Effect of Diesel Exhaust on the Host Response to Respiratory Viral Infection: Involvement of Toll-like Receptor 3

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ABSTRACT

Jonathan Ciencewicki: Effect of Diesel Exhaust on the Host Response to Respiratory Viral Infection: Involvement of Toll-like Receptor 3
(Under the direction of Dr. Ilona Jaspers)

Adverse effects resulting from exposure to air pollutants have become an increasing problem worldwide. Particularly levels of air pollutants have been associated with increases in the susceptibility and response to many respiratory diseases. Of the numerous air pollutants, diesel exhaust (DE) has become a major concern since it can account for a significant amount of the pollutants generated by motor vehicles in many areas. Previous results in our laboratory have demonstrated that prior exposure of respiratory epithelial cells to DE enhances the susceptibility to influenza infections. In this work, these results were confirmed and expanded upon both *in vitro* and *in vivo*. The effects of DE on the expression and signaling of toll-like receptor 3 (TLR3), which has previously shown to be needed for a complete innate immune response to influenza infections as well as other respiratory viral infections, were examined as well as mechanistic aspects involved in the observed effects. Increased expression and signaling of TLR3 was observed in respiratory epithelial cells exposed to DE prior to infection with influenza, resulting in an enhancement of the influenza-induced inflammatory and interferon (IFN) responses. A positive-feedback loop involving type I IFNs and DE-induced effects on epithelial cell tight junctional complexes played a role in the observed effects. *In vivo* exposure of mice to DE enhanced the susceptibility to influenza infections and decreased expression of surfactant proteins A and D, which are an important part of the innate immune defense against influenza and other

respiratory viruses. Together, these results demonstrate that exposure to an oxidant air pollutant, such as diesel exhaust, can enhance the susceptibility and response to respiratory viral infection and elucidate potential mechanisms behind some of these effects.

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ABBREVIATIONS

BAL: Bronchoalveolar lavage

DE: Diesel exhaust

DE_{as}: Aqueous-trapped solution of diesel exhaust

DEP: Diesel exhaust particle

dsRNA: double-stranded RNA

HAU: Hemagglutination unit

IL: Interleukin

IFN: Interferon

IFNAR: Interferon alpha receptor

IRF: Interferon regulatory factor

ISGF: Interferon-stimulated gene factor

ISRE: Interferon-stimulated response element

JAK1: Janus-activated kinase 1

LDH: Lactate dehydrogenase

NF-κB: Nuclear factor kappa B

PI3K: Phosphatidylinositol-3 kinase

PKR: Eukaryotic translation initiation factor 2-alpha kinase 2

PM: Particulate matter

PMN: Polymorphonuclear cell

Poly(I:C): polyinosinic acid:polycytidylic acid

RANTES: Regulated upon activation, normal T-cell expressed, and secreted

RIG-I: Retinoic acid-inducible protein I

RSV: Respiratory Syncytial Virus

SP: Surfactant protein

STAT: Signal transducer and activator of transcription

TLR: Toll-like receptor

TRAF6: Tumor necrosis factor (TNF) receptor-associated factor 6

Tyk 2: Tyrosine kinase 2

TBK1: TANK-binding kinase 1

TIR: Toll-IL-1 receptor

TRIF: TIR domain-containing adaptor inducing IFN-β

α: alpha

β: beta

γ: gamma

μ: micro

ω: omega

Chapter 1

Introduction

Air pollution has become an increasing concern in many areas worldwide. Despite current regulations, which limit the levels of certain air pollutants, there are still a number of adverse health effects that result from exposure to these agents. Numerous epidemiological studies have noted an association between the levels of air pollution and hospital admissions for a variety of different health reasons, including various respiratory diseases, such as asthma, chronic obstructive pulmonary disease (COPD), and respiratory infections (6, 48, 58, 106, 211, 251). In addition to an increase in hospital admissions, studies have also noted an association between the levels of air pollution and mortality (4, 12, 17, 45, 96, 168, 182, 186). Together these studies provide compelling evidence that the health risks from exposure to ambient air pollution are serious, especially for susceptible populations such as children and the elderly (58, 96, 182, 211).

"Criteria" Air Pollutants

The United States Environmental Protection Agency (U.S. EPA) is required by the Clean Air Act to set National Ambient Air Quality Standards (NAAQS) for certain pollutants, which are considered to be harmful to human health and the environment. The six "criteria" pollutants, for which there are NAAQS regulated by the U.S. EPA are carbon monoxide (CO), lead (Pb), nitrogen dioxide (NO_2), particulate matter (PM), ozone (O_3), and sulfur dioxide (SO_2) (232). Epidemiological studies have noted associations between the levels of many of these pollutants and hospital admissions for respiratory causes (58, 136, 211, 219) as

well as increased respiratory morbidity and mortality (12, 61, 168, 182, 185, 186). Additionally, levels of some of these pollutants have also been associated with decrements in lung function and exacerbations of allergic diseases, such as asthma (59, 136, 140, 216, 219, 231). Furthermore, experimental studies have shown that exposure to pollutants causes an inflammatory response in the lungs (139, 142, 164, 188).

In addition to the effects mentioned above, exposure to several of these pollutants has also been shown to enhance the susceptibility or alter the response to respiratory viral infections. Epidemiological evidence has demonstrated that increased levels of certain air pollutants are associated with the incidence of and hospital admissions for chronic cough, bronchitis, croup, pneumonia, influenza, as well as other upper respiratory infections (46, 58, 211, 252). Experimental studies examining the effects of air pollutant exposure on the susceptibility and response to viral infection have shown that exposure to air pollutants can increase the susceptibility to infection, enhance the morbidity, including an increased inflammatory response, associated with infection, as well as decrease macrophage function (54, 83, 176, 210). Together these data provide evidence of the broad range of effects induced by exposure to air pollutants.

Diesel Exhaust

A particular air pollutant that has become a concern is diesel exhaust (DE), since it can account for a significant percentage of air pollutants generated by motor vehicles in many places (42, 234, 235) and epidemiological data and experimental studies suggest that DE is a major contributor to adverse health effects associated with exposure to particulate air pollutants (235, 249). Diesel engines are widely used for land and sea transport, electrical power, farming, and construction, due to their fuel efficiency and durability. In fact, at full

load diesel engines use about 70% of the amount of fuel that a gasoline engine would consume under the same conditions, and at partial loads the advantage is even greater (52, 220). However, PM emissions from diesel engines are relatively high compared with those from gas engines (34, 248). A majority of the diesel PM and NO_x emissions are produced by off-road and heavy-duty on-road vehicles (233) and studies from California have shown that while on-road diesel vehicles contribute about 5% of daily vehicle miles traveled they are responsible for about 35% and 56% of the NO_x and PM produced by vehicle emissions, respectively (23). Ambient DE particle (DEP) concentrations range from ~5-50 $\mu g/m^3$, whereas occupational concentrations, such as mechanics, truck drivers, or bus garages, have been shown to be anywhere from ~25-200$\mu g/m^3$ and even as high as 1-2 mg/m^3 in some mining occupations. Furthermore, off-road diesel engines emit about 2 times the amount of PM as on-road vehicles, making them a serious concern for PM-induced health effects (23)

DE contains both particulate phase as well as gas phase components such as CO, SO_2, NO_x, hydrocarbons, transition metals, and formaldehyde (194). The exact composition depends on operating conditions such as speed, load, fuel, engine and vehicle type, air temperature, and humidity. DEPs consist of a carbon core with many organic compounds adsorbed onto them. Due to their small mass median diameter (0.05-1.0μm), DE particles are easily respirable and capable of being deposited in the lower airways and alveoli to a greater extent than larger particles (32, 209). It has been estimated that at least 80% of freshly emitted DEPs are $\leq 0.1\mu m$, which translates into a much greater surface to volume ratio (184). Therefore, a given mass of DEPs has, more particles, a larger surface area to adsorb compounds onto their surface, and can affect a greater surface area of lung tissue, than the equivalent mass of larger particles (151). Additionally, DEPs have the potential to travel

greater distances than those generated by gasoline engines, allowing them to disseminate over a much broader area (145).

There is abundant epidemiological and experimental evidence indicating that exposure to DE can cause adverse health effects, particularly in occupational exposures and susceptible populations. Studies have shown associations between levels of PM, of which DEPs can be a significant contributor, and cardiopulmonary mortality (47, 96, 168, 185). Other studies have shown an association between exposure to ambient PM and hospital admissions for cardiac and respiratory causes (4, 6, 106, 136, 219). There are a number of chemicals in DE known as human carcinogens and the National Institute of Occupational Safety and Health (NIOSH) recommends that DE be regarded as a potential carcinogen. Epidemiological evidence has noted an association between long-term occupational exposure to DE and increased risk for lung cancer (24, 119). In addition, both *in vitro* and *in vivo* studies have shown that exposure to DE or DEPs results in increased pulmonary inflammation and the production of reactive oxygen species (9, 43, 150, 179, 180, 215). Taken together, this evidence shows a wide range of adverse health effects caused by exposure to DE.

Diesel Exhaust and Respiratory Viral Infections

DE has also been implicated in having adverse effects on host immunity. It has been shown that DE increases cellular responsiveness to histamine (75, 100) and acts as an adjuvant to IgE production in human nasal exposures and murine models (44, 55, 143, 149). In addition, epidemiological evidence has noted an association between ambient particulate matter and pulmonary infections (166), including exacerbation of respiratory symptoms associated with these infections (30, 58, 155). Exposure to DE can also enhance the susceptibility to respiratory viral infections. The first study to examine the effects of chronic

DE exposure on the susceptibility to respiratory viral infection was conducted by Hahon et al. (69). Mice were exposed to 2mg/m^3 of diesel exhaust for 1, 3, or 6 months followed by infection with influenza virus. Higher virus growth levels and decreased interferon levels were observed in mice exposed to DE for 3 and 6 months compared to air-exposed mice. Additionally, antibodies against hemagglutinin, an influenza-associated protein, were lower in these mice. Another study examined the effect of short-term DE exposure on the susceptibility and response to RSV infection in mice (71). Mice were exposed to 300μg/m^3 or 1,000μg/m^3 of DE for 6 hours/day for seven consecutive days followed by infection with RSV. DE-exposed mice were more susceptible to RSV infection, shown by an increase in RSV gene expression in the lungs of mice exposed to either dose of DE compared to air controls. Prior exposure to DE also caused an enhancement of the inflammatory response to RSV infection. Additionally, studies in our laboratory have shown that exposure of human respiratory epithelial to DE enhances the susceptibility to influenza infections (85). Together, these studies demonstrate that exposure to DE enhances the susceptibility and response to respiratory viral infections, and may result in an increase of the resulting morbidity of infection. However, the mechanism(s) whereby exposure to DE modulates the host response to respiratory virus infections is not yet clear.

Influenza

Influenza infections in the U.S. account for approximately 36,000 deaths and over 100,000 hospitalizations each year, despite large-scale vaccination and antiviral treatments (226, 227). Influenza is a single-stranded, segmented RNA virus that is a member of the orthomyxoviridae family. The virus is classified as Influenza A, B, and C, with Influenza A being the most pathogenic. Influenza A consists of eight RNA segments that encode for 10

viral proteins: hemagglutinin (HA), neuramindase (NA), nucleoprotein (NP), matrix protein (M1), 3 polymerase proteins, an ion channel protein, and 2 nonstructural proteins. The outer part of the virus consists of a lipid bilayer coated with protein "spikes" which are HA and NA. These two proteins are the common epitopes for host antibody recognition. Influenza A usually binds to and infects epithelial cells of the respiratory tract, but can also infect monocytes/macrophages and leukocytes. The virus binds to sialic acid-containing receptors on the host cell through it's HA molecules. Once the virus has attached to the cell it gains entry through clathrin-dependent endocytosis. Recent studies have also shown that influenza can enter the cell by other endocytic pathways as well (204). Infected cells respond to Influenza by releasing chemokines and cytokines. Epithelial cells commonly produce RANTES, IL-6, IL-8, and MCP-1 to recruit and activate inflammatory cells as well as type I interferons (IFN-α and IFN-β), which activate anti-viral responses within the cell.

Innate Immune Response to Influenza Infection

The host's first line of defense against invading pathogens is the innate immune response. It keeps the infection under control until the adaptive immune response is mobilized. Many of the cytokines released upon infection have pleiotropic effects, but for the purpose of this review only their actions in response to viral infection will be discussed. IL-6 is a potent pyrogen that activates T cells and stimulates Ig production in B cells. IL-8 is a neutrophil attractant, MCP-1 recruits monocytes, and RANTES attracts T lymphocytes and monocytes. Neutrophils and macrophages recruited to the site of infection mediate an inflammatory response and phagocytose virus and dead or dying cells. Secreted type I interferons activate macrophages and NK cell function and enhance antigen presentation through upregulation of MHC expression and stimulation of maturation of antigen presenting cells. This important

antiviral cytokine also induces the expression of a number of genes involved in inhibiting viral replication. The type I IFN system plays a critical role in the innate immune response to viral infection and will be discussed in further detail later on. IL-18, another cytokine produced in response to influenza infection, has pro-inflammatory properties and also activates NK and T cell IFN-γ production. IFN-γ also activates NK cells and macrophages, and functions in the long-term control of viral infections. Recently another group of IFN-like cytokines were identified and termed IL-28 and IL-29. These cytokines are induced upon viral infection and display antiviral properties (203). Many of the cytokines and chemokines mentioned thus far are the end result of signaling cascades elicited by pattern recognition receptors (PRRs), which recognize molecular patterns associated with the influenza virus. The PRRs that are thought to play a role in the innate immune response to influenza infection are toll-like receptor 3 (TLR3), retinoic acid-inducible protein I (RIG-I), and eukaryotic translation initiation factor 2-alpha kinase 2 (PKR).

Another important part of the host's innate immune defense against influenza are the surfactant proteins (SP). These hydrophilic proteins, SP-A and SP-D, are secreted by alveolar type II cells and clara cells in the lung and facilitate phagocytosis, as well as regulate inflammation (124, 125). Defensins are another group of proteins, which mediate antiviral responses. These proteins are produced by a variety of cell types, including epithelial cells, and induce pro-inflammatory mediator production as well as the release of other cytokines important for innate antiviral defenses (237, 255). Again, this summary of the innate immune response to influenza is in no way a comprehensive review of the host's antiviral response. Instead it covers the main aspects of antiviral defenses and gives a general understanding of the topics, which will be further investigated herein.

Toll-like Receptors

Toll-like receptors (TLRs) are an important first recognition step in response to bacterial and viral infection. These receptors are members of the Interleukin-1 receptor (IL-1R) superfamily and share homology in the cytoplasmic region referred to as the Toll/IL-1R (TIR) domain. The TIR domain serves as a scaffold for other proteins to initiate a common signaling process. To date, 11 members of this family have been identified in mammals. The receptors can be located on the cell surface bound to the plasma membrane or intracellularly associated with endosomal membranes. TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) and downstream signaling leads to the production of innate immune defense mediators as well as activation of adaptive immune responses (1, 2). Toll-like receptor 3 (TLR3) is activated by double-stranded RNA (dsRNA), a molecular pattern commonly associated with viral infection, and mediates the activation of numerous downstream signaling proteins and transcription factors, ultimately eliciting production of inflammatory cytokines and type I interferons (3, 132).

TLR3 mRNA has been found in human lung, liver, heart, placenta, pancreas, as well as brain (174). It is also expressed in dendritic cells, NK cells, intestinal epithelial cells, as well as airway and respiratory epithelial cells (27, 67, 79, 144, 201, 240). Localization of TLR3 within the cell is variable. Surface or cytoplasmic expression of TLR3 has been demonstrated in various cell types (67, 131, 132, 201). The most likely explanation for these differences is that TLR3 expression as well as its localization is cell type specific. Additionally, expression of TLR3 has been shown to be up-regulated in dendritic cells as well as in bronchial and respiratory epithelial cells by viral infection or dsRNA treatment in the form of polyinosinic:polycytidylic acid (poly(I:C)) (67, 103, 131, 177, 223). There is also

recent evidence that in addition to causing up-regulation, viral infection also causes a shift in the localization of TLR3 from cytoplasmic to surface expression (65).

TLR3 Signaling

The actual signal cascade elicited by TLR3 differs between cell types and viruses. For the purpose of this review a simplified overview of the TLR3 signal cascade will be given (see figure 1A) and discrepancies that may exist will be pointed out in the text. It is generally accepted that once TLR3 is activated by dsRNA it recruits the adaptor protein TIR domain-containing adaptor inducing IFN-β (TRIF) through an interaction of TIR domains in each protein (154, 254). Phosphorylation of TLR3 on tyrosine residues is an important step for this recruitment (189, 190). The TLR3 signaling cascade diverges at TRIF with one branch of the signaling cascade leading to the activation of nuclear factor-kappa B (NF-κB) with subsequent of pro-inflammatory cytokines and the other branch of the signaling cascade leading to the activation of interferon regulatory factor (IRF) 3 and IFN-β production. The inflammatory pathway involves tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6 which is recruited and bound by TRIF (90, 191). TRAF6 forms a complex with TAK1, TAB 1 and 2, and possibly eukaryotic translation initiation factor 2-alpha kinase 2 (PKR), which leads to the activation of NF-κB (90). There is also evidence that receptor interacting protein (RIP) -1 is recruited by TRIF and is needed for NF-κB activation (134). The other branch of the TLR3 signaling pathway results in the production of type I interferons resulting in the activation of IRF3, which translocates to the nucleus and binds the interferon response element (ISRE) of the IFN-β promoter thereby activating its transcription (49, 247). Activation of IRF3 is achieved by a dual phosphorylation. It is generally accepted that TANK-binding kinase (TBK) 1, which is also recruited by TRIF, is one of the kinases

that phosphorylates IRF3 (53, 202). The additional phosphorylation of IRF3 is thought to be mediated by Akt or one of its downstream components. Upon activation, TLR3 is phosphorylated on tyrosine residues and recruits Phosphatidylinositol-3 kinase (PI3k), which then activates Akt (190). Akt or a downstream component of Akt then phosphorylates IRF3 leading to its complete activation, dimerization, and nuclear translocation. There is also evidence that IKKε provides the second phosporylation event (53, 202), but its function may be redundant with that of TBK1 (158).

Type I interferon signaling

Interferons (IFNs) are cytokines that have anti-viral, anti-proliferative, and immunomodulatory effects. These molecules are important role in the innate immune defenses against invading pathogens and also activate adaptive responses. There are a number of type I IFNs found in humans, including IFN-α, of which there are 13 subtypes, IFN-β, IFN-ε, IFN-κ, and IFN-ω. Once secreted, type I IFNs bind the IFNα receptor (IFNAR), which activates a signaling cascade that results in the transcription of numerous interferon-stimulated genes (ISGs) (see figure 1B). Binding of type I IFNs to the IFNAR results in a dimerization of the receptor subunits, IFNAR1 and 2, leading to autophosphorylation and activation of associated proteins: Janus activated kinase 1(JAK1) and tyrosine kinase 2 (TYK2) (reviewed in 165). These in turn activate signal transducer and activator of transcription (STAT) 1 and 2, which form a complex along with IRF9 known as IFN-stimulated gene factor 3 (ISGF3). ISGF3 then translocates to the nucleus where it binds with ISREs to initiate gene transcription. Recently, there has been evidence of alternative pathways and protein components, but the scope of this information is beyond this review.

The pathway described here is the main type I IFN signaling pathway and will be the one we focus on in elucidating the involvement of type I interferons in TLR3 expression.

Summing Up

Epidemiological studies have noted an association between levels of air pollutants and the incidence of respiratory infections. Furthermore, experimental studies have shown that exposure various air pollutants can enhance the susceptibility and response to a number of respiratory diseases. Considering that the most common form of illness in the U.S. and Europe is respiratory allergies and infections, which account for more missed work as well as school days than any other disease, it is important to understand the associations between, the potential effects of, and the mechanisms involved in air pollution-induced exacerbation of respiratory infections. Therefore, this dissertation will focus on elucidating the effects of diesel exhaust exposure on the host response to respiratory viral infection. Specifically, the effect of DE exposure on the susceptibility and response to influenza infections was examined both *in vitro* and *in vivo* as well as some of the mechanistic aspects involved in the observed effects. The results presented herein provide evidence that exposure to DE can enhance the susceptibility to influenza infections as well as the innate immune response to viral infection through an increase in the expression and signaling of TLR3.

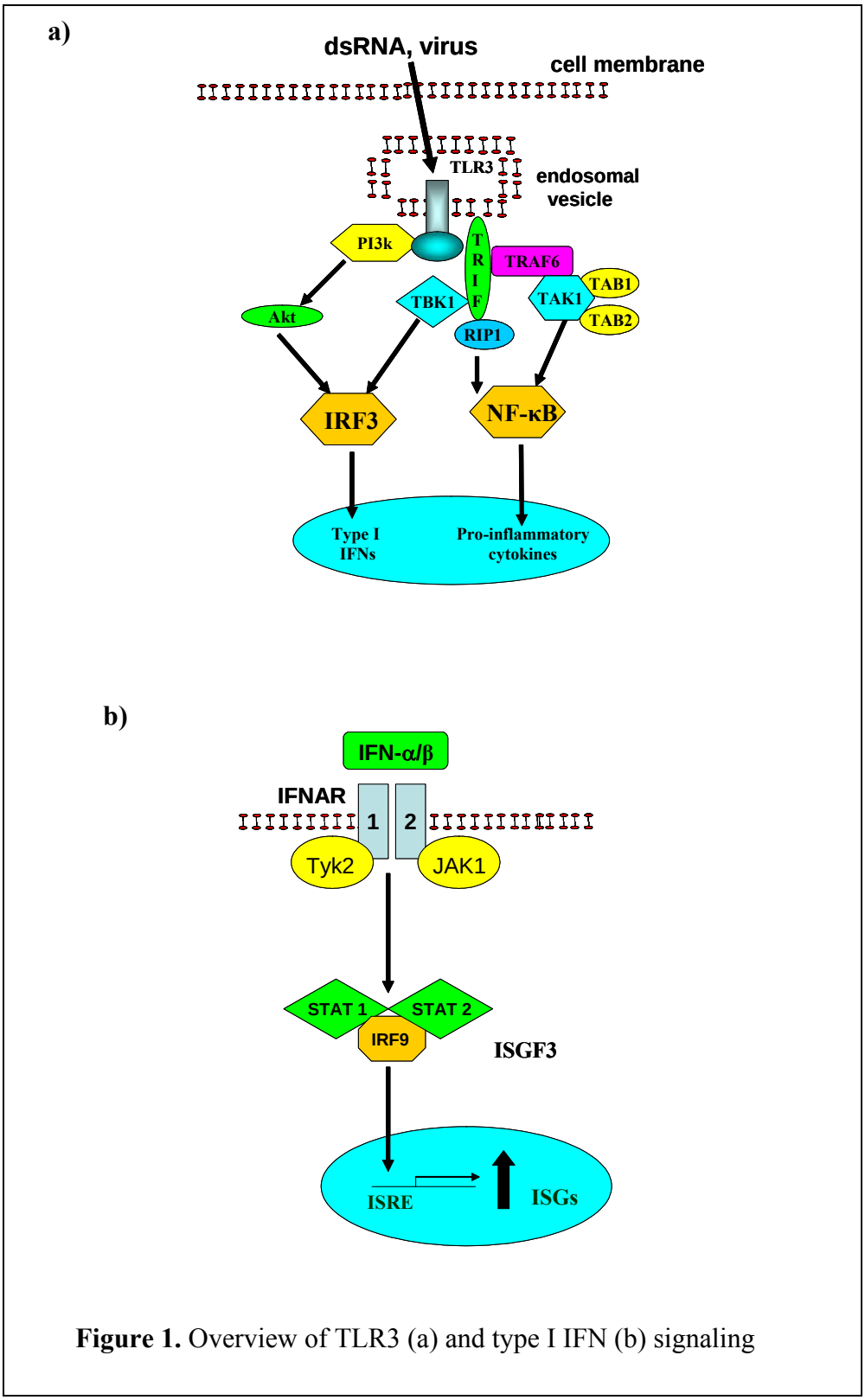


Figure 1. Overview of TLR3 (a) and type I IFN (b) signaling

Chapter 2

Diesel Exhaust Enhances Virus- and poly(I:C)-induced Toll-like Receptor 3 Expression and Signaling in Respiratory Epithelial Cells

Introduction

Adverse health effects induced by exposure to air pollution have become an increasing problem in many areas worldwide. Although many different emission sources account for the complex air pollution mixture in urban environments, diesel exhaust (DE) has raised a fair amount of concern, since it can account for a significant percentage of air pollutants generated by motor vehicles in many places (42, 234). In addition, epidemiological data and experimental studies suggest that DE is a major contributor to adverse health effects associated with exposure to particulate air pollutants (235, 249). Due to their small mass median diameter (0.05-1.0μm), DE particles are easily respirable and capable of being deposited in the lower airways and alveoli (32, 209). The carbonaceous core of these particles can absorb around 18,000 chemical compounds onto it, including polyaromatic hydrocarbons, which are suspected to play a major role in the adverse health effects induced by exposure to DE (250). Additionally, it has been shown that exposure to DE and DE particulates increases neutrophil recruitment and pulmonary inflammation in both rodents and humans (93, 148, 150, 178, 179, 180, 183, 257). Furthermore, DE has also been implicated in having adverse effects on host immunity. It has been shown that DE increases the cellular responsiveness to histamine (75, 100) and acts as an adjuvant to IgE production

(44, 55, 143, 149, 230). In addition, epidemiological evidence has noted an association between ambient particulate matter and pulmonary infections (166), including exacerbation of respiratory symptoms associated with infection (30, 58, 155). Exposure to DE can also enhance the susceptibility to respiratory virus infections. Specifically, our previous studies have demonstrated that exposure to an aqueous-trapped solution of DE (DE_{as}) enhances the susceptibility to influenza infections in human respiratory epithelial cells (85). Other studies have shown that mice repeatedly exposed to DE showed an increased susceptibility to infection with RSV, as shown by increased inflammatory responses and levels of viral titers following infection with RSV (71). These studies indicate that exposure to DE can increase the susceptibility and exacerbate responses to respiratory virus infections. However, the mechanism by which exposure to DE modulates the host response to respiratory virus infections is not yet clear.

The host's first line of defense against an invading pathogen is the innate immune response. It activates the secondary immune response and keeps the infection under control until the adaptive response is mobilized. An integral role in the innate immune response is played by toll-like receptors (TLRs). These receptors are members of the superfamily of Interleukin-1 receptors (IL-1R) and share homology in the cytoplasmic region referred to as the Toll/IL-1R (TIR) domain. TLRs recognize conserved pathogen-associated molecular patterns (PAMPs); recognition leads to the production of innate immune defense mediators as well as activation of the adaptive immune response (1, 2, 84). Toll-like receptor 3 (TLR3) recognizes double-stranded RNA (dsRNA), a molecular pattern commonly associated with viral infection. DsRNA stimulates TLR3 signaling, which culminates in the activation of numerous downstream signaling proteins and transcription factors and ultimately results in

production of inflammatory cytokines and type I interferons (3, 132). Expression of TLR3 has been shown to be up-regulated in dendritic cells as well as in bronchial and respiratory epithelial cells by viral infection or dsRNA treatment in the form of polyinosinic:polycytidylic acid (poly(I:C)) (67, 103, 131, 177, 223).

The respiratory epithelium is a common target for both DE as well as invading respiratory viruses. Since TLR3 plays such an integral role in the recognition and primary response to viral pathogens the purpose of this study was to examine the effects that pre-exposure to DE had on TLR3 expression and signaling in respiratory epithelial cells. Specifically, we examined whether exposure to an aqueous-trapped solution of DE (DE_{as}) enhances TLR3 expression and TLR3-dependent signaling in response to dsRNA or influenza virus infections in human respiratory epithelial cells. The results demonstrate that exposure to DE_{as} significantly increased TLR3 expression as well as TLR3-mediated innate immune responses in epithelial cells infected with influenza virus or treated with dsRNA.

Materials and Methods

Cell Culture

A549 cells, a human pulmonary type II epithelial-like cell line were cultured in F12K medium plus 10% fetal bovine serum and 1% penicillin and streptomycin (all from Invitrogen, Carlsbad, CA). For treatment with DE_{as} and stimulation with poly(I:C), A549 cells were grown in 6 or 24-well plates. When the cells reached about 80% confluency and about 18-24h before exposure to DE_{as} and stimulation with poly(I:C), the cell culture media was exchanged for serum-free F12K plus 1.5μg/ml BSA plus antibiotics. In some experiments 1μM wortmannin (Calbiochem, La Jolla, CA) was added 30 minutes before treatment with DE_{as}. Primary human bronchial epithelial cells were obtained from healthy nonsmoking adult volunteers by cytologic brushing at bronchoscopy. Primary human nasal epithelial cells were obtained from healthy nonsmoking adult volunteers by gently stroking the inferior surface of the turbinate several times with a Rhino-Probe curette (Arlington Scientific, Arlington, TX), which was inserted through an otoscope with a large aperture. The protocols for the acquisition of both primary human bronchial and nasal epithelial cells were reviewed by the University of North Carolina Institutional Review Board. Both primary human bronchial and nasal epithelial cells were expanded to passage 2 in bronchial epithelial growth medium (BEGM, Cambrex Bioscience Walkersville, Inc., Walkersville, MD) and then plated on collagen-coated filter supports with a 0.4 μM pore size (Trans-CLR; Costar, Cambridge, MA) and cultured in a 1:1 mixture of bronchial epithelial cell basic medium (BEBM) and DMEM-H with SingleQuot supplements (Cambrex), bovine pituitary extracts (13mg/ml), bovine serum albumin (BSA, 1.5 μg/ml), and nystatin (20 units). Upon confluency, all-trans retinoic acid was added to the medium and air liquid interface (ALI)

culture conditions (removal of the apical medium) were created to promote differentiation. Mucociliary differentiation was achieved after 18-21 days post-ALI.

Generation of A549-TRAF6 DN cells

A549 cells stably transduced with dominant negative TRAF6 were prepared as follows. The sequence encoding a truncated form of TRAF6 was digested out of the plasmid pCIneo-TRAF6 DN (TRAF6 289-530), kindly provided by Dr. R. Medhzitov's lab (Howard Hughes Medical Institute, Yale University Medical School, New Haven, CT, U.S.A.) (26) using *EcoR*I and *Sal*I restriction enzymes (New England Biolabs, Ipswich, MA) and ligated into *EcoR*I and *Sal*I digested pBabe-puro, which was a kind gift from Dr. A. Baldwin (Lineberger Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, U.S.A.). The pBabe-puro-TRAF6 DN retroviral vector was transfected into retroviral packaging PT67 cells and stably transfected cells were selected using 1μg/ml puromycin (Sigma). As a control, pBabe-puro alone was transfected into PT67 cells. Retroviral-laden supernatants were harvested periodically and stored at -80^{o}C until use. Immediately before use, supernatants were filtered through 0.45μm filters and supplemented with 8μg/ml polybrene (Sigma). A549 cells grown to about 50% confluency were transduced using the retrovirus containing supernatants and stably transduced A549 cells were selected using 1μg/ml puromycin for about two weeks. Expression of TRAF6 DN in stably transduced A549 cells was confirmed by Western blotting.

Exposure to aqueous-trapped solution of Diesel Exhaust (DEas)

DE_{as} was generated as described before (123). Briefly, emissions were taken from a Caterpillar diesel engine, model 3304, which was used to power a 113 KW generator. This type of engine was chosen because it is used in non-road vehicles, which are significant

contributors to ambient diesel exhaust levels and because the projected trend for emissions from non-road diesel engines was expected to remain at the same level or decline slowly in the future at the time of experimental design (235). The diesel exhaust emissions from this Caterpillar diesel engine were passed through a tubing system with a filter impactor and 2 impinger tubes (containing 100 ml PBS each) submerged in an ice bath. Impinger glassware had been washed and heated to remove and destroy endotoxin. Of the two impinger tubes, the emissions (at 10 L/min) that entered and remained in the first (primary) tube, but not the secondary tube, were utilized for the cell exposure studies. Extracts were generated and collected during a one-hour period when the engine was under high load (HL). This type of preparation was chosen, because it contains both DE particles as well as polar and thus water-soluble DE gas-phase components. To determine the mass of the emissions retained within the PBS in an impinger tube, an aliquot was dried overnight at 56ºC, and corrected for the mass of the PBS contribution (which was determined in a similar manner by overnight drying) and dilution with water from the exhaust. Aliquots of the DE_{as} were kept at - 80ºC until use. For all cell types used in this study, DE_{as} was added 2 hours before infection with influenza or treatment with poly(I:C). Specifically, for the differentiated human nasal and bronchial epithelial cells, DE_{as} was diluted in 200µl media to achieve 22 or 44µg DE_{as} per cm^2 of cell layer and added to the apical side. After the 2 hours incubation with DE_{as}, the diluted DE_{as} was removed and influenza virus or poly(I:C) was diluted in the same volume of media was added to the apical side for 2 hours, after which it was removed to establish ALI culture conditions again. For the experiments using A549 cells, DE_{as} was diluted in F12K media plus BSA plus antibiotics to achieve 25µg/cm^2 and added to the cells. After 2 hours incubation with DE_{as}, poly(I:C) was added to the cells. The effects of exposure to DE_{as} on

cell viability were assessed by analyzing cell culture supernatants for lactate dehydrogenase (LDH) activity using a commercially available kit according to the supplier's instructions (CytoTox 96®, Promega, Madison, WI).

Exposure to different diesel exhaust preparations, extractable organic material (EOM), and particle solutions

Diesel Exhaust was collected as previously described (25). Briefly, standard reference material (SRM) 2975 was obtained from the National Institute of Sciences and Technology (NIST, Minneapolis, MN) and was generated from a heavy-duty diesel forklift engine and collected under "hot" conditions. Comp-DEP was obtained from Dr. Ian Gilmour (U.S. EPA) and was generated from a 30kW (40hp) 4 cylinder diesel engine powering a compressor. Particles were weighed then dissolved in PBS + 0.005% BSA. Mt. St. Helens Dust (MSHD) and ultrafine carbon particles were used as particle controls. For A549 cells, particle solutions were diluted in F12K media plus BSA plus antibiotics to achieve 25μg/cm^2 and added to the cells. After 2 hours incubation with particle solutions, poly(I:C) was added to the cells. For experiments involving the EOM of Comp-DEP, cells were treated with an amount of extract that was equivalent to the amount, which would be present in a dose of 25μg/cm^2 of Comp-DEP assuming that this preparation contains 30% organic material. For these experiments vehicle control cells were exposed to the equivalent volume of DMSO. EOM or DMSO was diluted in the media and added to the cells for 2 hours prior to the addition of poly(I:C).

Infection with influenza or treatment with poly(I:C)

Throughout this study we used influenza A/Bangkok/1/79 (H3N2 serotype) which 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 described before (11). Stock virus was aliquoted and stored at -80^{o}C until use. Unless otherwise indicated, for infection of differentiated bronchial or nasal cells approximately 3 X10^{5}cells were infected with 320 (hemagglutination units) HAU of influenza A Bangkok 1/79. Cells were treated with 100 μg/ml of polyinosinic acid:polycytidylic acid (poly(I:C)) (Calbiochem; La Jolla, CA) 2 hours after exposure to DE_{as}.

Transfection with siRNA

siRNA for TLR3, PKR, and Lamin was obtained from Dharmacon (Layfayette, CO) and cells were transfected according to the supplier's instructions. Briefly, A549 cells were transfected with 500 μg of siRNA, which was added to the cell media, 24 hours prior to infection with influenza. Targeted knock-down was confirmed by real-time RT-PCR of the specific gene product.

RT-PCR

Total RNA was extracted using TRizol (Invitrogen) as per the supplier's instruction. First-strand cDNA synthesis and real-time RT-PCR was performed as described previously (86, 87). The sequences for the primers and probes used in this study are as following: HA: probe, 5'-FAM-TGATGGGAAAAACTGCACACTGATAGATGC-TAMRA-3'; sense, 5'-CGACAGTCCTCACCGAATCC-3'; antisense, 5'-TCACAATGAGGGTCTCCCAATAG-3'; IFN-β: probe, 5'-FAM-AGCAGCAATTTTCAGTGTCAGAAGCTCCTG-TAMRA-3'; sense, 5'- CAACTTGCTTGGATTCCTACAAAG-3'; antisense, 5'-AGCCTCCCATTCAATTGCC-3';IL-6: probe, 5'-FAM-TGTTACTCTTGTTACATGTCTCCTTTCTCAGGGCT-TAMRA-3'; sense, 5'-GGTACATCCTCGACGGCATCT-3'; antisense, 5'- GTCCCTCTTTGCTGCTTTCAC- 3';

TLR3: probe, 5'-FAM-ATGCAGTTCAACAAGCTATTGAACAAAATCTGGA-TAMRA-3'; sense, 5'- ATTAAAAGACCCATTATGCAAAAGATTC- 3'; antisense, 5'-CCTCAAGGAAAACCAATATAATGGA-3'; MxA: probe, 5'-FAM-AGGCCAGCAAGCGCATCTCCAG-TAMRA-3'; sense, 5'-CAGCACCTGATGGCCTATCAC-3', antisense, 5'-CATGAAGAACTGGATGATCAAAGG-3'; GAPDH: probe, 5'-JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA-3'; sense, 5'-GAAGGTGAAGGTCGGAGTC-3'; antisense, 5'-GAAGATGGTGATGGGATTTC-3'. mRNA analyses for β-actin, RIG-I, and PKR were performed using commercially available primer/probe sets (inventoried Taqman® Gene Expression Assays) purchased from Applied Biosystems (Foster City, CA).

ELISA

Supernatants were analyzed for IL-6 using commercially available ELISA kits as per the supplier's instructions (BD Biosciences; San Diego, CA).

Western blotting

Whole cell lysates were prepared by lysing the cells in RIPA buffer containing 1% Nonidet P (NP)-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors (Cocktail Set III; Calbiochem, San Diego, CA). Nuclear extracts were prepared as described before (87). 20-100 μg of whole cell lysate or 20-50μg of nuclear extract was separated by SDS-PAGE as described before (87). This was followed by immunoblotting using specific antibodies to TLR3 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), IRF3 (1:1000, Santa Cruz Biotechnology), phospho-Akt (1:1000, Cell Signaling, Beverly, MA), or Akt (1:1000, Santa Cruz Biotechnology). β-actin (1:2000, USBiological, Swampscott, MA) was used as a loading control for TLR3. Antigen-antibody complexes were stained with anti-rabbit or anti-

mouse, horseradish peroxidase-conjugated antibody (1:4000, Santa Cruz Biotechnology) and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The chemiluminescent signals were acquired using a 16-bit CCD camera (GeneGnome system; Syngene, Frederick, MD) and visualized using the GeneSnap software (Syngene). Densitometric analysis of the optical densities was performed using GeneTools Software (Syngene).

Immunohistochemistry/Confocal Microscopy

A549 cells were grown on chamber slides (Lab-Tek® Chamber slides, Nalge Nunc International,Naperville, IL). Cells were prepared as described previously (57) with slight modifications. Briefly, cells were fixed with 4% paraformaldehyde in PBS for 30 min, and then permeabilized with 0.5% saponin in 1% BSA in PBS for 30 min. Samples were washed with PBS then blocked in 1% BSA in PBS for 1 hour. Cells were washed in PBS then treated with anti-TLR3 mAB (TLR3.7) (1:10; ebioscience, San Diego, CA) overnight at 4º C. A no primary control was also done. Cells were washed in TBS then treated with goat anti-mouse Alexa 488 conjugated secondary antibody (5µg/ml, Molecular probes) in TBS/Triton for 1 hour at room temperature. Cells were then washed in TBS and cover slipped with VectaShield with DAPI (Vector Labs, Burlingame, CA). Immunofluorescence was visualized using a Zeiss 510 laser scanning confocal microscope at the Michael Hooker microscopy core facility at the University of North Carolina at Chapel Hill.

Flow Cytometry

A549 or differentiated NHBE cells were detached by incubating with 0.53mM EDTA/PBS at 37^{o}C for 10 minutes and were washed HBSS. For the analysis of TLR3 the cell pellets were resuspended at 10^{6} cells/ml. For intracellular staining of TLR3, cells were permeabilized and

fixed by incubating them with Cytoperm/Cytofix (BD Bioscience; San Diego, CA) as per the supplier's instructions and then washed twice with staining solution containing 1.0% bovine serum albumin and 0.02% sodium azide in PBS. This step was omitted for cells that were stained for surface expression of TLR3. Cells were then incubated with Phycoerthrin (PE)-conjugated anti-TLR3 (TLR3.7) (1 μg/10^6 cells, Ebioscience; San Diego, CA) or PE-mouse IgG1 (1 μg/10^6 cells) for 30 min at 4 ^oC then washed twice with staining solution. Cells stained for surface expression of TLR3 were then fixed by incubating them with 1% paraformaldehyde for 20 min at 4ºC. Data acquisition and analysis was performed on a flow cytometer (FACScan using the Cell Quest software; Becton Dickinson, Mountain View, CA). Ten thousand cells were examined for each determination.

Statistical Analysis

Data are expressed as means $\pm$ S.E.M. of at least three separate experiments. Data from experiments involving primary human nasal or bronchial epithelial cells were analyzed using the Wilcoxon matched-pairs test. Data generated from experiments using A549 cells were analyzed using two-way analysis of variance to determine if there was a significant interaction between the exposure (DE_{as}) and treatment (poly(I:C)), followed by the Tukey-Kramer HSD *post hoc* test for multigroup analysis, except for the data in figure 7, which were analyzed using the Wilcoxon Signed Rank Test, assuming a theoretical mean of 1.00 for the control group. A value of $p < 0.05$ was considered to be significant.

Results

TLR3 Expression and Localization

The TLR's involved in recognition of non-viral pathogen-associated molecular patterns (PAMPs) are anchored in the cell membrane, with the PAMP recognizing portion on the extracellular side and the TIR domain on the cytoplasmic side (200). However, the exact cellular location of TLR3 in respiratory epithelial and other cell types is still controversial, with the most recent data indicating that TLR3 is located in cytoplasmic vesicles. Therefore, the first objective was to characterize the expression and localization of TLR3 in respiratory epithelial cells. Using a PE-conjugated anti-TLR3 antibody the surface and cytoplasm were both stained for TLR3 in A549 cells and flow cytometric analysis was performed to identify TLR3-stained cells. Permeabilization followed by fixation was used to analyze cytoplasmic expression of TLR3, while surface expression was assessed in cells that did not receive the permeabilizing treatment. The results indicate that TLR3 was located predominantly in the cytoplasm with little or no surface expression evident (Fig 2A). This was also confirmed in differentiated human bronchial epithelial cells and similar results were observed (data not shown). To further identify cytoplasmic compartments in which TLR3 is localized, indirect immunofluorescent staining of A549 cells for TLR3, followed by visualizing the fluorescent staining using confocal microscopy was performed. Figure 2B indicates that the staining for TLR3 was distributed throughout the cytoplasm, without any staining concentrating along the cell membrane or the nucleus. In addition, the staining pattern seemed granular, confirming previous observations of TLR3 being located in cytoplasmic vesicles (57, 131).

Effect of DE_{as} on TLR3 expression

After identifying the localization of TLR3, it was examined whether the expression of TLR3 was affected in cells exposed to DE_{as} prior to viral infection or treatment with dsRNA, which are both stimuli that have previously been shown to enhance TLR3 mRNA levels (67, 177, 201, 223). DE has previously been shown to exacerbate inflammatory mediator production in response to RSV in mice (71) and IFN-β expression in response to influenza in respiratory epithelial cells (85), yet the role of TLR3 in these responses is unknown. Therefore, we wanted to determine if exposure to DE_{as} could enhance virus-induced expression of TLR3. To do this, levels of TLR3 mRNA were examined in differentiated human respiratory epithelial cells exposed to DE_{as} and subsequently infected with influenza. Similar to previous results (223), Figure 3 shows that infection with Influenza enhanced TLR3 mRNA levels as compared to uninfected control cells, although this effect was not statistically significant in the bronchial cells. As expected, exposure to DE_{as} alone did not significantly enhance TLR3 mRNA levels in either differentiated human nasal or bronchial epithelial cells. However, when cells were exposed to DE_{as} prior to infection with Influenza there was a significant enhancement of the Influenza-induced up-regulation of TLR3 mRNA levels in bronchial epithelial cells and an increase that was approaching statistical significance in nasal epithelial cells ($p=0.06$).

These experiments were repeated using poly(I:C), a synthetic form of double-stranded RNA, instead of the Influenza virus to induce TLR3 expression in A549 cells, a human respiratory epithelial cell line. Figure 4A shows that, although not significant, treatment with poly(I:C) alone caused a modest up-regulation of TLR3 mRNA in A549 cells and exposure to DE_{as} alone had no effect on TLR3 mRNA levels in this cell type. When cells are pre-exposed to DE_{as} before stimulation with poly(I:C) however, there was a significant

interaction between DE exposure and poly(I:C) treatment resulting in a significant up-regulation of TLR3 mRNA levels above each single treatment. To determine whether the enhanced TLR3 mRNA levels correspond to enhanced TLR3 protein expression, TLR3 protein levels were analyzed in whole cell lysates by Western blotting. Figure 4B shows that the up-regulation of TLR3 seen at the mRNA level in cells exposed to DE_{as} prior to stimulation with poly(I:C) translated into increased TLR3 protein expression in A549 cells. Densitometric readings expressed as fold induction over the media control show that exposure of cells to DE_{as} prior to stimulation with dsRNA enhanced TLR3 expression. Taken together, the results demonstrate that exposure to DE_{as} significantly enhances virus or dsRNA-induced TLR3 expression in different human respiratory epithelial cell types.

DE_{as} increases poly(I:C)-induced inflammatory mediator production

Stimulation of TLR3 by dsRNA or viral infection results in the activation of two separate signaling pathways, one culminating in the activation of Interferon regulatory factors (IRFs) and the production of type I IFNs and the other in the activation of NF-κB and the production of pro-inflammatory mediators. Production of inflammatory mediators is a common response of respiratory epithelial cells to viral infections and can be exacerbated by a number of environmental pollutants (71, 205). After observing an up-regulation of TLR3 expression by prior exposure to DE_{as}, it was determined whether exposure to DE_{as} also enhanced TLR3-mediated pro-inflammatory mediator production by analyzing the expression of IL-6 in cells exposed to DE_{as} and stimulated with poly(I:C). Using real-time RT-PCR, IL-6 mRNA levels were analyzed 24 hours after exposure to DE_{as} and stimulation with poly(I:C) in A549 cells. Figure 5A shows that levels of IL-6 mRNA were slightly elevated in response to treatment with poly(I:C) alone. Exposure to DE_{as} alone had no effect IL-6 mRNA levels. When cells

were exposed to DE_{as} prior to treatment with poly(I:C), IL-6 mRNA levels were significantly increased above the media control despite the fact that there was not a significant interaction between exposure and treatment. To validate the IL-6 mRNA levels, IL-6 protein levels were also measured in cell culture supernatants and a similar overall effect was observed (Fig 5B). Specifically, there was a significant interaction between exposure and treatment resulting in a significant increase in secretion of IL-6 above all other groups in cells exposed to DE_{as} prior to treatment with poly(I:C). These findings along with the mRNA data show that exposure to DE_{as} increases the TLR3-mediated inflammatory response.

To assure that the effect of DE_{as} on poly (I:C)-induced IL-6 expression was mediated by TLR3 and not other TLR3-independent pathways, such as PKR-dependent activation of NF-κB and production of inflammatory cytokines (156), IL-6 production was examined in cells overexpressing dominant negative mutant form of TNF receptor-associated factor 6 (TRAF6) lacking the N-terminal zinc binding structures (26). Although several other signaling pathways converge at TRAF6 to subsequently activate NF-κB and enhance inflammatory cytokine production, TRAF6 is also one of the main signaling proteins involved in the TLR3-mediated inflammatory response (89). Using retroviral expression vectors, A549 cells were stably transduced with the control vector or a vector expressing a truncated form of TRAF6 (26). Both cell lines were exposed to DE_{as}, stimulated with poly(I:C), and IL-6 mRNA levels were quantified 24 hours after stimulation with poly(I:C) using real time RT-PCR. Figure 6 shows that cells transduced with a control vector responded similarly to those in Figure 5A, whereby IL-6 mRNA levels were significantly increased above control in cells exposed to DE_{as} prior to treatment with poly(I:C), despite the absence of a significant interaction effect. However, when cells expressing the dominant negative form of TRAF6 (dnTRAF6) were

exposed to DE_{as} prior to treatment with poly(I:C) there was no significant up-regulation of IL-6 mRNA levels as seen in cells expressing the control vector. Additionally, the IL-6 levels observed in the dnTRAF6 cells treated with DE_{as} + poly(I:C) were decreased compared to control cells treated in the same manner. Interestingly, IL-6 expression was not completely abrogated in cells expressing dnTRAF6, which could be mediated by the endogenously expressed wild type form of TRAF6 or TRAF6 independent pathways. Nevertheless, the results show that the effects of DE_{as} on poly (I:C)-induced IL-6 production were largely dependent on TRAF6, an integral component of the TLR3 signaling pathway.

DE_{as} enhances the poly(I:C)-induced IFN-β expression

After elucidating the effects of DE_{as} on poly (I:C)-induced IL-6 expression, we examined how exposure to DE_{as} affected IFN-β expression after stimulation with poly(I:C). IFN-β is a type I interferon with anti-viral properties produced early on during viral infections (114, 225). The same exposure and treatment regimen was used as before and levels of IFN-β mRNA were quantified 24 hours after treatment with poly(I:C) using real-time RT-PCR (Fig 7A). No significant increase in mRNA levels were seen in cells exposed to DE_{as} or poly(I:C) alone. However, there was a significant interaction with DE_{as} exposure and poly(I:C) resulting in a significant increase in IFN-β mRNA levels, indicating that TLR3-mediated IFN-β production is also enhanced by exposure to DE_{as}.

TLR3-mediated expression of IFN-β is dependent on Interferon regulatory factor 3 (IRF3), which is the main transcription factor controlling IFN-β transcription (127, 146, 224). To determine whether exposure to DE_{as} enhances poly(I:C)-induced IFN-β expression at the transcriptional level, nuclear levels of IRF3 were determined. A549 cells were exposed and treated as before and western blotting was used to assay levels of IRF3 in nuclear

fractions harvested 4 hours after stimulation with poly(I:C) (Fig 7B). Treatment with DE_{as} or poly(I:C) alone resulted in a mild increase in the levels of nuclear IRF3. Similar to the IFN-β mRNA levels, cells exposed to DE_{as} prior to stimulation with poly(I:C) had greater levels of nuclear IRF3 compared to cells only stimulated with poly (I:C). Together these results show how pre-exposure to DE_{as} can up-regulate IFN-β levels through enhanced activation of IRF3.

Role of AKT in IFN-β production

Since an increase in IFN-β mRNA levels and increased nuclear levels of IRF3 were observed in cells exposed to DE_{as} and subsequently stimulated with poly (I:C), the upstream components of the TLR3-mediated interferon pathway were examined. Specifically, the role of the phosphatidylinositol-3 kinase (PI3K) pathway in the DE_{as}-induced up-regulation of IFN-β was of interest since evidence suggests that Akt, the downstream component of PI3K, is responsible for phosphorylating IRF3, which is required for complete activation of the transcription factor (180). Prior to exposure to DE_{as} and stimulation with poly(I:C), cells were pre-treated with wortmannin to inhibit activation of PI3K and thus Akt. These cells were then analyzed for IFN-β mRNA levels by real-time RT-PCR. Figure 8A shows that in cells pre-treated with the vehicle control, exposure to DE_{as} prior to treatment with poly(I:C) significantly increased levels of IFN-β mRNA. However, when cells were pre-treated with the PI3K inhibitor wortmannin, IFN-β mRNA levels were not significantly enhanced. To obtain further support for the participation of Akt in the effects of DE_{as} on poly(I:C)-induced IFN-β expression, levels of phosphorylated and thus activated Akt were analyzed in whole cell lysates using Western blotting (Fig 8B). Exposure to DE_{as} increased the levels of phosphorylated Akt compared to control and poly(I:C)-treated cells. Taken together, the

results suggest that the effects of DE_{as} on poly(I:C)-induced IFN-β expression are partially mediated by the effects of DE_{as} on Akt activity.

Effects of different diesel exhaust preparations on the expression of pattern recognition receptors, pro-inflammatory cytokines, and IFN-β

As stated earlier, the composition of DE can vary depending on the source it was derived from. Furthermore, there is evidence that organic components adsorbed onto DEPs are responsible for many of the adverse effects associated with exposure to DE (28, 221, 222). Given the previous observations with DE_{as}, which was generated from an engine used to power a generator, and its effect on TLR3 expression and signaling, the effect of different diesel exhaust and particle preparations on the expression of TLR3 was examined. To expand the focus of the effects of DE on influenza-induced expression and function of pattern recognition receptors (PRRs) and other antiviral mediators, changes in the expression of retinoic acid-inducible protein I (RIG-I), and eukaryotic translation initiation factor 2-alpha kinase 2 (PKR) were also examined. In addition, mRNA expression of the pro-inflammatory cytokines/chemokines IL-6, IL-8, and regulated on activation, normal T-cell expressed, and secreted (RANTES), as well as the type I interferon, IFN-β were examined. The preparations used were: 1) Standard reference material (SRM) 2975, which is derived from an industrial forklift and has a low percentage of organic material; 2)Compressor DEP (Comp-DEP), which was obtained from a diesel engine powering a compressor and has an intermediate percentage of organic material; 3) extracted organic material (EOM) from Comp-DEP; as well as 4) volcanic dust from the Mt. St. Helen's eruption (MSHD) and 5) ultrafine carbon (UF carbon) for use as particle controls. DE from an automobile, having a high percentage of

organic material, was also tested, but caused cytotoxicity at the doses examined in this study (data not shown) so subsequent analyses were not performed.

A549 cells were exposed to 25 μg/cm^2 of each preparation for 2 hours prior to stimulation with poly(I:C). Levels of TLR3 (Fig 9A), RIG-I (Fig 9B), PKR (Fig 9C), IL-6 (Fig 10A), IL-8 (Fig 10B), RANTES (Fig 10C), and IFN-β (Fig 10D) mRNA were quantified 24 hours after stimulation with poly(I:C). Exposure to each preparation alone or prior to stimulation with poly(I:C) had different effects on the expression of the genes examined and the results are summarized in Table 1. Specifically, cells exposed to either MSHD or Comp-DEP prior to treatment with poly(I:C) showed a significant increase in mRNA levels of all genes examined while cells exposed to UF carbon alone showed a significant decrease in the mRNA levels of all genes. Cells exposed to the EOM of Comp-DEP either alone or prior to stimulation with poly(I:C) showed no changes in TLR3 or IL-6 mRNA levels (Fig 11) so further analyses were not pursued.

Effect of siRNA on the expression of antiviral mediators

In an attempt to knock-down expression of the PRRs in order to determine their contribution to the innate immune response to influenza infection, cells were treated with siRNA to TLR3, PKR, or Lamin as a control. However, an increase in the antiviral mediator MxA was observed in cells treated with any of the siRNAs (Fig 12A). This resulted in a significant decrease in the susceptibility to infection in cells treated with siRNA for TLR3 and PKR shown by decreased HA RNA levels (Fig 12B), and prevented a sufficient pro-inflammatory response upon influenza infection in all siRNA treated cells shown by decreased IL-6 mRNA levels (Fig 12C). Thus, antiviral mediator production in response to

siRNA prevented the application of this technique to assess the role of TLR3 or other PRRs in the effects of DE on the response to influenza virus infections.

Discussion

We have recently demonstrated that exposure of respiratory epithelial cells to an aqueous-trapped solution of diesel exhaust (DE_{as}) increased the susceptibility to influenza virus infections (85). Similarly, exposure to DE has previously been shown to alter the response to and exacerbate respiratory symptoms associated with viral infection in mice (69, 71). However, the mechanism(s) by which these effects occur has not yet been fully elucidated. TLR3 has emerged as a key receptor eliciting pro-inflammatory mediator and type I IFN production in response to viral infections (130, 200). Based on current knowledge and previous findings in our laboratory we hypothesized that exposure to DE_{as} affects the TLR3-mediated, viral response pathway and show evidence here that exposure to DE_{as} prior to infection with Influenza A or stimulation with the dsRNA synthetic analog poly(I:C), up-regulates TLR3 expression in respiratory epithelial cells obtained from different regions of the human respiratory tract. This results in increased inflammatory cytokine and type I IFN response as demonstrated by increased levels of IL-6 and IFN-β, respectively. Taken together these results provide evidence for the ability of DE to alter the innate immune defense response against viral infection through an increase in TLR3 expression and signaling.

The cellular localization of TLR3 on both the cell surface and in the cytoplasm has been documented and appears to depend, at least in part, on the cell type (57, 67, 131, 132, 201, 223). Using flow cytometry as well as confocal microscopy we demonstrated that in respiratory epithelial cells, TLR3 was located in the cell cytoplasm most likely in endoplasmic vesicles, as has been suggested previously (57, 131). Since uptake of RNA-viruses, such as Influenza A, depends on the host cell's endosomes, it is plausible that virus-induced signaling mediating the innate immune defense response originates from this region.

It has been shown previously that TLR3 levels can be upregulated by viral infection in respiratory epithelial cells (67, 177, 223). We observed similar effects when primary, human, differentiated bronchial and nasal epithelial cells were infected with Influenza A virus, although a significant increase was not observed in bronchial cells. Additionally, when A549 cells were stimulated with poly(I:C) to mimic the viral by-product dsRNA, we also observed an increase in TLR3 mRNA levels (see figure 4). This effect, although not significant, mirrors what has been seen previously (67, 201) and may represents part of the epithelial cells' anti-viral response mechanism to enhance the production and release of mediators aimed at preventing neighboring cells from becoming infected with the invading pathogen. Prior exposure to DE_{as} caused a significant up-regulation of virus or dsRNA-induced TLR3 mRNA levels that was two-four times greater than what was seen with Influenza or poly(I:C) alone. This effect was observed in the primary cells derived from the nasal and bronchial epithelium as well as the cell line derived from the alveolar region of the lung, suggesting that this effect occurs throughout the respiratory tract. Given the fact that the entire pulmonary epithelium is the primary target for both inhaled vehicle emissions, i.e. DE, as well as many viral infections, interaction of DE exposure with a viral infection leading to an up-regulation of TLR3, as demonstrated here, could significantly alter the host's response to many other respiratory viral infections.

Stimulation of TLR3 culminates in the activation of two separate signaling cascades: A TRAF6-dependent pathway that mediates the activation of NF-κB and the expression of pro-inflammatory mediators, such as IL-6, and a TANK-binding kinase-1 (TBK-1)-dependent pathway that mediates the activation of IRF-3 and the expression of type I IFNs. The results demonstrate that prior exposure to DE_{as} caused a significant increase in both the mRNA and

protein levels of the pro-inflammatory cytokine IL-6 in response to stimulation with poly(I:C). This response was hindered in A549 cells stably transduced with a dominant negative form of TRAF6, which had significantly lower IL-6 mRNA levels than cells transduced with a control vector. The IL-6 response was not completely abrogated in the dnTRAF cells due to the fact that these cells still exogenously express the wild-type form of TRAF6 as well as TLR3-independent pathways that may contribute to the inflammatory response. Nevertheless, these data further support the notion that enhanced IL-6 expression in cells exposed to DE_{as} prior to stimulation with poly(I:C) was more than likely dependent on TLR3. Inflammatory mediators, such as IL-6 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. Enhancement of the IL-6 response in an infected host could lead to increased inflammation, recruitment and activation of immune cells, and fever, thereby increasing the morbidity of a respiratory infection. Thus, enhanced expression and function of TLR3 in cells exposed to DE_{as} prior to viral infection is likely to increase lung injury.

As indicated above, stimulation of TLR3 also results in type I IFN expression. Similar to the effects seen on IL-6 expression, the data show there is a significant interaction between DE_{as} exposure and treatment with poly(I:C) resulting in significantly greater IFN-β mRNA levels. DE_{as} also induced increases in the nuclear translocation of the transcription factor IRF3, which mediates IFN-β transcription. In addition, previous studies have shown that activation of the protein kinase Akt is needed to fully activate IRF3 (127, 146, 190, 224). Akt is activated by the upstream kinase PI3K, which is recruited and activated by TLR3 through phosphorylated tyrosine residues on the cytoplasmic domain of TLR3 (190). The results

shown here indicate that exposure to DE_{as}, but not stimulation with poly(I:C), increase levels of phosphorylated and thus activated Akt. Additionally, when Akt activity was inhibited in A549 cells prior to exposure to DE_{as} and stimulation with poly(I:C), there was a reduction in IFN-β mRNA levels as compared to the vehicle control. Taken together these results suggest that the effects of DE_{as} on virus-induced production of IFN-β are mediated by DE_{as}-enhanced Akt activity. It is possible, that the effects were not entirely dependent on TLR3, since several signaling pathways lead to phosphorylation of Akt and activation of PI3K. This notion is supported by the result showing enhanced levels of phosphorylated Akt in cells exposed to DE_{as} alone and by previous data demonstrating that in mouse epidermal cells, exposure to DE particulates enhanced the levels of phosphorylated Akt and activated NF-κB in a PI3K-dependent manner (120). Thus, exposure to DE_{as} potentially affects virus-induced IFN-β expression via two, possibly interdependent, pathways: a) through enhancing the virus-induced expression and activity of TLR3 and b) through enhancing IRF3 activity by activating PI3K. Future analysis of these pathways and their interaction will provide a greater understanding of the mechanisms involved.

Furthermore, it is possible that production and release of IFN-β, activates a positive feedback loop, resulting in increased TLR3 expression and further IFN-β production. Specifically, stimulation with the type I IFN's, IFN-α and IFN-β, resulted in enhanced expression of TLR3 and increased poly(I:C)-induced signaling in A549 cells (228). We have previously shown that treatment with DE_{as} significantly enhances Influenza-induced IFN-β expression in both primary respiratory epithelial cells and A549 cells (85). Similarly, we show here that treatment with DE_{as} significantly enhances the poly(I:C)-induced IFN-β response and that this response may be caused by DE_{as}-induced activation of Akt and

subsequent activation of IRF3 (see figures 6 and 7). Based on the findings published by Tissari et al. (228), it is plausible that the enhanced expression of IFN-β in cells exposed to DE_{as} and stimulated with poly(I:C) activates such a positive feedback loop, resulting in the increased expression of TLR3. Consequently, IFNβ-dependent enhanced expression of TLR3 would result in increased binding of dsRNA and therefore enhanced TLR3 signaling, which in turn would culminate in increased inflammatory mediator and IFN production, as was observed here.

The results presented here describe a mechanism by which exposure to DE can alter the innate immune defense responses against viral infections in respiratory epithelial cells, namely through an increase in the expression of TLR3. By increasing TLR3 levels, DE enhances part of the innate immune response that is thought to provide a bridge between the innate and adaptive immune responses against viral infections (1, 2, 84). Within epithelial cells this translates into increased recruitment and activation of inflammatory and immune cells, which can have detrimental consequences if left unchecked.

Since TLR3 is largely responsible for the inflammatory response to influenza (3, 67, 103, 104), it is likely that increased TLR3 expression results in increased inflammation during an influenza infection. Increased inflammation in turn would enhance the morbidity of the infection. Additionally, studies using TLR3-/- mice have shown that the TLR3-mediated inflammatory response has a detrimental contribution to the infected host and that morbidity and mortality are reduced in mice lacking TLR3 (103, 245). These data indicate that the DE-induced increase in TLR3 expression and signaling will ultimately have a detrimental effect on an infected host.

We were unable to utilize siRNA in order to further examine the role of TLR3 and other PRRs during an influenza infection due to the antiviral response elicited by the cell in response to siRNA transfection. Cells transfected with siRNA showed enhanced production of the anti-influenza mediator MxA, which prevented an adequate infection and subsequent antiviral response in these cells. Therefore, the use of siRNA technology may not be of use when studying cellular responses to certain viruses, particularly influenza.

The comparison of the different diesel exhaust preparations indicates that the effects observed with the aqueous-trapped solution of DE are not specific to this particular preparation, but are also observed when cells are exposed to DE from a compressor engine as well as particles from a volcanic eruption. Furthermore, another preparation of DEP did not cause similar effects indicating that DE from different sources may have differing effects depending on chemical composition as well as number of other factors. Studies have suggested that the organic components adsorbed onto the carbon core of DEPs are responsible for many of the adverse effects caused by exposure to DE (28, 221, 222). To determine if organic components adsorbed onto the core of DEPs were responsible for the observed effects in our study cells were treated with EOM from Comp-DEP and compared to cells treated with UF carbon, which mimics the carbon core of DEPs. Interestingly, neither exposure to the EOM of Comp-DEP nor UF carbon prior to stimulation with poly(I:C) caused the same effects as were observed when cells were exposed to Comp-DEP. These data indicate that the observed effects may be a consequence of both the particle and the adsorbed components acting together. Furthermore, DE with a low organic content, SRM 2975, also did not elicit the same effects as the Comp-DEP. One possibility is that the particle provides the adsorbed components with a route of entry into the cell. In fact,

previous studies have shown that respiratory epithelial cells can internalize these particles (133). When organic material from DEPs is not adsorbed onto a particle it may be unable to penetrate the cell and elicit the same effects as it would when it gains entry via cellular uptake of DEPs. Another possibility is that inorganic components of DE are responsible for the observe effects. Further studies will be needed to determine what components of DE are responsible for eliciting certain effects and responses in the cell and how different types of diesel engines running conditions, as well as collection methods, will influence the levels of these chemical components.

Since epidemiological evidence has noted an association between exposure to ambient particulate matter and pulmonary infections (166), including exacerbation of respiratory symptoms associated with infection (29, 58, 155), the results presented here combined with previous findings in our laboratory (85) provide a potential mechanism by which exposure to particulate air pollutants, such as DE, enhances the susceptibility to respiratory virus infections. It will be of great interest and public concern to elucidate further mechanisms involved in these processes as well as to identify the effects of air pollutants on other aspects of host defense responses against respiratory viruses.

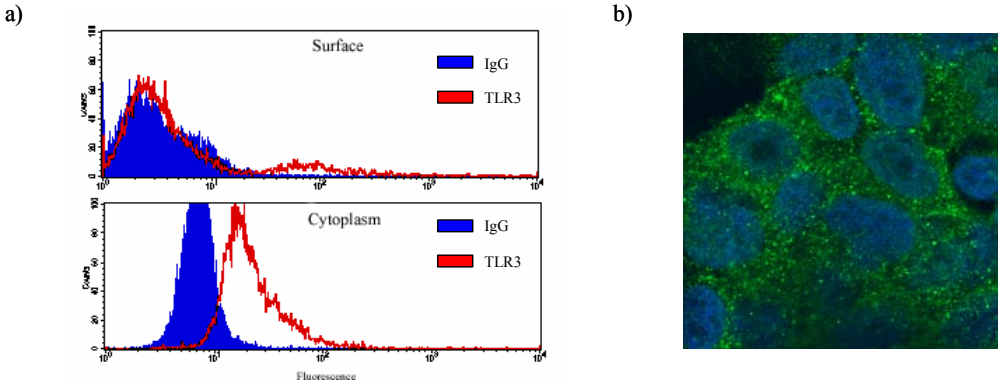


Figure 2 Localization of TLR3
a) Surface and cytoplasmic staining for IgG and TLR3 was performed in A549 cells. Levels of TLR3 were measured using flow cytometry and compared to IgG for control. **b)** A549 cells were immunohistochemically stained for TLR3 (FITC) and nuclei were counterstained with DAPI. Immunofluorescence was visualized using confocal microscopy.

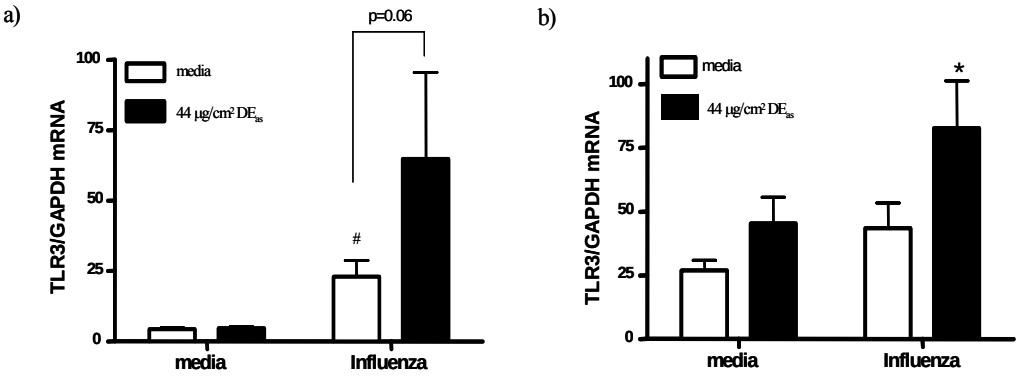


Figure 3 Effect of DE_{as} and Influenza A on TLR3 mRNA levels
Changes in mRNA were measured 24 hours after infection with Influenza A. Differentiated **(a)** nasal or **(b)** bronchial epithelial cells were treated with 0 or 44 µg/cm^2 of DE_{as} for 2 hours prior to infection with 320 HAU/10^5 cells of Influenza A. TLR3 mRNA was quantified using real time RT-PCR. Values are normalized to GAPDH mRNA. * significantly different from influenza infected cells, # significantly different from media control; $p<0.05$.

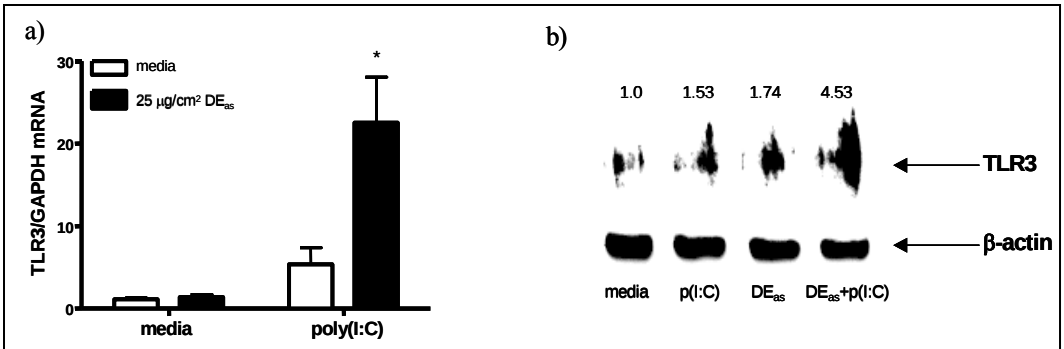


Figure 4. Effect of DE_{as} and poly(I:C) on TLR3 levels
Changes in mRNA were measured 24 hours after stimulation with poly(I:C).
(a) A549 cells were treated with 0 or 25 μg/cm^2 of DE_{as} 2 hours prior to stimulation with 100μg/ml of poly(I:C). TLR3 mRNA was quantified using real time RT-PCR. Values are normalized to GAPDH mRNA. * significantly different from all other groups; $p<0.01$.
(b) Whole cell lysates prepared from A549 cells treated with 0 or 25 μg/cm^2 of DE_{as} prior to stimulation with 100 μg/ml poly(I:C) were analyzed for TLR3 protein levels. Nitrocellulose membranes were stripped and re-probed with anti-β-actin antibody. Optical densitometry readings for TLR3 were normalized to β-actin levels and shown as fold induction over the media control (numbers on top) to facilitate comparison.

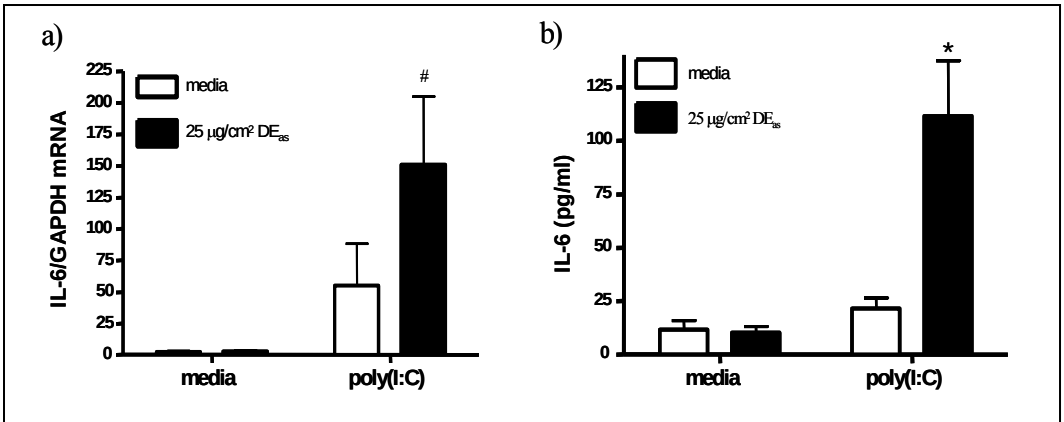


Figure 5 Effect of DE_{as} on IL-6 levels
Changes in IL-6 mRNA (a) and protein (b) levels were measured 24 hours after stimulation with poly(I:C). A549 cells were treated with 0 or 25 μg/cm^2 of DE_{as} 2 hours prior to stimulation with 100μg/ml of poly(I:C). IL-6 mRNA was quantified using real time RT-PCR and values were normalized to GAPDH mRNA. IL-6 protein levels were measured in cellular supernatants by ELISA. * significantly different from all groups; $p<0.01$. # significantly different from media control; $p<0.05$.

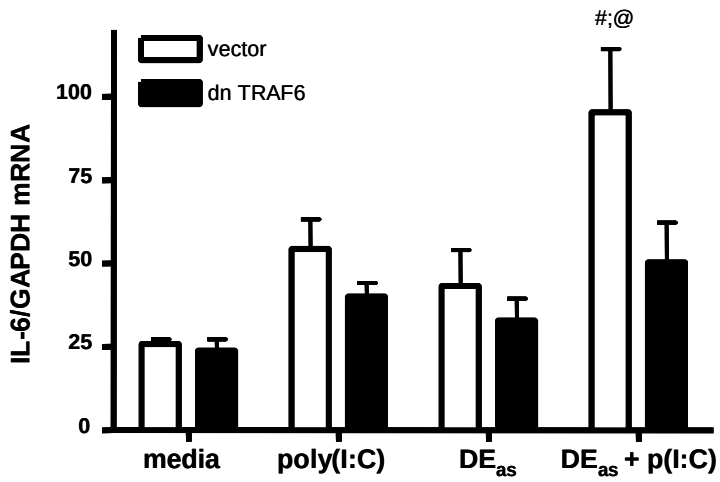


Figure 6 Effect of dnTRAF6 on DE_{as}-induced effects on IL-6 levels
A549 cells were stably transduced with a control vector or a truncated form of TRAF6 (dnTRAF6) and treated with 0 or 25 μg/cm^2 of DE_{as} 2 hours prior to stimulation with 100μg/ml of poly(I:C). Changes in IL-6 mRNA levels were measured 24 hours after stimulation with poly(I:C) using real-time RT-PCR. Values were normalized to GAPDH mRNA. # significantly different from media control; p<0.001, @ significantly different from dnTRAF6-DE_{as} + p(I:C) group; p<0.05.

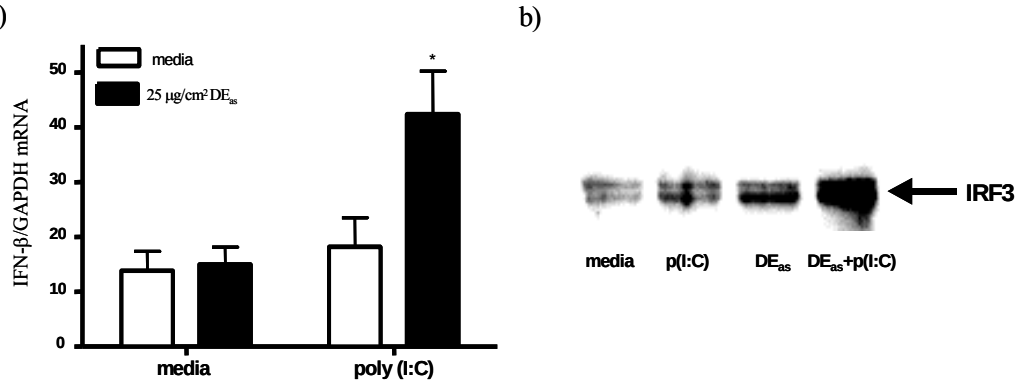


Figure 7 Effect of DE_{as} on IFN-β transcription
A549 cells were treated with 0 or 25 μg/cm^2 of DE_{as} 2 hours prior to stimulation with 100 μg/ml poly(I:C). **(a)** Changes in IFN-β mRNA levels were measured 24 hours after stimulation with poly(I:C). IFN-β mRNA was quantified using real time RT-PCR and values were normalized to GAPDH mRNA. * significantly different from all other groups; p<0.05 **(b)** Nuclear extracts prepared 4 hours after stimulation with poly (I:C) were examined for IRF3 levels by Western blotting. Representative immunoblot for nuclear IRF3.

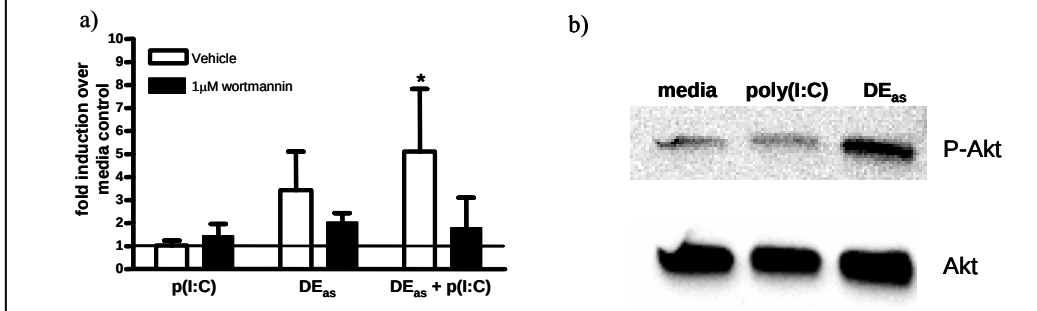


Figure 8 Effect of DE_{as} on levels of activated AKT and its role in IFN-β transcription **(a)** Changes in mRNA were measured 24 hours after stimulation with poly(I:C). A549 cells were treated with DMSO or 1μM wortmannin for 30 min prior to treatment with 0 or 25 μg/cm^2 of DE_{as}. 2 hours after treatment with DE_{as} cells were stimulated with 100 μg/ml poly(I:C). IFN-β mRNA was quantified using real time RT-PCR and values were normalized to GAPDH mRNA. Values are expressed as fold induction over the respective media control. * significantly different from control; $p<0.05$. **(b)** Whole cell lysates prepared from A549 cells 1 hour after treatment with 0 or 25 μg/cm^2 of DE_{as} or stimulation with poly(I:C) were analyzed for levels of phosphorylated Akt. Nitrocellulose membranes were stripped and re-probed using an anti-Akt antibody. Representative immunoblots for phosphorylated (top) and total (bottom) Akt.

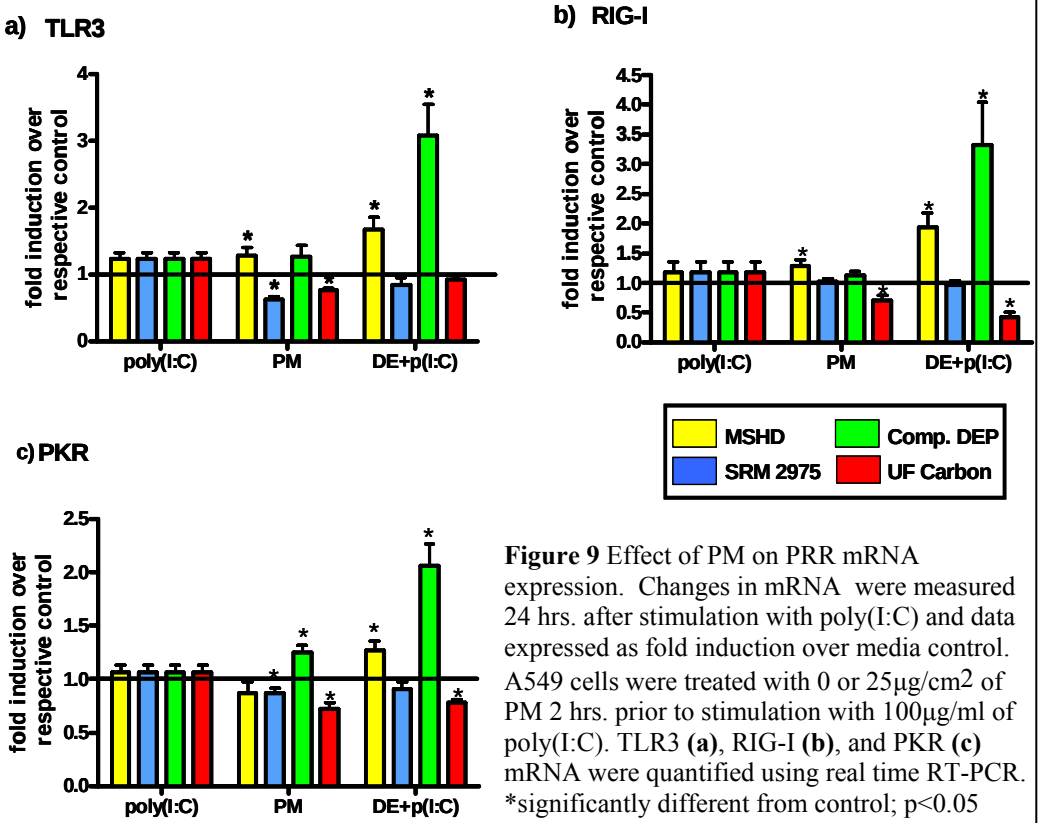


Figure 9 Effect of PM on PRR mRNA expression. Changes in mRNA were measured 24 hrs. after stimulation with poly(I:C) and data expressed as fold induction over media control. A549 cells were treated with 0 or 25μg/cm2 of PM 2 hrs. prior to stimulation with 100μg/ml of poly(I:C). TLR3 **(a)**, RIG-I **(b)**, and PKR **(c)** mRNA were quantified using real time RT-PCR. *significantly different from control; p<0.05

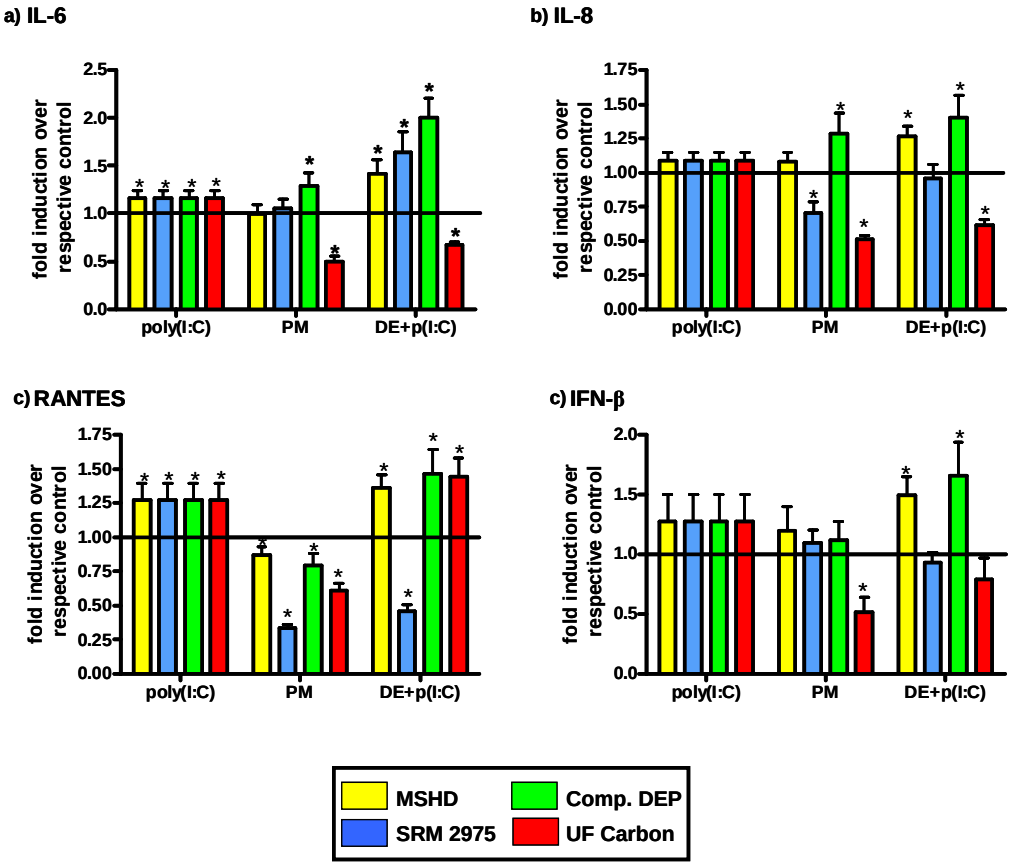


Figure 10 Effect of PM on cytokine mRNA expression.
Changes in mRNA were measured 24 hrs. after stimulation with poly(I:C) and data expressed as fold induction over media control. A549 cells were treated with 0 or 25µg/cm2 of PM 2 hrs. prior to stimulation with 100µg/ml of poly(I:C). IL-6 **(a)**, IL-8 **(b)**, RANTES **(c)**, and IFN-β **(d)** mRNA were quantified using real time RT-PCR. *significantly different from control; $p<0.05$

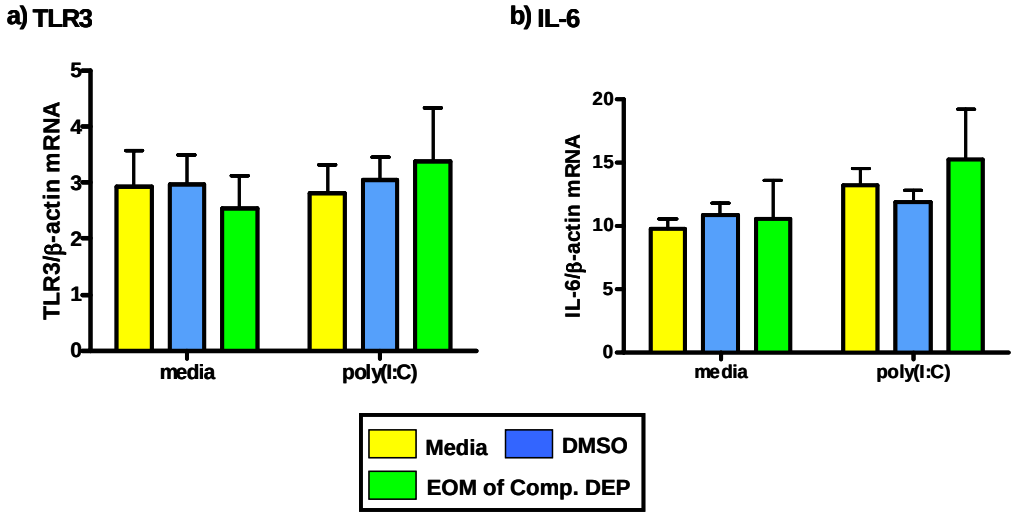


Figure 11. Effect EOM on TLR3 and IL-6 mRNA expression.
Changes in mRNA were measured 24 hrs. after stimulation with poly(I:C). A549 cells were treated with a dose of EOM that would be equivalent to the amount of organic material from a 25μg/cm2 exposure of Comp. DEP. Cells were treated with EOM for 2 hrs. prior to stimulation with 100μg/ml of poly(I:C). TLR3 **(a)** and IL-6 **(b)** mRNA were quantified using real time RT-PCR.

Summary of Effects

	MSHD		UF Carbon		SRM2975		Comp. DEP	
	alone	poly(I:C)	alone	poly(I:C)	alone	poly(I:C)	alone	poly(I:C)
TLR3	↑	↑	↓	—	↓	—	—	↑
RIG-I	↑	↑	↓	↓	—	—	—	↑
PKR	—	↑	↓	↓	↓	—	↑	↑
IL-6	—	↑	↓	↓	—	↑	↑	↑
IL-8	—	↑	↓	↓	↓	—	↑	↑
RANTES	↓	↑	↓	↑	↓	↓	↓	↑
IFN-β	—	↑	↓	—	—	—	—	↑

Table 1. Summary of Effects of different particulate preparations

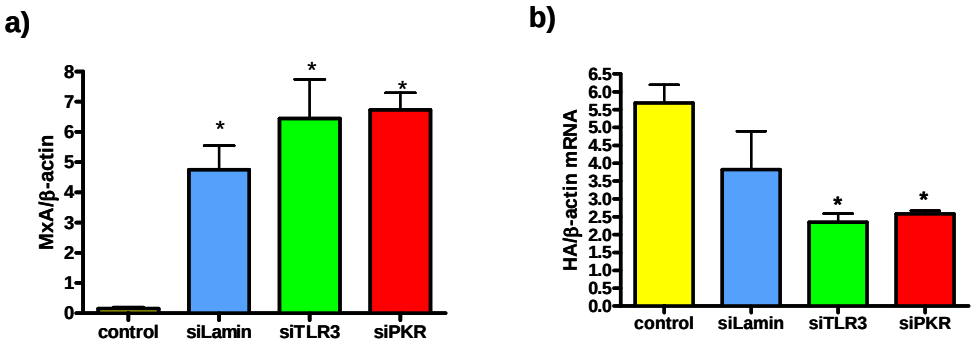


Figure 12. Effect of siRNA on antiviral responses
Changes in mRNA were quantified in A549 cells using real-time RT-PCR. **(a)** Changes in MxA mRNA were quantified 24 hrs after transfection with siRNA. Changes in HA RNA **(b)** and IL mRNA **(c)** were quantified 24 hrs. after infection with influenza

Chapter 3

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

Introduction

Diesel exhaust (DE) is an important contributor to particulate air pollution in urban air (42, 234). 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) (235). DE particles (DEPs) are made up of an elemental carbon core that behaves as an adsorbtive surface onto which a multitude of organic chemical and metals bind. Diesel exhaust particles are typically emitted as a mono-dispersed aerosol of around 0.2 μm (Mass median aerodynamic diameter [MMAD]) and may grow or accumulate up to around 1 μm in the atmosphere. These particles are thus easily respirable and capable of being deposited in the lower airways and alveolar region of the lung (116). Animal and human *in vitro* and *in vivo* studies have shown that exposure to DE increases neutrophil recruitment, nitric oxide production, and production of pro-inflammatory cytokines (76, 93, 148, 150, 178, 183).

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 (44, 56, 238). Additionally, numerous studies have reported that DE increases susceptibility to

respiratory infections. For example, several studies have demonstrated that DEP decreases phagocytosis and clearance of both gram negative and gram positive bacteria (28, 181, 214, 256, 258). 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) (69, 71, 85).

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 (226, 227). 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 (13, 72). 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 (124, 125). Previous studies have shown that uptake and clearance of Influenza A is reduced in SP-A- and SP-D-deficient mice (113). 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 (71).

Given our previous findings, which 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. Our results demonstrate that repeated exposure to DE increases the susceptibility to influenza infections in mice and this was associated with increased lung 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). All of the animals were housed in AAALAC-approved animal facilities with high-efficiency particulate air filters and received access to food and water ad libitum. 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. 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 $\mu g/m^3$ (high) and 500 $\mu g/m^3$ (low). Control animals were housed in a third chamber supplied with the same HEPA filtered room air. DEP concentrations in the low (500 $\mu g/m^3$) 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), sulfur dioxide (SO_2, Thermo Electron Corp, Waltham, MA), and total hydrocarbons (THC, California Analytical, Orange, CA). Samples were extracted through fixed stainless steel probes in the exposure chambers. Gas samples, with the exception of THC, 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 2. Chamber temperatures, relative humidity, and noise were also monitored, and maintained within acceptable ranges. Mice were exposed to HEPA filtered room air or to 0.5, or 2.0 mg/m^3 DEP 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 injections 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 (11). 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

Eighteen hours post infection, mice from each treatment group (n = 5-11) 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 electrophoresis using a10% 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 (86, 87). The sequences for the primers and probes used in this study are as follow: HA: probe 5'-FAM-TGATGGGAAAAACTGCACACTGATAGATGC-TAMARA-3'; sense 5'- CGACAGTCCTCACCGAATCC- 3'; antisense 5'-TCACAATGAGGGTCTCCCAATAG -3'; IL-6: probe 5'-FAM-CCAGCATCAGTCCCAAGAAGGCAACT-TAMRA-3'; sense 5'-TATGAAGTTCCTCTCTGCAAGAGA-3'; antisense 5'-TAGGGAAGGCCGTGGTT-3'; IFN-α: probe 5'-FAM-CTGCATCAGACAGCCTTGCAGGTCATT-TAMRA-3'; sense 5'-TGCAACCCTCCTAGACTCATTCT-3'; antisense 5'-CCAGCAGGGCGTCTTCCT-3';

IFN-β: probe 5'-FAM-AGGGCGGACTTCAAGATCCCTATGGA-TAMRA-3'; sense 5'-TGAATGGAAAGATCAACCTCACCTA-3'; antisense 5'-CTCTTCTGCATCTTCTCCGTCA-3'; INF-γ: probe 5'-FAM-CCTCAAACTTGGCAATACTCATGAATGCATCC-TAMRA-3'; sense 5'-AGCAACAGCAAGGCGAAAA-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).

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 control was also performed for each protein. The slides were evaluated under light microscopy.

Statistical Analysis

Data were pooled from three replicate experiments and are expressed as means ± SEM of three separate experiments. 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

Exposure to DE enhances 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 13A shows that mice exposed to 0.5 mg/m^3 of DE had significantly greater levels of HA mRNA compared to air exposed mice. 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 13B 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.

Exposure to diesel exhaust increases the influenza-induced inflammatory response

Cells infected with influenza respond by releasing various cytokines and chemokines to aid in fighting the invading pathogen. The purpose of many of these mediators released by the infected cell is to recruit and activate inflammatory cells. 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 14). Figure 14A 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 14B). 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 inflammatory response.

Exposure to DE does not increase 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 (table 3) in addition to differential cell counts to quantify levels of macrophages (table 3) and polymorphonuclear cells (PMNs) (fig 15A) in the BAL fluid 18 hours post-infection. As expected, Figure 15A 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 15A). 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 15B). Similarly, other markers of injury, such as NAG and MIA were not significantly affected by either DE

exposure or influenza virus infections (Table 3). These results show that the increase in PMNs caused by influenza infection were not accompanied by an increase in pulmonary injury and although exposure to DE prior to infection with influenza increases susceptibility to viral infection and pro-inflammatory mediator production, the exposure regimen used here does not result in significant increase in inflammatory cell recruitment or pulmonary injury 18 hours post-infection.

Effect of DE on the influenza-induced upregulation of TLR3

We have previously observed a significant increase in TLR3 mRNA expression in respiratory epithelial cells exposed to DE prior to infection with influenza (31). Therefore, the next objective was to determine the effect of DE exposure on TLR3 expression. To determine if exposure to DE prior to influenza infection would increase the expression of TLR3 *in vivo*, levels of TLR3 mRNA were quantified 18 hours post-infection in whole lung homogenates using real-time RT-PCR. Figure 16A shows that infection with influenza caused a significant increase in TLR3 mRNA levels in the lungs of mice exposed to air or 0.5 mg/m^3 DE, but not in mice exposed to 2.0 mg/m^3 DE. DE exposure did not significantly enhance TLR3 mRNA expression in response to infection. To confirm and expand upon these results lung sections were immunohistochemically stained for TLR3 (figure 16B). The left panel shows that there is no change in TLR3 staining observed in the airways, while the right panel suggests that there is increased TLR3 expression in the alveolar region of the lungs of mice exposed to 0.5 mg/m^3 DE prior to infection.

The enhanced susceptibility to infection caused by exposure to DE is not due to a decreased antiviral 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 (69). 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 17A), 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 17B). 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 17C) compared to controls. There was no effect on any of the IFNs observed in uninfected mice exposed to DE (data not shown).

The DE-induced effects on the susceptibility to infection are associated with changes in the expression of surfactant proteins

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 18A). 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 18B 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 immunohistochemical 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 (fig 18C).

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 19A and 19B). Immunohistochemical analysis (Figure 19C) 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 19A and 19, Figure 19C 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. Taken together these data suggest a strong association between enhanced susceptibility to infection observed in mice exposed to 0.5 mg/m^3 of DE and decreased expression of SP-A and SP-D in these animals.

Discussion

Various reports have linked DE to adverse effects on host immunity. Specifically, studies by us and others have shown that *in vitro* exposure of human respiratory epithelial cells and repeated *in vivo* exposure of mice to DE increases the susceptibility to respiratory viral infections (28, 69, 71, 85). 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. The results obtained in this study demonstrate that repeated exposure of mice to 0.5 mg/m^3 of DE enhances 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 infections. 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 (124, 125). These proteins contribute to the innate defense responses against influenza through their ability to bind and neutralize the virus (13, 72). 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 (113). 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 (78, 242). 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 (71). 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. 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 (38, 39), and NFAT in

turn is negatively regulated by Akt (259). Previous studies in our laboratory have shown that exposure of respiratory epithelial cells to DE results in an increased expression of activated Akt (31). 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* (107, 111, 112). 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 (107, 111, 112, 260). 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) (64, 169, 244). 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 (71). 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 (16, 103). 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 (69). 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 (85). 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 (71).

Expression of pro-inflammatory cytokines and type I interferons in response to viral infection can result from TLR3 activation, through its recognition of dsRNA, and subsequent signaling (3, 132). As stated earlier, dsRNA is commonly produced during a viral infection. Our previous results demonstrated that exposure of respiratory epithelial cells to DE enhanced TLR3 expression and signaling in response to influenza infection (31). A small, although not significant, increase in TLR3 mRNA was observed in the lungs of mice exposed to 0.5 mg/m^3 DE prior to infection. The lack of a significant increase in TLR3 mRNA may be due to the fact that the whole lung homogenates used to quantify levels of mRNA contain many cell types that have little or no expression of TLR3 as well as non-cellular material that is preventing us from detecting significant increases in certain cell types. It is possible that only certain cells are upregulating TLR3 in response to DE exposure and influenza infection. This notion is supported by the immunohistochemical staining for TLR3, which appears to show an increase in TLR3 expression in the alveolar region of the lung. Specifically, it appears that type II epithelial cells are the main cell type expressing TLR3. Further studies will be needed to determine how exposure to DE affects TLR3 expression *in vivo*.

Many of the endpoints examined in this study show an unusual biphasic response 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 (35) 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 (171). 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 (253). 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 (85, 115, 116, 253). Interestingly, apoptosis in epithelial cells creates an environment, which is not conducive for infection and replication of influenza (reviewed in 7). 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 22). 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 (236). 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 (212). 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.

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.

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
NO_2	ppm	<0.25	1.13±0.04
NO	ppm	<2.5	10.8±0.51
SO_2	ppm	0.06±0.02	0.32±0.1

Table 2. 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; NO_2, nitrogen dioxide; NO nitrogen oxide; SO_2, sulfur dioxide.

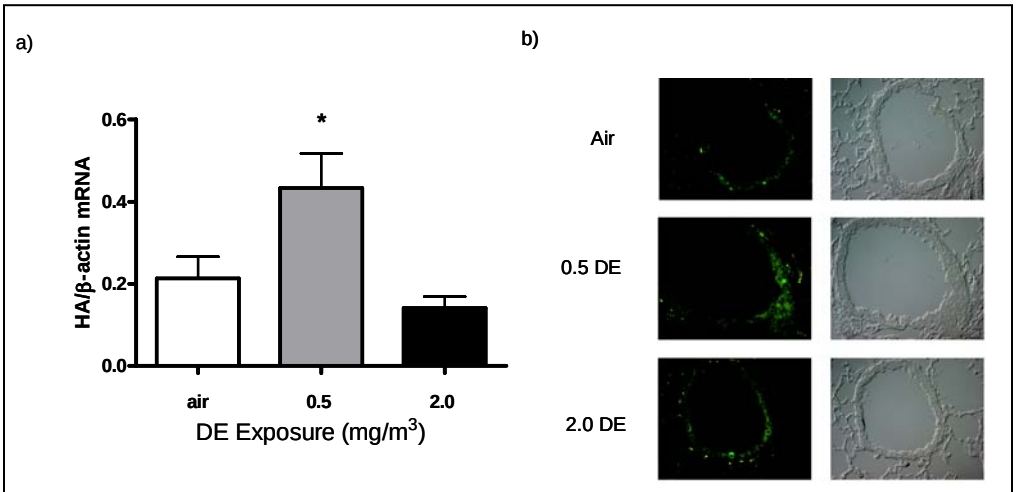


Figure 13. Effect of DE on the susceptibility to influenza infection.
a) Levels of HA mRNA were quantified in lung homogenates 18 hrs after infection with influenza by real-time RT-PCR. Values are normalized to β-actin and expressed as mean ± SEM. *significantly different from air/influenza mice; $p<0.05$. **b)** Mouse lung sections were immunohistochemically stained for influenza and visualized using confocal microscopy.

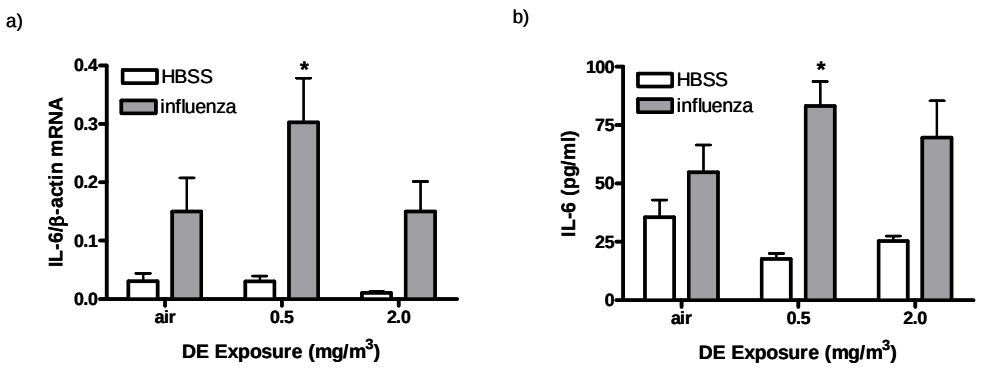


Figure 14. Effect of DE on the influenza-induced inflammatory response. Measurements were taken 18 hrs. post-infection and all data is expressed as mean ± SEM**. a)** Levels of IL-6 mRNA were quantified in lung homogenates by real-time RT-PCR and normalized to levels of β-actin. **(b)** Levels of IL-6 protein were quantified in the BAL fluid by ELISA. *significantly different from air/influenza mice; $p<0.05$.

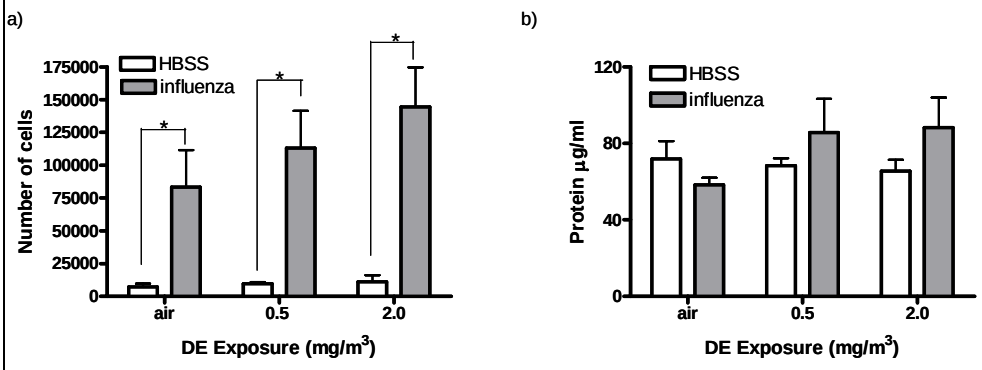


Figure 15. Neutrophil recruitment and levels of protein in the BAL fluid. Measurements were taken 18 hrs. post-infection and all data expressed as mean ± SEM. **a)** The number of neutrophils was determined by differential cell counts. **(b)** Levels of protein were quantified in the BAL fluid. *significantly different from indicated groups, # significantly different from all groups; $p<0.05$.

	HBSS			Influenza		
	air	0.5 mg/m3 DE	2.0 mg/m3 DE	air	0.5 mg/m3 DE	2.0 mg/m3 DE
Total Cells (10^4)	14 ± 2.3	7.8 ± 1.3	6.9 ± 0.8	14 ± 3.4	16 ± 3.3	19 ± 3.5
Macrophages (10^4)	13 ± 2.2	6.9 ± 1.3	5.9 ± 0.6	5.9 ± 1.1	4.8 ± 0.7	4.6 ± 0.8
NAG (U/L)	6.30 ± 0.57	5.20 ± 0.28	5.22 ± 0.17	5.65 ± 0.46	5.3 ± 0.28	7.57 ± 1.1
MIA (ug/ml)	13.07 ± 1.09	11.8 ± 1.61	12.56 ± 0.82	12.33 ± 0.95	20.52 ± 3.84	12.46 ± 0.93

Table 3. Summary of cell counts and markers of injury
Measurements were taken 18 hrs post-infection and all data expressed as mean. Total cell counts in the BAL fluid of each mouse were obtained with a Coulter Counter and number of macrophages was determined by differential cell counts. BAL fluid MIA concentrations were determined with the MALB SPQ II kit and NAG activity was determined from a commercially available kit.

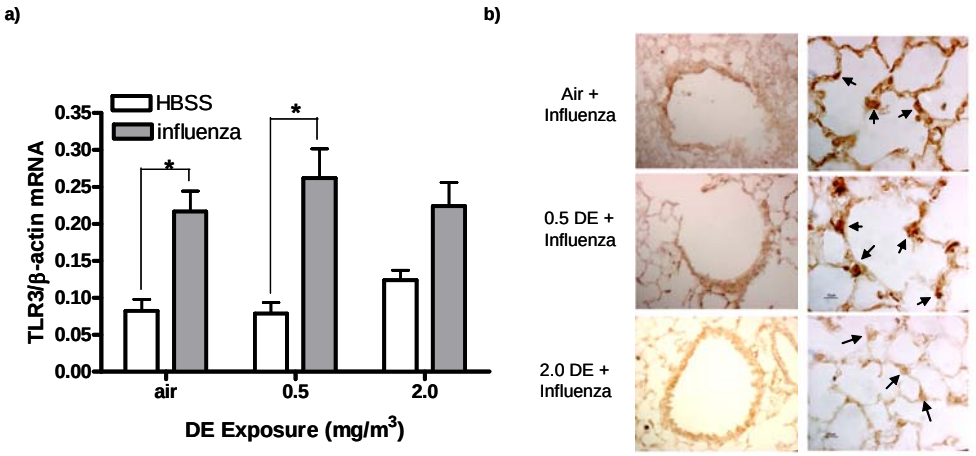


Figure 16. Effect of DE on TLR3 expression.
a) Levels of TLR3 mRNA were quantified in lung homogenates 18 hrs after infection with influenza by real-time RT-PCR. Values are normalized to β-actin and expressed as mean ± SEM. *significantly different uninfected mice; $p<0.05$.
b) Mouse lung sections were immunohistochemically stained for TLR3 and visualized using confocal microscopy. Airway (left) and alveolar (right) regions are shown. Arrows denote positively stained cells.

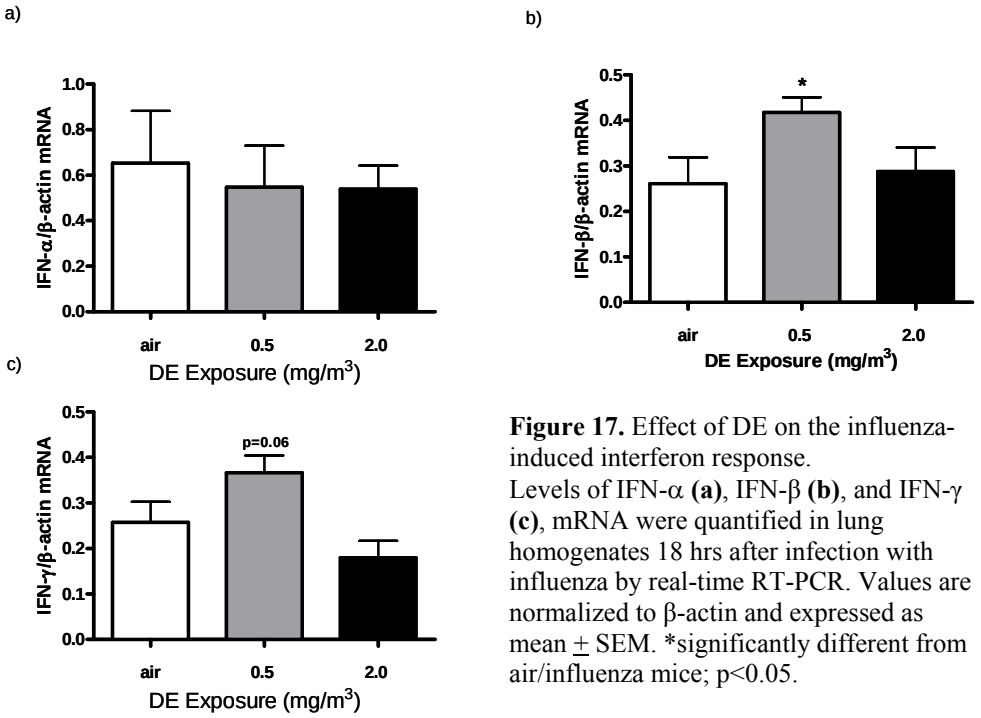


Figure 17. Effect of DE on the influenza-induced interferon response.
Levels of IFN-α **(a)**, IFN-β **(b)**, and IFN-γ **(c)**, mRNA were quantified in lung homogenates 18 hrs after infection with influenza by real-time RT-PCR. Values are normalized to β-actin and expressed as mean ± SEM. *significantly different from air/influenza mice; $p<0.05$.

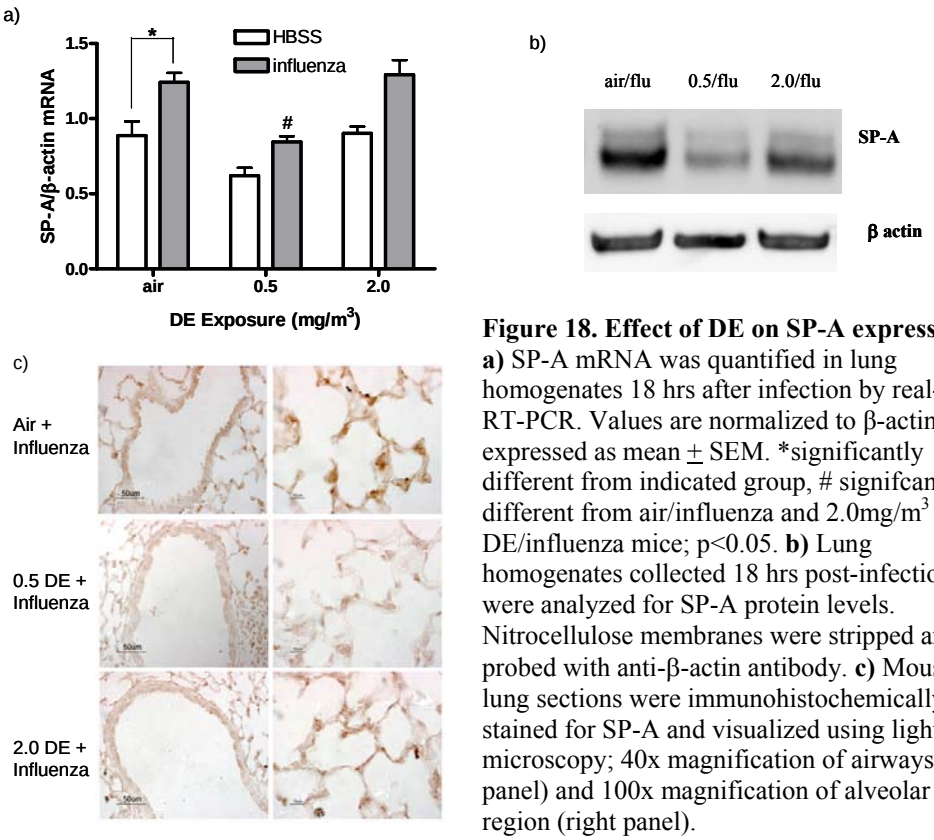
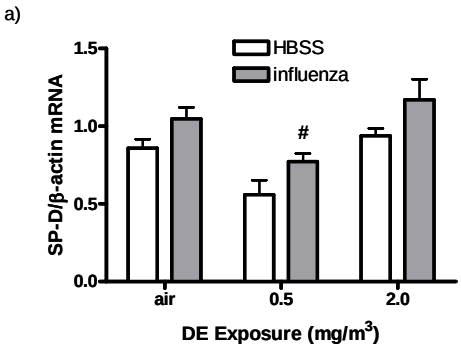
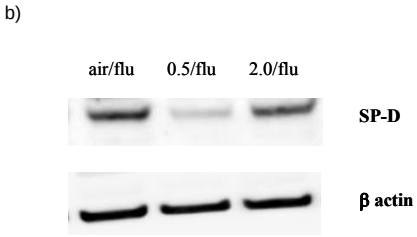


Figure 18. Effect of DE on SP-A expression.
a) SP-A mRNA was quantified in lung homogenates 18 hrs after infection by real-time RT-PCR. Values are normalized to β-actin and expressed as mean ± SEM. *significantly different from indicated group, # signifcantly different from air/influenza and 2.0mg/m^3 DE/influenza mice; $p<0.05$. **b)** Lung homogenates collected 18 hrs post-infection were analyzed for SP-A protein levels. Nitrocellulose membranes were stripped and re-probed with anti-β-actin antibody. **c)** Mouse lung sections were immunohistochemically stained for SP-A and visualized using light microscopy; 40x magnification of airways (left panel) and 100x magnification of alveolar region (right panel).





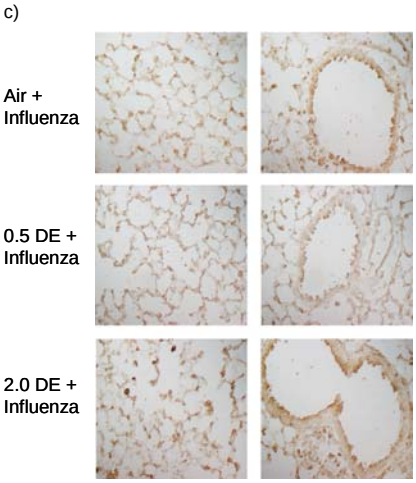


Figure 19. Effect of DE on SP-D expression.
a) SP-D mRNA was quantified in lung homogenates 18 hrs after infection by real-time RT-PCR. Values are normalized to β-actin and expressed as fold mean ± SEM. # significantly different from air/influenza and 2.0 mg/m^3 DE/influenza mice; $p<0.05$. **b)** Lung homogenates collected 18 hrs post-infection were analyzed for SP-D protein levels. Nitrocellulose membranes were stripped and re-probed with anti-β-actin antibody. **c)** Mouse alveolar (left panel) and airway (right panel) lung sections were immunohistochemically stained for SP-D and visualized using light microscopy.

Chapter 4

Influenza-induced TLR3 Upregulation in Pulmonary Epithelial Cells Occurs through a Positive-feedback Loop Involving Type I Interferons and can be Enhanced by Exposure to Diesel Exhaust

Introduction

The host's first line of defense against an invading pathogen is the innate immune response. It activates the secondary immune response and keeps the infection under control until the adaptive response is mobilized. An integral role in the innate immune response is played by Toll-like receptors (TLRs). These receptors are members of the superfamily of interleukin-1 receptors (IL-1R) and share homology in the cytoplasmic region referred to as the Toll/IL-1R (TIR) domain (200). TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) and binding of their respective ligands leads to the production of innate immune defense mediators as well as activation of the adaptive immune response (1, 2, 84). Toll-like receptor 3 (TLR3) recognizes double-stranded RNA (dsRNA), a molecular pattern commonly associated with viral infection. dsRNA stimulates TLR3 signaling, which culminates in the activation of numerous downstream signaling proteins and transcription factors and ultimately results in the production of inflammatory cytokines and type I interferons (3, 67, 132).

The respiratory epithelium is the target of a number of invading pathogens, including influenza. Once infected, these cells secrete various chemokines and cytokines, which elicit an innate antiviral response including the recruitment and activation of inflammatory cells as

well the production of a number of antiviral mediators to help limit the spread of infection to neighboring cells. Of these, type I interferons (IFNs) play an important role in inducing an antiviral state in cells through their induction of numerous genes involved in viral defense, which helps to limit the infection until other responses are mobilized. Type I IFNs in humans consist of IFN-α, β, ε, κ, and ω, of which IFN-α and β have been the most extensively studied and are known for their potent antiviral effects (165, 187). The type I IFNs all bind to a common receptor, the IFN-α/β receptor (IFNAR) (159, 160, 161), which elicits a signal cascade upon activation resulting in the transcription of interferon stimulated genes (ISGs). The IFNAR is composed of 2 subunits (IFNAR1 and IFNAR2), which dimerize upon ligand binding. These subunits are each associated with a member of the Janus activated kinase (JAK) family (36, 80), which are responsible for activating the downstream signaling proteins signal transducers and activators of transcription (STATs) (36, 213). Once activated the STAT proteins form homodimers or heterodimers and act as transcription factors for a variety of ISGs (165, 187).

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 (226, 227). Epithelial cells recognize viral pathogens through pattern recognition receptors (PRRs), such as TLR3, 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. While these responses are important for fighting off the invading pathogen, they are also the cause of much of the resulting morbidity and mortality associated with infection.

For this reason these responses must be tightly regulated by the host so that an adequate antiviral response can be mounted without causing excessive damage to the host.

Both influenza infections and type I IFNs have been shown to upregulate TLR3 expression (67, 74, 103, 135, 228) and virus-induced IFN-β has been proposed to play a role in a positive-feedback loop whereby viral infection causes increased TLR3 expression (223). Furthermore, studies using $TLR3^{-/-}$ mice have shown that the TLR3-mediated inflammatory response has a detrimental contribution to the infected host, and that morbidity and mortality was decreased in $TLR3^{-/-}$ mice (103, 245). Taken together, these studies suggest that the presence of TLR3 is critical for the induction of antiviral responses, but that expression and signaling needs to be tightly regulated. Additionally, previous results in our laboratory have shown that exposure of respiratory epithelial cells to DE prior to infection with influenza increases the expression of IFN-β as well as the expression and signaling of TLR3. Given these previous findings this study was designed to elucidate the potential role of influenza-induced IFN-β in the upregulation of TLR3 in respiratory epithelial cells and the contribution, if any, of DE exposure to this response. The results shown here demonstrate that influenza-induced IFN-β is responsible for the upregulation of TLR3 in influenza infected respiratory epithelial cells. Interestingly, IFNAR expression is limited to the basolateral side of airway epithelial cells. Exposure to DE affects the integrity of the epithelial tight junctional complexes, thereby allowing apically released IFN to access the basolateral portion of the cell where the IFNAR is localized in these cells.

Materials and Methods

Cell Culture

A549 cells, a human pulmonary type II epithelial-like cell line were cultured in F12K medium plus 10% fetal bovine serum and 1% penicillin and streptomycin (all from Invitrogen, Carlsbad, CA). For treatment with IFNs or DE_{as} and stimulation with poly(I:C), A549 cells were grown in 6 or 24-well plates. When the cells reached about 80% confluency and about 18-24h before exposure to DE_{as} and stimulation with poly(I:C), the cell culture media was exchanged for serum-free F12K plus 1.5μg/ml BSA plus antibiotics. For some experiments cells were treated with 1 μM of the tyrosine kinase inhibitor JAK inhibitor I (calbiochem, La Jolla, CA), which is specific to the JAK family. Primary human bronchial epithelial cells were obtained from healthy nonsmoking adult volunteers by cytologic brushing at bronchoscopy. Primary human nasal epithelial cells were obtained from healthy nonsmoking adult volunteers by gently stroking the inferior surface of the turbinate several times with a Rhino-Probe curette (Arlington Scientific, Arlington, TX), which was inserted through an otoscope with a large aperture. The protocols for the acquisition of both primary human bronchial and nasal epithelial cells were reviewed by the University of North Carolina Institutional Review Board. Both primary human bronchial and nasal epithelial cells were expanded to passage 2 in bronchial epithelial growth medium (BEGM, Cambrex Bioscience Walkersville, Inc., Walkersville, MD) and then plated on collagen-coated filter supports with a 0.4 μM pore size (Trans-CLR; Costar, Cambridge, MA) and cultured in a 1:1 mixture of bronchial epithelial cell basic medium (BEBM) and DMEM-H with SingleQuot supplements (Cambrex), bovine pituitary extracts (13mg/ml), bovine serum albumin (BSA, 1.5 μg/ml), and nystatin (20 units). Upon confluency, all-trans retinoic acid was added to the medium

and air liquid interface (ALI) culture conditions (removal of the apical medium) were created to promote differentiation. Mucociliary differentiation was achieved after 18-21 days post-ALI.

Transfection with the TLR3 promoter constructs

The wild-type, mutant ISRE, and mutant STAT TLR3 promoter constructs were generous gifts from Dr. Rehli (Universtity Hospital, Regensburg, Germany) and were designed as previously described (74). A549 cells grown to 60-80% confluence in 24-well tissue culture dishes were co-transfected with 250ng of the pTLR3-luc and 25ng of pSV-β-galactosidase using FuGENE 6 transfection reagent (3µl fugene:2µg DNA, Roche). 24 hours after transfection cells were then treated. Luciferase and β-galactosidase activity was determined using the Dual Light™ reporter gene assay system (Perkin-Elmer) and an AutoLumat LB953 luminometer (Berthold Analytical Instruments, Nashua, NH). Promoter activity was estimated as specific luciferase activity (luciferase counts per unit β-galactosidase counts) and expressed as fold induction over the respective media control.

Exposure to aqueous-trapped solution of Diesel Exhaust (DE_{as})

DE_{as} was generated as described before (123). Briefly, emissions were taken from a Caterpillar diesel engine, model 3304, which was used to power a 113 KW generator. This type of engine was chosen because it is used in non-road vehicles, which are significant contributors to ambient diesel exhaust levels and because the projected trend for emissions from non-road diesel engines is expected to remain at the same level or even increase in the future (235). The diesel exhaust emissions from this Caterpillar diesel engine were passed through a tubing system with a filter impactor and 2 impinger tubes (containing 100 ml PBS each) submerged in an ice bath. Impinger glassware was washed and heated to remove and

destroy endotoxin. Of the two impinger tubes, the emissions (at 10 L/min) that entered and remained in the first (primary) tube, but not the secondary tube, were utilized for the cell exposure studies. Extracts were generated and collected during a one-hour period when the engine was under high load (HL). This type of preparation was chosen, because it contains both DE particles as well as polar and thus water soluble DE gas-phase components. To determine the mass of the emissions retained within the PBS in an impinger tube, an aliquot was dried overnight at 56ºC, and corrected for the mass of the PBS contribution (which was determined in a similar manner by overnight drying) and dilution with water from the exhaust. Aliquots of the DE_{as} were kept at - 20ºC until use. For all cell types used in this study, DE_{as} was added 2 hours before infection with influenza or treatment with poly(I:C). Specifically, for the differentiated human nasal and bronchial epithelial cells, DE_{as} was diluted in 200µl media to achieve 22 or 44µg DE_{as} per cm^2 of cell layer and added to the apical side. After the 2 hours incubation with DE_{as}, the diluted DE_{as} was removed and influenza virus or poly(I:C) was diluted in the same volume of media was added to the apical side for 2 hours, after which it was removed to establish ALI culture conditions again. For the experiments using A549 cells, DE_{as} was diluted in F12K media plus BSA plus antibiotics to achieve 25µg/cm^2 and added to the cells. After 2 hours incubation with DE_{as}, poly(I:C) was added to the cells. The effects of exposure to DE_{as} on cell viability were assessed by analyzing cell culture supernatants for lactate dehydrogenase (LDH) activity using a commercially available kit according to the supplier's instructions (CytoTox 96®, Promega, Madison, WI).

Infection with influenza or treatment with poly(I:C) or IFNs

Throughout this study we used influenza A/Bangkok/1/79 (H3N2 serotype) which 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 described before (11). Stock virus was aliquoted and stored at -80^{o}C until use. Unless otherwise indicated, for infection of differentiated bronchial or nasal cells approximately 3 X10^{5}cells were infected with 320 (hemagglutination units) HAU of influenza A Bangkok 1/79. Cells were treated with 100 μg/ml of polyinosinic acid:polycytidylic acid (poly(I:C)) (Calbiochem; La Jolla, CA) 2 hours after exposure to DE_{as}. In some experiments cells were treated with 1 ng/ml of IFN-α, -β, -ω, or -γ (PBL Biomedical Laboratories, Piscataway, NJ) by adding them to the culture medium.

Epithelial Permeability Measurements

Trans-epithelial resistance (TER) was measured using an epithelial voltohmmeter (EVOM) (World Precision Instruments, Sarasota, FL) according to the supplier's instructions. Trans-epithelial flux of FITC-labeled dextran was used as another indicator of TER. Briefly, FITC-labeled dextran was added to the apical side of cells at a concentration of 100 μM. Fluorescent intensity of the basolateral media was measured at 0, 2, 4, and 6 hours using a fluorescent plate reader (HTS 700) (Perkin-Elmer, Norwalk, CT).

RT-PCR

Total RNA was extracted using TRizol (Invitrogen) as per the supplier's instruction. First-strand cDNA synthesis and real-time RT-PCR was performed as described previously (86, 87). The mRNA analyses were performed using commercially available primer/probe sets

(inventoried Taqman® Gene Expression Assays) purchased from Applied Biosystems (Foster City, CA).

Immunohistochemistry/Confocal Microscopy

DE-exposed cells were fixed for analysis of tight junctional protein complexes at 8, 16, and 24 hours post-exposure. Cells were fixed with acetone for 20 min, washed with TBS and blocked with Powerblock (Biogenex, San Ramon, CA) for 1 hr at RT. Following this, cells were incubated with primary antibody overnight at 4°C. Primary antibodies: anti-IFNAR2 used at 1:200 (Santa Cruz, Santa Cruz, CA); anti-ZO1 used at 1:100 (Zymed, San Francisco, CA); acetylated α-tubulin used at 1:1000 (Zymed, San Francisco, CA). After incubation with primary antibody samples were washed with TBS followed by incubation with secondary antibody at 1:200 for 1 hour at RT. Secondary antibodies: donkey anti-rabbit (fluor 555); goat anti-mouse (fluor 405); donkey anti-goat (fluor 488); all from molecular probes (Invitrogen). Following incubation with secondary antibody samples were washed with TBS and visualized by confocal microscopy using a Nikon C1Si.

Statistical Analysis

Data are expressed as means $\pm$ S.E.M. of at least three separate experiments. Raw data were analyzed using one-way ANOVA followed by the Newman-keuls post-hoc test. Data expressed as fold induction were analyzed using the Wilcoxon Signed Rank Test, assuming a theoretical mean of 1.00 for the control group. A value of $p < 0.05$ was considered to be significant.

Results

Soluble mediators released by influenza-infected cells can upregulate TLR3 expression.

Previous studies conducted *in vitro* using human pulmonary epithelial cells and *in vivo* in mice have show that infection with influenza causes increased expression of TLR3 (67, 103). However, to our knowledge it has not been shown if TLR3 expression is only upregulated in influenza-infected cells or if non-infected cells are also upregulating TLR3 in response to mediators released by infected cells. To determine if mediators released by infected cells affects TLR3 expression, TLR3 mRNA was quantified in cells, which were treated with conditioned media from influenza-infected cells (see diagram in Fig 20A). In this experiment primary nasal epithelial cells were first infected with influenza. Basolateral media was collected 24 hours post-infections and cells were assessed for viral infection, using RT-PCR to quantify HA RNA. The infection-conditioned medium was used to expose new, uninfected naïve nasal epithelial cells for 8 hours. TLR3 mRNA expression was then quantified 8 hours after placing the cells in the conditioned media using real-time RT-PCR. Figure 20B shows that cells cultured in the virus-conditioned media had higher levels of TLR3 mRNA ($p=0.06$) than cells cultured in control-conditioned media. The expression of HA was also quantified in these cells to ensure that no residual influenza virus was left in the media and had infected these cells, thereby causing the upregulation of TLR3 (data not shown).

As stated before, type I IFNs have also been shown to upregulate TLR3 expression (135, 207, 228). To determine if IFNs had the ability to upregulate TLR3 expression in respiratory epithelial cells, A549 cells were treated with 1ng/ml of type I and II IFNs IFN-α, β, ω, and γ for 6 hours upon which TLR3 mRNA expression was quantified using real-time RT-PCR. All of the type I IFNs significantly upregulated TLR3 mRNA, while IFN-γ caused a non-

significant increase in the levels of TLR3 mRNA (Fig 21A). Time-course experiments showed that the IFN-induced increase in TLR3 mRNA levels was maximal between 4-6 hours (Fig 21B). Once it was confirmed that type I IFNs could increase TLR3 expression in our model, the amount of each type I IFN produced in response to influenza infection was quantified 24 hours after infection. Levels of IFN-α, -β, and -ω mRNA were quantified in nasal epithelial cells using real-time RT-PCR (Fig 21C). IFN-β mRNA was by far the most highly expressed in the cells at this time-point.

Transcriptional regulation of TLR3 in response to IFN-β treatment

Since IFN-β was the predominant IFN expressed in response to infection and an increased expression in TLR3 mRNA was observed in cells treated with IFN-β, the transcriptional control of TLR3 in response to IFN-β was examined. A549 cells were co-transfected with the TLR3 promoter (pTLR3) linked to a luciferase reporter gene and pSV-β-galactosidase prior to treatment. Cells were then stimulated with 1 ng/ml of IFN-β for 6 hours and specific luciferase activity was determined using β-galactosidase as a normalizing factor. As shown if figure 21D, treatment with IFN-β significantly enhanced TLR3 promoter-reporter activity.

The next objective was to confirm the involvement of the IFN signaling pathway in the influenza-induced upregulation of TLR3. As stated earlier binding of type I IFNs to the IFNAR elicits a signaling cascade involving JAKs. These kinases are responsible for phosphorylating and activating STATs, which ultimately leads to transcription of ISGs. A549 cells were treated with 1μM of a JAK1 inhibitor (see figure 22), or vehicle control prior to infection with influenza. TLR3 mRNA levels were then quantified 24 hours post-infection using real-time RT-PCR (Fig 23A). Cells treated with the vehicle control showed a

significant increase in TLR3 mRNA levels in response to infection. However, this response was abrogated in cells treated with the JAK1 inhibitor. Cells were also stimulated with IFN-α after treatment with the JAK1 inhibitor to ensure that it was the IFN pathway that was being blocked. IFN-α increased TLR3 mRNA levels only in cells treated with the vehicle control confirming that the type I IFN signaling pathway was in fact inhibited (Fig 23B). Vehicle control or JAK inhibitor was then administered in cells co-transfected with the TLR3 promoter (pTLR3) linked to a luciferase reporter gene and pSV-β-galactosidase to examine TLR3 expression at the transcriptional level. As observed before, vehicle-treated cells infected with influenza showed significantly increased activation of the TLR3 promoter-reporter, while this effect was significantly reduced in cells treated with the JAK1 inhibitor prior to infection (Fig 23C). Finally, to determine the response elements in the TLR3 promoter regulating transcription of the gene, cells were transfected with the wild-type pTLR3 or a pTLR3 containing a mutation in either the interferon-stimulated response element (ISRE) or STAT binding sites of the TLR3 promoter. Following transfection with the respective promoter-reporter constructs, cells were then infected with influenza and luciferase activity was determined 24 hours post-infection. Figure 23D shows that activation of the TLR3 promoter-reporter was significantly reduced in cells containing the pTLR3 with the mutated ISRE site. Activation of the TLR3 promoter-reporter was also reduced, albeit not significantly, in cells containing the pTLR3 with the mutated STAT site.

Polarized response of pulmonary epithelial cells to IFN stimulation

It is well known that the airway epithelium is polarized, with the apical and basolateral membranes having different morphological as well as biochemical characteristics and functions. Furthermore, studies have shown that certain receptors predominantly localize to

the basolateral domain of epithelial cells (60, 239) in order to regulate their function. To determine if there was a polarized response in airway epithelial cells to IFN-β stimulation, TLR3 mRNA levels were quantified in bronchial epithelial cells treated for 6 hours with 1 ng/ml of IFN-β from either the apical or basolateral side or a combination of both. Figure 24A shows that only cells receiving basolateral IFN-β stimulation showed increased expression of TLR3 mRNA. After observing the polarized response of epithelial cells to IFN-β, the next objective was to determine the directionality of IFN-β secretion by these cells. Levels of IFN-β protein secreted to the apical and basolateral sides were quantified in bronchial epithelial cells 24 hours after infection with various doses of influenza. Figure 24B shows that bronchial epithelial cells secrete IFN-β at both the apical and basolateral surfaces in response to influenza infection in a dose-dependent manner.

Basolateral localization of the IFNAR in airway epithelium

Given the polarized response of airway to epithelial cells to IFN-β stimulation it seemed likely that the observed responses were due to a polarized expression of the INFAR in these cells. To test this hypothesis human tracheal tissue was immunohistochemically stained for IFNAR expression. As expected, IFNAR staining was predominantly localized to the basolateral portion of the tracheal epithelium (Fig 25A). To confirm these results in our cell model bronchial epithelial cells were also immunohistochemically stained for INFAR expression and visualized using confocal microscopy. Figures 25B and 25C show that the same expression pattern observed in the trachea is also seen in bronchial epithelial cells.

Diesel exhaust enhances epithelial cell permeability

Previous work in our laboratory has demonstrated that exposure of respiratory epithelial cells to DE prior to infection with influenza causes an increase in TLR3 expression (31, figures 3 and 4). However, the mechanism whereby an air pollutant, such as DE, can alter the response to a respiratory viral infection remains unknown. The data showing the involvement of the type I IFN signaling pathway in the influenza-induced upregulation of TLR3 expression, along with the polarization of the IFNAR led us to investigate the effects of DE on the permeability of the epithelium, which could allow access of apically released mediators to receptors on the basolateral portion of the cell. In fact previous studies have shown that air pollutants such as ozone and cigarette smoke can increase airway epithelial permeability through an effect on epithelial cell tight junctions (15, 18, 20, 62, 152). To examine if DE could exert similar effects on epithelial permeability bronchial epithelial cells were treated with 22 or 44 $\mu g/cm^2$ of DE for 2 hours followed by the addition of fluorescently labeled dextran to the apical surface. Trans-epithelial flux was determined by measuring fluorescent intensity of the basolateral media at 0, 2, 4, and 6 hours after the addition of the fluorescent-labeled dextran (Fig 26A). The fluorescent intensity at 6 hours was significantly greater in the basolateral media of cells treated with 44 $\mu g/cm^2$ of DE compared with untreated cells, indicating that exposure to DE increased trans-epithelial flux. Trans-epithelial electrical resistance was also reduced in cells exposed to 44 $\mu g/cm^2$ of DE (data not shown). To confirm and expand upon these results the effect of DE exposure on the disruption of tight junctional complexes was examined in bronchial epithelial cells using immunohistochemical staining for ZO-1. Cells were treated with 44 $\mu g/cm^2$ of DE for 2 hours and then fixed and immunohistochemically stained for ZO-1, a protein present in the tight junctional complexes of epithelial cells (81, 208), 16 hours after DE exposure. Cells

were then visualized using confocal microscopy. Figure 26B shows that disruption of tight junctional complex integrity, denoted by a thickening and interruption of the fluorescent staining, was observed in DE-exposed cells.

Effect of DE on IFNAR expression

Given the previous results, in which increased TLR3 expression was observed in respiratory epithelial cells exposed to DE prior to infection with influenza, the effect of DE exposure on the expression of the IFNAR was examined. Levels of IFNAR mRNA were quantified 24 hours post-infection in bronchial epithelial cells exposed to 44 $\mu g/cm^2$ of DE prior to infection with influenza. Figure 27A shows that cells exposed to DE prior to infection had significantly greater levels of IFNAR mRNA. These experiments were repeated in A549 cells exposed to 25 $\mu g/cm^2$ of DE prior to stimulation with poly(I:C). Figure 27B shows that expression of IFNAR mRNA is significantly increased in cells exposed to DE prior to stimulation with poly(I:C).

Discussion

Previous studies have shown that infection with influenza results in enhanced TLR3 expression (67, 103), but the mechanism whereby this upregulation occurs had not been completely elucidated. Based on previous studies it was hypothesized that influenza-induced IFN production played a role in the upregulation of TLR3 and that the enhancement of this response by DE involved effects on epithelial permeability. The results of this study show that the influenza-induced upregulation of TLR3 in respiratory epithelial cells is dependent on type I IFNs, which can act in a paracrine manner to induce TLR3 expression in neighboring cells. Furthermore, under normal conditions, the receptor for type I IFNs, the IFNAR, is localized on the basolateral membrane of airway epithelial cells and exposure to DE can increase the expression of the IFNAR as well as the access of apically secreted mediators to this receptor. Together these data provide a mechanism whereby the host can enhance the recognition of and response to influenza infections and how exposure to a common air pollutant like DE can alter this response.

Despite previous observations that influenza infection upregulates TLR3 expression, it was unknown if this was a direct effect of the infection or induced by mediators secreted by infected cells. Given the function of TLR3, which is to sense dsRNA produced during the viral replication cycle and to elicit signaling ultimately resulting in the production of pro-inflammatory cytokines and type I IFNs, it seemed the more effective response would be an upregulation of TLR3 not only in infected cells, but also in cells that have yet to be infected. This would provide a more robust response if the infection continued to spread and neighboring cells became infected. Given this reasoning the ability of influenza-infected cells to signal an upregulation of TLR3 in neighboring cells was examined. Nasal epithelial cells,

which were infected with influenza, did in fact secrete mediators that caused an upregulation of TLR3 in uninfected cells. This response was not due to residual virus left in the cellular media from infected cells, which was confirmed by the absence of HA RNA in the uninfected cells. The fact that mediators released by epithelial cells in response to influenza infection can act in a paracrine manner to 'prime' neighboring cells amplifies innate defense responses against viral infection.

Previous studies have also demonstrated that both IFN-α and IFN-β have the ability to stimulate TLR3 expression (135, 228). To confirm and expand upon these findings the ability of the type I IFNs α, β, and ω as well as the type II IFN γ, to stimulate TLR3 expression in A549 cells were examined. Similar to previous results treatment with IFN-α and -β resulted in a significant increase in TLR3 mRNA expression. Interestingly, IFN-ω was also able to significantly induce TLR3 mRNA expression indicating this effect is not specific to just IFN-α and -β, but that other type I IFNs exert a similar effect. This implies the involvement of the type I IFN signaling pathway in this response. IFN-γ was not able to induce a significant induction of TLR3 expression at the levels used in our experiments. It may be that higher levels of IFN-γ are needed to stimulate TLR3 expression in these cells, or the induction of TLR3 may occur at a later time-point than the ones examined in this study. Since IFN-γ plays a more important role in cell-mediated immunity through its activation of NK cells and macrophages, and its production is actually stimulated by type I IFNs it might not be necessary for INF-γ to exert this type of response in respiratory epithelial cells.

Given the ability of all of the type I IFNs tested to upregulate TLR3 expression it was necessary to determine which type I IFNs were produced by epithelial cells in response to influenza infection. The mRNA expression of IFN-α, -β, and -ω were examined 24 hours

post-infection. The mRNA levels of IFN-β were by far the highest observed at this time-point. Given these data, we focused on IFN-β for the remainder of the TLR3 expression studies. Transcriptional regulation of TLR3 was then examined using a variety of different TLR3 promoter constructs. Treatment with IFN-β caused significant TLR3 promoter activity indicating that type I IFNs enhance TLR3 expression at the transcriptional level. Next the transcriptional regulation of TLR3 and the involvement of the type I IFN pathway in this response were examined. Inhibition of the JAK/STAT pathway, using a JAK1 inhibitor, confirmed that type I IFN signaling was indeed necessary for both increased TLR3 promoter activation and TLR3 mRNA expression. These data indicate that type I IFN binding to the IFNAR activates a signaling cascade, which ultimately results in increased TLR3 mRNA expression. Next transcription factor binding sites within the TLR3 promoter were examined for their role in TLR3 transcriptional activity following IFN-β stimulation. The ISRE, but not the STAT binding site, was required for TLR3 promoter-reporter activity. This was not surprising considering the ISRE is found in the promoters of numerous interferon-stimulated genes (37**,** 105, 195). Activation of the pTLR3 was also reduced, although not significantly, in the promoter containing the mutated STAT site indicating that this site may not be essential for activation, but may be required for maximum response to be elicited.

The involvement of type I IFNs in the influenza-induced upregulation of TLR3 is not surprising considering the role of these cytokines is to induce the expression of genes involved in antiviral defenses. In order for the type I IFNs to exert their effects they must bind to IFNAR, which then activates a signaling cascade that ultimately results in the expression of various interferon-stimulated genes. The regulation of certain receptors' ligand binding and subsequent activation in epithelial cells is achieved through a polarized

localization of these receptors (60, 239). An increase in TLR3 expression was only observed in cells stimulated with IFN-β from the basolateral side suggesting a similar polarization of IFNAR localization in airway epithelial cells. Immunohistochemical staining confirmed the basolateral localization of the IFNAR in the airway epithelium. A possible explanation for this polarized localization is that under normal circumstances, epithelial cells may only receive IFN stimulation from the basolateral side *in vivo*. Dendritic cells, which produce a large amount of type I IFNs after viral infections, usually reside on the basolateral side of the epithelium. Alternatively, sequestration of the IFNAR to the basolateral membrane may serve to limit the amount of stimulation these receptors receive or it may be a way of regulating their activation. Given their localization, IFNs released to the apical side of epithelial cells would be unable to act on these receptors under basal conditions. However, under certain circumstances epithelial cells may increase apical expression of the IFNAR or allow access of apically released IFNs to the basolateral side of the cell through changes in cell permeability. A similar regulatory mechanism is employed by airway epithelial cells to control binding of the growth factor heregulin to its receptors (239). Under normal conditions, heregulin, which is located on the apical surface, cannot bind to its receptors, which are located on the basolateral membrane. However, when the integrity of the epithelium is compromised heregulin can bind its receptors on the basolateral portion of the cell and promotes repair of the damaged epithelium. Thus, the cell is able to control receptor ligand binding so that signaling is elicited only when necessary. In the case of the INFAR and type I IFNs epithelial cells may prevent excess stimulation through sequestration of receptor and ligand, but when epithelial integrity is compromised, such as would be the case

during more severe infections, there may be a need for enhanced antiviral responses to prevent viruses from permeating the epithelial barrier.

Previous work in our laboratory has demonstrated that exposure of respiratory epithelial cells to DE prior to infection with influenza causes an increase in TLR3 expression (31). However, the complete mechanism whereby DE caused an enhancement of the influenza-induced upregulation of TLR3 remains unknown. In addition, studies by other groups have shown that exposure of the airway epithelium to air pollutants such as cigarette smoke and ozone can increase permeability by affecting tight junctions (15, 18, 20, 62, 152). Given the polarization of the IFNAR and it's involvement in the influenza-induced upregulation it seemed plausible that DE's enhancement of this response could in part be mediated by a DE-induced increase in the permeability of the epithelium. Exposure of epithelial cells to DE did in fact reduce trans-epithelial resistance and increased trans-epithelial flux of the epithelium. To elucidate the mechanism behind this response, the effects of DE on the opening of epithelial tight junctions were examined. Immunohistochemical staining for ZO-1, a protein in tight junctional complexes of airway epithelial cells, confirmed our assumptions that exposure to DE caused a disruption of epithelial cell tight junctional complex integrity and thus increased permeability of the epithelium. These data provide a plausible explanation for our previously observed effects, demonstrating that exposure to DE increases influenza-induced effects on TLR3 expression in response to influenza infection. Specifically, increased permeability of the airway epithelium would allow apically released IFNs to access the basolateral portion of the cell where the IFNAR is located. Increased activation of the IFNAR would then result in increased TLR3 expression as was originally observed in epithelial cells exposed to DE prior to infection (see figures 3 and 4). The effect of DE on the

expression of the IFNAR itself was also examined. Surprisingly, expression of IFNAR was also increased in cells exposed to DE prior to infection with influenza. This effect would further amplify the upregulation of TLR3. DE exposure not only results in increased access of IFNs the INFAR, but also increased the expression of the IFNAR thereby providing additional receptors for the IFNs to bind. This enhancement of a tightly regulated cellular mechanism could potentially have a detrimental impact on the host.

The data presented here demonstrate that mediators secreted by airway epithelial cells in response to influenza infection can act in a paracrine manner to upregulate TLR3 expression in uninfected cells. Additionally, type I IFNs can increase the transcription and mRNA expression of TLR3 through signaling elicited upon their binding to the IFNAR, which is localized on the basolateral portion of airway epithelial cells. Furthermore, exposure to DE can enhance this response through its effects on tight junctional complexes, resulting in increased access to IFNAR, and enhanced IFNAR expression. Taken together, these results provide a further understanding of the epithelial innate antiviral responses and how exposure to air pollutants can alter these responses.

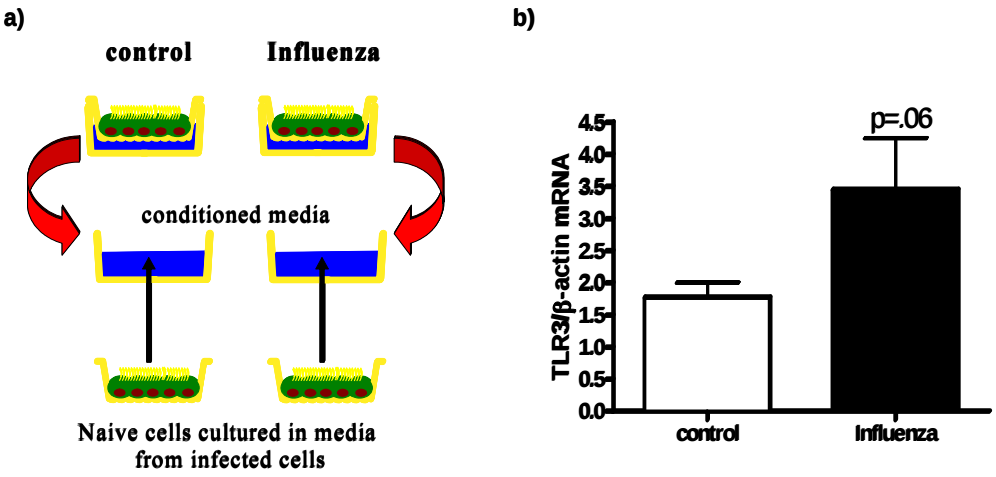


Figure 20. Effect of soluble mediators on TLR3 expression.
a) Schematic of experimental design. **b)** Nasal epithelial cells were left untreated or infected with ~320 HAU/10^5 cells of Influenza A. 24 hrs after infection media from cells was taken and used to culture new cells. TLR3 mRNA was quantified 8 hrs after the 2nd set of cells was cultured in the conditioned media by real-time RT-PCR.

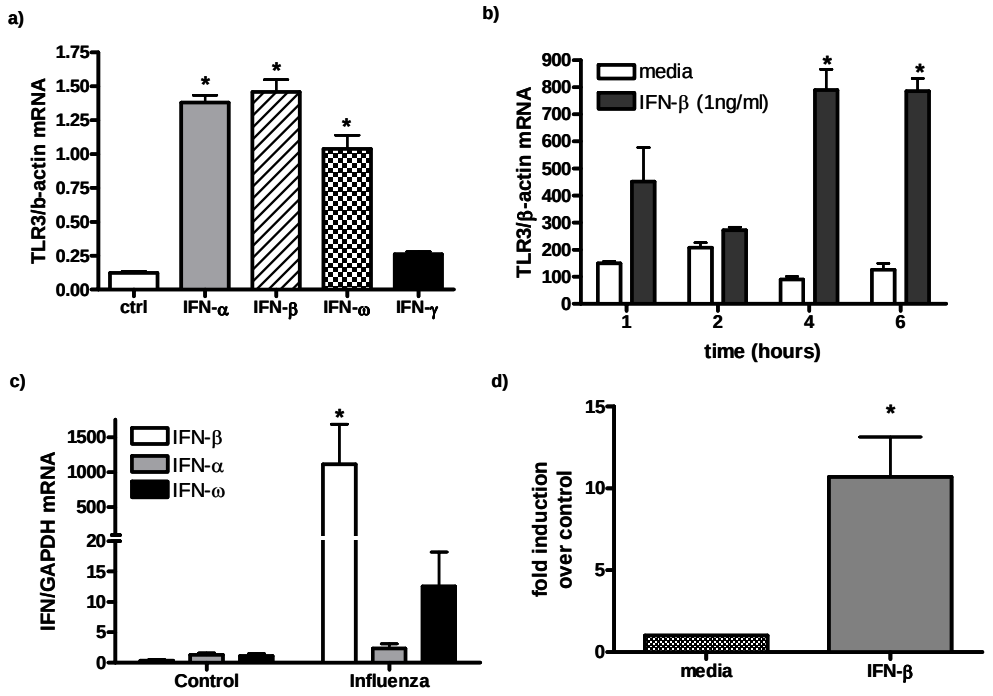


Figure 21. Effect of IFNs on TLR3 expression and promoter activation**a)** A549 cells were treated with 1ng/ml of IFNs and TLR3 mRNA expression was quantified 6 hrs after treatment by real-time RT-PCR. **b)** A549 cells were treated with 1ng/ml of IFN-β and TLR3 mRNA levels were quantified 1, 2, 4, and 6 hrs after treatment by real-time RT-PCR. **c)** Nasal epithelial cells were treated with ~640 HAU/10^5 cells and IFN levels were quantified 24 hrs after infection. **d)** A549 cells were co-transfected with the TLR3 promoter (pTLR3) linked to a luciferase reporter gene and pSV-β-galactosidase 24 hrs prior to treatment. Cells were then stimulated with 1 ng/ml of IFN-β for 6 hours and specific luciferase activity was determined using β-galactosidase as a normalizing factor and data expressed as fold induction over control. *significantly different from control; $p<0.05$

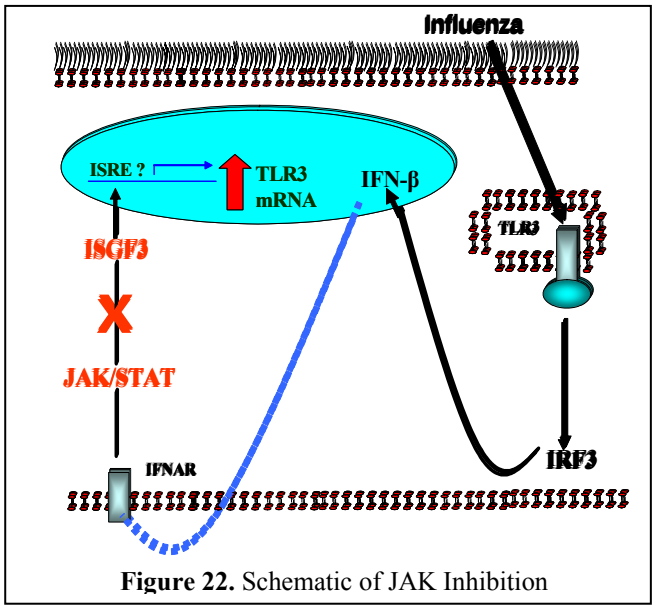


Figure 22. Schematic of JAK Inhibition

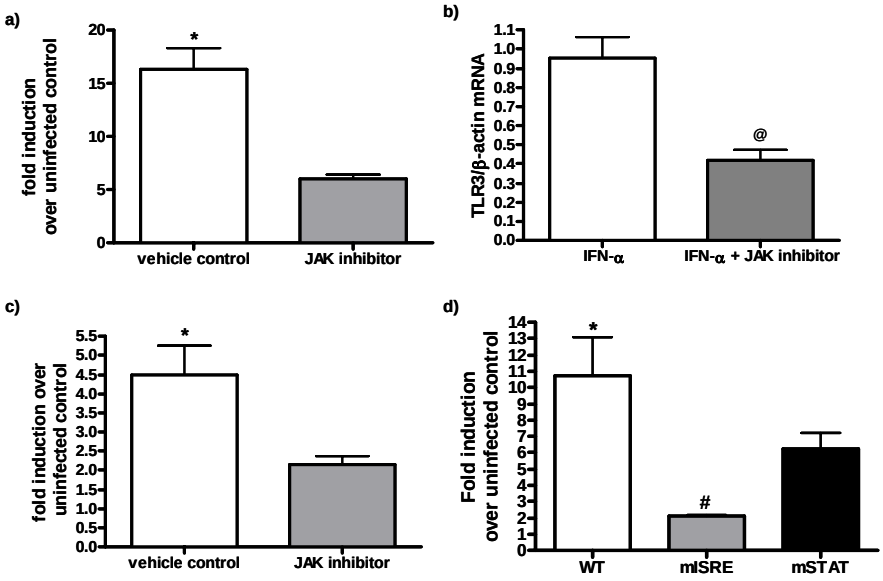


Figure 23. Effect of JAK inhibition on influenza-induced TLR3 mRNA expression and promoter activation.**a)** A549 cells were treated with 1μM of a JAK1 inhibitor, or vehicle control (DMSO) prior to infection with ~320HAU/10^5 cells of influenza. TLR3 mRNA levels were then quantified 24 hours post-infection using real-time RT-PCR. **b)** A549 cells were treated with 1μM of a JAK1 inhibitor, or vehicle control prior to treatment with 1ng/ml of IFN-α. TLR3 mRNA levels were then quantified using real-time RT-PCR. **c+d)** A549 cells were co-transfected with the TLR3 promoter (pTLR3) linked to a luciferase reporter gene or a pTLR3 with mutations in the ISRE or STAT binding sites, and pSV-β-galactosidase 24 hrs prior to treatment. Cells were then infected with ~320HAU/10^5 cells of influenza. Specific luciferase activity was determined 24 hrs post-infection using β-galactosidase as a normalizing factor. Values for a, c, and d are expressed as fold induction over uninfected control. *significantly different from control, @significantly different from uninhibited, #significantly different from WT; $p<0.05$

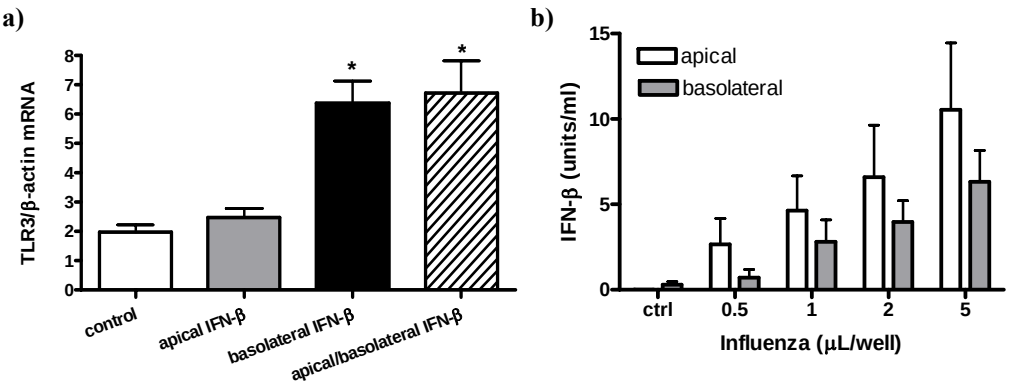


Figure 24. Effect of IFN-β on TLR3 expression
a) Bronchial epithelial cells were treated from the apical and basolateral sides with 1ng/ml of IFN-β. TLR3 mRNA was quantified 6 hrs after treatment using real-time RT-PCR. *significantly different from control, $p<0.05$ **b)** Levels of IFN-β protein were quantified by ELISA in apical washes and basolateral media of bronchial epithelial cells infected with varying doses of influenza at 24 hours post-infection.

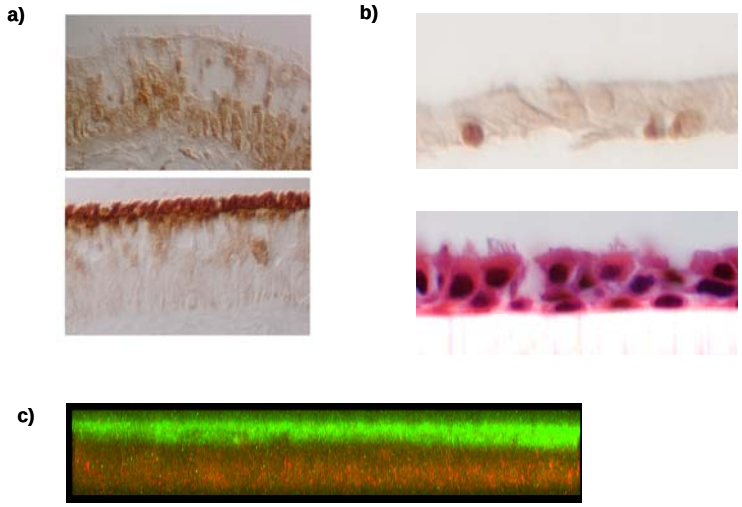


Figure 25. Localization of IFNAR
a) Human trachea immunohistochemically stained for IFNAR (top) and acetylated α-tubulin (bottom). **b)** Differentiated bronchial epithelial cells immunohistochemically stained for IFNAR (top). H+E staining of bronchial epithelial cells (bottom). **c)** Differentiated bronchial epithelial cells immunohistochemically co-stained for IFNAR (red) and acetylated α-tubulin (green). Staining was visualized by confocal microscopy.

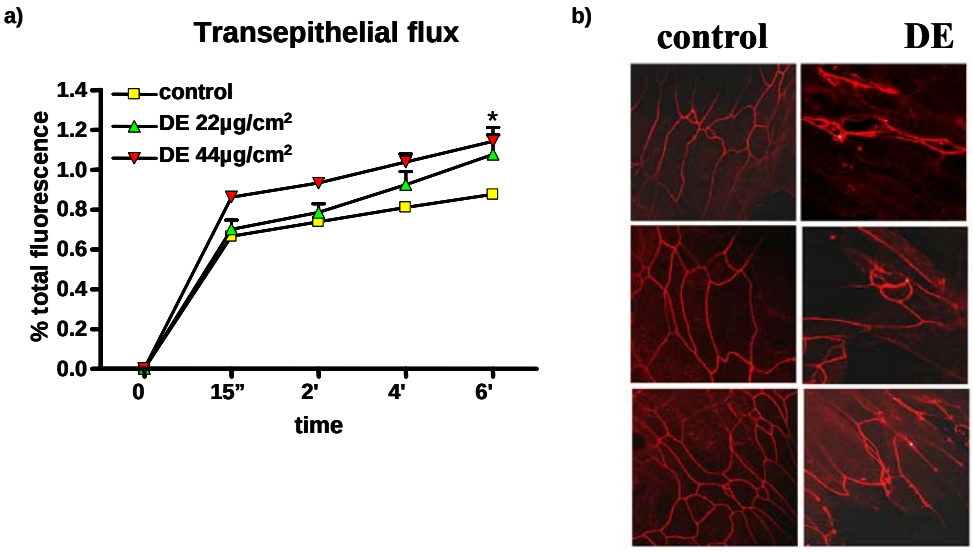


Figure 26. Effect of DE on epithelial cell tight junctional complexes
a) Differentiated bronchial epithelial cells were treated with 22 or 44 μg/cm^2 of DE for 2 hours followed by the addition of fluorescently labeled dextran to the apical surface. Trans-epithelial flux was determined by measuring fluorescent intensity of the basolateral media at 0, 2, 4, and 6 hrs after the addition of the fluorescent-labeled dextran. Data is expressed as % fluorescence of total amount added. *significantly greater than control, $p<0.05$. **b)** Differentiated bronchial epithelial cells were treated with 44 μg/cm^2 of DE for 8 hours. Cells were then immunohistochemically stained for ZO-1 and visualized by confocal microscopy.

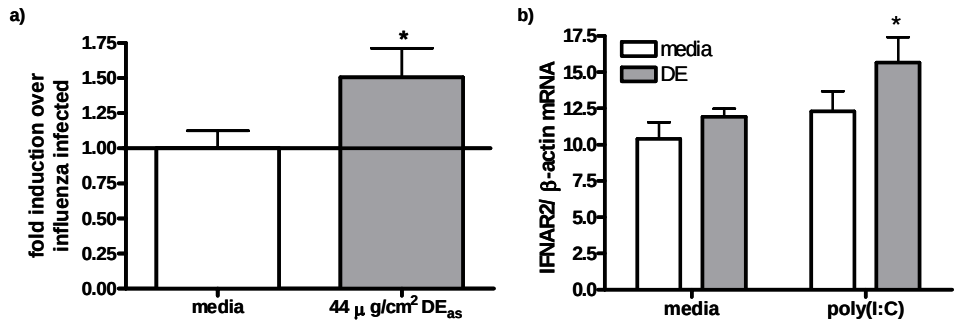


Figure 27. Effect of DE on IFNAR expression
IFNAR mRNA was quantified 24 hrs after infection with influenza or stimulation with poly(I:C) using real-time RT-PCR. **a)** Differentiated bronchial epithelial cells were treated with 44 μg/cm^2 of DE for 2 hours prior to infection with ~320 HAU/10^5 cells of influenza A. Data expressed as fold induction over influenza infected samples. *significantly different from non-DE exposed, $p<0.05$. **b)** A549 cells were treated with 25 μg/cm^2 of DE for 2 hours prior to stimulation with 100 μg/ml of poly(I:C). *significantly different from control, $p<0.05$.

Chapter 5

Overall Discussion

The data presented here demonstrate how exposure to a common air pollutant, like diesel exhaust can enhance the susceptibility and response to a respiratory viral infection through an alteration of the host's innate immune responses. Exposure to DE enhances a tightly controlled positive-feedback loop in pulmonary epithelial cells and results in an increase in the expression and function of TLR3. Since TLR3 is largely responsible for the inflammatory response to influenza (3, 67, 103, 104), it is likely that increased TLR3 expression will result in increased inflammation during an influenza infection. Additionally, studies using $TLR3^{-/-}$ mice have shown that the TLR3-mediated inflammatory response has a detrimental contribution to the infected host and that morbidity and mortality are reduced in mice lacking TLR3 (103, 245). Taken together, these data indicate that the DE-induced increase in TLR3 expression and signaling are likely to have a detrimental effect on an infected host.

Increased susceptibility and responses to influenza infections were observed both *in vitro* and *in vivo*, which provides compelling evidence that the same effects observed in the laboratory may also be occurring in humans. Therefore, it is possible that individuals exposed to DE are more susceptible to influenza virus infections and that the resulting infection will present with increased inflammation and morbidity in certain sub-populations, such as children and the elderly. For these reasons, the data presented here could have important public health implications for all of the areas in which exposure to DE is a concern.

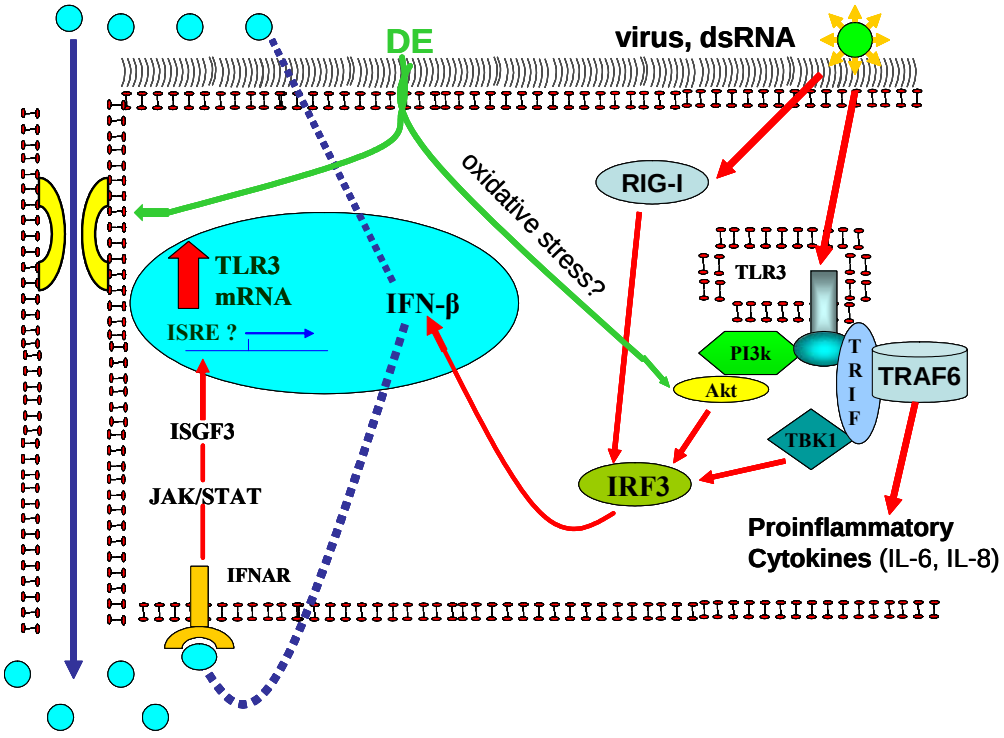


Figure 28 Overall hypothesis of the effects of DE on the influenza-induced upregulation of TLR3

DE has become a concern in many areas since it can account for a significant percentage of air pollutants generated by motor vehicles (42, 234, 235) and epidemiological data and experimental studies suggest that DE is a major contributor to adverse health effects associated with exposure to particulate air pollutants (235, 249). A majority of the diesel PM emissions are produced by off-road and heavy on-road vehicles (233), which is significant because off-road diesel engines emit about 2 times the amount of PM as on-road vehicles, making them a serious concern for PM-induced health effects (23). In fact, one study showed that average personal exposure to PM was 9 times higher for those working around forklift powered by diesel compared to those working around forklifts powered by other fuels (262). Other occupations for which, an increased exposure to DEPs have been shown include

mechanics, truck drivers, and mine workers, where concentrations can range from 25 $\mu g/m^3$ to 1mg/m^3 (175, 192, 262). These data indicate that a particularly susceptible population to the DE-induced effects on the susceptibility and response to viral infection would be those who receive occupational exposures to DE.

Individuals who are occupationally exposed to higher concentrations of DE do not represent the only sub-population for which, an increased susceptibility to the effects of DE exposure exist. Those who have pre-existing allergic and pulmonary diseases are also at higher risk, not because they are exposed to higher concentrations of DE, but because their condition renders them more susceptible. As mentioned earlier, studies have shown that DE increases cellular responsiveness to histamine (75, 100) and acts as an adjuvant to IgE production (44, 55, 143, 149). DEPs have also been shown to adsorb allergens, which could cause a further enhancement of the DE-mediated responses (153). Additionally, studies have demonstrated that patients with obstructed airways show increased lung deposition of PM (137, 179, 194) and that a 30% decrease in airway cross-sectional area results in approximately a 100% increase in particle deposition in the bifurcating airways (184, 99). Thus, DE particle deposition may be significantly higher in the lungs of individuals with obstructed or restricted airways.

The enhanced susceptibility to respiratory viral infections caused by exposure to DE could have even more serious implications for individuals with pre-existing pulmonary conditions. Several studies have shown that respiratory viral infections are associated with asthma exacerbations in both children and adults and that there may be a synergistic effect when individuals are infected with a respiratory virus and exposed to certain allergens (63, 92, 98, 147, 170, 246). Furthermore, another study showed that exposure to air pollution

increased the severity of virus-induced asthma in children (29). In addition to asthma, enhanced frequency and morbidity of chronic obstructive pulmonary disease (COPD) has been associated with respiratory viral infections, including influenza (157, 172, 199, 198). Therefore, DE-induced modifications of the susceptibility to respiratory infections would be particularly detrimental to these individuals.

Two other susceptible populations that may be affected by DE exposure are children and the elderly. Individuals over 65 years old are more likely to die from influenza infections and along with those under 2 years are more likely to be hospitalized due to influenza infection (193, 227). Due to their age these 2 groups are at a higher risk for adverse health effects associated with viral infection or exposure to air pollutants. In fact, numerous studies have demonstrated that these 2 populations show increased susceptibility and response to respiratory infections, as well as other adverse health effects, upon exposure to various air pollutants, including PM (29, 46, 82, 117, 136, 140, 167, 196, 197, 251). Altered susceptibility or immunity to viral infections in these 2 groups could potentially result in severe health effects. Children usually spend more time outdoors than adults so they may be exposed for a longer duration, which is of particular concern for those children living in close proximity to freeways or other sources of air pollutants.

In addition to DE, children are also particularly susceptible to the effects of other particulate air pollutants, such as environmental tobacco smoke (ETS) and woodsmoke or other biomass fuel emissions. These pollutants are similar to DE in the respect that they consist of particulates having chemicals, such as polyaromatic hydrocarbons (PAHs), adsorbed onto them. PAHs result from the incomplete combustion of organic materials and are thought to be responsible for some DE-induced effects (28). Epidemiological studies

have shown that children exposed to woodsmoke or PM from other biomass fuels are at greater risk for becoming infected with a respiratory virus as well as dying from the subsequent infection (5, 33, 40, 51, 91, 101, 138, 141, 173). Studies have also shown that exposure to ETS is associated with an increased frequency and severity of respiratory viral infections in children, yet the mechanisms behind these effects are still largely unknown (19, 66, 68, 77, 118). The mechanisms whereby DE can alter the response to influenza infection as shown here may provide insight into the effects elicited by other oxidant gaseous and particulate pollutants.

While extensive epidemiological and experimental human studies will be needed to elucidate how factors such as length and frequency of exposure will affect the host immune response to respiratory viral infections, the mechanistic aspects can be efficiently studied using *in vitro* techniques, which allow for the control of many confounding factors present in other types of studies. In addition to demonstrating the effects of DE on the innate immune response to influenza infections, this work has also begun to elucidate the underlying cellular and molecular mechanisms driving these effects. The results presented here provide insight into the cellular mechanisms whereby exposure to an air pollutant such as DE can modulate the host response to respiratory viral infection. Pulmonary epithelial cells are the primary targets of both air pollutants and a number of respiratory viruses, and employ similar responses and defenses upon exposure and infection. Therefore, the mechanisms described herein provide a model of cellular responses to environmental insults and how these influence innate immune responses, particularly at the cellular level.

Previous findings in our laboratory as well as others' have shown that exposure to DE enhances the susceptibility to influenza infections both *in vitro* and *in vivo* (28, 69, 85).

However, the mechanism whereby exposure to DE increases the susceptibility to infection is still not completely understood. Various explanations have been proposed, such as decreased antiviral and immune mediators, altered function or reduced expression of antiviral proteins, or even oxidative stress, and there is conflicting evidence in the literature to support or dispute each one. Due to the differences in exposure regimens as well as other experimental parameters, such as animal, virus strains, or timepoints, the differences in observed effects and proposed mechanisms are not surprising. Despite certain differences in their findings and experimental protocols, the overall observation that exposure to DE enhances the susceptibility to respiratory viral infections is consistent. It is the mechanism behind this effect that is still questionable and a further understanding of how this effect is occurring will provide valuable information.

The most plausible explanation seems to be that exposure to DE results in a suppression of the host's innate antiviral defenses. The host employs IFN-dependent responses, which involve various antiviral proteins mainly involved in inhibiting viral replication, as well as IFN-independent responses such as collectins and defensins, which play a role in virus binding and phagocytosis (see chapter 1). However, with a variety of antiviral defenses involved in the innate immune response and differences between cell types and species as well as differences in which responses are essential for different types of infections, there are various ways whereby DE may exert its effects. One aspect of the innate antiviral defense system that DE may alter is the expression or function of surfactant proteins (SPs). These proteins contribute to innate antiviral defenses through their ability to bind and neutralize virus (13, 72) and previous studies have shown that SP-A and SP-D play an important role in the uptake and clearance of influenza as well as other respiratory viruses such as respiratory

syncytial virus (RSV) and adenovirus (71, 70, 108, 109, 110, 113). Therefore, these results indicate that a decrease in expression or alteration of function of SP-A or SP-D would render the host more susceptible to respiratory virus infection. In fact, findings in our laboratory as well as others' have shown that exposure of mice to DE results in decreased expression of SP-A and SP-D, which is associated with enhanced susceptibility to respiratory virus infection (71, see figures 18 and 19).

The decreased expression of SP-A and SP-D caused by exposure to DE may also have other effects not relating to viral infection. In addition to their roles in antiviral and antibacterial responses, SPs have also been shown to play a role in allergic responses and other pulmonary diseases. Several experimental studies have shown that both SP-A and SP-D can bind and aggregate many different allergens, inhibit IgE binding to these allergens, as well as prevent allergen-dependent histamine release and mast cell degranulation (50, 121, 126, 243, 244). Furthermore, studies have shown that SPs can reduce airway hyperresponsiveness, and mice deficient for SP-A or SP-D were more susceptible to pulmonary hypersensitivity after exposure to allergen (122, 206, 217). Therefore, a decrease in SP-A or SP-D expression caused by exposure to DE could lead to increased frequency or exacerbations of allergic responses. Experimental studies have demonstrated that mice deficient for SP-A or SP-D show pulmonary changes consistent with emphysema (73). Finally, one study has shown that SP-A was deleted from the majority of cancer tissues in patients with non-small cell lung cancer and deletion in tumors or adjacent bronchial tissue was associated with increased risk of disease relapse (88). Taken together, these studies demonstrate how decreased surfactant protein expression not only has an impact on the host

defense against invading pathogens, but also on other aspects of host immunity as well as other pulmonary disease states.

Another possible driving force for the enhanced susceptibility to infection, as well as increased inflammation, caused by exposure to DE is oxidative stress. Exposure to DE has been shown to induce oxidative stress and can result in the production of free radicals, which can have damaging effects on the lung (9, 41, 85). The epithelial lining fluid (ELF) of the lung contains various antioxidants such as mucins, glutathione (GSH), ascorbate, uric acid, α-tocopherol, as well as other molecules that protect the lung from oxidative damage and there is evidence that oxidant pollutants react with some of these antioxidant molecules (97). Since epithelial cells lining the respiratory tract are the primary targets for invading pathogens as well as air pollutants, there may be a connection between the oxidative state of these cells and their susceptibility and response to viral infection. In fact, one study observed a decreased susceptibility to influenza infections in mice supplemented with GSH (21). Furthermore, studies conducted in our laboratory support this notion and have shown that pre-treatment of respiratory epithelial cells with GSH reverses the DE-induced enhancement of influenza infections (85). Therefore, it seems that oxidative state is an important determinate of the ability of the virus to infect the host cell. In addition to enhanced susceptibility, oxidative stress may also contribute the enhanced inflammatory response caused by exposure to DE. Generally, antioxidants have been suggested to be protective against respiratory virus-induced inflammation and injury. Specifically, studies by Beck et al. showed that the pathogenesis of a viral infection was exacerbated in mice that had a nutritionally-induced state of oxidative stress (10). Another study showed that transgenic mice with enhanced expression of extracellular superoxide dismutase showed less lung injury

and inflammation after infection with influenza compared with infected wild-type mice (218). Further examination will be necessary to elucidate the exact contribution of oxidative stress to viral infection and the underlying mechanisms that may be ultimately responsible for the observed effects.

Evidence presented here suggests that the major driving force behind the influenza-induced upregulation of TLR3 is a positive-feedback loop involving type I IFNs. Previous studies in our laboratory as well as others' have shown that type I IFNs can increase TLR3 expression (135, 228, see figure 21). In addition, exposure to DE prior to influenza infection results in significantly greater levels of IFN-β both *in vitro* and *in vivo* (see figures 7 and 17). The mechanism behind the enhanced IFN-β response most likely involves other DE- or virus-induced effects besides the increase in TLR3 expression and signaling. Previous studies have shown that exposure to DE can activate the PI3k/Akt signaling pathway, which plays a role in IFN-β transcription (31, 120, 190, see figure 8). This activation is most likely mediated by ROS, since the PI3k/Akt pathway is involved in oxidative stress signaling and ROS can react with several components of this pathway at multiple levels (8). An additional source of IFN-β may also be provided by RIG-I. Previous studies have shown that RIG-I signaling results in the production of IFN-β in response to influenza infection (104, 129, 162). Additionally, exposure to certain types of DE prior to stimulation with dsRNA can increase the expression of RIG-I (see figure 9). Based on studies demonstrating that RIG-dependent IFN-β production is evident as early as 2 hours post-infection (94, 95, 102), one possibility is that RIG-I provides the early stimulation for TLR3 transcription and increased expression caused by influenza infection. Furthermore, DE may enhance or contribute to this

effect through an activation of the PI3k/Akt pathway, which has been suggested to play a role in IFN-β production (31, 120, 190, see figure 8).

Another way in which DE may contribute to the IFN-induced upregulation of TLR3 is through disruption of epithelial tight junctions resulting in increased permeability of the epithelium (see figure 26). Increased permeability of the epithelium would allow apically released IFNs to access the basolateral portion of epithelial cells where the IFNAR is located, resulting in increased signaling and upregulation of TLR3. In addition to increasing the permeability of the epithelium, exposure to DE also resulted in enhanced expression of the IFNAR (see figure 27), which would further enhance the effect of IFN-β on the expression of TLR3.

Disruption of epithelial cell tight junctions caused by exposure to DE leading to increased permeability of the epithelium could impact the activation of other receptors, which are also located on the basolateral portion of epithelial cells. The coxsackievirus and adenovirus receptor (CAR) mediates infection by group B coxsackieviruses and subgroup C adenoviruses (14, 229). The CAR has been shown to be localized on the basolateral portion of polarized airway epithelial cells, thereby limiting the infectivity of these viruses (163, 241, 261). However, exposure to DE or other air pollutants which disrupt epithelial cell tight junctions, such as ozone or ETS (15, 18, 20, 62, 152), could enhance the susceptibility to infection by coxsackieviruses or adenoviruses by allowing increased access to the basolateral portion of airway epithelial cells where the CAR is located. The epithelial growth factor receptors (EGFR) have also been shown to be localized to the basolateral portion of airway epithelial cells while their ligand is located on the apical surface (239). This arrangement assures that receptor activation only occurs when epithelial integrity is disrupted. However,

disruption of the epithelial tight junctional complexes by DE or other pollutants may allow for unnecessary activation of the EGFR leading to increased cell proliferation where it is not needed. Yet another receptor which has been shown to be localized to the basolateral portion of epithelial cells is the IL-6 receptor (128). Similar to the IFNAR, increased access of IL-6 to its receptor resulting from an enhanced permeability of the epithelium, could lead to an exacerbation of the inflammatory response leading to increased morbidity of respiratory viral infections. This is one way in which exposure to DE may increase the viral inflammatory response in a TLR3-independent manner. As stated earlier, ETS and ozone have also been shown to increase the permeability of the epithelium (15, 18, 20, 62, 152), therefore exposure to either of these agents may have similar consequences as DE exposure.

The studies mentioned within this dissertation demonstrate how exposure to a common pollutant can alter host immunity to respiratory viral infections. Considering that the most common form of illness in the U.S. and Europe is respiratory allergies and infections, which account for more missed work as well as school days than any other disease, it is important to understand the associations between, the potential effects of, and the mechanisms involved in air pollution-induced exacerbation of respiratory infections. As more studies are conducted we will continue to improve our understanding of how these effects are elicited and who, if anyone, is most susceptible. An important challenge for the future will be to further our understanding of cellular and molecular mechanisms mediating pollutant-induced effects on the susceptibility to viral infections and potential therapeutic strategies to overcome these effects.

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