Effect of GSTM1 Genotype on Ozone-induced Allergic Airway Inflammation

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Abstract

Epidemiological data suggest that persons with asthma may have greater morbidity as measured by health care utilization after O_3 exposure than normal, healthy persons. Animal toxicological data provide evidence that O_3 exposure can affect immune function, including enhancement of allergic inflammatory responses in the lungs. Previous controlled human exposure studies have confirmed that O_3 exposure can enhance both the early and late bronchoconstrictor responses to inhaled antigen in allergic asthmatic subjects. The effects of O_3 exposure on lower airway and late-phase inflammatory responses have not been adequately studied. Recently, data from both controlled human exposure and epidemiological studies have suggested that a common genetic polymorphism in an antioxidant enzyme, glutathione S-transferase μ1 (GSTM1), is an important determinant of susceptibility to the respiratory effects of inhaled O_3. We designed an experiment to determine whether persons with allergic asthma have increased susceptibility to O_3 as a consequence of enhanced airway inflammatory responses to local endobronchial allergen challenge. This experiment was also designed to determine whether the effects of inhaled O_3 on the specific airway inflammatory responses to allergen were enhanced in asthmatic individuals with the GSTM1 null genotype. The experiment used a repeated-measures design, each subject completing both O_3 and filtered air (FA) exposures within the experiment, with the order of the exposures counter-balanced. Subjects were screened prior to beginning the experimental protocol so that 50% had the GSTM1 null genotype. Ten asthmatic subjects with specific sensitization to the house dust mite, *Dermatophagoides pteronyssinus* (DP), were exposed separately to 0.16 ppm O_3 and FA control for 4 hr with intermittent exercise. At 20 hr post-exposure, subjects underwent a challenge bronchoscopy during which DP allergen was instilled in a sub-segmental bronchus of the right middle lobe and saline was instilled in a sub-segmental bronchus of the right upper lobe. Six hours later, a second sampling bronchoscopy was performed to collect samples of airway lining fluid from each challenged bronchus for analyses of cellular and biochemical markers of non-specific and specific allergic inflammatory responses. Subjects underwent lung function testing pre- and immediately post-exposure, 18 hr post-exposure prior to and then hourly after the challenge bronchoscopy until the sampling bronchoscopy. Exposure to O_3 induced an expected decrease in lung function. The decrease in lung function 6 hours after O_3-allergen was greater than that after FA-allergen. While the neutrophilic inflammatory response was non-significantly greater after O_3-allergen compared to that after FA-allergen, the levels of multiple cytokines (GM-CSF, IL-1β**,** IL-4, IL-5, IL-8**,** IL-10, IL-13, and TNFα**)** were lower after O_3-allergen than after FA-allergen. These results suggest that while prior exposure to O_3 may enhance the bronchoconstrictor response to allergen, it has somewhat conflicting effects on the airway inflammatory response to allergen. Neutrophil chemotaxis to the airways may be increased, but at least some cytokine responses may be decreased. While there were no significant differences in lung function to O_3 based on GSTM1 genotype, the inflammatory response to allergen was consistently lower in the GSTM1-null subjects. Because of the small sample size, caution should be applied in interpretation of these results.

Executive Summary

Background

Ozone (O_3) is a major gaseous component of air pollution in urban environments. Epidemiological evidence suggests that people with asthma are at increased risk for exacerbation when exposed to elevated levels of ambient O_3. Controlled human exposure studies have not consistently shown subjects with asthma to be more sensitive to O_3 in terms of lung function response, although the neutrophilic airway inflammatory response does appear to be greater in asthmatic than in non-asthmatic subjects. In addition, there is evidence that lung function and airway inflammatory responses to O_3 are not well-correlated in healthy subjects. Asthma is a disease characterized by airway inflammation, particularly during the late-phase response to allergen, and the degree of airway inflammation is an important predictor of asthma severity. Thus, one possible explanation for the epidemiological findings is that O_3 exposure may enhance the inflammatory response to triggers of asthma, such as allergen, and was not reflected in prior controlled human studies measuring lung function parameters alone.

Animal toxicological data provide evidence that O_3 exposure can enhance allergic inflammatory responses in the lungs. Controlled human exposure studies have confirmed that O_3 exposure can enhance both the early and late bronchoconstrictor responses to inhaled antigen in some, but not all allergic asthmatic subjects. Most of these studies did not assess potential changes in airway inflammation during the late-phase response. However, in the two studies that did, significant O_3-induced enhancement was not consistently observed.

Oxidative stress, with the formation of reactive oxygen species (ROS) is a key component of inflammation. Although innate antioxidant defenses are available to detoxify ROS in the airway, individuals differ in their ability to deal with an oxidant burden, such as inhaled O_3, and such differences are in part genetically determined. Decreased ability to detoxify ROS may lead to enhanced airway inflammation, and thus potentially to increased bronchoconstriction and asthma symptoms. The glutathione S-transferase (GST) enzymes comprise a large supergene family located on at least seven chromosomes that are critical to the protection of cells from ROS. GSTM1 is a polymorphic gene with a common null allele. The null allele is unable to produce a functional enzyme, which would in turn be expected to affect response to oxidative stress. From 30-50% of the general population is GSTM1 null. The results of several studies have suggested that individuals who are GSTM1 null have greater lung function responses to O_3 exposure compared to individuals with the form of the gene that produces functional enzyme. Another study using a high concentration of O_3 (0.4 ppm) showed that the airway inflammatory response varied according to whether or not the individual had the null or functional form of the GSTM1 gene.

This research project was designed to provide information on the following two questions: 1) whether O_3 exposure enhances the specific airway inflammatory responses of asthmatic subjects during late-phase reactions to local endobronchial allergen challenge, and 2) whether asthmatic subjects with the GSTM1 null genotype have greater allergic inflammatory responses than subjects who have the functional form of the GSTM1 gene.

Methods

The experiment used a repeated-measures design, each subject completing both exposures within the experiment, with the order of the exposures counter-balanced. Subjects were screened prior to beginning the experimental protocol so that 50% had the GSTM1 null genotype. Ten asthmatic subjects with specific sensitization to the house dust mite, *Dermatophagoides pteronyssinus* (DP), were exposed separately to 0.16 ppm O_3 and filtered air (FA) control for 4 hr with intermittent exercise. At 20 hr post-exposure, subjects underwent a challenge bronchoscopy during which DP allergen was instilled in a sub-segmental bronchus of the right middle lobe and saline was instilled in a sub-segmental bronchus of the right upper lobe. Six hours later, a second sampling bronchoscopy was performed to collect samples of airway lining fluid from each challenged bronchus for analyses of cellular and biochemical markers of non-specific and specific allergic inflammatory responses. Subjects performed lung function testing pre- and immediately post-exposure, 18 h post-exposure prior to and then hourly after the challenge bronchoscopy until the sampling bronchoscopy. The differences in lung function, airway cells, and airway inflammatory proteins after O_3-allergen exposure and FA-allergen exposure were compared.

Results

The results of this study suggest that O_3, at least at the concentration (0.160 ppm) and exposure duration (4 hr) tested, appears to have mixed effects on allergen-induced airway inflammation. While airway neutrophils were non-significantly increased after O_3-allergen exposure compared to FA-allergen exposure, airway concentrations of all cytokines assayed (IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, GM-CSF, TNFα**)** were non-significantly lower after O_3-allergen exposure. The absence of GSTM1 appears to be associated with a smaller inflammatory response to endobronchial allergen challenge after O_3 exposure. These results must be interpreted with caution, however, given our small sample size (n=10). Despite the small sample size, however, we did find that O_3 exposure significantly enhanced the lung function response to allergen at 3 h-post local endobronchial challenge, consistent with previously published research that used the whole lung inhalation challenge method.

The original research proposal had planned for a larger sample size (n=30), but multiple problems contributed to our inability to recruit and enroll subjects. First, the UCSF Committee on Human Research (CHR) expressed considerable concern about the safety of the protocol. Second, the multiple bronchoscopies and local endobronchial allergen challenge (LEAC) procedure made recruitment and retention of subjects exceptionally difficult. Finally, one of the subjects did, in fact, experience a severe anaphylactic reaction after his initial LEAC; fortunately, this subject recovered without long-term sequelae.

Conclusions

The results of this study confirm previous reports that O_3 pre-exposure enhances the lung function response to allergen in specifically sensitized asthmatic subjects. The novel finding of this study, however, is that O_3 exposure appears to decrease the cytokine component of the airway inflammatory response to allergen in these subjects. Moreover, the absence of the antioxidant enzyme GSTM1, does not seem to increase the lung function or airway inflammatory response to allergen following O_3 exposure. Because of the small sample size, caution should be applied to the interpretation of these results.

Body of Report

Introduction

Epidemiological data suggest that people with asthma are at increased risk for exacerbation when exposed to elevated levels of ambient O_3 (1-11). Contrary to expectations, controlled human exposure studies have not consistently shown people with asthma to be more sensitive to O_3 in terms of physiologic response (12-16). The results of three earlier studies suggested that asthmatics did not have greater lung function responses to O_3 (12-14), while two later studies showed greater decreases in forced expired volume in 1 s (FEV1) in asthmatics, but not greater deficits in forced vital capacity (FVC) (15-16). In prior work with asthmatic and non-asthmatic subjects, we demonstrated that FEV1 and forced vital capacity FVC measurements do not correlate with O_3-induced cellular and biochemical indices of lung injury and inflammation, and that asthmatic subjects have greater O_3-induced inflammatory responses than normal subjects (17, 18). Asthma is a disease characterized by airway inflammation, particularly during the late-phase response to allergen, and the degree of airway inflammation is an important predictor of asthma severity (19). Thus, one possible explanation for the epidemiological compared to the controlled human exposure study findings is that O_3 exposure may enhance the inflammatory response to triggers of asthma, such as allergen, not reflected in prior controlled human studies measuring lung function parameters alone.

Ozone is a relatively water-soluble gas that is highly reactive as an oxidizing agent. A large percentage of inhaled O_3 is absorbed in the respiratory tract (up to 90%) (20). Absorption occurs along the entire tracheobronchial tree and in the alveoli, but the greatest dose to tissue is delivered to the peripheral airways at the junction between the conducting and respiratory (i.e., gas-exchange) airways (21). The primary determinant of O_3 uptake is surface reactivity, i.e., direct interaction with airway lining fluid constituents and/or cellular components (22). Ozone does not penetrate through the airway epithelium unreacted; it reacts directly with lipids and/or proteins in cells, producing reactive oxygen species (ROS), i.e., oxidative stress. Respiratory toxicity is likely related to the effects of O_3 and its reaction products on alveolar macrophages and airway epithelial cells, and airway neuroreceptors.

Genetic variants in antioxidant defense and asthma:
The presence of inflammation in the airway is an important feature of asthma (23-30). Oxidative stress, with the formation of ROS is a key component of inflammation. Although innate antioxidant defenses are available to detoxify ROS in the airway, individuals differ in their ability to deal with an oxidant burden, such as inhaled O_3, and such differences are in part genetically determined. Decreased ability to detoxify ROS may lead to enhanced airway inflammation, and thus potentially to increased bronchoconstriction and asthma symptoms. The glutathione S-transferase (GST) enzymes comprise a large supergene family located on at least seven chromosomes that are critical to the protection of cells from ROS (31, 32). The specific GST enzymes that have been proposed as candidate genes for asthma risk are those of the mu (GSTM1), theta (GSTT1), and pi (GSTP1) classes. The enzymes encoded by these gene classes preferentially use different ROS products as substrates. For example, quinone metabolites of catecholamines are used by GSTM1 (but not by GSTP1 or GSTT1) (33). GSTM1 and GSTT1 demonstrate activity toward a phospholipid hydroperoxide (33). The ROS-derived products of GSTs are essential in the

mobilization of arachidonic acid, with subsequent production of proinflammatory eicosanoids that may be important mediators of airway inflammation in asthma (31). Several studies have shown differential risk of asthma with GSTM1 and GSTP1 polymorphisms (34-37).

An allele is one of two or more forms of a gene; GSTM1 is a polymorphic gene with a common allele that results in a complete lack of the enzyme (designated GSTM1 null). Individuals who are GSTM1 null would be expected to have altered responses to oxidative stress because they lack this important antioxidant enzyme. From 30-50% of the general population is GSTM1 null, depending on ethnic background (37). In a study of Italian cyclists, risk of acute lung injury in response to ambient O_3 exposure was found to be increased in those with the GSTM1 null genotype [this was also dependent on the presence of the common genetic form of another antioxidant enzyme, NAD(P)H:Quinone Oxidoreductase (NQO1)] (38). The results of a second study by the same team of investigators using a chamber exposure to a low-level of O_3 confirmed that increases in biomarkers of oxidative stress in exhaled breath condensate were mainly accounted for by a subgroup who were both GSTM1 null and had the more common NQO1 Pro187Pro genotype (39). An epidemiological study in Mexico City confirmed a strong association between asthma risk in children with a high lifetime exposure to O_3 and the GSTM1 null genotype; the presence of a serine-containing allele at position 187 in NQO1 (Pro187Ser; i.e., non-wildtype) provided a protective effect among GSTM1 null subjects (40). A separate group of Mexico City children with asthma were followed with serial spirometry for 3 months in a cross-over trial with vitamin C and E supplementation; GSTM1 null children receiving placebo had significant O_3-related decrements in lung function, while GSTM1 positive children did not. Conversely, the effect of the antioxidant vitamins was stronger in children with the GSTM1 null genotype (41). The GSTM1 null children also had more respiratory symptoms with O_3 exposure (42). We showed an effect of the combined GSTM1 null/NQO1 Pro187Pro genotype on the chronic lung function response to O_3 in women, but not men (43). In a report from the Children's Health Study, the risks of both asthma and lifetime wheezing were decreased in children with the TNF-308 GG polymorphism in relation to O_3 exposure; the protective effects of the GG genotype were of greater magnitude in communities with lower ambient O_3 levels compared to communities with higher O_3 levels. The reduction of the protective effect from the -308 GG genotype with higher O_3 exposure was greater in the children who were GSTM1 null, suggesting that the lack of GSTM1 lowered antioxidant capacity. (44). In a second report from the Children's Health Study, GSTM1 null status was associated with increased risk of asthma (45). Although a recent *ex vivo* study in which primary airway cells obtained from both GSTM1-sufficient and null individuals were exposed to O_3 showed differential production of IL-8, two recent controlled human exposure studies did not demonstrate an effect of GSTM1 status on the airway inflammatory and lung function responses to O_3 in asthmatic or non-asthmatic adult subjects (46, 47). A third study of non-asthmatic subjects using a higher O_3 concentration (0.4 ppm) found that the GSTM1 null genotype was associated with increased airways inflammation 24 hours after exposure (48).

Considered together, the human studies on the effects of GSTM1 null genotype provide reasonably strong, although somewhat conflicting evidence for a role of the GSTM1 gene polymorphism in determining the response to oxidative stress in airway cells, and thus susceptibility to O_3-induced toxicity. GST enzymes are important in the first tier of antioxidant defense and their function is crucial to prevent the second tier of responses that lead to airway inflammation. Should there be deficiencies or malfunction in

antioxidant defenses, the possibility of developing airway inflammation is enhanced. We focused on the GSTM1 null genotype because it is sufficiently common in the general population for its effect to be studied in a controlled human exposure study (in addition to this specific genotype having already been linked in humans to susceptibility to ozone and tobacco smoke).

Epidemiological Studies of O_3 and asthma:
As noted above, there are considerable epidemiological data that indicate that persons with asthma are more sensitive to the respiratory effects of ambient O_3 (1-11). Several studies have demonstrated associations between O_3 levels and emergency department visits or hospital admissions for asthma (2, 3, 7, 8). Other studies have shown associations between O_3 exposure and respiratory symptoms, medication use, and/or lung function (1, 4-6, 10, 11). A report from the Children's Health Study showed that exposure to O_3 was associated with increased school absences for respiratory illness among both asthmatic and non-asthmatic children, although children with asthma appeared to be at greater risk (9).

Some evidence also exists that exposure to O_3 can contribute to the development of asthma (49). Another report from the Children's Health Study suggests that frequent exposure to O_3 while playing outdoor sports regularly in smoggy areas in southern California increases the risk of asthma more than 3-fold (50).

Animal Toxicological Studies:
Several studies using experimental animals have shown that O_3 exposure can enhance sensitization to allergens delivered to the respiratory tract (51-56). All of these studies used relatively high doses of O_3. For example, one of these studies exposed infant rhesus monkeys to 0.5 ppm O_3 for 8 hours/day for 5 days (56). Extrapolation of these high-dose exposure studies to the effects of ambient exposures of humans remains problematic.

There are also limited data to support the concept that O_3 exposure can enhance specific allergic responses in previously sensitized animals (57). In a dog model of *Ascaris suum* sensitivity, a single exposure to 3 ppm of O_3 increased the specific immune responses to subsequent inhaled antigen (46). However, two other dog studies produced conflicting results (58, 59). A study using trimellitic anhydride (TMA)-sensitized mice showed that exposure to 3 ppm O_3 for 3 hours enhanced bronchoconstriction, but did not enhance either airway responsiveness or airway inflammation, after subsequent TMA inhalation (60).

A recent study attempted to address the effect of exposure to ambient-level O_3 on both the induction of allergic sensitization and the enhancement of antigen-induced airway inflammatory responses on already sensitized animals (61). Mice exposed to 0.1 ppm O_3 for 4 hours for 2 days prior to and 2 days after intratracheal instillation of ovalbumin (OVA) for did not have enhanced sensitization to OVA compared to mice exposed to filtered air prior to and after OVA instillation. However, in previously sensitized mice, exposure to 0.1 ppm O_3 for 4 hours immediately prior to OVA instillation for 7 consecutive days had enhanced airway inflammation compared to mice exposed to filtered air prior to OVA instillation.

Controlled Human Exposure Studies:
Several, but not all, studies of the airway inflammatory responses of subjects with asthma have documented increased inflammatory cells in bronchoalveolar lavage (BAL) fluid after O_3 exposure (18, 47, 48, 62-64). In addition, even in a study that did not find enhanced airway inflammatory cell influx in BAL fluid after O_3 exposure in asthmatic subjects, epithelial expression of the pro-inflammatory cytokines, interleukin (IL)-5, granulocyte and macrophage-stimulating factor (GM-CSF), epithelial neutrophil-activating peptide 78 (ENA-78), and IL-8, was still increased in these subjects (65). In asthmatic subjects with allergen-induced nasal inflammation, exposure to 0.4 ppm O_3 enhanced the late-phase eosinophilic response to allergen; there was increased eosinophilic cationic protein (ECP), but not increased eosinophils in nasal lavage fluid (66). Ozone exposure (0.27 for 2 hours) increased the percentage of eosinophils in induced sputum in sensitized asthmatic subjects 24 hours after an inhaled allergen challenge (67).

Multiple studies have evaluated the effect of O_3 on the early bronchoconstrictor response to inhaled allergen in asthmatic subjects, but the results have been conflicting (67-75), and even repeat studies in the same laboratory have produced conflicting results (68, 69, 71, 75). Taking the data from the published studies together, there appears to be both a dose effect and considerable inter-subject variability (i.e., some subjects do not respond to exposure to O_3 with enhanced bronchoconstriction to allergen).

Late-phase bronchoconstriction 4-8 hours after allergen inhalation is believed to be due to acute airway inflammation as a result of cytokine [e.g., IL-5, IL-8, regulated upon activation, normal T-cell expressed and secreted (RANTES), GM-CSF] and other mediator release from airway mast cells and alveolar macrophages with specific IgE antibody on their cell surfaces (19, 76). Th2-like cytokine release from sensitized T-lymphocytes may also play a role in the late-phase inflammatory response (19, 76). Induced sputum or BAL fluid samples obtained during late-phase reactions show increases in neutrophils and eosinophils, as well as the products of their degranulation [e.g., myeloperoxidase (MPO) and ECP, respectively]. Given that O_3 exposure has been repeatedly found to cause enhancement of late-phase lung function changes, one would expect that enhancement of airway inflammatory responses should occur as well.

Most of the studies designed to determine whether O_3 exposure enhances bronchoconstrictor responses to inhaled allergen did not assess potential changes in airway inflammation during the late-phase response. However, in the two that did, significant O_3-induced enhancement was not consistently observed (74, 75); the results of these studies provided further evidence of a dose effect and considerable inter-subject variability.

Local Endobronchial Allergen Challenge:
The technique of local endobronchial allergen challenge has been shown to be safer and more effective at inducing a measurable allergic response than whole lung inhalational challenge because bronchoconstriction is localized and a relatively larger amount of allergen can be delivered to the challenged lung segment and a second lung segment can be sham-challenged with saline (77, 78).

Summary:
The epidemiological data reviewed above suggest that persons with asthma may be more sensitive to O_3 exposure than normal, healthy persons. The animal toxicological

data provide evidence that O_3 exposure can enhance allergic inflammatory responses in the lungs. Controlled human exposure studies have confirmed that O_3 exposure can enhance both the early and late bronchoconstrictor responses to inhaled antigen in some, but not all allergic asthmatic subjects. Controlled human exposure data on the effect of O_3 on the late-phase airway inflammatory response to inhaled allergen are sparse and somewhat conflicting.

This research project was designed to provide information on the following two questions: 1) whether O_3 exposure enhances the specific airway inflammatory responses of asthmatic subjects during late-phase reactions to local endobronchial allergen challenge, and 2) whether asthmatic subjects with the GSTM1 null genotype have greater allergic inflammatory responses than subjects who have GSTM1 present.

Materials and Methods

Study Design: This study had a repeated measure design in which subjects were exposed to either clean filtered air (FA) or 160 ppb O_3 for 4 hours in a climate-controlled chamber followed by a challenge bronchoscopy approximately 20 hours later and a sampling bronchoscopy 6 hours after the endobronchial challenge. Spirometry was performed immediately before exposure (0-h), immediately after exposure (4-h), and on the following morning prior to bronchoscopy (24-h). In addition, spirometry was performed on an hourly basis after the challenge bronchoscopy through discharge of the subject approximately 2 h after the sampling bronchoscopy. Each subject returned and underwent the second exposure type with a minimum of 2 weeks in between exposure sessions to allow for recovery from any inflammation or injury sustained during the prior session. The order of exposures was counterbalanced and randomized.
Subjects: Ten subjects were recruited via advertisements placed in University of California San Francisco (UCSF) campus newsletters, local San Francisco newspapers, and internet websites (e.g., www.craigslist.org). A total of 542 individuals responded to the Craig's List postings and all were contacted by email. Of these, 20 consented to participate in the study, of which 10 completed the study. Of the other 10, one was discontinued because of a severe hypotensive episode with syncope secondary to anaphylaxsis, one was ineligible due to lack of airway hyperresponsiveness, three were lost to follow-up, two withdrew consent due to work scheduling issues, one was ineligible due to a pulmonary interstitial lung disease diagnosis, and two were ineligible due to a negative *D. pteronyssinus* (house dust mite) skin test. The severe anaphylactic reaction that occurred with one subject caused a major delay in recruitment because once this severe adverse event was reported to the UCSF Committee on Human Research, a lengthy review process was initiated that culminated in required changes to our approved protocol.

The inclusion/exclusion criteria included: (1) age between 18 to 50 years; (2) ability to perform moderate-intensity exercise; (3) being healthy with no history of cardiovascular, hematologic, or pulmonary diseases other than mild asthma; (4) specific sensitization to the house dust mite, *Dermatophagoides pteronyssinus* (DP); (5) no history of acute infection within the 6 weeks prior to start of the study; (6) non-smoker as defined as having a history of less than ½ pack-year lifetime tobacco use and no history of any tobacco use in the past 6 months; and (7) no history of illicit drug use. The subjects were asked to stop their asthma and allergy medications in a sequential manner based on the duration of action of each medication (inhaled corticosteroids for 2 weeks, anti-histamines and leukotriene inhibitors for 3 days, long-acting bronchodilators for 2 days, and short-acting bronchodilators for 8 hr). The subjects were informed of the risks of the experimental protocol and signed a consent form that had been approved by the UCSF Committee on Human Research. All subjects received financial compensation for their participation.

Allergy Skin Testing: (Pre-enrollment) To determine allergy status, and sensitivity to *Dermatophagoides pteronyssinus* (DP) an allergy skin testing with a set of 10 common aeroallergens [DP, birch mix, Chinese elm, cat, dog, mountain cedar, mugwort sage, olive tree, perennial rye, *aspergillus fumigatus*] and controls of saline and histamine was performed inside the forearm. Sensitivity was defined as a >2 x 2 mm skin wheal response, except for DP (> 3 x 3 mm skin wheal). If the subject was sensitive to DP on the initial skin-prick test, a dilutional skin test using log concentrations (1.5 AU to 15,000 AU) of DP allergen was also be performed, to determine the dose of DP allergen to be

used for the allergen bronchoscopy.

Methacholine Challenge Testing: (Pre-enrollment) To assess asthma status, a methacholine inhalation test was be performed following a protocol modified from the American Thoracic Society guidelines (79), using a nebulizer (DeVilbiss) and dosimeter (Rosenthal) set to deliver 9 µL per breath. Subjects inhaled aerosol from the nebulizer in five breaths, (one every 12 seconds over a 1-minute period) and spirometry was measured 3 min after each dose. The next dose was administered within 30 seconds of completing the spirometry. Increasing doses of methacholine (0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 mg/mL) were given, until a 20% decrease in FEV_1 from saline FEV_1 was achieved. A positive methacholine test was defined as a 20% decrease in FEV_1 at <8 mg/mL.

Climate-Controlled Chamber and Atmospheric Monitoring: The experiments took place in a ventilated, climate-controlled chamber at 20°C and 50% relative humidity. The chamber is a stainless steel-and-glass room of 2.5 × 2.5 × 2.4 m (Model W00327-3R; Nor-Lake, Hudson, WI) that was custom-built and designed to maintain temperature and relative humidity within 2.0°C and 4% from the set points, respectively (WebCtrl Software; Automated Logic Corporation, Kennesaw, GA). Temperature and relative humidity were recorded every 30 s and displayed in real-time (LabView 6.1; National Instruments, Austin, TX).

Exposure Session: After a telephone interview, subjects were scheduled for an initial visit to the laboratory, where a medical history questionnaire was completed. A 30-min exercise test designed to determine a workload that generated the target ventilatory rate was also completed on the initial visit. Each exposure session was 4 hr long, with subjects exercising for the first 30 min and then resting for the following 30 min of each hour in the climate-controlled chamber. The exercise consisted of running on a treadmill or pedaling a cycle ergometer. Exercise intensity was adjusted for each subject to achieve a target expired minute ventilation (VE) of 20 L/min/m^2 body surface area. During exercise, VE was calculated (LabView 6.1; National Instruments, Austin, TX) from tidal volume and breathing frequency measured using a pneumotachograph at the 10-min and 20-min intervals of each 30-min exercise period. Subjects remained inside the chamber for the entire 4-h exposure period. The type of exposure (FA or O_3) was chosen randomly prior to each session and was not revealed to the subjects.

Spirometry: Each subject's spirometry and peak expiratory flow were measured at each of the 0-hr, 4-hr, and 24-hr time points. Spirometry was performed on a dry rolling-seal spirometer (S&M Instruments, Louisville, CA) following American Thoracic Society (ATS) performance criteria (80). The best values for FVC and FEV_1 from three acceptable FVC maneuvers were used in data analysis. After the challenge bronchoscopy, the subjects performed spirometry on an hourly basis using a portable spirometer (EasyOne, ndd Medical Technologies Inc., Andover, MA), again according to ATS performance criteria.

Bronchoscopy, Endobronchial Allergen Challenge, and Lavage Procedures: Allergen challenge bronchoscopies were performed 20 ± 2 hr after exposure. This time was chosen because previous studies have documented the presence of an ozone-induced inflammatory response in many subjects at this time point (81). Our laboratory's procedures of bronchoscopy and bronchoalveolar lavage (BAL) have been previously discussed in detail (81). Briefly, intravenous access was established, supplemental O_2 was delivered, and the upper airways were anesthetized with topical lidocaine. Sedation

with intravenous midazolam and fentanyl was used as needed for subject comfort. In addition, the local endobronchial allergen challenge (LEAC) bronchoscopies were conducted according to the guidelines of the European Respiratory Society (78). The bronchoscope was first directed into the right upper lobe anterior segment orifice (RUL), where a control challenge was performed with 20 mL of sterile 0.9% saline pre-warmed to 37°C. The bronchoscope was then advanced to the right middle lobe medial segment orifice (RML), where the allergen challenge was performed with 20 mL of pre-warmed DP allergen solution. The concentration of DP chosen for LEAC was 1/10 the dilution that elicited a 3 mm diameter skin wheal response. The bronchoscope was then withdrawn and the subject taken back to the clinical research center for monitoring and recovery. After the challenge bronchoscopy, the subject was monitored continuously and underwent hourly spirometry prior to the sampling bronchoscopy.

The sampling bronchoscopy was performed 6 hr after the challenge bronchoscopy. The bronchoscope was first directed into the RUL where lavage was performed with two 50-ml aliquots of 0.9 % saline warmed to 37° C. The bronchoscope was then directed to the RUL where again lavage was performed with two 50-ml aliquots of 0.9 % saline warmed to 37° C. The RUL and RML fluids returned were immediately put on ice. After the sampling bronchoscopy, the subject was observed for an approximate 2-hr recovery period.

Total cells were counted on uncentrifuged aliquots of BAL using a hemocytometer. Differential cell counts were obtained from slides prepared using a cytocentrifuge, 25 g for 5 min, and stained with Diff-Quik as previously described (81). Cells were counted by two independent observers; the average of the two counts was used in data analysis. BAL fluid was then centrifuged at 180 g for 15 min, and the supernatant was separated and re-centrifuged at 1,200 g for 15 min to remove any cellular debris prior to freezing at -80° C.

Concentrations of BAL cytokines were measured using a Milliplex human 9-plex cytokine assay (Millipore Corporation, St. Charles, MO). Cytokines measured included the following: granulocyte macrophage colony-stimulating factor (GMCSF), interleukin-1β (IL-1β), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 13 (IL-13), and tumor necrosis factor alpha (TNF-α). The lower limit of detection for GMCSF, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, and TNF-α was 3.2 pg/ml and for IL-8 was 16.0 pg/ml.

GSTM1 Genotyping: DNA was isolated from whole blood using a Qiamp Blood DNA Maxi kit (Qiagen, Inc., Santa Clarita, CA). The assessment of GSTM1 genotype was done by multiplex polymerase chain reaction (PCR) using the following primers: 5'-CTGGATTGTAGCAGATCATGC-3' and 5'-TACTTGATTGATGGGGCTCAC-3'. Briefly, 100 ng of DNA was added to 50 uL reaction containing 0.1 uM of primers, 0.2 mM each dNTP, 2.5 units of Taq polymerase, and 1.5mM magnesium chloride. Amplification was performed up to 40 steps. Products for the polymorphisms were identified on 3.5% agarose gel.

Data Management and Statistical Analysis: Student's t-test was used for initial pair-wise comparisons of spirometric and BAL analyte values between the two exposure types. The change in spirometric parameters over the course of each exposure was calculated linearly using the 0-h value as the baseline. All data were entered into a database (Microsoft Excel 2003; Microsoft; Redmond, WA) and then analyzed using STATA

statistics software (STATA IE, version 10.0; StataCorp; College Station, TX). Each subject served as their own control. Data are presented as mean±SD. A p-value of 0.05 was considered to be statistically significant in all analyses.

Results

Subject Characteristics: Subject characteristics are shown in Table 1. Of the 10 subjects who completed the study protocol, all 10 had mild asthma. Five were GSTM1-present and five were GSTM1-null.

Climate-Controlled Chamber Conditions: The mean temperature and relative humidity in the climate-controlled chamber were (mean ± SD) 18.9 ± 2.9 °C and 46.7 ± 11.9%, respectively. The mean O_3 concentrations for the FA and O_3 exposures were 0.0145 ± 0.003 ppm and 0.1607 ± 0.005 ppm, respectively (see Table 2)..

Ozone-induced Changes in Spirometric Indices: The mean pre- and post-exercise spirometric values for FEV_1, FVC, and FEV_1/FVC at are shown in Table 3 and Figure 1. Both FEV_1 and FVC declined immediately after O_3 exposure while FEV_1 increased and FVC did not change immediately after FA exposure; these differences between the FA and O_3 exposures exposures were statistically significant (see Figure 2). No statistically significant differences were seen 18 hr after the two types of exposure, prior to the challenge bronchoscopies (see Figure 2). There were also no differences in lung function response to O_3 between GSTM1-wild type and GSTM1-null subjects (Table 4).

Endobronchial Allergen Challenge-induced Changes in Spirometric Indices: The mean post-LEAC hourly spirometric indices are also shown in Table 3. At 3 h post-LEAC, the magnitude of decrease in FEV_1 was significantly greater after O_3-allergen than after FA-allergen ($p=0.04$). Again, there were no differences in lung function response to O_3-allergen or FA-allergen between GSTM1-wild type and GSTM1-null subjects (Table 5).

Ozone-induced Changes in BAL Inflammatory Indices: Bronchoalveolar lavage data are shown in Tables 6 and 7. As expected, BAL neutrophil and eosinophil counts were increased in segments challenged with allergen compared to segments that received sham normal saline challenges (although it should be noted that normal saline lavage is known to induce neutrophil influx into the airways). Neutrophil counts were non-significantly higher after O_3-allergen exposure compared to after FA-allergen exposure (see Table 6). As expected, BAL macrophage counts went down as neutrophil concentrations increased. Contrary to our hypothesis, GSTM1-wild type subjects had consistently greater inflammatory cell responses to O_3-allergen exposure than GSTM1-null subjects (see Figure 3). Concentrations of the following cytokines were non-significantly decreased in BAL after O_3-allergen exposure compared to FA-allergen exposure taking all subjects together: IL-1β, IL-4, IL-5, IL-8, IL-10, IL-13, GM-CSF, and TNF-α (see Figure 4). No significant differences in airway inflammatory cellular responses to O_3-allergen exposure compared to FA-allergen exposure based on GSTM1 genotype were noted. Contrary to our hypothesis, however, GSTM1-wild type subjects had consistently greater inflammatory cytokine responses to O_3-allergen exposure than GSTM1-null subjects; for GM-CSF, this difference was significant ($p=0.02$)

Discussion

In this research project, we attempted to address the following two questions: 1) whether O_3 exposure enhances the specific airway inflammatory responses of asthmatic subjects during late-phase reactions to inhaled local endobronchial allergen challenge, and 2) whether asthmatic subjects with the GSTM1 null genotype have greater allergic inflammatory responses than subjects who have GSTM1 present. The results of our study suggest that O_3, at least at the concentration (0.160 ppm) and exposure duration (4 h) tested, appears to have mixed effects on allergen-induced airway inflammation. While BAL neutrophils were non-significantly increased after O_3-allergen exposure compared to FA-allergen exposure, BAL concentrations of most cytokines assayed were non-significantly lower after O_3-allergen exposure. The absence of GSTM1 appears to be associated with decreased magnitude of the inflammatory response to endobronchial allergen challenge after O_3 exposure. These results must be interpreted with caution, however, given our small sample size (n=10). Despite the small sample size, however, we did find that O_3 exposure significantly enhanced the lung function response to allergen at 3 h-post local endobronchial challenge, consistent with previously published research that used the whole lung inhalation challenge method (68, 69, 72, 73).

The original research proposal had planned for a larger sample size (n=30), but multiple problems contributed to our inability to recruit and enroll subjects. First, the UCSF Committee on Human Research (CHR) expressed considerable concern about the safety of the protocol. The committee was concerned that the risks associated with the multiple bronchoscopies required (four in total, with two occurring on each LEAC day) and the LEAC procedure itself were excessive for allergic asthmatic subjects. It took almost 1 year and a special expert review of our protocol before the CHR gave its approval of the protocol. Because of the multiple bronchoscopies and LEAC procedure, recruitment and retention of subjects were also exceptionally difficult. Five hundred forty two contacts were required to enroll 20 subjects into the study and, of these, only 10 were able to complete the protocol. One of the subjects who could not complete the protocol experienced a severe anaphylactic reaction after his initial LEAC; fortunately, the subject recovered without long-term sequelae. After we reported this reaction to the CHR as required, a protocol review process was initiated during which we could not enroll any new subjects or conduct any further experimentation with already enrolled subjects for several months.

As expected from previous research in our laboratory and elsewhere, O_3 exposure did induce a significant decrease in lung function (17, 18, 74, 81). Despite the allergic asthmatic status of the subjects, there was no evidence of exercise-induced bronchoconstriction during or after the FA exposure. Although previous reports in the literature have suggested that the GSTM1 genotype enhances lung function responses to O_3 (38, 41, 43), we found no evidence for such an effect. In fact, the subjects with GSTM1 present had the largest decreases in FEV1 and FVC after 4-h exposure to O_3.

The endobronchial instillation of allergen to which the subjects were sensitized did induce an airway inflammatory response as evidenced by BAL leukocytosis and increases in selected cytokine concentrations after LEAC following FA exposure. While there was some suggestion of an enhanced neutrophilic response after LEAC following O_3 exposure, levels of all inflammatory cytokines assayed, except IL-6, were actually lower after O_3-allergen compared to FA-allergen.

No previous controlled human exposure study has investigated the effect of O_3 on allergic inflammation using BAL to study the late-phase reaction, although one study has used nasal lavage. Ours is the first study to apply the LEAC technique to the study of the airway inflammatory effects of O_3 or any other pollutant.

The mechanism underlying our primary finding, that exposure to O_3 caused a significant increase in the late-phase lung function response to allergen, is probably enhanced local bronchoconstriction of the allergen-challenged lung segment. We directly observed narrowing of the lumen of the previously challenged segment at the time of the sampling bronchoscopy 6 hr after allergen challenge bronchoscopies following both O_3 and FA exposures. Ozone exposure itself is known to cause some bronchoconstriction even in non-asthmatic subjects, possibly due to airway edema and/or neuroreceptor stimulation. It is likely that the direct effects of O_3 on the airways are additive to those of specific allergen challenge.

The novel finding of our study, a suggestion that the airway inflammatory cytokine response to specific allergen challenge is decreased after O_3 exposure, also requires mechanistic explanation. One possibility is that O_3 exposure leads to activation of innate immunity which may, in turn, dampen Th2 responses to allergen. However, there is evidence that O_3 activation of innate immunity actually enhances such responses. Thus, our finding of decreased cytokine responses after O_3 pre-exposure to allergen, including the Th2 cytokines IL-4 and IL-13, must be considered preliminary until confirmed in another study.

Despite previous reports that the GSTM1-null genotype enhanced lung function responses to O_3 exposure (38, 41, 43), we found no evidence of this in our small sample. We also found no evidence of an enhanced airway neutrophilic inflammatory response after O_3-allergen exposure in the GSTM1-null subjects. To our surprise, the GSTM1-null subjects had lower airway cellular and cytokine responses to O_3-allergen exposure than GSTM1-present subjects. We had hypothesized that GSTM1-null subjects would experience greater oxidative stress after O_3 pre-exposure than GSTM1-present subjects, and thus would have greater airway cellular and cytokine inflammatory responses to subsequent allergen challenge. Although we actually found a suggestion of a decreased airway inflammatory response to allergen after O_3 pre-exposure in the GSTM1-null subjects, this finding should also be considered preliminary until confirmed in another study.

Our study has both strengths and limitations. The strengths include the first controlled human exposure study of an air pollutant to use endobronchial allergen challenge followed by measurement of biomarkers of airway inflammation in BAL. It is also the first study to assess the impact of the common GSTM1 null genetic variant on airway responses to allergen after O_3 exposure. Limitations include relative lack of power to study small changes (e.g., the trend toward an increase in BAL neutrophils after O_3 exposure might have become significant with a larger sample size) and study subjects with relatively mild allergic asthma. We chose to recruit such subjects for safety reasons, given that the effects of O_3 inhalation on LEAC in allergic asthmatic subjects had not been previously studied. It is possible that only patients with more severe asthma are at greater risk for O_3-induced effects on allergic inflammatory responses. We chose to study asthmatic subjects who were otherwise healthy as a potentially susceptible subgroup because of pre-existing airway inflammation. We and others have

shown that asthmatic subjects have greater airway neutrophilic responses to O_3, but this may not translate to an increased inflammatory response to specific allergen.

Summary and Conclusions

Epidemiological data from multiple studies suggest that persons with asthma may have greater morbidity as measured by health care utilization after O_3 exposure than normal, healthy persons. The animal toxicological data provide evidence that O_3 exposure can enhance allergic inflammatory responses in the lungs. Controlled human exposure studies have confirmed that O_3 exposure can enhance both the early and late bronchoconstrictor responses to inhaled allergen in some, but not all allergic asthmatic subjects. Controlled human exposure data on the effect of O_3 on the late-phase airway inflammatory response to inhaled allergen are sparse and somewhat conflicting.

This research project was designed to provide information on the following two questions: 1) whether O_3 exposure enhances the specific airway inflammatory responses of asthmatic subjects during late-phase reactions to allergen, and 2) whether asthmatic subjects with the GSTM1 null genotype have greater allergic inflammatory responses than subjects who have GSTM1 present.

The study used a novel method to assess the airway inflammatory response to allergen, local endobronchial allergen challenge (LEAC). Subjects were screened prior to beginning the experimental protocol so that 50% had the GSTM1 null genotype. Ten asthmatic subjects with specific sensitization to the house dust mite, *Dermatophagoides pteronyssinus* (DP), were exposed separately to 0.16 ppm O_3 and filtered air (FA) control for 4 hr with intermittent exercise. At 20 hr post-exposure, subjects underwent a challenge bronchoscopy during which DP allergen was instilled in a sub-segmental bronchus of the right middle lobe and saline was instilled in a sub-segmental bronchus of the right upper lobe. Six hours later, a second sampling bronchoscopy was performed to collect samples of airway lining fluid from each challenged bronchus for analyses of cellular and biochemical markers of non-specific and specific allergic inflammatory responses. Subjects underwent lung function testing pre- and immediately post-exposure, 18 hr post-exposure prior to and then hourly after the challenge bronchoscopy until the sampling bronchoscopy.

The original research proposal had planned for a larger sample size (n=30), but multiple problems contributed to our inability to recruit and enroll subjects. First, the UCSF Committee on Human Research (CHR) expressed considerable concern about the safety of the protocol. Second, the multiple bronchoscopies and LEAC procedure made recruitment and retention of subjects exceptionally difficult. Finally, one of the subjects did, in fact, experience a severe anaphylactic reaction after his initial LEAC; fortunately, this subject recovered without long-term sequelae.

The results of this study confirm previous reports that O_3 pre-exposure enhances the lung function response to allergen in specifically sensitized asthmatic subjects. The novel finding of this study, however, is that O_3 exposure appears to decrease the cytokine component of the airway inflammatory response to allergen in these subjects. Moreover, the absence of the antioxidant enzyme, GSTM1, does not seem to increase the lung function or airway inflammatory response to allergen following O_3 exposure. Because of the small sample size, caution should be applied in interpretation of these results.

Recommendations

1) Because the results of this study confirm previous reports that O_3 pre-exposure enhances the lung function response to allergen in specifically sensitized asthmatic subjects, this can be considered one mechanism for why asthmatic individuals require increased health care utilization during smog episodes. Media messages during such episodes advising asthmatic individuals to stay indoors should be continued.

2) The novel finding of this study that O_3 exposure appears to decrease the cytokine component of the airway inflammatory response to allergen in allergic asthmatic subjects needs to be confirmed in a larger study.

3) The suggestion that the absence of the antioxidant enzyme, GSTM1, does not increase the lung function or airway inflammatory response to allergen following O_3 exposure should also be confirmed

4) The plan had called for a second experiment to be performed in which endobronchial allergen challenge would precede O_3 or FA exposure, the reverse order of the study reported here. A study of the effect of pre-exposure to allergen on the subsequent response to O_3 should still be done, but perhaps with the more traditional whole lung inhalational allergen challenge technique.

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Figure Legends

Figure 1. Range of FEV1 values for each hour of the protocol: panel A for O_3 exposures; panel B for filtered air exposures.

Figure 2. Time vs. FEV1: filtered air and O_3 exposures.

Figure 3. Mean values and standard deviations of cell counts in bronchoalveolar lavage fluid obtained 6 h after local endobronchial allergen challenge: panel A, all subjects; panel B, GSTM1-null subjects; panel C, GSTM1-present subjects. AG=antigen; NS=normal saline control.

Figure 4. Mean values and standard deviations of cytokine concentrations in bronchoalveolar lavage fluid obtained 6 h after local endobronchial allergen challenge: panel A, GSTM1-null subjects; panel B, GSTM1-present subjects; panel C, all subjects. AG=antigen; NS=normal saline control.

Table 1: Baseline characteristics of participants

Characteristic	GSTM-null	GSTM1-wild type	p-Value
N=	5	5	---
Male/Female	2/3	3/2	---
Age	41 ± 5.92	29.6±5.22	.01
Height (cm)	173.54 ± 7.14	167.64 ± 10.48	.32
Weight	102.52 ± 27.27	89.1 ± 22.97	.42
BMI	34.12 ± 9.09	31.39 ± 6.34	.59
BSA (m^2)	2.15 ± .26	1.98 ± .30	.35
PC_{20}	1.79 ± 2.23	.81 ± .87	.38
FEV1(L)	3.19 ± .77	3.16 ± .55	.94
FEV1 Percent Predicted	85.4 ± 21.41	89.8 ± 13.01	.70
FVC (L)	4.46 ± .76	4.07 ± .73	.43
FVC Percent Predicted	94.8 ± 9.93	97 ± 9.46	.72
Ratio (FEV1/FVC)	.72 ± .14	.78 ± .07	.41
FEV1(L)	3.03 ± .69	3.22 ± .58	.65
FEV1 Percent Predicted	78.8 ± 16.8	88.2±10.89	.32
FVC (L)	4.17 ± .75	4.30 ± .75	.79
FVC Percent Predicted	87.4 ± 8.91	97 ± 8.34	.11
Ratio (FEV1/FVC)	.73 ± .15	.75 ± .05	.82

Data shown are mean ± standard deviation. Participants were all asthmatic and atopic. Abbreviations: BMI=body mass index; BSA=body surface area; FEV_1=forced expiratory volume in 1 second; FVC=forced vital capacity

Table 2: Exposure Conditions

Characteristic	Genotype	Filtered Air	Ozone	p-Value◦
Ozone Concentration		.0145 ± .003	.1607 ± .005	0.00
	GSTM1-null	.015 ± .004	.162 ± .005	0.00
	GSTM1-WT	.014 ± .001	.159 ± .004	0.00
	p-Value*	.61	.42	--
Temperature (◦C)		18.46 ± 3.03	19.24 ± 2.89	.56
	GSTM1-null	18.94 ± 3.12	18.92 ± 3.12	.99
	GSTM1-WT	17.98 ± 3.21	19.56 ± 2.95	.44
	p-Value*	.64	.74	--
Relative Humidity		46.75 ± 13.65	46.78 ± 10.18	.99
	GSTM1-null	46.94 ± 15.99	45.2 ± 11.15	.84
	GSTM1-WT	45.56 ± 12.78	48.36 ± 10.14	.81
	p-Value*	.96	.65	--

Data shown are mean ± standard deviation. WT=wild type.
◦p-Value column: compares means listed in the same row
*p-Value rows: compare means listed in same column

Table 3: Serial measurement of lung function: comparison of ozone-allergen vs. filtered air-allergen

Hour	Lung Function	Filtered Air	Ozone	p-Value
0	FEV1	3.15 ± .21	3.11 ± .18	.63
	FVC	4.25 ± .24	4.22 ± .21	.71
	Ratio FEV1/FVC	.75 ±.03	.74 ± .03	.84
4	FEV1	3.22 ±.22	3.00 ± .21	**.03**
	FVC	4.25 ± .23	4.03 ± .23	**.03**
	Ratio FEV1/FVC	.76 ±.03	.75 ± .03	.24
22	FEV1	3.04 ± .21	3.12 ± .18	.33
	FVC	4.05 ± .24	4.18 ± .22	.21
	Ratio FEV1/FVC	.75 ± .03	.75 ± .03	.78
ez22	FEV1	2.87 ± .19	2.98 ± .16	.25
Pre-1st bronch	FVC	3.84 ±.18	3.99 ± .21	.09
	Ratio FEV1/FVC	.75 ± .03	.75 ± .03	1.00
ez23	FEV1	2.55 ± .17	2.44 ± .21	.18
	FVC	3.43±.20	3.21 ± .25	.18
	Ratio FEV1/FVC	.77 ± .03	.76 ± .03	.65
ez24	FEV1	2.55 ± .18	2.31 ± .17	.08
	FVC	3.36 ± .23	3.01 ± .26	.20
	Ratio FEV1/FVC	.76 ± .03	.77 ± .03	.78
ez25	FEV1	2.57 ± .18	2.30 ± .19	**.04**
	FVC	3.46 ± .25	3.02 ± .24	.13
	Ratio FEV1/FVC	.75 ± .04	.77 ± .03	.61
ez26	FEV1	2.37 ± .23	2.46 ± .24	.41
	FVC	3.33 ± .36	3.32 ± .29	.91
	Ratio FEV1/FVC	.72 ± .04	.75 ± .04	.47
ez27	FEV1	2.42 ± .17	2.36 ± .26	.67
	FVC	3.31 ± .22	3.23 ± .26	.60
	Ratio FEV1/FVC	.73 ± .03	.72 ± .04	.73
ez28	FEV1	2.30 ± .21	2.29 ± .27	.87
Pre-2nd bronch	FVC	3.11 ± .25	3.11 ± .32	.98
	Ratio FEV1/FVC	.74 ± 0.4	.74 ± .03	.83

Data shown are mean ± SEM. Abbreviations: $FEV1_1$=forced expiratory volume in 1 second; FVC=forced vital capacity; ez=EasyOne spirometer

Table 4: Serial measurement of lung function across ozone and filtered air exposures: comparison of GSTM1-null vs. GSTM1-wild type genotypes

Hour	Condition	Lung Function	GSTM1-null	GSTM1-wild type	P-value
0	Filtered Air	FEV1	3.13 ± .84	3.17 ± .50	.94
		FVC	4.25 ± .92	4.25 ± .68	1.00
		Ratio FEV1/FVC	.74 ± .15	.75 ± .05	.97
0	Ozone	FEV1	3.02 ± .72	**3.21 ± .42 a, b**	.62
		FVC	4.11 ± .67	**4.32 ± .71 c, d**	.64
		Ratio FEV1/FVC	.74 ± .14	.75 ± .06	.88
4	Filtered Air	FEV1	3.12 ± .82	3.33 ± .66	.67
		FVC	4.18 ± .79	4.33 ± .79	.77
		Ratio FEV1/FVC	.75 ± .06	.77 ± .02	.74
4	Ozone	FEV1	3.02 ± .86	**2.99 ± .46 a**	.43
		FVC	3.98 ± .84	**4.01 ± .71 c**	.86
		Ratio FEV1/FVC	.76 ± .13	.74 ± .03	.75
22	Filtered Air	FEV1	2.98 ± .79	3.11 ± .59	.77
		FVC	3.96 ± .72	4.14 ± .88	.73
		Ratio FEV1/FVC	.75 ± .13	.76 ± .07	.90
22	Ozone	FEV1	3.19 ± .75	**3.05 ± .43 b**	.72
		FVC	4.19 ± .80	**4.17 ± .66 d**	.97
		Ratio FEV1/FVC	.76 ± .12	.73 ± .06	.61

Data shown are mean ± SEM. Abbreviations: FEV_1=forced expiratory volume in 1 second; FVC=forced vital capacity.
a: p=0.08; b: p=0.0005; c: ; p=0.03; d: p=0.006.

Table 5: Serial measurement of lung function following local endobronchial allergen challenge: comparison of GSTM1-null vs. GSTM1-wild type genotypes

Hour	Condition	Lung Function	GSTM1-null	GSTM1-wild type	P-value
ez22	Filtered Air	FEV1	2.78 ± .79	2.97 ± .39	.66
	Pre-1st bronch	FVC	3.77 ± .61	3.92 ± .59	.72
		Ratio FEV1/FVC	.74 ± .15	.76 ± .04	.71
ez22	Ozone	FEV1	**3.02 ± .65 z**	**2.95 ± .43 a**	.84
		FVC	**3.97 ± .69 y**	**4.02 ± .70 b**	.91
		Ratio FEV1/FVC	.76 ± .11	.73 ± .06	.64
ez23	Filtered Air	FEV1	2.68 ± .60	2.43 ± .47	.47
		FVC	3.53 ± .65	3.15 ± .62	.37
		Ratio FEV1/FVC	.76 ± .12	.77 ± .03	.91
ez23	Ozone	FEV1	2.53 ± .74	2.34 ± .64	.67
		FVC	3.29 ± .85	3.12 ± .79	.74
		Ratio FEV1/FVC	.77 ± .13	.75 ± .02	.69
ez24	Filtered Air	FEV1	2.7 ± .66	2.39 ± .53	.44
		FVC	3.57 ± .79	3.15 ± .64	.39
		Ratio FEV1/FVC	.76 ± .12	.76 ± .05	.97
ez24	Ozone	FEV1	2.93 ± .44	2.22 ± .66	.64
		FVC	3.19 ± .66	2.96 ± 1.04	.68
		Ratio FEV1/FVC	.76 ± .13	.77 ± .06	.92
ez25	Filtered Air	FEV1	2.58 ± .27	2.56 ± .26	.95
		FVC	3.39 ± .31	3.53 ± .43	.79
		Ratio FEV1/FVC	.77 ± .13	.74 ± .10	.69
ez25	Ozone	FEV1	2.31 ± .25	2.29 ± .31	.96
		FVC	3.06 ± .33	2.98 ± .39	.89
		Ratio FEV1/FVC	.76 ± .11	.77 ± .04	.97
ez28	Filtered Air	FEV1	2.51 ± .58	1.93 ± .47	.19
		FVC	3.36 ± .64	2.70 ± .76	.23
		Ratio FEV1/FVC	.75 ± .13	1.49 ± 1.09	.17
ez28	Ozone	FEV1	**2.52 ± .78 z**	**1.99 ± .58 a**	.30
		FVC	**3.36 ± .91 y**	**2.74 ± .79 b**	.31
		Ratio FEV1/FVC	.75 ± .10	.73 ± .04	.75

Data shown are mean ± SEM. Abbreviations: FEV_1=forced expiratory volume in 1 second; FVC=forced vital capacity; ez=EasyOne spirometer.
a: p=0.04; b: p=0.04; z: p=0.009; y: p=0.01.

Table 6: Mean values and standard deviations of cell counts in bronchoalveolar lavage fluid obtained 6 h after local endobronchial challenge

	Condition	All Subjects	GSTM1-null	GSTM1-wild type	p-value
Total WBC	1	49.4 + 39.2	57.0 + 46.2	41.9 + 34.3	0.34
	2	120.2 + 121.6	93.3 + 72.7	147.1 + 161.8	0.51
	3	47.3 + 36.3	48.9 + 39.7	45.8 + 37.2	0.90
	4	147.3 + 266.6	56.8 + 37.7	237.7 + 371.5	0.31
MAC	1	20.9 + 11.0	20.7 + 7.6	21.1 + 14.5	0.46
	2	17.9 + 9.0	19.9 + 10.4	15.9 + 8.2	0.34
	3	17.5 + 8.7	21.4 + 10.7	13.7 + 4.1	0.16
	4	14.8 + 7.5	14.7 + 7.9	14.9 + 7.9	0.91
LMP	1	7.4 + 5.2	9.4 + 6.3	5.5 + 3.2	0.25
	2	14.0 + 10.0	14.7 + 11.3	13.4 + 9.9	0.60
	3	5.7 + 3.9	3.4 + 1.4	8.0 + 4.3	0.02*
	4	11.8 + 10.0	10.6 + 6.1	13.1 + 13.6	0.71
PMN	1	19.5 + 29.4	24.0 + 36.6	15.0 + 23.6	0.65
	2	49.5 + 10.2	24.6 + 28.3	74.4 + 145.5	0.47
	3	22.1 + 29.8	21.1 + 31.0	23.0 + 32.2	0.60
	4	94.3 + 239.8	23.2 + 23.8	165.4 + 340.8	0.37
EOS	1	0.84 + 1.90 **a**	1.36 + 2.70	0.32 + 0.41	0.41
	2	38.16 + 56.81 **a**	32.95 + 32.25	43.36 + 78.44	0.75
	3	2.05 + 3.33	2.92 + 4.71	1.19 + 0.93	0.44
	4	26.38 + 41.93	8.34 + 9.84	44.42 + 55.19	0.17

1 = Filtered + normal saline
2 = Filtered air + allergen
3 = O_3 + normal saline
4 = O_3 + allergen

Within groups

a = p-value ≤ 0.01 between 1 & 2

WBC = white blood cell
MAC = macrophage
LMP = lymphocyte
PMN = polymorphonuclear cell
EOS = eosinophil

Table 7: Mean values and standard deviations of cytokine concentration, stratified by genotype

	Condition	All Subjects	GSTM1-null	GSTM1-wild type	p-value
Il-1b	1	4.8 + 7.0	2.7 + 3.5	6.9 + 9.3	0.37
	2	10.3 + 26.0	1.7 + 1.2	17.2 + 34.9	0.40
	3	2.5 + 4.1	2.7 + 5.8	2.3 + 2.1	0.89
	4	7.3 + 20.6	0.9 + 1.2	13.6 + 29.2	0.36
Il-4	1	5.2 + 3.9 **a**	6.4 + 5.0	4.1 + 1.1 **d**	0.38
	2	148.2 + 140.3 **a**	174.4 + 177.5	127.2 + 120.3 **d**	0.64
	3	5.4 + 5.7 **e**	3.0 + 2.9	7.7 + 7.2 **h**	0.21
	4	63.6 + 121.9 **e**	11.7 + 19.2	115.6 + 162.2 **h**	**0.02***
Il-5	1	2.8 + 3.0 **a**	2.5 + 3.2	3.1 + 3.2 **d**	0.77
	2	98.7 + 97.5 **a**	105.8 + 124.9	93.0 + 85.0 **d**	0.85
	3	2.1 + 2.6 **b**	1.1 + 1.8 **e**	3.1 + 3.0 **e**	0.24
	4	63.9 + 122.3 **b**	20.0 + 37.9 **e**	107.9 + 165.5 **e**	**0.07**†
Il-6	1	199.8 + 168.5	199.1 + 159.4	200.5 + 196.2	0.99
	2	255.5 + 182.9	300.0 + 200.0	219.8 + 182.6	0.54
	3	210.3 + 188.2	113.5 + 162.3	307.1 + 173.0	**0.10**†
	4	234.8 + 178.3	203.9 + 198.9	265.7 + 172.0	0.61
Il-8	1	184.2 + 179.2	147.4 + 183.7	213.6 + 191.0	0.61
	2	291.2 + 156.4 **i**	400.0 + 0 **i**	204.2 + 166.1	**0.03***
	3	248.4 + 165.9	215.0 + 167.8	281.9 + 176.1	0.55
	4	159.4 + 175.4 **i**	138.2 + 167.0 **i**	180.5 + 200.6	0.72
Il-10	1	16.3 + 1.1 **d**	16.7 + 1.5	16.0 + 0	0.34
	2	109.1 + 165.4 **d**	124.24 + 184.5	97.1 + 169.6	0.82
	3	31.2 + 39.5	41.0 + 55.8	21.3 + 12.0	0.46
	4	68.4 + 121.4	22.5 + 14.6	114.2 + 166.4	0.25
Il-13	1	0.3 + 0.3 **a**	0.5 + 0.4	0.2 + 0.1 **d**	0.20
	2	94.9 + 130.9 **a**	83.1 + 127.8	104.4 + 147.5 **d**	0.82
	3	0.2 + 0.2 **b**	0.2 + 0.1	0.3 + 0.2 **e**	0.18
	4	37.0 + 80.3 **b**	12.6 + 27.8	61.4 + 110.8 **e**	**0.07**†
TNFa	1	78.4 + 123.6	35.1 + 38.3	121.7 + 168.0 **g**	0.29
	2	89.1 + 105.3	121.2 + 133.1	63.4 + 83.9 **g**	0.45
	3	93.3 + 139.0	63.1 + 136.9	123.5 + 150.0	0.52
	4	52.9 + 78.5	30.4 + 32.7	75.4 + 107.4	0.39
GM-CSF	1	8.3 + 9.5	3.4 + 2.2	13.1 + 9.5	**0.02***
	2	15.7 + 19.8	22.3 + 29.3	10.5 + 8.2 **i**	0.40
	3	8.2 + 5.4	4.7 + 5.0	11.7 + 3.3	**0.04***
	4	10.1 + 10.5	2.8 + 1.6	17.4 + 10.5 **i**	**0.02***

Between groups (GSTM1-null vs. GSTM1-wild type)

*** p-value < 0.05**
† p-value ≤ 0.10

1 = FA + NS
2 = FA + AG
3 = O3 + NS
4 = O3 + AG

Within groups

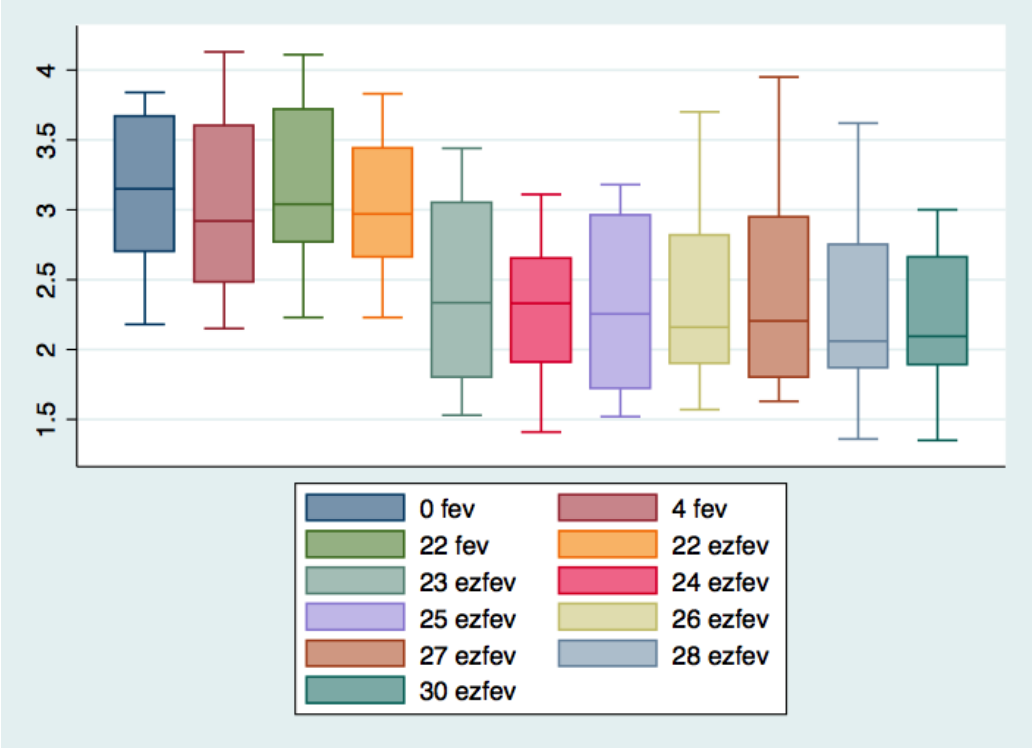
a = p-value ≤ 0.01 between 1 & 2
b = p-value ≤ 0.01 between 3 & 4
c = p-value ≤ 0.01 between 2 & 4

d = p-value < 0.05 between 1 & 2
e = p-value < 0.05 between 3 & 4
f = p-value < 0.05 between 2 & 4

g = p-value ≤ 0.08 between 1 & 2
h = p-value ≤ 0.08 between 3 & 4
i = p-value ≤ 0.08 between 2 & 4

Figure 1: Range of FEV1 values for each hour of the protocol: panel A for O_3 exposures; panel B for filtered air exposures.

A



B

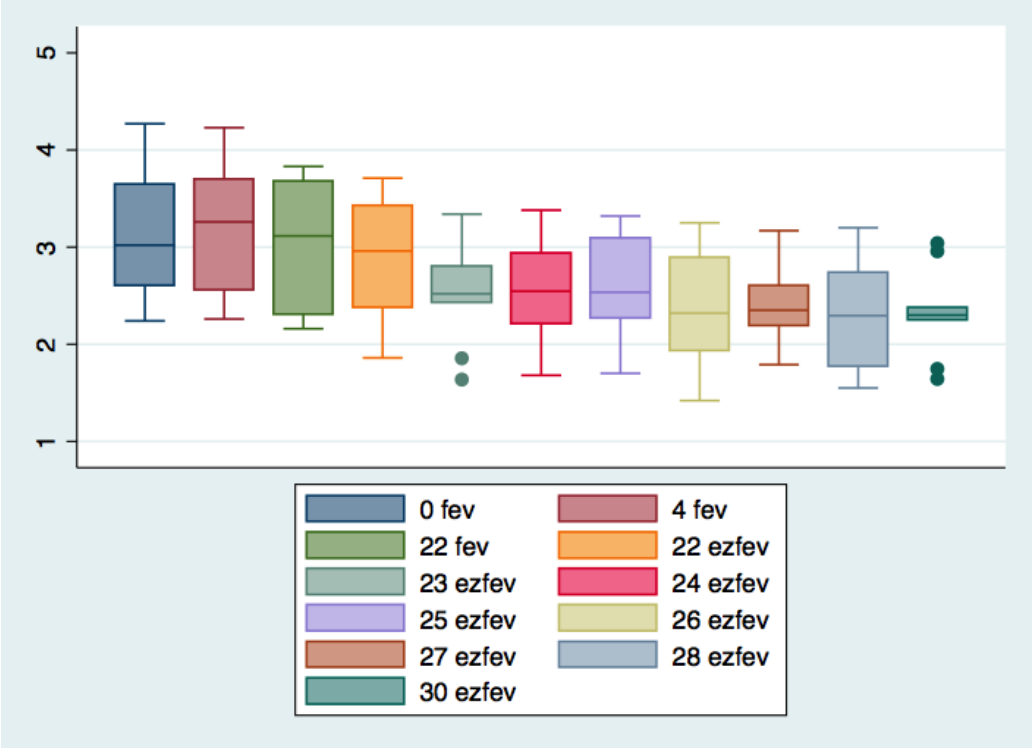


Figure 2:

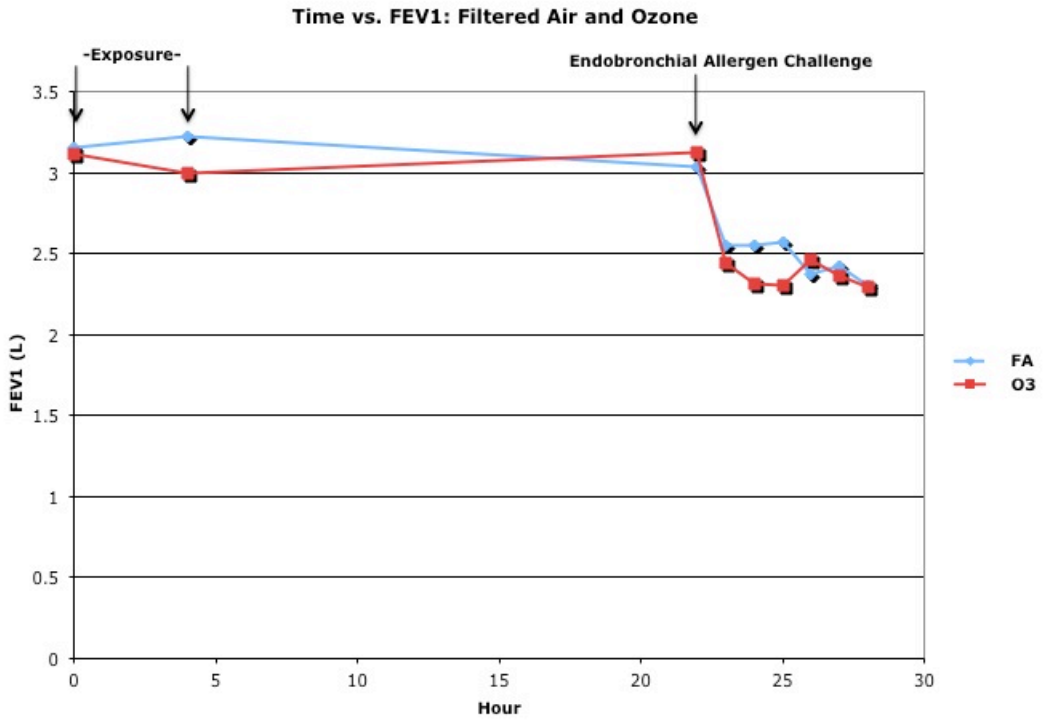
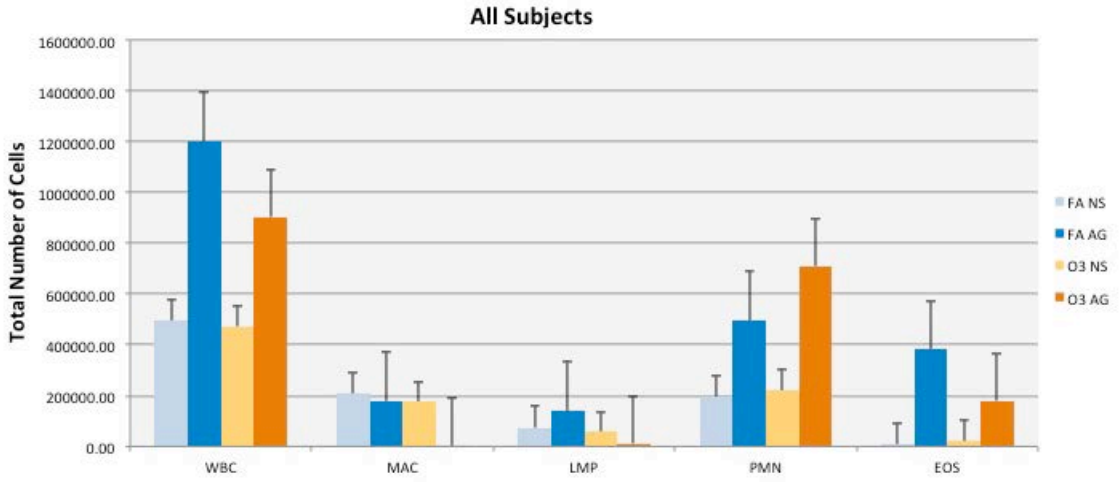
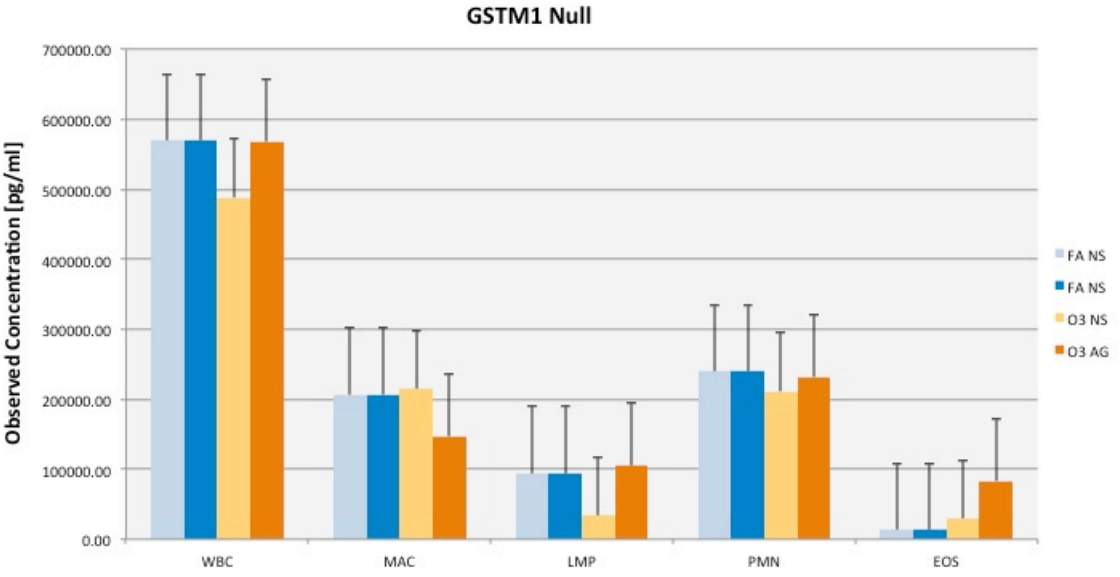

Time vs. FEV1: Filtered Air and Ozone
-Exposure-
Endobronchial Allergen Challenge
FEV1 (L)
Hour
FA
O3
0
0.5
1
1.5
2
2.5
3
3.5
0
5
10
15
20
25
30

Figure 3: **Mean values and standard deviations of cell counts in BAL fluid**

A



B



C

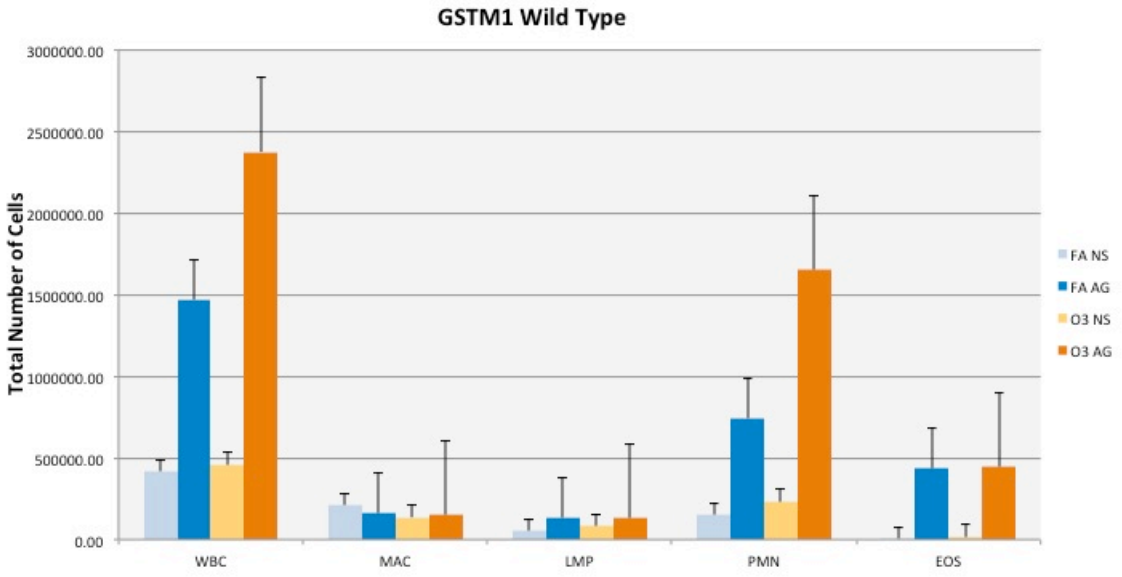
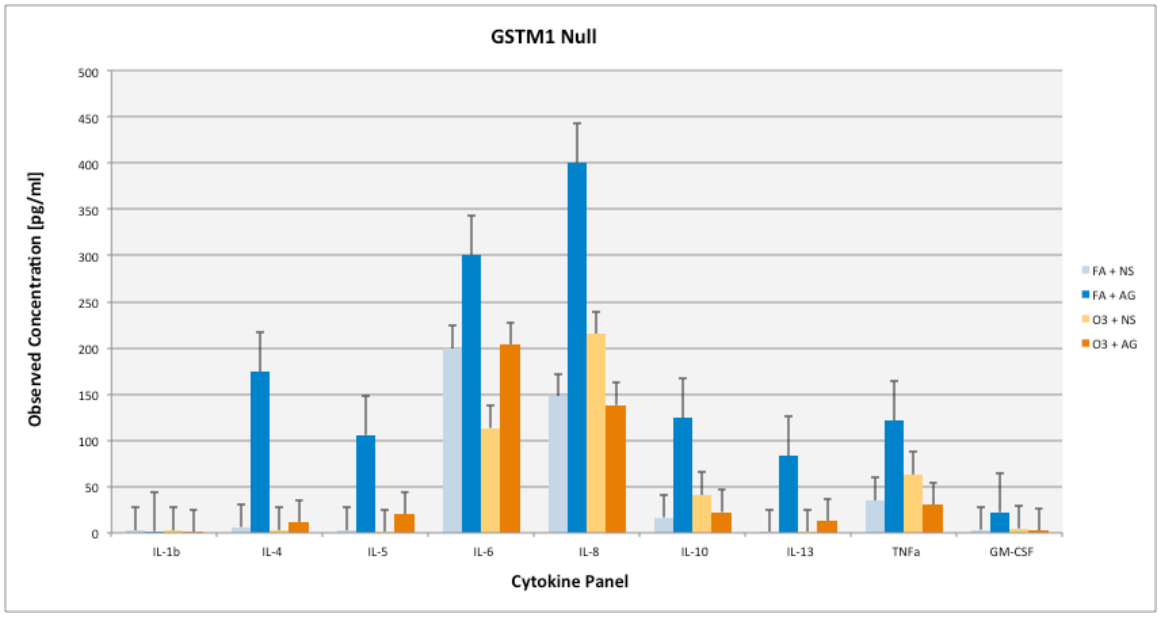
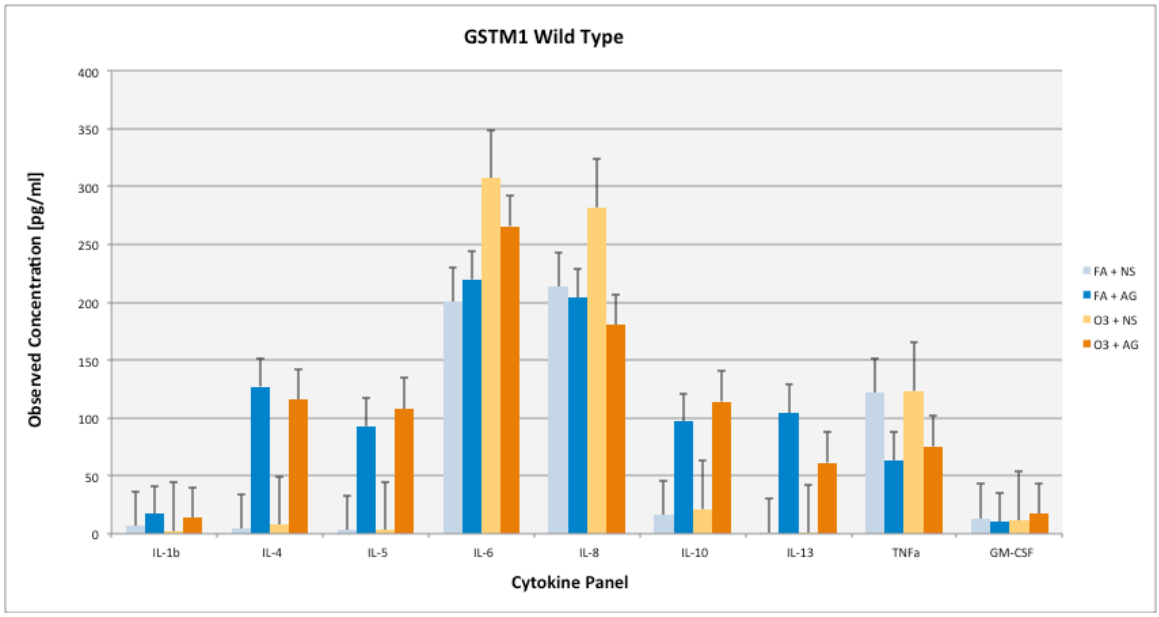

GSTM1 Wild Type
Total Number of Cells
3000000.00
2500000.00
2000000.00
1500000.00
1000000.00
500000.00
0.00
WBC
MAC
LMP
PMN
EOS
FA NS
FA AG
O3 NS
O3 AG

Figure 4: Mean values and standard deviations of cytokine concentrations in BAL fluid

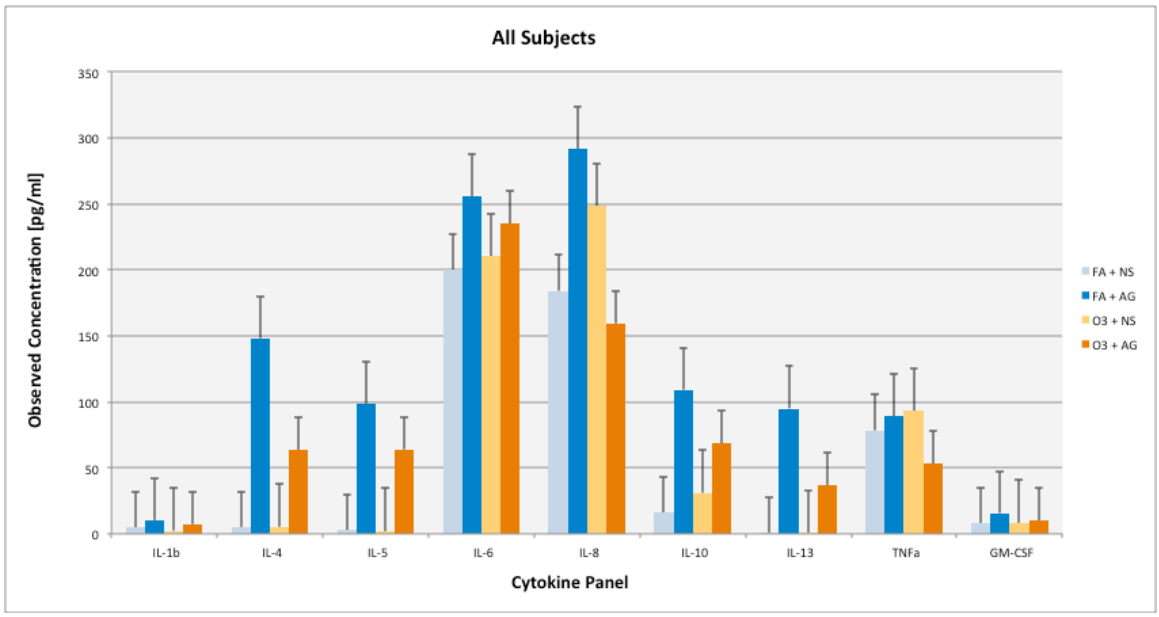
A



B



C


All Subjects
Observed Concentration [pg/ml]
350
300
250
200
150
100
50
0
IL-1b
IL-4
IL-5
IL-6
IL-8
IL-10
IL-13
TNFa
GM-CSF
Cytokine Panel
FA + NS
FA + AG
O3 + NS
O3 + AG

Glossary of Terms, Abbreviations, and Symbols

BAL	bronchoalveolar lavage
CHR	UCSF Committee on Human Research
DP	*Dermatophagoides pteronyssinus*
ECP	eosinophilic cationic protein
ENA-78	epithelial neutrophil-activating protein
FA:	filtered air
FEV_1:	forced expiratory volume in 1 second
FVC:	forced vital capacity
GM-CSF:	granulocyte macrophage colony-stimulating factor,
GSTM1	glutathione S-transferase mu
GSTP1	glutathione S-transferase pi
GSTT1	glutathione S-transferase theta
IL-1β:	interleukin-1β
IL-4	interleukin-4
IL-5:	interleukin 5
IL-6:	interleukin 6
IL-8:	interleukin 8
IL-10:	interleukin 10
IL-13:	interleukin 13
LEAC	local endobronchial allergen challenge
MPO	myeloperoxidase
NQO1	]NAD(P)H:Quinone Oxidoreductase
OVA	ovalbumin
RANTES	regulated upon activation, normal T-cell expressed and secreted
ROS:	reactive oxygen species
RML	right middle lobe
RLL	right upper lobe
Th2	T-helper cells 2
TMA	trimellitic anhydride
TNF-α:	tumor necrosis factor-alpha