Title: CXCL10/CXCR3 Regulation of Ozone-Induced Epithelial Permeability

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Purpose of the Study –

Ozone (O_3), a highly recognized cause of environmental lung injury, contributes to exacerbations of chronic pulmonary diseases and overall mortality. Despite efforts to reduce ambient O_3 levels, these are expected to rise with global warming. Addressing this public health concern requires focus on pulmonary mechanism(s) of O_3-induced host-responses to identify candidate pathways that can be targeted with precision in susceptible individuals. O_3 inhalation is known to compromise barrier integrity of respiratory epithelial surfaces, an initial step in pulmonary injury. Although frequently overlooked, a compromised epithelium compounds susceptibility to subsequent exposures with airborne infectious and/or toxic agents. Epithelial barrier preservation requires coordinated signaling between the epithelium and resident immune cells, *principally macrophages*. Identifying the specific cellular mechanisms critical to this interaction would identify individuals with heightened susceptibility to O_3 inhalation and potential targets for intervention.

In healthy adult subjects, we find that acute O_3 exposure significantly increases expression of the interferon-γ (IFN-γ) inducible chemokines CXCL9, CXCL10 and CXCL11 in lung (BAL) macrophages. In addition, human CXCL10 expression significantly associates with increased BAL albumin, a marker of epithelial permeability, suggesting that IFN-γ inducible chemokines mediate O_3-induced epithelial barrier dysfunction. CXCL9, 10 and 11, released from macrophages, signal on lung epithelial cells via their receptor CXCR3. Consistent with our human data, CXCR3 null mice are protected from O_3-induced epithelial permeability and have altered epithelial barrier proteins expression. To translate the CXCR3-/- finding, a common human polymorphism of CXCR3 exists wherein individuals with the minor allele have reduced CXCR3 gene expression/function. Therefore, this polymorphism may identify individuals with decreased susceptibility to O_3-derived health effects. This provides an opportunity to define a specific mechanism to explain O_3-induced airway epithelial leak. **We plan to test the hypothesis that O_3 induces the production and release of IFN-γ inducible chemokines by airway macrophages, activating CXCR3 on epithelial cells, which leads to O_3-induced permeability via modulation of epithelial barrier proteins**. Our overall research plan is translational (murine and human studies) relying on unique mouse models, primary macrophage and epithelial co-cultures, and BAL samples obtained from human exposure studies segregated by a CXCR3 polymorphism. For the purpose of this IRB proposal, our specific aim for the human studies are as follows:

Aim: To define if a human intronic CXCR3 polymorphism that reduces CXCR3 functionality defines genetic susceptibility to O_3-derived alterations in airway inflammation, and epithelial permeability. Using the National Institute of Environmental Health Sciences (NIEHS) Environmental Polymorphism Registry (EPR), we will identify and recruit subjects with the major or minor allele of the **CXCR3 polymorphism rs2280964,** and challenge them with filtered air (FA) vs O_3 (200 ppb) for 135 minutes, the equivalent of a code orange ozone day. 24h later, bronchoscopy with BAL and epithelial cell brushings will be performed to define O_3-induced alterations in airway epithelial permeability, and modifications of blood/BAL mononuclear cell populations. Harvested epithelial cells grown at air-liquid interface (ALI) and the resulting cultures will be used to determine direct effects of the polymorphism on O_3-induced epithelial permeability and barrier protein expression.

The completion of these human studies will support translational of mechanistic animal studies that will clearly determine the extent to which the CXCL10/CXCR3 axis mediates increased O_3-induced airway epithelial permeability and whether a functional CXCR3 polymorphism is associated with O_3 susceptibility. Furthermore, it provides a means to identify O_3-susceptible individuals and define novel therapeutics to limit O_3-induced epithelial permeability.

Background & Significance –

Ozone (O_3) is an ambient gas formed through a chemical reaction between oxides of nitrogen and volatile organic compounds in the presence of sunlight. Since the Clean Air Act of 1970, the EPA has identified ambient O_3 as one of six “criteria” pollutants with adverse health effects that significantly contribute to human morbidity and mortality. At risk populations include individuals with underlying respiratory disease (asthma, COPD), children, and seniors. O_3 is estimated to account for approximately 800 premature deaths, 4,500 hospital admissions and 900,000 school absences annually. These absences, and the more than a million days of restricted activity, have an annual economic burden estimated at $5 billion (1). Furthermore, ambient O_3 levels are expected to increase with climate change (2), with substantial impact on global health. These well-designed epidemiologic studies clearly demonstrate that a singular focus on regulation of O_3 levels will be insufficient to prevent all adverse health effects. An improved understanding of the basic mechanisms and susceptibility factors that contribute to O_3-derived health effects will be necessary to develop targeted therapeutic strategies in susceptible populations.

It is clear from studies of human subjects and inbred mouse strains that the biological response and susceptibility to O_3 depend on host factors (3-6). O_3 pathophysiologic changes include: development of airway hyperresponsiveness (AHR); activation of the innate immune system; and enhancement of epithelial cell permeability (7-10). Epithelial barrier dysfunction is a common feature of debilitating lung diseases including asthma, COPD, sarcoidosis, and pulmonary fibrosis. The maintenance of epithelial integrity limits access of xenobiotic materials (such as allergens, irritants or infectious agents) to the lung interstitium. Bi-directional epithelial leak can function to initiate and perpetuate inflammatory responses by reduction in the surface levels of endogenous anti-

inflammatory proteins and/or increases in serum and inflammatory mediators. Since O_3 enhances epithelial permeability, it raises the possibility that O_3 exposure is a disease susceptibility factor. In support of this, recent epidemiologic studies associated increased incidence of asthma and COPD emergency room visits with higher short-term O_3 concentrations (11, 12). Though less well defined, a study also suggested an association between short-term increases in O_3 levels and incidence of asthma in children (13). Multiple mechanisms could explain these associations; however, they support a causal link between acute exposure to O_3 and failure of epithelial integrity as a pathologic feature. To elucidate mechanisms, human-based studies are essential to defining host susceptibility to O_3 and acute epithelial injury.

Normal healthy humans undergoing laboratory exposure to O_3 exhibit varied biologic responses. These include: acute changes in Forced Expiratory Volume in 1 second (FEV1), enhanced airway hyperresponsiveness (AHR) to provocative challenge, inflammatory cell influx into the airway/alveolar space, and enhanced epithelial permeability. Well-designed human exposure studies demonstrate that individuals can be characterized as high or low responders to O_3 (3, 14, 15); O_3 responders typically exhibit only one specific biologic phenotype (15); and this response is highly reproducible for a given individual (10, 15-17). The reproducibility and specificity of biological responses in an individual subject strongly support the notion that host genetic factors contribute to the variability in O_3 sensitivity. To define susceptibility to O_3-induced alterations of epithelial integrity, markers of epithelial integrity such as serum CCSP and BAL total protein have been used in human exposure studies (9, 18). In work by our group, human subjects undergoing acute laboratory exposure to O_3 demonstrate enhanced ability to clear a low MW (490 Da) hydrophilic radio marker, 99mTc-DTPA, from the airway at ~20 h post-exposure (functional marker of epithelial permeability) (15). This effect was highly reproducible with repeated measures of the same subject; supporting that O_3-induced epithelial permeability is likely driven by host-genetic susceptibility factors. In preliminary studies, we measured BAL albumin from humans at ~20 h post FA or O_3 exposure. We calculated fold change of albumin responses. Plotting a distribution of the log of albumin (O_3/FA), it is likely that there are two major phenotypic responses (increased vs decreased albumin), each of which had a Gaussian distribution (Figure 9). Based on this analysis, 36% of people decrease BAL albumin after O_3, and 64% of people increase BAL albumin after O_3 supporting the use of this measure to define genetic susceptibility.

With clear associations between O_3 responses and host genetics, defining genetic susceptibility factors are needed to develop targeted interventions in susceptible individuals. Single nucleotide polymorphisms (SNP) are heritable single gene sequence changes that can alter gene expression/function, and could explain variability in human exposure responses. To explore potential disease modifying CXCR3 SNPs, Cheong et al sequenced the CXCR3 gene in a Korean cohort. They identified a prevalent intronic polymorphism rs2280964 (minor allele frequency= 0.445), which associated with asthma development risk (19). In follow up studies, homozygotic expression of the rs2280964 minor allele reduced CXCR3 expression and function (T cell chemotactic activity) (20, 21). Given these observations and our own preliminary data, we hypothesized the rs2280964 polymorphism segregates susceptibility for O_3-induced

Caucasian Female		Caucasian Male	
Genotypes	Observed #	Genotypes	Observed #
Homozygote Major Allele:	75	Hemizygote Major Allele:	46
Heterozygote:	45		
Homozygote Minor Allele:	6	Hemizygote Minor Allele:	16
Mean Allelic Frequency	**0.226**	**Mean Allelic Frequency**	**0.258**
African American Female		**African American Male**	
Genotypes	Observed #	Genotypes	Observed #
Homozygote Major Allele:	78	Hemizygote Major Allele:	30
Heterozygote:	54		
Homozygote Minor Allele:	11	Hemizygote Minor Allele:	13
Mean Allelic Frequency	**0.266**	**Mean Allelic Frequency**	**0.302**

Figure 1: Mean allelic frequency of CXCR3 polymorphism rs2280964 in the NIEHS Environmental Polymorphism Registry (EPR). EPR subjects (n=374) were genotyped for the major and minor allele of an intronic CXCR3 polymorphism. Since CXCR3 is X-linked male subjects are hemizygous for the allele. The female subjects are in Hardy Weinberg equilibrium.

epithelial permeability in humans. Since the SNP frequency in a US population is unknown, we collaborated with investigators at NIEHS to query the Environmental Polymorphisms Registry (EPR). The EPR is a NIEHS-sponsored cohort study to explore interactions between environmental stimuli and host-genetics. The cohort is comprised of approximately 15,000 North Carolina residents with associated DNA samples. In addition to baseline clinical information, EPR subjects agreed to be contacted for enrollment in future clinical studies. Approximately, 6000 subjects live within 50 miles of the Raleigh/Durham area. Using a random sampling of the cohort (n=374), we defined a mean allelic frequency for rs2280964 (Figure 10). In males this was 0.258 while in females this was 0.226 suggesting that approximately a quarter of this cohort expresses the minor allele, which prior studies suggested reduces CXCR3 expression and function. This offers the potential to determine if rs2280964 defines genetic susceptibility to O_3-induced airway epithelial permeability.

The **primary goal** of this proposal is to conclusively determine central factors that affect epithelial barrier function in oxidant mediated lung injury. We hypothesize that the CXCL10/CXCR3 axis is a central regulator of this response and will focus on the environmentally relevant and ubiquitous urban air pollutant, O_3. To carefully dissect how O_3 modifies the CXCL10/CXCR3 axis and its contribution to dysregulated epithelial permeability, we will combine *in vivo* modeling of genetically altered mice with *in vitro* co-cultures of macrophages and epithelial cells and then link these observations to human translational studies. The information provided in this proposed study would allow us to define critical susceptibility factors to human responses to O_3, which could have important impacts on public health.

Design & Procedures

Plan for Specific AIM:

We propose to prospectively recruit healthy human subjects from the NIEHS sponsored Environmental Polymorphism Registry (22) based on homo/hemizygotic expression of the major and minor allele. The EPR core at NIEHS will perform the genotyping of these subjects. The EPR has an established protocol for the cohort of individuals and for the use of their genetic information to screen for additional studies. All of the genotyping and initial contact to the potential subjects is under the EPR protocol and procedures, not this protocol. Based on the individual's genotype, the EPR will contact the subjects to update contact information and see about their interest in the study. If the individual

subject is interested, then contact information will be provided to Duke Investigators for recruitment based on this genotype following established protocols at NIEHS. Following informed consent, these subjects will undergo laboratory O_3 exposure to determine if expression of the minor allele protects against O_3-derived permeability of the respiratory epithelium. Additionally, we will use a novel flow cytometry panel and define immune cells in human blood and BAL (23). We will determine how O_3 modifies immune cell populations in the blood and BAL, determine which cells express CXCL10 and define how the CXCR3 polymorphism alters this response. Using the subjects' epithelial cells grown at ALI *in vitro* exposure studies will assess direct effects of the polymorphism on epithelial integrity and test CXCR3 pharmacologic agents (antagonists and biased agonists) as potential therapeutics. The specifics are as follows:

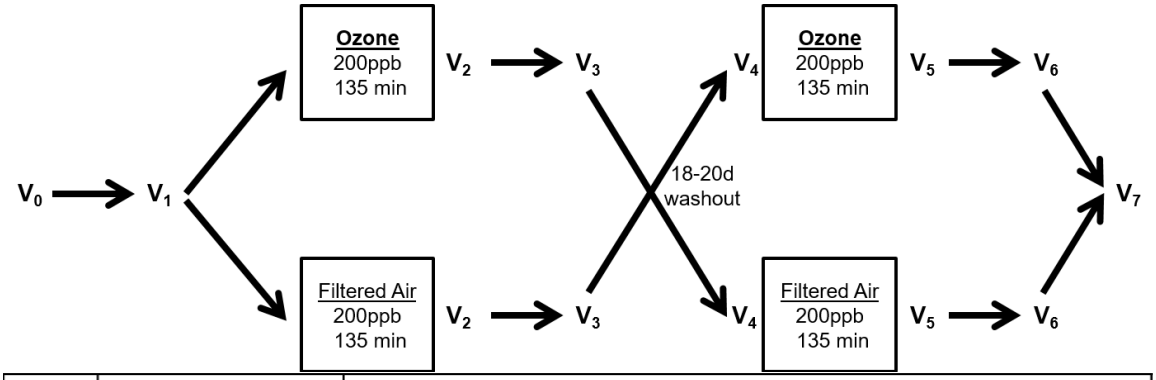
Sub AIM1: We will assess the effect of a CXCR3 polymorphism (rs2280964) on human O_3 biological responses by prospectively enrolling healthy adults based on homo/hemizygous expression of the major or minor alleles. Samples collected after each exposure to FA and O_3 will include: 1) epithelial cells from bronchial brushings; 2) bronchoalveolar lavage fluid; 3) purified alveolar macrophages; and 4) peripheral blood cells/serum. Our primary outcome will be to define the polymorphism's effect on injury to epithelial integrity (BAL albumin and serum CCSP) and pulmonary and systemic immune responses (flow cytometry of BAL and whole blood cells). These responses will be compared based on homo/hemizygotic expression of the major or minor allele to define if the minor allele protects against O_3-induced epithelial permeability and inflammation. In secondary analyses, BAL fluid and serum will be assessed by multiplex for cytokine release (IFN-γ and CXCL9-11) and cells by RT-PCR for cell specific expression (brushed epithelial cells and alveolar macrophages) of IFN-γ, CXCL9-11, and CXCR3. This will allow us to clearly define the expression of IFN-γ/CXCL10/CXCR3 axis in humans based on cell type; define the effect of O_3 on this expression; and identify if this is altered based on the polymorphism.

Sub AIM2: To determine if homo/hemizygotic expression of rs2280964 directly effects epithelial integrity we will use epithelial brushings obtained by bronchoscopy following FA exposure. Using an established protocol (24), we will expand the epithelial cells to passage 3, transfer them to transwells and differentiate them at ALI. Epithelial cells will be exposed to FA or O_3 (200 ppb) *in vitro* for 1 h. Following exposure, markers of epithelial integrity will be assessed (TEER and transit of FITC dextran) immediately and 1, 2 and 3 h post-exposure. RT-PCR and western blots will be performed for epithelial barrier proteins. The data will be analyzed to assess differences in *in vitro* responses based on expression of the major or minor allele. To assess potential therapeutic effects, *in vitro* exposures will be performed with pharmacologic grade CXCR3 antagonist/biased agonists to determine effects on epithelial integrity and barrier proteins. These studies will define direct epithelial-specific effects of the polymorphism and provide preliminary studies on the efficacy of the antagonist and biased agonists in human O_3-induced alterations of epithelial integrity.

Protocol for Specific AIM:

The overall study protocol will be as follows (see figure 2). The NIEHS EPR will sequence potential subjects located within 50 miles of the Raleigh-Durham area. Based on their genotype individuals will be approached by the EPR for consideration in the

study. Following initial consent to be contacted, contact information will be provided and potential subjects will be contacted to recruit for the study. Following recruitment, subjects will present for a screening visit (V_0). At that time, they will undergo an assessment of medical history/physical exam, baseline pulmonary function with a methacholine challenge (to define airway hyper-responsiveness) and urine pregnancy testing if a woman is of child bearing potential. If there are no observed exclusion criteria and the patient agrees to undergo the study, informed consent will be obtained and the patient will be scheduled for V1 (±14d). On V1 subjects will be exposed to either FA and O_3 using a cross-over challenge design and thus each subject will serve as his/her own control. Subjects will be randomized to the first challenge (FA or O_3) and will be blinded to the exposures during the study.



Visit	Description	Testing
V_0	Screening (recruited from the EPR)	History and Physical; Venous Blood Draw (DNA, CBC with Diff and creatinine); Spirometry; Methacholine Challenge, Serum Pregnancy Test (if female)
V_1	Pre-exposure	Venous Blood Draw; Spirometry; Urine Pregnancy Test (if female)
V_2	Post-exposure	Venous Blood Draw; Spirometry
V_3	18-20 hrs. post-exposure	Spirometry; Epithelial Permeability Measurements; Bronchoscopy; Urine Pregnancy Test (if female)
V_4	Pre-exposure	Venous Blood Draw; Spirometry; Urine Pregnancy Test (if female)
V_5	Post-exposure	Venous Blood Draw; Spirometry
V_6	18-20 hrs. post-exposure	Spirometry; Epithelial Permeability Measurements; Bronchoscopy; Urine Pregnancy Test (if female)
V_7	Post-protocol phone call	

Figure 2: Study Design of Human Exposure. Healthy subjects will be exposed to O_3 (200 ppb) or FA in a randomized crossover study design with each subject as his/her own control and an 18-20 day washout period between exposures. Immediately pre- and post-exposure we will measure pulmonary function (PFT) and collect venous blood. At 20-24 h post-exposure, we will again measure lung function, and venous blood. Then subjects will undergo bronchoscopy with BAL and epithelial cell brushing.

The specifics for each visit are listed in the figure. In brief, at V1 subjects will be assessed for baseline spirometry and venous blood analysis prior to exposure. The venous blood

will be used to assess clara cell secretory protein (CCSP) as a measure of epithelial integrity. Additionally, this will be used to obtain cells for flow cytometry and to measure cytokines and growth factors. At each visit, if the subject is female, there will be a urine pregnancy test performed. Following this initial assessment, subjects will be challenged with FA or O_3 and then spirometry will be performed, and venous blood will be obtained immediately following the exposure (V2). The subjects will then return 20 hours ±4h later for follow up studies (V3). There will be spirometry, a venous blood draw and urine pregnancy testing (if female) followed by bronchoscopy. At bronchoscopy, vital signs will be determined, including O_2 sat. Patient then undergo an 18-20 day washout period before they are brought back in for V4 for the alternate challenge. This will follow the same protocol as outline above in the initial exposure and use the same series of analysis as the first set of visits. Therefore, we will fully characterize the biological response to ozone and filtered air in these same subjects.

Specific Procedures:

Pulmonary Function Testing (PFT):
Spirometry: Spirometry breathing assessment will be performed in accordance with American Thoracic Society / European Respiratory Society (ATS/ERS) recommendations and the schedule of events. Testing will be performed at screening, and pre, and post study exposures. See Figure 2 for the schedule of PFT's. Parameters to be measured at each time point are (FVC) forced Vital Capacity, (FEV1), Forced Expiratory Volume in one second, (PEF), Peak expiratory flow rate, and (FEF25-75%) forced expiratory flow rate at 25 – 75% of exhalation. Measured results will be compared to the Crapo predicted set.

Methacholine Bronchoprovocation: Methacholine challenge will be performed at the screening following ATS/ERS recommendations. A seven –step procedure will be performed starting with diluent then increasing doses of Methacholine using a nebulizer, (from 0.0625 mg/ml up to maximum of 16 mg/ml). A physician and emergency medications will be within close proximity in case of any adverse response. Historical Methacholine challenge tests performed by study team are accepted.

Ozone versus Filtered Air: Exposures (135 min in duration) are performed in the Duke South human exposure facility, with recording and monitoring of minute ventilation, frequency of ventilation, pulse rate and transcutaneous O2-saturation. The O_3 level during the exposures will be 200 ppb, which has previously been used in human exposure studies without short or long term, untoward side effects (and comparable to peak levels attained during the summer in the Raleigh-Durham area of North Carolina) (REF – Bromberg review). As ambient O_3 levels can be a confounder for these studies, O_3 levels in the Raleigh-Durham area will be monitored during the study. If levels reach "Code Red" designation, then subjects will not undergo controlled laboratory studies for 2 weeks as a washout and until ambient O_3 levels return to baseline. For exposure to O_3, the dose will be controlled to within 10 ppb of 200 ppb. The exposure chamber (500 ft^3) is capable of 35 turnovers (passes)/hr and includes a conditioned air ventilation system. Supply air is hepa-filtered, and conditioned to desired temperature (25°C) and relative humidity (45-55%). Chamber air is monitored continuously for O_3; O_3 is

generated from 100% O_2 source by cold plasma corona discharge (Ozotech, Inc, Yreka, CA), pre-mixed with filtered air, and added to the chamber. For all exposures, the physical activity level during exposure of the subject will alternate between 15 min periods of rest and treadmill walking. This is similar to an individual performing light activity under ambient conditions.

Bronchoscopy Procedure in Humans: The Duke South bronchoscopy suite is available for research protocols. It is equipped for all safety concerns, for collection of lavage, and bronchial brushed epithelial cells. Lavage and brushing procedures follow standard clinical practice of the Duke University Medical Center. Subjects are pre-medicated intravenously (versed and fentanyl), but inhaled bronchodilators and minor sedatives may be administered as needed. An electrocardiogram and pulse oximeter are constantly monitored by the investigator and dedicated nurse providing sedatives. Intravenous access maintained at all times and nasal supplemental O_2 is available. After inhalation of aerosol anesthetic, a fiberoptic bronchoscope is inserted and gently wedged within lower subsegmental airway of the right middle lobe. Lung lavage is accomplished using four 50 ml aliquot of prewarmed (37 C) isotonic saline, with immediate aspiration after each installation. Following lavage, bronchial segment(s) are gently brushed (2 separate brushes) for collection of airway epithelial cells. After each brushing, the brush is rinsed into a sample tube with sterile PBS and chilled; brush tips are also snipped with sterile technique and placed in chilled PBS. Brush tips are rinsed and together with initial brush rinses, brush fluid is centrifuged for separation and cell counts of epithelial cells. At conclusion of the procedure, subjects remain under observation for another 90 min.

Lavage fluid returns are kept on ice, after which small amounts are removed for cell counts, viability determinations and differential staining. Sample processing will occur in the PI's laboratory (Medical Science Research Building I room 271).

Selection of Subjects –

Inclusion Criteria

- Individuals between 18-45 yrs. of age (No subject will be excluded from the study on the basis of gender or ethnicity)
- Prior enrollment in the NIEHS Environmental Polymorphism Registry located in the greater Raleigh-Durham area
- Wild type or homozygous (female)/hemizygous (male – since sex-linked) expression of the CXCR3 polymorphism rs2280964

Exclusion Criteria

- Current smokers of tobacco products including e-cigarettes or those with previous smoking history within the prior 5 years
- BMI > 35 kg/m2
- Pregnant women and women who are presently lactating.
- Subjects that have received antibiotic administration or an upper respiratory infection within the previous 4 weeks

- College and graduate students or employees who are under direct supervision by any of the investigators in this protocol
- Alcohol or illicit substance abuse
- Chronic cardio/pulmonary respiratory disorders or other medical conditions as determined by the investigator
- Increased airway hyperresponsiveness at baseline as measured by a positive methacholine challenge response (methacholine PC20 FEV1 < 8 mg/ml) in doses less than 4mg/mL
- Subjects will be requested to refrain from *antihistamines, nonsteroidal anti-inflammatory agents, antioxidants (e.g. beta-carotene, selenium, and lutein) and supplemental vitamins* (e.g. C and E), for 1 week prior to, and during testing.

Subject Recruitment and Compensation
The EPR will genotype individuals for the rs2280964 CXCR3 polymorphism using DNA obtained from subjects within a 100 mile radius of the research triangle area. From this, we will tentatively pre-screen 100 subjects from the EPR cohort based on gender, ethnicity and expression of the polymorphism with the goal of recruiting 46 subjects for the protocol based on our power calculations. Additional, subjects will be pre-screened if we do not reach our subject numbers or if there are subjects that drop out of the study. Up to 80 people may be consented to achieve the target enrollment.

Subjects will be recruited directly from the EPR with a preference for individuals in the Raleigh-Durham area. Following identification of subjects, by their genotype for the CXCR3 polymorphism, the EPR study team will initiate conversation with the subject about the research trial and assess their interest in participating. If they express interest, the subjects contact information will be provided to the Duke based study coordinator and the coordinator will contact the patient via telephone. A general description will be provided during this phone call prior to their initial visit. The screening of subjects will be conducted by the study coordinator and will include discussion of the informed consent process. If the potential enrollees are interested in participating, they will meet with the study coordinator and the co-investigators (Dr. Robert Tighe and/or Dr. Loretta Que and/or Dr. Devon Paul) to review the study and to ask questions. They will be asked to sign consent, and undergo the screening procedures. The consent form will be subject to approval by the Duke University IRB. A copy of the consent form will be given to each participant, and the signed original will be kept in the participant's research chart.

Subjects will be reimbursed $100 for the initial screening visit to compensate for time and travel. If subjects meet the eligibility criteria and undergo informed consent, they will receive $450 per exposure and bronchoscopy for a total of $1000 for completion of the whole study.

Consent Process – see Section 14 of the e-IRB submission form and complete the questions in that section.

Subject's Capacity to Give Legally Effective Consent – Subjects who do not have the capacity to give legally effective consent will not be included in this study.

Risk/Benefit Assessment –

Potential Risks and Procedures to Minimize Risks:

a. Pain and/or hematoma formation may occur at intravenous line site. This is not a serious complication.

b. Risks associated with drawing blood from participant's arm include momentary discomfort and/or bruising. Infection, excess bleeding, clotting, or fainting are also possible, although unlikely. These risks are minimized by the use of disposable single use needles, trained personnel, and application of pressure after the blood draw.

c. As part of the pre-screening procedures, along with a clinical history, the subject's pulmonary function will be measured for comparison to predicted normal values. Spirometry may exacerbate bronchospasm, but in the human physiology laboratory, this has not been a serious problem. Subjects will be monitored closely during the procedure and rescue medications are available if needed. To assess for evidence of baseline airway hyper-responsiveness, a methacholine challenge will be performed during screening. This test is carefully performed with precise administration of aerosolized methacholine at defined dosages and rescue medications are available if airflow obstruction is excessive (FEV1 exceeds a 20% decline from baseline response to saline aerosol).

d. Acute airflow obstruction can occur during or acutely after exposure to ozone. The proposed exposure concentration of 200 ppb is near to the 8 h federal standard of 70 ppb. For the most recent experience period of the human physiology laboratory involving approximately 150 healthy subjects, 18-45 yr. of age, there have been no subjects with excessive acute airflow obstruction at this ozone concentration (200 ppb). As part of the protocol design, subjects are monitored at baseline, during, and post-exposure when leaving the exposure room, and upon returning at 24 h post-exposure. If excessive airflow obstruction is observed, rescue medications are available if needed.

e. Research bronchoscopy risks: although the flexible bronchoscopy is a safe clinical procedure, there are some risks. Overall, the risk of a major complication during this procedure is 0.08%. Although complications from bronchoscopy are rare, some of the potential complications are described below. Topical anesthetic can numb the throat and vocal cords, which limits one's ability to prevent food or drink from passing into the airways of the lung. For this reason, participants are required not to eat or drink prior to the procedure and until administered lidocaine wears off after the procedure. Sedating medications can cause an individual's blood pressure to become low, suppress a person's respiratory (breathing) drive, or limit the ability to protect the airway. For this reason, a nurse trained in conscious sedation continuously monitors the subject during the procedure and can administer medications to reverse these effects. A history of liver or kidney disease might increase the risk of these complications and therefore these subjects would be excluded. Uncommonly, damage to the nose, vocal cords, airways, or lungs can occur during flexible bronchoscopy. The risk of developing a collapsed lung as a result of this procedure is less than 1%. The risk of significant bleeding as a result of trauma to the airways is less than 1%. Less than 1% of individuals will have a low-grade fever the night after a bronchoscopy, which typically does not represent infection. Overall, these risks are not considered to be very common.

f. Bronchoscopy may exacerbate bronchospasm. Subjects undergo spirometry before the procedure to establish baseline is within normal predicted values and will not undergo bronchoscopy if less than 80% of the predicted value. After bronchoscopy

takes place, the subject will be monitored via continuous pulse oximetry and physician examination, while in the bronchoscopy recovery area. In addition, bleeding and/or fever may occur after BAL collection and bronchial brushing; thus subjects are monitored post bronchoscopy and contacted each day for the following day to certify that no problems have arisen.

g. Clinical data obtained for the study. All data are maintained either in secured files in the Human Inhalation Facility or on a secured RedCap database. However, if information will aid in treatment of a subject, it will be released with the subject's approval.

h. Vulnerable populations:
1) Participants must be able to provide legally effective consent and be within the age range for the study (18-45)
2) Pregnant and lactating women will be excluded due to the risks of bronchoprovocation, and medications given during the bronchoscopy for conscious sedation. Any participants that become pregnant during the study must be withdrawn from study participation. A pregnancy test will be performed on women of childbearing potential prior to at risk study procedures as defined on the schedule of events.
3) No student reporting to or employees of the study investigators may be a participant on the study.

To summarize risks/benefits: Dr. Tighe and Que have been performing patient and research bronchoscopy for > 10 years. The clinical support personnel, physicians, and technical assistants are experienced in studying subjects with research protocols and all staff are aware of the potential complications associated with the procedures. The benefits resulting from this research includes a better understanding of the pathobiology that occurs to pulmonary tissues after exposure to O_3. Given our experience and safety record, we feel the risk/benefit ratio is acceptable.

At this time, there are no known benefits to a subject for participating in the research. Benefits to society and the volunteer from the proposed research are uncertain; although the potential is good for collecting useful information on air pollution hazards. During screening procedures and history, information may develop on an aspect of well-being that may be of benefit to the subject. For example, observations that the subject's lung function at baseline is within predicted normal ranges for gender, height and ethnicity, and confirm that are in good health with respect to lung homeostasis.

The importance of the knowledge to be gained from the research has a high potential to predict gene/environment interactions related to lung response to the air pollutant, ozone, that will enable with precision, assessment of risk to young adults from exposure and development of airway disease(s).

Costs to the Subject

There will be no additional costs to subjects for participating in this study, but routine medical care will be charged to the subject or their insurance.

Data Analysis & Statistical Considerations –

Using preliminary data of human BAL albumin responses, we identified that subjects could be segregated into two groups: 64% with increased BAL albumin and 36% with decreased BAL albumin. For sample size calculations, we assume that the two groups correspond to subjects with or without the rs2280964 polymorphism, and that exposure

to O_3 would result in at least a 50% increase in functional response in responders. Using the effect size from the preliminary data, we estimate that 23 subjects per genotype are required for a power of 80% with a two-tailed significance of 0.05. The 80% power accounts for our expectation that multiple genetic factors may associate with biologic responses to O_3. Therefore, for proposal, we anticipate that a total of N=46 subjects will be recruited during the 5-year period of the proposed research for exposure studies. We account for potential drop out of subjects during the study protocol to make sure that we achieve the 46 subjects needed in the study (therefore the increased number of screening). Data analysis will be performed only on subjects who complete both exposures (FA and O_3). For associations between gene/protein expression (IFN-γ or CXCL10) and O_3 phenotypic responses, we will use linear regression (method of least squares) modeling for fitting expression levels in response to O_3 exposure (expressed as fold-change from FA) to severity of O_3-induced changes in specific response phenotypes (% of response to FA) (15). Statistics will be performed with the assistance of Dr. Cliburn Chan an Associate Professor of Biostatistics and Bioinformatics at Duke University who is a co-investigator on this proposal.

Data & Safety Monitoring

As this proposal includes bronchoscopy for the subjects, a plan for monitoring is needed. All of the protocols in this proposal will be approved by our Institutional Review Board and reviewed periodically (every 6-12 months). In addition, a three member (Dr. Momen Wahidi, Dr. Scott Shofer and Dr. Neil MacIntyre) Data Safety and Monitoring Committee (DSMC) has been assembled through the Division of Pulmonary, Allergy and Critical Care at Duke University for other bronchoscopy-based protocols and will monitor this protocol. The individuals are pulmonologists with knowledge of bronchoscopy protocols and research design. This DSMC will monitor the studies in this proposal as the use of bronchoscopy is planned. The frequency of monitoring depends on the study but generally ranges between 3 and 12 months.

Protocol suspension and subject discontinuation

a) Protocol Stopping Rules.

This protocol will be stopped and re-evaluated if any of the following occur: 1) any serious adverse event as defined in 21 CFR 312.12 (a). Serious adverse experience: Any adverse experience occurring during study procedures that results in any of the following outcomes: Death, a life-threatening adverse experience, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse experience when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. An example of such medical events includes allergic bronchospasm requiring intensive treatment in an emergency room or at home. 2) >Two subjects experience the same severe adverse events 3) any other event that poses undue risk in the opinion of the investigator or DSMC. These stopping rules

apply to all aspects of this study including the screening evaluation and the inhalation challenge procedure.

b) Individual Stopping Rules.

The occurrence of any of the events described below will result in suspension of the study procedures (excluding safety monitoring) and discharge of the subject from the study after the adverse event(s) have resolved.

Methacholine Challenge

1. Subject requests that procedure be stopped
2. Reduction in FEV1 to <50% of baseline or FEV1 less than 1.0 liter (study-related endpoint is >20% decrease in FEV1).
3. Severe bronchospasm requiring more than two puffs of albuterol MDI to resolve or resulting in delay of discharge.
4. Any serious or unexpected adverse event.

Ozone and filtered air Challenge

1. Subject requests that procedure be stopped.
2. A severe adverse event.
3. Any serious or unexpected adverse event.

Bronchoscopy, Bronchoalveolar Lavage, Brush Biopsy

1. Subject requests that procedure be stopped.
2. Any serious or unexpected adverse event

In accordance with federal regulations the PI will monitor for, review, and promptly report to the IRB, appropriate institutional officials, sponsor, coordinating center and the appropriate regulatory agency head all unanticipated problems involving risks to subjects or others that occur in the course of a subject's participation in a research, all AE reports will be reported per the DUHS IRB policies.

Privacy, Data Storage & Confidentiality – see Section 12 of the e-IRB submission form and complete the questions in that section.

All study participants will be referred from the NIEHS from the National Institute of Environmental Health Sciences (NIEHS) Environmental Polymorphism Registry (EPR), We will identify and recruit subjects with the major or minor allele of the CXCR3 polymorphism rs2280964, A data transfer agreement will be completed after IRB approval which will allow for the transfer of PHI from the EPR to the DUKE study team.

Dr. Shepherd Schurman the Associate Medical Director at NIEHS is to be added as an outside investigator to the study at Duke. His role will be to facilitate the exchange of information from the EPR to Duke. In addition, for this study, a data exchange will also occur from Duke to the EPR based on the exposure data and phenotyping performed in this research protocol.

Research Specimens: Research material, which will be collected from human subjects and includes; serum, lavage fluids, BAL cells, epithelial brushing and data archiving of

recorded lung function. These data will be collected for research purposes, but remain available, if need and agreed to, by the subject for future research use. DNA will be stored indefinitely. Samples will be de-identified by assigning a code only accessible to PIs and study team, DNA will primarily be used for this study but could also be used for future studies (outlined in the consent using standardized language). If sample is provided to outside investigators, the DNA and clinical information will only be identified by the assigned code.

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Appendix A

Visit	V1	V2	V2b	V3		V4	V4b	V5	
					I day post V3	18-20 day min post V3			1 day post V5
Description	Screen –EPR recruits	Pre-exposure	Post exposure	18 – 20 hours post exposure	Post bronch phone call	Pre-exposure	Post Exposure	18 – 20 hours post exposure	Post Protocol phone call
Informed consent, eligibility review	•								
Anthropometrics-Ht & Wt	•								
Vital Signs – BP, HR, RR, Sp02	•	•	•	•		•	•	•	
Baseline medical Hx	•								
Physical Exam	•								
Blood draw for serum HCG-WOCBP	•								
Urine HCG for WOCBP		•		•		•		•	
Blood draw for DNA, CBC w Diff, creatinine	•								

Spirometry	•	•	•	•		•	•	•	
Methacholine Challenge	•								
Blood draw for serum		•	•			•	•		
Blood draw for Cells and serum factor		•		•				•	
Bronchoscopy with BAL and brush				•				•	
Questionnaire	•				•				•