

RESEARCH ARTICLE

Effects of simulated downwind coal combustion emissions on pre-existing allergic airway responses in mice

Edward G. Barrett¹, Kimberly C. Day¹, Andrew P. Gigliotti¹, Matthew D. Reed¹, Jacob D. McDonald¹, Joe L. Mauderly¹, and Steven K. Seilkop²

¹Lovelace Respiratory Research Institute, Albuquerque, NM, USA and ²SKS Consulting Services, Siler City, NC, USA

Abstract

Context: Coal-fired power plant emissions can contribute a significant portion of the ambient air pollution in many parts of the world.

Objective: We hypothesized that exposure to simulated downwind coal combustion emissions (SDCCE) may exacerbate pre-existing allergic airway responses.

Methods: Mice were sensitized and challenged with ovalbumin (OVA). Parallel groups were sham-sensitized with saline. Mice were exposed 6h/day for 3 days to air (control, C) or SDCCE containing particulate matter (PM) at low (L; 100 µg/m³), medium (M; 300 µg/m³), or high (H; 1000 µg/m³) concentrations, or to the H level with PM removed by filtration (high-filtered, HF). Immediately after SDCCE exposure, mice received another OVA challenge (pre-OVA protocol). In a second (post-OVA) protocol, mice were similarly sensitized but only challenged to OVA before air/SDCCE. Measurement of airway hyperresponsiveness (AHR), bronchoalveolar lavage (BAL), and blood collection were performed ~24 h after the last exposure.

Results: SDCCE significantly increased BAL macrophages and eosinophils in OVA-sensitized mice from the post-OVA protocol. However, there was no effect of SDCCE on BAL macrophages or eosinophils in OVA-sensitized mice from the pre-OVA protocol. BAL neutrophils were elevated following SDCCE in both protocols in nonsensitized mice. These changes were not altered by filtering out the PM. In the post-OVA protocol, SDCCE decreased OVA-specific IgG₁ in OVA-sensitized mice but increased levels of total IgE, OVA-specific IgE and OVA-specific IgG₁ and IgG_{2a} in non-sensitized animals. In the pre-OVA protocol, SDCCE increased OVA-specific IgE in both sensitized and non-sensitized animals. Additionally, BAL IL-4, IL-13, and IFN-γ levels were elevated in sensitized mice.

Conclusion: These results suggest that acute exposure to either the particulate or gaseous phase of SDCCE can exacerbate various features of allergic airway responses depending on the timing of exposure in relation to allergen challenge.

Keywords: Air pollution, coal, particulate matter, sulfur dioxide, nitrogen dioxide, nitrogen oxides, lung, respiratory, allergy, asthma, inflammation

Introduction

Emissions from coal-fired power (CPE) plants can contribute significantly to ambient air pollution by releasing sulfur dioxide (SO₂), nitrogen oxides (NO_x), carbon dioxide, hydrocarbons, particulate matter (PM) and secondary sulfates–nitrates, into the atmosphere (Chang et al., 2004; Hao et al., 2007). Epidemiological data and controlled clinical trials show strong associations between exposure to ambient air pollution and adverse

pulmonary effects such as exacerbations of allergic symptoms (wheeze and cough), increased sensitization to allergens, and increased hospital admissions for pulmonary diseases (Brunekreef et al., 1997; van Roosbroeck et al., 2006; Zanobetti et al., 2000). Further, childhood asthma has been reported to have a significant association with exposure to air pollutants (Sinclair et al., 2010). While there is convincing evidence that acute exposure to traffic-related air pollution has a detrimental effect

Address for Correspondence: Edward G. Barrett, PhD, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr. SE, Albuquerque, NM 87108, USA. Tel: 505-348-9417. Fax: 505-348-4980. E-mail: tbarrett@lrri.org

(Received 29 March 2011; revised 06 July 2011; accepted 29 July 2011)

on lung function and lung inflammation in asthmatics (Morgenstern et al., 2008) the specific impact of CPE or its components is less well-characterized with respect to asthma exacerbation.

With regards to evaluating the health effects of CPE, many epidemiology studies have utilized SO_2 levels and proximity to a source as potential markers of exposure to CPE or other industrial plant emissions. Short-term episodes of increased SO_2 exposures from refinery stack emissions have been associated with a higher number of asthma episodes in children living nearby (Smargiassi et al., 2009). Proximity to air pollution from a CPE station in Israel had a negative effect on children's lung function development (Dubnov et al., 2007). Additionally, controlled human exposures to SO_2 and/or NO_2 can induce acute bronchoconstriction in asthmatic subjects at concentrations well below those required to induce this response in healthy subjects (Balmes et al., 1987; Devalia et al., 1994; Horstman et al., 1986; Linn et al., 1987; Rusznak et al., 1996; Sheppard et al., 1980). However, more recent analyses of studies that were able to better separate out specific sources of emissions suggest that emissions from CPE plants are not associated with adverse health effects, but rather vehicular, industrial, and/or residual oil emissions or urban masses are so associated (Grahame and Schlesinger, 2005). Few studies, however, have specifically evaluated the effects of CPE on pre-existing asthma symptoms.

There are limited experimental studies in animals that have explored the effects of simulated downwind coal-fired power plant emissions (SDCCE) on allergic airway responses. Most studies have examined coal fly ash (CFA) which consists of a primary particle, along with soot as a solid from the power plant stack. Further, CFA exposure would be considered more of an occupational rather than an environmental concern. In general, these studies have shown that exposure to CFA leads to CFA particles being retained in the lung (Raabe et al., 1982), reduced lung function (Chen et al., 1990), increased immune cell activity (Dormans et al., 1999) and inflammation in the lung (Gilmour et al., 2004; Smith et al., 2006). In contrast, other studies did not find any effect of instilled CFA on lung inflammation (Walters et al., 2001). None of these studies addressed whether CFA exacerbated respiratory allergic responses. Moreover, none exposed the animals to the gas-phase components of SDCCE. A small number of studies have evaluated the effects of SO_2 or NO_2 exposure on allergic airway responses in various animal models of allergic airways disease (Abraham et al., 1981; Park et al., 2001; Poynter et al., 2006; Riedel et al., 1988). Those studies have shown that allergen-sensitized animals are more sensitive to the effects of SO_2 and generally exhibited more hyperresponsive airways following a range of exposure levels (0.1–43.5 ppm) and exposure durations (4 h–5 days).

We evaluated the effect of short-term (3-day) exposure to SDCCE and whether this exposure leads to increased allergic airway responses in BALB/c mice that had been pre-sensitized to albumin (OVA) and then exposed to

SDCCE. We also evaluated the allergic airway response of a separate group of mice who received a second OVA challenge following the SDCCE exposure. This study was part of a larger investigation by the National Environmental Respiratory Center (NERC; www.nercenter.org) of the health effects of SDCCE (Mauderly et al., 2011).

Materials and methods

Animals

Male BALB/c mice (8–10 weeks old; 15–16 animals per treatment group) were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed in wire rack cages inside whole-body exposure chambers (H-2000; Hazleton Systems, Inc., Maywood, NJ, USA). Chamber temperatures were maintained at 20–24°C and lights were on a 12-h on/off cycle. Excreta trays in the chambers were cleaned twice daily, and chambers were washed weekly. Mice were fed Teklad certified rodent diet (Harlan Teklad, Madison, WI, USA) *ad libitum* except during the 6-h exposure period. Water was available *ad libitum* throughout the duration of the study.

Experimental design

Mice were exposed to air, OVA, and SDCCE according to two different protocols, one in which exposure to the test atmospheres occurred after the initial OVA challenge (post-OVA) and one in which exposure to the test atmospheres occurred before the final OVA challenge, (pre-OVA). Separate whole-body exposure chambers were used for the OVA challenge and SDCCE exposures. In both protocols mice were sensitized by intraperitoneal (IP) injection with OVA (10 µg) and aluminum hydroxide (2mg) in sterile water or sham-sensitized by injection of saline on days 0 and 7. In the post-OVA protocol (Figure 1A), mice received an aerosolized OVA challenge on day 14, 1 day prior to 3 days (6 h/day) of clean air (control, C) or to SDCCE at three dilutions (low, L; medium, M; or high, H) or to the H level with PM removed by filtration (high-filtered, HF). Approximately 24 h after the last day of C or SDCCE exposures, methacholine (MCh)-induced changes in pulmonary function (PFT) were measured and the mice were immediately sacrificed. In the pre-OVA protocol, (Figure 1B), mice received an aerosolized OVA challenge on day 14. On days 26–28 mice were exposed to clean air (control, C) or to SDCCE. Immediately after SDCCE or C exposures on day 28, mice were placed in a separate whole-body exposure chamber and again challenged with aerosolized OVA. Mice were tested for MCh-induced changes in PFT 18–24 h after the end of the SDCCE or C exposures and then immediately sacrificed. All animal procedures were reviewed and approved by the Institute's Institutional Animal Care and Use Committee.

Generation of simulated downwind coal emissions

Briefly, the SDCCE were created to simulate the composition of the major particle and gas-phase constituents present downstream (hundreds of miles) of a coal

utility plant (McDonald et al., in press). The nature of the NERC protocol and limited resources precluded conducting exposures at an actual power plant or using a pilot-scale coal combustor that accurately simulated power plant operation. The intent to simulate key pollutants contributed by coal combustion at locations downwind from power plants precluded exposure solely to fresh combustion emissions. General compositional targets were established by a workshop of atmospheric scientists, coal combustion experts, and other participants from academia, government, and industry. The recommended mixture was not intended to reproduce processed emissions from a single plant, type of plant, or coal feedstock, but rather to include components in ratios commonly present in downwind locations. The exposure mixture was generated using a combination of an electric drop-tube furnace to burn coal, a vaporization-condensation method for generating sulfate aerosol, and SO_2 , NO and NO_2 from compressed gas tanks. Low-sulfur sub-bituminous coal from the Black Thunder Mine in Campbell County, WY (Powder River Basin), was selected to provide the small ash component of the mixture because it proved in preliminary trials to be more readily combusted than bituminous coal in the system used. The coal was pulverized, sieved at 50 μm , and stored under nitrogen at room temperature. The coal was fed by a screw feeder into a pre-heated stream of air into the furnace which was maintained at 1400°C.

The furnace effluent was cooled to quench reactions and routed through a primary ash mixing chamber and a cyclone to remove any large particles. After mixing the coal emissions, sulfate, and gases in a 1 m³ chamber, the mixture was diluted in parallel with filtered, conditioned air to target PM mass concentrations of nominally 1000, 300, and 100 $\mu\text{g}/\text{m}^3$ (Table 1). The PM mass concentrations were chosen to match the levels used in previous similarly designed NERC studies (diesel engine exhaust, hardwood smoke and gasoline engine exhaust) in order to allow for future detailed statistical analyses that will try to disentangle the complex exposure (mixtures)-response relationships.

OVA exposure

Exposure to aerosolized OVA was carried out using whole-body plethysmographs as exposure chambers (Buxco Electronics, Sharon, CT, USA). OVA aerosols were generated for 10 min by ultrasonic nebulization (DeVibiss Pulmosonic®, Model 5500) of 1% heat-aggregated OVA (chicken egg, grade V; Sigma, St. Louis, MO, USA) in water, passed through a 25-cm diffusion dryer, and then distributed to the plethysmograph exposure chambers. OVA aerosols were sampled directly from the exhaust port of the exposure chambers at a flow rate of 0.45 L/min (the total flow through the chambers was maintained at 0.85 L/min) onto 47-mm Zeflour (Pall-Gelman, Port Washington, NY) filters. The mass concentration of OVA

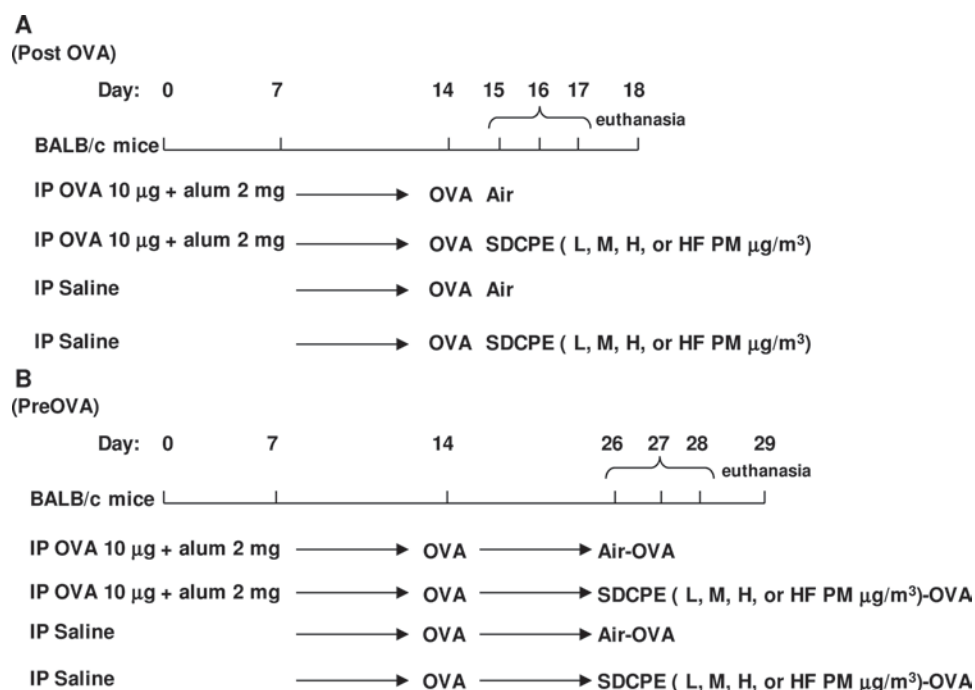


Figure 1. Mice (15–16 animals per treatment group) were immunized by intraperitoneal (IP) injection with ovalbumin (OVA) and aluminum hydroxide (alum) or saline only (no OVA). Mice were then exposed to air, OVA and simulated downwind coal-fired power plant emissions (SDCCE) according to two different protocols, (A) post-OVA and (B) pre-OVA. Post-OVA mice received an aerosolized OVA or air challenge on day 14, 1 day prior to 3 days (6 h/day) of filtered air or SDCCE (L, M, H or HF PM/ m^3) exposure. Approximately 24 h after the last day of SDCCE or air exposure, MCh-induced changes in PFT were measured and the animals were euthanized. Pre-OVA mice received an aerosolized OVA or air challenge on day 14. On days 26–28, groups of mice were exposed to filtered air or SDCCE (C, L, M, H or HF particulate matter [PM]/ m^3) 6 h/day. Immediately after SDCCE exposure on day 28 mice were challenged with aerosolized OVA or air. After 24 h, mice were tested for MCh-induced changes in pulmonary function (PFT) 24 h and then immediately euthanized.

Table 1. Composition of SDCCE exposure atmosphere composition.

Analyte	Units	Control Mean $\pm$ SE	Low Mean $\pm$ SE	Mid Mean $\pm$ SE	High filtered Mean $\pm$ SE	High unfiltered Mean $\pm$ SE
PM Mass	$\mu\text{g}/\text{m}^3$	7.2 $\pm$ 6.8	117.2 $\pm$ 20.02	313.4 $\pm$ 38.9	3.6 $\pm$ 3.6	1014.7 $\pm$ 104.2
PM number	$\#/\text{cm}^3$	3.8×10^2	2.3×10^5	5.9×10^5	3.0×10^2	14.8×10^5
Non-methane VOC	$\mu\text{g}/\text{m}^3$	86.54	61.21	65.45	101.10	85.71
SO ₂	$\mu\text{g}/\text{m}^3$ (ppm)	2.02 $\pm$ 3.3 (0.001)	39.2 $\pm$ 13.04 (0.02)	126.4 $\pm$ 26.9 (0.06)	465.0 $\pm$ 68.8 (0.21)	477.9 $\pm$ 70.1 (0.2)
CO	$\mu\text{g}/\text{m}^3$ (ppm)	20 $\pm$ 0.2 (0.02)	70 $\pm$ 0.2 (0.07)	10 $\pm$ 0.2 (0.01)	40 $\pm$ 0.2 (0.04)	20 $\pm$ 0.2 (0.02)
NO	$\mu\text{g}/\text{m}^3$ (ppm)	0.41 $\pm$ 1.1 (~0)	64.7 $\pm$ 12.7 (0.06)	189.1 $\pm$ 24.4 (0.18)	623.6 $\pm$ 59.8 (0.61)	603.8 $\pm$ 82.1 (0.59)
NO ₂	$\mu\text{g}/\text{m}^3$ (ppm)	1.3 $\pm$ 2.3 (~0)	59.6 $\pm$ 12 (0.04)	157.6 $\pm$ 23.1 (0.1)	487.5 $\pm$ 65.4 (0.31)	461.9 $\pm$ 62.6 (0.29)

was determined by gravimetric analysis of filter samples taken during test runs. The average mass concentration of OVA was $\sim 225 \text{ mg}/\text{m}^3$ during a 10-min exposure. OVA particle size was measured directly from the exposure chamber with a Mercer-style impactor (size range 0.6–12 μm) operating at a flow rate of 2 L/min. The mass median aerodynamic diameter (μm) and geometric standard deviation were 1.1 ± 2.0 .

Airway hyperresponsiveness

Airway Hyperresponsiveness (AHR) to increasing concentrations of aerosolized MCh was measured using the forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada) 24 h following the last particle exposure. Briefly, each mouse was anesthetized with an IP injection of Avertin (250 mg/kg; 0.02 mL/g). The trachea was cannulated and attached to the FlexiVent apparatus. Ventilation was performed through the cannula by positive pressure maneuvers and recorded on a personal computer. Mice received an IP injection of pancuronium (0.5 mg/kg) to stop respiration and the heart rate was monitored. Following baseline measurements of resistance, aerosolized saline (0.9%) or increasing MCh concentrations (6, 12, 25, and 50 mg/mL) were nebulized and delivered to the animals for 10 s. Changes in airway resistance were recorded for 3 min after each challenge and were used to compare responses between treatment groups.

Pathologic analysis

Following AHR measurements, animals were euthanized by injection of a lethal dose of pentobarbital-based euthanasia solution. Blood was collected from a renal artery, and serum was prepared. Bronchoalveolar lavage (BAL) fluid cells were obtained by inserting a catheter into the trachea and lavaging the lungs three times with 0.8 mL of PBS. Total BAL cells were determined using a hemacytometer. BAL cells were spun onto slides by cytocentrifugation and stained with a modified Wright-Giemsa stain. Four hundred cells were counted and the percentage of specific cell types was determined for each animal.

On animals designated for histopathologic examination, lungs were instilled via the trachea with 10% neutral buffered formalin (NBF) followed by immersion in NBF for at least 48 h. Lungs were processed routinely, paraffin

embedded, sectioned at a nominal 5 μm , stained with hematoxylin and eosin (H&E), and examined by a board certified pathologist (AG). Alcian blue/Periodic acid Schiff (AB/PAS) staining was performed on a serial section to evaluate mucous metaplasia of bronchial epithelium. A “read-down” approach was employed in which tissues from the control and high exposure groups were examined, and tissues from other exposure groups were to be examined only if abnormalities were found at the high level.

Analysis of OVA-specific IgG₁ and IgG_{2a}

Anti-OVA-specific immunoglobulin (Ig) G₁ and IgG_{2a} antibody levels in serum were measured with an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with 5 $\mu\text{g}/\text{mL}$ heat-aggregated OVA (Sigma) in PBS and incubated overnight at 4°C. Plates were washed three times (0.1% Tween 20 in PBS) and then blocked with 2.5% skim milk for 1 h. Samples diluted (IgG₁ [1:500, 1:2500 and 1:12500] and IgG_{2a} [1:100, 1:500, and 1:2500] in skim milk were added (100 μL) and incubated for 1 h at 37°C. Plates were washed three times and goat anti-mouse IgG₁-HRP or IgG_{2a}-HRP (Pharmingen, San Diego, CA, USA) was added (1:2000; incubation 30 min at 37°C). Next, plates were washed five times and ABTS peroxidase substrate (KPL, Gaithersburg, MD, USA) was added (incubation 30 min 37°C). Finally, peroxidase stop solution (KPL) was added and the plates were read in a microplate reader at 410 nm. Results are presented as units per milliliter, where the relative units represent the optical density of a sample multiplied by the sample dilution factor.

Analysis of OVA-specific IgE

OVA-specific IgE antibody levels in serum were measured by ELISA. Briefly, 96-well plates were coated overnight at 4°C with 2 $\mu\text{L}/\text{mL}$ anti-mouse IgE (Pharmingen). Plates were washed 3 times (0.1% Tween 20 in PBS) and then blocked with 10% fetal bovine serum (Hyclone, Logan, UT, USA) in PBS for 1 h. Plates were washed three times and then 100 μL of sample at multiple dilutions (1:15, 1:30, and 1:60) was added and incubated for 2 h at room temperature. Again, plates were washed five times and then 100 μL of OVA conjugated with biotin (4 $\mu\text{g}/\text{mL}$; EZ-link Biotinylations Kit; Pierce Biotechnology, Rockford, IL,

USA) plus avidin-horseradish peroxidase (HRP) (1/1000; Pharmingen) was added to each well and incubated at room temperature for 1 h. Plates were washed seven times and 100 μ L of TMB substrate (Pharmingen, San Diego, CA) was added (incubation for 30 min in the dark at room temperature). Finally, stop solution (2 N H_2SO_4) was added and the plates were read at 450 nm on a microplate reader. Results are presented as units per milliliter. Total IgE in serum samples was measured using an ELISA kit (IgE BD OptEIA; Pharmingen) following the manufacturer's protocol. Antibody measurements were compared between treatment groups.

Cytokine analysis

Cytokine levels for IL-2, -4, and -5, and IFN- γ in the BAL ($n=8-11$ /group based on availability of sufficient lavage fluid) were measured using an ELISA kit following the manufacturer's protocol (OptEIA; Pharmingen). Levels of IL-13 in BAL fluid were determined using monoclonal anti-mouse IL-13, biotinylated anti-mouse IL-13, and recombinant murine IL-13 in an ELISA following the manufacturer's recommended protocol (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate SDCCE-related effects on BAL parameters, cytokines, and antibody measurements. When an ANOVA indicated significant ($p < 0.05$) differences in mean responses across experimental groups, Dunnett's multiple comparison procedure (Dunnett, 1955, 1980) was performed to compare SDCCE-exposed group means with those of controls the linear term of the ANOVA (with equal dose spacing) was used to assess evidence of exposure-related trends in response across control and exposed groups. Because of differing variance structures, four separate ANOVAs were applied for each endpoint, corresponding to the two different allergen challenges (OVA and air) in the two protocols (pre- and post-OVA).

AHR measurements, which were repeated on each animal at differing levels of MCh, were analyzed using a repeated measures ANOVA model in which slope estimates for increasing MCh response in treated groups were compared to controls. ANOVA calculations were performed using the general estimating equations approach with an autoregressive (order 1) correlation matrix for random errors. The appropriateness of the autoregressive correlation assumption was verified by examination of correlations among model residuals.

For most endpoints, there was significant heteroscedasticity, with exposure group variances functionally related to their associated sample means. Logarithmic transformation of these data after application of an estimated offset constant (Rocke and Durbin, 2003) was employed prior to analysis to stabilize variances and satisfy the homogeneity of variance assumption underlying the ANOVA regression model. Data are summarized and plotted as mean $\pm$ standard error

(SE), and all reported p values are two-sided. Statistical computations were performed with SAS software system (Cary, NC, USA).

Results

Post-OVA

Sensitization and challenge with OVA in the post-OVA model led to an allergic airways phenotype that was generally characterized by an increase in BAL eosinophils (Figure 2), AHR (Figure 3), tissue inflammation-pathology (Figure 4) and serum OVA-specific antibody levels (Figure 5) when compared to non-sensitized controls. SDCCE exposures led to a dose-dependent increase in BAL total cells in OVA-sensitized and non-sensitized animals (Figure 2A). More specifically, SDCCE exposure led to a significant dose-dependent increase in BAL neutrophils-macrophages in non-sensitized mice and an increase in eosinophils-macrophages in sensitized mice (Figure 2B-D). None of the increases in BAL cell numbers were impacted by filtering out the particulate fraction (e.g. H vs. HF groups; Table 1) suggesting the increases in BAL cell numbers were due to the gaseous components. While there were apparent increases in AHR in OVA-sensitized mice at the H and HF levels these increases did not reach the level of statistical significance (Figure 3). Additionally, there was no effect of SDCCE exposure on AHR in non-sensitized animals (data not shown). Histopathology demonstrated differences between non-sensitized (Figure 4A and B) mice vs. OVA-sensitized mice (Figure 4C and D), primarily an expected peribronchovascular inflammation with eosinophils and mucous metaplasia of the bronchial epithelium. However, there were no substantive light microscopic differences between H SDCCE exposure animals and their respective controls (Figure 4E-F).

OVA sensitization and challenge led to an expected increase in serum antibody levels (Figure 5A-D; Total E and OVA-specific IgE, IgG₁, and IgG_{2a}). In sensitized mice, SDCCE exposure did not have a significant effect on total IgE, OVA-specific IgE, or OVA-specific IgG_{2a} (Figure 5A, B, and 5D). However, SDCCE at the L, M, and H levels led to a significant decrease in serum OVA-specific IgG₁ (Figure 5C). Interestingly, when the PM fraction was filtered out (HF vs. control or H)) SDCCE led to a significant reduction in total IgE, OVA-specific IgG₁, and OVA-specific IgG_{2a} in the sensitized animals. In contrast, SDCCE exposure led to an increase in total IgE (H), OVA-specific IgE (L, M, and H) and OVA-specific IgG₁ (H) and IgG_{2a} (H) in non-sensitized animals. However, when the PM fraction was filtered out the increase was no longer present suggesting the PM fraction may be mediating this response. Levels of IL-2, IL-4, IL-5 and IFN γ were negligible in the BAL fluid from OVA-sensitized and non-sensitized animals regardless of SDCCE exposure and there were no significant differences (results not shown). Levels of IL-13 in the BAL fluid were detectable in OVA-sensitized animals and were decreased following SDCCE

Post OVA

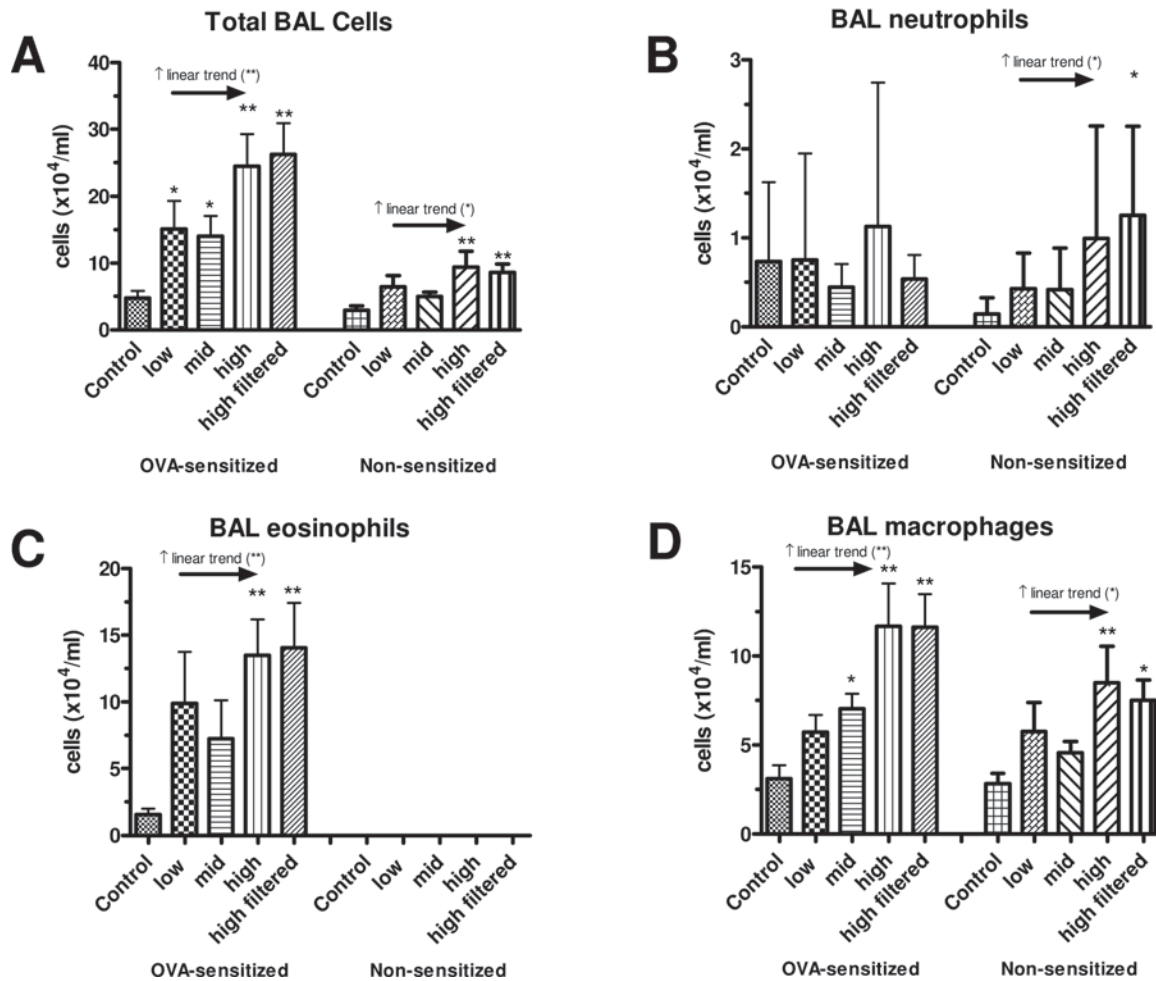


Figure 2. Bronchoalveolar lavage (BAL) cell numbers following post-OVA exposure protocol: (A) total cells, (B) neutrophils, (C) eosinophils, and (D) macrophages (mean $\pm$ SE). Asterisk indicates significant at $*p < 0.05$ or $**p < 0.01$ vs. control. Results of linear trend analyses examining dose-dependent trends are indicated below each treatment group; arrow sign indicates the direction of the trend.

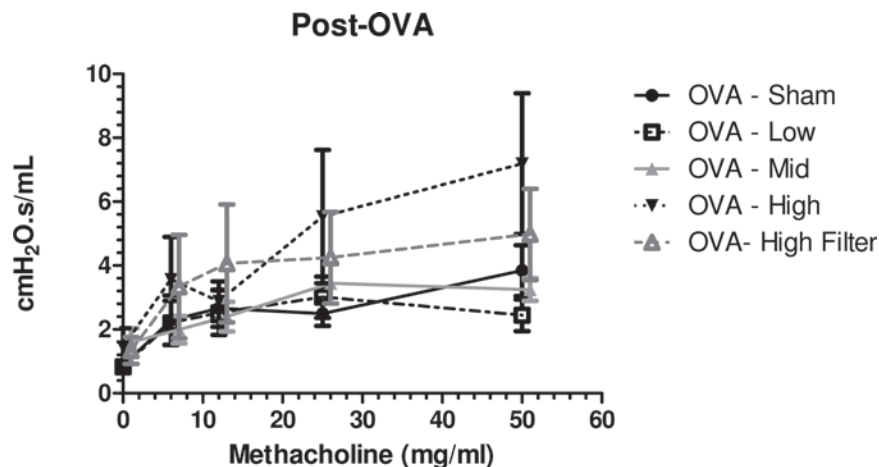


Figure 3. Changes in airway hyperresponsiveness from post-OVA-sensitized exposed to SDCCE according to the post-OVA protocols (mean $\pm$ SE). Pulmonary function was measured by the forced oscillation technique in response to MCh.

Post OVA

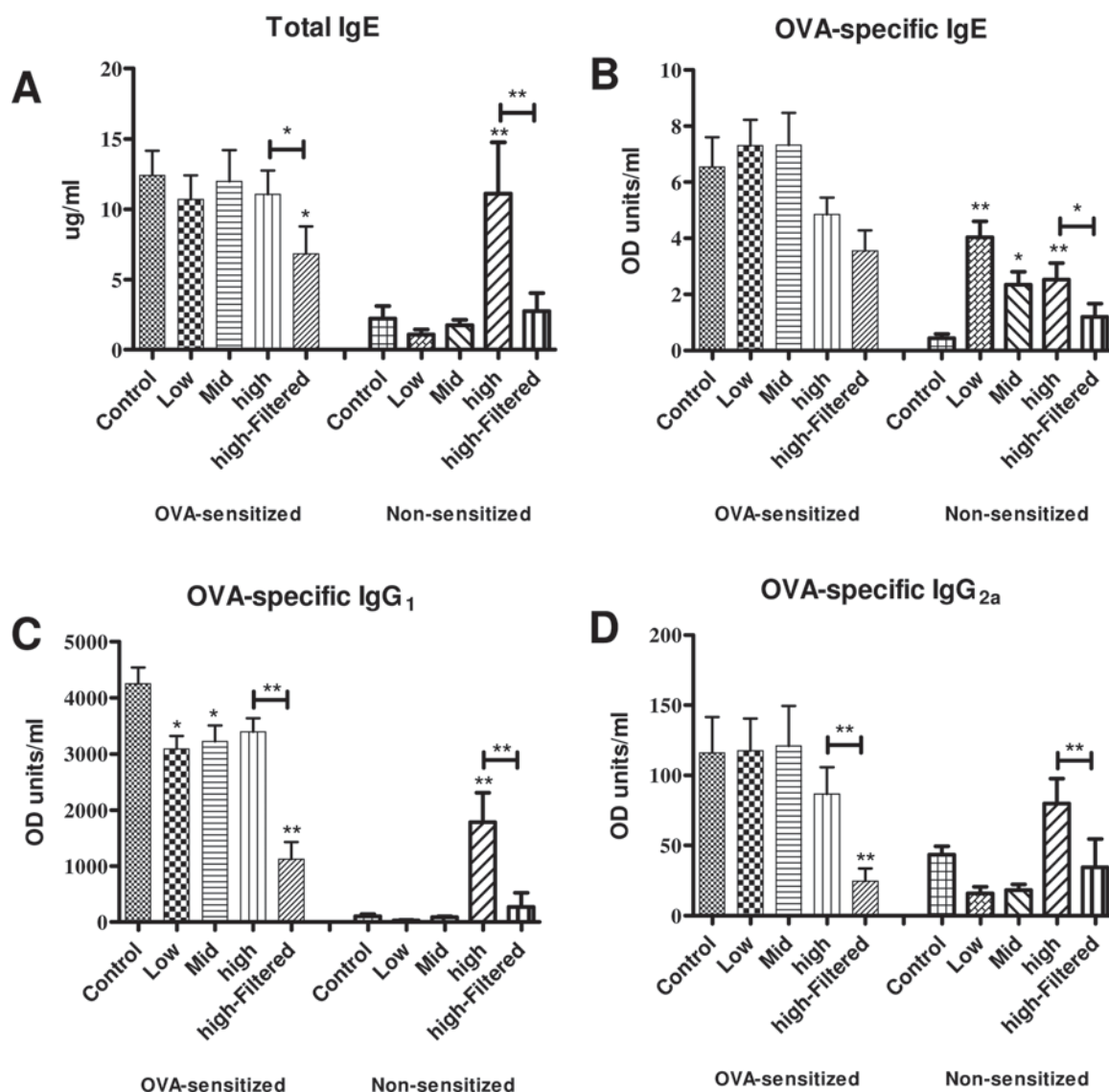


Figure 4. Serum antibody levels following post-OVA exposure protocol: (A) Total IgE, (B) OVA-specific IgE, (C) OVA-specific IgG₁, and (D) OVA-specific IgG_{2a} (mean $\pm$ SE), Asterisk indicates significant at $p < 0.05$ or $p < 0.01$ vs. control or H vs. HF as indicated. .

exposure C (21.1 ± 11.1 ng/mL), L (2.18 ± 1.32 ng/mL), M (0.35 ± 0.35 ng/mL), H (1.10 ± 1.10 ng/mL), and HF (14.0 ± 13.5 ng/mL) ($*p < 0.05$ vs. C).

Pre-OVA

Sensitization and challenge with OVA in the pre-OVA model led to an allergic airways phenotype that was generally characterized by an increase in BAL eosinophils and neutrophils (Figure 6), AHR (Figure 7), tissue inflammation-pathology and serum OVA-specific antibody levels (Figure 8) when compared to non-sensitized controls. SDCCE exposure in OVA-sensitized animals did not have any effect on BAL inflammatory cell numbers. However, SDCCE exposure did lead to a significant increase in BAL total cells, macrophages (M, H and HF) and neutrophils (increasing

dose-dependent linear trend) in non-sensitized mice (Figure 6). These increases were not inhibited by filtering out the particulate fraction. While there was an apparent increase in AHR in OVA-sensitized mice at the H and HF levels, only the HF group increase reached statistical significance ($p < 0.05$) (Figure 7). Additionally, there was no effect of SDCCE exposure in non-sensitized animals (data not shown). As in the post-OVA group, histopathology demonstrated differences between non-sensitized mice vs. OVA-sensitized mice, primarily the expected peribronchovascular inflammation with eosinophils and mucous metaplasia of bronchial epithelium. However, there were no substantive light microscopic differences between high SDCCE exposure animals and their respective controls (data not shown).

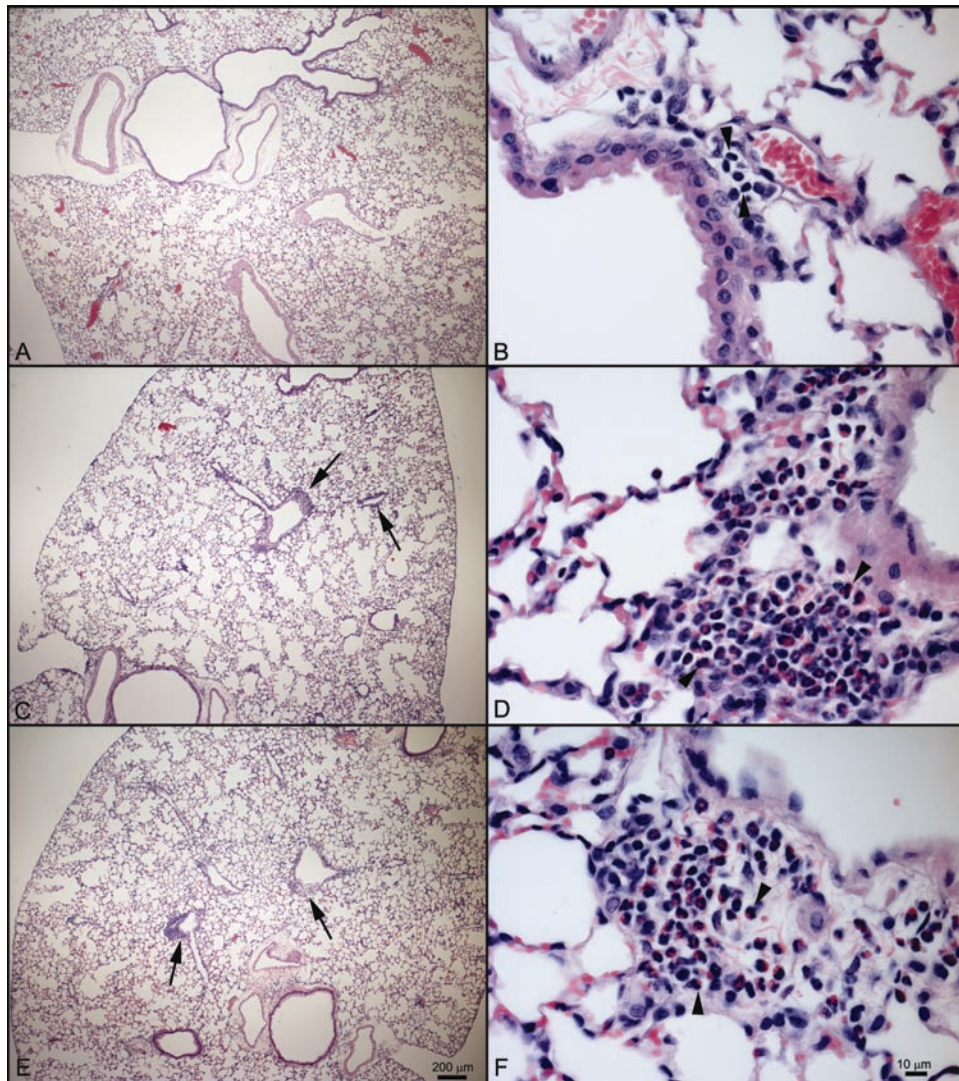


Figure 5. Representative pulmonary histopathology of post-OVA group animals: (A) Non-sensitized (IP saline), high SDCCE-exposed animal. Low power view of unremarkable lung. (B) High power view from same animal as in "A". As in normal, untreated lungs (not shown), only rare mononuclear cells are present adjacent to airways or vessels (arrowheads). (C) OVA-sensitized, air control animal. Low power view with mild peribronchovascular inflammatory infiltrates (arrows). (D) High power view of same animal as in "C". Note prominent contribution of eosinophils to peribronchovascular inflammation (arrowheads). (E) OVA-sensitized, High SDCCE-exposed animal. Low power view with mild peribronchovascular inflammatory infiltrates (arrows). Histopathologic appearance is similar to that of "C". (F) High power view of same animal as in "E". Note prominent contribution of eosinophils to peribronchovascular inflammation (arrowheads). Note: similar findings were observed in the pre-OVA animals (data not shown).

In sensitized mice, SDCCE exposure at the L and M levels led to a significant decrease in serum total IgE (Figure 8). In contrast, serum OVA-specific IgE levels were increased in the H group. As in the post-OVA model filtering out the PM fraction (HF vs. H) appeared to lead to a reduction in antibody levels, although only the differences in OVA-specific IgG₁ were statistically significant. In non-sensitized animals, levels of serum OVA-specific IgE and OVA-specific IgG₁ were increased in a dose-dependent manner. In contrast to the post-OVA model removing the PM fraction did not have an effect on the increase in antibodies in the non-sensitized animals. SDCCE exposure in OVA-sensitized animals led to a dose-dependent increase in IL-4, IL-13 and IFN γ levels in the BAL fluid (Table 2). The increases in BAL cytokines were attenuated when the particulate fraction was removed. (H vs. HF).

Similar increases in BAL fluid IL-13 and IFN γ levels in non-sensitized animals following SDCCE exposure were also present.

Discussion

In the present study, we found that exposure to SDCCE led to an increase in some allergic airway responses depending on the timing of the exposure with respect to allergen challenge. Some changes (BAL inflammation and AHR) were independent of the presence of the particulate fraction (HF) while some (serum antibody changes) were dependent on the particulate fraction. Also, SDCCE led to an increase in BAL inflammation (macrophages and neutrophils) in naïve (non-sensitized) animals that was independent of the particulate fraction being present.

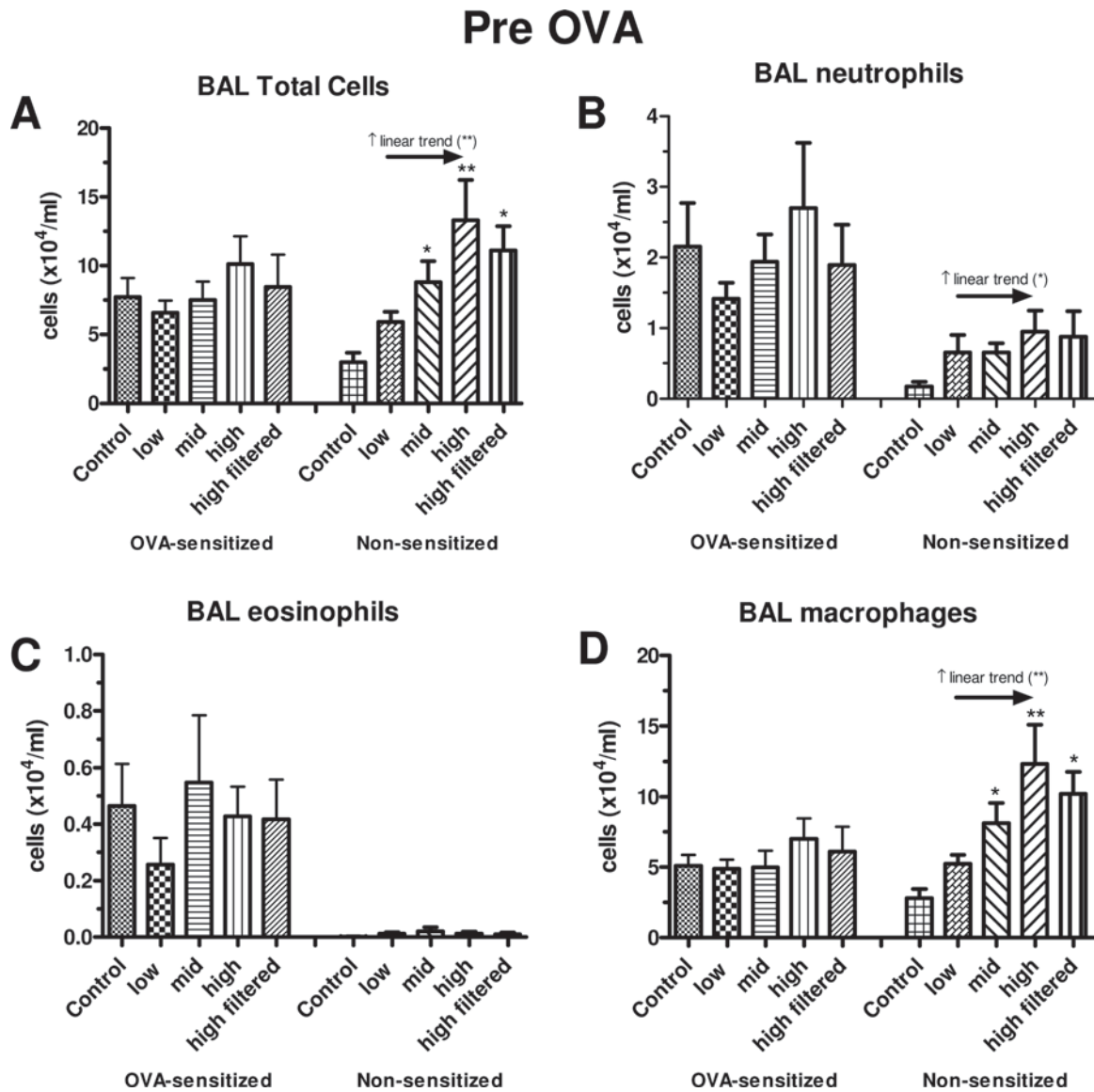


Figure 6. Bronchoalveolar lavage cell numbers following pre-OVA exposure protocol: (A) total cells, (B) neutrophils, (C) eosinophils, and (D) macrophages (mean $\pm$ SE). Asterisk indicates significant at $*p < 0.05$ or $**p < 0.01$ vs. control. Results of linear trend analyses examining dose-dependent trends are indicated below each treatment group; arrow sign indicates the direction of the trend.

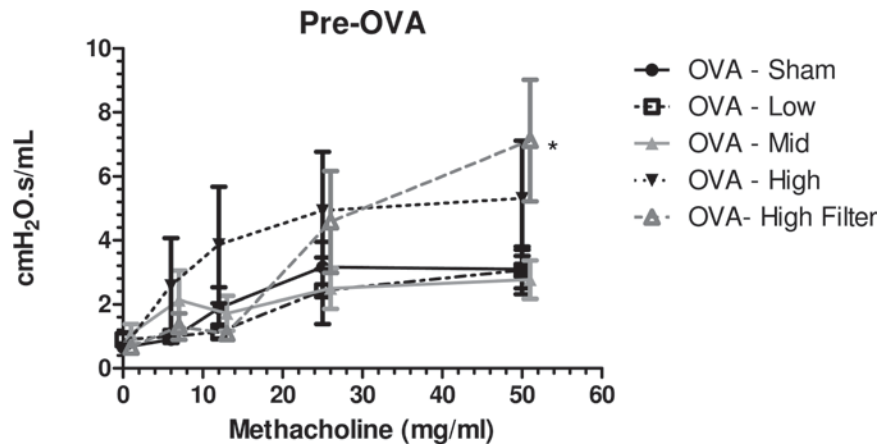


Figure 7. Changes in airway hyperresponsiveness from pre-OVA-sensitized exposed to SDCCE according to the post-OVA protocols (mean $\pm$ SE). Pulmonary function was measured by the forced oscillation technique in response to MCh. $*p < 0.05$ vs. Control.

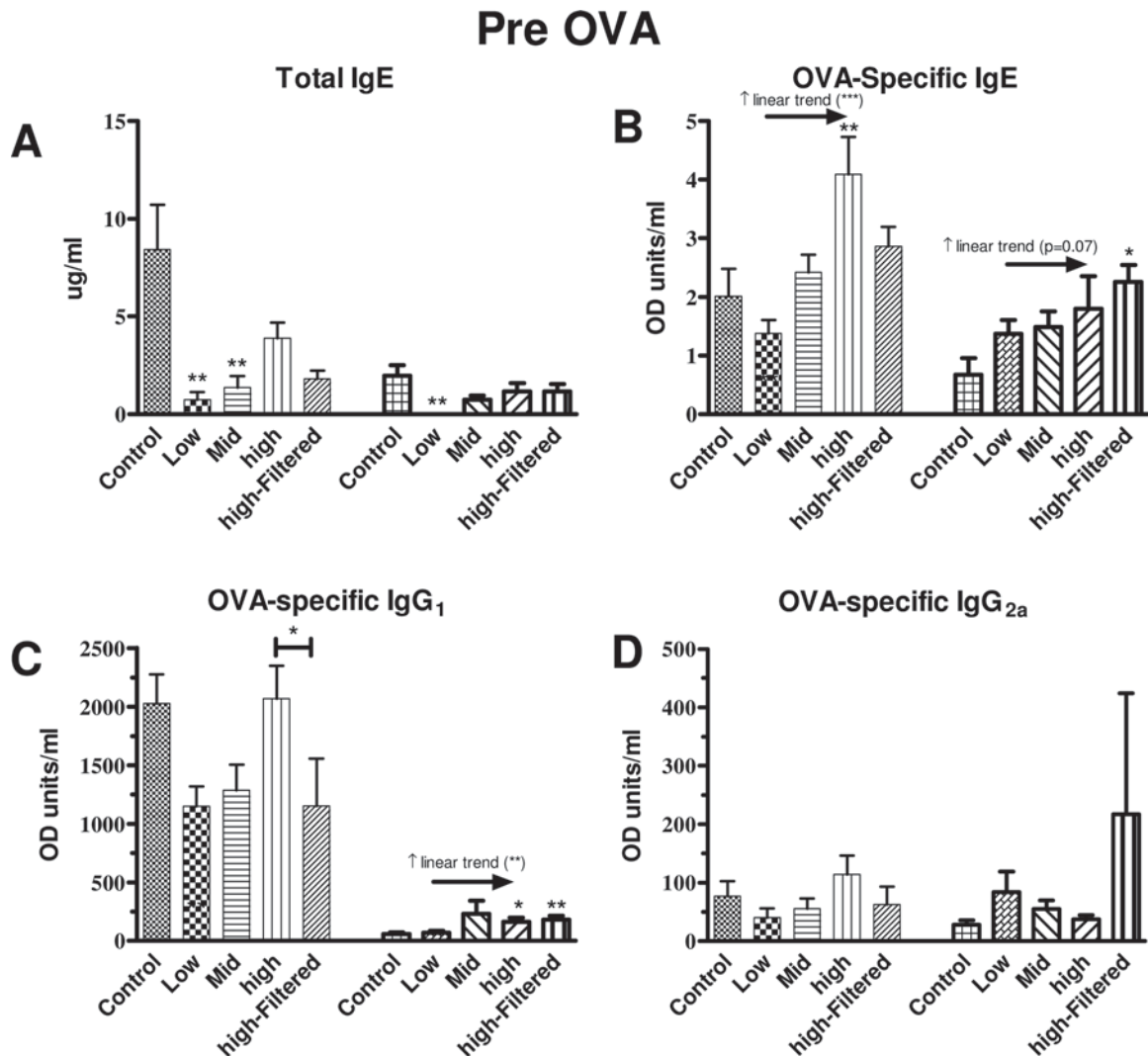


Figure 8. Serum antibody levels following pre-OVA exposure protocol: (A) Total IgE, (B) OVA-specific IgE, (C) OVA-specific IgG₁, and (D) OVA-specific IgG_{2a} (mean $\pm$ SE), Asterisk indicates significant at * $p < 0.05$ or ** $p < 0.01$ vs. control or H vs. HF as indicated.

Table 2. Pre OVA BAL cytokine levels.

Analyte	Units	C Mean $\pm$ SE	L Mean $\pm$ SE	M Mean $\pm$ SE	H Mean $\pm$ SE	HF Mean $\pm$ SE
OVA						
IL-4	ng/mL	8.14 $\pm$ 5.09	1.16 $\pm$ 0.73	5.83 $\pm$ 2.45	38.5 $\pm$ 19.0* ^{##}	1.52 $\pm$ 0.61 [‡]
IL-5	ng/mL	7.72 $\pm$ 5.86	1.06 $\pm$ 1.02	4.36 $\pm$ 1.65	19.6 $\pm$ 16.1	0.00 $\pm$ 0.00 [†]
IL-13	ng/mL	90.3 $\pm$ 45.4	10.7 $\pm$ 9.28	128 $\pm$ 18.5	197 $\pm$ 42.8* ^{##}	72.2 $\pm$ 21.8 [‡]
IFN γ	ng/mL	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	32.6 $\pm$ 5.00**	28.0 $\pm$ 5.61* ^{##}	19.0 $\pm$ 5.90*
No OVA						
IL-4	ng/mL	0.02 $\pm$ 0.01	0.01 $\pm$ 0.00	0.03 $\pm$ 0.01	0.03 $\pm$ 0.00 ^{##}	0.01 $\pm$ 0.00 [‡]
IL-5	ng/mL	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00 [†]
IL-13	ng/mL	0.00 $\pm$ 0.00	8.89 $\pm$ 8.89	84.2 $\pm$ 4.40**	84.2 $\pm$ 3.59* ^{##}	0.00 $\pm$ 0.00 [‡]
IFN γ	ng/mL	0.00 $\pm$ 0.00	5.18 $\pm$ 5.18	31.2 $\pm$ 5.14**	24.2 $\pm$ 5.93* ^{##}	8.97 $\pm$ 8.97

*Significantly different from control group ($p < 0.05$).

**Significantly different from control group ($p < 0.01$).

^{##} $p < 0.01$ -Value and direction $\uparrow$ of trend in exposure groups C-H.

[†]Group HF mean differs from Group H mean ($p < 0.05$).

[‡]Group HF mean differs from Group H mean ($p < 0.01$).

Finally, regardless of whether animals were sensitized or not, all the observed changes were generally dose-dependent.

Exposure for 3 days to SDCCE immediately following OVA challenge (post-OVA) led to a greater increase in BAL inflammatory cells (eosinophils and macrophages)

than if exposure preceded OVA challenge (pre-OVA). These differences may be due to several factors including (1) presence of active allergen-induced inflammation at the time of SDCCE exposure, and (2) timing of euthanasia with respect allergen/SDCCE exposure. We have previously shown in the same OVA challenge model that there is a greater increase in BAL neutrophils in the pre-OVA protocol and conversely a larger increase in eosinophils in the post-OVA protocol that are due to the timing of the animal sacrifice after OVA challenge (Barrett et al., 2006; Day et al., 2008). Following acute (single) OVA exposure, BAL neutrophils typically peak at 24–48 h and eosinophils peak at 48–72 h. Thus, there were minimal eosinophils present at the time of BAL collection in the pre-OVA model at ~ 16–18 h post challenge. It is possible that waiting for 72 h post-OVA challenge would have shown an SDCCE exposure effect on BAL cells in the pre OVA model.

Another possible explanation for the SDCCE-induced inflammation in the post-OVA model may be related to the nature of the SDCCE exposure itself and the requirement for the presence of robust allergen-induced inflammation. The effects of SDCCE may be driven more by the gas-phase components as is evident by the fact that filtering out the PM fraction had no effect on the increase in BAL cells. The effects of gas-phase constituents are likely more transient and only persist as long as the exposure and inflammation are present together. Whereas an exposure that is comprised of a significant nonsoluble particulate fraction may have a longer residence time in the lung and thus greater potential for impact even after the end of exposure (e.g. pre-OVA exposure scenario). In the case of SDCCE, the PM fraction is comprised of approximately 95% neutralized sulfate that is readily soluble in the lung and which has previously been shown to have relatively low toxicity in studies of inhaled sulfate aerosols (Schlesinger, 2007). In contrast, we have previously shown that NERC exposures with a significant nonsoluble PM fraction (diesel engine emissions and hardwood smoke) increase BAL inflammatory cells regardless of pollutant-OVA exposure order (Barrett et al., 2006; Barrett et al., 2002).

Interestingly, SDCCE exposure in naïve (non-sensitized) animals resulted in a dose-dependent increase in BAL neutrophils and macrophages regardless of exposure protocol (pre-OVA vs. post-OVA) that was independent of the presence of the PM fraction (HF=H group). However, this effect was not clearly observed in the OVA-sensitized animals. In the case of the neutrophilia this may be the result of background levels in the OVA-sensitized animals masking the lesser effect of the SDCCE exposure. It was not abundantly clear why there was not an increase in BAL macrophages in the pre OVA sensitized animals despite an increase in the corresponding non-sensitized controls. In contrast, naïve F344 rats exposed for 1 week or 6 months as part of the broader NERC SDCCE studies did not show any increase in BAL inflammatory cells (Mauderly et al.,

2011). Whether species differences or exposure timing explain these differential responses is not known.

Besides changes in inflammatory cells there were also SDCCE-induced effects on serum antibody levels in both naïve and OVA-sensitized animals. The effects varied depending on the exposure timing, presence of the PM fraction, or whether the animals were OVA-sensitized. It was apparent that the PM containing fraction seemed to be important in increasing antibody levels in the non-sensitized animals (Post-OVA model). When the PM fraction was filtered out, antibody levels were reduced (sensitized animals) or did not increase (non-sensitized animals). There is limited evidence in the literature to suggest a specific mechanism whereby SDCCE might be interacting with the different exposure protocols to drive divergent responses (e.g. increase vs. attenuation). However, there is evidence from other environmental pollutants, such as diesel exhaust, indicating that PM can have adjuvant properties and can enhance/induce allergic responses in rodents/humans (Saxon and Diaz-Sanchez, 2000; Suzuki et al., 1996). Whether similar mechanisms are involved with SDCCE is not clear especially since the characteristics of the PM fractions are different. The PM fraction of SDCCE (~95% neutralized sulfate) has relatively low toxicity as previously mentioned, although we acknowledge that the ash component of the PM fraction could be involved. The current experimental design did not allow for the evaluation of whether the PM fraction played an independent or synergistic role with other components of the exposure.

It's of particular interest to note that many of the effects of SDCCE (inflammation, airway function, antibodies and cytokines) were not dependent on the PM fraction of the exposure. This is similar to the findings using other response models in the broader NERC SDCCE studies (Mauderly et al., 2011). This suggests that gas-phase constituents, which are dominated by oxides of sulfur and nitrogen, may have caused the observed effects. The exposure levels of NO₂ and SO₂ in the current study were within the realm of previous studies with asthmatics (up to 6 h of 0.1–2 ppm) and allergen-sensitized animals (4 h–5 days of 0.1–43.5 ppm) that showed some exposure effects (reviewed in (Environmental Protection Agency (EPA), 2008b) and (Environmental Protection Agency (EPA), 2008a)). However, except in a few studies, most did not evaluate the combined effects of NO₂ and SO₂, making specific comparisons with the even more complex mixture of SDCCE even more complicated. The combination of exposure to 0.2 ppm SO₂ and 0.4 ppm NO₂ for 6 h in human asthmatics led to an enhanced airway responsiveness to an inhaled allergen challenge, whereas, exposure to the single gases showed no significant effect (Devalia et al., 1994; Rusznak et al., 1996).

As many of the observed results occurred in the absence of the PM fraction it is important to consider the results in the context of relevant human SO₂ and NO₂ exposure levels. Since the SDCCE exposures in the present study occurred over several days for 6 h/day it

may be most appropriate to compare the exposure concentrations to the annual average National Ambient Air Quality Standards (NAAQS) for SO₂ (0.03 ppm) and NO₂ (0.053 ppm). If the daily exposures are averaged over 24 h, the SO₂ and NO₂ exposures at the H level in this study were ~1.7 and 1.4 times the NAAQS levels, respectively. Whereas the M (0.06 ppm SO₂ and 0.1 ppm NO₂) and L (0.02 ppm SO₂ and 0.04 ppm NO₂) levels in the study would be below the NAAQS levels if averaged over 24 h. Thus, exposure levels in the present study covered a range of plausible exposures that might be observed in short- and long-term environmental setting.

Exposure to NO₂ or SO₂ alone in allergen-sensitized animals has been associated with various effects on the allergic response. OVA-sensitized mice (C57BL/6) exposed to 25 ppm but not 5 ppm NO₂ (6 h/day; 1, 3, or 5 days) exhibit enhanced eosinophilic inflammation and terminal bronchial lesions (Poynter et al., 2006). In the same study, naïve mice exposed to 25 ppm NO₂ exhibited acute lung injury that peaked after 3 days and was characterized by increases in BAL protein, LDH, and neutrophils as well as lesions within the terminal bronchioles. This is a similar response pattern (sensitized and naïve animals) albeit at much higher NO₂ levels than in the SDCCE exposures. Exposure to 5 ppm NO₂ for 3 h after both immunization and challenge procedures in Brown Norway rats results in significantly higher levels of antigen-specific serum IgE, local IgA, IgG, and IgE antibody than air controls, and increased numbers of inflammatory cells in the lungs (Gilmour et al., 1996). In contrast, while 20 ppm NO₂ in OVA challenged BALB/c mice induced a significant increase in airway hyperreactivity there was no effect on BAL eosinophils (Proust et al., 2002). A reduction in BAL eosinophils was unexpectedly observed at 5 ppm NO₂ in the same study.

Exposure to SO₂ alone in allergen-sensitized animals shows a similar response pattern as NO₂ exposures. OVA-sensitized guinea pigs exposed to 0.1 ppm SO₂ for 5 h per day for 5 days led to increases in enhanced pause (Penh), increases in BAL eosinophils, and tissue inflammation (Park et al., 2001). Similar increases in airway hyperreactivity were observed in ascaris-sensitized sheep exposed to 5 ppm SO₂ for 5 h (Abraham et al., 1981). Thus, there is reasonable evidence to suggest that NO₂ and/or SO₂ may be causing the observed effects with SDCCE although we acknowledge that other gas-phase constituents or PM may be also contributing to the responses.

The OVA-allergen challenge model used in the present study has been used for over 100 years as a standard approach for inducing allergic airway responses. At the time of the initial NERC studies it was decided that all exposure atmospheres (diesel engine, SDCCE, wood smoke and gasoline engine) would be utilized in the context of the same allergen challenge model in order to allow for comparisons across studies. We understand that as time has gone by there is a growing appreciation for the shortcomings of the acute OVA-challenge

models (reviewed (Stevenson and Birrell, 2011)). While the various perturbations of the model can replicate a number of the features that are consistent with asthma the model is not always indicative of the clinical situation. In fact, models using more relevant allergens, such as house dust mite, in more chronic challenge scenarios provide a more robust model that may be more translatable to the human (Stevenson and Birrell, 2011). These chronic models may be more susceptible to the subtle effects of SDCCE. However, in reality, the strength of the overall NERC approach is not necessarily in the direct translation of the results to human health endpoints but rather the ability to ultimately determine the relative contribution of different components of the exposure mixture.

In comparison to previous NERC exposures, the present exposure resulted in a moderate number of significant endpoint responses (diesel engine > SDCCE > wood smoke > gasoline engine). It is interesting to note that in the previous NERC gasoline engine emissions study that utilized the same animal model described herein there was a significant contribution of SO₂ (0.6 ppm) and NO₂ (0.9 ppm) to the gas phase components of the exposure but no significant effects (Day et al., 2008). However, caution should be taken in directly comparing the relative health hazards of these exposures based on any one exposure constituent because the complex interplay amongst components of the mixture likely drives the ultimate response.

Acknowledgements

The authors wish to thank many LRRI staff members who developed and operated the exposure system, maintained the animals and performed the biological measurements.

Declaration of interest

This work was supported by the National Environmental Respiratory Center, which was funded by numerous industry, state, and federal sponsors, including the U.S. Environmental Protection Agency, U.S. Department of Energy (Office of Freedom Car and Vehicle Technologies) and U.S. Department of Transportation. This manuscript does not represent the views or policies of any sponsor. As noted above, this research was funded jointly by multiple government and industry sponsors. Decisions and approvals regarding experimental strategy and design were vested in an advisory committee independent from sponsorship. Neither the committee nor the sponsors reviewed this manuscript before submission.

References

- Abraham WM, Oliver W Jr, Welker MJ, King MM, Wanner A, Sackner MA. 1981. Differences in airway reactivity in normal and

- allergic sheep after exposure to sulfur dioxide. *J Appl Physiol* 51:1651-1656.
- Balmes JR, Fine JM, Sheppard D. 1987. Symptomatic bronchoconstriction after short-term inhalation of sulfur dioxide. *Am Rev Respir Dis* 136:1117-1121.
- Barrett EG, Henson RD, Seilkop SK, McDonald JD, Reed MD. 2006. Effects of hardwood smoke exposure on allergic airway inflammation in mice. *Inhal Toxicol* 18:33-43.
- Barrett EG, Reed MD, Espindola T, Henson RD. 2002. Allergic airway responses following diesel exhaust exposure: Exacerbation vs. attenuation. *Am J Respir Crit Care Med* 165:A414.
- Brunekreef B, Janssen NA, de Hartog J, Harssema H, Knape M, van Vliet P. 1997. Air pollution from truck traffic and lung function in children living near motorways. *Epidemiology* 8:298-303.
- Chang MC, Chow JC, Watson JG, Hopke PK, Yi SM, England GC. 2004. Measurement of ultrafine particle size distributions from coal-, oil-, and gas-fired stationary combustion sources. *J Air Waste Manag Assoc* 54:1494-1505.
- Chen LC, Lam HF, Kim EJ, Gutty J, Amdur MO. 1990. Pulmonary effects of ultrafine coal fly ash inhaled by guinea pigs. *J Toxicol Environ Health* 29:169-184.
- Day KC, Reed MD, McDonald JD, Seilkop SK, Barrett EG. 2008. Effects of gasoline engine emissions on preexisting allergic airway responses in mice. *Inhal Toxicol* 20:1145-1155.
- Devalia JL, Rusznak C, Herdman MJ, Trigg CJ, Tarraf H, Davies RJ. 1994. Effect of nitrogen dioxide and sulphur dioxide on airway response of mild asthmatic patients to allergen inhalation. *Lancet* 344:1668-1671.
- Dubnov J, Barchana M, Rishpon S, Leventhal A, Segal I, Carel R, Portnov BA. 2007. Estimating the effect of air pollution from a coal-fired power station on the development of children's pulmonary function. *Environ Res* 103:87-98.
- Dunnett CW. (1955). A multiple comparison procedure for comparing several treatments with a control. *J Am Stat Assoc* 50:1096-1121.
- Dunnett CW. (1980). Pairwise multiple comparisons in the unequal variance case. *J Am Stat* 75:796-800.
- Environmental Protection Agency (EPA) (2008a). Integrated Science Assessment for Sulfur Oxides - Health Criteria (and annexes). National Center for Environmental Assessment, Office of Research and Development, Research Triangle Park, NC.
- Environmental Protection Agency (EPA) (2008b). Integrated Sciences Assessment for Oxides of Nitrogen - Health Criteria (and annexes). National Center for Environmental Assessment, Office of Research and Development, Research Triangle Park, NC.
- Gilmour MI, O'Connor S, Dick CA, Miller CA, Linak WP. 2004. Differential pulmonary inflammation and *in vitro* cytotoxicity of size-fractionated fly ash particles from pulverized coal combustion. *J Air Waste Manag Assoc* 54:286-295.
- Gilmour MI, Park P, Selgrade MJ. 1996. Increased immune and inflammatory responses to dust mite antigen in rats exposed to 5 ppm NO₂. *Fundam Appl Toxicol* 31:65-70.
- Grahame T, Schlesinger R. 2005. Evaluating the health risk from secondary sulfates in eastern North American regional ambient air particulate matter. *Inhal Toxicol* 17:15-27.
- Hao J, Wang L, Shen M, Li L, Hu J. 2007. Air quality impacts of power plant emissions in Beijing. *Environ Pollut* 147:401-408.
- Horstman D, Roger LJ, Kehrl H, Hazucha M. 1986. Airway sensitivity of asthmatics to sulfur dioxide. *Toxicol Ind Health* 2:289-298.
- Linn WS, Avol EL, Peng RC, Shamoo DA, Hackney JD. 1987. Replicated dose-response study of sulfur dioxide effects in normal, atopic, and asthmatic volunteers. *Am Rev Respir Dis* 136:1127-1134.
- Mauderly JL, Barrett EG, Gigliotti AP, McDonald JD, Reed MD, Seagrave J, Mitchell LA, Seilkop SK. 2011. Health effects of subchronic inhalation exposure to simulated downwind coal combustion emissions. *Inhal Toxicol* 23:349-362.
- McDonald JD, White RK, Holmes T, Mauderly JL, Laumb J, Zielinska B, Chow, JC, Grosjean E. Simulated downwind coal emissions for laboratory inhalation exposure atmospheres. *J Air Waste Manag Assoc*. (In Press).
- Morgenstern V, Zutavern A, Cyrus J, Brockow I, Koletzko S, Krämer U, Behrendt H, Herbarth O, von Berg A, Bauer CP, Wichmann HE, Heinrich J; GINI Study Group; LISA Study Group. 2008. Atopic diseases, allergic sensitization, and exposure to traffic-related air pollution in children. *Am J Respir Crit Care Med* 177:1331-1337.
- Park JK, Kim YK, Lee SR, Cho SH, Min KU, Kim YY. 2001. Repeated exposure to low levels of sulfur dioxide (SO₂) enhances the development of ovalbumin-induced asthmatic reactions in guinea pigs. *Ann Allergy Asthma Immunol* 86:62-67.
- Poynter ME, Persinger RL, Irvin CG, Butnor KJ, van Hirtum H, Blay W, Heintz NH, Robbins J, Hemenway D, Taatjes DJ, Janssen-Heininger Y. 2006. Nitrogen dioxide enhances allergic airway inflammation and hyperresponsiveness in the mouse. *Am J Physiol Lung Cell Mol Physiol* 290:L144-L152.
- Proust B, Lacroix G, Robidel F, Marliere M, Lecomte A, Vargaftig BB. 2002. Interference of a short-term exposure to nitrogen dioxide with allergic airways responses to allergenic challenges in BALB/c mice. *Mediators Inflamm* 11:251-260.
- Raabe OG, Tyler WS, Last JA, Schwartz LW, Lollini LO, Fisher GL, Wilson FD, Dungworth DL. 1982. Studies of the chronic inhalation of coal fly ash by rats. *Ann Occup Hyg* 26:189-211.
- Riedel F, Krämer M, Scheibenbogen C, Rieger CH. 1988. Effects of SO₂ exposure on allergic sensitization in the guinea pig. *J Allergy Clin Immunol* 82:527-534.
- Rusznak C, Devalia JL, Davies RJ. 1996. Airway response of asthmatic subjects to inhaled allergen after exposure to pollutants. *Thorax* 51:1105-1108.
- Saxon A, Diaz-Sanchez D. 2000. Diesel exhaust as a model xenobiotic in allergic inflammation. *Immunopharmacology* 48:325-327.
- Schlesinger RB. 2007. The health impact of common inorganic components of fine particulate matter (PM_{2.5}) in ambient air: A critical review. *Inhal Toxicol* 19:811-832.
- Sheppard D, Wong WS, Uehara CE, Nadel JA, Boushey HA. 1980. Lower threshold and greater bronchomotor responsiveness of asthmatic subjects to sulfur dioxide. *Am Rev Respir Dis* 122:873-878.
- Sinclair AH, Edgerton ES, Wyzga R, Tolsma D. 2010. A two-time-period comparison of the effects of ambient air pollution on outpatient visits for acute respiratory illnesses. *J Air Waste Manag Assoc* 60:163-175.
- Smargiassi A, Kosatsky T, Hicks J, Plante C, Armstrong B, Villeneuve PJ, Goudreau S. 2009. Risk of asthmatic episodes in children exposed to sulfur dioxide stack emissions from a refinery point source in Montreal, Canada. *Environ Health Perspect* 117:653-659.
- Smith KR, Veranth JM, Kodavanti UP, Aust AE, Pinkerton KE. 2006. Acute pulmonary and systemic effects of inhaled coal fly ash in rats: Comparison to ambient environmental particles. *Toxicol Sci* 93:390-399.
- Stevenson CS, Birrell MA. 2011. Moving towards a new generation of animal models for asthma and COPD with improved clinical relevance. *Pharmacol Ther* 130:93-105.
- Suzuki T, Kanoh T, Ishimori M, Ikeda S, Ohkuni H. 1996. Adjuvant activity of diesel exhaust particulates (DEP) in production of anti-IgE and anti-IgG1 antibodies to mite allergen in mice. *J Clin Lab Immunol* 48:187-199.
- van Roosbroeck S, Wichmann J, Janssen NA, Hoek G, van Wijnen JH, Lebret E, Brunekreef B. 2006. Long-term personal exposure to traffic-related air pollution among school children, a validation study. *Sci Total Environ* 368:565-573.
- Walters DM, Breyse PN, Wills-Karp M. 2001. Ambient urban Baltimore particulate-induced airway hyperresponsiveness and inflammation in mice. *Am J Respir Crit Care Med* 164:1438-1443.
- Zanobetti A, Schwartz J, Dockery DW. 2000. Airborne particles are a risk factor for hospital admissions for heart and lung disease. *Environ Health Perspect* 108:1071-1077.