

Effects of Allergen Challenge on Airway Epithelial Cell Gene Expression

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Allergen exposure induces the airway epithelium to produce chemoattractants, proallergic interleukins, matrix-modifying proteins, and proteins that influence the growth and activation state of airway structural cells. These proteins, in turn, contribute to the influx of inflammatory cells and changes in structure that characterize the asthmatic airway. To use the response of the airway epithelium to allergen to identify genes not previously associated with allergic responses, we compared gene expression in cytokeratin-positive cells before and after segmental allergen challenge. After challenge with concentrations of allergen in the clinically relevant range, 755 (6%) of the detectable sequences had geometric mean fold-changes in expression, with 95% confidence intervals that excluded unity. Using a prospectively defined conservative filtering algorithm, we identified 141 sequences as upregulated and eight as downregulated, with confirmation by conventional polymerase chain reaction in all 10 sequences studied. Using this approach, we identified asthma-associated sequences including interleukin (IL)-3, IL-4, and IL-5 receptor subunits, the p65 component of nuclear factor- κ B, and lipocortin. The genomic response of the human airway to concentrations of allergen in the clinically relevant range involves a greater number of genes than previously recognized, including many not previously associated with asthma that are differentially expressed after airway allergen exposure.

Keywords: asthma; genomics; lung disease; segmental allergen challenge

Asthma is a serious health problem that has a significant and growing impact on society and is associated with substantial morbidity, mortality, and economic cost (1). Asthma symptoms account for 130 million days of restricted activity among Americans, and exposure to allergen is one of the most common triggers of these symptoms (2). Much of our knowledge of the biology of this disease has been derived from studies of airway inflammatory cells and bronchial smooth muscle; by contrast, the full scope of the response of the airway epithelium to allergen has been underappreciated. This polarized, stratified structure of basal, columnar ciliated, and secretory cells was once believed to serve primarily a barrier function. However, the anatomic position of airway epithelial cells below the gas-airway lining fluid interface makes them the first cell type to be exposed to inhaled allergen, and there is increasing evidence that this epithelium responds to environmental stimuli by signaling to and otherwise interacting with cells of the innate and adaptive

immune systems (2). In the absence of disease, the bronchial epithelium is involved in mucosal defense by secreting cytoprotective molecules and contributing to biofilms that trap and remove inhaled debris, toxins, and allergens. On exposure to allergen, however, activation of mast cells, basophils, eosinophils, and other allergen-responsive cells in the airway perturbs the resting state of airway epithelial cells and induces within them changes in the expression of a number of stress-response mediators, including tumor necrosis factor- α , interleukin-1 β (IL-1 β), nuclear factor- κ B activator proteins, signal transducer and activator of transcription-6 (STAT-6), heat shock proteins, and cyclin-dependent kinase inhibitor p21^{waf} (3–5). Comparisons of the differential expression of these activation markers in epithelial cells have led to important advances in our understanding of the roles of the pathways associated with these markers in asthma pathogenesis.

In addition to responding to allergens and to the activation of inflammatory cells, under some circumstances, functional characteristics of the airway epithelium itself are sufficient to create key features of the asthma phenotype. For example, selective reconstitution of responsiveness to the proallergic cytokine IL-13 in the epithelium allows the expression of bronchial smooth muscle hyperresponsiveness and mucus hypersecretion after IL-13 exposure (6). Thus, a better understanding of the changes in airway epithelial cells after routine allergen exposure is fundamental to our ability to design novel preventive strategies, diagnostic tools, and therapies (7).

Despite increasing evidence that airway epithelial cell activation plays an important role in the response to allergens and the asthma phenotype, the breadth of the genomic response of basal and columnar ciliated airway epithelial cells to concentrations of allergen in the clinically relevant range for humans has not been fully explored. Segmental allergen challenge can provide information on the effects of such concentrations of allergen on airway epithelial cell activation. Furthermore, the ability to measure the effect of environmental stressors on gene expression has expanded considerably with the advent of cDNA microarray technology. For example, use of this technology to measure changes in expression of RNA sequences has been shown to provide useful and unexpected insights into cellular responses to environmental stimuli (8–11). This study reports the results of studies using cDNA microarrays to identify *in vivo* responses of cytokeratin-positive airway epithelial cells to segmental allergen challenge. This study tests the hypothesis that segmental airway challenge produces changes in gene expression that involves both known mediators of the asthma phenotype as well as other molecules not previously associated with asthma.

METHODS

General

This is a study of mRNA expression by airway epithelial cells that were obtained bronchoscopically before and after a segmental airway challenge with allergen. Each of the five subjects reported here served as his or her own control. The primary outcome was the relative expression of airway epithelial mRNA sequences, measured as an "expression ratio"

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for each sequence (the signal obtained after allergen challenge divided by the signal obtained before allergen challenge). Expression analysis was performed using Affymetrix U95Av2 chips (Affymetrix, Santa Clara, CA); 10 hybridizations were performed (five for samples obtained before segmental challenge and five for samples obtained after segmental challenge). The replicate sets thus derived were analyzed using published statistical methods based on the geometric mean and 95% confidence intervals (CIs) of individual sequence expression ratios (9, 11, 12).

Phenotype of Subjects

We studied five subjects: three European Americans (two men and one woman), one African American man, and a Native American woman. Each subject met the American Thoracic Society's definition of asthma (13) and had spirometrically determined disease of mild severity (14). Each provided written informed consent. Asthma was treated only with short-acting β agonists, and nasal symptoms only with loratidine. Segmental allergen challenge was performed at least 4 weeks after measurements of atopy and any symptoms of infection. Methacholine sensitivity, skin testing, and spirometry were performed as previously described (15). Subjects were required to have a baseline FEV₁ at least 65% of their predicted FEV₁ (16) and a history of lower airway symptoms in response to an aeroallergen to which they had a positive skin test. All of our subjects had baseline percent predicted FEV₁ in the normal range, with a mean of $88 \pm 3\%$. Each subject had a positive skin-prick test to standardized cat or dust mite allergen, which they had identified as an asthma trigger. The mean number of positive skin tests was 3 ± 0.7 of 12 tested. Each subject had an abnormal methacholine challenge test, with the provocative concentration causing a 20% fall in FEV₁ values ranging from 0.02 to 2 mg/ml.

To determine the segmental allergen challenge concentration of allergen, the lowest threefold dilution of allergen extract that produced a skin-prick wheal with a sum of orthogonals 3 mm greater than that produced by allergen diluent alone was determined and replicated on the opposite forearm.

Segmental Allergen Challenge

A detailed description of the methods appears in the online supplement. Serial standard bronchoscopic procedures were used to obtain bronchoalveolar lavage (BAL) fluid and airway epithelial cells and to deliver allergen. Specimens were obtained from a lingular subsegment before allergen was delivered to a right middle lobe subsegment. Specimens were obtained from the allergen-challenged segment 4 hours later. Allergen diluent delivered to a right upper lobe subsegment served as a control for the effects of diluent on inflammatory cell recruitment. Each subject had visible airway mucosal changes, including vascular engorgement, erythema, and airway narrowing, similar to those previously photographed (15), after the delivery of 2 ml of a solution of 2.2, 61, or 61 bioequivalent allergy units/ml standardized cat allergen or 61 or 185 allergy units/ml standardized dust mite allergen (*Dermaphagoides pteronyssinus*). Mucosal changes were not observed in any of the subjects after airway delivery of 2 ml of the diluent used to prepare the standardized allergen solutions or after 10-fold lower "test doses" of allergen.

RNA Extraction

After being strained through gauze, cells pelleted by centrifugation and RNA were extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed as previously described (11). The RNA yield was estimated by absorbance spectrophotometry and assessed for degradation by visual inspection of an ethidium bromide-stained agarose gel subjected to electrophoresis and ultraviolet transillumination. In these experiments, we recovered more than 2 million cells from six endobronchial cytology brushings from each site that yielded 70 ± 14 μ g of mRNA from cells before allergen challenge and 76 ± 15 μ g from cells after segmental allergen challenge.

Detection of Proteins

Cytospins were prepared from BAL and stained for keratin using an avidin-biotin technique, as previously described (17). Rabbit polyclonal antisera against the epithelial cell marker keratin (Dako Corp., Carpinteria, CA) at a 1:300 dilution were used as the primary antisera, and

negative controls consisted of substituting phosphate-buffered saline for the primary antisera. IL-1 β and IL-8 were measured in BAL using commercially available ELISA kits (Assay Designs, Inc., Ann Arbor, MI).

DNA Microarray Hybridization

DNA microarray hybridization was performed using Affymetrix U95Av2 gene chips, as described previously (11), following the procedures recommended in the Affymetrix technical manual, including the use of external controls (spikes) as recommended by Affymetrix. The specifications of these chips are listed in detail on the manufacturer's Web site (www.affymetrix.com). The U95Av2 arrays contain probes for 12,625 sequences representing approximately 11,300 unique GeneBank accession numbers. Each sample obtained was run on a separate chip; accordingly, five chips were used for the control samples and another five for the samples obtained after allergen challenge.

Detection of Differential Expression by Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed using techniques analogous to those that we have described previously (10), using primer sequences and conditions detailed in the online supplement (Table E1).

Data Analyses

Image scanning and preprocessing were performed using an Affymetrix Model G2500A scanner and Affymetrix software (MAS 5.0; Affymetrix). Statistical analyses and *post hoc* data filtering were performed using Microsoft Excel and Access. An expression ratio was derived on each pair of samples by dividing the signal obtained after allergen challenge by the signal obtained in the control sample. The resulting expression ratios from each of the five paired sets of samples were then used to determine whether there had been a statistically significant change in expression, by computing means and 95% CIs (derived using the T-distribution) on natural log-transformed data, as described previously (11). The data presented in the tables thus represent geometric means and 95% CIs on the geometric mean.

Where indicated in the manuscript, sequences that showed a statistically significant change in expression were filtered by two *post hoc* criteria (9). First, sequences were excluded if they were designated "absent" by the MAS 5.0 software in more than half of the control samples (for downregulated genes) or in more than half of the samples obtained after allergen exposure (for upregulated genes). In other words, to be included in the final list, a sequence had to be designated "present" or "marginal" by the MAS 5.0 software in at least three of the five paired samples, either at baseline (for downregulated genes) or after allergen exposure (for upregulated genes). Second, sequences were excluded if the changes in geometric mean of the expression ratios were less than twofold.

BAL protein measurements and cell counts were compared using the rank sum test, ANOVA, or ANOVA based on ranks as appropriate, *post hoc* analysis using Student-Newman-Keuls or Dunn's test, and presented as arithmetic mean and SE. A two-tailed p value of less than 0.05 was considered significant.

RESULTS

Proximal and Distal Effects of Segmental Allergen Challenge

Segmental airway challenge produced bronchoscopically visible airways mucosal changes that were not observed in segments exposed to either diluent or doses of allergen that were 10-fold lower than challenge doses. Consistent with the notion that allergen challenge is associated with disruption of the airway epithelium, we recovered significantly more cells from the allergen-challenged airway than from the diluent-exposed or prechallenge segment ($p < 0.05$; $1.7 \pm 0.16 \times 10^6$ cells/brush after allergen challenge, $0.41 \pm 4 \times 10^6$ cells/brush after diluent, and $0.48 \pm 4 \times 10^6$ cells/brush before allergen). The morphology of $96 \pm 1.2\%$ of the cells was that of ciliated and nonciliated airway epithelial cells. Cytokeratin staining revealed that $95 \pm 1.0\%$ of the cells were cytokeratin-positive; and vital staining revealed

that $3.2 \pm 0.1\%$ were neutrophils, $0.2 \pm 0.02\%$ were lymphocytes, and $0.1 \pm 0.1\%$ were eosinophils. Segmental allergen challenge produced no significant effect on these percentages. Thus, despite visual evidence of airways inflammation, the material obtained by cytology brush for gene expression analysis was minimally contaminated by inflammatory cells.

Although few inflammatory cells were recovered from airways accessible to cytology brush sampling, the results of BAL suggested that the applied segmental allergen challenge did produce an inflammatory response typical of asthma. There was a statistically significant increase in the number of eosinophils recovered after allergen challenge (0.55 ± 0.3 million before allergen, 1.3 ± 1.1 million after diluent, 279 ± 220 million after allergen, respectively; $p < 0.01$). Similar to our prior findings (15), there was a significant increase in the number of neutrophils recovered 4 hours after allergen challenge (0 ± 0.0 million before allergen, 1.2 ± 0.6 million after diluent, and 169 ± 100 million after allergen, respectively; $p < 0.05$). These differences were not caused by differences in BAL fluid recovery, which was similar among all samples (66 ± 11 ml in the prechallenge samples, 65 ± 12 ml in the diluent-exposed samples, and 73 ± 14 ml in the allergen-challenged samples).

Changes in Airway Epithelial Cell Gene Expression Ratio

The primary outcome measure of this study was gene expression ratio: the intensity of expression for each gene in airway epithelial cells taken 4 hours after exposure to allergen divided by expression in that gene measured in cells taken from the same subject before allergen challenge. A significant difference in expression was considered to have occurred if the 95% CIs of the geometric mean expression ratio excluded unity. Of the 12,625 sequences with the potential to be detected by the U95Av2 array, 755 (6%) showed a statistically significant change in expression, of which 635 showed a significant increase in expression, and 120 showed a significant decrease in expression. Of the 635 showing significant changes, fewer than half showed a change of twofold or greater (307 total: 255 increased and 52 decreased). Of the remaining sequences, 141 upregulated sequences and only 8 downregulated sequences met our prospectively identified *post hoc* criteria for consideration based on presence/absence determinations in a majority of the samples obtained before or after allergen challenge (as described in METHODS and in Reference 9). Thus, of 755 sequences that showed a statistically significant change in expression, only about one fifth (i.e., $\sim 1.2\%$ of all the sequences represented on the array) met all of our *post hoc* filtering criteria for identification as differentially expressed.

We found that the expressions of 12 control sequences, which are known to be constitutively expressed over a wide range of conditions, were not significantly affected by allergen challenge (Table 1).

We detected 141 sequences with increased expression after allergen challenge, including IL-3, IL-4, and IL-5 receptor subunits, the p65 component of nuclear factor- κ B, and lipocortin-1 (increased 2.4-fold [95% CI 1.1–5.3]). We identified 24 sequences (representing 21 genes) that had fourfold or greater increased expression (Table 2). The corresponding genes were assigned functional classes as previously described (10). We found that the largest number of sequences with fourfold or greater increased expression had been assigned to the following classes: cell growth, proliferation, and differentiation function (eight sequences representing seven genes), cytoskeleton (one sequence), extracellular proteins (one sequence), immune function (seven sequences representing five genes), metabolism (one sequence), protein degradation (three sequences), signal transduc-

TABLE 1. THE EFFECT OF ALLERGEN CHALLENGE ON THE EXPRESSION OF CONSTITUTIVELY EXPRESSED CONTROL SEQUENCES

Sequence	GenBank Identifier	Expression Ratio Allergen/Control ($n = 5$) Geometric Mean (95% CI)
Alu consensus sequence	U14573	1.0 (0.91–1.2)
β -Actin		
5' region	X00351	1.7 (0.78–3.6)
Middle region	X00351	1.6 (0.84–3.1)
3' region	X00351	1.6 (0.91–2.7)
GAPDH		
5' region	M33197	1.3 (0.63–2.7)
Middle region	M33197	1.4 (0.87–2.3)
3' region	M33197	1.4 (0.88–2.2)
Cyclophilin-A	X52851	1.2 (0.71–2.1)
Ribosomal protein L37a	L06499	1.0 (0.73–1.5)
Ribosomal protein L41	Z12962	1.0 (0.77–1.3)
Ribosomal protein S20	L06498	0.97 (0.63–1.5)
H3F3A	M11353	1.3 (0.78–2.1)

Definition of abbreviations: CI = confidence interval; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; H3F3A = H3 histone, family 3A.

tion (two sequences), and transcription factors (one sequence; Table 2).

In addition to sequences with increased expression, we also detected eight with significantly decreased airway epithelial expression after allergen challenge (Table 3). The sequences of known function represented the following functional classes: apoptosis; cell growth, proliferation, and differentiation; cytoskeleton; extracellular matrix; metabolism; plasma proteins; and signal transduction. We also identified a downregulated sequence of unknown classification with sequence similarity to flavin-containing monooxygenases.

PCR Detection of Differential Expression

Six upregulated sequences (IL-1RN, IL-1 β , GOS2, DAF, MMP-10, and PDE4B) with fourfold or greater increased expression and four downregulated sequences (RTP801, SPARC, RBP4, and HLF [Table 3]) were selected for confirmatory PCR. In each instance, paired PCR reactions that yielded equivalent detection of cyclophilin-A demonstrated differential expression in the direction detected by the microarray (Figure 1). PCR assay yielded consistent results in each of five samples available for study.

BAL Protein Levels

Levels of IL-1 β were significantly higher in BAL from segments before allergen challenge than from those after (preallergen 7.7 [range 7.5–8.2] pg/ml, allergen challenge 10.9 [range 8.6–12.5] pg/ml; $n = 5$; $p < 0.01$; Figure 2). Similarly, levels of IL-8 were significantly higher in BAL from segments before allergen challenge than from those after (preallergen 16 [range 13–29] pg/ml, allergen challenge 900 [range 140–1,400] pg/ml; $n = 5$; $p < 0.01$; Figure 2).

DISCUSSION

We identified 149 genes as differentially expressed (141 increased, 8 decreased) in cytokeratin-positive airway epithelial cells after segmental allergen challenge, including several genes previously implicated in the pathophysiology of asthma, as well as a number of genes not previously known to be involved in this condition. The breadth of the epithelial gene expression response to concentrations of allergen that are near the threshold of perception

TABLE 2. BRONCHIAL EPITHELIAL CELL mRNA WITH FOURFOLD OR GREATER UPREGULATED EXPRESSION AFTER ALLERGEN CHALLENGE

Gene Class	Common Name(s)	GenBank Identifier	Expression Ratio Allergen/Control (n = 5) Geometric Mean (95% CI)
Cell growth, proliferation, and differentiation	G0S2	M69199	13 (3.9–44)
	S100A8 (calgranulin A)	A1126134	5.3 (1.8–16)
	TM4SF1	M90657	4.8 (2.7–8.6)
		A1445461	4.5 (2.0–10)
	EMP1	Y07909	4.7 (2.3–9.9)
	S100A9 (calgranulin B)	W72424	4.7 (1.4–16)
	RAI3	AF095448	4.6 (2.5–8.3)
	CYR61	Y11307	4.0 (1.3–13)
	KRT6A	L42611	6.9 (1.5–33)
	TFF1	AA314825	5.3 (1.7–16)
Cytoskeleton			
Extracellular protein			
Immune function	IL-1RN	X52015	16 (1.9–132)
	IL-1 β	X04500	13 (3.8–42)
		M15330	7.9 (2.2–29)
	IL-8	M28130	10 (3.1–35)
		M17017	5.2 (1.6–16)
	DAF (CD55, Cromer blood group system)	M31516	7.3 (2.5–21)
	TNFAIP6, TSG-6	M31165	5.5 (2.2–14)
Metabolism	ALDH1A3	U07919	4.6 (2.0–10)
Protein degradation	SCCA2, leupin	U19557	10 (2.4–45)
	PAI-2	Y00630	9.0 (2.0–41)
	MMP-10 (stromelysin 2)	X07820	7.1 (2.7–19)
Signal transduction	PDE4B	L20971	7.7 (1.9–31)
	LCP2	U20158	4.3 (1.5–13)
Transcription	MAFF, v-maf	AL021977	6.2 (2.4–16)

Definition of abbreviations: ALDH1A3 = aldehyde dehydrogenase 1 family, member A3; CI = confidence interval; CYR61 = cysteine-rich angiogenic inducer 61; DAF = decay accelerating factor for complement; EMP1 = epithelial membrane protein 1; G0S2 = putative lymphocyte G0/G1 switch gene; IL = interleukin; IL-1RN = IL-1 receptor antagonist; KRT6A = keratin 6A; LCP2 = lymphocyte cytosolic protein 2; MAFF = v-maf musculoaponeurotic fibrosarcoma (AVIAN) oncogene family, protein F; MMP-10 = matrix metalloproteinase 10; PAI-2 = plasminogen activator inhibitor 2; PDE4B = phosphodiesterase 4B, cyclic adenosine monophosphate-specific; RAI3 = retinoic acid-induced 3; S100A8 = S100 calcium-binding protein A8; S100A9 = S100 calcium-binding protein A9; SCCA2 = squamous cell carcinoma antigen 2; TFF1 = trefoil factor-1; TM4SF1 = transmembrane 4 superfamily member 1; TNFAIP6; tumor necrosis factor α -induced protein 6; TSG-6 = tumor-specific glycoprotein-6.

of airway constriction for many patients with asthma is thus substantially larger than previously recognized. In addition, few prior studies have identified sequences with decreased expression. We detected eight sequences with significantly decreased expression after segmental allergen challenge.

The products of these differentially expressed genes are associated with diverse functional classes, including the following (Table E2): immune function, apoptosis, cell adhesion, cell growth and proliferation, membrane function, metabolism, tran-

scription factors, protein degradation, hormones, chaperonins, oxidant pathways, signal transduction, RNA processing and stability, transcription, translation, coagulation, cytoskeleton, DNA replication and repair, extracellular matrix proteins, and several sequences of unknown function. This suggests that the airway epithelial response to segmental allergen challenge involves both genes likely to affect epithelial cell function *per se* as well as genes (e.g., cytokines) that alter the behavior of other cell types in the airway. The picture that emerges from this study and

TABLE 3. BRONCHIAL EPITHELIAL CELL mRNA SEQUENCES WITH DOWNREGULATED EXPRESSION AFTER ALLERGEN CHALLENGE

Gene Class	Common Name(s)	GenBank Identifier	Expression Ratio Allergen/Control (n = 5) Geometric Mean (95% CI)
Apoptosis	RTP801, HIF-1 responsive RTP801	AA522530	0.41 (0.23–0.72)
Cell growth, proliferation, and differentiation	HLF	M95585	0.49 (0.36–0.67)
Cytoskeleton	KRT15	X07696	0.46 (0.26–0.83)
Extracellular matrix	SPARC (osteonectin)	J03040	0.25 (0.12–0.52)
Metabolism	ACP5	J04430	0.29 (0.095–0.90)
Plasma protein	RBP4	X00129	0.39 (0.17–0.88)
Signal transduction	PTGFR (FP), prostaglandin F $_{2\alpha}$ receptor	AF004021	0.38 (0.18–0.81)
Unknown	dj127D3.2	AL021026	0.39 (0.20–0.79)

Definition of abbreviations: ACP5 = acid phosphatase 5, tartarate resistant; CI = confidence interval; dj127D3.2 = flavin-containing monooxygenase family protein; HIF-1 = hypoxia-inducible factor-1; HLF = hepatic leukemia factor; KRT15 = keratin 15; PTGFR = prostaglandin F receptor; RBP4 = retinol-binding protein 4, plasma; SPARC = secreted protein, acidic, cysteine-rich.

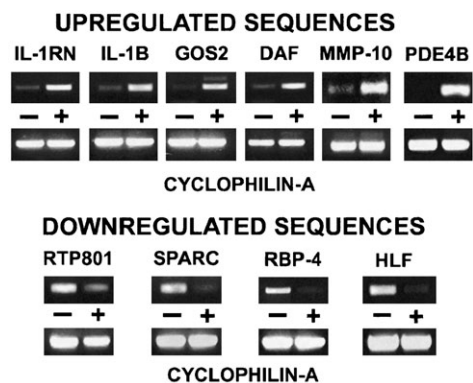


Figure 1. Polymerase chain reaction detection of differential gene expression for samples having equivalent detection of constitutively expressed cyclophilin-A. Each of five pairs of preallergen (–) and segmental allergen challenge samples (+) studied yielded equivalent results. IL-1RN = interleukin-1 receptor antagonist; GOS2 = putative lymphocyte G0/G1 switch gene; DAF = decay accelerating factor for complement; HLF = hepatic leukemia factor; MMP-10 = matrix metalloproteinase 10; PDE4B = phosphodiesterase 4B; RBP4 = retinal-binding protein 4; RTP801 = hypoxia-inducible factor-1 responsive RTP801; SPARC = secreted protein, acidic, cysteine-rich.

others is that the airway epithelium is not simply a mechanical barrier lining the airways but rather a functionally complex tissue that adapts to routine allergen exposure by altering gene expression to favor cell growth and proliferation as well as modifications in membrane function, oxidant pathways, and cytoskeletal properties likely to enhance survival. These cells not only survive but express genes that are known to influence both immune and nonimmune cells near the airway epithelium and thus contribute to asthma immunopathogenesis and to the structural changes that characterize the asthmatic airway.

One of the promises of genomic technology is the identification of proteins that contribute to asthma immunopathogenesis. To discern the power of differential airway epithelial gene expression to identify known asthma mediators using the segmental allergen challenge model, we examined a list of genes previously classified as influencing immune function. Of the 16 genes with twofold or greater differential expression assigned to the immune function category (Table E2), nine are known to promote

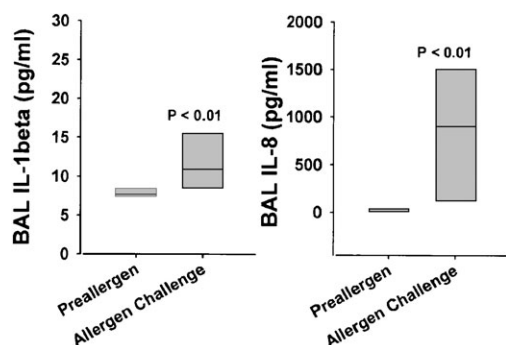


Figure 2. Concentrations of IL-1 β and IL-8 in bronchoalveolar lavage (BAL) fluid were significantly higher after segmental allergen challenge ($n = 5$; $p < 0.01$ by rank sum test). Boxes present the median, 25th, and 75th percentiles.

expression of the asthma phenotype (Table 4; this table also lists three genes associated with asthma that were not classified in the immune function category). These include the IL-1 β gene, which was among those showing the largest fold-increases in expression. This primary cytokine is known to have increased expression in segmental allergen challenge studies, including the current study (18, 19), is believed to mobilize the chemokines and effector molecules of the asthmatic diathesis, and is widely recognized as a mediator of asthma (20). The increased expression of the tumor necrosis factor α -induced protein-6, which is also induced by IL-1 β (21), suggests that the primary cytokines induced in this model had detectable influences on transcript expression. We also identified the IL-1 receptor antagonist gene as having increased expression. In asthma, the effects of IL-1 β are believed to be modulated by the secretion of IL-1 receptor antagonist, which increases with asthma severity (22) and is increased in the BAL of patients with life-threatening asthma (23).

The IL-4 receptor had significantly increased expression after allergen challenge. This receptor is required for expression of the asthma phenotype in animal models (24) and has been investigated as a therapeutic target (25). It is widely accepted as important for the pathogenesis of asthma (2). Similarly, the expression of the colony-stimulating factor 2 β receptor, which is required for the action of the asthma-associated IL-3 and IL-5 (2), was increased. We detected significant increases in IL-8 mRNA expression and BAL protein levels 4 hours after segmental allergen challenge, consistent with prior reports (20, 26). IL-8 has been directly associated with neutrophil recovery and is a chemoattractant for neutrophils. Furthermore, the increases in IL-8 expression we observed were associated with increased recovery of neutrophils from BAL, similar to our previous report (15). We detected increased expression of the gene for lipocortin-1, the product of which comprises 23% of the protein present in BAL from healthy subjects and is present in BAL from patients with asthma (27). Its increased secretion after prednisolone therapy has been associated with improvement in asthma control (28).

We detected significant increases of CCL2 (monocyte chemoattractant protein-1) and CCL3 (macrophage inflammatory protein-1 α) expression in airway epithelial cells after allergen challenge. In this model, CCL2 and CCL3 protein levels increase 4 hours after allergen challenge and return to baseline by 24 hours (29). The pulmonary expression of CCL2 is increased after allergen challenge, and its immunoneutralization in animal models limits expression of the asthmatic phenotype (30). In addition, we detected increased expression of the p65 subunit of the nuclear factor- κ B system, which is required for expression of the asthmatic phenotype in a mouse model (31). We have identified the histamine receptor and phosphodiesterase 4 genes, which have been investigated as therapeutic targets relevant to asthma treatment (32). Moreover, plasminogen activator inhibitor 2, or PAI-2, a gene not previously linked to the disease, has been identified by this method and associated with asthma (33).

Our findings support the contention that the delivery of concentrations of allergens in the clinically relevant range to the mild asthmatic airway elicits changes in gene expression signature consistent with a tissue repair and proliferation response. We found that a large fraction of genes with fourfold or greater differential expression were classified in the tissue repair and cellular proliferation pathways category. These findings further support the increasingly well established tenet that allergen exposure in mild asthma leads to airway remodeling. Our findings represent a modest advance in this area, because they identify several genes that had not previously been implicated in remodeling.

In addition to identifying sequences with increased expression, we identified eight sequences with significantly decreased expression, including RTP-801. Limiting the expression of this

TABLE 4. EFFECT OF ALLERGEN CHALLENGE ON EXPRESSION OF SEQUENCES THAT ARE KNOWN TO HAVE INCREASED EXPRESSION IN ASTHMA

Functional Class	Sequence	GenBank Identifier	Expression Ratio Allergen/Control (n = 5) Geometric Mean (95% CI)
Immune function	ANXA-1, lipocortin-1	X05908	2.4 (1.1–5.3)
	CCL2, MCP-1	M26683	2.3 (1.1–5.1)
		M28225	3.3 (1.9–5.9)
	CCL3, LD78 α precursor, MIP-1 α	D90144	3.0 (1.0–8.9)
	CSF2RB, IL-3 receptor, β chain; IL-5 receptor, β chain	H04668	3.3 (1.1–10)
	CXCL2, MIP-2 α , GRO β	M36820	2.5 (1.4–4.6)
	IL-1 receptor antagonist	X52015	16 (1.9–132)
	IL-1 β	M15330	7.9 (2.2–29)
		X04500	13 (3.8–42)
	IL-4 receptor	X52425	2.0 (1.1–3.6)
	IL-8	M17017	5.2 (1.6–16)
		M28130	10 (3.1–35)
	HRH1	Z34897	2.8 (1.3–6.0)
	NF- κ B, subunit p65	L19067	2.3 (1.2–4.4)
Other	PDE4B	L20971	7.7 (1.9–31)

Definition of abbreviations: ANXA-1 = annexin A1; CCL2 = chemokine (C-C motif) ligand 2; CI = confidence interval; CSF2RB = colony-stimulating factor receptor β ; CXCL2 = chemokine (C-X-C motif) ligand 2; HRH1 = histamine receptor H1; IL = interleukin; NF- κ B = nuclear factor- κ B; PDE4B = phosphodiesterase 4B, cAMP-specific.

gene, which can induce lung cell death, may increase the survival of airway epithelial cells that have been stressed and disrupted after allergen exposure (34). We also identified SPARC, which plays a role in branching morphogenesis during airway development and can limit fibrosis in the bleomycin-induced fibrosis mouse model (35). The novel association of these genes with allergen exposure is an initial step toward understanding their role in the allergic airway.

An important criticism of microarray experiments is that some popular analytic methods can produce large amounts of data that are not verifiable by alternative methods. In particular, approaches that inappropriately apply grouping methods, such as cluster analysis, or that do not apply inferential statistics (e.g., by pooling samples on a single array) are, in principle, particularly vulnerable to this problem. By contrast, we used a separate array for each of the samples analyzed (a total of 10 for the 5 pairs of samples) and applied statistical inference and *post hoc* filtering to identify genes whose changes in expression are likely both to be detectable by alternative techniques (e.g., PCR) and to be observable in future studies. The analytic approach here used *post hoc* filtering rather than adjustment of individual CIs to reduce the number of false-positive reports that occur when an inferential statistical test is applied repeatedly to thousands of different variables. This general approach been applied with accurate results in a number of contexts (9, 11, 12) and can lead to findings that are both congruent with the published literature and highly reproducible by conventional PCR. In this study, after *post hoc* filtering, the lower limit of the 95% CIs on the mean for each of the highly upregulated genes reported in Table 2 was substantially greater than unity (2.2 on average; range 1.3–3.9). For all upregulated genes (Table E2), the lower limit of the 95% CI on the mean was 1.5 on average (range 1.0–3.9), also well removed from unity. Thus, for a large number of the genes reported in this study, and in particular for the highly upregulated genes reported in Table 2, the probability that the observed changes occurred by chance alone is small. In addition, we sought to demonstrate the validity of our findings both by internal consistency (using alternative techniques for detecting differences in expression) and external consistency (by comparing our findings with those of others using the segmental

allergen challenge model). We identified 12 control sequences whose expression was unaffected by a diverse set of conditions and found that the fold-change in expression for each of these sequences was near unity (Table 1). To determine whether our findings could be replicated by other methods, we performed conventional PCR assays for 10 differentially regulated sequences, representing 6.7% of the 149 differentially expressed sequences identified on the array and approximately one-third of the genes listed in Tables 2 and 3. Although qualitative rather than quantitative, the results of these confirmatory PCR assays were consistent with those predicted by the microarray data in every instance, providing additional evidence that the changes in expression observed are not merely the product of random error. Our ability to identify genes known to promote expression of the asthma phenotype demonstrates the effectiveness of this analytic technique; the substantial percentage of immune function genes that represent externally validated true positives suggests that it is also efficient. These findings in a modest number of subjects thus met the standards of inferential statistical probability, internal validation, and external corroboration.

One of the limitations of analytic methods designed to reduce false-positive results is that they also tend to increase the number of false-negative reports. Furthermore, the declining expression of many asthma-associated chemokine genes expressed in the epithelium with burst-suppression kinetics, such as CCL11 (eotaxin-1) and CCL5 (regulated on activation normal T-cell expressed and secreted, or RANTES), led to variations in the expression of their mRNA sequences that prevented them from being identified as differentially expressed 4 hours after segmental allergen challenge. It is possible that sequences corresponding to these genes might have been found to be significantly upregulated had we studied more subjects or more time points.

Although each subject in this study served as his or her own control, a second important methodologic limitation of our study was the lack of a control for the effects of the instrumentation and allergen diluent. In concordance with many other studies, the inflammatory cellular response to diluent and instrumentation was modest compared with allergen (29, 36, 37), and we note that, in another study, the effects of diluent on gene expression appear to be small (15). Preliminary studies demonstrated that

allergen challenge in the absence of diluent can induce changes (including changes in gene expression) in adjacent segments of the lung. These observations prevented us from using an analytic approach in which gene expression ratios from the diluent-exposed segment could be used to control for the effects of diluent on gene expression ratios. We therefore cannot rule out the possibility that some of the effects we observed on differential gene expression may have been produced by the allergen diluent.

Our findings in the airway epithelial cells of patients with asthma are divergent from those obtained in lung biopsy tissue in monkeys challenged with *Ascaris suum* (38) and mice challenged with ovalbumin or *Aspergillus fumigatus* (39). Although these findings may be because of differences in species, antigen, or time after challenge, the organization of the tissue studied is also methodologically relevant. The whole-lung biopsy technique has the advantage of assessing all of the cell types in the lung. However, allergen-induced recruitment of inflammatory cells becomes a confounding factor, as increased expression of a sequence could be caused by either increases in its expression in resident cells or by its constitutive expression in recruited cells. The endobronchial brush variant of segmental allergen challenge addresses this issue by measuring changes predominantly in a single cell type. We found that approximately 95% of the cells that we processed were cytokeratin-positive, thus minimizing confounding by cellular admixture. Although it is possible that the 5% of cells that were not cytokeratin-positive may have influenced gene expression profile, this is unlikely to have affected many of the genes we report as differentially expressed. A gene present in only 5% of the cells would only be classified as differentially expressed if the intensity of its expression was of extraordinary magnitude.

We also compared our results to a list of sequences differentially expressed by cultured human airway epithelial cells exposed to IL-13 *in vitro*. This previous study used a more limited Affymetrix array (HuGeneFL, including ~ 5,000 sequences; Affymetrix) (40). To maximize the number of sequences identified as differentially regulated in both studies, we compared all of the sequences that we identified as significantly affected by allergen challenge without regard to *post hoc* filtering. Of 53 sequences reported in this manuscript for which corresponding probes were identified on our arrays, we found only 2 that showed similar differential regulation in both studies: serine protease inhibitor leupin (upregulated 4.8-fold *in vitro*, 10-fold in the present *in vivo* study [95% CI 2.4–45]) and kinesin-2 (upregulated 3.6-fold *in vitro*, 1.8-fold *in vivo* [95% CI, 1.0–3.2]). It appears that this *in vitro* model is not a robust predictor of the response of the asthmatic epithelium to allergen. This may be from the action of substances other than IL-13 that alter epithelial cell transcript expression in the context of allergen challenge or differences in the response of cells derived from subjects with and without asthma, or may reflect fundamental differences in the responses of airway epithelial cells in culture as compared with those *in vivo*.

In conclusion, measuring changes in the expression of the human genome in airway epithelial cells *in situ* after proallergic stimuli is one way to identify the genes involved in the asthmatic epithelial response to concentrations of allergen in the clinically relevant range. The identification of these genes is the first step in understanding their role in the airway response to allergen. In the present study, changes in mRNA expression profile after segmental allergen challenge included both molecules known to be involved in asthma as well as molecules not previously associated with this disease. Furthermore, we detected an extracellular response to allergen challenge that included signaling molecules and tissue repair enzymes and an underrecognized intracellular response that involved the expression of prolifera-

tion and survival response genes. The airway epithelium would thus appear to be a physiologically complex tissue that has a more extensive response to allergen challenge than previously recognized.

Conflict of Interest Statement: C.M.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; E.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; L.A.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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