

Increased Transcription of Immune and Metabolic Pathways in Naïve and Allergic Mice Exposed to Diesel Exhaust

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Diesel exhaust (DE) has been shown to enhance allergic sensitization in animals following high-dose instillation or chronic inhalation exposure scenarios. The purpose of this study was to determine if short-term exposures to diluted DE enhance allergic immune responses to antigen, and identify possible mechanisms using microarray technology. BALB/c mice were exposed to filtered air or diluted DE to yield particle concentrations of 500 or 2000 $\mu\text{g}/\text{m}^3$ 4 h/day on days 0–4. Mice were immunized intranasally with ovalbumin (OVA) antigen or saline on days 0–2, challenged on day 18 with OVA or saline, and all mice were challenged with OVA on day 28. Mice were necropsied either 4 h after the last DE exposure on day 4, or 18, 48, and 96 h after the last challenge. Immunological endpoints included OVA-specific serum IgE, biochemical and cellular profiles of bronchoalveolar lavage (BAL), and cytokine production in the BAL. OVA-immunized mice exposed to both concentrations of DE had increased eosinophils, neutrophils, lymphocytes, and interleukin-6 (high dose only) post-challenge compared with OVA control, whereas DE/saline exposure yielded increases in neutrophils at the high dose only. Transcriptional microarray analysis 4 h after the last DE exposure demonstrated distinct gene expression profiles for the high-dose DE/OVA and DE/saline groups. DE/OVA induced oxidative stress and metabolism pathways, whereas DE in the absence of immunization modulated cell cycle control, growth and differentiation, G-proteins, and cell adhesion pathways. This study shows for the first time early changes in gene expression induced by the combination of DE inhalation and mucosal immunization, which resulted in stronger development of allergic eosinophilia.

Key Words: diesel; genomics; mice; lung; allergy.

The prevalence of allergic asthma has risen over the last four decades and has been linked to increased urbanization and exposure to airborne pollutants (Brauer *et al.*, 2003; Delfino *et al.*, 2003; Hwang *et al.*, 2005). Recent studies have shown that the actual incidence of asthma could be related to residential highway proximity (Heinrich and Wichmann,

2004; Lwebuga-Mukasa *et al.*, 2004) or average ozone exposure concentrations (McConnell *et al.*, 2002; McDonnell *et al.*, 1999). A large contributor to urban air pollution and ambient particulate matter (PM) is diesel exhaust (DE). Human occupational exposures to DE range from 1 to 100 $\mu\text{g}/\text{m}^3$ (8-h averages) in the trucking and transportation industry, average 225 $\mu\text{g}/\text{m}^3$ (time-weighted average with adjustment for background levels) for Boston and New York firefighters, and 100–1700 $\mu\text{g}/\text{m}^3$ for underground miners (Froines *et al.*, 1987; Nauss, 1997). Experiments in mice have shown that inhalation of fresh DE or intrapulmonary instillation of DE particles (DEPs) results in adjuvant activity that increases the sensitization to allergens (Matsumoto *et al.*, 2006; Miyabara *et al.*, 1998; Steerenberg *et al.*, 2003). Most of these studies used high-dose instillation or chronic inhalation exposure scenarios to demonstrate this detrimental health effect, whereas some limited instillation studies showed similar effects in humans (Diaz-Sanchez *et al.*, 1997; 1999).

The mechanisms by which DE acts as an adjuvant are complex. The adjuvant potential of DE has been attributed to the generation of reactive oxygen species (ROS) by pro-oxidative organic chemical compounds on the surface of the particles (Hiura *et al.*, 1999; Li *et al.*, 2000; 2002; Xia *et al.*, 2004). There is good evidence, however, that the carbon core of DEPs also imparts adjuvant activity through ROS production, as can accompanying gases such as NO_2 (Fujimaki and Kurokawa, 2004; Gilmour and Selgrade, 1996; Lovik *et al.*, 1997; Maejima *et al.*, 2001; Nilsen *et al.*, 1997). ROS generation by any of these components of DE may lead to a three-tier hierarchical oxidative stress response described by Li *et al.* (2003a). Initial responses to oxidative stress first lead to the induction of antioxidant and detoxification mechanisms, which restore cellular homeostasis (tier 1). If the capacity of these systems is overwhelmed, the ensuing inflammation (tier 2) leads to apoptosis (tier 3) (Li *et al.*, 2003a). The importance of oxidative stress in promoting allergic immune responses is supported by reversal of these effects with thiol antioxidants (Li *et al.*, 2002; Whitekus *et al.*, 2002).

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After DE exposure in the context of antigen, components of the immune response responsible for allergic sensitization, such as interleukin (IL)-4, IL-5, and IL-13, are upregulated. This immune skewing results in a bias toward T-helper 2 immune activity, and increased development of IgE antibodies (Peden, 2000). Following antigen challenge there are subsequent increases in clinical indicators of asthma such as eosinophilic lung inflammation, airway hyperresponsiveness, and airway mucous production (Peden, 2000). Although the chemical components of DE that cause these adjuvant effects under high-dose conditions are diverse, demonstrating significant effects with more realistic inhalation exposure scenarios has been challenging because resultant changes in response are much smaller in magnitude.

Inhalation exposure studies are important from a dosimetry perspective for risk assessment calculations. Because low levels of DE exposure cause minimal changes in disease over short exposure periods, we sought to investigate more sensitive measures of altered immune function and early signaling pathways. The field of toxicogenomics has allowed simultaneous comparison of thousands of genes following experimental perturbations. Accompanying data sets and analytical software packages have been critical in identifying pathways as opposed to comparing single genes (Blalock *et al.*, 2005). Although some *in vitro* genomic studies of DEPs have been reported, no data are currently available for *in vivo* inhalation exposures. Furthermore, the interaction with antigen sensitization has not been studied with a broad toxicogenomic pathway analysis approach.

In this study mice were exposed by whole-body inhalation to filtered air or DE diluted to yield 500 or 2000 $\mu\text{g}/\text{m}^3$ of DEP. Exposures were conducted for 4 h/day over 5 consecutive days (days 0–4). On days 0, 1, and 2, mice were intranasally instilled with 100 μg of ovalbumin (OVA) or saline. Day 18 mice were either challenged with OVA or saline and all mice were challenged with OVA on day 28. Effects were assessed after the 2^o challenge to confirm that mild adjuvancy was accomplished. Lung tissues taken 4 h after the last DE exposure on day 4 were assessed for alterations in global gene expression as an indicator of changes associated with later development of clinical disease.

MATERIALS AND METHODS

Animals. Pathogen-free BALB/c female mice, 10–12 weeks old, weighing 17–20 g, were purchased from Charles River (Raleigh, NC). All of the animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facilities with high-efficiency particulate air (HEPA) filters and received access to food and water *ad libitum*. The studies were conducted after approval by the laboratory's Institutional Animal Care and Use Committee.

DE exposure and monitoring. DE was generated in-house using a 30 kW (40 hp) four-cylinder Deutz BF4M1008 diesel engine connected to a 22.3-kW Saylor Bell air compressor to provide a load. Diesel fuel was purchased from a local (Research Triangle Park, NC) service station and stored in drums.

Replicate analysis (ultimate, elemental, heating value, and specific gravity) of multiple batches of fuel purchased over time indicated consistent fuel properties and composition (data not shown). Engine lubrication oil (Shell Rotella, 15W-40) was changed before each set of exposure tests. The engine and compressor were operated at steady-state to produce 0.8 m^3/min of compressed air at 400 kPa. This translates to approximately 20% of the engine's full-load rating. From the engine exhaust, a small portion of the flow (14 l/min) was educated by an aspirator (3:1 dilution) to a second cone diluter (10:1 dilution), and then through approximately 15 m of flexible food grade polyvinyl chloride tubing (7.62 cm inside diameter) to two stainless steel 0.3- m^3 Hinner's inhalation exposure chambers housed in an isolated animal exposure room. The dilution air used was drawn from the animal exposure room through a HEPA filter. Target DEP concentrations in the two chambers were 2000 $\mu\text{g}/\text{m}^3$ (high) and 500 $\mu\text{g}/\text{m}^3$ (low). Control animals were housed in a third chamber supplied with the same HEPA filtered room air. DEP concentrations in the low (500 $\mu\text{g}/\text{m}^3$) chamber were achieved by additional dilution using HEPA filtered room air just prior to entering the chamber. All three chambers were operated at the same flow rate (142 l/min), which resulted in 28 air exchanges per hour.

Integrated 4-h filter samples (14.1 l/min) were collected once daily from each chamber and analyzed gravimetrically to determine particle concentrations. In addition, 8- and 20-min quartz filter samples (14.1 l/min) were collected from the high and low chambers, respectively, and analyzed using a thermal/optical carbon analyzer (Sunset Laboratory Inc., model 107, Tigard, OR) to determine organic carbon/elemental carbon (OC/EC) partitioning of the collected DEP. Continuous emission monitors were used to measure chamber concentrations of PM by tapered element oscillating microbalance (TEOM, Rupprecht and Patashnick Co., series 1400, Albany, NY); oxygen (O_2 , Beckman Corp., model 755, La Habra, CA); and carbon monoxide (CO , model 48, Franklin), nitric oxide and nitrogen dioxide (NO and NO_2 , model 42c, Franklin), and sulfur dioxide (SO_2 , model 43c, Franklin) by Thermo Electron Corp., Franklin (Waltham, MA). Samples were extracted through fixed stainless steel probes in the exposure chambers. Gas samples were passed through a particulate filter prior to the individual gas analyzers. Dilution air was adjusted periodically to maintain target PM concentrations as measured by the TEOM. Particle size distributions (PSDs) were characterized using a scanning mobility particle sizer (SMPS, TSI, Inc., model 3080/3022a, St Paul, MN) and an aerodynamic particle sizer (APS, TSI, Inc., model 3321). Chamber temperatures, relative humidity, and noise were also monitored, and maintained within acceptable ranges.

Experimental design. Figure 1 depicts the exposure regimen utilized for DE exposure and intranasal OVA immunization and challenge. Mice were exposed to HEPA filtered air or DE at a particle concentration of 500 or 2000 $\mu\text{g}/\text{m}^3$ 4 h/day for 5 consecutive days. The intranasal antigen exposure regimen used was a modification of that used by Farraj *et al.* (2004). Mice were anesthetized in a small plexiglass box using vaporized isoflurane (Webster Veterinary Supply Inc., Sterling, MA). Anesthetized mice were treated with an intranasal instillation of 100 μg of OVA (Sigma-Aldrich Inc, St Louis, MO) in 20 μl of sterile saline (Hospira Inc., Lake Forest, IL) or saline only (as negative control) divided evenly between each nare. The immunization phase consisted of a single instillation of OVA or saline once per day, 40 min after DE exposure, for 3 consecutive days (days 0–2). Immunized mice were challenged on days 18 and 28 with the same volume and concentration of antigen as the instillations during the immunization phase and naïve mice were instilled with saline on day 18 and OVA on day 28. Mice were necropsied either 4 h after the final chamber exposure on day 4, or 18, 48, or 96 h after the 2^o OVA challenge.

Bronchoalveolar lavage. Mice from each treatment group were euthanized with sodium pentobarbital and the trachea was exposed, cannulated, and secured with suture thread. The left mainstem bronchus was isolated and clamped with a microhemostat. The right lungs lobes were lavaged three times with a single volume of warmed Hanks balanced salt solution (Invitrogen, Grand Island, NY) (35 ml/kg). The resulting lavage was centrifuged ($717 \times g$, 15 min, 4°C) and 150 μl was stored at 4°C (for biochemical analysis) or –80°C (for cytokine measurement). The pelleted cells were resuspended in 1 ml of

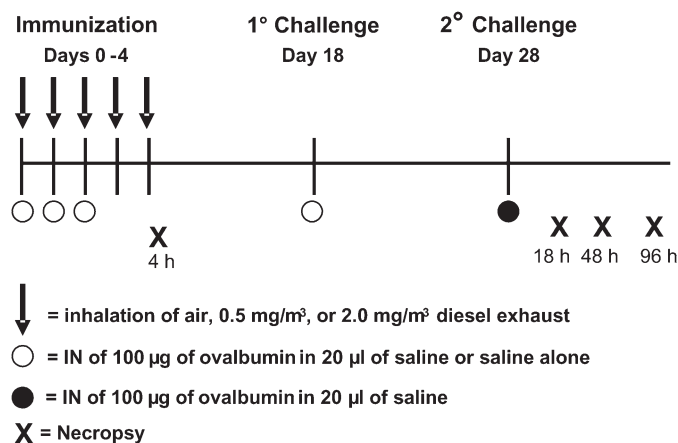


FIG. 1. Schematic of exposure regime. BALB/c mice were exposed to filtered air, 500, or 2000 µg/m³ of DE on days 0–4. After the first three days of exposure, mice were treated with an intranasal instillation of 100 µg in 20 µl of saline or saline alone. Mice were challenged with the same dose and concentration of either OVA or saline alone on day 18 and all mice were challenged with OVA on day 28. Necropsies were either 4 h ($n = 4$) after the last air or DE exposure or 18, 48, or 96 h ($n = 6$ –8) after the last challenge.

RPMI 1640 (Gibco, Carlsbad, CA) containing 2.5% fetal bovine serum (Gibco). Total cell counts in the lavage fluid of each mouse were obtained with a Coulter Counter (Beckman Dickson). Each sample (200 µl) was centrifuged in duplicate onto slides using a Cytospin (Shandon, Pittsburgh, PA) and subsequently stained with Diff Quik solution (American Scientific, McGraw Park, PA) for cell differentiation determination, with at least 200 cells counted from each slide. The left lobe was removed for RNA isolation at the 4-h time point postimmunization.

Cytokine measurements. Macrophage inflammatory protein-2 (MIP-2), IL-4, IL-5, IL-6, IL-10, IL-12, and tumor necrosis factor- α (TNF- α) concentrations in bronchoalveolar lavage (BAL) were measured by enzyme-linked immunosorbent assay (ELISA) with commercially available paired antibodies per manufacturer's instructions (Pharmingen, Franklin Lakes, NJ).

Cellular biochemistry. Lactate dehydrogenase (LDH) and total protein were modified for use on a Konelab 30 clinical chemistry analyzer (Thermo Clinical Lab systems Espoo, Finland). Activity for LDH was determined using a commercially available kit from Thermo DMA Corp (Cincinnati, OH). Total protein concentrations were determined with the Coomassie plus protein Reagent (Pierce Chemical, Rockford, IL) with a standard curve prepared with bovine serum albumin from Sigma-Aldrich.

Antigen-specific serum IgE. Antigen-specific serum IgE production was measured by ELISA. Briefly, 96-well flat-bottom ELISA plates were coated with 100 µl of OVA at a concentration of 2 µg/ml and incubated overnight at 4°C. The following day, after a nonspecific protein blocking step using bovine serum albumin and washing, 100 µl of each serum sample and an OVA-specific IgE antibody (Serotec, Ltd, Oxford, UK) for the standard control was added in duplicate wells to the plates. Following an overnight incubation at 4°C and washing, the plates were treated successively with 100 µl/well of biotinylated rat anti-mouse IgE (Serotec, Ltd), horseradish peroxidase–streptavidin (diluted 1:1500), with washes and incubation for 1 h at room temperature between each of these steps. Finally, 100 µl/well TM Blue (Dako Corporation, Carpinteria, CA) was added as a substrate for horseradish peroxidase and reactions were allowed to develop at room temperature for at least 10 min. Plates were read at 650 nm by a Spectromax ELISA plate reader (Molecular Devices, Menlo Park, CA).

RNA isolation. RNA from frozen lung tissue was isolated using RNeasy (Qiagen, Valencia, CA) following manufacture's protocol. Quantity and quality

of the RNA was measured using a Nanospot and Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA), respectively.

Microarray. RNA samples were prepared, processed, and hybridized to the Affymetrix Mouse 430A gene chip at Expression Analysis (Durham, NC), as described in the GeneChip Expression Analysis Manual (Affymetrix; Santa Clara, CA). The Mouse 430A Genome chip contains over 22,000 probe sets representing over 14,000 well-characterized mouse genes. A detailed description can be found at <http://www.affymetrix.com/products/arrays/specific/mouse430.affx>. A total of 24 gene chips representing 4-h samples from 24 individual mice (six treatments, $n = 4$) were used in this study. The microarray data have been deposited at Genome Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO series accession number GSE9383.

Statistical analysis of inflammatory, biochemical, and immune endpoints. The data were analyzed using a two-way analysis of variance (ANOVA) model. The two independent variables were exposure (DEP at levels 0, 500, and 2000 µg/m³) and treatment (at levels saline and OVA). Pairwise comparisons were performed as subtests of the overall ANOVA, subsequent to a significant main or interactive effect. If the usual ANOVA assumptions were not satisfied, either the data were log transformed so as to satisfy the assumptions or a distribution free test was substituted for the ANOVA. The level of significance was set at 0.05. No adjustment was made to the significance level as a result of multiple comparisons.

Overall data analysis strategy. The analysis of this data set consisted of six groups (filtered air, 500 µg/m³ [low], or 2000 µg/m³ [high] DE with or without OVA). Steps were made to (1) evaluate the data quality; (2) perform principal components analysis (PCA) for a global inspection of within-group sample correspondence and to examine model and dose effects; (3) perform gene set enrichment analysis (GSEA) to determine differentially expressed gene sets between groups; (4) extract core genes responsible for a particular gene set identified as significant from the GSEA analysis; and (5) map core genes to functional pathways using MetaCore GENEGO to identify altered pathways unique or in common among the treatments.

Principal component analysis. PCA transforms microarray data from all gene chips to a new coordinate system using an orthogonal linear transformation, which reduces the data to a three-dimensional coordinate system while retaining those characteristics of the data set that contribute most to the variance. This analysis was employed to survey the data for within-group outliers and model and dose effects using Rosetta Resolver (Rosetta Inpharmatics, Agilent Technologies) following linear weighting normalization ($p < 0.001$). Each individual gene chip or gene expression profile was represented by a single data point and the variance between each gene chip was comparable to the distance between the data points whereby two similar gene expression profiles were projected as two adjacent points and vice versa. This analysis was employed as a visual tool to initially inspect the data for within-group and across group similarities and dissimilarities.

Gene set enrichment analysis. GSEA is a powerful computational method that utilizes an *a priori* defined set of genes to determine statically significant, concordant differences between two phenotypes. For this analysis, raw data from 24 gene chips were normalized using Robust Multichip Average in Gene Pattern (www.genepattern.org) to generate estimated expression summaries. The molecular signature database (MSigDB) C2 provided on the website <http://www.broad.mit.edu/gsea/msigdb/msigdbindex.html>, which contains 1687 gene sets, was queried for association with a particular treatment in each pairwise comparison (low DE/OVA vs. air/OVA, high DE/OVA vs. air/OVA, high DE/saline vs. air/saline, air/OVA vs. air/saline, low DE/OVA vs. low DE/saline, and high DE/OVA vs. high DE/saline). Only gene sets with a minimal gene set size of 15 genes per pathway and a maximum of 500 were queried. To determine the significance of a gene set for each pairwise comparison, GSEA ranked all genes according to the difference in expression using a signal-to-noise metric. A running sum statistic termed the enrichment score (ES) was determined for each gene set and the maximum ES (MES) over all gene sets in

TABLE 1
Summary of Concentrations and Characteristics of the DEP and Gases within the Animal Exposure Chambers^a

Constituent	Units	Low exposure	High exposure
Particle mass concentration (TEOM)	μg/m ³	561 ± 9	2136 ± 63
Particle mass concentration (filter) ^b	μg/m ³	546 ± 29	1983 ± 55
Particle number concentration ^c	No./cm ³	5.6 × 10 ⁵ ± 4.1 × 10 ³	2.0 × 10 ⁶ ± 9.9 × 10 ³
Oxygen (O ₂)	%	20.7 ± 0.11	20.3 ± 0.07
Carbon monoxide (CO)	ppm	< 1.0	4.3 ± 0.07
Nitric oxide (NO)	ppm	< 2.5	9.2 ± 0.30
Nitrogen dioxide (NO ₂)	ppm	< 0.25	1.1 ± 0.05
Sulfur dioxide (SO ₂)	ppm	< 0.06	0.2 ± 0.10
Number median D _p ^d	nm	80 ± 2	86 ± 2
Volume median D _p ^d	nm	184 ± 2	195 ± 2
OC/EC ^e	wt ratio	0.7 ± 0.05	0.7 ± 0.05

^aTEOM, O₂, CO, NO, NO₂, and SO₂ data represent mean values from continuous measurements taken over the 5-day exposure ± SE.

^bFilter data represent mean values from one measurement per day taken over the 5-day exposure ± SE.

^cParticle number concentration data represent mean values from two measurements (low exposure) and three measurements (high exposure) taken over one representative exposure day ± SE.

^dD_p indicates particle geometric number and volume median diameters for a single representative PSD ± geometric standard deviation. Note that volume information is calculated from number based mobility diameters and assume spherical particles.

^eOC/EC (organic carbon to elemental carbon ratio) data represent mean values from one measurement per day taken over the 5-day exposure ± SE.

the actual data was recorded. The ES reflects the degree to which a gene set is overrepresented at the top or bottom of the ranked genes. To determine the significance of the MES, a comparison was made between the actual MES and that seen in 1000 permutations that shuffled the gene set labels creating a null distribution. In addition the data were normalized based on the size of the gene set (normalized expression set [NES]). A false discovery rate (FDR) was calculated corresponding to each NES. Gene sets with a FDR of < 25% were considered significant. Heat maps were generated from the top 50 genes that were most strongly associated with the DE or DE/OVA treatment. GSEA software and (MSigDB are available at <http://www.broad.mit.edu/gsea/> (Subramanian *et al.*, 2005).

Pathway level analysis. The gene sets with an FDR of < 25% were used to create a core gene list. The core gene list comprised genes responsible for a gene set being considered significant. These genes were then applied to a pathway analysis program called MetaCore GENEGO (<http://www.genego.com/metacore>), which maps genes to pathways and determines significance. All pathways with a *p*-value of < 0.01 were reported.

RESULTS

Diesel Exposures

Diesel concentration data. Table 1 shows a summary of the 5-day average exposure data for the low (500 μg/m³) and high (2000 μg/m³) DE concentrations. These target chamber concentrations, determined and adjusted based on continuous TEOM measurements were achieved with relatively low variability both within a particular 4-h exposure and between different days. Chamber particle concentrations determined gravimetrically from integrated filter samples (one 4-h sample per exposure day), agreed with the TEOM measurements within 10%. CO, NO, NO₂, and SO₂ concentrations in the high chamber averaged 4.3, 9.2, 1.1, and 0.2 ppm, respectively. Concentrations in the low chamber were below detection

limits, as indicated. Particle number concentrations were relatively high, and corresponded to PSDs with a well established accumulation mode and little evidence of notable nuclei or coarse modes. Geometric median number and volume (assuming spherical particles) diameters of 86 and 195 nm, respectively, were measured in both chambers. OC/EC wt ratios of 0.7 from both chambers indicate that approximately 41% of the DEP was comprised of organic carbon.

Post-challenge Endpoints

OVA-specific IgE antibodies. Mice exposed to the low and high DE during local immunization produced increasing OVA-specific IgE antibodies over time. Mice exposed to the high dose of DE (2000 μg/m³) had a mild but nonsignificant increase (relative to OVA control) in these antibodies at the 48-h time point (Fig. 2). In the absence of immunization with OVA, OVA-specific IgE antibodies were not detected (data not shown).

BAL cell differential counts. To evaluate the effect of DE exposure with or without OVA immunization on airway inflammation post-challenge, the cellular profile of BAL fluid 18, 48, and 96 h after OVA challenge was assessed. Cell profiles at the 18- and 96-h time points did not differ across treatment groups. At the 48-h time point however, eosinophils, neutrophils, and lymphocytes were statistically increased in OVA-immunized mice exposed to both concentrations of DE (Fig. 3). With DE exposure alone, only neutrophils were statistically increased in the high DE concentration.

Cytokine production in the BAL fluid. To characterize the role of local cytokine production on the effects of DE in an

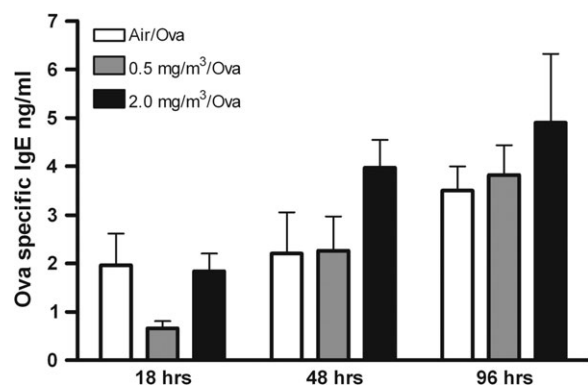


FIG. 2. Kinetic development of OVA-specific serum antibodies in mice exposed to DE during allergic immunization. Mice were exposed to filtered air or DE at a particle concentration of 500 or 2000 $\mu\text{g}/\text{m}^3$ on days 0–4 for 4 h/day, intranasally instilled with OVA on days 0, 1, and 2, challenged with OVA on day 18 and 28, and necropsied 18, 48, and 96 h later. OVA-specific IgE antibody levels were measured in the serum by ELISA. $n = 6$ –8.

OVA immunization model, the production of MIP-2, IL-4, IL-5, IL-6, IL-10, IL-12, and TNF- α were quantified. IL-6 production was significantly increased in mice exposed to the high-dose DE (2000 $\mu\text{g}/\text{m}^3$) at the 96-h time point (Fig. 4A). Although not significant, IL-10 was seen to increase in mice exposed to the high-dose DE for the 48- and 96-h time points (Fig. 4B). All other cytokines measured were unchanged compared with controls.

Postimmunization Endpoints

Pulmonary inflammation and lung injury. Mice were exposed to filtered air or DE at a concentration of 500 or 2000 $\mu\text{g}/\text{m}^3$ on days 0–4, given an intranasal instillation of either saline or 100 μg of OVA 40 min after the chamber exposure on days 0–2, and necropsied 4 h after the last chamber exposure as depicted in Figure 1. Cell differential counts in the BAL were quantified to assess pulmonary inflammation. No differences among the groups were observed for macrophage, lymphocyte, neutrophil, and eosinophil counts (data not shown). Protein and LDH levels were quantified to determine if DE and/or antigen exposure induced cellular lung injury. These biomarkers were not found to be increased in BALF of any group (data not shown).

Principle component analysis. PCA was applied to provide a multidimensional gene expression profile of each gene chip in a three-dimensional space to reveal clusters in the experimental data. All data from the 24 gene chips were analyzed with each dot representing a gene chip (Fig. 5). The first three PCs combined reflected approximately 40% of the variance among all samples. After analysis the gene chips were then highlighted in either blue (OVA treatment) or red (saline control). Good separation of the two groups was observed reflecting different expression profiles illustrating a model effect between antigen and saline (Fig. 5A). To determine if there was a diesel dose

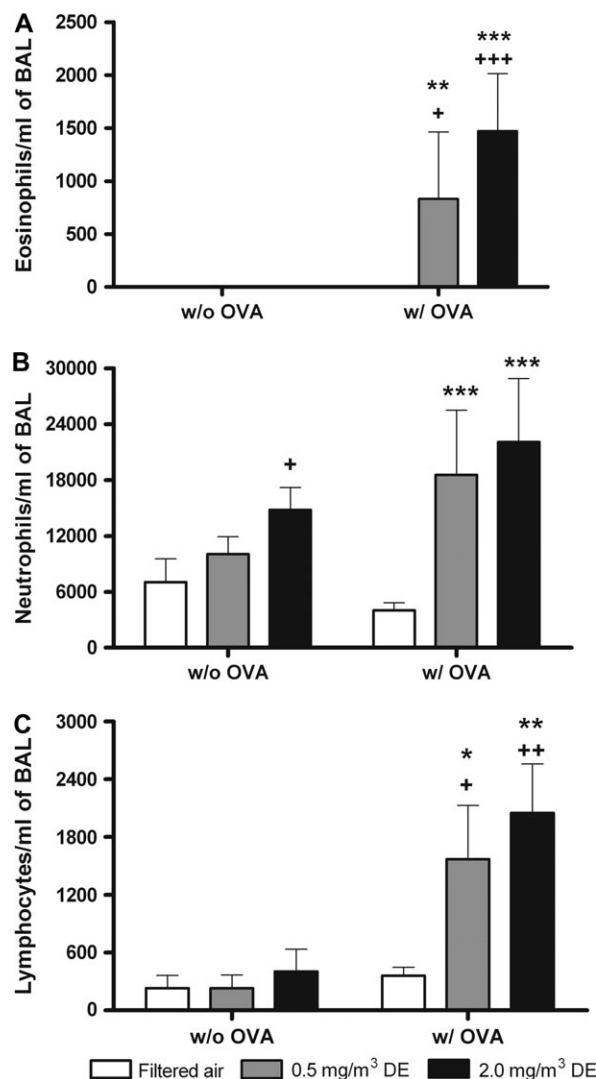


FIG. 3. Allergen-induced pulmonary inflammatory cells in mice exposed to filtered air or DE with or without OVA. Mice were exposed to filtered air or DE at a particle concentration of 500 or 2000 $\mu\text{g}/\text{m}^3$ on days 0–4 for 4 h/day, intranasally instilled with OVA or saline on days 0, 1, and 2 and challenged with OVA or saline on day 18. All groups were challenged with OVA on day 28 and necropsied 18, 48, and 96 h later. Data is shown for 48-h time point only. Eosinophils (A), neutrophils (B), and lymphocytes (C) were measured in BAL. $n = 6$ –8; significantly different from saline control: $^+p < 0.05$; $^{++}p < 0.01$. Significantly different from OVA control: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

effect, the gene chips were highlighted according to diesel concentrations (blue: air/saline and air/OVA, red: 500 μg DE/ m^3 /saline and 500 μg DE/ m^3 /OVA, green: 2000 μg DE/ m^3 /saline and 2000 μg DE/ m^3 /OVA) (Fig. 5B). The plot was rotated to reveal clustering among the 2000 $\mu\text{g}/\text{m}^3$ DE exposure groups.

Gene set enrichment analysis. To test for sets of related genes that might be altered in the lungs of mice exposed to the various treatments we employed GSEA. In contrast to conventional microarray analysis programs, the algorithm

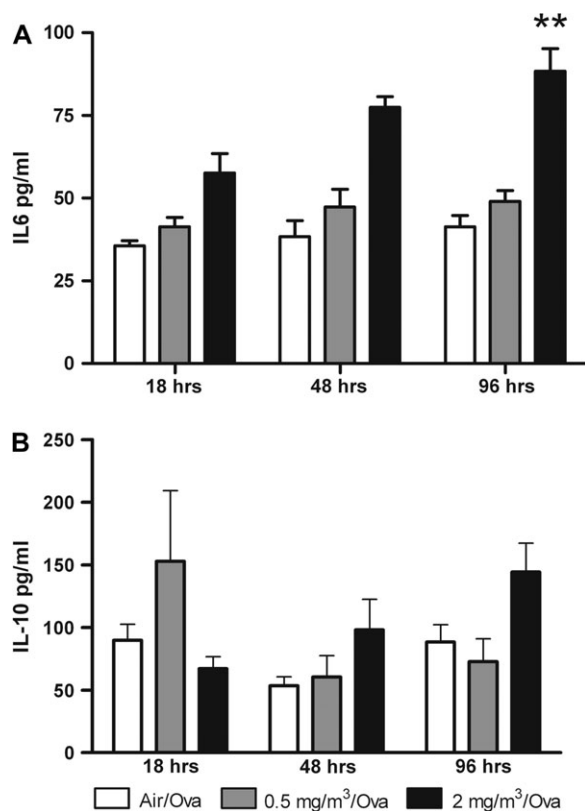


FIG. 4. Quantification of IL-6 and IL-10 protein levels. Mice were exposed to filtered air or DE at a particle concentration of 500 or 2000 $\mu\text{g}/\text{m}^3$ on days 0–4 for 4 h/day, intranasally instilled with OVA or saline on days 0, 1, and 2 and challenged with OVA or saline on day 18. All groups were challenged with OVA on day 28 and necropsied 18, 48, and 96 h later. IL-6 (A) and IL-10 (B) protein levels were measured by ELISA in the BAL of the same mice as described in Figure 1. $n = 6$ –8; significantly different from OVA control: ** $p < 0.01$.

employed by GSEA derives its power by focusing on gene sets with biological relevance rather than individual genes (Bild and Febbo, 2005; Subramanian *et al.*, 2005). Pairwise comparisons revealed significant gene set differences between high DE/saline versus air/saline and high DE/OVA versus air/OVA. For each comparison, a heat map was constructed of the top 50 genes associated with the treatment of interest. The degree of correlation in each chip is represented by a range of colors reflecting the strength of correlation (red: high, pink: moderate, light blue: low, and dark blue: lowest). Visual inspection of the high DE/saline heat map (Fig. 6) showed striking contrast of the top 50 genes between the diesel and air treatments. A similar map was also constructed for the high DE/OVA versus air/OVA comparison, where the top 50 genes associated with DE in the context of OVA are shown (Fig. 7). Although the contrast was less strong in this comparison, a binary response was still evident. For both heat maps there was good consistency across each of the four animals per group represented by individual gene chips (individual animals). Ten genes from the top 50 genes, 1190003K14RIK, CD14,

CXCL1, CXCL5, IFITM1, LCN2, PIGR, PROM1, REG3G, and RETNLA, were in common between both heat maps.

The C2 collection of curated gene sets from the MSigDB were queried and a detailed description of each gene set can be found on the website http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html. A table was constructed for the high DE/saline vs. air/saline comparison of the top 20 gene sets associated with the DE phenotype, as ranked by normalized ESs (Supplemental Table 1). The core genes in these gene sets are involved in cellular proliferation and inflammatory effects. The top 20 gene sets associated with the DE/OVA phenotype generated from the high DE/OVA versus air/OVA comparison (Supplemental Table 2) contained genes involved in oxidative stress responses. The gene set WANG_MLL_CBP_VS_GMP_UP was associated with both high DE/saline and high DE/OVA phenotypes.

Pathway analysis. A total of 49 enriched gene sets with a FDR < 25% were identified in the high DE/saline compared with air/saline. The combined 619 core genes from these significant gene sets were extracted and imported into a pathway analysis program MetaCore GENEGO. The list of pathways significantly altered by DE compared with air in the absence of antigen was clearly related to immune function and cell signaling pathways (Table 2). Specifically pathways included those for cell adhesion, cell cycle control, apoptosis, growth and differentiation, and cytokine signaling among others.

The pairwise comparison of high DE/OVA versus air/OVA yielded 23 enriched gene sets with a FDR < 25%. The 412 core genes were imported into the MetaCore pathway program. The pathways associated with the high DE/OVA phenotype were distinct from those associated with the DE/saline phenotype. The majority of these pathways could be functionally classified under metabolic processes with oxidative stress systems including oxidative phosphorylation, mitochondrial and peroxisomal oxidation, ubiquinone, glutathione, vitamin E, and PPAR regulation of lipid metabolism, being very prominent (Table 3).

DISCUSSION

DE has been shown to act as an adjuvant for allergic inflammation in animals and humans but the precise signaling pathways are not clear. Many studies have used instillation boluses of DEP or DEP extracts to explore the mechanisms of diesel-enhanced allergic immune responses. Although these methods are simpler and cheaper than inhalation, and can be used for hazard identification, they are not representative of real world exposures. Previous inhalation studies have used chronic exposures, between 5 and 34 weeks, and antigen administration has often been systemic or in combination with adjuvants such as alum. These studies have shown increases in neutrophils and eosinophils in the BAL (Ichinose *et al.*, 1998;

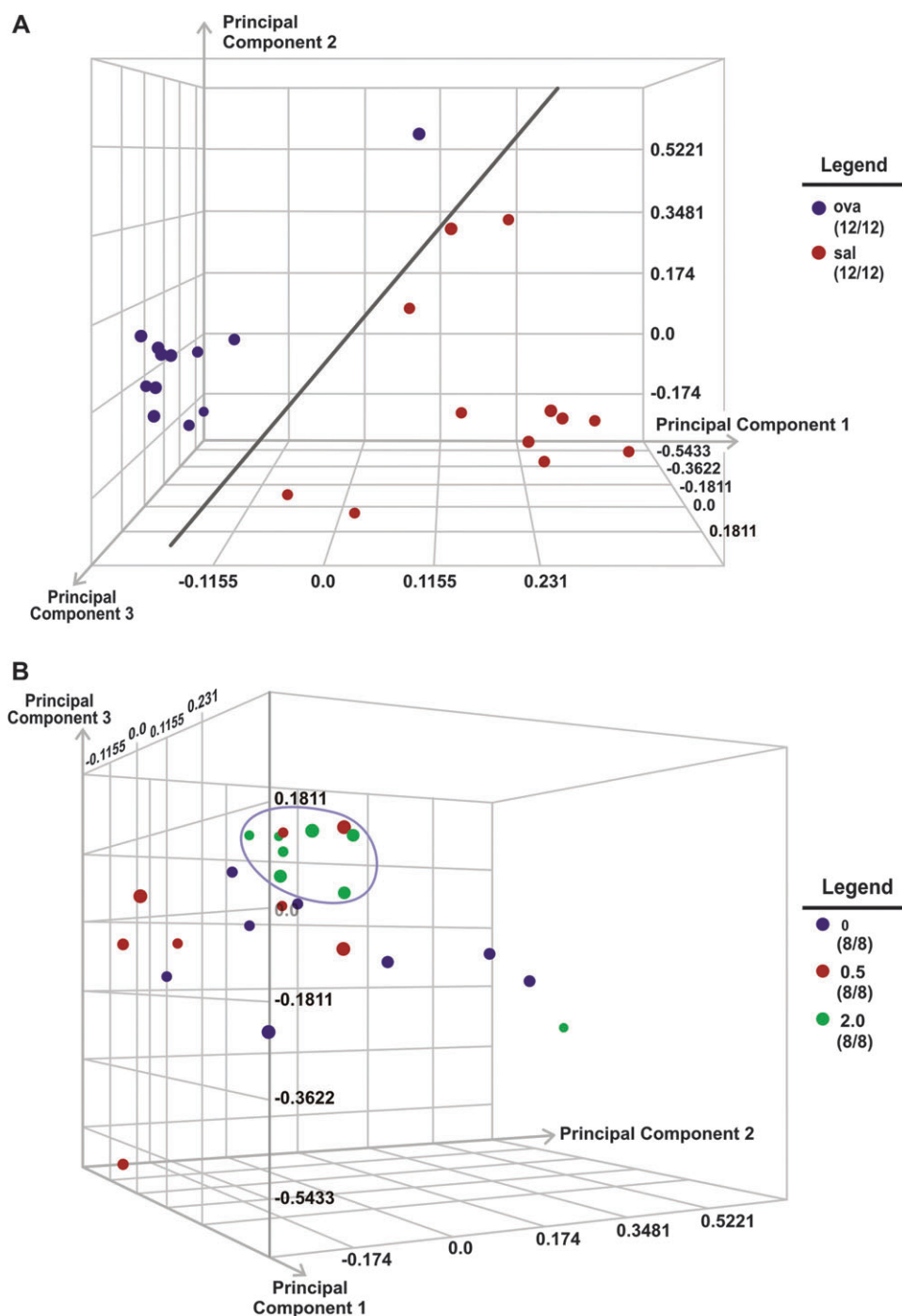


FIG. 5. Principle component analysis plot from microarray data. Mice were necropsied 4 h after the last diesel exposure on day 4. RNA was isolated from the lungs and hybridized to Affymetrix Mouse 430A gene chips ($n = 4$). PCA plots were created in Rosetta Resolver. Each plot contains gene chips from all mice and each dot represents a gene chip. Gene chips were highlighted according to the immunization protocol (blue: OVA treatment or red: saline treatment) (A) or the diesel concentration (blue: air/saline and air/OVA, red: 500 $\mu\text{g DE}/\text{m}^3$ /saline and 500 $\mu\text{g DE}/\text{m}^3$ /OVA, green: 2000 $\mu\text{g DE}/\text{m}^3$ /saline and 2000 $\mu\text{g DE}/\text{m}^3$ /OVA) (B).

Miyabara *et al.*, 1998) but it is not clear if short-term, moderate dose exposures would have the same effect on mucosal immunization. Moreover, these inhalation studies have not investigated the signaling mechanisms responsible for increased immunity and clinical disease. The present study had

two goals: (1) to investigate the adjuvant effects, post antigen challenge, of a short-term inhalation exposure to moderate doses (500 and 2000 $\mu\text{g}/\text{m}^3$) of DE; and (2) to identify global gene changes associated with altered immune function. Our data demonstrate for the first time that even short-term

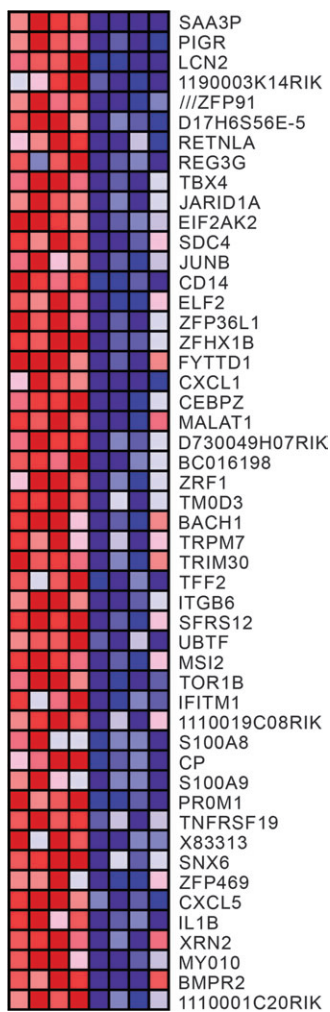


FIG. 6. Heat map of top 50 genes associated with high DE in nonallergic mice. In the heat map, expression values for the top 50 genes in each chip is represented as a color, where the range of colors (red, pink, light blue, dark blue) shows the range of expression values (high, moderate, low, lowest). Lanes 1–4 are high DE/saline and 5–8 are air/saline.

inhalation exposure to moderate concentrations of DE, when given with an antigen, can induce allergic lung disease after antigen challenge. Furthermore, these adjuvant effects were associated with changes in gene expression 4 h after DE exposure in mice given antigen that were distinct from changes in animals exposed to DE alone.

The post-challenge results demonstrate DE exposure with antigen resulted in mild adjuvancy as evidenced by significant increases in eosinophils, neutrophils, lymphocytes, and IL-6 in the BAL. Saline and OVA control mice did not induce an asthma phenotype after the 2° OVA challenge and DE alone only increased neutrophils, indicating the combination exposure of DE and antigen was essential to promote the development of allergic lung disease. In life measures showed that none of the animals lost weight or experienced any indicators of the lung injury.

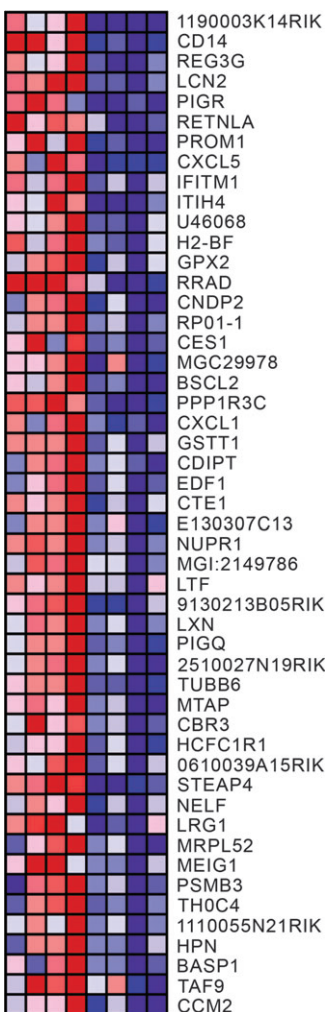


FIG. 7. Heat map of top 50 genes associated with high DE in OVA-allergic mice. In the heat map, expression values for the top 50 genes in each chip is represented as a color, where the range of colors (red, pink, light blue, dark blue) shows the range of expression values (high, moderate, low, lowest). Lanes 1–4 are high DE/OVA and 5–8 are air/OVA.

For the second goal, microarray analysis was employed to examine global gene changes 4 h after the last DE exposure to understand the mechanisms involved in promoting adjuvancy. Although cellular and biochemical measures showed no changes in clinical indicators of inflammation, PCA of the gene expression data revealed a model (OVA) and a high-dose DE (2000 $\mu\text{g}/\text{m}^3$) effect. GSEA was applied to further investigate gene changes associated with adjuvancy. The pairwise comparison of air/OVA versus air/saline yielded no significant gene sets. A plausible explanation for this is the last OVA dose was given on day 2 while the lungs were harvested for gene expression analysis on day 4 (Fig. 1) when the mild immune stimuli could have subsided. The GSEA comparison of low DE (500 $\mu\text{g}/\text{m}^3$)/OVA versus air/OVA resulted in no significant gene sets associated with the low DE/OVA treatment. Comparison of the high (2000 $\mu\text{g}/\text{m}^3$) DE/OVA

TABLE 2
Significantly Altered Pathways by High DE/Saline compared with Air/Saline

Pathway	p-Value	Genes
Cell adhesion		
Chemokines and adhesion	6.98E-05	18/153
Cytoskeleton remodeling	7.32E-03	15/177
FAK signaling	2.43E-03	9/70
PLAU signaling	3.14E-03	7/47
Cell cycle control		
Regulation of G1/S transition (p.1)	4.08E-03	8/62
Cell death/apoptosis		
FAS signaling cascades	9.45E-03	6/44
Cell survival		
EPO-induced Janus kinase signal transducer and activator of transcription (Jak-STAT) pathway	7.37E-04	7/37
Cyto/chemokines		
CXCR4 signaling pathway	1.68E-03	8/54
IL-4 antiapoptotic action	1.20E-03	7/40
IL-6 signaling pathway	2.18E-03	6/33
G-proteins		
A3 receptor signaling	1.60E-03	10/79
A2B receptor: action via G-protein alpha s	9.23E-03	7/57
Cyclic adenosine monophosphate signaling	9.29E-04	13/114
CREB pathway	9.48E-03	10/101
M-RAS regulation pathway	7.07E-03	4/19
RAB5A regulation pathway	6.97E-03	3/10
Regulation of CDC42 activity	2.74E-03	5/24
Growth and differentiation		
Insulin-like growth factor type I receptor (IGF-RI) signaling	7.54E-06	13/72
Membrane-bound ESR1: interaction with G-proteins signaling	2.96E-03	9/72
platelet-derived growth factor (PDGF) signaling via STATs and NF-κB	5.91E-03	6/40
Leptin signaling via JAK/STAT and MAPK cascades	6.45E-03	5/29
Membrane-bound ESR1: interaction with growth factors signaling	7.53E-03	6/42
TPO in cell process	4.83E-05	10/52
Transcription regulation of granulocyte development	4.84E-04	8/45
PIP3 signaling in cardiac myocytes	1.19E-03	10/76
Growth factors		
Prolactin receptor signaling	4.08E-03	8/62
Hormones		
Androgen receptor nuclear signaling	1.19E-03	10/76
Insulin signaling:generic cascades	7.61E-03	7/55
Nongenomic (rapid) action of androgen receptor	2.43E-03	9/70
Kinases		
AKT signaling	2.39E-03	8/57
Metabolism—lipid		
Insulin regulation fatty acid metabolism	2.29E-04	9/51
Insulin regulation of glycogen metabolism	9.23E-03	7/57
Metabolism—regulators		
Transcription regulation of amino acid metabolism	2.96E-04	8/42
Insulin receptor signaling pathway	1.86E-03	7/43

TABLE 2—Continued

Pathway	p-Value	Genes
Neuroscience		
Regulation of CDK5 in central nervous system	1.30E-03	6/30
GDNF signaling	3.31E-03	5/25
Phosphatases		
PTEN pathway	4.13E-04	9/55
Transcription factors		
PPAR pathway	7.58E-04	8/48
Translation regulation		
Insulin regulation of the protein synthesis	7.61E-03	7/55
Regulation activity of EIF2	8.39E-03	7/56

versus air/OVA, however, showed significant changes in 23 gene sets. For this reason subsequent analyses were focused on the high DE/OVA versus OVA comparison and the high DE/saline versus saline comparison.

DE inhalation has been shown to induce lung inflammation in humans (Salvi *et al.*, 1999) and in rodents (Miyabara *et al.*, 1998; Saito *et al.*, 2002). *In vitro* studies have demonstrated DEP exposure induces release of inflammatory cytokines, IL-1β, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Bayram *et al.*, 1998; Boland *et al.*, 1999; 2000; Sydbom *et al.*, 2001). Here the diesel exposures caused an upregulation of neutrophil homing chemokines genes (CCL4, CXCL1, -5, and -6) and inflammatory cytokines (IL-1β, CXCL2 [mouse equivalent to IL-8], and GM-CSF). In addition, 32 other signaling molecules were also associated with diesel exposures including numerous interleukins and TNF subtypes, and an array of CC and CXC chemokines.

Chronic DE exposures induce epithelial cell proliferation in the airways and alveoli, and increase the number of resident macrophages (Barnhart *et al.*, 1981; Kato *et al.*, 1992; Wright, 1986). An *in vitro* study reported that serum starved A549 cells proliferated in response to a low-dose (up to 10 μg/ml) DEP exposure (Bayram *et al.*, 2006). Analysis of diesel exposed lungs revealed increases in growth and differentiation pathways such as IGF-RI and PDGF signaling, and granulocyte development. Jak-STAT cascades involved in cell growth and survival, as well as genes in G1/S transition cell cycle control were also altered. Although cell cycle control genes such as cyclin E2, cell division cycle associated 7, cyclin-dependent kinase 8, E2F transcription factor 5, mitogen-activated protein kinase kinase kinase 5, and mitogen-activated protein kinase 6 (MAPK6), and retinoblastoma 1 were increased, we also observed upregulation of several genes upstream of this pathway such as Jun-B oncogene, trans-acting transcription factor 1 (Sp1), and early growth response 1 that could be driving this proliferative response.

A substantial amount of evidence has shown that PM including DEP acts as an adjuvant when given with antigen. The proposed mechanism is a hierarchical model whereby low levels of oxidative stress induce antioxidant defense

TABLE 3
Significantly Altered Pathways by High DE/OVA compared with Air/OVA

Pathway	p-Value	Genes
Cell cycle control		
Role of Brca1 and Brca2 in DNA repair	1.60E-06	9/40
Metabolism—amino acid		
Leucine, isoleucine, and valine metabolism p.1	4.00E-04	4/13
Leucine, isoleucine, and valine metabolism p.2	4.92E-06	7/25
Phenylalanine metabolism	1.12E-05	7/28
TCA	8.99E-07	7/20
Metabolism—carbohydrates		
Glycolysis and gluconeogenesis (short map)	3.55E-03	5/36
Glycolysis and gluconeogenesis	5.53E-03	3/13
Propionate metabolism p.1	5.48E-04	4/14
Propionate metabolism p.2	1.88E-06	7/22
Pyruvate metabolism	5.39E-03	4/25
Metabolism—energy		
Oxidative phosphorylation	7.31E-36	40/99
Metabolism—lipid		
Mitochondrial long chain fatty acid beta-oxidation	2.57E-10	9/17
Mitochondrial unsaturated fatty acid beta-oxidation	2.46E-09	8/15
Mitochondrial ketone bodies biosynthesis and metabolism	4.41E-06	5/10
Peroxisomal branched chain fatty acid oxidation	1.55E-05	6/20
Peroxisomal straight-chain fatty acid beta-oxidation	5.48E-04	4/14
Peroxisome proliferator-activated receptor regulation of lipid metabolism	3.02E-09	10/28
Regulation of fatty acid synthesis	1.77E-03	3/9
Metabolism—nucleotide		
ATP metabolism	1.66E-03	7/60
ATP/ITP metabolism	1.79E-08	14/73
CTP/UTP metabolism	1.75E-15	20/67
dATP/dITP metabolism	6.99E-04	7/52
dCTP/dUTP metabolism	3.05E-06	9/43
dGTP metabolism	9.40E-04	6/40
GTP-XTP metabolism	1.41E-07	12/61
TTP metabolism	1.04E-05	8/38
Metabolism—vitamin and cofactor		
Glutathione metabolism	7.64E-13	14/37
Heme metabolism	8.48E-03	5/44
Ubiquinone metabolism	3.30E-18	23/73
Vitamin E (α -tocopherol) metabolism	9.16E-05	5/17
Regulation of transcription		
Ligand-dependent transcription of retinoid-target genes	4.24E-03	10/129

mechanisms to restore redox balance in the cell. Intermediate levels of oxidative stress activate MAPK and nuclear factor-kappa B (NF- κ B) cascades, which induce inflammation, whereas high levels of oxidative stress disrupt the mitochondrial permeability transition pore and electron transport chain resulting in cell death (Li *et al.*, 2003a). *In vitro* studies have shown that DEP extracts and ultrafine particles induce ROS production and oxidative stress by interfering with the mitochondrial electron transport chain (Li *et al.*, 2003b; Xia *et al.*, 2004). The study presented here confirmed similar

effects *in vivo*. Global transcriptional analysis of lung tissue from mice in the high DE/OVA treatment group expressed increased transcription of 45 genes involved in the nicotinamide adenine dinucleotide (reduced) (NADH) and FADH₂ respiratory chain located in the inner membrane of the mitochondria. These include 6 adenosine triphosphate (ATP) synthases, 6 ATPases, 8 cytochrome *c* oxidases, 20 NADH dehydrogenases, and 2 ubiquinol-cytochrome *c* reductases, the majority of which were upregulated. The genomic profile for the DEP/OVA group also altered a significant number of genes reflecting phase I metabolism, including cytochrome P450s, dehydrogenases, carboxylesterases, and reductases, and a consistent induction of phase II transferases. These data confirm *in vitro* findings that have shown Polycyclic aromatic hydrocarbons induce oxidative stress indirectly, through biotransformation by cytochrome P450, epoxide hydrolase, and dihydrodiol dehydrogenase to generate redox active quinones (Penning *et al.*, 1999).

In conclusion mice exposed to high DE alone had altered inflammatory, cell cycle control, growth and proliferation, and cell adhesion pathways. Consistent with the Li *et al.* premise, DE exposure in the context of antigen immunization induced oxidative stress pathways, possibly through disruption of the inner mitochondrial membrane. These effects were associated with mild adjuvancy as evidenced by increases in eosinophils, neutrophils, and lymphocytes as well as IL-6 post-challenge. Genomic alterations in lung tissues after both high DE/saline and high DE/OVA exposures are more likely to reflect molecular changes within the resident lung cell population rather than the infiltration of new cells because the cell differential counts were unchanged compared with saline and OVA controls at that time point. This comprehensive approach using gene expression analysis to examine changes at a cellular and molecular level combined with more traditional immunotoxicity endpoints provide a clearer picture of the events occurring in the lung after DE exposure in the presence or absence of antigen. The results show that relatively short exposures to DE, at concentrations seen in severe occupational environments, cause mild increases in immunologic sensitization to allergen. Genomic analysis revealed a wide range of altered pathways suggesting this method may be more sensitive and can be used for identifying mechanisms involved in adverse effects of inhaled pollutants.

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Supplemental Table 1. Significantly altered gene sets by high DE/saline compared to air/saline.

ES = enrichment score; NES = normalized enrichment score

Geneset Name	SIZE	ES	NES	NOM p-val
WANG_MLL_CBP_VS_GMP_UP	42	0.5829	1.8836	0.00000
HSC_MATURE_SHARED	169	0.4647	1.8517	0.00000
CARIES_PULP_HIGH_UP	68	0.5137	1.8385	0.00000
HSC_MATURE_ADULT	232	0.4448	1.8207	0.00000
NEMETH_TNF_DN	26	0.5976	1.7882	0.00430
SA_MMP_CYTOKINE_CONNECTION	15	0.6905	1.7827	0.00938
UVB_NHEK3_C2	35	0.5643	1.7761	0.00546
GSK3PATHWAY	26	0.5953	1.7462	0.00147
ZHAN_PCS_MULTIPLE_MYELOMA_SPKD	20	0.6155	1.7248	0.00436
TAKEDA_NUP8_HOXA9_16D_DN	126	0.4403	1.7221	0.00000
CORDERO_KRAS_KD_VS_CONTROL_DN	48	0.5109	1.7068	0.00000
LE_MYELIN_UP	91	0.4602	1.6978	0.00122
NI2_MOUSE_UP	40	0.5196	1.6940	0.00409
YANG_OSTECLASTS_SIG	39	0.5290	1.6935	0.00955
IGF1MTORPATHWAY	19	0.6039	1.6913	0.01351
CHEN_HOXA5_TARGETS_UP	135	0.4345	1.6912	0.00000
UVB_NHEK3_C5	30	0.5573	1.6886	0.00980
HOHENKIRK_MONOCYTE_DEND_DN	100	0.4442	1.6822	0.00122
TENEDINI_MEGAKARYOCYTIC_GENES	47	0.5038	1.6754	0.00530
HCC_SURVIVAL_GOOD_VS_POOR_DN	118	0.4318	1.6661	0.00118

Supplemental Table 2. Significantly altered gene sets by high DE/OVA compared to air/OVA.

ES = enrichment score; NES = normalized enrichment score

Gene Set Name	SIZE	ES	NES	NOM p-val
ELECTRON_TRANSPORT_CHAIN	86	0.5388	1.8950	0.0000
MOOTHA_VOXPHOS	73	0.5432	1.8901	0.0000
CANTHARIDIN_DN	45	0.5841	1.8698	0.0000
WANG_MLL_CBP_VS_GMP_UP	42	0.5782	1.8590	0.0000
FETAL_LIVER_VS_ADULT_LIVER_GNF2	53	0.5481	1.8214	0.0000
LVAD_HEARTFAILURE_DN	33	0.5996	1.8206	0.0000
MITOCHONDRIA	355	0.4661	1.7823	0.0000
GLUTATHIONE_METABOLISM	28	0.5990	1.7704	0.0012
FLECHNER_KIDNEY_TRANSPLANT_REJECTION_UP	72	0.5018	1.7340	0.0000
CMV_HCMV_TIMECOURSE_14HRS_UP	33	0.5748	1.7278	0.0012
WIELAND_HEPATITIS_B_INDUCED	71	0.4953	1.7061	0.0000
INNEREAR_UP	34	0.5598	1.7037	0.0012
HUMAN_MITODB_6_2002	352	0.4416	1.7015	0.0000
ROSS_MLL_FUSION	60	0.5003	1.6858	0.0022
AMINOACYL_TRNA_BIOSYNTHESIS	18	0.6183	1.6649	0.0076
PYRIMIDINE_METABOLISM	55	0.5008	1.6546	0.0011
FATTY_ACID_DEGRADATION	23	0.5875	1.6440	0.0036
OXIDATIVE_PHOSPHORYLATION	55	0.4895	1.6302	0.0011
ABBUD_LIF_UP	45	0.5048	1.6222	0.0045
ROS_MOUSE_AORTA_UP	23	0.5807	1.6160	0.0086

