

The glutathione-S-transferase Mu 1 null genotype modulates ozone-induced airway inflammation in human subjects

Neil E. Alexis, PhD,^{a,c} Haibo Zhou, PhD,^{a,d} John C. Lay, PhD,^{a,c} Bradford Harris, MD,^{a,c} Michelle L. Hernandez, MD,^{a,c} Tsui-Shan Lu, PhD,^{a,d} Philip A. Bromberg, MD,^{a,b} David Diaz-Sanchez, PhD,^e Robert B. Devlin, PhD,^e Steven R. Kleeberger, PhD,^f and David B. Peden, MD^{a,b,c} Chapel Hill and Research Triangle Park, NC

Background: The glutathione-S-transferase Mu 1 (*GSTM1*) null genotype has been reported to be a risk factor for acute respiratory disease associated with increases in ambient air ozone levels. Ozone is known to cause an immediate decrease in lung function and increased airway inflammation. However, it is not known whether *GSTM1* modulates these ozone responses in vivo in human subjects.

Objective: The purpose of this study was to determine whether the *GSTM1* null genotype modulates ozone responses in human subjects.

Methods: Thirty-five healthy volunteers were genotyped for the *GSTM1* null mutation and underwent a standard ozone exposure protocol to determine whether lung function and inflammatory responses to ozone were different between the 19 *GSTM1* wild type and 16 *GSTM1* null volunteers.

Results: *GSTM1* did not modulate lung function responses to acute ozone. Granulocyte influx 4 hours after challenge was

similar between *GSTM1* normal and null volunteers. However, *GSTM1* null volunteers had significantly increased airway neutrophils 24 hours after challenge, as well as increased expression of HLA-DR on airway macrophages and dendritic cells.

Conclusion: The *GSTM1* null genotype is associated with increased airways inflammation 24 hours after ozone exposure, which is consistent with the lag time observed between increased ambient air ozone exposure and exacerbations of lung disease. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Glutathione-S-transferase Mu 1, ozone, pollution, inflammation, polymorphonuclear neutrophil, macrophage, dendritic cell

A number of pro-oxidant air pollutants, including particulate matter, tobacco smoke, diesel exhaust, and ozone, have been linked to exacerbation of asthma and lung disease.¹⁻⁵ Glutathione-S-transferase Mu 1 (*GSTM1*) is a phase II antioxidant enzyme regulated by the transcription factor nuclear factor E2-related factor 2.⁶ The *GSTM1* null (*GSTM1*⁻) genotype results in a failure to produce the *GSTM1* protein. As noted in many reviews, the *GSTM1*⁻ genotype confers increased risk for adverse health effects because of exposure to these air pollutants.¹⁻⁵ As reviewed by Giesler and Olshan,⁷ case-control studies conducted in the United States of various tumors report frequencies of the *GSTM1*⁻ genotype that range from 23% to 41% in African Americans, 32% to 53% in Asians, 40% to 53% in Hispanics, and 35% to 62% in white subjects. Furthermore, prevalence of the *GSTM1*⁻ genotype ranges from 48% to 57% in white subjects, as reported in several population studies. Thus this gene variant occurs at high frequency in most ethnic populations.

Many investigators have reported increased susceptibility to lung disease after exposure to environmental tobacco smoke associated with the *GSTM1*⁻ genotype. One study of children exposed to environmental tobacco smoke found that those children who either had the *GSTM1*⁻ genotype or were homozygous for the GSTP1Val105 allele had increased risk of asthma at younger ages, with an increased risk for decreased lung function in adolescence.⁸ Others have reported that children with the *GSTM1*⁻ genotype are significantly more likely to have increased risk of asthma or acute respiratory illnesses if they are born to mothers who smoke during pregnancy⁹ or if they had been exposed to environmental tobacco smoke.¹⁰ Human challenge studies have been conducted to investigate the role of the *GSTM1*⁻ genotype on the interaction of second-hand tobacco smoke exposure with diesel exhaust exposure on response to allergen in allergic

From ^athe Center for Environmental Medicine, Asthma and Lung Biology, the Departments of ^bMedicine and ^cPediatrics, UNC School of Medicine, and ^dthe Department of Biostatistics, Gillings School of Global Public Health, Chapel Hill; ^ethe Environmental Public Health Division, National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park; and ^fthe Laboratory of Respiratory Biology, National Institute of Environmental Health Sciences, Research Triangle Park.

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Reprint requests: David B. Peden, MD, Center for Environmental Medicine, Asthma, and Lung Biology, 104 Mason Farm Road, CB#7310, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7310. E-mail: peden@med.unc.edu.

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Abbreviations used

APC:	Allophycocyanin
CC16:	Clara cell protein 16
FITC:	Fluorescein isothiocyanate
FVC:	Forced vital capacity
<i>GSTM1</i> :	Glutathione-S-transferase Mu 1
MFI:	Mean fluorescence intensity
<i>NQO1</i> :	NAD(P)H:quinone oxidoreductase
PE:	Phycoerythrin

volunteers. In these studies *GSTM1*[−] volunteers have increased allergen-induced histamine release and IgE production in the nasal airway after exposure to these pollutants.^{11,12}

A series of reports of a cohort of children with asthma in Mexico City have demonstrated an effect of ambient air ozone exposure on asthma exacerbation. This group reported that asthmatic children with the *GSTM1*[−] genotype have increased risk for ozone-induced asthma exacerbation and are more likely to benefit from antioxidant supplementation to prevent such exacerbations. They have also reported that the Pro/Pro homozygous genotype (vs Pro/Ser or Ser/Ser genotypes) at the 187 position for the NAD(P)H:quinone oxidoreductase (*NQO1*) increases risk for asthma in *GSTM1*[−] children.^{13–16} Another study examined the effect on lung function and serum levels of Clara cell protein 16 (CC16), which is a marker for increased respiratory cell permeability, of ozone levels of greater than 0.08 ppm in 24 bicyclists during a 2-hour ride.¹⁷ Eight of these 24 persons had both the wild-type *NQO1* genotype and the *GSTM1*[−] genotype and were found to have a strong correlation between both serum CC16 levels and decreased lung function and ambient air ozone levels of greater than 0.08 ppm. Taken together, these observations indicate that the *GSTM1*[−] genotype is an important determinant of susceptibility to ozone-induced lung disease.

Human challenge studies of both asthmatic and nonasthmatic adults revealed 3 distinct responses after ozone challenge: an immediate decrease in lung function, which is most likely mediated by neural reflexes; increased bronchial reactivity; and increases in airway inflammation typified by increased influx of PMNs.¹⁸ The effect of ozone challenge on macrophage and monocyte populations is still being defined.

Acute ozone challenge has been reported to decrease macrophage numbers in the airway,^{18,19} whereas others have found that repeated exposure to ozone augments airway macrophage populations.²⁰ Additionally, we have reported that exposure to ozone causes increased expression of a number of innate and acquired immune surface proteins on airway monocytes and macrophages, including CD11b, CD14, CD16, CD86, and HLA-DR, that occur in concert with increases in airway PMN numbers.¹⁹ We hypothesized that an important mechanism by which ozone exerts adverse respiratory effects in human subjects involves recruitment of activated innate immune cells to the airway with enhanced expression of cell-surface proteins, such as CD14, CD86, and HLA-DR. These cells would likely enhance response to allergen in allergic persons, as well as responses to particulates containing biologic components, such as endotoxin, which might have health effects in allergic and nonallergic populations.

Healthy volunteers who had the *GSTM1*⁺ or *GSTM1*[−] genotype underwent inhalation challenge to 0.4 ppm of ozone for 2 hours to test this hypothesis. Changes in lung function,

PMN and macrophage numbers, expression of cell-surface markers on immune cells, macrophage function, and cytokine levels were examined. If the *GSTM1*[−] genotype is associated with decreased lung function or increased airway neutrophil and macrophage responses to ozone, then this would be an important risk factor for acute disease morbidity associated with ozone.

METHODS**Volunteer recruitment and inclusion criteria**

This protocol was reviewed and approved by the University of North Carolina Committee on the Rights of Human Subjects (institutional review board). All subjects underwent a physical examination, a routine blood panel with complete blood cell count and differential, allergy skin testing, and genotyping for the *GSTM1*[−] or *GSTM1*⁺ genotype by using previously reported techniques.²¹ Subjects were required to have a negative methacholine challenge result. Female subjects had to have a negative urine pregnancy test result before challenge, and all volunteers were required to be free of chronic cardiovascular or respiratory illness and be free of acute respiratory illness within 4 weeks of ozone challenge. All subjects had FEV₁ and forced vital capacity (FVC) values of 80% or greater of predicted value and FEV₁/FVC ratios of 75% or greater of predicted normal value for height and age and were non-smokers with no smoking history. All subjects were screened for their ability to provide an adequate induced sputum sample during their training sessions. Further recruitment criteria are detailed in the Methods section of this article's Online Repository (available at www.jacionline.org).

Ozone exposure and testing protocol

The ozone exposures were conducted in an ozone exposure chamber at the US Environmental Protection Agency Human Studies Facility on the campus of the University of North Carolina (Chapel Hill, NC). Each subject was exposed to ozone (0.4 ppm) for 2 hours while performing four 15-minute sessions of intermittent moderate exercise (expiratory minute ventilation, 30–40 L/min) on a treadmill, separated by 15 minutes of seated rest. Lung function, breath sounds, and vital signs were assessed before and after exposure.

Sputum was obtained 4 and 24 hours after exposure and processed as previously described.^{22,23} This is further outlined in the Methods section in this article's Online Repository. Sputum was assessed for total and differential cell counts and immunophenotyping of neutrophils, macrophages, monocytes, and dendritic cells,^{19,24} and functional assays for oxidative burst activity and phagocytosis of opsonized zymosan were assessed by means of flow cytometry on sputum macrophages.^{22,25} Sputum supernatants were also assessed for cytokine concentration, as described in the Methods section in this article's Online Repository. Lung function and genotyping procedures are also described in the Methods section in this article's Online Repository.

Statistical analysis

Our *a priori* primary hypotheses were that persons with the *GSTM1*[−] genotype would have increased effects of ozone on lung function (greater decrements in lung function) and airway inflammation (increases in airway neutrophils and decreases in airway macrophages). The effect of ozone on airway cell immunophenotypes and macrophage function were also determined to test the exploratory hypotheses that persons with the *GSTM1*[−] genotype would have enhanced airway macrophage activation and cell function.

Paired *t* tests were used to compare each effect of ozone on lung function immediately and 24 hours after challenge, and sputum end points at 4 and 24 hours after challenge were compared with baseline measures within each genotype cohort (*GSTM1*[−] and *GSTM1*⁺) to first determine the effect of ozone on these end points. To test the primary hypothesis that persons with the *GSTM1*[−] genotype have enhanced responses to ozone, we compared the change from baseline at 4 or 24 hours for a given end point after ozone

TABLE I. Study demographics

Cohort	No.	Age (y)	Sex	Race	Atopic*
<i>GSTM1</i> ⁺	19	23.8 ± 3.1	13 F/6 M	10 white, 8 African American, 1 Asian	6
<i>GSTM1</i> [−]	16	24.5 ± 5.1	7 F/9 M	15 white, 1 African American	6

F, Female; M, male.

*All subjects were nonasthmatic, as confirmed by means of methacholine challenge.

challenge between the *GSTM1*[−] and *GSTM1*⁺ groups using the 2-sample *t* test. Spirometric data immediately and 24 hours after challenge were normalized and expressed as the percentage change from baseline. Other end points were transformed by using natural logarithmic transformation to achieve a normal distribution before *t* test analysis. For primary hypotheses, significance was set at an α value of .05. For exploratory hypotheses, nominal *P* values of less than .10 are reported.

Although the ability to produce adequate sputum was an entrance criterion, some sputum samples recovered at baseline or after challenge did not yield a sufficient numbers of cells for recovery of all end points. This occurred randomly across the baseline, 4-hour postexposure, and 24-hour postexposure sputum collections. To account for the missing data points and maximize statistical power for hypothesis testing, we used multiple imputation methods.²⁶ Data imputation was not performed on any end point for which a subject had missing data at all time points because of inadequate sputum cell recovery. For all primary end points, the sample size is 19 for *GSTM1*⁺ and 16 for *GSTM1*[−] volunteers. For exploratory end points, the assessed sample size is noted in the Results section. Thirty imputations were performed for each set of sputum end points (eg, baseline PMNs per milligram sputum values in *GSTM1*⁺ volunteers) that contained missing data. Only mean and SE values from actual data are depicted and reported in the Results section, with imputed data used only for hypothesis testing. A detailed description of the imputation methodology appears in the Methods section in this article's Online Repository.

RESULTS

Demographics

Demographic data for the 35 healthy volunteers, ranging in age from 18 to 35 years, recruited for challenge to 0.4 ppm of ozone are presented in Table I. Twelve (32.3%) of the 35 were atopic, as determined by means of skin testing, although all were nonasthmatic, as determined by means of methacholine challenge. The *GSTM1* genotype cohorts are described in Table I.

Effect of 0.4 ppm of ozone on lung function

The effect of ozone on lung function was a primary end point for this study. Fig 1 shows that ozone exposure caused significantly ($P < .05$) decreased FEV₁ and FVC values immediately after challenge in *GSTM1*⁺ ($n = 19$) and *GSTM1*[−] ($n = 16$) subjects, and values are expressed as percentages of baseline value. No significant differences in lung function responses to ozone were found between the *GSTM1*⁺ and *GSTM1*[−] cohorts, with both having significant and similar decreases in FEV₁ and FVC values immediately and 24 hours after exposure.

Effect of 0.4 ppm of ozone on airway PMN and macrophage numbers

The effect of ozone on airway PMN and macrophage numbers was also a primary end point for this study. Fig 2 shows that the

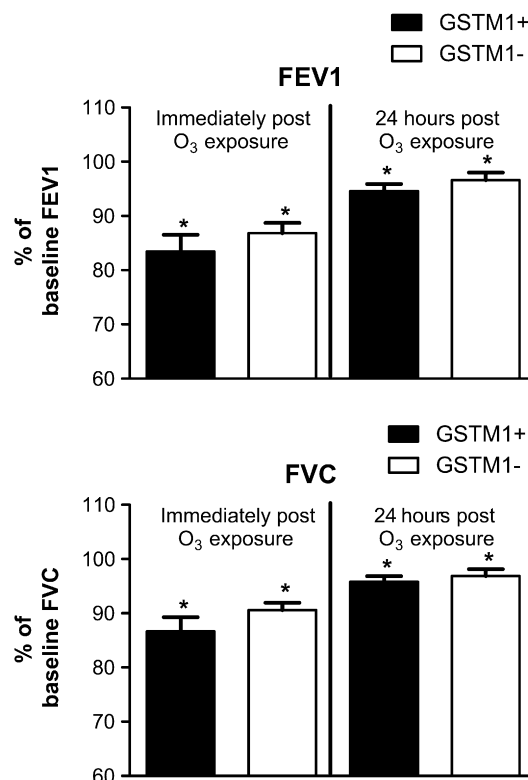


FIG 1. Effect of *GSTM1* genotypes on lung function response to ozone: FEV₁ (top) and FVC (bottom) expressed as mean (SEM) percentages of baseline values 4 and 24 hours after ozone exposure in *GSTM1*⁺ ($n = 19$, solid bars) and *GSTM1*[−] ($n = 16$, open bars) subjects.

GSTM1[−] cohort ($n = 16$) had significantly increased sputum PMN numbers 4 ($P = .021$) and 24 ($P = .001$) hours after challenge. The *GSTM1*⁺ cohort had significantly increased sputum PMN numbers 4 hours after challenge ($P = .01$), with a relative decrease in PMN numbers at 24 hours such that they were not significantly increased compared with baseline numbers. A comparison of the changes from baseline to 24 hours after challenge in the *GSTM1*⁺ and *GSTM1*[−] cohorts demonstrated that the *GSTM1*[−] cohort had a significantly increased PMN influx 24 hours after challenge ($P = .03$).

The bottom panel in Fig 2 shows that macrophage numbers were significantly decreased from baseline values 4 ($P = .013$) and 24 ($P = .001$) hours after ozone exposure in the *GSTM1*⁺ group. In the *GSTM1*[−] group a small nonsignificant decrease in macrophage numbers was found at 4 hours after challenge compared with baseline numbers, with the mean value increasing to a level greater than that seen at baseline at 24 hours. A comparison of the 24-hour macrophage response in the *GSTM1*⁺ and *GSTM1*[−] cohorts demonstrated that the *GSTM1*[−] cohort had a significantly increased macrophage number 24 hours after challenge ($P = .002$). For macrophages and neutrophils, data were recovered from 17 of 19 *GSTM1*⁺ volunteers at baseline, all volunteers 4 hours after challenge, and 17 of 19 volunteers 24 hours after challenge. For *GSTM1*[−] volunteers, data were recovered from 15 of 16 volunteers at baseline and 4 hours and 13 of 16 volunteers 24 hours after challenge. Multiple imputation techniques were used to estimate missing values for each data set, as described in the Methods section.

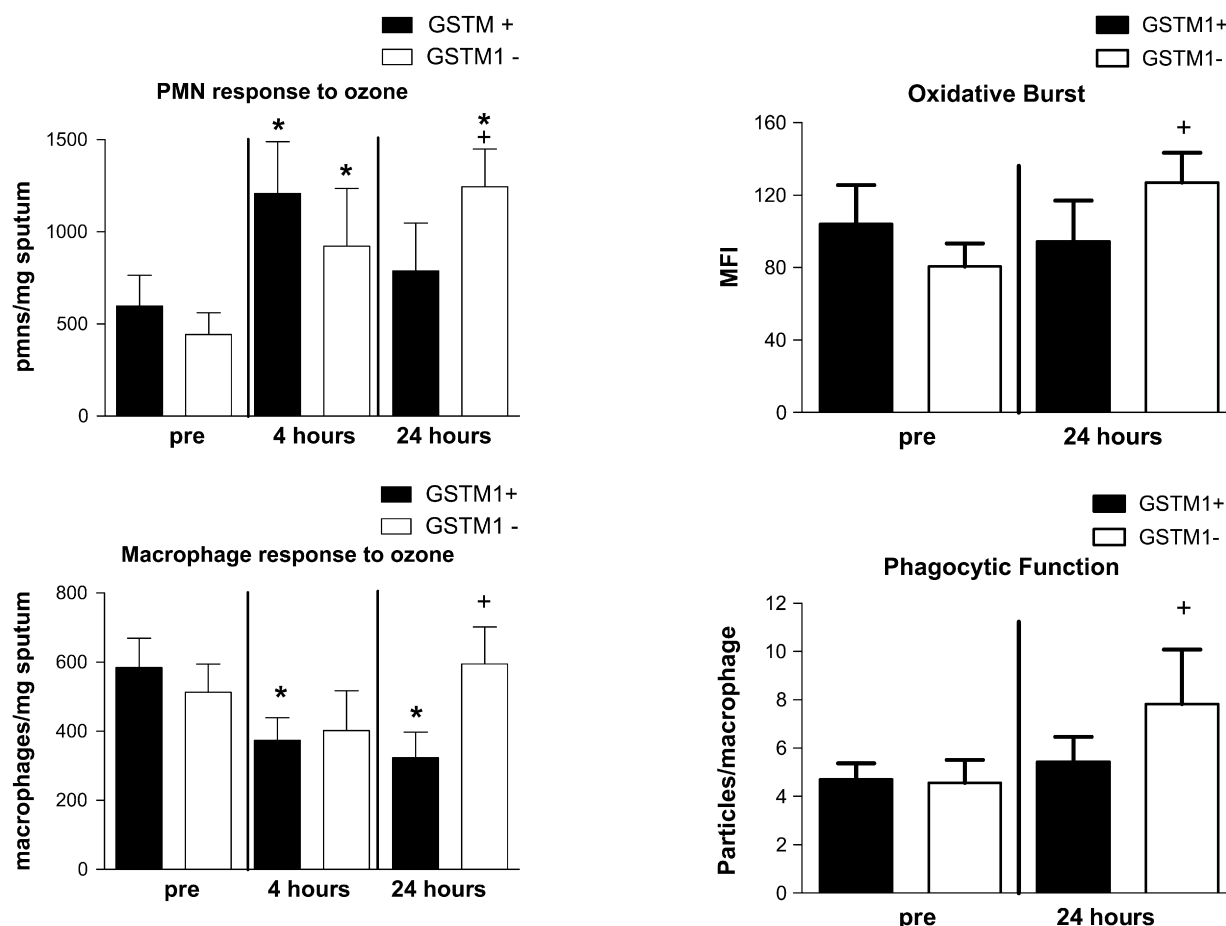


FIG 2. Effect of *GSTM1* genotypes on PMN and macrophage numbers in sputum in response to ozone: mean (SEM) PMNs per milligram of sputum (*top*) and macrophages per milligram of sputum (*bottom*) before (baseline) and 4 and 24 hours after ozone exposure in *GSTM1*⁺ (n = 19, *solid bars*) and *GSTM1*⁻ (n = 16, *open bars*) subjects. **P* < .05 versus baseline. +*P* < .05, *GSTM1*⁺ versus *GSTM1*⁻.

Effect of 0.4 ppm of ozone on airway macrophage and dendritic cell function

Two measures of macrophage function were assessed as exploratory end points: oxidative burst activity after activation with zymosan particles (*Fig 3*, *top*) and uptake of opsonized zymosan particles, phagocytosis (*Fig 3*, *middle*). Oxidative burst was increased in airway macrophages recovered from *GSTM1*⁻ volunteers (n = 16) 24 hours after ozone challenge when compared with macrophages from 17 *GSTM1*⁺ volunteers (*P* = .03). Macrophage phagocytosis was increased 24 hours after ozone challenge in *GSTM1*⁻ (n = 15) versus *GSTM1*⁺ (n = 19) volunteers (*P* = .05).

Another exploratory end point we examined was the effect of ozone on cell-surface phenotypes on monocyte and macrophage populations, as defined by using flow cytometry. We focused on the effect of ozone on the expression of CD11b, CD14, CD16, CD64, CD86, and HLA-DR in monocytes, immature macrophages, mature macrophages, PMNs, and dendritic cells. We have previously reported that ozone exposure enhances expression of these markers on airway monocytes after ozone challenge and confirmed this observation in the present study, with the exception of CD16 (data not shown). When we analyzed the effect of ozone on surface marker expression in the *GSTM1*⁺ (n = 9) and

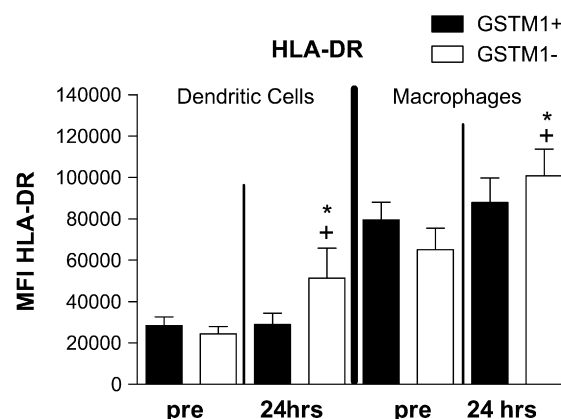


FIG 3. Effect of *GSTM1* genotypes on cell function 24 hours after ozone exposure: mean (SEM) oxidative burst activity MFI (*top*, macrophages, *GSTM1*⁺ n = 17, *GSTM1*⁻ n = 16), phagocytosis (*middle*, macrophages, *GSTM1*⁺ n = 19, *GSTM1*⁻ n = 15), and HLA-DR expression MFI (*bottom*, *GSTM1*⁺ n = 9, *GSTM1*⁻ n = 11) at 4 and 24 hours after ozone exposure in *GSTM1*⁺ and *GSTM1*⁻ subjects. **P* < 0.05 versus before. +*P* < .05 for 24-hour response in *GSTM1*⁺ versus *GSTM1*⁻ subjects.

GSTM1⁻ (n = 11) groups (*Fig 3*, *bottom*), we found 3 significant differences at 24 hours for *GSTM1*⁻ subjects: (1) increased expression of CD14 on airway PMNs (see *Fig E1* in this article's Online Repository at www.jacionline.org); (2) increased expression of HLA-DR on airway dendritic cells at 24 hours after exposure

($P = .003$); and (3) increased HLA-DR expression on macrophages ($P = .03$) 24 hours after exposure. The 24-hour HLA-DR response was significantly different between $GSTM1^{+}$ and $GSTM1^{-}$ volunteers for dendritic cells ($P = .02$) and macrophages ($P = .03$).

Airway cytokines

We also examined sputum supernatants for a number of proinflammatory cytokines (IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, macrophage inflammatory protein 1 α , and TNF- α). Of these, we observed significant increases in IL-1 β levels at 4 ($P = .01$) and 24 ($P = .05$) hours after ozone and IL-8 levels 24 hours after ozone in the $GSTM1^{-}$ cohort, whereas the $GSTM1^{+}$ cohort also showed increased levels, but these were not statistically significant. No difference between genotypes was observed. These results are further described in the Results section of this article's Online Repository (available at www.jacionline.org).

DISCUSSION

In this study we tested the hypothesis that the $GSTM1^{-}$ genotype would increase sensitivity to inhaled challenge to 0.4 ppm of ozone, with the primary end points being change in lung function (FEV₁ and FVC) and influx of neutrophils to the airway 4 and 24 hours after acute challenge. We found decreased mean FEV₁ and FVC values after ozone challenge in $GSTM1^{+}$ and $GSTM1^{-}$ volunteers but no difference in lung function response between these groups. We likewise found no difference in airway neutrophil numbers 4 hours after challenge between the genotype groups, with both groups demonstrating increased counts. However, at 24 hours after ozone challenge, we found a very notable and significant increase in airway neutrophil numbers in the $GSTM1^{-}$ cohort that was not found in the $GSTM1^{+}$ group.

We also found that there was a significant decrease in the macrophages per milligram of sputum recovered in the $GSTM1^{+}$ volunteers at 4 and 24 hours that was not observed in the $GSTM1^{-}$ group. Macrophage-mediated clearance of apoptotic luminal cells would depend on the presence of apoptotic PMNs in the airways, and hence PMN resolution resulting in fewer PMNs at 24 hours in the $GSTM1^{+}$ cohort might explain fewer macrophages at that time point. Persistent inflammation, however, as observed in the $GSTM1^{-}$ cohort at 24 hours, might render the need for increased macrophage numbers and enhanced macrophage-mediated clearance. Thus comparing the differences in the change from baseline values between the groups at 24 hours after challenge, the $GSTM1^{-}$ volunteers had a significantly greater number of macrophages present in the airway. When compared with baseline values, oxidative burst and phagocytic capability of airway macrophages recovered 24 hours after ozone challenge were increased in the $GSTM1^{-}$ versus $GSTM1^{+}$ volunteers. We also observed increased HLA-DR expression on macrophages and dendritic cells recovered from $GSTM1^{-}$ volunteers 24 hours after challenge. We have previously reported that concurrent with ozone-induced cell changes in the airway, namely neutrophil and monocytic cell influx, there are increases in expression of innate and acquired immune cell-surface proteins on airway macrophages and monocytes. These include CD11b, CD14, CD86, and HLA-DR.¹⁹ The persistence of macrophages in the airways of $GSTM1^{-}$ volunteers 24 hours after challenge is therefore likely biologically significant because these cell-surface proteins are

central in mediating innate and acquired immune responses that can mediate exacerbations of lung disease.

Adverse events associated with increases in ambient air ozone exposure generally occur 24 to 48 hours after the ozone event has occurred, as reviewed by Peden.¹⁸ As described in the introduction, the $GSTM1^{-}$ genotype is associated with increased risk for such adverse events. If these effects were primarily due to an immediate effect of ozone on lung function, air pollution events should result in immediate (same-day) exacerbation of disease. Our observations that $GSTM1^{-}$ volunteers have increased neutrophilic inflammation in the airway, increased presence of functionally activated macrophages, and increased expression of HLA-DR on airway macrophages and dendritic cells 24 hours after ozone challenge suggest that these inflammatory and innate immune changes likely account for these effects. These changes could directly modify the airway or perhaps, in the case of allergic asthmatic subjects, enhance response to inhaled allergen, which has been reported by many investigators, as noted previously.¹⁸

It is unclear whether the increases in neutrophil and macrophage numbers in the airways 24 hours after ozone challenge in $GSTM1^{-}$ volunteers reflects a primary difference in these cells or increased chemoattraction and activation of inflammatory and immune cells by mediators secreted by structural tissues of the airway after exposure to ozone. There is ample evidence that ozone stimulates airway epithelial cells in ways that might influence inflammation. Ozone upregulates cytokine and eicosanoid secretion by epithelial cells, as noted previously^{27,28} and reported here in the $GSTM1^{-}$ cohort, because they demonstrated significantly increased levels of the proinflammatory cytokines IL-1 β and IL-8 after ozone, the latter being a potent chemoattractant for PMN influx. Epithelial cells stressed with ozone also release ATP, which is a proinflammatory signal for a number of inflammatory cells.²⁹ Ozone actions on epithelial lining fluid might also result in production of lipid peroxidation products that have proinflammatory actions.^{27,28,30,31}

There are suggestions that the airway epithelium of $GSTM1^{-}$ subjects has increased susceptibility to ozone. A study of the effect of *in vitro* ozone exposure on nasal mucosal explants obtained from $GSTM1^{+}$ and $GSTM1^{-}$ volunteers reveals that tissues from $GSTM1^{-}$ volunteers have significantly increased superoxide dismutase activity relative to that seen in $GSTM1^{+}$ tissues.³² In the study by Bergamaschi et al¹⁷ examining the effect of ambient ozone on bicyclists, the $GSTM1^{-}$ genotype was a component of the haplotype associated with a correlation between ozone and CC16, a marker of epithelial cell injury. Taken together, these observations suggest that airway epithelial cells from $GSTM1^{-}$ subjects have increased susceptibility to ozone. We hypothesize that $GSTM1$ genotype-mediated differences in epithelial cell secretion of proinflammatory cytokine response to ozone might account, at least in part, for the effect of the $GSTM1^{-}$ genotype on ozone-induced airway inflammation.

It is important to note limitations to the current study. We did not assess changes in airway hyperresponsiveness, and we acknowledge that such changes could also account for increased risk for exacerbation of ozone-induced disease associated with the $GSTM1^{-}$ genotype. Also, in contrast to our study, Bergamaschi et al¹⁷ found that bicyclists exposed to lower levels of ambient ozone (0.08 ppm) experienced a significant effect on lung function in those with the *NQO1* wild-type/ $GSTM1^{-}$ haplotype. It is possible that the level of ozone we used in this study might

have been so high that a *GSTM1* genotype modulation of lung function response might have been masked.

Additionally, 16 of 16 of our *GSTM1*[−] volunteers were white, whereas 8 of the 19 *GSTM1*⁺ volunteers were African American, 1 was Asian, and 10 were white. The sample size of this study limits the ability to adjust for ethnicity, and it is possible that the differences we observed between the *GSTM1*⁺ and *GSTM1*[−] populations in this study might be due to other genetic factors that stratify by race rather than *GSTM* genotype. If factors other than differences in the *GSTM* genotype accounted for the differential responses to ozone we observed, then mechanisms other than impaired antioxidant capability would need to be explored as potential risk factors for pollutant-induced lung inflammation. However, as noted in the introduction, the prevalence of the *GSTM1*[−] genotype is high across most studied ethnic groups. As future studies enhance our sample size, we will need to confirm that the effect of the *GSTM1*[−] genotype on ozone-induced inflammation is true across multiple ethnic groups, which would make it less likely that other factors account for the differences we report herein.

In summary, we report that volunteers with the *GSTM1*[−] genotype have no differential lung function response to 0.4 ppm of ozone compared with the *GSTM1*⁺ group but do have a notable increase in airway PMN and activated macrophage numbers at 24 hours after ozone challenge compared with *GSTM1*⁺ volunteers. The *GSTM1*[−] genotype has been associated with increased risk for exacerbations of airway disease and asthma caused by pollutant exposure. Our observations on inflammatory response to ozone in *GSTM1*[−] volunteers temporally matches the epidemiologic observations that show ambient ozone-associated adverse effects 24 hours after exposure. Taken together, these observations are consistent with the hypothesis that *GSTM1* is an important risk factor for ozone-induced exacerbations of respiratory disease due in part to *GSTM1*-associated differences in inflammatory response to ozone.

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Clinical implications: These observations suggest that the *GSTM1*[−] genotype likely confers increased risk for exacerbation of ozone-induced lung disease through promoting an enhanced neutrophilic and monocytic inflammatory response to ozone.

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METHODS

Additional subject recruitment and ozone exposure monitoring criteria

Minimum requirements for an acceptable sputum sample included a total cell count of at least 100,000 cells, less than 40% squamous epithelial cells, and cell viability of at least 50%. Subjects unable to provide adequate sputum samples were excluded from participation. Our *a priori* smoking entrance criterion was that individuals with a smoking history of greater than 10 cigarettes (1 half-pack) a month during the 3 years before enrollment were excluded (although in fact all were true nonsmokers). Subjects using anti-inflammatory medications or medications suggesting a diagnosis for asthma were excluded. During the course of participation, all subjects were required to abstain from ingestion of vitamins C and E, ingestion of aspirin or any other anti-inflammatory medication, and exposure to cigarette smoke or other irritants. Female subjects provided a menstrual cycle history and a urine sample for pregnancy testing. During the exposure, cardiorespiratory performance was monitored and recorded during each exercise period. After the postexposure sputum induction procedure, subjects remained under medical supervision until pulmonary function (spirometric) results returned to within 5% of pre-exposure baseline values before leaving the laboratory.

Lung function testing

Spirometric testing was performed according to American Thoracic Society/European Respiratory Society recommendations by using the Viasys VMax 229 series spirometers (VIASYS Healthcare Respiratory Technologies, Yorba Linda, Calif). All subjects were seated, and at least 3 maneuvers were obtained, with the best of the 3 reported. Testing was performed before the subject entered the exposure chamber and then again immediately after exposure.

Genotyping

DNA extraction was performed on 100- μ L blood samples by using the DNeasy blood & tissue isolation kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A PCR method was used to detect the presence or absence of the *GSTM1* gene; the β -globin gene was coamplified as an internal positive standard. One hundred nanograms of DNA was added to a PCR mixture composed of PCR buffer, 10 μ mol/L of each *GSTM1* primer (forward: 5' - GAA CTC CCT GAA AAG CTA AAG C- 3'; reverse: 5' - GTT GGG CTC AAATAT ACG GTG G- 3'), 10 μ mol/L of β -globin primer (forward: 5' - GAA GAG CCA AGG ACA GGT- 3'; reverse: 5' - CAA CTT CAT CCA CGT TCA CC- 3'), and 1 U/ μ L FailSafe Taq DNA polymerase and buffer F (Epicentre Biotechnologies, Madison, Wis). The reaction mixture was placed on a thermocycler 9700 (Applied Biosystems, Foster City, Calif) for 4 minutes at 95°C and then subjected to 35 cycles of 95°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, followed by a final step at 72°C for 7 minutes. PCR products were submitted to electrophoresis on a 3% ethidium bromide-stained agarose gel (NuSieve/agarose, 3:1) and photographed with FluorChem SP Imager (Alpha Innotech, San Leandro, Calif). The absence of the amplifiable 215 bp (in the presence of the 268-bp β -globin PCR product) indicates the *GSTM1*⁻ genotype.

Sputum induction and processing

Subjects provided an induced sputum sample during the screening visit and at 4 and 24 hours after ozone exposure. Sputum induction and processing methods have been previously described in detail and are referenced in the print article. In brief, three 7-minute inhalation periods of nebulized hypertonic saline (3%, 4%, and 5%; UltraNeb 99 ultrasonic nebulizer; DeVilbiss, Jackson, Tenn) were followed by expectoration of sputum into a sterile specimen cup. A cell-enriched "select" sample was obtained by plucking visible clumps of cells and cell-rich mucus "plugs" from the raw sample to separate them from noncellular portions of the sample. This was then treated with a dilute (0.1%) solution of dithiothreitol (Sputolysin; Calbiochem, San Diego, Calif) in Dulbecco PBS to digest mucus. After centrifugation (500g for 10 minutes), aliquots of sputum supernatant were immediately frozen and stored at -80°C, and the cell pellet was suspended in a small volume of HBSS. Cell viability (trypan blue exclusion) and total cell counts were

assessed in a Neubauer hemacytometer, and differential cell counts were performed on cytocentrifuged cells stained with a modified Wright stain (Hema-Stain-3; Fisher Scientific, Hampton, NH). A portion of the cells was immediately used for flow cytometric assays.

Flow cytometry

Expression of selected cell-surface molecules on sputum and blood leukocytes was quantified by means of multicolor flow cytometry with a BD LSR-II flow cytometer (BD Immunocytometry Systems, San Jose, Calif). These included molecules associated with antigen presentation and specific immunity (CD80/B7.1, CD86/B7.2, and HLA-DR/MHC-II), as well as innate immune function (CD11b/CR3, CD14/LPS receptor, CD16/Fc γ RIII, and CD64/Fc γ RI). Sputum leukocytes were differentiated from cellular debris, bacteria, yeast, and squamous and bronchial epithelial cells by gating on CD45⁺ cells. Leukocyte populations were differentiated by using various specific gating strategies based on a combination of light scatter properties and the differential expression of CD45 (panleukocyte marker) and select surface lineage markers. Sputum dendritic cells were identified as lineage dim/negative HLA-DR^{high}/CD11c(+) leukocytes. Sputum macrophages were somewhat subjectively subcategorized as either "immature" or "mature" based primarily on light scatter properties, with the latter characterized by greater forward and side scatter (ie, larger and more granular) relative to the immature macrophages. Upregulation or downregulation of specific surface molecules was quantified as a change in the mean fluorescence intensity (MFI) of the gated population. Fluorochrome-labeled antibodies were obtained from BD Biosciences (Franklin Lakes, NJ; CD11b-phycoerythrin (PE)-CY5, CD11c-allophycocyanin [APC], CD45-APC-Cy7, CD206-APC, HLA-DR-peridinin chlorophyll protein, Lineage Cocktail-1 fluorescein isothiocyanate [FITC]) and Beckman Coulter (Fullerton, Calif; CD14-APC, CD16-PE, CD64-FITC, CD80-FITC, CD86-PE). Appropriate, nonspecific, labeled isotypic control antibodies were also obtained from these sources.

Phagocyte functional assays

Phagocytic function and respiratory burst activity of airway and blood phagocytes were assessed by means of flow cytometry with a BD FACSort flow cytometer, as previously described. Briefly, *in vitro* phagocytosis of FITC-labeled opsonized *Saccharomyces cerevisiae* zymosan-A bioparticles (3-4 μ m in physical diameter; Molecular Probes, Inc, Eugene, Ore) by sputum phagocytes was assessed by measuring a shift in MFI (relative to control autofluorescence) of gated populations as an index of phagocytic activity. The number of particles engulfed per phagocytic cell was estimated as the ratio of the MFI of the phagocytic cell population and that of fluorescent zymosan particle. Respiratory burst activity was assessed after incubation (45 minutes at 37°C) of macrophages with Fc-OxyBurst reagent (dichlorodihydrofluorescein; Molecular Probes/Invitrogen, Eugene, Ore) and was measured as increased MFI, as described above.

Mediator measurement

Cytokines were measured with multi-plex technology (Meso ScaleDiscovery/MSD, Gaithersburg, Md). Each sample was analyzed with the Human MIP-1 alpha Ultra Sensitive Kit (lot no. K0031370) and the Human TH1/TH2 10-Plex Ultra Sensitive Kit (lot no. K0031431). All supernatant samples were diluted 1:4 and had a final dithiothreitol concentration of less than 1 mmol/L where no deleterious effects have been observed with the MSD platform.

Statistical procedures: Multiple imputation process

Multiple imputation is a statistical procedure for analyzing incomplete data by replacing each missing value multiple times with an imputed value^{E1}. Then the completed data sets can be created from the vectors of imputations. Because the sets of imputations are completed with repeated random draws under 1 model, we can integrate the complete-data analysis results, each using the same complete-data method to make 1 inference that reflects uncertainty because of missing values under that model. We illustrate the process with the following example.

Suppose that we have 15 subjects stratified by *GSTM1* genotype, each being supposed to have the measurements at the baseline and 24 hours. In this data set (Table E1) subject 2 in the *GSTM1*⁺ cohort had a missing value for the baseline, and subject 15 in the *GSTM1*[−] cohort missed the 24-hour measurement. We are interested in conducting a *t* test to assess whether there is a difference in the changes between 24-hour values and baseline values between the *GSTM1*⁺ and *GSTM1*[−] cohorts.

The multiple imputation procedure is as follows:

Step 1. Replace missing values with imputed data. To replace the missing observation for subject 2, make a random draw from {X1, X3, X4, X5, X6, X7, X8}, the available data at baseline within the *GSTM1*⁺ cohort. For subject 15, we replace the missing value with a draw randomly from {Y9, Y10, Y11, Y12, Y13, Y14}, the available data at 24 hours within the *GSTM1*[−] cohort. Thus the first complete data set, including the *GSTM1*⁺ and *GSTM1*[−] cohorts, is obtained.

Step 2. Perform the *t* test. For the completed data set obtained in step 1, calculate the difference from baseline to 24 hours for each subject: d1=Y1−X1, d2=Y2−X2, ..., d15=Y15−X15. We then test the null hypothesis that there is no difference in changes from baseline to 24 hours between the *GSTM1*⁺ and *GSTM1*[−] cohorts using an independent *t* test. Calculate the *t* statistic and the associated variance.

Step 3. Repeat steps 1 and 2 for *M* times. We therefore obtain *M* *t* test statistics and *M* associated variances from the *M* completed data sets. Let $\hat{\theta}_l, \hat{W}_l, l=1, \dots, M$, be *M* completed-data estimates and the corresponding variances (ie, $\hat{\theta}_l$ is the *t* test statistic calculated from the *l*th completed data set and $\hat{W}_l$ is the variance associated with $\hat{\theta}_l$).

Step 4. Combine the results from the *M* analyses and make statistical inference. The combined estimate is

$$\bar{\theta}_M = \frac{\sum_{l=1}^M \hat{\theta}_l}{M}.$$

The variability associated with this estimate has 2 components: the average within-imputation variance,

$$\bar{W}_M = \frac{\sum_{l=1}^M \hat{W}_l}{M},$$

and the between-imputation component,

$$B_M = \frac{\sum_{l=1}^M (\hat{\theta}_l - \bar{\theta}_M)^2}{M-1};$$

the total variability associated with $\bar{\theta}_M$ is

$$T_M = \bar{W}_M + \frac{M+1}{M} B_M,$$

where $(M+1)/M$ is an adjustment for *M*. With scalar θ , *t* distribution is used as the reference distribution for interval estimates and significance tests,

$$(\theta - \bar{\theta}_M) T_M^{-1/2} \sim t_v,$$

where *v* is the degrees of freedom:

$$v = (M-1) \left[1 + \frac{1}{M+1} \frac{\bar{W}_M}{B_M} \right]^2.$$

RESULTS

PMN expression of CD14

We also observed a significant increase in CD14 expression on airway PMNs 24 hours after challenge when compared with baseline values in *GSTM1*[−] volunteers, with the change from baseline being significantly increased between *GSTM1*[−] and *GSTM1*⁺ volunteers. However, these differences appear to be due primarily to low-level CD14 expression on PMNs in *GSTM1*[−] volunteers before challenge (*P* = .02 vs prechallenge CD14 expression in *GSTM1*⁺ volunteers) rather than notable differences 24 hours after challenge (Fig E1).

Airway cytokines

We also examined supernatants recovered from airway sputum for a number of proinflammatory cytokines (IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, macrophage inflammatory protein 1 α , and TNF- α). Many of these were less than the level of detection in a majority of samples or were not significantly affected by ozone challenge. However, for IL-1 β , there were measurable cytokine amounts in samples from 17 subjects of the *GSTM1*⁺ cohort and 12 subjects of the *GSTM1*[−] cohort. For IL-8 samples, there were measurable cytokine amounts in samples from 16 subjects of the *GSTM1*⁺ cohort and 13 subjects of the *GSTM1*[−] cohort. These results are shown in Fig E1, with IL-1 β showing an increased trend in sputum recovered 4 hours after challenge in the *GSTM1*⁺ group (*P* = .08) and significantly increased in the *GSTM1*[−] group (*P* = .01). IL-8 showed a similar response pattern at 24 hours in both groups (*P* = .06 and .05 in the *GSTM1*⁺ and *GSTM1*[−] groups, respectively). There were no differences in cytokine response between the genotypes for either cytokine.

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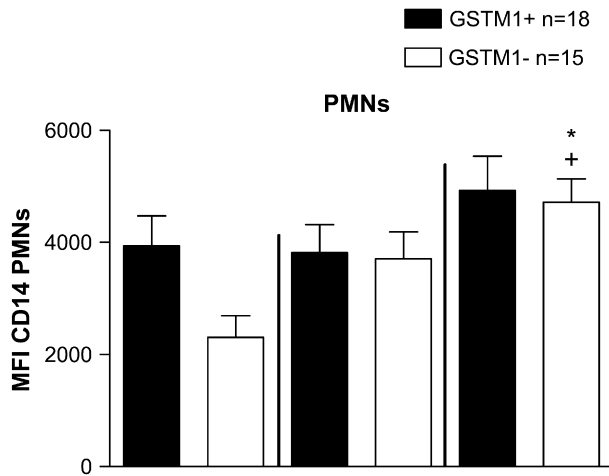


FIG E1. Effect of *GSTM1* genotypes on CD14 expression on airway PMNs 24 hours after ozone exposure. * $P = .002$ for the *GSTM1*⁻ cohort comparing baseline with 24-hour values within this genotype. + $P = .05$ change from baseline values at 24 hours in *GSTM1*⁻ versus *GSTM1*⁺ volunteers.

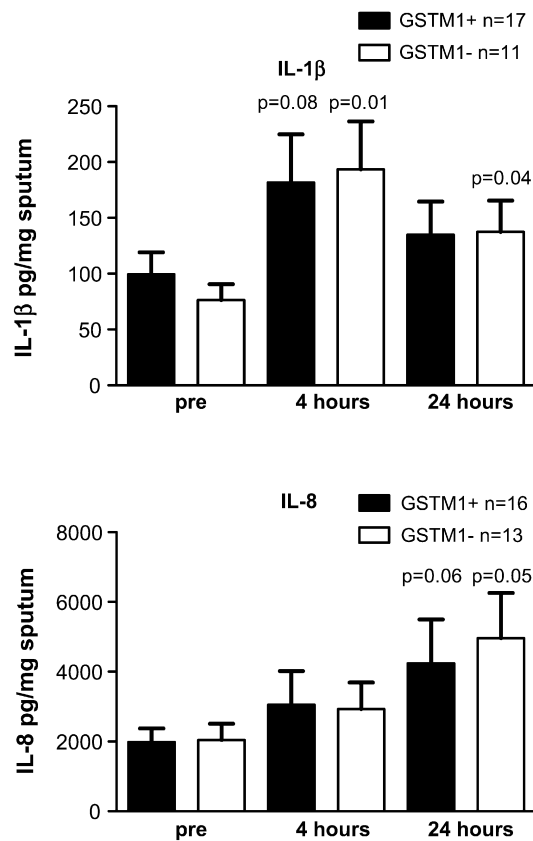


FIG E2. Effect of *GSTM1* genotypes on IL-1 β and IL-8 levels in airway sputum recovered after exposure to ozone: *GSTM1*⁻ volunteers had a significant increase in IL-1 β levels 4 and 24 hours after completion of the ozone exposure protocol, as well as IL-8 levels 24 hours after ozone exposure. Nominal *P* values are noted in the body of the figure.

TABLE E1. Example data set for imputation of missing data

Subject no.	<i>GSTM1</i> (+=1, -=2)	Baseline	After 24 h
1	1	X1	Y1
2	1		Y
3	1	X3	Y3
4	1	X4	Y4
5	1	X5	Y5
6	1	X6	Y6
7	1	X7	Y7
8	1	X8	Y8
9	2	X9	Y9
10	2	X10	Y10
11	2	X11	Y11
12	2	X12	Y12
13	2	X13	Y13
14	2	X14	Y14
15	2	X15	—