
Development of an inhaled endotoxin challenge protocol for characterizing evoked cell surface phenotype and genomic responses of airway cells in allergic individuals

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Background: Environmental exposure to endotoxin is a known cause of exacerbation of asthma. Inhaled endotoxin protocols have been used to evaluate airway cell surface phenotypes associated with antigen presentation and innate immunity in healthy volunteers, but not in allergic volunteers.

Objectives: To establish the safety of challenge with low-dose endotoxin (10,000 endotoxin units) (lipopolysaccharide [LPS]) inhalation in allergic individuals, to measure airway cell surface phenotypes associated with antigen presentation and innate immunity in induced sputum (IS) after LPS challenge, and to conduct gene expression profiling in IS cells to determine which host genetic networks are modified by LPS inhalation.

Methods: Induced sputum was obtained before and 6 hours after LPS inhalation in 10 allergic volunteers (8 with asthma and 2 with rhinitis). Flow cytometry was used to examine cell surface phenotypes on IS cells. Genomic expression was analyzed on a subset of IS samples (n = 10) using microarray and ingenuity pathway analysis.

Results: A total of 10,000 endotoxin units of LPS induced significant up-regulation of membrane CD14, CD11b, CD16, HLA-DR, CD86, and Fcε receptor 1 on sputum phagocytes and increased expression of genes that influence antigen-presenting surface molecules (HLA-DR, chemokine ligand 2 or monocyte chemoattractant protein 1, v-rel reticuloendotheliosis viral oncogene homolog, prostaglandin-endoperoxide synthase 2 or cyclooxygenase 2, and transforming growth factor β), immune activation (CD14, interleukin 1β, and regulated upon activation, normal T cell expressed and secreted), and inflammation (intracellular adhesion molecule 1 and inhibitory κBα). Gene profiles for nuclear factor κB, interleukin 1, and tumor necrosis factor pathways were also significantly affected.

Conclusions: Low-dose inhaled endotoxin challenge is safe in allergic individuals with mild to moderate disease. It enhances airway cell surface phenotypes and expression of genes associated with antigen presentation, innate immunity, and inflammation. Microarray with ingenuity pathway analysis can be successfully applied to sputum cells to characterize genetic responses to inhaled exacerbants.

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INTRODUCTION

Environmental exposure to endotoxin is a known cause of exacerbation of asthma.^{1,2} It was previously reported that

challenge with doses of endotoxin similar to those that may be encountered environmentally is an adjuvant for subsequent response to allergens in the nasal³ and lower airways⁴ of allergic patients with asthma. Although the mechanisms by which endotoxin induces exacerbation of airway disease are incompletely understood, it is likely that innate immune activation and enhanced antigen presentation by airway monocytic cells are important in this process. Endotoxin challenge may be a useful tool for translational research of asthma exacerbation in human volunteers, especially in evaluating the role that innate immune processes have in mediating an

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asthma exacerbation. However, sound research practices require that inhalation challenge protocols be conducted according to Food and Drug Administration investigational standards in which the safety of the challenge doses is established in healthy and then asthmatic volunteers.

In previous studies of nonallergic healthy volunteers, it was reported that a relatively low dose (10,000 endotoxin units [EUs]) of inhaled endotoxin,⁵ vs inhalation studies using doses as high as 50 μ g (approximately 200,000 EUs),^{6,7} up-regulates membrane CD14 (mCD14) and CD11b or CR3 expression and phagocytosis of zymosan particles by airway macrophages. This dose also promotes a T_H2 type airway cytokine profile with increased interleukin (IL) 13 and decreased interferon γ (IFN- γ) levels in sputum. It has also been reported that a higher dose of endotoxin (20,000 EUs) that is associated with neutrophil influx causes an enhanced expression of dendritic cell (DC) surface molecules (CD80, CD86, and HLA-DR) on sputum mononuclear cells recovered from healthy volunteers.⁸ In allergic mouse models, induction of T_H2 responses by inhaled antigen after low-level endotoxin exposure also involves the activation of antigen-containing DCs.⁹ Together, these studies in healthy volunteers suggest that cell surface phenotypes associated with innate immune activation and antigen presentation may be modified after exposure to moderate levels of endotoxin.

In this pilot study, we applied an endotoxin inhalational challenge model^{5,8} (using clinical center reference endotoxin [CCRE] *Escherichia coli* O:113 obtained from the National Institutes of Health) to examine the effect of low-dose (10,000 EUs) CCRE on airway monocyte responses of allergic volunteers and allergic volunteers with asthma to first evaluate the safety of this challenge protocol in this population. We next tested the feasibility of using microarray analysis of messenger RNA (mRNA) recovered from induced sputum (IS) cells to conduct gene expression profiling and signaling pathway analysis using paradigms developed by ingenuity pathway analysis (IPA). We hypothesized that, in our patient group, inhalation of low-dose endotoxin (10,000 EUs) will result in modified airway cell surface phenotypes associated with antigen presentation, innate immune activation, and IgE-mediated response. We further hypothesized that inhaled lipopolysaccharide (LPS) will affect a number of biological pathways in the airways that regulate innate immunity, antigen presentation, and inflammatory processes. Ultimately, differences in such responses between healthy volunteers and patients with asthma can be used to identify novel molecular mechanisms that mediate asthma exacerbations.

METHODS

Subjects

Ten (4 men and 6 women) nonsmoking, allergic (8 with asthma and 2 with allergic rhinitis) volunteers between the ages of 19 and 48 years were recruited for the study. A medical screening examination that included a medical history positive for asthma or allergic rhinitis, a physical exam-

ination, blood tests, a methacholine challenge test (provocation concentration 20, <10 mg/mL for asthma), and an allergy skin prick test was completed for all subjects on a separate visit before the study. All subjects had a positive skin test result (wheal size, >4 mm vs the negative control [saline]) to house dust mite extract (*Dermatophagoides pteronyssinus* or *Dermatophagoides farinae*). All female subjects provided a urine sample for pregnancy testing, and a positive pregnancy test result concluded in exclusion from the study. All subjects had a forced expiratory volume in 1 second (FEV₁) of greater than 80% of predicted values for a population of similar height, weight, sex, age, and race. No subjects were receiving oral corticosteroid treatment during the study or within 3 months before study enrollment. The study was approved by the Committee on the Protection of the Rights of Human Subjects, UNC School of Medicine, The University of North Carolina at Chapel Hill, the General Clinical Research Center Ethics Review Board at The University of North Carolina at Chapel Hill, and the Food and Drug Administration under IND BB9998. Informed written consent was obtained from all subjects before participation in the study.

LPS Inhalation Challenge Protocol

This protocol was described previously in detail.^{5,8} In brief, the study protocol consisted of 3 consecutive days, where day 1 consisted of baseline measurements of spirometry, venesection for complete blood cell count analysis, vital signs, symptom score, and IS end points. On day 2, baseline measurements were repeated immediately before inhalation challenge. Endotoxin inhalation was performed using an ultrasonic nebulizer (DeVilbiss Ultraneb 99; DeVilbiss, Heston, England) for 10 minutes at tidal breathing. Endotoxin (10,000 EUs) was provided by the National Institutes of Health Clinical Center, is referred to as CCRE, and was extracted from *E coli* O:113 (US standard reference endotoxin). Endotoxin (10,000 EUs) was reconstituted in 5 mL of sterile water for injection, US Pharmacopeia (preservative free). Spirometry was performed 1 time each hour for 6 hours after the inhalation challenge. Venesection for complete blood cell count analysis and IS was performed 6 hours after the inhalation challenges. All subjects were observed overnight in the clinical unit of the General Clinical Research Center at The University of North Carolina Hospitals. Follow-up spirometry, vital sign measurements, and symptom scores were recorded on the following morning (day 3).

IS, Differential Cell Counts, and Fluid-Phase Analysis

Sputum induction and processing were performed according to previously published methods.^{5,8,17} In brief, subjects inhaled increasing concentrations (3%, 4%, and 5%) of hypertonic saline for 7 minutes each, for a total of 21 minutes. Manual selection of plug material was weighed and treated with 0.1% dithiothreitol (DTT) for cell dispersion. Differential cell counts were analyzed from Romanowski (Diff-Quik)–stained slides, based on 400 cells, and expressed as a per-

centage of total nonsquamous nucleated cells. Acceptable slides had a minimum of 50% viability (trypan blue staining) and less than 20% squamous epithelial cell content. Fluid-phase components were analyzed using bead-based multiplex assays (Luminex¹⁰⁰ total system; Luminex Corp, Austin, Texas): IL-1 receptor antagonist (IL-1Ra), IL-4, IL-6, IL-8, IL-12p40, IL-13, granulocyte-macrophage colony-stimulating factor, macrophage inflammatory protein 1 α , macrophage inflammatory protein β , IL-10, monokine induced by IFN- γ , eotaxin, and monocyte chemoattractant protein (MCP) 1. To control for the effect of DTT on the analytes measured, a standard curve was also generated with 0.1% DTT. Comparison to the normal standard curve (no DTT) showed that some analytes were affected by the presence of DTT; as a result, all analyte values were taken and reported from the standard curve with DTT. Other cytokines that were analyzed had several missing values that were below the lower limit of detection of the assay and were not included in the analysis. These included IL-1, tumor necrosis factor (TNF) α , IL-5, and IFN- γ .

Flow Cytometry and Immunofluorescent Cell Surface Phenotype Staining

Flow cytometry was performed using a sorter (FACSort; Becton Dickinson, Franklin Lakes, New Jersey), as previously described.^{5,8} Macrophages, monocytes, neutrophils, and lymphocytes in sputum were identified and gated based on light scatter properties and positive expression for CD45 (pan leukocyte marker), HLA-DR or CD14 (macrophages or monocytes), CD16 or Fc γ receptor 3 (neutrophils), and CD3 (lymphocytes). Dendritic cell features were further analyzed by examining B7 costimulatory molecules CD80 and CD86. Surface receptors for low (CD23) and high (Fc ϵ receptor 1) IgE affinity were measured along with CD11b or CR3 and CD64 or Fc γ receptor 1. Analysis was performed using computer software (Cell Quest; Becton Dickinson), and receptor expression was given as mean fluorescence intensity (MFI).

Phagocytosis Assay

Phagocytosis of fluorescein isothiocyanate conjugated-labeled IgG-opsonized *Saccharomyces cerevisiae* zymosan A bioparticles (Molecular Probes Inc, Eugene, Oregon) was performed, as previously described.^{5,8} All samples were analyzed by flow cytometry within 24 hours of fixation, and particle uptake was identified as a rightward shift in fluorescence into the zymosan-exposed region from the control (non-zymosan-exposed region).

RNA Preparation and Probe Labeling

RNA was prepared on a subset of sputum samples ($n = 10$) in which adequate cells were available for analysis. No other samples had adequate cells remaining for microarray analysis. Sputum samples were homogenized in reagent (Trizol; Invitrogen, Carlsbad, California), and total RNA was purified according to manufacturer's instructions. RNA integrity was evaluated by comparing 18S and 28S ribosomal RNA species

using gel electrophoresis or a bioanalyzer (Agilent Technologies, Inc, Santa Clara, California). Fluorescent-labeled Cy5 (red) amplified RNA (aRNA) sample probes were prepared by reverse transcription with a reverse transcriptase (SuperScriptIII; Invitrogen), 1 round of amplification with amino-allyl nucleotides according to manufacturer's protocol (Epicenter Biotechnologies, Madison, Wisconsin), and coupling to Cy5 dye (GE Healthcare, Piscataway, New Jersey). Likewise, a Cy3 (green)-labeled aRNA probe was prepared using the universal human reference RNA composed of RNA pooled from 10 human cell lines (Stratagene, La Jolla, California). Each Cy5 sample aRNA probe was paired with a Cy3 reference aRNA probe and hybridized to microarrays in formamide-containing buffer overnight at 42°C using a system (Maui Hybridization System; BioMicro Systems Inc, Salt Lake City, Utah). For this study, expression profiling was completed for 4 pre-CCRE sputum samples and 6 post-CCRE samples.

Microarray Hybridization

The custom-designed microarray consisted of 70-mer oligonucleotides representing 628 inflammation-related gene targets. Commercially available and custom-designed oligos were purchased from Operon Biotechnologies Inc (Huntsville, Alabama) and printed onto glass slides by the Duke Microarray Facility (Duke University, Durham, North Carolina). Each microarray slide contained replicate arrays in which each target was spotted 4 times (up to 8 replicates per gene target). The gene target pool also included housekeeping genes used as the reference to normalize signals between slides, negative controls, and spiked RNA oligos (Applied Biosystems, Austin, Texas). The hybridized spot intensities were acquired using a scanner (GenePix 4000B; Molecular Devices Corp, Sunnyvale, California) and quantified using software (GenePix Pro 6.0; Molecular Devices Corp). Subsequently, the intensities were normalized, filtered, and assembled into data sets using a software package (GeneSpring 7; Agilent Technologies Inc). Last, the data sets of normalized expression values plus their associated gene identifiers were uploaded into IPA software (Ingenuity Systems, Redwood City, California) to generate biological networks. This was done by mapping values and gene identifiers (GenBank accession) to their corresponding gene objects in the pathways knowledge base (Ingenuity Systems) developed from published sources.

Data Analysis

Differences between pre-CCRE and post-CCRE 10,000-EU inhalation in cell surface phenotypes and differential cell counts were tested using nonparametric paired t tests (Wilcoxon signed rank test), with the overall α level set at .05. Data are presented as mean $\pm$ SEM unless otherwise noted. Correlation analysis was performed using the Pearson correlation coefficient (R) with the α level set at .05. For genomic analyses, a right-tailed Fisher exact test was used to produce

a significance *P* value that described how likely genes from a specific data set participated in a desired function.

RESULTS

Subject demographic data are as follows. Their mean $\pm$ SEM age was 25.7 ± 2.5 years. There were 7 white and 3 African American subjects. Their predicted mean $\pm$ SEM FEV₁ was $106.2\% \pm 3.8\%$ (range, 85%–125%). The allergic subjects with asthma had mild well-controlled disease, and all used β -agonist therapy on an as-needed basis. One asthmatic subject was receiving inhaled corticosteroid treatment, and one was receiving nasal corticosteroid (fluticasone propionate [Flonase]; GlaxoSmithKline, Research Triangle Park, North Carolina) treatment. All 10 allergic subjects were sensitive to house dust mite (skin test positive result to *D pteronyssinus* or *D farinae*) and tolerated the IS procedure without incident. No adverse events or changes in vital signs (heart rate, respirations, oxygen saturation levels, or blood pressure) were observed at any time.

Lung Function, Symptoms, and Blood Variables

We measured lung function (spirometry) immediately before and once an hour for 6 hours after CCRE inhalation. Compared with pre-CCRE inhalation, we observed no significant (*P* = .34) change in lung function (percentage predicted FEV₁, forced vital capacity, and forced vital capacity–FEV₁ ratio) at any post-CCRE time point. Symptoms such as malaise, fatigue, headache, shortness of breath, cough, chills, and fever were recorded in symptom diaries before and after CCRE inhalation. There was no significant (*P* = .44) increase in the frequency or severity of any of the recorded symptoms for any subject after CCRE inhalation. Before and after CCRE blood samples were obtained and examined for white blood cell count and differential leukocyte count (neutrophils, monocytes, eosinophils, and lymphocytes). Clinical center reference endotoxin inhalation resulted in no significant (*P* = .39) changes in white blood cell or blood leukocyte differential for any subject.

Sputum Differential Cell Counts

Differential cell counts (neutrophils, macrophages, and eosinophils) before and after CCRE inhalation are reported in Table 1. As expected, the percentage of neutrophils was statistically significantly increased after CCRE inhalation,

with a corresponding decrease in the percentage of macrophages. No statistically significant changes were observed, however, in the absolute number of cells per milligram of sputum for any of the examined cell types.

Fluid-Phase Components and Correlations With the CCRE-Induced Neutrophil Response

Secreted cytokines were measured to examine the specificity of cellular responses to CCRE challenge. These included IL-4, IL-5, IL-8, IL-13, IP10, IL-12 p40, granulocyte-macrophage colony-stimulating factor, eotaxin, and MCP-1. Interleukin 1Ra, a potent anti-inflammatory cytokine, was statistically significantly reduced after CCRE inhalation (mean $\pm$ SEM, $67,142 \pm 14,932$ ng/mL vs $94,072 \pm 23,838$ ng/mL; *P* < .05). No other cytokines were significantly changed before vs after CCRE inhalation. Some cytokines, including IL-1 and TNF- α , had several nondetectable values and, therefore, were not included in the analysis. Several cytokines at baseline (pre-CCRE inhalation) were predictive of the neutrophil response (percentage of polymorphonuclear leukocytes) after CCRE inhalation, as demonstrated by the Pearson correlation coefficient. These statistically significant (*P* < .05) correlations included IL-6 (*r* = 0.80), IL-8 (*r* = 0.60), macrophage inflammatory protein 1 β (*r* = 0.90), and MCP-1 (*r* = 0.80).

Cell Surface Markers

As shown in Figure 1A-F, we analyzed cell surface phenotypes on sputum cells (monocytes, macrophages, and neutrophils) associated with innate immunity and inflammation (CD11b or CR3, mCD14, CD16 or Fc γ receptor 3, and CD64 or Fc γ receptor 1), the DC phenotype (CD86 and CD80), antigen presentation (HLA-DR), and IgE activity (Fc ϵ receptor 1). After CCRE inhalation, we observed a statistically significant increase in the expression of CD11b or CR3 (*P* = .01), mCD14 (*P* = .004), CD16 or Fc γ receptor 3 (*P* = .04), CD86 (*P* = .05), HLA-DR (*P* = .01), and Fc ϵ receptor 1 (*P* = .04) (Figure 1A-F, respectively). CD23 (*P* = .22), CD64 or Fc γ receptor 1 (*P* = .34), and CD80 (*P* = .69) were not significantly changed after CCRE inhalation (data not shown), and limited numbers of CD3⁺ lymphocytes in sputum precluded the measurement of CD4⁺ T lymphocytes.

Table 1. Sputum Differential Cell Count^a

CCRE exposure	Polymorphonuclear neutrophils		Eosinophils		Macrophages	
	%	No./mg ^b	%	No./mg ^b	%	No./mg ^b
Before	26 $\pm$ 4.6	1529 $\pm$ 1161	1 $\pm$ 0.5	10 $\pm$ 3	64 $\pm$ 7.2	1697 $\pm$ 1113
After	47 $\pm$ 4.8 ^c	2601 $\pm$ 2203	2 $\pm$ 0.9	57 $\pm$ 43	50 $\pm$ 7.2 ^c	1139 $\pm$ 718

Abbreviation: CCRE, clinical center reference endotoxin.

^a Data are given as mean $\pm$ SEM.

^b Indicates milligram per wet weight of sputum.

^c *P* < .05 vs before CCRE exposure.

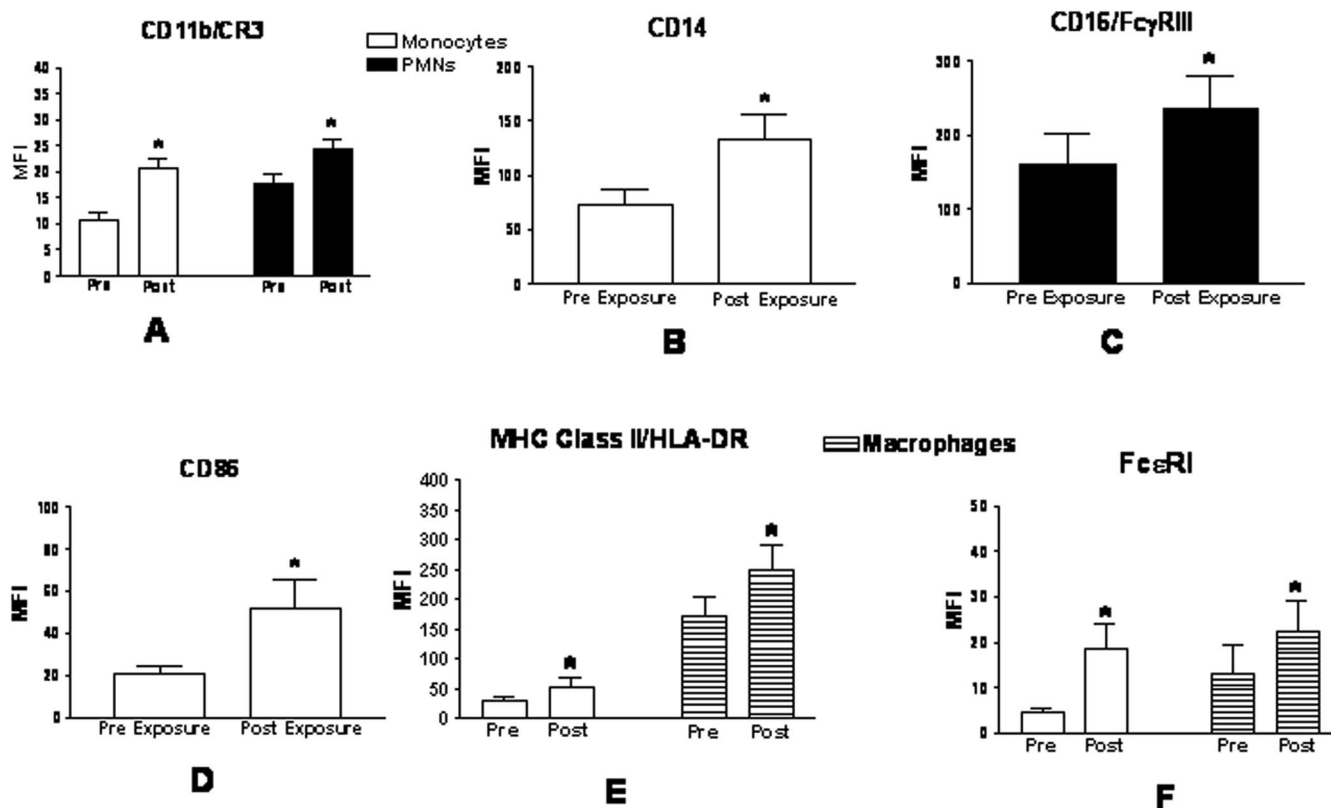


Figure 1. Cell surface phenotype expression (mean fluorescence intensity [MFI]) for CD11b or CR3 (A), mCD14 (B), CD16 or Fcγ receptor 3 (C), CD86 (D), major histocompatibility complex class II or HLA-DR (E), and Fcε receptor 1 (F) pre- and post-clinical center reference endotoxin (CCRE) inhalation exposure. All cell surface phenotypes showed significantly increased expression with CCRE inhalation ($P < .05$). PMN indicates polymorphonuclear leukocyte.

Phagocytosis

Phagocytic activity in IS samples was examined to investigate the functional state of phagocytic cells resulting from CCRE challenge. No statistically significant change in phagocytosis after CCRE inhalation was observed on any cell type (macrophages, 624 ± 109 MFI vs 1015 ± 224 MFI [$P = .69$]; monocytes, 293 ± 46 MFI vs 368 ± 41 MFI [$P = .31$]; and neutrophils, 290 ± 38 MFI vs 310 ± 31 MFI [$P = .72$]).

Profiling of Factors Involved in Immune Cell Activation and Antigen Presentation

To broadly profile differentially regulated genes because of exposure to inhaled CCRE, microarray analysis was performed on 10 available sputum samples before ($n = 4$) and 6 hours after ($n = 6$) CCRE inhalation. Table 2 provides normalized ratios (to human universal reference) of RNA expression of several genes associated with immune activation before and after CCRE exposure. In particular, transcripts encoding cell surface proteins HLA-DRA, HLA-DPA, CD14, CD32 (Fcγ receptor 2B), intracellular adhesion molecule 1, and TNF superfamily, member 1A, were elevated more than 2-fold in allergic subjects before vs after CCRE exposure. To confirm and

explore biological networks altered with exposure to CCRE, expression profile data sets were subjected to IPA. Figure 2 shows the antigen presentation network, including the cell surface proteins previously described and demonstrating additional up-regulated factors (expression of genes normalized to human universal reference), such as IL-1β, chemokine ligand 5 or regulated upon activation, normal T cell expressed and secreted (RANTES) chemokine ligand 2 or MCP-1, v-rel reticuloendotheliosis viral oncogene homolog (RELB), prostaglandin-endoperoxide synthase 2 or cyclooxygenase 2, and transforming growth factor β, that influence antigen-presenting surface proteins after CCRE exposure.

Profiling of Inflammatory Mediators in the Lung

Figure 3 shows a network of genes associated with airway inflammation. Normalized expression of several genes was markedly enhanced with CCRE exposure. For example, expression of IL-1β, a key regulator of innate immunity, was up-regulated 16-fold in our allergic cohort exposed to CCRE. Other genes were also enhanced (2- to 6-fold) after CCRE exposure, including caspase 1, heme oxygenase 1, intracellular adhesion molecule 1, B-cell lymphoma 6, and RELB.

Table 2. Genomic Profiling of Expression of Immune Cell Activation Targets

Name	Pre-CCRE ^a	t Test P value ^b	Post-CCRE ^a	t Test P value ^b	Fold change for post-CCRE vs pre-CCRE
CD14	2.23	<.001	100.50	<.001	45.07
IL-1 β	12.57	<.001	200.99	<.001	15.99
CCL5 (RANTES)	2.14	.11	15.77	<.001	7.37
CCL2 (MCP1)	0.74	.26	3.88	<.001	5.24
HLA-DRA	71.39	<.001	348.67	<.001	4.88
HLA-DPA1	34.23	<.001	158.65	<.001	4.64
ICAM1	0.72	.01	3.09	<.001	4.31
Fc γ receptor 2B (CD32)	5.11	<.001	20.43	<.001	4.00
RELB	2.43	<.001	7.07	<.001	2.91
TNFRSF1A	1.45	.03	2.95	<.001	2.04
PTGS2	5.00	.02	8.85	<.001	1.77
CCL3 (MIP1 α)	35.83	<.001	61.88	<.001	1.73
TGF- β 1	1.58	.007	2.66	<.001	1.69
LTB	16.45	<.001	21.46	<.001	1.30
IL-10	8.24	<.001	10.31	<.001	1.25
CAMP (CAP18)	11.31	<.001	9.20	<.001	-1.23

Abbreviations: CAMP, cathelicidin antimicrobial protein; CCL, chemokine ligand; CCRE, clinical center reference endotoxin; ICAM, intracellular adhesion molecule; IL, interleukin; LTB, lymphotoxin β ; MCP, monocyte chemoattractant protein; PTGS2, prostaglandin-endoperoxide synthase 2; RANTES, regulated upon activation, normal T cell expressed and secreted; RELB, v-rel reticuloendotheliosis viral oncogene homolog; TGF, transforming growth factor; TNFRSF1A, tumor necrosis factor receptor superfamily, member 1A.

^a Normalized ratios of RNA expression compared with human universal reference.

^b The *t* test indicates significant agreement of expression values between biological replicates for either the pre-CCRE (*n* = 4) or post-CCRE (*n* = 6) group.

Profiling of Factors Involved in Nuclear Factor κ B Modulation of Innate Immunity

Because nuclear factor κ B (NF- κ B) is known to regulate a variety of downstream targets and biological processes, including inflammation, we focused our analysis on the expression of NF- κ B signaling components. As given in Table 3, expression of initiating factors (eg, IL-1 β and TNF receptor superfamily, member 1A) and downstream regulatory components (NF- κ B1 or p105, NF- κ BIA or inhibitory κ B α , and RELB) was enhanced after CCRE exposure. Other signaling mediators, such as AKT1 (murine thymoma viral [v-akt] oncogene homolog 1), IRAK3 (IL-1 receptor–associated kinase 3) or IRAKM (IL-1 receptor–associated kinase M), TAB1 (TAK1-binding protein), and I κ B kinase changed less than 2-fold with CCRE, whereas expression of the noncanonical NF- κ B component NF- κ B2 or p100 was reduced more than 2-fold with CCRE exposure. Ingenuity pathway analysis (Fig 4) showed that the overall expression of the NF- κ B network of regulatory factors was increased after exposure to CCRE. This supports our finding that exposure to even a low dose of CCRE up-regulates inflammatory activity in allergic patients.

DISCUSSION

In the present pilot study, we demonstrated that inhalation challenge with 10,000 EUs of endotoxin is a safe dose in allergic individuals with mild to moderate disease severity. We further observed that 10,000 EUs of CCRE inhalation

challenge induces increased expression of airway cell surface phenotypes associated with innate immunity (CD11b or CR3 and mCD14), antigen presentation (CD86, HLA-DR, and Fc ϵ receptor 1), and inflammation (CD16 or Fc γ receptor 3) in allergic volunteers. Furthermore, microarray analysis on sputum samples, which derive from the local surfaces of the central airways,¹⁰ revealed corresponding changes in a number of genes associated with innate immunity, antigen presentation, and inflammation, with notable increases in genes for mCD14, IL-1 β , and major histocompatibility complex class II molecules (HLA-DR). Furthermore, we demonstrated the feasibility of using IPA with our gene array data and found that low-level endotoxin inhalation induced significant increases in expression of gene networks associated with the innate immune antigen presentation and inflammation pathways. Sputum samples contain an admixture of cell types, mainly macrophages and neutrophils, that can contribute to the mRNA signal, with macrophages contributing more RNA than neutrophils. Although we cannot completely rule out the possibility that the altered genetic expression we observed after CCRE exposure was related to cell composition rather than LPS-induced activation, it is unlikely because the total number and proportion of macrophages was actually lower after vs before CCRE exposure, despite our observed increases in expression of several genes of interest.

Within the inflammation gene network, caspase 1, heme oxygenase 1, and B-cell lymphoma 6 were each modified by CCRE challenge, indicating their potential importance to the

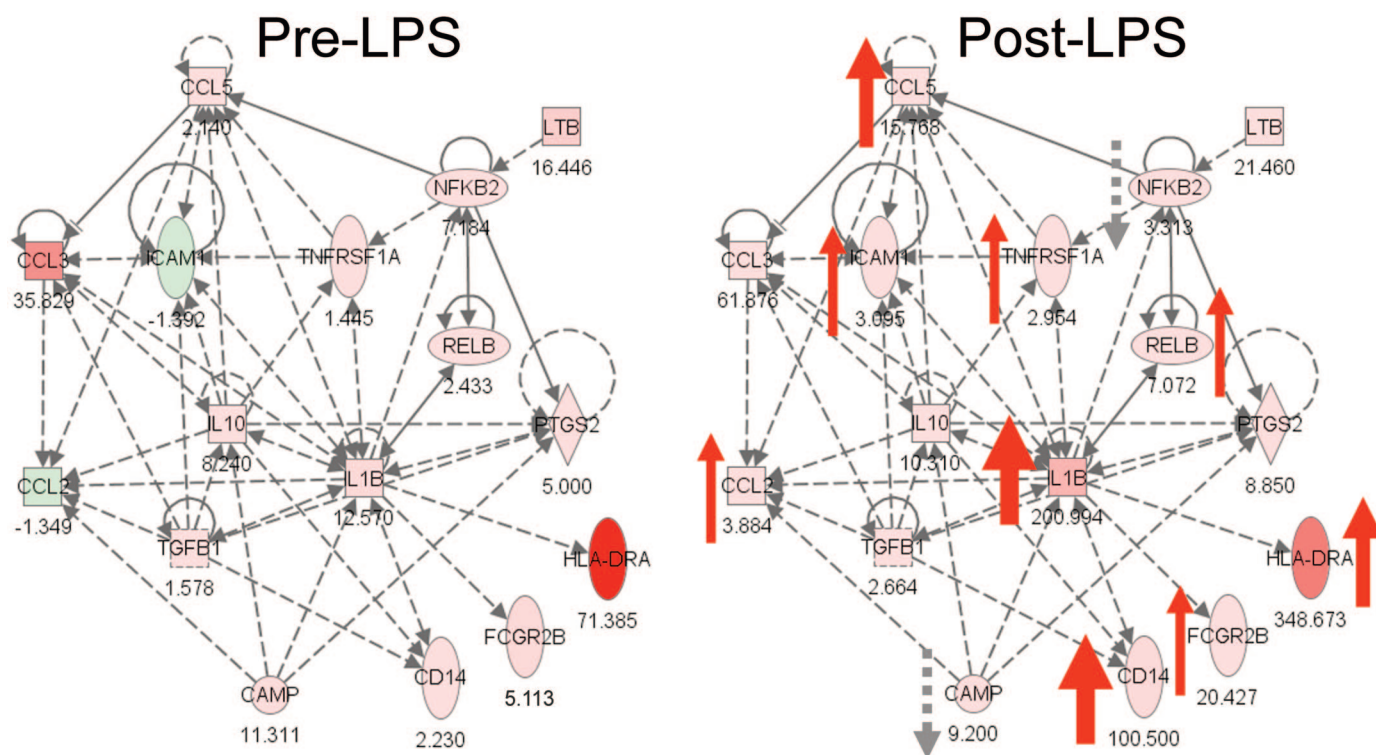


Figure 2. Normalized (to human universal reference) gene expression profile associated with activation of antigen presentation in sputum cells before (n = 4) (A) vs after (n = 6) (B) clinical center reference endotoxin (CCRE) exposure. The collection of gene products (represented as nodes) found in this network is significantly ($P < .001$) up-regulated vs human universal reference. Biological relationships between nodes are direct (solid line) or indirect (dotted line). The color of the node indicates regulation, with red being up-regulated and green being down-regulated, with corresponding normalized values listed below each gene symbol. The shape of the node indicates the type of target: square (cytokine), diamond (enzyme), triangle (kinase), trapezoid (protease), oval (transmembrane receptor), and circle (other). The arrows indicate changes in expression with CCRE exposure: thick solid up arrows, increased expression; and dotted down arrows, decreased expression. LPS indicates lipopolysaccharide.

CCRE-induced airway inflammatory response. Caspase 1, in particular, which showed a 6-fold increase with CCRE challenge, has been important in resolving LPS-induced acute lung injury.¹¹ Our genomic profiling analysis demonstrated an overall increase in the NF- κ B network of regulatory and initiating factors after exposure to CCRE, with a 3-fold notable increase in expression of the NF- κ BIA (I κ B α) gene. This was coupled with a significantly decreased concentration of the potent anti-inflammatory cytokine IL-1Ra. Loss of an IL-1Ra response in asthma may facilitate increased inflammation, with exacerbants such as rhinovirus leading to acute disease exacerbation. Furthermore, evidence suggests that the balance between proinflammatory IL-1 β and anti-inflammatory IL-1Ra may be more important than the individual cytokine levels themselves in modulating airway inflammation in asthma.^{12,13} If IL-1Ra plays a role in controlling allergen-induced inflammation, then reduction of this protein by endotoxin might contribute to the enhancement of allergen-induced inflammation by LPS.

When we examined surface phenotypes of sputum mononuclear phagocytes, we found enhanced expression of

HLA-DR and the B7 costimulatory molecule CD86, with no concomitant significant increase in phagocytosis. These features are consistent with a DC phenotype, and although we did not directly measure T-cell responses, it is possible that these changes may promote antigen presentation capability that can lead to increased T-cell activity and recall responses. We plan to implement antigen presentation assays in future studies to determine whether changes in DC surface molecules after endotoxin inhalation correspond to increased T-cell activity.

The other interesting finding was the increased expression of Fc ϵ receptor 1 (high-affinity IgE receptor) on airway monocytes and macrophages after CCRE inhalation. Monocytes, macrophages, and DCs express Fc ϵ receptor 1 on their surface, but whereas mast cells and basophils express the Fc ϵ receptor 1 β subunit, monocytic cells express only the $\alpha\gamma_2$ species.^{14,15} Relative to mast cells, monocytes and macrophages express lower densities of Fc ϵ receptor 1, but monocyte Fc ϵ receptor 1 expression has been up-regulated in atopic vs nonatopic individuals.¹⁵ Furthermore, the activation state of the monocyte determines the functionality of Fc ϵ receptor 1. Indeed, our observation of increased expression of

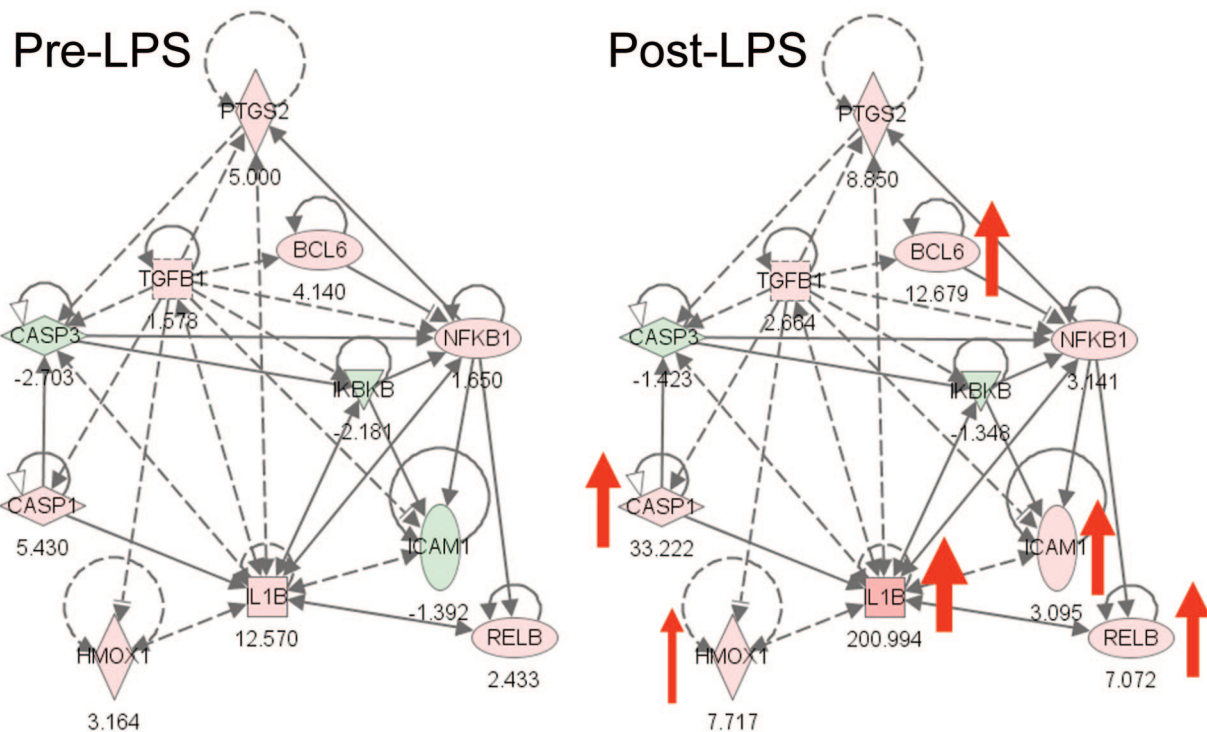


Figure 3. Normalized gene expression profile associated with inflammation in the lung before (A) vs after (B) clinical center reference endotoxin (CCRE) exposure. Known inflammatory mediators in the lung comprise this inflammation network, which are significantly ($P < .001$) up-regulated compared with human universal reference. Solid up arrows represent up-regulated expression (>2-fold) after vs before CCRE exposure. LPS indicates lipopolysaccharide.

Table 3. Genomic Profiling of Expression of NF- κ B Signaling Components

Name	Pre-CCRE ^a	<i>t</i> Test <i>P</i> value ^b	Post-CCRE ^a	<i>t</i> Test <i>P</i> value ^b	Fold change for post-CCRE vs pre-CCRE
NF- κ BIA (I κ B α)	3.52	<.001	11.17	<.001	3.18
TNFAIP3 (A20)	3.05	.004	9.69	<.001	3.18
IL-1R2	11.07	<.001	30.74	<.001	2.78
TLR1	4.50	.02	12.01	<.001	2.67
TLR8	12.37	<.001	26.84	<.001	2.17
NF- κ B1 (p105)	1.65	<.001	3.14	<.001	1.90
IKBKB	0.46	<.001	0.74	.002	1.62
IRAK1	0.51	.005	0.67	.002	1.30
IRAK3	11.36	<.001	13.01	<.001	1.15
MAP3K7IP1 (TAB1)	0.38	.01	0.53	<.001	1.14
AKT1	0.50	<.001	0.37	<.001	-1.22
NF- κ B2 (p100)	7.18	<.001	3.31	<.001	-2.17

Abbreviations: AKT1, murine thymoma viral oncogene homolog 1; CCRE, clinical center reference endotoxin; IKBKB, inhibitor of κ light polypeptide gene enhancer in B cells, kinase B; IL-1R2, interleukin 1 receptor 2; IRAK1, interleukin 1 receptor-associated kinase 1; MAP3K7IP1, mitogen-activated protein kinase kinase 7 interacting protein 1; NF- κ B, nuclear factor κ B; TAB1, TAK1-binding protein; TLR1, toll-like receptor 1; TNFAIP3, tumor necrosis factor α -induced protein 3.

^a Normalized ratios of RNA expression compared with human universal reference.

^b The *t* test indicates significant agreement of expression values between biological replicates for either the pre-CCRE ($n = 4$) or post-CCRE ($n = 6$) group.

Fc ϵ receptor 1 after CCRE challenge occurred in conjunction with increased monocyte expression of CD11b or CR3 and mCD14, both markers of monocyte activation. Likewise, we

observed that pre-CCRE mCD14 expression was positively correlated with monocyte HLA-DR expression after CCRE inhalation ($r = 0.80$, $P < .05$), indicating again that the

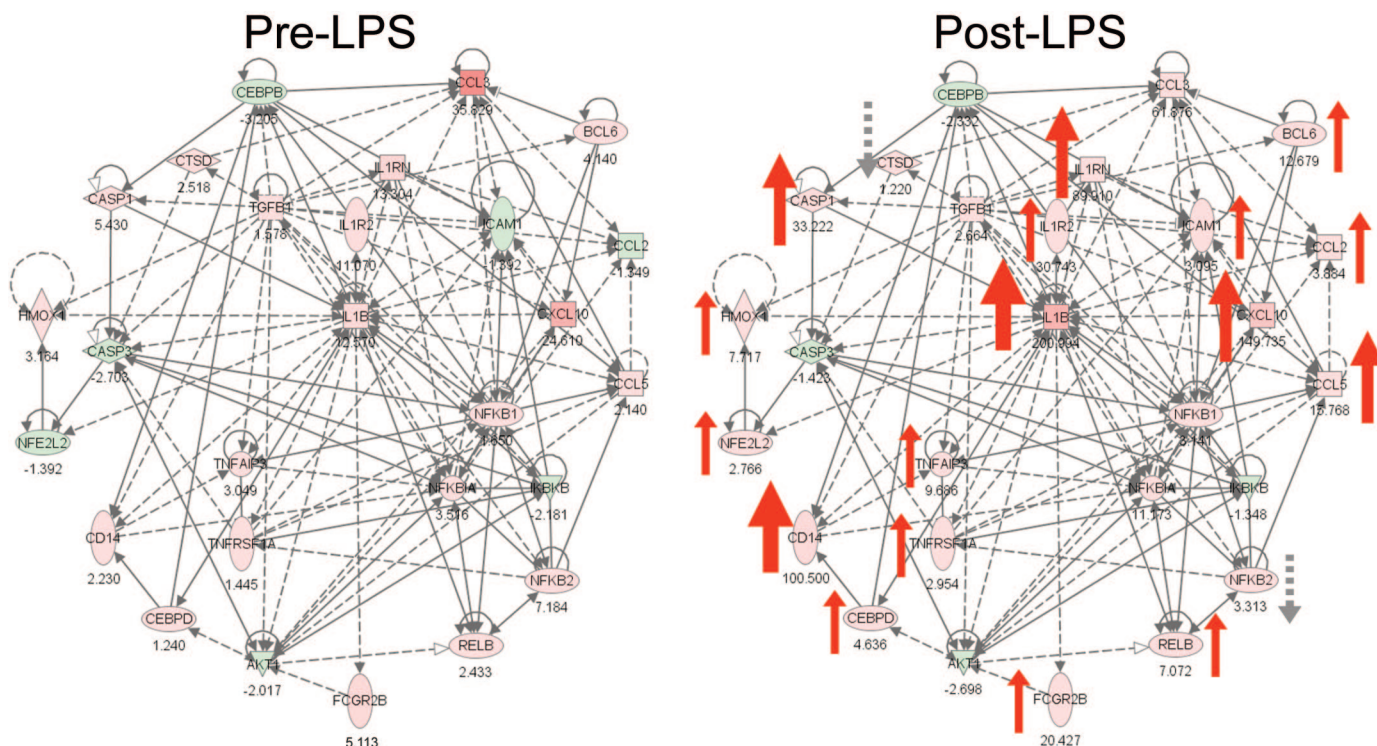


Figure 4. Normalized gene expression profile of inflammatory factors associated with cell signaling, downstream targets, and biological regulators before (A) vs after (B) clinical center reference endotoxin (CCRE) exposure. These networks of inflammatory factors are significantly ($P < .001$) up-regulated compared with their human universal reference. Solid up arrows represent up-regulated expression (>2 -fold) after vs before CCRE exposure. LPS indicates lipopolysaccharides.

activation state of the monocyte before CCRE stimulation plays an important role in antigen presentation. Levels of inflammatory cytokines (ie, IL-8 and IL-6) before CCRE were also positively correlated with the magnitude of the neutrophil response after CCRE inhalation, suggesting that higher baseline airway inflammation may modify or prime responses to subsequently inhaled CCRE. To our knowledge, this is the first report of increased Fc ϵ receptor 1 expression (together with increased CD86 and HLA-DR expression) on airway (sputum) monocytes and macrophages in vivo in allergic individuals after inhaled endotoxin. It is interesting to speculate whether increased Fc ϵ receptor 1 expression on airway monocytes plays a role in reported observations of increased sensitivity of allergic patients with asthma to inhaled allergen after exposure to LPS.⁴ Our data may offer some initial insight showing that, in allergic individuals, endotoxin-driven IgE-mediated allergic processes, together with enhanced antigen presentation in the airways, may act synergistically with traditional mast cell and basophil-mediated responses. Altogether, these processes may lead to increased air pollutant-induced morbidity in the allergic population.

Finally, to increase our understanding of the mechanisms and factors involved in allergic airway responses after inhaled LPS, we investigated the biological significance of genomic profiling of sputum cells using IPA. Although our array data

were based on a relatively small cohort of subjects and were not compared with a nonallergic cohort, this is the first report, to our knowledge, of the successful application of these techniques to recovered sputum cells in humans. One limitation of mRNA analysis in sputum cells is the heterogeneous population of cells present in the recovered sample. To this end, methods for isolating macrophages from airway sputum samples without cell activation have recently been examined and successfully applied.¹⁶ For our future studies, we will recover mRNA from purified cell types in sputum such that a more refined resolution of molecular events initiated in airway cells by endotoxin can be achieved. This pilot study, however, demonstrates a basic first step in genomic profiling of sputum cells in which a powerful evaluation tool can be applied to the measurement of inflammatory responses to environmental stimuli, particularly those that induce disease exacerbation in allergic individuals. Indeed, gene expression profiling may be a sensitive tool to evaluate biomarkers of response to real-life levels of environmental pollutants, such as endotoxin, particulates, and ozone.

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