RUTGERS
School of Public Health

Title: Activated Macrophages and Ozone Toxicity

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1. *Purpose/Specific Aims*

The purpose of the study is to better understand the mechanisms of lung injury from ozone exposure. Ozone is a ubiquitous urban air pollutant that causes adverse health effects in both healthy and susceptible individuals. The study will focus on the inflammatory mechanisms which follow ozone exposure. Specifically, the study will determine the role of different types of macrophages, proinflammatory M1 macrophages and anti-inflammatory reparative M2 macrophages, in lung injury.

The study will incorporate a pilot study to refine protocols and measure novel soluble inflammatory markers in sputum supernatant including cytokine, chemokines, eicosanoids and prostaglandins following ozone exposure. Samples from the pilot will be used to identify which markers will be measured in the main study.

1.1 Objectives

1. The phenotype of macrophages responding to ozone exposure will be assessed (main study).
2. The objective of the pilot study is to pilot the study protocols. Additionally, the response of novel inflammatory markers to ozone exposure will be assessed.
3. The response of the respiratory microbiome to ozone exposure will be assessed.

1.2 Hypotheses

The hypothesis for the main study is that ozone toxicity is a result of cytotoxic proinflammatory macrophages overwhelming the activity of anti-inflammatory reparative macrophages.

2. *Background and Significance*

Ozone is a ubiquitous air pollutant and the main component of photochemical smog. It remains one of the most problematic air pollutants to control because it is formed from intermediates that arise from multiple sources [1-3]. Inhaled ozone has been shown to irritate and damage the lung in both healthy and susceptible individuals, including children and the elderly [3, 4]. Ozone causes inflammation and constriction of the airways, reducing pulmonary function. Ozone also exacerbates asthma and suppresses nonspecific immunity increasing the susceptibility of exposed individuals to respiratory infections [5, 6]. Epidemiologic studies in the U.S. have demonstrated that for every 10 ppb increase in peak ozone levels, the total death rate for the same day increases by 0.41% [4], while a more recent US/European study estimated 0.56% [7]. Adverse effects of ozone are even greater in the developing world where ozone levels can be significantly higher than in the U.S. with a concomitantly greater health burden. In this regard, despite control attempts and much lower standards, daily maximum ozone concentrations have recently reached 128 ppb in Shanghai and 225 ppb in Mexico City [8, 9].

Thus, new strategies to reduce lung injury from ozone and other air pollutants by elucidating specific pro- and anti-inflammatory mechanisms that contribute to ozone toxicity are key to public health.

Accumulating evidence suggests that tissue injury induced by ozone is due, not only to direct effects of ozone on the lung, but also indirectly to the actions of resident and infiltrating macrophages [10-12]. These cells are activated to release a myriad of inflammatory mediators that fight infection, protect against xenobiotics, activate adaptive immunity, and promote wound healing. These diverse activities of macrophages are thought to be mediated by distinct subpopulations that develop in response to inflammatory signals within their microenvironment. Two major subpopulations have been identified: classically activated M1 macrophages which release cytotoxic/proinflammatory mediators and alternatively activated M2 macrophages, which release mediators that suppress inflammation and initiate wound repair [13, 14].

Our overall goal is to analyze the distinct contributions of M1 and M2 macrophage subpopulations to ozone-induced lung injury. Preliminary data demonstrate that both macrophage populations play a role in ozone toxicity. Whereas initially, classically activated M1 macrophages contribute to lung injury by releasing cytotoxic/proinflammatory mediators, subsequent release of anti-inflammatory and mitogenic mediators by M2 macrophages promotes tissue repair. We hypothesize that ozone-induced injury and altered lung mechanics ensue when M1 macrophage proinflammatory/cytotoxic activity predominates over the antiinflammatory/reparative functions of M2 macrophages. To test this hypothesis, the phenotype of macrophages responding to ozone-induced injury will be analyzed in animals and humans. Additionally, mice with specific defects in M1 or M2 macrophages, or monocyte precursors, will be used to assess their contribution to toxicity. Lineage tracking strategies will be used to analyze the origin of macrophage subpopulations in the lung and mechanisms mediating their accumulation.

The hypothesis that inhaled ozone causes a sequential accumulation of M1 and M2 macrophages in humans and animals has not been previously tested. Findings that results in humans are comparable to mice would provide strong support for the relevance of our mouse models to human toxicity resulting from ozone exposure. For translational human studies, we will use induced sputum as a source of macrophages [15-17]. The advantage of induced sputum is that it is a safe and non-invasive technique; additionally, induced sputum selectively samples the surfaces of the large conducting airways, a primary target for ozone effects in humans [15, 18].

The supernatant from the induced sputum samples will be analyzed for markers of inflammation, including chemokines, cytokines, prostaglandins and eicosanoids. Eicosanoids, including 20-HETE and 15-HETE, have been shown to induce airway hyper-responsiveness and to be increased by oxidant exposure in animals [19]. In addition to directly inducing inflammation, oxidants, such as ozone, can generate free rad icals altering the oxidant/antioxidant balance within the respiratory tract. The release of ROS leads to transcription of pro-inflammatory cytokine and chemokine genes, increased release of pro-inflammatory mediators and up-regulation of adhesion molecules [20, 21]. Mononuclear cells

isolated from induced sputum in subjects with asthma express IL-4 and IL-10 levels to a greater degree than cells from healthy controls, suggesting an enhanced immunologically mediated inflammatory response [22]. Exhaled breath condensate samples, blood samples and urine samples will also be analyzed for markers of inflammation.

The characterization of the lung microbiome is currently the focus of an ongoing study at Rutgers (Pro20140000953). The study compares microbial communities in numerous samples from the respiratory tract (including lung cells collected by bronchoalveaolar lavage, induced sputum, oral wash, and oropharyngeal samples). Environmental exposures may alter the lung microbiome, affecting disease progression [23-25]. Samples will be collected by all of the above methods except bronchoalveolar wash to determine the response of the microbiome to ozone exposure.

The current USEPA standard for ozone is 0.070 ppm averaged over an 8 hour day. This is meant to protect not only healthy individuals, but those more sensitive to ozone's effects such as infants, children, those with asthma, and those with other chronic lung conditions such as COPD. The proposed exposure concentration of ozone (0.2 ppm, 3 hours) is almost twice maximal levels reported in the world's most polluted cities (i.e., Mexico City); however, this concentration and higher concentrations (up to 0.4 ppm, 2 hours) with exercise have been used extensively for mechanistic studies at United States Environmental Protection Agency (USEPA), the University of Southern California (USC), the University of California, San Francisco (UCSF), the University of California, Davis (UC Davis), the University of Rochester, and other institutions for over 40 years with no adverse effects in hundreds of subjects, including asthmatics, beyond transient and expected physiologic declines in spirometry, reported [26-34]. For instance, one recent study at University of Rochester exposed healthy exercising subjects to 0.2 ppm of ozone for 3 hour, identical to our proposed protocol [27]. 7-10%declines in FEV1 were observed 0.5 hours after exposure had largely resolved by 4 hours following exposure. Another study using the roughly equivalent 0.3 ppm ozone for 2 hours with exercise showed transient FEV1 declines of 7-15% [26]. Our exposures will be limited to 3 hours and will only occur once. Based on published studies, subjects are not expected to experience more than transitory effects including symptoms of mucosal irritation, cough or headache, but these are expected to be mild and dissipate within an hour after the exposure is terminated.

3. Research Design and Methods

The study is a single blind randomized cross-over design. Subjects (up to 68 healthy men and women) will participate in the study (48 non-smokers and 10 smokers in the main study and 5-10 in the pilot study). Eligible subjects will have 5 clinic visits: one screening visit, 2 exposure visits, and 2 post-exposure sample collection visits. Each subject will participate in 2 exposure sessions, one to clean air (ozone<5ppb) and one to 0.2 ppm ozone in the Controlled Exposure Facility (CEF) at the Environmental and Occupational Health Sciences Institute (EOHSI). The order of the exposure sessions will be randomized. Up to 2 subjects may participate in each exposure session. Induced sputum samples will be collected at the baseline (during screening) then at one of three pre-selected time periods (24, 48 or 72 hours) following each exposure session. Breath, blood, oral washes, oropharyngeal swabs, and urine samples will be collected at each visit.

Subjects will undergo telephone screening. If eligible they will be scheduled for the screening visit. Subjects will be asked to refrain from antioxidants, over the counter anti-inflammatory medications, strenuous exercise, alcohol, home air purifiers, unvented household combustion sources, gases and fumes for 48 h prior to the screening visit and before each exposure visit through the post-sample collection period. Non-smokers will also be asked to avoid tobacco smoke.

Subjects will be consented then scheduled for a screening visit to determine eligibility. Subjects will be asked to refrain from eating for 2 hours prior to the screening visit. On the day prior to the screening visit, subjects will be called/emailed/texted to confirm their appointment. If the subject reports a respiratory or other acute illness, including active allergies, during the four prior weeks or if they have taken any medication for allergies or respiratory illness, their visit will be rescheduled to a later date. The screening visit will include a complete medical history and physical examination (including height, weight, and three blood pressure measurements), lab tests (CBC with differential and platelet count, urinalysis, and standard metabolic screen), spirometry, impulse oscillometry (IOS), and ECG. A pregnancy test (urine hCG) will be administered to females at enrollment and before each exposure session. Exhaled breath condensate (EBC) samples will also be collected using an RTube™ (the condensate collection system manufactured by Respiratory Research, Inc.) or using the Jaeger EcoScreen. With the RTube™, each subject uses a new and separately packaged device to eliminate any risk of infection transmission. The samples are collected by having patients breathe through a one-way valve into a cooled condenser for 5 to 15 minutes. As much as 1 ml of condensed liquid can be collected in a 5-minute period. There is virtually no risk and no spuriously induced inflammation in the lung from this procedure, and it may be serially repeated [35]. Subjects will be asked for permission to store excess EBC sample for future use by the study investigators.

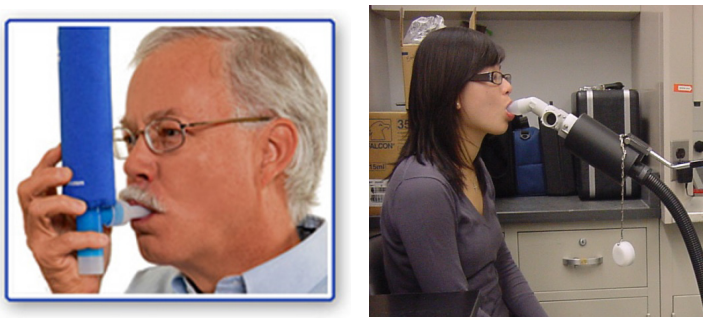

Figure 1. R-tube (left); Ecoscreen (right)

Impulse oscillometry (IOS) is an additional noninvasive and effort-independent technique to measure airway resistance and capacitance. The airway resistance and capacitance, reflecting airway compressibility, is measured at normal tidal breathing. This physiologic technique also allows measurement of peripheral and central airway resistance. IOS waves are measured for 30 seconds of tidal breathing and the maneuver is repeated three times. This technique has no known side effects and is a safe test providing a great deal of information regarding peripheral airway resistance.

Oral washes and oropharyngeal swabs will be collected for microbiome analyses. For the oral wash (OW), participants will gargle with 10 ml sterile 0.9% saline and expel the sample into a specimen container. Oropharyngeal swabs (OS) will be collected using a new, sterile cotton

swab. While wearing gloves and depressing the tongue with a sterile tongue depressor, the swab will be inserted into the back of the oral cavity. The participant will be asked to say "aahh" to elevate the uvula and with a sweeping motion the posterior pharyngeal wall and tonsilar pillars will be swabbed. By applying a little force a large quantities of mucosa will be collected. Care will be exercised to avoid swabbing the soft palate or touching the tongue with the swab tip. The swab will immediately placed in a sterile tube and stored with the other samples for transport.

In addition to the EBC, oral washes, and oropharyngeal swab samples, urine and blood (approximately 10 ml) samples will be collected at the screening visit. The urine samples will be analyzed for eicosanoids and other markers of inflammation. The urine samples of the smokers will also be analyzed for cotinine to confirm their smoking status. The blood samples will be processed by Dr. Laumbach's lab at EOHSI. Plasma from the blood samples will be stored in the principal investigator's private freezer for future analysis of markers of ozone response; no genetic analyses will be performed on these stored samples. Only study investigators will have access to these samples.

At screening, subjects will be asked to provide an induced sputum specimen (baseline sample), which will be immediately analyzed for the presence of plugs. Using the method described by Pin et al. [36] and Alexis et al., [37] subjects inhale increasing concentrations of saline (3%, 4% and 5%) for 3 consecutive 7-minute periods through a mouthpiece without a valve. To reduce the risk of bronchospasm, following the recommendations of the European Respiratory Commission [38], albuterol (180 mcg; 2 puffs of a standard metered inhaler) will be given to subjects 15 minutes prior to the start of the induction. Post-dose spirometry is performed to obtain a baseline FEV-1. The subject will be asked to breathe through his/her mouth during the induction. If the subject is observed to breathe through his/her nose, he/she will be asked to monitor his/her breathing and will be offered a nose clip to use during the induction. After each period of inhalation, an FEV-1 is measured for safety. If the FEV-1 decreases by 20% or more from the post-dose FEV-1, the procedure is stopped. After rinsing the mouth, swallowing water and blowing the nose to minimize contamination by saliva or post nasal drip, subjects will cough sputum into a sterile container. Subjects unable to provide an adequate specimen (70-75 mg of plugs) will be excused from the study. In the main study, subjects will be assigned to a post-exposure sample collection time (24, 48, or 72 hours) to be used for both exposure sessions. In the pilot study and for all smokers, all subjects will be assigned to the 24 hour post-exposure sample collection. The exposure sessions will be scheduled.

Subjects may be screened (telephone and screening visits) year-round. For the main study, the exposure visits will be scheduled between October and June, consistent with low ambient ozone months in New Jersey. For the pilot study, since only acute responses are examined (24 hours of the exposure), the screening and exposure visits will take place year-round. If over six months have passed between the screening visit and scheduling the exposures, those subjects will be re-consented and the screening visit will be repeated.

Each exposure session will be 3 hours in duration. Subjects will be called the day prior to the visit to confirm the appointment. If unable to be reached by phone, reminder texts or emails may be sent from a Rutgers-authenticated computer. Subjects will be asked to report any

current or recent respiratory symptoms. If subjects report any symptoms within the previous 4 weeks, the visit will be rescheduled. Subjects will be asked to refrain from eating for 2 hours prior to the exposure visit. At the start of the visit, the subject's blood pressure, pulse, and temperature will be measured. IOS and spirometry will be performed before and after the exposure session. Two questionnaires will also be administered before the exposure session: a stress questionnaire and a symptom questionnaire. If the subject reports the presence of any respiratory symptoms, the exposure session will be canceled. During the exposure, heart rate will be measured using a wireless monitor (H7; Polar USA). Subjects will be asked to alternate (every 15 minutes) rest and mild/moderate exercise periods on a cycle ergometer to maintain an average heart rate of approximately 60% maximum heart rate (220-age). The exercise is necessary as healthy human subjects are relatively insensitive to ozone [39, 40]. The exercise schedule may be modified to maintain the ventilation rate or for the subject's comfort. After the exposure session, the stress and symptom questionnaires will be administered. An exposure type questionnaire will also be administered immediately after the exposure. If the subject reports any physical symptoms or has a 20% decrease in his/her FEV1 after the exposure session, the study physician will be consulted. Subjects will be given verbal and written instructions to contact the Robert Wood Johnson Medical School Pulmonary Division if they experience any breathing problems after leaving EOHSI. Subjects will inform the responding pulmonologist that he/she is a study subject. As an additional precaution, subjects will be given a rescue inhaler to use only if directed to do so by the responding pulmonologist. Subjects will be shown how to use the inhaler prior to leaving the EOHSI clinic. The pulmonologist will inform Dr. Kipen of any calls made to the pulmonologist and any treatment. The symptom questionnaire will be administered to each subject the day after exposure. For subjects returning at 24 hours for the post-exposure sputum sample, the questionnaire will be administered in person. For all other subjects the questionnaire will be administered by phone. As a secondary check, the questionnaire will ask the study subject if he/she contacted the Pulmonary Division. If the subject reports the presence of any symptoms or called the Pulmonary Division, the study physician will be consulted, prior to the collection of any samples.

Immediately before and after each exposure session EBC samples will be collected using the same methods as in the screening visits. In addition to the EBC samples, urine, oral washes, oropharyngeal swabs, and blood samples will be collected before and after each exposure session. During the second exposure visits, an additional blood sample will be collected for genotyping of GST polymorphisms. The urine samples will be analyzed for eicosanoids and other markers of inflammation. The blood samples will be processed by either Dr. Laumbach's lab (research blood for future use) or the Rutgers University Cell and DNA Repository facility core at EOHSI (sample for genetic analysis).

Subjects will be asked to return for a follow-up visit either 1, 2, or 3 days after each exposure visit. Subjects will be asked to refrain from eating for 2 hours prior to the visit. The blood pressure, pulse rate, and oral temperature will be checked. IOS and spirometry will be performed and an induced sputum sample will be collected. The symptom and stress questionnaire will be administered. The blood, urine, oral washes, oropharyngeal swabs, and EBC samples will also be collected.

Sputum samples obtained after the ozone exposure may be compared with samples collected either at the screening visit or after the clean air exposure. If the subject is randomized to ozone exposure first and is unable to produce a sputum sample after the ozone exposure, the subject will be withdrawn from the study.

3.1. Duration of Study

The study duration is expected to be four years. Each person will have 5 visits (one screening visit, two exposure visits, and two post-exposure visits) to the EOHSI clinic, for a total time of approximately15 hours.

3.2 Study Sites

The consent, screening, exposure sessions and sputum collection will take place at EOHSI. All consent forms and identifiable data will also be stored at EOHSI. Analysis of de-identified data will take place at the Ernest Mario School of Pharmacy and at the School of Public Health.

3.3 Sample Size Justification

Calculations are based on published human exposure studies [41, 42] demonstrating 2.3-fold and 1.6-fold increases (SE=8%) in mean fluorescence intensity of sputum leukocyte expression of markers of M1 (CD14) and M2 (CD16) macrophages following ozone exposure at 24 h compared to clean air. The SDs for changes [the square root of $2(1\text{-rho})SD^2$] were calculated where rho is the intra-person correlation (0.5) between measurements. Based on this, 16 subjects/exposure group are needed to achieve 90% power using 0.05 level two-group t-tests.

3.4 Subject Selection

Two subject populations will be studied: smokers (n=10) and non-smokers (n=70). The pilot study will recruit up to 10 people (non-smokers) and the main study will recruit 70 people (10 smokers & 60 nonsmokers).

3.4.1 Inclusion Criteria – both smokers and non-smokers.

Healthy men and women (n= up to 80) between the ages of 18 and 40 will be recruited for the study. Subjects who participate in the pilot study will also be eligible to participate in the main study.

Subjects must have been fully vaccinated for COVID-19 – people are considered fully vaccinated for COVID-19 ≥2 weeks after they have received the second dose in a 2-dose series (e.g., Pfizer-BioNTech or Moderna), or ≥2 weeks after they have received a single-dose vaccine (e.g., Johnson and Johnson).

Additional Inclusion Criteria for Smokers: currently smoking at least one cigarette each day.

3.4.2 Exclusion Criteria

- Cardiovascular disease

- Respiratory disease
- Recent (within 4 weeks) respiratory or COVID-19 symptoms
- Diabetes
- Pregnancy
- HIV Infection
- Orthopedic or rheumatologic conditions which would interfere with cycle use
- Inability to produce a sputum plug
- Daily use of antioxidant supplements, excluding those in a multivitamin. These supplements include Vitamin C or E, selenium, beta-carotene, lycopene, lutein, zeaxanthin and ginkgo biloba. Supplements taken less frequently but at least once a week will be reviewed by the principal investigator for eligibility determination.

Additional Exclusion Criteria for Non-smokers: history of smoking within the past 5 years.

4. Study Variables

4.1 Independent Variables or Interventions

The independent variable is the ozone exposure. The exposure will take place in the Controlled Exposure Facility at EOHSI. The CEF consists of three major components: 1) the exposure chamber; 2) the air pollutant generation system; and 3) the control systems. The CEF is a large stainless steel room (7.3 ft. high x 13.5 ft. wide x 9 ft. deep; volume= 887 cubic feet) in which the air flow, temperature and humidity can be varied and well controlled. A divider separates the chamber into two compartments, so that two subjects can be exposed simultaneously. There is a lavatory in the chamber for extended exposure studies. For the clean air exposure, ambient air that supplies the exposure chamber will be treated using a series of processes, which include cooling/heating, humidification/dehumidification, and filtration through a carbon filter, a KMnO4 purifier, and HEPA filters. The clean air enters the CEF through two diffusers located in the ceiling and exits through the perforated stainless steel floor without recirculation.

For ozone exposure sessions, ozone will be generated in situ by an ozone arc-generator (Ozone Research & Equipment Corporation, Phoenix, AZ), mixed with the filtered clean air and will enter the CEF through the two air diffusers. The ozone concentration will be achieved by adjusting the ozone output and the air mixing flow through the chamber. Based on previous studies [43], ozone can be precisely controlled during an exposure session. The ozone concentration will be monitored in real time using an API Model 450 ozone analyzer (API, Inc., San Diego, CA). The sensitivity of the instrument is 1 ppb, and response time is less than 20 sec. Noise is less than 0.7 ppb.

Additionally, during each exposure session, additional air pollutants will be monitored. CO concentration will be measured using a real-time Langan T15 CO gas monitor. NO/NO_2 concentration will be monitored by a TEI Model 42C TL Trace Level $NO-NO_2-NOx$ real-time Analyzer (Thermo Electron Corporation, Franklin, MA). $PM_{2.5}$ mass concentration will be measured using a real-time personal PM monitor (SidePak, TSI Inc., MN).

4.2 Dependent Variables or Outcome Measures

Induced sputum samples will be collected at the Environmental and Occupational Health Sciences clinic. The relative concentrations of M1 and M2 macrophages in the induced sputum sample will be assessed in the Rutgers University Flow Cytometry/Cell Sorting and Confocal Microscopy Core facility (http://www.flowcyt.rutgers.edu/). Cells will be separated from mucus plugs, stained with fluorescent antibodies and then analyzed by flow cytometry. Leukocytes will initially be separated from epithelial cells by light scatter and by CD45 expression, and macrophages identified by HLA-DR expression [44]. Flow cytometry will also be used to assess macrophage phagocytosis and oxidative burst. To confirm their phenotype, sputum macrophage subpopulations will be sorted and expression of inflammatory proteins and additional M1/M2 macrophage markers not detectable by flow cytometry will be analyzed by RT-PCR and immunocytochemistry.

Macrophage response may vary by genotype, specifically GST polymorphisms. The genotyping will be done by the Rutgers University Cell and DNA Repository (RUCDR) on a fee for service basis. De-identified samples will be sent to RUCDR. Any sample remaining after the genetic analyses will be destroyed.

Supernatant from the induced sputum sample, EBC, and urine will be analyzed for cytokines, chemokines, prostaglandins and novel eicosanoids. The analysis will first be done in samples from the pilot study to identify markers of interest for the main study. All analyses will be done at the Rutgers Flow Cytometry Lab and in Dr. Laumbach's lab in EOHSI. Subjects will be asked for permission to store excess samples for future use by the study investigators.

Oral washes and oropharyngeal swabs as well as aliquots of induced sputum and EBC samples will be given to Dr. Lee Kerkhof for microbiome analysis.

Specimens (induced sputum, exhaled breath condensate, and blood samples) will be analyzed for oxidized and nitrosylated variants of surfactant protein D. All analyses will take place in the Rutgers Flow Cytometry Lab.

Data from the stress questionnaires, collected before and after the exposure and before the follow-up sputum induction, will be examined as possible modifiers of the response. Subject fitness, calculated by their heart rate recovery during the exposure visit, will also be examined as a possible modifier.

4.4 Risks of Harm

Ozone exposure: The proposed exposure concentration of ozone (0.2 ppm, 3 hours) is almost twice maximal levels reported in the world's most polluted cities (i.e., Mexico City); however, this concentration and higher concentrations (up to 0.4 ppm, 2 hours) with exercise have been used extensively for mechanistic studies at EPA, USC, and other institutions for over 40 years with no adverse effects, beyond transient physiologic declines in spirometry, reported. Our exposures will be limited to 3 hours and will only occur once. Based on published studies, subjects are not expected to experience more than transitory effects including symptoms of mucosal irritation, cough or headache, but these are expected to be mild and dissipate within an hour after the exposure is terminated. Based on prior experiments looking at monitored

chamber exposures of smokers to ozone, it appears there is no increased risk of acute deterioration in pulmonary function, respiratory pattern, heart rate or symptomatology, even at ozone doses >3 times the concentration proposed in this protocol [45]. Thus, there does not appear to be an increased risk of harm by exposing smokers to ozone as described in this protocol. Prior to the exposure, subjects will complete a symptom questionnaire. If subjects report the presence of any symptoms, the exposure session will be canceled. During the exposure sessions, the participants are continually observed through a window to assure that they are not exhibiting any visual signs of physical problems and wear a sensor for continuous monitoring of heart rate. A physician with a code cart is on-call during the entire procedure to respond to any subject complaints or situations. If a subject develops any symptoms of chest pain, dizziness, or other symptoms/signs of concern to the subject or the study technician, the physician will speak with and examine the subject and decide if the exposure session may continue. The covering physician will examine the subject, including EKG if indicated, and make an appropriate triage decision, including referral via ambulance to the emergency department. Regardless of the physician's recommendation, the study subject may choose to end the session at any time. At the end of each exposure session, the subject will complete a symptom questionnaire. This questionnaire will be reviewed by the study technician and a physician to ensure that the subject is ready to resume daily activities. The subject will also be asked to complete the symptom questionnaire the next day (by phone or in person). If the subject reports any symptoms, he/she will be referred to the study physician. All subjects will be asked to return for their scheduled follow-up visit.

As a precaution against delayed bronchospasm after an exposure session, after-hours response has been arranged with the pulmonary division Robert Wood Johnson Medical School. After each exposure session, subjects will be given written and verbal instructions to call the pulmonary division for any delayed respiratory problems. A physician will be available 24 hours a day/7 days per week. Subjects will also be provided a rescue inhaler to be used only if advised by the responding pulmonologist. Prior to leaving EOHSI, subjects will be instructed in the use of the inhaler and asked to return the inhaler at their follow-up visit. At each follow-up visit, the meter on the inhaler will be checked for use. Any use will be reported to the study physician to determine if the subject can continue with the study. The inhaler will be discarded once the subject has completed the study.

The study has a single ozone exposure and a single clean air exposure. If the subject experiences bronchospasm either during or after the ozone exposure, the subject will be asked to return for his/her scheduled follow up visit for the sputum collection. Since no further ozone exposure will occur, the subject will be permitted to continue with the study and participate in the clean air exposure, if not done previously. In the unlikely event that the subject experiences bronchospasm either during or after the clean air exposure and has not yet had the ozone exposure, the subject will be considered ineligible and his/her participation will stop. If the ozone exposure session has been completed, the subject will be asked to continue in the study and return for a follow-up visit.

Sputum induction: Sputum induction with hypertonic saline may cause reversible bronchoconstriction in some individuals. Bronchoconstriction has been observed primarily in asthmatic subjects who have pre- existing bronchial hyperreactivity. In order to reduce the risk

of bronchoconstriction, potential subjects who have a history of asthma, a history of symptoms consistent with bronchial hyperreactivity, or abnormal spirograms will be excluded from participation in the study. The sputum induction will be done only during clinic hours in EOHSI, when a physician is available. As a further precaution against bronchospasm, all subjects will be pretreated with albuterol 180 mcg or 2 puffs of a standard metered inhaler) prior to the start of induction. Post-albuterol spirometry will be done to determine a baseline FEV-1 during induction. Our induction protocol consists of 3 segments of inhalation of nebulized saline at increasing concentrations. After each of the 3 segments spirometry is done. If the FEV-1 decreases from the baseline by more than 20% after a segment, , the procedure will be stopped. An additional 180mcg of albuterol will be administered and spirometry will be repeated 15 minutes afterward. If the FEV-1 has recovered (>=80% baseline FEV-1) the subject will be permitted to leave EOHSI. If the FEV-1 is still depressed (<80% baseline FEV-1), the subject will be referred to care at the EOHSI clinic and the referral will be documented as an adverse event. At any point during the induction, if the subject complains of any breathing difficulties, the induction will be stopped and the subject will be referred to care at the EOHSI clinic. The episode will be reported as an adverse event.

Albuterol: Albuterol, a short-acting bronchodialator, will be administered before induction via a rescue inhaler to prevent bronchoconstriction and possibly afterward to treat difficulty breathing, wheezing or shortness of breath due to bronchoconstriction. Temporary side effects from albuterol include tremor, palpitations, and headache. These effects may last four to six hours.

Genotyping: The selected assays currently have no immediate application to the diagnosis or treatment of any disease, but may relate to the inflammatory response. The primary risk from the testing is the loss of confidentiality. Every effort will be made to keep the results confidential.

Venipuncture: Slight pain, some bleeding, or bruising may occur when blood is drawn for the routine laboratory tests.

Oropharyngeal Swabs: The swabbing will be performed by a clinic nurse or medical assistant to reduce the possibility of a gag reflex.

Oral wash: No physical risk but subjects may experience a salty taste.

IOS: Subjects may experience brief, temporary lightheadedness during the IOS measurement.

4.5 Potential for Benefit

There are no direct benefits to individual subjects participating in this study, except to receive the results of the physical examination and routine lab tests to share with their personal physician. Ozone is a ubiquitous air pollutant and the main component of photochemical smog. It remains one of the most problematic air pollutants to control because it is formed from intermediates that arise from multiple sources. Inhaled ozone has been shown to irritate and damage the lung in both healthy and susceptible individuals, including children and the elderly.

This mechanistic public health research study has great potential to help us understand and ultimately prevent disease associated with common exposures such as ambient ozone.

5. Subject Recruitment and Enrollment Considerations

5.1 Subject Recruitment

Subjects will be recruited through posting flyers at approved locations on the Rutgers University Piscataway/New Brunswick campuses. Flyers will also be posted in nearby public and commercial buildings, including retail stores and community centers and made available at community events, with permission from the appropriate authority. Additionally the study will be posted on the EOHSI website (http://eohsi.rutgers.edu/content/research-volunteers) and the EOHSI Facebook page. Announcements about the study will be posted periodically on the EOHSI Facebook page. The announcements will link to the website. EOHSI maintains a volunteer mailing list, for persons who wish to be notified when a new study is posted on the website. An email announcement will be sent to the mailing list subscribers. Through the website and the flyers, interested candidates will be instructed to call the study coordinator. The study coordinator or a research assistant will call the subject to explain the study and answer any questions. If the candidate wishes to participate, a brief telephone screening questionnaire will be administered. The screening questionnaire will ascertain the presence of major exposure-study exclusions such as HIV infection, cardiovascular disease, stroke, diabetes, or asthma. The recruiter will ask about the COVID-19 vaccination status of the candidate, only fully vaccinated volunteers are eligible. The recruiter will inquire about a personal history of cigarette or other smoking within 5 years, regular use of vitamin E or C, selenium, or other supplements beyond a multivitamin, ingested herbal preparations, or any medication prescribed by a physician. The candidate will be asked to provide a list of his/her vitamins, supplements, and herbal preparations and their frequency of use. The supplements will be checked for antioxidant content and the candidate will be informed if he/she is eligible. If the candidate is not eligible, no identifying information will be recorded. If the candidate is eligible, contact information will be recorded and an appointment will be made for the screening visit. The candidate will be given, mailed or emailed (based on his/her preference) a copy of the consent form to review prior to the screening visit. A copy of the medical history questionnaire will also be given to the subject. The subject will be asked to bring the completed questionnaire to the screening visit. The subject will also be asked to bring proof of vaccination to the screening visit – this can be their physical vaccine card or a photo of it. If the subject is a Rutgers student or employee, they may show their myRutgers Dashboard vaccine status.

5.2 Consent Procedures

When subjects come to the EOHSI Clinical Center for screening visit, they will be provided the written informed consent to read and sign. The study coordinator or a research assistant will review the consent form with the subject and answer any questions. If the subject wishes to participate, he/she will be asked to sign the consent form. The coordinator or research assistant will also sign the consent form. A copy of the signed consent form will be given to the subject. After the consent form is signed, the medical history questionnaire will be collected and the screening visit will begin.

After the screening visits, if the subject does not meet the research criteria, the recruiter will thank the individual and explain that for safety reasons, he/she cannot participate. The specific reason will be explained. For individuals who do not become subjects, personal identification information will be removed from the screening questionnaires, although demographic information and reasons for exclusion will be maintained for research purposes. If the subject does meet the research criteria, the first exposure and follow-up visit will be scheduled.

5.3 Subject Costs and Compensation

No costs are expected to be incurred by the subject.

The subject will be compensated, in cash, on a pro-rated basis:
Screening visit - $50
Exposure visit - $125
Follow-up visit (for sputum collection) - $75

If the subject completes all part of the study protocol, the total compensation will be $450. Subjects will receive compensation after the completion of their screening visit, and if applicable, at the conclusion of the study, according to the pro-rated schedule above.

6. *Data Handling*

Confidentiality of participants will be protected by using identification numbers for all participants. All hard copies of forms with personal data will be kept in a locked file in a private office (Room 127) at EOHSI. All data and specimens will be coded only by the study identification number. The link to identification of all participants will be kept in a password protected file on a secure shared drive at EOHSI and will be accessible to project staff directly involved in contacting participants and the Rutgers-RWJMS Institutional Review Board. The link will be destroyed when data entry is completed. All data (consent forms, etc.) will be kept for 6 years. De-identified data will be kept indefinitely.

De-identified data will be entered into Excel worksheets. The data will be stored on the EOHSI secure shared drive. All data will be double-entered by two different investigators. All discrepancies in the data will be resolved by a third investigator. Only de-identified data will be transmitted between study investigators..

7. *Statistical Analysis*

Descriptive statistics, including means, standard deviations, and histograms will summarize the distributions of markers stratified by time point and exposure. Analysis of covariance (ANCOVA) will be used to compare the means of markers of inflammation. Specifically, with baseline level as a covariate in the model, F-tests will test whether the means are significantly different following exposure to ozone or clean air at each follow-up time-point. The mean fold-changes (or log-fold-changes if necessary to meet normality assumptions) in M1 and M2 macrophages will also be assessed at each time-point (24, 48 or 72 h) following ozone and compared to clean air in humans.

8. *Data and Safety Monitoring*

All study procedures are conducted during regular business hours when a physician is available at the EOHSI clinic. All study staff are trained in study procedures by the principal investigator. During inductions, lung function will be assessed using spirometry. A significant decrease in lung function (FEV-1<80% baseline) will be treated with albuterol. If lung function does not recover, the subject will be referred to clinical care and the adverse event will be reported to the IRB. The pulmonary fellow on call at RWJBUH is available for backup if needed. During the exposure sessions, the participants are continually observed through a window to assure that they are not exhibiting any visual signs of physical problems and wear a heart rate sensor (Polar.com) for continuous monitoring of rate. Regular voice communication between the study staff and the subject is maintained through an intercom system. A physician with a code cart is on-call during the entire procedure to respond to any subject complaints or situations. At the end of each exposure session, the subject will complete a symptom questionnaire and a spirometry. This questionnaire will be reviewed by the clinical technician and a physician to ensure that the subject is ready to resume daily activities. If a subject develops symptoms of chest pain, dizziness, or other symptoms of concern to the subject or the physician, the study will be terminated and the covering physician will examine the subject, perform an EKG, and make an appropriate triage decision, including referral to the emergency department. In the case of a delayed reaction, subjects will be instructed to call the pulmonary division of RWJMS and be given a rescue inhaler. Any respiratory reaction requiring referral to the emergency department will be considered an adverse event and will be reported to the IRB within 1 week of occurrence. Although interim analyses are not appropriate for the study, a single occurrence of an adverse event will result in a suspension of exposure visits and sputum collection while procedures are reviewed. The visits will resume only after IRB review of the event and approval of any modified procedures.

9. Reporting Results

9.1 Individual Results

All subjects will be given the results of their screening blood tests. For subjects who do not pass the physical examination or have significant findings on the blood tests, the physician will send a letter to them describing the reason for exclusion and to make diagnostic or treatment recommendations if needed. The physician will also be available to discuss findings if needed.

9.2 Aggregate Results

Aggregate results will be posted on the Center for Environmental Exposures and Disease website. The webpage is under development.

9.3 Professional Reporting

The results will be published in peer-reviewed journals and presented at professional conferences.

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