *et al.*, 2004). Influenza primarily replicates in the epithelial cells of the respiratory tract, but can also infect macrophages and monocytes. The clearance of influenza primarily relies on the production of anti-viral type I interferons and a variety of Th1 cytokines produced by multiple cells (Sarawar *et al.*, 1994). In contrast, the release of the Th2 cytokine IL-4, delays the clearance of an influenza infection (Moran *et al.*, 1996; Lopez *et al.*, 2002; Seneviratne *et al.*, 2005).

Animal and human *in vitro* and *in vivo* studies have shown that exposure to DE increases neutrophil recruitment, nitric oxide production, and pro-inflammatory cytokines (Kato *et al.*, 1992; Hiura *et al.*, 1999; Rudell *et al.*, 1999; Salvi *et al.*, 1999; Nightingale *et al.*, 2000; Nordenhall *et al.*, 2000; Singh *et al.*, 2004). DE alone or in the context of antigen exposure has also been reported in humans to induce the expression of Th2 cytokines such as IL-4 and IL-13 with a decrease in IFN-γ (Diaz Sanchez *et al.*, 1994; Diaz-Sanchez, 1997; Diaz-Sanchez *et al.*, 2000). The mechanism by which DE promotes a Th2 bias remains unknown but it is thought to be associated with oxidative lung injury and inflammation. Exposure to DE induces oxidative stress in target cells (Hiura *et al.*, 1999; Xiao *et al.*, 2003) with reactive oxygen species (ROS) inducing the transcription of phase II enzymes including heme-oxygenase 1 (HO-1) and catalase (Li *et al.*, 2002; Xiao *et al.*, 2003). ROS and oxidative stress regulate the immune response by controlling signal transduction in antigen-presenting cells (APCs) and lymphocytes (Peterson *et al.*,

1998). ROS can also interfere will the polarity of the immune response with oxidative stress favoring a Th2 skewing (Murphy and Reiner, 2002). Depletion of glutathione in DCs, an important APC for stimulating naïve T cells, downregulates IL-12 production and increases IL-4, favoring a Th2 phenotype (Kim *et al.*, 2007).

This present study was designed to address how diesel exhaust (DE) affected the course of an ongoing influenza infection in mice. We hypothesized DE increased IL-4 while downregulating cytokines critical for clearing influenza would be downregulated in addition it was of interest to determine whether boosting antioxidant levels would ameliorate the diesel enhanced influenza infection. Defining the mechanism for these effects could lead to control strategies and therapeutics for reducing the health impact of particulate air pollution on respiratory infections.

Materials and Methods

Animals

Pathogen-free BALB/c female mice, 10-12 wk old, weighing 17-20 g, were purchased from Charles River (Raleigh, NC). Once at the U.S. EPA animal care facilities (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care), animals were housed in groups of five in polycarbonate cages with hardwood chip bedding (Beta Chip, Northeastern Products, Warrensburg, NY), provided a 12-hour light (0600 hours) to dark (1800 hours) cycle, maintained at 22.3 ± 1.1°C and 50 ± 10% humidity, and given access to both food (5P00 Prolab RMH 3000, PMI Nutrition International, Richmond, IN) and water *ad libitum*. Animals were acclimated for at least

ten days before the study began. Sentinel animals were housed in the same location. The studies were conducted after approval by the laboratory's Institutional Animal Care and Welfare Committee.

Influenza Virus

The influenza A/HongKong/8/68 (H3N2 serotype) used in this study was obtained from Dr. Dori Germolec (Laboratory of Respiratory Biology, NIEHS, NIH, RTP, NC 27709). The virus was used to prepare dilutions in sterile saline containing 50 PFUs in 50 µl. Viral titers were determined using influenza infection of Madin-Darby canine kidney cells. Stock virus was aliquoted and stored at -80° C until use.

Oropharyngeal Aspiration of Virus

Immediately before the first DE exposure, mice were anesthetized in a small Plexiglass box using vaporized isofluorane (Webster Veterinary Supply Inc., Sterling, MA). The needle used for oropharyngeal apsiration was a 24 –gauge intragastric feeding needle, with a 1.25 mm-diameter ball on tip, attached to a 1 ml syringe. The mice were then suspended vertically by their front incisors on a small wire attached to a support. The tongue was extended with forceps and 50 µl of either sterile saline (Hospira Inc., Lake Forest, IL) or 50 plague forming units (PFUs) ($10^{2.4}$ $TCID_{50}$) of influenza A/Honkong/8/68 (H3N2 serotype) in 50 µl of saline was instilled into the oro-pharynx. The nose of the mouse was then covered, causing the liquid to be aspirated into the lungs.

Diesel Exhaust Exposure and Monitoring

Diesel exhaust for animal inhalation exposure experiments was generated using a 134 kW (180 hp) 8-cylinder 6.5 liter displacement indirect injection Detroit Diesel engine mounted in a 1994 Chevrolet Cheyenne 2500 pickup truck equipped with a manual transmission and oxidation catalyst. The engine and transmission were connected directly to a Land &Sea (model DYNOmite 300) eddy current dynamometer to provide a load. The equipment was operated in an attempt to simulate steady-state highway operation. The engine and transmission were operated at 2500 rpm in third (1:1 ratio) gear, respectively. The dynamometer was operated at 7 amps, providing approximately 100 ft/lbs of torque after a warm-up period. The projected load was equivalent to approximately 25% of the maximum engine load (at 2500 rpm). The truck speedometer (measuring drive shaft rpm) indicated a steady speed of 55 miles/h. Road taxed diesel fuel was purchased from a local (Research Triangle Park, NC) service station and stored in 55 gal drums. Replicate analysis (ultimate, elemental, heating value, and specific gravity) of multiple batches of fuel purchased over time indicated consistent fuel properties and composition (data not shown). Engine lubrication oil (Shell Rotella, 15W-40) was changed before each set of exposure tests.

From the engine exhaust, a small portion of the flow (14 L/min) was educted by an aspirator (3:1 dilution) to a second cone diluter (10:1 dilution), and then through approximately 10 meters of stainless steel tubing (7 cm inside diameter) to a stainless steel Hazelton (model 1000) exposure chamber housed in an isolated animal exposure

room. The dilution air used was drawn from the animal exposure room through an activated carbon bed and high efficiency particulate air (HEPA) filter. The target diesel emission particle (DEP) concentration in the chamber (500 $\mu g/m^3$) was continuously monitored using a tapered element oscillating microbalance (TEOM, Rupprecht and Patashnick Co., series 1400, Albany, NY). Dilution air was periodically adjusted to control DEP concentrations. Control animals were housed in a separate chamber supplied with the same activated carbon/HEPA filtered room air. The two chambers were operated at the same flow rate (280 L/min) which resulted in 16 full air exchanges per hour.

Integrated 4 h filter samples (14.1 L/min) were collected daily from each chamber and analyzed gravimetrically to determine particle concentrations. In addition, 20-min quartz filter samples (14.1 L/min) were collected from the DEP exposure chamber each day and analyzed using a thermal/optical carbon analyzer (Sunset Laboratory Inc., model 107, Tigard, OR) to determine organic carbon/elemental carbon (OC/EC) partitioning of the collected DEP. In addition to TEOM measurements, continuous emission monitors (CEMs) were used to measure chamber concentrations of oxygen (O_2, Beckman Corp., model 755, La Habra, CA), carbon monoxide (CO, Thermo Electron Corp, model 48, Franklin, MA), nitrogen oxides (NO_x, Teledyne Technology Co., model 200A4, San Diego, CA), and sulfur dioxide (SO_2, Thermo Electron Corp, model 43c, Franklin, MA). Samples were extracted through fixed stainless steel probes in the exposure chambers. Gas samples were passed through a particulate filter prior to the individual gas analyzers.

Particle size distributions were characterized during each exposure using a scanning mobility particle sizer (SMPS, TSI Inc., model 3080/3022a, St. Paul, MN) and an aerodynamic particle sizer (APS, TSI Inc., model 3321, St. Paul, MN). Chamber temperatures, relative humidity, and noise were also monitored, and maintained within acceptable ranges. Mice were exposed to DEP or filtered air for 4 h/day for 13 consecutive days.

Bronchoalveolar Lavage

On day 1, 4, 8, and 14 p.i. mice from each treatment group were euthanized with sodium pentobarbital and the trachea was exposed, cannulated, and secured with suture thread. The left mainstem bronchus was then isolated, clamped with microhaemostats after the trachea was cannulated. The right lungs lobes were lavaged 3 times with a single volume of warmed Hanks balanced salt solution (HBSS) (Invitrogen, Grand Island, NY) (35ml/kg). The resulting lavage was centrifuged (717 x g, 15 min, 4° C) and stored at -80° C for cytokine measurement or 4° C for protein measurement. The pelleted cells were resuspended in 1 ml of RPMI 1640 (Gibco, Carlsbad, CA) containing 2.5 % fetal bovine serum (FBS; Gibco, Carlsbad, CA). Total cell counts in the lavage fluid of each mouse were obtained with a Coulter Counter (Beckman Dickson, Fullerton, CA). Each sample (200 µl) was centrifuged in duplicate onto slides using a Cytospin (Shandon, Pittsburgh, PA) and subsequently stained with Diff Quik solution (American Scientific, McGraw Park, PA) for cell differentiation determination, with at least 200 cells counted from each slide. The left lobe was snap frozen in liquid nitrogen and subsequently stored at -80° C

for isolation of RNA and protein or homogenized in 250 µl of DMEM containing 1µg/ml of TPCK treated trypsin and 1% BSA for viral titers.

BAL fluid Biochemistry

Total protein assay was modified for use on the Konelab 30 clinical chemistry analyzer (Thermo Clinical Labsystems Espoo, Finland). Total protein concentrations were determined with the Coomassie Plus Protein Reagent (Pierce Chemical, Rockford, IL) with a standard curve prepared with bovine serum albumin from Sigma-Aldrich (St. Louis, MO.).

Pulmonary Function Measurements

Pulmonary function changes to increasing concentrations of inhaled methacholine (Mch) were measured in a 12-chamber whole-body plethysmograph system (Buxco Electronics, Troy, NY) on day 1, 4, 8, and 14 after influenza infection. Pressure signals were analyzed with BioSystem XA software (SFT3812, version 2.0.2.4, Buxco Electronics) to derive whole-body flow parameters that were used to calculate enhanced pause (Penh). Penh was used as an index of airflow obstruction, which has been correlated with changes in airway resistance (Metzger and Peterson, 1988). After measuring baseline parameters for 7 min, an aerosol of saline or Mch in increasing concentrations (6.25, 12.5, and 25 mg/ml) was nebulized through an inlet of the chamber and mice were exposed for 10 minutes to each concentration. The recorded Penh values were averaged during the baseline periods and the 10 minute Mch challenges to obtain

mean values for each event and were represented as change from the mean during the baseline period to the mean during each Mch challenge.

Pulmonary Virus Quantification

Virus titers were determined by $TCID_{50}$. Briefly, on days 1, 4, 8, and 14 p.i. the left lung was homogenized in 250 µl of DMEM containing 1µg/ml of TPCK treated trypsin and 1% BSA. The homogenates were spun at 1000 x g to remove cellular debris and supernatants were used to determine tissue culture infectious dose that kills 50 percent of the cells ($TCID_{50}$). Supernatants were plated on confluent MDCK cells in 96 well plates in log_{10} dilutions. After 3-5 days of incubation at 37° C, the cytopathic effect was observed and $TCID_{50}$ was calculated using Reed-Muench method (LaBarre and Lowy, 2001)

Histopathology

Lung tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Five µm thick sections were placed on Superfrost/plus slides (Fisher Scientific) and stained with H&E. The slides were evaluated under light microscopy at 10x and 40x objective by a veterinary pathologist (Dr. Mac Law) from NCSU College of Veterinary Medicine. Five to ten fields of at least 2 sections per animal and 2 animals per experimental group were evaluated. Tissue sections were photographed using an Olympus DP25 digital camera.

Real Time PCR

Total RNA was extracted from lung tissue with TRIzol (Invitrogen, Grand Island, NY) as per the supplier's instructions. First strand cDNA synthesis and real-time RT-PCR were performed as previously described (Jaspers *et al.*, 1999; Jaspers *et al.*, 2001). Genbank mRNA primers were IFN-γ NM_008337.1; IL-12p40 NM_008353.1; IL-4 NM_021283.1; IFN-β NM_008336.2; HO-1 NM_010442.1 purchased from Applied Biosystems (Foster City, CA). Expression changes were calculated using the relative quantification method. The housekeeping gene β actin was used as an endogenous reference to normalize target gene Ct values. Gene transcription was expressed as an n-fold difference relative to the control.

Antioxidant Administration

Animals were treated with 320 mg/kg N-acetylcysteine (NAC) (Sigma-Aldrich, St. Louis, MO) in sterile saline intraperitoneally (i.p.) immediately before each inhalation exposure for the first four days as previously described (Whitekus *et al.*, 2002).

Glutathione levels

Perchloric acid (PCA, 60% solution) was added to lung homogenates to a final concentration of 3% and samples were stored at -80° C. Reduced glutathione (GSH) and oxidized glutathione (GSSG) in the PCA supernatants were labeled with dansyl chloride by the method of (Jones *et al.*, 1998) and analyzed by HPLC using the method of (Gan *et al.*, 2005).

Statistical Analysis

Data were pooled from three replicate exposure studies/experiments and expressed as means ± SEM. Data generated from experiments were analyzed using nonparametric one-way ANOVA (Kruskal-Wallis test), followed by the Student Newman Kuehls comparison post hoc test. A value of $P<0.05$ was considered to be significant.

Results

DE Chamber Concentrations

Table 1 shows a summary of the 14-day average exposure data for the control and (0.5 mg/m^3) DE concentration. These target chamber concentrations, determined and adjusted based on continuous TEOM measurements were achieved with relatively low variability either within a particular 4 hour exposure or between different days. Chamber particle concentrations determined gravimetrically from integrated filter samples (one 4 h sample per exposure day), agreed with the TEOM measurements within 15%. CO and NO_x concentrations in the chambers averaged 12 and 17.6 ppm, respectively. SO_2 concentrations were very low and below detection levels for the DE chamber. Particle number concentrations were relatively high and corresponded to particle size distributions (PSDs) with a well established accumulation mode and little evidence of notable nuclei or coarse modes. Geometric median number and volume (assuming spherical particles) diameters of approximately 53 and 194 nm, respectively, were measured in both chambers. It should be noted, however, that the SMPS system (with long column) limited measurements to particles greater than approximately 15 nm, and a

small increase in the number counts in channels less than 25 nm may indicate the presence of a small nuclei mode below the instrument's range. OC/EC wt ratios of 1.1 from both chambers indicate that approximately 52.4% of the DEP was comprised of organic carbon.

Viral Quantification and Type I Intereferon Production

The purpose of this study was to determine how DE modulates an ongoing viral infection. The normal course of infection for this strain of influenza A, which causes a mild amount of inflammation, occurred with peak viral titers between day 4 and 8 p.i. with clearance by day 14 p.i (Figure 1A). Exposure to 0.5 mg/m^{3}of DE during infection resulted in significantly greater levels of virus compared to air exposed mice at day 4 and 8 post infection however DE exposure did not delay clearance at day 14.

Previous studies have demonstrated that chronic exposure of mice to DE resulted in increased viral titers, which correlated with decreased lung IFN levels (Hahon *et al.*, 1985). These data suggest that the enhanced influenza infection caused by exposure to DE was due to a suppression of the host's IFN-dependent, innate antiviral response resulting in a reduced ability to limit and clear the invading pathogen. In the present study the increased viral titers observed in mice exposed to 0.5 mg/m^3 of DE was not due to a suppression of the host's IFN-dependent antiviral responses. In fact, levels of IFN-β mRNA were increased although not significantly in the lungs of mice exposed to 0.5 mg/m^3 of DE during infection (Fig 1B) and corresponded to the level of virus (Fig 1A). In addition there was not a significant change in the levels of IFN-α observed in the lungs

of mice exposed to 0.5 mg/m^3 of DE during infection and no differences in expression of either type I interferons were seen in mice exposed to DE in the absence of influenza infection (data not shown).

Neutrophil Recruitment and Pulmonary Inflammation

Differential cell counts from air or DE exposed mice with or without influenza infection were assessed in the BAL (Fig 2A). DE exposure alone caused a significant increase in PMNs at day 4, 8, and 14 (data not shown) however this was orders of magnitude less than the PMN influx in the influenza infected animals. Influenza infection alone induced influx of PMNs at day 4 and day 8 p.i. and this was significantly increased by DE exposure at day 4 and persisted at day 8 ($p=0.06$) (Fig 2A). No residual PMNs were seen in the BAL at day 14 in either treatment group.

The amount of protein in the BAL was also assessed as a marker of pulmonary edema. DE alone did not significantly increase protein in the BAL at any time point (data not shown). Influenza infection alone increased the amount of protein on day 4 and 8 p.i. with a return to baseline by day 14 p.i. (Fig 2B). Exposure to DE caused a significant increase in the amount of protein in the BAL on day 4 which persisted to day 8 compared to flu infected mice exposed to air (Fig 2B).

Histopathological examination showed patchy areas of mild interstitial inflammation in the lungs from influenza infected mice (Fig. 2C), and these lesions were judged to be more severe in the DE/flu animals as early as day 1 and more prominately at day 4 p.i.(Fig. 2C). Inflammation was noted as multifocal alveolar septa that were

prominent and thickened by edema and capillary congestion, and infiltrated by mild to moderate numbers of neutrophils, histiocytes, lymphocytes, and plasma cells. In the more severely affected areas, small amounts of lumenal exudate comprised of proteinaceous fluid with fibrin, cell debris, and often large (activated) foamy macrophages. In the diesel exposed groups (DE or DE/flu), macrophages contained multiple phagocytosed particles after 1 or 4 DE exposures.

Pulmonary Function

Previous studies have shown that DE exposure (Li *et al.*, 2007) and influenza infection in mice (Dye *et al.*, 1996) can cause an increase in pulmonary responsiveness (PR) with a methacholine challenge. Therefore it was of interest to examine the PHR in the DE enhanced influenza infected mice as another indicator of lung disease and decreased pulmonary function. DE exposure did not increase PR at any time point when compared to air controls (Fig 3). Influenza infection alone significantly increased PR on day 4 and 8 p.i (data not shown). Mice exposed to DE during influenza infection had a significant increase in PR at day 1 p.i. that was not seen with influenza alone or DE exposure (Fig 3). At day 4 and 8 p.i. the PR of DE/flu exposed mice was not significantly different from air/flu controls but both groups were significantly higher than that of air or DE alone exposed animals (data not shown). No differences amongst any of the treatments were observed at day 14 p.i indicating no residual PR (data not shown).

IL-4 Expression in Lung

DE alone has been reported to induce the production of Th2 cytokines (Diaz-Sanchez, 1997) and since IL-4 decreases clearance of influenza (Moran *et al.*, 1996), we hypothesized that the DE enhanced influenza infection was caused by an increase in IL-4. DE alone significantly increased IL-4 expression after 4, 8, and 14 days of exposure when compared to the air controls (Fig 4A). Influenza infection alone significantly increased IL-4 expression at day 8 and 14 p.i. compared to the air controls, however with DE exposure during influenza infection IL-4 mRNA was significantly increased at day 1 and 4 p.i. compared to DE alone, air/flu or air controls (Fig 4B).

Pulmonary Th1 Cytokine Expression

In addition to establishing that DE exposure during influenza infection increased IL-4, it was of interest to examine if DE decreased Th1 cytokines during an influenza infection. Previous studies indicated that Th1 cytokines such as IL-12 and IFN-γ were essential to clear an influenza infection (Baumgarth and Kelso, 1996). The expression of IL-12p40 and IFN-γ was measured in lung homogenates by RT-PCR. DE exposure alone did not alter expression of either cytokine at any necropsy time point compared to air control (data not shown). Influenza significantly increased IFN-γ at day 1, 4, and 8 p.i and IL-12p40 expression was enhanced on day 4 and 8 (Fig 5A and B). Mice exposed to DE during an influenza infection had a significant decrease in IFN-γ expression at day 4 and 8 p.i. and a significant decrease in IL-12p40 expression at day 8 p.i when compared to air exposed infected mice.

Effects of NAC on Glutathione and Hemeoxygenase-1(HO-1)

Thiol antioxidants such as NAC have been reported to reverse the DEP- induced oxidative stress. To determine whether or not the increase in viral titers associated with DE exposure was a result of oxidative stress, mice were injected i.p. with 320 mg/kg NAC or vehicle 2 hours before each exposure as previously described (Whitekus *et al.*, 2002). One of the mechanisms for NAC-mediated decrease in oxidative stress is to upregulate the amount of reduced glutathione (GSH) available for detoxifying reactive species (Li *et al.*, 2000). Mice given NAC before all treatments had an increase in lung GSH levels although this was only significant with mice exposed to DE or DE/flu at day 1 and 4 p.i.(Figure 6A).

Another marker of oxidative stress that has been reported to change with NAC treatment is the antioxidant enzyme heme oxygenase 1 (HO-1). HO-1 levels after 1 day of DE exposure were only elevated in the mice exposed to DE during influenza infection when compared to the air control indicating an oxidative stress response (Fig 6B). This increase was less with NAC treatment decreasing HO-1 expression to a comparable level of the air control. After 4 days of DE exposure with or without infection, HO-1 mRNA was again only significantly increased with DE/flu treatment compared to air or air/flu controls (Fig 6C). This trend again was abrogated with 4 days of NAC treatment indicating a decrease in oxidative stress response.

Effect of NAC on Viral Titers

Previous studies have shown that an increase in GSH in an *in vitro* DE/flu model decreased the amount of influenza virus attaching to the epithelium (Jaspers *et al.*, 2005). Whole lung homogenates again were evaluated by $TCID_{50}$ to determine if NAC decreased the DE enhanced viral titers at day 4 p.i. As before DE increased viral titers on day 4 p.i. compared to air/flu mice (Fig 7A). Even though NAC increased GSH and decreased HO-1 in mice exposed DE during an influenza infection, viral titers were not affected by this treatment (Fig 7A).

Effect of NAC on DE Enhanced Influenza Inflammation

DE-induced proinflammatory effect has previously been reported to be mediated through the generation of oxidative stress (Li et al., 2007). The data reported thus far showed that DE exposure during an influenza infection increased viral titers and pulmonary inflammation and that NAC treatment caused an increase in molecules important in detoxifying reactive oxygen species that could drive the DE enhanced pulmonary inflammation. To determine if NAC treatment decreased pulmonary inflammation, the biomarkers that were previously measured were assessed. Neutrophil counts that were significantly increased with influenza infection and DE exposure were decreased at day 4 p.i. in mice treated with NAC (Fig 7B).

Another marker of inflammation that was increased in the DE enhanced influenza infected mice was the amount of protein in the BAL. No differences amongst treatments

were seen at day 1. NAC reversed DE enhanced exudation in the lungs of infected mice back to baseline levels at day 4 p.i. (data not shown).

As shown above, DE exposure increased PR in mice infected with influenza on day 1 p.i. (Fig 3). However, with NAC treatment the DE/flu effect was no longer significant at the highest methacholine challenge, indicating a decrease in inflammation that was reflected in pulmonary function (Fig 7C). Even though multiple markers of inflammation were decreased with NAC, no significant change was seen by histological examination (data not shown).

Effect of NAC on DE Enhanced IL-4 Expression.

DE induced oxidative stress has been shown to downregulate IL-12 and IFN-γ production and increase IL-4 favoring a Th2 phenotype (Kim *et al.*, 2007) and this effect can be reversed with NAC (Li *et al.,* 2007). Therefore the effects of NAC on DE-enhanced IL-4 expression were measured in the lung. Results shown in Figure 8 indicate that treatment with NAC significantly decreased IL-4 expression in mice exposed to DE during influenza infection on both day 1 and 4 p.i. This effect was also seen with mice exposed to DE alone after 4 days of NAC dosing. No differences in IL-4 expression were seen between NAC treated and untreated mice infected with influenza but not exposed to DE. Interestingly, NAC alone slightly increased IL-4 expression in untreated mice (air alone) as previously reported (Monick *et al.*, 2003).

A response to IL-4 can decrease expression of Th1 cytokines such as IFN-γ through transcriptional regulation (reviewed in (Bot *et al.*, 2004). Since, IL-4 expression

was decreased with NAC dosing it was of interest to examine if this resulted in restoration of the Th1 cytokine profile. No difference was seen in IFN-γ mRNA with NAC in mice exposed to air or influenza alone (Fig 9A). However on day 1 and 4 p.i. mice exposed to DE or DE/flu had a significant increase in IFN-γ expression when compared to saline controls (Fig 9A and B). No difference was seen with IL-12p40 expression at the early time points chosen for the NAC study although examining the day 8 time point would have been more appropriate since that was when a DE effect was observed (data not shown).

Discussion

Previous reports by this and other groups have demonstrated that pre-exposure to DE increases susceptibility to respiratory viral infections (Hahon *et al.*, 1985; Harrod *et al.*, 2003; Ciencewicki *et al.*, 2007). None of these studies ever addressed the question of how an air pollutant such as DE might alter the immune response to an ongoing established infection. We hypothesized that DE exposure during influenza infection would increase viral titers and/or reduce clearance resulting in more severe pulmonary disease. The results showed that exposure to DE enhanced pulmonary inflammation and viral titers during the course of infection but did not affect eventual clearance of the virus. The increased pulmonary edema and damage was associated with overexpression of IL-4 and reduced expression of IFN-γ and IL-12. Furthermore the results with NAC treated mice suggested that oxidative stress induced a less protective Th2 immune polarization in association with increased viral infection.

In general the influenza infection produced a greater degree of lung injury and inflammation; however this was potentiated with the DE exposure. Mouse adapted influenza infections alone caused an influx of PMNs by day 4 p.i but is not residual at day 10 p.i upon normal clearance of the virus (Bohn *et al.*, 2005). DE alone also has been reported to induce a significant influx of PMNs after just 1 day of exposure (Gowdy *et al.*, 2008). A significant influx of PMNs was not seen with mice exposed to DE during an influenza infection on day 1 however on day 4 and 8 p.i. an increase PMNs was seen, indicating pulmonary inflammation that was also reflected in the amount of protein in the BAL and pathological changes in the lung tissue. Normally influenza infected cells produce inflammatory mediators to induce antiviral responses but excessive inflammation can be detrimental to the host by causing morbidity. This was reflected in the PR data in which the mice exposed to DE during an influenza infection had an increase in sensitivity to a methacholine challenge. Respiratory infections have been a source of complications with underlying respiratory diseases such as asthma (reviewed in (Hogan *et al.*, 2008). In this current study we report that PR was exacerbated with DE exposure in association with increased lung injury and inflammation.

A balance between Th1 and Th2 immunity is required for optimum recovery from influenza virus infection. Both Th1 and Th2 cytokines are induced in influenza infections in mice (Carding *et al.*, 1993) and humans (Mbawuike *et al.*, 1997). The role of Th2 cytokines such as IL-4 has been reported to be deleterious (Moran *et al.*, 1996; Bot *et al.*, 2000) compared to Th1 cytokine production that is required to ultimately clear

the infection. It has been shown that the induction of IL-4 during a viral infection diminishes MHC class I-restricted T cells and polarizes CD4+ T cells away from an IFN-γ dominated response (Seneviratne *et al.*, 2005). Also, mice that lack functional IL-4 genes have been shown to clear sublethal doses of influenza (Bot et al., 2000) while IL-12 or IFN-γ knock out mice can not (Sarawar *et al.*, 1994; Monteiro *et al.*, 1998). In the present study DE exposure during an influenza infection significantly increased the expression of IL-4 during day 1 and 4 p.i. These data do not completely correlate with the increase in viral titers at day 4 and 8 p.i. but the early presence of this Th2 cytokine could be responsible for increased titers later on since the overexpression of IL-4 has been shown to increase influenza titers (Moran et al., 1996; Bot et al., 2000). The increase in IL-4 was also associated with a decrease in IFN-γ and IL-12p40 expression on day 4 and 8 p.i. (Hanlon *et al.*, 2002). Downstream of the IL-4 receptor activation is the transcription factor GATA-3 that initiates chromatin remodeling, increases accessibility of Th2 cytokine loci, and blocks the Th1 cytokine transcription factor Tbet from binding (Li-Weber and Krammer, 2003). Therefore the DE induction of IL-4 dominated response may increase Th2 transcription factors that then block the production of Th1 cytokines.

DE exposure has been shown in numerous studies to increase the skewing of T cell responses toward a Th2 profile (reviewed by (Diaz-Sanchez, 1997). Intranasal administration of an allergen and DEPs increases not only Th2 cytokine production results in increased antigen specific IgE with a decrease in IFN-γ production (Diaz-Sanchez *et al.*, 1994; Diaz-Sanchez *et al.*, 2000). It has been hypothesized that this

increase in Th2 phenotype with DE exposure is a result of ROS and oxidative stress releasing nuclear factor-erythoid 2 (NF-E2) – related factor 2 (Nrf2) which initiates expression of phase II enzymes such as HO-1 (Chan *et al.*, 2006). The expression of these enzymes drives the immune system to produce more of an anti-inflammatory Th2 cytokine profile (Peterson *et al.*, 1998) that can be blocked by the administration of a thiol specific antioxidant NAC (Whitekus *et al.*, 2002; Li *et al.*, 2007). NAC can be converted into GSH to be utilized by enzymes such as glutathione peroxidase to convert ROS into H_20 (Jones *et al.*, 1998; Xiao *et al.*, 2003). This was reflected with a significant increase in the amount of GSH in mice treated with NAC and exposed to DE or DE/flu. HO-1, which is downstream of superoxide dismutase and glutathione peroxidase, was significantly decreased at day 1 and 4 p.i. with DE and DE/flu mice. Administration of NAC blocked the DE-enhanced pulmonary inflammation and PR to levels comparable to the air/flu control. NAC did not however improve the viral titers or pathology of mice exposed to DE during influenza. This indicates that oxidative stress is not the only mechanism behind DE enhanced influenza infection. It has previously been reported that DE exposure to human airway epithelium increases the amount of influenza virus that attached (Jaspers *et al.,* 2005), this could be applicable to the model in this study.

NAC reversed the polarization towards an IL-4 dominated response at day 1 and 4 p.i. in DE/flu mice in association with a subsequent increase in IFN-γ expression on day 4 p.i. The polarization towards a Th1 cytokine profile could be explained by increasing glutathione levels that have previously been shown to interfere with IL-4 production and

favor a Th1 phenotype (Bengtsson *et al.*, 2001). Likewise, glutathione depletion has been reported to shift the immune system in the opposite direction leading to an IL-4 dominated response (Peterson *et al.*, 1998). This phenomenon could be explained by DE promoting a Th2 differentiation by changing dendritic cell (DC) characteristics through pulmonary inflammation and oxidative stress (Devouassoux *et al.*, 2002). Previous studies have shown that DEP co incubated with DCs induce pro-oxidative responses that inhibit stimulation of Th1 CD4 T cells (Chan *et al*., 2006). Further studies of the DE enhanced flu model are needed to assess DC function and T cell stimulation to better understand the increase in an IL-4 dominated response.

In summary we have shown that the oxidative stress induced by DE exposure during an influenza infection increased viral titers and subsequent inflammation. This triggered an IL-4 dominated response that could be reversed by thiol antioxidants. Based on these findings it is evident that a delicate balance exists in the immune response to a viral infection and that exposure to a stressor such as an air pollutant can alter the course of disease. However if the mice used in this study were not otherwise healthy or if a secondary bacterial challenge was given, the effect of DE on viral clearance may be different. It has been well established that an increase in viral titers during an influenza infection causes an increase in airway epithelium damage that promotes an increase in bacterial binding sites therefore increasing the risk for secondary infection (reviewed in (McCullers, 2006). Given the number of individuals that have enhanced morbidity and mortality after an influenza infection each year, this study may provide insight into how

air pollution events result in increased morbidity and mortality to respiratory disease including viral illness. Future mechanistic studies are needed to further understand the type of pollutant which causes these effects and the mechanisms by which they occur.

References

Baumgarth, N. and A. Kelso (1996). In vivo blockade of gamma interferon affects the influenza virus-induced humoral and the local cellular immune response in lung tissue. *J Virol,* **70**,(7), 4411-8.

Bengtsson, A., M. Lundberg, J. Avila-Carino, G. Jacobsson, A. Holmgren and A. Scheynius (2001). Thiols decrease cytokine levels and down-regulate the expression of CD30 on human allergen-specific T helper (Th) 0 and Th2 cells. *Clin Exp Immunol,* **123**,(3), 350-60.

Bohn, A. A., K. S. Harrod, S. Teske and B. P. Lawrence (2005). Increased mortality associated with TCDD exposure in mice infected with influenza A virus is not due to severity of lung injury or alterations in Clara cell protein content. *Chem Biol Interact,* **155**,(3), 181-90.

Bot, A., A. Holz, U. Christen, T. Wolfe, A. Temann, R. Flavell and M. von Herrath (2000). Local IL-4 expression in the lung reduces pulmonary influenza-virus-specific secondary cytotoxic T cell responses. *Virology,* **269**,(1), 66-77.

Bot, A., K. A. Smith and M. von Herrath (2004). Molecular and cellular control of T1/T2 immunity at the interface between antimicrobial defense and immune pathology. *DNA Cell Biol,* **23**,(6), 341-50.

Carding, S. R., W. Allan, A. McMickle and P. C. Doherty (1993). Activation of cytokine genes in T cells during primary and secondary murine influenza pneumonia. *J Exp Med,* **177**,(2), 475-82.

Carey, M. A., J. A. Bradbury, J. M. Seubert, R. Langenbach, D. C. Zeldin and D. R. Germolec (2005). Contrasting effects of cyclooxygenase-1 (COX-1) and COX-2 deficiency on the host response to influenza A viral infection. *J Immunol,* **175**,(10), 6878-84.

Castranova, V., J. Y. Ma, H. M. Yang, J. M. Antonini, L. Butterworth, M. W. Barger, J. Roberts and J. K. Ma (2001). Effect of exposure to diesel exhaust particles on the susceptibility of the lung to infection. *Environ Health Perspect,* **109 Suppl 4**,(609-12.

Chan, R. C., M. Wang, N. Li, Y. Yanagawa, K. Onoe, J. J. Lee and A. E. Nel (2006). Pro-oxidative diesel exhaust particle chemicals inhibit LPS-induced dendritic cell

responses involved in T-helper differentiation. *J Allergy Clin Immunol,* **118**,(2), 455-65.

Ciencewicki, J., K. Gowdy, Q. T. Krantz, W. P. Linak, L. Brighton, M. I. Gilmour and I. Jaspers (2007). Diesel exhaust enhanced susceptibility to influenza infection is associated with decreased surfactant protein expression. *Inhal Toxicol,* **19**,(14), 1121-33.

Ciencewicki, J. and I. Jaspers (2007). Air pollution and respiratory viral infection. *Inhal Toxicol,* **19**,(14), 1135-46.

Devouassoux, G., A. Saxon, D. D. Metcalfe, C. Prussin, M. G. Colomb, C. Brambilla and D. Diaz-Sanchez (2002). Chemical constituents of diesel exhaust particles induce IL-4 production and histamine release by human basophils. *J Allergy Clin Immunol,* **109**,(5), 847-53.

Diaz-Sanchez, D. (1997). The role of diesel exhaust particles and their associated polyaromatic hydrocarbons in the induction of allergic airway disease. *Allergy,* **52**,(38 Suppl), 52-6; discussion 57-8.

Diaz-Sanchez, D., A. R. Dotson, H. Takenaka and A. Saxon (1994). Diesel exhaust particles induce local IgE production in vivo and alter the pattern of IgE messenger RNA isoforms. *J Clin Invest,* **94**,(4), 1417-25.

Diaz-Sanchez, D., M. Penichet-Garcia and A. Saxon (2000). Diesel exhaust particles directly induce activated mast cells to degranulate and increase histamine levels and symptom severity. *J Allergy Clin Immunol,* **106**,(6), 1140-6.

Dye, J. A., K. T. Morgan, D. L. Neldon, J. S. Tepper, G. R. Burleson and D. L. Costa (1996). Characterization of upper respiratory disease in rats following neonatal inoculation with a rat-adapted influenza virus. *Vet Pathol,* **33**,(1), 43-54.

Falsey, A. R. and E. E. Walsh (2000). Respiratory syncytial virus infection in adults. *Clin Microbiol Rev,* **13**,(3), 371-84.

Gan, J., T. W. Harper, M. M. Hsueh, Q. Qu and W. G. Humphreys (2005). Dansyl glutathione as a trapping agent for the quantitative estimation and identification of reactive metabolites. *Chem Res Toxicol,* **18**,(5), 896-903.

Garozzo, A., G. Tempera, D. Ungheri, R. Timpanaro and A. Castro (2007). N-acetylcysteine synergizes with oseltamivir in protecting mice from lethal influenza infection. *Int J Immunopathol Pharmacol,* **20**,(2), 349-54.

Gowdy, K., Q. T. Krantz, M. Daniels, W. P. Linak, I. Jaspers and M. I. Gilmour (2008). Modulation of pulmonary inflammatory responses and antimicrobial defenses in mice exposed to diesel exhaust. *Toxicol Appl Pharmacol,* **229**,(3), 310-9.

Hahon, N., J. A. Booth, F. Green and T. R. Lewis (1985). Influenza virus infection in mice after exposure to coal dust and diesel engine emissions. *Environ Res,* **37**,(1), 44-60.

Hanlon, A. M., S. Jang and P. Salgame (2002). Signaling from cytokine receptors that affect Th1 responses. *Front Biosci,* **7**,1247-54.

Harrod, K. S., R. J. Jaramillo, C. L. Rosenberger, S. Z. Wang, J. A. Berger, J. D. McDonald and M. D. Reed (2003). Increased susceptibility to RSV infection by exposure to inhaled diesel engine emissions. *Am J Respir Cell Mol Biol,* **28**,(4), 451-63.

Hiura, T. S., M. P. Kaszubowski, N. Li and A. E. Nel (1999). Chemicals in diesel exhaust particles generate reactive oxygen radicals and induce apoptosis in macrophages. *J Immunol,* **163**,(10), 5582-91.

Hogan, S. P., H. F. Rosenberg, R. Moqbel, S. Phipps, P. S. Foster, P. Lacy, A. B. Kay and M. E. Rothenberg (2008). Eosinophils: biological properties and role in health and disease. *Clin Exp Allergy,* **38**,(5), 709-50.

Jaspers, I., J. M. Ciencewicki, W. Zhang, L. E. Brighton, J. L. Carson, M. A. Beck and M. C. Madden (2005). Diesel exhaust enhances influenza virus infections in respiratory epithelial cells. *Toxicol Sci,* **85**,(2), 990-1002.

Jaspers, I., J. M. Samet and W. Reed (1999). Arsenite exposure of cultured airway epithelial cells activates kappaB-dependent interleukin-8 gene expression in the absence of nuclear factor-kappaB nuclear translocation. *J Biol Chem,* **274**,(43), 31025-33.

Jaspers, I., W. Zhang, A. Fraser, J. M. Samet and W. Reed (2001). Hydrogen peroxide has opposing effects on IKK activity and IkappaBalpha breakdown in airway epithelial cells. *Am J Respir Cell Mol Biol,* **24**,(6), 769-77.

Jones, D. P., J. L. Carlson, P. S. Samiec, P. Sternberg, Jr., V. C. Mody, Jr., R. L. Reed and L. A. Brown (1998). Glutathione measurement in human plasma. Evaluation of sample collection, storage and derivatization conditions for analysis of dansyl derivatives by HPLC. *Clin Chim Acta,* **275**,(2), 175-84.

Kato, A., H. Kyono and N. Kuwabara (1992). [Electron-microscopic observations on rat lungs after long term inhalation of diesel emissions--non-neoplastic lesions]. *Nihon Kyobu Shikkan Gakkai Zasshi,* **30**,(2), 238-47.

Kim, H. J., B. Barajas, R. C. Chan and A. E. Nel (2007). Glutathione depletion inhibits dendritic cell maturation and delayed-type hypersensitivity: implications for systemic disease and immunosenescence. *J Allergy Clin Immunol,* **119**,(5), 1225-33.

Klimov, A., L. Simonsen, K. Fukuda and N. Cox (1999). Surveillance and impact of influenza in the United States. *Vaccine,* **17 Suppl 1**,(S42-6.

LaBarre, D. D. and R. J. Lowy (2001). Improvements in methods for calculating virus titer estimates from TCID50 and plaque assays. *J Virol Methods,* **96**,(2), 107-26.

Li, N., S. Kim, M. Wang, J. Froines, C. Sioutas and A. Nel (2002). Use of a stratified oxidative stress model to study the biological effects of ambient concentrated and diesel exhaust particulate matter. *Inhal Toxicol,* **14**,(5), 459-86.

Li, N., M. I. Venkatesan, A. Miguel, R. Kaplan, C. Gujuluva, J. Alam and A. Nel (2000). Induction of heme oxygenase-1 expression in macrophages by diesel exhaust particle chemicals and quinones via the antioxidant-responsive element. *J Immunol,* **165**,(6), 3393-401.

Li, Y. J., T. Kawada, A. Matsumoto, A. Azuma, S. Kudoh, H. Takizawa and I. Sugawara (2007). Airway inflammatory responses to oxidative stress induced by low-dose diesel exhaust particle exposure differ between mouse strains. *Exp Lung Res,* **33**,(5), 227-44.

Li-Weber, M. and P. H. Krammer (2003). Regulation of IL4 gene expression by T cells and therapeutic perspectives. *Nat Rev Immunol,* **3**,(7), 534-43.

Lopez, C. B., T. M. Moran, J. L. Schulman and A. Fernandez-Sesma (2002). Antiviral immunity and the role of dendritic cells. *Int Rev Immunol,* **21**,(4-5), 339-53.

Mbawuike, I. N., C. L. Acuna, K. C. Walz, R. L. Atmar, S. B. Greenberg and R. B. Couch (1997). Cytokines and impaired CD8+ CTL activity among elderly persons and the enhancing effect of IL-12. *Mech Ageing Dev,* **94**,(1-3), 25-39.

McCullers, J. A. (2006). Insights into the interaction between influenza virus and pneumococcus. *Clin Microbiol Rev,* **19**,(3), 571-82.

Metzger, J. M. and L. B. Peterson (1988). Cyclosporin A enhances the pulmonary granuloma response induced by Schistosoma mansoni eggs. *Immunopharmacology,* **15**,(2), 103-15.

Monick, M. M., L. Samavati, N. S. Butler, M. Mohning, L. S. Powers, T. Yarovinsky, D. R. Spitz and G. W. Hunninghake (2003). Intracellular thiols contribute to Th2 function via a positive role in IL-4 production. *J Immunol,* **171**,(10), 5107-15.

Monteiro, J. M., C. Harvey and G. Trinchieri (1998). Role of interleukin-12 in primary influenza virus infection. *J Virol,* **72**,(6), 4825-31.

Monto, A. S., J. Rotthoff, E. Teich, M. L. Herlocher, R. Truscon, H. L. Yen, S. Elias and S. E. Ohmit (2004). Detection and control of influenza outbreaks in well-vaccinated nursing home populations. *Clin Infect Dis,* **39**,(4), 459-64.

Moran, T. M., H. Isobe, A. Fernandez-Sesma and J. L. Schulman (1996). Interleukin-4 causes delayed virus clearance in influenza virus-infected mice. *J Virol,* **70**,(8), 5230-5.

Murphy, K. M. and S. L. Reiner (2002). The lineage decisions of helper T cells. *Nat Rev Immunol,* **2**,(12), 933-44.

Nightingale, J. A., R. Maggs, P. Cullinan, L. E. Donnelly, D. F. Rogers, R. Kinnersley, K. F. Chung, P. J. Barnes, M. Ashmore and A. Newman-Taylor (2000). Airway inflammation after controlled exposure to diesel exhaust particulates. *Am J Respir Crit Care Med,* **162**,(1), 161-6.

Nordenhall, C., J. Pourazar, A. Blomberg, J. O. Levin, T. Sandstrom and E. Adelroth (2000). Airway inflammation following exposure to diesel exhaust: a study of time kinetics using induced sputum. *Eur Respir J,* **15**,(6), 1046-51.

Peterson, J. D., L. A. Herzenberg, K. Vasquez and C. Waltenbaugh (1998). Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proc Natl Acad Sci U S A,* **95**,(6), 3071-6.

Pope, C. A., 3rd, R. T. Burnett, G. D. Thurston, M. J. Thun, E. E. Calle, D. Krewski and J. J. Godleski (2004). Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. *Circulation,* **109**,(1), 71-7.

Pope, C. A., 3rd and D. W. Dockery (1992). Acute health effects of PM10 pollution on symptomatic and asymptomatic children. *Am Rev Respir Dis,* **145**,(5), 1123-8.

Rudell, B., A. Blomberg, R. Helleday, M. C. Ledin, B. Lundback, N. Stjernberg, P. Horstedt and T. Sandstrom (1999). Bronchoalveolar inflammation after exposure to diesel exhaust: comparison between unfiltered and particle trap filtered exhaust. *Occup Environ Med,* **56**,(8), 527-34.

Salvi, S., A. Blomberg, B. Rudell, F. Kelly, T. Sandstrom, S. T. Holgate and A. Frew (1999). Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am J Respir Crit Care Med,* **159**,(3), 702-9.

Sarawar, S. R., M. Sangster, R. L. Coffman and P. C. Doherty (1994). Administration of anti-IFN-gamma antibody to beta 2-microglobulin-deficient mice delays influenza virus clearance but does not switch the response to a T helper cell 2 phenotype. *J Immunol,* **153**,(3), 1246-53.

Seneviratne, S. L., L. Jones, A. S. Bailey, R. V. Samuel, A. P. Black and G. S. Ogg (2005). Interleukin-4 induced down-regulation of skin homing receptor expression by human viral-specific CD8 T cells may contribute to atopic risk of cutaneous infection. *Clin Exp Immunol,* **141**,(1), 107-15.

Singh, P., D. M. DeMarini, C. A. Dick, D. G. Tabor, J. V. Ryan, W. P. Linak, T. Kobayashi and M. I. Gilmour (2004). Sample characterization of automobile and forklift diesel exhaust particles and comparative pulmonary toxicity in mice. *Environ Health Perspect,* **112**,(8), 820-5.

Steerenberg, P., A. Verlaan, A. De Klerk, A. Boere, H. Loveren and F. Cassee (2004). Sensitivity to ozone, diesel exhaust particles, and standardized ambient particulate matter in rats with a listeria monocytogenes-induced respiratory infection. *Inhal Toxicol,* **16**,(5), 311-7.

Takizawa, H., S. Abe, T. Ohtoshi, S. Kawasaki, K. Takami, M. Desaki, I. Sugawara, S. Hashimoto, A. Azuma, K. Nakahara and S. Kudoh (2000). Diesel exhaust

particles up-regulate expression of intercellular adhesion molecule-1 (ICAM-1) in human bronchial epithelial cells. *Clin Exp Immunol,* **120**,(2), 356-62.

Takizawa, H., T. Ohtoshi, S. Kawasaki, S. Abe, I. Sugawara, K. Nakahara, K. Matsushima and S. Kudoh (2000). Diesel exhaust particles activate human bronchial epithelial cells to express inflammatory mediators in the airways: a review. *Respirology,* **5**,(2), 197-203.

Thompson, W. W., D. K. Shay, E. Weintraub, L. Brammer, C. B. Bridges, N. J. Cox and K. Fukuda (2004). Influenza-associated hospitalizations in the United States. *Jama,* **292**,(11), 1333-40.

Thompson, W. W., D. K. Shay, E. Weintraub, L. Brammer, N. Cox, L. J. Anderson and K. Fukuda (2003). Mortality associated with influenza and respiratory syncytial virus in the United States. *Jama,* **289**,(2), 179-86.

Whitekus, M. J., N. Li, M. Zhang, M. Wang, M. A. Horwitz, S. K. Nelson, L. D. Horwitz, N. Brechun, D. Diaz-Sanchez and A. E. Nel (2002). Thiol antioxidants inhibit the adjuvant effects of aerosolized diesel exhaust particles in a murine model for ovalbumin sensitization. *J Immunol,* **168**,(5), 2560-7.

Xiao, G. G., M. Wang, N. Li, J. A. Loo and A. E. Nel (2003). Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. *J Biol Chem,* **278**,(50), 50781-90.

Yang, H. M., J. M. Antonini, M. W. Barger, L. Butterworth, B. R. Roberts, J. K. Ma, V. Castranova and J. Y. Ma (2001). Diesel exhaust particles suppress macrophage function and slow the pulmonary clearance of Listeria monocytogenes in rats. *Environ Health Perspect,* **109**,(5), 515-21.

Yin, X. J., C. C. Dong, J. Y. Ma, J. M. Antonini, J. R. Roberts, C. F. Stanley, R. Schafer and J. K. Ma (2004). Suppression of cell-mediated immune responses to listeria infection by repeated exposure to diesel exhaust particles in brown Norway rats. *Toxicol Sci,* **77**,(2), 263-71.

Table 1. Summary of Concentrations and Characteristics of the Diesel Exhaust Particles and Gases within the Animal Exposure Chambers.

Constituent	*Units*	*Exposure*
Particle mass concentration (TEOM)	$\mu g/m^3$	500±9
Particle mass concentration (filter)[b]	$\mu g/m^3$	701±16
Particle number concentration[c]	$\#/cm^3$	1.0×10^8 $\pm4.7\times10^6$
Oxygen (O_2)	%	19.7±0.5
Carbon monoxide (CO)	ppm	12.0±1.0
Nitrogen oxides (NO_x)	ppm	17.6±0.7
Sulfur dioxide (SO_2)	ppm	<3.0
Number median D_p[d]	nm	53±2
Volume median D_p[d]	nm	194±2
OC/EC[e]	wt ratio	1.1±0.1

[a]These data represent averaged results from three exposure studies performed from 8/1-13/06 (13 days), 10/2-14/07 (13 days), and 2/11-19/08 (9 days). Tapered element oscillating microbalance (TEOM), O_2, CO, and NO_x data represent mean values from continuous measurements taken over the 35 days of exposure ± SE.
[b]Filter data represent mean values from one measurement per day taken over 34 days of exposure ± SE.
[c]Particle number concentration data represent a mean value from 13 days of exposure ± SE.
[d]D_p indicates particle geometric number and volume median diameters for 13 days of particle size distribution measurements ± geometric standard deviation. Note that volume information is calculated from number based mobility diameters and assume spherical particles.
[e]OC/EC (organic carbon to elemental carbon ratio) data represent mean values from one measurement per day taken over 13 days of exposure ± SE.

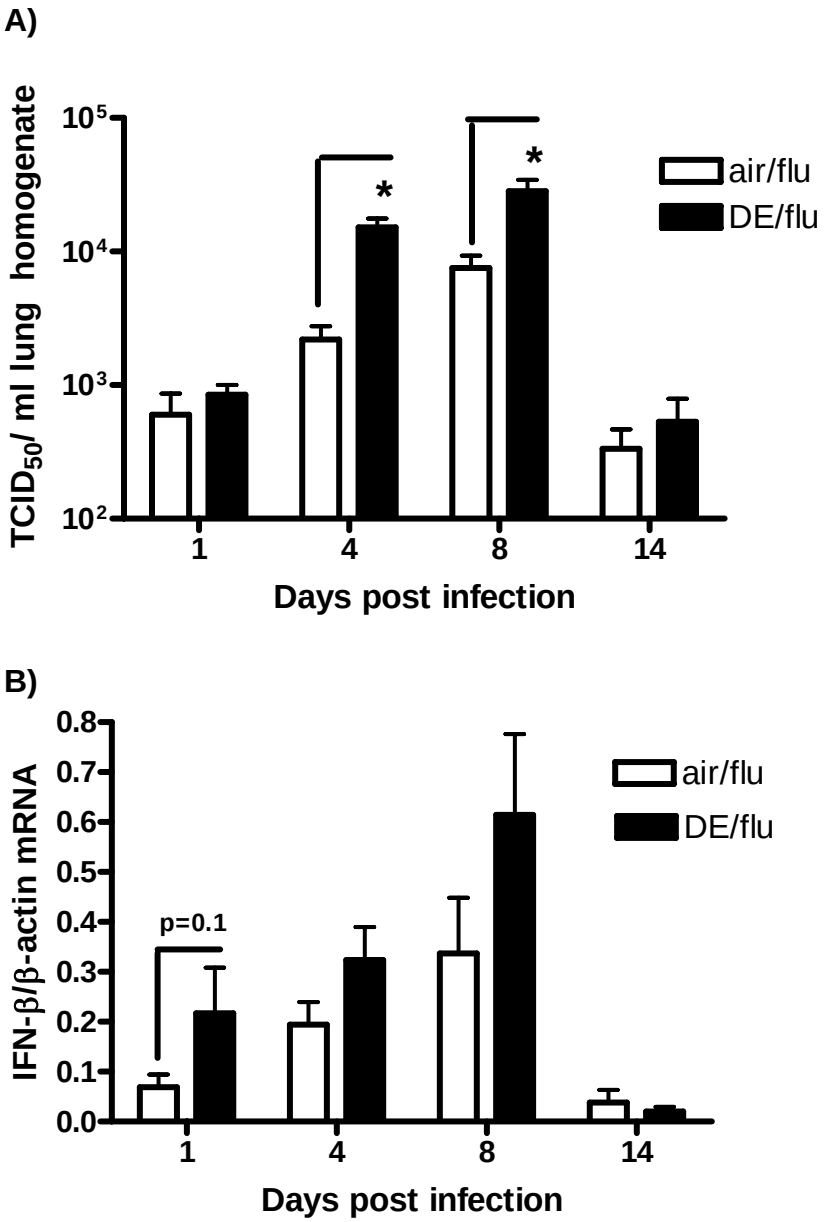
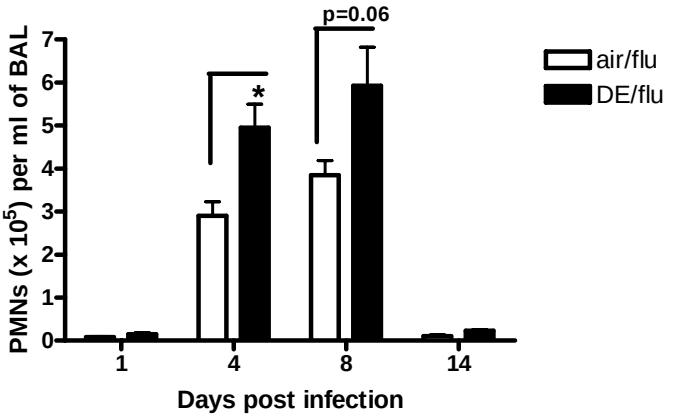


Figure 1. Exposure to DE enhances influenza titers and IFN-β expression.
A)Viral titers were quantified in lung homogenates by $TCID_{50}$ on day 1,4, 8, and 14 p.i. **B)**IFN-β mRNA expression was quantified in lung RNA by RT-PCR. Values are normalized to β-actin and expressed as relative quantification. *significantly different from air exposed influenza infected mice ($p<0.05$, $n=11$).

A)



B)

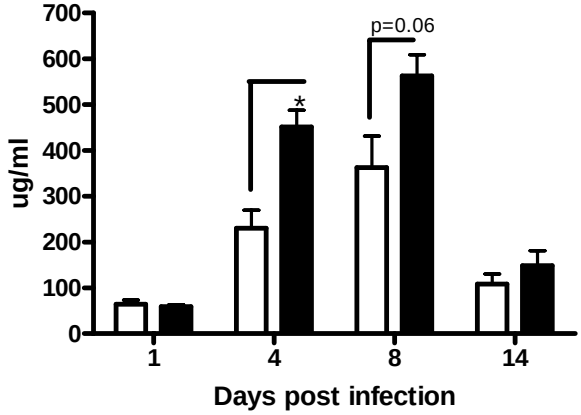
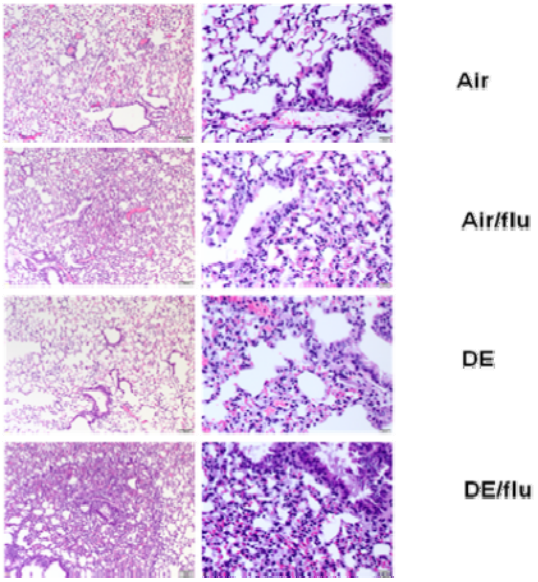


Figure 2. Exposure to DE enhances influenza induced pulmonary inflammation. BALF were obtained day 1, 4, 8, and 14 p.i. **A)** Neutrophil counts per ml of BAL. **B)** Protein production (μg/ml) in BAL. *significantly different from air exposed mice ($p<0.05$, n=8 for non- infected; n=11 for influenza infected).

A)



B)

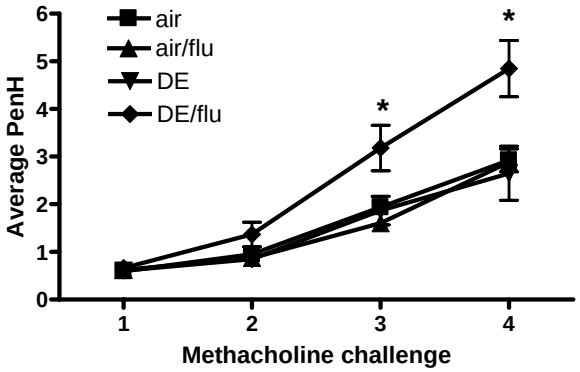


Figure 3. Exposure to DE enhances influenza induced pathology and pulmonary responsiveness (PR). A) Pathology scores of mouse lung sections were stained with H & E and visualized and scored using light microscopy from day 4 p.i. Left column is representative of 100x magnification and right column is representative of 400x magnification. **B)** PR measured by Buxco systems at day 1 p.i. reported as PenH values with increasing doses of aerosolized methacholine. *significantly different from air and air/flu exposed mice ($p<0.05$, $n=6$).

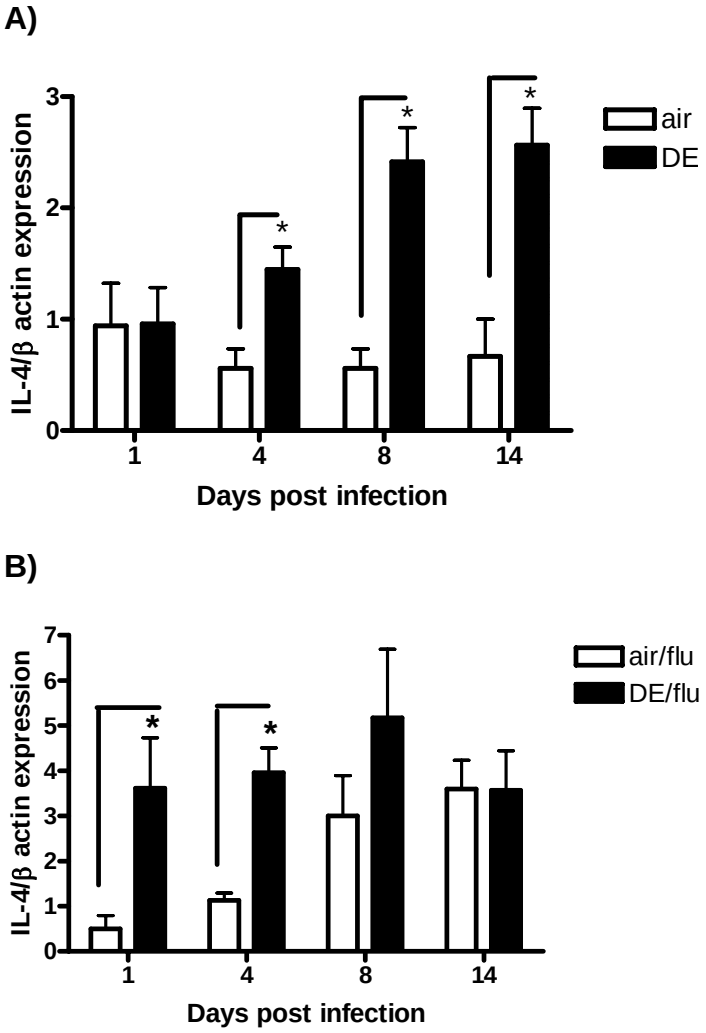


Figure 4. Exposure to DE or DE during an influenza infection increases the message of Interluekin 4 (IL-4).
IL-4 expression was analyzed in lung RNA on day 1, 4, 8, and 14 p.i. **A)** Levels of IL-4 mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin in mice exposed to air or DE. **B)** Levels of IL-4 mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin in mice exposed to air or DE during an influenza infection.*significantly different from air or air/flu exposed mice ($p<0.05$, n=8 for non- infected; n=11 for influenza infected).

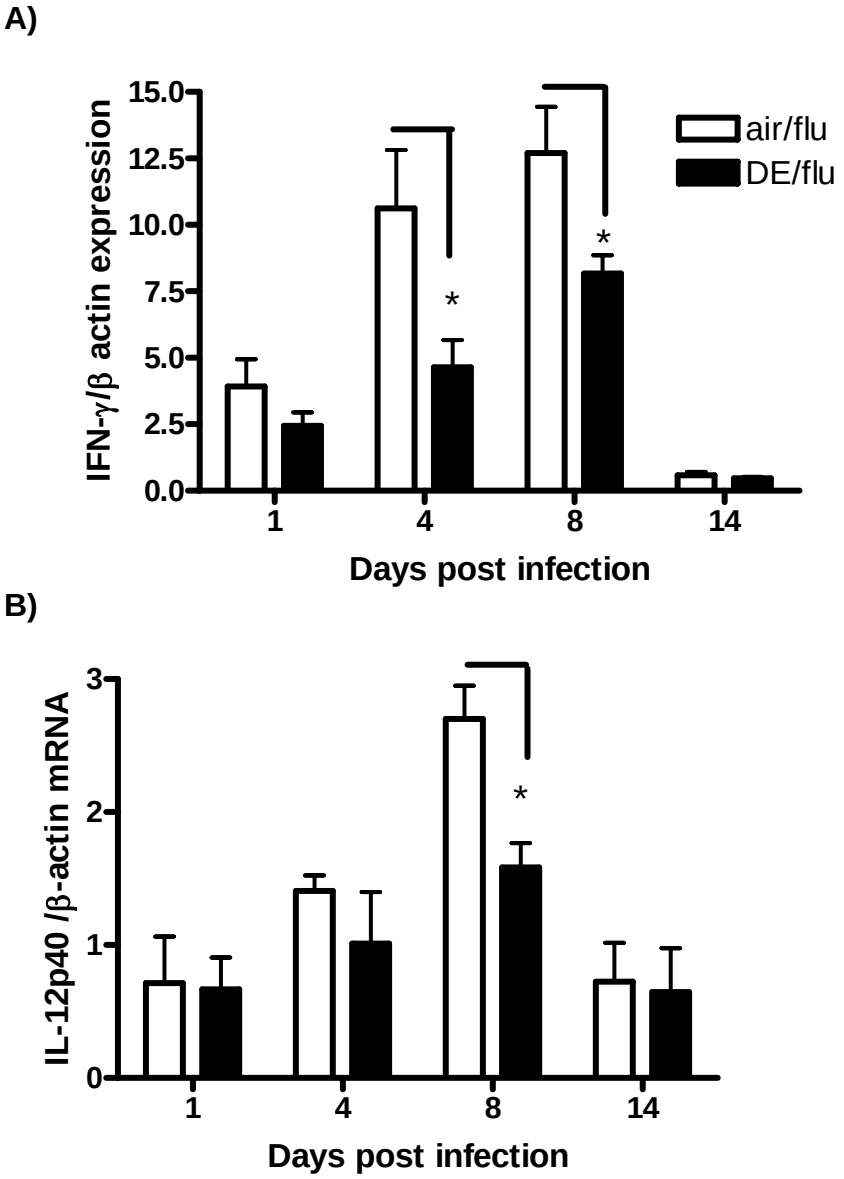
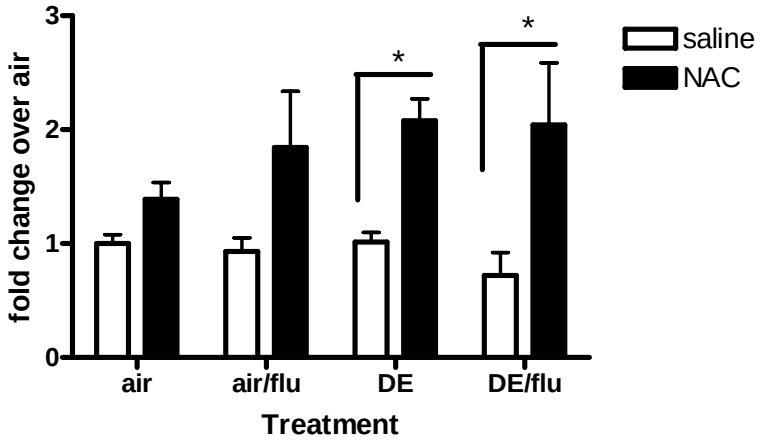


Figure 5. Exposure to DE decreases the expression of IFN-γ and IL-12p40 cytokines. Levels of Th1 cytokine mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin on day 1, 4, 8 and 14 p.i. **A)** IFN-γ; **B)** IL-12p40 subunit. *significantly different from air or air/flu exposed mice ($p<0.05$, $n=11$).

A)



B)

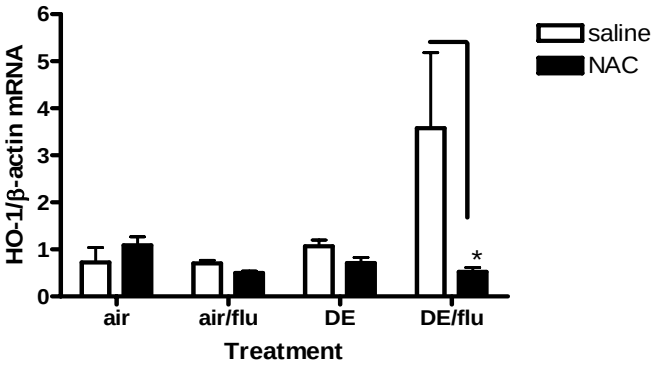
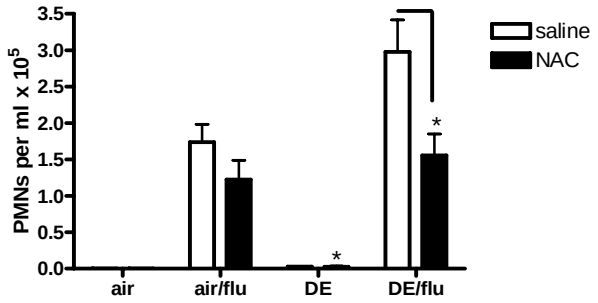


Figure 6. Antioxidants can increase glutathione (GSH) and decrease hemeoxygenase 1 (HO-1) expression during DE enhanced influenza infection. **A)** Levels of GSH were quantified in PCA lung homogenates by HPLC and normalized to levels of air exposed saline injected mice. Graph is representative of day 4 p.i. **B)** Levels of HO-1 mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin in mice exposed to air on day 4 p.i. ($p<0.05$, $n=6$).

A)

B)



C)

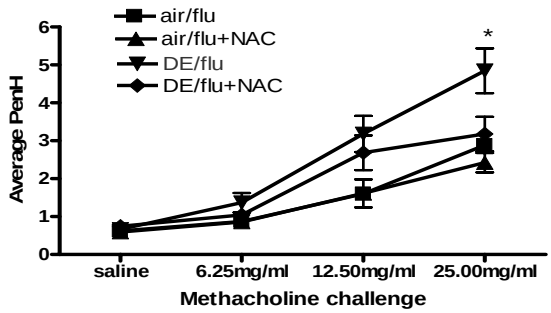


Figure 7. Antioxidants have no effect on DE enhanced influenza titers but decrease DE enhanced pulmonary inflammation. A) Viral titers were quantified in lung homogenates by $TCID_{50}$ on day 1 and 4 p.i. *significantly different from air/flu ($p<0.05$, n=6) **B)** Neutrophil counts per ml of BAL on day 4 p.i. **C)** PR measured by Buxco systems at day 1 p.i. reported as PenH values with increasing doses of aerosolized methacholine. *significantly different from saline controls ($p<0.05$, n=6).

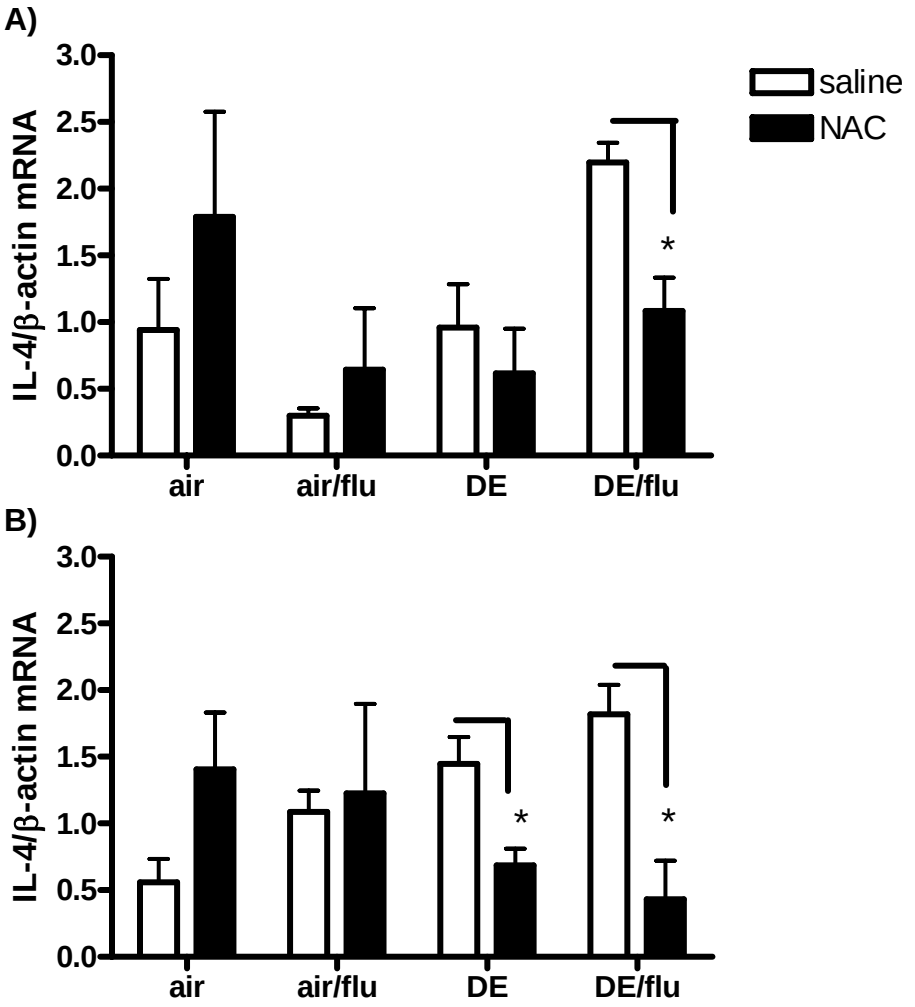


Figure 8. Antioxidants decrease DE or DE during an influenza infection increases in Interluekin 4 (IL-4).

IL-4 expression was analyzed in lung RNA on day 1 and 4 p.i. **A)** Levels of IL-4 mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin in mice exposed to air on day 1 p.i. **B)** Levels of IL-4 mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin in mice exposed to air on day 4 p.i. *significantly different from saline control ($p<0.05$, $n=6$).

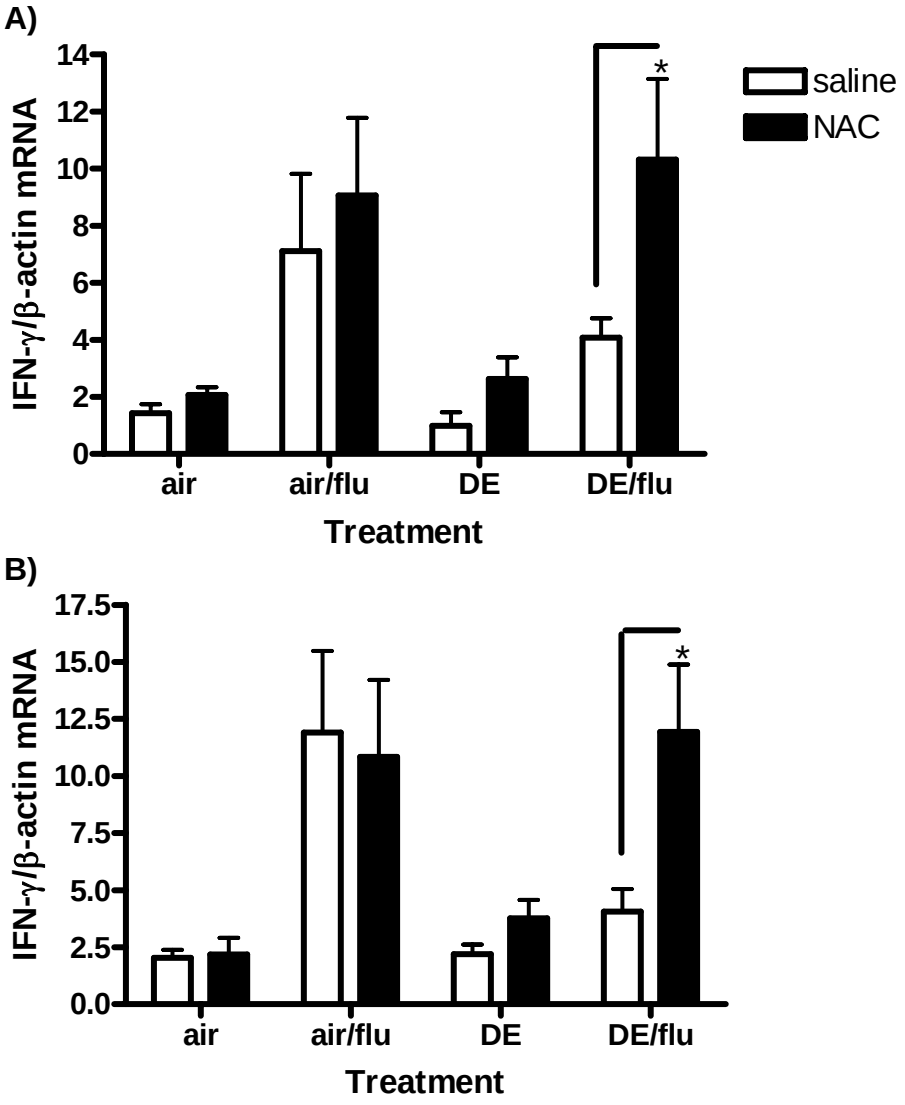


Figure 9. Antioxidants increase the expression of IFN-γ during a DE-enhanced influenza infection.

Levels of Th1 cytokine mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin on day 1 and 4 p.i. **A)** IFN-γ on day 1 p.i.; **B)** IFN-γ on day 4 p.i. *significantly different from saline control ($p<0.05$, $n=6$).

Chapter 5

Overall Discussion

Influenza virus is one of the most infectious human pathogens and worldwide epidemics occur each year. Influenza causes an average of 20,000 deaths annually in the United States (Fedson, 1996) and despite years of research, there is still no cure or completely reliable vaccine against influenza. Infection with influenza induces a cascade of immune responses that leads to damage of the airway epithelium, pulmonary inflammation, edema and decreased pulmonary function. A number of cytokines induced by the virus have immunoregulatory and antiviral properties while others contribute the severity of disease. A common cause of death from influenza is secondary bacterial pneumonia and/or an overwhelming pulmonary inflammatory response to the virus (Rothberg *et al.*, 2008). This most often occurs in susceptible populations such as children, the elderly, and individuals with preexisting pulmonary disease (Nicholson, 1996).

Multiple epidemiological studies have noted that air pollution increases the occurrence of respiratory infection and viral pneumonia. The data presented here demonstrate how exposure to a common air pollutant such as DE can 1) alter pulmonary host defenses important for clearing pathogens, 2) increase susceptibility to influenza by decreasing innate immune responses 3) enhance the severity of an ongoing influenza infection in association with altered cytokine homeostasis in the lung. Since both innate and adaptive immunity are required to clear influenza infection, a stressor such as DE altering either of these responses can change the course of the virus infection. Additionally, DE can

add to the inflammatory burden either prior to or during infection which ultimately can add to the severity of secondary pneumonia. Taken together exposure to DE has the potential to alter the susceptibility to infection or potentiate symptoms by augmenting inflammation and pathology associated with the viral illness.

It is important to study the health effects of DE since it can account for a significant percentage of air pollutants generated by mobile sources (Environmental Protection Agency, 2002). Diesel emissions are also produced by off-road and stationary engines which are sources since they can emit twice the DEPs as on road sources (California, 2000). DE also poses an occupational hazard since people with jobs such as transit workers, highway police, bus drivers, coal miners, and mechanics can be exposed daily to levels between from 0.25 to 1 mg/m^3 (Environmental Protection Agency, 2002).

DE exposure alone induced pulmonary edema and inflammation as well as a decrease in the expression and production of host defense molecules, SP-A, SP-D, and CCSP. Interestingly this was not in a dose dependent manner indicating that exposure level is an important factor in potential health effects. DE exposure significantly decreased not only the protein expression of host defense molecules but also the message level. Therefore, DE effects can be severe enough to affect genetic regulation of molecules important for clearing viral and bacterial pathogens. Mice lacking functional SP-A or SP-D can not clear a sublethal influenza infection (reviewed in (Crouch *et al.*, 2000) and have increased susceptibility to bacterial pathogens (Hartshorn *et al.*, 1998). Taken together if DE exposure can decrease the expression of surfactant proteins and CCSP this may provide a mechanism

to explain how air pollutant exposure increases the severity of respiratory infections. It should be borne in mind however, that not all DE contains the same amount of organic or elemental carbon, and the amount of noxious gases in the exhaust may vary. DE composition is dependent on the engine's characteristics, load, fuel used, and even its age. The biological activities of different DEPs have been shown to be variable in mutagenicity assays (DeMarini *et al.*, 2004), proinflammatory potential (Singh *et al.*, 2004); and allergic adjuvancy (Stevens *et al.*, 2008). Therefore even though this source of DE decreased SP-A, SP-D and CCSP expression this may be more or less apparent depending on the DE source, fuel, and other variable factors.

The influence of DE on the production of innate molecules such as SP-A, SP-D, and CCSP could explain how it may affect other pulmonary diseases besides influenza infection. Mice deficient in surfactant proteins have an increase in sensitivity to allergens (Wright, 1997). Therefore after exposure to DE an increase in pulmonary hypersensitivity and exacerbation of allergic asthma may occur. Deficiencies in surfactant proteins have also been reported to promote pulmonary changes similar to emphysema (Zhang *et al.*, 2002). Furthermore, mice lacking in CCSP are more susceptible than mice with normal CCSP production to oxidant injury and pulmonary inflammation (Stripp *et al.*, 2000) indicating that this molecule functions not only in host defense but also as an anti-inflammatory mediator. Since DE downregulates CCSP, the lung tissue would be more susceptible to cellular injury and inflammation. Taken together the impact on these host defense mechanisms not only

increases susceptibility to pathogens but also affects pulmonary disease status and ability to repair damage to the lungs.

Previous investigations have shown that DE exposure can increase susceptibility to viral infections such as influenza and RSV (Hahon *et al.*, 1985; Harrod *et al.*, 2003). However, the mechanisms for this immunomodulation occurs are still not fully understood. Data reported here indicate that mice exposed to DE for 5 days prior to infection with influenza had a significant increase in viral HA mRNA expression after 1 day of infection and this was associated with increased pulmonary inflammation. Previous findings have shown that DE alone can decrease phagocytosis of bacteria and alter T lymphocyte recruitment, therefore delaying clearance of the infection (Castranova *et al.*, 2001; Yang *et al.*, 2001; Yin *et al.*, 2005). The results presented here provide insight into the cellular mechanisms whereby exposure to an air pollutant such as DE can modulate innate responses to respiratory viral infection and lead to increased rates of secondary bacterial pneumonia.

Exposure to DE during an established ongoing influenza infection has yet to be examined by laboratory reports even though epidemiological studies have found that air pollution episodes increased the rate of respiratory infections (Pope *et al.*, 2004; Pope and Dockery, 2006). The research presented here indicated that DE exposure during an influenza infection increased viral titers and pulmonary inflammation. This DE enhanced infection was also associated with an increase in IL-4 expression and a concomitant decrease in Th1 cytokines that are important for viral clearance. IL-4 has previously been implicated in decreased clearance of influenza (Moran *et al.*, 1996; Bot *et al.*, 2004). DE is known to

increase polarization towards an IL-4 dominated response alone or in the context of antigen (Diaz-Sanchez *et al.*, 1994; Diaz-Sanchez, 1997; Devouassoux *et al.*, 2002). DE enhanced Th2 cytokine production leads to upregulated antigen specific IgE production and reduced IFN-γ production. It is still not fully clear why DE induces a Th2 polarization but it has been hypothesized that it is a result of oxidative stress. Studies have reported that DE exposure results in the production of free radicals, which can have damaging effects on the lung tissue (Li *et al.*, 2000; Li *et al.*, 2002; Whitekus *et al.*, 2002; Li *et al.*, 2007). The airway epithelium is an important source of detoxifying enzymes such as intra and extracellular glutathione (Anderson, 1985) that protect the lung from oxidative damage, and there is evidence that oxidant pollutants react with these antioxidant molecules (Behndig *et al.*, 2006). Injury to the epithelium can result in danger signals to different interstitial immune cells such as lymphocytes and dendritic cells. Oxidative stress has also been reported to influence the polarization of $CD4^+$ T cells towards an IL-4 dominated response (Kim *et al.*, 2007). Thus, exposure to an air pollutant such as DE could consequently enhance virus-induced exacerbation of allergies and asthma.

The data reported here was generated in a healthy mouse model. However, there are many susceptible populations that are at a higher risk for adverse health effects from viral illness and pollutant exposure. Individuals over 65 and those with preexisting lung disease have a lower antioxidant potential and higher ROS production than individuals between the ages of 18-45, which makes them more sensitive to cellular injury and pulmonary

inflammation (Meydani *et al.*, 1990). Further studies are needed to examine if these effects can be reversed with antioxidants as reported here.

Even though multiple epidemiological studies have reported an association between airborne PM and respiratory infections, more reports are needed to examine length of exposure, frequency of exposure and what components are responsible for increased susceptibility. More mechanistic studies in rodents using whole body exposures are also required to examine different sources and mixtures of air pollutants. The research presented here identified several innate host defense markers that were affected by exposure to DE either before or during an influenza infection. However, fewer studies have considered the impact of DE on the adaptive immune response including T and B cell function. These two components of the adaptive immune response are responsible for development of antigen-specific protection against re-infection with the same pathogen. Although DE can alter pulmonary immune responses to a primary infection it is not clear if this will result increase susceptibility to a subsequent viral challenge. Considering that PM in the air is still a significant environmental and public health issue its health effects are of great concern in the aspect for disease control and vaccination strategy. Further research in this field will lead to a greater understanding of how pollutants modulate immune responses and the molecular mechanisms responsible for PM enhancing the rate and severity of respiratory viral infections.

References

Anderson, M. E. (1985). Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol,* **113**, 548-55.

Behndig, A. F., I. S. Mudway, J. L. Brown, N. Stenfors, R. Helleday, S. T. Duggan, S. J. Wilson, C. Boman, F. R. Cassee, A. J. Frew, F. J. Kelly, T. Sandstrom and A. Blomberg (2006). Airway antioxidant and inflammatory responses to diesel exhaust exposure in healthy humans. *Eur Respir J,* **27**,(2), 359-65.

Bot, A., K. A. Smith and M. von Herrath (2004). Molecular and cellular control of T1/T2 immunity at the interface between antimicrobial defense and immune pathology. *DNA Cell Biol,* **23**,(6), 341-50.

California Air Resources Board (2000). EMFAC2000 Emissions Factor Model. Release 2.02.

Castranova, V., J. Y. Ma, H. M. Yang, J. M. Antonini, L. Butterworth, M. W. Barger, J. Roberts and J. K. Ma (2001). Effect of exposure to diesel exhaust particles on the susceptibility of the lung to infection. *Environ Health Perspect,* **109 Suppl 4**, 609-12.

Crouch, E., K. Hartshorn and I. Ofek (2000). Collectins and pulmonary innate immunity. *Immunol Rev,* **173**, 52-65.

DeMarini, D. M., L. R. Brooks, S. H. Warren, T. Kobayashi, M. I. Gilmour and P. Singh (2004). Bioassay-directed fractionation and salmonella mutagenicity of automobile and forklift diesel exhaust particles. *Environ Health Perspect,* **112**,(8), 814-9.

Devouassoux, G., A. Saxon, D. D. Metcalfe, C. Prussin, M. G. Colomb, C. Brambilla and D. Diaz-Sanchez (2002). Chemical constituents of diesel exhaust particles induce IL-4 production and histamine release by human basophils. *J Allergy Clin Immunol,* **109**,(5), 847-53.

Diaz-Sanchez, D. (1997). The role of diesel exhaust particles and their associated polyaromatic hydrocarbons in the induction of allergic airway disease. *Allergy,* **52**,(38 Suppl), 52-6; discussion 57-8.

Diaz-Sanchez, D., A. R. Dotson, H. Takenaka and A. Saxon (1994). Diesel exhaust particles induce local IgE production in vivo and alter the pattern of IgE messenger RNA isoforms. *J Clin Invest,* **94**,(4), 1417-25.

Environmental Protection Agency, U. S. A. (2002). Health risk assessment document for diesel exhaust.

Fedson, D. S. (1996). Evaluating the impact of influenza vaccination. A North American perspective. *Pharmacoeconomics,* **9 Suppl 3**, 54-61.

Hahon, N., J. A. Booth, F. Green and T. R. Lewis (1985). Influenza virus infection in mice after exposure to coal dust and diesel engine emissions. *Environ Res,* **37**,(1), 44-60.

Harrod, K. S., R. J. Jaramillo, C. L. Rosenberger, S. Z. Wang, J. A. Berger, J. D. McDonald and M. D. Reed (2003). Increased susceptibility to RSV infection by exposure to inhaled diesel engine emissions. *Am J Respir Cell Mol Biol,* **28**,(4), 451-63.

Hartshorn, K. L., E. Crouch, M. R. White, M. L. Colamussi, A. Kakkanatt, B. Tauber, V. Shepherd and K. N. Sastry (1998). Pulmonary surfactant proteins A and D enhance neutrophil uptake of bacteria. *Am J Physiol,* **274**,(6 Pt 1), L958-69.

Kim, H. J., B. Barajas, R. C. Chan and A. E. Nel (2007). Glutathione depletion inhibits dendritic cell maturation and delayed-type hypersensitivity: implications for systemic disease and immunosenescence. *J Allergy Clin Immunol,* **119**,(5), 1225-33.

Li, N., S. Kim, M. Wang, J. Froines, C. Sioutas and A. Nel (2002). Use of a stratified oxidative stress model to study the biological effects of ambient concentrated and diesel exhaust particulate matter. *Inhal Toxicol,* **14**,(5), 459-86.

Li, N., M. I. Venkatesan, A. Miguel, R. Kaplan, C. Gujuluva, J. Alam and A. Nel (2000). Induction of heme oxygenase-1 expression in macrophages by diesel exhaust particle chemicals and quinones via the antioxidant-responsive element. *J Immunol,* **165**,(6), 3393-401.

Li, Y. J., T. Kawada, A. Matsumoto, A. Azuma, S. Kudoh, H. Takizawa and I. Sugawara (2007). Airway inflammatory responses to oxidative stress induced by low-dose diesel exhaust particle exposure differ between mouse strains. *Exp Lung Res,* **33**,(5), 227-44.

Meydani, S. N., M. Meydani and J. B. Blumberg (1990). Antioxidants and the aging immune response. *Adv Exp Med Biol,* **262**, 57-67.

Moran, T. M., H. Isobe, A. Fernandez-Sesma and J. L. Schulman (1996). Interleukin-4 causes delayed virus clearance in influenza virus-infected mice. *J Virol,* **70**,(8), 5230-5.

Nicholson, K. G. (1996). Socioeconomics of influenza and influenza vaccination in Europe. *Pharmacoeconomics,* **9 Suppl 3**, 75-8.

Pope, C. A., 3rd, R. T. Burnett, G. D. Thurston, M. J. Thun, E. E. Calle, D. Krewski and J. J. Godleski (2004). Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. *Circulation,* **109**,(1), 71-7.

Pope, C. A., 3rd and D. W. Dockery (2006). Health effects of fine particulate air pollution: lines that connect. *J Air Waste Manag Assoc,* **56**,(6), 709-42.

Rothberg, M. B., S. D. Haessler and R. B. Brown (2008). Complications of viral influenza. *Am J Med,* **121**,(4), 258-64.

Singh, P., D. M. DeMarini, C. A. Dick, D. G. Tabor, J. V. Ryan, W. P. Linak, T. Kobayashi and M. I. Gilmour (2004). Sample characterization of automobile and forklift diesel exhaust particles and comparative pulmonary toxicity in mice. *Environ Health Perspect,* **112**,(8), 820-5.

Stevens, T., Q. T. Krantz, W. P. Linak, S. Hester and M. I. Gilmour (2008). Increased transcription of immune and metabolic pathways in naive and allergic mice exposed to diesel exhaust. *Toxicol Sci,* **102**,(2), 359-70.

Stripp, B. R., S. D. Reynolds, C. G. Plopper, I. M. Boe and J. Lund (2000). Pulmonary phenotype of CCSP/UG deficient mice: a consequence of CCSP deficiency or altered Clara cell function? *Ann N Y Acad Sci,* **923**, 202-9.

Whitekus, M. J., N. Li, M. Zhang, M. Wang, M. A. Horwitz, S. K. Nelson, L. D. Horwitz, N. Brechun, D. Diaz-Sanchez and A. E. Nel (2002). Thiol antioxidants inhibit the adjuvant effects of aerosolized diesel exhaust particles in a murine model for ovalbumin sensitization. *J Immunol,* **168**,(5), 2560-7.

Wright, J. R. (1997). Immunomodulatory functions of surfactant. *Physiol Rev,* **77**,(4), 931-62.

Yang, H. M., J. M. Antonini, M. W. Barger, L. Butterworth, B. R. Roberts, J. K. Ma, V. Castranova and J. Y. Ma (2001). Diesel exhaust particles suppress macrophage function and slow the pulmonary clearance of Listeria monocytogenes in rats. *Environ Health Perspect,* **109**,(5), 515-21.

Yin, X. J., C. C. Dong, J. Y. Ma, J. M. Antonini, J. R. Roberts, M. W. Barger and J. K. Ma (2005). Sustained effect of inhaled diesel exhaust particles on T-lymphocyte-mediated immune responses against Listeria monocytogenes. *Toxicol Sci,* **88**,(1), 73-81.

Zhang, L., K. L. Hartshorn, E. C. Crouch, M. Ikegami and J. A. Whitsett (2002). Complementation of pulmonary abnormalities in SP-D(-/-) mice with an SP-D/conglutinin fusion protein. *J Biol Chem,* **277**,(25), 22453-9.

APPENDIX I

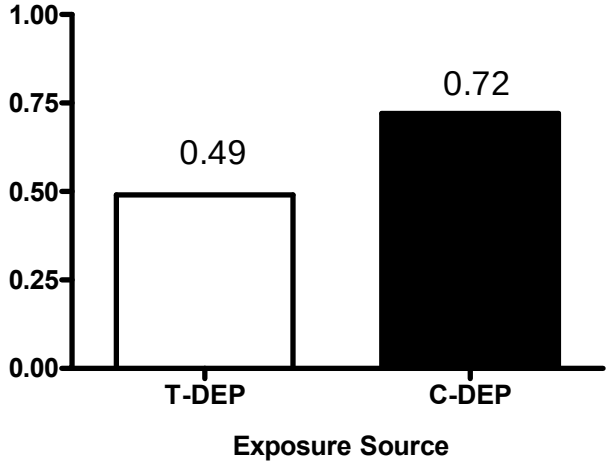
Comparison of On and Off Road Diesel Exhaust Sources on Susceptibility to Influenza Infection in Mice.

Abstract

Diesel exhaust particulate (DEP) is a major component of urban air pollution and its influence on susceptibility to respiratory infection is of concern. DEP is emitted from various types and ages of engine and the toxicity is dependent upon fuel, and combustion conditions which influence the physico-chemical characteristics of the emission. The purpose of this study was to evaluate the effects of an on- and off-road source of DEP exposure on the severity of acute influenza infection in mice. BALB/c mice were exposed (4 hrs/day, 5 days) to air or 0.5 mg/m3 DEP from engines powering a compressor (C-DEP) or a diesel truck (T-DEP) (Figure 1). One hour after the final diesel exposure, mice were intra-tracheally instilled with 10 HA units of influenza A/Bangkok/1/79 virus. 18 hours later mice were euthanized and lung tissue was assessed by RT-PCR for mRNA expression of viral hemagglutinin antigen, IFN-β, IL-6, and surfactant protein A and D (SP-A and SP-D). Lung injury was measured by differential cell counts and the production of LDH, microalbumin, and protein in lung lavages (BAL). Exposure to 0.5 mg/m3 DEP from either source in conjunction with infection caused increased protein levels and neutrophil numbers as well as enhanced IFN-β expression relative to air/flu controls (Figure 3). Mice exposed to C-DEP had significantly higher viral HA mRNA suggesting greater viral proliferation that was not seen with mice exposed to T-DEP (Figure 2). This effect was also associated with decreased expression of SP-A and SP-D (Figure 4), which is an important in defense against respiratory

infections. We conclude that while exposure to the on road (T-DEP) emission produced additive effects of lung injury during influenza infection, exposure to the DEP from the off road source (C-DEP) also enhanced susceptibility to infection in association with decreased production of SP-A and SP-D. Further studies will clarify the physico-chemical elements associated with this effect.

A)



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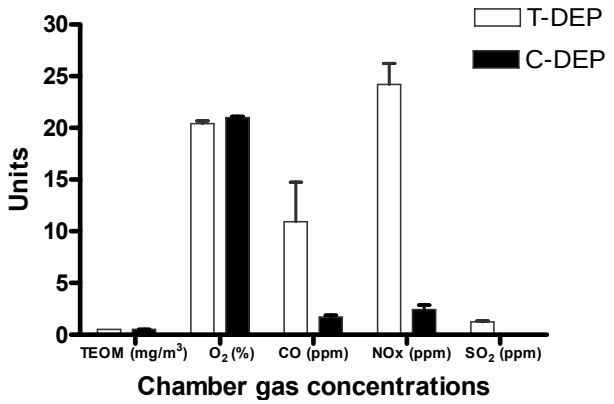
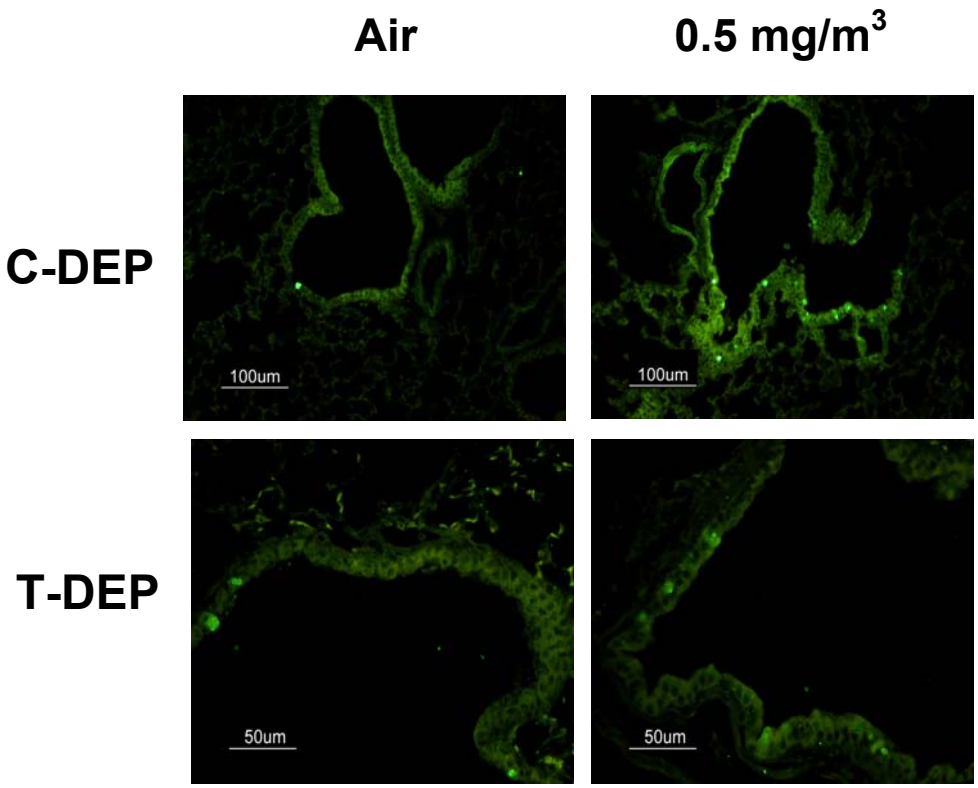


Figure 1: Comparison of diesel exhaust from a truck (T-DEP) and a compressor (C-DEP). A) Comparsion of organic carbon to elemental carbon (OC/EC) ratio in both sources over a 5 day exposure**. B)** Comparison of gas concentrations in exhaust.

A)



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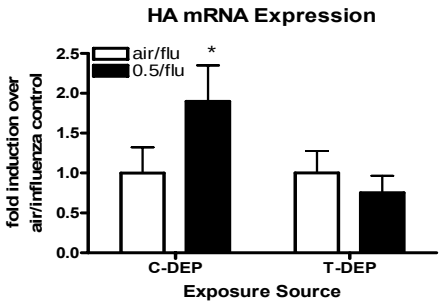
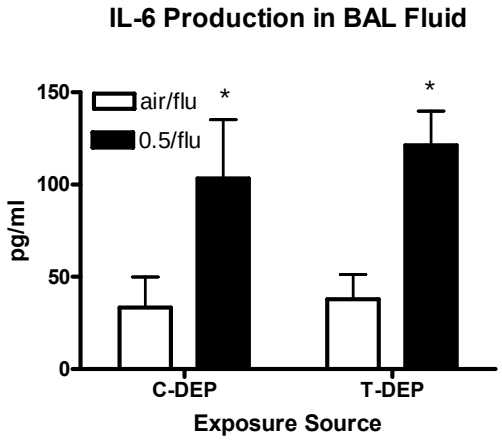
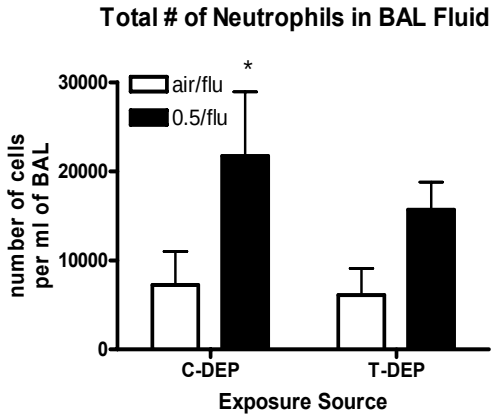


Figure 2: Exposure to C-DEP, not T-DEP, increases influenza HA mRNA and influenza nuclear protein staining in the lung. **A)** Staining for nuclear protein of Influenza. Positive cells fluoresce green. **B)** Hemaglutanin mRNA levels in whole lung RNA measured by PCR. * is significantly different from air/flu control $p < 0.05$.

A)





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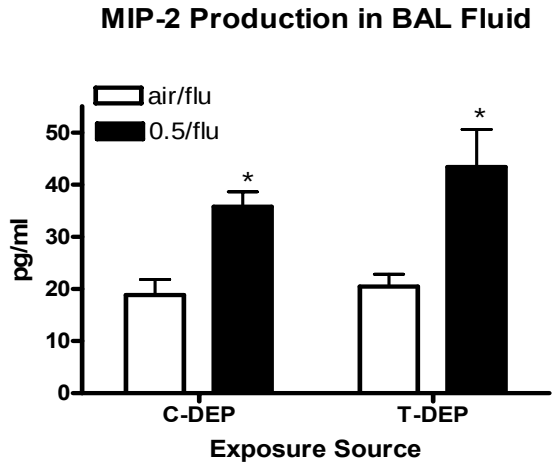


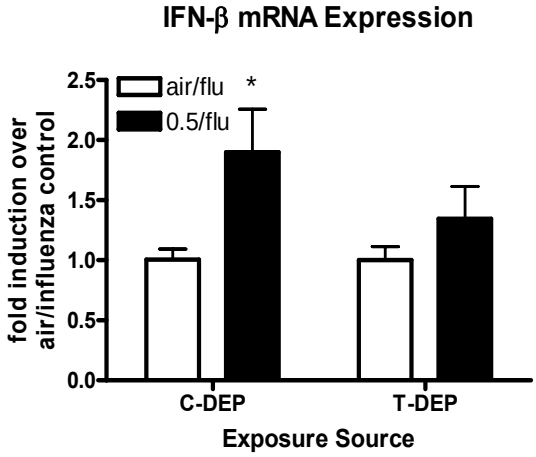


Figure 3: Exposure to C-DEP or T-DEP increases inflammatory biomarkers and Type I Interferon production associated with influenza. **A)** IL-6 production in BAL measured by ELISA. **B)** MIP-2 production in the BAL measured by ELISA. **C)** PMN counts per ml of BAL. **D)**IFN-β mRNA levels in whole lung RNA measured by Real Time PCR. * is significantly different from air/flu control $p < 0.05$.

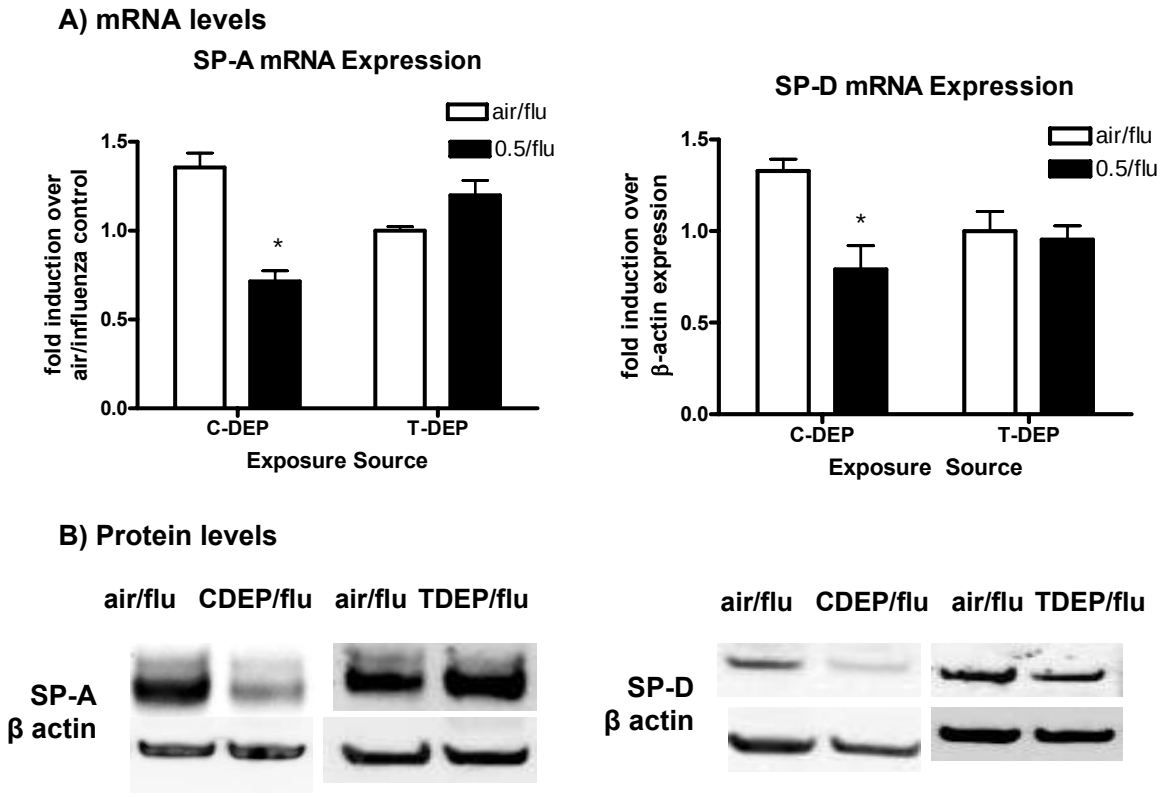


Figure 4: Exposure to C-DEP decreases the mRNA and protein expression of SP-A and SP-D, but no decrease with exposure to T-DEP. **A)** SP-A and SP-D mRNA levels measured in whole lung RNA by Real Time PCR. **B)** Protein levels measured in whole lung homogenates by Western Blot. Membranes were stripped and re-probed for β actin.

Appendix II

Diesel Exhaust Exposure Increases Susceptibility to Influenza Infection and Induces Dendritic Cell Migration and Maturation.

Abstract

Numerous studies have shown that diesel exhaust (DE) decreases resistance to respiratory infection and can alter the maturation and migration of dendritic cells (DCs). The purpose of this study was to evaluate the effects of DE exposure on susceptibilty to influenza infection in mice and to determine if this correlated with changes in the pulmonary DC populations. BALB/c mice were exposed to air or 0.5 mg/m^3 DE from a diesel-powered generator (Figure 1) (4 hrs/day, 5 days) and after the final diesel exposure, were intra-nasally instilled with 10^{-3} LD50 of influenza A/PR/8/34 virus. Mice were necropsied at day 1, 4, 8 and 14 post-infection and lung tissue was assessed for virus titers by $TCID_{50}$, lung injury and inflammation. Lung and lymph node DC populations ($CD11c^+$, MHCII, $CD45^+$, $CD80^+$ and $CD86^+$) were identified by flow cytometry. Prior exposure to DE significantly increased viral titers in the lung at 1 and 4 days post infection (Figure 2) in association with increased neutrophil influx and lung injury (Figure 4). Pro-inflammatory cytokines including IP-10, MCP-1, GM-CSF, and IL-1β (Figure 8), and the antiviral cytokine IFN-β (Figure 3) were also increased at days 1, 4 and 8 post infection compared to air/flu controls. The number of DCs in the lung was not affected with previous exposure to DE (Figure 5), however the lymph nodes had increased number of mature DCs at 1, 4, and 8 days post infection compared to the air/flu controls (Figure 6 and 7). We conclude that exposure to DE prior to an influenza infection increases pulmonary inflammation, viral titers, and stimulates more

DCs to migrate to the lymph nodes and mature as a consequence of the DE-enhanced influenza infection.

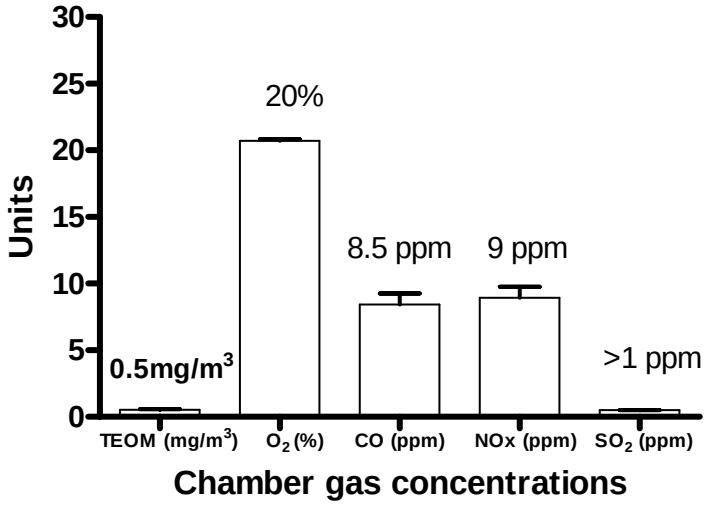
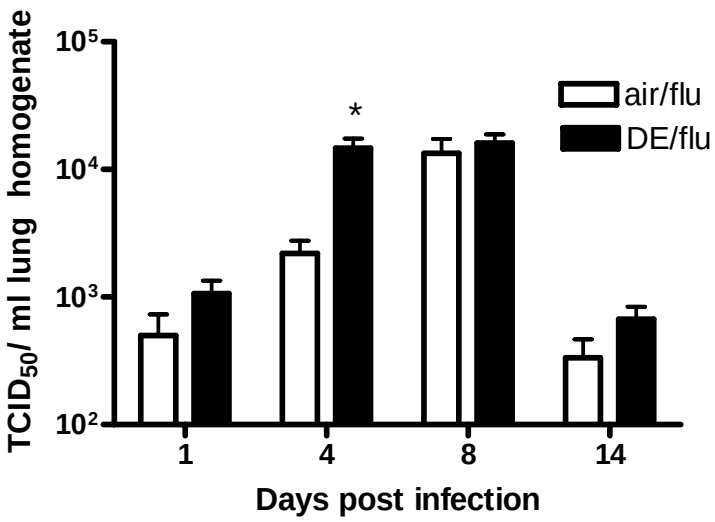


Figure 1: Concentrations of gases in diesel exhaust from a generator (G-DEP).
Not included in Figure 1 OC/EC ratio: 0.4 and Particle Size (GM): 74±1.7 nm.

A)



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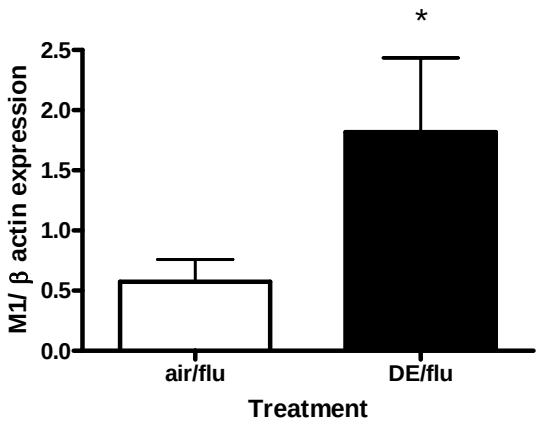


Figure 2: Effect of DE on the susceptibility to influenza infection. **A)** $TCID_{50}$ in whole lung homogenates. **B)** Matrix 1 (M1) mRNA levels in whole lung RNA measured by PCR. * is significantly different from air/flu control $p < 0.05$.

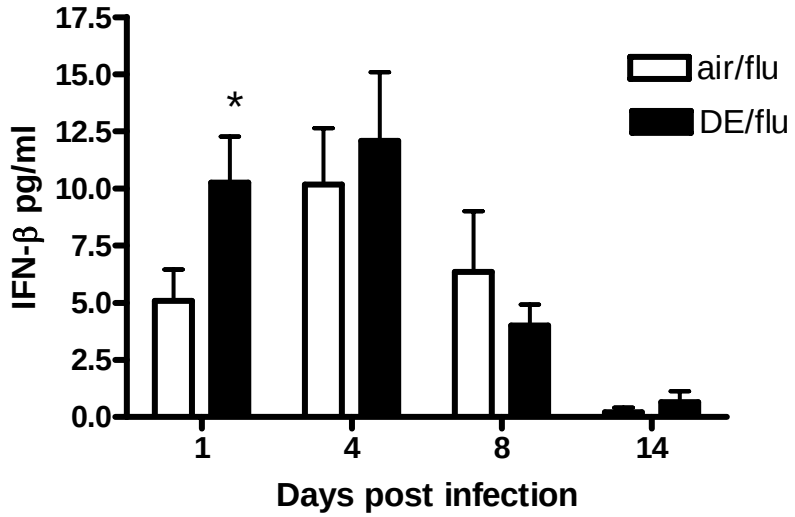
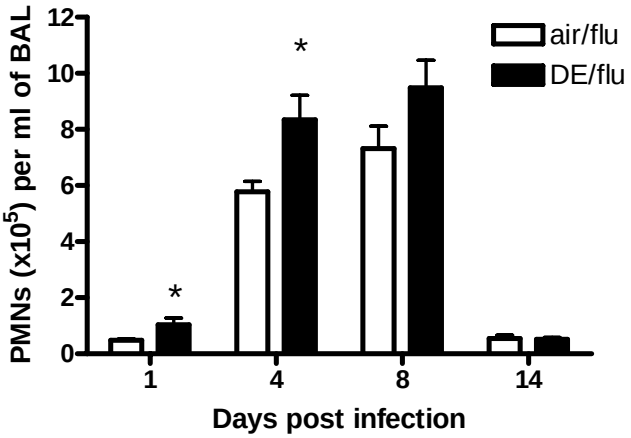


Figure 3: Effect of DE on the influenza-induced interferon response. IFN-β in BAL measured by ELISA. * is significantly different from air/flu control $p < 0.05$.

A)



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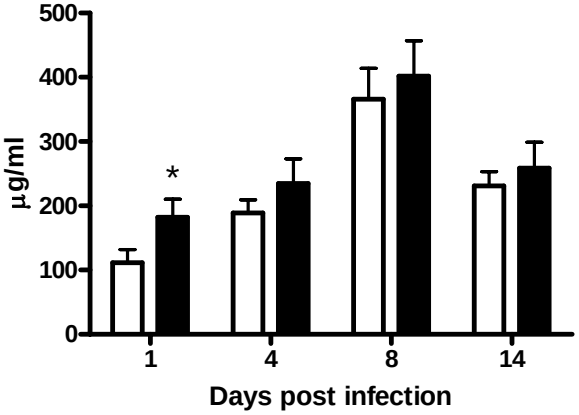

Figure 4: Effect of DE on the influenza-induced inflammatory response. **A)** PMN counts per ml of BAL. **B) A)** Protein production in BAL measured by ELISA. * is significantly different from air/flu control $p < 0.05$.

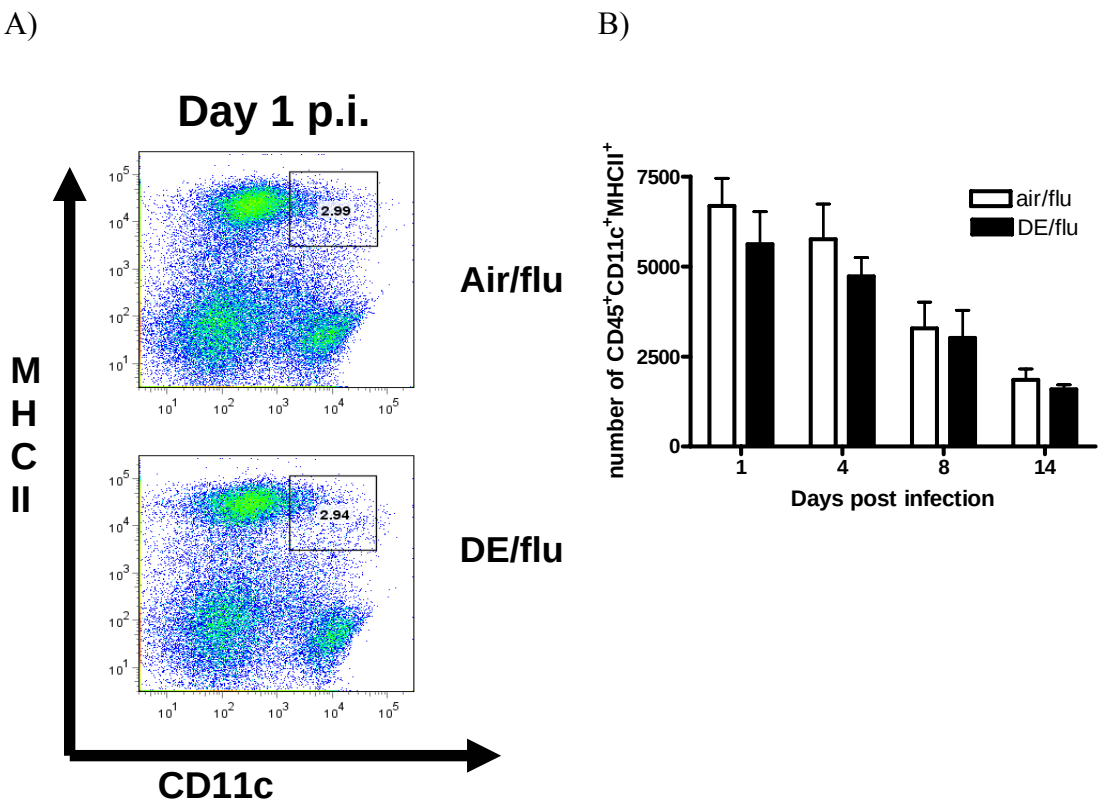

Figure 5: Effect of DE dendritic cell numbers in the lung. **A)** Scatter plot of total lung cells gated on $CD45^+$ population. **B)** Total number of cells in lung that are $CD45^+$, $CD11c^+$, $MHCII^+$. * is significantly different from air/flu control $p< 0.05$.

A) B)

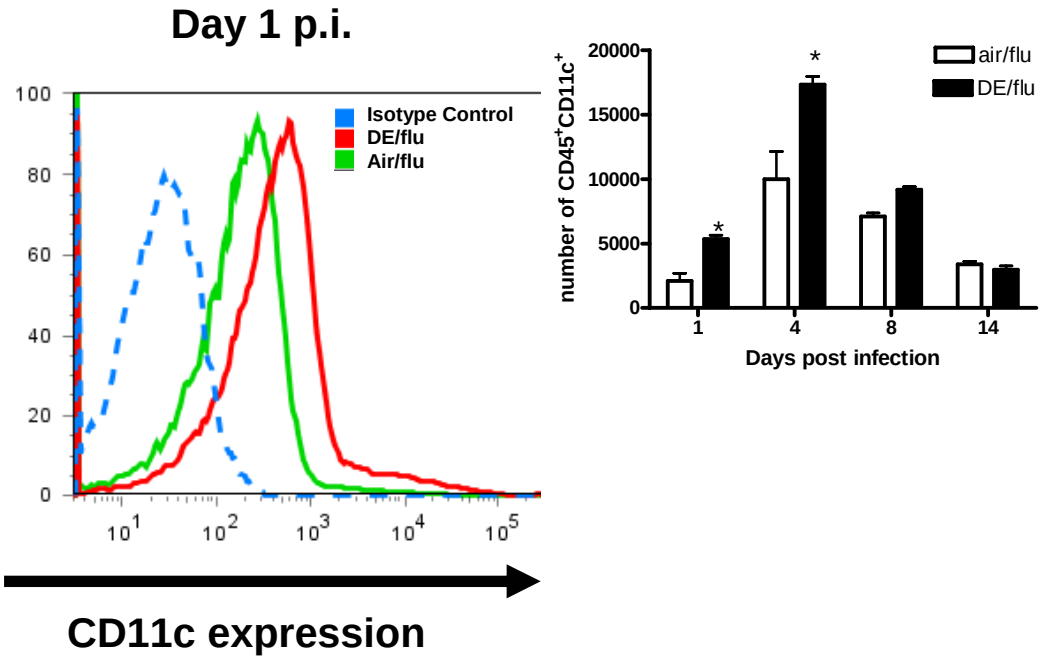

Figure 6: Effect of DE dendritic cell numbers in the draining lymph node. **A)** Scatter plot of total lymph node cells gated on $CD45^+$ population. **B)** Total number of cells in lung that are $CD45^+$, $CD11c^+$, $MHCII^+$. * is significantly different from air/flu control $p< 0.05$.

A) B)

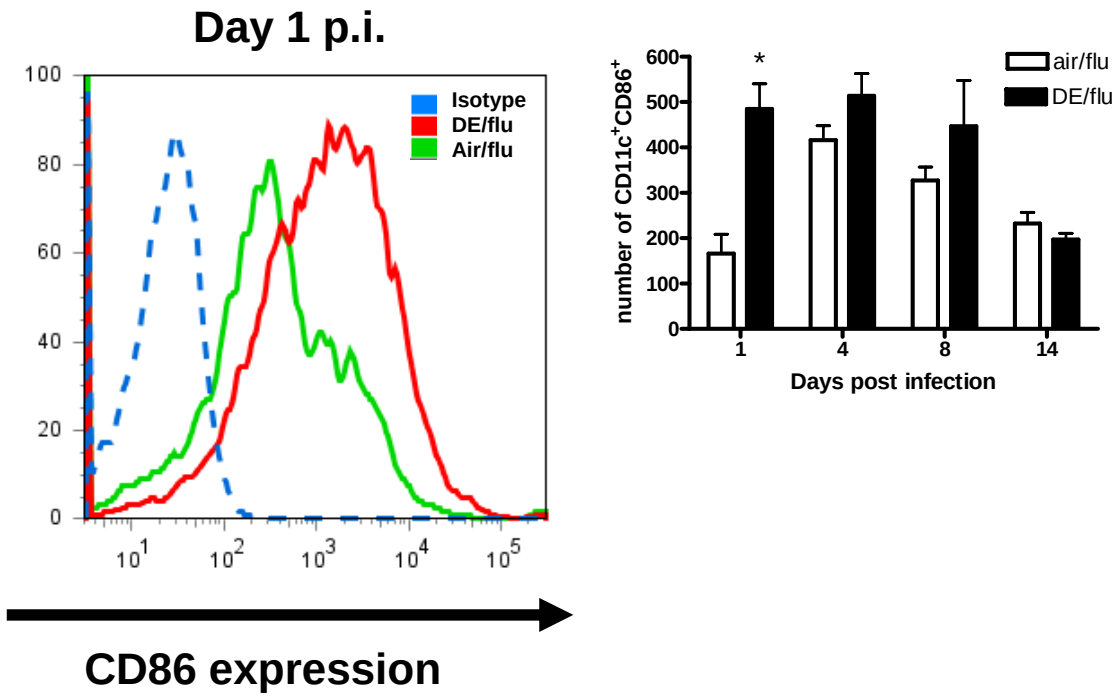

Figure 7: Effect of DE dendritic cell maturation in the draining lymph node. **A)** Scatter plot of total lung cells gated on $CD45^+$ population. **B)** Total number of cells in lung that are $CD45^+$, $CD11c^+$, $MHCII^+$. * is significantly different from air/flu control $p< 0.05$.

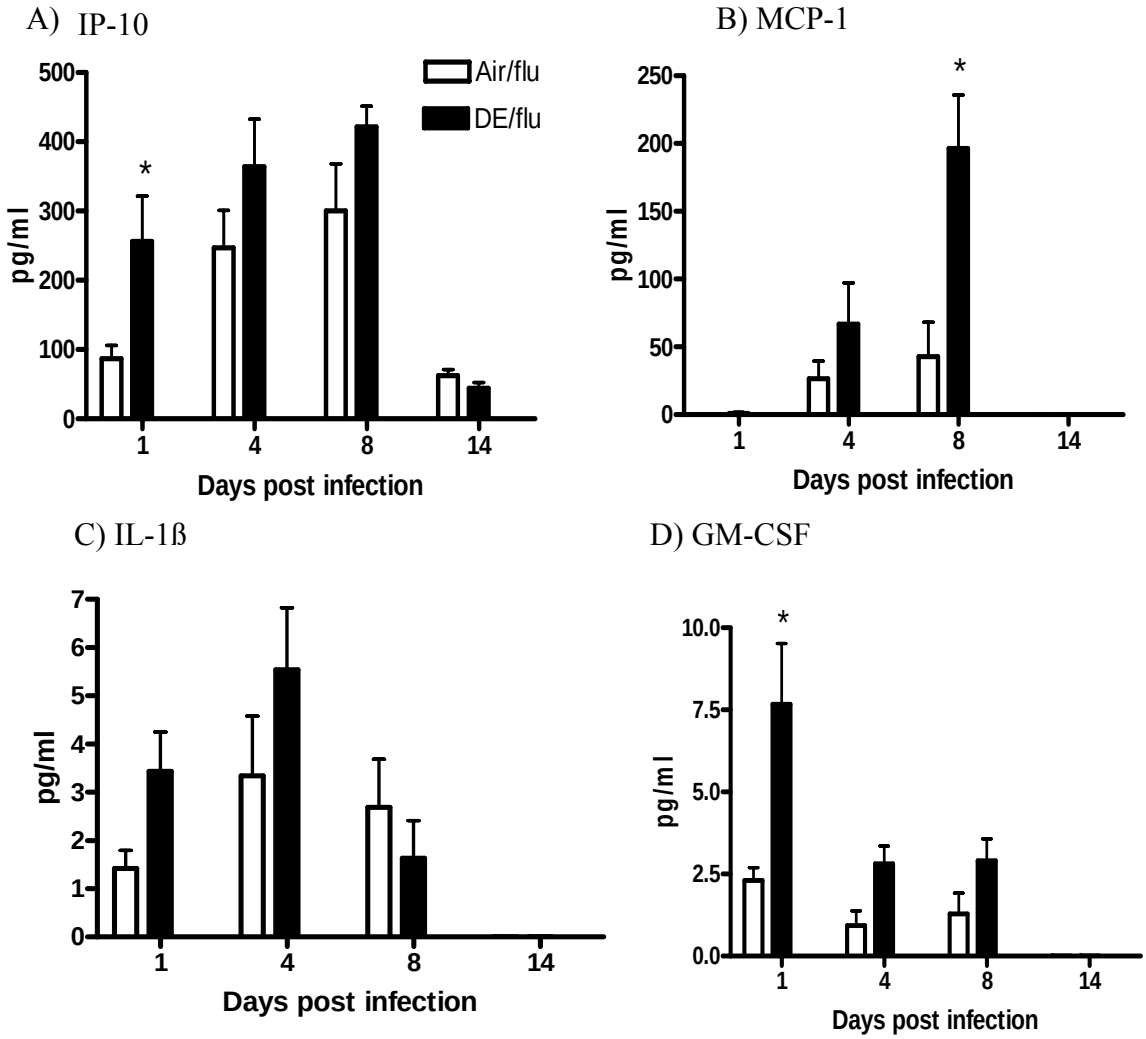

Figure 8: Effect of DE on inflammatory cytokines and chemokines associated with influenza. **A)** IP-10 **B)** MCP-1 **C)** IL-1B **D)** GM-CSF measured in the BAL by multiplex kit. * is significantly different from air/flu control $p < 0.05$.