

Atopic asthmatic subjects but not atopic subjects without asthma have enhanced inflammatory response to ozone

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Background: Asthma is a known risk factor for acute ozone-associated respiratory disease. Ozone causes an immediate decrease in lung function and increased airway inflammation. The role of atopy and asthma in modulation of ozone-induced inflammation has not been determined.

Objective: We sought to determine whether atopic status modulates ozone response phenotypes in human subjects.

Methods: Fifty volunteers (25 healthy volunteers, 14 atopic nonasthmatic subjects, and 11 atopic asthmatic subjects not requiring maintenance therapy) underwent a 0.4-ppm ozone exposure protocol. Ozone response was determined based on changes in lung function and induced sputum composition, including airway inflammatory cell concentration, cell-surface markers, and cytokine and hyaluronic acid concentrations.

Results: All cohorts experienced similar decreases in lung function after ozone. Atopic and atopic asthmatic subjects had increased sputum neutrophil numbers and IL-8 levels after ozone exposure; values did not significantly change in healthy volunteers. After ozone exposure, atopic asthmatic subjects had significantly increased sputum IL-6 and IL-1 β levels and airway macrophage Toll-like receptor 4, Fc ϵ RI, and CD23 expression;

values in healthy volunteers and atopic nonasthmatic subjects showed no significant change. Atopic asthmatic subjects had significantly decreased IL-10 levels at baseline compared with healthy volunteers; IL-10 levels did not significantly change in any group with ozone. All groups had similar levels of hyaluronic acid at baseline, with increased levels after ozone exposure in atopic and atopic asthmatic subjects.

Conclusion: Atopic asthmatic subjects have increased airway inflammatory responses to ozone. Increased Toll-like receptor 4 expression suggests a potential pathway through which ozone generates the inflammatory response in allergic asthmatic subjects but not in atopic subjects without asthma. (*J Allergy Clin Immunol* 2010;126:537-44.)

Key words: Ozone, innate immunity, asthma, atopy, hyaluronic acid, environmental airways disease, IL-1 β , IL-10

Ozone is a commonly encountered environmental pollutant. Asthma morbidity (emergency department visits, hospitalizations, and rescue medication use) is clearly associated with exposure to increased levels of ambient air ozone.¹ Asthmatic subjects are thought to have increased susceptibility to ozone because of aggravation of underlying allergic airways inflammation. Ozone exposure studies have shown that ozone induces an airway granulocyte response characterized by sputum neutrophilia in healthy volunteers¹⁻³ and sputum neutrophilia and eosinophilia in atopic asthmatic subjects.⁴ Ozone challenge has also been found to enhance responses to subsequent inhaled allergen challenge in subjects with mild atopic asthma.⁵⁻⁷ The ability of ozone to enhance airway inflammation and enhance responsiveness to subsequent inhaled allergen likely plays an important role in asthma exacerbation. However, it is unclear whether nonasthmatic persons with atopy also have enhanced susceptibility to ozone, as has been shown in atopic asthmatic subjects. Khatri et al⁸ found associations between air quality and airway inflammation in both asthmatic and nonasthmatic subjects with allergies, suggesting that atopic status itself might contribute to ozone-induced inflammatory responses in the airway.

Our group has also observed that response to inhaled LPS yields airway inflammatory responses similar to those seen after ozone exposure. Like ozone exposure, airway LPS challenge induces airway neutrophilia, enhances the response to inhaled allergen in allergic subjects,⁹⁻¹¹ and induces airway proinflammatory cytokines.¹ We and others have also found that ozone and LPS induce selective increases in specific macrophage and monocyte populations in the airway¹² and that ozone induces influx of monocytes and macrophages with increased expression of CD11b and CD14,¹³ a coreceptor for LPS. Building on these observations, our group has recently found correlations in airway inflammatory responses in healthy subjects who underwent both ozone and LPS exposures.¹⁴ The similarities between responses

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Abbreviations used

BAL: Bronchoalveolar lavage
 DTT: Dithiothreitol
 FVC: Forced vital capacity
 HA: Hyaluronic acid
 TLR4: Toll-like receptor 4

of healthy volunteers and atopic asthmatic subjects to ozone and LPS suggest that atopy modifies the response to a number of innate stimuli and that common molecular mechanisms account for the response to both ozone and LPS.

Consistent with this idea, mechanistic studies in mice suggest that at least some ozone responses are mediated through the primary LPS receptor, Toll-like receptor 4 (TLR4).¹⁵⁻¹⁷ Because ozone is a reactive oxygen molecule, it is unlikely that a discrete receptor exists that modulates the response to this agent or that ozone would act directly on airway monocytes or macrophages to induce airway inflammation. Indeed, *in vitro* studies of airway macrophages or THP-1 cells exposed to ozone do not reveal an ozone-induced release of inflammatory mediators.¹⁸⁻²⁰ It seems more likely that ozone exerts a biological effect through interaction with components of airway lining fluid to produce secondary signaling molecules or danger signals, which cause inflammatory responses. Ozone challenge of airway epithelial cells in culture has been shown to result in nuclear factor κ B activation and production of a variety of mediators.²¹⁻²³ Proinflammatory mediators, such as IL-8, are among those associated with ozone-induced inflammation in asthmatic subjects,²⁴ and reduced levels of the anti-inflammatory cytokine IL-10 have been reported in asthmatic subjects at baseline.²⁴⁻²⁶ Hyaluronic acid (HA) is an endogenous proinflammatory ligand for CD44 and TLR4²⁷⁻²⁹ and might be an important mediator in asthma. HA has also been recently identified as a candidate host danger signal that mediates ozone-induced inflammatory responses.^{30,31}

At present, it is unclear whether ozone modifies levels of TLR4 in human subjects and whether atopic status itself modifies the ozone-induced airway inflammatory response. The present study tests the hypothesis that atopic status alone modifies lower airway inflammatory response to a 0.4-ppm ozone exposure through examination of airway inflammatory cells and airway epithelial cell lining fluid from induced sputum.

METHODS

Volunteer recruitment and inclusion criteria

These protocols were 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, routine blood panel with CBC and differential cell count, allergy skin testing, and methacholine challenge. Atopy was demonstrated by a positive immediate skin test response to one of the following allergen mixes: 2 species of house dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), cockroach, tree mix, grass mix, weed mix, mold mix 1, mold mix 2, rat, mouse, guinea pig, rabbit, cat, or dog. A positive methacholine challenge result was determined as having a decrease of 20% from postsaline FEV₁ at a level of 10 mg/mL or less methacholine. This study was an analysis of lung function data and that samples were obtained from a total of 50 subjects who were stratified into 3 groups based on the results of their allergy skin testing and methacholine challenge. Atopic asthmatic subjects (n = 11) had positive skin prick test responses and positive methacholine challenge results and were required

to have mild intermittent asthma, as defined by the National Heart, Lung, and Blood Institute's 2007 guidelines (without inhaled corticosteroid or leukotriene receptor antagonist therapy). Healthy volunteers (n = 25) had negative skin prick test responses and negative methacholine challenge results, and atopic volunteers (n = 14) had positive skin prick test responses and negative methacholine challenge results. All atopic asthmatic volunteers were newly recruited into this study protocol, whereas other subjects were derived from previously completed studies.^{12,32} Female subjects had to have a negative urine pregnancy test result before challenge, and all volunteers were required to be free of chronic cardiovascular illness and be free of acute respiratory tract illness within 4 weeks of ozone challenge. All subjects had FEV₁ and forced vital capacity (FVC) values of 80% or greater of predicted normal value for height and age³³ and FEV₁/FVC ratios of 0.75 or greater and were nonsmokers with no smoking history. All subjects were screened for their ability to provide an adequate induced sputum sample during their training session. Subjects provided an induced sputum sample during the screening visit and at 4 to 6 hours after the ozone exposure. Sputum induction and processing methods are found in the Methods section of this article's Online Repository at www.jacionline.org.

Lung function testing

Spirometric testing was performed according to American Thoracic Society/European Respiratory Society recommendations by using the Viasys VMax 229 series spirometers (CareFusion, San Diego, Calif). All subjects were seated, and at least 3 maneuvers were obtained, with the best of the 3 reported.

Ozone exposure protocol

For ozone inhalation challenge, 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, North Carolina. Each subject was exposed to ozone (0.4 ppm) for 2 hours while performing four 15-minute sessions of intermittent moderate exercise (minute ventilation = 30–40 L/min) on a treadmill separated by 15 minutes of seated rest. Spirometric results, breath sounds, and vital signs were assessed before and immediately after exposure. Sputum was obtained 4 to 6 hours after exposure and processed as previously described.^{12,32,34,35} Sputum was assessed for total and differential cell counts and flow cytometric assessment of CD11b, CD14, CD86, TLR4, Fc ϵ RI, CD23, and HLA-DR expression on macrophages and monocytes.^{12,36} Sputum supernatants were also assessed for cytokine concentration.

Flow cytometry

Expression of selected cell-surface molecules on sputum 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 (CD86/B7.2 and HLA-DR/MHC-II), as well as innate immune function (CD11b/CR3 and CD14/LPS receptor). First, sputum leukocytes were differentiated from cellular debris, bacteria, yeast, and squamous and bronchial epithelial cells by gating on CD45 (panleukocyte marker)-positive cells. Upregulation or downregulation of specific surface molecules was quantified as a change in the mean fluorescent intensity of the gated population. Fluorochrome-labeled antibodies were obtained from BD Biosciences, San Jose, Calif (CD11b-phycoerythrin-CY5, CD45-allophycocyanin-Cy7, HLA-DR-peridinin-chlorophyll-protein) and Beckman Coulter, Fullerton, Calif (CD14-allophycocyanin, CD86-phycoerythrin). Appropriate, nonspecific, labeled isotypic control antibodies were also obtained from these sources.

Mediator measurement

Cytokine levels from sputum supernatants were measured with Multiplex technology (MSD, Gaithersburg, Md). Each sample was analyzed with the Human MIP-1 α Ultra Sensitive Kit (lot no. K0031370) and the Human T_H1/

TABLE I. Study demographics

Cohort	No.	Age (y)	Sex	Race
Healthy volunteers	25	23.7 ± 3.8	14 F/11 M	19 White, 5 African American, and 1 Asian
Atopic subjects	14	25.2 ± 5	7 F/7 M	8 White, 5 African American, and 1 Asian
Atopic asthmatic subjects	11	26.3 ± 6.5	6 F/5 M	8 White and 3 African American

Atopy was determined by a positive skin prick test response to a panel of environmental allergens. Asthma status was determined by means of methacholine challenge.

TH2 10-Plex Ultra Sensitive Kit (lot no. K0031431). All supernatant samples were diluted 1:4 and had a final dithiothreitol (DTT) concentration of less than 1 mmol/L, at which no deleterious effects have been observed with the MSD platform.

HA measurement

HA levels from induced sputum supernatants were performed with a commercially available ELISA (Echelon, Salt Lake City, Utah) with minor modifications. The lowest concentration of available standard was 50 ng/mL. This was diluted 1:1 to make a 25 ng/mL standard, and the 25 ng/mL standard was diluted 1:1 to make a 12.5 ng/mL standard. In addition, standards were run with and without 0.1% DTT, without notable differences in standard concentrations. Concentrations of HA from sputum supernatants were compared with standards containing DTT. The standard curve generated was log linear from 12.5 to 1,600 ng/mL.

Statistical analysis

The primary hypothesis to be tested was that atopic asthmatic or atopic nonasthmatic subjects had increased susceptibility to ozone compared with healthy volunteers, with the primary end point being sputum neutrophil numbers. Changes in cell counts per milligram of sputum, differential cell count percentage (sputum), mean fluorescent intensity measures (flow cytometry), cytokine values, and HA levels before and after ozone exposures within each cohort were assessed. The nonparametric paired Wilcoxon signed-rank test was used to determine whether a particular cohort responded to ozone. Differences between either atopic asthmatic or atopic nonasthmatic subjects and healthy volunteers were analyzed by using nonparametric *t* tests (Mann-Whitney test). Nonparametric 1-way ANOVA (Kruskal-Wallis test) was used to confirm that baseline values among all 3 groups were not different. Significance was set at a *P* value of .05.

RESULTS

Demographics

Demographic data for the 50 volunteers, ranging in age from 19 to 39 years, recruited for challenge to 0.4 ppm of ozone are presented in Table I. Fourteen were atopic, although they could not exhibit symptoms of atopy at the time of exposure. Eleven were atopic asthmatic subjects, none of whom were receiving maintenance inhaled corticosteroid therapy or other scheduled drugs.

Effect of 0.4 ppm of ozone on lung function

The effect of ozone on lung function in cohorts of healthy volunteers (*n* = 25), atopic subjects (*n* = 13), and atopic asthmatic subjects (*n* = 11) was compared. Ozone caused similar significant decreases in mean FEV₁ (*P* < .001, all cohorts) and

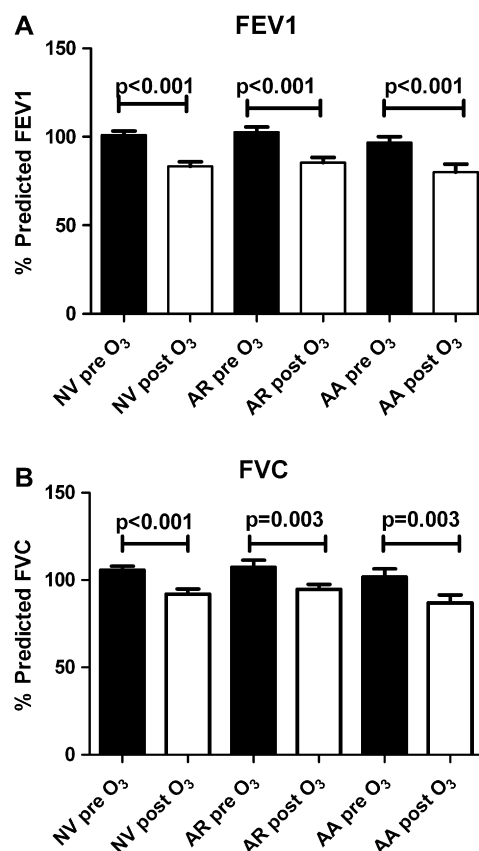


FIG 1. Baseline and post-ozone exposure FEV₁ (A) and FVC values (B): *n* = 25 healthy volunteers (NV), 13 atopic subjects (AR), and 11 atopic asthmatic subjects (AA). Data bars depict means and SEMs.

FVC (*P* < .001, healthy volunteers; *P* = .003, atopic and atopic asthmatic subjects) immediately after challenge in all cohorts (Fig 1) compared with baseline values.

Effect of 0.4 ppm of ozone on airway leukocyte numbers

The effect of ozone on airway neutrophil, macrophage, and eosinophil numbers was a primary end point for this study. Paired data (before and after ozone exposure) were available for 25 healthy volunteers, 13 atopic subjects, and 10 atopic asthmatic subjects. Fig 2, A, shows that the atopic and atopic asthmatic cohorts had significantly increased sputum neutrophil numbers (cells per milligram of sputum; *P* = .045, atopic subjects; *P* = .04, atopic asthmatic subjects) 4 hours after challenge. Although sputum neutrophil numbers increased in healthy volunteers, the increase in mean neutrophil numbers did not reach statistical significance, which might have been influenced by more variable baseline neutrophil numbers. Although macrophage numbers (cells per milligram of sputum; Fig 2, B) decreased in all cohorts, this was significant only among the atopic asthmatic volunteers (*P* = .01). Fig 2, C, shows that eosinophil numbers at baseline were increased in the airways of atopic (*P* = .0002) and atopic asthmatic (*P* = .0002) volunteers compared with very low values in healthy volunteers. After ozone exposure, eosinophil numbers

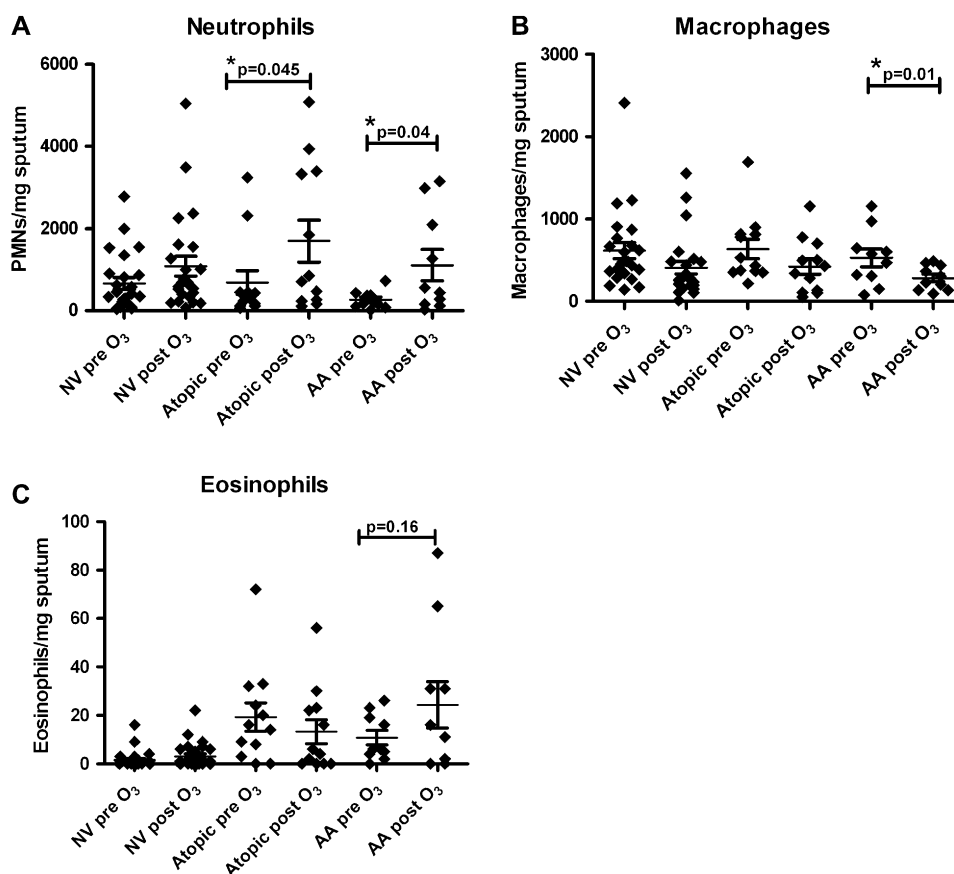


FIG 2. Changes in inflammatory cell infiltrate in sputum (cells per milligram of sputum) in response to ozone: $n = 25$ healthy volunteers (NV), 13 atopic subjects, and 10 atopic asthmatic subjects (AA). Lines depict means and SEMs.

did not significantly change in the atopic or atopic asthmatic subjects and remained very low in healthy volunteers.

Changes in airway cytokine levels after ozone

We also examined sputum supernatants for a number of immunoregulatory cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, macrophage inflammatory protein 1 α , TNF- α , and IFN- γ). Sputum supernatants were available for 24 healthy volunteers, 12 atopic subjects, and 10 atopic asthmatic subjects. Levels of 4 cytokines (IL-1 β , IL-8, IL-6, and IL-5) were significantly altered by ozone exposure, and this occurred only in the atopic and atopic asthmatic cohorts. Atopic asthmatic subjects had significant increases in IL-1 β ($P = .03$), IL-8 ($P = .01$), and IL-6 ($P = .01$) levels (Fig 3, A-C), whereas atopic subjects had increased IL-8 ($P = .02$; Fig 3, B) and IL-5 ($P = .03$) levels after ozone exposure (data not shown). Two atopic asthmatic subjects had higher baseline and postzone levels of IL-1 β and IL-8 compared with the rest of the cohort. Analysis of the data excluding these 2 subjects showed that the change in IL-8 and IL-1 β levels was significant ($P = .04$) and close to significant ($P = .08$) for these cytokines, respectively. We further analyzed the IL-1 β data as a percentage change from baseline; using this metric, we found a significant difference when comparing healthy volunteers versus atopic asthmatic subjects ($P = .009$, data not shown). When comparing baseline values, atopic asthmatic subjects had increased IL-1 β levels compared with those of healthy volunteers

($P = .02$) or atopic subjects ($P = .05$; Fig 3, A). No group experienced significant increases in IL-10 levels after ozone exposure. However, atopic asthmatic subjects had significantly decreased IL-10 levels at baseline compared with those seen in healthy volunteers ($P = .007$; Fig 3, D).

Change in sputum HA levels after ozone exposure

Preozone and postzone exposure sputum supernatant samples from 10 subjects in each cohort were available for analysis. There were no differences in HA levels at baseline or after ozone exposure among the cohorts (Fig 3, E). Ozone exposure caused increased HA levels in atopic subjects ($P = .004$) and atopic asthmatic subjects ($P = .002$) compared with their respective baseline values.

Changes in cell-surface markers after ozone

Macrophages from induced sputum were isolated as discussed in the Methods section, and changes in cell-surface markers are presented in Fig 4 ($n = 13$ healthy volunteers, 7 atopic subjects, and 6 atopic asthmatic subjects). Macrophages in the atopic asthmatic cohort had increased expression of CD23 ($P = .003$), Fc ϵ RI ($P = .03$), and TLR4 ($P = .03$) after ozone exposure (Fig 4, A-C). All cohorts showed increased CD11b expression after ozone, but this increase attained significance only in the atopic ($P = .02$) and atopic asthmatic ($P = .03$) cohorts (Fig 4, D). There were no other

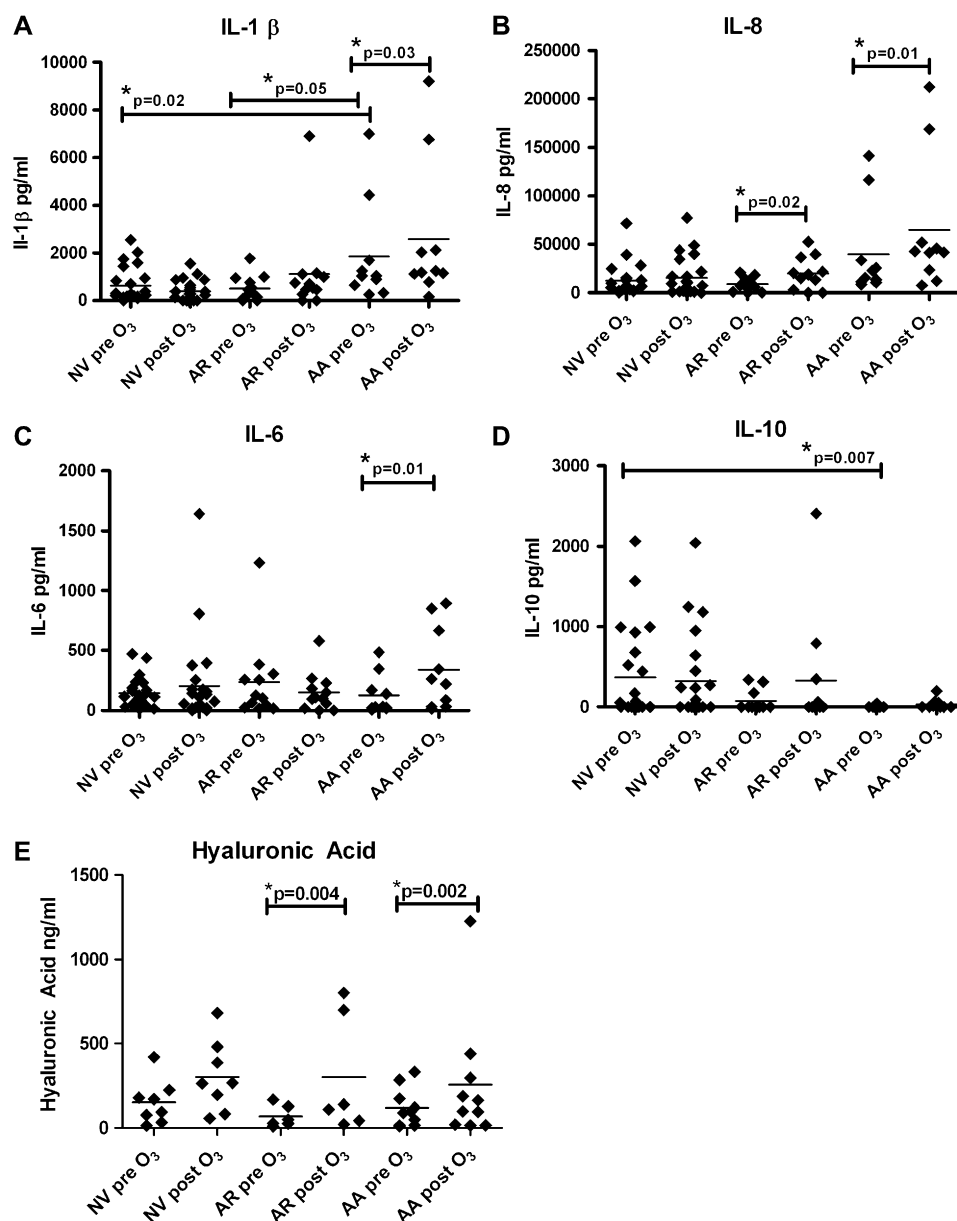


FIG 3. Changes in induced sputum cytokine and HA levels in response to ozone: $n = 24$ healthy volunteers (NV), 12 atopic subjects (AR), and 10 atopic asthmatic subjects (AA) for sputum cytokines; $n = 10$ each cohort for HA measurement in sputum. Lines depict means and SEMs.

significant changes in cell-surface marker expression (CD80, CD86, TLR2, CD14, and HLA-DR) after ozone in any cohort (data not shown).

DISCUSSION

There are numerous epidemiologic studies linking increased environmental ozone levels with asthma exacerbations. Asthmatic susceptibility to ambient ozone has been purported to be due to enhanced underlying allergic airways inflammation^{4,37}; however, not all studies have observed modified allergic airways inflammation after exposure in atopic subjects.^{4,38}

In this study we examined whether atopic status alone or mild intermittent atopic asthma influenced the airways inflammatory

response to inhaled ozone. We found that all cohorts experienced similar decreases in lung function (FEV₁ and FVC) immediately after ozone exposure, with no differences among the cohorts. Atopy or atopic asthma did not appear to influence this immediate effect on lung function thought to be a reflex-mediated phenomenon triggered in part by eicosanoids.^{39,40} We did find that although all cohorts had increased sputum neutrophil numbers compared with their baseline values after ozone exposure, these differences were significant only in atopic and atopic asthmatic subjects. We note, however, that the atopic and atopic asthmatic cohorts had less variability in their baseline PMNs per milligram than the healthy volunteers. We can speculate that atopic status might have influenced the variability of baseline neutrophilia when compared with that seen in healthy volunteers. Levels of

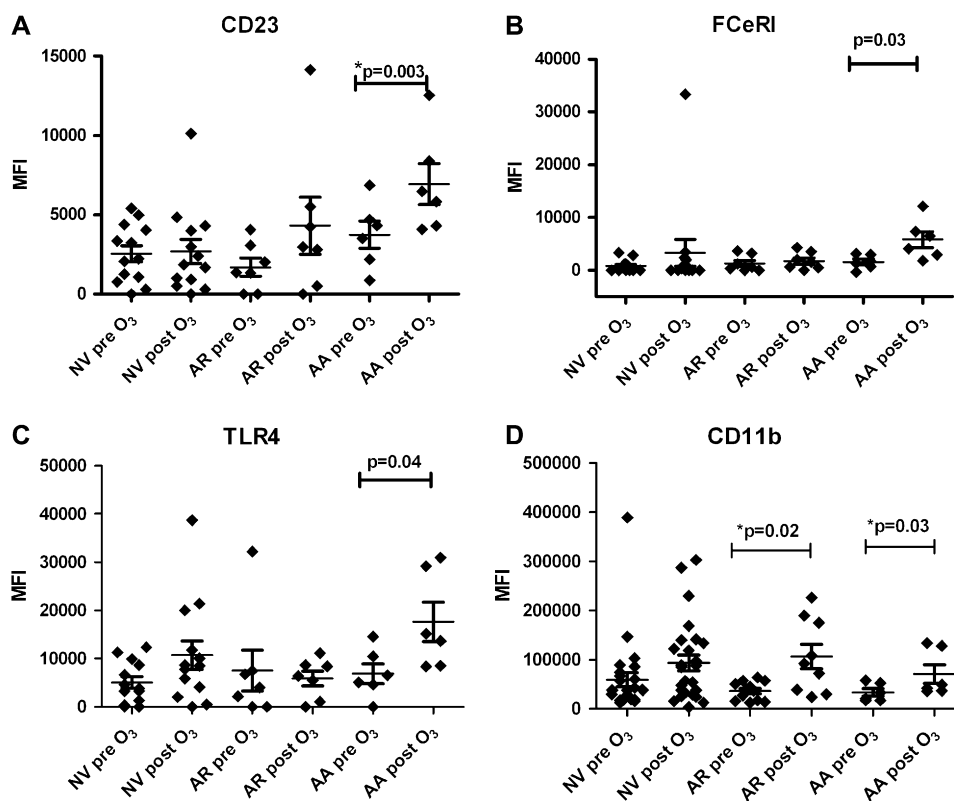


FIG 4. Changes in cell-surface marker expression (mean fluorescent intensity [MFI]) on induced sputum macrophages: *n* = 13 healthy volunteers (NV), 7 atopic subjects (AR), and 6 atopic asthmatic subjects (AA). Lines depict means and SEMs.

the potent neutrophil chemoattractant IL-8 were significantly increased only in the atopic and atopic asthmatic cohorts after ozone exposure, with no significant differences between them. Thus some markers of neutrophilia appear to be influenced by atopic status at the early time point sampled. However, the present study is limited in that we cannot comment on more chronic inflammatory changes, such as reduced lung function or sputum neutrophilia, that might selectively persist in some cohorts 24 hours after ozone exposure, the time frame with which ozone-induced asthma exacerbations are associated.

Although 0.4 ppm of ozone did not significantly increase sputum eosinophil numbers in this cohort with mild asthma, we did find that ozone exposure upregulated cell-surface expression of FcεRI and CD23, the high- and low-affinity IgE receptors, respectively, in the macrophages of atopic asthmatic subjects. This finding suggests that ozone-induced airway macrophages from atopic asthmatic subjects have enhanced the ability to participate in the antigen uptake process⁴¹ after ozone exposure. In the past, our group has shown that macrophages of allergic asthmatic subjects have enhanced particle uptake capability compared with those of healthy volunteers.³⁶

The lack of change in sputum eosinophil numbers might be influenced in great part by the time point sampled (4 hours after ozone) because previous studies by our group showed increased eosinophil numbers in atopic asthmatic subjects 18 hours after exposure.⁴ Of notable interest were the increased baseline sputum eosinophil numbers in the atopic nonasthmatic and atopic asthmatic cohorts. This observation has been documented by 2 other groups^{42,43} in bronchial biopsy specimens, in which baseline

numbers appear to reflect atopic status alone. What is unclear is whether and how these increased baseline eosinophil numbers influence eosinophil trafficking at later time points, eosinophil activation with consequent exacerbation of disease, or both.

In addition to ozone's enhancement of allergic airways inflammation, we found that subjects with mild atopic asthma had increased cell-surface expression of TLR4 on mature macrophages after ozone exposure. This finding corroborates murine studies of ozone challenge in which ozone exposure alone has been shown to upregulate TLR4 on murine alveolar macrophages.⁴⁴ Mechanistic studies in animals suggest that at least some responses to ozone are mediated through TLR4 and consequent elaboration of innate immune cytokines, such as IL-1β.³¹ Hollingsworth et al¹⁶ have shown that compared with wild-type mice, TLR4-deficient mice had reduced airway hyperresponsiveness after subacute ozone exposure and that inflammatory cytokine expression was altered in a TLR4-dependent manner.³¹ The role of TLR4 in ozone-induced inflammation is further highlighted by our HA observations. Hyaluronan has recently been identified as an endogenous ligand of TLR4²⁷⁻²⁹ and has been found to mediate ozone-induced airway hyperresponsiveness^{30,31} and proinflammatory cytokine production.³¹ Our finding of significantly increased HA levels in atopic and atopic asthmatic subjects but not in healthy subjects makes the HA-TLR4 link an interesting mechanism to explore in future ozone studies.

We hypothesize that atopic asthmatic subjects expressed more surface TLR4 on airway macrophages because of a combination of factors: increased potential ligands stimulating increased TLR4 expression, the baseline cytokine environment in the atopic

asthmatic cohort with increased IL-1 β and decreased IL-10 levels, or both. Our HA assay was limited in that we could not distinguish between low- and high-molecular-weight forms, with the low-molecular-weight form serving as a putative TLR4 ligand. Atopic asthmatic subjects might have had increased endogenous TLR4 ligands that were not assayed for in the present study. The baseline cytokine environment might also influence susceptibility to augmenting TLR4 expression because others have shown that IL-10 can downregulate TLR4 expression⁴⁵ and that IL-1 β overexpression is capable of provoking the release of endogenous TLR4 ligands,⁴⁶ enabling TLR4-mediated production of cytokines, such as IL-1 β , IL-6, and IL-8.

In addition to the increased IL-1 β levels at baseline in the atopic asthmatic cohort, we found that IL-1 β and IL-6 levels were increased after ozone exposure in atopic asthmatic subjects. This suggests an important role for IL-1 β and possibly activation of the NLRP3 inflammasome, which is central in the release of IL-1 β from macrophages. Administration of (human) IL-1 receptor antagonist before and after ozone exposure has been shown to prevent the development of airway hyperresponsiveness and blunt increases in proinflammatory cytokine levels, as well as to decrease neutrophilia in bronchoalveolar lavage (BAL) fluid in a murine model of ozone-induced lung injury.⁴⁷ Similar to our study, IL-1 β levels have been reported to be greater in BAL fluid and sputum of asthmatic subjects compared with levels seen in healthy volunteers,⁴⁸ with airway macrophages from asthmatic subjects also having increased expression of IL-1 β .⁴⁹ Most notably, IL-1 β levels have been shown to be increased in BAL fluid from persons with symptomatic asthma versus those with asymptomatic asthma.⁵⁰ Our findings highlight the heterogeneity of IL-1 β values at baseline and after ozone exposure in subjects with very mild asthma. Interestingly, Hastie et al⁵¹ recently analyzed induced sputum from asthmatic subjects stratified by granulocyte populations and found that those who had greater than 40% sputum neutrophils, independent of eosinophil level, had increased IL-1 β levels compared with sputum with less than 40% neutrophils. Therefore it is plausible that in asthmatic subjects with neutrophil-driven inflammatory responses, such as those evoked by ozone exposure or endotoxin inhalation challenge, IL-1 β might play a significant role in the disease state. However, the importance of IL-1 β in the response to ozone exposure will need to be confirmed in a follow-up study with a larger sample size. We hypothesize the increased TLR4 expression correlates with increased proinflammatory cytokine production, as has been shown in TLR4-transgenic animals.⁵² Taken together, our data on TLR4 and IL-1 β lead us to suggest that innate immune inflammatory pathways involving activation of TLR4 and subsequent release of IL-1 β might play an important role in driving ozone-induced asthma exacerbations. Release of an endogenous TLR4 ligand might activate the NLRP3 inflammasome, with consequent production and release of IL-1 β , as documented in other models of sterile inflammation.⁵³

Another possible explanation for increased inflammatory cytokine production and increased TLR4 and IgE receptor surface expression after ozone exposure in atopic asthmatic subjects might be the decreased level of IL-10 at baseline with no increase after ozone exposure. IL-10 is a potent anti-inflammatory cytokine, suppressing the production of a multitude of proinflammatory cytokines by activated macrophages, such as TNF- α , IL- β , IL-6, macrophage inflammatory protein 1 α , and IL-8.⁵⁴ Decreased levels of IL-10 have been reported in the BAL fluid²⁵

and induced sputum²⁶ of asthmatic subjects compared with levels seen in nonasthmatic subjects. In the present study we found that baseline IL-10 levels in sputum of allergic asthmatic subjects were significantly decreased compared with those seen in healthy volunteers ($P = .007$), and there was a similar trend for a difference with atopic nonasthmatic subjects ($P = .09$). Studies of IL-10 knockout mice indicate that IL-10 appears to be protective against ozone-induced neutrophilic inflammation and nuclear factor κ B activation.⁵⁵ Therefore suppressed baseline IL-10 levels in our atopic asthmatic cohort might have contributed to increased proinflammatory cytokine production after ozone exposure.

In conclusion, we report that atopic asthmatic subjects exposed to ozone exhibit an increased response of proinflammatory cytokines (IL-1 β , IL-6, and IL-8), TLR4, and IgE receptor expression in the airways. We suggest this airways milieu might confer increased reactivity to subsequently inhaled allergen and innate immune ligands. Low levels of IL-10 in the airways of atopic asthmatic subjects might underlie this reactivity to ozone. We propose that these findings have important health implications because clarifying the mechanisms underlying susceptibility to environmentally induced asthma and modulators of these inflammatory pathways, such as microRNAs,⁵⁶ might allow for the development of more targeted traditional or alternative therapies to prevent asthma exacerbations.

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Clinical implications: These observations suggest that mild atopic asthma confers increased risk for exacerbation of ozone-induced lung disease through promoting an enhanced innate immune inflammatory response to ozone.

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METHODS

To be eligible for any of our studies in which flow cytometry is not a primary end point (as for all the subjects used for the current study), an adequate sputum producer is defined as having at least 250,000 cells (to generate a total of 4 slides), at least 50% viability, and less than 40% squamous cell contamination. Those subjects who were not sputum producers during our screening process were disqualified from the study.

For sputum induction, three 7-minute inhalation periods of nebulized hypertonic saline (3%, 4%, and 5%; DeVilbiss UltraNeb 99 ultrasonic nebulizer, Sunrise Medical, Somerset, Pa) were followed by expectoration of sputum into a sterile specimen cup. A cell-enriched select sample was obtained by selecting 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 DTT (Sputolysin; Calbiochem, San Diego, Calif) in Dulbecco's PBS to

depolymerize mucins. 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 Hanks balanced salt solution. Cell viability (trypan blue exclusion) and total cell counts were assessed in a Hausser Neubauer hemocytometer (Hausser Scientific, Horsham, Pa), and differential cell counts were performed on cyto-centrifuged cells stained with a modified Wright stain, (Hema-Stain-3; Fisher Scientific, Waltham, Mass). A portion of the cells were immediately used for flow cytometric assays.

Although the ability to produce adequate sputum was an entrance criterion, some sputum samples recovered at baseline or after challenge did not yield sufficient numbers of cells for recovery of all end points. Assessment of macrophage function through assays of phagocytosis and oxidative burst were limited in that the sample size providing before and after samples for comparison was not adequate.