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Airway cells from atopic asthmatics exposed to ozone display an enhanced innate immune gene profile

Michelle Hernandez, MD[a,b], **Willie June Brickey, PhD**[c], **Neil E. Alexis N, PhD**[a,b], **Rebecca C. Fry, PhD**[d], **Julia E. Rager, MS**[d], **Baiming Zhou, MS**[e], **Jenny PY Ting, PhD**[e], **Haibo Zhou, PhD**[a,e,*], and **David B. Peden, MD,MS**[a,b,c,*]

[a]Center for Environmental Medicine, Asthma and Lung Biology The University of North Carolina at Chapel Hill School of Medicine Chapel Hill, North Carolina

[b]Department of Pediatrics The University of North Carolina at Chapel Hill School of Medicine Chapel Hill, North Carolina

[c]Department of Microbiology and Immunology The University of North Carolina at Chapel Hill School of Medicine Chapel Hill, North Carolina

[d]Environmental Sciences & Engineering, The Gillings School of Global Public Health

[e]Biostatistics, The Gillings School of Global Public Health

Capsule Summary

This study identifies transcriptional phenotypes of sputum samples from normal volunteers and atopic asthmatics exposed to ozone. Network analyses suggest that asthmatics elevate immune signaling following oxidative stress, while nonasthmatics attempt to mitigate the ozone-induced response.

Keywords

Asthma; Ozone; Induced Sputum; Profiling; Oxidative Stress

Respiratory complications caused by ozone (O_3) represent a significant public health burden[1–4]. Ozone and a range of other pollutants are thought to exert their effects via oxidative stress responses in the lower airway. Despite studies suggesting that pulmonary oxidative insults by air pollutants lead to deleterious health outcomes, treatment options for environmentally-induced oxidative stress are limited, partially due to the lack of understanding of exactly how these pollutants affect susceptible populations.

Corresponding Author: Michelle L. Hernandez, MD Center for Environmental Medicine, Asthma and Lung Biology 104 Mason Farm Road UNC School of Medicine Chapel Hill, NC 27516 Michelle_Hernandez@med.unc.edu phone 919-843-5383 fax 919-966-9863.
*These authors contributed equally to this effort as senior authors

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Our group recently reported that atopic asthmatics (AA) had enhanced sputum inflammatory responses compared to normal volunteers (NV) after a 2 hour, 0.4 ppm O_3 exposure, characterized by increases in macrophage TLR4 expression and concentrations of IL-1β, IL-6, and IL-8 in sputum samples[5]. This report expands on those results by examining gene expression profiles of sputum inflammatory cells from NV and AA volunteers as a means of identifying additional mechanisms that may explain the differences in inflammatory response between these two cohorts, and to confirm the airway inflammatory phenotype in a larger cohort of AA and NV.

Subject recruitment, sample collection and analysis techniques are identical to those we have recently reported[5]. Demographic data from a total of 51 subjects (34 NV, 17 AA) are presented in Table I. Statistical tests include paired t-tests to assess a specific cohort response to O_3 for cell differentials; non-parametric paired Wilcoxon signed rank test to assess O_3 responses for induced sputum cytokines; and non-parametric t tests (Mann-Whitney Test) to examine baseline differences in cytokines or cell differentials between AA and NV.

Data for induced sputum cell differentials and cytokines from 9 NV and 6 AA were added to our previously published cohort[5]. The composition of sputum cellularity was similar between the two cohorts, with the exception of increased sputum eosinophils in the AA cohort at baseline compared to NV (Table I). AA continued to show evidence of increased levels of IL-1β, IL-6, and IL-8 after O_3 exposure (Table I), with reduced levels of IL-10 and increased levels of IL-1β and IL-8 at baseline. The larger sample size supports the notion that despite similarities in sputum cellularity, AA display increased levels of airway pro-inflammatory cytokines after O_3 exposure compared to NV.

Of the 51 volunteers described above, adequate mRNA was recovered from 18 NV and 13 AA for microarray analysis using a custom-designed microarray representing more than 2000 immune response gene targets[6]. Raw expression data were first normalized using the down-weighting loess fit[7] to remove systematic ratio-intensity dependence. The normalized red/green expression ratio was $\log_2$ transformed and uploaded to Significant Analysis of Microarrays software package[8] in R (version 2.10) to identify differentially expressed genes. To control for multiple comparisons, we used false discovery rate (FDR), estimated by 5000 permutations for each target.

At FDR = 0.05, we found a total of 102 genes that showed differential expression after O_3 exposure compared to baseline conditions (Figure 1A). Fifty-five genes had significant expression changes with O_3 exposure in NV; 47 genes had significant changes in gene expression with O_3 exposure in AA. Only one gene overlapped between the two cohorts, aurora kinase B.

In order to identify potential biological pathways affected by O_3 exposure, the differentially expressed gene profiles for each cohort were overlaid onto protein-protein maps enabled through the Ingenuity Pathways' Knowledge Base[6]. Statistical significance of each network was calculated using a Fischer's exact test. This test generated a p-value signifying the probability that each network was associated with the mRNA targets by chance alone.

For each of the two gene lists, significant networks were identified. The set of 55 genes that showed O_3-induced changes in NV could be integrated into four networks ranging in significance from 10^{-16} to 10^{-44}. The most significant NV network is enriched for proteins involved in the ERBB2 (also known as HER-2) pathway, with most of the signaling predicted to be downregulated in this pathway (Table E1). The 47 differentially expressed genes associated with the O_3 challenge in AA were integrated into four networks ranging in

significance from 10^{-9} to 10^{-45}. The most significant AA network is enriched for genes involved in immune response signaling such as NFκB (Table E2).

Of the genes that showed the most statistically significant changes in gene expression, confirmatory quantitative PCR was performed on induced sputum mRNA from 6 NV and 8 AA using previously published methods[9]. A comparative CT approach was used to discern changes in relative expression due to O_3 with β-*Actin* as the housekeeping gene and pre-O_3 as untreated control (Figure 1B).

The expression levels of HER-2 pathway genes such as ERBB2, cyclin D1 (CCND1), and matrix metalloprotease II (MMP2) were significantly increased in AA compared to NV (Figure 1B). Furthermore, AA had significantly increased expression of genes involved in cellular detoxification after oxidative stress (*GPX3, GSTM4, NQO1)* and those involved in neutrophil oxidative burst (*NCF2*) compared to NV (Figure 1B). This is consistent with the idea that asthmatics have a differential oxidative stress response following O_3 exposure compared to NV. The expression levels of several innate immune genes, such as the MHC-II molecule *HLA-DPA1*, the integrin *ICAM-1*, and the pro-inflammatory cytokines *IL-6, IL-8, IL-18, and TNF*α were significantly increased in AA compared to NV upon exposure to O_3 (Figure 1B).

In summary, gene expression profiles for sputum cells are distinctly different in NV and AA despite similar neutrophil and macrophage proportions after acute O_3 exposure. Compared to NV, AA showed increased immune signaling, elevated pro-inflammatory cytokines, and up-regulated expression of the HER-2 gene network, indicative of enhanced epithelial cell proliferation. These data suggest that unlike NV, AA cannot limit epithelial cell proliferative responses following O_3 -induced oxidative stress. HER-2 blockade has been found to reduce lung fibrosis and remodeling in a model of bleomycin-induced lung injury[10]. Limitations of this study include small sample size and mixed sputum cellularity that prevented cell specific determination of oxidative stress responses in these populations. Future studies will focus on dissecting the contributions of distinct cell types on determining responses to O_3 in a larger cohort of individuals, as well as including baseline measures of inflammation. This information is important for the judicious development of therapeutics that will prevent or reduce the harmful effects of oxidant lung injury.

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Abbreviations

O_3	Ozone
AA	atopic asthmatic
NV	normal volunteer
ppm	part per million
IL-1β	interleukin -1 beta
IL-8	interleukin-8
IL-6	interleukin-6
TLR4	toll-like receptor 4

TLR2	toll-like receptor 2
TNFα	tumor necrosis factor alpha
ICAM1	Inter-Cellular Adhesion Molecule 1
HLA-DP1	major histocompatibility complex, class II, DP
GSTM4	glutathione S-transferase mu 4
GPX3	glutathione peroxidase 3
NCF2	neutrophil cytosolic factor 2
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
CCND1	cyclin D-1
MMP2	matrix metalloprotease II
NQO1	NAD(P)H dehydrogenase, quinone 1

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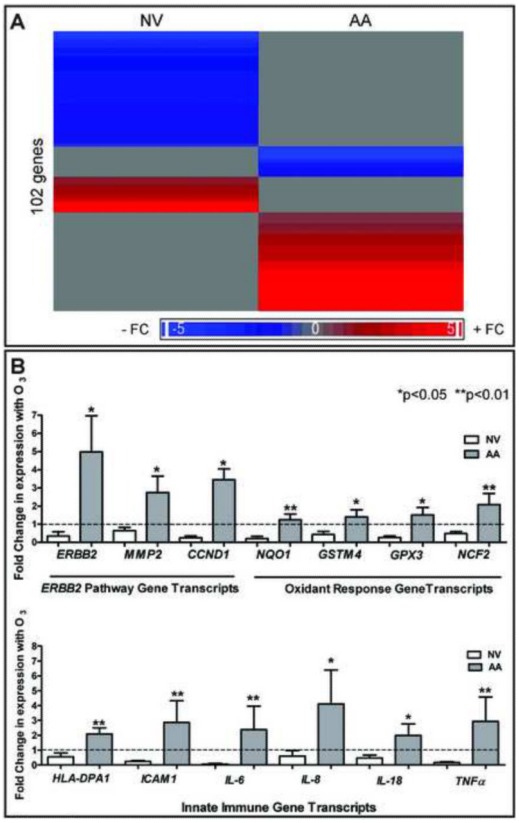

Figure 1. O_3-induced transcript profiles in NV and AA

A) Heat map of O_3-modulated genes in NV and AA with low (blue) and high (red) expression. **B)** Changes with O_3 in PCR-amplified ERBB2 pathway, oxidant response and innate immune transcripts relative to *ACTB* (*n*=6 NV, 8 AA). Mean changes in gene expression with O_3 (± SEM). Nonparametric t testing (Mann Whitney testing) was used to compare differences in NV and AA.

Table I

Characteristics of Participants

Clinical Characteristic	Normal Volunteer	Atopic Asthmatic
n	34	17
Age (y), mean (SD)	24.2 (3.9)	24.4 (5.5)
Gender	20 Female / 14 Male	10 Female / 7 Male
Race	27 White; 4 African American; 3 Asian	13 White; 3 African American 1 Asian

	Pre O_3	Post O_3	Pre O_3	Post O_3
Induced Sputum Cellularity				
% Neutrophils, Mean (SD)	40.9 (19.3)	64.3 (15.7)*	35.8 (19.4)	58.4 (23.4)*
% Eosinophils, Mean (SD)	0.12 (0.2)	0.5 (0.2)	1.95 (2)**	2.9 (4.7)
% Macrophages, Mean (SD)	55.6 (18.7)	32.1 (14.8)	59.6 (19.7)	34.2 (20.7)
Induced Sputum Cytokines (pg/ml sputum)				
IL-1β, Median (25%–75%)	208 (162–691)	221 (77–475)	648+ (244–1247)	1121++ (295–2018)
IL-8, Median (25%–75%)	4694 (1892–11373)	3622 (837–14071)	12496^ (8347–25669)	23278++ (7558–46020)
IL-6, Median (25%–75%)	110 (46–172)	123 (39–233)	32 (18–138)	139# (31–666)
IL-10, Median (25%–75%)	36 (0–307)	7 (0–240)	0** (0–6)	0 (0–53)

* p<0.001 comparing pre O_3 to post O_3 values

** p<0.01 comparing baseline values of AA to NV

+ p=0.03 comparing baseline values of AA to NV

++ p=0.03 comparing pre O_3 to post O_3 values

^ p=0.02 comparing baseline values of AA to NV

p=0.01 comparing pre O_3 to post O_3 values