DISSERTATION

DEVELOPMENT AND APPLICATION OF AN IMPROVED IN VITRO MODEL FOR AEROSOL TOXICOLOGY

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ABSTRACT

DEVELOPMENT AND APPLICATION OF AN IMPROVED IN VITRO MODEL FOR AEROSOL TOXICOLOGY

In vitro cellular studies offer an economical and rapid screening tool for assessing aerosol toxicity. Traditional submerged in vitro cell models and exposure techniques are often criticized for their inability to (1) simulate in vivo cellular morphology (2) maintain the chemical and physical characteristics of sampled aerosol and (3) estimate 'delivered' exposure levels. Further, the exposure levels applied in traditional submerged in vitro systems are often orders of magnitude above inhalational exposures that occur in vivo.

Improved airway cell culture models and direct air-to-cell exposure systems have been developed over the last few decades; these improvements offer greater 'real-world' significance to in vitro aerosol toxicology. Air-liquid interfaced airway cell cultures offer greater physiological relevance than previous, submerged cell cultures. Further, direct air-to-cell exposure systems offer the ability to (1) better maintain the chemical and physical characteristics of test aerosols and (2) more closely control and approximate exposure levels.

Presented here, are two improved direct air-to-cell aerosol exposure systems that rely upon electrostatic deposition or gravitational settling to directly expose well-differentiated airway cell cultures to three different aerosols of interest, with regard to

occupational and environmental health. The first and second study presented here used electrostatic deposition to expose well-differentiated normal human bronchial epithelial cells to diesel particulate matter and complete diesel exhaust. Cells were exposed to either (1) diesel particulate matter or (2) complete diesel exhaust from an engine run on either petro- or biodiesel, and with and without a diesel particulate filter. Cellular response was assessed by measuring transcripts associated with inflammation, oxidative stress, aromatic hydrocarbon response and overall cellular dysfunction at 1, 3, 6, 9, and 24 hours after exposure to diesel particulate matter. Cellular response to complete diesel exhaust was assessed by measuring transcripts associated with oxidative stress and aromatic hydrocarbon response at two hours after exposure. The main aims of these two studies were to (1) characterize the time course of the proinflammatory response of normal human bronchial epithelial cells after exposure to diesel particulate matter and (2) screen for the effects of exposure to petro- and biodiesel exhaust, with and without a diesel particulate filter. The third study presented here used gravitational settling to expose well-differentiated human bronchial or nasal epithelial cells to two different particle size fractions from inhalable dust collected at a local dairy parlor. Cellular response was assessed by measuring transcripts associated with inflammation at two hours after exposure. Cell compromise was also measured in all three studies by measuring percent lactate dehydrogenase release.

Significant airway cellular responses were observed in all three studies, at levels of exposure far lower than reported in previous traditional in vitro studies. Results from the

work presented here strongly support the use of improved airway cell models and direct air-to-cell exposure systems in future in vitro studies in aerosol toxicology.

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DEDICATION

To my parents, Barbara and Mike: two people who taught me the value of taking pride in one's work, determination, and gratitude for every opportunity in life; and to my grandfather, Roy Chewning, whose life and work history first began my fascination with occupational health and aerosol toxicology.

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$C_{i,down}$	Particle concentration measured downstream of EAVES with repeller plate on during collection efficiency tests
$C_{i,up}$	Particle concentration measured upstream of EAVES during collection efficiency tests
d_p	diameter of particle
Ei	Total particle collection efficiency in the EAVES chamber
PM_3	particulate matter with an aerodynamic diameter smaller than 3 micrometers
PM_{3-10}	particulate matter with an aerodynamic diameter larger than 3 micrometers and smaller than 10 micrometers
PM_4	particulate matter with an aerodynamic diameter smaller than 4 micrometers
$PM_{>10}$	particulate matter with an aerodynamic diameter larger than 10 micrometers
PM_{mass}	estimated PM mass delivered to cells per unit of cellular growth area

CHAPTER 1—INTRODUCTION

The respiratory tract is the first living epithelial layer within the human body to encounter airborne pathogens and hazardous gases and particulates. Because the respiratory tract is vulnerable to airborne pathogens and air toxics, millions of dollars are spent and thousands of animal lives are sacrificed in aerosol toxicology studies each year.(Bakand et al., 2005, Choi et al., 2009) This cost, in energy, resources, and animal lives, is greatly increased when pharmacological studies of aerosol therapeutics are also considered.

Studies that aim to elucidate the mechanisms of toxicity associated with aerosol exposure often rely upon rodent and canine models. Animal models are not only questionable ethically but are also costly in terms of space, energy, and waste. (Choi et al., 2009, Zurlo et al., 1994, Andersen and Krewski, 2009) Further, inherent anatomical differences between humans and rodents, the latter of which are obligate nasal breathers, complicate extrapolations from one species to another. (Miller, 2000) Yet, animals are often used to study inhalation toxicology because, until recently, in vitro models have lacked biological, physiological, and environmental relevance. *In vitro* models of aerosol exposure demonstrate potential to advance our understanding of aerosol health effects; however, traditional *in vitro* exposure techniques have been limited in application and public health relevance. Thus, there is a need for improved *in vitro* models to support aerosol toxicology research that has real-world applications.

Traditionally, the most widely used technique for in vitro lung cell exposures relies upon growing cells submerged in growth media and exposing the cells to particulate matter-extracts or particulate matter resuspended in a liquid.(Aufderheide et al., 2005) Traditional in vitro exposure techniques are limited in practical relevance by: (1) the dissimilarities between most in vitro cell models and the respiratory epithelium in vivo (2) the alteration of particle size, surface, and chemistry when particles are collected on a filter substrate and resuspended in a liquid medium and (3) the inability to control or calculate the 'dose' delivered to the submerged cells.(Volckens et al., 2009a, Holder et al., 2008) These limitations have largely stifled the translational potential of in vitro models for aerosol toxicology.

Primary airway cell culture dates back to the 1960s and 1970s when research interest began to surge in the study of disease processes like cystic fibrosis or lung cancer and environmental exposures like cigarette smoke.(Lamb and Reid, 1969, Boat et al., 1974, Gruenert et al., 1995, Frizzell et al., 1986) However, early cell culture models were limited by lack of a physiologically relevant in vitro system.(Adler and Li, 2001) Early primary cell cultures were typically grown submerged in cell media. Such cultures were observed as undifferentiated, exhibiting a flattened, stratified squamous structure rather than a columnar shape. (Bloemen et al., 1993, Whitcutt et al., 1988) The development of primary airway cell cultures that maintained their differentiated morphology remained in infancy until the late 1980's and 1990's. In the late 1980's, Whitcutt et al. developed a biphasic system that allowed human tracheal epithelial (HTE) cells to be cultured at an air liquid interface.(Whitcutt et al., 1988) Whitcutt et al. observed that when cell cultures

were exposed to air and fed basally through a porous membrane, the cells differentiated into a mucociliary phenotype similar to that observed in vivo.(Whitcutt et al., 1988) For comparison, Whitcutt et al. grew HTE cells in constantly submerged conditions and observed that HTE cells remained as stratified squamous epithelial cells in structure. By the late 1980's submerged airway cell cultures were regarded as an inappropriate model of the lung epithelium in vivo. (Gruenert et al., 1995)

Since the late 1980's, airway cell researchers have found that airway cell differentiation is largely dependent on three factors: growth media, extracellular matrix, and air-liquid interfaced cell culture techniques. Several researchers in the late 1990's observed that the addition of retinoic acid to growth media was particularly critical to the maintenance of the columnar, mucous phenotype in human tracheobronchial and bronchial cells.(Gray et al., 1996, Yoon et al., 1997, Koo et al., 1999) Gray et al. observed that without retinoic acid supplementation, normal human tracheobronchial epithelial (NHTBE) cells became squamous in morphology and decreased mucin secretions by 300-900 fold.(Gray et al., 1996) Further, it became widely observed that fibroblast contamination from explant cultures could be mitigated by growth in serum free medium and selective trypsinization.(Gruenert et al., 1995, Wu, 2004, Gruenert et al., 1990, Robinson and Wu, 1991) Alongside of retinoic acid and serum free growth media recommendations, the extracellular matrix (e.g. collagenous membrane support) was also stressed as important for maintaining airway cell differentiation.(Wu et al., 1985) Perhaps the most important factor in maintaining airway cell differentiation however, is growth at an air-liquid interface.(Gruenert et al., 1995) When cultured at an air-liquid

interface, primary airway cells form a pseudostratified columnar epithelium, complete with ciliated and mucus-secreting cells.(Ross et al., 2007, Gruenert et al., 1995) As Fulcher et al. points out, the development of well differentiated airway cell cultures are a 'quantum leap toward the in vivo biology, and are an excellent model to probe airway epithelial function.'(Fulcher et al., 2005) Because air-liquid interfaced airway cells exhibit a pseudo-stratified columnar epithelial structure complete with the mucociliary phenotype typical of the in vivo nasal and bronchial epithelium, air-liquid interfaced cultures offer airway cell biologists a more appropriate, and physiologically relevant in vitro model of the lung epithelium in vivo.

Described here, are two different aerosol *in vitro* exposure systems that allow the direct deposition of aerosols onto well-differentiated, air-liquid interfaced airway cells. These direct air-to-cell systems are relatively simple, more physiologically relevant, and can potentially reduce the number of animals needed for lung-related research. Presented here are three different studies that demonstrate the utility of these improved aerosol *in vitro* exposure systems. Two different systems were developed and evaluated here: electrostatic deposition and gravity deposition. Electrostatic deposition was used in the first two studies, to screen for the pro-inflammatory and carcinogenic potential of diesel particulate matter (study 1) and complete diesel engine exhaust (study 2). Gravitational settling was used for the third study, to screen for the pro-inflammatory potential of two different inhalable size fractions (PM_{10} and $PM_{>10}$) in dusts collected in an agricultural setting.

Several groups have developed novel in vitro exposure systems that use electrostatic force or gravitational settling to directly expose *air liquid interfaced (ALI)* airway cells to aerosols (Volckens et al., 2009a, de Bruijne et al., 2009, McDougall et al., 2008, Savi et al., 2008). Electrostatic deposition of aerosols onto cells grown at ALI has several advantages: (1) the physical and chemical characteristics of the aerosol (e.g., size, shape, surface area, chemical composition) are maintained (as compared to submerged cell models where particles are placed into suspension and delivered by a syringe spike) and (2) electrostatic force offers efficient deposition for particles in submicron range such that studies may be carried out in a timely fashion. Gravitational settling offers similar advantages, however, gravitational settling systems are limited to studies of particles with sufficient mass (PM > 1 μm) to deposit in a timely manner.

Electrostatic deposition was used in the first two studies described here because diesel particulate matter and diesel exhaust contain particles in the submicron range. A novel, simple, and improved electrostatic aerosol in vitro system that uses a well-differentiated model of the bronchial epithelium to study the response of healthy human lung tissue to submicron aerosol exposure is presented in Chapters 2 and 3. In contrast, because agricultural dusts are typically larger in size and mass (mass median diameters of $PM_{>10}$ are typical of agricultural environments, (Kullman et al., 1998, J. Schaeffer, 2013), gravitational settling was used for the third study presented here. A gravitational based in vitro system is described in Chapter 4.

A discussion of overall conclusions from, and limitations of, the work presented in Chapters 2-4 can be found in Chapter 5. Future experiments that might elicit potential mechanisms of action for these aerosol exposures (DPM, diesel exhaust, and dairy dust) are also discussed in Chapter 5. Future work with similar airway cell exposure systems can also be found in Chapter 5. Although the work presented here is limited to diesel particulate matter, diesel exhaust, and dairy parlor dust as the test agents, similar direct air-to-cell exposure systems could be used more broadly. Further, the work presented here is limited to cell culture with one cell type: human airway epithelial cells. Co-culture models, namely for airway epithelium with other cell types (e.g. macrophages, fibroblasts etc.) and the in vitro applications of assessing exposures to whole lung tissue slices are discussed further in Chapter 5.

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CHAPTER 2— TIME COURSE OF BRONCHIAL CELL INFLAMMATION FOLLOWING EXPOSURE TO DIESEL PARTICULATE MATTER USING A MODIFIED EAVES

SUMMARY

Electrostatic deposition of particles onto the surface of well-differentiated airway cells is a rapid and efficient means to screen for toxicity associated with exposure to fine and ultrafine particulate air pollution. This work describes development and application of an electrostatic aerosol in vitro exposure system (EAVES) with high-throughput and ease-of-use. The modified EAVES accommodates standard tissue culture plates and uses an alternating electric field to deposit a net neutral charge of aerosol onto air-interface cell cultures. Using this higher-throughput design, we were able to examine the time-dependent effects of diesel particulate exposures on human bronchial airway epithelial gene expression and cytotoxicity (1, 3, 6, 9, and 24 hours post-exposure) at levels with 'real-world' significance. Statistically significant responses were observed at exposure levels (~0.4 μg/cm^2) much lower than typically reported in vitro using traditional submerged/resuspended techniques. Levels of HO-1, IL-8, CYP1A1, COX-2, and HSP-70 transcripts increased immediately following diesel particulate exposure and persisted for several hours; increased cytotoxicity was observed at 24 hours. The modified EAVES provides a platform for more efficient and representative testing of aerosol toxicity in vitro.

INTRODUCTION

Elevated exposure to ambient air pollution increases one's risk for chronic lung inflammation & fibrosis (Henderson et al., 1988, Mauderly et al., 1988), allergic immune responses (Sydbom et al., 2001), asthma (Pandya, 2002), lung cancer (NIOSH, 1988), and cardiovascular morbidity (Lucking et al., 2008, Peters et al., 2001). Ambient air pollution has recently been identified as the eighth leading cause of death worldwide, outpacing the risks posed by all other environmental hazards.(Lim et al., 2013) The U.S. EPA recently reduced the annual standard for fine particulate matter ($PM_{2.5}$) from 15 to 12 $\mu g/m^3$ in an effort to reduce mortality rates, incidents of heart attacks, stroke, and childhood asthma resulting from exposure. (EPA, 2012) Despite the increased regulatory action, human exposures to emissions from motor vehicle traffic, coal burning, and other forms of combustion remain prevalent. Human exposure to ultrafine particles is increasing due to increased traffic density and to the practice of biomass burning in developing countries.(Bruce et al., 2000) The advent of nanotechnology, and nanotoxicology, has also created renewed interest in ultrafine particles.(Oberdörster et al., 2005) As a result, a need exists to improve our understanding of the mechanisms associated with ultrafine PM toxicity.(Oberdörster et al., 2005, Teeguarden et al., 2007b)

Airway cell cultures offer an economical means to study the toxicological effects of exposure to aerosol inhalation hazards. However, 'traditional' submerged models of aerosol exposure/response in vitro that date back to the 1960s, have been limited in practical relevance by their dissimilarities to the respiratory epithelium in vivo. The

alteration of particle size, surface, and chemistry are also problems when particles are collected via filtration, extracted, and resuspended in liquid, and then applied to submerged cell cultures. Furthermore, the calculation of particulate 'dose' delivered to cells that are submerged in media is also problematic.(Teeguarden et al., 2007b, Lenz et al., 2009, Liu, 2011) Recent advancements in the last 20 to 30 years, however, have enabled the development of more representative models of aerosol toxicology. Two significant advances are the advent of air-liquid interface (ALI) cultures and direct air-to-cell exposure systems.

When cultured at an air-liquid interface, normal human bronchial epithelial (NHBE) cells progressively differentiate into a mucociliary phenotype that resembles the bronchial epithelium in vivo. Cultured NHBE cells exhibit a pseudo-stratified columnar orientation and show evidence of goblet, basal, and ciliated cell types.(Ross et al., 2007) Because these cells are cultured at an air-liquid interface, they are amenable to direct aerosol exposure, provided that airborne particles can be delivered to the culture surface. Direct air-to-cell exposure systems are advantageous because they tend to preserve the physical and chemical characteristics of the aerosol (e.g., size, shape, surface area, chemical composition) during an exposure (as opposed to when particles are collected on filters, extracted into solution, and delivered to cultures via syringe spike). Direct air-to-cell systems also allow more accurate estimation of the dose delivered to cells.(Teeguarden et al., 2007b)

Direct air-to-cell exposure systems have typically employed three mechanisms to deposit aerosol directly onto cell surfaces: diffusion, gravitational settling, and electrostatic forc(Lenz et al., 2009, Amy, 2007, de Bruijne et al., 2009, Savi et al., 2008, Aufderheide, 2005) Impaction of particles has also been considered. However, air velocities necessary for fine and ultrafine particle impaction are often on the order of meters per second, which can dry out and/or shear apart the cell. Diffusion-based systems are limited to gases and ultrafine particles; above 0.1 μm particle diffusion velocities are too low to support efficient deposition, although a recently-proposed thermophoretic device may improve this rate.(Broßell et al., 2013) Gravity-based systems, on the other hand, are limited only to very large particles (d_p > 10 μm) with sufficient settling velocities. Alternatively, electrostatic force can be used to deposit charged particles of nearly all sizes directly onto the cell surface. Electrostatic air-to-cell exposure systems offer far greater deposition efficiency for fine and ultrafine particles than systems that rely upon gravitational settling or diffusion.(Savi et al., 2008)

Electrostatic force is a popular means to deposit aerosol directly onto the surface of cells cultured at an air-liquid interface. (Volckens et al., 2009a, de Bruijne et al., 2009, Savi et al., 2008) Several systems have used corona discharge (a type of non-thermal plasma) to increase particle electrical mobility, as this technique exhibits high charging (and deposition) efficiencies. (Volckens et al., 2009a, de Bruijne et al., 2009, McDougall et al., 2008) However, corona discharge also creates pollutant byproducts, such as ozone and oxides of nitrogen, at levels that rival regulatory limits for outdoor air.(Nashimoto, 1988) These oxidant byproducts, which are known to generate a pro-

inflammatory response in airway cells, can confound aerosol toxicology experiments.(Becker and Koren, 1994) Studies using corona-based systems have not widely reported corona-related effects on cells,(de Bruijne et al., 2009, Volckens et al., 2009a) but such investigations have been limited in scope. For example, well-differentiated NHBE cultures were not used; instead, an adenocarcinoma cell line (A549) (de Bruijne et al., 2009) or NHBE cells after only three days of growth at ALI were used to screen for corona-related effects.(Volckens et al., 2009a) Further, only one electric field strength (+1.5 kV), two cell markers (IL-8 and LDH), and only one time point following exposure, have been evaluated.(de Bruijne et al., 2009) The corona plasma may also affect particle composition, as has been reported for various organic aerosols. (Volckens and Leith, 2002)

This work had two primary objectives. The first objective was to investigate modifications to an electrostatic aerosol in vitro exposure system (EAVES) that increased sample throughput and ease-of-use while also eliminating potential artifacts associated with corona-based charging. A secondary objective was to apply this system to study the time course of bronchial cell inflammation following exposure to diesel particulate matter. Previous work by Parsanejad et al. evaluated the time course of human bronchial cell inflammation following cigarette smoke exposure, however, they chose to use traditional submerged cell cultures which would have altered the temporal rate of deposition of cigarette PM and potentially the observed temporal response of bronchial cells to cigarette PM. (Parsanejad et al., 2008)

A diagram of this system is shown in Figure 2-1. The new EAVES accommodates a standard, disposable cell culture plate (e.g., 12, 24, or more wells of a transwell or standard tissue culture plate) to increase sample throughput and ease-of-use. Previously described systems have been limited by a lack of sample throughput (using only 3-9 cell wells) and have used non-disposable, custom machined plates for cell culture inserts.(de Bruijne et al., 2009, Hawley and Volckens, 2013, Mertes et al., 2013) Our modified system uses neutralized aerosol (i.e., the native, bi-polar charge distribution present on particles) in conjunction with an alternating electric field to effectively deposit fine and ultrafine aerosol onto the surface of air-liquid interfaced airway cells. The modified EAVES exhibits reproducible particle deposition at levels that more closely resemble those reported for human inhalation studies.(Winkler-Heil and Hofmann, 2002)

We applied this system to evaluate the time-course (1, 3, 6, 9, and 24 hours post-exposure) of transcript production and cytotoxicity in well-differentiated human bronchial cells exposed to diesel particulate matter (DPM) at levels of 'real-world' significance. To the authors' knowledge, the time-course of mRNA production of interleukin-8 (IL-8), heme-oxygenase 1 (HO-1), cyclooxygenase-2 (COX-2), cytochrome p4501A1 (CYP1A1), and heat shock protein 70 (HSP-70) in well-differentiated human bronchial epithelial cells exposed to DPM has never before been evaluated. We sought to identify peak-cellular-response time points after exposure to DPM because these time points could then be used for cellular response measurements in future work with complete

diesel exhaust. We also evaluated the effects of airflow, electric field strength, and exposure duration on multiple markers of cellular inflammation and dysfunction.

MATERIALS AND METHODS

Improved Electrostatic Aerosol in Vitro Exposure System (EAVES)

We modified a previously described EAVES (Volckens et al., 2009a) by replacing the corona-charger with a bipolar aerosol neutralizer (model 3012, TSI Inc.),(Savi et al., 2008) by redesigning the housing to accommodate a standard (disposable) plastic culture dish (85.5 x 128 mm), and by adding a programmable, dual-polarity voltage source to vary repeller plate field strength from -4 to +4 kV at constant periodicity (10 seconds at each polarity). By cycling the polarity of the repeller voltage over time, a net neutral bolus of charged particles is delivered to cell cultures (e.g., positive particle deposition, followed by negative, and then positive, etc.), which also enables the use of plastic (non-conductive) culture dishes. By accepting standard culture dishes, the modified system is more user-friendly and higher-throughput (accommodating 6-24 ALI wells in a single exposure). The EAVES also operates with programmable flow, temperature, and humidity control as described previously.(de Bruijne et al., 2009, Volckens et al., 2009b, Volckens et al., 2009a) A schematic of the EAVES system can be seen in Figure 2-1.

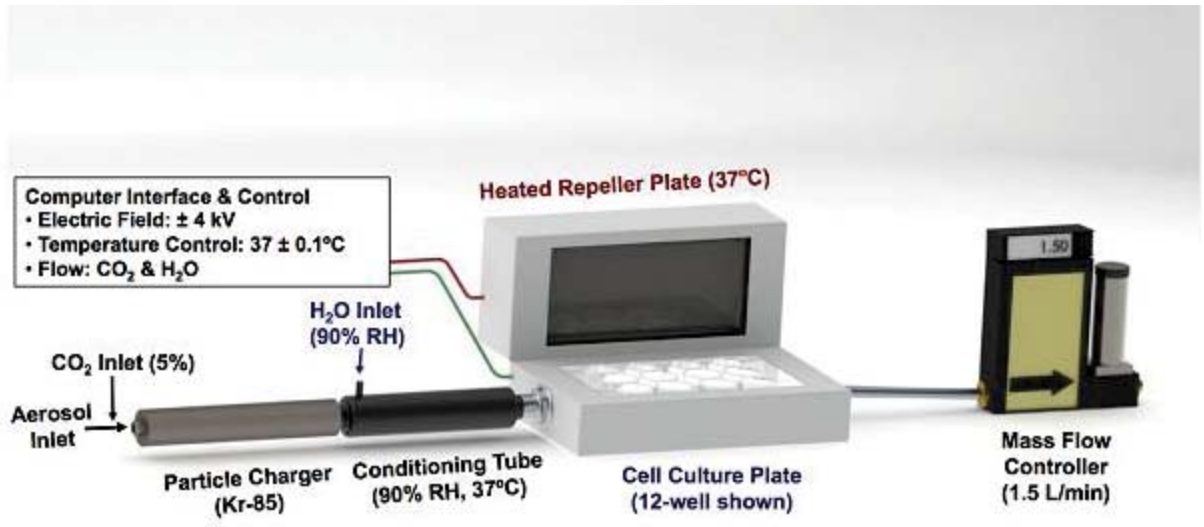

Figure 2-1. Schematic of the modified electrostatic aerosol exposure system.

Particle Deposition and Collection Efficiency

The rate and uniformity of particle deposition was evaluated in the laboratory using fluorescently-labeled particles from 10 to 1000 nm in diameter. A series of ammonium fluorescein solutions (0.13%, 1%, 7% by mass, respectively) was nebulized to generate three polydisperse aerosols with count median diameters of approximately 75, 120, and 220 nm and geometric standard deviations of 2.2, 2.1, and 2.9, respectively. Each well of a standard 12-well tissue culture dish was filled with 1.5 mL ammonium hydroxide solution (pH = 12) and the dish was then placed in the exposure chamber with operating conditions of 85-90% relative humidity, 37ºC, a flow rate of 1.5 L/min. The repeller plate was operated with an alternating electric field (±4 kV) at a periodicity of 10 seconds for each polarity. A reference filter was run in parallel to this system so that particle mass

deposited into each well could be compared to the total mass flowing into the system. Fluorescence was quantified with a microplate reader (FLX-800, BioTek Inc, Winooski, VT) and blanks were carried for each test. Deposition efficiency tests were repeated four times for each electric field operating condition. The linearity and precision of the reader was confirmed with a five point calibration curve spanning the range of ammonium fluorescein concentrations collected.

The rate and uniformity of particle deposition by particle count was evaluated by placing two TEM grids on each of the 12 transwells. A 0.5% aqueous solution of DPM was nebulized inside a 1.0 m^3 aerosol chamber. Nebulized DPM was pulled through the EAVES chamber for two hours. Operating conditions were maintained as described above. Total particle counts and particle size distributions were measured with a Sequential Mobility Particle Sizer (SMPS+C; Grimm Technologies, Douglasville, GA, USA). TEM grids with DPM were imaged using a scanning electron microscope (JEOL JSM-6500F) with magnifications at 2000x and 4000x. Five to ten fields of view were randomly chosen per grid for particle size and count analysis. ImageJ software (NIH, Bethesda, MD) was used to calculate particle sizes and counts per grid. Deposition fractions per cm^2 cellular growth area (deposited counts/total N from SMPS readings) were calculated for particles between 11.1 and 700 nm in size.

Particle collection efficiency, as a function of particle mobility diameter, was estimated by measuring aerosol number size distributions upstream and downstream of the exposure chamber. These measurements were repeated four times with the EAVES

operated at the same conditions as those listed for the deposition efficiency tests. Particle collection efficiency (E_i), where *i* denotes particle size, was determined using Equation 1,

$$E_i = \left[1 - \left(\frac{C_{i,down}}{C_{i,up}}\right)\right] \times 100 \qquad (1)$$

where $C_{i,down}$ represents the concentration of particles exiting the chamber with the repeller on, and $C_{i,up}$ represents the concentration of the particles entering the EAVES chamber.

Cell Culture

Normal human bronchial epithelial (NHBE) cells were obtained by brush biopsy from two healthy, non-smoking human volunteers (EPA, Research Triangle Park, NC, USA) in accordance with a human studies protocol approved by the Institutional Review Board at the University of North Carolina. Cell populations were expanded through two passages in Petri dishes with Bronchial Epithelial Growth Media (BEGM kit; Lonza, Walkersville, MD, USA) before being plated onto collagen-coated, porous, polycarbonate membranes (0.4-μm Snapwell membrane; Corning, Inc., Corning, NY, USA) at a seeding density of approximately 110,000 cells per cm^2. Air–liquid interface (ALI) cultures were carried for a minimum of 21 days (prior to exposure) to allow progressive differentiation into basal, ciliated, and mucin-producing cell types within a pseudo-stratified columnar epithelium.(Ross et al., 2007) Mucus production was visually apparent by day 10 of ALI and excess mucus was removed with a PBS wash, every five days thereafter.

Culture Progression of Air-Liquid Interfaced Cells

On days 1, 10, 24, and 28 of air-liquid interface, one culture well was fixed with formalin and paraffin embedded to observe signs of progressive cell differentiation. Paraffin-embedded cultures were sectioned on a microtome, affixed to a microscope slide, and stained with hematoxylin and eosin using a standard procedure (Veterinary Diagnostic Lab, Colorado State University). Slides were then imaged using transmitted light microscopy at 312x magnification (Leitz Light Microscope, Orthoplan, Germany; SPOT RT Slider Camera, SPOT imaging solutions, Sterling Heights, MI). The live cells were visualized on days 1, 10, 21, and 24 with bright field microscopy at 400x (day 1) or 200x (days 10, 21, 24) magnification.

Cell Exposures to Diesel Particulate Matter

Well-differentiated NHBE cells (cultured at ALI for a minimum of 21 days) were placed in the EAVES chamber and exposed to nebulized diesel particulate matter (DPM; NIST SRM 2975) for a duration of either 90 or 180 minutes. An exposure matrix for NHBE DPM cell exposures can be seen in Table 2-1. For these tests, the EAVES was operated with an alternating field strength of ±4 kV at a flow rate of 1.5 L/min. A 0.5% aqueous solution of DPM was nebulized inside a 1.0 m^3 aerosol chamber. Particle size distributions were measured with a Sequential Mobility Particle Sizer (SMPS+C; Grimm Technologies, Douglasville, GA, USA) for the duration of the cell exposures. Elemental/organic carbon content of SRM 2975 was analyzed by collecting DPM onto quartz filters and then storing filters at -80ºC until carbon samples could be later processed with an OC-EC Lab Analyzer (OC-EC Lab Instrument, Sunset Laboratories

Inc.) Upon entering the EAVES, the DPM aerosol was conditioned to physiological temperature (37ºC) and humidity (80-90%). Each exposure was repeated twice. Particle number concentrations were converted into mass concentrations using the density correction methods of Park et al.(Park et al., 2002) Park et al.'s density corrections were based on an aerosol particle mass analyzer's mass measurements of mobility-sized diesel exhaust particles. Particle density was assumed to decrease with increasing particle size.(Park et al., 2002) We compared the response of DPM-exposed cells to (1) incubator controls and (2) HEPA filtered room air and electric field exposed cells at 1, 3, 6, 9, and 24 hours after exposure.

Table 2-1. Exposure matrix for DPM NHBE Cell Exposures

Experimental Variable	Number of Variables	Level of Variation
Normal Human Bronchial Epithelial Cells (NHBE)	2	Donors 1, 2
Exposure Types	3	NIST DPM ALI (2), NIST DPM submerged (2) Incubator Control (1)
Exposure Level	2	Duration: 90, 180 minutes (N = 12 per exposure condition)
Experimental Repetitions	2	NHBE cells exposed to each exposure duration (2) on two separate days
Cellular Marker Timepoints	5	1, 3, 6, 9, 24 hrs Following Exposure
Cellular Markers	6	IL-8, CYP1A1, HO-1, HSP70, COX-2, LDH

Positive Controls: NHBE Cells Exposed to DPM using 'Traditional' Method

A subset of NHBE cells were exposed to NIST DPM using the traditional submerged method. A DPM solution [50µg per mL BEBM media] was applied to the surface of cells for 90 or 180 minutes (200 mLs total volume). The mRNA transcript levels for IL-8, HO-1, CYP1A1, COX-2, and HSP-70 were then immediately quantified by qPCR, according to MIQE guidelines.

Cell Exposures to an Electric Field

Cultured ALI NHBE cells were placed into the exposure chamber operating at optimal conditions (80-90% relative humidity, 37ºC, 1.5 L/min) with an electric field strength of

either 0 kV (no electric field) or an alternating 4 kV electric field. An exposure matrix for cell exposures to the electric field can be seen in Table 2-2. Under these conditions we exposed the cells to HEPA filtered room air for durations of 45, 90, and 180 minutes. Each exposure was repeated twice. We then evaluated cellular response relative to incubator controls.

Table 2-2. Exposure Matrix for Electric Field NHBE Cell Exposures

Experimental Variable	Number of Variables	Level of Variation
Normal Human Bronchial Epithelial Cells (NHBE)	2	Donors 1, 2
Exposure Types	3	Electric Field + Room Air (3), Room Air (3) Incubator Control (1)
Exposure Level	3	Duration: 45, 90, 180 minutes
Experimental Repetitions	2	NHBE cells (2) exposed to each exposure duration (2) on two separate days
Cellular Marker Timepoints	2	1, 6 hrs Following Exposure (N = 12 per exposure, per timepoint)
Cellular Markers	3	IL-8, HSP70, LDH

Transcript Production in ALI NHBE Cells

Because combustion-derived particles can sequester cytokines produced by epithelial cells, a negative bias can occur when these cytokines are quantified using ELISA.(Totlandsdal et al., 2010, Seagrave et al., 2004, Seagrave, 2008, Kocbach et al., 2008) Previous work by Wyatt et al. observed good correlation between transcript (at 1 HR) and cytokine production (at 6-24 hours) in human bronchial cells exposed to feedlot

dusts, suggesting that mRNA transcripts can be used as a proxy for cytokine production in NHBE cells.(Wyatt et al., 2007) Therefore, we chose not to quantify protein levels directly but instead to assess levels of mRNA transcripts as indicators for later cytokine production.

We chose to quantify mRNA transcripts coding for a panel of five proteins. The production of transcripts associated with inflammation (Interleukin-8, IL-8), oxidative stress (Heme oxygenase-1, HO-1), pain-signaling (Cyclooxygenase-2, COX-2), PAH-adduct formation (Cytochrome p450 1A1, CYP1A1), and overall cellular dysfunction (Heat-shock protein 70, HSP70) were quantified with RT-PCR according to MIQE guidelines.(Bustin et al., 2009) Expression profiles for each transcript were normalized to GAPDH.(Barber et al., 2005) Transcript levels of IL-8, HMOX, COX-2, CYP1A1, and HSP70 were measured in cells exposed to DPM at 1, 3, 6, 9, and 24 hours after exposure. The production of transcripts for inflammation (IL-8) and cellular dysfunction (HSP-70) were measured in cells exposed to HEPA filtered room air and EAVES operating conditions with an alternating 4 kV electric field at 1 and 6 hours post exposure. All transcript expression profiles were normalized to the incubator controls' expression levels of each transcript.

Cytotoxicity in ALI NHBE Cells

Lactate dehydrogenase (LDH) is expressed universally in NHBE cells. The loss of membrane integrity during cell injury and death causes extracellular release of LDH, an indicator of cytotoxicity.(Allan and Rushton, 1994) Extracellular LDH was assayed at 1

and 6 hours post-exposure to an alternating electric field using a standard kit (Promega Cytotox96 Non-radioactive Cytotoxicity Assay, Promega Corporation, Madison, WI, USA). Cellular LDH release was also measured at 1, 3, 6, 9, and 24 hours following exposure to DPM as discussed above. Percent cytotoxicity was calculated by following the standard protocol established by Promega for an assay with a single cell type.

Statistical Analysis

All statistical analyses were conducted with SAS software (v9.0, SAS Institute Inc., Cary, NC, USA) with a type I error rate of $\alpha = 0.05$. Transcript data were log-transformed to satisfy model assumptions of normality and homoscedasticity. The effects of exposure duration, exposure type, time post-exposure, donor phenotype, experimental repeat (and their interactions) were evaluated relative to the expression of IL-8, HO-1, COX-2, CYP1A1, and HSP-70 transcripts and extracellular LDH using the MIXED procedure in SAS. The MIXED procedure in SAS was chosen because it accounts for the repeated measures in time-series data. Cell donor and experimental replicate were treated as random effects.

RESULTS

EAVES Collection and Deposition Efficiency

The relative magnitude and uniformity of particle deposition, by mass, is shown in Figure 2-2A for a 12-well culture plate placed within the EAVES. Particle mass deposition and overall collection efficiencies were consistent between tests. The percentage of incoming aerosol deposited per cm^2 of cellular growth area ranged from

0.29% to 0.43% by mass, for particles between 10 and 700 nm. Particle collection efficiency as a function of size, is shown in Figure 2-2B. The average collection efficiency for particles between 10 and 700 nm in size ranged from 40 to 70% when the repeller plate was run with a 4KV alternating field. The relative magnitude and uniformity of particle deposition, by count, is shown in Figure 2-3.

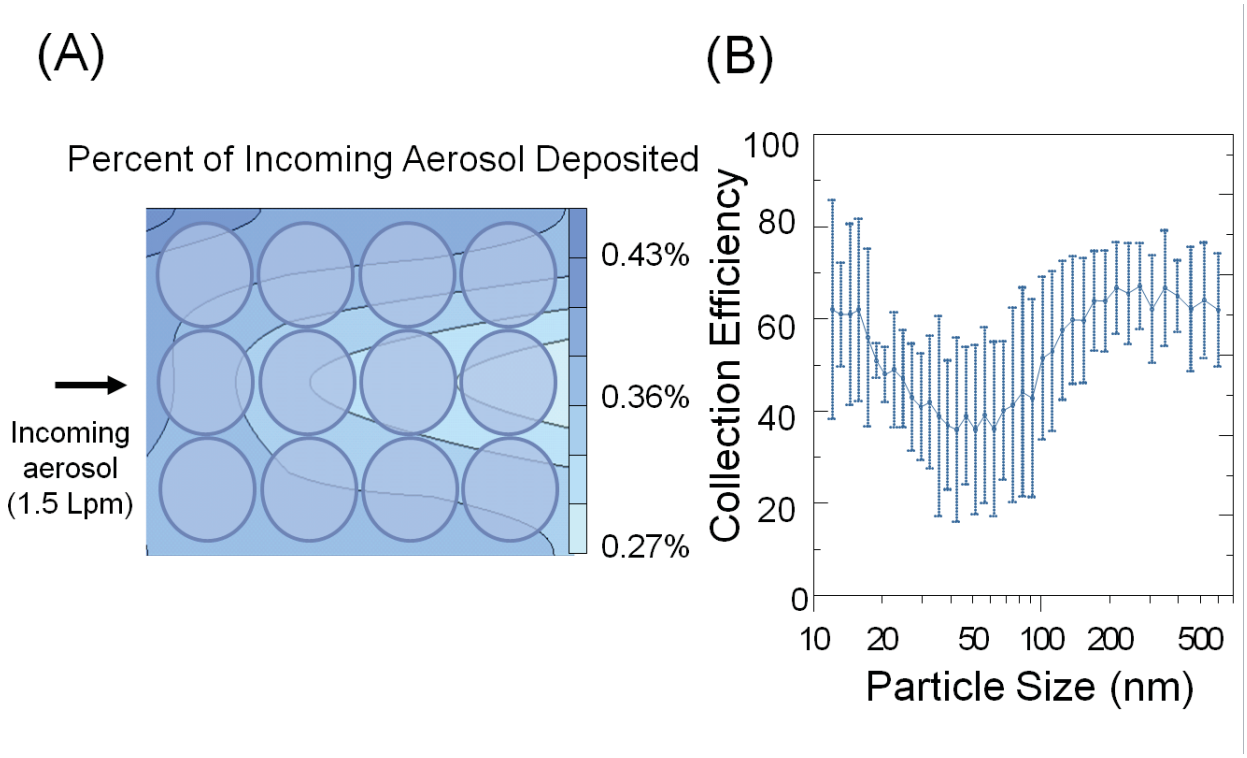

Figure 2-2. (A) Contour plot of deposition efficiency per cm2 of cellular growth area in a cell culture plate. (B) Collection efficiency for particles with diameters of 10-700 nm in size. Circles indicate cell wells in a 12-well plate.

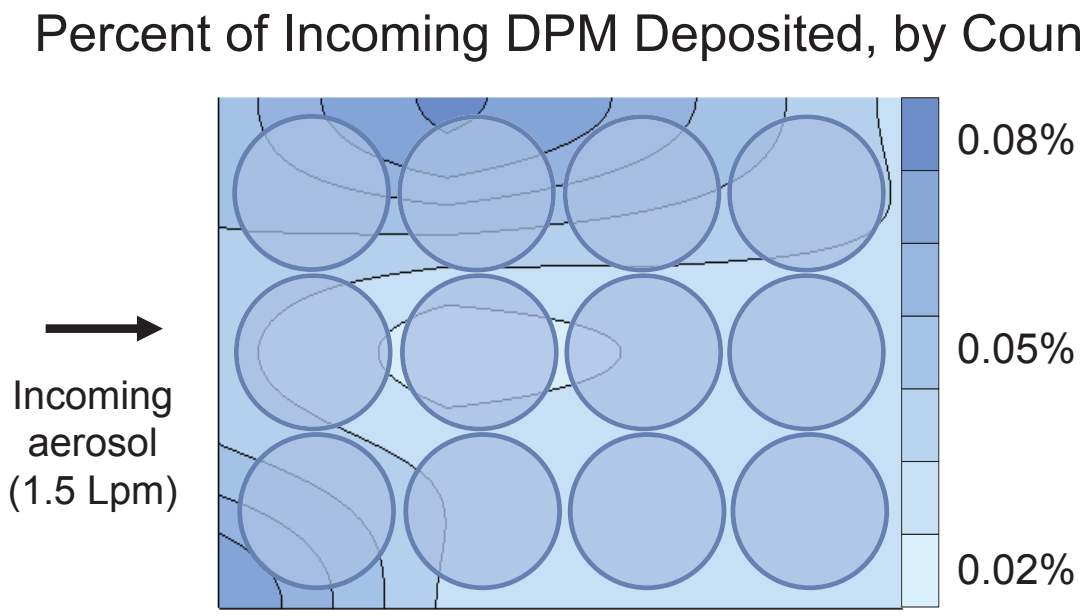

Figure 2-3. Contour plot of percent diesel particulate matter deposited, by count.

Air-Liquid Interfaced NHBE Cells

The physical progression of the NHBE cells grown at an air-liquid interface (ALI) can be seen in Figures 2-4 and 2-5. The NHBE cells underwent progressive differentiation over the course of 28 days. Day 24 and 28 ALI images capture cell cultures with a pseudo-stratified columnar epithelium with signs of basal, goblet, and ciliated cell types.

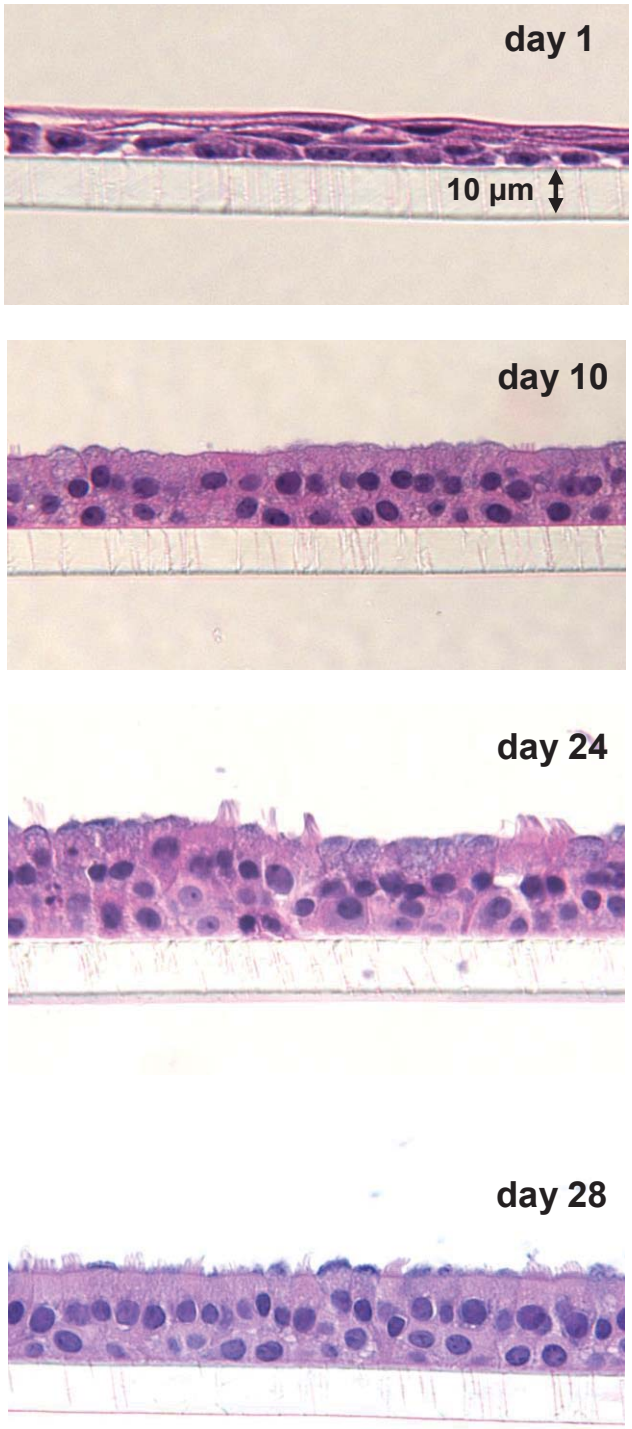

Figure 2-4. Formalin-fixed and paraffin-embedded sections from air-liquid interface cultures of NHBE cells grown upon a collagen-coated membrane. Cells were stained with hematoxylin and eosin. Images are shown at 312x magnification.

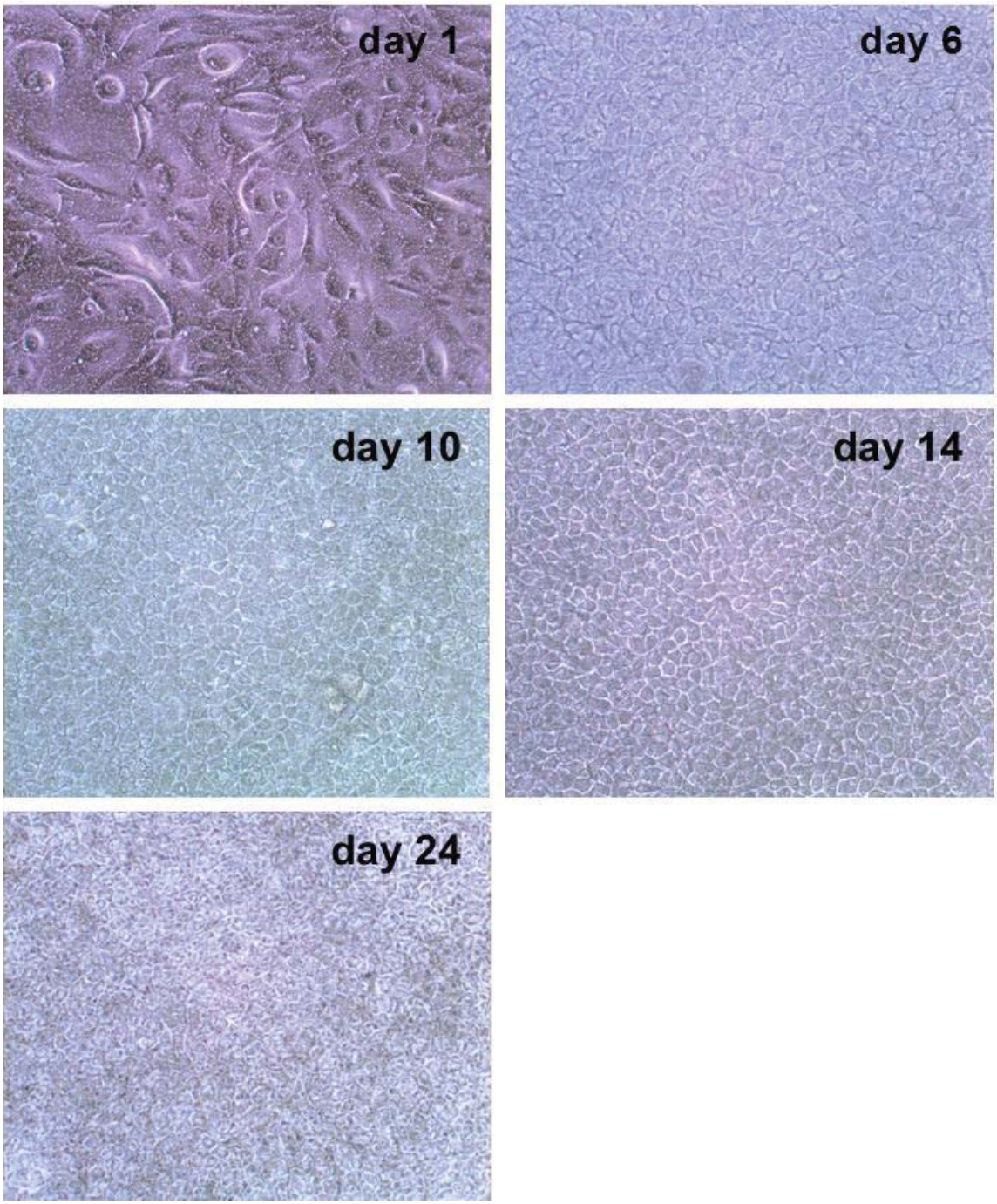

Figure 2-5. Bright field microscopy images of air-liquid interface cultures of NHBE cells. Day 1 image is shown at 400x magnification. Day 6, 10, 14, and 24 images are shown at 200x magnification

Size Distribution, Carbon Content, and PAH Content of Diesel Particulate Matter

The size distribution by count of aerosolized DPM is shown in Figure 2-6. This size distribution was relatively stable across individual experiments and between replicates. The count median diameter (CMD) for the 90 minute cell exposures was 59.2 nm with a GSD of 2.4. A slightly larger CMD was observed during the 180 minute exposures (67 nm), with a GSD of 2.4. . Elemental carbon content of collected aerosol was 88.7 ± 2.7% by mass. The PAH content of this aerosol is provided in Table A.1 (NIST SRM 2975 Certificate of Analysis).

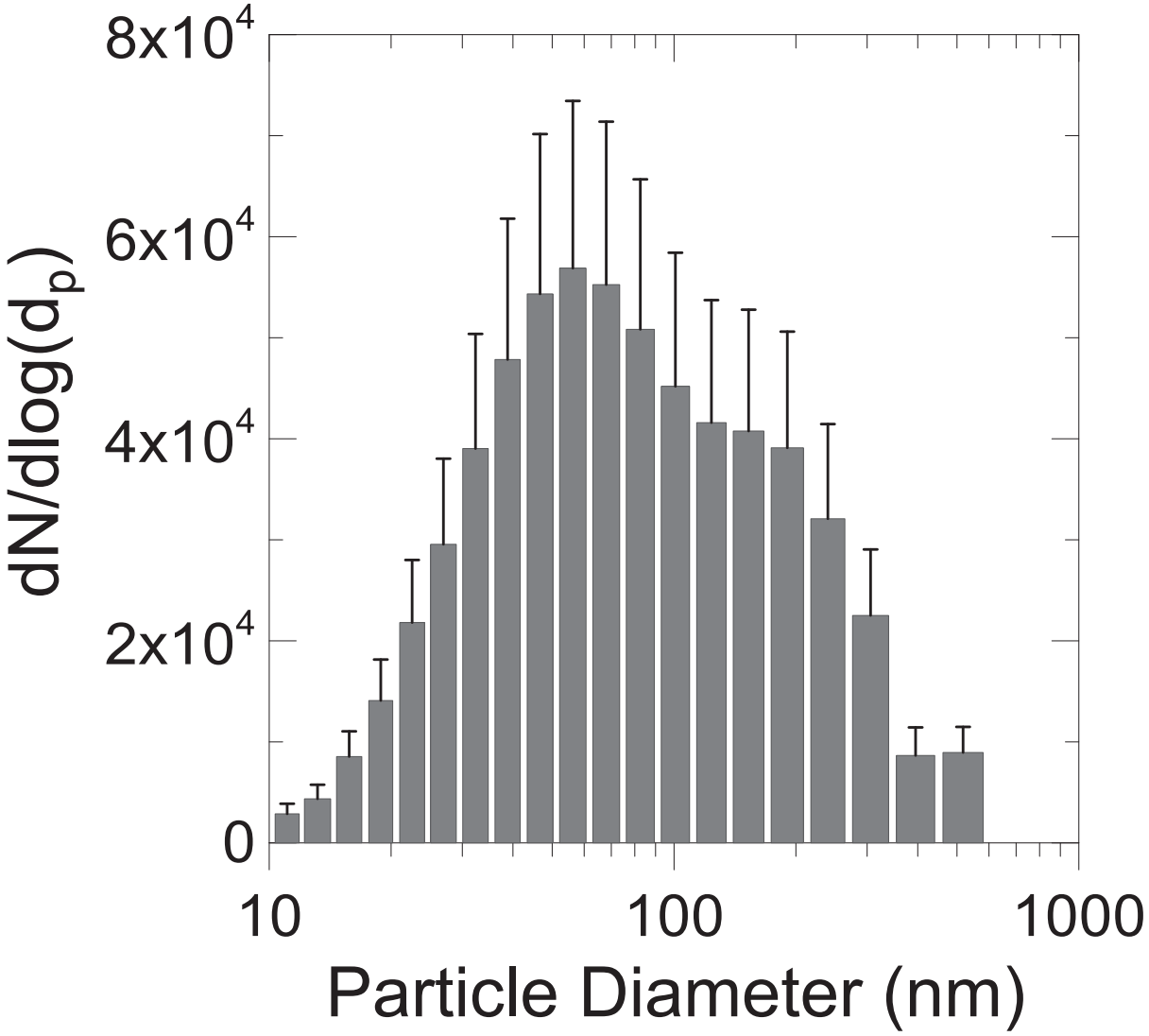

Figure 2-6. DPM Size Distribution during 180 minute cell exposure. Error bars indicate one standard deviation.

ALI NHBE Cell Response to Diesel Particulate Matter

Levels of diesel particulate matter (DPM) mass delivered to NHBE cell cultures during the 90 exposures were approximately 0.36 (± 0.17) µg per cm^2 of cellular growth area; exposure levels for the 180 minute experiments were approximately 1.10 (± 0.52) µg per cm^2 of cellular growth area. Although the DPM size distribution remained relatively stable, the chamber mass concentration varied slightly from test-to-test. During the 180 minute tests the DPM mass concentration ranged from 1200-1350 $\mu g/m^3$; during the 90 minute tests the mass concentration was slightly more stable, ranging from 900-1000 $\mu g/m^3$. As a result, the mass levels deposited onto cells did not scale linearly between the 90 and 180 minute exposures. The estimated diesel particulate matter counts delivered to each cell well with NHBE cell cultures during the 90 or 180 minute exposures can be seen in Tables 2-3 and 2-4. The average counts were $7.11E+10^6$ (± $3.56E+10^6$) and $1.92E+10^7$ (± $9.59E+10^6$) deposited DPM particles per cm^2 of cellular growth area (during the 90 and 180 minute tests, respectively).

Table 2-3. Estimated diesel particulate matter deposited by count, per cm^2 cellular growth area per cell well, during the 90 minute cell exposures.

Cell Culture Plate, Rows		Cell Culture Plate, Columns			
		1	2	3	4
	A	8.05E+06 (± 2.83E+06)	1.39E+07 (± 1.78E+06)	9.43E+06 (± 2.69E+06)	5.28E+06 (± 1.42E+06)
	B	6.29E+06 (± 2.15E+06)	3.16E+06 (± 1.02E+06)	4.40E+06 (± 4.73E+05)	5.08E+06 (± 3.11E+06)
	C	1.27E+07 (± 4.24E+06)	5.41E+06 (± 7.99E+05)	4.27E+06 (± 1.69E+06)	4.80E+06 (± 3.15E+06)

Table 2-4. . Estimated diesel particulate matter deposited by count, per cm^2 cellular growth area per cell well, during the 180 minute cell exposures.

Cell Culture Plate, Rows		Cell Culture Plate, Columns			
		1	2	3	4
	A	2.24E+07 (± 7.88E+06)	3.87E+07 (± 4.96E+06)	2.62E+07 (± 7.47E+06)	1.47E+07 (± 3.95E+06)
	B	1.75E+07 (± 5.985E+06)	8.77E+06 (± 2.84E+06)	1.22E+07 (± 1.31E+06)	1.41E+07 (± 8.64E+06)
	C	3.53E+07 (± 1.18E+06)	1.50E+07 (± 2.22E+06)	1.19E+07 (± 4.71E+06)	1.34E+07 (± 8.75E+06)

One potential disadvantage of the reduced deposition efficiency reported in this system, as compared to previous, corona-charging based systems, is that there may not be sufficient particulate matter delivered to the cellular surface to produce an observable cellular response. However, when exposed over 90 minutes to DPM concentrations similar to those documented in underground mines (900-1350 μg/m^3),(Attfield et al., 2012) NHBE cells produced significantly higher levels of transcripts coding for inflammation (IL-8), oxidative stress (HO-1), prostanoid-signaling (COX-2), tumorigenesis (CYP1A1), and overall cellular dysfunction (HSP-70) (Figure 2-7A-F). Exposed cells gave an immediate rise in transcription of HO-1, HSP-70, COX-2, IL-8, and CYP1A1 following DPM exposures at levels 20-400 times lower than previous studies using submerged cultures(IARC, 2012, McDougall et al., 2008, Ji and Khurana Hershey, 2012, Schwarze et al., 2013) (Figure 2-7F).

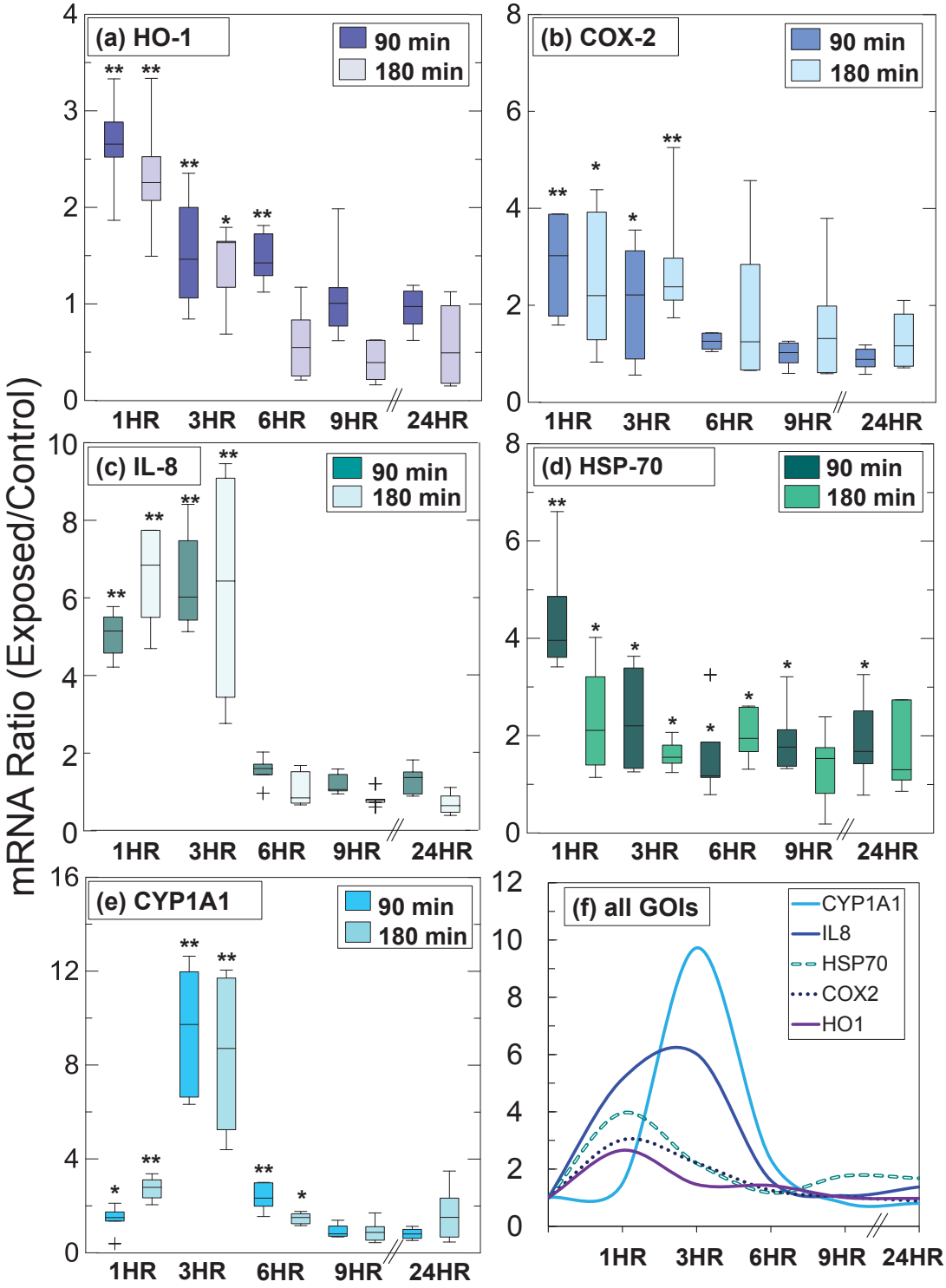

Figure 2-7. (A-E) Box-whisker plots of transcript production in ALI NHBE cells exposed to nebulized NIST DPM for 90 or 180 minutes. All transcript levels are normalized to incubator controls. (+) signifies outliers. * signifies p<0.05, ** signifies p<0.0001, when compared with incubator controls. (F) Modeled time-course (polynomial fit) of transcript production by NHBE cells exposed to DPM (median values shown).

Response of NHBE cells exposed to DPM using 'Traditional' Method

NHBE cells exposed to DPM at a concentration of 50 µg/mL for either 90 or 180 minutes, had significantly higher mRNA transcript levels for IL-8, HO-1, CYP1A1, COX-2, and HSP70 when compared with controls (Figure 2-8). Because submersion in aqueous media induces stress in well-differentiated airway cells, the immediate increase in HO-1 and HSP70 (regardless of duration of exposure) was unsurprising. Cellular levels of mRNA transcript levels for IL-8, CYP1A1, and COX-2 increased with increased exposure duration. This too was unsurprising, as an increase in exposure duration allows for more suspended DPM to settle or diffuse onto the cellular surface.

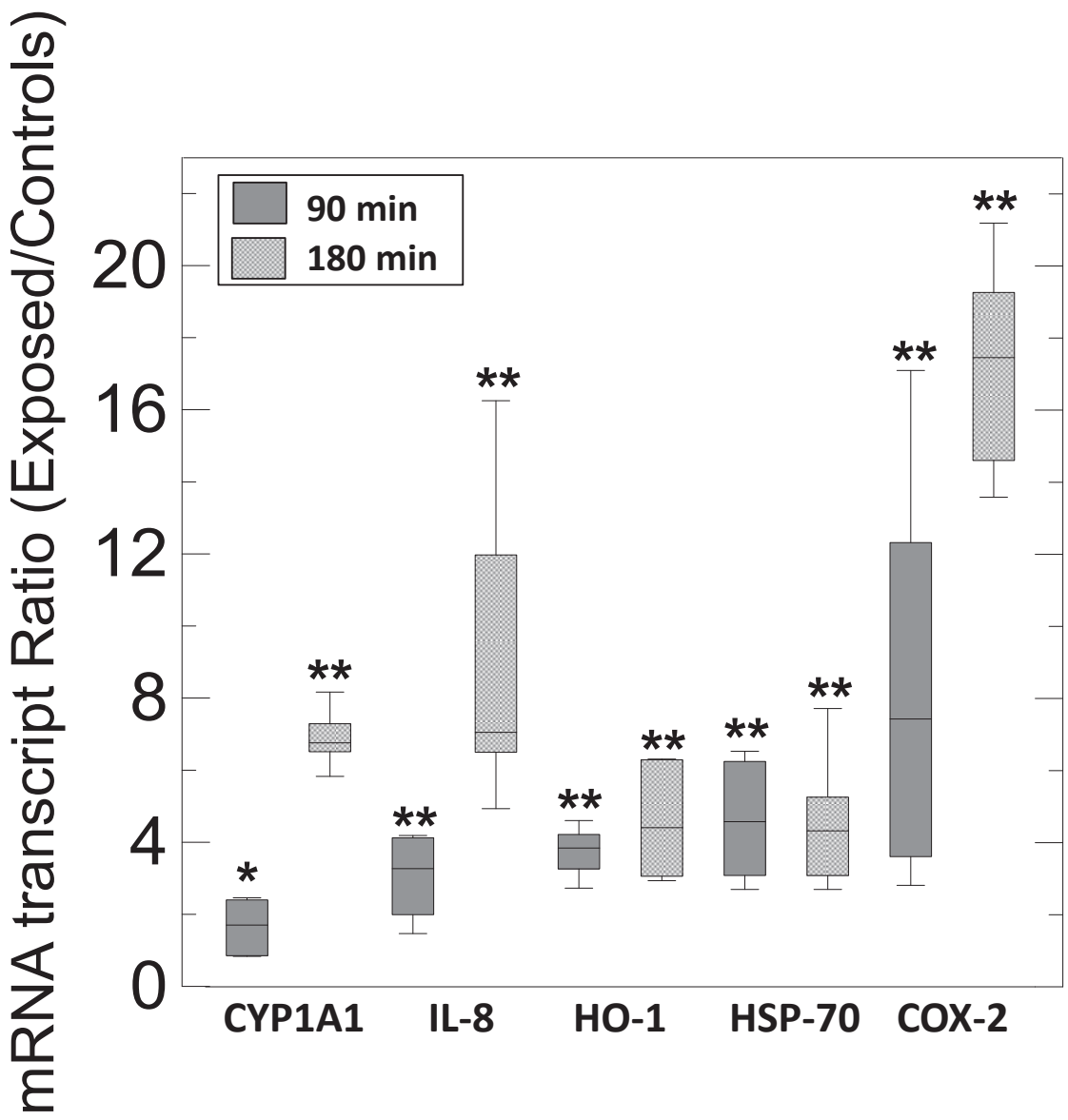

Figure 2-8. Box-whisker plots of positive controls (DPM liquid submersion [50 µg DPM/mL BEBM])

ALI NHBE Cell Response to Electric Field

The alternating 4 kV electric field did not significantly increase the production of transcripts for inflammation (IL-8), or cellular dysfunction (HSP-70), when compared to ALI NHBE cells exposed to HEPA-filtered air alone (Figure 2-9). However, the ALI NHBE cells exposed to HEPA-filtered room air for 180 minutes, *both with and without the electric field*, did increase transcript production of IL-8 at one hour post exposure when compared to incubator controls; albeit, this increase was significantly less than the increase in IL-8 observed in DPM-exposed cells ($p = 0.0004$ and $p = 0.01$, for cells exposed without, and with the electric field, respectively).

ALI NHBE Cytotoxicity after Exposure to DPM and Electric Field

Significant increases in cytotoxicity were observed at 24 hours after a 90 minute exposure ($p<0.0001$) and at 6 and 24 hours after a 180 minute exposure ($p < 0.05$, and $p < 0.0001$, respectively) (Figure 2-10).

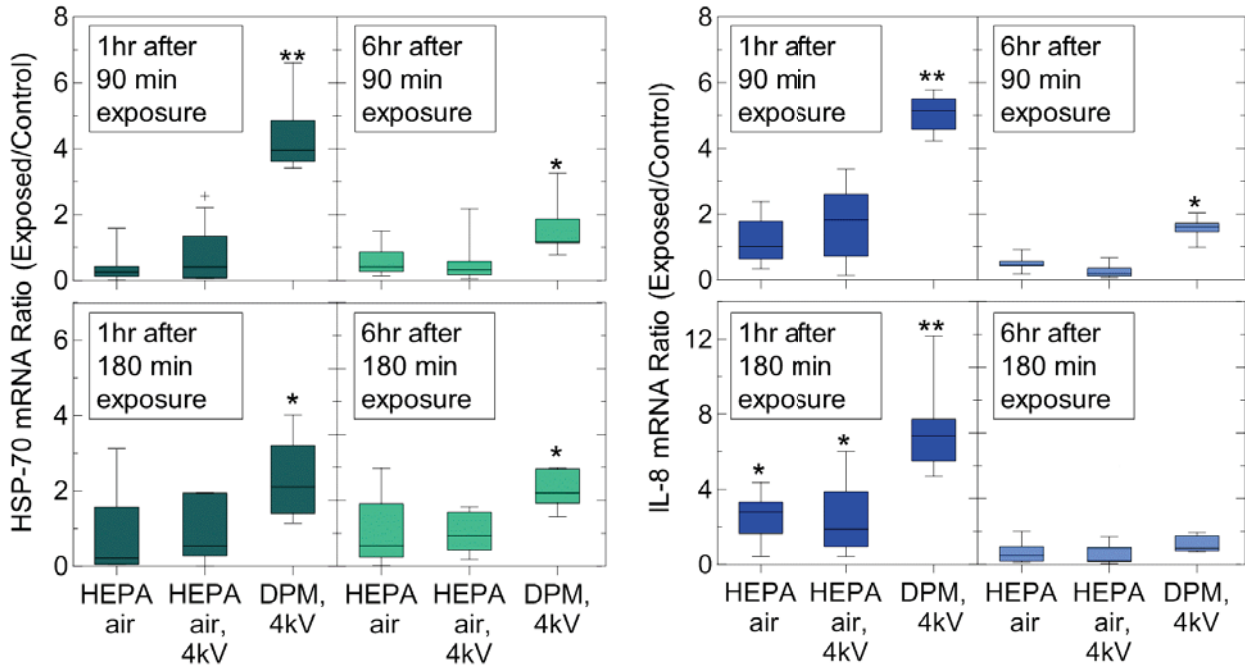

Figure 2-9. Box-whisker plots of the mRNA transcript production by ALI NHBE cells exposed to (1) HEPA-filtered room air (2) HEPA-filtered room air and an alternating 4 kV electric field and (3) NIST DPM and an alternating 4 kV electric field at one and six-hours post exposure. Levels shown are normalized to incubator controls. (+) signifies outliers. * signifies p<0.05, ** signifies p<0.0001, when compared with incubator controls.

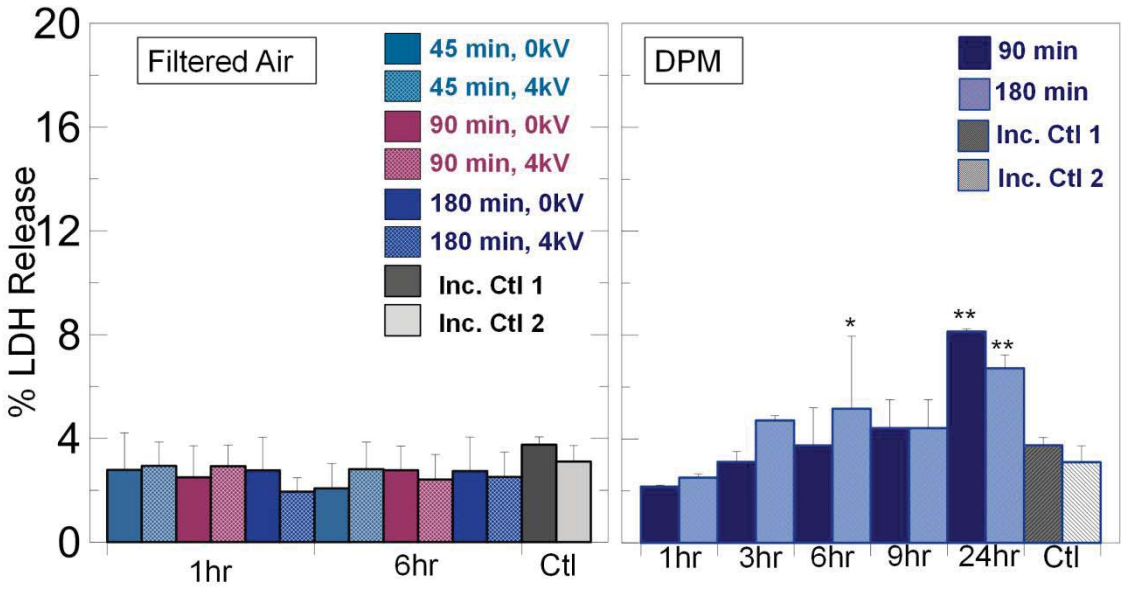

Figure 2-10. LDH release from ALI NHBE cells exposed to (1) NIST DPM or (2) HEPA-filtered room air with and without an alternating 4 kV electric field. Incubator controls: left and right bars indicate incubator controls isolated on days 1 and 2 of exposure test repetitions. * signifies $p<0.05$, ** signifies $p<0.0001$, when compared with incubator controls. Error bars indicate one standard deviation.

DISCUSSION

We chose to evaluate the response of well-differentiated human bronchial cells because the airway epithelium is the first line of defense for inhalation hazards. We observed the greatest increases in transcript production of IL-8, HO-1, COX-2, CYP1A1, and HSP-70 at 1 and 3 hours after exposure, suggesting that the bronchial cellular response to diesel particulate matter is fast-acting.

No single particle 'dose' parameter is likely to adequately describe the biological effects of fine and ultrafine particulate matter.(Paur et al., 2011, Teeguarden et al., 2007b) Diesel particulate matter, is not uniform;(Shi et al., 2000) the large variation in size, shape, and density (Figure A.1) make 'dose' estimates for DPM number or surface area difficult. Therefore, we report DPM deposition rates as a function of both mass and count.(Cauda et al., 2012, Park et al., 2002)

Gangwal et al. provide estimates of ultrafine aerosol deposition in the lungs of a human exposed acutely (24 hours) or over the course of a working lifetime (45 years). Using an multiple pathway particle dosimetry (MPPD) model, Gangwal et al. estimate the total PM deposited in a human's lungs would range from 2.0-4.9 μg per cm^2 of lung cellular surface, when exposed to ultrafine PM at 0.1 mg per m^3, over the course of 45 years. Estimates provided for an acute, 24 hour exposure are dramatically less (0.006-0.02 μg per cm^2 of lung cellular surface).(Gangwal et al., 2011) Previous 'traditional' resuspended PM in vitro studies report significant responses in airway cells exposed at levels 100-1000 fold, or 10-100 fold higher than what might be observed in a human

exposed acutely, or over the course of a working lifetime, respectively (20-200 μg per cm^2 or 20-200 μg per mL).(Baulig et al., 2003, Totlandsdal et al., 2010, Schwarze et al., 2013, Cao et al., 2007) Our system more closely resembles the in vivo dose-level estimated by Gangwal et al., than previous 'traditional' submerged or previous ALI systems. (Baulig et al., 2003, Totlandsdal et al., 2010, Schwarze et al., 2013, Cao et al., 2007) The cellular exposures reported here are comparable to an individual after one-week of exposure to ultrafine particles at 1 mg per m^3. (Gangwal et al., 2011) When compared with other novel, *air-liquid* interface systems, we observed an increase in cellular response to DPM at levels 1.25-300 times lower than previously reported.(Seagrave et al.; 2007, de Bruijne et al., 2009; Aufderheide amd Mohr, 2000) The greater sensitivity of cells exposed to DPM in our exposure system may be due to (1) the direct deposition of DPM onto the cellular surface (2) the improved ability to approximate dose using direct, ALI deposition (as opposed to submerged systems) and (3) measuring the immediate cellular response by quantifying transcripts for oxidative stress, polycyclic aromatic hydrocarbon exposure, overall cellular dysfunction, and prostanoid and pro-inflammatory signaling in well-differentiated human bronchial epithelial cells.

Exposure to DPM results in oxidative stress and inflammation in the human airways.(Li et al., 2002) Disruption of the mitochondrial membrane and redox-cycling chemicals in DPM (e.g. polycyclic aromatic hydrocarbons, quinones, ketones, metals, and aldehydes) contribute to the formation of reactive oxygen species (ROS) in the cell. Increased ROS can then create reactive metabolites that lead to the deleterious,

cytotoxic effects from DPM exposure.(EPA, 2012) DPM also activates the unfolded protein response in the endoplasmic reticulum.(Adair-Kirk et al., 2008) A primary cellular defense against the cytotoxic effects of oxidative stress and the unfolded protein response (UPR) of the endoplasmic reticulum is activation of the Nuclear factor-like 2 (Nrf2) and the NF-kβ pathways.(Zhang et al., 2005, Nguyen et al., 2009, Morgan and Liu, 2011, Madamanchi et al., 2001) Increases in cellular ROS following DPM exposure also activate the Nrf2 pathway, which then activates many cytoprotective genes, including HO-1, as well as pro-inflammatory mediators, like IL-8.(Swanson, 2009) The p38 MAP kinase pathway is also sensitive to DPM exposure (and to the redox state of the cell) and also increases the production of IL-8. (Hashimoto et al., 2000) NFkβ and JAK-STAT pathways are also activated by an increase in cellular ROS and the UPR, and these pathways, too, increase the transcription of HO-1 and IL-8, as well as the pro-inflammatory prostaglandin, COX-2 and the cytoprotective protein, HSP-70.(EPA, 2013, Schwarze et al., 2013) A diagram of the two most often cited pathways in particulate-induced lung inflammation (Nrf2 and NF-kβ) (Adair-Kirk et al., 2008) can be seen in Figure 2-11 below. In Figure 2-11 we highlight some of the major genes involved in the antioxidant, detoxification, and inflammatory response after DPM exposure. As expected, we observed an increase in transcripts for HO-1, IL-8, HSP-70, and COX-2 immediately after exposure to DPM (1 hr; Figure 2-7F). Because IL-8 transcription is increased through multiple cellular pathways (Nrf2, NFkβ, and p38 MAP kinase pathways), the steady rise in IL-8 production (1-3 hours) after exposure to DPM was not surprising (Figure 2-7C). We note that due to the differing exposure durations, the timepoints do not sync wholly between the 90 min and 180 min cell responses. Our

results suggest that the timing of response tends to sync up with when the exposure ceased.

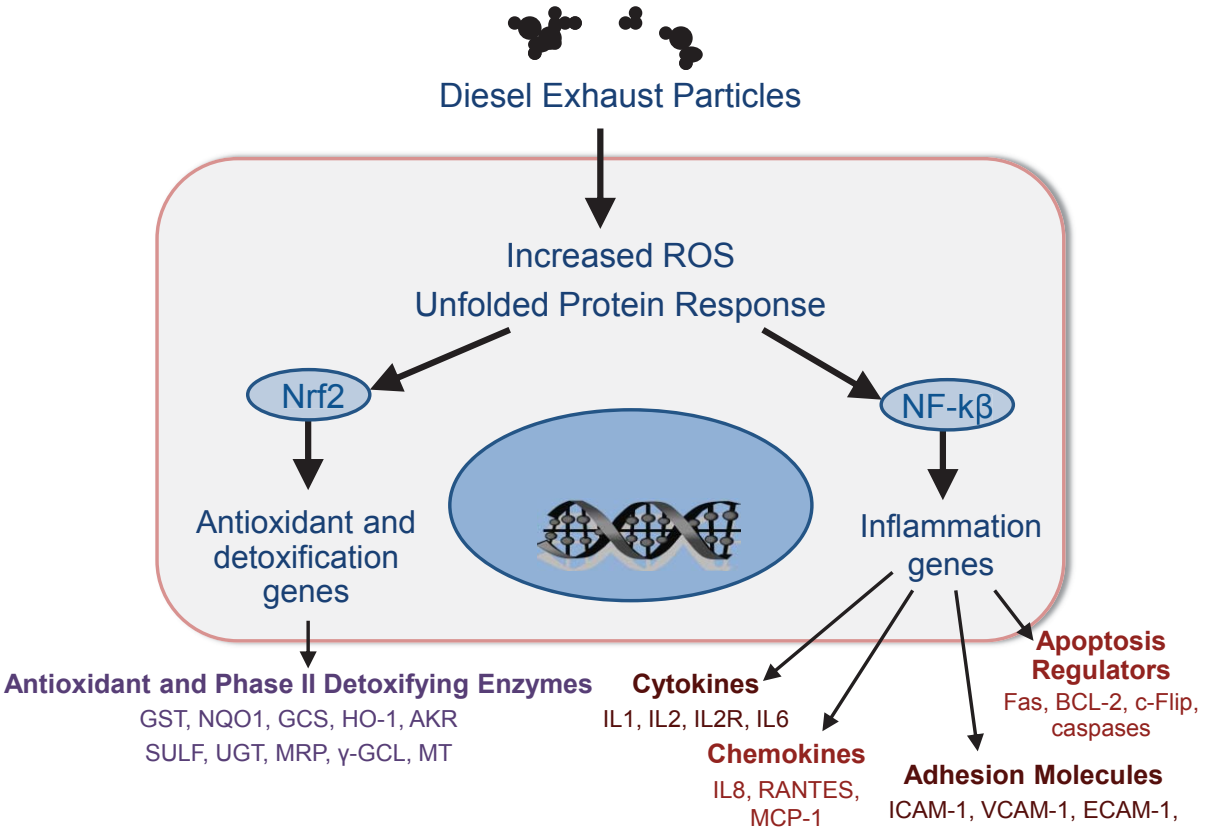

Figure 2-11. Diagram of Nrf2 and NF-kβ Cellular Response to Diesel Particulate Matter

The World Health Organization's International Agency for Research on Cancer recently announced that exposure to diesel exhaust causes lung cancer in humans.(Benbrahim-Tallaa et al., 2012) Many of the polycyclic aromatic hydrocarbons (PAHs) and nitro-substituted PAHs found in DPM are implicated in the risk for lung cancer because PAH metabolites are capable of forming DNA adducts and mutations in exposed cells and tissues.(Lewtas, 2007; Ma and Lu, 2007) The toxic PAH metabolites are formed as a result of cytochrome p450 1A1 (CYP1A1) activity. CYP1A1's function is to increase the

solubility of PAHs, thereby allowing for greater excretion of PAHs from the body. However, in making PAHs more soluble, CYP1A1 creates highly reactive, carcinogenic metabolites.(A, 1988, Totlandsdal et al., 2010) CYP1A1 transcription is increased after exposure to exogenous ligands (PAHs, halogenated aromatic hydrocarbons (HAHs), dioxin, curcumin, carotenoids, indole 3-carbinol, 7,8-digydrorutacarpine, flavonoids, isoflavones).(Denison and Nagy, 2003, Li et al., 1996) These exogenous can activate the aryl hydrocarbon receptor (AhR) which then translocates into the nucleus, binds with high affinity to its specific DNA recognition site, and increases transcription of CYP1A1.(Denison and Nagy, 2003) Of the AhR ligands identified, PAHs and HAHs are the only ones found in diesel exhaust.(Li et al., 1996) The induction of CYP1A1 by PAHs and HAHs in diesel exhaust is thought to contribute to DPM's carcinogenic potential.(Chaudhuri, 2013, Promega, 2012, Bonvallot et al., 2001) Even at the low doses tested here, exposure to DPM led to a large increase in CYP1A1, with a peak in expression nine- to ten-fold above controls at 3 hours after exposure (Figure 2-7E).

If the cell is not able to recover from the insults due to increased cellular ROS and DNA- and protein-adducts, apoptosis and/or necrosis will occur.(Simon et al., 2000, Park et al., 2010) Evidence of overall cell compromise was observed at 6 and 24 hours after exposure (Figure 2-10).

Although the well-to-well deposition in the EAVES varied by as much as 30%, this variability was consistent between experiments. Because typical exposure levels in vitro vary from 2- to 10-fold between treatments, a variation of 30% between wells will be

less impactful for in vitro toxicity screening. Our results demonstrate that a bipolar charging system with an alternating 4 kV electric field can be used to evaluate the human bronchial cellular response to fine and ultrafine particulate matter. Further, the use of a bipolar charger and alternating electric field offers the advantage of creating an equilibrium charge distribution, similar to what is observed for ambient aerosols, while depositing a net neutral charge onto the cells over time.(Savi et al., 2008) Thus, the modified EAVES offers the advantage of greater 'real-world' significance than previously described corona-based systems that use highly-charged particles of one polarity.

Our work reported here was limited to investigating the effects of the particulate matter in diesel exhaust. Gaseous and semivolatile species in complete diesel exhaust also contribute to cellular toxicity and inflammation.(Holder et al., 2008; Steiner et al., 2012) Future experiments with the EAVES system will investigate these mixtures. Based on the findings of Holder et al., we suspect that future work with complete diesel exhaust will demonstrate significant cellular responses at exposure levels even lower than those reported here.

Although NHBE cells constitute the majority of the bronchial epithelium, our in vitro model is limited. At present, our system does not capture the entirety of the response that would be observed in vivo. Our results suggest that the EAVES can be used as an effective, cost-reducing tool for screening the toxicity associated with exposure to fine and ultrafine aerosols. We did not observe an increase in cellular response as a result of electric field exposure, however, our results from NHBE cells exposed to HEPA-

filtered air suggest that systems employing direct-air exposure should include a control group of filtered-air exposed cells (as opposed to simply using incubator controls), especially for studies that use IL-8 as an endpoint and for exposures lasting greater than three hours in duration. We used diesel particulate matter as the test agent in this study, but the EAVES could be applied to many exposure scenarios, for example: airborne pathogens, like viruses or microorganisms; pharmacological aerosol therapeutics; toxic gases; and many other fine and ultrafine aerosols.

CONCLUSIONS

Statistically significant responses were observed at exposure levels 20-400x lower (~0.4 $\mu g/cm^2$) than typically reported in vitro using traditional submerged/resuspended techniques. Levels of transcripts associated with oxidative stress (HO-1), inflammation (IL-8, COX-2), aromatic hydrocarbon response (CYP1A1), and overall cellular dysfunction (HSP-70) increased immediately following diesel particulate exposure and persisted for several hours; cytotoxicity was increased at 24 hours. The modified EAVES described here provides a platform for higher throughput, more efficient and representative testing of aerosol toxicity in vitro.

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CHAPTER 3— OXIDATIVE STRESS AND AROMATIC HYDROCARBON RESPONSE OF HUMAN BRONCHIAL EPITHELIAL CELLS EXPOSED TO PETRO- OR BIODIESEL EXHAUST TREATED WITH A DIESEL PARTICULATE FILTER

SUMMARY

The composition of diesel exhaust has changed over the past decade due to the increased use of alternative fuels, like biodiesel, and to new regulations on diesel engine emissions. Given the changing nature of diesel fuels and diesel exhaust emissions, a need exists to understand the human health implications of switching to “cleaner” diesel engines run with particulate filters and engines run on alternative fuels like biodiesel merit investigation. We exposed well-differentiated normal human bronchial epithelial cells to fresh, complete exhaust from a diesel engine run (1) with and without a diesel particulate filter and (2) using either traditional petro- or alternative biodiesel. Despite the lowered emissions in filter-treated exhaust (a 67-85% reduction in mass), significantly increased expression of genes associated with oxidative stress and polycyclic aromatic hydrocarbon response were observed in all exposure groups and no significant differences were detected between exposure groups. Our results suggest that biodiesel and filter-treated diesel exhaust elicits as great, or greater a cellular response as unfiltered, traditional petrodiesel exhaust in a representative model of the bronchial epithelium.

INTRODUCTION

Ambient air pollution has recently been identified as the eighth leading cause of death worldwide (Lim et al., 2013) and previous studies have reported that diesel emissions are a leading source of particulate matter air pollution. (Chow et al., 1992, Particles, 2013) Human exposure to diesel exhaust (DE) is widespread due to the combustion of diesel fuel for construction, agriculture, mining, and transportation. Inhalation of DE has been associated with chronic lung inflammation and fibrosis (Henderson et al., 1988, Mauderly et al., 1988), allergic immune responses in the lungs (Sydbom et al., 2001), asthma (Pandya, 2002, Strickland, 1999), lung cancer (NIOSH, 1988, Cancer, 1989), and cardiovascular health effects (Lucking et al., 2008, McDonald et al., 2011). Diesel particulate matter was also recently classified as a human carcinogen.(IARC, 2012) Although the health effects from exposure to traditional forms of diesel combustion exhaust have been well-studied, the potential effects from exposure to (1) filtered and (2) biodiesel exhaust have not been extensively evaluated. (Swanson et al., 2007) Further, very few studies have evaluated the human airway cell response to filtered biodiesel exhaust, and of the studies that have, most have evaluated the effects of diesel exhaust particles alone and not the complete exhaust mixture.(Gerlofs-Nijland et al., 2013, Swanson, 2009, Schwarze et al., 2013)

The U.S. EPA recently reduced the annual standard for fine particulate matter ($PM_{2.5}$) from 15 to 12 $\mu g/m^3$ in an effort to reduce mortality rates, incidents of heart attacks, stroke, and childhood asthma resulting from exposure. (EPA, 2012) In an effort to reduce diesel emissions in particular, the U.S. EPA initiated a “National Clean Diesel

Campaign" in 2006, which required refiners to produce low-sulfur diesel fuel.(EPA, 2013) Further, the campaign requires engine producers to reduce emissions by 90% in on-road engines by 2007, and in off-road engines by 2010. (EPA, 2013) After-treatment technologies like diesel particulate filters are often used to meet these emission reduction requirements. However to date, the number of studies that have assessed the toxicity of "clean" diesel exhaust (i.e., emissions that are treated with a diesel particulate filter), are few, and existing studies present conflicting results. (Karthikeyan et al., 2013, Rudell et al., 1999, McDonald et al., 2004)

Concern over rising petroleum costs and limited resources have resulted in a push towards more stable, renewable energy supplies. With the widespread availability of crop surpluses in the U.S., the use of biodiesel is forecasted to increase as a fuel in the transportation, mining, industrial, and agricultural sectors. (Chaudhuri, 2013) Between 2010 and 2011, biodiesel production in the U.S. increased almost threefold from an estimated 350 million gallons to 1 billion gallons. Biodiesel production capacity for 2014 is forecasted to be 2.2 billion gallons, with 112 biodiesel plants currently in operation.(EIA, 2013, Chaudhuri, 2013) Although biodiesel emissions have been well studied and characterized, the human health impacts from exposure to these emissions are uncertain. (Swanson et al., 2007)

The objective of this work was to assess acute cellular responses following exposure to 'clean' and 'green' diesel exhaust in a model of the human airway epithelium. We investigated the response of well-differentiated human bronchial epithelial (NHBE) cells

following exposure to various forms of diesel exhaust: with and without a diesel particulate filter and using either traditional petrodiesel or biodiesel fuel. Transcripts associated with oxidative stress (HO-1) and polycyclic aromatic hydrocarbon response (CYP1A1) were measured 2 hours after exposure. Cytotoxicity was measured as well. To our knowledge, this is the first study to examine oxidative stress and polycyclic aromatic hydrocarbon responses in airway cells exposed to complete exhaust from an engine run with (1) petro- or biodiesel and (2) a diesel particulate filter.

MATERIALS AND METHODS

Electrostatic Aerosol in Vitro Exposure System (EAVES)

We used an electrostatic aerosol in vitro exposure system (EAVES) that has been described previously. (Hawley et al., 2014) A schematic of the EAVES system is shown in Figure 2-1. Fresh diesel exhaust was diluted, charge neutralized (Kr-85 neutralizer, TSI Inc.), conditioned to physiological temperature and humidity (37ºC, 80-90% relative humidity), and then drawn through the EAVES chamber at 1.5 l/min. In the cell chamber, charged particles were forced onto the cultures by an electric field that cycled from -4 to +4 kV at constant periodicity (10 seconds at each polarity). By cycling the polarity of the electric field over time, a net neutral charge of particles was delivered to cell cultures. Aerosol deposition in the EAVES has been characterized previously. (Hawley et al., 2014) For particles between 10 and 700 nm in size, the percentage of incoming aerosol deposited per cm^2 of cellular growth area ranged from 0.29% to 0.43% by mass. (Figure 2-2A)

Cell Culture

Normal human bronchial epithelial (NHBE) cells were obtained by brush biopsy from two healthy, non-smoking human volunteers (EPA, Research Triangle Park, NC, USA) in accordance with a protocol approved by the Institutional Review Board at the University of North Carolina at Chapel Hill. Cell populations were expanded through two passages in Petri dishes with Bronchial Epithelial Growth Media (BEGM kit; Lonza, Walkersville, MD, USA) before being plated onto collagen-coated, porous, polycarbonate membranes (0.4 μm Snapwell membrane; Corning, Inc., Corning, NY, USA) at a seeding density of approximately 140,000 cells per cm^2. Air–liquid interface (ALI) cultures were carried for a minimum of 21 days (prior to exposure) to allow progressive differentiation into basal, ciliated, and mucin-producing cell types within a pseudo-stratified columnar epithelium.(Ross et al., 2007) Mucus production was visually apparent by day 10 of ALI and excess mucus was removed with a gentle saline rinse, every three days thereafter.

Cell Exposures to Diesel Exhaust

Tests were performed using a 4.5 L John Deere 4045H PowerTech Plus diesel engine. The PowerTech Plus engine is a 4-cylinder, common rail, direct injection diesel engine that includes exhaust gas recirculation and a catalyzed exhaust filter that contains diesel oxidation catalyst (DOC) and a diesel particulate filter (DPF). The DOC reacts with exhaust gases to reduce carbon monoxide, hydrocarbons and a portion of the PM. The DPF (Tenneco, Grass Lake, MI, model number: TENN NO H0084737A), located downstream of the DOC, traps the remaining PM wherein the particles are

oxidized via a passive regeneration process that utilizes heat from the exhaust gases. As described herein, experiments were performed with and without installation of DPF/DOC into the engine exhaust system. An eddy current dynamometer (Midwest Inductor Dynamometer, 1014A) and dynamometer controller (Dyn-LocIV) were used to maintain a constant torque on the engine at a given engine speed. Prior to the test campaign, the engine lubrication oil was changed and the same lubrication oil charge was used for all tests

Well-differentiated NHBE cells (cultured at ALI for a minimum of 21 days) were placed in the EAVES chamber and exposed to fresh, complete diesel exhaust for a duration of 5, 20, or 60 minutes (n = 12 per treatment group). An experimental matrix is provided in Table 3-1. On each test day, a heavy-duty diesel engine (John Deere model number 4045H, 4.5 L, 4 cylinder, 107 bhp at 2400 rpm) was brought to steady temperature, load, and speed while running on either petrodiesel (Team Petroleum, LLC, Fort Collins, CO) or biodiesel (B99, Suncor Refining, Commerce City, Colorado) fuel. Tests with a diesel particulate filter (*DPF+*; Tenneco, Grass Lake, MI, model number: TENN NO H0084737A) and tests without a diesel particulate filter (*DPF-*) were conducted in a similar manner. For all cell exposures, fresh exhaust was pulled through a dilution tunnel (Bennett et al., 2008) at a ratio of 1:20 of fresh exhaust to supplemental, heated, filtered air. Engine exhaust was pulled from the dilution tunnel through a cyclone with a $PM_{2.5}$ cutpoint (GK2.05, BGI Inc., Waltham, MA) and then into the EAVES cell exposure system. Particle size distributions were measured with a Sequential Mobility Particle Sizer (SMPS+C; Grimm Technologies, Douglasville, GA, USA) immediately upstream of

the EAVES for the duration of the cell exposures. Control cells were exposed to the same conditions stated above, except that incoming room air was HEPA filtered to remove >99% of particles. Each exposure condition was repeated on two separate days. We compared the response of complete diesel exhaust exposed cells to the response of control cells, two hours after exposure, as previous work suggests that mRNA production reaches a peak at this time point.(Hawley et al., 2014)

Table 3-1. Exposure matrix for DE NHBE Cell Exposures

Experimental Variable	Number of Variables	Level of Variation
Normal Human Bronchial Epithelial Cells (NHBE)	2	Donors 1, 2
Exposure Types	5	DE: Petro- or Biodiesel (2), DE: -DPF or +DPF (2) HEPA Air + Electric Field Controls (1)
Exposure Level	3	Duration: 5, 20 or 60 minutes
Experimental Repetitions	2	NHBE cells (2) exposed to each exposure duration (2) on two separate days
Cellular Marker Timepoints	1	2 hrs Following Exposure
Cellular Markers	3	CYP1A1, HO-1, LDH

Characterization of Engine Exhaust

Emissions were collected directly from the dilution tunnel used to supply fresh exhaust for cell exposures. Mass emissions during each test condition were measured by collecting fresh diesel exhaust onto pre-weighed Teflon filters following the methods of Subramanian et al.(Subramanian et al., 2004). Elemental/organic carbon content was

analyzed by collecting fresh diesel exhaust onto pre-baked quartz filters and then storing filters at -80ºC until analysis (EC/OC Lab Instrument, Sunset Laboratories Inc.). Elemental, organic, and total carbon emissions were calculated according to NIOSH method 5040 . Gaseous emissions were measured in the main exhaust line using a five-gas analyzer (Rosemount Analytical, Houston, TX), which reported levels of total hydrocarbons (HC), oxygen (O_2), carbon monoxide (CO), carbon dioxide (CO_2), and nitrogen oxides (NO_x). Measured gaseous emissions were then multiplied by 0.05 to account for the dilution factor (1:20, exhaust to supplemental, heated, filtered air) applied to the exhaust used for cell exposures.

Transcript Production in ALI NHBE Cells

Combustion-derived particles can sequester cytokines produced by epithelial cells, and a negative bias can occur when these cytokines are quantified using ELISA.(Totlandsdal et al., 2010, Seagrave et al., 2004, Seagrave, 2008, Kocbach et al., 2008) Previous work by Wyatt et al. suggests that mRNA transcripts can be used as a proxy for cytokine production in NHBE cells.(Wyatt et al., 2007) Therefore, we chose to quantify levels of mRNA transcripts as indicators for subsequent cytokine production.

We quantified transcripts coding for proteins that characterize cellular oxidative stress (Heme oxygenase-1, HO-1) and PAH-adduct formation (Cytochrome p450 1A1, CYP1A1). All transcript analyses were quantified by RT-PCR (CFX96, Bio-Rad Laboratories, Hercules, CA) according to MIQE guidelines.(Bustin et al., 2009) Expression profiles for each transcript were normalized to GAPDH.(Barber et al., 2005)

All transcript expression profiles were normalized to controls' expression levels of each transcript.

Cytotoxicity in ALI NHBE Cells

Lactate dehydrogenase (LDH) is expressed universally in NHBE cells. The loss of membrane integrity during cell injury and death causes the extracellular release of LDH, which can be used as an indicator of cytotoxicity.(Allan and Rushton, 1994) Extracellular LDH was assayed at two hours post-exposure using a standard kit (Promega Cytotox96 Non-radioactive Cytotoxicity Assay, Promega Corporation, Madison, WI, USA). Percent cytotoxicity was calculated by following the manufacturer's protocol.(Promega, 2012)

Statistical Analyses

Transcript data were log-transformed to satisfy model assumptions of normality and homoscedasticity. The effects of exposure duration, exposure type, donor phenotype, and experimental repeat (and their interactions) were evaluated relative to the expression of HO-1 and CYP1A1 transcripts and extracellular LDH using one-way ANOVAs. Cell donor and experimental replicate were treated as random effects. Statistical analyses were conducted with SAS software (v9.0, SAS Institute Inc., Cary, NC, USA) with a type I error rate of 0.05.

RESULTS

Exhaust Characteristics

The size distribution by particle count of the diesel exhaust is shown in Figure 3-1. This size distribution was relatively stable within a given treatment and between replicates. The count median diameters (CMD) of each exhaust treatment can be seen in Table 3-2. Particle emissions were different between each exhaust treatment. Combustion of biodiesel produced consistently smaller size distributions (CMDs that were 50% and 58% smaller for DPF- and DPF+ treated, respectively), and fewer in number than petrodiesel PM emissions. A similar trend was observed with the DPF treated exhaust. DPF treated (DPF+) exhaust resulted in smaller CMDs (33% and 22% smaller for petro- and biodiesel exhaust, respectively) and particles that were fewer in number than unfiltered exhaust.

Table 3-2. Count median diameter (geometric standard deviation in parentheses), count and mass concentration in each exhaust treatment. Data shown are averages; one standard deviation is shown in parentheses.

Exhaust Treatment	CMD, nm	Count Concentration, N/cm^3	Mass Concentration, $\mu g/m^3$
Petrodiesel, DPF-	64 (± 2)	6.9E7 (± 1.8E6)	782.5 (± 100.9)
Petrodiesel, DPF+	43 (± 2)	1.4E5 (± 3.2E4)	15.0 (± 11.4)
Biodiesel, DPF-	32 (± 2)	5.9E5 (± 3.7E5)	159.4 (± 0.6)
Biodiesel, DPF+	25 (± 2)	5.8E4 (± 3.4E4)	9.8 (± 7.7)

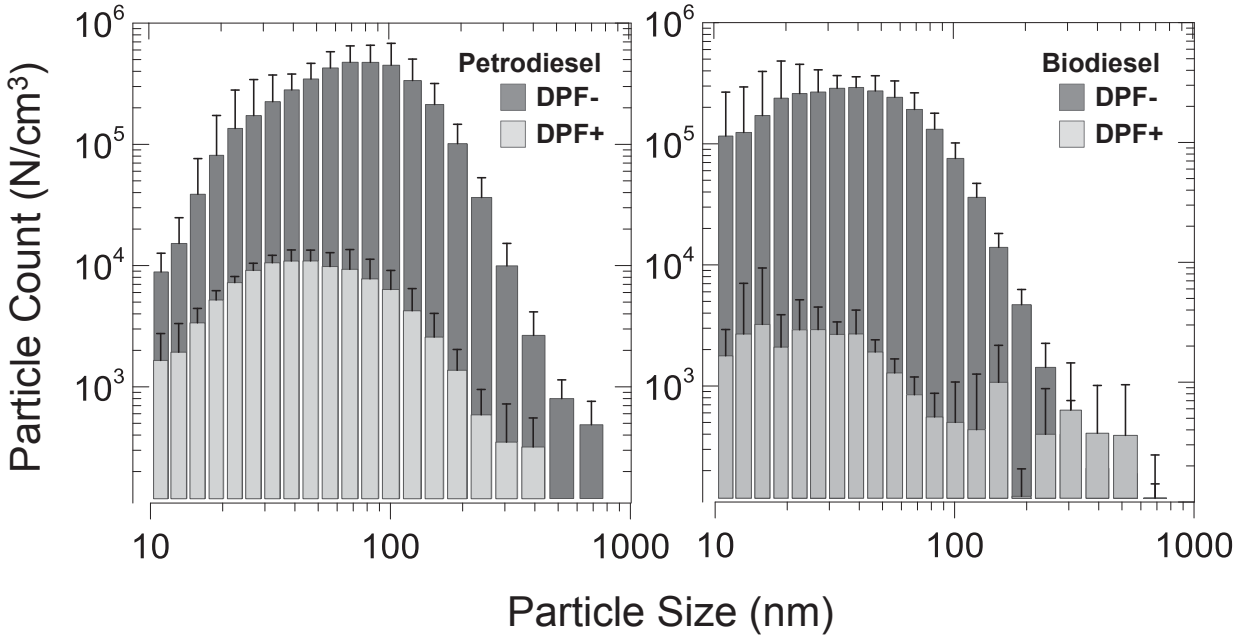

Figure 3-1. Particle size distributions for each test condition during cell exposures.

Particle mass concentrations of each exhaust treatment are shown in Table 3-2. Petrodiesel and unfiltered (DPF-) exhaust contained higher $PM_{2.5}$ mass concentrations compared to biodiesel, and DPF treated exhaust, respectively. Use of a DPF reduced $PM_{2.5}$ mass emissions by 98% and 94% for petro- and biodiesel exhaust, respectively. Concentrations of particulate elemental and organic carbon are shown in Table 3-3 as a function of each exhaust treatment. Elemental carbon emissions were substantially higher in the unfiltered petrodiesel exhaust than in the unfiltered biodiesel exhaust. Concentrations of elemental carbon were greatly reduced in the DPF+ treated exhaust (99% and 97% reductions for petro- and biodiesel exhaust, respectively). Biodiesel exhaust contained 66% (DPF+) and 89% (DPF-) less elemental carbon when compared to unfiltered petrodiesel. Organic carbon content was not different between fuel types, in

each filtered exhaust treatment groups. However, organic carbon content was reduced substantially in the unfiltered biodiesel exhaust, as compared to the unfiltered petrodiesel exhaust (50% reduction, by organic carbon mass). However, the DPF significantly reduced organic carbon content (reductions of 95% and 91% in petro- and biodiesel exhaust, respectively). Gaseous emissions in each exhaust treatment are shown in Table 3-4. Total hydrocarbons were reduced by 86-87% in the filtered exhaust (petro- and biodiesel exhaust, respectively). Biodiesel exhaust contained fewer total hydrocarbons than petrodiesel exhaust. Although nitrous oxide (NO) was decreased in the filtered exhaust from petrodiesel (-44%) ($p=0.0014$), NO increased in filtered biodiesel exhaust (10%) ($p=0.86$). Nitrogen dioxide (NO_2) increased significantly in exhausts from both fuel types, when treated with a DPF (+357% ($p=0.0011$) and +572% ($p=0.0225$) for petro- and biodiesel exhaust, respectively). Total NO_x increased slightly in filtered petrodiesel exhaust (1%). On the other hand, DPF treatment caused a 70% increase in NO_x in biodiesel exhaust, mostly attributable to a significant increase in NO_2 (9.55 ppm in DPF+ versus 1.41 ppm in DPF- exhaust).

Table 3-3. Carbon content of emissions in each exhaust treatment. Note: Values are averages, and one standard deviation is shown in parentheses. OC and EC content represent the organic and elemental carbon content, respectively.

Exhaust Treatment	Mass Concentration		Carbon Fractions	
	OC, $\mu g/m^3$	EC, $\mu g/m^3$	Percent OC, OC/TC	Percent EC, EC/TC
Petrodiesel, DPF-	186.7 (± 88.2)	595.8 (± 2.5)	24% (± 18%)	76% (± 14%)
Petrodiesel, DPF+	8.8 (± 11.0)	6.2 (± 5.7)	59% (± 33%)	41% (± 40%)
Biodiesel, DPF-	93.5 (± 13.3)	65.9 (± 7.2)	59% (± 8%)	41% (± 24%)
Biodiesel, DPF+	7.7 (± 8.8)	2.1 (± 2.5)	79% (± 43%)	21%(± 18%)

Table 3-4. Gaseous pollutant concentrations during cell exposures. Values are averages; one standard deviation is shown in parentheses.

Exhaust Treatment	CO, ppm	CO_2, ppm	NO_x, ppm	NO, ppm	NO_2, ppm	HC, ppm
Petrodiesel, DPF-	5.7 (± 0.50)	0.31 (± 0.017)	21.0 (± 0.0428)	19.0 (± 0.411)	2.30 (± 0.368)	3.1 (± 0.048)
Petrodiesel, DPF+	0.13 (± 0.015)	0.30 (± 0.0022)	20.2 (± 0.206)	10.1 (± 0.197)	10.1 (± 0.0088)	0.42 (± 0.029)
Biodiesel, DPF-	3.4 (± 0.036)	0.26 (± 0.05)	13.1 (± 11.3)	11.7 (± 9.60)	1.41 (± 1.67)	2.0 (± 0.0077)
Biodiesel, DPF+	0.08 (± 0.001)	0.29 (± 0.0051)	22.6 (± 0.0939)	13.0 (± 0.628)	9.55 (± 0.534)	0.26 (± 0.055)

Cell exposures

Levels of diesel particulate matter (DPM) mass delivered to NHBE cell cultures during the 5, 20, and 60 minute exposures are shown in Table 3-5. These deposition rates were calculated based on mass concentrations, exposure duration, and the known deposition efficiency in the EAVES (0.29-0.43% of incoming particle mass delivered per cm^2 cellular growth area.) (Hawley et al., 2014) The PM mass delivered to each exhaust treatment group varied significantly. Within each exhaust treatment exposure group, PM mass delivered increased by four- (20 minute exposure) and twelve-fold (60 minute exposure). In the highest exposure groups (60 minute exposure duration), PM mass delivered varied by 16 (B99, DPF-) to 80 fold (Petro, DPF-), relative to the lowest exposure group, the filtered biodiesel exhaust.

Table 3-5. Estimates of particle deposition, by mass, during each cell exposure treatment. Values shown represent the range of deposited PM mass per cellular growth area.

Exhaust Treatment	Deposited DPM Mass (ng per cm^2)		
	5 min exposure	20 min exposure	60 min exposure
Petrodiesel, DPF-	20-30	80-110	230-340
Petrodiesel, DPF+	0.23-0.34	1.0-1.5	3.0-4.0
Biodiesel, DPF-	3.7-4.0	10-20	40-50
Biodiesel, DPF+	0.12-0.23	0.7-0.8	2.0-4.0

Cellular Response to Diesel Exhaust Exposures

Relative transcript production by NHBE cells exposed to whole diesel exhaust is shown in Figure 3-2. An exposure-response relationship is evident, with no significant increases in transcript production at 5 min and substantially higher production at 60. No significant differences were observed between the responses of petrodiesel or biodiesel exhaust exposed cells. The response in DPF treated exhaust exposed cells was as great, or greater, than the unfiltered exhaust exposed cells; the high (60 min) exposure group showed greater increases in the filtered (DPF+) exposure groups. On average, a 60 min exposure to filtered, petrodiesel, and unfiltered, petrodiesel exhaust produced 14.1 ($p < 0.0001$) and 8.8 ($p < 0.0001$) fold increases in CYP1A1 (Figure 3-2C), respectively, and 8.3 ($p < 0.0001$) and 4.5 ($p < 0.0001$) fold increases in HO-1, respectively (Figure 3-2A). Filtered petrodiesel exhaust resulted in transcript production levels that were 1.6 (CYP1A1) to 1.8 (HO-1) fold greater than transcript production levels in unfiltered exhaust. Filtered biodiesel exhaust followed a similar pattern. Significant differences in transcript production were also observed after 20 minutes of exposure. HO-1 was significantly increased by a factor of 2.2 ($p=0.03$) in NHBE cells exposed to unfiltered biodiesel exhaust (Figure 3-2B). CYP1A1 increased by a factor of 2.1 ($p=0.004$) in unfiltered petrodiesel tests (Figure 3-2D). No significant increases in cytotoxicity were observed at 2 hours after exposure in any of the treatment groups (Figure 3-3).

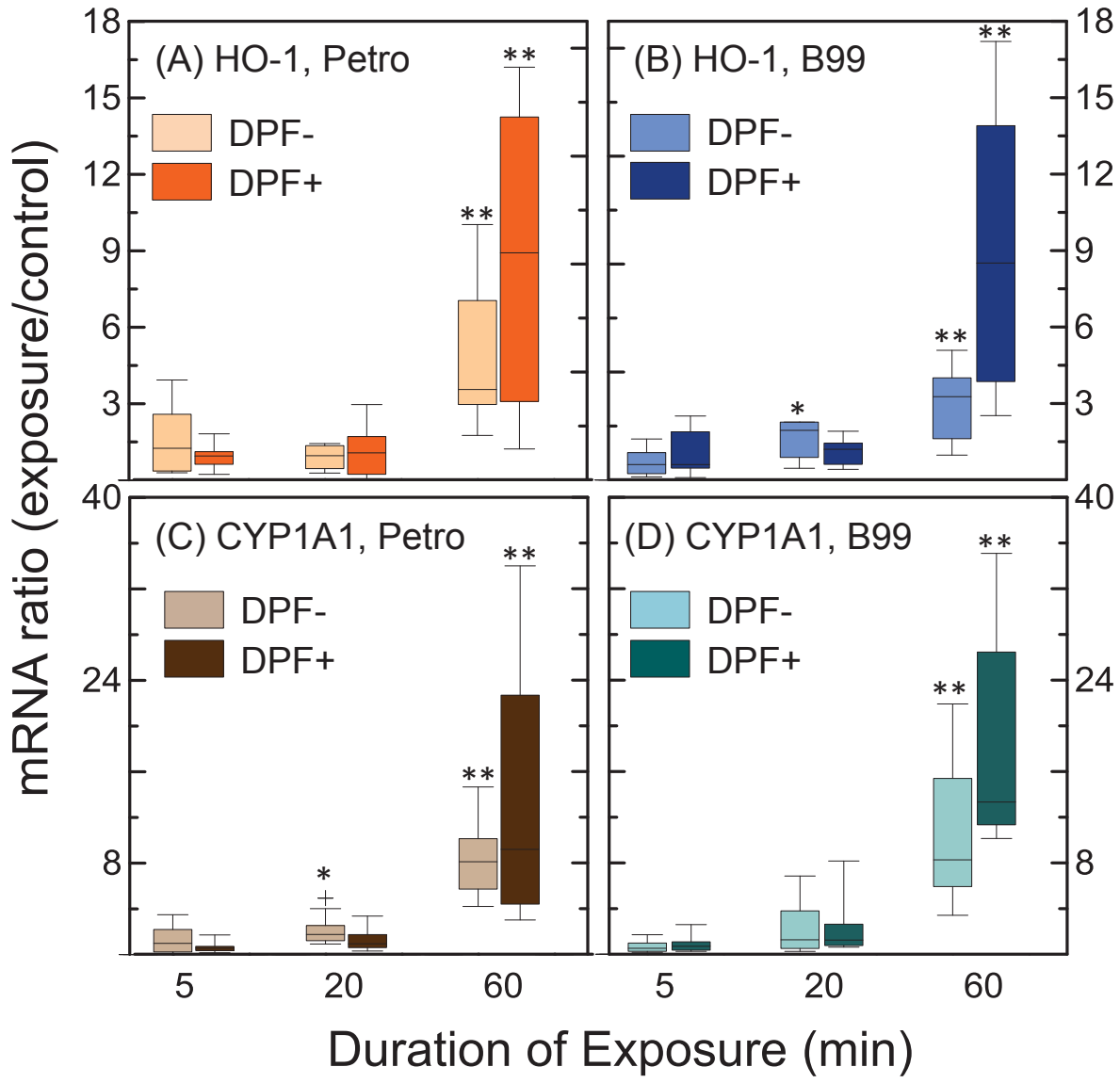

Figure 3-2. Box-whisker plots of transcript production in ALI NHBE cells exposed to fresh diesel exhaust for 5, 20, or 60 minutes. Panel A and B: HO-1 transcript production in Petro- or B99 exposed cells, respectively. Panel B and C: CYP1A1 transcript production in Petro- or B99 exposed cells, respectively. All transcript levels are normalized to HEPA-air controls. * signifies p<0.05, ** signifies p<0.0001, when compared with controls.

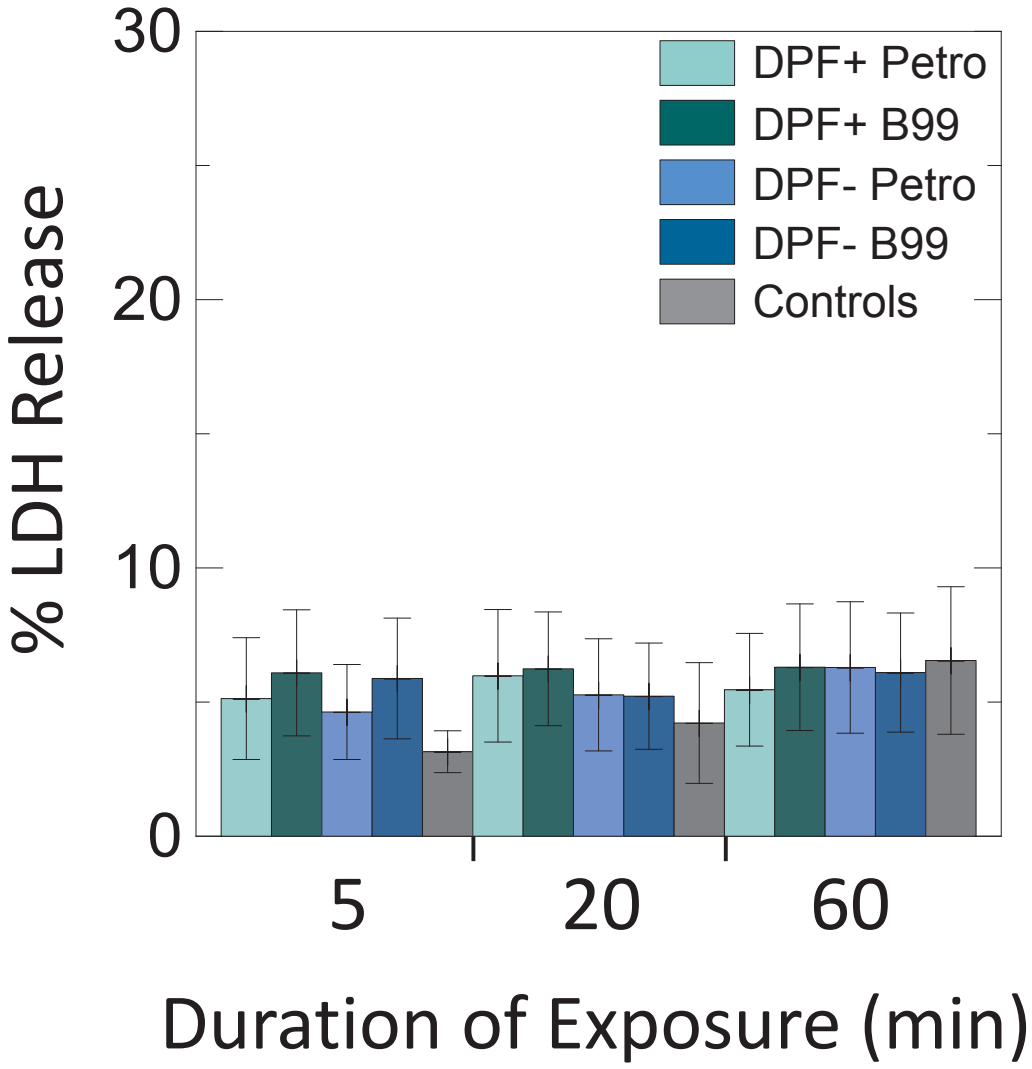

Figure 3-3. LDH release from ALI NHBE cells exposed to fresh, complete diesel exhaust or HEPA- room air with and without an alternating 4 kV electric field. Error bars indicate one standard deviation.

DISCUSSION

The cellular exposure levels and the exposure system used for this work were more representative of 'real-world' conditions than previously reported in vitro. We observed statistically significant cellular responses at levels of PM exposure that were 60 to 100,000 fold less than previous studies that exposed cells to resuspended extracts of PM collected onto filters (20-200 µg per cm^2 or 20-200 µg per mL). (Totlandsdal et al., 2010, Baulig et al., 2003, Schwarze et al., 2013, Cao et al., 2007) Further, the cellular PM exposure levels reported here, are similar to the estimates reported by Gangwal et al., for PM deposition in the lungs of a human exposed acutely to a PM concentration of 0.1 mg per m^3.(Gangwal et al., 2011) Using an MPPD model, Gangwal et al. estimated that an acute, 24 hr exposure to PM concentration of 0.1 mg per m^3, would result in PM deposition of 0.006 to 0.02 µg per cm^2 of lung cellular surface.(Gangwal et al., 2011) Our work also aligns with the recent in vivo study by Karthikeyan et al., who found that ultrafine particulate matter, and increased nitrogen dioxide in DPF treated exhaust may heighten the injury and inflammation after inhalational exposure.

Diesel exhaust is a generalized term for thousands of gaseous and particulate matter species emanating from a diesel engine. The composition of diesel exhaust can vary greatly with engine loading, fuel type, and exhaust after-treatment. We observed changes in particle size distribution, particle mass concentration, relative carbon content, and gaseous emissions from a heavy-duty diesel engine operated with varying fuel and exhaust treatment, but at similar speed and load (2400 rpm, 107 bhp). Particle emissions, by mass, count, and size were greatly reduced in both biodiesel and DPF

treated exhaust, when compared with petrodiesel and unfiltered exhaust, respectively. With the exception of nitrogen dioxide, hazardous gaseous emissions were found to decrease in DPF treated exhaust as well.

Previous in vitro studies with petrodiesel or biodiesel particulate matter (PM) have reported increased pro-inflammatory responses in cells exposed to biodiesel PM when compared to cells exposed to petrodiesel PM. Gerlofs-Nijland et al. observed increases in IL-6 and cytotoxicity in BEAS-2B cells exposed to B50 PM (50% biodiesel, 50% petrodiesel) at exposure levels of 100 µg per mL, or approximately 16 µg per cm^2 cellular growth area (assuming total deposition of all suspended PM). (Gerlofs-Nijland et al., 2013) Similarly, Fukagawa et al. observed increases in IL6, Interferon-Gamma-induced Protein 10, and Granulocyte Stimulating Factor (G-CSF) in BEAS-2B (an immortalized bronchial cell line) and THP-1 cells (a human monocyte cell line) exposed to B20 (20% biodiesel, 80% petrodiesel) particulate matter at exposure levels of 10-20 µg per mL. (Fukagawa et al., 2013) Further, Fukagawa et al. also exposed to mice to the same petrodiesel or B20 PM (84 µg per oropharyngeal aspiration treatment, repeated three times over 72 hours) and observed similar increases in pro-inflammatory mediators in the bronchoalveolar lavage fluids of mice exposed to B20, when compared with petrodiesel.(Fukagawa et al. 2013) In a study of the effect of the organic extracts from petrodiesel or biodiesel PM, Swanson et al. observed that soluble organic fraction exposure levels of 0.27-50 µg per mL resulted in a dose-dependent increased expression of IL-8 and IL-6 in BEAS-2B cells.(Swanson et al., 2009) Our results reported here did not suggest significant differences in the responses of bronchial cells

exposed to biodiesel or petrodiesel exhaust, however we note several important differences in the design of our experiments with previous studies in vitro. First, previous work has been performed predominantly with (1) submerged cells (2) an immortalized cell line (BEAS-2B) (3) exposure levels 5-1800 fold greater than the exposure levels used in our work. Despite the differences in exposure techniques and exposure levels between our work and previous in vitro studies, the overall conclusions were similar. Similar to previous in vitro studies, we found that although the particle emissions were greatly reduced in biodiesel the cellular response was not mitigated relative to such reductions. Assuming a power level of 0.8 and a p-value of 0.05, future work with a similar cell population would need to have a sample size of (1) 40 or 120 N per exposure group to detect differences between unfiltered Petro- or B99 exhaust exposed cells or (2) 15000 or 100 N per exposure group to detect differences between filtered Petro- or B99 exhaust exposed cells for HO-1 or CYP1A1, respectively.

Our work suggests that current exhaust treatment technologies, and alternative biobased fuels, may not be sufficient in mitigating the negative health outcomes associated with diesel exhaust inhalational exposure. Similar to the results reported by Karthikeyan, the results reported here also highlight the importance of including gas-phase and semi-volatile organics when studying the toxicity of diesel exhaust in vitro and in vivo. We observed elevated production of transcripts associated with oxidative stress and tumorigenic potential following NHBE exposure to DPF-treated exhaust even though these levels were eight-fold lower than the unfiltered exhaust. Interestingly, most of the PM mass decrease in DPF treated exhaust came from a decrease in elemental

carbon (99% to 97% for petro- and biodiesel, respectively). Previous work by Bonvallot et al. found that the elemental carbon core of diesel particulate matter produced a very weak effect in HBE cells, with regard to oxidative stress, inflammatory response, and PAH metabolism.(Bonvallot et al., 2001) On the other hand, the organic carbon fraction, and 'native' (carbon core with organic carbon surface) diesel particulate matter produced strong effects on NHBE oxidative stress, inflammatory response, and PAH metabolism. (Bonvallot et al., 2001) Similar to the observations by Bonvallot et al., we found that DPF-treated exhaust produced substantially lower PM mass emissions. However we found that the reduction in mass emissions, due mostly to a reduction in elemental carbon mass emissions, did not coincide with a similar decrease in cellular production of HO-1 and CYP1A1.

We chose to report cellular exposure levels in terms of deposited DPM mass, because particulate mass is an often used metric by which engines are deemed "clean" or "dirty". This metric also allows comparisons to be drawn between our results and previous studies. (Attfield et al., 2012, Silverman et al., 2012) In reality, no single exposure parameter is likely adequate to describe the effective dose and subsequent biological effects of diesel exhaust. (Paur et al., 2011, Teeguarden et al., 2007) Although previous work suggests that PM mass emissions in diesel exhaust may not be the best metric for predicting human health outcomes, metrics such as particle number or surface area concentration also have issues. (Karthikeyan et al., 2013, Rudell et al., 1999, Cauda et al., 2012, Park et al., 2010)

We found that median particle size and number concentration decreased with DPF treatment and with biodiesel fuel. As seen in Table 3-2, the count median diameter (CMD) for DPF treated exhaust was 43 nm (± 1.92) for petro-diesel and 25 nm (± 1.85) for biodiesel exhaust; the CMDs for unfiltered exhaust were larger: 64 nm (± 1.82) and 32 nm (± 1.68), respectively. Surface reactivity increases exponentially with decreasing particle size. (Oberdörster et al., 2005) Surface reactivity is attributed to trace metals and redox-active organic chemicals which cause cellular dysfunction and oxidative stress (Geller et al., 2006) and exposure to diesel exhaust results in oxidative stress in the human airways. (Li et al., 2002, Benbrahim-Tallaa et al., 2012)

Disruption of the mitochondrial membrane and redox-cycling chemicals in diesel exhaust (e.g. polycyclic aromatic hydrocarbons, quinones, ketones, metals, and aldehydes) contribute to the formation of reactive oxygen species (ROS) in the cell. Increased ROS can then create reactive metabolites that lead to the harmful, cytotoxic effects from diesel exhaust exposure.(Li et al., 2003) The Nuclear factor-like 2 (Nrf2) pathway is a primary cellular defense against the cytotoxic effects of oxidative stress.(Nguyen et al., 2009) Diesel exhaust exposure, and subsequent ROS formation, activates the Nrf2 pathway, which then activates many cytoprotective genes, including HO-1.(Zhang et al., 2005) Increased reactive oxygen species also activate NFkβ and JAK-STAT pathways and these pathways also result in an increased production of HO-1.(Liu, 2011, Madamanchi et al., 2001) Further, NO_2 has been observed to be a potent pro-inflammatory mediator and oxidant in previous studies with both bronchial cells and mice. (Ayyagari et al., 2007, Carl J. Johnston, 2000) Increased production of transcripts

for heme oxygenase-1 has previously been observed in mice exposed to NO_2 at levels three times greater than the high end reported here (30 ppm) (Carl J. Johnston, 2000) Unsurprisingly, we observed an increase in transcript production for HO-1 immediately after exposure to diesel exhaust (Figure 3-2A). We also observed an enhanced response in cells exposed to DPF-treated exhaust. Previous work by Li et al., suggests that the polar organic fraction (which contains quinones and oxygenated PAH compounds) was responsible for a subsequent increase in HO-1 in human macrophages.(Li et al., 2002) We also note that nitrogen dioxide, a potent oxidant, was increased in DPF treated exhaust. We believe that the increased nitrogen dioxide, and the remaining ultrafine PM and organic carbon content in filtered exhaust likely contributed to the increased production of HO-1 in NHBE cells exposed to DPF treated exhaust.

Although we did not measure trace metals in the exhaust particles used for this study, previous studies have observed potassium (K), magnesium (Mg), calcium (Ca), aluminum (Al), iron (Fe), zinc (Zn), nickel (Ni) and chromium (Cr) in both petro- and biodiesel PM, while copper is predominantly observed in biodiesel PM.(Brito et al., 2010, Cheung et al., 2010, Betha and Balasubramanian, 2013) Betha et al. measured the size distributions and metal constituents of diesel exhaust from petrodiesel, B50 (50% biodiesel), or B100 (100%) and observed greater amounts of ultrafine PM and higher amounts of the toxic metals, Zn, Cr, and Ni in biodiesel exhaust. Because Cr and Ni are established as class I carcinogens by the IARC, Betha et al. suggest that biodiesel exhaust exposure results in a greater human health risk than exposure to

ultra-low sulfur diesel exhaust. (Betha and Balasubramanian) Further, Zn and Ni are well-known redox cyclers and Zn and Ni in the exhaust here may have also contributed to the observed cellular oxidative stress response.

Organic carbon present on the surface of DPM can also carry mutagenic and carcinogenic compounds such as polycyclic aromatic hydrocarbons (PAHs). (Shah et al., 2004) The World Health Organization's International Agency for Research on Cancer recently announced that exposure to diesel exhaust causes lung cancer in humans.(Rudell et al., 1999, IARC, 2012) We chose to measure CYP1A1 because toxic PAH metabolites are formed as a result of cytochrome p450 1A1 (CYP1A1) activity. CYP1A1's function is to increase the solubility of PAHs, and thereby increase excretion of PAHs from the body. However, in the conversion of PAHs into more soluble molecules, CYP1A1 creates highly reactive, carcinogenic metabolites.(Totlandsdal et al., 2010) CYP1A1 is induced after exposure to PAHs and nitro-PAHs in diesel exhaust, and the induction of CYP1A1 is thought to contribute to DPM's carcinogenic potential.(Ma and Lu, 2007, Jacquet et al., 1996, Mollerup et al., 1999)

CYP1A1 is upregrulated when exogenous ligands enter the cell (PAHs, halogenated aromatic hydrocarbons (HAHs), flavonoids, curcumin, carotenoids, indoles, dioxin) and bind to the aryl hydrocarbon receptor (AhR).(Denison and Nagy, 2003) Activated AhR then translocates into the nucleus, binds with high affinity to its DNA recognition site, and increases the transcription of CYP1A1. (Denison and Nagy, 2003) Of the exogenous AhR ligands identified, only PAHs and HAHs are found in diesel exhaust.(Li

et al., 1996) Binding affinities required for AhR activation are reported to be as low as in the pM to nM and nM to μM range for HAHs and PAHs, respectively. (Denison and Nagy, 2003) Further, polycyclic aromatic hydrocarbons (PAHs) and nitro-substituted PAHs found in diesel are thought to increase the risk for lung cancer via the formation of PAH-DNA adducts and mutations in exposed cells and tissues.(Lewtas, 2007, Chaudhuri, 2013) Here, exposure to filtered and unfiltered diesel exhaust led to a large increase in CYP1A1, with greater increases observed in DPF treated exhaust. Lu et al. observed that gas-phase PAHs make-up the largest fraction of total PAHs in filtered exhaust and that the gas-phase PAHs are often ignored in studies that rely on particle extractions.(Hu et al., 2013) Here, exposure to complete, fresh filtered and unfiltered diesel exhaust led to a large increase in CYP1A1, with greater increases observed in DPF treated exhaust. Similar to the results observed for transcript production of HO-1, we hypothesize that the increase in CYP1A1 transcript production in NHBE cells exposed to DPF treated exhaust, was due to the remaining unbound, gas-phase PAH compounds, alongside of an increase in the potent gaseous oxidant, nitrogen dioxide. Future work designed to measure the gas-phase PAHs and nitro PAHs in unfiltered exhaust and exhaust filtered with a similar DPF, and passive regeneration process, is needed to elucidate the role of the unbound aromatic hydrocarbons in the cellular responses observed here..

Although NHBE cells constitute the majority of the surface of bronchial epithelium, our in vitro model is limited. In the bronchial lung, the immediate epithelial response would be followed by the recruitment of neutrophils, macrophages, and fibroblasts. At present,

our culture model does not capture the entirety of the response that would be observed in vivo. However, we note that our observations here support the observations observed by several in vivo studies. (Rudell et al., 1999, Karthikeyan et al., 2013) Unlike 'traditional' in vitro studies, which rely on particle extractions from filters as a source of DPM, our system was capable of exposing cells to complete exhaust (gas-phase PAH and nitro-PAH, quinones, and gaseous emissions).(Gerlofs-Nijland et al., 2013, Pezzulo et al., 2010) Our results differ with those of Gerlofs-Nijland et al., who similarly observed that a biodiesel blend did not reduce the observed cellular response, but alternatively, Gerlofs-Nijland et al. observed that DPF treated DPM contained less cytotoxic, oxidative, and pro-inflammatory potential.(Gerlofs-Nijland et al., 2013) However, their study used only extracted DPM, not *complete* exhaust. A growing body of evidence suggests that semi-volatile organic compounds (PAHs, nitro-PAHs, and PAH-quinones) and gaseous emissions (NO_2) are critical to the toxicity observed following exposure to DPF treated exhaust.(Rudell et al., 1999) For example Karthikeyan et al., and Rudell et al., who exposed rats and humans, respectively, to treated or unfiltered exhaust both reported that a DPF was not sufficient in abolishing the pro-inflammatory effects of the exhaust.(Karthikeyan et al., 2013, Rudell et al., 1999) Although our in vitro model is limited to the human bronchial epithelium, our results from exposing a representative model of the bronchial lung epithelium to complete, fresh diesel exhaust, support previous in vivo studies. Our results suggest that biodiesel and filter treated diesel exhaust elicits as great, or greater a cellular response as unfiltered, traditional petrodiesel exhaust.

CONCLUSIONS

Recent regulations on diesel engine emissions have resulted in changes to the composition of diesel exhaust. In addition, new forms of diesel fuel, like biodiesel, are emerging. Given the changing composition of diesel fuels and diesel exhaust emissions, a need exists to understand the human health implications of switching to “cleaner” diesel engines run with particulate filters and engines run on alternative fuels like biodiesel. To the extent of the author’s knowledge, the work presented here is the first to examine oxidative stress and polycyclic aromatic hydrocarbon responses in airway cells exposed to complete exhaust from an engine run with (1) petro- or biodiesel and (2) a diesel particulate filter. Despite the lowered emissions in filter-treated exhaust (a 67-85% reduction in mass), significant increases in transcripts associated with oxidative stress and polycyclic aromatic hydrocarbon response were observed in all NHBE cell exposure groups and were not significantly different between exposure groups. Surprisingly, our results suggest that biodiesel and filter-treated diesel exhaust elicits as great, or greater a bronchial cellular response as untreated, traditional petrodiesel exhaust in a representative model of the bronchial lung epithelium.

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CHAPTER 4— DIFFERENTIAL EFFECTS OF DAIRY DUST EXPOSURE ON HUMAN NASAL VS. BRONCHIAL EPITHELIAL CELLS

SUMMARY

Exposure to organic dusts is associated with increased respiratory morbidity and mortality in agricultural workers. Dairy workers, in particular, have elevated risks for negative respiratory outcomes. Organic dusts in dairy farm environments are complex, polydisperse mixtures of toxic and immunogenic compounds. Although previous toxicological studies have focused primarily on exposures to the respirable size fraction, these dusts are known to contain larger particles extending well above 10 μm in size. Such larger particles tend to deposit in the upper airways (i.e., the nasal and bronchial regions); thus, the nasal and bronchial epithelia represent targets of interest following organic dust exposures in agricultural settings. Here, we exposed well-differentiated (a) normal human bronchial epithelial cells and (b) normal human nasal epithelial cells to two different size fractions (PM_{10} and $PM_{>10}$) of dairy parlor dust. Levels of pro-inflammatory transcripts (IL-8, IL-6, and TNF-α) were measured two hours after exposure. Lactate dehydrogenase release was also measured as an indicator of cytotoxicity. We characterized cell exposure to dairy dust as a function of total mass, endotoxin, and muramic acid content delivered in each size fraction. To our knowledge, this is the first study to evaluate the effect of agricultural dust size on human airway epithelial cell response. Our results suggest that both PM_{10} and $PM_{>10}$ size fractions elicit an inflammatory response in airway epithelial cells and that the entire inhalable size fraction should be considered in future agricultural dust studies. Further, our results

suggest that human bronchial and human nasal epithelial cells do not respond similarly to similar dust exposures and should be considered separately in airway cell models of agricultural dust toxicity.

INTRODUCTION

Dust exposure is a major source of respiratory morbidity and mortality amongst agricultural workers. (Schenker, 2000, Reynolds et al., 2013, ATS, 1998, Linaker and Smedley, 2002) Dairy workers, in particular, have an increased risk for asthma, rhinitis, sinusitis, mucus membrane inflammation syndrome, bronchitis, chronic obstructive pulmonary disease, hypersensitivity pneumonitis, and organic dust toxic syndrome.(Kullman et al., 1998, May et al., 2012) Agricultural aerosols typically span particle sizes from the respirable (d_p < 4 μm) to inhalable range (d_p < 100 μm). For example, Kullman et al. reported a mass median aerodynamic diameter of dairy farm dusts to be 13.5 μm (with a geometric standard deviation of 2.1). (Kullman et al., 1998) The respirable size fraction ($PM_{<4}$) is often focused upon in epidemiological and toxicological studies because of the ability of $PM_{<4}$ to penetrate to the deepest regions of the lungs. Historically, he $PM_{>10}$ size fraction was not studied extensively in epidemiologic or toxicological contexts. (Rask-Andersen et al., 1989, Kateman et al., 1990)As evidenced by Kullman et al., and in our work reported here, particles larger than 10 μm in diameter ($PM_{>10}$) contribute substantially PM exposures in dairy farm environments.(Kullman et al., 1998) Because of the substantial contribution of $PM_{>10}$ to dust exposures in dairy farm environments, a shift to include the entire inhalable dust

fraction is evidenced in recent epidemiological exposure studies.(Garcia et al., 2013b, Reynolds et al., 2013, Burch et al., 2010)

Dairy farm dusts are complex particulate mixtures that contain both toxic and immunogenic constituents. (Kullman et al., 1998) The organic dust fraction in dairy farm environments can contain yeasts, molds, mesophilic and thermophilic bacteria, histamine, cow urine antigen, mite antigen, endotoxins, pharmaceutical compounds, and pesticides. (Kullman et al., 1998, Donham, 1986, Kemper, 2008) The inorganic dust fraction can contain silicates, clays, pesticides, and metals. (Schenker, 2000) Of the potential microbial etiologic factors found in dairy farm dusts, endotoxin is the most often studied constituent.

A universal dose-response for endotoxin has yet to be established because previous studies have reported conflicting results. Rask-Andersen et al. observed high endotoxin exposure (5000 EU m^{-3}) without symptoms,(Rask-Andersen et al., 1989) while Kateman et al. observed a dose-response in workers exposed to much lower doses (18, 19, and 64 pg m^{-3}).(Kateman et al., 1990) Both research groups analyzed only a fraction of the total inhalable dust (PM < 5 μm or 8.5 μm for Rask-Andersen et al. or Kateman et al., respectively), and Rask-Andersen notes that 75% of the endotoxin activity was observed in the non-respirable (PM > 5 μm) fraction of inhalable dust. Previous studies in swine workers have suggested an exposure limit of 100 EU per m^3. (Donham et al., 2000) Alternatively, the Netherlands proposed an occupational exposure limit of 50 EU per m^3 in 1998, however, this was shortly thereafter increased to 200 EU per m^3 by the

Dutch social affairs ministry, before being eventually abandoned. (Duquenne et al., 2012, Heederik and Douwes, 1997) The Dutch Expert Committee on Occupational Safety has recently (2010) proposed a new exposure limit of 90 EU per m^3. To date, no occupational exposure limit for endotoxin has been established in the U.S., or Internationally.(Duquenne et al., 2012) Differences in sampling techniques and analysis, as well as the failure to consider the total inhalable dust size fraction, may contribute to a lack of a clear dose-response for endotoxin exposure.(Reynolds et al., 1996, Reynolds et al., 2002, Reynolds et al., 2005, Duquenne et al., 2012) Furthermore, recent work suggests that the inflammatory potential of such dusts does not depend solely on endotoxin. (Poole et al., 2010)

Previous studies have suggested that human nasal epithelial cells might serve as a reliable (and more accessible) surrogate for bronchial epithelial cells in respiratory toxicology studies.(McDougall et al., 2008) Here, we examine acute responses of human bronchial and nasal airway cells following exposure to two different particle size fractions collected from within a dairy parlor. We compared the response of well-differentiated human bronchial epithelial (NHBE) cells and human nasal epithelial (HNE) cells following exposure to dairy parlor dust in the size range of PM < 10 μm (PM_{10}) and PM > 10 μm ($PM_{>10}$). Exposure levels were designed to achieve similar PM mass loadings between each particle size and cell type exposure. Transcripts associated with pro-inflammatory cytokines and chemokines (IL-8, IL-6, and TNF-α) were measured 2 hours after exposure. Cytotoxicity was measured as well. To our knowledge, this is the first study to look at the (1) pro-inflammatory response of airway epithelial cells after

exposure to dairy parlor dusts across the inhalable size fraction and (2) differences in the pro-inflammatory response between two different airway cell populations, after exposure to two different particle size fractions.

MATERIAL AND METHODS

Cell Culture

Normal human bronchial epithelial (NHBE) cells were obtained by brush biopsy from two healthy, non-smoking human volunteers (EPA, Research Triangle Park, NC, USA) in accordance with a human studies protocol approved by the Institutional Review Board at the University of North Carolina. Cell populations were expanded through two passages in Petri dishes with Bronchial Epithelial Growth Media (BEGM kit; Lonza, Walkersville, MD, USA) before being plated onto collagen-coated, porous, polycarbonate membranes (0.4 μm Snapwell membrane; Corning, Inc., Corning, NY, USA) at a seeding density of approximately 150,000 cells per cm^2. Air–liquid interface (ALI) cultures were carried for a minimum of 21 days (prior to exposure) to allow progressive differentiation into basal, ciliated, and mucin-producing cell types within a pseudo-stratified columnar epithelium.(Ross et al., 2007) Mucus production was visually apparent by day 10 of ALI and excess mucus was removed with a gentle saline rinse, every three days thereafter.

Human nasal epithelial (HNE) cells from two different human donors were obtained from Celprogen Inc. (Celprogen Inc., San Pedro, CA). The HNE cells were cultured following the same protocol described above for the NHBE cells. The only protocol difference to

note was a saline rinse every two days (as opposed to three) due to increased mucus production by the HNE cells.

Dairy Dust Collection and Extraction

Airborne dust from a local dairy parlor was size-slectively sampled using a high-volume cascade impactor (IESL v2, (J. Collett Jr, 1995) over the course of single 72 hr period. The cascade impactor operated at an airflow of 1500 L min^{-1} and collected dust onto Teflon collection plates at three aerodynamic size cuts of 3, 10, and 30 µm. Particulate matter less than 3 µm in aerodynamic diameter (PM_3) was collected downstream of the impactor on an 8"x11" filter (Zeflour, Pall Inc, Ann Arbor, MI). The PM_3 filter was replaced every 12 hours to prevent overloading. An annotated image of the cascade impactor sampler can be seen in Figure 4-1.(J. Schaeffer, 2013) Relative humidity and temperature inside of the parlor varied from 45-80% and 47-57ºF, respectively. Immediately after sampling, the impactor was transported to the laboratory and collected PM was scraped from each plate. Each size fraction was then placed in cryovials and stored at -20ºC until use. Each PM_3 filter was placed into 100 mL of acetone in non-pyrogenic glass vials and allowed to soak for ten minutes. The filters were then vortexed for two minutes and finally shaken for two hours at 100 RPM and 22ºC. Acetone was used as the solvent for particle extractions because we desired to extract both the polar and nonpolar constituents from the PTFE filter. The filters were carefully removed from the acetone solution, and the acetone was allowed to evaporate in a fume hood overnight. The remaining PM was then lyophilized to ensure complete solvent removal. To ensure the extraction process had no effect upon endotoxin levels,

rFC analysis was performed with a standardized assay kit and protocol and 1 mg/mL solutions of untreated PM_{3-10} dairy dust in acetone or tween and (Figure A.2) (Pyrogene Recombinant Factor C Assay; Lonza Group Ltd., Walkersville, MD).(Thorne et al., 2010) The PM_3 fraction was combined with PM_{3-10} at approximately a 1:2 mass ratio in sterile, pyrogen free H_2O, to achieve a 1.25% stock solution PM_{10} for cell exposures. Standard curves for endotoxin units (EU) as a function of PM mass were prepared for each particle size fraction (Figure A.3).

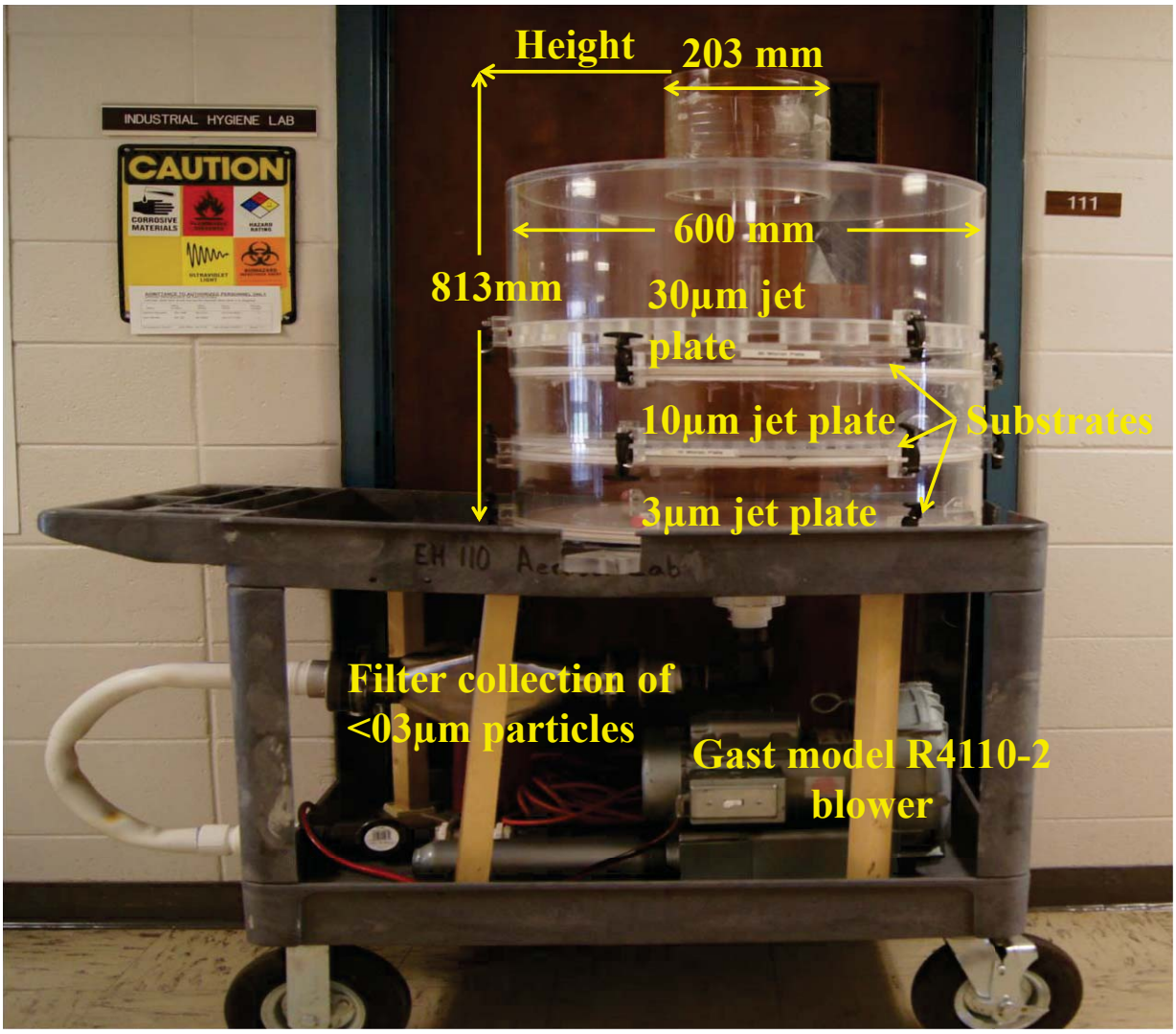

Figure 4-1. High Volume Multi-stage Impactor (IESL v2) used to Collect $PM_{>30}$, PM_{10-30}, PM_{3-10} µm, and PM_3.

Cell Exposures to Dairy Parlor Dust

Well-differentiated NHBE or HNE cells (cultured at ALI for a minimum of 21 days) were exposed to resuspended dust samples in a gravity settling chamber (n = 10 per treatment group). A schematic of the settling chamber can be seen in Figure 4-2. A heated water bath served to maintain temperature (37ºC) and humidity (85-90%) inside the chamber at near-physiologic conditions. Supplemental CO_2 (5% by volume) was provided to the chamber volume to maintain cellular pH. An experimental matrix for the cellular exposures is provided in Table 4-1. Dairy dust was nebulized for five minutes, until a stable, steady-state, mass concentration of either 1.4, or 2.8 mg per m^3 was achieved inside the settling chamber. The mass concentrations used for cell exposures are typical of the mass concentrations measured for personal, inhalable dust exposures in dairy farm environments. (Burch et al., 2010, Garcia et al., 2013b, Reynolds et al., 2013, J. Schaeffer, 2013) Particle mass concentrations inside the settling chamber were monitored with a DustTrak Aerosol Monitor (DustTrak Aerosol Monitor 8520; TSI, Shoreview, MN, USA) that was calibrated by gravimetric analysis. An Aerodynamic Particle Sizer (APS; TSI, Shoreview, MN, USA) was used to measure the particle size distributions immediately after the steady-state concentration was reached. Cells were exposed for two hours to allow each dust sample to settle completely onto samples. Control cells were exposed to the same conditions stated above except that no dairy dust was nebulized. Each exposure condition was repeated on two separate test days. We compared the response of PM_{10} exposed cells to the response of control cells, two hours after exposure.

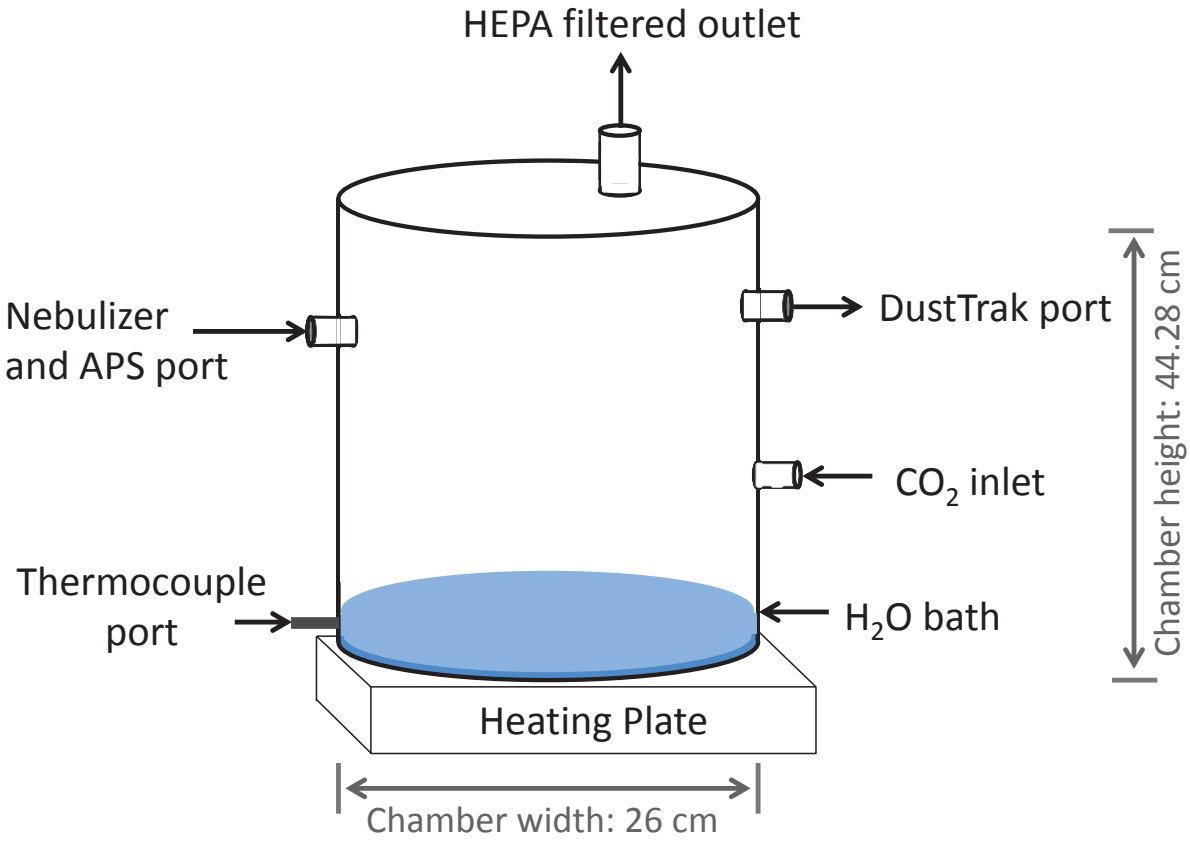

Figure 4-2. Gravitational Settling Chamber used in PM_{10} Cell Exposures

Table 4-1. Exposure matrix for Cell Exposures to Dairy Parlor Dust

Experimental Variable	Number of Variables	Level of Variation
Airway Cell Type	2	Normal human bronchial epithelial cells and normal human nasal epithelial cells
Biological Variability	2	Donors 1, 2
Exposure Types	3	PM_{10} exposed cells, $PM_{>10}$ exposed cells, HEPA Air + Gravity Settling Chamber Controls (1)
Exposure Level	5	PM_{10} µm 0.1 and 0.4 µg per cm^2, $PM_{>10}$ µm 0.1, 0.4, 1.0 µg per cm^2 exposure levels
Experimental Repetitions	2	NHBE and HNE cells (2) exposed to each exposure duration (2) on two separate days
Cellular Marker Timepoints	1	2 hrs Following Exposure
Cellular Markers	4	IL-8, IL-6, TNF-α, LDH

A similar approach was used for cell exposures to $PM_{>10}$. However, a larger settling chamber (Figure 4-3; dimensions: 1 m x 1 m x 2 m) was used to disperse and settle these dusts. Because the $PM_{>10}$ size fraction contained less endotoxin per mg of dust than the PM_{10} size fraction (Figure A.3) we included an additional $PM_{>10}$ exposure level of higher mass. Preliminary studies with this chamber indicated that an aspirator mass loading of 8 mg corresponded to a deposition level of approximately 1 µg cm^{-2}. Therefore, an 8 mg mass loading was used to establish the highest $PM_{>10}$ exposure group and 0.8 and 4 mg mass loadings were used to establish the low and medium exposure groups, respectively. On each test day, NHBE or HNE cells were placed in the

chamber and then $PM_{>10}$ dust was aspirated and allowed to settle for ten minutes. Given the high terminal settling velocities of $PM_{>10}$, ten minutes was sufficient to allow complete settling of all aspirated dust. The cell plate was then transferred to the same gravitational settling chamber used for the PM_{10} exposures and cells were exposed to the same conditions (physiological temperature, humidity, CO_2) and same total duration of exposure (two hours) as used for the PM_{10} exposed cells. Control cells were treated similarly, except that no $PM_{>10}$ was aspirated. We compared the response of $PM_{>10}$ exposed cells to the response of control cells, two hours after exposure.

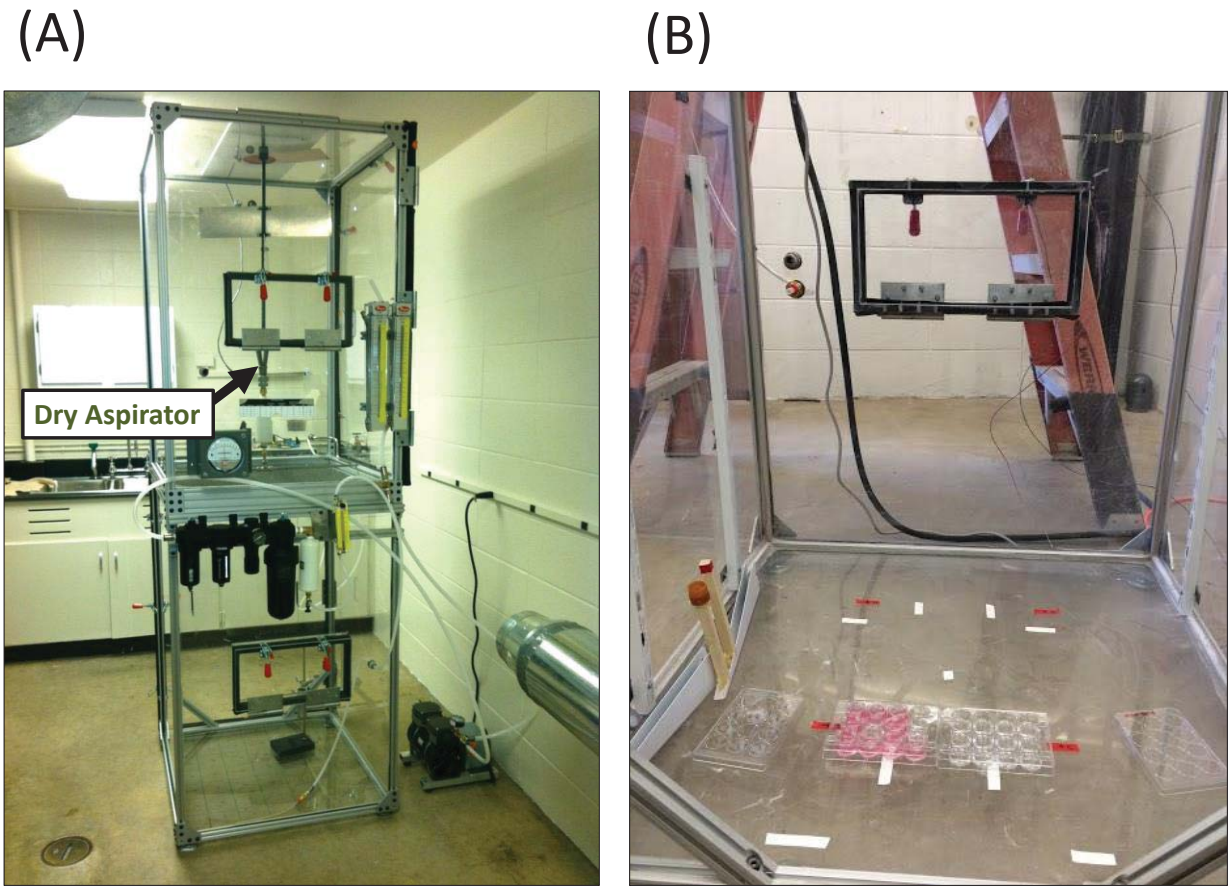

Figure 4-3. (A) Gravitational Settling Chamber used in $PM_{>10}$ Cell Exposures (B) Cell Plate Placement during Ten Minute Settling Exposures. Dimensions: 1 m x 1 m x 2 m

Levels of PM mass deposited to cells were calculated by direct measurement of endotoxin present on a 12-well plate that was co-located with cell cultures in the exposure chamber. Each culture plate also contained two empty cell wells that were analyzed for quality control. Unseeded wells were filled with 1.5 mL of non-pyrogenic tween solution (0.05% by volume). Dust PM was collected into these fourteen wells during cell exposures, and total EUs per mL were measured in triplicate with a commercial rFC assay and standardized protocol (Pyrogene Recombinant Factor C Assay; Lonza Group Ltd., Walkersville, MD).(Thorne et al., 2010) Sample coefficient of variations were less than 25%. Total mass deposited was estimated with the standard curve equations for EU per mg of PM for either the PM_{10} or $PM_{>10}$ size fractions (Figure A.3). Equations 1 (PM_{10}) and 2 ($PM_{>10}$) below were used to estimate total mass deposited,

Equation (1) $$PM_{mass} = \frac{0.36 \cdot EU \cdot V}{A}$$

Equation (2) $$PM_{mass} = \frac{5.2 \cdot EU \cdot V}{A}$$

where PM_{mass} represents mass of PM deposited per cellular growth area (μg per cm^2), *EU* represents measured endotoxin concentration in each well (EU per mL), *V* represents total volume Tween solution per cell well (1.5 mL) and *A* represents the total area of a single well from a standard 12-well plate (3.83 cm^2). Integers on the right-hand side of equations 1 and 2 account for units conversion between endotoxin mass content and total dust mass for each size fraction (taken from serial calibration curves of known mass content).

Muramic acid content of the two dust size fractions were measured with gas chromatography mass-spectrometry using an Agilent 6890 gas chromatograph (Agilent Technologies, Loveland, CO) with a Micromass Quattro Micro mass spectrometer (Waters Corporation, Milford, MA) and a standardized protocol (Poole et al., 2010). A 1.25% (by mass) solution of each particle dust size fraction (PM_{10} and $PM_{>10}$) was aliquoted with volumes 150 µL (120 µg total mass) and frozen at -80ºC until GC-MS analysis could be performed. Samples were thawed, lyophilized, and GC-MS analysis was performed for muramic acid. Measured levels of muramic acid were reported as ng per µg of dust.

Transcript Production in ALI NHBE Cells

We chose to quantify mRNA transcripts coding for proteins that characterize the cellular pro-inflammatory response (Interleukin 8, IL-8; Interleukin 6, IL-6, and Tumor Necrosis Factor alpha, TNF-α). These transcripts were chosen based upon the inflammation markers observed by (1) Burch et al. in an epidemiological study of agricultural workers, and (2) Poole et al. in an in vitro study with similar dust exposures. (Burch et al., 2010, Poole et al., 2010) All transcript analyses were quantified by RT-PCR (CFX96, Bio-Rad Laboratories, Hercules, CA) according to MIQE guidelines.(Bustin et al., 2009) Expression profiles for each transcript were normalized to GAPDH.(Barber et al., 2005) Transcript levels of IL-8, IL-6, and TNF-α were measured two hours after exposure. All transcript expression profiles were normalized to controls' expression levels of each transcript.

Cytotoxicity in ALI NHBE Cells

Lactate dehydrogenase (LDH) is expressed constituently in NHBE and HNE cells. The loss of membrane integrity during cell injury and death causes the extracellular release of LDH, which can be used as an indicator of cytotoxicity.(Allan and Rushton, 1994) Extracellular LDH was assayed at two hours post-exposure to dairy dust sing a standard kit and protocol (Promega Cytotox96 Non-radioactive Cytotoxicity Assay, Promega Corporation, Madison, WI, USA). Percent cytotoxicity was calculated by following the standard protocol established by Promega for an assay with a single cell type.(Promega, 2012)

Statistical Analysis

Transcript data were log-transformed to satisfy model assumptions of normality and homoscedasticity. The effects of exposure type, exposure level, donor phenotype, and experimental repeat (and their interactions) were evaluated relative to the expression of IL-8, IL-6, and TNF-α transcripts and extracellular LDH (cytotoxicity) using a PROC MIXED procedure in SAS. Cell donor and experimental replicate were treated as random effects. Statistical analyses were conducted with SAS software (v9.0, SAS Institute Inc., Cary, NC, USA) with a type I error rate of 0.05.

RESULTS

Dairy Parlor Dust Characteristics

The size distribution for nebulized PM_{10} was consistent across all exposure groups (mass median diameters were 5.8 μm (GSD = 1.5 μm) d_p and 5.6 μm (GSD = 1.5 μm)

d_p for the low and high exposure groups, respectively; count median diameters were 4.2-4.4 µm with a GSD of 1.42, Figure 4-4). The bimodal particle size distribution is typical of what has previously been observed in dairy parlor environments. (J. Schaeffer, 2013)Mass concentrations inside the gravity settling chamber ranged from 1.3-1.5 mg m^{-3} to 2.6 to 3.0 mg m^{-3} at the start of cell exposures, for the low and high exposure groups, respectively. After two hours, these starting concentrations resulted in 0.1-0.2 and 0.3-0.4 µg of settled PM_{10} per cm^2 cellular growth area. Cells exposed to $PM_{>10}$ were exposed to starting PM mass loading of 0.8, 4, and 8 mg into the aspirator, which resulted in 0.1-0.2, 0.4-0.5, and 1.0-1.2 µg per cm^2 exposure levels, as estimated by following Equation 2 and using the EU levels measured directly in the cell wells following each test. The average size distribution for the $PM_{>10}$ cell exposures can be seen in Figure 4-4 (CMD = 11.1 µm, GSD = 1.20). Endotoxin content by mass was approximate 14.5 fold greater in the PM_{10} fraction than in the $PM_{>10}$ µm size fraction (Figure A.3, Equations 1 and 2). Muramic acid content varied from 0.057 (± 0.003) to 0.044 (± 0.006) ng per µg in the PM_{10} and $PM_{>10}$ dust fractions, respectively.

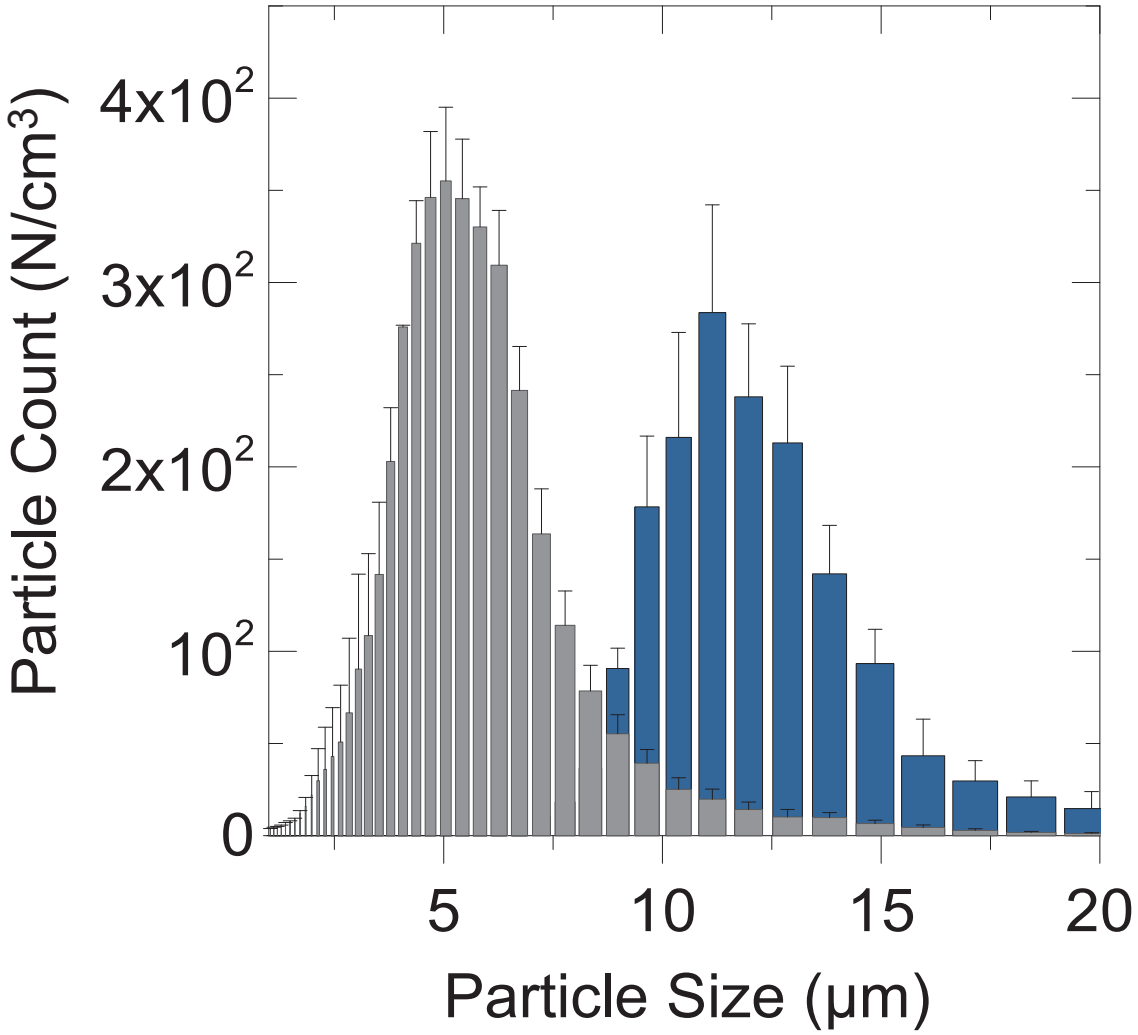

Figure 4-4. Particle size distributions for nebulized PM_{10} and $PM_{>10}$.

Cell Exposure Levels

Levels of endotoxin measured in cell wells after the PM_{10} cell exposures ranged from 0.7 (± 0.12) EU per mL in the low exposure group and 2.8 (± 0.15) EU per mL during the high exposure group. These levels corresponded to an endotoxin loading that varied significantly between exposure groups with approximately 0.3 (± 0.07) EU deposited per cm^2 in the low exposure group and 1.1 (± 0.1) EU per cm^2 in the high exposure group. The endotoxin deposition measurements resulted in PM mass exposure estimates of 0.1-0.2 µg per cm^2 for the low exposure group and 0.35-0.45 µg per cm^2 for the high exposure group.

Levels of endotoxin measured in the cell wells after $PM_{>10}$ exposure ranged from 0.11 (± 0.03), to 0.19 (± 0.05), to 0.52 (± 0.12) EU per mL for the low, mid, and high exposure groups, respectively. These levels corresponded to endotoxin loadings that ranged from 0.04 (± 0.01), to 0.08 (± 0.03), to 0.2 (± 0.04) EU per cm^2 for the low, mid, and high exposures, respectively. Levels of endotoxin deposited varied significantly between the high and low $PM_{>10}$ exposure groups. The EU deposition measurements resulted in $PM_{>10}$ mass exposure estimates of 0.1-0.2, 0.4-0.5, and 1.0-1.2 µg per cm^2 for the low, mid, and high exposure groups, respectively.

Muramic acid content did not vary significantly between the two particle size fractions. In PM_{10} exposed cells, muramic acid exposure levels varied from approximately 0.006 to 0.01 and 0.020 to 0.023 ng per cm^2 in the low and high exposure groups, respectively. In $PM_{>10}$ exposed cells, muramic acid exposure levels varied from 0.005 to 0.01, to

0.015 to 0.020, and 0.045 to 0.050 ng per cm^2 in the low, mid, and high exposure groups, respectively.

Cellular Response after Exposure to Dairy Parlor Dust

Differences between NHBE and HNE cell responses were observed after exposure to each dairy dust size fraction and mass exposure level. In cells exposed to the smaller particle size fraction (PM_{10}), a 0.4 µg per cm^2 exposure level resulted in significantly increased transcript levels for IL-8, IL-6, and TNF-α in NHBE cells, but not in HNE cells (Figure 4-5). Significant differences were observed between NHBE and HNE cells exposed to 0.4 µg per cm^2 PM_{10} for IL-8 ($p = 0.001$), IL-6 ($p = 0.001$), and TNF-a ($p = 0.007$). In the larger PM size fraction exposed cells ($PM_{>10}$), a 0.4 and 1.0 µg per cm^2 exposure resulted in significant increases in IL-8 HNE cells but not in NHBE cells (Figure 4-5). These same exposure levels resulted in increased levels of IL-6 in both NHBE and HNE cells (Figure 4-5) and increased levels in TNF-a in NHBE but not in HNE cells.

Cytotoxicity, as estimated by measured lactate dehydrogenase release, was not significantly increased above controls in any exposure group (Figure 4-6).

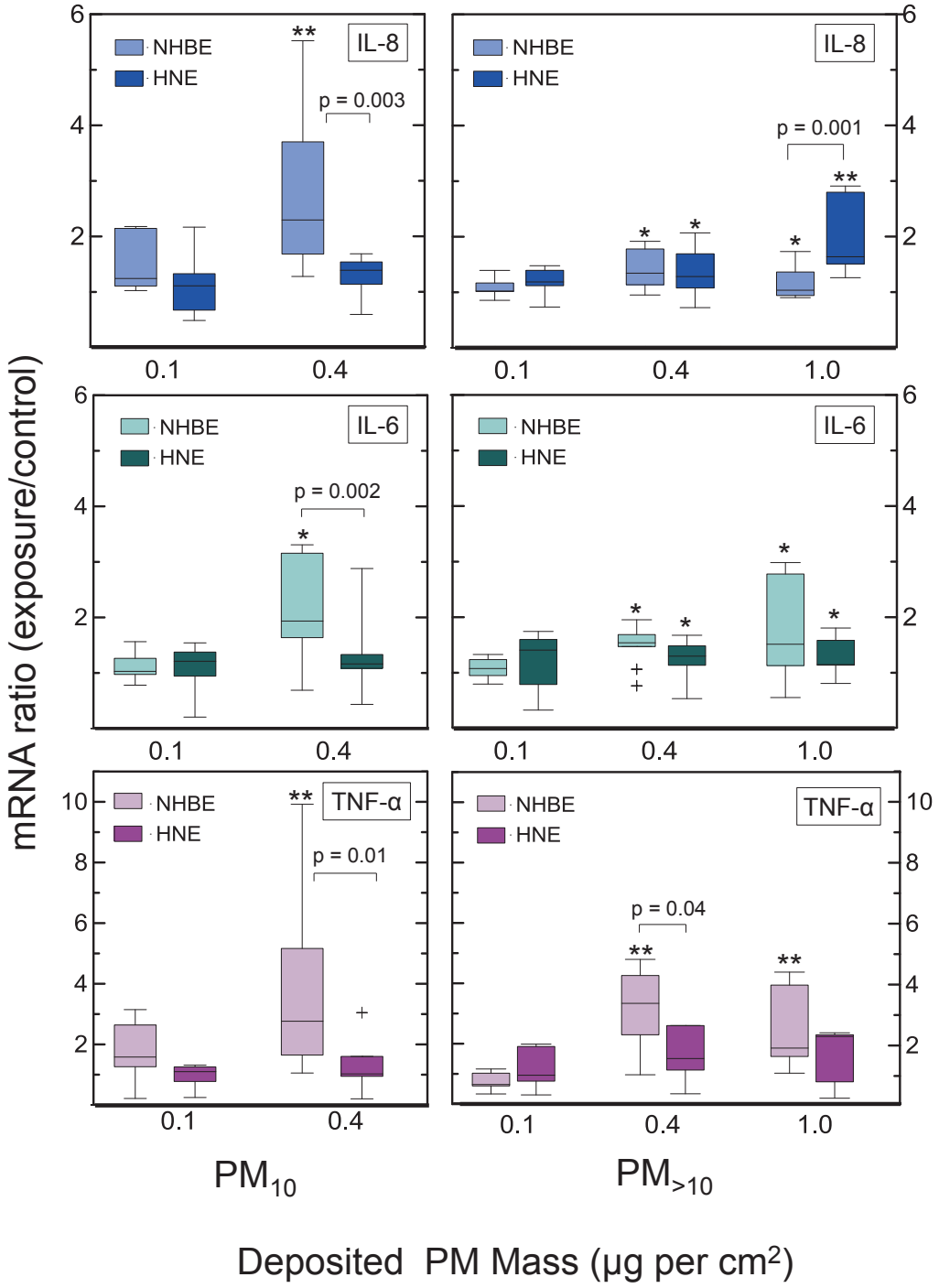

Figure 4-5. Box-whisker plots of transcript production in ALI NHBE and HNE cells exposed to PM10 (left panel) and PM>10 (right panel) size fractions. All transcript levels are normalized to HEPA-air controls. (+) signifies outliers. * signifies p<0.05, ** signifies p<0.0001, when compared with controls. P-values shown in plots are p-values for cell type differences.

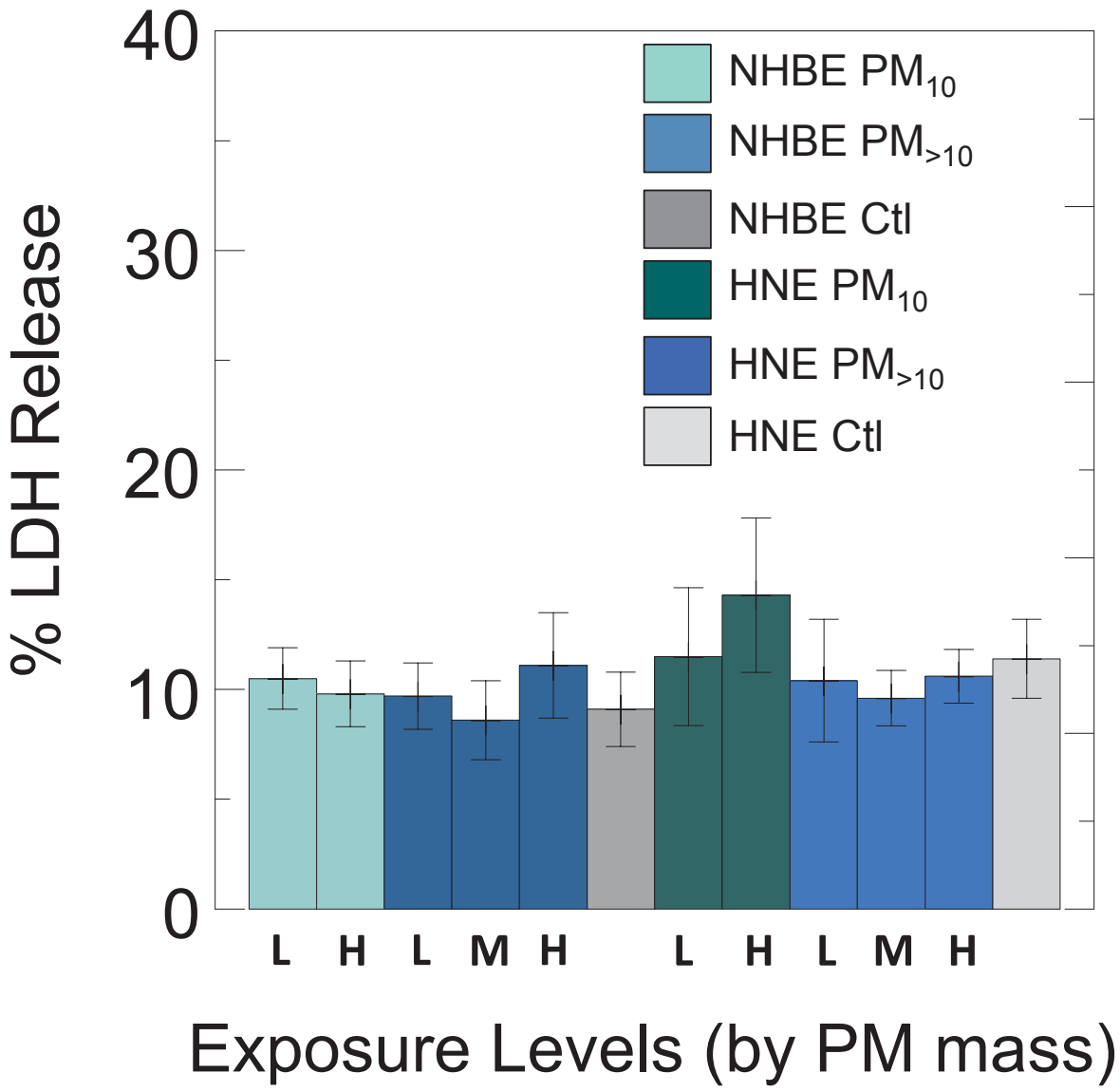

Figure 4-6. LDH release from ALI NHBE cells exposed to PM10 and PM>10 µm size fractions. L, M, H, signify low, mid, and high exposure groups by PM_{mass}, respectively. Error bars indicate one standard deviation.

DISCUSSION

We observed significant differences in the accumulation of pro-inflammatory transcripts within NHBE and HNE cells following exposure to dairy dust (Figure 4-5). The responses of each cell type were significantly different and varied with particle size and by transcript type. For example, in $PM_{>10}$ exposed cells, IL-8 was increased in HNE cells but not in NHBE cells whereas TNF-a was increased in NHBE cells, but not in HNE cells (Figure 4-5, right panel). These results suggest that IL-8 transcript production in NHBE and HNE cells may not be driven via the same stimuli and/or pathways, and that if IL-8 is a cytokine of interest, HNE cells may not be a good surrogate for NHBE cellular response. Further, TNF-α transcript production was also observed to be different between NHBE and HNE cells. NHBE cells produced significantly greater levels of TNF-α than HNE cells after exposure to PM_{10} and $PM_{>10}$ mass loadings of 0.4 μg per cm^2 (Figure 4-5, $p = 0.007$ and $p = 0.01$, respectively).

Our results differ from those reported by McDougall et al., who concluded that nasal epithelial cultures represent an acceptable surrogate for studies of lower airway inflammation.(McDougall et al., 2008) Some obvious differences are noteworthy between our work presented here, and the work presented by McDougall et al. For example, we exposed cells to an exogenous stressor (dairy dust) known to generate a proinflammatory response in bronchial airway epithelia (Poole et al., 2010), whereas, McDougall et al. used endogenous cytokines (IL-1β and TNF-α) as a stimulant. Although McDougall et al. observed that human nasal and bronchial airway epithelial cells respond similarly to cytokine stimulation, our work here suggests that the

similarities observed by McDougall et al. do not transfer to human bronchial and nasal epithelial cell responses to exogenous stimuli like agricultural dusts. Our results suggest that HNE and NHBE cells have distinctly different responses to dairy parlor dust.

The bimodal size distribution (Figure 4-4) and mass concentrations used for the cell exposures here are similar to the size distribution and inhalable mass concentrations observed in dairy farming environments.(J. Schaeffer, 2013, Reynolds et al., 2013, Garcia et al., 2013a, Reynolds et al., 2012) The particle size fractions used here appeared to play a role in eliciting differential responses among HNE and NHBE cells. For example, PM_{10} elicited a significant increase in IL-8 transcript levels in NHBE cells, whereas $PM_{>10}$ did not (Figure 4-5). Alternatively, IL-8 and IL-6 transcript levels in HNE cells increased with increased $PM_{>10}$ mass loadings while IL-8 transcript levels did not increase after PM_{10} exposure (Figure 4-5). Overall, NHBE cells responded more strongly to PM_{10}, whereas HNE cells responded more strongly to the $PM_{>10}$ size fraction. In vivo, $PM_{>10}$ deposits primarily in the extrathoracic region (20-40% of inhaled $PM_{>10}$ is deposited in the human nasal, mouth, larynx, and pharynx, versus 0-1% deposition in the bronchial region of the lungs, (ICRP, 1994)). To that end, primary nasal cells from human donors may be more sensitized to $PM_{>10}$ size fractions (as opposed to PM_{10} size fractions). Similarly, NHBE cells may be more sensitive PM_{10} due to increased deposition of these particles in the conducting airways. Anywhere from 1-20% of inhaled PM_{10} is deposited in the human bronchial airways, whereas 5% or less of $PM_{>10}$ is deposited in this same region.(James et al., 1991) The mechanism for sensitizing HNE and NHBE cells to specific particle sizes is unclear but may involve

epigenetic or immunogenic factors. Previous studies have found that exposure to PM_{10} air pollutants induces gene-specific and global methylation in the lungs.(Holloway et al., 2012)

Because muramic acid levels did not vary significantly between each particle size fraction exposure group, we do not believe muramic acid to be responsible for the observed differences between PM_{10} and $PM_{<10}$ exposed cells. The higher endotoxin content in PM_{10} (Figure A.3) could suggest that the higher endotoxin content in PM_{10} played a role in the increased NHBE IL-8 response to PM_{10}. However, previous work by Poole et al. showed that endotoxin removal did not eliminate NHBE production of IL-8. (Poole et al., 2010) Because we did not explicitly control for endotoxin exposure levels, we cannot conclude that the higher endotoxin content in PM_{10} was responsible for the NHBE IL-8 response to PM_{10}. Further, because HNE cells responded significantly to the $PM_{>10}$ but not to PM_{10}, the higher endotoxin content in PM_{10} had no observable effect on HNE cell response.(Parsanejad et al., 2008)

Kullman et al. reported that agricultural dust exposures involve particle sizes that extend into the inhalable size fraction (i.e., $PM_{>10}$; (Kullman et al., 1998)). Historically, the $PM_{>10}$ size fraction has been largely ignored in previous epidemiologic or toxicological contexts (Rask-Andersen et al., 1989, Kateman et al., 1990) however increased interest in measuring the entire inhalable particle size fraction has greatly increased in recent years. (Burch et al., 2010, Garcia et al., 2013b, Basinas et al., 2012, Reynolds et al.,

2013) Our results support the inclusion of large, inhalable particles in future studies of dairy dust toxicity, given the inflammatory potential of such particles studied here.

Our exposure model is limited to the human airway epithelium response, which does not fully mimic the nasal or bronchial lung. Future work should consider whether these results are reproduced with immune cells (e.g. mast cells, eosinophils, lymphocytes) present in co-culture. Our sample size was also limited; however, our study was powered (successfully) to detect significant differences in the responses by cell type and particle size. Strengths of this study include the use of a direct, air-to-cell exposure system, the use of primary cells, and the application of distinct (and previously unstudied in airway cells) particle sizes at environmentally-relevant exposure levels. Many traditional in vitro studies rely on the use of particle extracts (or suspensions) with unrealistic doses.(Frampton et al., 1999, Veranth et al., 2004, Romberger et al., 2002, Wyatt et al., 2007) Our work here reports for the first time, differences in the pro-inflammatory (1) response of human airway cells to two different particle size fractions across the inhalable size range of agricultural dusts and (2) response of two airway cell populations after exposure to agricultural dust.

CONCLUSIONS

Our results offer preliminary insight into the relatively unstudied toxicity of two different particle size fractions present within agricultural dusts. Human airway cell pro-inflammatory responses varied with cell type, particle size fraction, and particle mass loading. Our work suggests that HNE cells would not be a good surrogate for NHBE

cellular response in future work with agricultural dusts. Our results also suggest that when selecting a 'screening' cell type for evaluating the pro-inflammatory response of airway cells to agricultural dusts, NHBE cells offer greater sensitivity to PM_{10} than HNE cells. Alternatively, both HNE and NHBE cells were sensitive to $PM_{>10}$ agricultural dusts. Further, because we observed significant responses in cells exposed to $PM_{>10}$, our work suggests that particles in the inhalable size fraction should continue to be considered alongside of particles in the respirable and thoracic size fractions in future agricultural dust studies.

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CHAPTER 5 – CONCLUSIONS, LIMITATIONS, AND FUTURE WORK

CONCLUSIONS

The work presented here further supports the replacement of traditional, submerged cell cultures and resuspended particle exposure techniques with improved airway cell models and aerosol exposure techniques. The results from the studies presented here suggest that well-differentiated ALI cell cultures combined with direct air-to-cell exposure systems allow for greater sensitivity and provide more physiological relevance to aerosol toxicology in vitro than former traditional submerged techniques.(Gruenert et al., 1995, Ross et al., 2007, Hawley et al., 2014, Volckens et al., 2009a, Volckens et al., 2009b)

The electrostatic aerosol exposure system detailed in Chapters 2 and 3 allowed for the observation of a human bronchial epithelial cell response at levels of exposure that were 20-400 and 60-100,000 fold lower than previous, traditional in vitro studies of diesel particulate matter or diesel exhaust, respectively.(Totlandsdal et al., 2010, Cancer, 1989, Cao et al., 2007, IARC, 2012) Further, the direct air-to-cell gravitational system detailed in Chapter 4 allowed for the observation of a proinflammatory response in human nasal and bronchial epithelial cells at levels of exposure that were 1000 to 2000 fold less than previous work with submerged airway cells and extracts from settled dairy parlor dusts.(Poole et al., 2010)

When compared with former traditional in vitro techniques the exposure levels used in the studies reported here more closely approximate an in vivo exposure. Former in vitro

exposure techniques typically use exposure levels that varied anywhere from 20-200 μg per cm^2 or 20-200 μg per mL for diesel particulate matter to as high as 10 mg per mL for large animal farming dusts.(Schwarze et al., 2013, Poole et al., 2010, 1999, Romberger et al., 2002) When the former DPM exposure levels reported for traditional submerged studies are compared with estimates of ultrafine aerosol deposition in the lungs of a human exposed acutely (24 hours) or over the course of a working lifetime (45 years), the former in vitro exposure techniques deliver exposure levels that are 100 to 10,000 fold greater than estimated exposure levels in vivo.(Gangwal et al., 2011) Gangwal et al. estimate that the total PM deposited in a human's lungs would range from 2.0-4.9 μg per cm^2 of lung cellular surface after exposure to ultrafine PM at 0.1 mg per m^3 over the course of 45 years. Estimates provided for an acute 24 hour exposure are dramatically less (0.006-0.02 μg per cm^2 of lung cellular surface) and these lower deposition levels associated with an acute exposure further highlight the unrealistically high exposure levels associated with former traditional submerged exposure techniques. (Gangwal et al., 2011)

Unlike 'traditional' in vitro studies, which rely on particle extractions from filters as a source of DPM, our system was capable of exposing cells to complete exhaust (gas-phase PAH and nitro-PAH, quinones, and gaseous emissions).(Gerlofs-Nijland et al., 2013, Pezzulo et al., 2010) Surprisingly, our results from Chapter 3 suggest that filtered and biodiesel exhaust may be as toxic as untreated, traditional petrodiesel exhaust. Despite the lowered emissions in biodiesel exhaust (lowered 27-30% by mass) and in diesel particulate filter treated exhaust (lowered 67-85% by mass), increases in

transcripts associated with oxidative stress and polycyclic aromatic hydrocarbon response were observed in all exposure groups, and were not significantly different. The work presented in Chapter 3 further supports the importance of using *complete* aerosol sources (complete with ultrafine particulate matter and gases) in future studies of complex aerosols like diesel exhaust. Because ultrafine particulate matter and gases like nitrogen dioxide are thought to contribute to the observed toxicity associated with diesel exhaust (Karthikeyan et al., 2013) the *complete* exhaust should not be excluded in future in vitro exposure matrices.

Former in vitro toxicological studies of dairy farm dusts often fail to include consideration of particles larger than the respirable size fraction. The inhalable fraction is often ignored in dairy dust exposure assessments despite the observation that particles larger than 10 μm in diameter ($PM_{>10}$) contribute substantially PM exposures in dairy farm environments.(Kullman et al., 1998) Because significant responses in human airway cells after exposure to $PM_{>10}$ were observed in the work reported here, future toxicological studies of dairy farm dusts should include respirable, thoracic, and inhalable particle size fractions.

LIMITATIONS

The work presented here was designed to assess the immediate effect of aerosol exposure on the first responders in the human respiratory tract: airway epithelial cells. Human airway epithelial cells not only provide a structural boundary between the body and the environment, but airway epithelial cells have also been observed to play an

active, significant role in the inflammatory response, and AECs are critical actors in the innate immune response.(Mathew et al., 2007) However, because only two cell types were assessed in the studies presented here, the cell models used here are limited with regard to in vivo relevance. In vivo the initial acute inflammatory response of the human airway epithelial cells would potentially be followed by (1) recruitment of macrophages (2) activation of fibroblasts and (3) changes in cardiovascular endothelium. Future work is warranted with co-culture systems.

FUTURE WORK

Although a panel of genes was carefully selected as indicators of the cellular response each respective exposure (diesel particulate matter, diesel exhaust, and dairy parlor dusts) for the generalizability and ease of comparison with previously published in vitro and in vivo studies, many additional genes might be added to future studies to further understand the toxicity associated with each respective exposure. For example, many more genes might be used in future work that could highlight the extent of Nrf2 and NF-kβ pathway activation. The magnitude of the oxidative stress and detoxification (Nrf2) response of cells exposed to diesel exhaust particulates (DEPs) could be further characterized by including measurement of: GST, NQO1, GCS, AKR, SULF, UGT, MRP, γ-GCL, MT. (Figure 2-11) The role of the Nrf-2 pathway in the oxidative stress response of airway cells exposed to DEPs could be further assessed with a Nrf-2 knock out mouse model. Further, the effects of DEPs on oxidative stress in vitro or in vivo could be further assessed by (1) pretreating cells with superoxide dismutase (2) reactive oxygen species assays (e.g. Oxyselect ROS assay) (3) protein oxidation assays (e.g.

Oxyblot assay) or (4) measurement of serum glutathione (in vivo only). Alternatively, the magnitude of the pro-inflammatory response of cells exposed to DEPs, or dairy parlor dusts, could be further understood with includion of the genes that encode for: IL1, IL2, IL2R, RANTES, MCP-1, ICAM-1, VCAM-1, ECAM-1, Fas, BCL-2, c-Flip, and caspases. (Figure 2-11)

The contribution of various constituents in either in diesel exhaust or dairy parlor dusts to the observed cellular response could be further understood with future experiments designed to remove the constituent in question. For example, a selective catalytic reduction catalyst could be added to the diesel exhaust system to reduce exhaust levels of nitrogen dioxide. Alternatively, cells could be exposed solely to nitrogen dioxide at similar exposure concentrations as reported in this work here (exposure levels for future work: 1, 5, 10 ppm). These experiments would further characterize the role of NO_2 in the observed cellular responses after exposure to diesel exhaust from petro- or biodiesel, and with and without a DPF (Chapter 3). Similarly, the role of bacterial and endotoxin loading in the observed cellular response after dairy parlor dust exposure (Chapter 4) could be further understood with experiments designed to block either overall bacterial or, more specifically, endotoxin effects. The effect of bacterial loading could be assessed by irradiating the dust used for cell exposures, thereby killing active bacteria and eliminating the cellular response due to bacterial loading. Alternatively, the effect of endotoxin could be assayed by treating the dusts with polymyxin B. Because polymyxin B has been observed to bind and inhibit the effects of endotoxin, differences in the

responses of airway cells exposed to either untreated or dusts treated with polymyxin B could be attributed to endotoxin exposure. (Poole et al., 2010)

Recent work by several research groups has suggested that monocytes can be successfully co-cultured with well-differentiated air-liquid interfaced airway epithelial cells.(Mathew et al., 2007, Schwab et al., 2013) Additionally, Schwab et al. demonstrated that well-differentiated airway epithelial cell and macrophage phagocytic activity could be tracked with live confocal microscopy. (Schwab et al., 2013) Schwab et al. were able to observe the live movement of both phagocytes and bacteria alongside of ciliary beating by the bronchial epithelial cells. (Schwab et al., 2013) A similar 3-D model applied in future studies of airborne particulate matter and (2) airborne pathogens would be a powerful in vitro tool in aerosol toxicology. A similar co-culture system with live imaging could increase understanding of the toxicity associated with and clearance mechanisms involved in the removal of particulate matter or pathogens.

Previous studies have successfully co-cultured human AECs with human airway myofibroblasts.(Zhang et al., 1999a, Nakamura et al., 1995, Mio et al., 1998) Often, the myofibroblasts will be cultured in a collagen gel, and human AECs will be seeded onto the surface. (Zhang et al., 1999b, Mio et al., 1998) A co-culture system like that used by Zhang et al. would offer insights into airway epithelial damage and response and may be most helpful when applied in a study of occupational or environmental aerosol agents associated with increased risk for asthma. (Holgate, 2000) Although a co-culture system of human AECs and fibroblasts was not used here, future work with similar test

aerosols and a co-culture system of AECs and fibroblasts would further our understanding of diesel exhaust or separately, agricultural dusts, in the development of obstructive and restrictive airway diseases like pulmonary fibrosis, chronic bronchitis, COPD, or asthma.(Nakamura et al., 1995, Hunninghake et al., 1984, Brewster et al., 1990, Zhang et al., 1999b, Knight, 2001, Attfield et al., 2012, Reynolds et al., 2013)

Perhaps the most exciting advancements in co-culture models have occurred with the development of 'lung on a chip' technologies. Co-culture models of human alveolar epithelial cells with human microvascular cells allowed for the development of alveolo-capillary barriers in vitro.(Hermanns et al., 2004) Microscale engineering allowed for the development of microfluidic devices that contain co-cultures of human alveolar and microvascular cells in advanced 3-D models of the alveolar-capillary interface.(Huh et al., 2010, Long et al., 2012) Further, breathing movements can be reproduced with these 'lung on a chip' devices, allowing for an advanced in vitro model of the alveolo-capillary barrier.(Huh et al., 2010, Huh et al., 2011) Future work with a similar advanced in vitro model of the alveolo-capillary barrier would contribute especially to the work presented in Chapter 3, in the diesel exhaust study. Because treatment of diesel exhaust with a diesel particulate filter did not completely remove ultrafine particles, future studies with an alveolo-capillary barrier in vitro model could screen for deep airway and cardiovascular effects after exposure to filtered diesel exhaust. More broadly, lung on a chip devices offer an enticing ability to rapidly screen for the toxicity associated with ultrafine and nanoparticles, cosmetics, or alternatively, potential benefits of newly developed pharmaceutical therapeutics. Further, these devices could

be employed in the field to assess ‘real-world’ occupational or environmental exposures..

Although the three studies presented in Chapters 2, 3, and 4 use (1) diesel particulate matter (2) diesel exhaust and (3) dairy parlor dust as the test agents, similar direct air-to-cell in vitro exposure systems have a wide variety of applications in future work. Electrostatic or gravitational based air-to-cell exposure systems similar to the two described here could be used to screen for the cellular response after exposure to (1) airborne pathogens, like viruses or microorganisms (2) pharmacological aerosol therapeutics and (3) environmental or occupational gases and ultrafine particulate matter. Long term, these systems especially when combined with recent advancements in coculture models, have great potential to reduce the heavy reliance upon animal models in aerosol toxicology.

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APPENDIX – SUPPLEMENTARY INFORMATION AND FIGURES

RNA isolation and qPCR Analysis

Total mRNA transcripts from cell samples were isolated using a standardized kit and protocol (RNeasy mini kit, Qiagen, Valencia, CA). A DNase digestion step was added to minimize the risk of DNA contamination (RNase Free DNase Set, Qiagen, Valencia, CA). The purity and quantity of mRNA was assessed by spectrophotometry at wavelengths of 260 and 280 nm (Nanodrop ND-1000, ThermoScientific, Wilmington, DE). Transcripts were stored at -80ºC prior to analysis with real-time RT PCR. A starting quantity of 50 ng RNA was used to follow a standard real-time RT PCR protocol (Biorad One-Step RT-PCR Kit with SYBR Green, Bio-Rad Laboratories, Hercules, CA) to amplify five genes of interest: Interleukin-8 (IL-8), Cytochrome p450 1A1 (CYP1A1), Heme oxygenase-1 (HO-1), Cyclooxygenase-2 (COX-2), and Heat Shock Protein-70 (HSP70). The Livak ΔΔCt method was used to normalize copy numbers with gylceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. A water blank and a non-reverse transcriptase control were run to identify any DNA contamination. The following primer sets were synthesized by Integrated DNA Technologies (Coralville, IA) for the qPCR analysis [forward; reverse]: *IL-8* [TTGGCAGCCTTCCTGATTTC; TATGCACTGACATCTAAGTTCTTTAGCA]; *CYP1A1* [TCCCAGCTCAGCTCAGTACC; GCCGACATGGAGATTGGGAA]; *HO-1* [CAGCAACAAAGTGCAAGATTCTG; AGTGTAAGGACCCATCGGAGAAG]; *COX-2* [GAATCATTCACCAGGCAAATTG; TCTGTACTGCGGGTGGAACA]; *HSP-70* [CCATGGTGCTGACCAAGATGAAG; CACCAGCGTCAATGGAGAGAACC]; *GAPDH* [GAAGGTGAAGGTCGGAGTC; GAAGATGGTGATGGGATTTC].

Gas Chromatography-Mass Spectrometry Analysis of Filter Extractions

Because we desired to extract both the polar and nonpolar constituents from the PTFE filter, we performed PM_3 filter extractions in acetone. To ensure that our extraction process did not alter the chemistry of the PM constituents, we compared PM extracts from acetone dissolved filters, and extracts from filters that were dissolved in a 0.05% tween extraction. GC-MS analysis was performed by Greg Dooley at Colorado State University's Center for Environmental Medicine.

Endotoxin Standard Curves for PM_{10} and $PM_{>10}$

PM_{10} and $PM_{>10}$ particulate matter were serially diluted by mass into sterile, non-pyrogenic water and vortexed for fifteen minutes. Endotoxin content (EU per mL) for each serial dilution was then analyzed with a commercial rFC assay and standardized protocol (Pyrogene Recombinant Factor C Assay; Lonza Group Ltd., Walkersville, MD). Measured endotoxin levels (EU per mL) versus PM mass (mg per mL) were plotted for each size fraction and used as the standard curves for cell exposures.

RESULTS

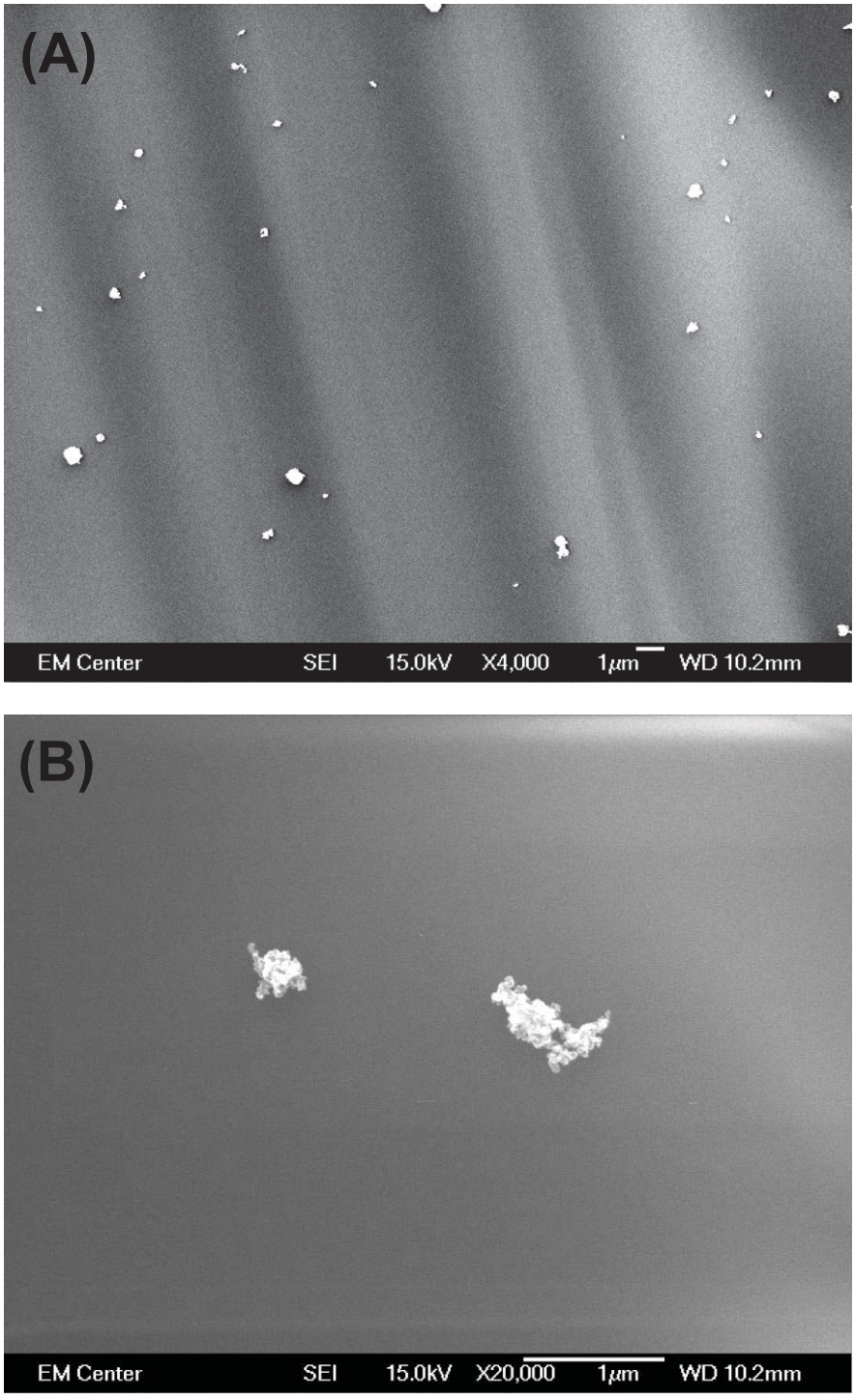

Figure A.1. Scanning electron microscopy images of diesel particulate matter collected onto TEM grids placed within cell wells in the EAVES. Image A is shown at 4000x magnification. Image B is shown at 20000x magnification.

Table A.1. PAH Content of Diesel Particulate Matter (NIST SRM 2975, Certificate of Analysis)

Polycyclic Aromatic Hydrocarbon	**Mass Fraction (mg/kg)**
Phenanthrene	17.0 ± 2.8
Fluoranthene	26.6 ± 5.1
Pyrene	0.90 ± 0.24
Benz[α]anthracene	0.317 ± 0.066
Chrysene	4.56 ± 0.16
Triphenylene	5.22 ± 0.20
Benzo[*j*]fluoranthene	0.82 ± 0.11
Benzo[*k*]fluoranthene	0.678 ± 0.076
Benzo[*e*]pyrene	1.11 ± 0.10
Benzo[*a*]pyrene	0.0522 ± 0.0053
Benzo[*ghi*]perylene	0.498 ± 0.044
1-Methylphenanthrene	0.89 ± 0.11
2-Methylphenanthrene	2.0 ± 0.2
3-Methylphenanthrene	1.0 ± 0.2
4- and 9-Methylphenanthrene	0.44 ± 0.09
1,2-Dimethylphenanthrene	0.05 ± 0.02
1,6-, 1,7-, 2,5-, and 2,9-Dimethylphenanthrene	0.57 ± 0.08
1,8-Dimethylphenanthrene	0.06 ± 0.02
2,6-Dimethylphenanthrene	0.25 ± 0.05
2,7-Dimethylphenanthrene	0.23 ± 0.05
3,6-Dimethylphenanthrene	0.18 ± 0.02
Anthracene	0.038 ± 0.008
Benzo[*ghi*]fluoranthene	10.2 ± 0.5
8-Methylfluoranthene	0.068 ± 0.004
1-, 3-, and 7-Methylfluoranthene	0.53 ± 0.03
2-Methylpyrene	0.040 ± 0.008
4-Methylpyrene	0.022 ± 0.005
Benzo[*c*]phenathrene	1.0 ± 0.4
Benzo[*a*]fluoranthene	0.06 ± 0.02
Benzo[*b*]fluoranthene	11.5 ± 3.6
Perylene	0.054 ± 0.009
Indeno[1,2,3-*cd*]pyrene	1.4 ± 0.2
Indeno[1,2,3-*cd*]fluoranthene	1.1 ± 0.2
Dibenz[*a,j*]anthracene	0.37 ± 0.07
Dibenz[*a,c*]anthracene/Dibenz[*a,h*]anthracene	0.52 ± 0.08
Pentaphene	0.038 ± 0.007
Benzo[*b*]chrysene	0.08 ± 0.03
Picene	1.0 ± 0.2
Coronene	1.1 ± 0.2

Gas Chromatography-Mass Spectrometry Analysis of Filter Extractions

The GC-MS analysis results can be seen in Figure A.2. No obvious differences were observed in the chemical profiles for either the acetone and 0.05% Tween PM extracts.

Endotoxin Standard Curves for PM_{10} and $PM_{>10}$

As can be seen in Figure A.3, the PM_{10} size fraction had approximately 15x greater amounts of endotoxin units per mg of dust than the $PM_{>10}$ size fraction. As reported in the methods section of Chapter 4, the standard curves seen in Figure A.3 were used to estimate the PM mass deposited during cell exposures.

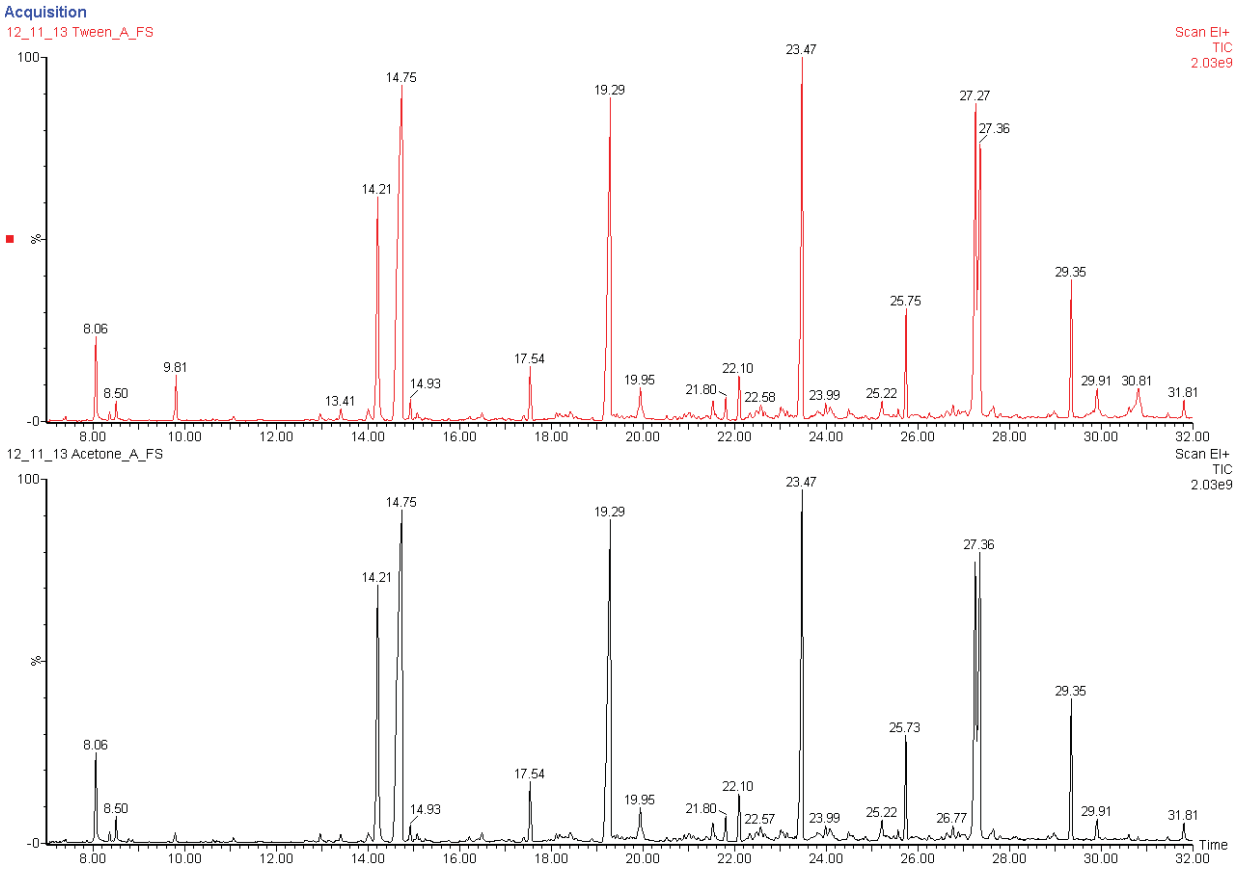

Figure A.2. Gas chromatograph-mass spectrometry results for PM_3 extracted into 0.05% Tween (top panel) or acetone (bottom panel)

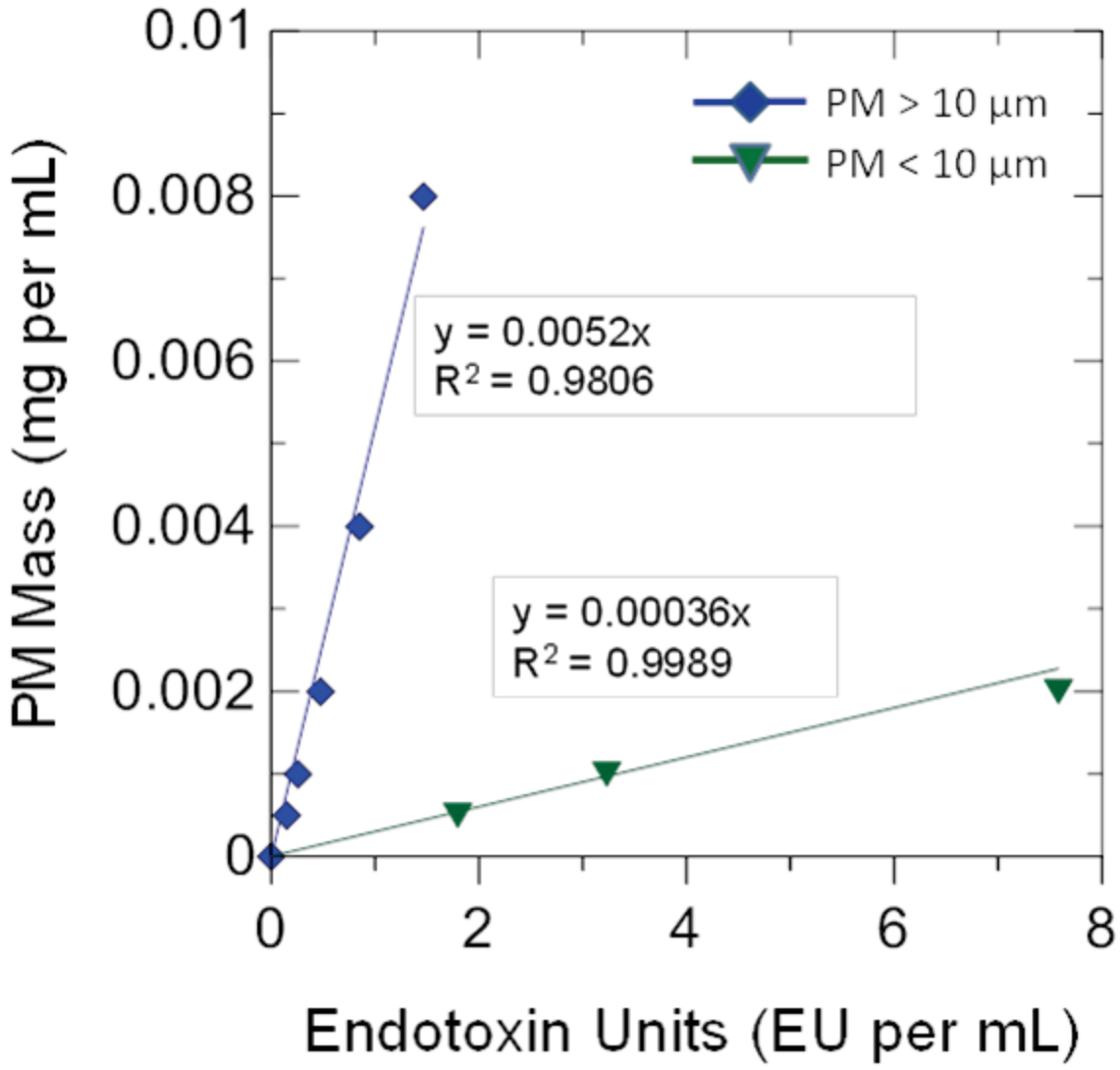

Figure A.3. Endotoxin Standard Curves for PM_{10} and $PM_{>10}$.

LIST OF ABREVIATIONS

3-D	Three dimensional
AECs	airway epithelial cells
AKR	aldo-keto reductase
ALI	air-liquid interface
ANOVA	analysis of variance
BEBM	Bronchial Epithelial Basal Medium
BEGM	Bronchial Epithelial Growth Medium
B99	Commercially available 100% biodiesel
cm	centimeter
cm^3	cubic centimeters
CMD	count median diameter
CO	carbon monoxide
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
CPC	condensation particle counter
CYP1A1	cytochrome p450 1A1
DMEM-H	Dulbecco's Modified Eagle Medium, high glucose
DPF	diesel particulate filter
DPM	diesel particulate matter
EAVES	electrostatic aerosol in vitro exposure system
EECL	Engines and Energy Conversion Laboratory
EPA	Environmental Protection Agency
EU	Endotoxin Unit
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
γ-GCL	glutamate cysteine-ligase
GCS	Gamma-glutamyl cysteine synthetase
GM-CSF	granulocyte-macrophage colony stimulating factor
GST	glutathione S-transferase
HAH	halogenated aromatic hydrocarbons
HC	total hydrocarbons
HEPA	high efficiency particulate air
HR	hour
HO-1	hemeoxygenase-1
HSP-70	heat-shock protein 70
IARC	International Agency for Research on Cancer
ICAM-1	intercellular adhesion molecule-1
ICRP	International Commission on Radiological Protection
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
JAK-STAT	Janus-kinase signaling pathway
kg	kilograms
kV	kilovolts

L	liters
LDH	lactate dehydrogenase
m	meters
m^3	cubic meters
mm	Millimeters
MMD	mass median diameter
MPPD	Multiple-path Particle Dosimetry Model
min	minutes
MIP-1	macrophage inflammatory protein 1
mRNA	messenger ribonucleic acid
MRP	multi-drug resistance protein
MT	metallothionein
NHBE	normal human bronchial epithelial
Nfkβ	Nuclear factor kappa beta pathway
NIOSH	National Institute for Occupational Safety and Health
ng	nanograms
nm	nanometers
nM	nanomolar
NO	nitrogen oxide
NO_x	nitrogen oxides
NO_2	nitrogen dioxide
NQO1	NAD(P)H dehydrogenase [quinone] 1
Nrf2	nuclear factor-like 2 pathway
OSHA	Occupational health and safety administration
p38 MAP	p38 mitogen-activated protein kinase pathway
PAH	polycyclic aromatic hydrocarbon
ppb	parts per billion
ppm	parts per million
RA	retinoic acid
RNA	ribonucleic acid
qPCR	real-time reverse transcriptase polymerase chain reaction
rFC	Recombinant Factor C
RH	relative humidity
ROS	reactive oxygen species
RT PCR	reverse transcriptase polymerase chain reaction
SMPS	sequential mobility particle sizer
TEM	Transmission electron microscopy
µg	Micrograms
UGT	UDP glucuronosyltransferases
µm	micrometers
ultrafine PM	particulate matter with a diameter smaller than 100 nm
WHO	World Health Organization