

**Mechanisms of Ozone-Induced Alterations in
Efferocytosis and Phagocytosis
(MOZEPH)**

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Study Title: Mechanisms of Ozone-Induced Alterations in Efferocytosis and Phagocytosis

Purpose of the Study –

Ozone (O₃) is a criteria air pollutant that induces epithelial injury, exacerbates chronic pulmonary disease, and unfavorably increases susceptibility to respiratory infections. Reductions in ambient O₃ are essential to public health as ambient levels directly correlate with emergency visits, hospitalizations, and school absences within 24 hours of exposure. Beyond acute effects, epidemiologic findings show a relationship between air pollution, including O₃, and increased incidence of respiratory infections. O₃ is known to decrease pulmonary host defense; however, the specific biologic mechanism(s) wherein O₃ alters susceptibility to respiratory pathogens is fairly unstudied, and far from being understood. Defining a mechanism is an unmet public health need and would favor the development of targeted strategies to reverse O₃-induced injury to host-pathogen defenses.

Scavenger receptors are critical mediators of host defense and are protective in O₃ induced lung inflammation, suggesting they may modulate O₃-induced susceptibility to respiratory infection. CD163 is a class B scavenger receptor, expressed on monocytes and macrophages, that exists in both membrane-bound and soluble (sCD163) forms. During inflammation, cell surface CD163 is cleaved into sCD163. Membrane CD163 clears cell-free hemoglobin (CFH) by recognizing and internalizing hemoglobin-haptoglobin complexes. Once recognized by CD163, the complex is degraded by heme-oxygenase-1 (HO-1) into anti-inflammatory byproducts. Recent studies have demonstrated that delayed CFH clearance augments inflammatory responses and exacerbates lung diseases. Excessive CFH also reduces macrophage phagocytosis and apoptotic cell clearance (efferocytosis) resulting in persistent inflammation and defective pathogen clearance. In human subjects following an acute laboratory O₃ exposure, we identified a subset of subjects with increased expression of CD163 in bronchoalveolar lavage (BAL) macrophages as compared to filtered air (FA) exposure. In a mouse model of O₃ exposure, we found that lung CD163 expression and BAL CFH were increased. BAL CFH was further augmented in CD163^{-/-} mice after O₃ exposure. Furthermore, in CD163^{-/-} mice O₃ enhanced lung inflammation/injury and increased numbers of BAL apoptotic neutrophils. These data suggest that CD163 facilitates the resolution of O₃-induced lung injury, mediates clearance of CFH and maintains effective efferocytosis. Based on our preliminary data, **we hypothesize that macrophage dependent clearance of CFH is mediated by CD163, which limits acute lung injury after O₃, and maintains pulmonary host defense through effective efferocytosis, and pathogen phagocytosis.**

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The purpose of this human study is to perform translational studies in support of animal mechanistic studies to define a potential role for CFH, CD163 and sCD163 in ozone-induced alterations in efferocytosis and phagocytosis. This will be accomplished in the following human study aims. **Aim 1: To determine if healthy human subjects undergoing acute laboratory exposure to O₃ alter CD163, CFH and macrophage efferocytosis. Aim 2: To determine if healthy human subjects undergoing acute laboratory exposure to O₃ alters CD163, CFH and macrophage phagocytosis.**

Overall, these human translational studies will allow us to define the role of CFH and CD163 in O₃-induced health effects. The completion of these human studies, we would define a novel mechanism of how ambient pollutants mediate critical macrophage inflammation resolving and pathogen clearance functions. Additionally, we have the potential to define sCD163 as biomarker of O₃-induced lung injury and susceptibility to infections.

Background & Significance –

Air pollution is a significant environmental health concern leading to approximately 800,000 premature deaths each year (1). Despite strict regulations, ozone (O₃), a criterion air pollutant, causes more than 3000 deaths annually in the United States (2). Epidemiologic studies clearly demonstrate that individuals living in high ambient O₃ areas are at increased risk of chronic lung diseases and respiratory infections such as community-acquired pneumonia (CAP) (3). CAP is the eighth leading cause of death in the USA and the leading cause of death from infection (4, 5). Since respiratory infections such as CAP are important to public health, a clear understanding of the relationship between air pollution exposure and susceptibility to bacterial infection are highly clinically relevant. Defining a potential mechanism(s) of overlap between pulmonary host-pathogen interactions and host-responses to ambient pollutants is an unmet, public health need. Since ambient O₃ levels are rising and an ongoing public health threat, these studies are critical and timely.

Prior animal studies suggest that O₃ exposure reduces host antibacterial defense and increases susceptibility to respiratory infections (6-8). The specific mechanisms involved remain poorly described. To define these mechanisms, we focus on the alveolar macrophage function. Alveolar macrophages are central mediators of pulmonary host-pathogen interactions and the biologic response to ambient air pollutants (9-12). They are one of the first lines of defense to both ambient pollutants and bacterial or viral pathogens. During initial exposure with pathogenic bacteria, alveolar macrophages clear pathogens from the airspace by phagocytosis. In addition to pathogen phagocytosis, macrophages are critical to the resolution of pulmonary inflammation and injury, which limits damage and prevents systemic bacterial translocation. A crucial anti-inflammatory function is

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performed by clearance of debris and dying/apoptotic immune cells in a process termed 'efferocytosis' (13). Efferocytosis is an essential cellular homeostatic process that removes dead cells before the onset of necrosis; thereby limiting stimuli that prolong and heighten inflammation. Once apoptotic cells are cleared, efferocytosis initiates a pro-resolving signaling cascade defined by the release of anti-inflammatory mediators (e.g. IL-10, TGF- β), and the production of proliferative growth factors to repopulate resident immune and structural cells (14-16). If efferocytosis is impaired, these factors are reduced, prolonging lung injury (14, 17, 18). Given critical alveolar macrophage functions in pathogen clearance and efferocytosis, ambient pollutant alterations can augment inflammation/injury and worsen the severity of respiratory infections with increased host morbidity and mortality.

Essential alveolar macrophage functions in pathogen phagocytosis and efferocytosis involve recognition of pathogens associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and scavenger receptors (SRs) (19-21). SRs are a broad family of multi-recognition receptors with non-redundant roles in initial immune responses to PAMPs and DAMPs (22). CD163 is a class B SR expressed on the surface of monocytes and macrophages, which recognize hemoglobin-haptoglobin (Hb-Hp) complexes to clear cell free hemoglobin (CFH) (23). CD163 is essential in host responses to biologic and pathogenic conditions where RBC lysis generates CFH (24). In this setting, CFH is released and scavenged by haptoglobin (Hp) to generate a hemoglobin (Hb)-Hp complex, which is recognized by CD163. After recognition, the CD163/Hb-Hp complex is internalized and degraded by heme oxygenase 1 (HO-1) into anti-inflammatory bi-products (bilirubin, carbon monoxide and ferritin). Once CD163 is activated, it initiates phosphorylation of PKC- α which feedbacks on CD163, to increase CD163 activation (25). CD163 internalization and metabolism of Hb-Hp also initiates a macrophage-signaling cascade leading to the production of the anti-inflammatory cytokine IL-10 (26). In addition to its cell surface functions, CD163 can be cleaved and released as a soluble form (sCD163) (27). Because sCD163 is released during inflammatory responses, plasma levels of sCD163 are used in diseases such as diabetes, obesity, and cardiovascular diseases (CVD) as a biomarker of disease activity (28-31). Despite its use as a biomarker, the functional relevance of sCD163 in scavenging CFH or Hb-Hp complexes is unknown. Furthermore, CD163 and sCD163 effects in air pollution-induced lung injury and host defense are presently unexplored.

The **primary goal** of this human study is to provide translational relevance to mechanistic animal studies exploring the novel relationship between CD163 and CFH in the context of oxidant-mediated environmental lung injury. We hypothesize that O₃ exposure releases CFH into the BALF dependent on

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CD163. In support of this hypothesis, we demonstrate that during whole body rodent exposure, CFH is dependent on CD163 expression. To explore how increased CFH enhances O₃-induced lung injury and susceptibility to bacterial infection, the translational studies will focus on CFH alterations of macrophage efferocytosis and phagocytosis. Loss of these functions allows persistence of inflammatory responses and enhances pulmonary susceptibility to respiratory bacterial pathogens. To carefully dissect this mechanism, we will perform human exposure studies to filtered air (FA) and O₃. By using a combination of in vivo assessments of CD163, CFH and sCD163 we will confirm if this pathway is altered by human O₃ exposure and if there is a relationship between other physiologic outcome. Furthermore, using alveolar macrophages obtained from subjects undergoing FA or O₃ exposure, we will perform in vitro studies to assess CFH and CD163 as determining factors of macrophage apoptotic PMN efferocytosis and bacterial pathogen clearance. These studies would define a novel mechanism for O₃-induced lung injury and susceptibility to respiratory bacterial infections.

Design & Procedures

Overall Plan for Specific Aims:

We will utilize acute laboratory O₃ exposure studies in healthy humans to define associations between CD163, CFH, and macrophage efferocytosis in the biologic response to O₃. This allows translation of our murine observations to explore potential impacts on human health. Using a cross-over challenge study design, we will recruit healthy human subject (age 18-30 years old) without recent respiratory infection and challenge them with FA or O₃ (200 ppb) for 135 minutes (Figure 1). The O₃ dose was selected, as it is a relevant level commonly associated with 'O₃-Action' days in the southeast US (e.g., Research Triangle area of NC) (37, 38). The experiments are designed to have each subject serve as their own control in the data analysis. This design accounts for biologic and genetic diversity in human O₃ responses. Using biologic samples and alveolar macrophages obtained from bronchoscopy prior to and following exposures we will assess for CD163, CFH and sCD163 and define in vitro effects on efferocytosis and phagocytosis. The specifics are as follows:

AIM1: Following the FA or O₃ exposure (~20 hrs), bronchoscopy will be performed to obtain BAL fluid and cells to determine CD163 expression by real time PCR and CFH/sCD163 (eBioscience, San Diego, CA) by ELISA. Additionally, blood will be collected for hematocrit and for serum to assess CFH and sCD163 levels (at V1-6 Figure 1). Alveolar macrophages from human BAL after FA or O₃ exposure will be used for ex vivo efferocytosis assays as previously described (63). Briefly, blood to generate apoptotic PMNs will be drawn from participants prior to their FA or O₃ exposure and kept at room temperature until the post-exposure BAL. PMNs are isolated by discontinuous

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percoll gradient. Blood PMN apoptosis will be determined by flow cytometry (Annexin V and PI). We anticipate >80% of PMNs will be apoptotic after an overnight incubation. 1×10^5 alveolar macrophages will be cultured and then incubated with approximately 5×10^5 apoptotic blood PMNs at 37°C for 40 min in media. Following incubation with apoptotic PMNs, the alveolar macrophages will be placed on cytopins and stained with Wright-Giemsa to determine the efferocytic index. Alveolar macrophages from the FA exposure samples will be used to define alterations in ex vivo efferocytosis in the presence of CFH, anti-CD163 blocking antibodies, and with or without sCD163. These studies will define if CFH directly effects human alveolar macrophage efferocytosis, if it is dependent on CD163, and if sCD163 can rescue efferocytic alterations. In a secondary analysis, a subset of BAL macrophages after efferocytosis will be used for RNA isolation to measure mRNA levels of HO-1 and PKC- α and anti-inflammatory cytokines TGF- β and IL-10. We will also define measures of lung inflammation and injury (BAL cytokines, cell influx, total protein/albumin) in the subjects and determine if these responses are predicted by the CD163 expression, CFH, sCD163 or ex vivo efferocytic responses. This will allow us to comprehensively assess the effect of CD163 in human O₃-induced lung injury.

AIM2: To translate observations of O₃-induced alterations in macrophage phagocytosis (a surrogate of pathogen clearance), we will obtain alveolar macrophages from healthy humans undergoing acute laboratory exposure to O₃. Aim2 will use BAL samples generated in Aim 1. An aliquot of alveolar macrophages will be used for ex vivo phagocytosis assays (1×10^6 alveolar macrophages). These data will be correlated with macrophage mRNA levels of CD163 and BAL/serum CFH and sCD163 to define a relationship between this axis and O₃-derived alterations in macrophage phagocytosis. To determine direct effects of CFH on phagocytosis, we will expose cultured alveolar macrophages from the FA exposure bronchoscopy to CFH in vitro and then assess phagocytosis. These in vitro experiments will also be performed in the presence of anti-CD163 blocking antibodies and/or sCD163. Optimal doses of CFH, sCD163 and anti-CD163 will be determined empirically by the maximum binding efficacy dose of sCD163 to CFH and the anti-CD163 neutralization dose of CD163. This will allow us to determine if CFH has a direct effect on human alveolar macrophage phagocytosis, if it is dependent on CD163, and if sCD163 rescues CFH-mediated alterations in phagocytosis.

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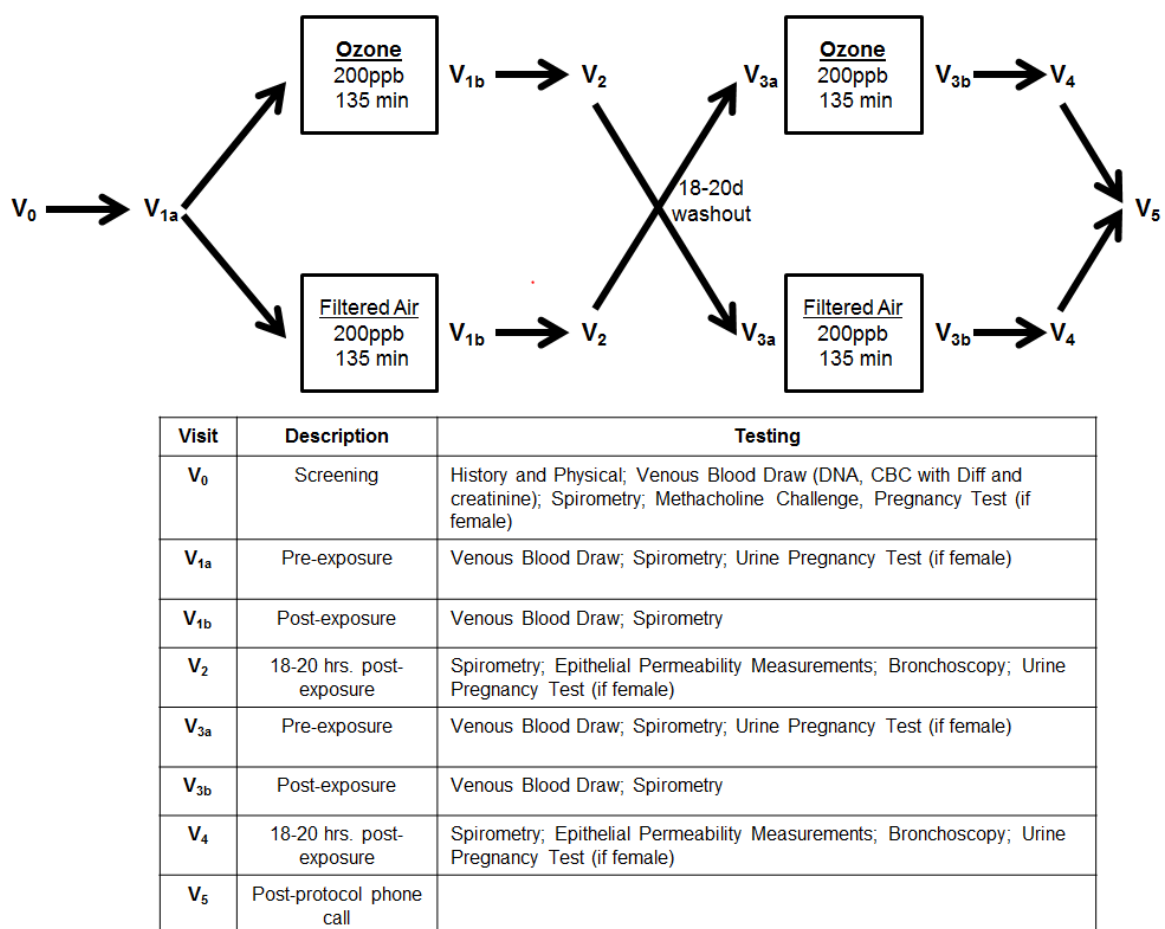


Figure 1. Exposure study design. Healthy subjects will be exposed to 200 ppb of ozone (O₃) or filtered air (FA) using a random crossover study design with each subject as his/her own control and an 18-20 day washout period between exposures. Pre and post exposures venous blood will be drawn for neutrophil isolation, hematocrit, cell free hemoglobin (CFH) and sCD163 measurements. Approximately 20-24hrs post-exposure, subjects will undergo bronchoscopy with bronchoalveolar lavage (BAL) for alveolar macrophages and measurements of CFH and sCD163.

The specifics for each visit are listed in Figure 1. 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 obtain PMNs for apoptosis assays, and serum for CFH and sCD163 levels. Additionally, this will be used 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 O₃ 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 O₂

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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 1 for the schedule of PFT's. Parameters to be measured at each time point are forced Vital Capacity (FVC), Forced Expiratory Volume in one second (FEV1), Peak expiratory flow rate (PEF), and forced expiratory flow rate at 25 – 75% of exhalation (FEF25-75%). 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 0.02 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 results from subjects that previously screened with study personnel will be accepted, and subject will be re-enrolled in study if they previously screen failed from increased hyperactivity in concentrations greater than or equal to 4mg/mL.

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 O₃ 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 O₃ levels can be a confounder for these studies, O₃ 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 O₃ levels return to baseline. For exposure to O₃, the dose will be controlled to within 10 ppb of 200 ppb. The exposure chamber (500 ft³) 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 O₃; O₃ is generated from 100% O₂ source by cold plasma corona discharge

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(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 O₂ 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 five 50 ml aliquot of prewarmed (37 C) isotonic saline, with immediate aspiration after each installation. Following lavage, bronchial segment(s) are gently brushed (3 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 18-35 yrs. of age (No subject will be excluded from the study on the basis of gender or ethnicity)

Exclusion Criteria

- Current smokers of tobacco products including e-cigarettes or those with previous smoking history within the prior 5 years
- 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

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- 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 when administered a dose with a concentration less than 4mg/mL (methacholine PC20 FEV1 < 8 mg/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

We will tentatively pre-screen 70 subjects and from this population we will recruit a total of 34 subjects to be evaluated based on our power calculations. Subjects will be recruited from Duke University campus and surrounding Durham, NC, community using advertisement in newspapers, mailings, and campus student newspaper. After informed consent is obtained, potential subjects will be screened to determine if they satisfy all inclusion and exclusion criteria. 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.

If subjects express interest, they will contact the study coordinator. The coordinator will then contact the patient via telephone. A general description of the study 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 \$75 for the initial screening visit to compensate for time and travel. If subjects meet the eligibility criteria and undergo informed consent, they will receive \$400 per exposure and bronchoscopy for a total of \$875 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.

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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 participants' 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). Subjects with a >20% decline after inhalation of a dose with a concentration less than 1mg/mL is exclusionary.

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

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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 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 O₃. 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.

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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 –

For human studies we estimate a sample size of $n=34$ which provides 80% power, a two tailed $\alpha=0.050$, if we assume that the alveolar macrophage efferocytosis would decrease by 50% below baseline. The endpoints for human participants are serum and BAL measurements of CFH and sCD163 as well as alveolar macrophage efferocytosis and phagocytosis. The differences between these variables will be assessed in terms of outliers and departures from normality. The one sample t-test with associated confidence intervals for the mean difference will be used. Nonparametric analysis will supplement these analyses if there are concerns with the use of normal-based inference. The statistical software R will be used for graphs and statistical analyses (R Foundation for Statistical Computing, www.R-project.org).

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-

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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.

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

This study is performed as a collaboration with Dr. Kymberly Gowdy PhD at East Carolina University. A data transfer agreement will be completed after IRB approval, which will allow for the transfer of de-identified data with the randomly assigned study ID from Duke to East Carolina for the purpose of data analysis. Dr. Kymberly Gowdy is an Assistant Professor of Pharmacology and Toxicology at the Browdy School of Medicine at East Carolina University. She is the principal investigator of the NIH grant providing the support for these human studies under a subcontract with Duke University. Dr. Gowdy will be added as an outside investigator to the study at Duke.

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 the Duke PI and Duke 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.

Bibliography: