HARNESSING INTER-INDIVIDUAL VARIABILITY TO IDENTIFY MOLECULAR MECHANISMS SHAPING AIRWAY EPITHELIAL CELL TRANSCRIPTIONAL RESPONSES TO OZONE EXPOSURE

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

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ABSTRACT

Emma Ciel Bowers: Harnessing Inter-Individual Variability to Identify the Molecular Mechanisms Shaping Airway Epithelial Cell Transcriptional Responses to Ozone Exposure
(Under the direction of David Diaz-Sanchez)

Millions of people are exposed to levels of the ambient air pollutant ozone that are known to produce pulmonary inflammation; however, inflammatory responses exhibit extensive inter-individual variability. Moreover, during multi-day exposures acute inflammatory responses are attenuated, resulting in a phenomenon known as "ozone adaptation." The mechanisms governing these phenomena are not understood, but their identification is essential in understanding the health impacts of air pollutant exposure. The ozone-mediated induction of pro-inflammatory genes is a key step in the release of cytokines and chemokines in the airway. Thus, differences in the regulation of transcription may be a potential source of ozone inflammatory response inter-individual variability and may explain ozone adaptation. To model ozone associated transcriptional inter-individual variability, primary human bronchial epithelial cells (phBECs) were collected from different individuals, cultured at air-liquid-interface, and exposed to ozone *in vitro*. I then examined the expression of the chemokine *IL-8*, a central mediator of pulmonary inflammation, in addition to other ozone-responsive genes. I found that ozone inductions exhibited inter-individual variability and were reproducible within donors even when phBECs were collected, cultured, and exposed at different times. This suggests that ozone-responsive gene induction adheres to a set of biological rules that remain to be defined. Recent findings suggest that the MAPKs p38 and ERK1/2 mediate ozone response in phBECs,

thus I investigated whether these kinases also controlled gene induction inter-individual variability. I found that phBECs with higher inductions of *IL-8* are distinguished by elevated activation of ERK1/2, but not p38, following ozone exposure. Upon repeated ozone exposure, ozone-responsive gene expression was suppressed and was paralleled by decreases in ERK1/2 activation, suggesting that this may be an important adaptive mechanism. In collaboration with other scientists, I also found that epigenetic modifications at pro-inflammatory gene promoters were strongly associated with ozone-associated gene expression, suggesting that the epigenome is critical part of epithelial cell response 'programming.' In summary, this work identifies novel molecular mechanisms that dictate responsiveness to ozone exposure. This information can be used to refine definitions of susceptible populations and better predict health outcomes associated with air pollutant exposures.

I dedicate this work to my parents,

Drs. Michael Bowers and Christine Flanagan, who raised me as their favorite

‘experiment.’

ACKNOWLEDGMENTS

This work would not have been possible without the exceptional guidance and mentorship of David Diaz-Sanchez and Shaun McCullough. David's experience and knowledge of the field were essential in helping me choose my battles and strategically plan my research. I would often arrive in David's office gushing ideas about experiments, but would leave with one resonating word: "FOCUS." David's insight and criticism were essential in preparation for committee meetings, presentations, publications, etc. David considers himself to be a harsh critic, but I tend to disagree. I think it's because when someone is ripping your work apart in an English accent, it distracts you from having your feelings hurt. Importantly, whenever I felt overwhelmed, downtrodden, or panicked, David's ability to put life (and science) into perspective always made me feel better.

I became Shaun McCullough's 'ward' when he was transitioning between a post-doctoral position and a principle investigator. Initially Shaun's role was to teach me laboratory techniques and chromatin biology; however, his role thereafter became much more of a friend and mentor. Shaun's attention to detail, technical knowledge, enthusiasm for science, and emphasis on professionalism were essential in my development as a scientist. Many of my accomplishments have been the result of his incessant goading and encouragement. I will never forget the hours we spent side-by-side on the computer honing my scientific writing skills. Doctoral students should be so lucky to have somebody like that in their corner.

One aspect that Shaun emphasized was teamwork, which I whole-heartedly subscribed to. For quite a few years our lab 'team' consisted of David Morgan, Doan On, and Lisa Dailey.

Doan and David were great friends, but also great technicians. Together we harvested cells, optimized ChIP protocols, performed clinical studies, and ran a lot of qPCR (I mean *a lot*). Doan even forgave me when I messed up our three-day protocol when I got too distracted by juicy gossip (it was the third day). Lisa Dailey, also part of our team, was essential in curating primary cells and growing phBEC cultures. Given the hundreds (likely *thousands)* of Transwells that I have used in my research and the months of care they required, I wouldn't have accomplished nearly as much if I had to manage it all on my own. Hannah Smith, a newer addition to our team, is a promising undergraduate from the UNC School of public health. Hannah performed several of the analyses included in this dissertation, which speaks to her impressive capabilities as a young researcher.

I would be remiss if I didn't thank the rest of the EPA family who helped me throughout my graduate work. Scott Meade and the rest of the TRC Engineering team built and maintain the exposure chambers that were critical to my research. I am also grateful to the nurses Tracey, Mary Anne, and Julie for their assistance, sense of humor, and for allowing me to be a co-investigator in clinical studies. I would also like to acknowledge the heroic efforts of Ilona Jaspers and Julie Cannefax. These ladies have gone out of their way to help me many times, especially with the STAR fellowship that I had to turn down, and I can't imagine what the program would be like without them (seriously, it would be scary).

I would like to think my mother and father for being great parents, being unapologetically eccentric, and thinking the world of me (somebody's got to). I would also like to think my husband, Aaron Wesley, for being a wonderful partner and super good looking. I would also like to thank my dogs Hope and Turk for providing unconditional love and making me exercise-something I don't think any other creature is capable of. My cat Bee-Bee is okay, too.

I would also thank the funding sources that have made my studies possible. My research was funded by the Toxicology T32 training grant T32-ES007126 and the Center for Environmental Medicine and Asthma Lung Biology (CEMALB).

PREFACE

Although I was the lead in this dissertation, much of this work was done in collaboration with other scientists. I would like to take this opportunity to acknowledge these individuals and describe their role in this research. Thought the dissertation, David Diaz-Sanchez and Shaun McCullough helped me design and plan experiments, hence they are the senior and second authors, respectively, on my publications. Other individuals also greatly contributed to this research. Lisa Dailey, our primary cell technician, was instrumental in curating phBEC donor libraries and preparing cell cultures. David Morgan and Doan On, both technicians in the McCullough lab, were helpful throughout my dissertation research. Doan, David, and Lisa assisted with sample collection during large harvests. David also helped process some of the RNA extractions and PCR reactions from Chapters 1 and 2. Hannah Smith, a bright undergraduate in our lab, was able to use my banked samples to perform several of her own experiments, which include the baseline transcript analysis described in Figure 2-9, the oxidative stress PCR panel featured in Figure 3-5, and the GSTM1 analysis in Figure 5-5.

Large portions of Chapters 2 and 3 have been or are currently in preparation for publication submission:

> Bowers EC, McCullough SD, Morgan DS, Dailey LA, Diaz-Sanchez D. (Under review 2017) Ozone-mediated IL-8 responsiveness is an intrinsic property of airway epithelial cells and is determined by the activation of the MAP kinase ERK1/2.
>
> Bowers EC, McCullough SD, Morgan DS, Smith HJ, Dailey LA, Diaz-Sanchez D. (June 2017). Using primary epithelial cells to model ozone adaptation: investigating the role of antioxidant capacity and MAP kinase signaling.

Chapter 4 represents unpublished data that is the result of a collaborative effort between myself and the aforementioned individuals. Doan On, David Morgan, and Shaun McCullough banked the material that was used in this chapter. Under the supervision of Shaun McCullough, I designed the ChIP experiments and performed the data analysis. To increase the throughput of our sample processing, Doan On and I performed the ChIP reactions in tandem. In order to generate this data our lab spent a significant amount of time deconstructing and optimizing our ChIP protocol. Our efforts resulted in a streamlined, efficient ChIP protocol that we were then invited to publish, which can be found at:

> McCullough, S.D., On, D.M., Bowers, E.C. Using Chromatin Immunoprecipitation in Toxicology: A Step-by-Step Guide to Increasing Efficiency, Reducing Variability, and Expanding Applications. Current Protocols in Toxicology, 3-14.

Several of the figures and discussion points in Chapters 1 and 4 were previously published in a review that I coauthored with Shaun McCullough:

> Bowers, E. C., & McCullough, S. D. (2017). Linking the Epigenome with Exposure Effects and Susceptibility: The Epigenetic Seed and Soil Model. Toxicological Sciences, 155 (2): 302-314. Used with permission.

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LIST OF ABBREVIATIONS

BALF	Bronchoalveolar lavage fluid
CGRP	Calcitonin gene-related peptide
ChIP-qPCR	Chromatin Immunoprecipitation- quantitative polymerase chain reaction
EGFR	Epidermal Growth Factor Receptor
ERK1/2	Extracellular Related Kinase 1/2
FA	Filtered Air
GSTM1	Glutathione S-transferase Mu 1
GSTP1	Glutathione S-Transferase Pi 1
H3K27me3	Histone H3 lysine 27 trimethyl
H3K4me3	Histone H3 lysine 4 trimethyl
H4Ac	Pan-acetylated histone H4
HMOX-1	Heme oxygenase 1
IL-6	Interleukin 6
IL-8	Interleukin 8
LPS	Lipopolysaccharide
MAPK	Mitogen Associated Protein Kinase
MEK	MAPK/ERK Kinase
NAAQS	National Ambient Air Quality Standards
NOx	Nitrogen oxides
NQO1	NAD(P)H Quinone Dehydrogenase 1
O_3	Ozone
ORAC	Oxygen radical antioxidant capacity assay

PTGS2/COX-2	Prostaglandin-endoperoxide synthase 2
ROS	Reactive oxygen species
RSK	Ribosomal S6 Kinase
TEER	Transepithelial Electrical Resistance
TNF	Tumor necrosis factor
TRPA1	Transient receptor potential cation channel, subfamily A, member 1
VOCs	Volatile organic compounds

CHAPTER 1: INTRODUCTION

Ozone: a major public health issue

Air pollutant exposure causes over 12 million deaths each year, and is a major risk factor in cardiovascular and pulmonary disease morbidity and mortality. As a result, air pollutant exposure is currently considered to be the largest single environmental health risk (WHO 2014). A major air pollutant of concern is ground level ozone. Ozone exposure is associated with thousands of pre-mature deaths from respiratory causes and may play a role in cardiovascular mortality (US EPA 2013). In addition, ozone exposure results in the exacerbation of pulmonary diseases (Figure 1-1), which result in increased hospitalizations, emergency room visits, lost school days, and lost productivity, all of which are estimated to cost billions of dollars (US EPA 2011). One of the key factors precipitating these adverse events is ozone-associated pulmonary inflammation (US EPA 2013). Currently a major problem in the environmental health field is understanding how ozone exposure causes inflammation and identifying individuals who are especially susceptible to exposure effects. This dissertation addresses these knowledge gaps by investigating the molecular mechanisms underlying the ozone-mediated pro-inflammatory response.

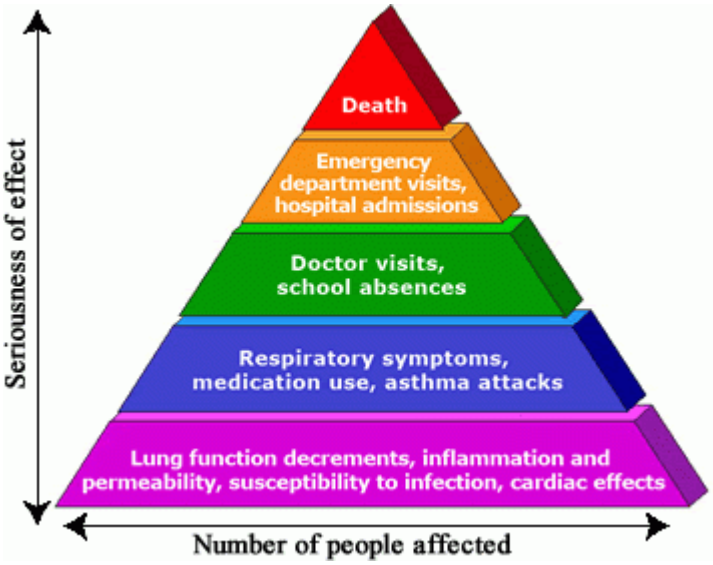

Figure 1-1. Health effects and repercussions of ozone exposure. This material was generated by the U.S. EPA and is not copyrighted. Available at https://www.epa.gov/ozone-pollution-and-your-patients-health/health-effects-ozone-general-population.

Ozone formation and pollution trends

Ozone is a ubiquitous oxidant pollutant that is often used to study the inflammatory response caused by air pollutant exposure. Unlike many other ambient air pollutants, ozone is not directly emitted, and is instead formed during secondary reactions between sunlight, oxygen, and other air pollutants such as volatile organic compounds (VOCs) and nitrogen oxides (NOx). Because sunlight and heat catalyze ozone formation, ozone concentrations often parallel sunlight intensity, peaking in the afternoon and dissipating in the evening. Ozone formation also exhibits seasonal variation, where high concentrations may occur more frequently during warm, sunny seasons such as summer and early fall. In addition to the previously described factors, other components such as topography, humidity, and wind patterns can play an important role in determining ambient ozone levels (US EPA 2013).

Ozone is currently a criteria air pollutant regulated by the National Ambient Air Quality Standards (NAAQS), which consider ozone exposures exceeding an average of 70 ppb/8 hours to

be unhealthy. The number of high ozone days in the past 35 years has steadily declined (US EPA 2016), likely due to clean technologies and regulations targeting precursor pollutants. Despite this reduction, ozone pollution is still a major public health concern in many areas. This is exemplified in Figure 1-2, which indicates the number of days in which ozone concentrations reached unhealthy levels in major United States cities. While nearly every area experiences occasional high ozone levels, some locations are far more likely to have recurring high ozone days than others. Urban areas typically have abundant precursor air pollutants and are therefore more conducive to ozone formation. Urban areas with warm, sunny, dry climate typically have the highest number of elevated ozone days, while areas that have cloudy and cool climate often have the least. The tendency of ozone to form in highly populated areas also increases the number of individuals who are exposed. It is estimated that in the United States 108 million people live in a county in which ozone exceeds air quality standards (US EPA 2017). Despite the recent decline in ozone concentrations, increasing temperatures and changing weather patterns associated with climate change may lead to increases in ozone formation (Ebi and McGregor 2008).

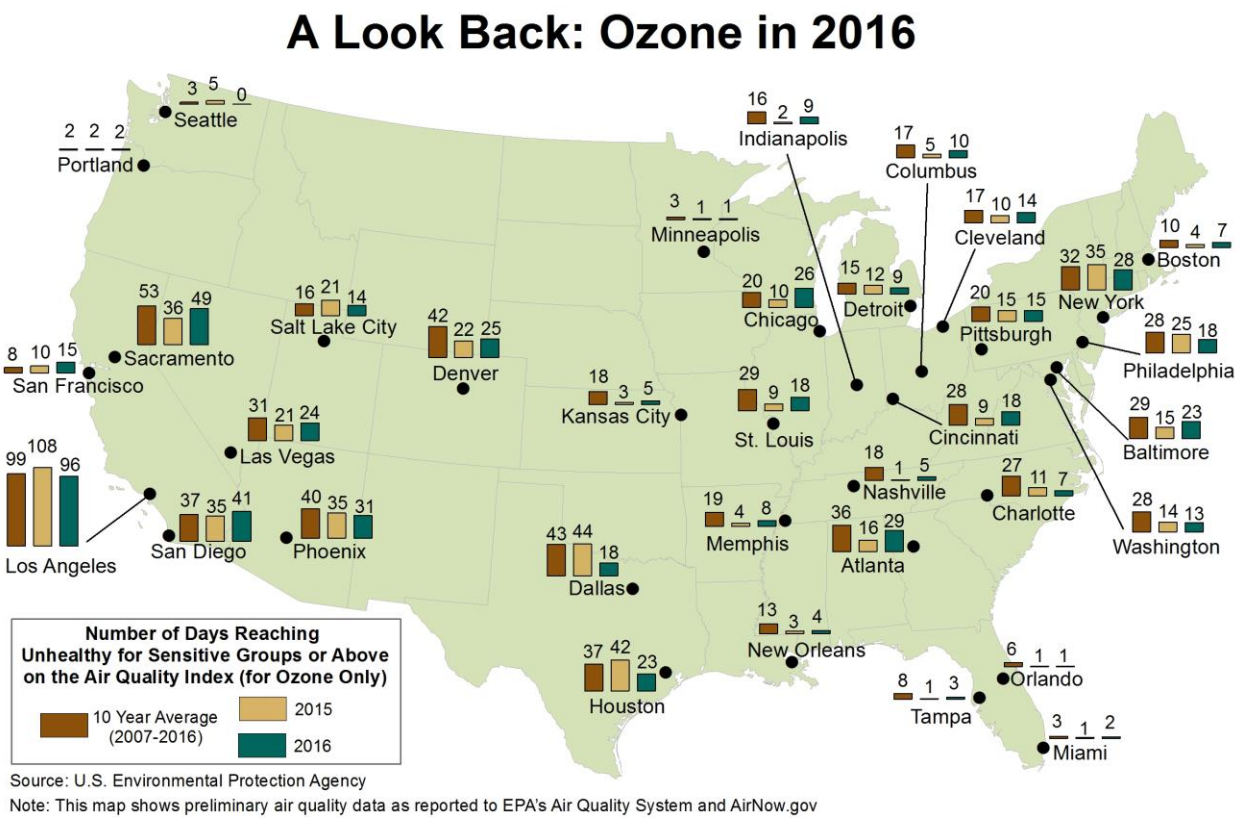

Figure 1-2. Number of days reaching unhealthy ozone levels in major U.S cities. Figure indicates the number of days exceeding the ozone NAAQS standard in major U.S. cities. This figure was published by the US EPA (2016) and is not copyrighted. Available at https://www3.epa.gov/airnow/airaware/trends.html.

Health effects of ozone inhalation

While ozone can interact with all surfaces of the airway, the tissues receiving the highest doses in humans are thought to be the terminal bronchioles and the centriacinar region, which is located between the tracheobronchial and gas exchange regions (Plopper *et al.,* 1998). Ozone interacts with extracellular lining fluid as well as the phospholipids comprising cell membranes, forming secondary oxidation products such as aldehydes, H_2O_2, and lipid ozonation products (Pryor *et al.,* 1995). Oxidative stress caused by ozone exposure can also lead to the production of pro-inflammatory eicosanoids via enzymatic and non-enzymatic processes (Kafoury *et al.,* 1998; US EPA 2013).

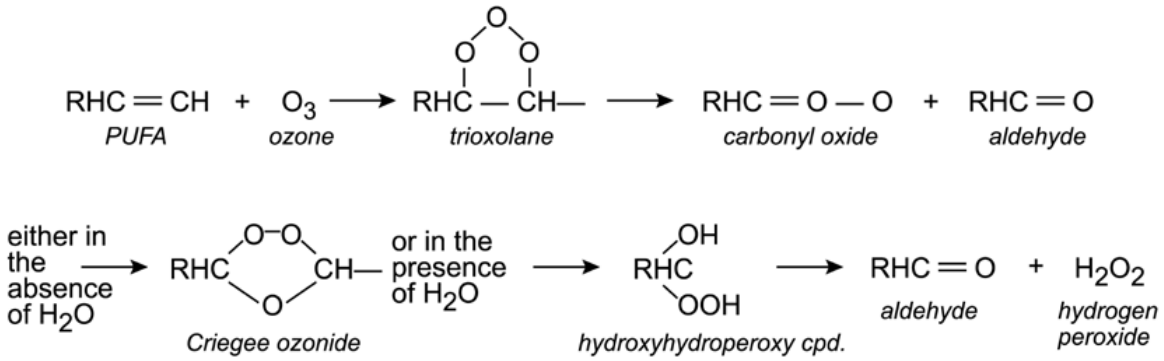

Figure 1-3. Reaction of ozone with organic molecules in the airway lining fluid and cell membranes. Ozone is thought to react with double bonds in polyunsaturated fatty acids (PUFA), producing lipid oxidation products, aldehydes, and hydrogen peroxide. These oxidation products mediate many of the health effects associated with ozone exposure. This image previously appeared in US EPA 2013 Integrated Science Assessment for Ozone and Related Photochemical Oxidants. This material is not copyrighted and can be found at https://cfpub.epa.gov/ncea/isa/recordisplay.cfm?deid=247492.

These ozonation products and pro-inflammatory eicosanoids are responsible for many of the adverse health effects associated with ozone exposure. Ozonation products activate transient receptor potential ankyrin 1 (TRPA1) receptors on vagal pulmonary C-fibers innervating the lungs, causing the release of various neurotransmitters including substance P, neurokinin A, and calcitonin gene-related peptide (CGRP) (US EPA 2013). The release of these neurotransmitters sensitizes the airway, leading to painful and truncated inspiration and restrictive decrements in lung function. Ozone-mediated lung function decrements are mostly restrictive in nature; however, the release of some neurotransmitters such as tachykinins and substance P may also cause bronchoconstriction and create obstructive lung function decrements (Verhein *et al.,* 2011). Vagal nerve stimulation is also responsible for several autonomic changes in the cardiopulmonary system, including changes in breathing rate and altered cardiac electrophysiology, which could contribute to cardiovascular morbidity and mortality (Farraj *et al.,* 2012).

In addition to lung function decrements ozone exposure also causes pulmonary inflammation, predominantly characterized by airway neutrophilia. The ozone inflammatory response is mediated by the release of pro-inflammatory mediators into the airway. These include pro-inflammatory eicosanoids as well as a variety of cytokines and chemokines, such as IL-8, IL-6, IL-1, and TNFα. The pathways and receptors that are associated with the release of these pro-inflammatory mediators are still being elucidated, and are discussed in subsequent sections. Cytokines and markers of vascular inflammation are also elevated in the blood following ozone exposure (Devlin *et al.,* 2012, Thompson *et al.,* 2010). Although the source of these circulating cytokines is unclear, this may be an important finding in determining whether there is a relationship between ozone exposure and cardiovascular mortality.

Airway neutrophil influx typically peaks six hours after exposure, and then declines 18-24 hours post exposure (Schelegle *et al.,* 1991; US EPA 2013). Infiltrating neutrophils are thought to play an important role in the removal of necrotic debris from the airway (Hu *et al.,* 1982); however, they may also cause further epithelial injury (Balmes *et al.,* 1996; Vesely *et al.,* 1999) via the release of bactericidal ROS. In addition to neutrophils, ozone exposure is also associated with the chemotaxis of other leukocytes into the airway, including lymphocytes, macrophages, and mast cells, which comprise a secondary component of the ozone inflammatory response (US EPA 2013). While changes in lung function are reversible, repeated episodes of inflammation may lead to tissue injury, permanent lung damage, airway remodeling, and metaplasia (Harkema *et al.,* 1999). Given these findings, individuals who are more susceptible to ozone-associated inflammation may be at a greater risk of long-term airway damage.

The connection between ozone-mediated lung function decrements and the inflammatory response is unclear. Currently it is thought that these responses arise from different mechanisms,

as they occur within different time frames and don't necessarily occur in the same individuals (Balmes *et al.,* 1996; Blomberg *et al.,* 1999). Regardless of their origin, their combined effect has a substantial impact on public health. Increases in ambient ozone levels are correlated hospitalizations and emergency department visits due to respiratory complications (Burnett *et al.,* 1997; Moore *et al.,* 2008), and are also associated with increased mortality (Jerrett *et al.,* 2009).

The health effects of ozone exposure must also be considered in the context of realistic exposure scenarios, which often involve multiple pollutants and can result in additive or synergistic effects. Previous ozone exposure sensitizes individuals to the effects of subsequent allergen exposure (Jorres *et al.,* 1996; Schelegle *et al.,* 2003), and has an additive effect with a variety of other air pollutants such as diesel exhaust, particulate matter, and NO_2 (Ehrlich et al., 1977; Kafoury and Kelley 2005; Madden *et al.,* 2014).

Ozone adaptation

Predicting the health effects of ozone exposure is complicated by the fact that outcomes are highly dependent on exposure history. While a single exposure is associated with lung function decrements and pulmonary inflammation, these effects are greatly reduced or even abolished during repeated exposures, an effect termed "ozone adaptation." Ozone adaptation can be divided into two distinct components: lung function adaptation and inflammatory adaptation.

Lung function adaptation was first documented in human clinical exposure studies where it was observed that subjects who were long-time residents of the Los Angeles area (which experiences frequent high ozone days) were less responsive to controlled clinical ozone exposure than those who were from areas with better air quality (Hackney *et al.,* 1976, 1977). Subsequent

studies monitoring the effects of repeated daily ozone exposures found that lung function decrements occur following one to two exposures, but in subsequent exposures these declines are abated or even disappear (Folinsbee *et al.,* 1980, 1994). Lung function adaptation lasts approximately one week, but its persistence varies between individuals (Linn *et al.,* 1982).

Inflammatory ozone adaptation has been observed in both animal and human clinical ozone exposure studies. In such studies the acute inflammatory response typically peaks after one to two exposure days, but after four days of repeated exposures the level of airway cytokines, such as IL-8 and IL-6, are reduced as are airway leukocytes (Christian *et al.,* 1998; Devlin *et al.,* 1996; Jörres *et al.,* 2000; Schelegle *et al.,* 2003; Van der Wal *et al.,* 1994). Unlike lung function adaptation, the persistence of inflammatory adaptation is more complex. Some inflammatory markers return to baseline levels within a week of exposure, while other markers remain altered for over 20 days (Devlin *et al.,* 1996).

The mechanisms responsible for lung function and inflammatory adaptation are unknown. A long-standing hypothesis is that in antioxidant capacity may be an important mechanism. While some studies support this hypothesis (Rahman *et al.,* 1991; Wiester *et al.,* 1996, 2000), findings from other studies are contradictory or are inconclusive (Kodavanti *et al.,* 1995; Nambu and Yokonama 1981; Tepper *et al.,* 1989). Another important but undefined aspect of ozone adaptation is its impact on public health. While some have dismissed the importance of lung function adaptation because it is very short-lived (Linn *et al.,* 1982), the health effects of inflammatory adaptation have not been addressed. A recent epidemiological study found that ozone-associated mortality exhibits an adaptive effect, where mortality rates are highest at the beginning of the high ozone season and decrease throughout the year, reaching a null effect by winter (Zanobetti and Schwartz 2008). While this pattern could be the result of a

'culling' effect, the role of adaptation in ozone-associated mortality needs to be further investigated.

Inter- and intra-individual variability in the ozone inflammatory response

Human clinical ozone exposure studies have demonstrated that ozone exposure results in an average increase in neutrophil (PMN) influx into the lungs; however, there is substantial inter-individual variability in this response (Figure 1-4). Some individuals exhibit no change in neutrophil infiltration, or even a reduction, while others have substantial increases.

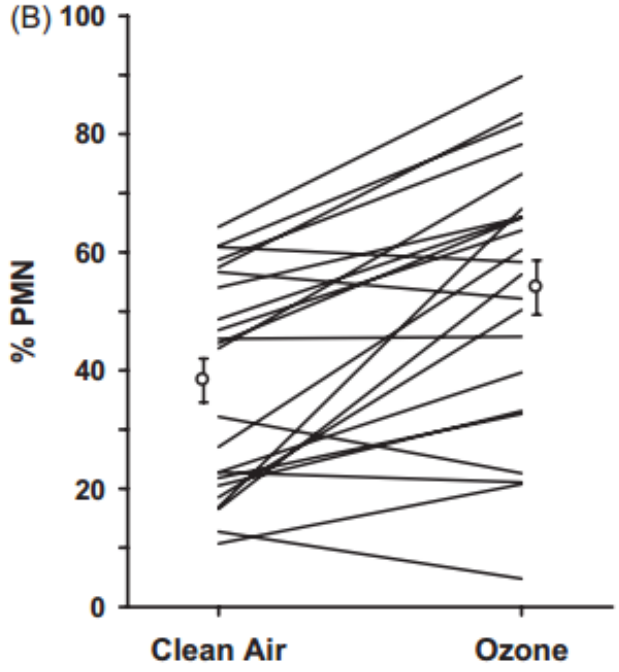

Figure 1-4. Inter-individual variability in the ozone inflammatory response. Twenty-four human subjects were exposed to 0.06 ppm ozone or clean air for six hours. Leukocyte infiltration was assessed 16-18 hours post exposure via bronchoalveolar lavage. The percent neutrophils present in the lavage fluid is indicated ("% PMN"). Mean and SEM are shown by bars while individual responses are indicated by lines. Reprinted with permission of the American Thoracic Society. Copyright © 2017 American Thoracic Society. Kim CS, Alexis NE, Rappold AG, Kehrl H, Hazucha MJ, Lay JC, *et al.,* 2011. Lung function and inflammatory responses in healthy young adults exposed to 0.06 ppm ozone for 6.6 hours. Am J Respir Crit

Care Med 183:1215-1221. The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society.

Risk factors such as genotype, age, and disease state (Alexis *et al.,* 2009; Scannell *et al.,* 1996; Vancza *et al.,* 2009), appear to be associated with increased inflammatory responses; however, they have poor predictive value and the mechanisms by which they exert their effects are unknown. Moreover, inflammatory responses in healthy individuals who don't exhibit any risk factors can exceed those in traditional risk groups (Holz *et al.,* 1999). While ozone responses are extremely variable between individuals, responses within an individual are highly reproducible (Figure 1-5), which suggests that the ozone inflammatory response adheres to a set of biological rules that remain to be discovered.

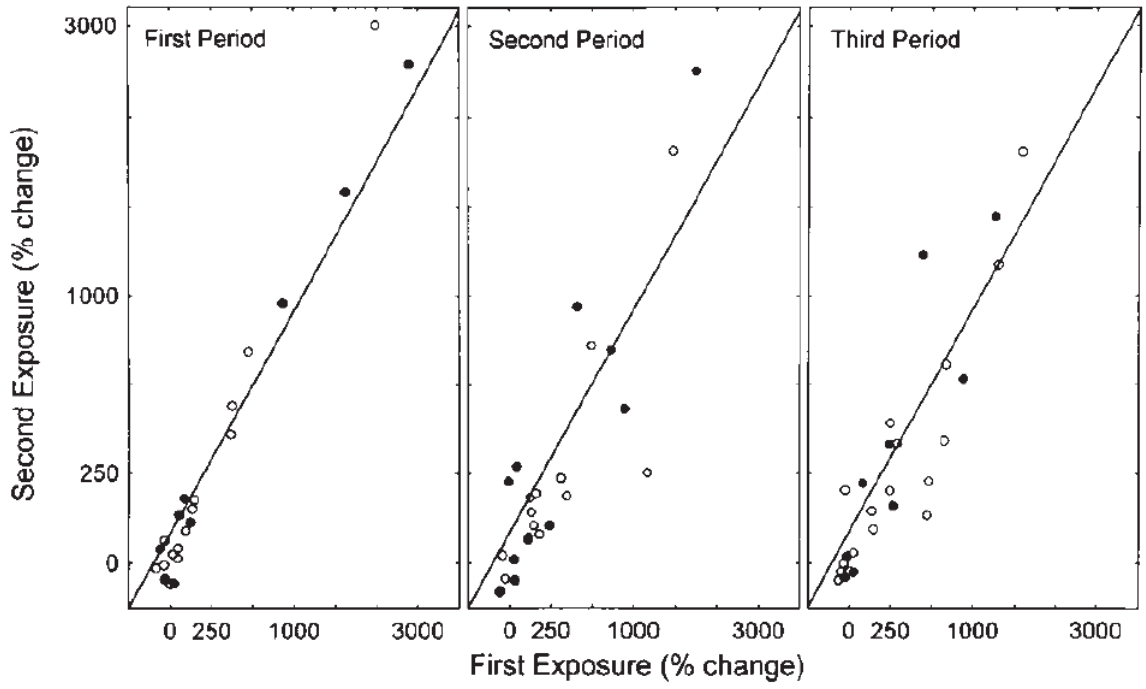

Figure 1-5. Ozone responses within individuals are highly reproducible. Human subjects were exposed to 0.25 ppm ozone/3h at two different times. Induced sputum was collected before exposure and three times after exposure. The percent change before and after exposure were calculated from each collection and percent changes from the first exposure (*x*-axis) were compared to changes from the second exposure (*y*-axis). Healthy subjects are indicated by open circles while asthmatics are indicated by closed circles. Reprinted with permission of the

IL-8 is a hallmark of the ozone inflammatory response

Inflammatory responses require the coordinate activity of many different chemical messengers and cell types. A critical mediator in this process is IL-8, a potent neutrophil chemokine that is produced in the airway in response to pollutants and other foreign substances. Like the ozone-associated neutrophil response, IL-8 levels are increased in the airway following ozone exposure and exhibit substantial inter-individual variability (Figure 1-6); however, these levels are also reproducible with individuals (Krishna *et al.,* 1998). Elevated IL-8 is linked to increased ozone-associated inflammatory response. Fry *et al.,* (2012) found that "ozone responders" with especially high neutrophil influx following ozone exposure also had elevated IL-8 in their sputum, while individuals with minimal neutrophil influx response did not. Furthermore, removal of IL-8 from the conditioned media of ozone-exposed epithelial cells abolishes neutrophil chemotaxis (Chang *et al.,* 1998). The importance of IL-8 in neutrophil recruitment suggests that the factors controlling IL-8 abundance may help explain inter-individual variability in the ozone inflammatory response.

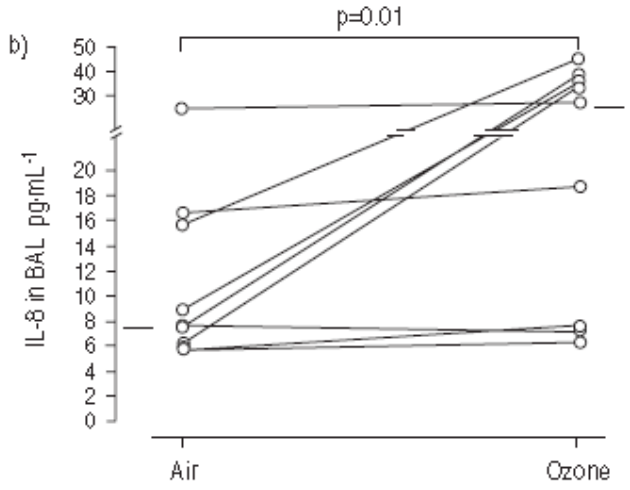

Figure 1-6. Inter-individual variability in airway IL-8 levels following ozone exposure. Twelve healthy humans were exposed to 0.2 ppm ozone and filtered air. Bronchoalveolar lavage fluid was collected six hours post exposure and IL-8 concentrations were assessed. Reproduced with permission of the European Respiratory Society ©. European Respiratory Journal Jun 1998, 11 (6) 1294-1300.

Although multiple cell types produce IL-8, airway epithelial cells may be the most important source of this chemokine during ozone exposure (Devlin *et al.,* 1994; Jaspers et al 1997; Chang *et al.,* 1998). The induction of the *IL-8* gene in response to ozone exposure is a key step in the release of IL-8 protein and may be an important source of inter-individual variability. While previous studies in epithelial cell lines have attributed ozone-mediated *IL-8* induction to NFκB, in physiologically-relevant primary human bronchial epithelial cells, the activation of the mitogen activated protein kinase (MAPK) pathway appears to be the most important (McCullough *et al.,* 2014). The activation of two MAPKs, extracellular related kinase (ERK) 1/2 and p38, appear to be particularly important in the ozone-mediated induction of pro-inflammatory genes such as *IL-8*, *IL-1B*, and *IL-1a*. McCullough *et al.,* (2014) demonstrated in polarized phBECs that inhibiting the activity of these kinases significantly reduces the ozone-associated induction of these genes. Ozone-associated MAPK activation has also been corroborated in other *in vitro* and *in vivo* studies (Bauer *et al.,* 2011, Williams *et al.,* 2008).

Molecular events linking ozone exposure to pro-inflammatory gene expression

There are several hypotheses regarding how ozone exposure leads to pro-inflammatory gene expression via the MAPK pathway. Ozone interacts with surface liquids and phospholipid membranes leading to the production of reactive oxygen species (ROS) such as hydroxyl radicals, aldehydes, and hydrogen peroxide (Pryor *et al.,* 1995). These ROS are thought to play a role in the activation of membrane proteins, specifically the epidermal growth factor receptor (EGFR; McCullough *et al.,* 2014; Wu *et al.,* 2015), which signals downstream to ERK1/2. ROS such as H_2O_2 are known to directly modify EGFR and increase its kinase activity (Paulsen *et al.,* 2012). Others have hypothesized that ozone-associated ROS may change lipid membrane raft formation (Park *et al.,* 2009), thereby encouraging EGFR receptor dimerization and autophosphorylation. ROS are also known to oxidize specific residues within phosphatases, thereby inactivating them and removing the 'brake' from MAPK pathway activation (Bonini *et al.,* 2014; Tal *et al.,* 2006; Yan *et al.,* 2016). Finally, ozone exposure is also thought to increase the production of heat shock proteins, which directly activate ERK1/2 (Bauer *et al.,* 2011). Less is known about the molecular events that lead to ozone-mediated p38 activation, but it is possible that the ROS-mediated deactivation of phosphatases is involved. Moreover, the activation of toll-like-receptor 4 (TLR4) may also play an important role (Bauer *et al.,* 2011; Williams *et al.,* 2007).

The epigenome: a potential source inter-individual variability in gene induction

While cellular signaling is important in determining the pro-inflammatory signals that enter the nucleus, inside the nucleus the epigenome at pro-inflammatory genes is another

important regulator of gene induction and a potential source of inter-individual variability. Unlike the DNA sequence, the epigenome is malleable- shaped from birth by environmental factors such as diet, stress, and chemical exposures, among others (Bowers and McCullough 2017; Bredfeldt *et al.*, 2010; Dolinoy *et al.*, 2010; Fiel and Fraga 2012). This duality - the ability to shape expression and be shaped by external forces- grants the epigenome predictive and/or explanatory power that other fixed predictors (i.e., genotype, sex, age) may not possess.

The epigenome consists of DNA methylation and chromatin modifications. DNA is packaged into chromatin by coiling around histone proteins, the tails of which are subject to a variety of covalent modifications. These modifications work in concert to regulate gene expression by mediating changes in chromatin architecture and thus governing the accessibility of DNA to transcription factors (Figure 1-7).

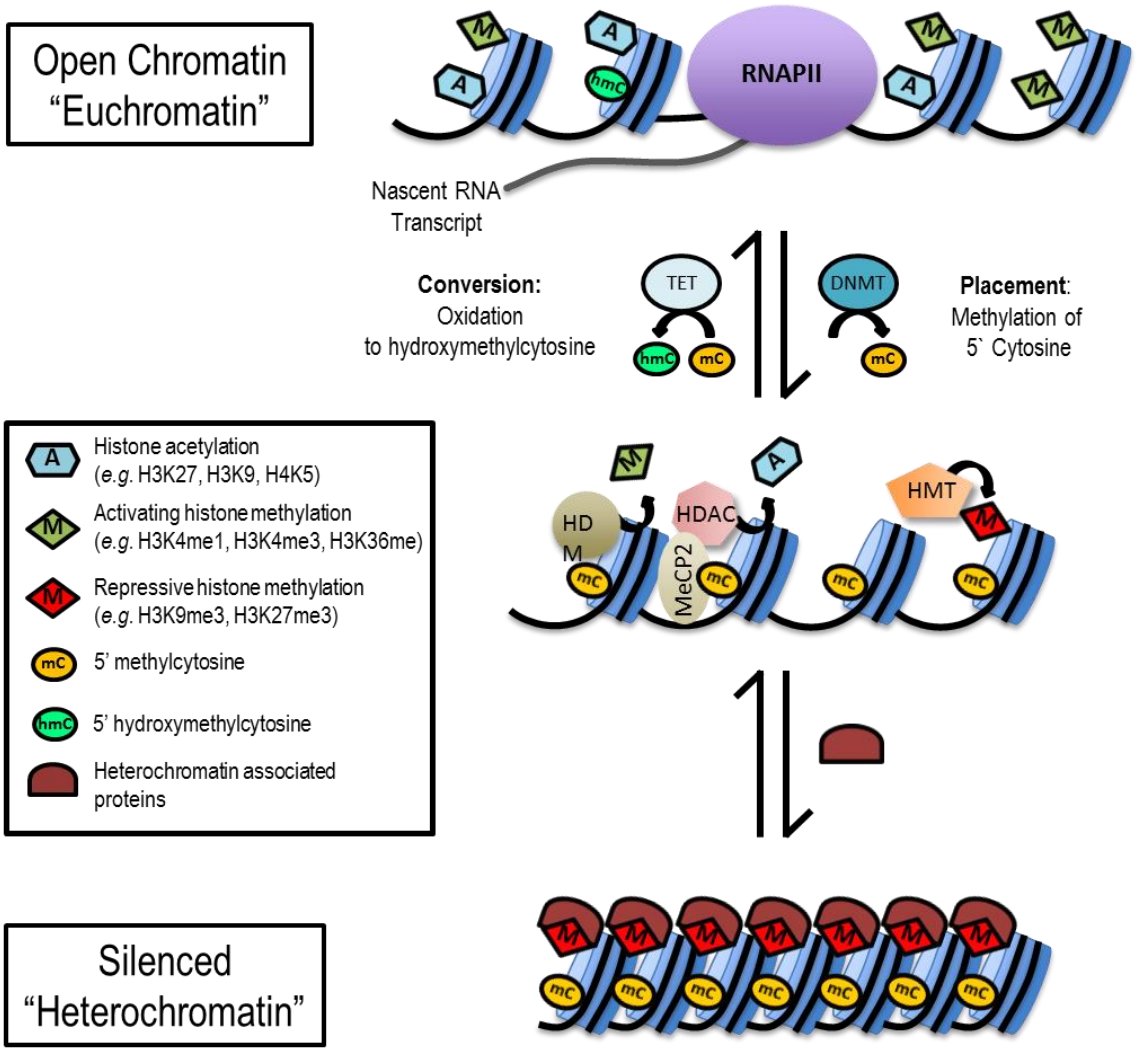

Figure 1-7. Histone modifications and DNA methylation: The epigenetic code that determines chromatin state.[1] Histone modifications and DNA methylation function cooperatively to regulate chromatin structure, accessibility to transcription factors, and gene expression. DNA methylation is the addition of a methyl group by a DNA methyltransferase (DNMT) to the cytosine residue of CpG dinucleotides in DNA. Methylation of DNA in gene regulatory regions (promoters and enhancers) often results in transcriptional repression; however, the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine by the ten-eleven translocation (TET) family of methylcytosine dioxygenases is associated with the activation of gene expression. The genome is packaged on a protein scaffolding composed of histone proteins arranged into repeating units known as nucleosomes. The unstructured tails of these histones extend outside of the core nucleosome and are subject to numerous modifications such as acetylation, methylation, phosphorylation, ubiquitination, et cetera. These modifications can be activating (e.g. H3K4me3 and acetylation) or repressive/silencing (e.g. H3K9me3 and

[1] This figure and caption previously appeared in Bowers, E. C., & McCullough, S. D. (2017). Linking the Epigenome with Exposure Effects and Susceptibility: The Epigenetic Seed and Soil Model. Toxicological Sciences, 155 (2): 302-314. Used with permission.

H3K27me3). Activating histone acetylation and methylation, modifications made by histone acetyltransferases (HATs) and histone methyltransferases (HMTs), facilitate chromatin accessibility (euchromatin), recruitment of the transcriptional machinery, including RNA polymerase II (RNAPII), and initiation/elongation of transcription. DNA methylation and repressive histone modifications function cooperatively, through proteins such as methyl-CpG binding protein 2 (MeCP2), histone deacetylases (HDACs), histone demethylases (HDMs), and repressive HMTs, in the recruitment of transcriptional co-repressors and the formation of repressed and inactive (heterochromatin) epigenetic states.

Chromatin modifications have been particularly implicated in controlling pulmonary inflammation (Adcock *et al.,* 2007; Barnes *et al.*, 2005; Saccani and Natoli 2012) and they are highly predictive of gene expression (Heintzman *et al.,* 2007; Karlić *et al.,* 2010; Wang *et al.,* 2008). Our lab recently found that patterns of chromatin modifications in unexposed phBECs were associated with ozone-associated gene induction, suggesting that pre-exposure chromatin states may be an important source of inter-individual variability (Figure 1-8). While pre-exposure chromatin modifications may be influential, epigenetic changes occurring as a result of ozone exposure may be just as important, as the induction of some genes may be dependent on the removal or placement of certain epigenetic modifications. Thus, profiling exposure-associated changes in chromatin modifications is an important corollary to the previously described experiments performed in McCullough *et al.* (2016).

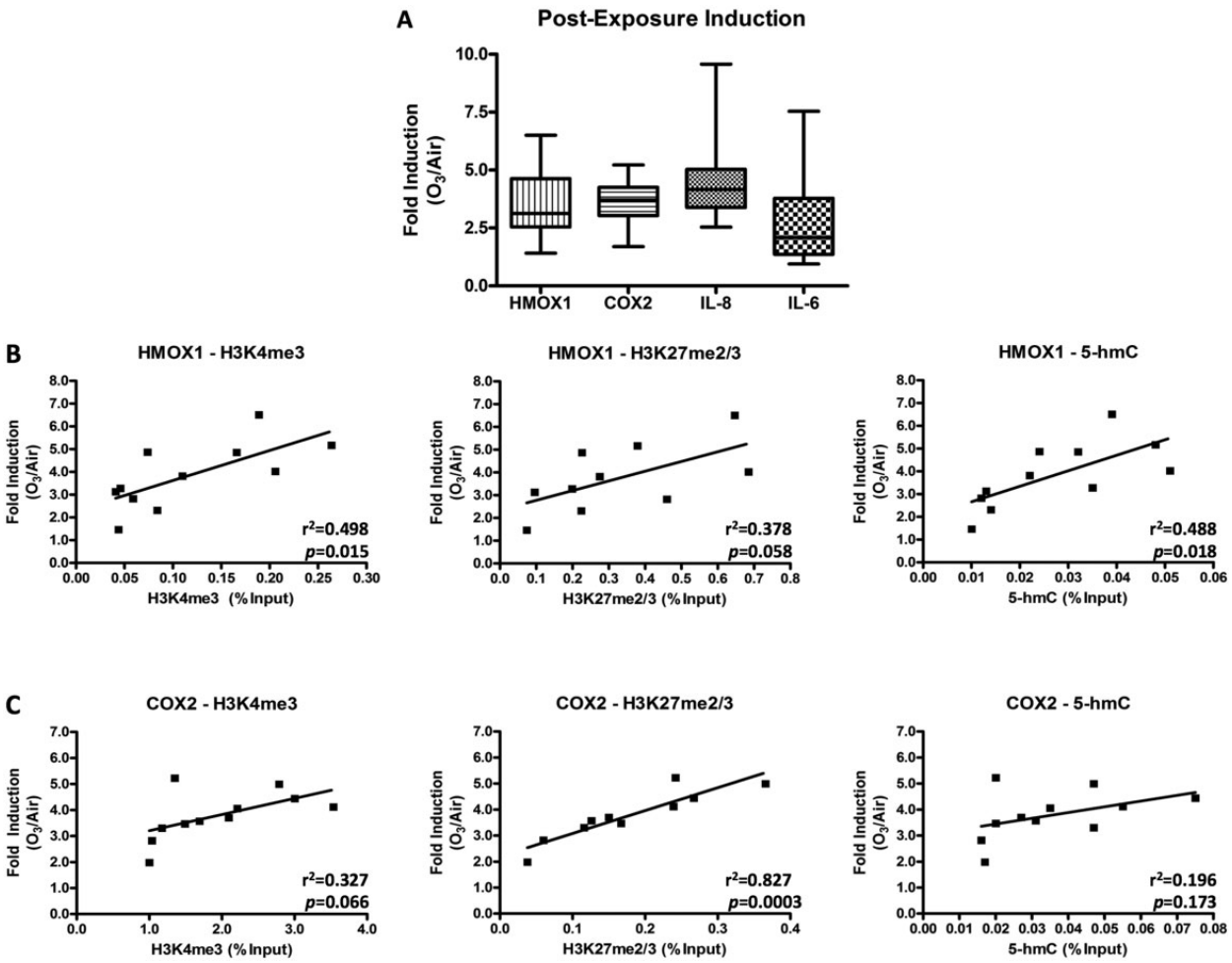

Figure 1-8. Correlations between specific baseline chromatin modification levels and O_3-induced gene expression.[2] (A) Induction of the pro-inflammatory genes *COX-2*, *IL-8*, and *IL-6* and the oxidative stress gene *HMOX-1* were measured in pHBECs following a two hour exposure to 0.5 ppm O_3. Baseline levels of H3K4me3, H3K27me2/3, and 5-hmC were compared to the peak induction of *HMOX-1* (B) and *COX-2* (C). Fold induction is shown as O_3/air and was normalized to corresponding fold change in the housekeeping gene *ACTB* according to the Pffafl method. R^2 and *p*-values were determined by simple linear regression.

Model System: Primary human bronchial epithelial cells (phBECs)

The majority of studies examining inflammatory response inter-individual variability and inflammatory adaptation have been human clinical exposure studies. While highly relevant,

[2] This figure and caption previously appeared in McCullough, S. D., Bowers, E. C., On, D. M., Morgan, D. S., Dailey, L. A., Hines, R. N., Devlin, R.B., & Diaz-Sanchez, D. (2016). Baseline chromatin modification levels may predict interindividual variability in ozone-induced gene expression. Toxicological Sciences 150 (1): 216-224. Used with permission.

clinical studies are inefficient and have a limited capacity for mechanistic investigation. While mechanisms are more easily studied in animal models, there is less genetic complexity to model inter-individual variability and the question of applicability to human biology is ever present. Alternatively, *in vitro* research using cell lines has a limited capacity to study inter-individual variability, as each cell line represents only one genotype. Moreover, most epithelial cell lines do not exhibit contact inhibition, and therefore become over-confluent during multiple days of exposures. Many airway epithelial cell lines also require submersion in media, which complicates exposing cell surfaces to ozone gas.

To overcome these obstacles, I used primary human bronchial epithelial cells (phBECs) cultured at air-liquid interface (ALI). In this model system, cells are collected from healthy human donors, expanded several passages, plated on Transwell inserts, and then the apical layer of media is removed forming an air-liquid interface. We added retinoic acid to our ALI cultures for 24 days to facilitate differentiation of epithelial cells into a pseudostratified epithelium consisting of ciliated, goblet, and basal epithelial cells. Figure 1-9 depicts epithelial cell cultures created at US EPA Human Studies Facility using the collection, culture, and differentiation technique used in this dissertation, and previously described by Ross *et al.,* (2007). These cells are especially amenable to studying inter-individual variability because they can be collected from many different human donors. Because these cultures exhibit contact inhibition, they are also well-suited for use in multi-day exposure studies required for ozone adaptation research.

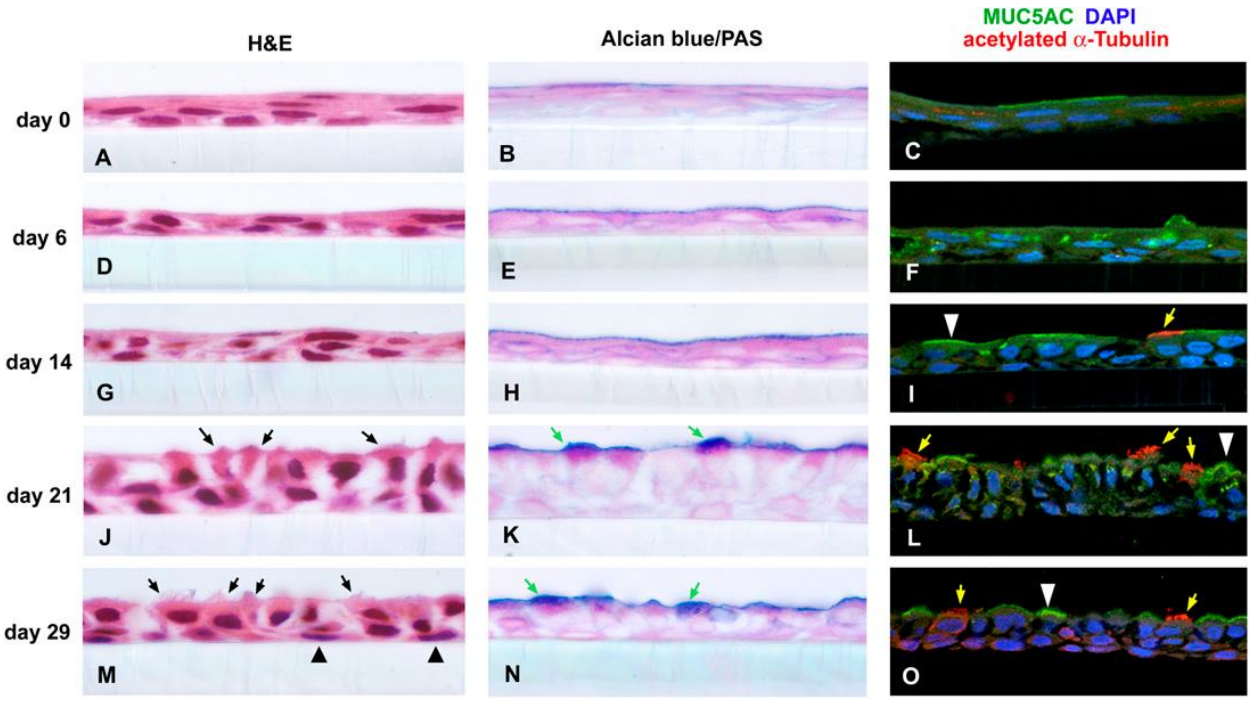

Figure 1-9. Mucociliary differentiation of phBECs at US EPA using technique described by Ross *et al.*, (2007). ALI cultures were fixed at Day 0 (A–C), Day 6 (D–F), Day 14 (G–I), Day 21 (J–L), and Day 29 (M–O). Sections were stained with H&E, Alcian blue/PAS reagent, or immunostained to label acetylated α-tubulin (red) and MUC5AC (green) with DAPI nuclear staining (blue). J, M: Ciliated cells=black arrows; basal cells=black arrowheads. K, N: Cells with PAS and Alcian blue staining, indicating the presence of mucins, are indicated with green arrows. I, L, O: Acetylated α-tubulin staining indicates ciliated cells (yellow arrows) and mucin staining indicates secretory cells (white arrowheads). Reprinted with permission of the American Thoracic Society. Copyright © 2017 American Thoracic Society. Ross *et al.,* 2007. Am J Respir Cell Mol Biol Vol 37. pp 169–185. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.

Scope of Dissertation

Two long-observed but poorly characterized phenomena may hold the key to understanding susceptibility to ozone exposure effects: inflammatory response inter-individual variability and inflammatory ozone adaptation. Inflammatory responses to ozone exposure exhibit marked heterogeneity where the range of responses in healthy individuals can exceed that observed in populations deemed "susceptible" by conventional risk factors. Furthermore, some but not all individuals exhibit an ability to suppress inflammatory responses during repeated

ozone exposure, which may prevent excessive inflammation and permanent lung damage. These phenomena have been observed in human clinical studies and exposed populations for nearly fifty years, but is unclear if these responses are a feature of the airway environment specific to each individual or they are mediated on a cellular level. If we can identify the mechanisms that dictate inflammatory response inter-individual variability and adaptation, we may be able use this information to both engineer protective interventions and also refine predictions of susceptible populations.

The inflammatory response to ozone exposure exhibits extensive inter-individual variability, but responses within an individual are highly reproducible. This indicates that the inflammatory ozone response obeys biological rules that have yet to be discovered. Epithelial cells line the airway and are the first to encounter inhaled pollutants. These cells act as sentinels by sensing pollutants and/or tissue damage and releasing pro-inflammatory mediators such as IL-8. IL-8, a potent neutrophil chemokine, is an important driver of the ozone-associated neutrophil response. A key step in the release of IL-8 from epithelial cells is the ozone-mediated transcription of the *IL-8* gene. By understanding the factors dictating ozone-mediated *IL-8* transcription in epithelial cells, we may be able to understand a critical component of ozone inflammatory response 'programming.'

Using the phBEC model system, the goal of my dissertation is to understand the molecular programming that dictates the expression of *IL-8* in airway epithelial cells (Figure 1-10). Given the central role of MAPK signaling in polarized phBECs as previously described by McCullough *et al.* (2014), I hypothesize that the activation of the MAPKs ERK1/2 and p38 are an important component of this programming in differentiated phBEC cultures. Furthermore, I hypothesize that the differential activation of these kinases shapes *IL-8* transcriptional responses

to acute ozone exposure (Chapter 2) and may be an important mechanism underlying adaptive responses during repeated ozone exposure (Chapter 3). While ozone-associated MAPK activation is an important component of pro-inflammatory signaling, focusing on cellular signaling alone does not take into account the role of the epigenome at pro-inflammatory gene promoters. Thus, I also tested the hypothesis that changes in promoter epigenetic modifications may explain inter-individual variability in ozone-mediated *IL-8* induction (Chapter 4).

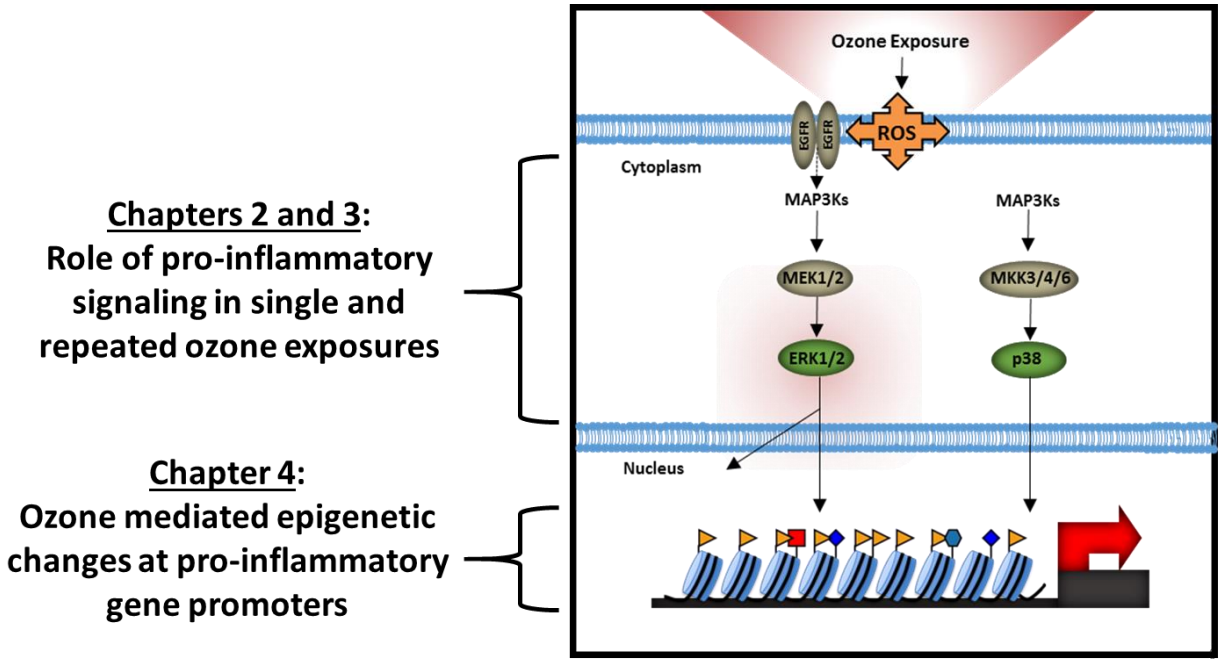

Figure 1-10. Diagram of dissertation approach. Chapters 2 and 3 address the contributions of cellular signaling in response inter-individual variability and ozone adaptation. While examining MAPK signaling accounts for one of the main pro-inflammatory signals entering the nucleus, it does not take into consideration the role epigenome, which is explored in Chapter 4.

The findings from my dissertation provide compelling evidence that phBEC *IL-8* transcriptional responses are indeed 'programmed,' and that the MAPKs ERK1/2 and p38 are an integral part of this programming. I found that differences in MAPK activation were critical mediators of ozone response inter-individual variability, where high *IL-8* responding phBEC

cultures had elevated activation of ERK1/2. Moreover, when phBECs were subjected to repeated ozone exposure, many cultures exhibited suppression of ozone-responsive gene expression, recapitulating the *in vivo* adaptive response. Upon further investigation, I discovered that antioxidant capacity is not increased during repeated ozone exposure as was previously hypothesized. Instead, phBECs that exhibited suppressed *IL-8* induction showed reductions in ERK activation, suggesting this is an important mechanism driving the adaptive response. While MAPK signaling is an important component of the ozone response, epigenetic changes at pro-inflammatory gene promoters may also shape response inter-individual variability. I observed that the magnitude of pro-inflammatory gene induction is associated with changes in activating and repressive chromatin modifications.

Inflammatory response inter-individual variability and adaptive responses are currently major obstacles in anticipating health effects in populations exposed to air pollutants and predicting individuals who might be especially susceptible to exposure effects. This research will help overcome these obstacles by providing novel insights into the mechanisms underlying these phenomena.

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CHAPTER 2: OZONE-MEDIATED IL-8 RESPONSIVENESS IS AN INTRINSIC PROPERTY OF AIRWAY EPITHELIAL CELLS AND IS DETERMINED BY THE ACTIVATION OF THE MAP KINASE ERK1/2 [3]

Introduction

Millions of individuals are exposed to levels of the ambient air pollutant ozone that are known to produce pulmonary inflammation; however, inflammatory responses exhibit extensive inter-individual variability, making it difficult to anticipate health effects of exposed individuals. The variability of ozone associated inflammation is not reliably explained by biological factors, nor can it be predicted by traditional risk groups; for example, the magnitude of inflammatory response in healthy individuals may be greater than that observed in asthmatics (Holtz *et al.*, 1999; US EPA 2013). While ozone inflammatory responses are poorly understood and exhibit a high degree of inter-individual variability, they also exhibit low *intra*-individual variability, as inflammatory responses are reproducible if the same individual is exposed to ozone at different times (Holz *et al.*, 1999 and 2005). This indicates that there are biological factors dictating the ozone inflammatory response that have yet to be defined, and suggests that their discovery could facilitate the reliable identification of individuals who may be particularly susceptible to ozone exposure.

[3] This chapter has been submitted for publication in April of 2017. Bowers, E.C., McCullough, S.D., Morgan, D.S., Dailey, L.A., Diaz-Sanchez, D. *Ozone-mediated IL-8 responsiveness is an intrinsic property of airway epithelial cells and is determined by the activation of the MAP kinase ERK1/2.*

IL-8 is an important pro-inflammatory chemokine that is a hallmark of environmentally-induced airway inflammation (Aris *et al.,* 1993; Sundeep *et al.,* 2000). Multiple studies have shown that ozone exposure results in the release of IL-8 protein into the airways and that these levels exhibit extensive variation between individuals (Fry *et al.,* 2012; Krisha *et al.,* 1998). Given the importance of IL-8 in airway inflammation, we sought to further understand the molecular mechanisms driving IL-8 response variability, as these may lead to the discovery of novel ozone susceptibility factors.

We examined *in vitro* IL-8 response in parallel with the activation of cellular signaling pathways that are known to be important in mediating the ozone pro-inflammatory response. We modeled *IL-8* expression in epithelial cells as they are one of the first cells to respond to inhaled pollutants and are a major source of IL-8 following ozone exposure (Devlin *et al.,* 1994; Jaspers et al 1997; Chang *et al.,* 1998). While *IL-8*-associated gene transcription has previously been shown to occur through the NFκB pathway in cell lines, our lab demonstrated that in primary human bronchial epithelial cells (phBECs), the mitogen-activated protein kinases (MAPKs) are central signaling mediators (McCullough *et al.,* 2014). These kinases are activated by phosphorylation at specific residues and, in turn, phosphorylate other downstream effectors, which can include kinases, transcription factors, stress-associated proteins, etc. Within the MAPK pathway, the activity of extracellular-signal related kinases (ERK) 1 and 2, as well as p38 have an essential role in ozone pro-inflammatory signaling, as inhibiting these signaling pathways diminishes ozone-induced pro-inflammatory gene expression in polarized phBECs (McCullough *et al.,* 2014). Thus, we hypothesized ERK and p38 phosphorylation pays an equally important role in differentiated phBEC cultures and drives inter-individual variability in ozone-induced *IL-8* transcription.

To determine if *IL-8* transcriptional variability occurred *in vivo*, we first assessed IL-8 transcription in epithelial cells collected from human subjects following controlled ozone exposure. We then investigated the role of MAPKs in modulating *IL-8* transcriptional response using differentiated phBECs cultured at air-liquid interface (ALI). Here we report that both *in vivo* and *in vitro*, ozone exposure results in inter-individual variability in epithelial cell *IL-8* transcription. PhBEC *IL-8* transcriptional responses from individual donors were consistent across repeated collections, suggesting that the magnitude of *IL-8* induction is an intrinsic property of phBECs and may be donor-specific. By extension, this epithelial cell 'programming' could partially explain the reproducibility of ozone responses observed *in vivo* (Holz *et al.*, 1999 and 2005). When we examined ozone-associated pro-inflammatory signaling pathways in phBECs, we found that MAPK activity differed between cultures with high and low *IL-8* transcriptional responses, with highly responsive cultures exhibiting elevated ERK1/2 pathway activation. These results suggest that an individual's epithelial cells have intrinsic properties that regulate their response to environmental pollutants, and that one mechanism of this programming may be the differential regulation of the ERK1/2 pathway.

Materials and Methods

In vivo epithelial cell ozone responses: Clinical ozone exposures and epithelial cell collection

Nine subjects participated in a randomized single-blind crossover study as described in Devlin *et al.,* (2012). The median age was 24 years (range 21-32 years) and all were male (1 bi-racial, 7 Caucasian, 1 Hispanic). Briefly, young healthy volunteers gave a detailed medical history and underwent a physical examination. All subjects were lifetime nonsmokers. Each subject was exposed twice for 2 hours: once to filtered air (FA) and once to 0.3 ppm ozone. This ozone dose is frequently used in human exposure studies and is equivalent to an exposure at the 2005 National Ambient Air Quality Standard (NAAQS) of 75 ppb over an eight-hour period, which is slightly higher than the current NAAQS of 70 ppb/8h. Exposures were separated by at least 2 weeks. During each 2-hour exposure, subjects alternated between 15 minutes of rest and 15 minutes of exercise on a cycle ergometer and exposure exercise levels were adjusted to obtain a target minute ventilation of 25 $L \cdot min^{-1} \cdot m^{-2}$ body surface area. One hour after the exposure concluded, epithelial cells were collected from the bronchi of subjects via bronchial brushing, mixed with Trizol (Life Technologies, Carlsbad, CA), and the extracts were stored at -80 °C until they were ready for processing. The informed consent and collection protocol were approved by the UNC School of Medicine Committee on the Protection of the Rights of Human Subjects and by the US EPA.

In vitro epithelial cell ozone responses: Primary cell collection, plating, and ozone exposure

Primary human bronchial epithelial cells were obtained via bronchial brushing from healthy non-smokers with no more than a 1-pack year smoking history. Donors gave their informed consent after being informed of risks and procedures. The consent and collection

protocol were approved by the UNC School of Medicine Committee on the Protection of the Rights of Human Subjects and by the US EPA. To determine if phBECs from the same donor had consistent responses to ozone over time we compared *IL-8* inductions between cells from the same donor that were collected during two different bronchoscopies, which were a minimum of three weeks apart. Cells were cultured and differentiated as described by Ross *et al.*, (2007). Briefly, cells were expanded for three passages and then plated on 24 mm Transwell inserts with 0.4 µm pores (Corning Life Sciences, Tewksbury, MA). Three inserts per treatment per donor were used. Once confluent, cells were submerged for 48 hours with 500 nM retinoic acid. Afterward the apical layer of medium was removed creating an air-liquid interface (ALI). Cells were maintained for 24 days at ALI and supplemented with 100 nM retinoic acid to promote differentiation into a pseudostratified columnar epithelium. Prior to each ozone exposure, the basolateral medium was replaced and the apical surface was washed with 500 µL Dulbecco's PBS (Life Technologies, Carlsbad, CA) to remove cellular debris and secretions. To investigate the role of MAPK activity in ozone-associated *IL-8* induction, the ERK1/2 and p38 kinase inhibitors SCH772984 and LY2228820 (Cayman Chemical, Ann Arbor, MI), respectively, were used. LY2228800 and SCH772984 both inhibit these kinases by competitively binding the ATP binding domain, but SCH772984 also induces a conformation change that prevents the phosphorylation of ERK1/2 by upstream kinases (Campbell *et al.*, 2014; Morris *et al.*, 2013). These inhibitors were added to cell media to a final concentration of 250 nM with 0.2% DMSO during media change two hours prior to ozone exposure and remained throughout exposure until harvest. After a two-hour acclimation period cells were exposed to a filtered air (FA) control or 0.5 ppm ozone for two hours. This dose is conventionally used *in vitro* and is similar to doses used in clinical ozone exposure studies (Hatch *et al.*, 2014). Immediately following exposure

cells designated for MAPK analysis were harvested as described below. Two hours following exposure, cells designated for gene expression analysis were harvested in RNA lysis buffer (Life Technologies) and stored at -80° C until they were ready for processing. As a positive control for NFκB pathway activation, primary cells were stimulated with 10 ng/mL TNFα for 20 minutes.

RT-qPCR

We used *IL-8* transcription as our primary read-out, as protein levels may be influenced by further regulatory processes such as degradation. For *in vivo* samples, RNA was extracted from lysed samples using RNeasy kit (Qiagen, Valencia, CA), while for *in vitro* samples, an RNA Mini Kit (Life Technologies) was used. RNA was quantified using a Nanodrop ND1000. For *in vivo* and *in vitro* samples 100 ng and one (1) μg, respectively, was used to synthesize cDNA using iScript Reverse Transcription Kits (Bio-Rad, Hercules, CA). Gene expression was assessed in technical triplicates using TaqMan RT-qPCR primers and probes (Supplementary Methods) and the CFX96 qPCR system (Bio-Rad). Target gene expression was normalized to β-actin (ACTB) and then expressed as a fold changes between filtered air and ozone exposure treatments using the Pfaffl method (Pfaffl 2001).

It has been shown that in individuals who have elevated IL-8 protein in the airway after ozone exposure also exhibit increased IL-8 protein prior to exposure (Fry *et al.*, 2010); thus, we wanted to determine if there was a relationship between baseline *IL-8* transcription and ozone-induced transcription. To answer this question, we performed absolute quantification of the *IL-8* transcript by generating standard curves with known copy numbers of closed circular pUC57-

based plasmids (synthesized by Genewiz, Inc.) contained the *IL-8* cDNA sequence. The resulting values were normalized to the absolute quantity of *ACTB* transcript in the same PCR reaction.

MAPK Pathway Analysis

We compared ozone-associated MAPK activation between donors that had high versus low *IL-8* inductions by examining phosphorylation at specific residues. Cellular extracts were prepared in RIPA buffer (50mM Tris, pH 8.0; 150mM NaCl; 1% Triton X-100; 400μM EDTA; 10% glycerol; 0.1% SDS; 0.1% deoxycholate) with 1X protease (cOmplete EDTA-free, Roche, Indianapolis, IN) and 1X phosphatase (PhosSTOP, Roche) inhibitors. Cellular debris was then centrifuged and aliquots RIPA extract were removed for protein quantification via BCA assay (ThermoFisher, Waltham, MA). The remaining supernatant was supplemented with Laemmli buffer to a final concentration of 1X (60mM Tris, pH 6.8; 200 mM DTT; 10% glycerol; 2% SDS; 0.05% bromophenol blue) incubated at 95°C for five minutes, aliquoted, and stored at -80°C. For each sample, equal amounts of protein were loaded into SDS-PAGE gels, electrophoresed, and transferred to nitrocellulose membranes (Bio-Rad) via tank transfer. Following primary antibody binding (Supplementary Methods), horseradish peroxidase (HRP)-conjugated secondary antibodies and Pierce Enhanced Chemiluminescence (ECL) Western blotting substrate (ThermoFisher) were used to generate chemiluminescence. Immunoblots were imaged on a LAS-3000 detection system (Fuji/GE Healthcare, Pittsburgh, PA). Densitometry analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD). The pixel density from the phosphorylated protein was normalized to the pixel density from total protein levels, after which the fold changes between treated and filtered air conditions were calculated.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism 6.07 (GraphPad Software, La Jolla, California, USA). For all analyses a *p*-value of less than 0.05 was considered statistically significant. Simple linear regression was used to relate *IL-8* responses between repeated bronchoscopies. To determine if inhibitor treatments significantly reduced IL-8 induction from the ozone-vehicle (O_3-V) control, a two-way ANOVA with Dunnett's Multiple Comparisons was used. Kinase phosphorylation in high and low responders was compared using a non-parametric Mann-Whitney test.

Results

Inter-individual variability in ozone induced epithelial derived *IL-8* response

Epithelial cells collected from human subjects exposed to ozone demonstrated variable *IL-8* expression following exposure (Table 2-1; Figure 2-1A). When normalized to matched filtered air control exposures, the mean *IL-8* induction (± SD) was 1.30 ± 0.69. In phBEC cultures exposed to ozone *in vitro* (Table 2-2; Figure 2-1 B), the mean *IL-8* induction was 4.18 ± 2.29. Although the magnitude of *IL-8* inductions differed *in vitro* and *in vivo*, the coefficients of variability were nearly identical between model systems, 53% and 55%, respectively, indicating that the data were similarly distributed. For downstream analysis using the phBEC *in vitro* system, we used the arithmetic mean value of 4.2 fold change from FA to separate donor cultures into "high responders" and "low responders" depending on whether they fell above or below this value.

***In vivo* exposure subjects**				
Subject	Sex	Age	Ethnicity	Mean *IL-8*
1	M	23	White	0.52
2	M	26	White	2.55
3	M	32	White	1.23
4	M	24	Hispanic	1.27
5	M	24	White	0.4
6	M	21	White	1.19
7	M	23	White	1.86
8	M	22	Bi-racial	0.88
9	M	26	White	1.81

Table 2-1. Characteristics of human subjects who underwent *in vivo* ozone exposure. Donor characteristics from the data depicted in Figure 2-1A. *IL-8* inductions from each subject's bronchial epithelial cells are shown in the last column.

phBEC donors				
Donor	Sex	Age	Ethnicity	Mean $1XO_3$ *IL-8*
1	F	35	Black	5.8
2	M	32	White	7.9
3	M	39	White	2.8
4	F	29	White	6.5
5	M	26	White	2.7
6	F	18	Black	5.0
7	M	26	White	9.2
8	M	20	Black	2.4
9	M	34	White	2.9
10	M	21	Asian	1.9
11	M	27	White	3.1
12	M	33	Black	2.4
13	M	39	White	5.7
14	F	35	White	4.5
15	M	28	White	1.4
16	F	21	Black	2.6

Table 2-2. Characteristics of phBEC donors. The characteristics of phBEC donors included in figure 2-1B are depicted. *IL-8* inductions from *in vitro* ozone exposure are included for reference.

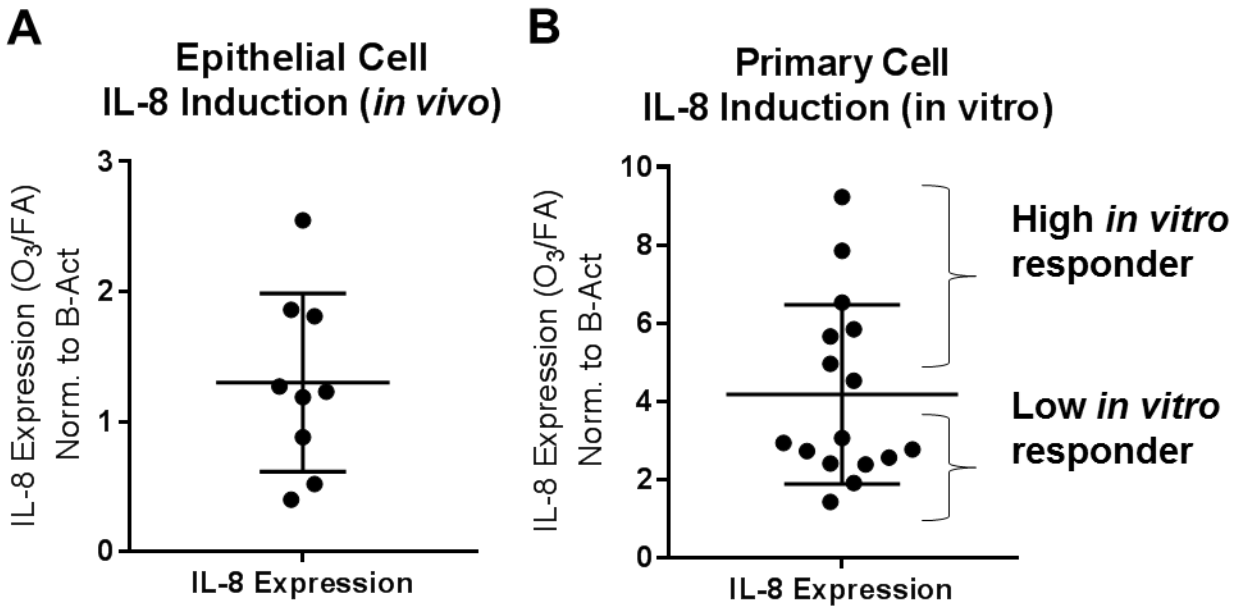

Figure 2-1. Inter-individual variability in epithelial cell *IL-8* induction from both controlled human ozone exposure studies (*in vivo*) and primary cell cultures exposure to ozone (*in vitro*). (A) *IL-8* induction in bronchial epithelial cells collected from ozone-exposed human subjects. Subjects were exposed to 0.3 ppm ozone for 2h and after a one-hour acclimation period subjects underwent bronchoscopy to collect epithelial cells via bronchial brushing. *IL-8* expression was first normalized to β-actin and then expressed as a fold change from matched filtered air (control) exposures from the same subjects. Mean ± SD shown, n=9 subjects. (B) *IL-8* expression in phBECs cultured at ALI exposed to ozone (0.5 ppm/2h). Each data point represents a phBEC culture collected from a different human donor. Expression was normalized to β-actin and expressed as fold change from filtered air exposures. Mean ± SD shown, n=16 donors. To further investigate the mechanisms underlying ozone responsiveness using phBECs, we classified cultures as being "high" or "low" responders based on whether *IL-8* inductions fell above or below the group mean (4.2 fold change).

Donor-specificity in the *IL-8* response

PhBECs collected from the same donor over time exhibited donor-specificity in ozone associated *IL-8* induction (Figure 2-2). Data were available for seven phBEC donors who had two epithelial cell collections. The time between bronchoscopies ranged from 49-453 days. Once again, we used the mean described in Figure 2-1B, 4.2-fold change from FA, to differentiate high and low responders. For all seven donors, responder status was recapitulated

between collections as indicated by the data points clustering in the shaded quadrants in Figure 2, as well as strength of linear regression (R^2=0.78, p=0.009). In addition, repeated platings from cells from the same bronchoscopy that were thawed and plated at different times also demonstrated reproducible *IL-8* responses (Figure 2-12). These data suggest that epithelial cell *IL-8* transcriptional response to ozone is an intrinsic property.

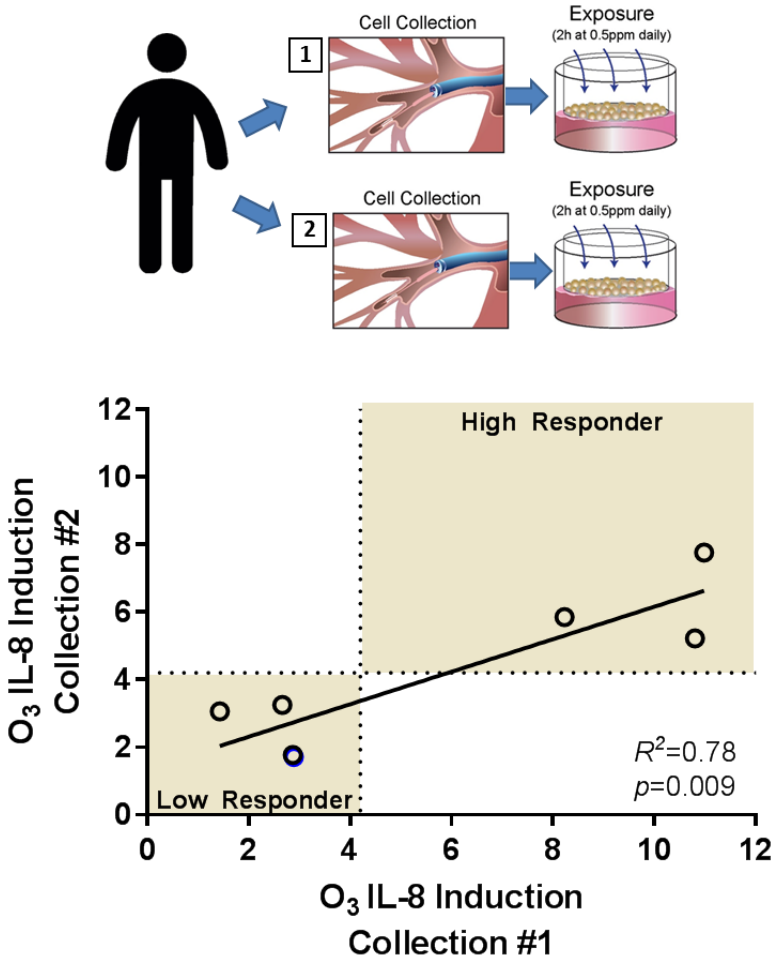

Figure 2-2. Donor-specificity of phBEC IL-8 induction following *in vitro* ozone exposure. We investigated whether phBEC cultures collected from the same cell donors at different times had reproducible *IL-8* responses. Seven phBEC donors were used (two data points overlap) with the time between collections ranging from 49-453 days. The consistency of ozone response was assessed by comparing the first exposure response (X-axis) with the second exposure response (Y-axis) via linear regression. Dotted lines are drawn at X=4.2, Y=4.2, which is the metric by which we differentiated high and low *in vitro* responders. The shaded areas indicate the quadrants the data points would cluster within if cultures were consistent in their response status (high or low) in both collections one and two.

The influence of ERK1/2 and p38 inhibition on the ozone-*IL-8* response

The addition of the ERK1/2 and p38 inhibitors SCH772984 and LY2228820, respectively, resulted in the reduced induction of *IL-8* (Figure 2-3). In *in vitro* high responders, the addition of either SCH772984 or LY2228820 alone reduced *IL-8* induction; however, the simultaneous addition of both inhibitors was required to achieve a statistically significant reduction from O_3-V levels (ANOVA with Dunn's Multiple Comparisons p=0.008). Mean *IL-8* induction (± SD) for the O_3-V treatment was 8.26 ± 4.5, which was reduced to 2.96 ± 1.69 during the O_3+SCH772984+LY2228820 treatment. Three low *in vitro* responders also received the inhibitor treatment (Figure 2-12) and no statistically significant changes in *IL-8* induction were observed. A representative Western blot depicting MAPK activation after the addition of these inhibitors is shown in Figure 2-4.

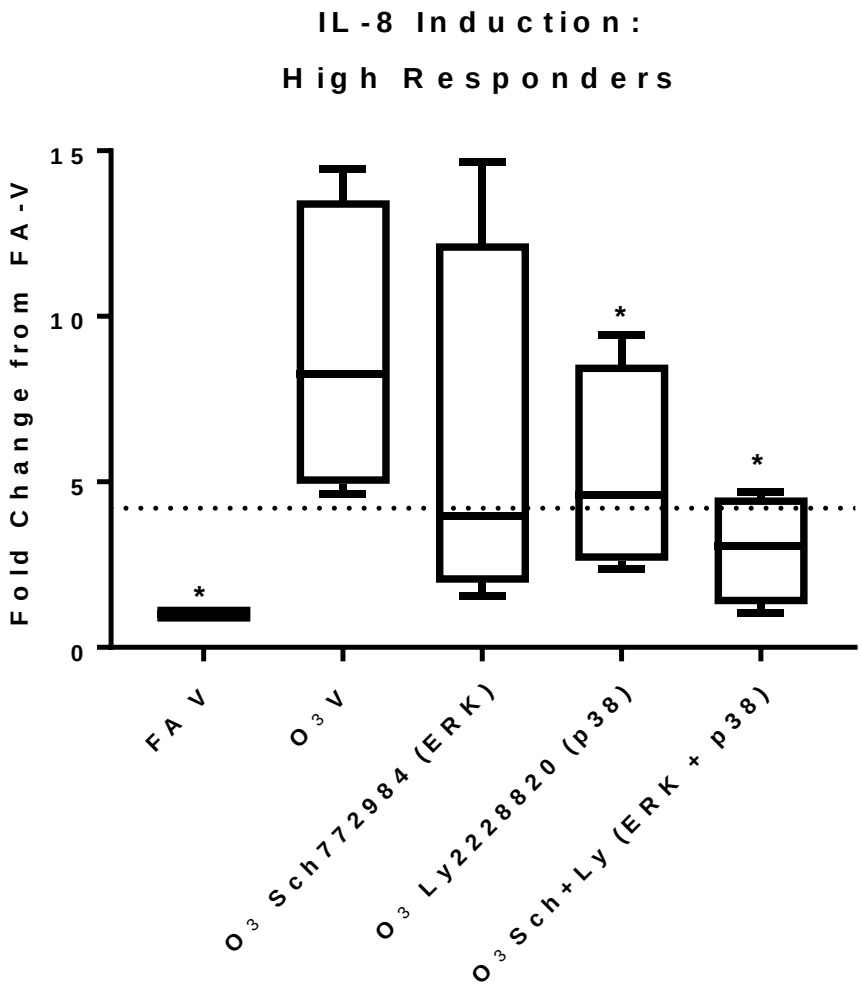

Figure 2-3. The influence of ERK1/2 and p38 inhibition on ozone associated IL-8 induction in high responders. To determine whether the activation of the MAP kinases ERK1/2 and p38 were required for ozone-mediated *IL-8* induction, inhibitors of these kinases (SCH772984 and LY2228820, respectively) were added to cell media two hours prior to ozone exposure. Seven donors were used, four high responders (shown above) and three low responders (Figure 2-13). *IL-8* inductions were normalized to a 0.2% DMSO filtered air vehicle control. *IL-8* inductions were compared between the ozone-vehicle control (O_3-V) and all other treatments via 2-way ANOVA. *p<0.05.

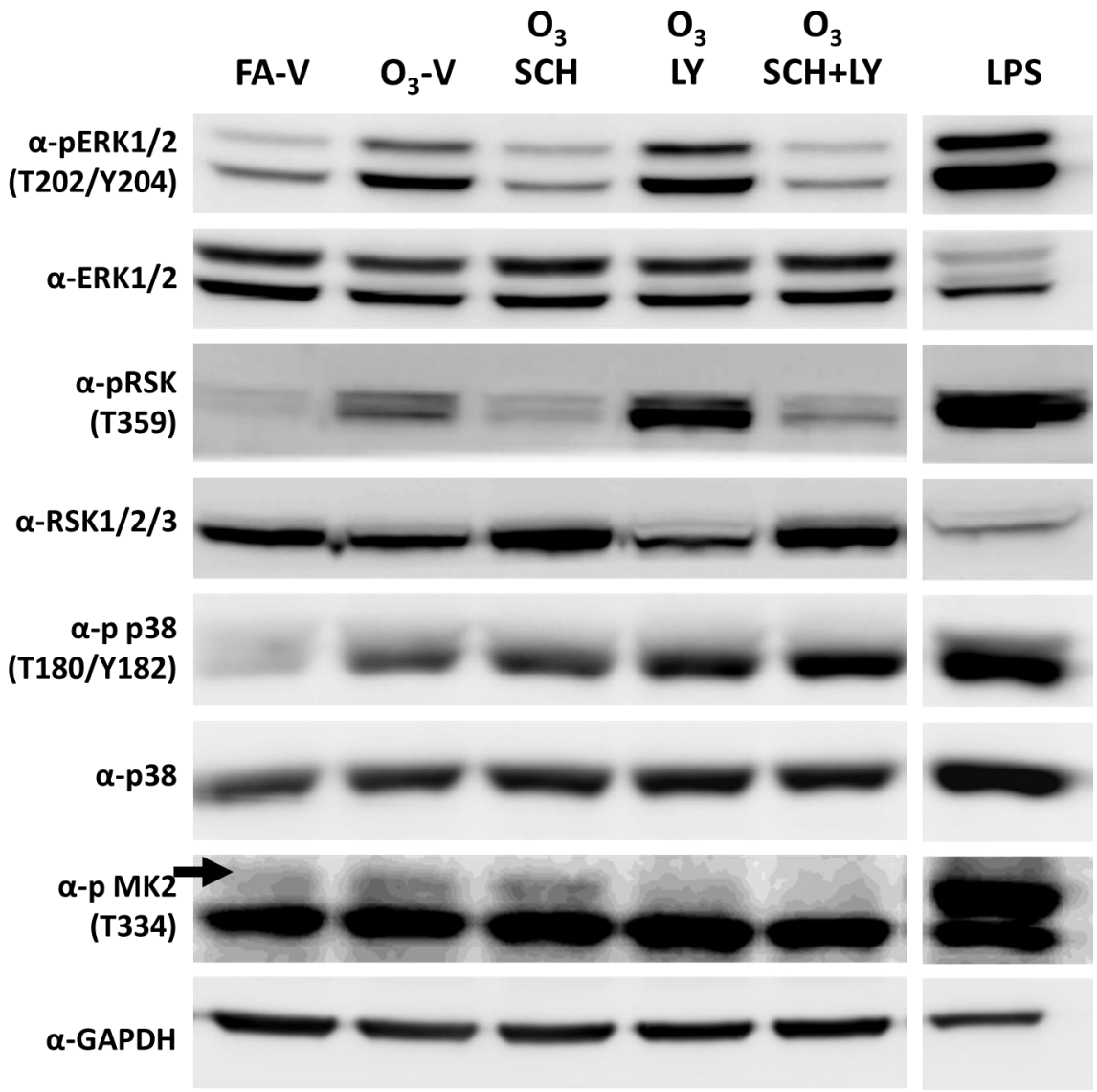

Figure 2-4. Representative Western blot showing the effects of SCH772984 and LY2228820 on ERK1/2 and p38 phosphorylation. The ERK1/2 and p38 inhibitors SCH772984 and LY2228820 (respectively, 250 nM each) were added to cell media with 0.2% DMSO (V) two hours prior to ozone exposure. Protein was harvested immediately following ozone exposure. The phosphorylation of ERK1/2, p38 and the downstream kinases RSK and MK2, respectively, are shown. Because LY2228820 does not inhibit p38 from being phosphorylated, but prevents p38 kinase activity, changes in MK2 phosphorylation are used as a read-out of p38 kinase activity. Extract from LPS-stimulated phBECs was used as a positive control. The LPS positive control for each mark was run on the same gel, immunoblotted, and imaged at the same time as the blots in the adjacent row. Because the LPS treatments were run in different lanes that did not align, the images were adjusted so they could be shown in the same lane.

Comparison of MAPK phosphorylation in high and low responding cultures

To test our hypothesis that MAPK phosphorylation might explain the inter-individual variability in *IL-8* ozone response, we compared ozone-associated *IL-8* induction with the activation of the ERK1/2 and p38 pathways following ozone exposure. Using the same cutoff of a 4.2-fold change to differentiate high and low *in vitro IL-8* responders, we obtained matched RIPA extracts and RNA samples from six high- and six low-responding phBEC cultures. Ozone-mediated *IL-8* induction in these donors (Figure 2-5) ranged from a 1.43-4.06 fold change in low responders and 4.53 to 14.45-fold change in high responders.

A non-parametric Mann-Whitney test was used to assess MAPK phosphorylation differences between high and low responders, as several distributions were skewed. Following ozone exposure, the mean fold change from FA (± SD) of ERK1/2 phosphorylation in low responders was 1.23 ± 0.21 and 2.39 ± 0.84 in high responders. After finding a significant difference in ERK1/2 phosphorylation between these groups (p=0.022), we then examined the phosphorylation of the kinases upstream (MEK) and downstream (RSK) from ERK1/2. MEK phosphorylation in low responders and high responders was found to be significantly different, with low responders having a mean of 1.43 ± 0.27 and high responders a mean of 2.39 ± 0.61 (p=0.004). RSK phosphorylation was also significantly different between these groups, with low responders having a mean of 1.28 ± 0.67 and high responders having a mean of 3.32 ± 2.01 (p=0.026).

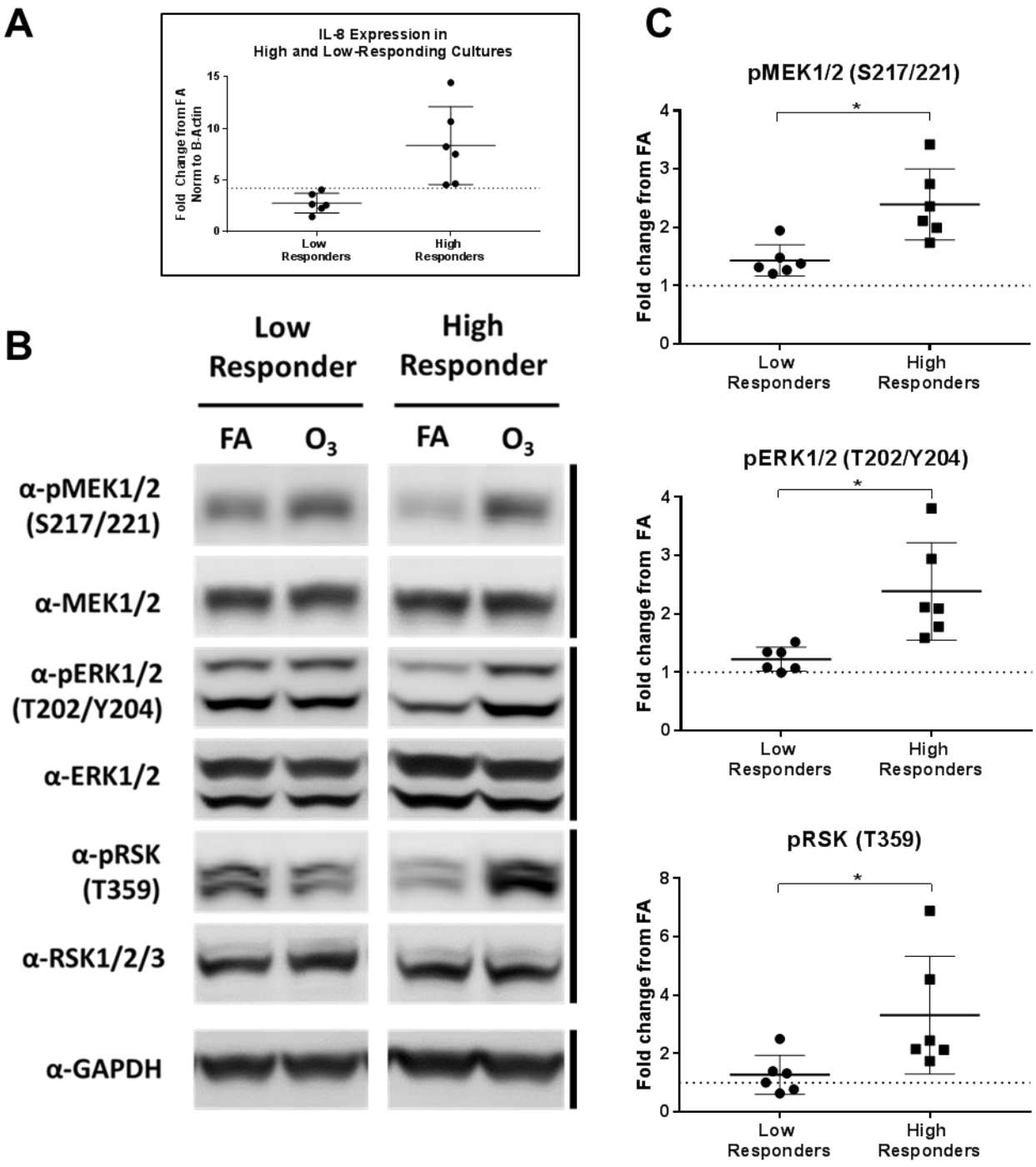

Figure 2-5. The activation of ERK1/2 and its associated kinases in high and low-responding cultures. The phosphorylation of the MAP kinase ERK1/2 and its association with *IL-8* response variability. Cells were considered "high responders" if their *IL-8* induction was above the group mean (4.2 fold change) and low responders were below this group mean. Protein was collected from twelve donors (n=12), six high and six low responders. The distribution of *IL-8* inductions as well as the group means ± SD is shown in **(A)**. **(B)** Blots from two representative donors (one

high, one low) show the phosphorylation of ERK1/2 and an upstream (MEK1) and a downstream (RSK) kinase. **(C)** Densitometry analysis was used to calculate the fold change in activation (normalized to filtered air control) for each donor. Mean activation (±SD) shown for each group. Differences between high and low responders were determined via Mann-Whitney test. *$p<0.05$.

After determining that high responders had elevated ERK1/2 pathway activation, we then assessed the phosphorylation of p38 (Figure 2-6). Low responders had a mean p38 phosphorylation of 1.47 ± 0.44 and high responders had a mean of 1.43 ± 0.52. The phosphorylation of p38 was not found to differ between these groups (p=0.94). As an additional check of the p38 pathway, we also examined the phosphorylation of MKK4, which is a MAP kinase kinase upstream of p38 that is activated by ozone exposure (McCullough *et al.*, 2014). Low responders had a mean MKK4 phosphorylation of 1.20 ± 0.14 and were not significantly different from high responders, which had a mean of 1.37 ± 0.29 (p=0.13). This indicated that the activation of the p38 pathway does not differ between high and low *IL-8* responders.

In addition to p38 and ERK1/2 phosphorylation, we also assessed the phosphorylation of the p65 NFκB subunit (Figure 2-7). While the NFκB pathway is not influential in ozone-mediated *IL-8* signaling in polarized phBECs, given its importance in cell lines, we wanted to confirm these findings in differentiated phBECs. We found that p65 phosphorylation did not differ between these groups, with low responders having a mean of 0.86 ± 0.26 and high responders a mean of 1.26 ± 0.35.

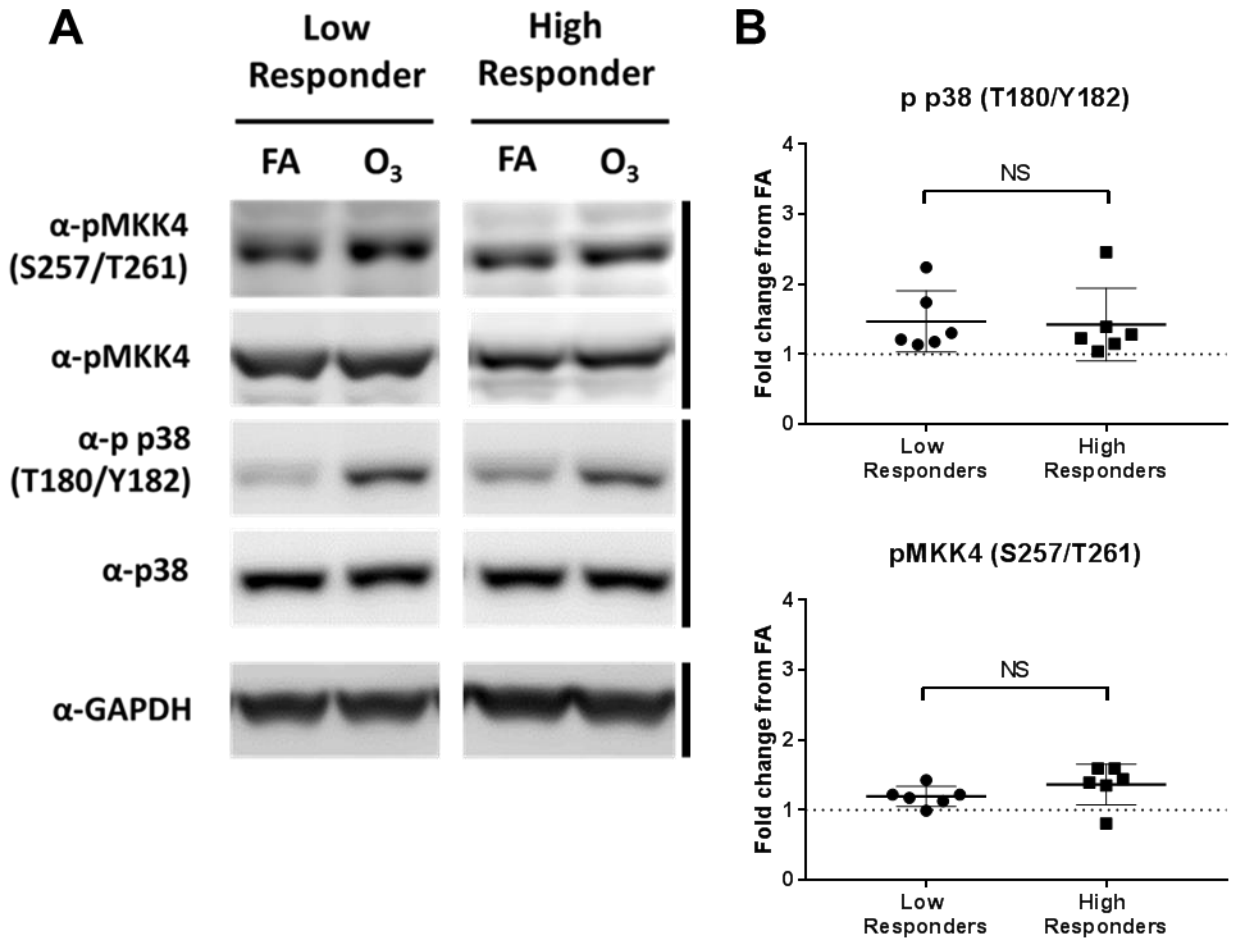

Figure 2-6. The activation of p38 and MKK4 in high and low-responding cultures. In addition to ERK1/2, we also examined the phosphorylation of p38 and its upstream kinase MKK4 to determine if this pathway was associated with phBEC *IL-8* response variability. Blots were generated using the same donors as described in Figure 2-5. **(A)** Representative blots from one high, one low responding donor- the same donors depicted in 2-5B. **(B)** Densitometry analysis was used to calculate the fold change in phosphorylation (normalized to filtered air control) for each donor. Mean activation (±SD) shown for each group. No differences between high and low responders were found (Mann-Whitney test. $p>0.05$).

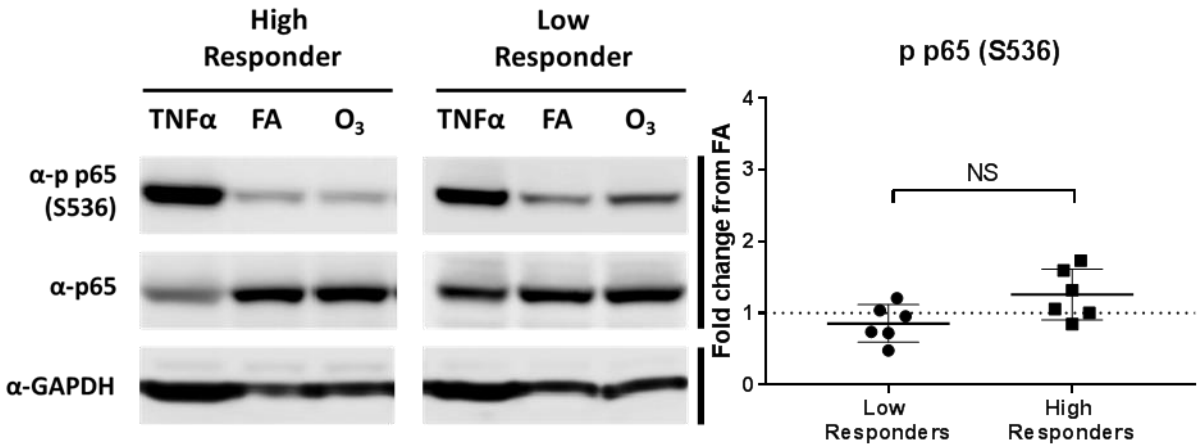

Figure 2-7. Comparison of p65 activation in high and low-responding cultures. Using the same panel of donors as Figures 2-5 and 2-6, we examined the p65 phosphorylation at S536, an indicator of canonical NFκB activation. Primary cells were stimulated with 10ng/mL TNFα for 20 minutes as a positive control. Representative donors shown are the same donors depicted in Figures 2-5 and 2-6. Densitometry analysis shows average (± SD) fold change in activation (normalized to filtered air control) across 6 high and 6 low responders (n=12). No significant difference was found between these two groups.

Continuous analysis of *IL-8* induction and MAPK activation

In addition to analyzing MAPK activation based on and high and low responder status, the data were analyzed in a continuous format in which the ratio of phosphorylated protein (Fold change between O_3 and FA) was plotted against ozone *IL-8* induction (Figure 2-8) and compared using simple linear regression. A significant correlation was observed for ERK1/2 (R^2=0.46, p=0.016), but not for p38 (R^2=0.11, p=0.28).

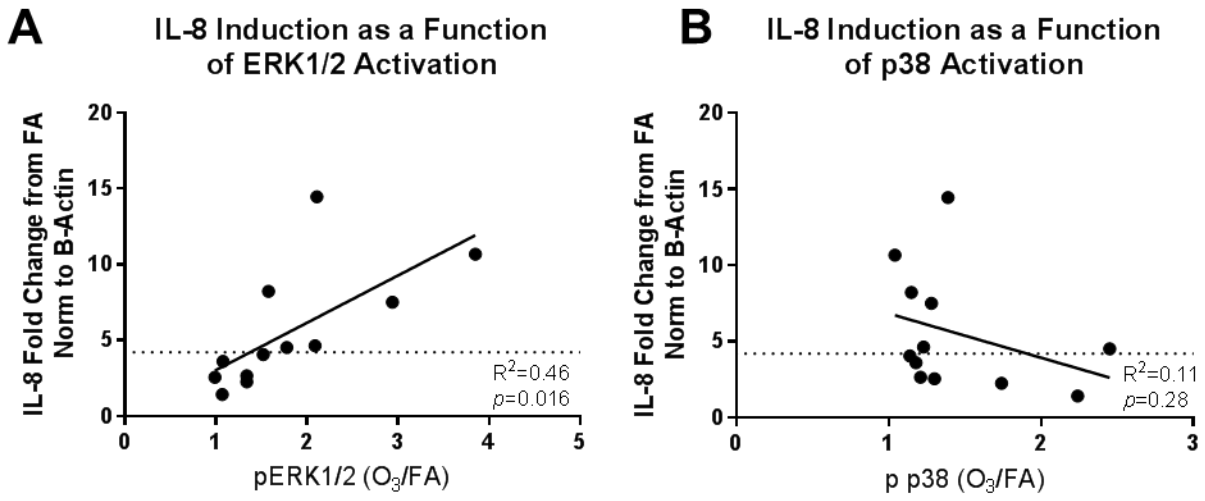

Figure 2-8. *IL-8* expression as a function of ERK1/2 and p38 activation. ERK1/2 and p38 phosphorylation was plotted against *IL-8* induction for both ERK1/2 (A) as well as p38 (B). Figures were created using the same donors as depicted in Figure 2-5 and 2-6 (*n*=12; 6 high and 6 low responders). R^2 and *p*-values shown in each panel.

Baseline versus induced *IL-8* expression and MAPK activation

It has been previously observed that individuals who have relatively higher ozone-associated neutrophilia and IL-8 release exhibit higher levels of airway IL-8 prior to exposure (Fry *et al.*, 2012). We wanted to determine if a similar relationship existed between basal and ozone-associated *IL-8* expression. *IL-8* transcript abundance was quantified in FA conditions and plotted against the *IL*-8 transcript abundance in ozone-exposed cells (Figure 2-9A). We found that phBECs with elevated ozone-mediated *IL-8* inductions also had higher levels of basal transcript (R^2=0.32, *p*=0.014). We also examined whether there was a similar relationship between ERK1/2 and p38 phosphorylation in ozone-exposed and unexposed cells. Consistent with the previously observed pattern in transcript abundance, baseline ERK1/2 and p38 phosphorylation was positively correlated with phosphorylation following ozone exposure (Figures 2-9 B and C; R^2=0.76, *p*<0.0001; R^2=0.63, *p*=0.0007).

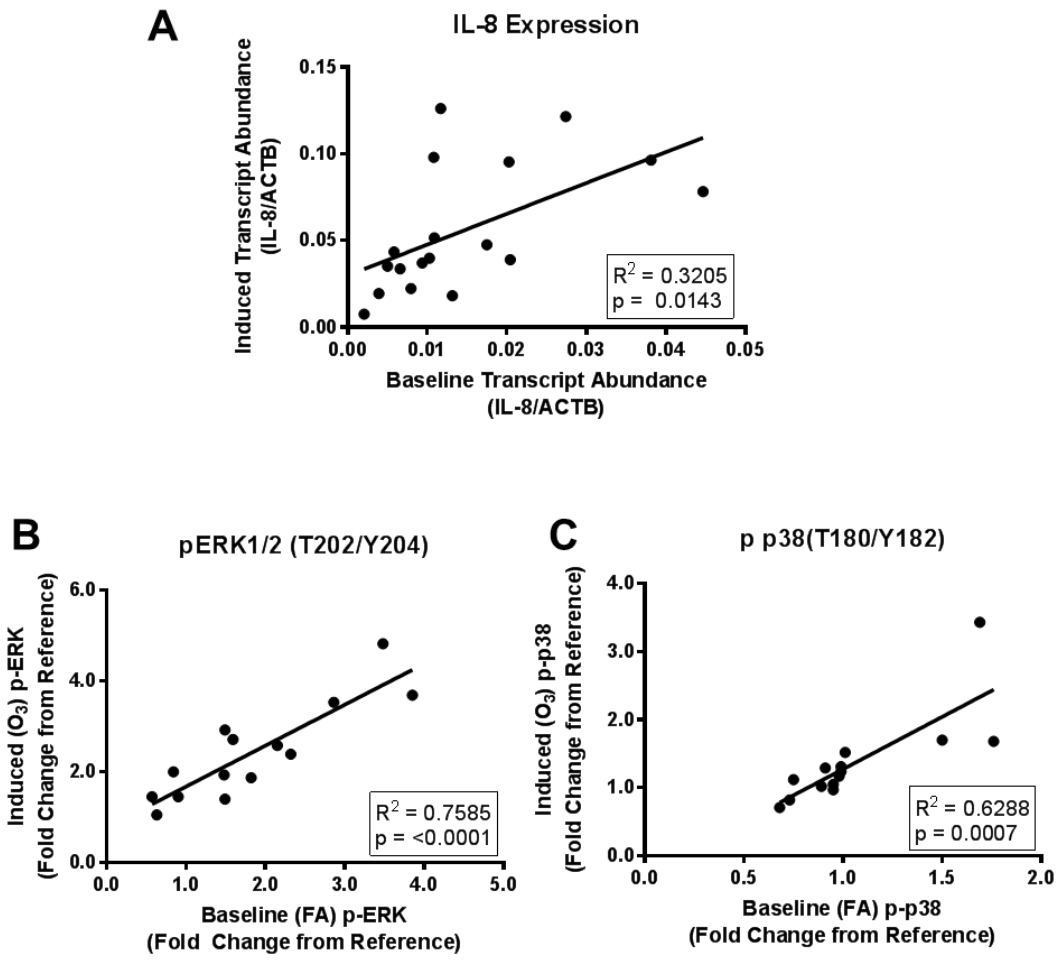

Figure 2-9. Comparison of baseline with induced values of *IL-8* expression and MAPK phosphorylation. We assessed whether basal levels of *IL-8* transcript (A) and MAPK phosphorylation (B, C) were predictive response to ozone exposure. (A) Transcript abundance was measured by making a standard curve using known copy numbers of transcript. These were then normalized to the number of copies of *B-Actin* transcript. (B) To allow for comparison across Western Blots samples, the ratio of phosphorylated to total ERK1/2 (B) and p38 (C) were calculated and normalized to a reference sample and expressed as a fold change of reference.

Heterogeneity and the effect of MAPK inhibition in other ozone-responsive genes.

Response inter-individual variability and the role of MAPK activation in gene induction were also assessed in several other ozone-responsive genes: two pro-inflammatory genes (*IL-6, COX-2*) and one oxidative stress associated gene (*HMOX-1*; Figure 2-10). Gene expression was assess using the same samples that were used to generate Figure 2-1. The mean (±SD) *HMOX-1*

induction was 4.63 ± 3.45. *IL-6* induction was 6.27 ± 5.0, and *COX-2* was 2.82 ± 1.34. Response heterogeneity was also observed in the expression of these genes, with approximately 4-5 donors stratified above the mean. The donors that had phBEC cultures that were considered to be *in vitro* high *IL-8* responders in Figure 2-1 are indicated by the red circles. As these donors are often the highest responders in these other genes, this suggests that *IL-8* expression may be indicative of responses in other important ozone-responsive genes.

We also examined the expression of these three genes after addition of the MAPK inhibitors, as detailed in the experiment in Figure 2-3. Similar to the pattern observed with *IL-8*, *HMOX-1* and *IL-6* exhibited reduced expression with the addition of both ERK1/2 and p38 inhibitors, although results were not statistically significant.

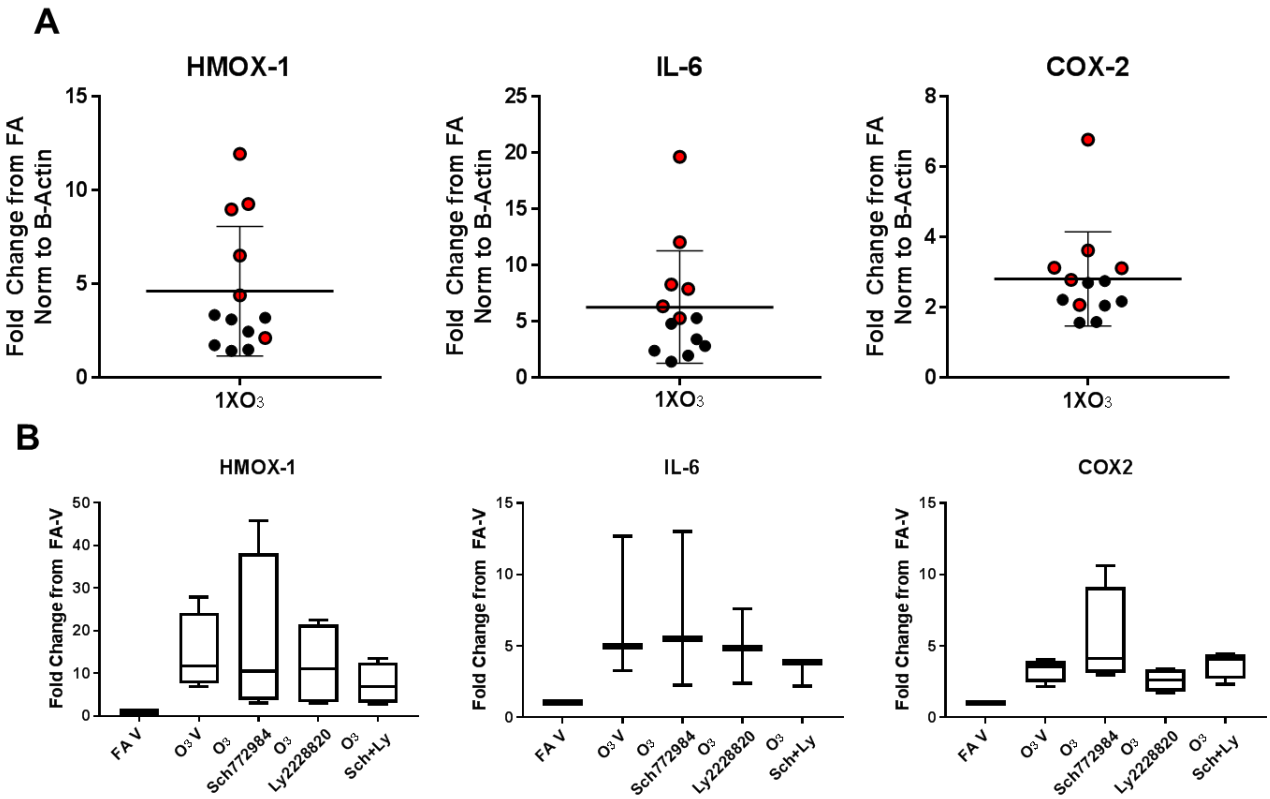

Figure 2-10. Induction and MAPK inhibition in other ozone-responsive genes. (A) Using the same donor pool and techniques as previously described in Figure 2-1, the expression of heme oxygenase 1 (*HMOX-1), IL-6,* and prostaglandin-endoperoxide synthase 2- (*PTGS2* or *COX-2)* were assessed. To enable comparison across genes, the donors that were high *IL-8* responders are indicated by the red circles. (B) The expression of these genes was assessed in the inhibitor treatments as described in Figure 2-3. No statistically significant differences from the O_3-V treatment were found.

Discussion

Inter-individual variability in exposure responses makes it difficult to predict the health effects of environmental pollutants. Ozone is one such pollutant, where for unknown reasons certain individuals exhibit heightened inflammatory responses compared to their less responsive counterparts. IL-8, a potent chemokine, is elevated in the airways following ozone exposure, but its abundance exhibits inter-individual variability, implying that the differential regulation of this gene could be an important factor underlying ozone inflammatory response variability. Here we show that an important source of IL-8 variability may be differences in transcriptional responses in airway epithelial cells. Our data suggest that the magnitude of *IL-8* induction may be due to the intrinsic properties of these cells. Examination of cellular signaling pathways revealed that the level of *IL-8* induction was related to the magnitude of ERK1/2 pathway activation. These findings suggest that the ERK1/2 pathway may be regulated differently in high and low *IL-8 in vitro* responders, a finding that may provide important information regarding ozone response inter-individual variability.

Our data indicate that the ozone pro-inflammatory response of cells harvested from a particular individual are highly reproducible, which suggests that the ozone-mediated inflammatory response adheres to a set of biological rules that have yet to be characterized. Respiratory epithelial cells play a key role in eliciting inflammatory responses, and understanding their biology may offer unique insights into the factors underlying variability in response to ozone exposure. When we exposed these cells to ozone, we observed inter-individual transcriptional *IL-8* variability both *in vitro* and *in vivo.* These responses were similarly distributed but had different magnitudes of induction, which could be explained by differences in ozone dosage, the timing of sample collection, or differences in model system.

Previous *in vivo* studies have shown that individuals who have greater inflammatory response to ozone exhibit heightened airway IL-8 protein levels compared to their less responsive counterparts (Fry *et al.*, 2012). Thus, epithelial cell transcriptional processes may be an important factor in explaining the variability in IL-8 abundance.

Because cells are sensitive to slight variations in culture environment (Hartung *et al.*, 2002), we questioned whether the transcriptional variability we observed in the phBEC system was the result of intrinsic cellular properties or 'noise' introduced by slight variations in culture environment. We confirmed that if *IL-8* responses to ozone could be recapitulated in phBECs collected and cultured independently, suggesting that epithelial cell responses are intrinsic and that these 'programs' drive phBEC response variability. This novel finding supports the growing use of primary cells in environmental health research, as it suggests that cells collected from various donors can be used to investigate inherent cellular differences underlying inter-individual variability.

While our data suggest that epithelial cell responses are conserved, the basis of this 'programming' needs to be determined. While it may simply reflect genetic variability, it is possible that epithelial cells may retain an epigenetic imprint of their donor's life history that can determine future responsiveness (Bowers and McCullough 2017). Although the factors that ultimately determine ozone-associated *IL-8* induction in epithelial cells are unclear, our findings suggest that the differential activation of the MAPK pathway is likely a part of this programming.

Here we propose a model in which ERK1/2 modulates the level of *IL-8* induction (Figure 2-11).

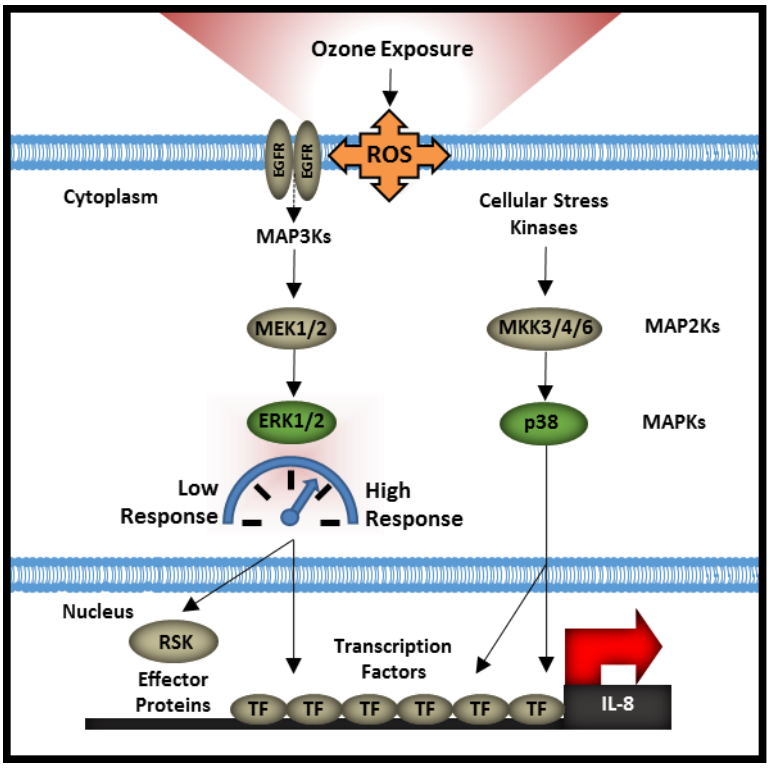

Figure 2-11. Paradigm describing how differences in MAPK signaling lead to inter-individual variability in phBEC ozone-mediated IL-8 induction. Previous studies have demonstrated that ozone exposure results in the production of ROS, which lead to the activation of the MAP Kinases ERK1/2 and p38 by various mechanisms including the activation of the EGFR receptor. PhBEC cultures that have high and low *IL-8* transcriptional responses have similar levels of p38 activation; however, high responders have increased ERK1/2 pathway activation. Thus we propose a model in which the level of ERK1/2 activation modulates the magnitude of the *IL-8* response and differentiates high from low *IL-8* responding epithelial cells. ERK1/2 phosphorylates effector proteins such as RSK and additional kinases, transcription factors, and other stress-associated proteins, which ultimately converge on the *IL-8* promoter and regulate gene transcription.

This model raises important questions regarding why ERK1/2 is differentially activated in high and low-responding cultures. Ozone is known to react with the airway surface and generate reactive oxygen species (ROS) such as hydroxyl radicals, aldehydes, and hydrogen peroxide (Pryor *et al.*, 1995). These ROS, in turn, may directly or indirectly be responsible for activating effectors upstream of ERK1/2. Such proposed effectors include the activation of the EGFR receptor, via the alteration of cell membrane lipid raft composition (Park *et al.*, 2009).

Alternatively, EGFR and other MAPKs may become phosphorylated as a result of ROS directly deactivating phosphatases, which typically act as a brake on MAPK activation (Tal *et al.,* 2006; Yan *et al.*, 2016). Another proposed mechanism is that ozone exposure results in TLR4 signaling, which results in the activation of heat shock proteins, which in turn activate ERK1/2 (Bauer *et al.,* 2011). Thus, activation of the ERK1/2 pathway may vary between high and low responders *in vitro* due to any number of differences such as cell membrane properties, the ability to neutralize ROS, or the balance of kinase and phosphatase activity.

Our data suggest that the level of ERK1/2 activation modulates the magnitude of *IL-8* induction; however, they also suggest that a basal level of p38 has a synergistic effect. ERK1/2 mediates many functions related to proliferation and cell survival while p38 is more commonly associated with stress response and apoptosis. While these kinases have seemingly disparate roles, there is substantial crosstalk between these pathways and their coordinate activity is required for many cellular functions. Here we report that the response to the pro-inflammatory stimulus ozone is one such function, as evidenced by the fact that simultaneous inhibition of ERK and p38 is required to significantly reduce ozone-associated *IL-8* induction (Figure 2-3). These results recapitulate earlier findings from our lab, which assessed the role of p38 and ERK1/2 activation in polarized phBECs (phBECs that have not been differentiated) using a different set of MAPK inhibitors (McCullough et al, 2014). While these early studies established the importance of MAPKs in ozone response, it was unknown whether these findings would also be observed in fully differentiated phBECs and whether MAPKs played a role in ozone responsiveness. In addition to the phBEC system, other model systems have reported similar findings using inhibitors of ERK1/2 and p38. Cytokine expression from the e*x vivo* stimulation of human alveolar macrophages with lipopolysaccharide (LPS) is partially reduced with the

addition of either a p38 or ERK1/2 inhibitor; however, the addition of both inhibitors is necessary to achieve a near abrogation of expression (Carter *et al.,* 1999). The same observation was noted during LPS stimulation of the monocyte cell line THP-1 (Rutault *et al.,* 2000). The requirement for the activation of both kinases for full *IL-8* induction could be explained by the fact that p38 has an essential role in the stabilization of *IL-8* mRNA. The 3' untranslated region (UTR) of *IL-8* mRNA contains several AUUUA motifs which confer instability and result in its rapid degradation; however, the activation of the p38 pathway results in the stabilization of the *IL-8* mRNA transcript by increasing the binding of stabilization factors to these AU-rich regions, thus facilitating the accumulation of *IL-8* mRNA (Winzen *et al.,* 1999; Jijon *et al.,* 2002; Suswam *et al.,* 2005). Thus, p38 and ERK1/2 work in a coordinated fashion to both transcribe *IL-8* and stabilize its transcript.

While coordinate activity may be important for full ozone mediated *IL-8* induction, our data suggest that there is a component distinct to the ERK1/2 pathway, which determines the magnitude of response and is differentially regulated in high responders. A unique feature of the ERK1/2 downstream signaling is the activation of a family of p90 ribosomal S6 kinases (RSKs). RSKs have a multitude of cellular functions relating to growth, proliferation, translation, and also phosphorylate additional transcription factors (Anjum and Blenis 2008). Additionally, RSKs have been shown to bind and enhance the function of activating chromatin-modifying complexes (Nakajima *et al.,* 1996) and can phosphorylate immediate early gene products such as serum response factor (SRF), Fos, and Jun, promoting their stability (Anjum and Blenis 2008). Thus, the activation of downstream targets such as RSKs may augment ozone signaling, leading to greater transcriptional *IL-8* induction in high responders.

While RSK activation may be an important component of ozone responsiveness, there are other important molecular factors likely shaping *IL-8* transcriptional response. This is suggested by the fact that even when both p38 and ERK1/2 inhibitors were used, we did not achieve a complete reduction of expression; moreover, *IL-8* inductions in low responders were not influenced by kinase inhibition. This residual *IL-8* induction may be due to low-level inputs from other cellular effectors, such as NRF2 (Zhu *et al.*, 2016). Moreover, *IL-8* induction in other model systems has been associated with DNA methylation levels as well as post-exposure changes in histone acetylation and methylation (Angrisano *et al.*, 2010, Saccani and Natoli 2002). Together epigenetic factors and other signaling pathways may also influence *IL-8* induction and may account for expression that cannot be explained by MAPK activation.

Here we present an *in vitro* model of *IL-8* expression inter-individual variability that is linked to differential activation of the ERK1/2 pathway. This model can be used to further the understanding of molecular factors driving variability in the pro-inflammatory response to ozone, and could be used to investigate implications of the ozone pro-inflammatory response. However, this work is not without limitations. Our use of inhibitors was essential in this investigation, as fully differentiated phBECs are recalcitrant to transfection/transduction; yet off-target effects are always possible when using these compounds. We attempted to minimize this possibility by using SCH772984 and LY2228820, which are novel compounds noted for their specificity and efficacy even in nanomolar concentrations (Campbell *et al.*, 2014, Morris *et al.*, 2013). Here we use a phBEC model to examine molecular mechanisms underlying differences in *IL-8* expression. While our use of phBEC allowed us to model epithelial cell responses across many individuals, inflammatory responses *in vivo* involve complex interactions with many different cell types. Future studies could utilize co-culture techniques to assess these relationships and

whether similar patterns of ERK1/2 activation are observed in other cell types, such as neutrophils, macrophages, fibroblasts, etc. Future studies will also be required to address whether donors who exhibit heightened inflammatory responses *in vivo* also produce phBEC cultures that are high *IL-8* responders *in vitro*. It is possible that donor characteristics such as genotype, age, sex, and ethnicity may play a role in shaping ozone responses; however we were unable to detect the influence of these factors with only 16 subjects. Relative to many other *in vitro* toxicity studies we have a large number of primary cell donors; however if the characteristics of the phBEC donors are considered, we do not have adequate numbers to conduct this type of analysis. This study also has some unique strengths: whereas previous studies have relied heavily on cell lines, we have used the phBEC system which is more physiologically relevant, facilitates the exploration of inter-individual variability, which formerly was only possible using *in vivo* exposure studies.

Conclusions

Epithelial cells exposed to ozone exhibit inter-individual variability in *IL-8* transcriptional responses, which likely contribute to the inter-individual variability in inflammatory response observed in human clinical ozone exposure studies. The level of ozone-induced *IL-8* transcription is reproducible in phBECs from the same individuals even when collected and cultured at different times, suggesting that *IL-8* response is an intrinsic property of epithelial cells. This finding suggests that we may be able to better understand inflammatory response inter-individual variability by further defining the basis of this programming, and that primary cell models are an effective approach. As a first step to describing the molecular mechanisms underlying these differences, we propose a model where ERK1/2 activation acts as a modulator of *IL-8* induction. Because many different susceptibility factors may ultimately converge on the ERK1/2 pathway, our model could be used to synthesize previous work and streamline the understanding of susceptibility to ozone as well as many other pollutants.

Supplementary Figures

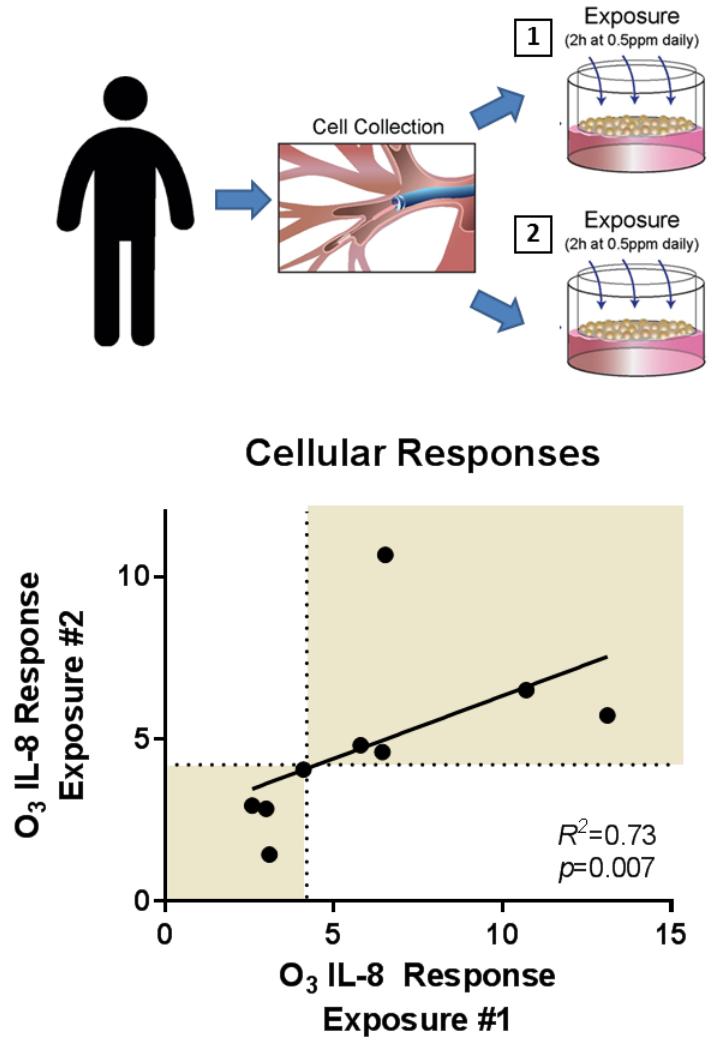

Figure 2-12. *In vitro* ozone *IL-8* induction in cells collected from single bronchoscopy and exposed to ozone at two different times. To assess whether *in vitro* ozone response was an inherent property of cells we performed ozone exposures on cells that were collected from the same bronchoscopy but thawed, plated, and exposed to ozone at different times; The consistency of ozone response was related by plotting the first exposure response on the X-axis and the second on the Y-axis and applying linear regression. *n*=9

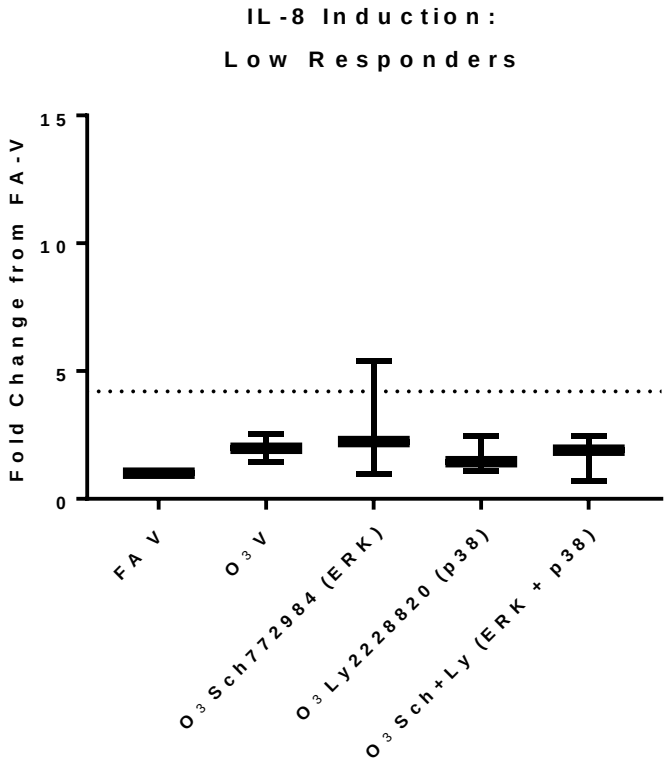

Figure 2-13. The influence of ERK1/2 and p38 inhibition on ozone associated *IL-8* induction in low responders. To determine whether the activation of the MAP kinases ERK1/2 and p38 were required for ozone-mediated *IL-8* induction, inhibitors of these kinases (SCH772984 and LY2228820, respectively) were added to cell media two hours prior to ozone exposure. Seven donors were used, four high responders (Figure 2-3) and three low responders (Figure 2-13). *IL-8* inductions were normalized to a 0.2% DMSO filtered air vehicle control. *IL-8* inductions were compared between the ozone-vehicle control (O_3-V) and all other treatments via 2-way ANOVA. *p<0.05.

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CHAPTER 3: USING PRIMARY EPITHELIAL CELLS TO MODEL OZONE ADAPTATION: INVESTIGATING THE ROLE OF ANTIOXIDANT CAPACITY AND MAP KINASE SIGNALING

Introduction

Ground-level ozone is formed from precursor ambient air pollutants during UV-catalyzed reactions. As a result, ozone levels typically parallel sunlight intensity, peaking in early afternoon and dissipating in the evening. Ambient ozone concentrations are regulated on an eight-hour basis, focusing on the concentrations that occur within a given day. The frequency of high ozone days, however, is also of public health concern because sporadic and repeated daily ozone exposures can produce different health effects. A single, acute ozone exposure causes decrements in lung function, nonspecific airway reactivity, and pulmonary inflammation characterized by increased cytokine levels and leukocyte infiltration (EPA 2013). Paradoxically, repeated daily ozone exposure results in "ozone adaptation" in which many acute exposure effects are reduced or abolished. During inflammatory ozone adaptation, airway cytokine levels and leukocyte infiltration are suppressed (Christian *et al.,* 1998; Devlin *et al.,* 1996; Jörres *et al.,* 2000; Schelegle *et al.,* 2003; Van der Wal *et al.,* 1994). Inflammatory ozone adaptation has been observed in both human clinical exposure studies and various animal models, but despite its extensive observation very little is known about its causal mechanisms and health implications.

Previous studies have hypothesized that ozone adaptation may be mediated by increases in the antioxidant capacity of the airway. This is proposed to occur either by the upregulation of

oxidative stress related genes (Rahman *et al.,* 1991) or by the increased abundance of antioxidants, particularly ascorbic acid, in the fluids lining the airway (Wiester *et al.,* 1996, 2000). While some studies support these hypotheses, findings from other studies are contradictory or are inconclusive (Kodavanti *et al.,* 1995; Nambu and Yokonama 1981; Tepper *et al.,* 1989); thus the role of oxidant/antioxidant balance in ozone adaptation remains unclear. In the time since inflammatory adaptation was first described, there have been significant advances in the understanding of how ozone initiates pro-inflammatory responses, which could provide promising leads in the search for the adaptive mechanism. Epithelial cells are the sentinel cells of the airway, as they are the first to encounter pollutants and play a key role in initiating inflammatory responses. Our lab recently found that in primary bronchial human epithelial cells (phBECs), an *in vitro* model of the airway epithelium, the ozone-mediated induction of pro-inflammatory genes such as *IL-8* occurred primarily through the mitogen-activated protein kinase (MAPK) pathway (McCullough *et al.,* 2014). Two MAPKs, extracellular-signal related kinases (ERK) 1/2 and p38 are critical for this signaling, as inhibition of these kinases greatly reduces pro-inflammatory gene induction in polarized phBECs (McCullough *et al.,* 2014) and fully differentiated phBECs (Chapter 2). While previous studies using cell lines identified NFκB activation as the central mediator of ozone-associated pro-inflammatory signaling, in the more physiologically-relevant phBEC model system, inhibiting NFkB activation does not influence the induction of these genes (McCullough *et al.,* 2014).

Because the airway epithelium plays an integral role in mediating pro-inflammatory responses, it is possible that ozone adaptation may be due to changes in how these cells respond to ozone exposure. Moreover, given the importance of ERK1/2 and p38 in mediating the induction of pro-inflammatory genes, the alteration of MAPK signaling might play an important

but undefined role in differential responses between single and repeated ozone exposure. We therefore hypothesized that inflammatory adaptation can be attributed to reduced ozone responsiveness in airway epithelial cells, which may be mediated by alteration of MAPK signaling.

To investigate both old and new hypotheses regarding adaptive mechanisms, we modeled single and repeated ozone exposures in differentiated phBECs cultured at air liquid interface. As a read-out of pro-inflammatory response we monitored the expression of interleukin 8 (*IL-8*), a potent neutrophil chemokine and hallmark of pulmonary inflammation, which is predominantly regulated by p38 and ERK1/2 in the phBEC ozone-exposure model. We also examined the expression of other genes that are known to contribute to ozone response to determine if transcriptional changes may also occur in these genes. These genes include prostaglandin-endoperoxide synthase 2- (*PTGS2* or *COX-2*), heme oxygenase 1 (*HMOX-1*), and *IL-6*. In parallel with gene expression, we also assessed whether repeated ozone exposure is associated with changes in MAPK activation, as well as increases in antioxidant capacity, measured both by oxidative stress gene expression and the antioxidant capacity of cellular secretions.

We observed peak inductions of all genes following a single ozone exposure and then suppression following repeated exposure, a response that mimics *in vivo* ozone adaptation. We examined both oxidative stress-related gene expression and the total antioxidant capacity of cell secretions and found no increases in cells repeatedly exposed to ozone. We did find, however, that cells exhibiting adaptation had reductions in ERK1/2 activation, which was not observed in non-adapted cells.

Materials and Methods

Cell Culture and Ozone Exposure

Primary human bronchial epithelial cells were obtained via bronchial brushing from healthy, non-smoking donors ages 18-40 with no more than a one pack-year smoking history (Figure 3-1A; Table 3-1). Donors gave their informed consent after being informed of risks and procedures. The consent and collection protocol were approved by the UNC School of Medicine Committee on the Protection of the Rights of Human Subjects and by the US EPA. Cells were cultured as previously described (Ross *et al.,* 2007). Briefly, cells were expanded three passages and then plated on 24 mm Transwell inserts with 0.4 μm pores (Corning Life Sciences, Tewksbury, MA). Once cells were confluent, the medium was supplemented with 500 nM retinoic acid for two days to initiate differentiation of polarized cells into a pseudostratified columnar epithelium. The apical layer of media was then removed forming an air-liquid-interface (ALI). One hundred (100) nM retinoic acid was then added to the medium and cells were cultured for 24 days to allow differentiation to occur. Exposures began on ALI day 24 and occurred daily for four days (Figure 3-1B). All treatments were harvested on the same day, exposure day four, to normalize for the number of days in culture. Prior to each daily exposure, the basolateral medium was replaced and the apical surface was washed with 500 uL Dulbecco's PBS (DPBS, Life Technologies, Carlsbad, CA) to remove cellular secretions. After a two-hour acclimation period, cells were exposed for two hours to a filtered air (FA) control or 0.5 ppm ozone, which has been used for other *in vitro* and *in vivo* studies and is 1.8 times the current safety standard (70 ppb/8h). On the day of harvest, cells designated for protein analysis were harvested immediately after exposure. Cells designated for gene expression analysis were

allowed to incubate for an additional two hours before being harvested using PureLink RNA Mini Kit (Life Technologies). Samples were stored in -80° C until ready for processing.

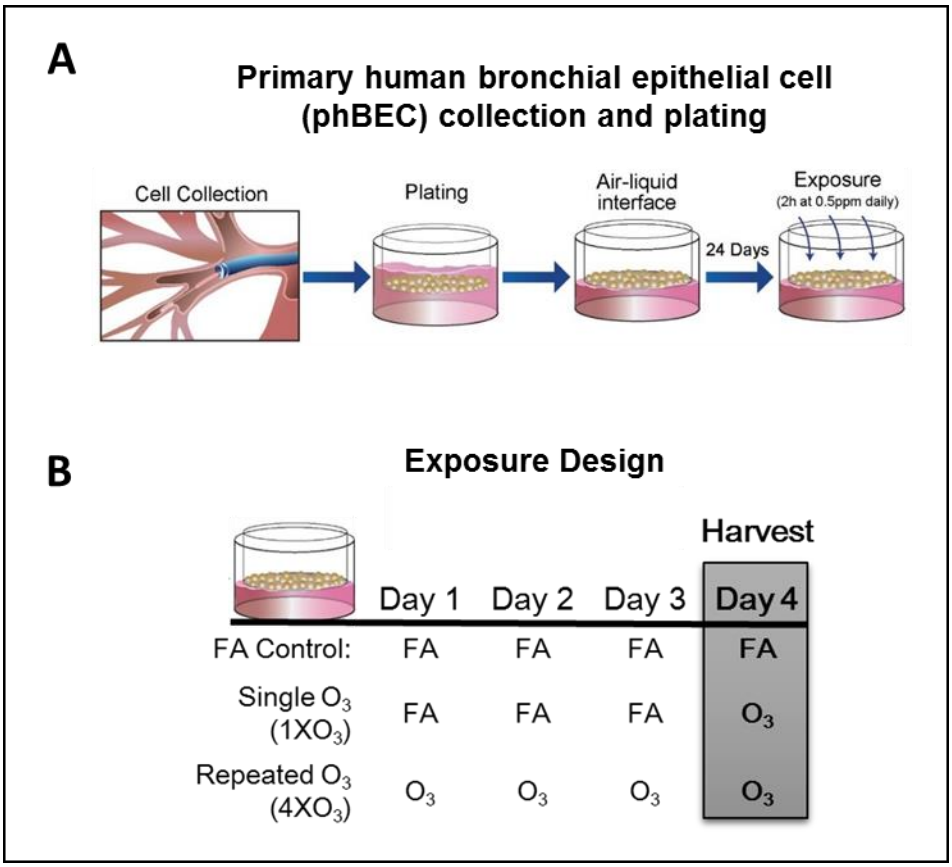

Figure 3-1. Collection, culture, and exposure of primary human bronchial epithelial cells. **(A)** Cells were collected from healthy human volunteers, expanded, and plated on Transwell inserts. After becoming confluent, the apical layer of media was removed forming an air-liquid interface (ALI). Cells were differentiated at ALI over 24 days to allow the formation of a pseudostratified columnar epithelium. On the 24th day, cells were used for exposures. **(B)** Cells from each donor were exposed to four days of FA (FA), three days of FA and a single ozone exposure ($1XO_3$), or four days of repeated ozone exposures ($4XO_3$). RNA and protein were harvested on day four of exposure.

TEER Measurement

Transepithelial electrical resistance (TEER), a measure of epithelial monolayer integrity, was assessed using an epithelial volt/ohm meter at the time of RNA harvest, two hours post exposure (World Precision Instruments, Sarasota, FL). Briefly, one mL of DPBS was added to the apical surface and electrical resistance was measured between the apical and basolateral compartments using a clean, calibrated probe tip. Each condition had a total of nine readings (three readings from three inserts per donor per treatment). Final TEER was calculated by subtracting the resistance of an empty Transwell insert from the reading of each cultured insert. A two-way ANOVA was used to compare mean FA, $1XO_3$, and $4XO_3$ TEER readings and Tukey's multiple comparisons use to measure donor-specific changes

Gene Expression Analysis

Gene expression was assessed using three Transwell inserts per treatment for each donor. RNA was extracted from lysed samples (Life Technologies), and quantified using a NanodropND1000. One μg of RNA was then used to synthesize cDNA using iScript Reverse Transcription Kits (BioRad, Hercules, CA). Target gene cDNA was quantified using TaqMan RT-qPCR primers and probes and the CFX96 qPCR system in triplicate reactions (BioRad). Target gene expression was normalized to the abundance of β-Actin (*ACTB*) and then fold changes between FA and ozone exposure treatments were calculated via the Pfaffl method (Pfaffl, 2001). An Oxidative Stress and Antioxidant Defense Prime PCR Assay (BioRad) was used to assess the expression of oxidative stress-related genes in six donors. For each donor, cDNA from three biological replicates from each treatment (FA, $1XO_3$, or repeated ozone) was

combined into one sample. Target gene expression was normalized to *ACTB* and a fold change between ozone and FA was calculated using the delta-delta Ct method. Genes that were not detected were excluded from analysis. A repeated measures ANOVA was used to identify genes that were differentially expressed between a single and repeated ozone exposure and had a greater than ±1.5 fold change from FA.

Assessing the Antioxidant Capacity of Cellular Secretions

Apical secretions were collected from cells during medium change prior to day four exposure and had therefore had been exposed to either three days of FA or three days of ozone. The apical surface of each Transwell was washed with 400 uL warm DPBS (Life Technologies). Cells and debris were centrifuged at 4°C at 1,000 rpm for 5 minutes. The resulting supernatant was supplemented to 5% glycerol, and frozen at -80° C until ready for processing. The antioxidant capacity of apical washes was quantified using an oxygen radical antioxidant capacity assay (ORAC) according to the manufacturer protocol (Cell Biolabs, San Diego, CA). This technique was chosen because it assesses total antioxidant capacity of all moieties present in the washes (e.g. proteins, lipids, glutathione, ascorbic acid, etc.), thus avoiding *a priori* decisions about which constituents to examine, which is a short-coming of previous studies. Antioxidant content from the apical washes of six donors was assessed. From each donor, each treatment (FA, repeated ozone) was assessed in triplicate.

Immunoblotting

We collected protein from ten phBEC cultures following single and repeated ozone exposures. Cellular extracts were prepared in RIPA buffer (50mM Tris, pH 8.0; 150mM NaCl;

1% Triton X-100; 400μM EDTA; 10% glycerol; 0.1% SDS; 0.1% deoxycholate) with 1X protease (cOmplete EDTA-free, Roche, Indianapolis, IN) and 1X phosphatase (PhosSTOP, Roche) inhibitors and incubated on ice for 20 minutes. Cellular debris was then precipitated via centrifugation and RIPA extract aliquots were removed for protein quantification via BCA assay (ThermoFisher, Waltham, MA). The remaining supernatant was then supplemented with Laemmli buffer to a final concentration of 1X (60 mM Tris, pH 6.8; 200 mM DTT; 10% glycerol; 2% SDS; 0.05% bromophenol blue), incubated at 95°C for five minutes, aliquoted, and stored at -80°C. Equal amounts of protein were loaded into SDS-PAGE gels, electrophoresed, and transferred to nitrocellulose membranes (BioRad). Following primary antibody binding, HRP-conjugated secondary antibodies and Pierce ECL Western blotting substrate (ThermoFisher) were used to generate chemiluminescence. Antibodies are detailed in Appendix 1. Immunoblots were imaged on a LAS-3000 detection system (Fuji/GE Healthcare, Pittsburgh, PA). Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD). The pixel density from the phosphorylated protein was normalized to the pixel density of the total parent protein. The fold change between FA and ozone treatments was then calculated.

Statistical Analysis

With the exception of the PCR Panel analysis, all statistical analyses were conducted using GraphPad Prism 6.07 (GraphPad Software, La Jolla, California, USA). PCR Panel analyses and heat map generation was performed in Partek Genomics Suite 6.0 (Partek Inc., St. Louis, MO). Mean differences between single and repeated ozone exposures were assessed using a Wilcoxon matched-pairs signed rank test. Linear regression was used to assess the

relationship between the magnitude of a single ozone exposure and the adaptive effect. A 2-way ANOVA was used to compare mean TEER between treatments and Tukey's multiple comparisons used to identify significant differences within individual donors. We used a 2-way ANOVA to identify genes that were differentially expressed between single and repeated ozone exposures in the oxidative stress PCR panel. To compare changes in the antioxidant capacity of secretions from ozone and FA-exposed cells, we used a 2-way ANOVA with Sidak's multiple comparisons. To assess differences in protein activation between cells exhibiting adaptation and those that did not, an ANOVA with Dunnett's multiple comparisons was used.

Results

Ozone-responsive gene induction following single and repeated ozone exposure

The inductions of four ozone-responsive genes were examined after single and repeated ozone exposure using cultures from 13 different donors (Figure 3-2). We found that in all four genes, mean inductions were higher following a single ozone exposure ($1XO_3$) compared to repeated ozone exposure ($4XO_3$; $p<0.05$, Wilcoxon matched-pairs signed rank test). The mean (±SD) *IL-8* induction following a single ozone exposure was 4.63 ± 2.45, which then declined to 2.49 ± 0.85 upon repeated exposure. *HMOX-1* induction declined from 4.63 ± 3.45 to 2.88 ± 0.52. The induction of *IL-6* declined from 6.27 ± 5.00 to 3.803 ± 2.00. The induction of *COX-2* declined from 2.82 ± 1.34 to 1.82 ± 0.51.

Donor	Sex	Age	Ethnicity	Mean $1XO_3$ *IL-8*
1	F	35	Black	5.8
2	M	32	White	7.9
3	M	39	White	2.8
4	F	29	White	6.5
5	M	26	White	2.7
6	F	18	Black	5.0
7	M	26	White	9.2
8	M	20	Black	2.4
9	M	34	White	2.9
10	M	21	Asian	1.9
11	M	27	White	3.1
12	M	33	Black	2.4
13	M	39	White	5.7

Table 3-1. Donor characteristics for phBECs depicted in Figure 3-2. Age, sex, ethnicity, and mean *IL-8 in vitro* inductions are shown for reference.

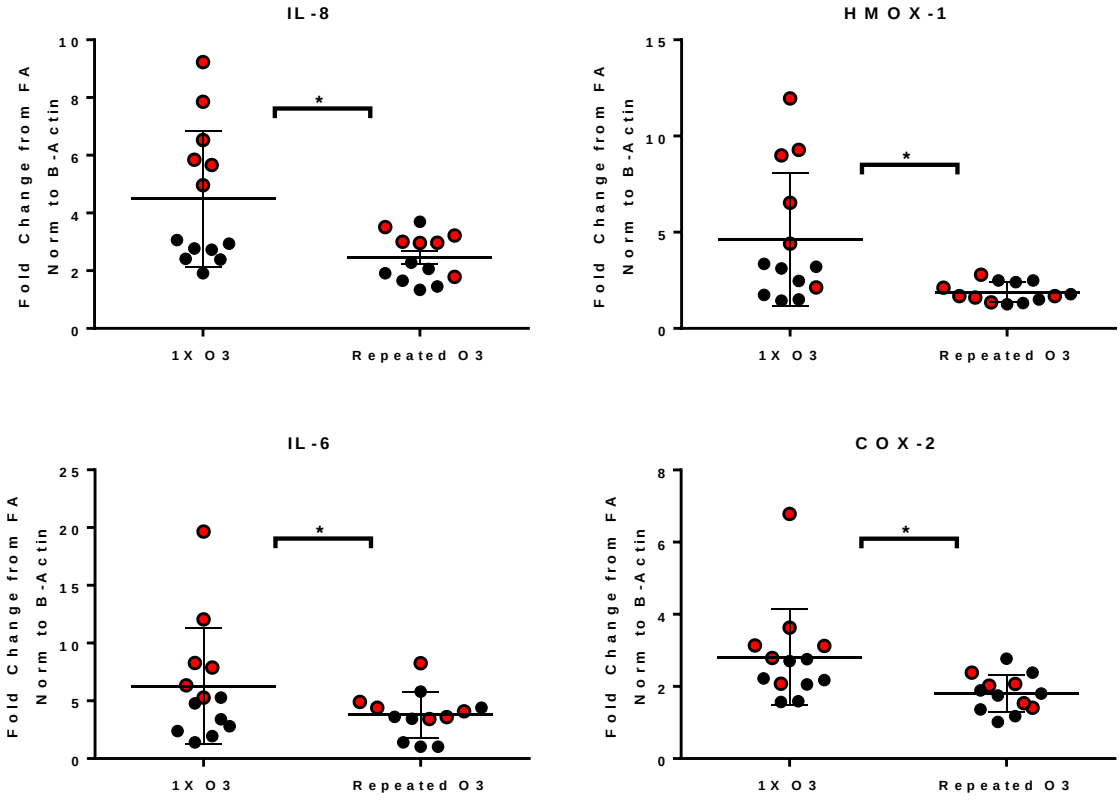

Figure 3-2. Ozone-responsive gene induction following single and repeated ozone exposure. The expression of *IL-8*, *HMOX-1*, *IL-6*, and *COX-2* was assessed via RT-qPCR. Target genes were first normalized to β-Actin and then expressed as a fold change from the FA (FA) control. PhBEC cultures with an *IL-8* induction above the mean are designated by red circles to allow for comparison across genes. A Wilcoxon matched-pairs signed rank test was used to compare means between single and repeated ozone exposures *p<0.05. Mean and SD shown. n=13 cell donors.

PhBEC responses to ozone exposure exhibited considerable donor-to-donor heterogeneity. In all four genes, the inductions of five to six cultures were distributed above the mean (approximately 40% of donors) while the rest of the cultures were clustered below the mean. For all genes the donor cultures that were the most highly responsive were typically from the same donors (indicated by the red dots in Figure 3-2). The widest response range was associated with a single ozone exposure, yet following repeated exposure the range of responses narrowed. We made the

observation that this reduction in range appeared to be driven by cultures with the highest $1XO_3$ inductions exhibiting reductions following repeated ozone exposure. To quantify this relationship, we assessed the correlation between single exposure gene inductions and the magnitude of the adaptive response, as measured by the fold change between single and repeated exposures (Figure 3-3). In all genes, there was a significant relationship between these two variables. The strongest relationships were found for *IL-8* (r^2=0.607, p=0.002) and *HMOX-1* (r^2=0.853, p<0.001). *IL-6* and *COX-2* both exhibited significant correlations (r^2=0.547, p=0.003; r^2=0.720, p=0.002, respectively); however, these distributions both contained an extreme data point that influenced these correlations. Although the Grubb's test identified these data points as 'outliers,' we did not omit them from our analysis because they represent responses that are physiologically possible. Thus cultures with elevated *IL-8, HMOX-1*, *COX-2*, and *IL-6* inductions to ozone exposure are likely to have decreased inductions following repeated exposure while less-responsive cultures will likely undergo little change.

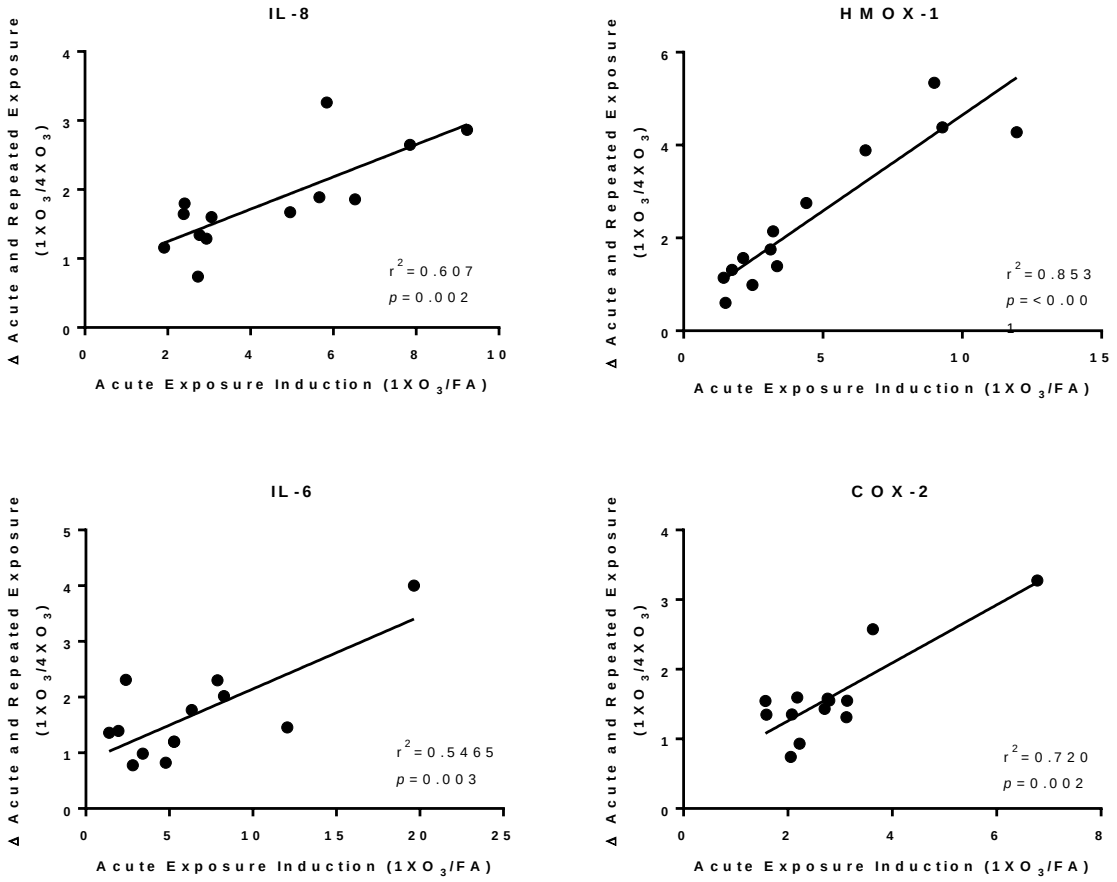

Figure 3-3. Relationship between single ozone exposure induction ($1XO_3$) and the magnitude of the adaptive effect. Linear regression was used to correlate single exposure induction ($1XO_3$) with the magnitude of the adaptive response, which was calculated by taking the ratio between a single and repeated exposure inductions ($1XO_3/4XO_3$). IL-6 and COX-2 both contained outliers as identified by the Grubbs Test. *n*=13 donors, the same donor set as depicted in Figure 3-2.

To ensure that the patterns we were observing were not a byproduct changes in monolayer integrity, we monitored transepithelial electrical resistance (TEER, in Ohms Ω; Figure 3-4). We compared TEER measurements between single and repeated ozone exposure in cells from seven different donors. These cultures had $1XO_3$ *IL-8* inductions ranging from 7.85-2.09. The mean (±SD) TEER following FA exposures across all donors was 304 ± 20.55. TEER declined to 109.9 ± 30.06 following a single ozone exposure and was 140.1 ± 29.6 following

four days of repeated exposure. Although the mean TEER of both single and repeated ozone exposures were significantly different from FA (p <0.05), there was no difference between single and repeated ozone exposures. In addition to comparing the mean TEER between treatment groups, we also analyzed changes within specific donor cultures. Of the seven donors, all had significant reductions in TEER between FA and $1XO_3$ and FA and $4XO_3$. Only one culture, Donor 5, differed between $1XO_3$ and $4XO_3$ exposures (p<0.05).

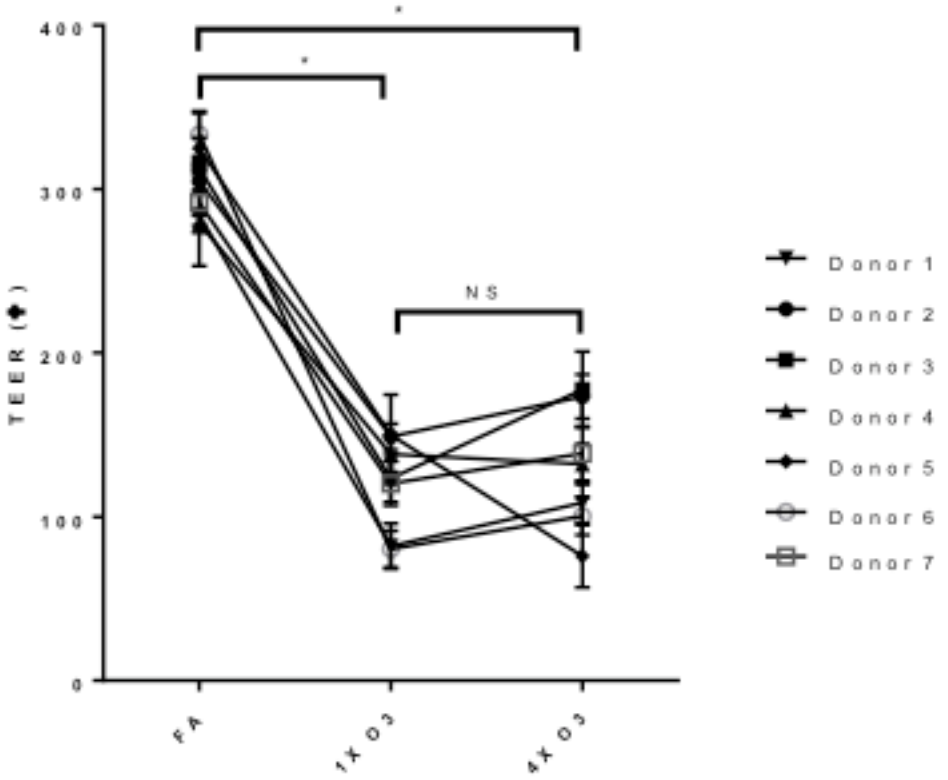

Figure 3-4. TEER after single and repeated ozone exposures. Transepithelial resistance (TEER), a measure of cell monolayer integrity, was measured 2 hours after ozone or FA exposures. Cultures from 7 donors were used (n=7) which had *IL-8* inductions ranging from 7.85-2.09. Treatment effects were compared using a 2-way ANOVA. The mean TEER following both $1XO_3$ and $4XO_3$ ozone exposures were significantly different from FA (*p<0.05); however, $1XO_3$ and $4XO_3$ were not significantly different from each other. Post-hoc multiple comparisons were used to compare TEER within individual donors. All donors had significant TEER reductions between FA and $1XO_3$ and FA and $4XO_3$; however only one donor, Donor 5, exhibited a difference in TEER between $1XO_3$ and $4XO_3$.

Assessing differences in antioxidant capacity in ozone-adapted cells

To test the hypothesis that oxidative stress and antioxidant genes are upregulated during repeated ozone exposure we examined the inductions of 86 antioxidant and oxidative-stress genes following single and repeated ozone exposures using cultures from six donors (Figure 3-5). These donors had $1XO_3$ *IL-8* inductions ranging from 9.26-3.25 fold change from FA. We were able to detect the expression of 75 genes. We expressed inductions as fold-changes between FA and $1XO_3$ and FA and $4XO_3$ and then compared inductions between single and repeated ozone exposures using a repeated measures ANOVA in Partek Genomics Suite. We identified five genes, nitric oxide synthase 2 (*NOS2*), heme oxygenase 1 (*HMOX1*), metallotheionein 3 (*MT3*), prostaglandin-endoperoxide synthase 2 (*PTGS2* or *COX2*), scavenger receptor class A member 3 (*SCARA3*), and heat shock protein family A (*Hsp70*) member 1A (*HSPA1A*), that were differentially regulated ($p<0.05$, FC>±1.5). All genes were down regulated less than three-fold change between single and repeated ozone exposure (Table 3-2). These findings indicate that many genes commonly associated with oxidative stress response are not upregulated as a result of repeated ozone exposure. Moreover, the majority of the genes we assessed exhibited no change in induction, indicating that reductions in our candidate genes are not the result of global gene expression declines.

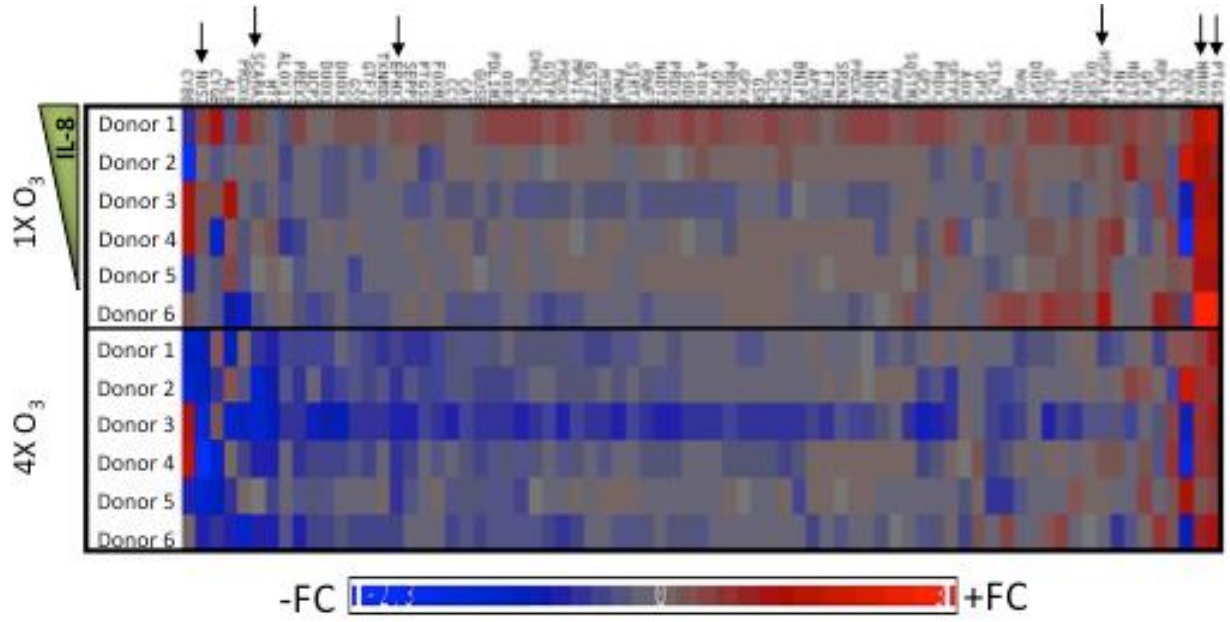

Figure 3-5. Comparing oxidative stress and antioxidant gene expression in single and repeated ozone exposures. To test the hypothesis that genes involved in the oxidative-stress response are upregulated during repeated ozone exposure, we used a PCR panel to examine the inductions of 86 oxidative stress and antioxidant genes following single and repeated ozone exposures. Six primary cell donors (noted as Donors 1 – 6) were used. For both single and repeated ozone exposures, target gene expression was normalized to β-Actin and expressed as a fold change from FA. Fold change values were then log2 transformed to show positive (red) and negative (blue) changes. In the top half of the heatmap donors were ranked in descending $1XO_3$ *IL-8* responses (9.26-3.25 fold change from FA). The same donor order was repeated in the bottom half of the heatmap, which shows inductions following repeated ozone exposure ($4XO_3$; ranging from 2.68-3.57). A two-way ANOVA was used to identify genes that were differentially expressed between $1XO_3$ and $4XO_3$ exposures. Six genes had >±1.5 fold change between treatments and all were downregulated. These genes are indicated by the black arrows in the top panel (Table 3-2).

Gene	Name	P-Value	Log2 Fold Change	Direction
NOS2	Nitric Oxide Synthase 2	4.30E-05	-2.68	1X up vs 4X
HMOX1	Heme Oxygenase 1	0.00616858	-1.97	1X up vs 4X
MT3	Metallothionein 3	7.36E-05	-1.72	1X up vs 4X
PTGS2	Prostaglandin-Endoperoxide Synthase 2	0.0428888	-1.68	1X up vs 4X
SCARA3	Scavenger Receptor Class A Member 3	0.00514513	-1.62	1X up vs 4X
HSPA1A	Heat Shock Protein Family A (Hsp70) Member 1A	0.0148525	-1.49	1X up vs 4X
EPHX2	Epoxide Hydrolase 2	6.43E-05	-1.43	1X up vs 4X

Table 3-2. Oxidative stress and antioxidant genes with differing expression between single (1X) versus repeated (4X) ozone exposure.

Next we assessed whether repeated ozone exposure could increase the antioxidant capacity of cellular secretions, which could putatively provide protection from ozone-generated ROS. To avoid making *a priori* decisions about which secreted components to examine, a potential drawback of previous studies, we used an oxygen radical absorbance capacity (ORAC) assay to measure total antioxidant potential. We performed this assay using phBECs from six donors, which ranged in $1XO_3$ *IL-8* inductions from 4.63 to 2.09 (Figure 3-6). Of the six cultures we assessed, cultures from only two donors had significant differences between FA and ozone treatments. Donor 2 was reduced from (in Troxlox Equivalents, the positive assay control) 12.32 to 6.62 and the second was reduced from 12.55 to 6.72 ($p<0.05$, 2-way ANOVA with Sidak's multiple comparisons). These results demonstrate that the antioxidant capacity of epithelial secretions are decreased, not increased, as a result of repeated ozone exposure.

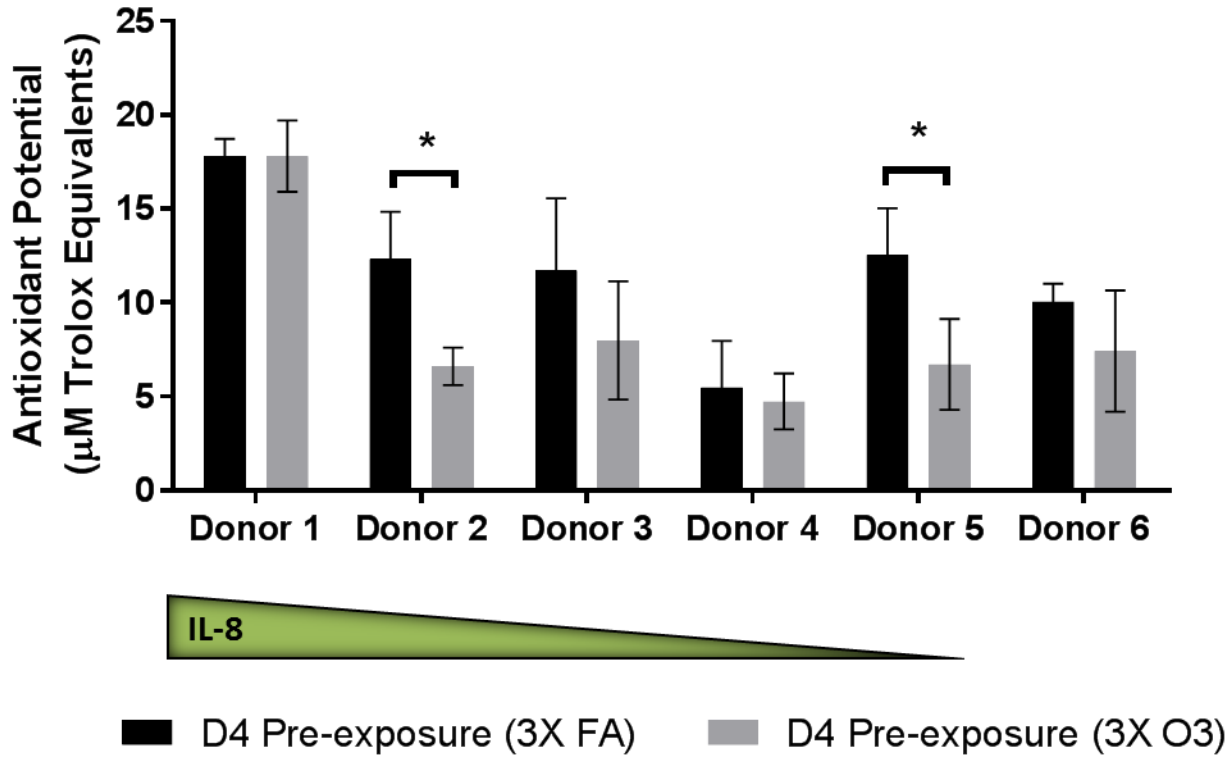

Figure 3-6. Antioxidant potential of apical cell secretions measured before Day 4 exposure. Apical washes were collected during the day four media change prior to exposure to assess if cells that had been exposed to ozone had increased antioxidant potential in their secretions. Antioxidant potential was assessed using the oxygen radical absorbance capacity (ORAC) assay and expressed in Trolox (antioxidant) µM equivalents. For comparison, donors are ordered on the X-axis from greatest to least $1XO_3$ *IL-8* induction (ranging from 4.63-2.09). Washes from three cell inserts per donor were assessed in duplicate. Mean ±SD shown. Differences in FA and O_3-exposed cells were assessed via 2-Way ANOVA with Sidak's multiple comparisons (*p<0.05). n=6 donors.

MAPK pathway activation following single versus repeated ozone exposure

We collected protein from 10 primary cell cultures that underwent single and repeated ozone exposures. Because ozone-associated *IL-8* induction is predominantly regulated by p38 and ERK1/2 activation in phBECs, we subdivided these cultures based on whether they exhibited *IL-8* adaptation to determine whether adapting and non-adapting cultures exhibited different patterns of MAPK activation. *IL-8* adaptation was determined by t-test (p<0.05) comparing

$1XO_3$ and $4XO_3$ *IL-8* inductions. Four donors exhibited a statistically significant reduction in *IL-8* induction between single and repeated ozone exposures, while six donors did not. Representative Western blots depicting a phBEC culture exhibiting *IL-8* adaptation and one that did not are shown in Figure 3-7A. ERK1/2 and p38 activation for each phBEC culture is shown by the line graphs in Figure 3-7B, and suggest that indeed *IL-8* adapted and non-adapted cells exhibit different patterns of MAPK activation during single and repeated exposures. We then compared the mean phosphorylation of ERK1/2 and p38 in both of these groups following single and repeated ozone exposures using a 2-way ANOVA with Dunnett's Multiple Comparisons. We observed that in cultures exhibiting *IL-8* adaptation, mean ERK1/2 phosphorylation significantly increased 2.6 fold (± SD, 1.0) from FA following a single ozone exposure, but then decreased to 1.45 fold (± 0.12) following repeated ozone exposure. ERK1/2 activation following repeated exposed was significantly different from both FA and single exposure levels. Alternatively, in donors that did not exhibit *IL-8* adaptation ERK1/2 activation significantly increased 1.99 (± 0.41) fold change from FA and then increased to 2.30 (± 0.54) following repeated exposure. Although for this group both single and repeated exposure activation levels were significantly different from FA, they were not significantly different from each other.

We also examined the mean activation of p38 in these groups. In the cultures exhibiting *IL-8* adaptation, p38 activation significantly increased to 1.28 (± 0.19) fold change from FA following a single ozone exposure. Following repeated exposure, activation fell to 1.16 fold change (± 0.14), which was not significantly different from FA levels. In cultures that did not exhibit adaptation, mean p38 activation following a single exposure was 1.22 (± 0.07) and following repeated exposure it was 1.25 (± 0.13), both of which were significantly greater than FA levels.

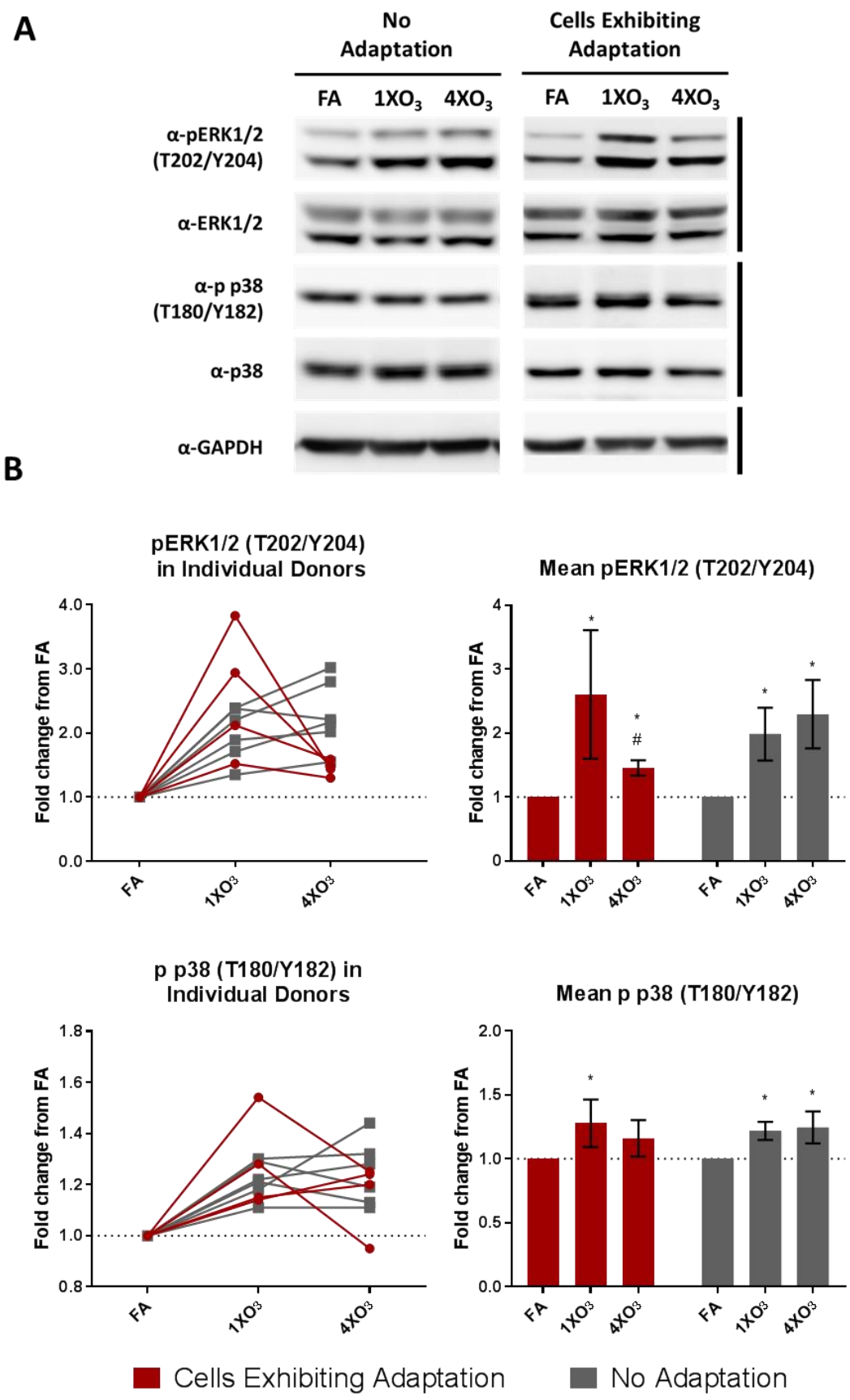
A
No Adaptation
Cells Exhibiting Adaptation
FA
1XO3
4XO3
FA
1XO3
4XO3
α-pERK1/2 (T202/Y204)
α-ERK1/2
α-p p38 (T180/Y182)
α-p38
α-GAPDH
B
pERK1/2 (T202/Y204) in Individual Donors
Fold change from FA
Mean pERK1/2 (T202/Y204)
Fold change from FA
p p38 (T180/Y182) in Individual Donors
Fold change from FA
Mean p p38 (T180/Y182)
Fold change from FA
Cells Exhibiting Adaptation
No Adaptation

Figure 3-7. MAPK pathway activation after single and repeated ozone exposures.
We collected protein from 10 primary cell cultures (n=10 donors) that underwent single and repeated ozone exposures. Because ozone-associated *IL-8* induction is predominantly regulated by p38 and ERK1/2 activation in phBECs, we subdivided these cultures based on whether they exhibited *IL-8* adaptation to determine whether adapting and non-adapting cultures exhibited different patterns of MAPK activation. *IL-8* adaptation was determined by t-test ($p<0.05$) comparing $1XO_3$ and $4XO_3$ *IL-8* inductions. (A) Representative western blots showing ERK1/2 and p38 activation. (B) Four donors exhibited a statistically significant reduction in *IL-8* induction between single and repeated ozone exposures (shown in red), while six donors did not (grey). The bar graphs depict the mean (± SD) change in ERK and p38 activation in both of these groups following single and repeated ozone exposures. Group means were compared using a 2-way ANOVA with Dunnett's multiple comparisons. The * indicates treatments that are significantly different from FA ($p<0.05$), while # indicates significant differences between $1XO_3$ and $4XO_3$ ($p<0.05$).

Discussion

Health outcomes resulting from exposure to the model air pollutant ozone are highly dependent on exposure history. An isolated exposure to ozone results in elevated inflammation, whereas repeated daily exposure results in inflammatory suppression, which has unknown health implications. Inflammatory ozone adaptation has been repeatedly observed in controlled human and animal exposure studies, yet it remains poorly understood due to a lack of efficient exploratory models. To overcome this obstacle, we modeled single and repeated ozone exposures using phBECs cultured at ALI and tested old and new hypotheses regarding adaptive mechanisms. Our findings suggest that inflammatory adaptation may be mediated by reduced pro-inflammatory gene expression in airway epithelial cells. While ozone adaptation was previously attributed to increases in antioxidant capacity, we did not observe this association. Instead, we found that suppression in ozone-responsive gene expression was paralleled by decreases in ERK1/2 activation, which is a central mediator of ozone-responsive gene induction. Interestingly, not all cultures exhibited pro-inflammatory suppression, suggesting that adaptation may be part of a more complex ozone response phenotype and could be an important component of air pollutant susceptibility.

One of the best ways to gain insight into the health implications of inflammatory adaptation is to understand its underlying mechanisms. Using the *in vitro* phBEC model, we discovered that reduced induction of pro-inflammatory genes in airway epithelial cells may be an important component of this response. We investigated two potential adaptive mechanisms: antioxidant capacity and MAPK activation.

A major theme within ozone research is the role of antioxidants in counterbalancing reactive oxygen species (ROS) and protecting against adverse exposure effects. Indeed, previous

studies have found that antioxidant supplementation can reduce the severity of ozone-mediated lung function impairment, albeit with a less-clear effect on inflammation (Romieu *et al.*, 1998; Samet *et al.*, 2001, Sienra-Monge *et al.*, 2004). Moreover, associations between repeated ozone exposure and the increased abundance of certain antioxidants in the airway lining fluid have been observed (Rahman *et al.*, 1991; Wiester *et al.*, 1996, 2000). In our model we failed to observe any increases in the expression of oxidative stress and antioxidant response genes following repeated ozone exposure. Moreover, the majority of surveyed gene inductions were unchanged, indicating that the effects we observed were not an artifact of global gene expression declines. Because analyzing gene expression may not account for changes in the amount or composition of cellular secretions, we also assessed the total antioxidant capacity of apical secretions and again, found no increases. While these findings are contrary to past hypotheses, they are corroborated by the observation that during repeated ozone exposure oxidative damage continues to accumulate in the lung, suggesting that ozone-generated ROS are not counterbalanced (Tepper *et al.*, 1989). Even if antioxidant capacity changes were contributing to inflammatory adaptation in a way not predicted by our findings, our results suggest that other mechanisms are involved with the inflammatory adaptive response which may be equally important.

While we did not find evidence of increased antioxidant capacity, we did find that MAPK activation was associated with inflammatory adaptation. The MAP kinases ERK1/2 and p38 are associated with a variety of essential cellular functions including proliferation, differentiation, survival, stress response, motility, and apoptosis. These kinases also have a critical role in regulating inflammatory responses. When we compared the activation of these kinases in our model, we found that ozone-adapted cells had reductions in ERK1/2 and p38 activation, while non-adapted cells did not. Previous studies in the phBEC model have shown that during ozone

exposure the induction of *IL-8* is predominantly controlled by the dual activity of p38 and ERK1/2, suggesting that the mechanism of *IL-8* adaptation is likely the reduced activation of these kinases. Given the critical role of IL-8 in attracting neutrophils and activating immune cells, this finding offers mechanistic insight into an important component of inflammatory ozone adaptation. Although the mechanisms controlling the ozone-mediated induction of our other candidate genes are not as well characterized in the phBEC model, studies in other systems have demonstrated that p38 and ERK1/2 signaling have central roles in the induction of *IL-6* (Heinrich *et al.*, 2003), *COX-2* (Tsatsanis *et al.*, 2006), and *HMOX-1* (Alam & Cook 2006). Thus, the reduced activation of p38 and ERK1/2 likely have regulatory implications in these genes as well.

Inflammatory ozone adaptation has been long-observed but critical questions remain regarding its mechanism and health implications. Part of the reason for this knowledge gap may be the fact that inflammatory ozone adaptation research was overshadowed by earlier findings on lung function adaptation, which some downplayed as a significant public health issue in the mid-1980`s. While regaining lung function is beneficial in the short term, researchers dismissed the overall importance of lung function adaptation (Linn *et al.*, 1982) because it does not prevent oxidative damage from occurring in the lung (Tepper *et al.*, 1989) and it is also relatively brief, lasting less than one week (Linn *et al.*, 1982). Moreover, it is thought that ozone-associated lung function impairment and inflammation arise from different mechanisms, as they occur within different time frames and don't necessarily occur in the same individuals (Blomberg *et al.*, 1999; Balmes *et al.*, 1996), thus researchers should be cautious about combining these distinct phenomena under the umbrella term "ozone adaptation."

The suppression of inflammation could be important in modulating the health effects of ozone exposure. Ozone-mediated increases in airway cytokines, such as IL-8 and IL-6, can

exacerbate diseases such as asthma and COPD and lead to increased hospitalizations (Burnett *et al.,* 1997; Moore *et al.,* 2008). Elevated levels of certain cytokines in the blood are also a risk factor for cardiovascular disease. IL-6, for example, has been found to promote atherosclerosis, hypertension, and can alter endothelial physiology (Yudkin *et al.,* 2006). While the source of increased circulating ozone-associated IL-6 is not clear, it is possible that IL-6 produced from airway epithelial cells could be an important contributor. Several of the pro-inflammatory mediators implicated in cardiopulmonary disease exacerbation also show adaptation during repeated ozone exposure, both in the airway (Jörres *et al.*, 2000; Devlin *et al.*, 1996), and putatively the blood (Thompson *et al.,* 2010). Thus, it could be hypothesized that ozone inflammatory adaptation could protect cardiovascular health and prevent pulmonary disease exacerbation by tempering the expression of pro-inflammatory genes which might otherwise be continually elevated. A recent epidemiological study supporting this hypothesis found that ozone-associated cardiopulmonary mortality exhibited adaptation, where the highest mortality rates occurred at the beginning of the high ozone season but then continuously declined, reaching a null effect by early fall (Zanobetti and Schwartz 2008). In addition to those with pre-existing diseases, inflammatory adaptation could benefit otherwise healthy individuals by limiting airway remodeling or lung damage caused by excessive or repeated bouts of inflammation.

While inflammatory adaptation could have cardiopulmonary benefits, it may also have detrimental health effects. Exposure to ozone damages respiratory tissue, which infiltrating leukocytes help to clear and heal. Inflammatory mediators and immune cells are also essential in host defense, thus individuals who exhibit inflammatory adaptation may be less able to remove and repair damaged tissue and may be more susceptible to infection. It is currently unknown whether inflammatory adaptation may have negative effects in individuals with impaired

mucociliary clearance or reduced host defense capability. Thus, the health effects of inflammatory ozone adaptation should be carefully weighed and susceptible subpopulations should be given special consideration.

Chronic inflammatory diseases such as asthma, COPD, Crohn's Disease, rheumatoid arthritis, and Alzheimer's disease have been associated with elevated MAPK activation, and can be treated by using clinically approved MAPK inhibitors (Bhavsar *et al.,* 2010; Hommes *et al.,* 2002; Kaminska 2005; Underwood *et al.,* 2000). While clinical intervention may be required in individuals with poorly controlled inflammation, there are inherent negative feedback systems for controlling MAPK activity. Ozone adaptation is likely a phenotype resulting from the actions of these systems, which reduce potentially excessive inflammation. Suppression of MAPKs and other pro-inflammatory pathways has been observed in other adaptive responses, for example, lipopolysaccharide (LPS) tolerance. LPS exposure induces a potent pro-inflammatory response, which is then attenuated during repeated exposure. LPS tolerance has been observed for decades on both cellular and organismal levels and unlike inflammatory ozone adaptation, many mechanisms underlying LPS tolerance have been identified. These repressive mechanisms target both the MAPK pathway and pro-inflammatory genes and include: the upregulation of microRNAs, alterations in the activity or abundance of cellular signaling proteins and receptors, and epigenetic alterations at pro-inflammatory gene promoters (Biswas and Lopez-Collazo 2009; Fan and Cook 200; Foster *et al.,* 2007). Borrowing from this literature, we are currently investigating how these mechanisms contribute to the adaptive response. Epigenetic alterations at target gene promoters may be of particular importance in this model, as our lab has previously shown that histone modifications can influence the induction of ozone-responsive genes (McCullough *et al.,* 2016).

Using the phBEC model system we have made the novel observation that pro-inflammatory ozone adaptation can be modeled *in vitro* and, furthermore, we are among the first to explore its associated mechanisms. This research, however, is not without limitations. Perhaps the greatest restriction is that we are only able to model part of the adaptive response, inflammatory adaptation, as lung function changes cannot be assessed by an *in vitro* model. Moreover, we are unable to model other potential contributors to the adaptive response, such as changes in breathing habits and the role of other cell types. Epithelial cells are among the first cells to encounter ozone and initiate immune responses; however, inflammatory responses involve a complex interplay with other cell types such as fibroblasts, neutrophils, macrophages, etc. While it is unclear what role these other cells may play in inflammatory adaptation, other models (i.e. LPS tolerance) have shown that adaptive responses are conserved across many cell types. Thus, many of the observations we have described could also apply to other cells in the airway. Future studies could examine this possibility using a combination of monoculture and co-culture techniques. Additional questions remain regarding how demographics such as age, sex, genotype, may influence responses to repeated ozone exposure and adaptation. Unfortunately we did not have a large enough sample size to conduct such as investigation.

Here we have taken the first steps toward investigating an adaptive mechanism, the reduction of MAPK signaling. However, further steps are required to discover why ERK activation is reduced. The LPS tolerance literature indicates that a number of overlapping mechanisms could be responsible, including altered expression of signaling proteins, microRNA-mediated inhibition, or epigenetic modulation. Future work can examine the relative contributions of these mechanisms to inflammatory ozone adaptation.

Conclusions

Taken together, our results demonstrate that inflammatory ozone adaptation is may be mediated by reduced pro-inflammatory gene expression in airway epithelial cells. The reduced activation of MAPKs likely mediates the adaptation of *IL-8* and possibly other ozone-responsive genes. Inflammatory adaptation may be an important yet unappreciated factor in determining how those with pre-existing diseases respond to ozone exposure. The *in vitro* model presented here can be used to further investigate the heterogeneity, mechanisms, and health implications of this response.

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CHAPTER 4: OZONE-ASSOCIATED HISTONE MODIFICATION CHANGES AT BIVALENT GENE PROMOTERS ARE ASSOCIATED WITH THE MAGNITUDE OF GENE INDUCTION[4]

Introduction

Ozone is an ambient air pollutant that produces pulmonary inflammation; however, the extent of this inflammatory response exhibits wide inter-individual variability, which is currently not well understood. Traditional risk factors such as age, disease state, sex, and genotype are poor predictors of susceptibility to exposure effects. Ozone-induced inflammation is propagated by the release of cytokines and chemokines into the airway, thus an important source of ozone inflammatory response variability may be differences in the induction of pro-inflammatory genes. Recently we described an application of primary human bronchial epithelial cell (phBEC) cultures in which responses across different human donors are compared to identify mechanisms of inter-individual variability. Using this system, we found that the epigenome may play an important but poorly characterized role in determining variability in the pro-inflammatory response to ozone exposure (McCullough *et al.*, 2016).

The epigenome – a suite of covalent modifications to DNA and its histone protein scaffolding – dictates chromatin structure, interactions between the transcriptional machinery

[4] Portions of this chapter previously appeared in Bowers, E. C., & McCullough, S. D. (2017). Linking the Epigenome with Exposure Effects and Susceptibility: The Epigenetic Seed and Soil Model. Toxicological Sciences, 155 (2): 302-314. Used with permission.

and DNA, and ultimately gene expression. While epigenetic modification of DNA is limited to methylation, histone proteins are decorated with a broad range of covalent modifications, including methylation, acetylation, phosphorylation, ubiquitination, and many others (Kouzarides, 2007). Patterns of activating modifications facilitate an open chromatin structure ("euchromatin") where DNA is accessible to transcription factors, while patterns of repressive modifications lead to compaction. Some genes contain both activating and repressive histone modifications in their promoters. Genes with these "bivalent" promoter regions can switch between active, poised, repressed, or silenced states depending on the balance of these modifications (Bernstein *et al.,* 2006, Mikkelsen *et al.,* 2007; Figure 4-1). While bivalency is often associated with developmental genes, many inducible genes also exhibit bivalent promoters, including the ozone responsive genes interleukin (*IL*) *-8, IL-6,* heme oxygenase 1 (*HMOX-1),* and prostaglandin-endoperoxide synthase 2- (*PTGS2* or *COX-2)* (Figure 4-2).

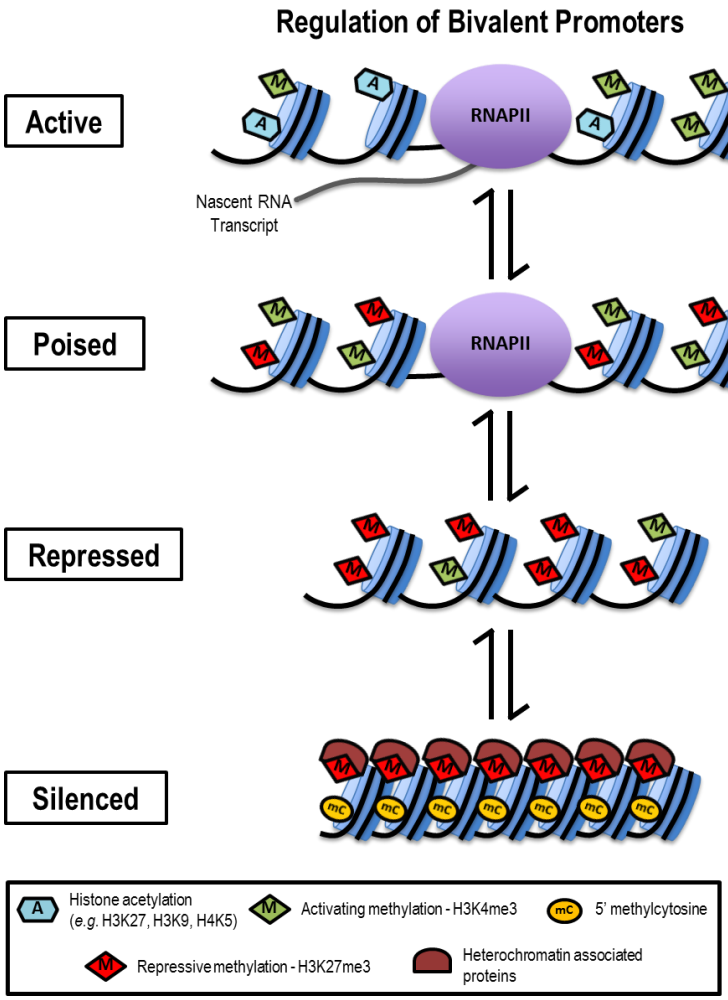

Figure 4-1. Bivalent gene promoters regulate expression based on the balance of activating and repressive histone modifications. [5]Bivalent modifications occur in gene promoters (H3K4me3/H3K27me3) and enhancers (H3K27ac/5mC) in both stem and somatic cells. The balance of otherwise opposing modifications determines whether a gene is repressed, poised (contains a paused polymerase ready to initiate transcription), or actively expressed. This figure is a representation of the generalized functions of certain epigenetic factors; however, the functionality of epigenetic modifications can vary based on the specific context in which they exist.

[5] This figure and caption previously appeared in Bowers, E. C., & McCullough, S. D. (2017). Linking the Epigenome with Exposure Effects and Susceptibility: The Epigenetic Seed and Soil Model. Toxicological Sciences, 155 (2): 302-314. Used with permission.

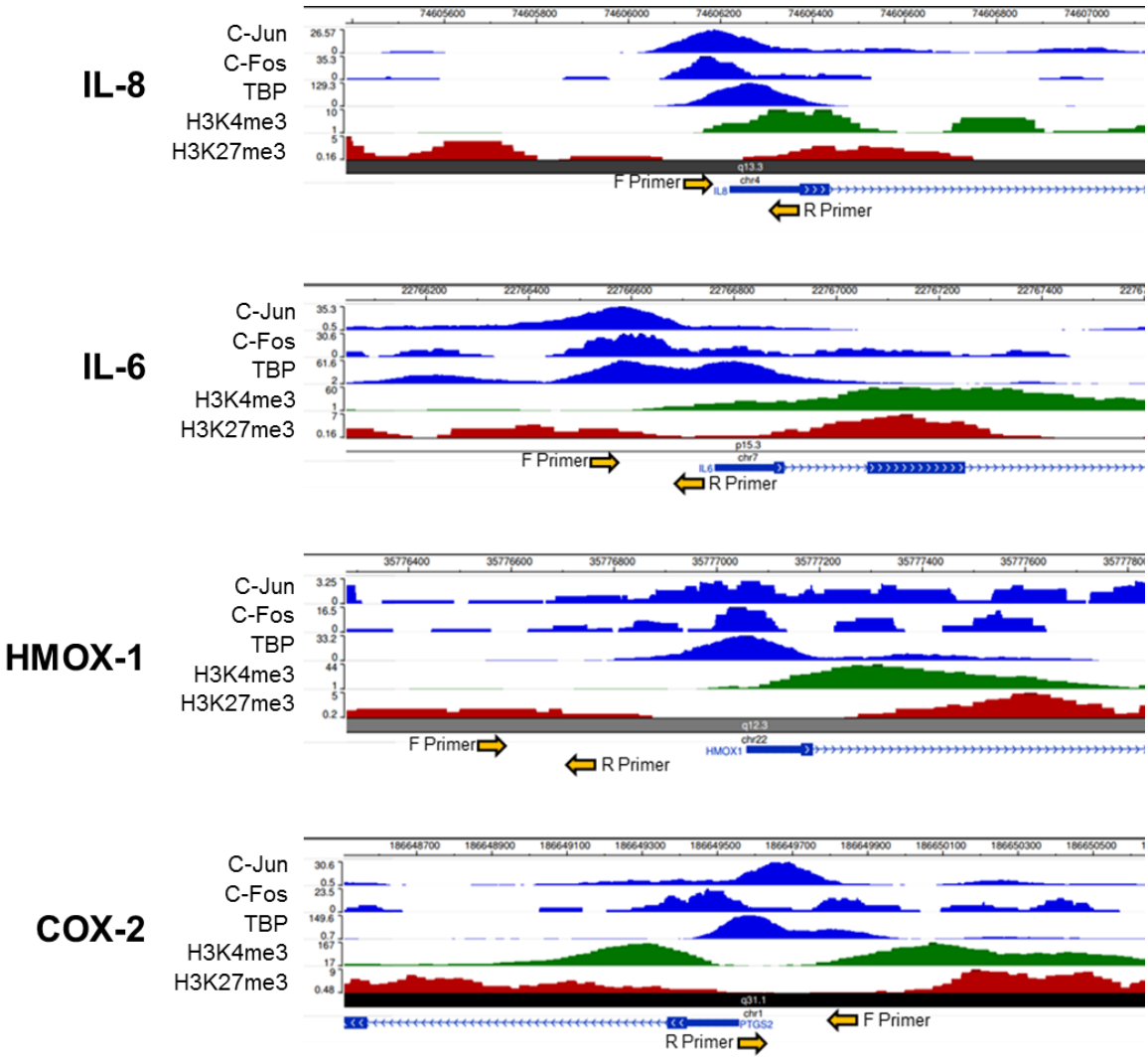

Figure 4-2. ENCODE ChIP-seq data showing selected epigenetic features of the promoter regions of ozone-responsive genes. Figures were generated using the Roadmap Epigenome Browser v 1.19 and the WashU Epigenome Browser (http://epigenomegateway.wustl.edu). Features include binding sites of TATA-Binding Protein (TPB), which is proximal to the transcription start site, as well two stress-associated transcription factors, c-Jun and c-Fos, which are known to be down stream of pathways activated by ozone exposure. Also shown are two chromatin modifications which are often present at bivalent promoters: H3K27me3, which is commonly associated with repression or silencing, and H3K4me3, an activating mark. We also examined the acetylation of Histone H4, but information regarding H4 acetylation is unavailable in the Epigenomics roadmap data. The binding sites of primers used in this study are indicated by yellow arrows.

Based on this premise, we hypothesized that changes in the abundance of activating or repressive modifications shape the transcriptional response to ozone exposure and may explain transcriptional inter-individual variability. To test this hypothesis, we collected phBECs from

different donors and performed *in vitro* ozone exposures. We used chromatin immunoprecipitation (ChIP) – qPCR to examine two marks that are commonly found at bivalent gene promoters, activating trimethyl histone H3 lysine 4 (H3K4me3) and repressive H3K27me2/3 (Bernstein *et al.,* 2006, Mikkelsen *et al.,* 2007). We also examined the abundance of pan-acetyl histone H4 (H4Ac), an activating modification that has been shown to be important in the regulation of pro-inflammatory genes (Foster *et al.,* 2007). We calculated the fold change in abundance of these modifications between ozone and filtered air exposures and then compared these values with the peak transcriptional responses of the four aforementioned genes. If our hypotheses is correct, then we would expect that phBECs with higher gene inductions would exhibit greater fold changes in the abundance of either activating (increases) or repressive (decreases) histone modifications. Because important regulatory epigenetic changes could putatively precede or co-occur with peak gene expression, we compared epigenetic changes during peak gene induction and one hour prior to peak induction (Figure 4-3).

We discovered that peak gene expression of *COX-2* and *IL-6* peak (2H) was associated with increases in activating histone modifications either directly before or coinciding with peak gene expression. Alternatively, we observed that peak *IL-8* expression was associated with reduction of repressive H3K27me2/3. We did not observe any associations with *HMOX-1*. These results suggest that ozone-associated changes in the chromatin landscape may be an important determinant of transcriptional response in pro-inflammatory genes; however, in other genes, such as *HMOX-1*, other regulatory factors may be more important.

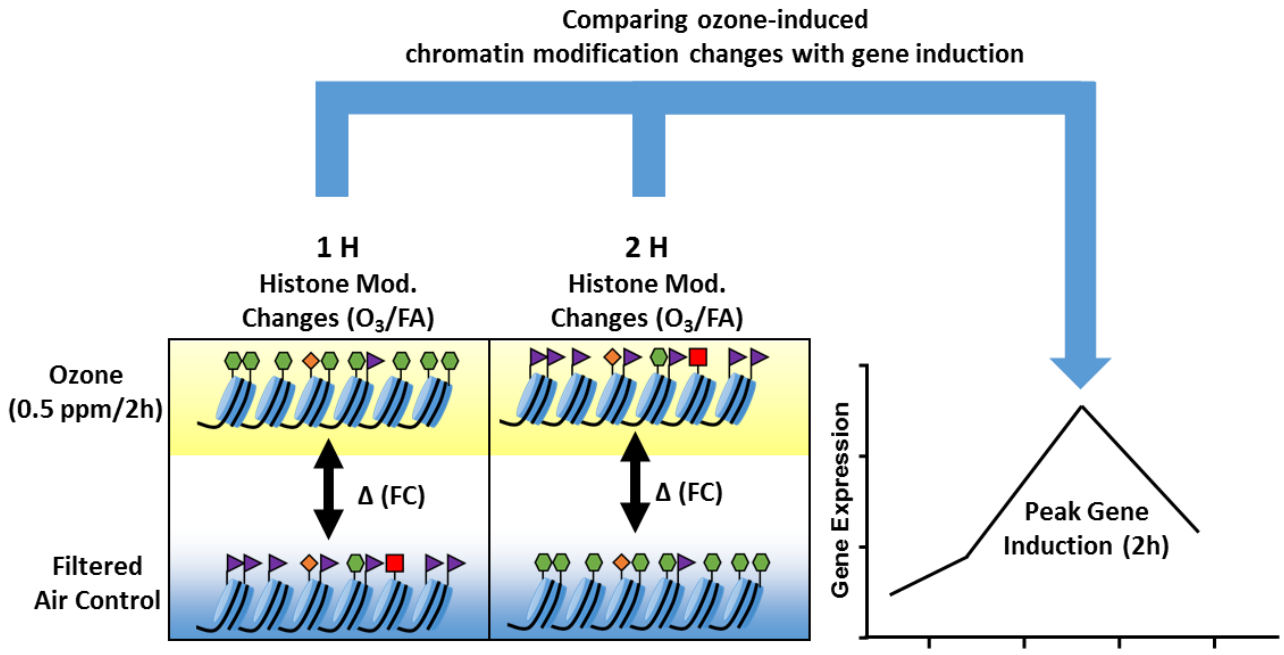

Figure 4-3. Exposure design: comparing ozone-associated changes in the chromatin landscape with peak gene induction. PhBECs were exposed to two hours of filtered air (FA) or ozone. Fixed cells for ChIP were collected after one and two hours of exposure (1H O_3, 2H O_3). Peak gene expression for all genes occurred at 2H O_3. To assess whether post-exposure chromatin modifications were related to the magnitude of gene induction, we compared peak gene induction (occurring after 2h of exposure) with changes in histone modifications occurring prior to and directly coinciding with peak induction (1H O_3, 2H O_3).

Materials and Methods

Cell Culture

Primary human bronchial epithelial cells were obtained via bronchial brushing from healthy, non-smoking donors ages 18-40. Donors gave their informed consent after being informed of procedures and associated risks. The consent and collection protocol were approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects and by the U.S. Environmental Protection Agency. After collection, cells were expanded three passages and plated at air-liquid interface (ALI) on 24 mm uncoated Transwell inserts with 0.4 μm pores (Corning) as previously described (Ross *et al.,* 2007). Cells were maintained in ALI culture for four days during which they became confluent and polarized.

In vitro Ozone Exposure

Two hours prior to exposure, the apical surface of Transwells were washed with Dulbecco's phosphate buffered saline (DPBS; Life Technologies) and the cell medium was replaced with ALI growth medium lacking hydrocortisone. Cells were then placed in *in vitro* exposure chambers and exposed to either a FA or 0.5 ppm ozone for two hours as previously described. At the indicated time points, cells designated for gene expression analysis were removed from the chambers and total RNA was harvested with using RNA Lysis buffer (Life Technologies) and stored at 80 °C until ready for processing.

Chromatin Immunoprecipitation (ChIP) qPCR

The constituents of all the following buffer can be found in Appendix 1. Cells designated for ChIP were detached from Transwells by the addition of Trypsin-EDTA (Life Technologies)

for five minutes followed by the addition of soybean trypsin inhibitor (Sigma-Aldrich). Cells from each condition were pooled, washed, and resuspended in DPBS. Cells were then fixed in 1% formaldehyde (Sigma), followed by quenching with 125 mM glycine. The cells were washed in 1X protease inhibitor mixture, and collected by centrifugation. The supernatant was aspirated, and the pellets were frozen in liquid nitrogen and storage at -80 °C. ChIP was performed as previously described (McCullough *et al.,* 2016). Briefly, cell pellets were sonicated in ChIP Lysis Buffer to shear the chromatin into approximately 500 base pair fragments. Following sonication, insoluble material removed via centrifugation and soluble chromatin was transferred to a clean tube and diluted in ChIP dilution buffer. The diluted soluble chromatin was subjected to immunoprecipitation overnight with Protein A agarose beads (Millipore) that had been conjugated with antibodies targeting H3K4me3, H3K27me2/3, H4ac, or total H3 (Appendix 1). The next morning beads were washed with each of the following buffers: Low Salt Wash Buffer, High Salt Wash Buffer, LiCl Wash Buffer, and TE Wash Buffer. After the final wash, the beads were resuspended in TE, transferred to a new tube, and subjected to RNaseA (Life Technologies) digestion. DNA-protein complexes were eluted from the beads with ChIP elution buffer. The crosslinks between DNA and chromatin proteins were reversed via four hour incubation at 65°C during which proteins were degraded with Proteinase K (Sigma-Aldrich). DNA was purified and precipitated by phenol-chloroform-isoamyl alcohol extraction and resuspended in TE Buffer. The abundance of target gene promoter DNA was quantified by TaqMan quantitative real time quantitative PCR (qPCR) in triplicate reactions (sequences in Appendix 1). The relative abundance of specific chromatin modifications at the promoters of target genes was normalized to the input material from the chromatin

immunoprecipitation ("% Input"). ChIP data was expressed as a fold change between filtered air and ozone treatments (% Input O_3 / % Input FA).

RT-qPCR

For each donor, total RNA was extracted and purified from three inserts per time point per condition using a Purelink RNA Kit (Life Technologies). RNA was quantified using a Nanodrop ND-1000. Complementary DNA (cDNA) was synthesized using 1000 ng of purified total RNA and an iScript cDNA synthesis kit (Bio-Rad). Transcript abundance was then quantified by TaqMan qPCR using a CFX96 Touch (Bio-Rad). Gene induction was determined by first normalizing to β-Actin transcript and then calculating the fold change between O_3 and FA treatments (Pfaffl 2001). Primer sequences for gene expression and ChIP-qPCR analysis are included in Appendix 1.

Statistical Analysis

Gene inductions were compared to FA treatments via paired t-test. Changes in histone H3 occupancy were assessed using a one-sample t-test. The relationship between the abundance of the indicated chromatin modifications and gene expression was determined by simple linear regression. All statistical analyses were conducted using GraphPad Prism software.

Results

The induction of ozone-responsive genes

We collected gene expression data after one and two hours of ozone exposure, as well as one hour post-exposure (Figure 4-4). We did not extend the time course further because we were primarily interested in peak gene expression and not resolution. Across donors the mean (±SD) *IL-8* induction after one hour of ozone exposure was 1.88 ± 0.42, which peaked at 4.86 ± 1.97 after two hours of exposure and then decreased to 3.13 ± 1.02 one hour post exposure. These inductions were all significantly different from FA induction ($p<0.05$). After one hour of ozone exposure, *IL-6* was induced 1.23 fold ± 0.36, which further increased to 2.78 ± 1.94 after two hours of exposure, and then subsequently declined to 1.52 ± 0.81 one hour post exposure. While the induction after one hour post exposure was not significantly elevated, inductions at two hours of exposure and one hour post exposure were significantly different from FA induction ($p<0.05$). *COX-2* was induced 1.57 fold ± 0.32 after one hour of ozone exposure, which further increased to 3.66 ± 0.93 after two hours, and then declined to 2.92 ± 1.02 one hour post exposure. All three inductions were significantly different from FA induction ($p<0.05$). After one hour of ozone exposure *HMOX-1* was induced 1.16 fold ± 0.27, which increased to 3.77 ± 1.41 after two hours of exposure and then slightly decreased to 3.44 ± 1.34 one hour post exposure. *HMOX-1* inductions after two hours of ozone exposure and one hour post exposure were significantly different from FA ($p<0.05$).

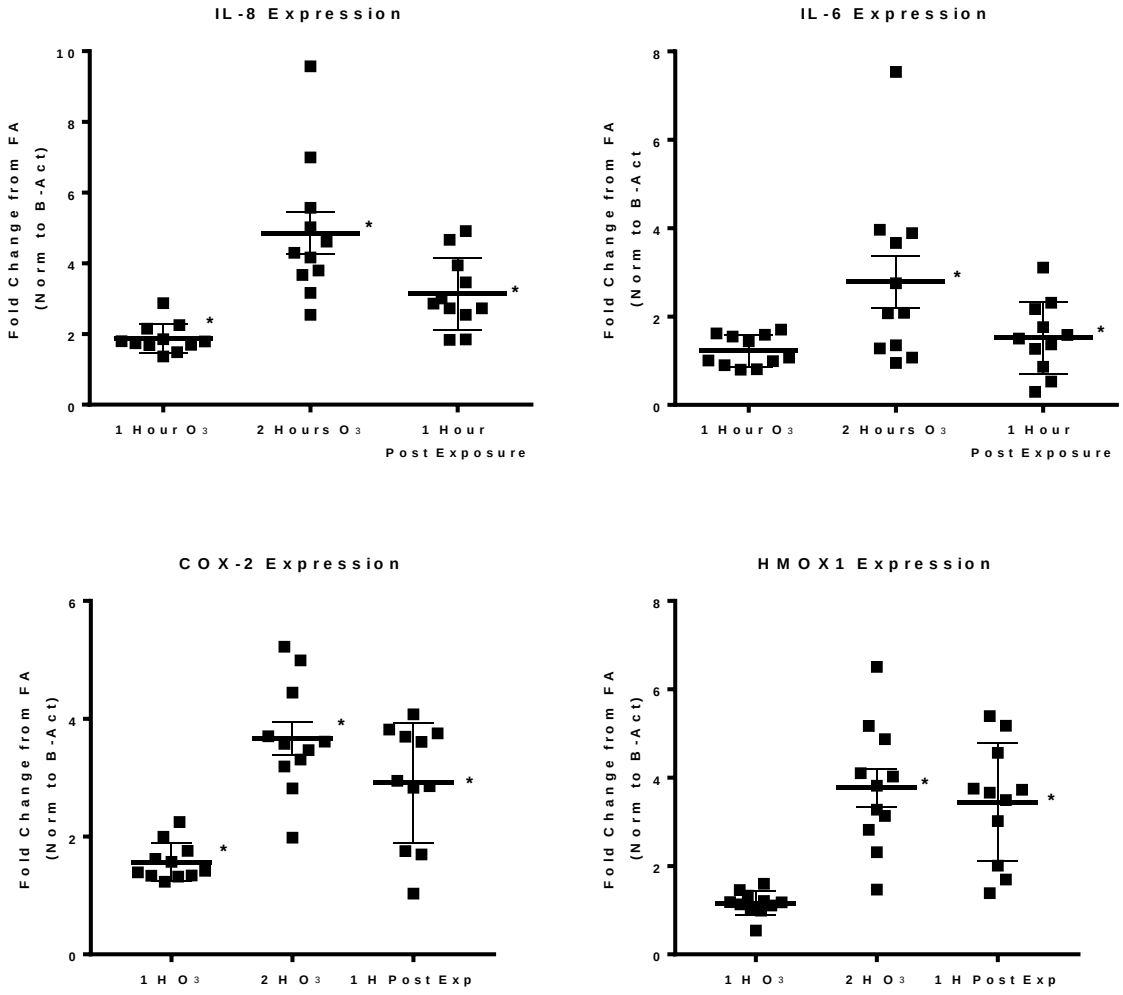

Figure 4-4. Inductions of ozone-responsive genes during ozone exposure and 1 hour post exposure. Cells were exposed to ozone (0.5 ppm) or filtered air for two hours and RNA was collected at the indicated time points. Target gene expression was calculated by normalization to β-Actin transcript levels and then calculating the fold change between ozone and FA exposures. Each data point is cultured from a different human donor (n=11 donors). Mean ± SD shown. For all genes, peak expression occurred at two hours of ozone exposure. *Indicates mean is significant different from FA (t-test; p<0.05).

Histone H3 occupancy at candidate gene promoters and negative control (IgG)

We performed a histone H3 ChIP to assess whether histone occupancy changed as a result of ozone exposure (Figure 4-5). We did not observe any instances in which histone H3 was significantly altered from FA (t-test). The mean fold changes across all gene promoters and

treatments ranged from 0.89 to 1.64, with a maximum standard deviation of ± 0.67. To verify that background genomic DNA levels were not confounding our ChIP results, we also performed immunoprecipitations using an IgG antibody. Fixed cell pellets from seven different donors were used. For all four genes, percent inputs were less than 0.02% (data not shown).

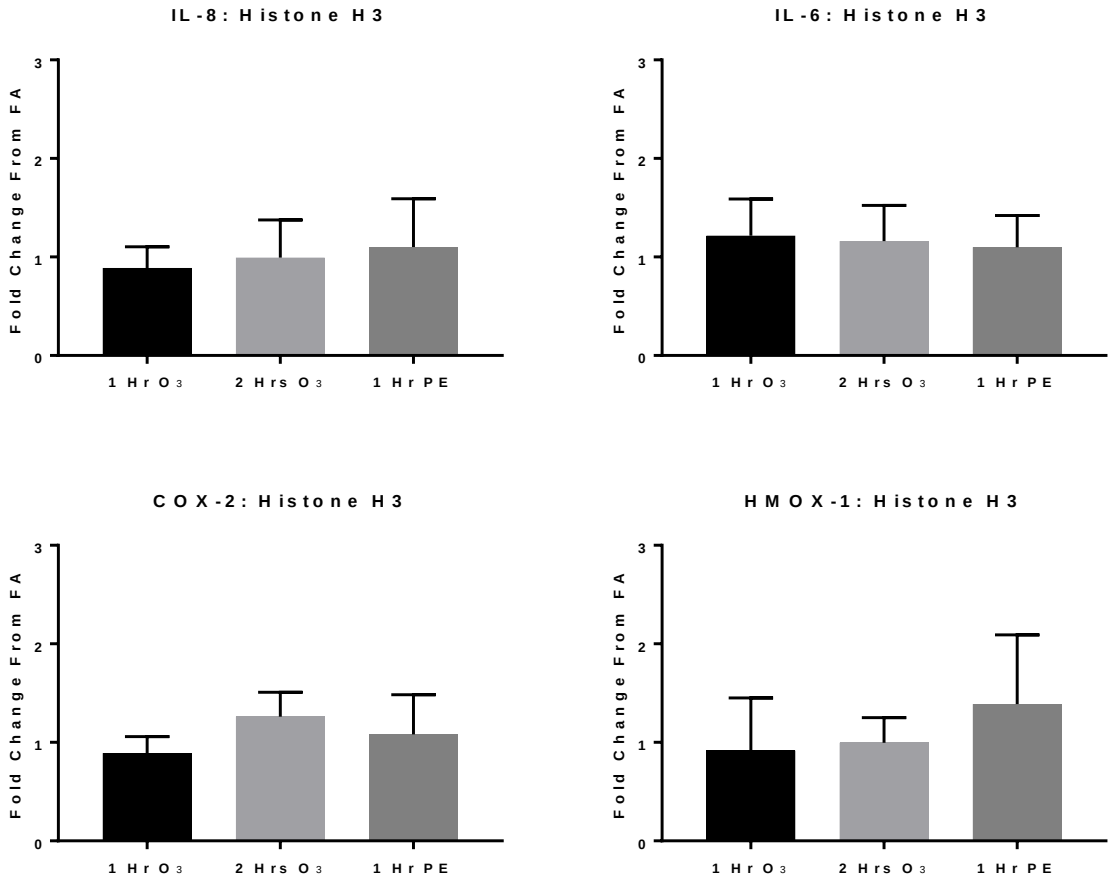

Figure 4-5. The abundance of total histone H3 at candidate gene promoters. To determine if changes in histone occupancy occurred with ozone exposure we examined histone H3 levels via ChIP-qPCR and expressed these levels as a fold change between ozone filtered air. No significant changes from FA exposures were found (t-test). Mean ± SD shown; *n*=5 donors.

Relationship between chromatin modifications and peak gene induction

To assess the relationship between the magnitude of peak gene expression and chromatin states, we performed simple linear regression comparing peak gene expression at two hours of

ozone exposure with chromatin modifications directly preceding (1H O_3) or coinciding (2H O_3) with this time point. R^2 values from these regressions ranged from $5.6x10^{-7}$ to 0.83 and are shown in the heat maps in Figure 4-6. The regressions with the highest R^2 values are depicted in Figure 4-7. Of the six regressions featured, four had *p*-values that were below 0.05. Peak *IL-8* expression was positively correlated with the abundance of the repressive mark K27me2/3after one hour of ozone exposure (R^2=0.71, *p*=0.018, y=0.27x + 0.02). Peak *IL-6* expression was positively correlated with the abundance of H4Ac (R^2=0.73, *p*=0.007, y=0.21x + 0.54) after one hour of ozone exposure. Peak *COX-2* expression was correlated with the abundance of K4me3 (R^2=0.83, *p*=0.012, y=0.12x + 0.57) after two hours of ozone exposure.

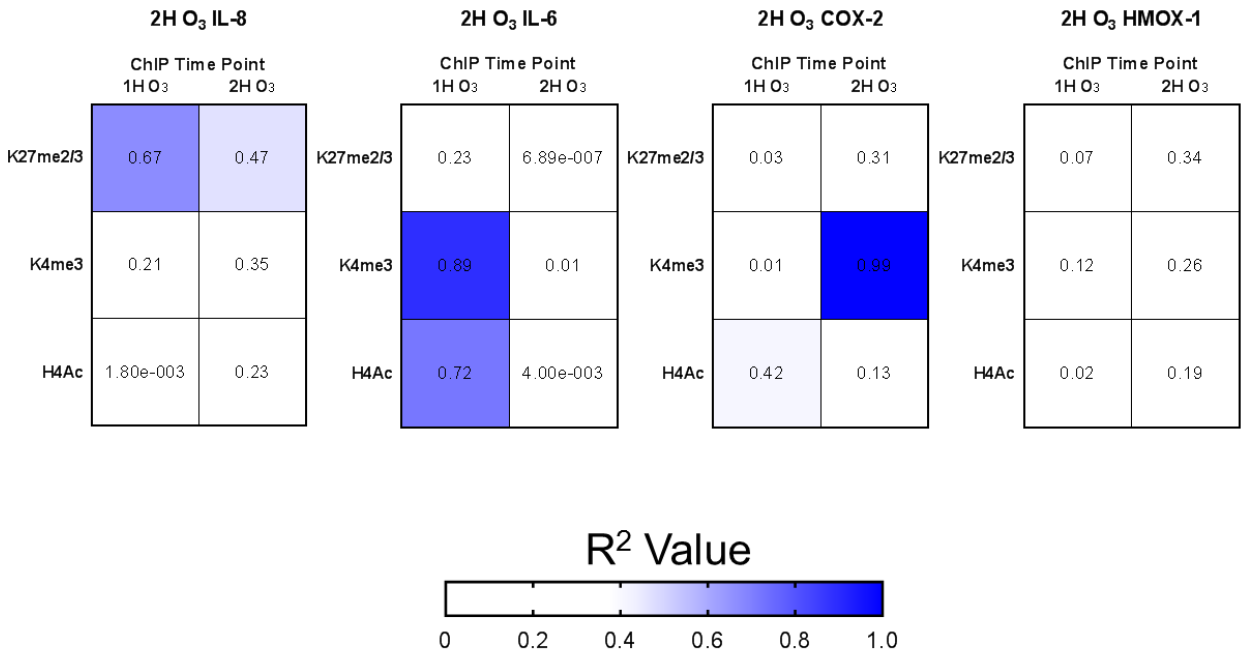

Figure 4-6. The magnitude of ozone-responsive gene expression is related to post-exposure chromatin modifications. ChIP-qPCR was used to assess the abundance of repressive H3K27me2/3 and activating K4me3 and H4Ac at candidate gene promoters. ChIP results at one and two hours of exposure were expressed as a fold change between ozone and filtered air and then compared to peak gene induction (2H O_3) using simple linear regression. The resulting R^2 values are shown in the heat maps above. A minimum of six donors were used for each comparison.

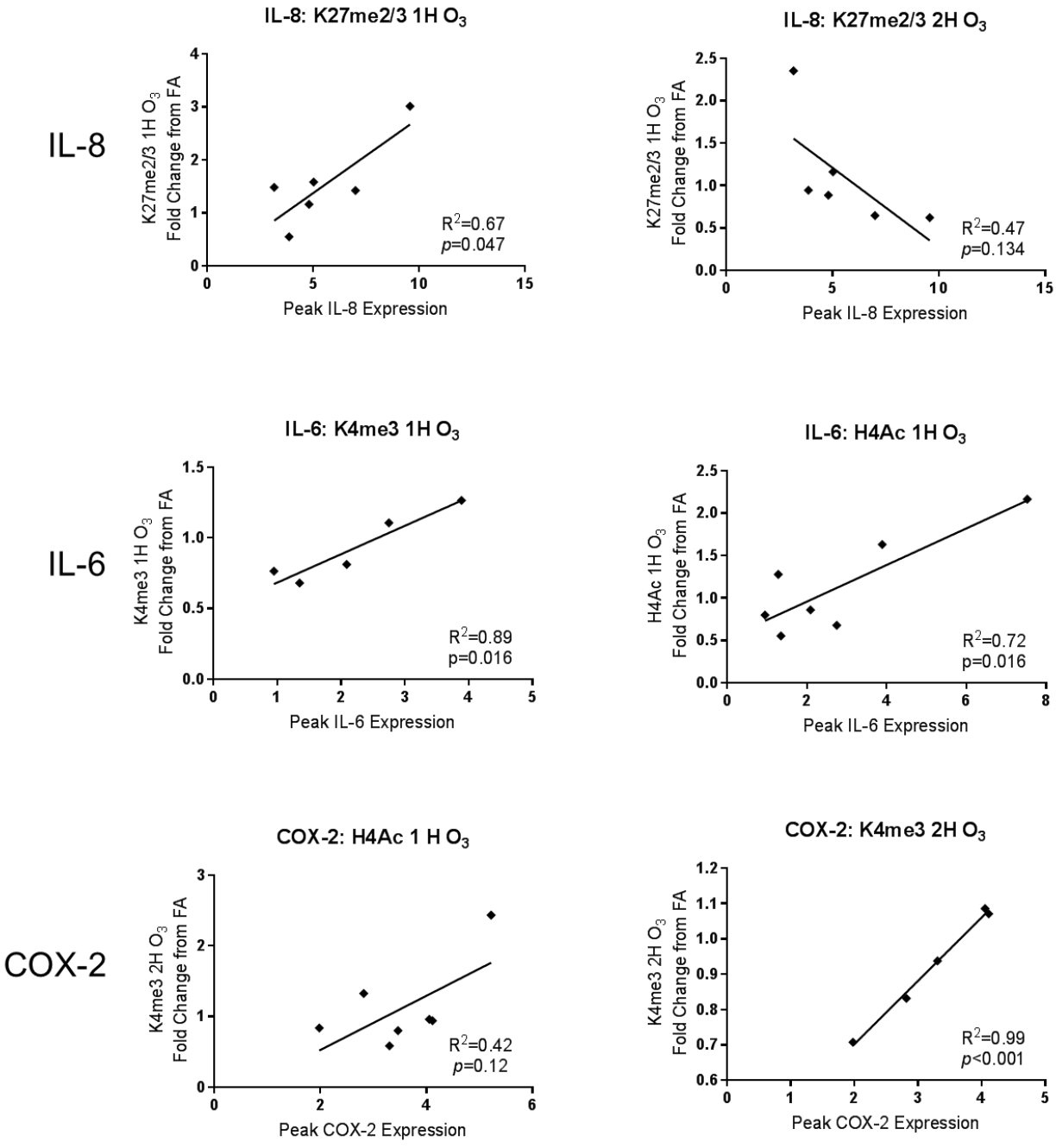

Figure 4-7. Relationships between peak gene induction and post-exposure chromatin modifications. Correlations from Figure 4-6 with the highest R^2 values are shown.

IL-8 promoter K27me2/3 is associated with the magnitude of induction

We observed that the magnitude of *IL-8* induction was associated with the abundance of K27me2/3 after one hour of ozone exposure. This was unexpected because K27 methylation is typically associated with gene repression or silencing. We hypothesized that perhaps this relationship could be explained by the removal of this mark after the one hour time point. To test this hypothesis, we quantified the change in K27me2/3, which we termed "ΔK27me," between one and two hours of ozone exposure (2H O_3/1 H O_3) and determined if ΔK27me2/3 was correlated with peak *IL-8* expression (Figure 4-8). We observed that phBEC cultures with higher *IL-8* inductions had ΔK27me2/3 levels less than one (indicating a reduction), while cultures with lower *IL-8* inductions had ΔK27me2/3 greater than one (an increase; R^2=0.57, p<0.05, y= -0.15x + 1.83). These results suggest that removal of repressive K27me2/3 may facilitate higher *IL-8* induction.

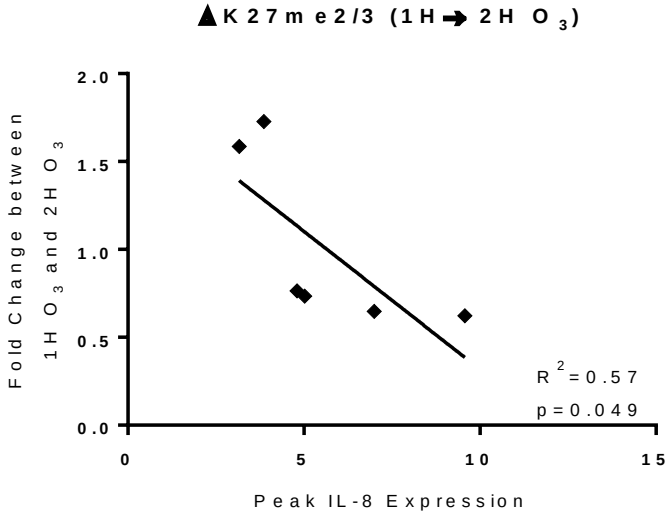

Figure 4-8. Changes in K27 methylation from 1H to 2H O_3 are associated with the magnitude of *IL-8* induction. We calculated the removal of K27me2/3 between one and two hours of ozone exposure, "ΔK27me," (2H O_3/1 H O_3) and compared this value to peak gene induction. n=6

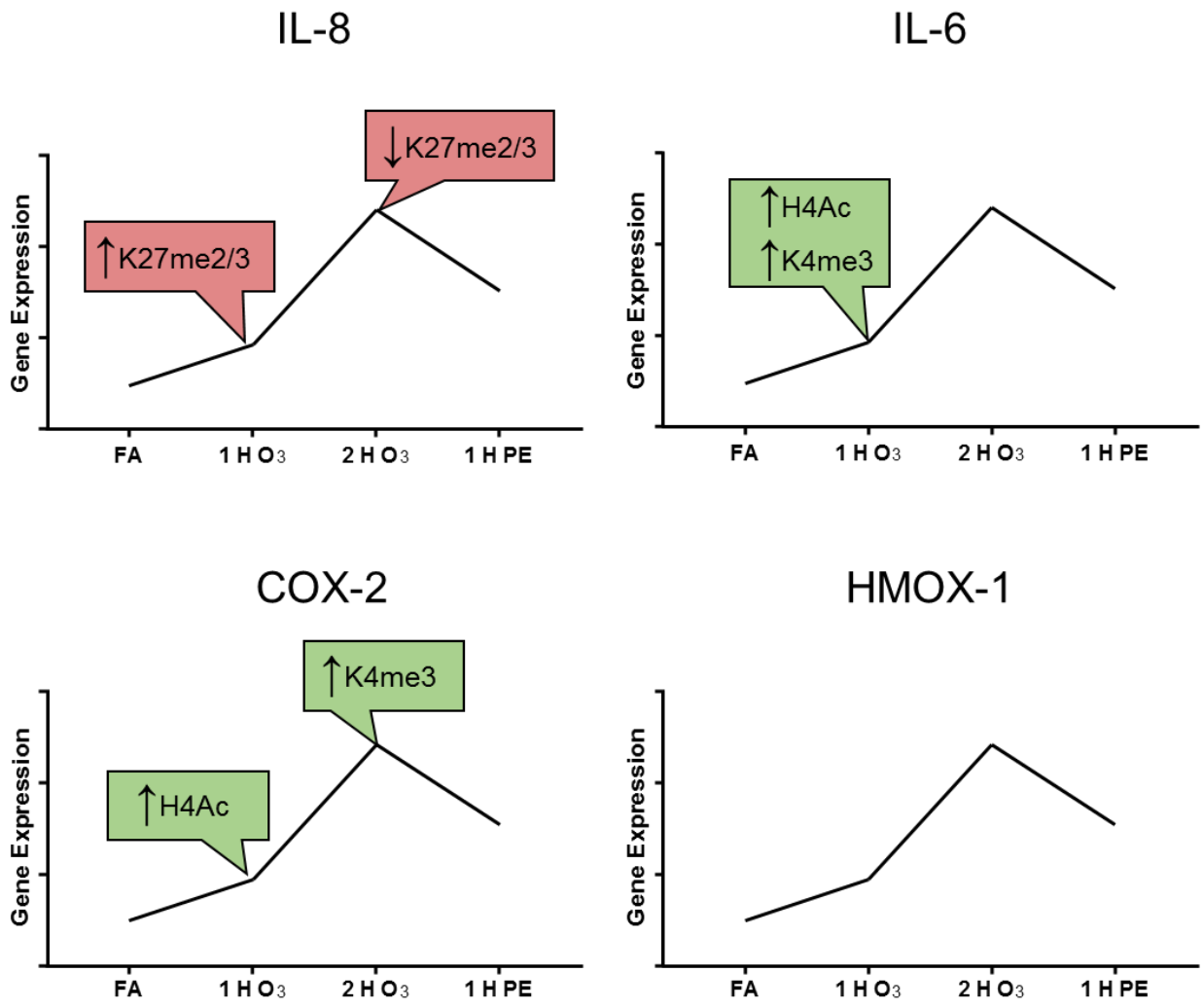

Figure 4-9. Summary of relationships between post-exposure chromatin modifications and gene expression. This figure summarizes the significant (p>0.05) correlations between post exposure chromatin modification changes and gene expression. The general pattern of gene expression is indicated for reference in the background. Green boxes indicate an association with an activating modification (H4Ac or K4me3) and the red boxes indicate an association with repressive K27me2/3. An up arrow indicates the mark is positively associated with gene expression, while a down arrow indicates the mark is negatively associated with gene expression. No relationships were identified for *HMOX-1*.

Discussion

The ozone-mediated induction of pro-inflammatory genes is a key step in the release of cytokines and chemokines in the airway and a potential source of ozone inflammatory response inter-individual variability. Transcriptional responses to ozone are reproducible (Chapter 2), which suggests that ozone-mediated gene induction adheres to an undiscovered set of biological rules. While the activation of MAPK signaling may be one important component of response inter-individual variability, the epigenome is also an important regulator of gene induction. We previously demonstrated that epigenetic states present prior to exposure may influence transcriptional ozone responses, but epigenetic changes occurring as *a result* of ozone exposure may also be an important response modulator. We used a panel of phBECs with varying ozone-associated gene inductions and epigenetic changes to determine if these two variables were related. We found that peak *IL-6* and *COX-2* expression were associated with increases in H3K4me3 and H4Ac after either one or two hours of ozone exposure (Figure 4-9). Alternatively, the removal of the repressive mark H3K27me2/3 was associated with increased *IL-8* expression. Our results suggest that differences in exposure-mediated epigenetic changes could be an important source of ozone response inter-individual variability.

The epigenome is a critical regulator of gene expression, but its role in environmental health is still developing. Currently many toxicoepigenetic studies explore how basal epigenetic states may be related to disease state or gene expression patterns. We recently published such a study in which we found that baseline epigenetic states in unexposed phBECs were associated with ozone-mediated gene induction (McCullough *et al.,* 2016; Figure 1-8). The aforementioned study suggests that the epigenome can be a predictor of exposure effects; however, the addition or removal of epigenetic modifications occurring during response propagation may be just as

important as those present prior to exposure. This principle is especially applicable to bivalent promoters where the balance of activating and repressive modifications ultimately determine gene expression patterns. Many ozone-responsive genes have bivalent promoters, thus the removal of repressive marks and/or the placement of activating modifications may drive ozone inductions and be an important source of inter-individual variability. The present study explores this principle by profiling exposure-associated changes in activating and repressive histone modifications at ozone-responsive gene promoters.

Here we show that, indeed, post exposure changes in the abundance of these marks is associated with both the magnitude of ozone-associated gene transcription. While our findings are novel with respect to ozone exposure, other model systems have found similar results using other pro-inflammatory stimuli. Angrisano *et al.,* (2010) stimulated intestinal epithelial cells with lipopolysaccharide (LPS) and examined changes in histone modifications at the *IL-8* promoter 30 minutes to 24 hours post exposure. They found that peak *IL-8* expression occurred one hour post exposure, and was accompanied by increases in activating pan-acetyl H3 and H3K4me2 and concomitant decreases in repressive H3K9 and H3K27 methylation. Decreases in *IL-8* induction also coincided with increased levels of H3K27me3. Other studies have observed similar epigenetic changes at gene promoters following pro-inflammatory stimulation (Saccani & Natoli 2002). Moreover, exposure to other air pollutants such as inhaled metals, diesel exhaust particles, and benzo-a-pyrene is associated with both global and locus-specific changes in the abundance of activating histone acetylation and methylation (Cantone *et al.,* 2011; Cao *et al.,* 2007; Liang *et al.,* 2011). A strong association has also been made between the severity of inflammatory lung diseases and increased histone acetylation at pro-inflammatory gene promoters (Ito *et al.,* 2005; Adcock *et al.,* 2007).

Our findings in combination with evidence presented by other studies suggest that the ozone-mediated induction of pro-inflammatory genes may be mediated by the placement of activating histone modifications and/or the removal of repressive modifications. This suggests that individuals with particularly elevated pro-inflammatory responses to ozone exposure may undergo unique epigenetic changes that may differentiate them from their less responsive counterparts. Discovering the underlying reasons for these differences could provide unique insights into response inter-individual variability.

While this general framework could be used to explain stimulus-induced transcription, our results demonstrate that this explanation does not apply to all inducible genes and for each modification the importance, kinetics of placement/removal, and functional implications may vary gene-to-gene. For example, we did not observe any post-exposure associations with *HMOX1*. Moreover, we observed that *IL-6* and *COX-2* had increases in activating modifications, but no changes in repressive H3K27me2/3. Alternatively, *IL-8* induction was associated with the removal of H3K27me2/3 but no associations with activating modifications were noted.

Our observations may offer novel insights into the transcriptional regulation of these genes; however this work is not without limitations. This research was designed to be a pilot study to identify important epigenetic modifications and time points that could be used in more expansive future studies. Given the limited abundance of primary cell material, a relatively small number of donors were used, thus sample size is a limitation. While were able to identify several significant associations, it is possible that we may have missed important relationships due to time point selection. We collected samples hourly; however, some chromatin changes may have occurred more rapidly than predicted could have been overlooked by our analysis. Alternatively, key events could have occurred in between time points. Another important caveat

is that we only examined one location within the promoter region near the transcription start site of each gene. We selected this region because this is where many transcription factors bind and epigenetic changes occur, which are important regulators of gene transcription; however, these areas can also be less nucleosome dense. Low nucleosome density could result in low ChIP signal or a lack of detection of epigenetic changes. This could be corrected in future studies by the incorporation of additional primer sets targeting more proximal or distal promoter regions.

While the epigenetic changes we describe are associated with ozone induction and agree with current literature regarding the activation of bivalent genes, further studies are required to determine whether these changes are associative or causal. Characterizing the complexes that place and/or remove these marks during ozone exposure will facilitate this process, as the identified proteins can be overexpressed, knocked-out, have their activity inhibited, etc. to determine the effect on the placement of histone modifications during ozone exposure. This may be more easily accomplished for some histone modifications than others because some marks (e.g. H3K27me2/3) have fewer writers/erasers than others (e.g., H4Ac).

While we have identified several epigenetic changes associated with ozone exposure, the molecular processes linking these two phenomena are currently unknown. Previous findings have established that ozone exposure activates cellular signaling cascades such as the MAPK pathway (Chapters 2, 3; McCullough *et al.*, 2014). The activation of MAP kinases leads to histone phosphorylation and acetylation at target genes by the direct phosphorylation of histone substrates, the activation of chromatin modifying enzymes such as CBP/p300, and the activation of transcription factors that bind to target gene promoters and, in turn, recruit additional chromatin modifying complexes (Cargnello and Roux 2011; Liu *et al.*, 1999). Future studies can identify specific transcription factors and chromatin modifiers that are recruited to pro-

inflammatory gene promoters as a result of ozone exposure. This information can then be used in the aforementioned mechanistic investigations.

Conclusions

We observed that phBEC cultures undergo epigenetic changes during ozone exposure which may be important drivers of pro-inflammatory response inter-individual variability. Higher pro-inflammatory gene inductions are associated with increases in activating marks and/or a reduction in repressive modifications. These results are consistent with current models of bivalent gene regulation in which induction is controlled by the balance of activating and repressive histone modifications. Our findings also demonstrate there is gene-to-gene variation in the types changes that occur, kinetics of placement/removal, and the relative importance of each modification. While these findings suggest that susceptible individuals could have unique epigenetic responses to environmental exposures, additional questions remain regarding the molecular events leading to these changes and the functional role of the epigenetic alterations described.

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CHAPTER 5: ADDITIONAL EXPERIMENTS

Cytotoxicity Assessment of Multiple Ozone Exposures

Introduction/Purpose:

Although inspection via light microscopy revealed that tissue monolayers and cilia appeared to be intact over four days of repeated ozone exposure, I wanted to perform a more quantitative assessment of cytotoxicity. I initially experimented with a Live/Dead (Invitrogen) flow cytometry-based technique; however, the sensitivity of this assay was confounded by the variability and complexity of the phBEC cell suspension. Others in the field have used the lactate dehydrogenase (LDH) assay, however I was skeptical about the information this assay would provide, given that ozone could damage the cell membrane and cause LDH release but may not necessarily cause cell death. I eventually used the classic trypan blue exclusion assay, which has previously been used by others assess exposure-associated phBEC toxicity (Hellerman *et al.,* 2002; Takeshi *et al.,* 2001).

Methods:

Cells at ALI Day 24 were exposed to ozone and filtered air on a daily basis for four days. Twenty-four hours following each ozone exposure, primary cell cultures were removed from Transwell inserts via trypsinization for approximately 5-7 minutes. Trypsin was inactivated with cell media and cells were centrifuged, washed with PBS, and resuspended in PBS. Cells were diluted and trypan blue was added according to the manufacturer's protocol (ThermoFisher).

Cells were counted using a hemocytometer. The number of trypan blue positive cells was expressed as a percentage of total counted cells. For each condition 6-8 hemocytometer fields were counted per treatment. Prior to cell counts, I was blinded to treatment conditions. A 2-way ANOVA was used to compare FA to ozone-exposed cells for each exposure day.

Results:

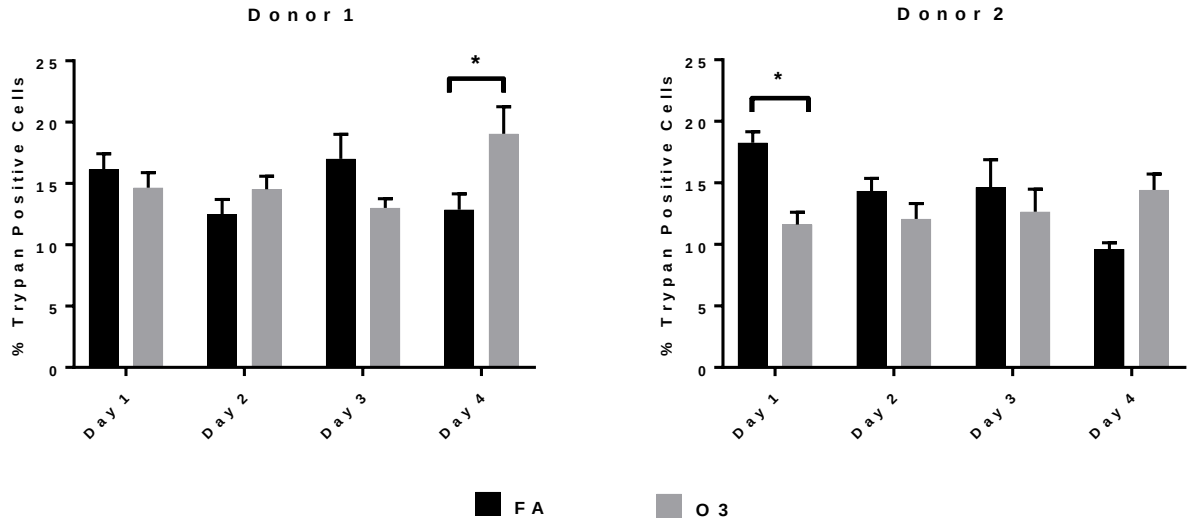

Figure 5-1. Cytotoxicity assessment of repeated ozone exposures using trypan blue exclusion assay. Mean ± SE. *Significantly different from FA (p<0.05).

Discussion/Conclusions:

During the initial days of exposure (Days 1-3), the number of trypan positive cells trended slightly higher in FA than ozone treatments; however, after four days of exposure, this trend was reversed. Initial increases in cytotoxicity in filtered air conditions may be explained by a 'culling effect,' where weakened/dying cells may be forced into apoptosis by ozone exposure prior to counting. Increased ozone-associated cytotoxicity on the fourth day is likely due to treatment effect; however, this effect appeared to be limited to a 5% increase, which argues that cytotoxicity is not a driver of the effects observed in this dissertation. An important

finding to note is that the background of trypan positive cells is higher than would be expected compared to the background in many cell lines. I think that this is because differentiated phBECs within a pseudostratified epithelium are more difficult to trypsinize than many cell lines, thus the mechanical action of washing and pipetting may be more likely to tear membranes, which would allow dye to enter the cells and create a higher background. I anticipate this effect would be uniform across days and treatments, thus the cytotoxic effect of ozone may still be discriminated.

Comparison of *in vitro IL-8* induction and the *in vivo* inflammatory response

Introduction/Purpose:

This dissertation presents evidence that the phBEC model may be useful in studying the mechanisms that control *IL-8* transcription. IL-8 is an important chemokine regulating neutrophil chemotaxis, which is a hallmark ozone exposure. However, a compelling question is whether phBEC cultures are predictive of ozone-mediated neutrophil responses in the donors that they are collected from. To investigate this question, I selected samples from a clinical ozone exposure study in which subjects had matched phBEC collections and bronchoalveolar lavages following ozone and FA exposure. I compared phBEC ozone-mediated *IL-8* inductions with previously recorded neutrophil recruitment data, hypothesizing that donors with elevated neutrophil influx would have higher phBEC ozone-mediated *IL-8* inductions.

Methods:

In the aforementioned clinical study, bronchoalveolar lavage fluid (BALF) was collected 18 post exposure from both filtered air and ozone treatments. Treatments were separated by at least two weeks. EPA Human Studies Facility staff performed the BALF cell differential analysis. From this donor pool I selected eight donors that had varying levels of neutrophil (polymorphonuclear cells - PMNs) influx following ozone exposure. For these eight donors, epithelial cells collected after the *in vivo* FA exposure were cultured and differentiated. At ALI Day 24, I carried out the phBEC *in vitro* ozone and filtered exposures and performed RNA extraction and RT-qPCR as previously described. To prevent unintentional bias, phBEC exposures were performed under a double-blind. After the RT-qPCR results were obtained, the

blind was removed and comparisons were made between phBEC *IL-8* inductions (indicated in orange in Table 5-1) and infiltration (blue) via simple linear regression.

Results:

			In vivo - Collected from BALF				
Subject	Exposure		Total Cells (x 10^6)	% PMNs		Raw PMNs	
1	Air		1.60	1.00		0.02	
2	Air		5.40	2.00		0.11	
3	Air		3.20	3.00		0.10	
4	Air		3.70	0.10		0.00	
5	Air		5.70	5.00		0.29	
6	Air		2.80	0.10		0.00	
7	Air		2.90	0.10		0.00	
8	Air		3.70	3.00		0.11	

		In vitro	In vivo - Collected from BALF				
Subject	Exposure	phBEC IL-8 induction	Total Cells (x 10^6)	% PMNs	PMN FC (O_3/FA)	Raw PMNs	Raw PMN FC (O_3/FA)
1	Ozone	3.92	1.70	3.00	3.00	0.05	3.19
2	Ozone	2.59	3.60	19.00	9.50	0.68	6.33
3	Ozone	2.3	3.20	12.00	4.00	0.38	4.00
4	Ozone	2.42	2.10	15.00	150.00	0.32	85.14
5	Ozone	2.09	5.10	20.00	4.00	1.02	3.58
6	Ozone	3.1	4.30	6.00	60.00	0.26	92.14
7	Ozone	4.63	2.60	8.00	80.00	0.21	71.72
8	Ozone	6.18	5.20	1.00	0.33	0.05	0.47

Table 5-1. Comparison of *in vitro* phBEC *IL-8* responses to *in vivo* acute inflammatory responses. The top panel shows PMN influx following filtered air exposure, while the bottom panel shows responses following ozone exposure. The bottom panel also contains fold change calculations (from FA) with respect to raw PMN number and percent PMNs.

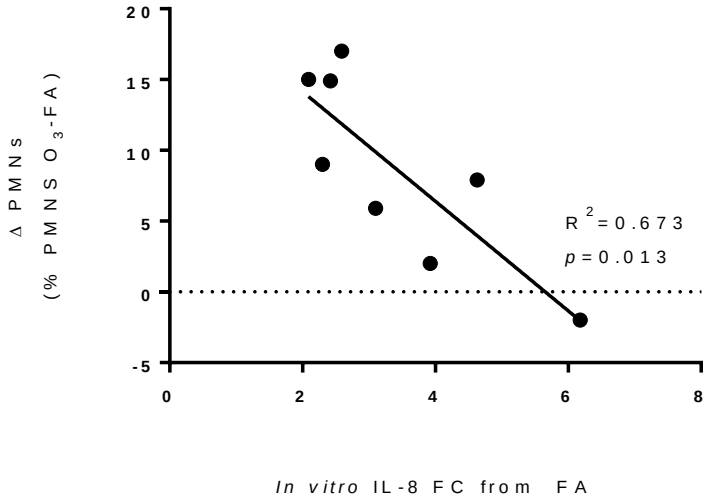

Figure 5-2. Relationship between *in vitro IL-8* induction and PMN infiltration. Percent PMN infiltration is inversely related to *in vitro IL-8* induction. n =8

Discussion/Conclusions:

I observed that *in vitro* IL-8 inductions were inversely related to *in vivo* neutrophil influx following ozone exposure. This pattern hold if *IL-8* inductions are compared with both percent PMNs (Figure 5-2) and raw PMNs (R^2=0.48, p=0.056; data not shown). These findings oppose my original hypothesis.

While phBEC IL-8 inductions and neutrophil influx may be inversely related 18 hours post exposure, it is possible that a different relationship could be observed if BALF had been collected at a different time point. Ozone associated neutrophil influx typically peaks around six hours post exposure, and begins to dissipate 18-24 hours post exposure (US EPA, 2013; Schelegle *et al.,* 1991). Thus, the inflammatory phenotypes observed are likely more representative of resolution phenotype than peak expression.

If earlier time points were assessed (*i.e*. during peak neutrophil recruitment) and this inverse trend continued to be observed, the phBEC model could still be used to study factors

dictating *IL-8* response, and it is still predictive of *in vivo* responses, but not in the way originally anticipated (low IL-8 *in vitro* responses would predict high *in vivo* responders).

In order to make a conclusion regarding the phBEC-*in vivo* response relationship, a study should be designed in which respiratory inflammation can be assessed at multiple time points. While BALF collection may be too invasive for repeated collections, induced sputum may be a viable option.

Additional Characterization of Ozone Adaptation

Introduction/Purpose:

In Chapter 3 the concept of *in vitro* ozone adaptation is introduced and potential mechanisms are explored; however, the characterization of *in vitro* ozone adaptation is limited to four repeated days of ozone exposure. Additional questions remain regarding the acquisition and persistence of *in vitro* ozone adaptation. Two important questions are: how many ozone exposures are required to produce ozone adaptation; and 2) if adapted cells are withheld from ozone exposure for several days, do they regain their responsiveness? The following experiments were performed to address these questions.

Methods:

PhBECs were used for exposures after 24 Days of differentiation at ALI as previously described in Chapter 3. Cells were exposed to ozone and filtered air according to the exposure schemes included with each figure. In the recovery experiment, phBEC cultures that exhibited *IL-8* adaptation after four days of repeated exposure (defined by significant t-test comparing $1XO_3$ and $4XO_3$ $p<0.05$) were withheld from exposures to see if they regained responsiveness to ozone. Cells had their media changed and washed each day that they were withheld from exposure. In both experiments, all exposures are compared to responses from a single ozone exposure control ($1XO_3$) to determine whether adaptation was present. Instead of fold change values, the ozone inductions in the recovery experiment were expressed as a percent inhibition. Percent inhibition was calculated by equating the induction of the cells that were re-exposed to ozone as a percentage of the daily $1XO_3$ control, and then subtracting that value from 100% (e.g.

if previously exposed cells had an induction that was equal to the $1XO_3$ control, they would have 0% inhibition).

Results:

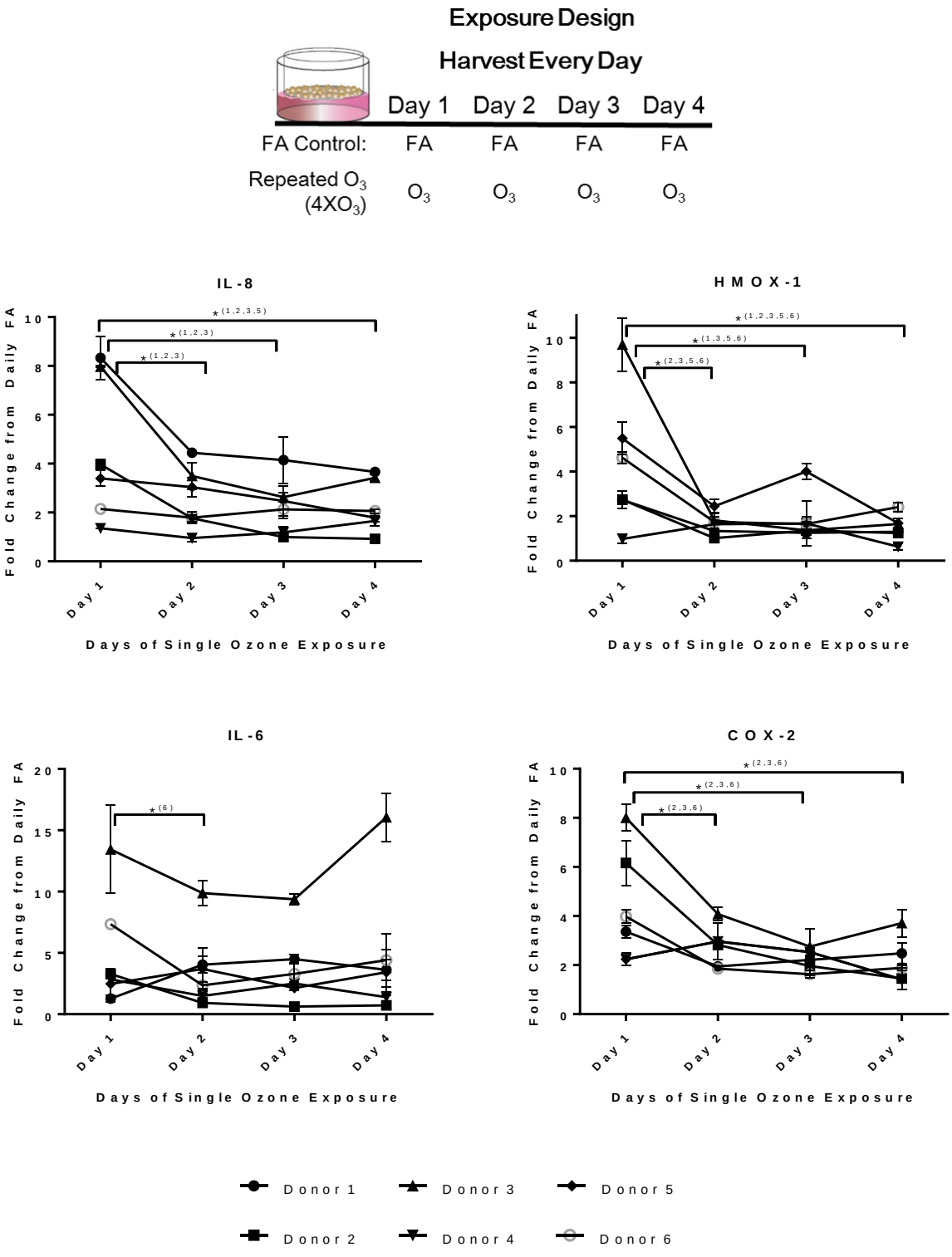

Figure 5-3. Number of exposure days required for ozone responsive genes to exhibit suppression. Cells were exposed to 0.5 ppm O_3 and filtered air as previously described. Cells were harvested for gene expression analysis on a daily basis. A 2-way ANOVA was used to compare all inductions to induction the first day of exposure (Day 1). The asterisks indicate the

donors that had a significant (p<0.05) declines from Day 1 ozone response, which would indicate that suppression had occurred (2-way ANOVA).

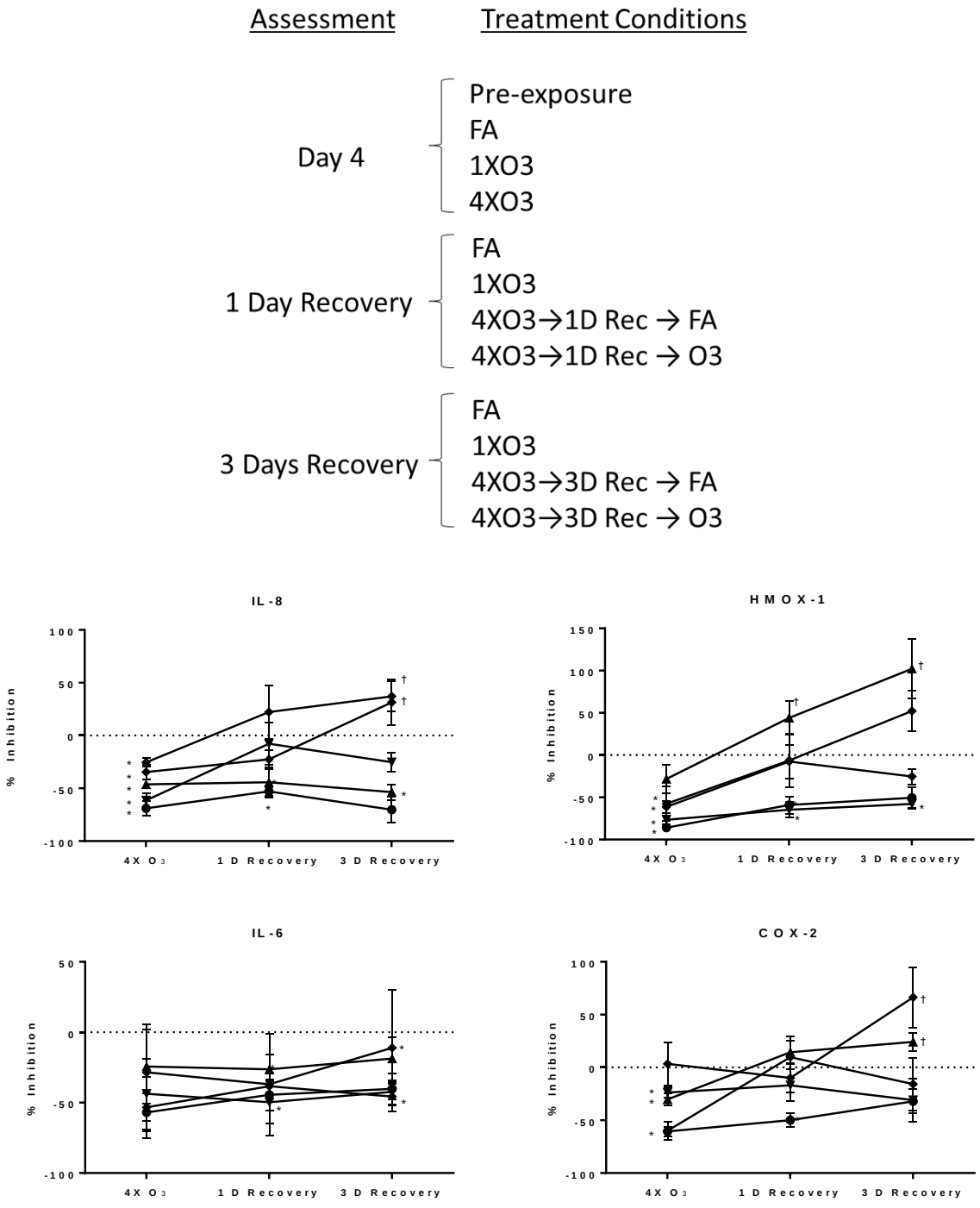

Figure 5-4. Do 'adapted' cells regain responsiveness? Cells that were exposed to ozone for four days and were '*IL-8 adapted*' were withheld from exposure for one or three days before being re-exposed to ozone. For each indicated day a 2-way ANOVA was used to compare the

single ozone exposure control ($1XO_3$) to each treatment. *Indicates significant reduction from $1XO_3$, while † indicates a significant increase from $1XO_3$ ($p<0.05$).

Because gene inductions in this dissertation are expressed as a fold change from filtered air, there was a conundrum of what FA control to use for cells that were re-exposed to ozone: cells that had been only exposed to filtered air, or those that were previously exposed to ozone ($4XO_3$), and then were not exposed to ozone again (and were only exposed to FA) during the recovery assessment (e.g., $4XO_3$→3D Rec → FA). For the calculations presented above, the FA only treatment was used; however, I used the $4XO_3$→ Rec → FA treatment to check if previous ozone exposure altered baseline expression of any of the above genes. To do this, I normalized all FA treatments to the "pre-exposure" value. Across all donors, baseline expression of these genes was either unchanged or exhibited a slight reduction (no more than 0.75 FC from pre-exposure; data not shown). This indicates that shifts in baseline expression do not account for the trends observed here.

Discussion/Conclusion:

The results depicted in Figures 5-3 and 5-4 are consistent with those presented in Chapter 3, where the most responsive donors had the most substantial reductions in expression. In donors exhibiting gene suppression, this reduction was often evident after two exposure days (Figure 5-3). Some phBECs, however, did not exhibit statistically significant reductions in gene induction until the fourth day of exposure. Many of the phBECs did not exhibit *IL-6* and *COX-2* adaptation; moreover, there was actually a U-shaped trend in gene expression patterns.

Recovery of responsiveness varied from donor-to-donor, where two of the five phBEC cultures regained responsiveness after a three-day recovery window. Not only did these donors

regain responsiveness, but their inductions actually exceeded the responses of the $1XO_3$ control cells. This trend was evident for several other genes, including *HMOX-1* and *COX-2*. No donors regained *IL-6* responsiveness.

These findings indicate that the dynamics of *in vitro* ozone adaptation are highly dependent on both the phBEC donor as well as the gene being examined. If phBECs are going to adapt to ozone exposure, most will do so after two exposure days. Among phBECs exhibiting adaptation, 40% (2/5) may regain responsiveness after several days of recovery, and could even have enhanced responses. While it is unclear whether these findings mimic *in vivo* adaptation dynamics, they suggest that *in vivo* inflammatory adaptation may exhibit a high level of inter-individual variability which could play an important role in ozone response susceptibility.

The Influence of phBEC Donor Characteristics on *IL-8* Induction

Introduction:

Previous epidemiological and *in vivo* studies have shown that characteristics such as age, sex, and genotype can influence the inflammatory response to air pollutant exposure (US EPA 2013). It is unclear, however, whether these factors can also influence pro-inflammatory responses in phBECs. If these relationships did exist, they could provide additional insight into response inter-individual variability and create new avenues for mechanistic phBEC research. To determine if donor characteristics are related to ozone responsiveness, phBEC *IL-8* inductions were stratified according to the characteristics of their donors. A brief background on each trait as it relates to air pollutant exposure is given below:

GSTM1 genotype: Approximately 40% of the population is homozygous for a polymorphism that prevents the expression of a gene called glutathione S-transferase mu 1 (*GSTM1*; Garte *et al.,* 2001). These *GSTM1* null individuals exhibit greater inflammatory response to ozone exposure then their wild type counterparts, which is thought to be due to a decreased ability to neutralize ozone-associated ROS (Alexis *et al.,* 2009).

Sex: Many studies have reported sex differences in air pollutant exposure responses. Although the outcomes of such studies are variable, the majority have reported that women are more susceptible to the adverse effects of air pollutant exposure (Clougherty 2010). Female mice exhibit more pronounced inflammatory responses following ozone exposure and are more susceptible to ozone-associated mortality and secondary infection (Mikerov *et al.,* 2008; Silveyra *et al.,* 2015).

Age: Air pollutant exposure effects are known to vary with age; however, the nature of this relationship is still being characterized. Young children and juvenile rodents are especially

susceptible to ozone exposure (Gunnison *et al.,* 1992; Silverman and Ito 2010). Age-dependent changes in exposure-associated inflammatory responses are highly dependent on genetics, and evidence suggests that once in adulthood inflammatory responses to air pollutants increase with age (Elder *et al.,* 2000; Vancza *et al.,* 2008).

Methods:

The phBECs used in experiments in this dissertation were cultured as they became available and were not pre-selected based on donor characteristics. These characteristics were requested from the EPA Clinical Research Branch upon the conclusion of experiments. *GSTM1* genotyping was performed by the EPA Human Studies Facility staff using PCR. Given that several distributions were skewed and were therefore not Gaussian, non-parametric analyses were used to compare *IL-8* inductions between groups.

Results:

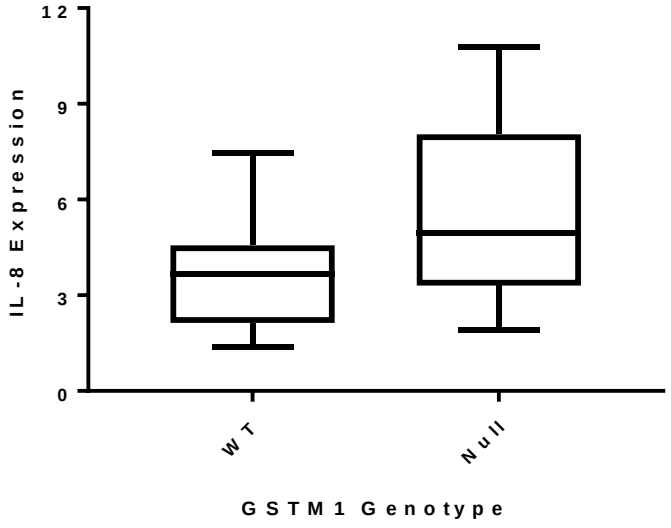

Figure 5-5. Ozone inductions in *GSTM1* wild type and *GSMT1* null phBEC donors. *IL-8* inductions are shown for nine wild type "WT" and nine null donors. The line in the middle of the box indicates the median, while the box represents the inter-quartile range, and the whiskers indicate the minimum and maximum values. A Mann-Whitney test was used to compare null and WT distributions. No significant differences were found.

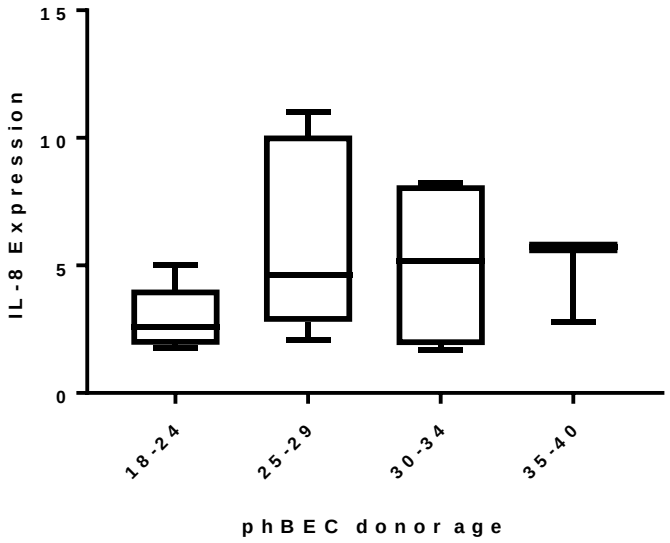

Figure 5-6. PhBEC *IL-8* induction in different donor age groups. Ozone-mediated *IL-8* inductions are shown. PhBEC donors were binned by age group. For 18-24 *n*=7; 25-29 *n*=11; 30-34 *n*=4; 35-40 *n*=3. A non-parametric ANOVA (Kruskall-Wallace) was used to compare groups. No significant differences were found.

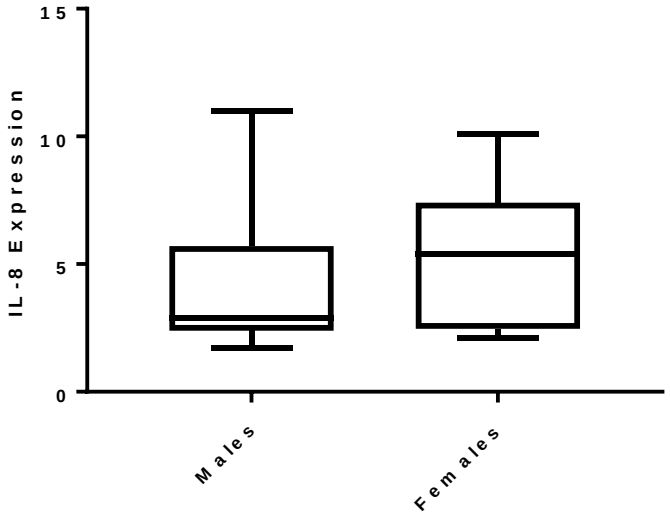

Figure 5-7. PhBEC *IL-8* induction in male and female donors. Ozone-mediated *IL-8* inductions are shown. Inductions between sexes were compared using a Mann-Whitney test. No of airway epithelial cells and is determined by the activation of the MAP kinases p38 and ERK1/2 significant differences were found. *n*=6 females and 19 males.

Discussion/Conclusions:

While this examination revealed no statistically significant findings, this is not entirely unexpected given the small number of donors included. If analyzing responsiveness according to demographic traits had been a central focus of the dissertation, I may have been able to recruit donors based on specific characteristics in order to balance subject demographics. For example, there were far more donors ages 25-29 (11) than 35-40 (3). Moreover, for unknown reasons the majority of phBEC donors are male (19 males versus 6 females). While I did observe several trends, it is difficult to tell if these trends are accurate representations or artifacts of unequal sample sizes.

With this important qualifier in mind, several of the observed trends are consistent with observations in epidemiological and/or *in vivo* studies. For example, *GSTM1* null phBEC donors had a much wider variation in *IL-8* induction, and the median induction was higher than in wild type cells. This agrees with the observation that *GSTM1* null individuals have higher inflammatory responses to ozone exposure compared to their wild type counterparts. Females have a wider range of *IL-8* induction than males and have a higher median response, which is concordant with prior findings regarding the ozone inflammatory response in mouse models. With respect to age, it appears that 18-24 year olds are the least responsive to ozone exposure and have the narrowest range in *IL-8* induction. Above this age group *IL-8* inductions are more widely distributed and have higher medians. This is consistent with observations in animal

studies that suggest that among adult mice, ozone-induced inflammation increases with age (Elder *et al.,* 2000; Vancza *et al.,* 2008).

The fact that the trends agree with prior published research is encouraging because this suggests that the mechanisms driving these associations may be able to be explored using the phBEC *in vitro* model. If indeed phBEC responses are influenced by donor age, this would be a particularly interesting finding because this effect would have to be epigenetically mediated. Donor age could be an important factor dictating the epigenetic patterns previously noted (McCullough *et al.,* 2016) and those discussed in Chapter 4.

The aforementioned trends will need to be verified in future studies in which the number of phBEC donors is increased and subject characteristics are balanced throughout the donor pool. The preliminary data presented here could be useful in designing future studies because they could be used to conduct power calculations.

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CHAPTER 6: CONCLUSIONS, PERSPECTIVES, AND FUTURE DIRECTIONS

While ozone mediated inflammatory responses exhibit extensive inter-individual variability, responses within individuals are highly reproducible. One of the key findings from this dissertation is that this reproducibility can be recapitulated on a cellular level, as the response status of a particular individual's phBECs can be recapitulated even if cultures are collected up to a year apart. This provides evidence that fundamental differences in an individual's airway epithelial cells may play a major role in ozone inflammatory response. This finding is exciting because it suggests that there are discrete rules governing epithelial cell responses, and that the identification of these rules can be accomplished by examining epithelial cell biology. Here I used phBEC ozone response inter-individual variability as a tool to describe how two novel susceptibility factors, MAPK signaling and epigenetic patterns, are important aspects of epithelial cell programming.

Epithelial cell programming: genetic and epigenetic considerations

While this dissertation makes many novel insights regarding ozone response inter-individual variability, the origin of these differences in phBEC cultures remains to be determined. Genetics likely play a major role, but it is also possible that epigenetic imprints could be retained from the phBEC donor's life history.

The human genome contains approximately 20,000 genes that encode the proteins which serve as building blocks and perform the tasks necessary for life. Variations in DNA sequences within protein-coding and non-coding regions likely account for a considerable amount of the

inter-individual variability in the inflammatory response to ozone exposure. Epidemiological studies, human clinical exposure studies, and model systems such as the collaborative cross have found that certain polymorphisms are associated with the severity of the ozone inflammatory response (Bauer and Kleeberger 2010).

A gene that is frequently discussed in the context of the ozone inflammatory response is *GSTM1*. Multiple epidemiological and human clinical exposure studies have demonstrated that individuals who are *GSTM1* null are more susceptible to ozone-associated lung function decrements and inflammation (Alexis *et al.,* 2009; Romieu *et al.,* 2006). Consistent with this finding, I found that GSTM1 null phBEC cultures may also have slightly higher pro-inflammatory gene inductions than wild type cultures (Figure 5-5). The influence of GSTM1 and the central role of MAPK signaling as demonstrated in this dissertation suggest that one of the ways GSTM1 could influence the pro-inflammatory response is by modulating the activation of the MAPK pathway. Indeed, it has been shown that GSTM1 impinges on the MAPK pathway by its interactions with apoptosis signal-regulating kinase 1 (ASK1). Under normal cellular conditions, GSTM1 binds to ASK1 thereby inhibiting its activity; however, during conditions of oxidative stress, GSTM1 dissociates from ASK1, freeing ASK1 to activate MKK3, 4 and/or 6, which are upstream of p38 (Brancho *et al.,* 2003; Dolado *et al.,* 2007; Kennedy *et al.,* 2007). TRAF6 and TRAF2, which are downstream effectors of TLR4 can also phosphorylate and activate ASK1 (Matsuzawa *et al.,* 2008; Nagai *et al.,* 2007). The information provides a putative link between ozone exposure, GSTM1, MAPK signaling, and toll-like receptor signaling, which are all known to have central roles in the ozone inflammatory response.

While GSTM1 status can be influential, it may be unrealistic to attribute response variability to one polymorphism alone. Instead the overlap of many different polymorphisms is

a much more powerful explanatory factor. Based on this logic, GSTM1 has been proposed to be a 'gatekeeper' during conditions of oxidative stress, where wild type individuals will be protected, but null individuals may be much more susceptible to polymorphisms in other genes associated with stress responses (David *et al,* 2003; Romeiu *et al.,* 2006). This could explain the fact that in the phBEC model, pro-inflammatory response variability was wider in the GSTM1 null donors (Figure 5-5). Other genes that have been implicated as modulators of the ozone inflammatory response include detoxification enzymes such as glutathione S-transferase Pi 1 (*GSTP1*), nicotinamide adenine dinucleotide (phosphate) reduced: quinone oxidoreductase (*NQO1*), and catalase (*CAT*) (Bauer and Kleeberger 2010; David *et al.,* 2003; Romeiu *et al.,* 2006). Genome-wide association studies (GWAS) in mice have identified that polymorphisms within *Jnk1* and genes upstream of MAPKs such as *Tnfa*, *Tlr2*, *Tlr4*, and *Myd88* are also important modulators of inflammation (Bauer and Kleeberger 2010).

Further experiments using the phBEC model system may be able to investigate how these polymorphisms are linked to MAPK signaling through mediators such as ASK1. Moreover, it would be informative to model ozone responses in phBECs possessing particular polymorphism combinations. While one approach could be finding phBEC donors possessing particular polymorphisms, genes could also be knocked out using recently developed gene editing techniques (i.e. CRISPR).

While the genome is akin to fixed hardware, the epigenome is the software that dictates where, when, and the extent to which genes will be expressed. Unlike the genome, the epigenome is malleable and can be influenced by a variety of intrinsic properties such as age, sex, genotype and extrinsic factors such as environmental exposures (Bowers and McCullough *et al., 2*017). Given the ability of environmental exposures to alter the epigenome, an important

question is whether exposures incurred by donors prior to phBEC collection could influence the responses of phBEC cultures to ozone via an epigenetic mechanism.

During the phBEC culture process, epithelial cells are collected and then basal epithelial cells undergo several divisions and eventually differentiate into the various cell types found in fully differentiated phBEC cultures. In order for prior exposures to impact *in vitro* phBEC responses, epigenetic imprinting would have to remain intact throughout all of these stages.

Currently, it is unknown how persistent exposure-associated epigenetic changes may be, but there are several experiments that could be performed to answer these questions. EPA currently possesses phBECs that were collected from the same individual following exposures to both FA and ozone. Both the FA and ozone-exposed phBECs could be cultured, exposed to ozone and filtered air *in vitro*. RNA samples could be collected to assess exposure-associated gene inductions and ChIP material could be collected prior to exposure to determine if the baseline epigenome differed as a result of prior *in vivo* exposure. While it is possible that a single exposure to ozone may not leave a detectable epigenetic imprint in phBECs, this same experimental approach could be used to assess outcomes from more extensive exposures, such as comparing the epigenomes of phBECs collected from smokers and non-smokers, or comparing outcomes in individuals who have different diets.

Additional characterization of the adaptive mechanism

This dissertation demonstrates that the reduction of ERK1/2 activation is an important component of *in vitro* ozone adaptation. Reductions in MAPK activation are also a central mediator of a similar phenomenon called LPS tolerance, which is characterized by inflammatory suppression during repeated exposure to LPS. LPS tolerance literature suggests that reduced

MAPK activation is accomplished by the increased expression of microRNAs targeting MAPK proteins, increased phosphatase abundance and activity, as well as decreased expression of MAPK proteins, receptors, and co-receptors (Biswas and Lopez-Collazo 2009; Fan and Cook 200; Foster *et al.,* 2007). While my current research has been based on a candidate gene approach, a more objective hypothesis-generating approach would useful to identify unknown mediators of the adaptive response. In such an analysis I would perform RNA-seq on both FA, $1XO_3$, and $4XO_3$ samples to determine which genes were alternatively regulated. If the results of this analysis identified genes that were related to MAPK regulation, this would be highly supportive of the findings in this dissertation and provide novel insights regarding how the MAPK is regulated during ozone adaptation.

The LPS tolerance literature suggests that adaptive responses are mediated by multiple overlapping mechanisms that target both MAPK signaling and the epigenome. At the beginning of my dissertation research I hypothesized that a central mechanism of ozone adaptation was the alteration of epigenetic modifications at pro-inflammatory gene promoters. As a first step to addressing this hypothesis, I needed to identify epigenetic modifications that were associated with ozone responsiveness so that I could then see if the abundance of these marks changed during adaptation. These initial steps consumed most of my time and resources, so unfortunately I didn't get the opportunity to investigate epigenetic contributions to *in vitro* ozone adaptation. If I were able to continue this research, I would use the information I had previously obtained regarding epigenetic modifications and time points to conduct ChIP-seq on cells that had been exposed to FA, $1XO_3$, and $4XO_3$. In such an experiment, RNA would be collected at the time of peak gene expression, and ChIP material would be collected prior to exposure (to see if repeated

ozone exposure altered baseline chromatin modifications), as well as during peak expression (to see if adaptation is related to a lack of a certain mark being placed).

While this analysis could just be focused on the four candidate genes described in this dissertation, performing ChIP-seq and RNA-seq would facilitate a more objective approach and could be used to grant a more global view of ozone adaptation. Other studies have noted that epigenetically-mediated suppression during repeated exposure can vary by gene function; for example, during repeated LPS stimulation, inflammatory genes are suppressed due to a lack of histone acetylation, while the acetylation and induction of antimicrobial response genes are unchanged (Foster *et al.,* 2007). It would be of interest to see if the same patterns could be observed during repeated ozone exposure.

Epigenetic influences: association vs. causation

While the epigenetic studies presented in this dissertation represent an initial screening to identify epigenetic modifications and time points that may be important, a short-coming of this work is that the data presented are predominantly associative. While it is known that many chromatin modifications play an active role in controlling gene transcription by altering nucleosome conformation and providing docking sites for effector proteins; the role of specific modifications may not always be straightforward and can be highly locus dependent (Berger et al., 2007; Kouzarides *et al.,* 2007). Thus, future studies using the phBEC model system will need to establish if chromatin modifications play a functional role in the ozone-mediated induction of each gene.

An epigenetic modification that I am particularly interested in is H3K27 methylation. Previous work suggests that the removal of H3K27 methylation at inducible gene promoters is

important for transcriptional elongation (Chen *et al.,* 2012). Indeed, the data presented in Chapter 4 suggests that the removal of H3K27 methylation may facilitate *IL-8* induction during ozone exposure. I considered, but did not have time to implement, an experiment in which phBEC cultures were treated with an inhibitor of a K27 demethylase, jumonji domain containing protein (JMJD3), to determine if an inability to remove this mark influenced ozone-associated transcription. Alternatively, overexpressing enhancer of zeste homolog 2 (*EZH2*), the catalytic component of the polycomb repressive complex 2 (PRC2) that confers H3K27 methylation, may be a way to determine if the enrichment of this mark decreases ozone-associated gene induction. Placement of activating histone modifications, such as histone acetylation, may be equally as important as the removal of repressive modifications. Similar experiments could be undertaken to assess the impact of HDAC/HAT inhibitors on ozone induction.

Unfortunately, such experiments in the phBEC model system require the use of small molecular inhibitors, as these cells are recalcitrant to transfection and transduction. Although valuable information may be obtained by the use of such inhibitors, off-target effects are always a concern. Despite previous difficulties in genetically manipulating phBECs, recent advances suggest that this may be possible using the CRISPR-Cas9 system, which would be a powerful tool in answering many of the questions posed in this chapter. We could potentially use this system to study the effect of the gene polymorphisms discussed earlier in this chapter. Moreover, we have considered tethering epigenetic effectors to dead Cas9, which could be then be targeted to a specific location within the genome (e.g. the *IL-8* promoter). This would be an ideal way to assess the role of certain epigenetic effectors while minimizing off-target effects.

Considerations in modeling the ozone pro-inflammatory response

Airway neutrophilia is a hallmark of ozone exposure. While neutrophils may help clear tissue damaged by exposure, they can also cause further damage to the epithelium by releasing bactericidal ROS. Inflammation is an important physiological process that must be tightly regulated, as too much or too little can be pathological. Thus it is important to emphasize that inflammation should not immediately be considered negative; however excessive, uncontrolled, or repeated bouts of inflammation can be detrimental as it can cause squamous cell metaplasia, airway remodeling, and permanent damage to the lung (US EPA 2013).

The transcriptional control of *IL-8* has been a major focus in this dissertation because *IL-8* is an important mediator of neutrophil chemotaxis following ozone exposure. As stated above, the induction of *IL-8* in phBECs does not necessarily equate to a negative health outcome, rather we are trying to understand the factors controlling *IL-8* induction because, putatively, if *IL-8* induction is excessive or prolonged, this could lead to an excessive neutrophil response. In this context, the adaptive response could be beneficial because it could prevent excessive inflammation.

While IL-8 is a crucial pro-inflammatory cytokine, there are other mediators that are released as a part of the ozone pro-inflammatory response. While we were able to attribute a large component of *IL-8* induction to ERK1/2 and p38 activation, the pathways upstream of other genes such as *IL-6* and *COX-2* are less characterized in the phBEC model. Future experiments could explore the role of other receptors and pathways ouch as Akt, and NFkB.

In addition to epithelial cells, other cell types contribute to the ozone inflammatory response, such as fibroblasts, macrophages, mast cells, dendritic cells, etc. Future experiments could utilize co-culture techniques to determine the role that these cells may play in modulating

inter-individual variability and ozone adaptation. While the phBEC system used in this dissertation grants insight into the pro-inflammatory response, it does not permit the study of lung function changes, which is a critical aspect of the ozone response.

While modeling the mechanics of lung function *in vitro* may not be a viable approach, decrements in lung function could be inferred by examining the ozone-mediated release of certain compounds. Ozone exposure causes the activation of TRPA1 receptors on C-fibers which then release compounds such as substance P, neurokinin A, and CGRP. These compounds then lead to painful inspiration and bronchial smooth muscle reactivity, which are the predominant causes of ozone-associated lung function decrements (US EPA 2103). It may be possible to use a neuronal cell line to study how ozone could lead to the release of these substances. With advancing iPSC technology, it may also be possible to differentiate fibroblasts collected from different individuals into neuronal cells, which could then be used to study inter-individual variability in the ozone-mediated release of these neurotransmitters.

While the primary goal of this dissertation was to examine the mechanisms that shape pro-inflammatory gene expression, an important aspect, which we were unable to address is the effect of phBEC donor characteristics on ozone responsiveness. The results depicted in Chapter 5 suggest that factors such as age, sex, and genotype may be important contributing factors, yet we were unable to identify statistically significant trends due to limitations in sample size. Future studies could use the data collected in this dissertation to conduct power calculations. Furthermore the donor pool described here could then expand upon and donors with specific characteristics could be recruited.

Conclusions: The MAPK-epigenome axis

The goal of this dissertation was to discover the basis of the molecular programming that shapes epithelial pro-inflammatory responses to ozone exposure. Using the phBEC model I discovered two important components of this programming: differences in MAPK pathway activation and patterns of epigenetic modifications at pro-inflammatory gene promoters. While it may be easy to think of these as discrete units, they are actually part of the same system, which allows cells to respond to their external environment by converting extracellular signals into gene induction. The activation of membrane receptors and cellular signaling cascades, such as ERK1/2, represents the top of this axis and epigenetic changes at gene promoters represents the bottom, which is the mechanism by which signaling leads to transcription. While the extreme ends of this system have been described in this dissertation, further research is required to describe events occurring in between, in particular the involvement of other kinases, transcription factors, chromatin-modifying complexes, etc. Conceptualizing this system as an interrelated axis is amenable to environmental health research because factors that shape ozone associated gene inductions can be easily incorporated (Figure 6-1). For example, the GSTM1 protein can influence the MAPK pathway as previously described, as can prior ozone exposures (Chapter 3). These factors may reprogram epigenetic patterns at pro-inflammatory gene promoters, which we have been shown to be highly associated with ozone induction (McCullough et al., 2016; Chapter 4).

The more we understand about this system, the more insight we will have into the mechanisms governing exposure response inter-individual variability. With this information we may be able to refine predictions of susceptible individuals and understand health effects in

exposed populations. This may be particularly helpful in understanding the health effects of real world exposures and complex responses such as ozone adaptation.

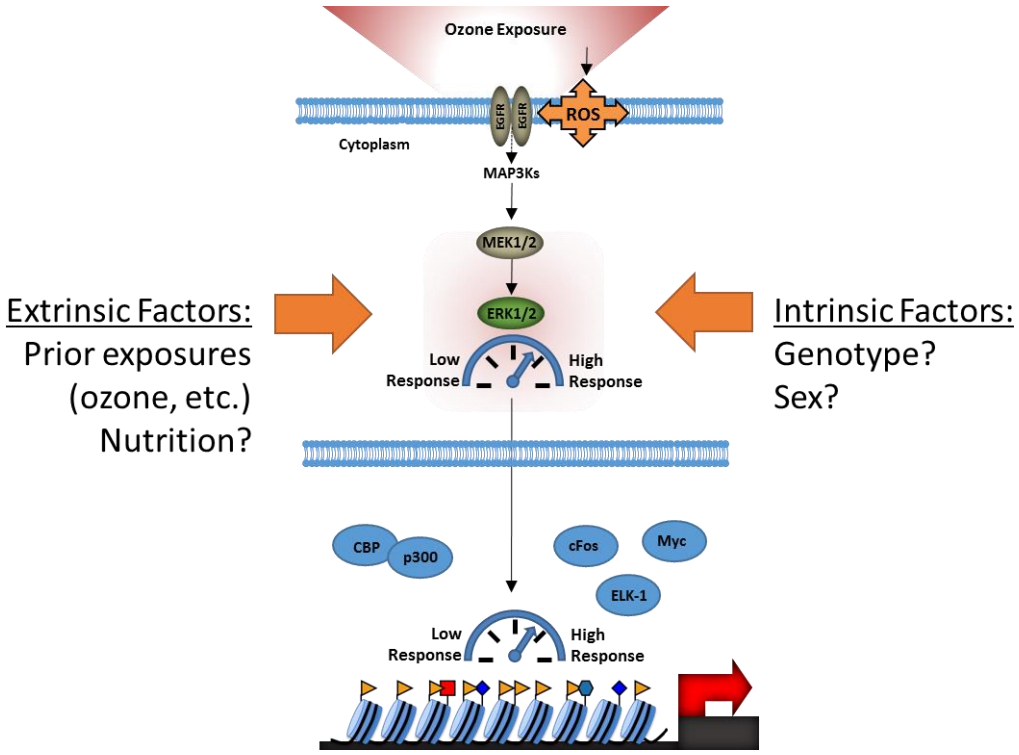

Figure 6-1. Bringing it all together: the MAPK-epigenetic axis. This dissertation has provided evidence that the activation of ERK1/2 and epigenetic patterns at pro-inflammatory genes may be important in mediating variability ozone-associated gene inductions. This 'ERK1/2-epigenetic axis' could also be influence by a variety of intrinsic factors, such as genotype and sex, and extrinsic factors, such as prior ozone exposure.

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APPENDIX 1: SUPPLEMENTARY METHODS

Target	Antibody Manufacturer	Product Number
GAPDH	Cell Signaling	5174
α-ERK1/2	Cell Signaling	4695
α-ERK1/2 p(T202/Y204)	Cell Signaling	4370
α-MEK1/2 p(S217/221)	Cell Signaling	9121
α-MEK1/2	Cell Signaling	9126
α-MKK4/SEK1 p(S257/T261)	Cell Signaling	9156
α-MKK4/SEK1	Cell Signaling	9152
α-p38	Cell Signaling	9212
α-p38 p(T180/Y182)	Cell Signaling	4511
α-p65 p(S536)	Cell Signaling	3033
α-p65	Cell Signaling	8242
Total H3	Active Motif	39163
H3K4me3	Active Motif	39915
H3K27me2/3	Active Motif	39535
H4ac	Active Motif	39925
Normal Mouse IgG	Santa Cruz Biotechnology	SC-2025

Table A1-1. Antibodies used for Western blotting and ChIP.

Solution	Composition
Protease Inhibitor Mixture	1X cOmplete protease inhibitor cocktail (Roche), 20 mM butryric acid, 25 mM NaF, 1mM $Na_4P_2O_7$
ChIP Lysis Buffer	50 mM Tris, pH 8.1; 10 mM EDTA; 1% SDS; 20 mM butryic acid; 1 mM PMSF; 1X cOmplete protease inhibitor
ChIP Dilution Buffer	16.7 mM Tris, pH 8.1; 167 mM NaCl; 1.2 mM EDTA; 1.1% Triton X-100; 0.01% SDS; 20 mM butyric acid; 1 mM PMSF; 1X cOmplete protease inhibitor
Low Salt Wash Buffer	20 mM Tris, pH 8.1; 150 mM NaCl; 2 mM EDTA; 1% Triton X-100; 0.1% SDS
High Salt Wash Buffer	20 mM Tris, pH 8.1; 500 mM NaCl; 2 mM EDTA; 1% Triton X-100; 0.1% SDS
LiCl Wash Buffer	10 mM Tris, pH 8.1; 1 mM EDTA; 1% IGEPAL CA-630 (NP-40) 250 mM LiCl; 1% sodium deoxycholate
TE Wash Buffer	10 mM Tris, pH 8.0; 1 mM EDTA
ChIP Elution Buffer	1% SDS, 0.1 M $NaHCO_3$

Table A1-2. ChIP buffer formulations.

ChIP Primer and Probe Sets	
Gene and Target	Sequence (5’ to 3’)
IL-8 Forward	CATCAGTTGCAAATCGTGGA
Reverse	GAAGCTTGTGTGCTCTGCTG
Probe	AAAGCCACCGGAGCACTCCA
IL-6 Forward	AGCCTCAATGACGACCTAAGCT
Reverse	ATTGTGCAATGTGACGTCCTTT
Probe	CACTTTTCCCCCTAGTTGTGTCTTGCCA
COX-2 Forward	CTGGGTTTCCGATTTTCTCATT
Reverse	GTACCCCCCACAAATTTTTCC
Probe	TGGGTAAAAAACCCTGCCCCCACC
HMOX-1 Forward	GACATTTTAGGGAGCTGGA
Reverse	TCCCAGAAGGTTCCAGAAAGC
Probe	CTGATGTTGCCCACCAGGCTATTGC

Table A1-3. Primer and probe sequences used for detection of ChIP DNA.

Gene Expression (cDNA) Primer and Probe Sets	
Gene and Target	Sequence (5’ to 3’)
IL-8 Forward	TTGGCAGCCTTCCTGATTTC
Reverse	TATGCACTGACATCTAAGTTCTTTAGCA
Probe	CCTTGGCAAAACTGCACCTTCACACA
IL-6 Forward	GGTACATCCTCGACGGCATCT
Reverse	GTGCCTCTTTGCTGCTTTCAC
Probe	TGTTACTCTTGTTACATGTCTCCTTTCTCAGGGCT
COX-2 Forward	GAATCATTCACCAGGCAAATTG
Reverse	TCTGTACTGCGGGTGGAACA
Probe	TCCTACCACCAGCAACCCTGCCA
HMOX-1 Forward	CAGCAACAAAGTGCAAGATTCTG
Reverse	AGTGTAAGGACCCATCGGAGAAG
Probe	AGGGAAGCCCCCACTCAACACCC
B-Actin Forward	CTGGCACCCAGCACAATG
Reverse	GCCGATCCACACGGAGTACT
Probe	ATCAAGATCATTGCTCCTCCTGAGCGC

Table A1-4. Primer and probe sequences used for quantification of expression