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Atopic Asthmatics but not atopics without asthma have enhanced inflammatory response to ozone

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Abstract

Background—Asthma is a known risk factor for acute ozone-associated respiratory disease. Ozone (O_3) causes an immediate decrease in lung function and increased airway inflammation. The role of atopy and asthma in modulation of O_3-induced inflammation has not been determined.

Objective—To determine if atopic status modulates O_3 response phenotypes in humans.

Methods—Fifty volunteers (25 normal volunteers, 14 atopic non-asthmatics, 11 atopic asthmatics not requiring maintenance therapy) underwent a 0.4 ppm O_3 exposure protocol. Ozone response was determined by changes in lung function and induced sputum composition, including airway inflammatory cell concentration, cell surface markers, cytokine and hyaluronic acid concentration.

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Results—All cohorts experienced similar decreases in lung function post O_3. Atopics and atopic asthmatics had increased sputum neutrophils and IL-8 after O_3 exposure; levels did not significantly change in normal volunteers. Following O_3 exposure, atopic asthmatics had significantly increased sputum IL-6 and IL-1 β, and airway macrophage TLR4, FceRI, and CD23 expression; levels in normal volunteers and atopic non-asthmatics showed no significant change. Atopic asthmatics had significantly decreased IL-10 at baseline compared to normal volunteers: IL-10 did not significantly change in any group with O_3. All groups had similar levels of hyaluronic acid at baseline, with increased levels after O_3 exposure in atopics and atopic asthmatics.

Conclusion—Atopic asthmatics have increased airway inflammatory responses to O_3. Elevated TLR4 expression suggests a potential pathway through which O_3 generates the inflammatory response in allergic asthmatics but not in atopics without asthma.

Clinical Implications—These observations suggest that mild atopic asthma confers increased risk for exacerbation of O_3-induced lung disease through promoting an enhanced innate immune inflammatory response to O_3.

Keywords

ozone; innate immunity; asthma; atopy; hyaluronic acid; environmental airways disease; interleukin-1 beta; interleukin-10

Introduction

Ozone (O_3) is a commonly encountered environmental pollutant. Asthma morbidity (ER visits, hospitalizations, rescue medication use) is clearly associated with exposure to increased levels of ambient air O_3 [1] Asthmatics are thought to have increased susceptibility to O_3 due to aggravation of underlying allergic airways inflammation. Ozone exposure studies have shown that O_3 induces an airway granulocyte response, characterized by sputum neutrophilia in healthy volunteers [1–3] and sputum neutrophilia and eosinophilia in atopic asthmatics [4]. Ozone challenge has also been found to enhance responses to subsequent inhaled allergen challenge in mild atopic asthmatics [5–7]. The ability of O_3 to enhance airway inflammation and enhance responsiveness to subsequent inhaled allergen likely play important roles in asthma exacerbation. However, it is unclear if non-asthmatic persons with atopy also have enhanced susceptibility to O_3 as has been shown in atopic asthmatics. Khatri and colleagues found associations between air quality and airway inflammation in both asthmatics and non-asthmatics with allergies [8], suggesting that atopic status itself may contribute to O_3-induced inflammatory response in the airway.

Our group has also observed that response to inhaled lipopolysaccharide (LPS) yields airway inflammatory responses similar to those seen after O_3 exposure. Like O_3 exposure, airway LPS challenge induces airway neutrophilia, enhances response to inhaled allergen in allergic subjects [9–11], and induces airway pro-inflammatory cytokines[1]. We and others have also found that O_3 and LPS induce selective increases in specific macrophage and monocyte populations in the airway [12] and that O_3 induces influx of monocytes and macrophages with increased expression of CD11b and CD14, [13]a co-receptor for LPS. Building on these observations, our group has recently found correlations in airway inflammatory responses in healthy individuals who underwent both O_3 and LPS exposures (Hernandez, in press). The similarities between responses of normal volunteers and atopic asthmatics to O_3 and LPS suggest that atopy modifies response to a number of innate stimuli and that common molecular mechanisms account for response to both O_3 and LPS.

Consistent with this idea, mechanistic studies in mice suggest that at least some O_3 responses are mediated through the primary LPs receptor, TLR4 [14–16]. As O_3 is a reactive oxygen

molecule, it is unlikely that a discrete receptor exists which modulates response to this agent, or that O_3 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 O_3 do not reveal an O_3-induced release of inflammatory mediators [17–19]. It seems more likely that O_3 exerts a biological effect via 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 NF-κB activation and production of a variety of mediators [20–22]. Pro-inflammatory mediators such as IL-8 are among those associated with O_3-induced inflammation in asthmatics 23 and reduced levels of the anti-inflammatory cytokine IL-10 have been reported in asthmatics at baseline 23–25. Hyaluronic acid (HA) is an endogenous pro-inflammatory ligand for CD44 and TLR4 [26–28] and may be an important mediator in asthma. Hyaluronic acid has also been recently identified as a candidate host danger signal which mediates O_3-induced inflammatory responses [29, 30].

At present, it is unclear if O_3 modifies levels of TLR4 in humans, and if atopic status itself modifies the O_3-induced airway inflammatory response. The present study tests the hypothesis that atopic status alone modifies lower airway inflammatory response to a 0.4 ppm O_3 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 positive immediate skin test response to one of the following allergen mixes: two 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 was determined as having a drop of 20% from post-saline FEV1 at a level of 10 mg/ml or less of methacholine. This study was an analysis of lung function data and samples obtained from a total of 50 subjects that were stratified into three groups based on the results of their allergy skin testing and methacholine challenge. Allergic asthmatics (AA, N=11) had positive skin prick testing and positive methacholine challenge and were required to have mild intermittent asthma, as defined by National Heart, Lung, and Blood Institute (NHLBI) 2007 guidelines (not on inhaled corticosteroid or leukotriene receptor antagonist therapy). Normal volunteers (NV, N=25) had negative skin prick testing and negative methacholine challenge, and atopic volunteers (N=14) had positive skin prick testing and negative methacholine challenge. All AA volunteers were newly recruited into this study protocol while other subjects were derived from previously completed studies [12, 31]. Female subjects had to have a negative urine pregnancy test prior to challenge and all volunteers were required to be free of chronic cardiovascular illness, and be free of acute respiratory illness within 4 weeks of O_3 challenge. All subjects had FEV_1 and FVC ≥ 80% predicted normal for height and age [32], and $FEV_1/FVC \geq 0.75$ 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 session. Subjects provided an induced sputum sample during the screening visit and at 4–6 h after the O_3 exposure. Sputum induction and processing methods are found in the online repository.

Lung Function testing

Spirometry testing was preformed according to ATS/ERS recommendations using the Viasys VMax 229 series spirometers. All subjects were seated, and at least 3 maneuvers were obtained, with the best of the three reported.

O_3 Exposure protocol

O_3 Inhalation Challenge: The O_3 exposures were conducted in an O_3 exposure chamber at the US-EPA Human Studies Facility on the campus of the University of North Carolina, Chapel Hill, NC. Each subject was exposed to O_3 (0.4 ppm) for 2 hours while performing four 15 minute sessions of intermittent moderate exercise (minute ventilation or VE_{min} = 30–40 L/min) on a treadmill, separated by 15 minutes of seated rest. Spirometry, breath sounds, and vital signs were assessed before and immediately after exposure. Sputum was obtained 4–6 hours post exposure and processed as previously described [12, 31, 33, 34]. Sputum was assessed for total and differential cell counts, flow cytometric assessment of CD11b, CD14, CD86, TLR4, FceRI, CD23, and HLA-DR on macrophages and monocytes [12, 35]. Sputum supernatants were also assessed for cytokine concentration.

Flow cytometry

Expression of selected cell surface molecules on sputum leukocytes was quantified via multicolor flow cytometry (FCM) using a BD LSR-II flow cytometer (BD Immunocytometry Systems; San Jose, CA). These included molecules associated with antigen presentation and specific immunity (CD86/B7.2, HLA-DR/MHC-II,), as well as innate immune function (CD11b/CR3, CD14/LPS receptor). First, sputum leukocytes were differentiated from cellular debris, bacteria, yeast, squamous and bronchial epithelial cells by gating on CD45 (pan leukocyte marker) positive cells. Up-or down-regulation of specific surface molecules was quantified as a change in the mean fluorescent intensity (MFI) of the gated population. Fluorochrome-labeled antibodies were obtained from BD Biosciences (CD11b-PE-CY5, CD45-APC-Cy7, HLA-DR-PerCP, Beckman-Coulter (CD14-APC, CD86-PE). Appropriate, nonspecific, labeled isotypic control antibodies were also obtained from these sources.

Mediator Measurement

Cytokines from sputum supernatants were measured using multi-plex technology Meso ScaleDiscovery/MSD, Gaithersburg, MD). Each sample was analyzed with the HumanMIP-1 alpha Ultra Sensitive Kit (LOT#: K0031370) and the Human TH1/TH2 10-Plex Ultra Sensitive Kit (LOT#: K0031431). All supernatant samples were diluted 1:4 and had a final DTT concentration of < 1mM where no deleterious effects have been observed using the MSD platform.

Hyaluronic Acid Measurement

Hyaluronic acid (HA) levels from induced sputum supernatants were performed with a commercially available enzyme-linked immunosorbent assay (Echelon, San Jose, CA) 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 to standards containing DTT. The standard curve generated was log linear from 12.5 ng/ml to 1600 ng/ml.

Statistical Analysis

The primary hypothesis to be tested was that atopic asthmatics or atopic non-asthmatics had increased susceptibility to ozone compared to normal volunteers, with the primary endpoint being sputum neutrophils. Changes in cell counts/mg sputum, differential cell count percentage (sputum), mean fluorescence intensity (MFI) measures (flow cytometry), cytokine values, and HA levels pre- and post-O_3 exposures within each cohort were assessed. To determine if a particular cohort responded to O_3, non-parametric paired Wilcoxon signed rank test was employed. Differences between either atopic asthmatics or atopic non-asthmatics and normal volunteers were analyzed using non-parametric t tests (Mann-Whitney Test). To confirm that baseline values between all three groups were not different, non-parametric one way analysis of variance (Kruskall Wallis Test) was employed. Significance was set at a p= 0.05.

Results

Demographics

Demographic data for the 50 volunteers, ranging in age from 19 to 39 years, recruited for challenge to 0.4 ppm of O_3 are presented in Table 1. Fourteen were atopic, though they could not exhibit symptoms of atopy at the time of exposure. Eleven were atopic asthmatics, none of whom were on maintenance inhaled corticosteroid therapy or other scheduled drug.

Effect of 0.4 ppm Ozone on Lung Function

The effect of O_3 on lung function in cohorts of normal volunteers (n=25), atopics (n=13), and atopic asthmatics (n=11) was compared. Ozone caused similar significant decreases in mean FEV1 ($p<0.001$ all cohorts) and FVC ($p<0.001$ normal volunteers, p=0.003 atopics and atopic asthmatics) immediately after challenge in all cohorts (Figure 1) compared to their baseline values.

Effect of 0.4 ppm Ozone on Airway Leukocyte Numbers

The effect of O_3 on airway neutrophil, macrophage, and eosinophil numbers was a primary endpoint for this study. Paired data (pre and post O_3 exposure) was available for 25 normal volunteers, 13 atopics, and 10 atopic asthmatics. Figure 2A shows that the atopic and the atopic asthmatic cohorts had significantly increased sputum neutrophil numbers (cells/mg sputum, p=0.045 atopics, p=0.04 atopic asthmatics) 4 hours after challenge. Although sputum neutrophils increased in normal volunteers, the increase in mean neutrophil numbers did not reach statistical significance, which may have been influenced by more variable baseline neutrophil numbers. Although macrophage numbers (cells/mg sputum) (Figure 2B) decreased in all cohorts, this was significant only among the atopic asthmatic volunteers (p=0.01). Figure 2C shows that eosinophil numbers at baseline were elevated in the airways of atopic (p=0.0002) and atopic asthmatic volunteers (p=0.0002) compared to very low values in normal volunteers. After O_3 exposure, eosinophil numbers did not significantly change in the atopics or the atopic asthmatics and remained very low in normal volunteers.

Changes in Airway Cytokines following Ozone

We also examined sputum supernatants for a number of immunoregulatory cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, macrophage inflammatory protein 1 alpha, TNF alpha and Interferon gamma). Sputum supernatants were available for 24 normal volunteers, 12 atopics, and 10 atopic asthmatics. Four cytokines (IL-1β, IL-8, IL-6, and IL-5) were significantly altered by O_3 exposure and this occurred only in the atopic and atopic asthmatic cohorts. Atopic asthmatics had significant increases in IL-1β (p=0.03), IL-8 (p=0.01), and IL-6 (p=0.01) (Figures 3A–C), while atopics had increased IL-8 (p=0.02) (Figure 3B) and IL-5 (p=0.03) following O_3 exposure (data not shown). Two atopic asthmatic subjects

had higher baseline and post O_3 levels of IL- β and IL-8 compared to the rest of the cohort. Analysis of the data excluding these two subjects showed that the change in IL-8 and IL-β was significant (p=0.04) and close to significant (p=0.08) for these cytokines, respectively. We further analyzed the IL-1β data as % change from baseline; using this metric we found a significant difference when comparing NV vs. AA (p=0.009) (data not shown). When comparing baseline values, atopic asthmatics had increased IL-1β compared to normal volunteers (p=0.02) or atopics (p=0.05) (Figure 3A). No group experienced significant increases in IL-10 after O_3 exposure. However, atopic asthmatics had significantly decreased IL-10 at baseline compared to normal volunteers (p=0.007) (Figure 3D).

Change in Sputum Hyaluronic Acid after Ozone Exposure

Pre and post O_3 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 O_3 exposure among the cohorts (Figure 3E). Ozone exposure caused increased HA levels in atopics (p=0.004) and atopic asthmatics (p=0.002) compared to their respective baseline values.

Changes in Cell Surface Markers following Ozone

Macrophages from induced sputum were isolated as discussed in the methods, and changes in cell surface markers are presented in Figure 4 (n=13 normal volunteers, 7 atopics, and 6 atopic asthmatics). Macrophages in the atopic asthmatic cohort had increased expression of CD23 (p=0.003), FceRI (p=0.03), and TLR4 (p=0.03) after O_3 exposure (Figure 4A–C). All cohorts showed increased CD11b after O_3, but this increase attained significance only in the atopic (p=0.02) and atopic asthmatic (p=0.03) cohort (Figure 4D). There were no other significant changes in cell surface markers (CD80, CD86, TLR2, CD14, and HLA-DR) following O_3 in any cohort (data not shown).

Discussion

There are numerous epidemiologic studies linking increased environmental O_3 levels with asthma exacerbations. Asthmatic susceptibility to ambient O_3 has been purported to be due to enhanced underlying allergic airways inflammation [4, 36], however, not all studies have observed modified allergic airways inflammation following exposure in atopic individuals [4, 37].

In this study we examined whether atopic status alone or mild intermittent atopic asthma influenced the airways inflammatory response to inhaled O_3. We found that all cohorts experienced similar decreases in lung function (FEV1 and FVC) immediately after O_3 exposure, with no difference among 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 [38, 39]. We did find that although all cohorts had increased sputum neutrophils compared to their baselines after O_3 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/mg than the normal volunteers. We can speculate that atopic status may have influenced the variability of baseline neutrophilia when compared to normal volunteers. The potent neutrophil chemoattractant IL-8 was significantly increased only in the atopic and atopic asthmatic cohorts following O_3 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 may selectively persist in some cohorts 24 hours after O_3 exposure, t he time frame with which O_3-induced asthma exacerbations are associated.

Although 0.4 ppm O_3 did not significantly increase sputum eosinophils in this mild asthmatic cohort, we did find that O_3 exposure upregulated cell surface expression of FceRI and CD23, the high and low affinity IgE receptors respectively, in the macrophages of atopic asthmatics. This finding suggests that O_3-induced airway macrophages from atopic asthmatics have enhanced the ability to participate in the antigen uptake process[40] following O_3 exposure. In the past our group has shown that macrophages of allergic asthmatics have enhanced particle uptake capability compared to normal volunteers [35].

The lack of change in sputum eosinophils may be influenced in great part by the timepoint sampled (4 hours post O_3), as previous studies by our group showed increased eosionphils in atopic asthmatics 18 hours post exposure [4]. Of notable interest were the elevated baseline sputum eosinophils in the atopic non-asthmatic and the atopic asthmatic cohorts. This observation has been documented by two other groups [41, 42] in bronchial biopsy specimens, where baseline numbers appear to reflect atopic status alone. What is unclear is if and how these elevated baseline eosinophil numbers influence eosinophil trafficking at later timepoints and/or eosinophil activation with consequent exacerbation of disease.

In addition to O_3's enhancing allergic airways inflammation, we found that mild atopic asthmatics had increased cell surface expression of TLR4 on mature macrophages after O_3 exposure. This finding corroborates murine studies of O_3 challenge, where O_3 exposure alone has been shown to upregulate TLR4 on murine alveolar macrophages [43]. Mechanistic studies in animals suggest that at least some responses to O_3 are mediated through TLR4 and consequent elaboration of innate immune cytokines such as IL-1 β [30]. Hollingsworth and colleagues have showed that compared to wild type mice, TLR4 deficient mice had reduced airway hyper-responsiveness following subacute O_3 exposure [15], and that inflammatory cytokine expression was altered in a TLR4 dependent manner [30]. The role of TLR4 in O_3-induced inflammation is further highlighted by our HA observations. Hyaluronan has recently been identified as an endogenous ligand of TLR4[26–28] and has been found to mediate O_3-induced airway hyper-responsiveness[29, 30] and pro-inflammatory cytokine production.[30] Our finding of significantly increased HA levels in atopic and atopic asthmatic subjects but not in normals, makes the HA-TLR4 link an interesting mechanism to explore in future O_3 studies.

We hypothesize that atopic asthmatics expressed more surface TLR4 on airway macrophages due to a combination of factors: a). increased potential ligands stimulating increased TLR4 expression, and/or b). the baseline cytokine environment in the atopic asthmatic cohort with increased IL-1β and decreased IL-10. Our HA assay was limited in that we could not distinguish between low and high molecular weight forms; the low molecular weight form serving as a putative TLR4 ligand. Atopic asthmatics may have had increased endogenous TLR4 ligands that were not assayed for in the present study. The baseline cytokine environment may also influence susceptibility to augmenting TLR4 expression, as others have shown that IL-10 can downregulate TLR4 expression [44], and that IL-1β overexpression is capable of provoking the release of endogenous TLR4 ligands [45], enabling TLR4-mediated production of cytokines such as IL-1β, IL-6, and IL-8.

In addition to the elevated IL-1β at baseline in the atopic asthmatic cohort, we found that IL-1β and IL-6 were elevated after O_3 exposure in atopic asthmatics. This suggests an important role for IL-1β and possibly activation of the NLRP3 inflammasome which is central in release of IL-1β from macrophages. Administration of (human) IL-1 receptor antagonist before and after O_3 exposure has been shown to prevent the development of airway hyper responsiveness and blunt increases in pro-inflammatory cytokines, as well as decrease neutrophilia in bronchoalveolar lavage (BAL) fluid in a mouse model of O_3-induced lung injury [46]. Similar to our study, IL-1β levels have been reported to be greater in BAL fluid and sputum of asthmatics compared to normal volunteers[47], with airway macrophages from asthmatics also

having increased expression of IL-1β[48]. Most notably, IL-1β has been shown to be elevated in BAL fluid from persons with symptomatic asthma vs. those with asymptomatic asthma [49]. Our findings highlight the heterogeneity of IL-1β values at baseline and following O_3 exposure in very mild asthmatics. Interestingly, Hastie et al. recently analyzed induced sputum from asthmatics stratified by granulocyte populations and found that those asthmatics who had >40% sputum neutrophils, independent of eosinophil level, had increased IL-1β compared to sputa with <40% neutrophils[50]. It is plausible then that in asthmatics with neutrophil-driven inflammatory responses, such as those evoked by O_3 exposure or endotoxin inhalation challenge, IL-1β may play a significant role in the disease state. However, the importance of IL-1β in response to O_3 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 pro-inflammatory cytokine production, as has been shown in TLR4 transgenic animals [51]. Taken together, our data on TLR4 and IL-1β lead us to suggest that innate immune inflammatory pathways involving activation of TLR4 and subsequent release of IL-1β may play an important role in driving O_3-induced asthma exacerbations. Release of an endogenous TLR4 ligand may activate the NLRP3 inflammasome, with consequent production and release of IL-1β, documented in other models of sterile inflammation [52].

Another possible explanation for increased inflammatory cytokine production and increased TLR4 and IgE receptor surface expression after O_3 exposure in atopic asthmatics may be the decreased level of IL-10 at baseline, with no increase after O_3 exposure. Interleukin-10 is a potent anti-inflammatory cytokine, suppressing the production of a multitude of pro-inflammatory cytokines by activated macrophages, such as TNF-α, IL-β, IL-6, macrophage inflammatory protein-α, and IL-853. Decreased levels of IL-10 have been reported in the BAL24 and induced sputum[25] of asthmatics compared to non-asthmatics. In the present study we found that baseline IL-10 levels in sputum of allergic asthmatics were significantly decreased compared to normal volunteers (p=0.007), and there was a similar trend for difference with atopic non-asthmatics (p=0.09). Studies of IL-10 knockout mice indicate that IL-10 appears to be protective against O_3-induced neutrophilic inflammation and NFkB activation[54]. Therefore, suppressed baseline IL-10 in our atopic asthmatic cohort may have contributed to increased pro-inflammatory cytokine production after O_3 exposure.

In conclusion, we report that atopic asthmatics exposed to O_3 exhibit an elevated response of pro-inflammatory cytokines (IL-1β, IL-6, IL-8), TLR4 and IgE receptor expression in the airways. We suggest this airways milieu may confer increased reactivity to subsequently inhaled allergen and innate immune ligands. Low levels of IL-10 in the airways of atopic asthmatics may underlie this reactivity to O_3. We propose that these findings have important health implications, as clarifying the mechanisms underlying susceptibility to environmentally-induced asthma and modulators of these inflammatory pathways, such as microRNAs [55] may allow for the development of more targeted traditional or alternative therapies to prevent asthma exacerbations.

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Abbreviations

O_3	ozone
HA	hyaluronic acid
TLR4	toll-like receptor 4
ppm	parts per million
NV	normal volunteer
AA	atopic asthmatic
IL	Interleukin
IL-1β	Interleukin-1 beta

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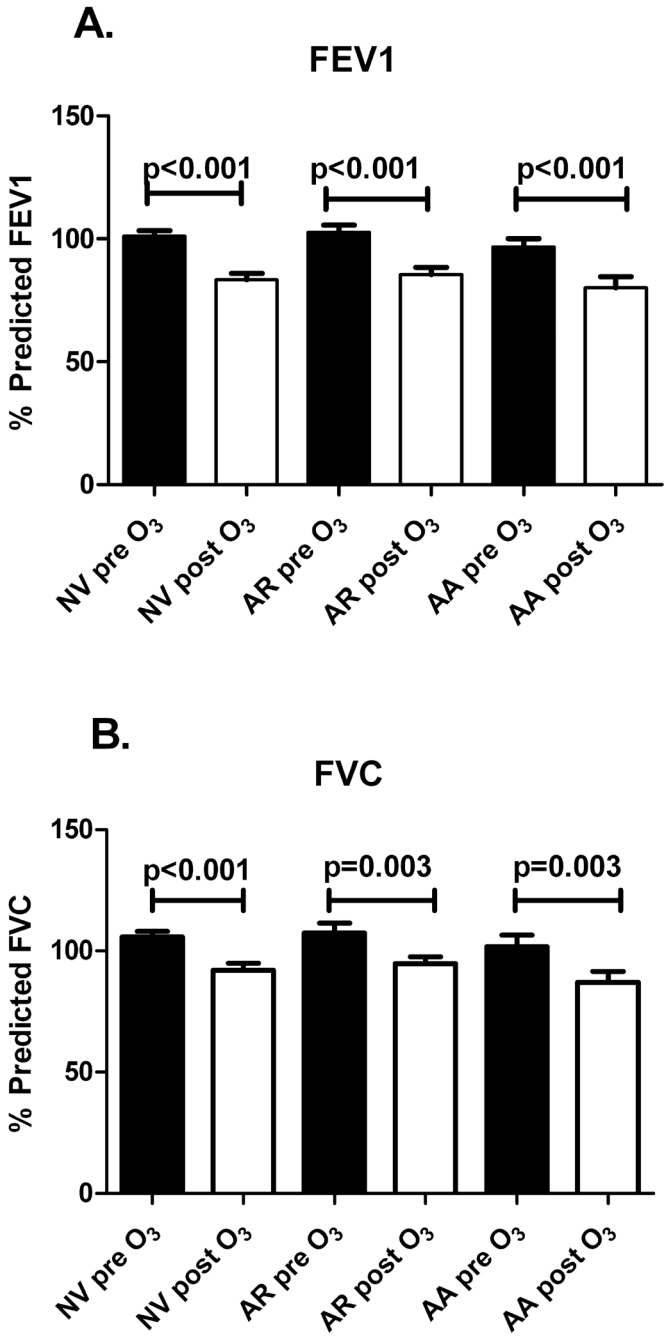

Figure 1.
Baseline and post O_3 exposure FEV1 (A) and FVC (B). n=25 normal volunteers (NV), 13 atopics, and 11 atopic asthmatics (AA). Data bars depict mean and standard error of the mean.

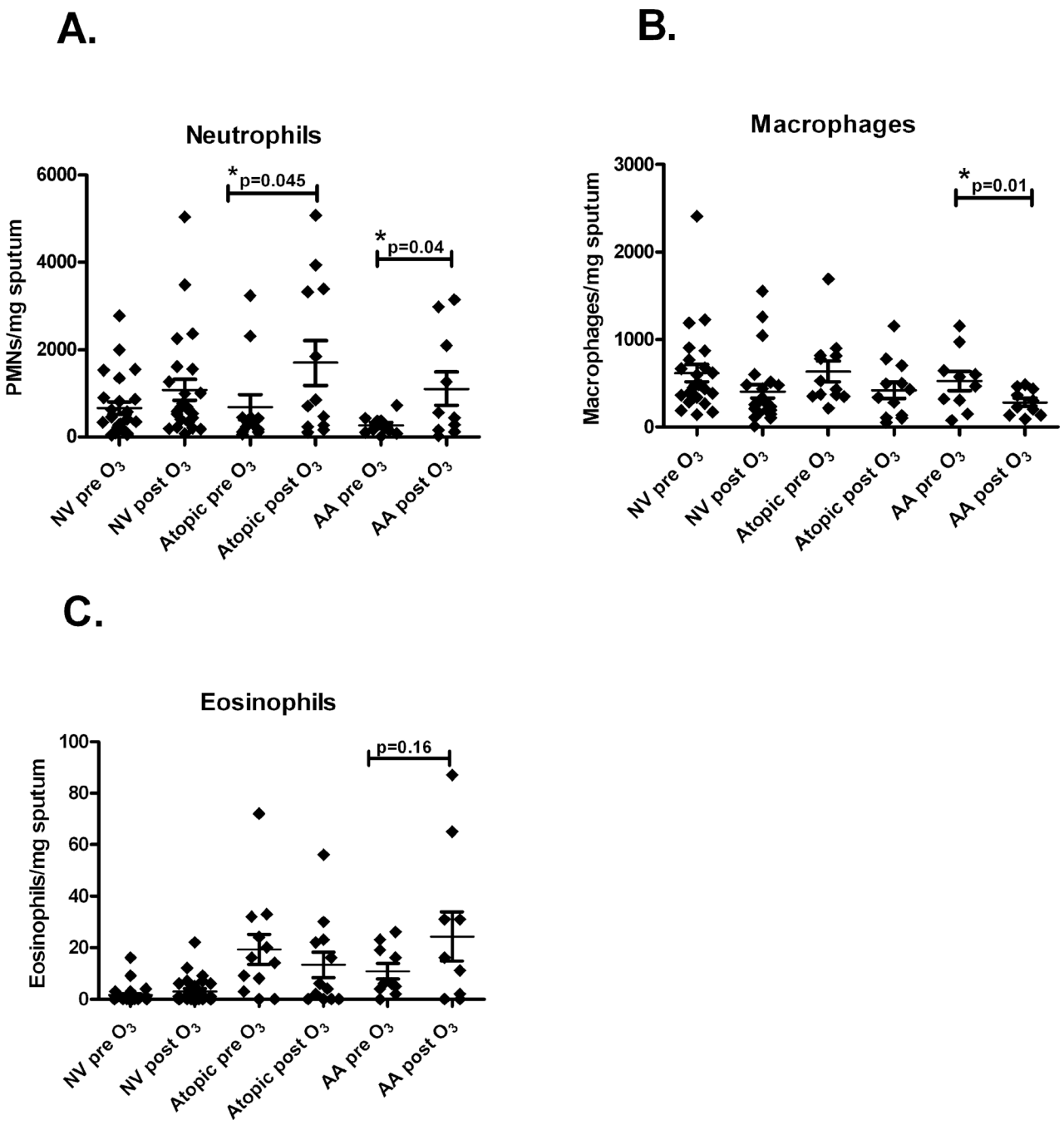

Figure 2.
Changes in inflammatory cell infiltrate in sputum (cells/mg sputum) in response to O_3. N=25 normal volunteers, 13 atopics, and 10 AA. Lines depict mean and standard error of the mean.

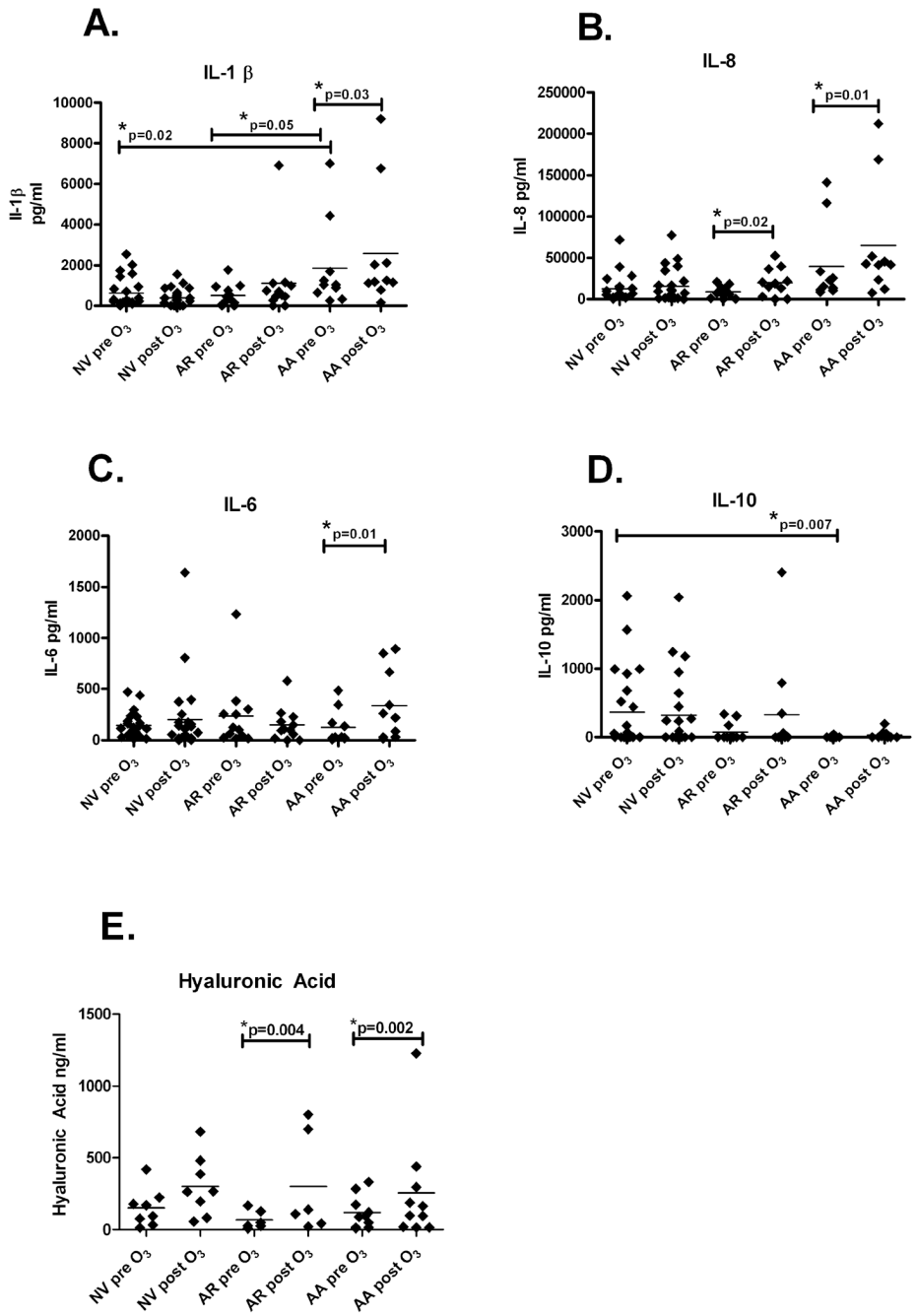

Figure 3.

Changes in induced sputum cytokines and hyaluronic acid (HA) in response to O_3. N=24 NV, 12 atopics, and 10 AA for sputum cytokines, N=10 each cohort for HA measurement in sputum. Lines depict mean and standard error of the mean.

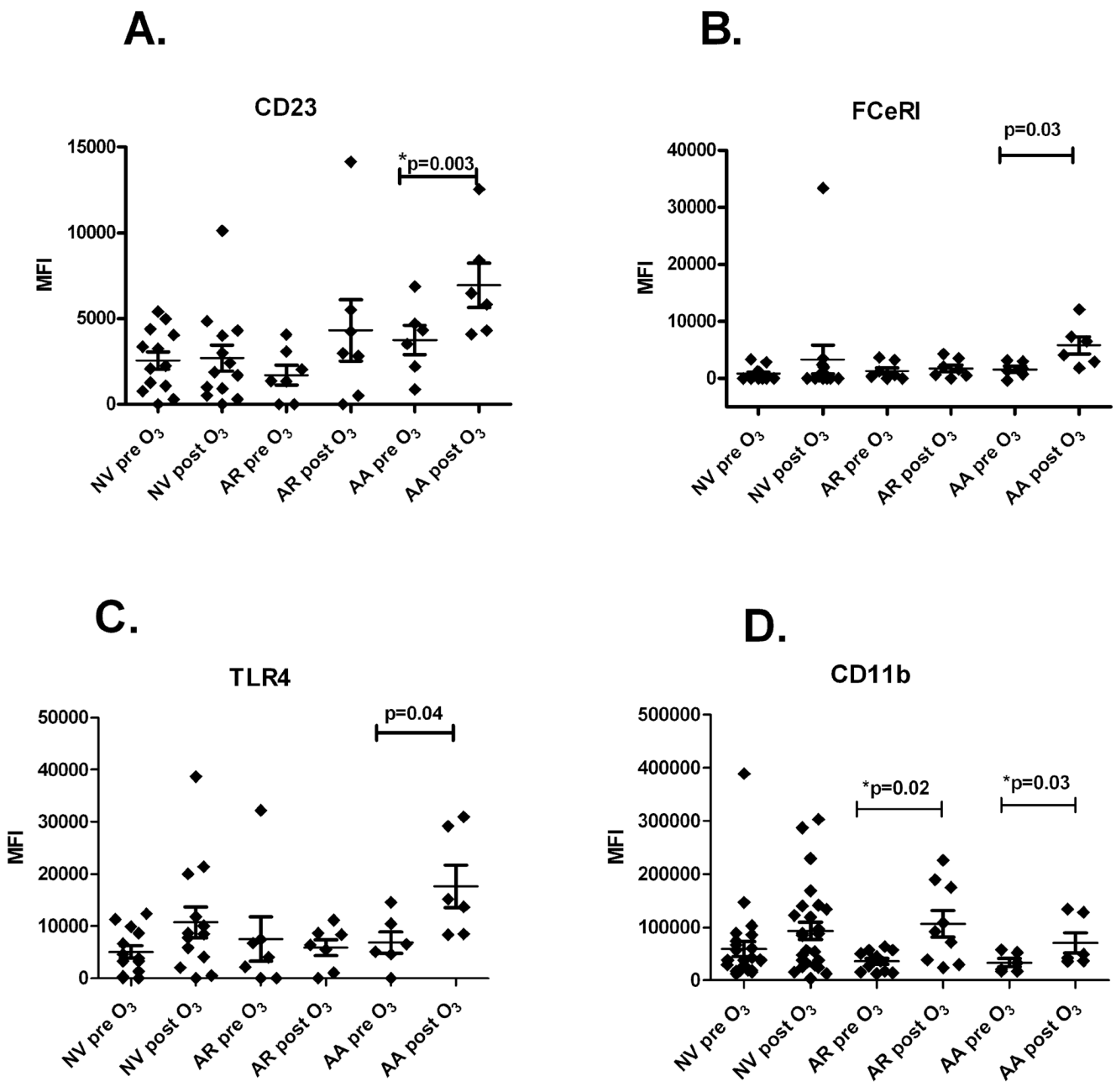

Figure 4.
Changes in cell surface marker expression (MFI) on induced sputum macrophages. N=13 normal volunteers (NV), 7 atopics, and 6 atopic asthmatics. Lines depict mean and standard error of the mean.

Table I

Study Demographics

Cohort	No.	Age (y)	Gender	Race
Normal Volunteer	25	23.7 ± 3.8	14 F / 11 M	19 white, 5 African American, 1 Asian
Atopic	14	25.2 ± 5	7 F / 7 M	8 white, 5 African American, 1 Asian
Atopic Asthmatic	11	26.3 ± 6.5	6 F / 5 M	8 white, 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.