

Comparative Microarray Analysis and Pulmonary Changes in Brown Norway Rats Exposed to Ovalbumin and Concentrated Air Particulates

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The interaction between air particulates and genetic susceptibility has been implicated in the pathogenesis of asthma. The overall objective of this study was to determine the effects of inhalation exposure to environmentally relevant concentrated air particulates (CAPs) on the lungs of ovalbumin (ova) sensitized and challenged Brown Norway rats. Changes in gene expression were compared with lung tissue histopathology, morphometry, and biochemical and cellular parameters in bronchoalveolar lavage fluid (BALF). Ova challenge was responsible for the preponderance of gene expression changes, related largely to inflammation. CAPs exposure alone resulted in no significant gene expression changes, but CAPs and ova-exposed rodents exhibited an enhanced effect relative to ova alone with differentially expressed genes primarily related to inflammation and airway remodeling. Gene expression data was consistent with the biochemical and cellular analyses of the BALF, the pulmonary pathology, and morphometric changes when comparing the CAPs-ova group to the air-saline or CAPs-saline group. However, the gene expression data were more sensitive than the BALF cell type and number for assessing the effects of CAPs and ova versus the ova challenge alone. In addition, the gene expression results provided some additional insight into the TGF- β -mediated molecular processes underlying these changes. The broad-based histopathology and functional genomic analyses demonstrate that exposure to CAPs exacerbates rodents with allergic inflammation induced by an allergen and suggests that asthmatics may be at increased risk for air pollution effects.

Key Words: ovalbumin; allergen; asthma; particulate matter; remodeling; inflammation; microarray.

The prevalence of asthma has increased in recent years, particularly in industrialized countries, making it an important public health concern. The air pollution and specific allergens that accompany urbanization are thought to be partly responsible for this increase (Busse and Mitchell, 2007). Diesel exhaust (DE) is a major contributor to particulate matter (PM) related air pollution, especially in urban areas. As DE emissions have increased globally, so has asthma prevalence (Keller and Lowenstein, 2002). Airborne PM has a number of detrimental health effects, particularly in asthmatics (Riedl and Diaz-Sanchez, 2005). Genetics are also a factor; they determine the susceptibility to asthma or other respiratory diseases that can be influenced by PM (Moller *et al.*, 2007).

PM from air pollution has several mechanisms of action for generating inflammation, damaging cells, and exacerbating airway hyperresponsiveness (Gilmour *et al.*, 2006). In particular, PM derived from a variety of sources induces the production of reactive oxygen species in inflammatory cells (Becker *et al.*, 2002) and oxidative DNA damage in human airway epithelial cells (Prahallad *et al.*, 2001). PM exposure also has specific allergy-related effects. For example, the polyaromatic hydrocarbons in DE particles have been shown to increase IgE production by human B cells (Takenaka *et al.*, 1995; Tsien *et al.*, 1997). Increases in coarse PM correlate with increases in circulating eosinophils, serum triglycerides, and decreased heart rate variability in asthmatics (Yeatts *et al.*, 2007). PM has also been shown to act as an adjuvant in ovalbumin (ova)-induced allergic reactions in mice and Brown Norway (BN) rats (Dong *et al.*, 2005; Harkema *et al.*, 2004a; Matsumoto *et al.*, 2006; Miyabara *et al.*, 1998; Takano *et al.*, 1997). BN rats challenged with ova have greater PM particle retention and eosinophilia in the lungs, compared with unchallenged BN rats (Morishita *et al.*, 2004). Humans with allergies have increased Th2 cytokines and ragweed-specific IgE expression after coexposure to ragweed allergen and DE

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particulates (Diaz-Sanchez *et al.*, 1997). The mechanism for the effect of PM on asthma remains unclear, due to its complex interactions with the immune system and other metabolic pathways (McCunney, 2005).

Examining the complex interaction between environmental exposures and genetic factors can provide vital information needed to better understand and treat respiratory diseases (Kleeberger and Peden, 2005). Because asthma genetics are complex and likely to involve many genes that vary by population, the use of emerging genomics tools such as expression profiling and pathway analyses are needed (Ober and Hoffjan, 2006). Using a rodent model of airway allergic inflammation or pulmonary allergy minimizes genetic variation so that allergy- and exposure-induced gene expression changes can be isolated and investigated. Gene expression profiling in animal models has yielded large numbers of differentially expressed genes, from which genes related to asthma susceptibility and pathogenesis have been distilled (Follettie *et al.*, 2006; Izuhara and Saito, 2006; Kuperman *et al.*, 2005; Walker *et al.*, 2006). However, there is little information regarding the precise mechanisms of gene-environment interactions in relation to asthma (London, 2007).

BN rats sensitized with ova are thought to resemble the clinically significant features of allergic asthma (Salmon *et al.*, 1999; Tarayre *et al.*, 1992; Underwood *et al.*, 1995) and often used as an animal model for pulmonary allergic conditions such as asthma because they have a high capacity for IgE production and exhibit airway hyperresponsiveness following exposure to inhaled allergens (Abadie and Prouvost-Danon, 1980; Pauwels *et al.*, 1979). A few studies have examined global gene expression profiles in rodents exposed to environmental agents, including concentrated ambient particulates (CAPs) (Gunnison and Chen, 2005; Sigaud *et al.*, 2007), environmental tobacco smoke (Izzotti *et al.*, 2005; Nadadur *et al.*, 2002), ozone (Leikauf *et al.*, 2001; Park *et al.*, 2004; Williams *et al.*, 2007), and PM (Kooter *et al.*, 2005; Nadadur and Kodavanti, 2002; Sato *et al.*, 1999; Wise *et al.*, 2006). BN rats coexposed to both lipopolysaccharide (LPS) and tobacco smoke (Meng *et al.*, 2006) or mice coexposed to LPS and DE (Yanagisawa *et al.*, 2004) have also been examined via microarray, as models of chronic obstructive pulmonary disorder and acute lung injury, respectively.

To our knowledge, this study is the first to use gene expression array profiling and traditional toxicological approaches to decipher and evaluate how the combination of susceptibility and exposure to environmentally relevant CAPs act together to perturb biological pathways that may be important to the exacerbation of asthma.

MATERIALS AND METHODS

Animals and exposure regimen. Figure 1 shows the experimental design for the four treatment groups BN rodents (Charles River, Indianapolis, IN) Each group consisted of eight male rats, aged 10–12 weeks. Rats were free of pathogens and respiratory disease, and used in accordance with guidelines set forth by the Institutional Animal Care and Use Committee at Michigan State University. All four groups were sensitized in the animal care facility, for three consecutive days to ova (0.5% in saline, intranasally). Two weeks later, two of the groups were then challenged with saline vehicle, the other two with ova, by intranasal instillation (0.5% ova in saline, 150 µl/nasal passage) for 3 consecutive days. Starting the day after the last challenge, two groups of rats (one each of the saline and ova-challenged groups) were exposed to CAPs from Grand Rapids, MI (8 h/day for 13 days). There was a second ova or saline challenge 9 days following the first ova challenge. Rats were sacrificed 24 h after the last CAPs exposure.

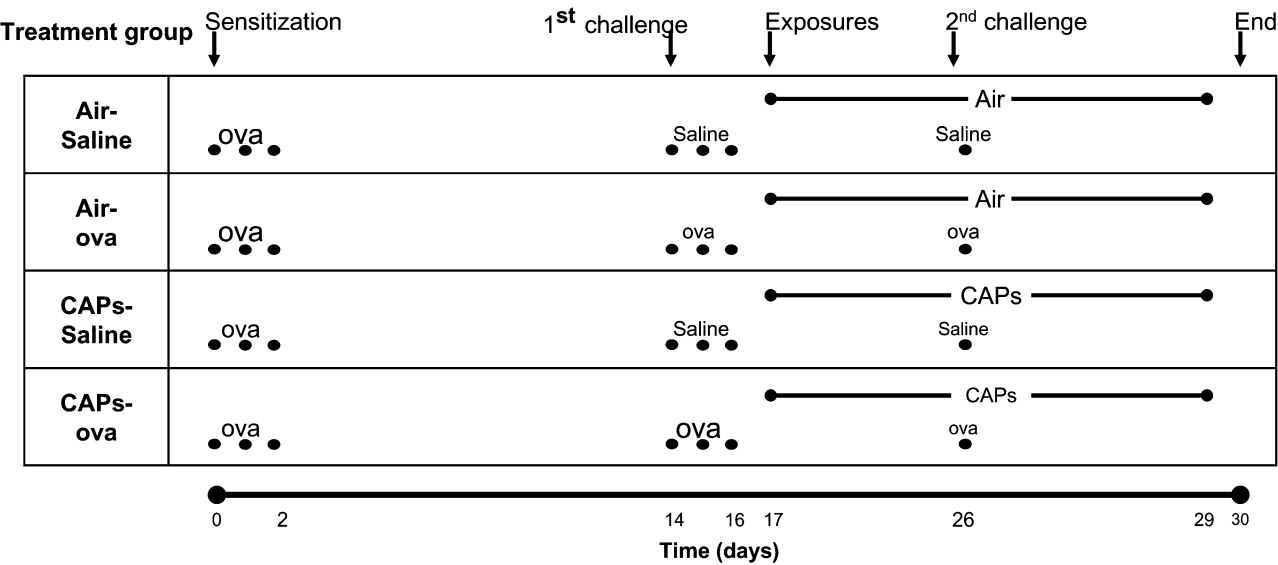


FIG. 1. Experimental design and exposure regimen. The initial ova sensitizations and first ova or saline challenges were administered intranasally over three consecutive days (0–2 and 14–16, respectively). Animals were exposed to CAPs or ambient filtered air on days 17–29. A second ova or saline challenge was administered intranasally on day 26. Lung tissue was harvested on day 30.

The CAPs exposure and ova challenge occurred via the mobile air research laboratory, AirCARE 1 (Harkema *et al.*, 2004b). AirCARE 1 contained whole body inhalation chambers with a Harvard/EPA ambient fine particle concentrator, a biomedical lab, an inhalation exposure lab, and an atmospheric monitoring lab (Harkema *et al.*, 2004b; Keeler *et al.*, 2007). The fine particle concentrator was a three-stage aerosol concentrator that utilizes virtual impactors to increase the concentration of particles (size range 0.1–2.5 μm) by an approximate factor of 30 (Sioutas *et al.*, 1997). The remaining two groups were exposed to HEPA-filtered ambient air, also in AirCARE 1.

Characterization of CAPs. CAPs were collected during each 8-h exposure period. The mass concentrations of CAPs were determined by placing 47-mm Teflon filters (Gelman Sciences, Inc., Ann Arbor, MI) in Teflon filter packs attached to the back of the animal exposure chambers at flow rates of 3 LPM. After the gravimetric determination, CAPs samples on Teflon filters were extracted in 10% nitric acid and analyzed for a suite of trace elements (including crustal/urban dust related elements (Fe, Si, Ca, Al) using inductively coupled plasma-mass spectrometry (ELEMENT 2, Thermo Finnigan, San Jose, CA). Pre-baked quartz filters (Gelman Sciences, Inc., Ann Arbor, MI) were also placed in Teflon filter packs mounted on the exposure chambers and were analyzed for carbonaceous (organic and elemental) aerosols by a thermal-optical analyzer (Sunset Labs, Forest Grove, OR). Annular denuder/filter pack samples were also collected and analyzed for acid gases and ion species by ion chromatography (Model DX-600, DIONEX, Sunnyvale, CA).

RNA isolation. Four animals from each group were randomly chosen for gene expression analysis. Total RNA was isolated from the right cranial lung lobe, using RNeasy Mini kits (Qiagen Inc., Valencia, CA) with DNase treatment. RNA quality was checked using an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA was quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and then 3.8 μg of each sample was sent to Expression Analysis (Durham, NC) for cDNA target generation and hybridization to rat R230 2.0 whole genome arrays (Affymetrix, Inc., Santa Clara, CA). RNA from one rat in the CAPs-saline exposure group failed to generate enough target to hybridize to a microarray chip.

Gene array data analysis. Invariant probe signals were removed using the REDI (reduction of invariant probes) method, proprietary to Expression Analysis. Array data were normalized using robust multiple-array averaging (RMA), and compared by group using the permutation analysis for differential expression (PADE, http://www.expressionanalysis.com/pdf/PADE_TechNote_2005.pdf) algorithm. Noise from the PADE was removed by setting probe signals that were < 128 to 128 (the level of noise) to prevent artificially high fold change differences, and fold changes were recalculated. Absolute fold changes < 1.5 and values which exceeded accumulated false discovery rates (FDRs) of < 0.05 were removed. The resulting significant probesets from each PADE analysis are shown in Supplemental Table S1. Probesets were annotated using NetAffx (Affymetrix Inc., Santa Clara, CA). Only three PADE analyses had a list of significant genes that met the cutoff values. The probeset lists generated by this procedure were uploaded into GeneSpring 7 (Agilent Technologies, Palo Alto, CA) for principal components and clustering analyses.

Pathways analysis with metacore. Probeset lists created using an FDR of ≤ 0.2 (Supplemental Table S2) rather than ≤ 0.05 were uploaded into MetaCore 4.5 (GeneGo, Inc., St Joseph, MI, <http://www.genego.com/metacore.php>) for functional and pathways analysis. Each gene identifier was mapped to its corresponding gene object in the MetaCore database. The genes were then compared with both gene ontology (GO) processes and GeneGo maps to determine processes or pathways which were significantly overrepresented in the differentially expressed gene list. The *p* values for maps and processes were calculated using a hypergeometric distribution.

Pathways analysis with ingenuity pathways analysis. Probeset lists created using an FDR of ≤ 0.2 (Supplemental Table S2) rather than ≤ 0.05 were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These

genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity. The Functional Analysis of the top scoring network identified the biological functions and/or diseases that were most significant to the genes in the network. The network genes associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fisher's exact test was used to calculate a *p* value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.

Confirmatory quantitative PCR. Total RNA was used to generate cDNA for confirmatory quantitative PCR (qPCR). Total RNA (350 ng) was reverse transcribed in a buffer containing $1\times$ reverse transcriptase polymerase chain reaction buffer, 25 μM random hexamers, 5mM dithiothreitol, 500 μM deoxynucleotide triphosphates, 20 U RNase OUT, and 200 U SuperScript III (all from Invitrogen Corp., Carlsbad, CA) for 10 min at 25°C, 60 min at 50°C, and 15 min at 75°C. Six genes of interest and one endogenous control (β -actin) were analyzed by confirmatory qPCR. TaqMan MGB probes (Applied Biosystems, Foster City, CA) for transforming growth factor $\beta 3$ (TGF- $\beta 3$), Fc receptor IgE high affinity I alpha polypeptide (Fc ϵ 1 α), complement component 4 binding protein (C4BP), vascular endothelial growth factor C (VEGF-C), chitinase 3-like 1 (Ch3L1), metallothionein 1a (Mt1a), and the β -actin endogenous control were used according to the recommended procedure on a 7500 Real-Time PCR machine (Applied Biosystems, Foster City, CA). Briefly, 5 μl of cDNA was mixed with 2.5 μl of 20 $\times$ TaqMan MGB probe mix, 17.5 μl of water, and 25 μl of 2 $\times$ TaqMan Universal PCR Master Mix for a total volume of 50 μl per well in a 96-well optical plate (Applied Biosystems, Foster City, CA). Reactions for each sample were performed in duplicate. The plate was run for 2 min at 50°C, then 10 min at 90°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The data were analyzed using the Auto C_T method to generate a standard curve, and the duplicate relative concentration measurements for each sample were averaged. Data were normalized to the control gene concentration by dividing the mean relative concentration for a gene of interest by the mean relative concentration of the β -actin control. Normalized values were then averaged among rats in each treatment group.

Statistical analysis of qPCR results. Data from qPCR ($n = 4$) were expressed as the mean group value $\pm$ the standard error of the mean. The data were subjected to ANOVA for factors of inhalation exposure type (e.g., CAPs or filtered air) and airway sensitization/challenge (ova or saline). Significant differences between experimental groups were identified using the Tukey honest significant difference *post hoc* test. The criterion for statistical significance was $p \leq 0.05$.

Lung tissue section and bronchoalveolar lavage. All animal sacrifices were conducted in the laboratory of Dr. Harkema at Michigan State University. At the time of sacrifice, animals were deeply anesthetized, 5 ml of whole blood was collected from the ascending vena cava, and the animal was exsanguinated. The trachea was cannulated, and the heart/lung block was removed from the thoracic cavity. The right extrapulmonary bronchus was ligated with suture and the right lung lobes were removed. The entire right cranial lobe was processed for isolation of total RNA (see above). The left lung lobe was lavaged with saline and the recovered lavage fluids (bronchoalveolar lavage fluid; BALF) from each rat were analyzed for total and differential cell counts, total and ova-specific IgE and secreted mucins by enzyme-linked immunosorbent assay (ELISA). After bronchoalveolar lavage the left lung lobe was perfusion-fixed with 10% neutral buffered formalin via the trachea at a constant pressure of 30 cm of fixative. After 2 h of intratracheal fixation, the trachea was ligated and the lung lobe was immersed in a large volume of the same fixative for at least 24 h before the left lung lobe was further processed for light microscopy as described below.

Analysis of BALF. Total cells recovered by bronchoalveolar lavages were determined manually using a hemacytometer. Cytospin preparations from eight rodents per treatment group were made with a cytocentrifuge and stained with Diff-Quick (IMB, Inc., San Marcos, CA). Differential cell counts

(e.g., neutrophils, macrophages, eosinophils, lymphocytes) were determined by counting 200 cells per animal. The BALF was centrifuged to remove cells and debris, and the supernatant was stored at -80°C . Cell-free supernatant was assayed for protein content by the bicinchoninic acid method (#23255, Pierce Chemical Co., Rockford, IL). Mucin glycoprotein 5AC in BALF was analyzed by ELISA using a monoclonal antibody (mucin5AC Ab-1; Neomarkers, Fremont, CA), a peroxidase-conjugated avidin/biotin complex (ABC Reagent; Vector Laboratories, Burlingame, CA), and a fluorescent substrate (Quanta-Blue; Pierce Chemical, Rockford, IL). Total and ova-specific IgE were determined in ELISA (colormetric capture assays) by coating plates with anti-rat IgE (Pharmingen, San Diego, CA) or ova, respectively. After incubation with BALF, bound samples were detected with biotinylated anti-rat IgE and quantified with a peroxidase system (Vector Laboratories, Burlingame, CA) using a Bio Tek Elx 808 plate reader.

Lung tissue collection and epithelial morphometry. The intrapulmonary airways of the fixed left lung lobe from each of eight rodents were microdissected. Beginning at the lobar bronchus, airways were split down the long axis of the largest daughter branches (i.e., main axial airway; large diameter conducting airway) through the 12th airway generation. Tissue blocks that transverse the entire lung lobe at the level of the fifth and eleventh airway generation of the main axial airway were excised and processed for light microscopy and morphometric analyses. The lung tissue blocks were embedded in paraffin, and 5- to 6- μm sections from the proximal face of each block were cut and placed on charged slides (Probe-on-plus; Fischer Scientific, Pittsburgh, PA). Tissue sections were histochemically stained with (1) hematoxylin and eosin (H&E) for evaluation of epithelial morphology and quantitation of epithelial cell numeric densities, or (2) Alcian Blue (pH 2.5)/Periodic Acid Schiff's sequence (AB/PAS) to detect acidic and neutral mucosubstances for quantitation of stored mucosubstances within the airway epithelium.

Morphometric Analysis of intraepithelial mucosubstances in airway. The volume density (V_s) of stored intraepithelial mucosubstances (IMs) in the surface epithelium lining the proximal and distal pulmonary axial airways (generations 5 and 11, respectively) were determined using image analysis and standard morphometric techniques previously described in detail (Harkema *et al.*, 1997a, b). The quantity of stored mucosubstances per unit area were determined as described by Harkema *et al.* (1987a, b) and expressed as the mean volume (nl) of ip mucosubstances (IM)/ mm^2 of basal lamina $\pm$ standard error of the mean (Fig. 8D).

Statistical analysis of BALF and morphometric endpoint. Data describing the type and magnitude of the pulmonary inflammatory response in BALF and morphometric changes in mucosubstances in airway epithelium ($n = 8$) were expressed as the mean group value $\pm$ the SEM. The data were subjected to ANOVA for factors of inhalation exposure type (e.g., CAPs or filtered air) and airway sensitization/challenge (ova or saline). Significant differences between experimental groups were identified using appropriate *post hoc* tests (Tukey's omega procedure). Transformation of data (e.g., log or arcsin $^{-1}$) was performed if needed to render variances homogeneous. The criterion for statistical significance was $p \leq 0.05$.

RESULTS

Characterization of CAPs

The average CAPs concentration during the 13-day exposure period was $493 \pm 391 \mu\text{g}/\text{m}^3$ (Table 1). As shown in Table 1, over half of the CAPs collected in Grand Rapids were organic and elemental carbon. Although the 13-day averaged concentration of sulfate was only about 10% of the CAPs, during the first week of the exposure study a large amount of secondary particles composed mostly of sulfate and organic carbon was observed (Fig. 2). The Hybrid Single Particle Lagrangian

TABLE 1
Average Mass and Major Composition of Concentrated Air Particles during 13-Day Exposure Period

Mass	493 $\pm$ 391
Organic carbon	244 $\pm$ 144
Elemental carbon	10 $\pm$ 4
Sulfate	79 $\pm$ 131
Nitrate	39 $\pm$ 67
Ammonium	39 $\pm$ 59
Urban dust ^a	18 $\pm$ 6

Note. All values are presented as mean $\pm$ SD ($\mu\text{g}/\text{m}^3$).

^aCrustal urban dust was estimated from Fe, Al, Ca, and Si.

Integrated Trajectories model (HYSPLIT, National Oceanic and Atmospheric Administration) indicated regional transport of an air mass that passed through Missouri, Illinois, and northwestern Indiana (Fig. 2). Cities in northwest Indiana, including Gary and East Chicago, have been home to heavy industry for the last century.

Differentially Expressed Genes

Criteria for the pairwise analyses were $\text{FDR} \leq 0.05$ and fold change ≥ 1.5 for expression over background. The centroid plot in Figure 3A is a visual representation of the relative

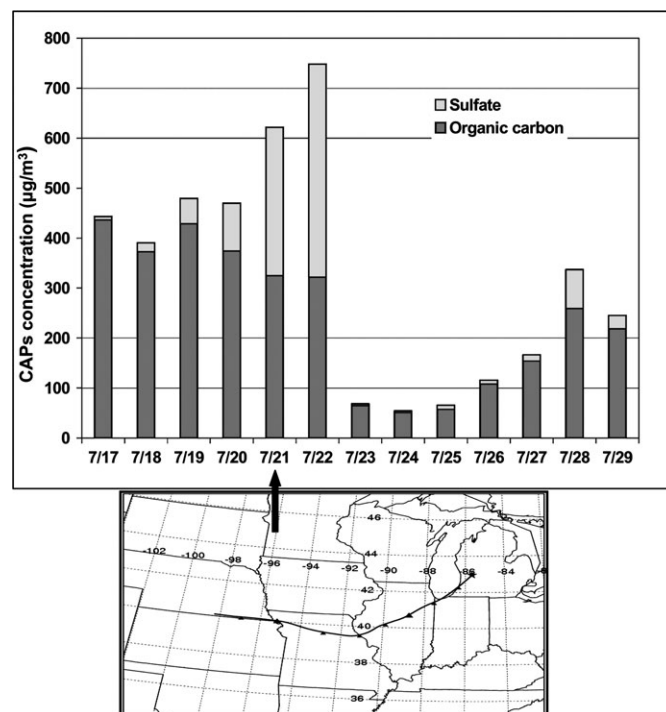


FIG. 2. Air mass contributing to the CAPs used for exposure. The HYSPLIT model (National Oceanic and Atmospheric Administration)—generated 48-h backward trajectories showing the history of an air mass arriving in Grand Rapids at noon on July 21. (Data are from National Oceanic and Atmospheric Administration.)

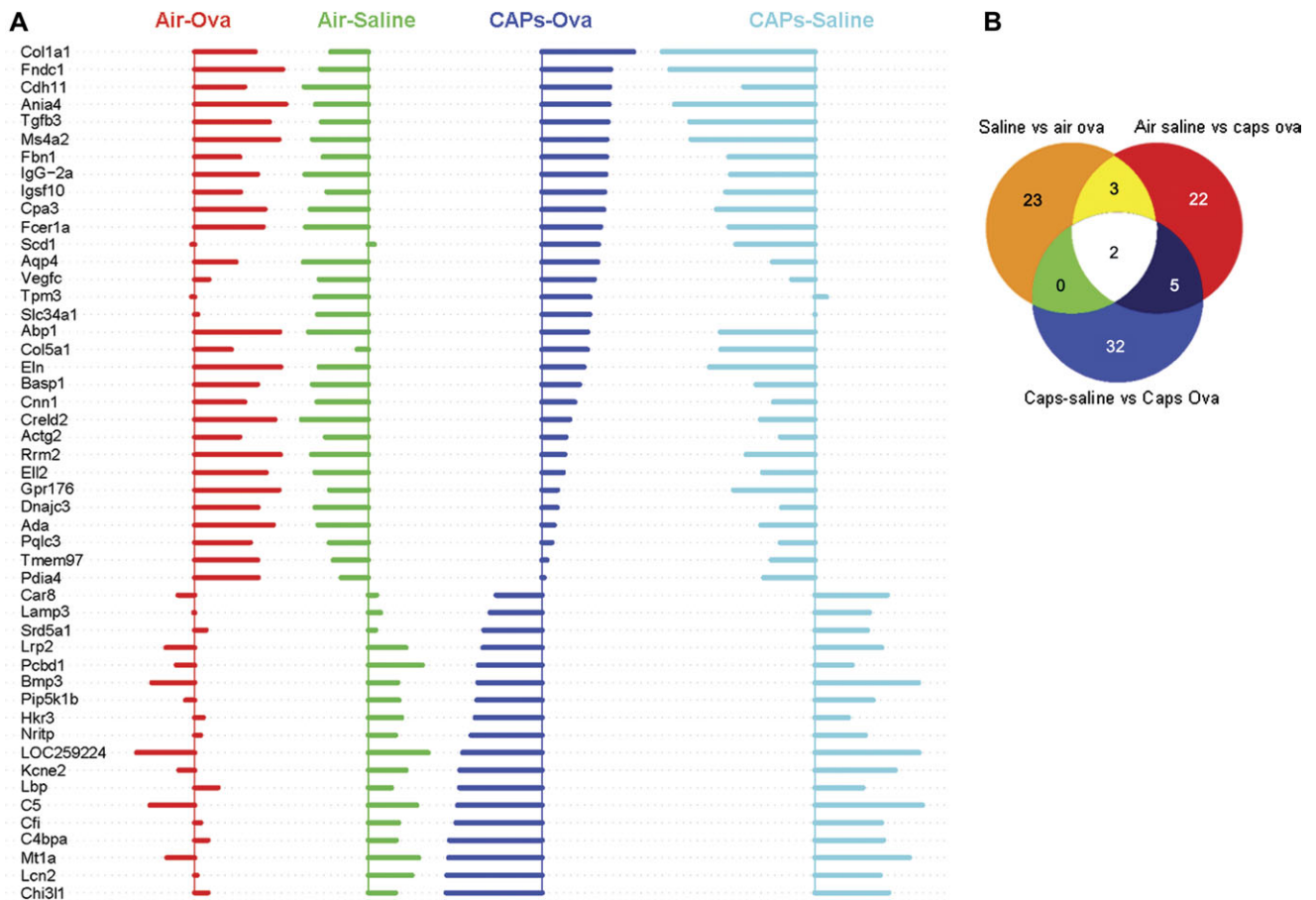


FIG. 3. Probesets with an absolute fold change > 1.5 and a FDR < 0.05 from the three permutation analyses for differential expression comparisons were considered significant, (total of 87 differentially expressed probesets). (A) Centroid plot of significant genes. The bars represent expression values relative to the centroid value for each gene considering all groups. Bars extending to the right of the vertical line in each treatment group represent elevated expression of that gene; bars extending to the left represent lower expression of that gene. Genes are plotted in descending order based on expression by the CAPs-ova treatment group. Red = air-ova, green = air-saline, dark blue = CAPs-ova, light blue = CAPs-saline. (B) Venn diagram of all 87 significant probesets.

class-wise expression of genes that met these criteria, not including probesets of limited or ambiguous annotation (for the full list of genes see Supplemental Table S1). The shrunken centroids were calculated using the method described in (Tibshirani *et al.*, 2002). It is clear in this centroid plot that the relative gene expression patterns for the CAPs-ova treatment group were opposite that of the air-saline and CAPs-saline treatment groups and more like that seen in the air-ova treatment group. Figure 3B shows a Venn diagram indicating the amount of overlap between the significant probesets from pairwise comparisons. The first pairwise comparison group studied was the air-exposed, saline-challenged rats compared with the air-exposed, ova-challenged rats (air-saline vs. air-ova). The 28 differentially expressed probesets in this comparison group were all upregulated; the greatest gene upregulation was 3.39-fold for immunoglobulin heavy chain gamma 2a (IgG-2a). The expression of 39 probesets was significantly changed in the CAPs-exposed, saline-challenged rats compared with the CAPs-exposed, ova-challenged rats (CAPs-saline vs. CAPs-

ova). The greatest increase in expression was 4.95-fold for stearoyl-coenzyme A desaturated 1 (Scd1); the greatest decrease was -4.23 -fold for complement component 5 (C5). A total of 32 probesets were differentially expressed in the air-saline rats compared with the CAPs-exposed, ova-challenged rats (air-saline vs. CAPs-ova). The greatest upregulation was 3.42-fold for IgG-2a, and the greatest downregulation was 2.49-fold for Mt1a. No significant differentially expressed probesets were found by comparing the air-saline and air-ova groups to the CAPs-saline and CAPs-ova, respectively (data not shown).

The relative upregulation/downregulation of genes was confirmed using qPCR (Fig. 4). Specifically, the PADE analysis indicated that TGF- β 3 is expressed 1.6- and 2.2-fold higher in CAPs-ova versus air-saline and versus CAPs-saline exposed rodents, respectively; Fcgr1a was expressed 2.1-fold higher in CAPs-ova versus air-saline rodents; C4BP was expressed 2.8-fold lower in CAPs-ova versus CAPs-saline rodents; VEGF-C was expressed 1.6-fold higher in CAPs-ova

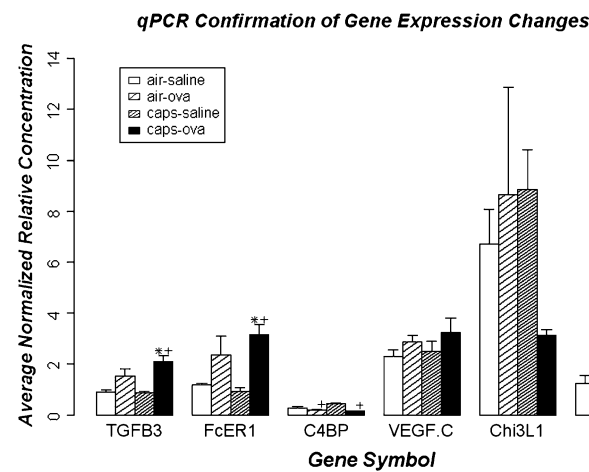


FIG. 4. Gene expression by confirmatory qPCR. The average relative concentration of each target gene (run in duplicate), divided by the corresponding β -actin endogenous control per rat, then averaged with all rats of the same treatment group. Error bars show the standard error of the mean. The relative expression of each gene by treatment group corresponds with microarray data outputs (compare to Supplemental Table 1). *Significantly different from air-saline treatment ($p < 0.05$); +significantly different from CAPs-saline treatment ($p < 0.05$); there were no significant differences between the air-ova & CAPs-ova groups.

versus air-saline rodents; Chi3L1 was expressed 3.5-fold lower in CAPs-ova versus CAPS-saline rodents; and Mt1a was expressed 3.3-fold lower in CAPs-ova versus CAPs-saline rodents and 2.5-fold lower in air-ova versus air-saline rodents. Due to small sample size, the changes for three of the six genes were not significantly different, illustrating the danger in relying upon measurements of single genes in studies like this. Microarray studies allow the simultaneous consideration of groups of functionally related genes, allowing smaller changes to be detected (as discussed below).

Principal Components and Cluster Analysis

PCA by treatment conditions was performed on array data using the list of 87 differentially expressed probesets described above (Fig. 5A). The saline-challenged animals were separated from the ova-challenged animals by the first principal component, which accounts for 55.92% of the variance between samples. The second principal component (accounting for 15.21% of variance) separated the ova-challenged rats between those exposed to laboratory air and those exposed to CAPs with one exception. The air-saline and the CAPs-saline groups were indistinguishable in the first two principal components. Hierarchical clustering of all samples by condition, based on the list of 87 differentially expressed probesets, separated the animals into groups of either saline-challenged or ova-challenged rodents (Fig. 5B). Individual probesets were normalized to the median value for coloring, and clustering indicated two main branches. The bottom branch showed lower levels in six of the eight ova-challenged samples versus higher

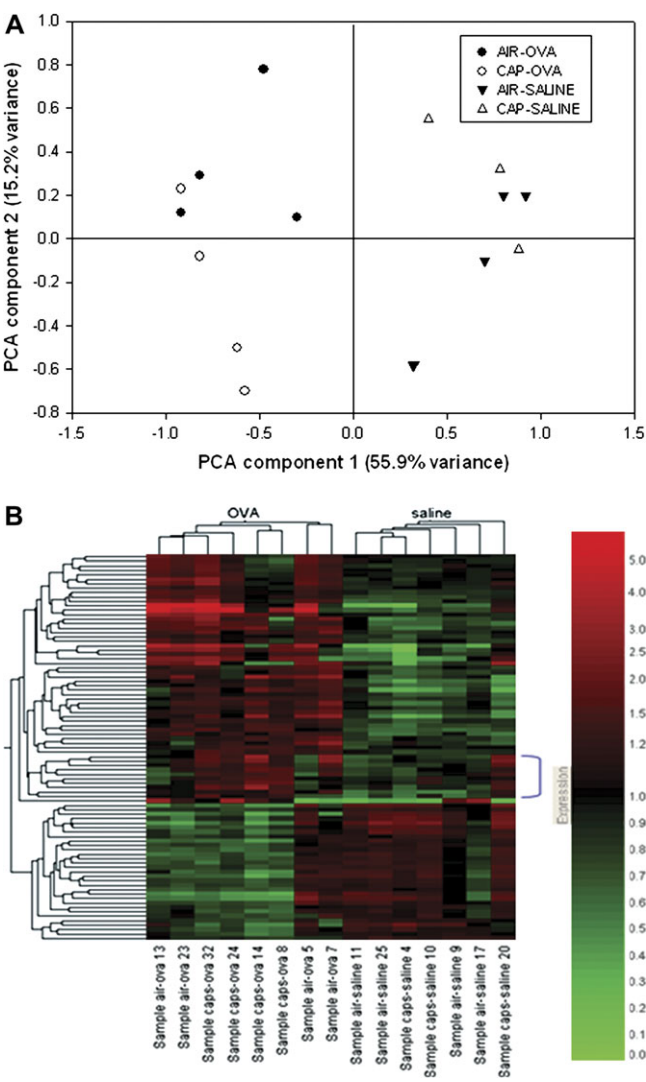


FIG. 5. Principal components analysis and hierarchical clustering of differentially expressed probesets. The list of 87 differentially expressed probesets was used for the analyses. (A) Two dimensional principal components analysis by conditions of treatment. x-axis: component 1, accounting for 55.92% of variance; y-axis: component 2, accounting for 15.21% of variance. Component 1 strongly separates the ova-challenged from the saline-challenged rats. (B) Hierarchical clustering of treatment conditions and genes. Expression levels were derived from REDI-adjusted and RMA-normalized signal values, which were then normalized to the per gene median in order to achieve the color scale (red indicates a high level of expression, green indicates a low level). Clustering shows that the rats fall into two main groups: ova-challenged and saline-challenged. The blue bracket indicates probesets in a subset of the top branch that are highly expressed in all of the CAPs-ova exposed rats and in one rat each from the air-ova and the CAPs-saline exposure groups.

expression levels in all but one of the saline-challenged animals. The top branch was the reverse: it showed mostly higher expression in the ova-exposed animals and lower expression values in all but one saline-exposed animal. One gene, Scd1, is shown between these two groups.

Functional Analysis

Functional analysis to screen the differentially expressed genes in the three group comparisons was performed in Metacore using a larger number of genes (air-saline vs. air-ova = 75, CAPs-saline vs. CAPs-ova = 93, and air-saline vs. CAPs-ova = 200) obtained via a relaxed FDR cutoff of ≤ 0.2 (Supplemental Table S2). The most significant Metacore maps (corresponding to known biological pathways curated by GeneGo) of the air-saline versus air-ova comparison were split into two main groups: nucleotide metabolism (maps 1–2, $p = 1.279 \times 10^{-2}$, 2.445×10^{-2} , respectively) and immune response/histamine metabolism (maps 3, 4, 6, 8, p value range = 3.227×10^{-2} to 4.501×10^{-2}) (data not shown). Maps 1 and 5 for the CAPs-saline vs. CAPs-ova comparison were related to cell adhesion and extracellular matrix (ECM) remodeling ($p = 2.726 \times 10^{-4}$, 8.206×10^{-3} , respectively), whereas maps 2–4 were related to immune response via complement pathways (p value range = 8.324×10^{-4} to 6.367×10^{-3}) (data not shown). The top 10 maps for the air-saline versus CAPs-ova comparison also broke into two main groups. Maps 1–3 were related to immune response via complement pathways (p value range = 3.229×10^{-5} to 9.242×10^{-5}), and maps 4–9 related to remodeling and cell adhesion (p value range = 1.841×10^{-4} to 3.405×10^{-3}) (data not

shown). GO processes for this comparison broke into similar groups, with the first and second processes relating to lung development ($p = 4.0489 \times 10^{-7}$ and 4.5838×10^{-7}) and processes 3–7 relating to the inflammatory response (p value range = 6.0503×10^{-7} to 2.2709×10^{-5}) (data not shown).

The most statistically significant gene ontology processes across all genelists are shown in Figure 6. The top three relate to tissue development and the fourth to the defense response (Fig. 6). It is of note that the top four categories were much more significant in the presence of CAPs than with ova treatment alone. Air-saline versus CAPs-ova had the most significant relationship to lung/respiratory tube development and defense response indicating both inflammatory responses as well as remodeling in response to this treatment. The CAPs-saline versus CAPs-ova comparison had the most significant relationship with skeletal development and the differentially expressed genes in the skeletal development category were predominantly regulated via TGF- β . This, along with the significant enrichment for the “regulation of TGF- β receptor signaling” category (Fig. 6), suggests that changes in TGF- β signaling are particularly significant when comparing these two groups. The expression changes for TGF- β 3 (Fig. 3) may explain this effect in that transcription is reduced by CAPs exposure in the absence of ova challenge but not in the presence of ovalbumin.

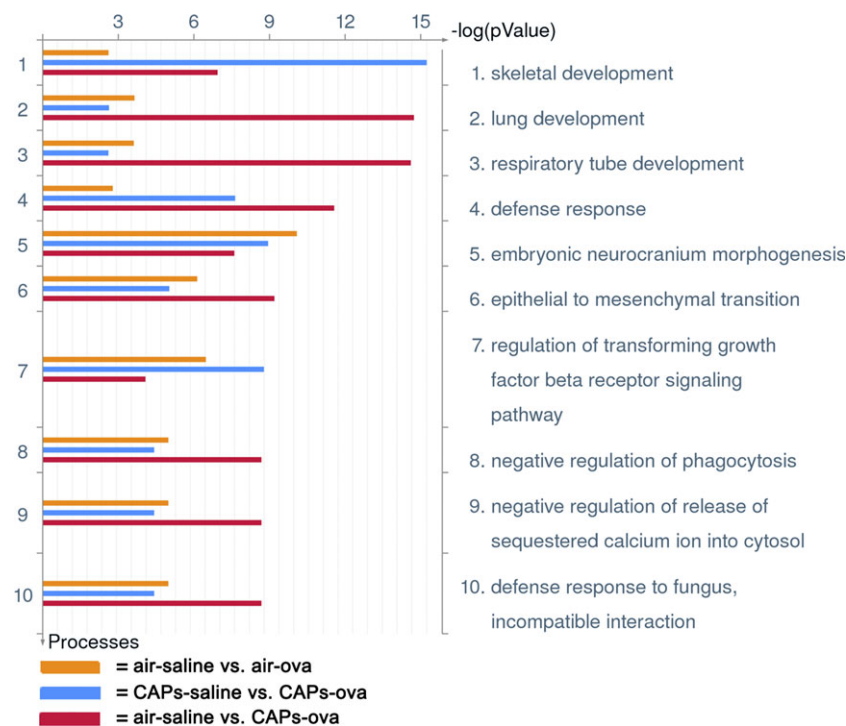


FIG. 6. Overrepresented gene ontology categories from Metacore for all three treatment comparisons. y-axis shows the top 10 GO processes sorted by the lowest p value for any single treatment comparison. Longer bars correspond to lower p values for enrichment of genes in a GO category within the differentially expressed genes in each treatment contrast with p values ranging from 10^{-3} to 10^{-15} . Categories 1 and 7 were most significantly enriched in the CAPs-saline versus CAPs-ova treatment. Categories 2–4, 6, and 8–10 were most significantly enriched in the air-saline versus CAPs-ova treatment. Category 5 was most significantly enriched in the air-saline versus air-ova treatment. Top, air-saline versus air-ova, middle—CAPs-saline versus CAPs-ova, bottom—air-saline versus CAPs-ova. p Values reported by MetaCore are not corrected for multiple testing.

The only genes that overlap between the skeletal and lung/respiratory tube development processes that are found in these two genelists were TGF- β 1 and TGF- β 3, however several of the other genes from the skeletal development process (Fbn1, Col1A1, Col1A2, and Cdh11) could be involved in lung/respiratory tube development as well. *p* Values reported by MetaCore are not corrected for multiple testing.

Network Analysis

The CAPs-ova synergy was investigated further with pathways analysis using Ingenuity IPA. The highest-scoring network generated for air-saline versus CAPs-ova had top

functions of cellular movement, immune response, cell-to-cell signaling and interaction (Fig. 7) and matched the results seen with the MetaCore analysis (Fig. 6). The genes in this network can be categorized as either related to remodeling (orange circle), inflammation (purple circle) or both. Overlaying data relating only to air-saline versus air-ova onto this network show three genes (Fcer1A, Ms4a2, Bcl6b) related to inflammation (Albrecht *et al.*, 2004; Galli *et al.*, 2008), two genes (cadherin 11 [Cdh11] and TGF- β 3) that fall under both inflammation and remodeling (Broide, 2008), and one gene (elastin [Eln]) that is consistent with remodeling only (Shi *et al.*, 2007). Overlaying data relating only to the air-saline versus CAPs-ova comparison show a dramatic increase in the

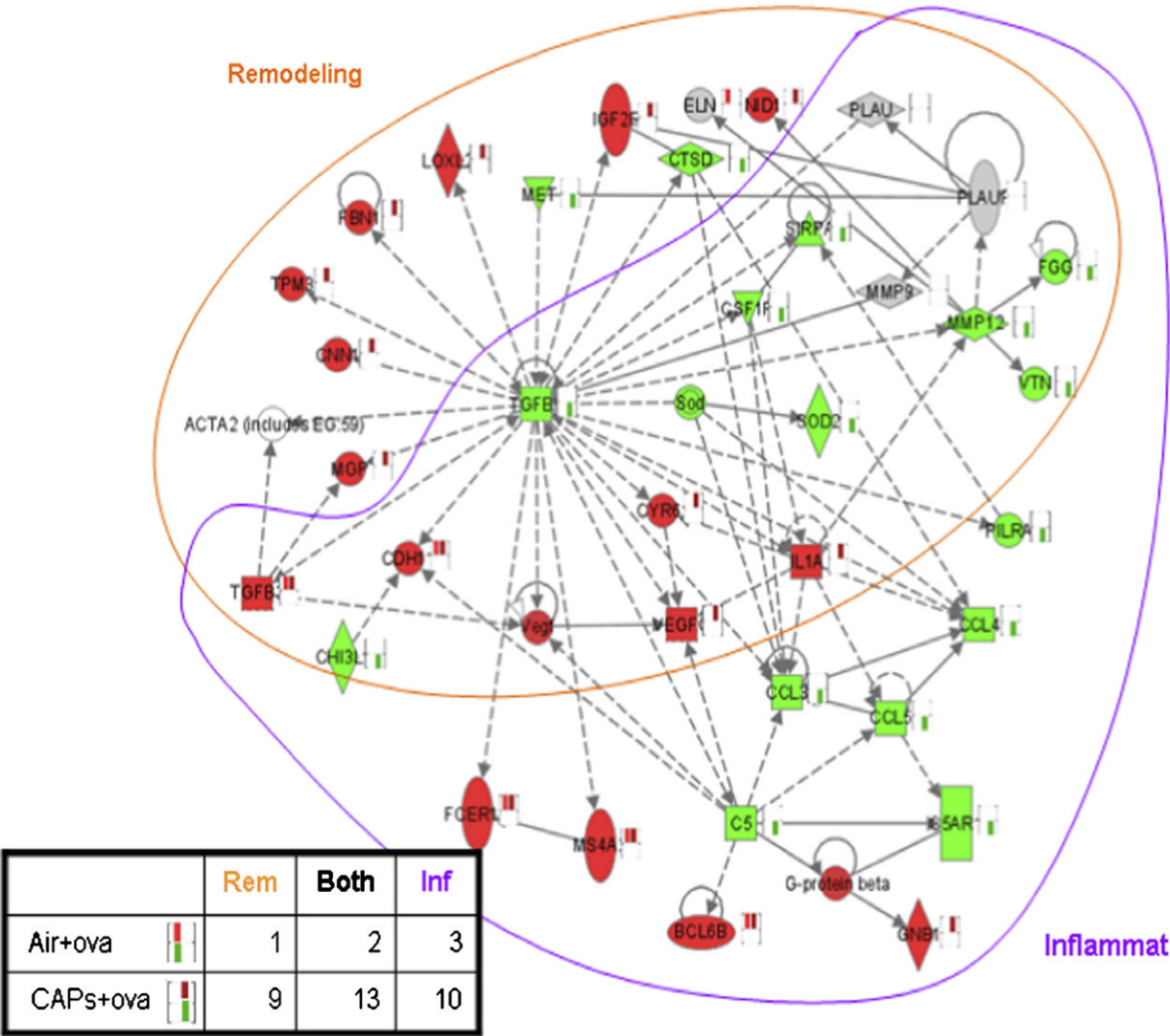


FIG. 7. Gene network derived from PADE data from the air-saline versus CAPs-ova comparison was uploaded into Ingenuity IPA. Cutoffs were set at ≥ 1.5 -fold for fold change and ≤ 0.2 for FDR. This network represents the highest-scoring network (score = 61), with genes involved in either inflammatory (purple circle) or remodeling (orange circle) mechanisms. For nodes, red = upregulated expression and green = downregulated expression in the CAPs-ova versus air-saline comparison. Bars adjacent to each node represent statistically significant expression (upregulated or downregulated) for air-saline versus air-ova (left) and air-saline versus CAPs-ova (right). The table (inset) shows the number of genes falling in each class showing the enhancement of effects by coexposure to CAPs. Note that three nodes (Sod, Vegf, G-protein beta) in the network correspond to gene families rather than genes and were not counted in the totals. (see Supplemental Table 4).

number of the genes falling into inflammation, remodeling, or both (Fig. 7, inset).

The genes in the remodeling category that do not overlap with inflammation are predominantly upregulated, indicating an increase in pathways that impact remodeling. Expression levels of the remaining genes in the remodeling category and those in the inflammation category are mixed. For example, the IgE receptors FCER1A and MS4A2/FCER1B and the cytokine IL1A are upregulated as expected, whereas genes (Mmp12 and CCL5/RANTES) that have been shown to be upregulated in asthma are

downregulated in this study. This is possibly due to mechanisms such as feedback inhibitory signaling at the level of transcription and may not reflect a decrease in the associated protein. The inflammatory and remodeling genes PLAUR, PLAUR, and Mmp9 were found in a screen for genes related to COPD (Wang *et al.*, 2007), and both PLAUR and MMP9 have been implicated in asthma (Begin *et al.*, 2007; Sampsonas *et al.*, 2007). Thus, these three genes were included in the network even though their fold changes and/or FDRs did not meet cutoff values. PLAUR came very close to significance, with an FDR of 0.146 and a fold

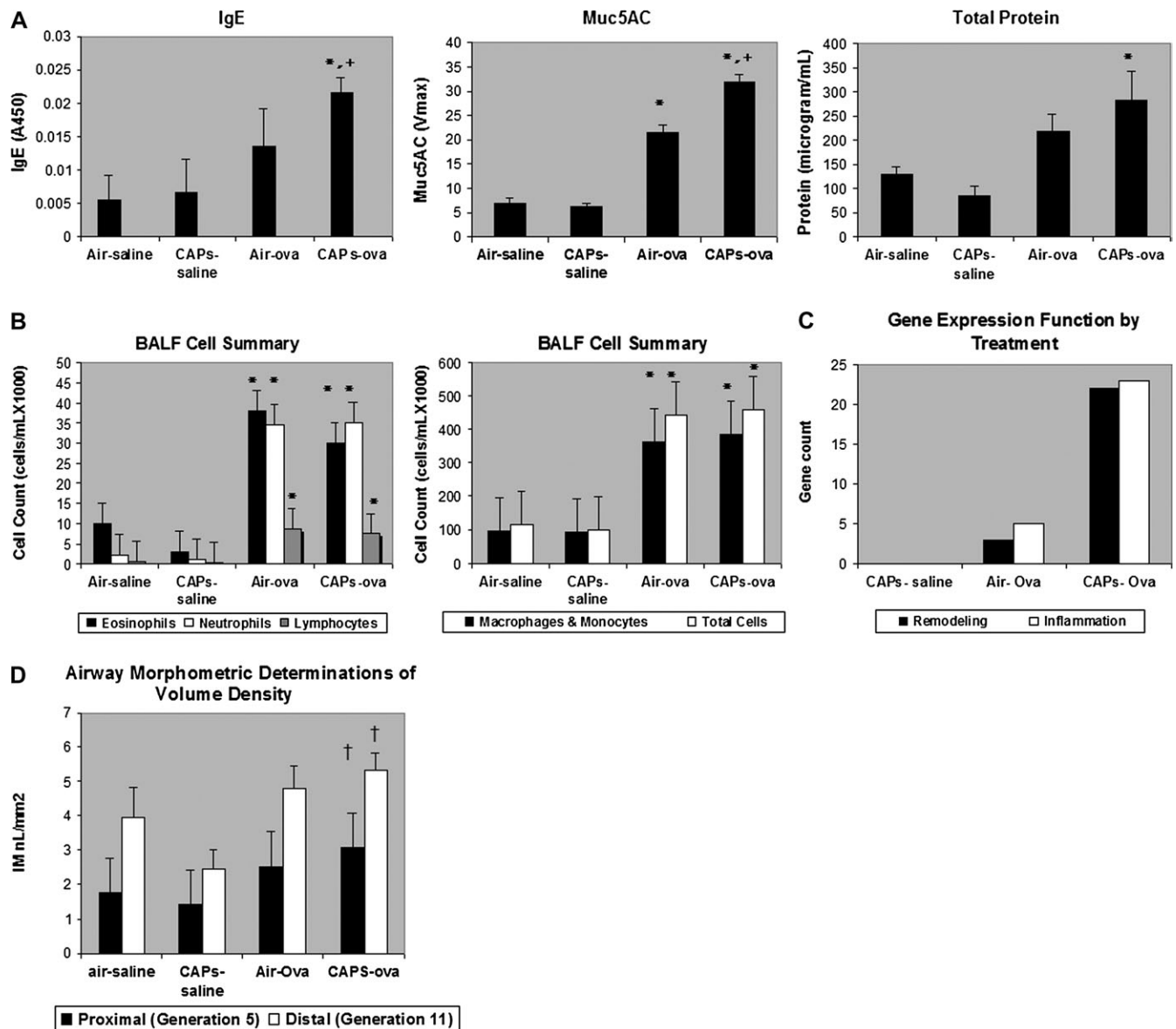


FIG. 8. Effect of ova sensitization and challenge (with and without coexposure to CAPs) on biochemical and cellular parameters in the BALF and gene expression and airway morphometry in lung tissue. (A) IgE Mucin 5AC and total Protein measured in BALF; (B) BALF cell differential; (C) number of genes significantly changed in each treatment group ($n = 4$) by functional category. Data represent the group mean ($n = 8$) and SEM in each treatment group for (A, B, D). *Significantly different from air-saline group ($p \leq 0.05$); +significantly different from air-ova group ($p \leq 0.05$). (D) Morphometric determinations of the volume density of AB/PAS-stained IMs lining the axial airways of the left lobe (Proximal airway generation 5 and distal airway generation 11). †Significantly different from CAPs-saline group ($p \leq 0.05$). Volume density (Vs; nl/mm²).

change of -1.3 in air-saline versus CAPs-ova group. Overall, the network analysis shows a perturbation of inflammatory genes in rodents challenged with allergen. Moreover, genes involved in the remodeling of lung tissue are altered by CAPs exposure only in the presence of allergen challenge.

BALF

Figure 8 contains the results of the biochemical and cellular analyses of the BALF and gene expression and lung morphometric determinations from each of the exposure groups (Supplemental Table S3 contains the BALF data in table form). Exposure to CAPs for 13 consecutive days produced no changes in BALF proteins or cellularity in saline-challenged rats. Sensitization and challenge with ova caused increases in total cells (fourfold), neutrophils (15-fold), lymphocytes (16-fold), eosinophils (fourfold) (Fig. 8B), and mucin glycoprotein (muc5AC) (threefold) (Fig. 8A). CAPs exposure did not affect ova-induced increases in BALF cellularity, but enhanced ova-induced increase in mucin glycoprotein by 50% (Fig. 8A). Furthermore, only ova-challenged rats exposed to CAPs had significant elevations in total BALF protein. CAPs exposure also enhanced ova-induced increases in ova-specific IgE in BALF (Fig. 8A). The gene expression changes reflected this same trend (Fig. 8C) with the number of inflammatory genes increasing from 5 (OVA alone) to 23 (CAPs plus OVA) and the number of remodeling genes increasing from 3 (OVA alone) to 22 (CAPs and OVA). Total IgE in BALF was not significantly increased by CAPs exposure or ova challenge. The small sample size for this study did not provide the power required to explicitly test for correlation, but the cellular & protein changes are consistent with the gene expression results (Figs. 7 and 8C) with the caveat that the cellular changes are less sensitive with regard to the enhancement of inflammation and remodeling by CAPs.

The number of eosinophils in the BALFs from our ova-sensitized and saline-challenged BN rats were higher than routinely seen in other strains/stocks of rats (e.g., F344, Sprague-Dawley), but not uncommon for the BN strain of rat, and well below previously published reports in non-infected BN rats that were similarly ova-sensitized and saline challenged (Noritake *et al.*, 2007). The levels of both eosinophils and neutrophils in the BALFs of our ova-challenged rats were markedly and significantly elevated compared with our saline-challenged controls, indicative of an allergen-induced hypersensitivity response.

Pulmonary Pathology/Morphometry

Light microscopic examination of the selected lung sections from all the rats in this study, substantiated and correlated well with the analytical observations of the BALF from these same rats. Furthermore, significant morphometric differences among the groups, described below, correlated with our findings in the BALF and histopathology. The lungs of control rats (ova-sensitized and saline-challenged rats) had a minimal influx of eosinophils widely scattered along with a few mononuclear

leukocytes (e.g., lymphocytes, plasma cells) in the interstitium surrounding some of the bronchiolar airways. In addition, there were occasional small foci of eosinophils and mononuclear cells (macrophages, monocytes, and lymphocytes) in the alveolar parenchyma of these control rats. These background findings are consistent with previous reports on the normal pulmonary morphology of pathogen-free BN rats (Noritake *et al.*, 2007). In contrast, the lungs of BN rats sensitized and challenged with ova had conspicuous allergic bronchiolitis and alveolitis (allergic bronchopneumonia). There was a noticeable proximal to distal decrease in the severity of the bronchiolitis in the left lung lobe of these rodents with more inflammatory lesions in the proximal lung section (G5 axial airway level; closest to the hilus of the lung lobe) compared with the more distal section (G11 axial airway level). Ova-induced inflammatory and epithelial lesions in the conducting airways involved the large diameter, proximal axial airways and the small diameter, distal pre-terminal and terminal airways. Inflammatory and epithelial lesions were usually more severe in the more proximal axial bronchioles compared with those in the more distal pre-terminal and terminal bronchioles. Ova-induced bronchiolitis was characterized by peribronchiolar edema associated with a mixed inflammatory cell influx of eosinophils, lymphocytes, plasma cells, and occasional neutrophils (Fig. 9).

Bronchiole-associated lymphoid tissues in the air-ova group airways were also enlarged due to lymphoid hyperplasia relative to the air-saline group. Perivascular interstitial accumulation of a similar mixture of eosinophils and mononuclear cells, along with perivascular edema, were also present in the lungs of air-ova rats (i.e., surrounding pulmonary arteries adjacent to bronchioles and pulmonary veins scattered throughout the alveolar parenchyma).

Air-ova rats had a mild-marked epithelial hypertrophy and mucous cell metaplasia/hyperplasia (MCM) with increased amounts of AB/PAS-stained mucosubstances in the mucous cells within the surface epithelium (i.e., IMs) lining the affected large diameter bronchioles, including the proximal and distal axial airways (Fig. 9). Saline-challenged rats (the air-saline group) had mucous cells with less IM compared with the ova-challenged (air-ova) rats (Fig. 10). There was no significant difference in the amounts of IM between air-saline and CAPs-saline rats (Fig. 8D).

In addition to the perivascular and peribronchiolar lesions, there were varying sized focal areas of allergic alveolitis in the lung parenchyma of the air-ova rats. These alveolar lesions were characterized by accumulations of large numbers of alveolar macrophages, epithelioid cells, and eosinophils, with lesser numbers of lymphocytes, monocytes, and plasma cells, in the alveolar airspace. Often the alveolar septa in these areas of alveolitis were thickened due to type II pneumocyte hyperplasia and hypertrophy, intracapillary accumulation of inflammatory cells, and capillary congestion.

Ova-challenged rats exposed to CAPs (the CAPs-ova group) had a more severe allergic bronchopneumonia than the air-ova group. This was reflected both in the severity and distribution of

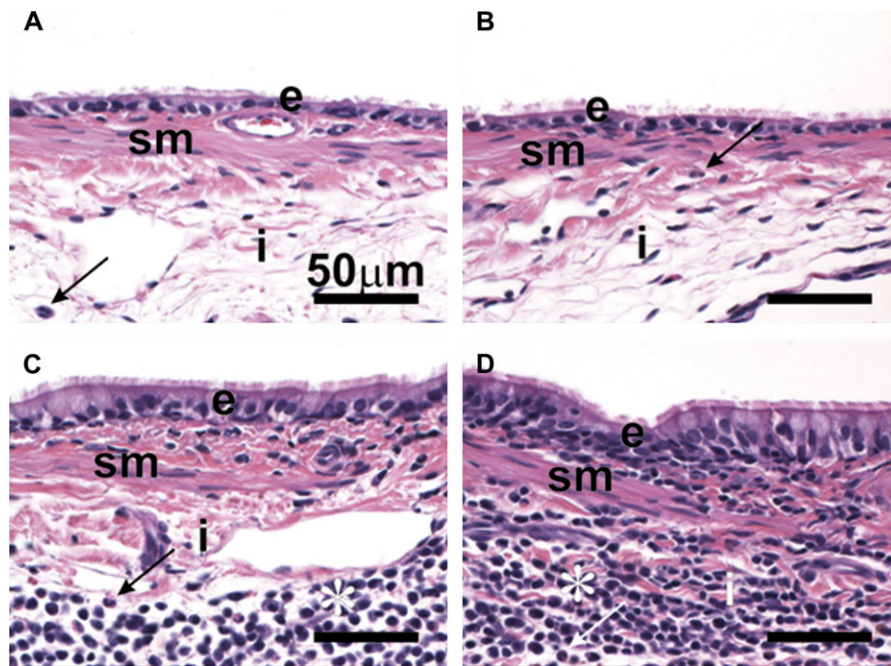


FIG. 9. Light photomicrographs of the respiratory epithelium (e) lining the proximal axial airway (generation 5) in the left lung lobe of rats exposed to (A) air/saline (control), (B) CAPs/saline, (C) air/ova, or (D) CAPs/ova. Compared with the normal airway in the control rats (A), significant morphologic changes are present only in rats challenged with ova (C, D). The most prominent histologic changes due to ova challenge included a thickened, hypertrophic, respiratory epithelium with increased numbers of mucous goblet cells, and a mixed inflammatory cell infiltrate (asterisks) consisting mainly of lymphocytes, plasma cells and widely scattered eosinophils (arrows) in the interstitium (i) of the airway wall. These histologic airway changes are slightly greater in the CAPs/ova rats (D) compared with those in the air/ova rats (C). All tissues stained with H&E. Sm, smooth muscle.

the allergic bronchiolitis and alveolitis. Air-ova rats had a mild to moderate allergic bronchopneumonia with the inflammatory and epithelial lesions in approximately one fourth to one third of the lung lobe. In contrast, CAPs-ova rats had a moderate to marked bronchopneumonia with lesions in approximately one half or more of the lung lobe. CAPs-ova exposed rats also had more severe MCM in the epithelium lining the large diameter axial airways compared with the air-ova group.

Thirteen consecutive days of CAPs exposure in saline-challenged rats did not cause changes in the morphometrically measured amounts of stored mucus in airway epithelium in either proximal or distal airways (Fig. 8D). The only significant increases in IMs were found in CAPs-ova rats (Fig. 8D). This CAPs-induced enhancement of ova-induced IM correlated with the increased amounts of muc5AC in BALF from these animals (Fig. 8A). There was a trend (though not statistically different) for a CAPs-related exacerbation of ova-induced increases in the stored intraepithelial AB/PAS-stained mucosubstances in both the proximal and distal axial airways of the left lung lobe (quantitative estimate of the severity of MCM).

DISCUSSION

Asthma is a complex airway disease involving gene-environment interactions. This study used comparative micro-

array analysis to investigate the effect that CAPs have on a BN rodent model of airway allergic inflammation. An enhanced effect on the type and magnitude of gene expression changes was observed with ova-challenged BN rats coexposed with CAPs. The genomic data were consistent with the observed histopathology. This combination of allergen challenge and CAPs exposure led to differential expression of genes related to airway remodeling that was not observed in rodents treated with either allergen or CAPs alone.

In agreement with the data presented here, air particulates have been shown to have an adjuvant effect on allergic reactions as determined by changes in histopathology. For example, BN rats exposed to both CAPs and ova showed adjuvant effects which resulted in increased airway mucus, mucin glycoprotein muc5AC, pulmonary inflammation, and airway epithelial remodeling (e.g., MCM) (Harkema *et al.*, 2004a). Other studies point to an adjuvant effect of diesel particulates on ova-challenged rodents which resulted in enhanced airway hyperresponsiveness and pulmonary inflammation (Dong *et al.*, 2005; Matsumoto *et al.*, 2006; Miyabara *et al.*, 1998; Takano *et al.*, 1997). At the molecular level, exposure to diesel particulates in ragweed-sensitized humans resulted in an increase in mRNA transcripts of asthma-related cytokines (Diaz-Sanchez *et al.*, 1997).

CAPs exposure alone had no significant effect on gene expression in the lung in this study. ApoE/LDL double

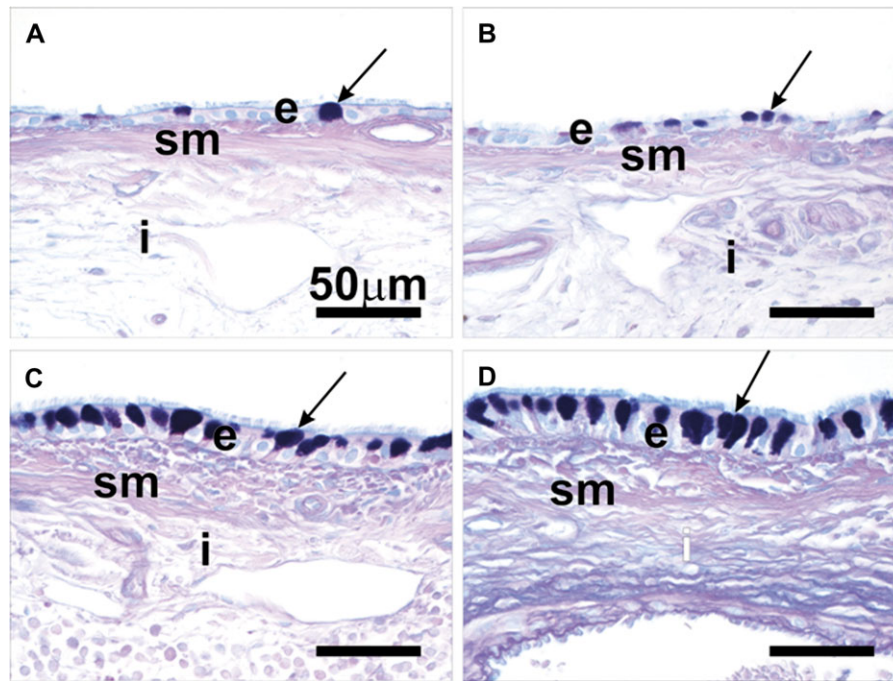


FIG. 10. Light photomicrographs of AB/PAS-stained mucosubstances (arrows; dark magenta stain) in mucous goblet cells of the respiratory epithelium (e) lining the proximal axial airway (generation 5) in the left lung lobe of rats exposed to (A) air/saline (control), (B) CAPs/saline, (C) air/ova, or (D) CAPs/ova. Compared with the normal airway in the control rat (A), significant increases in the amount of IMs are present only in rats challenged with ova (C, D). This ova-induced intraepithelial change is slightly greater in the CAPs/ova rats (D) compared with that in the air/ova rats (C). All tissues stained with AB (pH 2.5)/PAS sequence to detect acidic and neutral mucosubstances. Sm, smooth muscle; I, interstitial layer of the airway wall.

knockout mice exposed to a longer period of inhaled CAPs resulted in no significant changes in gene expression in lung tissue (Gunnison and Chen, 2005). Another study in which mice were intranasally instilled with CAPs showed increased gene expression in several cytokines and an increase in polymorphonuclear cells in BALF (Sigaud *et al.*, 2007), however the authors note the high dose of particles used for instillation was a useful for proof-of-principle but not applicable to realistic exposures. A recent toxicogenomics study found differential gene expression upon inhalation exposure of BALB/c mice to DE (Stevens *et al.*, 2008) in the presence or absence of ova. The genes identified in that study were involved in immune function, cell signaling, and metabolic and oxidative stress response. Taken together, this suggests that the response is dependent on the source of air pollutants, individual susceptibility and possibly route and duration of exposure.

Ova challenge alone resulted in the largest changes in magnitude of gene expression, albeit for a limited number of genes, in the present study (Fig. 5B). Two prominent genes upregulated by ova challenge were *Fcεr1a* and *Ms4a2/Fcεr1b*; receptors for IgE that factor into allergy mediated events. In the present study and in previous studies, cytological, and immunohistochemical methods for assessing ova-induced allergic airway inflammation in BN rats showed responses common to asthma such as increased levels of airway mucous glycoproteins, MCM in bronchiolar epithelium, increased numbers of eosinophils, neutrophils, and lymphocytes in both

lung tissue and BALF, and increased IgE in the serum (Salmon *et al.*, 1999; Tarayre *et al.*, 1992; Underwood *et al.*, 1995). Gene expression studies in ova-challenged mice have implicated a variety of genes that relate to inflammation, airway hyperresponsiveness, atopy, or mucus production and may impact asthma pathogenesis (Follettie *et al.*, 2006; Izuhara and Saito, 2006; Kuperman *et al.*, 2005; Walker *et al.*, 2006). Our study is consistent with these previous reports in that they all implicate genes involved in inflammation.

In contrast to the results seen with ova alone, the effects specifically due to CAPs/ova coexposure were more subtle though much more widespread with regard to number of genes effected. In the current study, CAPs exposure in the ova-challenged rats led to expression changes in remodeling genes, and a larger number of genes related to inflammation compared with ova challenge alone. Inflammation and remodeling pathways centered predominantly on TGF- β 1 (Fig. 7). Two other growth factors, TGF- β 3 and VEGF-C, were also identified in the network. Members of both the TGF family and the VEGF family are thought to be important for airway remodeling and inflammation (Lloyd and Robinson, 2007). There were 23 TGF- β related inflammatory genes significantly changed with CAPs-ova coexposure versus five with ova exposure alone (Figs. 7 and 8C). This corresponded with a significant increase in the amount of ova-specific IgE found in the BALF from CAPs-ova rats in our study when compared with either the air-saline or air-ova groups (Fig. 8A). This is

further evidence for an enhancement of ova challenge by CAPs via increased allergy mediators.

Airway remodeling is a hallmark of asthma referring to changes in structural cells, including the thickening of airway walls (Lloyd and Robinson, 2007). The findings of both bronchiolar and alveolar wall thickening in air-ova and CAPs-ova rats in the present study (due to epithelial hypertrophy and MCM) are characteristic histopathologic features of allergic airway disease that mimic those reported in asthmatic airways of humans. The MCM in the epithelium lining the large diameter axial airways was more severe in the CAPs-ova rats compared with the air-ova rats, consistent with the gene expression data implicating remodeling events in the CAPs-ova exposure group. Significant increases in IM and total BALF protein and elevated muc5AC were apparent only in the CAPs-ova group. Taken together, pulmonary histopathology (subjective assessment of inflammatory and epithelial responses), morphometric measurements (quantitative assessment of airway epithelial remodeling), and BALF analyses (quantitative assessment of inflammatory and mucosecretory responses) consistently demonstrated that CAPs exposure enhanced the allergic airway disease. Typically, inflammation and remodeling processes are viewed separately, however, there is emerging data that suggest that smooth muscle changes can contribute directly to proinflammatory changes that may perpetuate airway inflammation and the development of airway remodeling (Broide, 2008). Collectively, our data support this conclusion.

In total, 22 TGF- β related genes implicated in remodeling were significantly up- or downregulated in response to CAPs-ova coexposure (Figs. 7 and 8C). Several of these are components of the ECM—fibrinogen, vitronectin, nidogen 1, fibrillin 1, elastin, and matrix Gla protein, and three genes are related to smooth muscle and/or the cytoskeleton—calponin 1, α 2 actin, and tropomyosin 3. Altered ECM protein profiles in asthmatic lungs result in an increase in the proliferation rate of airway smooth muscle (ASM) cells, a feature of remodeling that causes the thickening of airway walls (Johnson *et al.*, 2004). TGF- β 1 can increase the proliferation of smooth muscle cells and the deposition of ECM proteins, whereas the smooth muscle cells aid in the downregulation of ECM-degrading matrix metalloproteinases such as Mmp9 and Mmp12 (Parameswaran *et al.*, 2006).

Because inflammatory cells in the BALF were not significantly different between the CAPs-ova and CAPs-saline groups, the changes in gene expression are most likely due to an intracellular transcriptional response from either the inflammatory cells, the resident alveolar cells, or both. Our results cannot distinguish whether the transcriptional changes shown in Figure 7 represent signaling in a single cell type or integrated responses across multiple cell types, but it does provide clues to the signaling changes taking place in response to ova and CAPs coexposure. TGF- β 1 and TGF- β 2 have previously been causally linked to allergen-induced proliferation of ASM cells and mucus-producing goblet cell hyperplasia (Lloyd and Robinson, 2007; McMillan *et al.*, 2005). TGF- β 2

treatment of primary bronchial epithelial cells in culture produced significant increases in MUC5AC mRNA and protein levels as well as elevated mucin as measured by AB/PAS staining (Chu *et al.*, 2004). The results from the current study show more pronounced changes in TGF- β 3 transcript levels suggesting that TGF- β 3 may also play a key role in lung remodeling. This is supported by reports of delays in pulmonary development in TGF- β 3 knockout mice (Kaarinen *et al.*, 1995). This study supports previous findings of TGF- β involvement in lung remodeling, implicates a third member of this gene family in the process, provides clues as to the downstream mediators of this process, and shows an enhancement of these effects upon coexposure to CAPs.

A concern from an air regulatory point of view is whether allergic individuals are more sensitive to adverse effects from exposure to air PM than nonallergic individuals. Application of new genomic technologies together with more traditional toxicological approaches provide a means to begin to address links among inhalation exposures to fine particles, genetic and metabolic changes in the lung cells in response to these exposures, and aggravation of the symptoms of asthma. The integration of these approaches can add important new information to the knowledge base regarding specific asthma mechanisms at more environmentally relevant doses of PM_{2.5} to help ensure adequate protection against adverse health effects, particularly for susceptible individuals.

Asthma is a disease that results from a variety of environmental factors acting on a background of genetic factors. There are likely subtypes of asthmatics with varying susceptibility to a wide array of environmental exposures. Using a rodent model of allergic airway disease, this study explores the application of emerging toxicogenomic tools in conjunction with bronchoalveolar lavage, pulmonary pathology and morphometric analyses to investigate the multifactorial etiology of allergy induced airway inflammation using ova-sensitized and challenged BN rats exposed to environmentally relevant CAPs. By using a sensitive model such as this, the mechanistic basis for the environmental influence can be more easily characterized. Although the contribution of genetics remains uncharacterized, this study provides a framework for interpreting human studies which incorporate the genetic variability most relevant for risk assessment.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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Supplemental Table S4: Genes from network in figure 7 are listed with the indication of the direction c

Annotation	Gene Symbol	Air+Ova	CAPs+Ova
Remodeling	TPM3		up
	FBN1		up
	LOXL2		up
	IGF2R		up
	ELN	up	
	NID1		up
	CNN1		up
	MET		down
	CTSD		down
	MGP		up
Both	TGFB3	up	up
	CHI3L1		down
	CDH11	up	up
	TGFB1		down
	CYR61		up
	VEGFC		up
	IL1A		up
	CSF1R		down
	SIRPA		down
	SOD2		down
	MMP12		down
	FGG		down
	VTN		down
Inflammation	FCER1A	up	up
	MS4A2	up	up
	BCL6B	up	up
	C5		down
	CCL3		down
	CCL5		down
	C5AR1		down
	GNB1		up
	CCL4		down
	PILRA		down

of change and associated annotation